

Original Article

Bone marrow stem cells delivered into the subarachnoid space via cisterna magna improve repair of injured rat spinal cord white matter

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Abstract: The influence of bone marrow stem cells on regeneration of spinal cord in rats was investigated. Young adult male Wistar rats were used (n=22). Focal injury of spinal cord white matter at Th10 level was produced using our original non-laminectomy method by means of high-pressured air stream. Cells from tibial and femoral bone marrow of 1-month old rats (n=3) were cultured, labeled with BrdU/Hoechst and injected into cisterna magna (experimental group) three times: immediately after spinal cord injury and 3 as well as 7 days later. Neurons in brain stem and motor cortex were labeled with FluoroGold (FG) delivered caudally from the injury site a week before the end of experiment. Functional outcome and morphological features of regeneration were analyzed during 12-week follow-up. The lesions were characterized by means of MRI. Maximal distance of expansion of implanted cells in the spinal cord was measured and the number of FG-positive neurons in the brain was counted. Rats treated with stem cells presented significant improvement of locomotor performance and spinal cord morphology when compared to the control group. Distance covered by stem cells was 7 mm from the epicenter of the injury. Number of brain stem and motor cortex FG-positive neurons in experimental group was significantly higher than in control. Obtained data showed that bone marrow stem cells are able to induce the repair of injured spinal cord white matter. The route of cells application via cisterna magna appeared to be useful for their delivery in spinal cord injury therapy.

Keywords: Bone marrow cells, cell transplantation, SCI, spinal cord regeneration, therapeutic strategies

Introduction

Spinal cord injuries are still a serious problem of regenerative medicine. The effects of various therapies are up to date unsatisfactory, though many trials are performed, examining, among others, the effectiveness of cellular therapies, including bone marrow stem cell transplants [1].

Characteristic features of stem cells are: ability to undergo unlimited cell divisions (to reconstruct the population) and possibility to differentiate into various cells. There are two sources (types) of stem cells, depending on the phase of ontogenesis: fetal (embryonic stem cells) and adult (somatic stem cells). Another important characteristic of stem cells determines their ability to differentiate into various cell

lines. There are toti-, pluri-, multi- and monopotent stem cells. Totipotent stem cells are able to form the whole organism and fetal membranes, in contrast to monopotential stem cells, which can give rise to one particular cell line only [2-4].

In adult animal organisms, most of the stem cells are mono- or multipotent somatic stem cells. These cells are characteristic to particular tissues and they have been already isolated from many body organs [4-6]. In the brain, stem cells are found in specific areas as hippocampal gyrus and periventricular zone. These cells can differentiate into neurons, oligodendrocytes, and astrocytes. In experimental medicine, as well as in clinical practice, two types of stem cells are currently most commonly used: embryonic and bone marrow stem cells. The

Bone marrow stem cells in spinal cord repair

first ones are obtained from the embryos by isolation of single blastomeres or by cloning [2]. There is also a possibility to reprogram differentiated somatic cells with help of genetic engineering to create induced pluripotential stem cells. Vectors are used in this case to introduce genes (transcription factors) typical for the pluripotential cell (e.g. Oct-4, Klf-4, c-Myc, Nanog) into fibroblasts line [7]. It is known, that embryonic pluripotential stem cells (neural stem cells) can differentiate in the injured spinal cord to form astrocytes, oligodendrocytes or, less frequently, neurons [3, 4, 6]. Furthermore, it was shown that in contrast to undifferentiated embryonic stem cells, neural and glial embryonic stem cells can be responsible for the remyelination of demyelination foci, caused by different factors like contusion, chemical injury and others [5, 8].

Another type of stem cells is mesenchymal stem cells. These cells can be found in bone marrow and many other tissues (e.g. adipose tissue, skin, bones, and blood-including umbilical cord blood) and organs (e.g. liver, lungs). Their number in bone marrow oscillates between 0.001% to 0.01% of all mononuclear cells and decreases with age [2]. These cells have the capacity to differentiate into bone, fat, cartilage and muscle. Differentiation into neuron-like cells expressing markers typical for mature neurons has been also reported [2, 8]. Recently, a great deal has been learned about the isolation and characterization of these cells, as well as about ability to control their differentiation. Walker *et al.* [9], in their fundamental paper, found that that bone marrow-derived mesenchymal stromal cells (MSCs), used as “MSC therapy” following traumatic CNS injury, act as remote “bioreactors” via stimulation of lung macrophages and augmentation of production by the spleen of T regulatory cells, leading to systemic increase in circulating anti-inflammatory cytokines and alteration of the milieu of the central nervous system.

In the presented work, we examined the use of MSCs delivered via cerebrospinal fluid in the repair of focal injury of spinal cord in rats.

Material and methods

All procedures were performed in accordance to the EU animal protection law and were

approved by the Local Animal Research Ethics Committee. Twenty five male Wistar C rats were used; twenty two adults (3 month-old, approximately 300 g b.w.) were randomly divided into two groups, control (C; n=10) and experimental (BM; n=12) and three 30-day-old rats were donors of bone marrow-derived stem cells.

Spinal cord injury

The focal spinal cord injury was performed using the device we designed and created, the pressure impactor, producing a precisely controlled air blast [10].

