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V.O. Piatykop, N.I. Zavgorodnia, V.Yu. Kaliuzhka, E.A. Shchegelskaya, O.O. Geletka, M.A. Markevych
Kharkiv National Medical University, Kharkiv

NEUROINDUCED MESENCHYMAL STEM CELLS EFFICIENCY FOR RECONSTRUCTION OF RAT SCIATIC NERVE DEFECTS

e-mail: vladislavkalyuzhka@gmail.com

Peripheral nerves damage is a frequent pathology with significant socio–economic significance. The aim of research was to study regeneration potential of transplanted biodegradable fibrin matrices filled with neuroinduced mesenchymal stem cells for peripheral nerves damage in rats and evaluate degree of anatomical and functional integrity. The study was carried out on 48 mongrel female rats aged 3–4 months (250±50 g) which were divided into 4 groups. Sciatic nerves of all rats were intersected with formation of 10 mm gap and then reconstituted using autografts, acellular fibrin matrix and fibrin matrix with neuroinduced mesenchymal stem cells. Functional, electrophysiological tests and histological evaluation were performed to analyze functional and anatomical recovery during 2 months after injury. It has been shown that using neuroinduced mesenchymal stem cells for restoration of large size gap and recovery of function of peripheral nerve after its damage is effective method according to functional, electrophysiological, histological results.

Key words: mesenchymal stem cells, sciatic nerve, fibrin matrix.

В.О. П'ятикоп, Н.І. Завгородня, В.Ю. Калюжка, О.А. Щегельска, О.О. Гелетка, М.А. Маркевич

ЕФЕКТИВНІСТЬ НЕЙРОІНДУКОВАНИХ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН ДЛЯ РЕКОНСТРУКЦІЇ ДЕФЕКТІВ СІДНИЧНОГО НЕРВУ ЩУРІВ

Пошкодження периферичних нервів є частою патологією, що має велике соціально–економічне значення. Метою дослідження було вивчення регенеративного потенціалу трансплантованих біодеградуємих фібрінових матриксов, заповнених нейроіндукованими мезенхімальними стовбуровими клітинами, для відновлення пошкоджених периферичних нервів у щурів і оцінки ступеня анатомічної та функціональної цілісності. Дослідження проводилося на 48 самках щурів лінії WAG у віці 3–4 місяців (250±50 г), які були розділені на 4 групи. Сідничні нерви всіх щурів перетинали з утворенням дефекту розміром 10 мм і потім відновлювали з використанням аутотрансплантатів, безклітинних фібрінових матриць і фібринових матриць заповнених нейроіндукованими мезенхімальними стовбуровими клітинами. Функціональні, електрофізіологічні тести та гістологічна оцінка проводилися для аналізу функціонального і анатомічного відновлення протягом 2 місяців. Було показано, що використання нейроіндукованих мезенхімальних стовбурових клітин для відновлення функції периферичного нерва після його пошкодження з утворенням дефекту великого розміру є ефективним методом за даними функціональних, електрофізіологічних, гістологічних результатів.

Ключові слова: мезенхімальні стовбурові клітини, сідничний нерв, фібринові матриці.

Damages of peripheral nervous system are pathology with a spread of 13 to 23 cases per 100 people in a year [4]. Injury of peripheral nerves after different musculoskeletal system traumas can be cause partial or complete loss of limb function. Due to increasing of technological progress and frequency of technogenic injuries number of victims are increasing annually Rapid urbanization leads to increasing of neurotraumatism on average by 2 % per year [2]. In Ukraine the number of injured people is 2,5–3,000 every year, 60–75 % of them are disabled [13]. There are different anatomical forms from nerve compression to complete nerve transection. Trauma can affect different nerves causing respective regional paralysis. Diseases patterns are characterized by reflexes changes, muscle weakness, numbness, severe pain, motor dysfunction and prolonged disability. Based on this functional recovery has paramount importance in the treatment of peripheral nerves injuries [7].

In recent years, fundamentally new opportunities are being developed in the treatment of compression–ischemic lesions of nerve trunks mainly through the improvement of surgical techniques [2]. In most cases, trauma of nerves is accompanied by damage of blood vessels, bones and soft tissues. Isolated nerve trunk injury is very rare condition [8]. Operative techniques for peripheral nerve restoration (neurolysis, autoneuroplasty, neurotization) don`t achieve the desired results especially in large nerve defects repair despite the rapid development of microsurgical technology [8, 15]. Peripheral nervous system (PNS) injuries require 200,000 operations in the United States and 300,000 in Europe each year [8].

