THE ASSESSMENT OF EFFICIENCY OF LOCAL DELIVERY PATHWAYS OF THERAPEUTIC GENES IN MURINE SPINAL CORD INJURY: CORRELATION OF STRUCTURE AND FUNCTION PARAMETERS

UDC 61–001:575
Received 12.10.2012

© **G.F. Shaymardanova,** PhD, Research Worker, the Laboratory of Molecular Basics of Pathogenesis[1];
Y.O. Mukhamedshina, Tutor, the Department of Histology, Cytology and Embryology[2];
Y.A. Chelyshev, D.Med.Sc., Professor, Head of the Department of Histology, Cytology and Embryology[2]

[1]Kazan Institute of Biochemistry and Biophysics of Kazan Scientific Centre of the Russian Academy of Sciences, Lobachevskogo St., 2/31, Kazan, Republic of Tatarstan, 420111;
[2]Kazan State Medical University, Butlerova St., 49, Kazan, Republic of Tatarstan, Russian Federation, 420012

The aim of the investigation was to assess the efficiency of posttraumatic regeneration of murine spinal cord in immediate single administration of human umbilical cord mononuclear blood cells transfected by pBud-VEGF-FGF2 plasmid, and direct injection of this plasmid in the damage area. Two problems were to be solved: to reveal the correlation between morphological and functional spinal cord indices and estimate the amount of S100B$^+$-cells in the conditions of local delivery of *vegf* and *fgf2* genes on cellular carriers or in direct gene therapy.

Materials and Methods. The rats after dosing contusion spinal cord injury (T_{VIII} level) were divided into four groups. One group animals were administered umbilical cord mononuclear blood cells transfected by pBud-VEGF-FGF2 plasmid in damage area, the animals of another group were administered the same cells transfected by pEGFP-N2 plasmid in similar conditions. The animals of other two groups were injected pBud-VEGF-FGF2 plasmid in the same area in one case, and another — the same amount of pEGFP-N2 plasmid.

Results. We established direct negative correlation between the damage area size and the motor function recovery index in experiments with a direct injection of pBud-VEGF-FGF2 plasmid. The highest correlation coefficient was obtained at the distance of 5 mm away from injury epicenter. In case of transplantation of cells transfected by this plasmid there was no correlation. The number of S100B$^+$-cells in exterior zones of white matter at the distance of 1.5 cm from the injury epicenter under the conditions of direct gene delivery increased by 46% ($p<0.05$). If umbilical cord blood cells transfected by pBud-VEGF-FGF2 plasmid were administered the index grew by 55% ($p<0.05$).

Conclusion. In the course of regeneration after contusion spinal cord injury, the damage area reduction and related motor function recovery is more effective in direct gene therapy compared to the delivery of the same genes on cellular carriers.

Key words: spinal cord; spinal regeneration; local gene delivery; umbilical cord blood cells; VEGF, FGF2 plasmids.

One of the promising directions in spinal cord injury therapy is local gene delivery of neurotrophic factors as part of plasmid vectors. pBud-VEGF-FGF2 plasmid we developed [1] simultaneously and independently expresses genes of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF2). The selection of genes of these factors is conditioned by the fact that these factors are at once neurotrophic and angiogenic. In their separate delivery to the area of spinal cord injury VEGF is known to stimulate neurogenesis, survival, neuronal migration and axonal growth [2], and FGF2 — axonal growth and motor function recovery [3, 4], promotes differentiation of microglia precursors in mature cells [5, 6]. In literature there are reports on effect synergism of these factors on angiogenesis *in vitro* and *in vivo* [7] that gives grounds for the study of their combined effect on posttraumatic regeneration. However, direct injection of protein molecules in the organism, in spinal cord particularly, is characterized

For contacts: Shaymardanova Gulnara Ferdinantovna, phone: (843)231-90-51, 8-917-867-36-92; gulnara_kzn@rambler.ru

by low efficiency and various complications [8, 9]. An injection of genes encoding necessary factors seems to be the safer method to increase the content of neurotrophic factors in a recipient's tissue. We have taken an interest in possible combined introduction of *vegf* and *fgf2* genes in a spinal cord injury area.

In our experiments we use two delivery methods of these genes: direct (an injection of DNA-containing vectors) and cell-mediated (consists in using cells as carriers of therapeutic genes).