After intraperitoneal anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), animals were placed on the heated plate and immobilized by means of the head holding bars and spine clamps at the vertebral levels of Th-9 and Th-11 to destroy selectively the segment of Th-10 of the spinal cord. For this purpose, Th-10 vertebra was stabilized and easy accessible for further steps. The skin was then incised over the spinous processes and the vertebral surfaces were exposed dorso-laterally from Th-9 to Th-11. Under the control of stereomicroscope (Nikon, Japan), a small hole (2 mm diameter) was drilled in the Th-10 vertebral arch on the right side. To avoid overheating and thermal lesion, trepan was cooled down with chilled phosphate-buffered saline (PBS). Impactor tip was placed close to the drilled opening and adjusted by means of micromanipulator. Penetration depth of the tip was set up in the way that a contact with the dura mater was established, however without exerting any pressure on the dura. After setting on impact parameters (150 kPa pressure, 0.1 s duration), the air blast system was activated. The “shot” was observed under the stereomicroscope and recorded by the attached camera (Nikon, Japan). After completing the procedure, the hole was secured with a bone wax, muscles were sutured in layers, skin was closed and the wound was treated with a sterile bandage.

To avoid dehydration, all animals were subcutaneously injected with 2 mL of sterile saline. Because the autonomic function of urinary bladder was impaired due to the spinal shock, it was emptied manually twice a day until the recurrence of bladder function. To prevent pain, paracetamol (suspension 120 mg/5 mL) was dissolved in drinking water (an average drug dosage of 5 mg/kg b.w.).

Bone marrow stem cells in spinal cord repair

Table 1. Percentage of different cell populations in mixed culture of rat bone marrow stem cells

Surface antigen	Cell % in culture	Cell characteristics
CD34+	3.5	mesenchymal/hematopoietic
CXCR4+	10	low differentiated mesenchymal
CXCR4+/CD34+	4.6	low differentiated mesenchymal/hematopoietic
CXCR4-/CD34-	81.9	mesenchymal
CD45+/Lin+	33.3	hematopoietic
CD45- /Lin-	55.1	non-hematopoietic

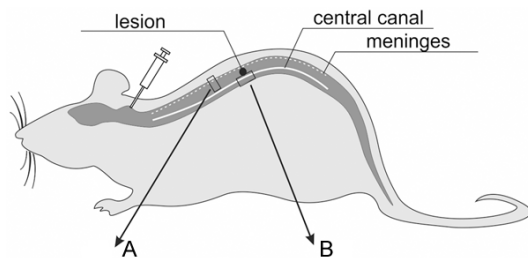


Figure 1. Schematic cells spreading along the spinal cord after their application into the *cisterna magna*. A and B areas of the spinal cord presented in **Figure 3**.

Bone marrow stem cell culture

Stem cells were obtained from red bone marrow isolated from femoral and tibial bones of three 30-day-old Wistar C rats. Prior to stem cells isolation animals were sacrificed by overdose of with Avertin (Tribromoethanol). Diaphysis of the bone was cut in sterile conditions in order to open the marrow cavity. Bone marrow was pushed out of the bone with a syringe with No. 9 needle containing alfa-MEM medium (Sigma-Aldrich, Germany). The marrow was dispersed by pushing it several times through the same needle containing stem cell culturing medium MesenCult (Stem Cell Technologies Inc., Canada). Obtained bone marrow suspension was transferred to T75 culture bottles (75 cm² surface area). After 24 hours of culturing in temp of 37°C, in air with 5% CO₂ content, the culturing medium with non-adherent cells was removed. Adherent cells were rinsed with phosphate buffered saline (PBS) and supplied with fresh MesenCult medium. When the cells covered whole surface of the culture bottles, they were dissociated with AccuMax solution (PAA, Austria), containing plant protease and passaged in culturing bottles with twice as large surface area.

Cultured cells (one passage each time) were used in the experiment or were frozen for later use. Cell banking solution contained 70% of alfa-MEM medium without antibiotics, 20% of non heat inactivated bovine fetal serum and 10% of DMSO (dimethyl sulfoxide). Cells were chilled gradually, spending 24 h in temperatures of

-20°C and -80°C to be finally frozen in liquid nitrogen where they were stored.

BrdU and Hoechst labeling

To enable visualization BM cells after their transplantation into the spinal cord, their nuclei were labeled with BrdU. Twenty four hours before transplantation, BrdU (Sigma, Germany) at the final concentration of 10 mg/mL was added to the culture of the bone marrow stem cells. The excess of tracer was washed out with PBS and cells were suspended in fresh culture medium to obtain approximately 300.000 cells in 10 µL.

In addition, for the cells devoted for transplantation, the cell culture was treated with Hoechst 33342 nuclear dye (Sigma, USA; 5 µg/ml in DMEM) for 1 hour at 37°C before cell harvest and then washed for several times in PBS. The fluorescent activity of these cells was checked before transplantation by viewing a sample in fluorescent microscope.

The cells pellet was resuspended in fresh culture medium to obtain approximately 300.000 cells in 10 µL.

Examination of cell culture purity

Stem cells isolated from rat bone marrow created a mixed population of adherent cells. Characteristic of adherent rat red bone marrow cells was determined with fluorescence-activated cell sorter (FACS) Aria BD (USA). Cell surface antigens, allowing for identification of specific cell populations, were detected by fluorochrome-conjugated antibodies. Low-differentiated stem cells are characterized by the presence of alpha-chemokine receptor specific for stromal-derived-factor-1, CXCR4. Hematopoietic stem cells, as well as mesen-

