

The effects of adipose-derived multipotent mesenchymal stromal cells transplantation on locomotor activity and function of the sciatic nerve in mice with peripheral neuropathy



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ABSTRACT

The Charcot-Marie-Tooth disease type 1A (CMT1A) is one of the most common hereditary motor-sensory peripheral neuropathies, which is caused by demyelination of motor and sensory nerve fibers and leads to nerve dysfunction. There are currently no effective treatments for hereditary neuropathies, but recent studies indicate a number of potentially effective therapeutic agents, including multipotent mesenchymal stromal cells (MMSCs).

The **PURPOSE** of the study was to evaluate the effect of adipose-derived MMSCs (ADSCs) transplantation on locomotor activity and sciatic nerve function of transgenic mice with peripheral neuropathy.

MATERIALS AND METHODS. The transgenic B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy were injected intramuscularly with MMSCs, which were isolated from the adipose tissue of FVB-Cg-Tg(GFP) mice transgenic by GFP. Locomotor activity of experimental animals was investigated in dynamics in 2, 4, 6, 8 and 10 weeks using the behavioral balance beam test. The functions of the sciatic nerve were analyzed according to the footprint test by calculating the sciatic functional index (SFI).

RESULTS. For 2-10 weeks in animals with neuropathy, disease progression was observed, which was expressed in a growing increase in the number of hindlimb paw slips from the beam and the latency to cross the beam. SFI in animals of this group decreased and at the 10th week it was -47.0 ± 2 units.

In contrast, from the 2nd week of the experiment, mice with neuropathy after ADSCs transplantation performed 20 % fewer paw slips and spent 11 % less time in the balance beam test compared to animals without cell transplantation. In the same period, there was an increase in the SFI up to -30.2 ± 2 against -34.6 ± 0.9 , respectively. On the 10th week after ADSCs injection, the SFI value was -10.1 ± 2.3 units and correlated with a decrease in the number of foot slips and the time to cross the beam.

CONCLUSION. ADSCs transplantation improves the sciatic functional index and fine locomotor skills in mice with peripheral neuropathy. ADSCs have the potential to be an effective therapeutic agent in the treatment of peripheral neuropathy at Charcot-Marie-Tooth disease type 1A.

KEY WORDS: adipose-derived multipotent mesenchymal stromal cells; demyelination; peripheral neuropathy; sciatic functional index.

Charcot-Marie-Tooth disease type 1A (CMT1A) is one of the most common inherited peripheral neuropathies. Its frequency is from 1:1500 to 1:10000 in different populations [1]. This pathology is caused by the processes of demyelination of motor and sensory nerve fibers, which are accompanied by atrophy of their axons that leads to nerve dysfunction [1]. The symptoms of CMT1A usually appear in the first twenty years of life in the form of symmetrical muscle weakness, rapid fatigue during exercise and decreased sensitivity of the distal parts of the limbs. In this case, the lower limbs are mainly affected more than the upper ones [2].

The cause of CMT1A is the duplication of a fragment of chromosome 17p.11.2 that leads to the appearance of the third allele of the gene of peripheral myelin protein 22 kDa (PMP22), which causes its overexpression [2]. PMP22 protein is an integral membrane protein of the tetraspan family, which accounts for 2-5 % of the total number of proteins of the compact myelin layer of the peripheral nervous system [1, 3]. The specific functions of PMP22 have not been established yet, but it is known that PMP22 is involved in maintaining the stability of lipid rafts on Schwann cell membranes and the processes of myelin membrane formation during axonal myelination [3, 4].

The mechanisms of demyelination induced by overexpression of PMP22 are not well established. However, there is a hypothesis that under normal conditions, the expression of PMP22 occurs at levels close to the saturation of the control system of protein folding in the endoplasmic reticulum. Therefore, the introduction of an additional copy of the PMP22 gene overloads the cellular folding systems and, as a consequence, leads to the accumulation of unfolded PMP22 molecules. This leads to stress damage to the endoplasmic reticulum and causes disruption of intracellular vesicular transport and calcium metabolism [4, 5]. This hypothesis is confirmed by the fact that the stimulation of autophagy enhances the degradation of such protein aggregates and improves myelination in mice [5].