Differentiated, stem, induced pluripotent and progenitor cells are studied for delivery of therapeutic genes on cell carriers [10, 11]. The criteria for selection of cells suitable for transplantation are the following: high survival rate, controlled differentiation, oncogenic and infectious safety, the possibility to transfect therapeutic genes with high-efficient expression in recipient's tissues. The use of umbilical cord blood cells as the source of stem and progenitor cells seems to be highly promising due to their low immunogenicity, availability, easy and safe preparation, the capability to withstand long-time storage, the possibility to use autologous material [12, 13]. Human umbilical cord blood cell transplantation in spinal cord injury suppresses inflammatory response, has neurotrophic effect, and stimulates neovascularization [14–16].

The previous study of the efficiency of these two delivery ways of *vegf* and *fgf2* genes directly to the zone of spinal cord injury in rats [17–19] showed that a direct injection of plasmid DNA ranks slightly below in delivery efficiency of the same therapeutic genes on cell carriers, and surpasses it in the parameters of motor function recovery, the change in dimensions of pathological cavities. However, those studies had no analysis of possible correlations between structural and functional parameters. Moreover, in spinal cord injury therapy it appears to be of importance to study the state of cells of certain populations to estimate neuroregeneration effectiveness. Astrocytes is the most numerous population of glial cells, which provide brain balance control and malfunction of which results in neurodegeneration [20, 21]. In combined delivery of *vegf* and *fgf2* genes, the population size of astrocytes in spinal cord injury has not yet been studied.

The aim of the investigation was to reveal possible correlations between morphological and functional spinal cord indices and estimate the number of astrocytes by the presence of specific protein S100B in the conditions of local delivery of *vegf* and *fgf2* genes as part of plasmid construction pBud-VEGF-FGF2 in injection mediated by umbilical cord blood cells and in direct plasmid injection into traumatic area.

Materials and Methods. The experiments were carried out on 52 white female and male rats weighing 200–250 g. The animals were kept in standard conditions, with easy access to water and food, according to ethical principles established in Kazan State Medical University (Russia). The work was performed in accordance with ethical principles established by European Convention for the protection of vertebrata used for experimental and other scientific purposes (the Convention was passed in Strasburg, Mar, 18, 1986, adopted in Strasburg, Jun, 15, 2006). The animals were anesthetized with an i.p. injection of chloral hydrate (80 mg/ml, 0.4 ml per 100 g). Dosed spinal cord contusion was simulated after laminectomy on T_{VIII8} level [22].

For experiments on cell-mediated gene delivery, human umbilical blood samples were drawn and mononuclear cell fraction was performed according to a previously described method [23]. The obtained cells were transfected by pBud-VEGF-FGF2 plasmid using electroporation and test group 1 animals were injected immediately after injury. The animals of control group 1 under the same conditions were injected the same umbilical blood cells transfected by pEGFP-N2 plasmid.

In experiments with direct gene therapy, the test group 2 animals were injected with pBud-VEGF-FGF2 plasmid in the same area, and control group 2 animals were injected with the same amount of pEGFP-N2 plasmid.

Beginning with the day 7 after injury, the animals of all groups were tested in the open plain every other day according to the method of D.M. Basso et al. [24]. 30 days after injury the animals were anesthetized and transcardially perfused with 4% paraformaldehyde solution (4°C). A spinal cord fragment was taken with vertebrae. Total injury area was measured on spinal cord cross sections [17–19]. To estimate a linear correlation between the values we used Pearson's coefficient and statistics package in Origin Pro 7.0.

On frozen cross spinal cord sections at a distance of 1.5 cm from injury epicenter using indirect immuno-peroxidase technique $S100B^+$-cells were detected (Sigma, dilution 1:100). The cells were counted on digital images in four fixed morphometric zones [18]. The preparations were studied and images were digitized on Axio Imager A1 microscope (Carl Zeiss, Germany).

Results. The comparison of total injury area in animal groups 30 days later using different delivery techniques of therapeutic genes showed the index in a direct plasmid injection at a 5 mm distance from the epicenter to be twice as little, and at a distance of 3 mm — 1.5 times less than in gene-cell therapy (Fig. 1).