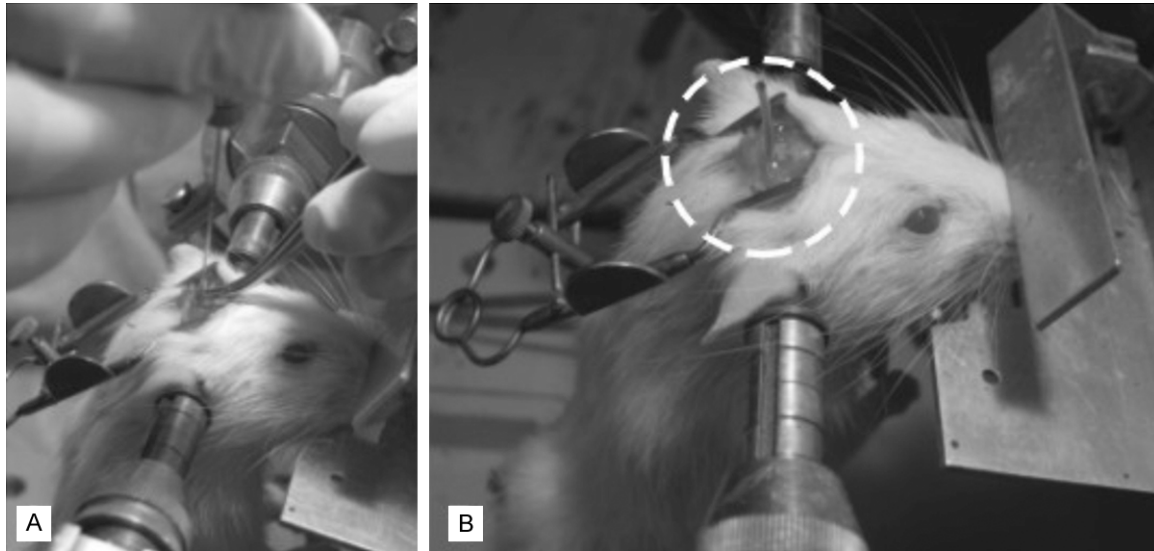


Figure 2. A. Cannulation of *cisterna magna*; B. Implanted cannula prior to bone marrow cells transfer.

chymal stem cells, present transmembrane sialomucin protein CD34. Anti-CXCR4 antibody was conjugated with allophycocyanin (APC) and anti-CD34 antibody was labeled with phycoerythrin conjugated with cyanine dye Pe-Cy7 (both- Chemicon, USA). CD45 and Lin antigens (labeled with mix of antibodies against CD11b/c, γ/δ TCR α/β TCR), are commonly used hematopoietic cell line markers. The mixture of these antibodies, conjugated with Pe-Cy7, was used for the identification purposes.

Analysis with flow cytometer showed presence of cells with various surface antigens. Most of the cells (~82%) in examined population were mesenchymal cells. Ten percent were CXCR4-positive, similarly like in other studies, showing that only small percentage of mesenchymal stem cells express this protein [11]. Only small fraction (3.5%) of CD34+ cells was found, and 55.1% of cells were CD45- and Lin-negative while 33.3% possessed these antigens, thus were of hemopoietic line (**Table 1**).

Cells transplantation

We decided to deliver cells via cerebrospinal fluid (**Figure 1**), what should provide the most physiologic way of their migration and protect the injured spinal cord from an extra trauma during direct delivery of these cells into the spinal cord parenchyma.

The cannulation of *cisterna magna* (CM) and the application of MSCs were performed

according to the technique of Solomon *et al.* [12] with our own modifications [13]. The animals were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg). The rat's head was immobilized in the stereotactic apparatus (Narishige, Japan), and the body slumped freely at the operative table, at right angle to the barrel. Parietal bone, occipital bone, the arch of the atlas and atlanto-occipital membrane were exposed after the medial incision of the skin and muscles in the parieto-occipital region. The 0.8 mm trephination hole was drilled sagittally over the parieto-occipital suture. Then the cannula (Venocath-18, Abbot) was placed *via* this hole into the CM (**Figure 2A**) and attached to the parietal bone with cyanoacrylic glue. The correct position of the cannula was achieved by observing the localization of its end in the CM through the atlanto-occipital membrane and by noting free minor CSF outflow through the cannula. The trephination hole was then protected from the CSF leakage with bone wax and cyano-acrylic glue (**Figure 2B**).

Ten microliters of BM cells suspension containing approximately 300.000 cells suspended in fresh medium (BM group) or 10 μ l saline (C group) were slowly injected through the previously inserted cannula immediately after spinal cord injury. This procedure was repeated 3 and 7 days later. In order to confirm successful delivery of transplanted cells into the injured spinal cord, spinal cord sections collected from 2 animals from BM cells group 24 h post trans-

Bone marrow stem cells in spinal cord repair

plantation were stained to visualize exogenous stem cells in the spinal cord.

Assessment of locomotor function

During 12 weeks postoperatively, animals were observed and analyzed regarding development or regression of neurological deficits. Behavioral observations included gait analysis (foot print test) and open field test according to the BBB (Basso, Beattie and Bresnahan) scale [14].

Foot print test

Foot print test was carried out in 1st, 4th, 7th, and 12th week postoperatively. Animals were tested on a 100 cm long and 7 cm wide runway with side walls and transparent bottom. The walk of a rat through the runway was recorded with digital camera and automatically analyzed frame by frame (Catwalk XT 8.1, Noldus, Holandia). Each animal was tested three times consecutively. Framed foot images were analyzed regarding the foot rotation angle (the angle made by two lines connecting the third toe and the stride line at the center of the paw) of the right hind paw (ipsilateral to the injury site), and interlimb coordination (smallest distance between the middle point of the hind paw and forepaw on the same side). Normal rotation angle is about 8° and after trauma increases up to 30°. Interlimb coordination is about 1.5 cm in healthy rat and increases to 3 cm or more after injury [15].

BBB open field test

Test was carried out in classical way on the plexiglass surface [14]. During the test, motor function of joints was analyzed to assess the stepping ability of the animal. Additionally, general coordination and stability of the body were evaluated. The value range was 0-22; 0 was related to complete lack of motor capability, and 22 indicated the best score in this aspect.