To date, there are no effective treatments for peripheral neuropathies caused by CMT1A disease. Recent studies indicate a number of therapeutic agents that can potentially have a positive effect in the treatment of CMT1A disorder [6]. These include purine P2X7 receptor antagonists, antisense oligonucleotides, and various types of multipotent mesenchymal stromal cells (MMSCs), in particular, from bone marrow, adipose tissue and umbilical cord [6-9].

In our study, adipose-derived multipotent mesenchymal stromal cells (ADSCs) were selected as an agent for sciatic nerve regeneration because subcutaneous adipose tissue is a rich source of MMSCs, and the secretome of these cells, including neurotrophin growth factors, promotes axonal growth and survival of Schwann cells [9, 10]. In addition, ADSCs show typical characteristics of MMSCs, such as multipotency, the ability to long-term cultivation and cryopreservation without negative consequences [11].

The **PURPOSE** of the study was to evaluate the effect of ADSCs transplantation on locomotor activity and sciatic nerve function in transgenic mice with peripheral neuropathy.

MATERIALS AND METHODS

All animal experiments were performed in compliance with the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Convention, Strasbourg, 1986), Article 26 of the Law of Ukraine «On Protection of Animals from Cruelty» (No. 3447-IV, 21.02.2006), as well as all norms of bioethics and biosafety. The animals were kept in standard vivarium conditions with free access to food and water *ad libitum*.

The study used 3 groups of mice aged 7 months, both sexes with a body weight of 23-29 g. Group I (control) – healthy C57Bl mice (n = 6). Group II (PN) – transgenic B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy (n = 6), which were obtained on the C57Bl/6J strain (purchased from The Jackson Laboratory, USA). Group III (PN+ADSCs) – B6.Cg-Tg(PMP22)C3Fbas/J mice (n = 10) after the transplantation of ADSCs.

To obtain ADSCs, we used male FVB-C-Tg(GFP)5Nagy/J mice, transgenic for green fluorescent protein (GFP), aged 5 months (n = 8). The mice were kindly provided by the European Molecular Biology Laboratory (Monterotondo, Italy).

Obtaining of MMSCs from adipose tissue. The FVB-C-Tg(GFP)5Nagy/J mice were euthanized by cervical dislocation under the anesthesia with 2.5 % solution of 2,2,2-tribromethanol (avertin at a dose 400 mg/kg). Under sterile conditions, subcutaneous adipose tissue was isolated, minced with scissors into 1x1 mm pieces in DMEM/F12 medium (Sigma, USA) and incubated in 0.1 % solution of collagenase type IA (Sigma, USA) for 90 min at 37 °C and constant stirring on a shaker at 100 rpm. The resulting cell suspension was washed in DMEM medium (Sigma, USA) by centrifugation at 300 xg. The supernatant with mature adipocytes and debris was removed and pellet passed through a sterile EASYstrainer nylon filter with a pore diameter of 100 µm (Greiner bio-one, Austria). Cells of the stromal-vascular fraction were cultured in a CO₂ incubator in humidified air with 5 % CO₂ at a temperature of +37 °C in complete nutrient medium DMEM-LG (Sigma, USA) supplemented with 15 % fetal bovine serum (FBS) (HyClone, USA), penicillin 100 U/mL, streptomycin 100 µg/mL (Sigma-Aldrich, USA), 1:100 nonessential amino acids (Sigma-Aldrich, USA). The nutrient medium was replaced in 3 days. Cells were subcultured to achieve 80 % monolayer confluence using 0.25 % trypsin solution (Sigma, USA) and 0.02 % versene (Bio-Test Laboratory, Ukraine).

On the 2nd passage, immunophenotyping and directed differentiation towards osteogenic and adipogenic lineages of the obtained cultures were performed according to standard methods, as described previously [12]. Cells were analyzed by flow cytometry with BD FACSAria cell sorter (Becton Dickinson, USA) using rat anti-mouse monoclonal antibodies against surface antigens CD44, CD73, CD90, CD105, CD34, and CD45 (BD Bioscience, USA). There was identified high expression of specific mesenchymal markers CD44, CD73, CD90, CD105, and, at the same time, low level of hematopoietic markers CD34, CD45 (Fig. 1 A). At the directed adipogenic differentiation, there were determined lipid droplets in a cytoplasm of cells (Fig. 1 B). At osteogenic differentiation, there was a deposition of calcium salts in an extracellular matrix. Thus, the obtained cultures met the minimal criteria to define MMSCs in terms of morphology, adhesive properties, immunophenotype and potential for directed differentiation [13].

