IMMUNOHISTOCHEMICAL STUDY OF RAT NERVE REGENERATION AFTER CRUSHING AND MSCs TRANSPLANTATION

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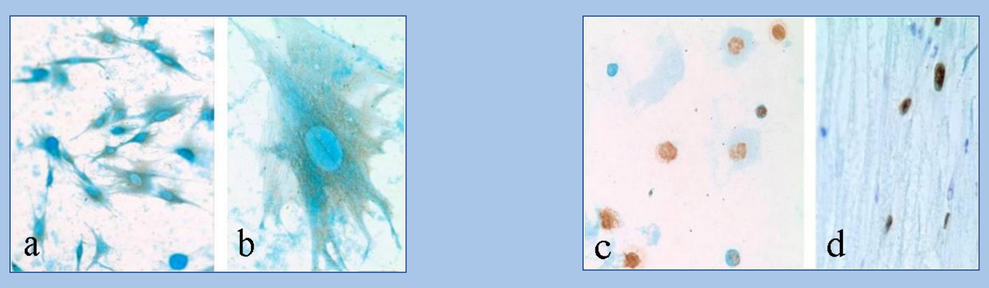

Figure 1. MSCs in culture (*a, b*), BrdU-containing MSCs in a smear (*c*) and in the sciatic nerve 1 day after transplantation (d) (*a, b* – vimentin-immunohistochemistry; *c, d* – BrdU-immunohistochemistry; toluidine blue staining (d) (×400; x1000 (b)).

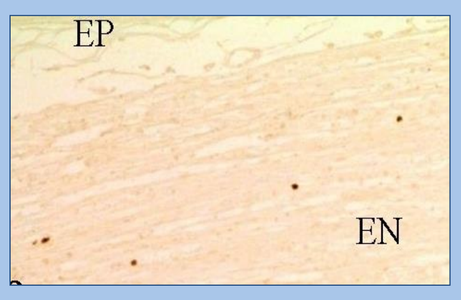

Figure 2. MSCs in endoneurium (EN) of the recipient rat nerve. Five days after the operation. BrdU-immunohistochemistry (×100).

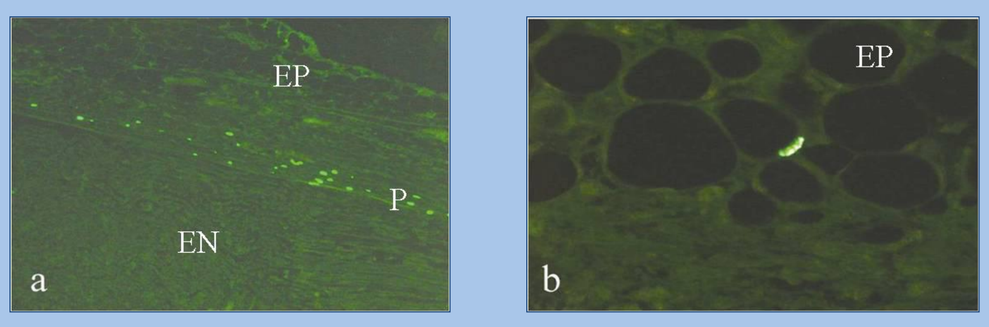

Figure 3. BrdU-labeled MSCs in the perineurium (P) and epineurium (EP)of recipient rats (a) and BrdU+ adipocyte in adipose tissue of the recipient epineurium (b) 7 days after transplantation MSCs in the rat sciatic nerve. Immunohistochemical reaction on BrdU. Fluorescence microscopy (×100, ×600).

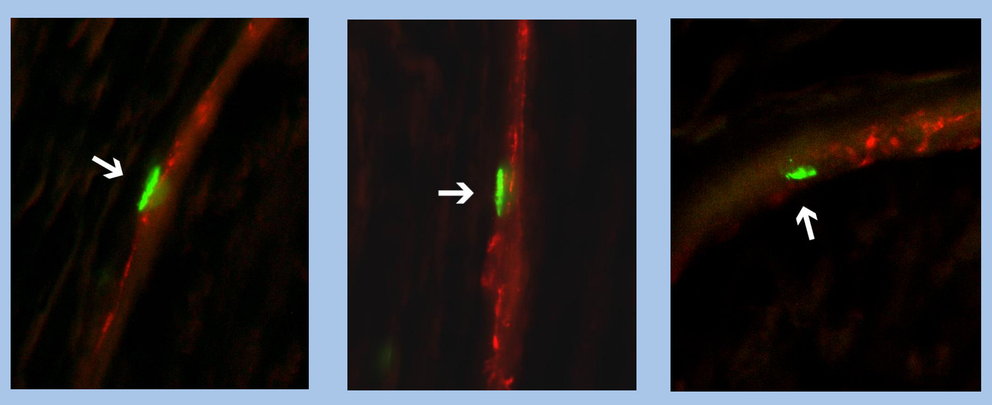

Figure 4. BrdU-labeled (arrows) transplanted cells in the recipient perineurium 7 days after transplantation in the rat sciatic nerve. Double positive staining for claudin1 (red) and BrdU (green) Fluorescence microscopy. ×600.