Neuroanatomical tracing

Retrograde neuroanatomical tracing was used to determine the extent to which supraspinal neurons regrew axons to reach spinal cord segments caudal to the injury. Four animals in each group were randomly selected for retrograde tracing. One week before the end of experiment, after anesthesia as above, the spinal cord was exposed by laminectomy below

the injury site, and 2 microcrystals of Fluorogold (FG) (Fluorochrome Inc., Englewood, NJ) were placed bilaterally inside the spinal cord 10 mm caudally from injury site. For quantification of the number of FG-positive neurons present in brain stem (red nucleus) and primary motor cortex on the last day of experiment, animals were rapidly perfused with a bolus of cold PBS, and whole brains were carefully dissected, dehydrated in 15% sucrose, embedded in TissueTek (Sakura, Japan), frozen and then cut coronally into 10 µm sections. Every sixth slide was analyzed, giving 18-22 sections per animal. The sections were viewed under UV excitation (at 365 nm wave specific for FG) in fluorescent microscope using appropriate filter (Labophot 2, Nikon, Japan) and photographed. The number of cells from each section was then summed for each rat and the number of cells per mm² was calculated and finally averaged for whole group.

Morphological evaluation

MRI analysis: MR images were obtained at the Institute of Nuclear Physics PAN (Cracow, Poland) using 4.7 T research MRI scanner, equipped with MARAN DRX digital console (Resonance Instruments, UK). Measuring probe-head with surface RF coil dedicated for rat spinal cord investigations was used. Animals were anesthetised with halothane (2%) mixed with air (60%/1.2 L/min) and oxygen (40%/0.8 L/min) Physiological conditions of the animal (temperature, breath rate and cardiac rate) were continuously monitored during experiment using MR-compatible Small Animal Monitoring & Gating System (SA Instruments, Inc., USA).

Spin echo pulse sequence was used for acquiring T2 weighted and diffusion weighted MR images in sagittal and axial projections. The following parameters of the spin echo sequence were applied: echo time (TE=32 ms), repetition time (TR=3 s), field of view (FOV=2.5 cm), image matrix size 128×128, slice thickness of 1.5 mm. The signal was accumulated (NA=6) in order to improve signal to noise ratio (SNR).

Diffusion weighted images were obtained with additional diffusion sensitive gradients (b=1000 s/mm², δ=5 ms and Δ=23 ms) added to spin echo sequence in transversal and longitudinal directions to the spinal cord axis.

Bone marrow stem cells in spinal cord repair

In order to minimize motion artifacts, all measurements were synchronized with animal breath cycle.

Histology

One (3 animals per group) or 12 weeks (3 animals per group) after injury, rats were re-anesthetized and perfused transcardially with 300 mL PBS (pH 7.4) followed by 300 mL of 4% paraformaldehyde solution in the same buffer. Fragments of spinal cord (~2 cm) containing the injury area were dissected and dehydrated in 20% sucrose in PBS for 24 h at 4°C, embedded in TissueTek (Sakura, Japan), frozen, and cut sagittally into 10 µm sections mounted on SuperFrost Plus slides (Menzel Glaeser, Germany) in 10-section-step manner. For immunostaining, the frozen sections were air dried at room temperature for 10 min. and washed with PBS for 10 min. Slides were subjected to hematoxylin-eosin staining, 1% toluidine blue staining or to immunohistochemical labeling, and mounted in VectaShield with DAPI (Vector, USA). Sections were examined under confocal laser scanning microscope FluoView (Olympus, Japan). The images were digitally stored and then analyzed.

To examine the ability of transplanted BM cells to survive in host spinal cord, 1 and 11 weeks following last transplantation, the number of Hoechst/BrdU-positive BM cells was determined for the 3 animals at each time point by counting all labeled cells present at the epicenter and nearest surrounding of injury site and expressed as number of cells per mm².

In the same group of animals, one week after transplantation maximal distance of expansion of implanted BM cells in injured spinal cord was measured 1 week after last transplantation as a maximal distance covered by Hoechst/BrdU-positive BM cells from the epicenter of the lesion into the spinal cord parenchyma.

Twelve weeks after injury we also measured the sizes of the lesion center (cavity area) using Image Pro Plus software to calculate automatically manually outlined contour (area borders) of the cavity lesion.

Nestin immunostaining for visualization of neuronal precursors

The sections were blocked with 1% BSA, 5% skim milk and 0.3% Triton X-100 in PBS for 1 h

at room temperature, and the primary antibody was applied in the same blocking solution overnight at 4°C. We used anti-rat rabbit anti-nestin antibody (1:100; Chemicon, UK). Then, slides were washed 3 times in PBS and incubated with secondary goat anti-rabbit IgG antibodies conjugated with Alexa 488 (1:400; Molecular Probes, USA). Mounted in VectaShield (Vector, USA) sections were examined under confocal laser scanning microscope FluoView (Olympus, Japan). The images were digitally stored and then analyzed.

BrdU-positive cells visualization

The sections were washed with PBS incubated in 2N HCL and 1% Triton X-100 in PBS for 15 min. at room temperature, and wash in sodium borate in PBS for 10 min. The slides were blocked with 1% BSA, 5% skim milk and 0.3% Triton X-100 in PBS for 1 h at room temperature and incubated with a mouse anti-BrdU antibody (1:100; BD-Immuno; Oxford, UK). In the same blocking solution overnight at 4°C. Slides were then washed 3 times in PBS and incubated with goat anti-mouse antibody conjugated with Alexa 594 (1:400; Molecular Probes, USA). Finally, sections were examined under confocal laser scanning microscope FluoView (Olympus, Japan). The images were digitally stored and then analyzed.

Maximal distance of expansion of transplanted cells in the injured spinal cord was measured at 2 weeks after operation and survival of transplants (number of cells seen in the epicenter of the injury) was examined 2 and 12 weeks following surgery (1 or 11 weeks after last cell transplantation).