Animal testing in the open plain revealed a motor nonfunction of lower limbs in both test groups as well as in both control groups up to 6 days after injury. The rats of control group 1 (injected by umbilical blood cells transfected by pEGFP-N2 plasmid) and control group 2 (with pEGFP-N2 plasmid injection) were recorded to have eventual partial self-recovery of voluntary movements from 3.3±0.9 and 7.7±0.5 scores for the first two weeks after the operation to 4.9±1.0 and 8.1±0.9 scores in the last two weeks of the experiment. In animals of test group 2 with direct pBud-VEGF-FGF2 plasmid injection the index of motor function recovery was 6.8±1.6 scores in the first two weeks after the operation and increased up to 14±1.7 scores in the last two weeks of the experiment that is higher by 2 scores than in the test group 1 animals with transplantation of umbilical blood cells transfected by pBud-VEGF-FGF2 plasmid.

The animals with direct pBud-VEGF-FGF2 plasmid injection (test group 2) by day 30 after injury were found to have negative correlation between injury area and a motor function recovery index (Fig. 2). A correlation coefficient appeared to be higher for sections at 5 mm distance from

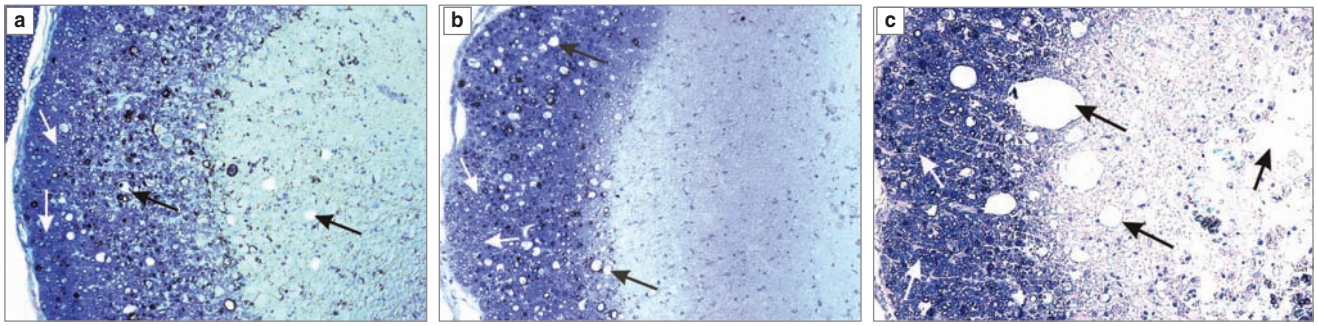

Fig. 1. White matter fragment of rat spinal cord (30 days after injury) at a 5 mm distance from injury epicenter on T_{VIII} level: *a* — an injection of umbilical blood cells transfected by pBud-VEGF-FGF2, *b* — in direct injection of pBud-VEGF-FGF2 plasmid, *c* — an injection of umbilical blood cells transfected by pEGFP-N2. Black arrows show the cavities resulted from an injury, white arrows indicate myelinated fibers. Cross sections are stained by methylene blue; ×20