The cells of the 2nd passage were resuspended at a concentration of 1•10⁶ cells/mL in a cryopreservation medium consisting of 90 % FBS and 10 % dimethyl sulfoxide (Sigma, USA), frozen to -80 °C in containers CoolCell® (Corning, USA) and stored in cryostorage with liquid nitrogen at -196 °C.

Transplantation of ADSCs. Prior to transplantation, the cells were thawed in a water bath at 37 °C, washed in 10 mL of DMEM/F12 medium containing 10 % FBS, and resuspended in phosphate buffered saline (PBS) (HyClone, USA). The B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy were transplanted with 0.5•10⁶ GFP-positive ADSCs in 50 µL of PBS intramuscularly in m. gastrocnemius on both sides under intra-abdominal anesthesia (calypsol + xylazine, 75 and 2 mg/kg body weight, respectively). The animals of group II (PN) were injected with 50 µL of PBS without cells intramuscularly in m. gastrocnemius on both sides.

At the start of the experiment and in 2, 4, 6, 8 and 10 weeks, locomotor activity and sciatic nerve function were examined in experimental animals using the balance beam test, the footprint test, and the sciatic functional index (SFI) was calculated as well.

Balance beam test. To perform this test, a special installation was constructed, which consisted of a cylindrical wooden beam with a diameter of 2 cm and a length of 110 cm and was placed at a height of 50-60 cm above the floor at an angle of 10° to the horizontal plate. The beam was connected to a darkened box of 13 x 22 cm, which had an entrance hole of 5 x 6 cm (Fig. 2) [14].

At the lower end of the beam, a white line indicated the starting position of the animal. Another line was drawn 100 cm from the start at the