Statistical analysis

ANOVA or nonparametric Kruskal-Wallis ANOVA or Student's test were used. Significance level was set at $P \leq 0.05$. All data are expressed as means \pm SD.

Results

Surviving and migration of transplanted cells within injured spinal cord

After grafting, BM cells (identified after Hoechst/BrdU-positive nuclei) were observed on the surface of the injured spinal cord and then they propagated along meninges in the direction of white matter. Later (1 week after

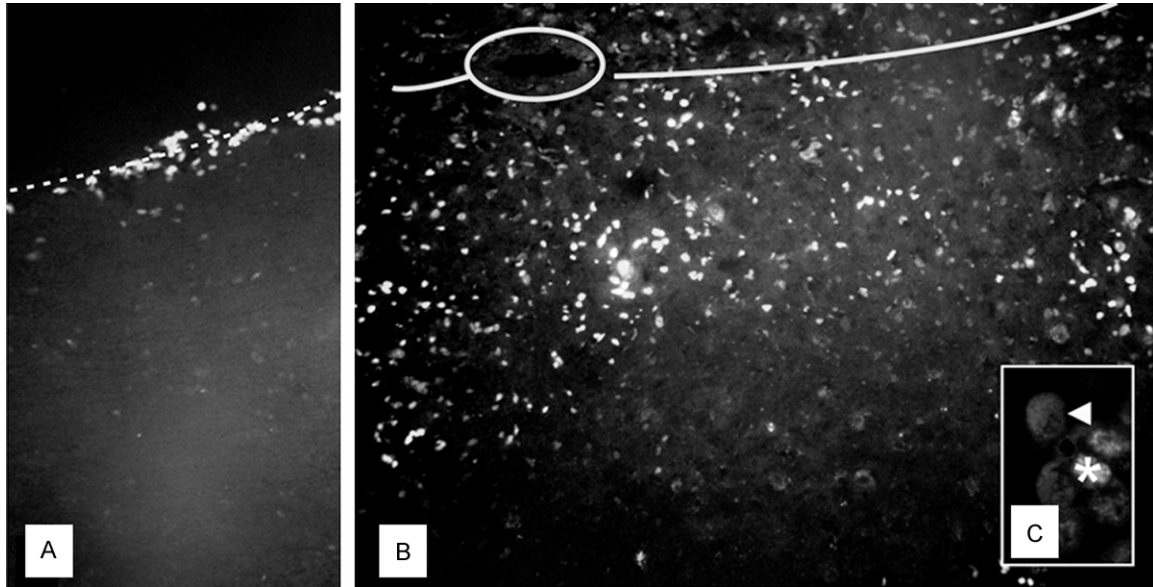


Figure 3. Fluorescent microscope images of rat spinal cord, 7 days after injury and triple application of bone marrow stem cells: A. Cells (nuclei labeled with Hoechst) migrating along the spinal meninges (dash line); B. Cells crossing the middle line of the spinal cord (solid line, central canal-circled); C. Double-labeled (asterisk-BrdU; arrowhead- nestin) implanted cells. Magnification: A, B. 100×; C. 200×.

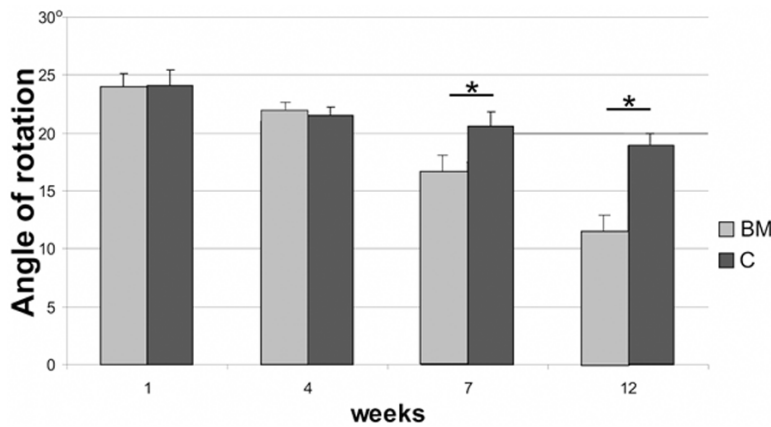


Figure 4. Angle of rotation of the hind paw after spinal cord injury in BM and C groups, respectively. Asterisks indicate statistical significance ($P \leq 0.05$).

last [third] injection), transplanted MSCs penetrated spinal cord parenchyma deep inside the spinal cord interior close to the central canal, very near to the injury region. Some of these cells invaded the other side of the spinal cord-not affected by injury (**Figure 3**). Their number decreased with time -1 week following last transplantation we found 967.2 ± 331.7 cells/ mm^2 , while at the end of experiment, it was 71.7 ± 47.1 cells/ mm^2 in analyzed fragments of spinal cord.

Behavioral analysis

Immediately after SCI, 17 animals (74%) were monoplegic. Rats in the experimental group presented significant improvement of locomotor performance when compared to the control group. The footprint pattern of animals one week after SCI showed strongly compromised both the interlimb coordination and the angle of rotation (2.5 ± 0.42 and $23.4 \pm 1.7^\circ$, respectively). From 7th week after SCI, the experimental group displayed a significant

reduction in the angle of rotation in hindlimb placement compared with the control group (12.3 ± 1.4 versus 18.9 ± 1.1 at the 12th week following injury) ($P < 0.05$) (**Figure 4**). From 4th week after injury, the BM group showed also a significant ($P < 0.05$) improvement in the interlimb coordination, reaching at the end of the experiment value of 1.53 ± 0.11 in contrast to the control animals (2.19 ± 0.21) (**Figure 5**). In addition, significant functional deficit, as measured with the BBB average score, was noted in