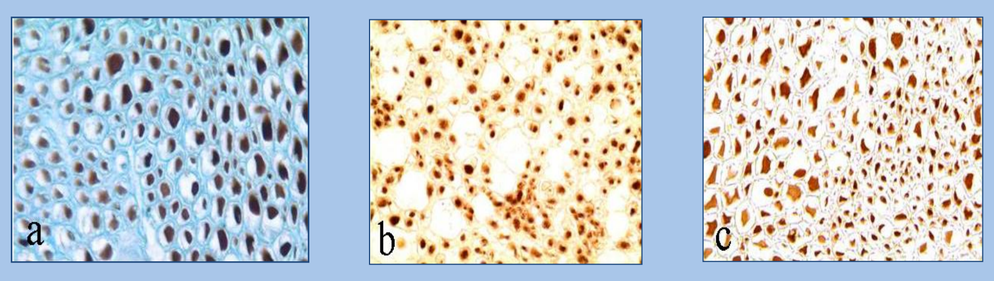

Figure 5. Fragments of transverse sections through the sciatic nerve of rats. A - intact nerve; b - the distal segment of the nerve 2 months after the ligature; c – the distal segment of the nerve 2 months after ligature and MSC transplantation. Immunohistochemical reaction to peripherin, astra blue (a). x 400

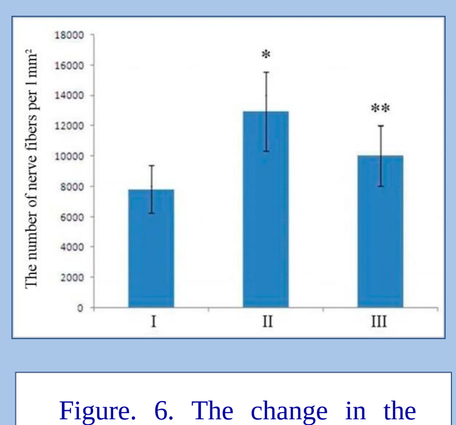

Figure. 6. The change in the density of the rat sciatic nerve fibers. The ordinate - the number of nerve fibers per 1 mm^2. I - intact nerve; II - ligature; III - ligature and the introduction of MSCs. *$p_{\ I,\ II} < 0.05$; **$p_{\ II,\ III} > 0.05$.

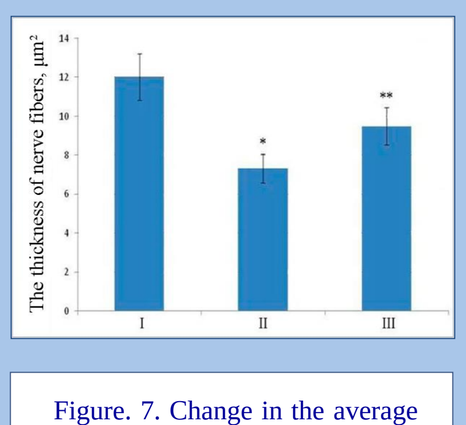

Figure. 7. Change in the average thickness of rat sciatic nerve fibers. I - intact nerve; II - ligature; III - ligature and the transplantation of MSCs. *$p_{\ I,\ II} < 0.05$; **$p_{\ II,\ III} < 0.05$.