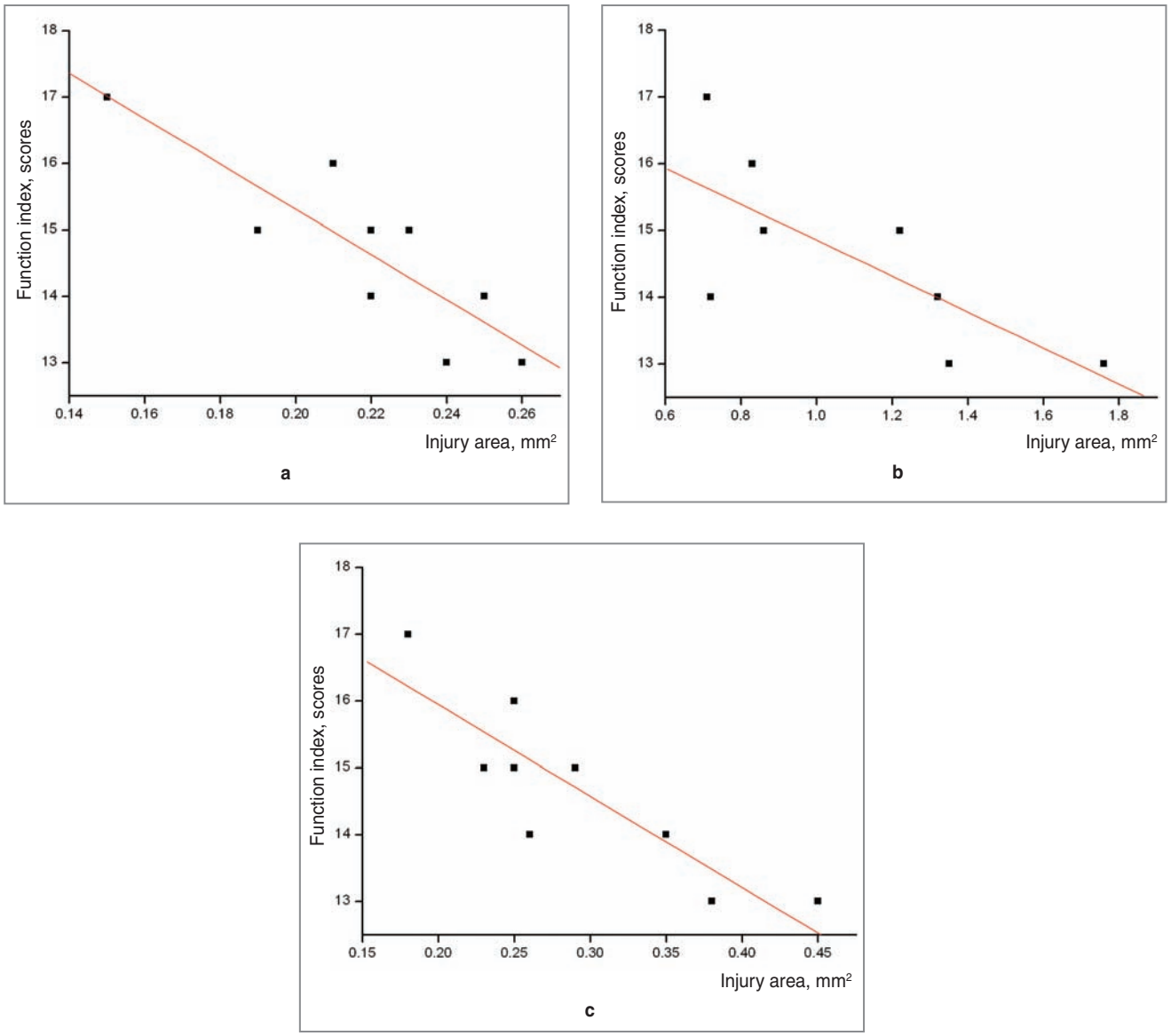

Fig. 2. Distribution of values of a motor function index and injury area at a 5 mm (*a*) and 3 mm (*b*) distance in rostral direction, and at a 5 mm distance in caudal direction (*c*) from an injury epicenter in direct pBud-VEGF-FGF2 plasmid injection in contusion spinal cord injury (by day 30 after injury)

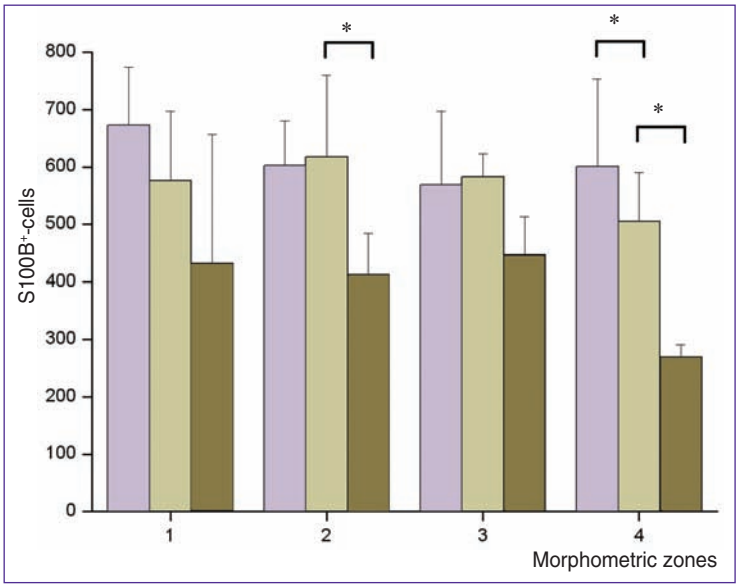

Fig. 3. S100B$^+$-cell count in spinal cord white matter at a distance of 1.5 cm from the epicenter on day 30 of the experiment. Lilac columns — test group 1 with injection of umbilical mononuclear blood cells transfected by pBud-VEGF-FGF plasmid; light green columns — test group 2 with pBud-VEGF-FGF2 plasmid injected; dark green columns — control group 1 with injected umbilical mononuclear blood cells transfected by pEGFP-N2 plasmid; * — $p<0.05$