Bone marrow stem cells in spinal cord repair

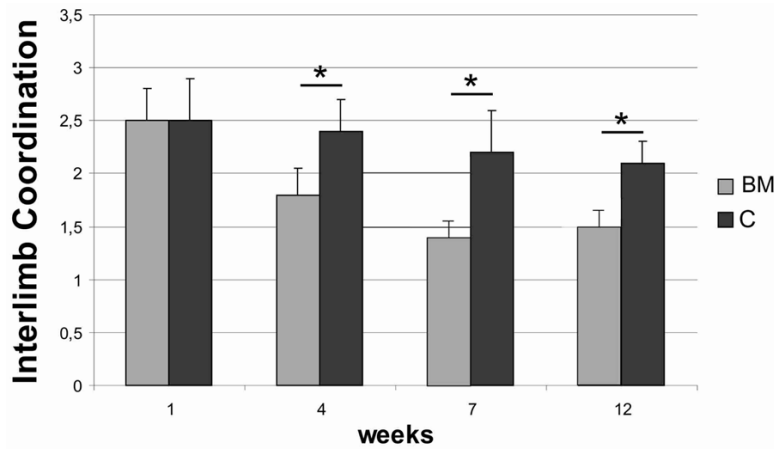


Figure 5. Inter-limb coordination after spinal cord injury in BM and C groups, respectively. Asterisks indicate statistical significance ($P<0.05$).

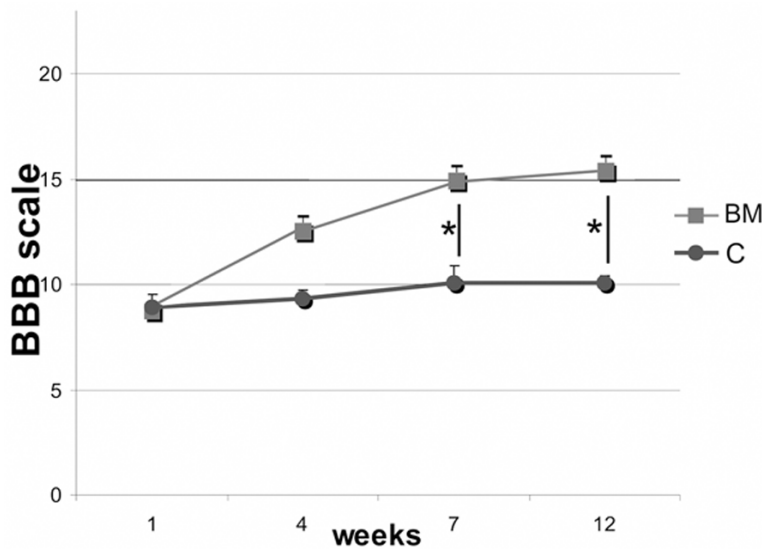


Figure 6. BBB open field score after spinal cord injury in BM and C groups, respectively. Asterisks indicate statistical significance ($P<0.05$).

all animals from the control group (10.1 ± 0.3) when compared to the experimental one (16.3 ± 0.7) ($P<0.05$) (**Figure 6**).

Magnetic resonance imaging

MR images showed no post-traumatic cyst development in the white matter of spinal cord in the BM group, while in the control group changes resembling typical post-traumatic syringomyelia were frequently found (**Figure 7**). Comprehensive analysis of diffusion data from white matter gives quantitative description of injury in terms of the spatial change of the diffusion parameters as shown on **Figure 8**. The

transverse diffusion gradients of the white matter (perpendicular to the spinal cord axis) showed no difference between BM and C group, while the longitudinal diffusion gradients were slightly, however non-significantly, higher in the BM group when compared to the control ($P>0.05$). These values were close to the reference values measured in healthy animals.

Assessment of cavity area

The average size of the lesion center (cavity area) was also significantly smaller in the experimental group (0.12 ± 0.14 mm²) than in the control one (1.02 ± 0.21 mm²) ($P<0.05$) (**Figure 9**).

Neuroanatomical tracing

Number of the brain stem and primary motor cortex FG-positive neurons (**Figure 10**) in BM group (248.1 ± 105.1) was significantly higher than in the control (14.3 ± 3.3) ($P<0.05$).

Neuronal precursors' number

Some transplanted bone marrow cells differentiated towards neurons, as confirmed by BrdU/nestin double labeling. Number of such double labeled cells found one week following transplantation was 41.4 ± 23.7 mm², while after 11 weeks it reached significantly smaller value of 7.1 ± 1.3 mm².

Discussion

We showed that mixed population of bone marrow derived stem cells delivered via cerebrospinal fluid may improve morphologic as well as functional deficits of injured spinal cord in rats.

A surprising finding of our study was that relatively small heterogeneous population of bone

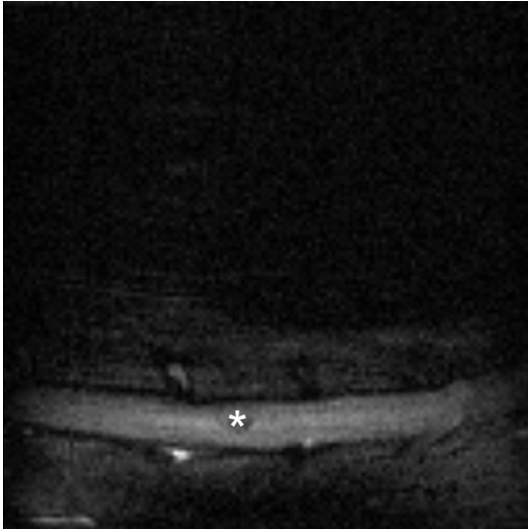


Figure 7. MR sagittal image of control rat's spinal cord 12 weeks after injury; post-traumatic cyst (asterisk) visible in the central part.

marrow stem cells repeatedly grafted via cerebrospinal fluid can overpass some limitation of current therapy of spinal cord injury in animals. This refers to elaboration of optimal delivery rout of grafted cells and their successful survival, migration, integration and finally differentiation within the host tissue as well as their putative regenerative influence on injured white matter. Unlike many other studies, we focus on therapy of focal injury of white matter of spinal cord consisting predominantly of axons, not neuronal cell bodies.