Nowadays development of cellular technologies and tissue engineering methods are underway to give new prospects for effective technique for the restoration of peripheral nerves. For this purpose scientists use new biopolymer materials, stem cells and tissue engineering structures [1–3, 8, 11, 14].

The purpose of the work was to study regenerative potential of transplanted biodegradable fibrin matrices filled with neuroinduced mesenchymal stem for peripheral nerves damage in rats and evaluate degree of anatomical and functional integrity.

Materials and methods. Research was carried out on 48 mongrel female rats aged 3–4 months weighing 250±50 g, which were kept in the standard conditions of the Kharkiv national medical university experimental biological clinic. Experimental animals were sacrificed in strict compliance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes"; (Strasbourg, 1986), as well as with the "General Ethical Principles of Animal Experiments" adopted by the First National Congress on Bioethics (Kyiv, 2001).

To obtain MSC culture of rats, bone marrow was flushed from the tibia, washed twice in Hanks' solution, centrifuged at 430g for 10 minutes. Cells were sowed in ratio of 50 million per culture flask (75 cm^2, Nunc) and cultured in DMEM/F12 medium (1/1) with 10 % FBS (fetal bovine serum) in a CO_2 incubator. After 24 hours the culture medium with non–adherent cells to the substrate was removed, fresh culture medium was added and the remaining fibroblasts-like MSCs had been cultivated for the next 14 days, changing the medium every three days [10]. After formation of the monolayer, MSC were removed from the bottom of the vials and resuspended in Hanks solution to the required concentration. All reagents, serums and media came from SIGMA–ALDRICH (USA).

Culture medium with 2 % fetal bovine serum and a solution of retinoic acid (10^{-6}M) were used for neuroinduction of bone marrow MSC of rats. MSCs were labeled with the Rhod Chol red fluorescent dye (λ_{em}=580 nm), which we used earlier for vital staining of cells [9].

For performing experiment model of sciatic nerve injury was chosen. Surgery was performed under general anesthesia (intraperitoneal administration of xylazine Sedazin, "Biowet", Poland, 15 mg/kg and ketamine – "Farmak", Ukraine, 70 mg/kg body weight).

The animal was fixed on the operating table in the mid–physiological position [14]. Left sciatic nerve (SN) was picked up, mobilized and cut. Surgical wound was closed with one row of nodular sutures. Nerve transection and repair was done on the left limb, and the contralateral limb served as a non–transected nerve control.

After it all animals were divided into 4 groups of 12 individuals each. Animals of the first group were observed without subsequent repair. After 3 days of observation 3 other groups sciatic nerves were restored with different methods.

The recovery results of the sciatic nerve were evaluated by neurological tests, which were performed 10, 20, 30, 40, 50, 60 days after the operation. To control the functional dynamics we used "Walking track analysis" by Johnston et al [12, 15]. The data were calculated using the Bain–Mackinnon–Hunter formula and then functional index of the sciatic nerve (SFI–sciatic functional index) was evaluated [12].

Electrophysiological tests were performed to evaluate reinnervation of the target muscles every 20 days up to 60 days postoperation (dpo). Sciatic nerve was stimulated percutaneously. The compound muscle action potential (CMAP) of the tibialis anterior and gastrocnemius muscles was recorded. The amplitude and the latency of the M–wave were measured [13, 15].

The animals were sacrificed from the experiment after 60 days from the operation as a result of an thiopental anesthesia overdose. The tissues samples from the site of different operations were stained with hematoxylin-eosin for a general cytomorphological evaluation of the tissues.

Statistical processing of the study results was carried out using the Statistica 6.0 software (StatSoft, USA). The average values were presented as (M±m), where M is the average value, m is the standard error of the average value. The statistical significance of the difference between the data of the right and left limbs within each group was assessed by Wilcoxon signed-rank test, homolateral limbs within each group at different observation times, and between different groups at the same observation time by the Mann-Whitney U-test. The difference was considered statistically significant at $p<0.05$.

Results of the study and their discussion. Nerve trunk integrity of the second group was restored by epineural end–to–end neurorrhaphy (atraumatic needle with a monofilament nylon thread No. 10/0 "Golnit", Ukraine) under operating microscope magnification (mag. ×12) (fig. 4) Nerves integrity of the third group restored with fibrin cell–free matrix. In animals of the fourth group the integrity of the nerve trunk was restored with fibrin matrices filled by neuroinduced mesenchymal bone marrow stem cells of rats. Ends of the nerve trunks were connected with biomatrices by fibrin glue which was prepared 15 minutes before application in experiments 3 and 4.

