

Axonal outgrowth stimulation after alginate/mesenchymal stem cell therapy in injured rat spinal cord

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Despite strong efforts in the field, spinal cord trauma still belongs among the untreatable neurological conditions at present. Given the complexity of the nervous system, an effective therapy leading to complete recovery has still not been found. One of the potential tools for supporting tissue regeneration may be found in mesenchymal stem cells, which possess anti-inflammatory and trophic factor-producing properties. In the context of transplantations, application of degradable biomaterials which could form a supportive environment and scaffold to bridge the lesion area represents another attractive strategy. In the present study, through a combination of these two approaches we applied both alginate hydrogel biomaterial alone or allogenic transplants of MSCs isolated from bone marrow seeded in alginate biomaterial into injured rat spinal cord at three weeks after spinal cord compression performed at Th8-9 level. Following three-week survival, using immunohistochemistry we studied axonal growth (GAP-43 expression) and both microglia (Iba-1) and astrocyte (GFAP) reactions at the lesion site and in the segments below and above the lesion. To detect functional improvement, during whole survival period we performed behavioral analyses of locomotor abilities using a classical open field test (BBB score) and a Catwalk automated gait analyzing device (Noldus). We found that despite the absence of locomotor improvement, application of both alginate and MSCs caused significant increase in the number of GAP-43 positive axons.

Key words: spinal cord injury, mesenchymal stem cells, alginate, biomaterials, axonal outgrowth

INTRODUCTION

Spinal cord injury is a serious health condition which in spite of extensive research in the field lacks any effective treatment leading to complete recovery of lost motor and sensory functions. Several approaches with reported potential therapeutic effect have been studied including surgical interventions (van Middendorp et al. 2012), pharmacology (Blight and Zimmer 2001), hypothermia (Grulova et al. 2013), physical exercise (Ying et al. 2008, Foret et al. 2009) or cell transplantation (Ritfeld et al. 2012). Experimental therapeutic interventions are intended to influence secondary damage processes such as inflammation, inhibition of molecule formation, edema or apoptosis to prevent further damage in the tissue (Tator 1995). Despite the necessity of taking all of these components into account as a single comprehensive condition for future therapeutic efforts, detailed studies of above-mentioned individual events still remain important. Inflammation, as a nat-

ural response of the tissue to injury, is one of the most important processes occurring after SCI, characterized by infiltration of activated immune cells and edema formation with consequent devastating impact on the intact nervous tissue (Donnelly and Popovich 2008). In general, with its prevailing negative effects, inflammation has become of key target of experimental and clinical treatment. Mesenchymal stem cells (MSCs) represent an interesting tool with considerable potential in this context. Several studies have produced evidence about the capacity of these cells not only to suppress immune response (Aggarwal and Pittenger 2005) but also to produce neurotrophic (Zhang et al. 2003) and vascular factors (Hamano et al. 2000). In the context of transplantation and pharmacological therapies, another attractive strategy has emerged in recent years. Application of degradable biomaterials enriched with cells or various factors has been described as very promising (Suzuki et al. 2002, Nomura et al. 2006, Grulova et al. 2015). There are several potential benefits of using bio-

materials, one of the most important being that biomaterials such as alginate have the capacity to fill cavities and thus offer physical support for newly-formed regenerating tissue including axonal processes (Suzuki et al. 2002, Nomura et al. 2006). Similarly, biomaterial scaffolds could form microenvironments for transplanted cell populations, enabling sustainable release of supporting factors (Wang et al. 2008).

In the present study we applied alginate hydrogel alone or seeded with MSCs isolated from bone marrow into the injured rat spinal cord at three weeks after SCI. To detect functional improvement, during the whole survival period we performed behavioral analyses of locomotor abilities in a classical open field test (BBB score) and with a Catwalk automated gait analyzing device (Noldus). After three weeks using immunohistochemistry we studied axonal growth and both microglia and astrocyte reactions at the lesion site and in segments below and above the lesion. We found that despite intergroup differences in axonal sprouting and both microglia and astrocyte numbers, these changes were not significantly reflected in behavioral outcomes.

METHODS

Animals

Adult male *Wistar albino* rats (300–330g), were divided into four groups: *intact controls* (n=5), *sci/saline* (n=5), rats treated with alginate – *sci/Alg/-* (n=5), rats treated with alginate+MSCs *sci/Alg/MSCs* (n=6).