The mechanism of an influence of bone marrow derived stem cells on an injured spinal cord of adult mammals is complex and has not been entirely explored. There are probably two possibilities: limitation of the extent of injury in the traumatic spinal cord, which seems to be more probable, or strictly neuro-regenerative effect leading to both cell survival and regrowth of their axons. It is possible that both abovementioned mechanisms overlap and the truth lies somewhere between. It seems possible, that bone marrow-derived stem cells through differentiating into oligodendrocytes restore the population of these cells, lost due to spinal cord injury. Demyelinated axons are very sensitive to further neuro-degradation and their prompt remyelination by oligodendrocytes is necessary to limit their progressing decomposition. The latter, in turn, can result in propaga-

tion of the damaged region to next parts of the spinal cord, not affected directly by the damage or merely injured [16]. Eventually, the white matter of the spinal cord becomes severely damaged and a classic post-traumatic cyst is formed, which is observed both in humans and rats [17]. Bone marrow derived stem cells may also influence the formation of new intercellular connections what lead to spinal cord plasticity as well as stimulation of axonal growth [4, 18].

In our experiment, we decided not to use selected homogenous population but mixed bone marrow cells. It made possible to reconstruct the entire spectrum of supposed activities of bone marrow stem cells in injured spinal cord. There are experiments showing that mixed populations of stem cells, composed usually of two or more cell lineages, transplanted in the injured spinal cord present higher vitality, differentiate better and cause more significant improvement of the functional outcome than any one of them used separately [1].

Currently, a tendency can be noted to examine this kind of complex therapies, including mixed cell therapies that are usually more effective than a mono-therapy [19]. Another reason to apply a mixed population of bone marrow derived stem cells are problems concerning the direction of their differentiation in the recipient. For instance, it was proven that highly purified mouse hematopoietic bone marrow-side population (SP) of stem cells [20] and CD34+ positive bone marrow cells [21] showed no ability to transdifferentiate and restore neurons or astroglia. Contrary, another type of hematopoietic bone marrow-side populations of stem cells, identified as Sca-1+, c-kit+ and Lin-, relatively easily differentiated into oligodendrocytes and astrocytes [22, 23]. The whole bone marrow derived stem cells, allo- [24] and xenogeneic [21], differentiated easily into glial cells and replaced the population of lost oligodendrocytes, retaining the ability to remyelinate damaged axons of the spinal cord, as opposed to hematopoietic stem cells that were not able to remyelinate the axons. An example that well illustrates this strategy is combined application of glial cells of various origins, namely olfactory bulb cells with adult Schwann cells, into the injured spinal cord. It permitted achievement of better functional tests results than separate application of these cells [25].

Bone marrow stem cells in spinal cord repair

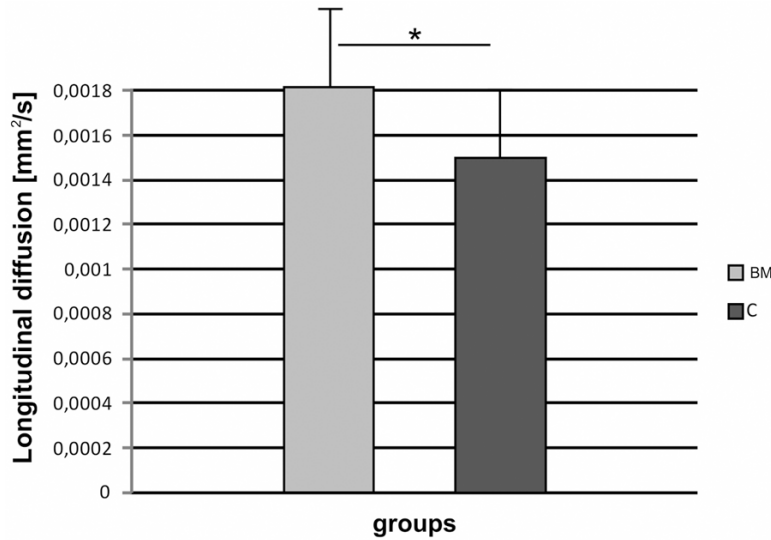


Figure 8. Longitudinal diffusion gradients of the spinal cord white matter in BM and C groups, respectively.

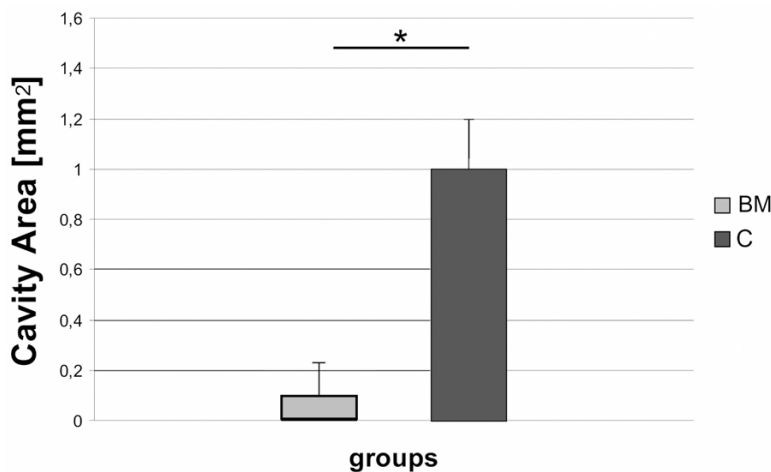


Figure 9. Areas of lesion cavities, 12 weeks after spinal cord injury in BM and C groups, respectively. Asterisk indicates statistical significance ($P \leq 0.05$).