injury epicenter than for those at 3 mm distance. Thus, at a distance of 5 mm in rostral direction r=–0.85; p=0.002, and in caudal direction r=–0.87; p=0.009; at 3 mm distance in rostral direction r=–0.73; p=0.024; and in caudal direction r=–0.21; p=0.56. There was no such correlation in the experiment with transplantation of umbilical blood cells transfected by pBud-VEGF-FGF2 plasmid. In control groups no correlation between injury area and a motor function recovery index was revealed.

By day 30 of the experiment the number of S100B$^+$-cells in external white matter zones at 1.5 cm distance from injury epicenter in direct gene delivery increases by 46% ($p<0.05$). And this index increases by 55% ($p<0.05$) in injected umbilical blood cells transfected by pBud-VEGF-FGF2 plasmid (Fig. 3).

Discussion. The revealed direct negative correlation between spinal cord injury area and a motor function recovery index in the experiments with direct pBud-VEGF-FGF2 plasmid injection indicates that matrix conspicuity factor of preserved tissues is of primary importance (among other numerous factors controlling neuroregeneration in spinal cord injury) for the growth of regenerating nerve fibers. Compared with the results of transgenic delivery on cell carriers these data support high efficiency of direct gene therapy and give reasons for its practical application.

The highest correlation coefficient was found at a 5 mm distance from injury epicenter. This observation is consistent with our earlier findings [18, 19] indicating that destructive changes are more marked at a 3 mm distance from injury epicenter compared to those revealed at a 5 mm distance. Direct plasmid injection experiments showed better integrity of grey and white matter. It promotes better intergrowth of myelinated fibers in regeneration area and motor function recovery.

There is no correlation between the injury area and a motor function recovery index by day 30 in transplantation of cells transfected by pBud-VEGF-FGF2 plasmid. The results can be related to weak expression of transgenes delivered with cells, insufficient secretion of molecular factors synthesized with the involvement of transgenes in these cells after transplantation in injury area.

Increased expression of specific protein of macroglia S100B in case of direct gene therapy and in injection of umbilical mononuclear blood cells transfected by therapeutic genes can have positive effect on spinal cord regeneration. More marked increase of S100B$^+$-cells count in cell-mediated therapy compared with direct plasmid injection can be associated with a known capacity of umbilical mononuclear blood cells to suppress inflammatory response and have neurotrophic effect, or with the production of specific factors supporting survival and differentiation of astrocytes [25, 26].

In local delivery of therapeutic genes *vegf* and *fgf2*, the modulation of astrocyte phenotype and population increase of S100B-immunopositive cells can reflect a positive process of injured tissue reconstruction in the course of neuroregeneration that is important for adverse cell-mediated response prevention, degeneration control, inflammatory response restriction, and neovascularization stimulation. Protein S100B secreted by astrocytes acts in physiological nanomolar concentrations having a neurotrophic effect and supporting neuronal survival and axonal growth [15, 27]. Moreover, a positive role of increased population of S100B$^+$-cells is proved by an idea that many molecules produced by astrocytes have neuritrophic paracrine effect on a target cell in injury area, and astrocytes themselves form an adequate matrix for axonal growth.

Conclusion. In the course of regeneration after contusion spinal cord injury, the damage area reduction and related motor function recovery is more effective in direct gene therapy compared to the delivery of the same genes on cellular carriers.

This work was carried out with the support of state contract of Federal Target Program of the Ministry of education and science of the Russian Federation No.16.512.11.2101, OPTEK grant 2012, Russian Foundation for Basic Research grant No.12-04-31092-мол_а.

The authors are grateful to R.R. Islamov, A.P. Kiyasov, A.A. Rizvanov for the generous gift of pBud-VEGF-FGF2 plasmid and I.I. Salafutdinov for preparation and transfection of human umbilical blood cells.