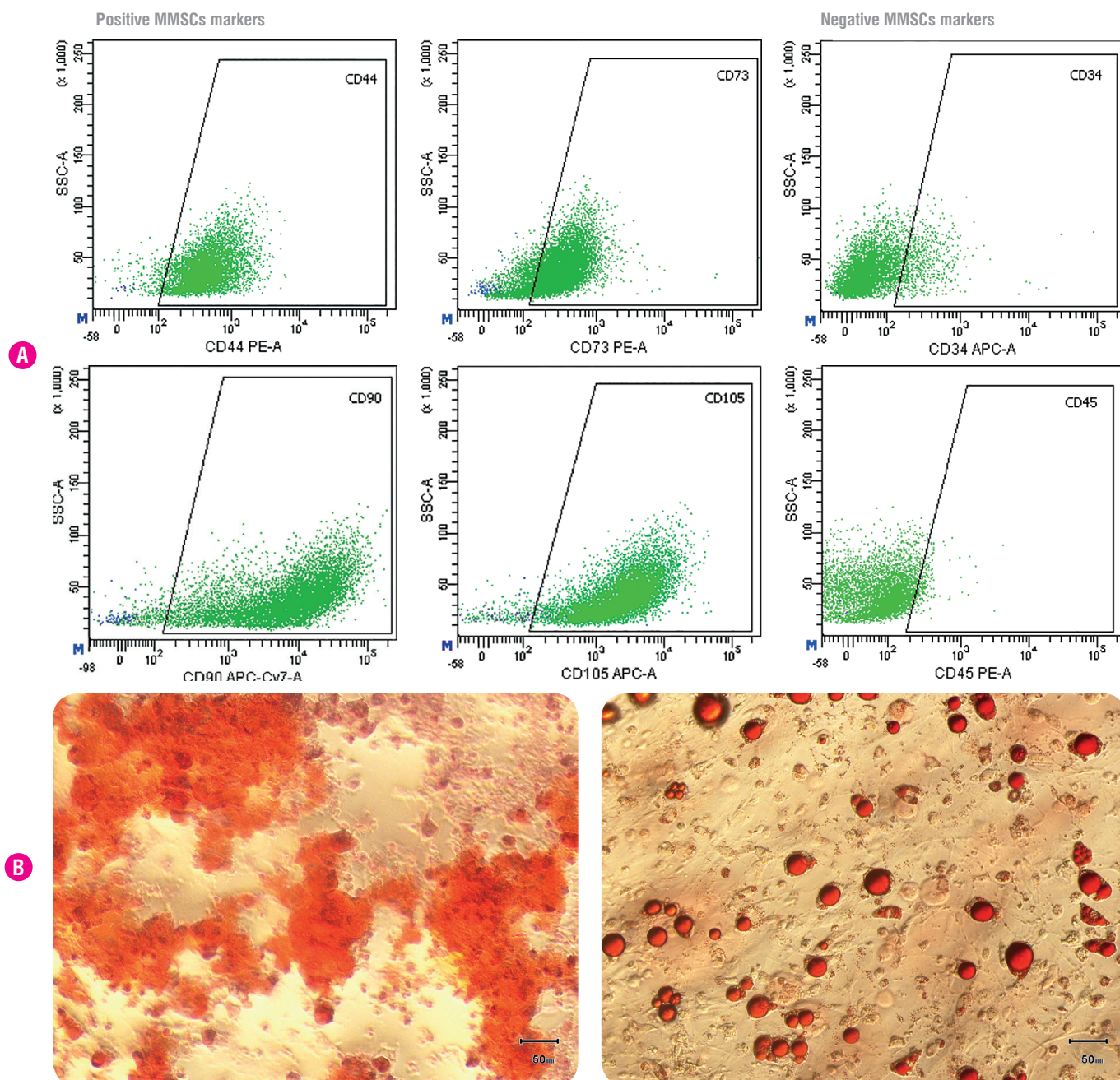


Fig. 1. Confirmation of the minimal MMSCs criteria for murine ADSCs cultures.

A – dot-plot histograms of CD44, CD73, CD90, CD105, CD34 and CD45 markers expression in the culture of murine ADSCs according to flow cytometry, 2nd passage.

B – a photomicrograph of murine ADSCs after directed osteogenic (left) and adipogenic (right) differentiation on the 14th and 21st day of culturing, respectively. Lipid droplets stained with Oil Red O (red), calcium deposits in mineralized extracellular matrix stained with Alizarin Red S (pink); light microscopy, scale – 50 microns.

upper end of the beam at the entrance to the box to show the finish line. In front of the beam, a video camera was placed to record the behavior of the mice at an angle to cover the entire length of the beam and ensure clear visibility of the start and finish lines.

The balance beam test measured the following parameters: the latency to cross the beam from the start to the finish line and the number of hindlimb foot slips during the distance. The test was performed for 3 days: 2 days for training and 1 day of testing. Each animal performed the test 7 times with a 40 s interval to rest.

Footprints test. To perform this test, the animals' hindlimbs were painted with ink and they were let to walk a corridor 60 cm long and 8 cm wide, covered with a white paper tape. In the footprints test, two param-

eters were measured: toe spread (TS) and print length (PL) (**Fig. 3**). Each animal performed the test 3 times, and at least 5 footprints were analyzed from each tape.

Calculation of the sciatic functional index. Sciatic functional index (SFI) was calculated according to the formula by R. Baine with the modification of Inserra et al. [15]:

$$SFI = 118,9 \left(\frac{ETS-NTS}{NTS} \right) - 51,2 \left(\frac{EPL-NPL}{NPL} \right) - 7,5$$

SFI – sciatic functional index, E – experimental, N – normal, TS – toe spread, PL – print length

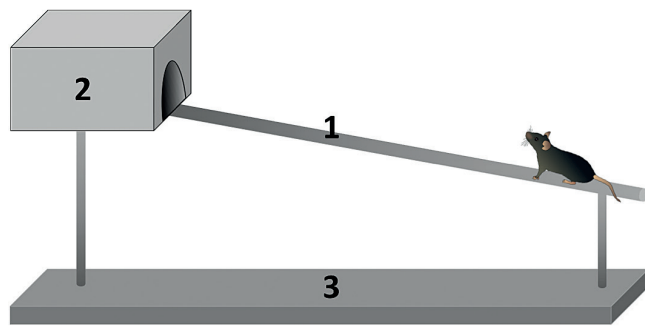


Fig. 2. Scheme of the balance beam test apparatus. 1 – a cylindrical beam with a diameter of 2 cm and a length of 110 cm; 2 – a darkened 13 x 22 cm box with a 5 x 6 cm entrance hole; 3 – a stand plate.