The abovementioned differences indicate that bone marrow contains populations of stem cells not completely described and considerably more diverse than one can suspect [26]. It is probable that the direction of differentiation of bone marrow derived stem cells is determined, apart from mutual cellular interactions, by other factors, including external ones, like for instance the nature of the spinal cord injury itself [27]. It is difficult to predict the behaviour of implanted stem cells in conditions where the white matter of the spinal cord was damaged, that took place in our model.

It is suspected that one of the most crucial characteristics of bone marrow derived mesenchymal stem cells that determines their main functions and clearly distinguishes them from other types of stem cells is their ability to form “bands of cells” similar to bands of Büngner present in the peripheral nervous system and connecting edges of post-traumatic changes (cysts) [4, 28]. Possibly, in our experiment, the almost complete lack of visible post-traumatic changes in the spinal cord in the BM group is related to an inflow and filling of the regions of potential cysts by these cells with a possible differentiation later into other spinal cord cell populations.

The ability of the implanted cells to penetrate into the area of post-traumatic changes and, first and foremost, invading the region of the glial scar in the rostral spinal segments is crucial for the recovery of the lost nerve functions. Interesting studies in this matter were carried out by Lu *et al.* [29]. They showed a possibility of penetrating even several week-old post-traumatic cyst with regenerating spinal axons

after application of mesenchymal stem cells into the cyst in rats.

A favorable feature of mesenchymal stem cells that facilitates their application in regenerative medicine is also their low immunogenicity or complete lack of it, what allows to eliminate immunosuppression in case of their application [17, 30]. Moreover, in a number of papers it was indisputably proven that application of bone marrow derived mesenchymal stem cells has a favorable influence on functional recovery (usually test BBB was applied) of the injured spinal cord. This effect was not related to cells differ-

Bone marrow stem cells in spinal cord repair

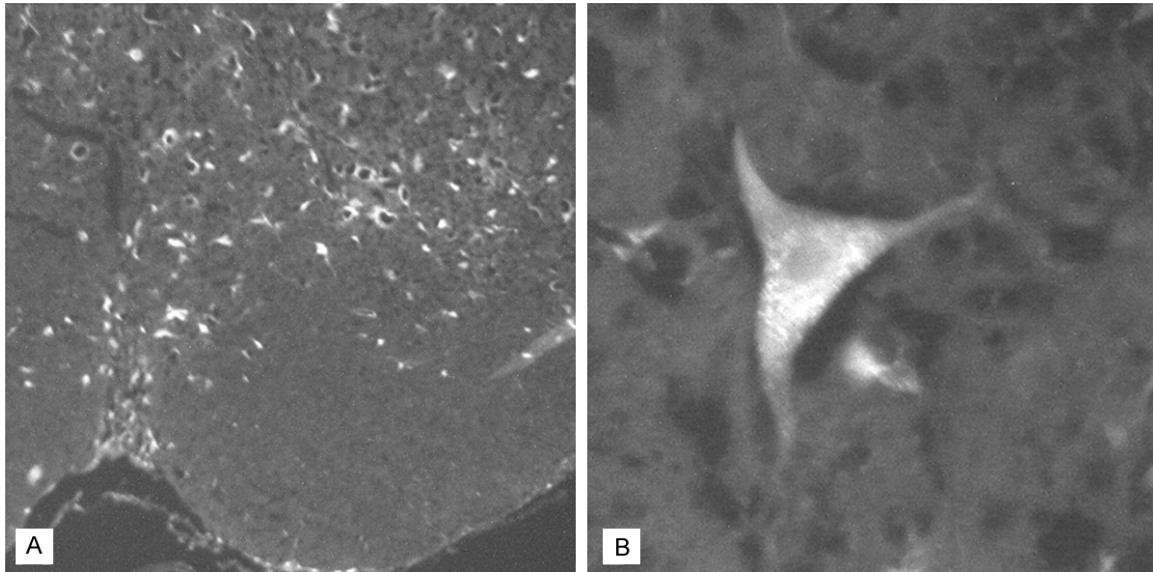


Figure 10. FluoroGold-positive cells in the rat's brain (BM group), 12 weeks following spinal cord injury and bone marrow cells transplantation. A. Brain stem cells. Magnification 100 \times ; B. Motor cortex neuron. Magnification 400 \times .

entiation into specific populations of cells and did not depend on spinal cord injury model [28, 31-33]. In *in vitro* conditions, whole bone marrow stem cells caused a significant improvement of nerve conduction [24]. In another study, behavioral tests showed an improvement in motor functions, despite the fact that among implanted cells only a small group differentiated into oligodendrocytes and astrocytes [23].

Only partial recovery of motor functions observed in experimental animals after MSC therapy, despite the lack of obvious morphological changes in the injured spinal cord, can be explained that even almost complete reconstruction of a structure does not always restore accurate function in the CNS. From experiments with other cells used in SCI therapy, Felts and Smith found that remyelination of spinal cord axons by Schwann cell is permanent, fully effective and in general restore almost normal impulse conduction in them [34]. It is also known, however, that normal conduction in remyelinated axon can be disturbed as a consequence of membrane ion channels rearrangement [35].

Results presented above show positive influence of bone marrow derived stem cells on injured spinal cord white matter. The route of delivery of these cells via cerebrospinal fluid

also appeared to be a useful method. However, the precise mechanisms of action of these cells in the repair of the nervous system require further examination.

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Disclosure of conflict of interest

None.

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Bone marrow stem cells in spinal cord repair

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