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Changes in the Thickness of Rat Nerve Sheaths after Single Subperineural Administration of Rat Bone Marrow Mesenchymal Stem Cells

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The sheaths of the damaged peripheral nerve of Wistar-Kyoto rats were studied after single subperineural administration of bromodeoxyuridine (BrdU)-labeled bone marrow mesenchymal stem cells (MSC) from the same rats. The sciatic nerve was damaged by ligation for 40 sec directly before MSC administration. $BrdU^+$ MSC were identified in the recipient nerve within 1 week after transplantation and were detected not only in the endoneurium, but also in the epineurium and perineurium. It was found that single administration of MSC into the damaged nerve trunk led to an almost 2-fold increase in the thickness of its sheaths (perineurium and epineurium) in comparison with the control group (ligation). It can be hypothesized that MSC induce thickening of nerve sheaths through the production of factors that stimulate angiogenesis and adipogenesis.

Key Words: *bone marrow MSC; rat nerve; epineurium; perineurium; cell technologies*

Much efforts are now focused on the search for new methods to stimulate the regeneration of damaged peripheral nerves using cell and gene therapy [3-6,24]. Mesenchymal stem cells (MSC) derived from various sources are widely used in modern experimental regenerative medicine [1,2,13,18,25]. MSC are also used in the cell therapy of damaged nerve [16,17,19]. Most studies in this field are focused on nerve fiber regeneration, while nerve sheaths of the recipient received little attention. However, the epineurium and perineurium perform several essential functions, in particular, mechanical protection of the nerve fibers. Their structural particular qualities allow controlling the local environment of nerve fibers and modulate physical stresses [27]. Perineural cells and vascular endothelial cells are involved in the formation of the blood—nerve barrier. In addition, the sheath cells (fibroblasts, perineural cells, endothelial cells, pericytes, and vascular smooth muscle cells) are involved in the processes of reparative regeneration of nerves and can affect the regeneration of damaged nerve fibers [15].

The study was designed to examine the nerve sheaths after single sub-perineural injection of MSC in the damaged sciatic nerve in rats.

MATERIALS AND METHODS

Bone marrow MSC of Wistar-Kyoto rats were provided by Trans-Technologies Company (St. Petersburg). The method of isolation and characteristics of MSC are previously described in detail [2]. The cells were cultured for 1 week in culture flasks (NEST Scientific) and αMEM culture medium (BioloT). Three days before the end of culturing, a thymidine analogue bromodeoxyuridine (BrdU) (Sigma) was added to the culture medium for labeling maximum number of MSC. The cell suspension was washed twice with BrdU-free culture medium and centrifuged for 15 min at 200*g*. The precipitate was resuspended in 1 ml fresh culture medium, cell viability was checked using 0.2% trypan blue (BioloT) by counting cells in the Goryaev's

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chamber. For transplantation in the nerve, a suspension containing at least 90% viable MSC was used. Smears were also prepared from the suspension, fixed in ethanol for 40 min, and immunohistochemical reaction for BrdU was performed.

The experiment was performed on Wistar-Kyoto male rats weighing 200-250 g (n=32). All experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental or Other Scientific Purposes. The study was approved by the Local Ethics Committee (Protocol No. 3/17, November 30, 2017).

The sciatic nerves at the level of the upper third of the thigh were damaged by ligation for 40 sec [20,21] under ether anesthesia. The cell suspension (5×10^4 in 5 µl medium) was injected subperineurally into the largest nerve trunk of the sciatic nerve using a thin glass cannula under a microscope. Control animals received injection of 5 µl culture medium. After the surgery, the animals were kept under standard vivarium conditions and were euthanized with ether overdose at different terms after surgery. For detection of transplanted cells, the material was fixed in 1 (n=4) and 7 days (n=4) after surgery. For measuring sheath thickness, the samples from the control (n=10) and experimental (n=10) groups were fixed in 21-30 days. The sciatic nerves of intact rats (n=4) were also studied.

The nerve segments were fixed in a zinc-ethanol-formaldehyde solution [10] and after appropriate processing were embedded in paraffin. Longitudinal and cross-sections (5 µm) were prepared (Rotary 3003 PFM microtome). MSC were studied on longitudinal sections 1 and 7 days after surgery. In the control and experimental groups, quantitative analysis of the thickness of the nerve sheath and the blood vessel density was carried out on cross-sections through the distal nerve segment.