Fig. 3. Photograph of the mouse footprint indicating the parameters that were analyzed: TS – toe spread, PL – print length.

The SFI value was assessed in points from 0-100. The values ≥ 0 indicate normal function, and -100 points is completely equal to the dysfunction of the sciatic nerve.

Statistical analysis. To compare the data of the experimental groups, the analysis of variance ANOVA using software Origin Pro 8.5 (Origin Lab. Corp., USA) was performed. The difference was considered significant at $p < 0.05$; all experiments were performed at least three times. The results of the behavioral tests are presented as mean \pm standard error of the mean (Mean \pm SEM).

RESULTS AND DISCUSSION

It was found that at the start of the experiment before ADSCs transplantation, the mice of the control group passed the balance beam test for 6.5 ± 0.8 s, and B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy (group PN) crossed the same distance in 15.6 ± 1.7 s. Animals of the experimental group PN+ADSCs traverse the beam in 15.8 ± 1.7 s (Fig. 4).

Also, during the balance beam test, we calculated the number of hindlimb foot slips in animals of the control and experimental PN and PN+ADSCs groups. Animals of the control and PN groups during the test distance had an average of 1.5 ± 0.6 and 8.5 ± 0.8 paw slips, respectively (Fig. 5). The number of paw slips in the group PN+ADSCs was 8.7 ± 1.0 .

Throughout the observation period (10 weeks), the time taken for the animals to cross the distance from the start line to the finish line and the number of hindlimb paw slips for the animals increased in the control group and PN group. In PN+ADSCs group there was a progressive decrease in these parameters, but at none of the time intervals this parameter reached the results of normal animals (Fig. 4, 5).

Statistically significant differences in traverse time and number of paw slips during the balance beam test between the experimental groups PN and PN+ADSCs began to appear on the second week of the experiment: animals of the group PN+ADSCs made 20 % paw slips, and spent 11 % less time compared to the group PN. At the same time, the animals of the control group made 86 % less paw slips and cross the beam 62 % faster than the animals of PN group. Whereas similar values in comparison with animals of PN+ADSCs group were lower by 81 % and 57.5 %, respectively.

The sciatic functional index was determined by calculating such footprint parameters as toe spread and print length. At the start of the study in animals of the control group, the value of SFI was 2.5 ± 0.9 (Fig. 6). Whereas in animals of PN and PN+ADSCs groups of the same age this value was -30.6 ± 2 and -31.2 ± 4 , respectively.