References: **1**.Southwell D.G., Nicholas C.R., Basbaum A.I. et al. (2014) Interneurons from embryonic development to cell-based therapy. *Science.* 344:1240622. **2.** Fairbairn N.G., Meppelink A.M., Ng-Glazier J. et al. (2015) Augmenting peripheral nerve regeneration using stem cells: A review of current opinion. *World J. Stem Cells.* 7: 11–26. **3**. Zinkova N.N., Sokolova I.B., Shvedova E.V. et al. (2007) Dynamics of morphological changes after transplantation of mesenchymal stem cells in rat brain provoked by stroke. *Cell and tissue biology*. 1(6): 482-490. **4**. Petrova E.S. (2015) Injured nerve regeneration using cell-based therapies: current challenges. *Acta Naturae*. 7: 38-47. **5**. Dezawa M., Takahashi I., Esaki M. et al. (2001) Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. *Eur. J. Neurosci* . 4: 1771-1776. **6**. Petrova E.S., Isaeva E.N. (2014). Study of effect of embryonic anlage allografts of the rat spinal cord on growth of regenerating fibers of the recipient nerve. *Biology Bull* . 41: 479-485. **7**. Petrova E., Isaeva E., Kolos E., Korzhevskii D. (2018) Allogeneic bone marrow mesenchymal stem cells in the epineurium and perineurium of the recipient rat. *Biological Communications*. 63(2): 123-132. **8**. Korzhevskii D.E., Sukhorukova E.G., Gilerovich E.G., Petrova E.S., Kirik O.V, Grigor'ev I.P. (2014) Advantages and disadvantages of zinc-ethanol-formaldehyde as a fixative for immunocytochemical studies and confocal laser microscopy. *Neurosci. Behav. Physiol*. 44: 542-545.

Introduction

Current investigations on regenerative neurobiology have demonstrated that transplantation of stem cells can have a stimulating effect on the processes of reparative regeneration of the nervous system organs [1, 2]. The experimental elaboration of cell technologies to stimulate nerve regeneration is carried out actively [2-6]. The mechanisms of their influence are not fully understood.

The aim of this work was to study the effect of single MSCs transplantation on the regenerating fibers of a rat damaged sciatic nerve.

Materials and Methods

Adult male Wistar-Kyoto rats were used (n = 36); the care and keeping of laboratory animals and all experiments were carried out according to the rules for work with experimental animals. The research was positively approved by the local Ethics Committee of the Institute of Experimental Medicine, (Protocol №3/17 of November 30, 2017). The sciatic nerves of recipient rats were damaged by application of a ligature for 40 s at the level of the upper third of the thigh. A small section (1 mm long) of epineurium and perineurium was made close to the site of the lesion. At this site, a cell suspension (5 x 10^4 in 5 µl medium) was infused under the perineurium of the nerve trunk. The method of MSCs transplantation was described in more detail earlier [6, 7].

MSCs derived from bone marrow of Wistar-Kyoto rats were provided by "Trans-Technologies" (Saint-Petersburg, General Director D.G. Polyntsev). The method of MSCs obtaining and its characteristics were described in more detail earlier by Zinkova et al. [3]. Cells were cultured for a week, using a culture flasks (NEST Scientific, USA) and culture medium MEM alpha (BioloT, Russia) supplemented with bovine serum. Three days prior to usage of culture, 5-bromo-2-deoxyuridine (BrdU) (Sigma, USA) was added to the medium. The MSCs suspension was washed twice with the medium without BrdU and centrifuged for 15 min (200 *g*). The precipitate was resuspended in 1 ml of fresh medium, and viability of the cells was tested using 0.2% trypan blue solution (BioloT, Russia) and by cell calculation in a Goryaev chamber. The cell suspension was used for transplantation if the viability of the latter was at least 90%.

Then, the animals were kept in standard conditions of vivarium and were euthanized after 1, 5, 60 days. The nerve segments containing the graft, as well as small fragments of the distal segment (at a distance of 5 mm from the site of injury) were fixed in a solution of zinc-ethanol-formaldehyde [8]. After dehydration of the material in alcohols of increasing concentration and pouring in paraffin, the sections (5 µm in thickness) were cut using rotary microtome (RM 2125RT Leica, Germany) and mounted on silane treated (HistoBondR, Germany) glass slides. Histological slides were counterstained with toluidine blue and astra blue.

Immunohistochemical reaction to vimentin was used to characterize cultured MSCs (figure 1 a,b). Immunohistochemical labeling was performed using murine monoclonal antibodies to vimentin (clone V-9) (Dako, Denmark). This immunohistochemical reaction was carried out on the MSCs culture directly in culture flasks. Monoclonal antibody to BrdU (Bu20a clone, Dako, Denmark) and polyclonal antibodies to peripherin (Abcam, UK) were also used (figure 5). For fluorescence microscopy, biotinylated anti-mouse antibodies and streptavidin, conjugated with fluorescent carbocyanine dye (Cy2, Jackson ImmunoResearch, USA) were applied (figure 3). Immunohistochemical marking of perineurium on longitudinal sections through the nerve was also performed, using polyclonal antibodies to Claudin-1 (Dako, Denmark) and The anti-rabbit secondary antibodies, conjugated with TRITC (Dako, Denmark).