After neurological tests results evaluation of animals, it was found that a total anatomical rupture (8–10 mm) of SN without treatment in animals of the 1st group leads to a persistent neurologic deficit that has been persisting for 60 days of observation (SFI=90) in all animals. At the other group we have noticed partical recovery of limb function. In the second experimental group partial restoration of SN function began on the 10th day of evaluation and increased to SFI=27 on 60th day after operative reconstruction of

nerve trunk. In the 3rd group of animals with a transplanted acellular fibrin matrix, the partial restoration of SN function (SFI=48) occurred in animals after 40 days and remained stable on 60th day after the operation.

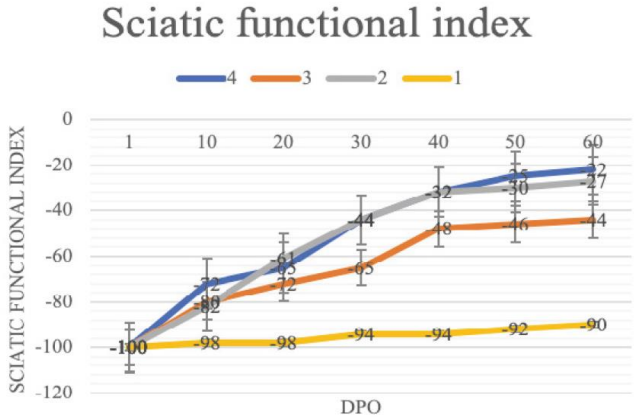

Fig. 1 Sciatic functional index of all groups in comparison.

In the 4th experimental group, partial restoration of SN function was started in 80 % of the animals on the 3rd day of observation, stably increased on the 20th, 40th and 60th days after the operation and reached values (SFI=22), comparable with the results in the 2nd group. The results of all groups are graphically displayed in comparison. (fig. 1).

The results of neurophysiological testing for all groups 8 weeks after surgery are shown in Fig 2. The results of electroneuromyography after 1 week of sciatic nerve injury demonstrated complete denervation of the muscles. Significant differences for the tibialis anterior and gastrocnemius CMAP ($p<0.0001$) were observed at the final examination after 60 days of observation between group with nMSC (15 2mV) versus the groups of animals that received acellular matrixes and surgical reconstruction (10 2mV). (fig. 2A, 2B)