To describe cultured MSC, astra-blue staining that allows characterizing cell nuclei and their shape and immunohistochemical (IHC) reaction for vimentin, an intermediate filament protein specific for MSC were used. The IHC reaction was conducted on MSC cultures directly in culture flasks after their fixation in zinc-ethanol-formaldehyde solution. To detect vimentin, mouse monoclonal anti-vimentin antibodies (clone V-9; Dako) were used at a dilution of 1:100. For identification of transplanted cells, monoclonal antibodies to BrdU (clone Bu20a; Dako) were used at a dilution of 1:100. Reagents from the Polymer_HRP AntiMouse EnVision+ System kit-(Dako) were used as secondary antibodies. Some sections were stained with toluidine blue and aniline blue. For detection of blood vessels, polyclonal rabbit antibodies to endotheliocyte marker von Willebrand factor (vWF) were used in a dilution of 1:250 (Dako). Reagents from the Reveal Polyvalent HRP/DAB Detection System kit (Spring Bioscience) were used as secondary antibodies.

Histological sections were analyzed using a Leica DM750 light microscope and Leica ICC50 digital camera. LAS EZ (Leica) software was used for image processing. Quantitative assessment of the studied characteristics of the recipient nerve (cross-sectional area of the nerve, nerve tissue (area occupied by the nerve trunks), and external nerve sheaths) were quantified as described previously [23]. The area of these structures and the density of blood vessels in the epineurium were measured using ImageJ software. The area was measured using 40× objective on nerve cross-section stained with toluidine blue. The number of blood vessels was counted on images of histological sections with IHC reaction for vWF without counterstaining using 40× objective, followed by calculation per unit area (1 mm^2).

Statistical processing of the results was carried out using Microsoft Excel software. The data are presented as $M\pm SE$. Significance of differences was evaluated using Student's t test at $p<0.05$.

RESULTS

The bone marrow MSC from Wistar-Kyoto rats used for transplantation are adherent cells and can be studied directly in culture flasks. MSC had a fibroblast-like shape with processes, large volume of cytoplasm, and round light nuclei. Vimentin intermediate filaments specific for MSC were detected in their cytoplasm (Fig. 1, *a*). Examination of smears prepared from MSC suspension before transplantation showed that most MSC were $BrdU^+$ (Fig. 1, *b*, *c*). One day after surgery, dense clusters of exogenous BrdU-labeled MSC were observed in the nerve trunks of the recipient rats (Fig. 2, *a*).

Seven days after, individual $BrdU^+$ MSC were detected both in the endoneurium of the nerve trunks (Fig. 2, *b*) and in the external nerve sheaths: perineurium and epineurium (Fig. 2, *c*). MSC can penetrate into the sheaths of the ligated recipient nerve only due to a breach of the blood—nerve barrier. The blood—nerve barrier is broken after an injury caused by ligation and cell transplantation. The presence of transplanted MSC in the recipient nerve sheaths suggested that they can affect the thickness and structural changes in the epineurium and perineurium after damage.

In this study, the thickness of the total external nerve sheaths (epineurium+perineurium) was measured, because the boundary between these sheaths is difficult to determine at the light-optical level. In intact sciatic nerve, the epineurium was formed by loose conjunctive tissue with all its components: collagen fibers, blood vessels, adipose tissue islands, fibroblasts,