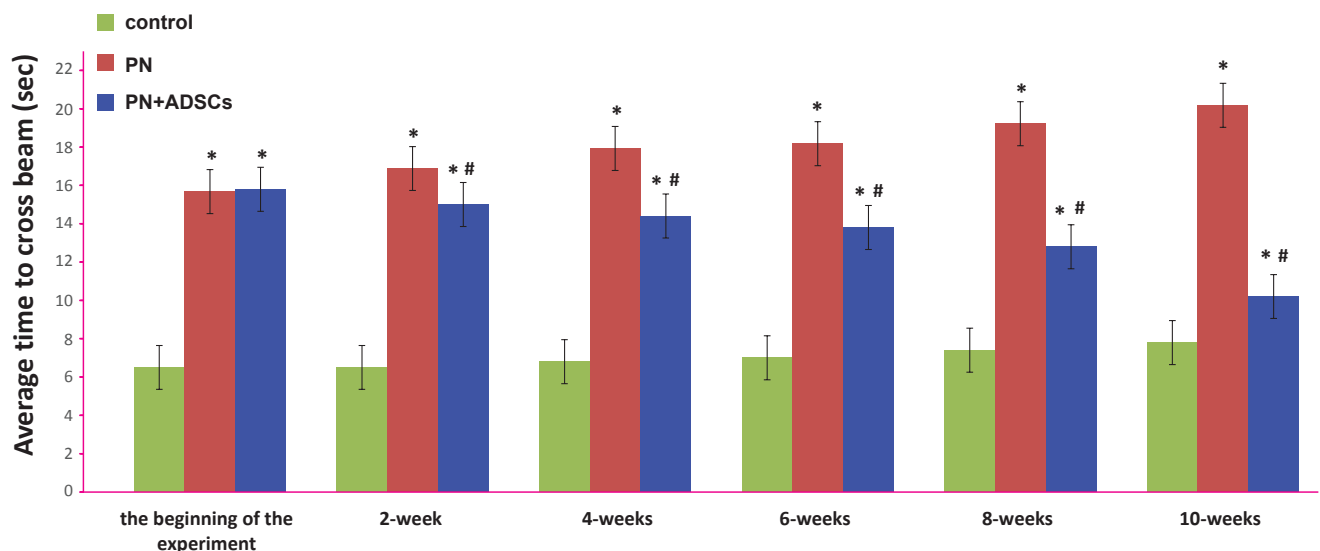


Fig. 4. Average time of traverse the beam for mice of each group: control – normal C57Bl mice (n = 6); PN – B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy (n = 6); PN+ADSCs – B6.Cg-Tg(PMP22)C3Fbas/J mice, which were transplanted with ADSCs (n = 10). X-axis is the time (in weeks) after ADSCs transplantation.

Notes: * – $p < 0.05$ compared to control, # – $p < 0.05$ compared to PN group.

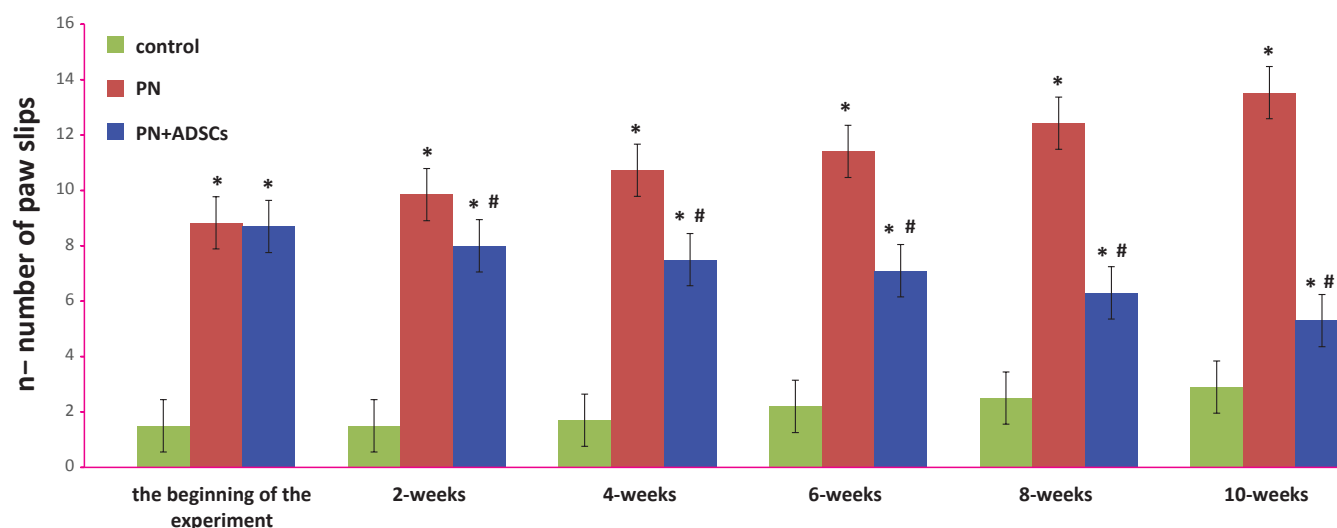


Fig. 5. The average number of hindlimb foot slips in mice of each group: control – normal C57Bl mice (n = 6); PN – B6.Cg-Tg(PMP22)C3Fbas/J mice with peripheral neuropathy (n = 6); PN+ADSCs – B6.Cg-Tg(PMP22)C3Fbas/J mice, which were transplanted with ADSCs (n = 10). X-axis is the time (weeks) after ADSCs transplantation.