Results and discussion

The transplanted mesenchymal stem cells of the graft were identified by immunohistochemical reaction to BrdU (figure 1-4). Bromodeoxyuridine-labeled MSCs were found in the recipient's nerve for a week and were discovered not only in the endonervium (figure 2), but also in the epineurium (figure 3,b) and perineurium (figure 3, a; 4). Apparently, a part of the transplanted MSCs migrated to the outer sheaths of the peripheral nerve after the barrier breaking by ligation.

A study of the regenerating nerve fibers in the distal segment of crushed nerves was performed using immunohistochemical detection of peripherin (figure 5). Peripherin is a 57 kDa type III intermediate filament protein involved in the process of nerve fibers elongation in damaged nerve. Detection of this protein is often used to study the structures of the peripheral nervous system. Two months after the operation, peripherin-immunopositive nerve fibers were counted and measured on transverse sections of the recipient's nerve distal segment (figure 6, 7). Morphometric analysis of regenerating fibers was performed using ImageJ software (NIH, USA). It was shown that the average thickness of nerve fibers in animals of the experimental group was increased. A study of the nerve fibers thickness distributions of the damaged nerve distal segment showed that in animals treated with MSCs, the percentage of larger diameter fibers is higher than in rats of the control group (figure 8).

Two hypotheses can be made for explanation of the obtained fact. The first hypothesis is associated with the features of the growth of regenerating axons to the periphery. After injury to the nerve trunk, Wallerian degeneration occurs in its distal segment, and thin regenerating axons begin to grow to the periphery. As they grow, their thickness increases, some of them undergo myelination. We believe that exogenous mesenchymal stem cells, producing neurotrophic and growth factors, can accelerate the growth of regenerating axons, an increase in their caliber, and their myelination. The second hypothesis concerns the neuroprotective effect of MSCs. Due to the paracrine effect on the endogenous cells of the recipient's nerve (neurolemmocytes, fibroblasts, perineurium cells, cells of the blood vessels wall, macrophages), MSCs transplantation can prevent the degeneration of some of the fibers after compression. To find out it, is necessary to further study the dynamics of growth of nerve fibers in the early stages after trauma, as well as to study the effect of exogenous MSCs on Wallerian degeneration.

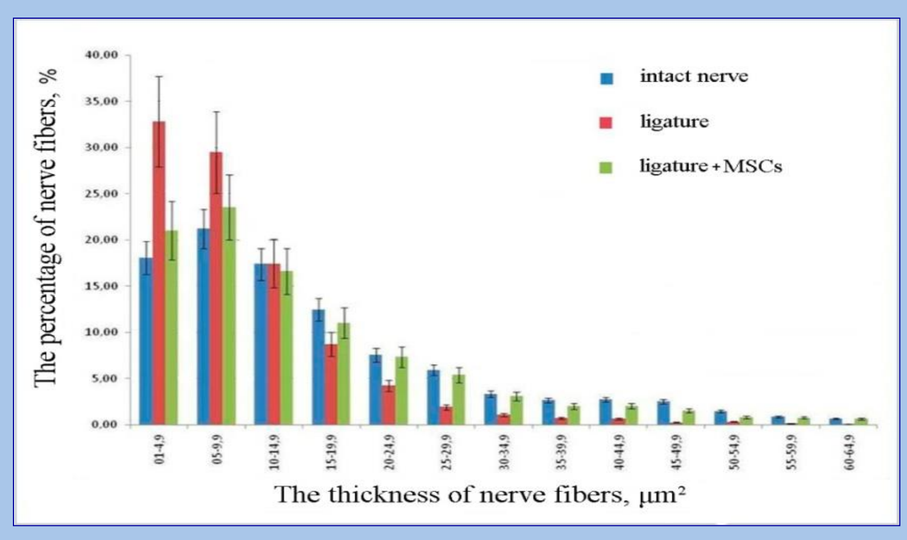

Figure. 8.
The distribution of nerve fibers thickness for the intact sciatic nerve of the rat (blue bars), for the nerve after ligature (red bars) and after ligature and MSC (green bars).

Conclusion

An analysis of the distribution of nerve fibers in the distal nerve segment showed that a single transplantation of rat bone marrow MSCs into the damaged nerve leads to an increase in the proportion of regenerating fibers with a large diameter compared to the control (damage without MSCs injection). Presumably, this is a consequence of the stimulating effect of MSCs on the growth of the recipient's nerve fibers at an earlier date. The data obtained should be taken into account in studies devoted to the search for new ways to stimulate nerve regeneration.