References

1. Chelyshev Yu.A., Shaymardanova G.F., Mukhamedshina Ya.O., Islamov R.R., Rizvanov A.A., Salafutdinov I.I. *Sposob stimulirovaniya neyroregeneratsii s pomoshch'yu geneticheskikh konstruktsiy* [The method of neuroregeneration stimulation using genetic makers]. Patent RF 2459630 C1. 2012.

2. Mackenzie F., MacRuhrberg C. Diverse roles for VEGF-A in the nervous system. *Development* 2012 Apr; 139(8): 1371–80.

3. Moftah M., Landry M., Nagy F., Cabelguen J.M. Fibroblast growth factor-2 mRNA expression in the brainstem and spinal cord of normal and chronic spinally transected urodeles. *J Neurosci Res* 2008 Nov 15; 86(15): 3348–3358.

4. Guzen F.P., Soares J.G., de Freitas L.M., Cavalcanti J.R., Oliveira F.G., Araújo J.F., Cavalcante Jde. S., Cavalcante J.C., do Nascimento E.S. Jr., de Oliveira Costa M.S. Sciatic nerve grafting and inoculation of FGF-2 promotes improvement of motor behavior and fiber regrowth in rats with spinal cord transaction. *Restor Neurol Neurosci* 2012; 30(3): 265–275.

5. Ohori Y., Yamamoto S., Nagao M., Sugimori M., Yamamoto N., Nakamura K., Nakafuku M. Growth factor treatment and genetic manipulation stimulate neurogenesis and oligodendrogenesis by endogenous neural progenitors in the injured adult spinal cord. *J Neurosci* 2006 Nov 15; 26(46): 11948–11960.

6. Yang Y.L. Effect of adenovirus-mediated basic fibroblast growth factor gene transfer in vivo on oligodendrocyte cell numbers throughout ventrolateral white matter following spinal cord injury in rats. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2012 Aug; 34(4): 348–352.

7. Seghezzi G., Patel S., Ren C.J., Gualandris A., Pintucci G., Robbins E.S., Shapiro R.L., Galloway A.C., Rifkin D.B., Mignatti P. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol* 1998 Jun 29; 141(7): 1659–1673.

8. Apfel S.C. Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? *Int Rev Neurobiol* 2002; 50: 393–413.

9. Chelyshev Yu.A., Mukhamedshina Ya.O., Shaymardanova G.F., Nikolaev S.I. Pryamaya dostavka terapevticheskikh genov dlya stimulirovaniya posttravmaticheskoy neyroregeneratsii [Direct delivery of therapeutic genes to stimulate posttraumatic neurogeneration]. *Nevrologicheskiy vestnik (zhurnal im. V.M. Bekhtereva) — Neurological Bulletin named after V.M. Bekhterev* 2012; 64(1): 76–83.

10. Chelyshev Yu.A., Viktorov I.V. Kletochnye tekhnologii remielinizatsii pri travme spinnogo mozga [Cellular technologies of remyelinization in spinal cord injury]. *Nevrologicheskiy vestnik (zhurnal im. V.M. Bekhtereva) — Neurological Bulletin named after V.M. Bekhterev* 2009; 1: 49–55.

11. Gerin C.G., Madueke I.C., Perkins T., Hill S., Smith K., Haley B., Allen S.A., Garcia R.P., Paunesku T., Woloschak G. Combination strategies for repair, plasticity, and regeneration using regulation of gene expression during the chronic phase after spinal cord injury. *Synapse* 2011 Dec; 65(12): 1255–1281.

12. Deng X.Y., Zhou R.P., Lu K.W., Jin D.D. Lithium chloride combined with human umbilical cord blood mesenchymal stem cell transplantation for treatment of spinal cord injury in rats. *Nan Fang Yi Ke Da Xue Xue Bao* 2010 Nov; 30(11): 2436–2439.

13. Kim J.Y., Jeon H.B., Yang Y.S., Oh W., Chang J.W. Application of human umbilical cord blood-derived mesenchymal stem cells in disease models. *World J Stem Cells* 2010 April 26; 2(2): 34–38.

14. Kaner T., Karadag T., Cirak B., Erken H.A., Karabulut A., Kiroglu Y., Akkaya S., Acar F., Coskun E., Genc O., Colakoglu N. The effects of human umbilical cord blood transplantation in rats with experimentally induced spinal cord injury. *J Neurosurg Spine* 2010 Oct; 13(4): 543–551.