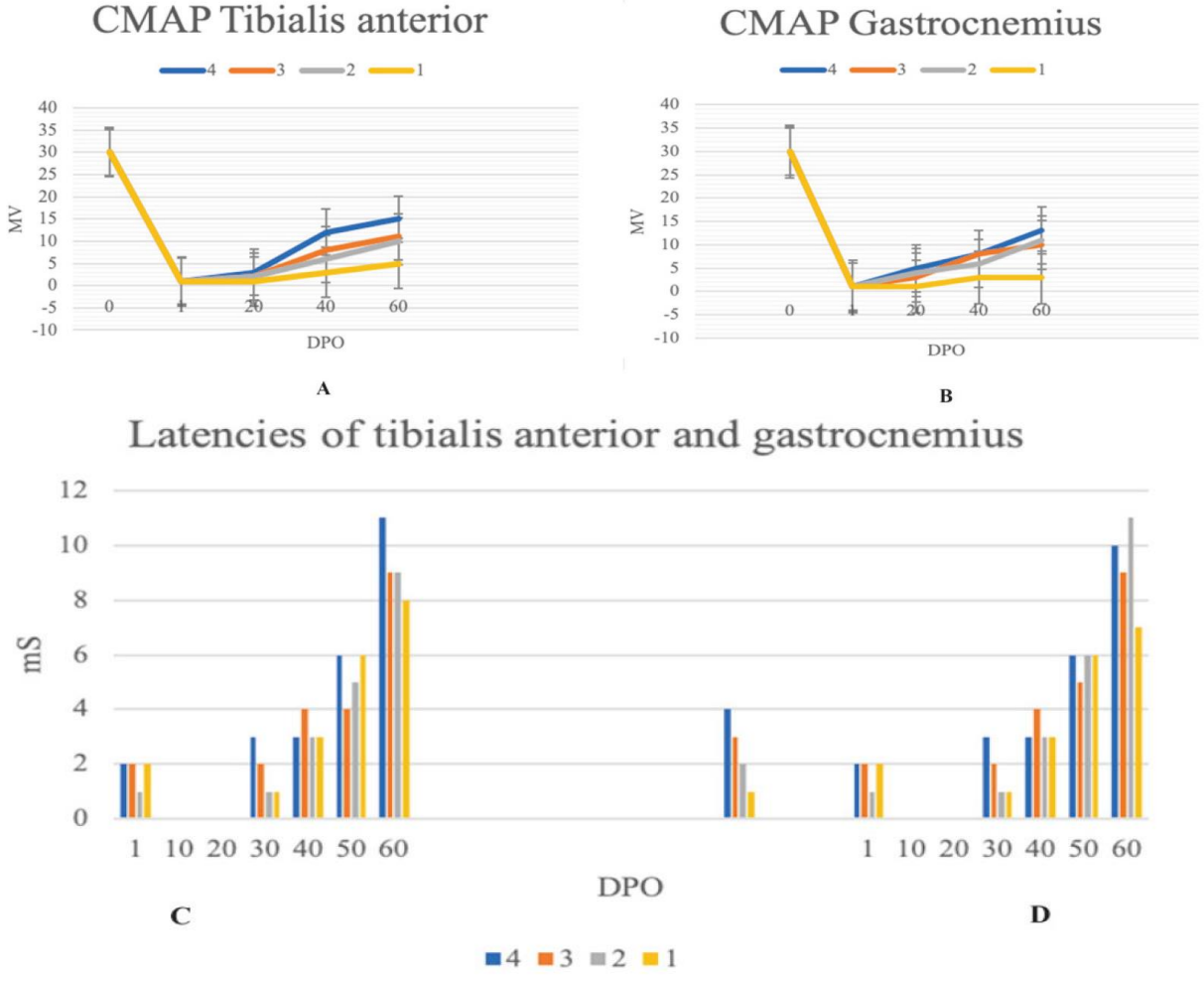

Fig. 2 Amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A), gastrocnemius (B), lantecy of the compound muscle action potential of tibialis anterior (C), gastrocnemius (D)

Latencies of CMAPs were longer than normal at the first stages of reinnervation and tended to shorten with time toward normal values. At the end of the postoperative observation period, the latencies of the CMAP recorded on tibialis anterior and gastrocnemius muscles were similar between groups. Results are shown in comparison between groups graphically (fig. 2C, 2D).

The tissue of the excised area of intact n.ischiadicus is represented by well-defined fibers alternating with glial and fibrocyte cells lying along the fibers. Eosinophilic fibers (axial cylinders) are in

places surrounded by an optically transparent layer in the form of local lesions of irregular shape, which can be considered as myelin sheath. Outside the capillary, cells with round nuclei, possibly monocytes, migrating into nerve tissue are detected.

Analysis of histological tissue samples from the trauma zone showed that in 1st group with complete anatomical rupture of SN spontaneous recovery of nerve fibers does not occur and connective tissue is formed at the site of SN disruption. On the tissue samples of the 2nd group there were alternating portions of connective tissue scar with portions of nerve fibers. Apparently, it can explain the partial restoration of SN function in many animals from this group. In most animals of 3rd group the formation of a large scar at the site of the transplanted fibrin matrix was observed. And only in 3 animals from this group among the fibroblasts clusters thin nerve fibers were found.

On the 60th day after transplantation of a biomatrix with neuroinduced MSC into the excision zone of n.ischiadicus, it is possible to see the formation of a cylindrical shape from parallelly arranged bundles of eosinophilic fibers with a large number of cells having a longitudinally extended nucleus. We can note the abundance of macrophages, as well as the presence of a very large number of vessels of the microvasculature, which is characteristic of intensively regenerating tissue. Interestingly, nerve regeneration along the transplanted matrix with MSC occurs at different rates. After 4 weeks, the initial stage of nMSC differentiation into cells with the Schwann-cell phenotype, which are still immature, was observed. The germination of nerve fibers and the direction of their growth with signs of myelinithation was revealed. After 8 weeks in the tissue-engineering matrix, there was an orderly organization of myelinated nerve fibers and nMSC have been differentiated into Schwann-cells.

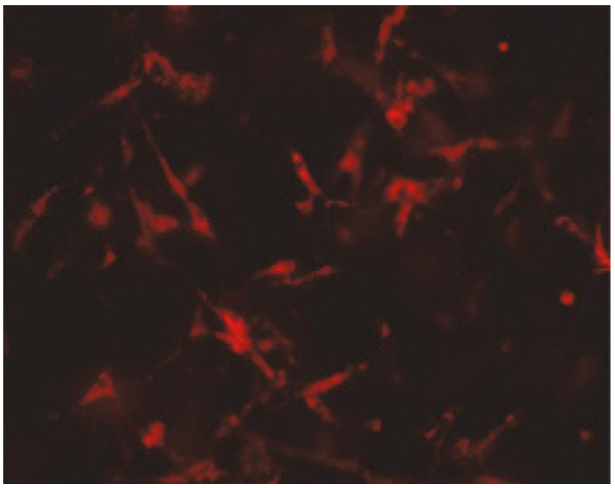

Fig. 3. Red fluorochrome labeled nMSCs in transplanted matrix at 10 day (mag.x200).