Compliance with Ethical Standards

This study was carried out with the approval and according to the guidelines of the Institutional Animal Care and Use Committee of the Slovak Academy of Sciences and with the European Communities Council Directive (2010/63/EU) regarding the use of animals in research and Slovak Law for Animal Protection 377/2012 and 436/2012. Totally 21 adult male *Wistar albino* rats were used in this study.

Spinal cord injury

Moderate spinal cord injury was induced by modified balloon-compression according to Vanicky 2001. Briefly, rats were first put under anesthesia with 1.5% to 2% isoflurane. Then 2-French Fogarty catheter was inserted epidurally at Th8–Th9 level of the vertebral

column and the balloon was inflated with 12.5 µl of saline for 5 minutes. After 5 minutes the catheter was deflated and removed from the vertebral canal. Selected volume of balloon compression caused initial complete paraplegia followed by gradual recovery of locomotion. First 3–7 days after surgery, manual bladder expression was performed twice a day until the bladder control was regained. Postoperational care did not involve antibiotic or analgetic treatment.

Preparation of alginate scaffold

Alginate biomaterial was prepared according to previously reported protocols (Tsur-Gang et al. 2009, Grulova et al. 2015). Briefly, solutions of sodium alginate (VLVG, 30–50 kDa, >65% guluronic acid content, NovaMatrix FMC Biopolymers, Drammen, Norway) and D-gluconic acid/hemi calcium salt were prepared by dissolving these components in double-distilled water and stirring at room temperature. Then, both solutions were filtered separately through a sterile 0.2 µm filter membrane into a sterile dish in a tissue culture hood. To prepare partially calcium-cross linked alginate, equal volumes from each stock solution (2.08% and 0.64% (w/v) for VLVG alginate and D-gluconic acid, respectively) were combined by extensive homogenization for several minutes to facilitate homogenous distribution of the calcium ions and cross linking of alginate chains.

MSCs isolation and cultivation

Bone marrow was isolated from the femur and tibia of adult male *Wistar* rats (300 g) after terminal anesthesia (thiopental, 50 mg/kg, i.p.) as described in our previous work (Nagyova et al. 2014). Whole bone marrow was flushed with ice-cold saline solution and dissected into small pieces on ice. The tissue was then homogenized and centrifuged at 400×g for 10 min. The obtained cell pellet (containing both hematopoietic cells and marrow mesenchymal cells) was resuspended; plated on a 75-cm² flask; cultured in 13 ml of culture medium containing Minimum Essential Medium (MEM) (Biowest, Nuaille, France), 15% fetal bovine serum (FBS) (Biowest), and 1% penicillin-streptomycin (Biochrom AG, Berlin, DE); and incubated at 37°C in a humidified atmosphere with 5% CO₂. Nonadherent cells were removed after 48 h by change of the medium. Upon reaching 90% confluence, the cells were passaged using 0.05% trypsin-EDTA (Gibco; Invitrogen, Carlsbad, CA) and plated on culture flasks at a density of 0.7×10⁶/75 cm².

Phenotypic characterization of bone marrow MSCs by flow cytometry

The phenotypic expression of CD90, CD29, and the absence of CD 45 at passage 3 was analysed. Following antibodies for flow cytometry were used according to the suppliers recommendations: PE anti-rat CD90 (BioLegend), PE anti-rat CD45 (BioLegend), PE anti-mouse/rat CD29 (BioLegend). Samples were processed through a FACS Calibur (BD Bioscience) operated by CellQuest software.

Membrane labeling of MSCs with PKH67

Prior to the intraspinal delivery, the MSCs were labeled with green fluorescent cell marker PKH67 according to the previously published protocol (Wallace et al. 2008, Nagyova et al. 2014). Briefly, immediately prior to staining procedure, PKH67 dye (Sigma) was prepared and added to 1 ml of resuspended MSCs (2×10^6 cells). After 20 min incubation at 25°C, an equal volume of α -MEM with 1% FBS was added to stop the staining reaction.

In vitro cultivation of PKH67 labeled MSCs in alginate

At day of *in vivo* application, PKH67 labeled MSCs in alginate (16 μ l) were separated and cultivated in 4 well dishes (4 μ l/well) with α -MEM, 10% FBS and 1% penicillin-streptomycin at 37°C for 3 weeks. After this period, to verify the intensity of PKH67 signal, cells were observed under inverted fluorescent microscope (Nikon Eclipse Ti).