Notes: * – $p < 0.05$ compared to control, # – $p < 0.05$ compared to PN group.

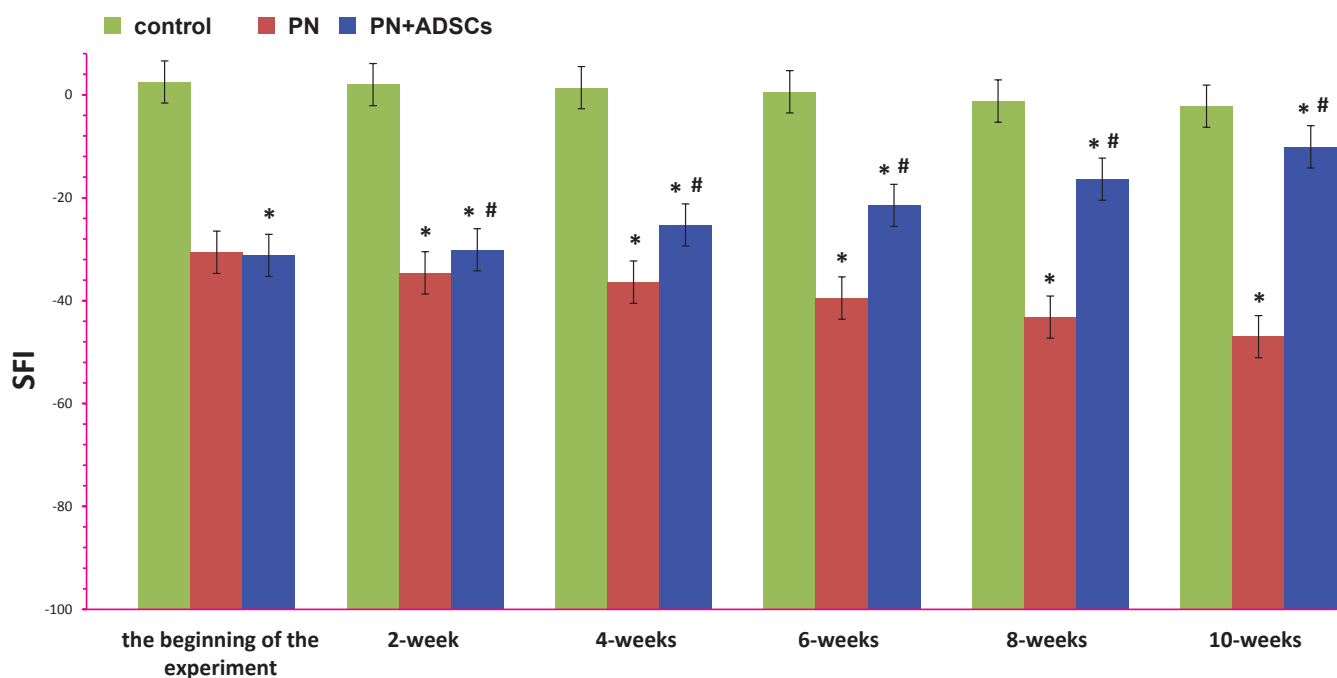


Fig. 6. The sciatic functional index (SFI) for mice of each group: control – normal C57Bl mice (n = 6); PN – B6.Cg-Tg (PMP22)C3Fbas/J mice with peripheral neuropathy (n = 6); PN+ADSCs – B6.Cg-Tg(PMP22)C3Fbas/J mice, which were transplanted with ADSCs (n = 10). X-axis is the time (weeks) after ADSCs transplantation.

Notes: * – $p < 0.05$ compared to control, # – $p < 0.05$ compared to PN group.

The analysis of the sciatic nerve function and the results of the balance beam test determined the improvement of motor activity of the animals of PN+ADSCs group in 2, 4, 6, 8 and 10 weeks after ADSCs transplantation. Within 2-10 weeks in animals of the control group, there was a slight decrease in locomotor function associated with age, which manifested in a gradual decrease in SFI from 2.5 ± 0.9 at the start of the study to -2.2 ± 1.5 in 10 weeks and a slight increase in the number of paw slips from 1.5 ± 0.6 to 2.9 ± 0.8 and, as a consequence, an increase in latency to cross the beam from 6.5 ± 0.8 s to 7.8 ± 0.9 s (Fig. 4-6).