Spindle-shaped cells with processes running from one end of SN to the other were found on the specimens of grafts with induced fluorochrome labeled MSC from the trauma zone on the 10 day after the operation (fig. 3).

Peripheral nerve regeneration is a complex process that involves the survival of damaged nerves, remyelination of regenerated axons, reinnervation of the corresponding nerves, and restoration of communication between regenerated axons and target organs.

Although the mechanisms underlying the increased regenerative efficiency of Schwann cells and axons have not been fully elucidated, various possible options are being discussed. Previous studies have reported that endogenous Schwann cells are major factors in peripheral nervous system regeneration when nerve damage occurs, they are extracted from damaged axons, and subsequently show low expression of several molecules, including nerve growth factor, BDNF, and p75 nerve growth factor receptor, which are crucial for axon regeneration. However, it has been shown that transplanted stem cells are able to secrete these factors after transplantation. Moreover, the cut axons have the phenomenon of “die-back”, which is a retraction from the original area by a few millimeters. Transplanted nMSCs can immediately produce neurotrophic factors for regenerating axons to reduce “die-back” phenomenon and direct the proximal nerve stump to improve regeneration. In addition, nMSC can prevent neuroinflammation and increase levels of anti-inflammatory cytokines (such as interleukin 10) and neurotrophic factors (such as BDNF and glial cell line-derived neurotrophic factor), which is beneficial for nerve regeneration. Thus, nMSC can improve the microenvironment, support the survival of Schwann cells and axons, and promote sciatic nerve regeneration.

Based on the results of functional tests, it can be concluded that the normal act of walking is restored more quickly. The obtained results do not contradict previously published works on the effect of stem cells on nerve restoration, but the use of neuroinduced mesenchymal bone marrow stem cells in a collagen matrix has a number of advantages over previously used techniques for repairing a damaged nerve and correletes with results of other reports [3, 8, 14]. nMSCs have a positive effect on the regeneration of PN in the experiment, which is confirmed by significantly better nerve recovery according to key ENMG parameters. nMSC ensure the recovery of more axons, and therefore the neuro-muscular connections and fiber. The tissue-engineering approach in PN defect plastics using fibrin matrix and nMSC provides a result not worse than the “gold standard” −autoneuroplasty [5]. Implanted nMSC as part of a tissue-engineered

matrix have a stimulating effect on nerve regeneration, mainly due to their differentiation into cells with Schwann-cell phenotype, which are able to myelinate nerve fibers. Therefore, the efficiency of restoring potential of transplanted fibrin matrices with nMSC in our work can be related not only with neuronal cells differentiating in the matrix and to their transformation into Schwann cells [11]. In addition, growth factors and chemokines which are produced by stem cells have a regenerative and anti-inflammatory effects. Collagen matrices may be a substrate for the growth of nerve fibers from one end of SN to the other in some cases [2]. The strategy used to improve the restoration of limb function in peripheral nerve damage with MSCs is attractive, technically feasible and promising. In our study, MSC therapy improved functional limb repair in peripheral nerve damage. Research to identify new approaches, such as stem cell therapy, is expected to have a significant impact on the clinical outcome of sciatic nerve injuries and limb transplantation. Our results allow a promising method of clinical recovery for peripheral nerves damage.

Conclusions

1. We confirm that using neuroinduced mesenchymal stem cells for restoration of large size gap and recovery of function of sciatic nerve in rats after its damage is effective method according to results of neurological, electrophysiological tests and histological examination.

2. Neuroinduced mesenchymal stem cells has been shown regenerative potential for restoration of sciatic nerves by differentiation into Schwann cell, formation of nerve fibers and producing biologically active substances that develops microenvironment. All these factors improve physiological growth of nervous tissue and formation of new nerve trunk.

3. Development of new tissue engineering techniques such as transplantation of mesenchymal stem cell is expected to improve regeneration of peripheral and central nervous system after traumatic injuries, strokes, and oncology and make an impact in the clinical outcome. In addition, we suggest that combination of nMSC with other tissue engineering methods may have be more effective for neural restoration than isolative MSC using.

4. However, additional evidence is needed to further characterize nMSC mechanisms of action for the neural repairing possibilities.

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