Intraspinal delivery of alginate and MSCs

Three weeks after SCI, animals were anesthetized with 1.5–2% isoflurane and to expose the spinal cord, a partial laminectomy at Th6–12 level was performed. Using a 50- μ l Hamilton syringe (27G needle, 9 Cole Parmer, Anjou, Quebec) connected to UltraMicroPump III with Micro4 Controller, 4-Channel (World Precision Instruments, Inc., Sarasota FL) and stereotactic device, 6 intraspinal injections with saline or alginate or alginate/MSCs per animal (4 μ l/injection) were applied into the lesion site that showed discreet signs of damage. The number of MSCs in alginate/MSCs group represented approximately 1.3×10^5 cells per microliter. Bilateral delivery of i) saline, ii) ALG, or iii) ALG/MSCs with delivery rate of 0.5 μ l/min was performed. Each injection was positioned 1mm from the spinal cord midline avoiding spinal blood vessels and applied at the depth of 1.8–2mm

from the surface of the spinal cord. The distance between injections was 1mm. After each injection, the needle was kept in the tissue for an additional 30 seconds. Rats received no antibiotic treatment after surgery.

Behavioral testing

Open field test – BBB score

Behavioral testing was performed by using BBB open field locomotor test (Basso et al. 1995), measuring recovery of locomotor functions before SCI procedure (baseline) and immediately after SCI at days 0, 7, 14, 21 post-injury and after treatment. BBB score represents 21-point open field locomotor scale, where 0 reflects no locomotion and 21 normal motor functions. Each rat was observed for 5 minutes by two blinded observers; rat's hindlimb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position were analyzed during evaluation period. The values of both hindlimbs were averaged.

Catwalk

In the present study we used the CatWalk gait analysis system (Catwalk XT version 10.0; Noldus) for more objective evaluation of hindlimb motor activity. Animals were familiarized and trained on the Catwalk glass walkway (62x11.3 cm) 2 weeks prior to surgery as previously described (Hamers 2001). Pre-operative measurements were performed one week before surgery. Then, each assessment was realized first week after surgery, when rats regained capacity of plantar stepping of the hindpaws. Three runs were analyzed from each animal in each studied period (7, 14 and 21 days after injury and at 7, 14 and 21 days after treatment). Only runs with the duration between 2–5 seconds were taken into account. The values of both hindlimbs were averaged. In our study we assessed these 8 parameters: stand duration, swing duration, swing speed, stride length, mean intensity and regularity index, which have been evaluated in previous experiments using various spinal cord injury models (Hamers et al. 2001, Vrinten and Hamers 2003, Joosten et al. 2004, Klapka et al. 2005, Kloos et al. 2005, Hendricks et al. 2006, Koopmans et al. 2007, Galvan et al. 2008). For statistical analysis one-way ANOVA followed by Tukey's post hoc tests was used.

Tissue processing and immunohistochemistry

After a 21 day survival period, animals were deeply anesthetized by intraperitoneal injection of thiopental (50 mg/kg) and transcardially perfused with 500 ml saline,

followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1M phosphate-buffer (PB). Spinal cords were removed from vertebral canals, postfixed in 4% PFA at 4°C overnight, embedded in gelatin-egg albumin protein matrix (10% ovalbumin, 0.75% gelatine) polymerized by glutaraldehyde (albumin from chicken egg white, grade II, Sigma-Aldrich) fixed in 4% PFA, and cryoprotected with 30% sucrose in 0.1M PB at 4°C. Cryostat transversal spinal cord sections (40 µm) were cut from rostral, lesion or caudal segments (each 1 cm long) and collected in 12-well plates with 0.1M PBS containing 0.1% sodium azide. For immunohistochemistry, free floating sections (40 µm) were incubated in PBS (0.1 M; pH 7.4) with 10% normal goat serum (NGS) and 0.2% Triton X-100 for 2 hours at room temperature to block non-specific reaction. Sections were then incubated overnight (4°C) with primary antibodies: mouse anti-synaptophysin (SYN; 1: 500, Merck-Millipore), mouse anti- glial fibrillary acidic protein (GFAP; 1: 1000, Merck-Millipore) rabbit anti-GAP-43 (1: 1000, Merck-Millipore), rabbit anti-Iba-1 (1: 500, Wako). Next, sections were rinsed in 0.1 M PBS and incubated with secondary fluorescent antibodies Texas Red (Alexa Flour 594) for 1 hour or fluorescein isothiocyanate (FITC) (Alexa Flour 488) at room temperature for 2 hours. For general