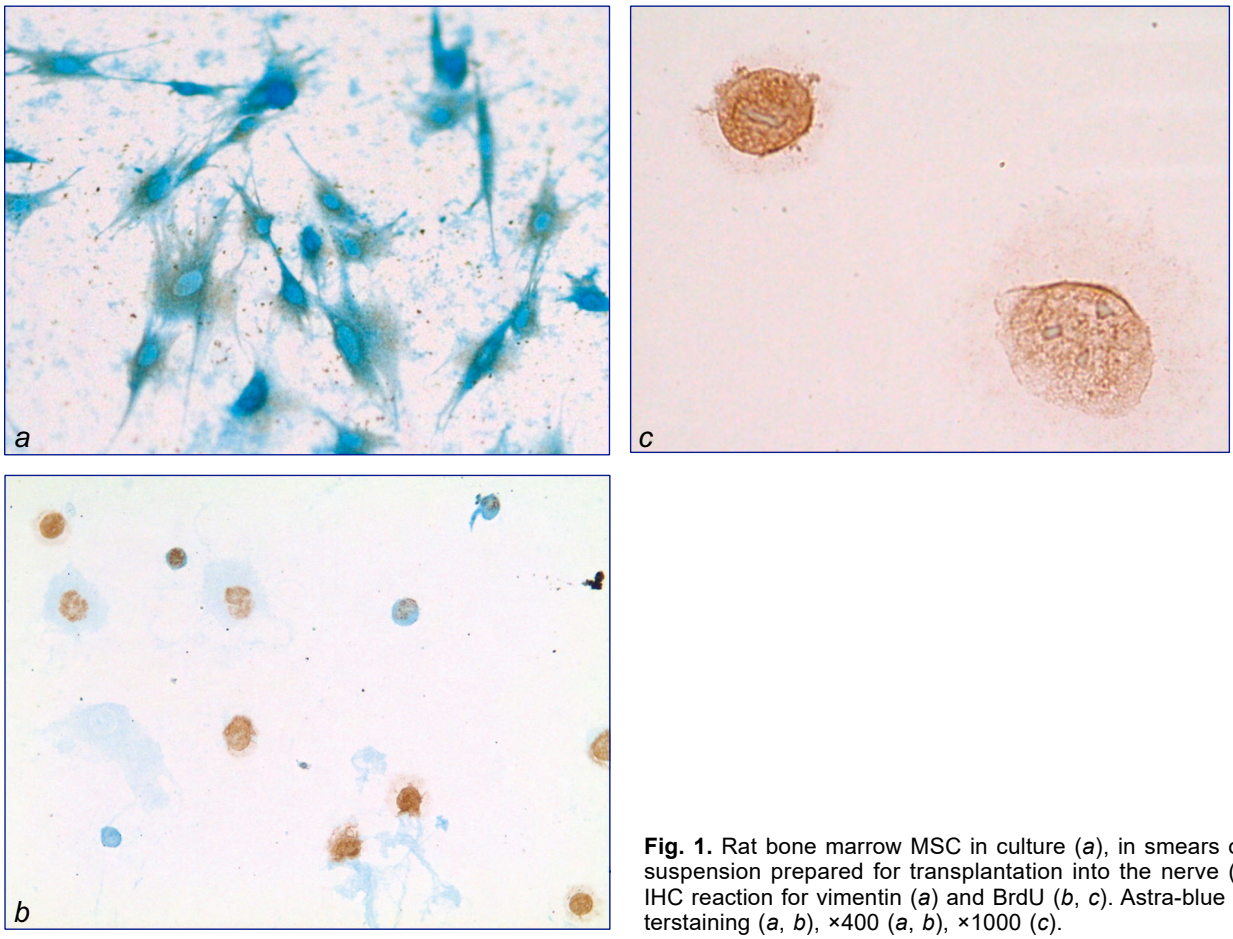

Fig. 1. Rat bone marrow MSC in culture (*a*), in smears of cell suspension prepared for transplantation into the nerve (*b*, *c*). IHC reaction for vimentin (*a*) and BrdU (*b*, *c*). Astra-blue counterstaining (*a*, *b*), ×400 (*a*, *b*), ×1000 (*c*).

smooth muscle cells, and mast cells. The part of the perineurium bordering on the endoneurium is presented by several layers of strongly flattened cells connected by tight junctions, while another part is a connecting tissue containing collagen fibers similar to the epineurium fibers. The cross-sectional area of the intact rat sciatic nerve and the area of its epineurium and perineurium were 1.23±0.30 and 0.56±0.07 mm^2, respectively. After damage caused by ligation, the thickness of the nerve sheaths increased by almost 2 times (p<0.05).

The effect of cell therapy on the damaged nerve sheaths was studied on day 21-30. This period was chosen because at earlier terms after surgery, the processes typical of Wallerian degeneration occur in the distal segment of the injured nerve: fragmentation of damaged axons, destruction of their myelin sheaths, macrophage migration, phagocytosis of myelin

TABLE 1. Changes in Structural Elements of Rat Sciatic Nerve after Ligation and Single Subperineural Administration of MSC (n=10; M±SE)

Parameter	Control group (ligation)	Experimental group (ligation and MSC transplantation)
Mean cross-sectional area of the nerve, mm^2	1.89±0.16	3.49±0.31*
Mean number of fascicles in the nerve	3.40±0.16	3.80±0.13
Mean cross-sectional area of the fascicle, mm^2	0.24±0.02	0.25±0.02
Mean cross-sectional area of the nerve tissue, mm^2	0.69±0.06	0.91±0.08
Mean cross-sectional area of the nerve epineurium and perineurium, mm^2	1.27±0.12	2.33±0.32*
Number of vessels in the epineural sheath per 1 mm^2	50.85±5.00	64.50±5.44

Note. *p<0.05.