15. Chua S.J., Bielecki R., Yamanaka N., Fehlings M.G., Rogers I.M., Casper R.F. The effect of umbilical cord blood cells on outcomes after experimental traumatic spinal cord injury. *Spine (Phila Pa 1976)* 2010 Jul 15; 35(16): 1520–1526.

16. Yao L., He C., Zhao Y., Wang J., Tang M., Li J., Wu Y., Ao L., Hu X. Human umbilical cord blood stem cell transplantation for the treatment of chronic spinal cord injury. *Neural Regen Res* 2013; 8(5): 397–403.

17. Shaymardanova G.F., Mukhamedshina Ya.O., Rizvanov A.A., Salafutdinov I.I., Chelyshev Yu.A. Primenenie plazmidnogo vektora s genami vegf i fgf2 pri travmaticheskom povrezhdenii spinnogo mozga krysy [The use of plasmid vector with VEGF and FGF2 genes in murine spinal cord injury]. *Kletochnye tekhnologii v biologii i meditsine — Cellular technologies in biology and medicine* 2012; 4: 200–204.

18. Shaymardanova G.F., Mukhamedshina Ya.O., Arkhipova S.S., Salafutdinov I.I., Rizvanov A.A., Chelyshev Yu.A. Posttravmaticheskie izmeneniya spinnogo mozga krysy pri transplantatsii mononuklearnykh kletok krovi pupoviny cheloveka, modifitsirovannykh genami vegf i fgf2 [Posttraumatic changes of murine spinal cord in the transplantation of human umbilical cord blood mononuclear cell modified by VEGF and FGF2 genes]. *Morfologiya — Morphology* 2011; 6: 36–42.

19. Shaymardanova G.F., Mukhamedshina Ya.O., Rizvanov A.A., Salafutdinov I.I., Chelyshev Yu.A. Effekt transplantatsii v oblast' travmaticheskogo povrezhdeniya spinnogo mozga krysy mononuklearnykh kletok krovi pupoviny cheloveka, ekspressiruyushchikh rekombinantnye geny VEGF i FGF2 [The transplantation effect of human umbilical cord blood cells expressing recombinant VEGF and FGF2 genes in the damage area of spinal cord injury in rats]. *Morfologiya — Morphology* 2012; 142(4): 31–36.

20. Matyash V., Kettenmann H. Heterogeneity in astrocyte morphology and physiology. *Brain Res Rev* 2010 May; 63(1–2): 2–10.

21. Allaman I., Bélanger M., Magistretti P.J. Astrocyte-neuron metabolic relationships: for better and for worse. Trends Neurosci 2011 Feb; 34(2): 76–87.

22. Lebedev S.V., Timofeev S.V., Zharkov A.V., Shipilov V.G., Chelyshev Yu.A., Masgutova G.A., Chekhonin V.P. Nagruzochnye testy i metod VVV pri otsenke dvigatel'nykh narusheniy posle kontuzionnoy travmy spinnogo mozga [Stress tests and VVV method in the assessment of motor disturbances after contusion spinal brain injury]. *Bull Eksp Biol Med — Bulletin of Experimental Biology and Medicine* 2008; 145(10): 471–476.

23. Rizvanov A.A., Kiyasov A.P., Gaziziov I.M., et al. Human umbilical cord blood cells transfected with VEGF and L(1)CAM do not differentiate into neurons but transform into vascular endothelial cells and secrete neuro-trophic factors to support neurogenesis — a novel approach in stem cell therapy. *Neurochem Int* 2008 Dec; 53(6–8): 389–394.

24. Basso D.M., Beattie M.S., Bresnahan J.C. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995 Feb; 12(1): 1–21.

25. Shen C.T., Foo N.H., Liu W.S., Chen S.H. Infusion of human umbilical cord blood cells ameliorates hind limb dysfunction in experimental spinal cord injury through anti-inflammatory, vasculogenic and neurotrophic mechanisms. *Pediatr Neonatol* 2008 Jun; 49(3): 77–83.

26. Adami C., Sorci G., Blasi E., Agneletti A.L., Bistoni F., Donato R. S100B expression in and effects on microglia. *Glia* 2001 Feb; 33: 131–142.

27. Rothermundt M., Peters M., Prehn J.H., Arolt V. S100B in brain damage and neurodegeneration. *Microsc Res Tech* 2003 Apr; 60(6): 614–632.