nuclear staining 4-6-diaminidino-2-phenylindol (DAPI; 1: 200) was added to the final secondary antibody solutions. In the final step, sections were mounted and coverslipped with Vectashield mounting medium (Vector Laboratories).

Quantification analyses

Sections were analyzed using confocal (Leica DM1500) or fluorescent microscope (Olympus BX-50) and quantification performed by ImageJ software. Five sections per animal were analyzed for each staining in rostral, lesion and caudal segments (15 sections per animal) except of GAP-43 staining in which only lesion segment was considered. Iba-1 positive microglia cells were counted manually in 5 randomly selected squares of a grid. GFAP positivity was evaluated as a percentage of black pixels in overall image. Images were first transformed into monochromatic 8-bit images and then threshold was adjusted to optimal value after visual comparison of the original images. The length of GAP-43 positive axons was measured manually in 5 sections from each animal and the result represents the average length for each experimental group.

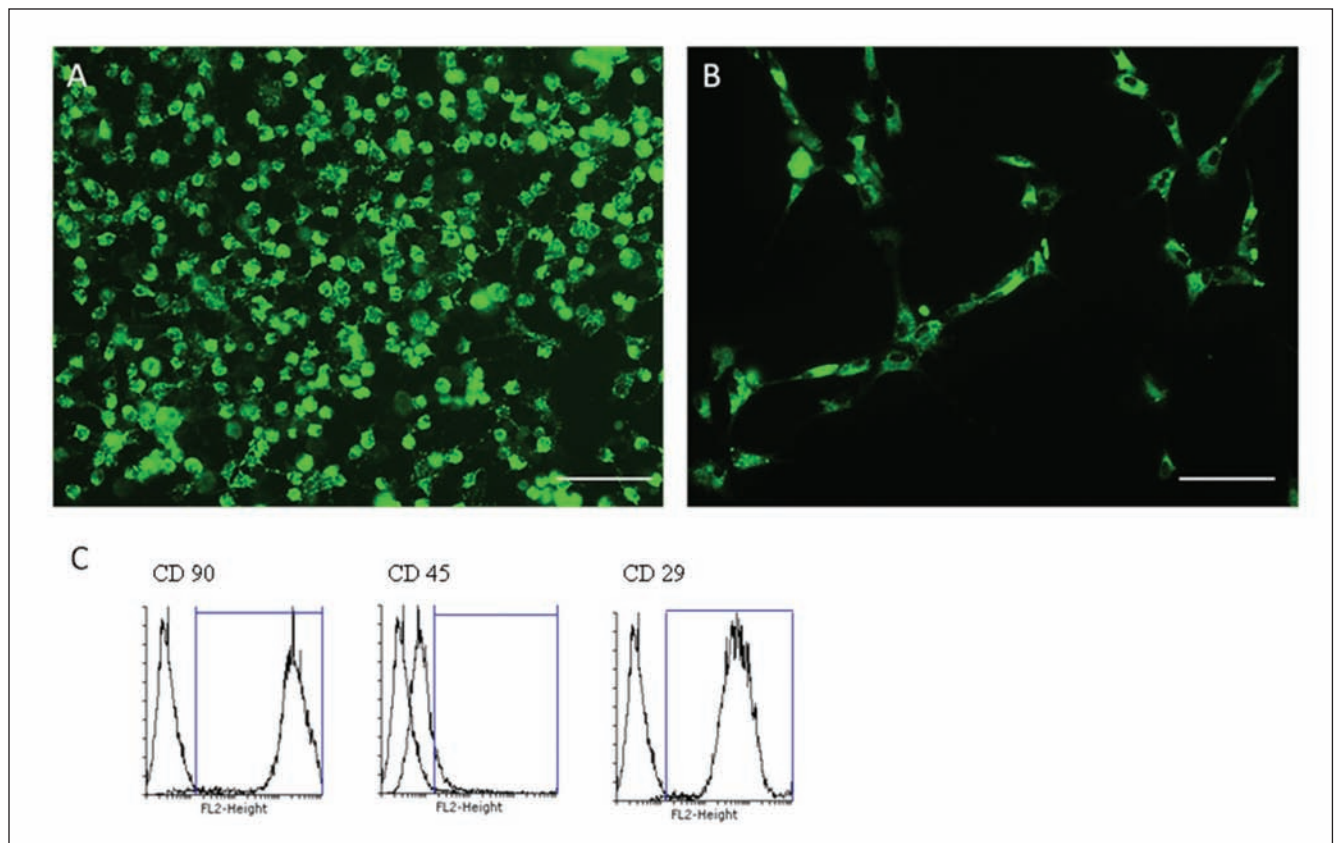


Fig. 1. PKH67 labeled floating cells after seeding in alginate *in vitro* at day of application (A) and after 3 weeks (B). Although cell density decreased in time, PKH67 signal intensity remained unchanged. Note the change in cell morphology due to cell adhesion. Scale bar 100 µm. C: Fenotypic characterization of bone marrow MSCs : expressed 99.37% CD90; 99.43% CD29 and 14.98% CD45.

The orientation of GAP-43 positive axons was analyzed by recently developed AngleJ plugin of ImageJ software (Gunther et al. 2015a) in 8-bit gray scale images at 20x magnification. Measured angles were pooled into 36 bins between -90° and 90° . The orientation of GAP-43 positive axons is expressed in a range of 180° , with 0° representing the longitudinal (rostral-caudal) axis of the spinal cord and $-90^{\circ}/90^{\circ}$ representing the mediolateral direction.