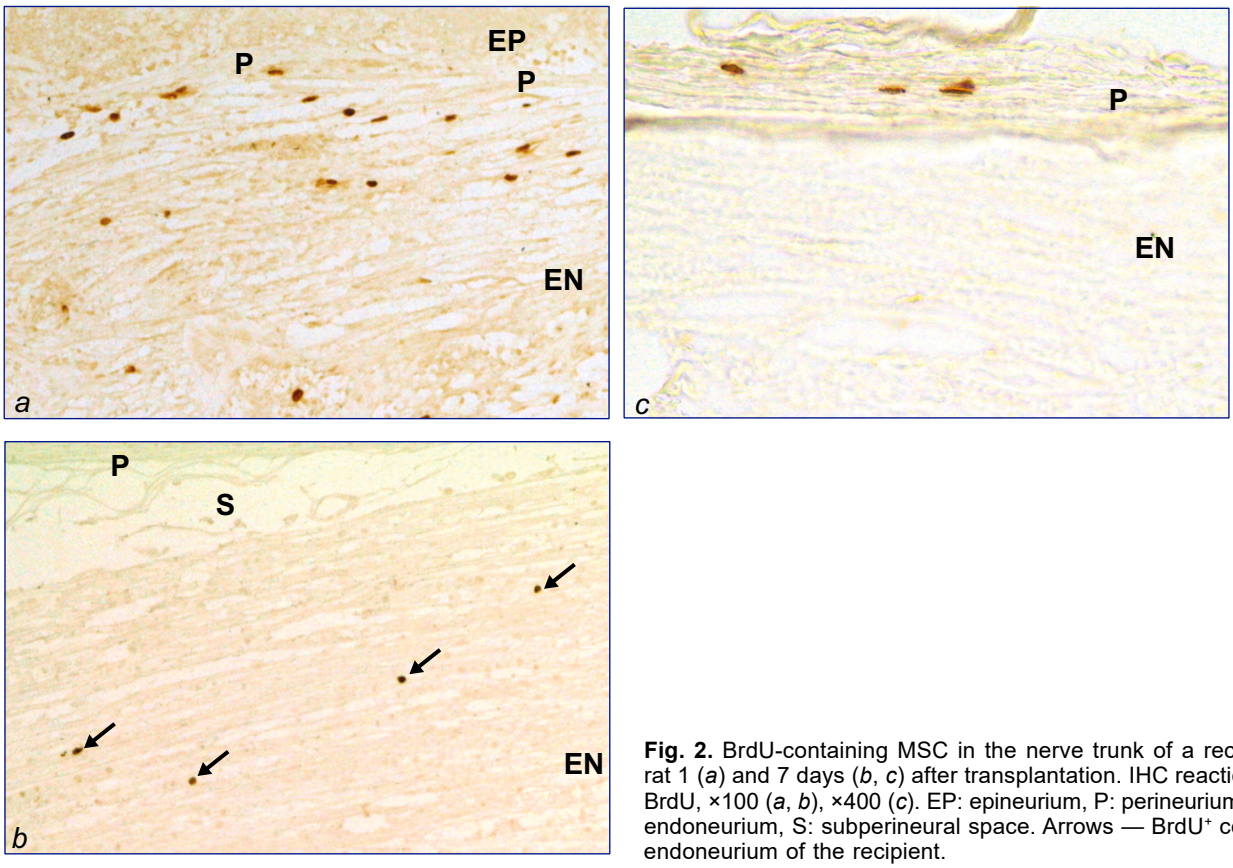

Fig. 2. BrdU-containing MSC in the nerve trunk of a recipient rat 1 (*a*) and 7 days (*b*, *c*) after transplantation. IHC reaction for BrdU, ×100 (*a*, *b*), ×400 (*c*). EP: epineurium, P: perineurium, EN: endoneurium, S: subperineural space. Arrows — $BrdU^+$ cells in endoneurium of the recipient.

breakdown products. There is also a reaction to trauma in nerve sheaths: vasodilation, tissue swelling, inflammatory cell infiltration. In 21-30 days, the reaction to injury becomes less pronounced, which allows studying the effect of transplanted MSC on the thickness of nerve sheaths (Fig. 3, *a*).

Histological analysis showed that in 21-30 days after surgery, the thickness of the sciatic nerve in animals receiving injection of MSC suspension after ligation increased in comparison with the control group (Table 1). The evaluation of the cross-sectional area of the nerve and its structures showed that the number of fascicles and the area of nerve tissue did not significantly differ in the control and experimental groups. However, the area of the external nerve sheaths (epineurium and perineurium) in the experimental group increased by almost 2 times (Table 1).