In animals of the group PN there was a further progression of the disease. This was expressed in an increase in the number of hindlimb foot slips from the beam and the time required to pass the test distance

(Fig. 4, 5). The sciatic functional index in animals of PN group decreased throughout the study and in 10 weeks of the study it was -47.0 ± 2 (Fig. 6). In animals of this group there were also significant impairments of somatosensory sensitivity of the distal regions of hindlimbs and locomotor coordination.

In animals of PN+ADSCs group, the increasing of the sciatic functional index relatively to animals of PN group was detected in 2 weeks after ADSCs transplantation and was -30.2 ± 2 against -34.6 ± 0.9 in the PN group (Fig. 6). In 10 weeks after ADSCs transplantation, the average value of SFI in animals of PN+ADSCs group was -10.1 ± 2.3 , while in PN group the average value of SFI was -47.0 ± 2 . The increase in the SFI value correlated with a decrease in the number of paw slips and the

latency to cross the beam in animals of PN+ADSCs group (Fig. 4-6). Thus, adipose-derived MMSCs transplantation induced an improvement in the sciatic functional index and fine locomotor skills in mice with peripheral neuropathy.

It is known that the development of neuropathy is accompanied by motor and sensory disorders, which manifest themselves in the form of progressive muscle weakness, incoordination and decreased tactile sensitivity of the distal regions of limbs. Morphologically, CMT1A manifests itself in the form of loss of the myelin sheath of nerve fibers, the formation of areas of abnormal myelination (onion bulbs), and axonal dystrophy [1]. With the progression of this disease there is a disorder of axonal transport and trophic functions of Schwann cells, which leads to atrophy of the distal axons [1, 4].

As noted above, the development of Charcot-Marie-Tooth disease is based on an increase in the number of alleles of the *PMP22* gene and, as a consequence, an increase in protein expression. Other common mutations in this gene that underlie pathological myelination include deletion of one of its alleles, which leads to hereditary neuropathy with pressure palsy (HNPP) due to insufficient expression of the protein PMP22, as well as insertions that cause replacement of individual amino acids in the protein PMP22, and is the cause of CMT1A [2, 16]. The severity of such neuropathies is quite different and depends directly on the functional role of the replaced amino acid in the protein molecule [16].

In our study, transgenic B6.Cg-Tg(*PMP22*)C3Fbas/J mice with three copies of the *PMP22* gene were selected as the CMT1A model because the course of peripheral neuropathy in these animals correlates well with the course of CMT1A in humans. As a therapeutic agent, we used adipose-derived multipotent mesenchymal stromal cells. To determine the effect of ADSCs on the state of sciatic nerve, we used the footprint test, because it allows to determine reliably the functionality of the affected nerve.

As shown by Bain et al. in rat models, sciatic nerve dysfunction due to an injury or demyelination causes changes in the walk of the affected limb and manifests itself in changes in the footprint length, as well as toe spread and intermediate parameters of toe spread [17].

Numerous *in vivo* and *in vitro* studies have demonstrated the paracrine activity of ADSCs, which is manifested by the secretion of a wide range of therapeutic and immunomodulatory cytokines, such as interleukins IL-1Ra, IL-10, IL-13, vascular endothelial growth factor (VEGF) and ciliary neurotrophic factor (CNTF) [18, 19]. In addition, MMSCs secrete growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which have an antiapoptotic effect and are able to promote axonal growth and remyelination [20].

Thus, it can be assumed that ADSCs can potentially become an effective therapeutic agent in the treatment of peripheral neuropathies, in particular Charcot-Marie-Tut type 1A disease.

CONCLUSION

- 1. Adipose-derived multipotent mesenchymal stromal cells transplanted into mice with peripheral neuropathy improve locomotor activity in animals according to the balance beam test.**
- 2. ADSCs transplantation improves sciatic nerve function in mice with peripheral neuropathy according to sciatic nerve index.**

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