Data and statistical analysis

Data from tissue analyses and behavioral testing were reported as mean \pm SEM. Mean values among different experimental groups were statistically compared by one-way ANOVA and Tukey's post hoc tests using Graph pad PRISM software. Values of $P < 0.05$ were considered statistically significant (* P value of < 0.05 , ** P value of < 0.01 , *** $P < 0.001$).

RESULTS

Characterisation of bone marrow MSCs

Flow cytometry analysis (Fig. 1C) have shown that MSCs at passage 3 were positive for mesenchymal stem cells markers (99.37% CD90 and 99.43% CD29) and negative for hematopoietic marker CD45 (14.98%).

Durability of PKH67 staining and the presence of transplanted PKH67+ mesenchymal stem cells at lesion site

To test and compare the durability of PKH67 staining *in vitro* vs. *in vivo*, cultivation of PKH67+ MSCs was performed in parallel with the *in vivo* experiment. After 3 week cultivation in alginate biomaterial, labeled MSCs exhibited strong PKH67 positivity although cell density significantly dropped (Fig. 1A, B). *In vivo*, PKH67+ MSCs exhibited round-shaped morphology and were mostly localized in clusters with slight signs of migration from the injection site in both rostral and caudal direction (Fig. 2).

Behavioral analysis

Two behavioral tests were performed during animal survival. In all groups, progressive locomotor recovery was seen throughout the survival period. In all treated groups, significant decrease in BBB scale and change in catwalk parameter was observed after treatment procedure.

BBB score

All animals were tested for their locomotor performance in an open field test 24 hours after SCI,