The study of the vascularization of the sciatic nerve sheaths using the endothelial cells marker vWF (Fig. 3, *b*) showed that the density of blood vessels per unit area of the epineurium in control and experimental rats did not differ (Table 1). Thus, the density of blood vessels did not decrease with increasing nerve sheath thickness due to the MSC administration.

The cell therapy affects the damaged tissues via two mechanisms: replacement of degenerated structures and paracrine induction [9]. In our case, the effect of exogenous MSC on the recipient nerve sheaths presumably follows the second way. This is evidenced by the fact that, despite previously established possibility of differentiation of some transplanted MSC into adipocytes and perineurial cells [21], the survival time of exogenous cells is short. A large portion of transplanted cells degenerates soon after surgery. According to the data obtained on different experimental models, the death of exogenous MSC can occur through autophagy and apoptosis [29].

Currently, much attention is focuses on such intercellular interactions as paracrine signaling, secretion of extracellular vesicles [8,30], and formation of tunneling nanotubes enabling exchange of cellular organelles (in particular mitochondria) between exogenous cells and recipient cells [18]. All these interactions significantly affect the behavior of endogenous cells of the recipient and suggest that the short period when MSC remain viable after transplantation into organs and tissues of the recipient is sufficient to stimulate reparative processes. In our opinion, the thickness of the recipient nerve sheaths and their structures (loose conjunctive tissue, adipose tissue, and blood vessels) increases under the effect of growth factors and cytokines synthesized by MSC [13]. It is known that bFGF, TGF-β, IGF-1, VEGF, and other bioactive substances produced by MSC can affect endogenous stem

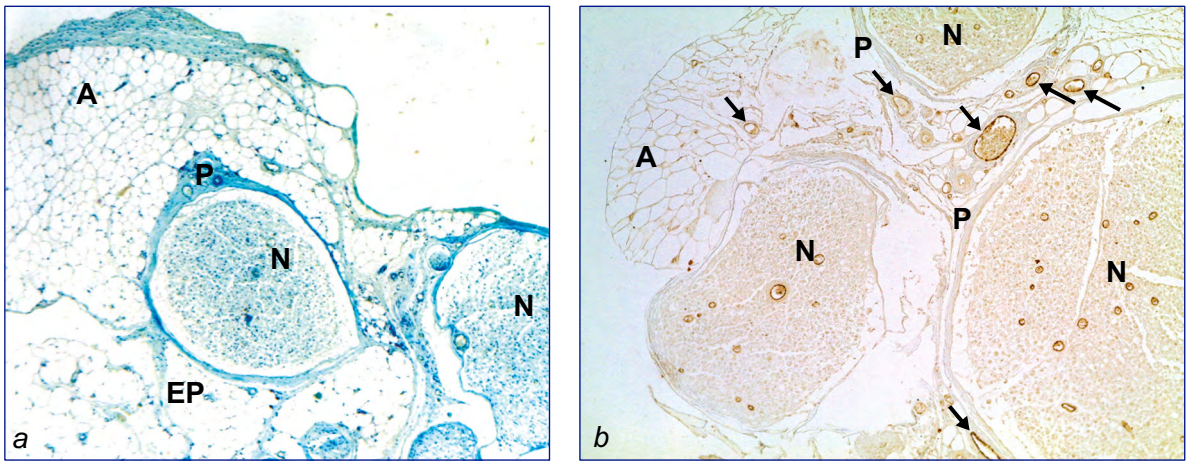

Fig. 3. Cross-section of the rat sciatic nerve 21 days after ligation and injection of MSC. Toluidine blue staining (*a*); IHC reaction for vWF (*b*), ×400. A: adipose tissue in the epineurium, P: perineurium, N: nerve bundles, EP: epineurium. Arrows show blood vessels in the epineurium.

cells in tissues and organs and stimulate angiogenesis [11,12] and adipogenesis [7,14,22,26,28]. For more accurate understanding of the molecular and cellular mechanisms of the influence of transplanted cells on the tissues of the nerve sheaths, cell reactions in the recipient nerve at the early stages after damage and transplantation of MSC should be studied.

Thus, single transplantation of rat bone marrow MSC into a damaged nerve affects the thickness of its external sheaths. On days 21-30 after injury and cell therapy, their thickness increases by almost 2 times in comparison with the control (ligation). MSC presumably affect the growth of the connective tissue of nerve sheaths through production angiogenic and growth factors, which should be taken into account when stimulating peripheral nerve regeneration using cell therapy. Currently, the significance of the detected thickening of the nerve sheaths for its regeneration remains unclear. Further research should contribute to understanding the biological significance of the established fact.

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