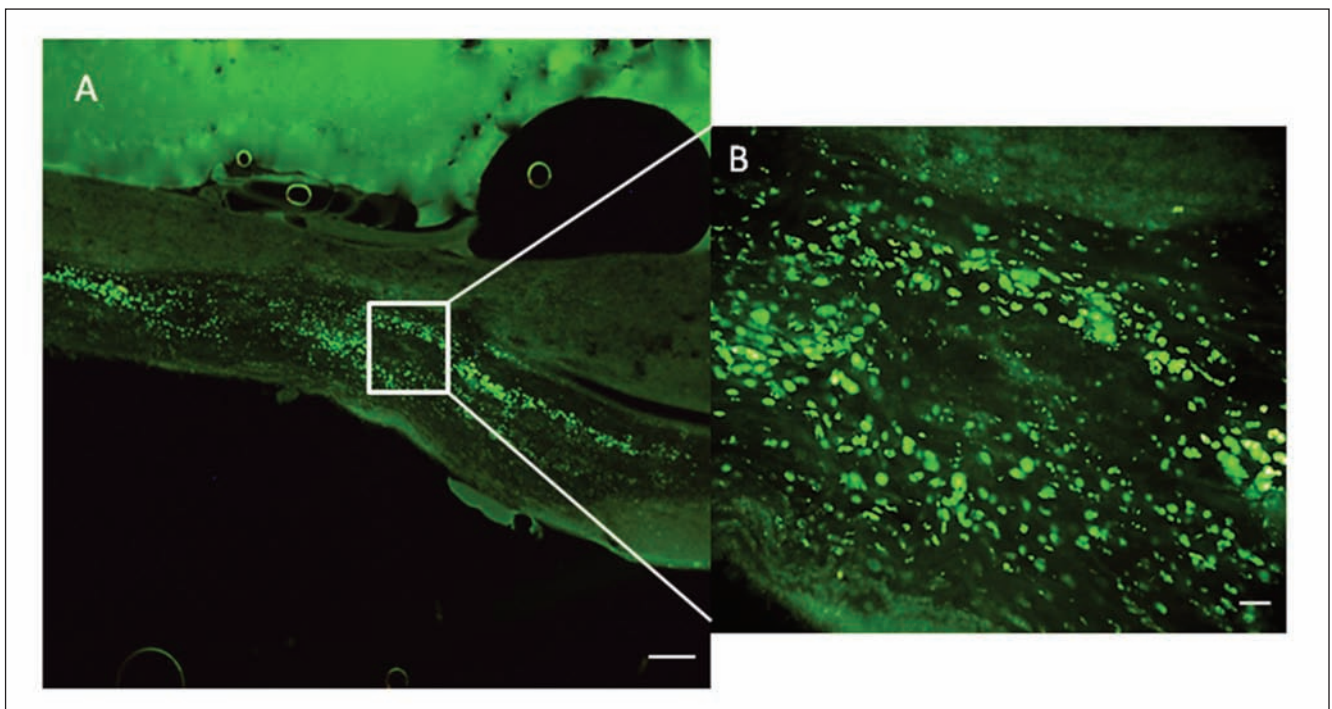


Fig. 2. (A, B) Transplanted MSCs PKH67+ cells at the lesion site three weeks after application. (A) Scale bar 200 μ m, (B) 40 μ m.

and then every 7th day. The outcomes expressed as BBB scores are shown in Fig. 3. One day after injury, compression caused total hindlimb paralysis with no movement. Following 21 days, animals in all three groups with injury showed similar gradual recovery of locomotion together with improved urination. On the 21st day, before application of either alginate or alginate with MSCs animals showed BBB score without significant difference between groups (*sci/saline* 12±1.4; *sci/Alg* 12.5±0.8; *sci/Alg/MSCs* 13±3.8; $p=0,806$). 7 days after treatment we found decrease in BBB scale (*sci/saline* 9±1.4; *sci/Alg* 9.5±2.1; *sci/Alg/MSCs* 10±5.1; $p=0,892$) due to treatment procedure which was typical for all animals. In next two weeks, animals showed gradual improvement of locomotion but without significant differences between groups. For each time point, one-way ANOVA followed by Tukey's post hoc tests was used.

Catwalk

Following gait parameters were evaluated in this study (Fig. 4):

Stand duration - the time (s) of contact of the hindpaw with the glass surface of the instrument. This parameter remained relative settled in *intact group* and without significant changes in *sci/Alg* or *sci/alg/MSCs* treated rats. The temporary increase in *sci/saline* (0.368 ± 0.02 s) group at 7 days after SCI was followed by gradual decrease with significant difference ($p=0.013$) at 28 (0.22 ± 0.09), 35 (0.17 ± 0.07) and 42 (0.19 ± 0.09) days after SCI compared to the 7 day interval. At 7 days after SCI, the values of stand duration were highest in *sci/saline* group when compared to *sci/Alg* or *sci/alg/MSCs* treated rats, however without significant differences (*intact control* 0.26 ± 0.08 , *sci/saline* 0.36 ± 0.02 ; *sci/Alg* 0.28 ± 0.08 ; *sci/Alg/MSCs* 0.28 ± 0.09 ; $p=0.834$). Alginate and alginate/MSCs group did not show any significant

fluctuations in stand duration parameter during the whole studied period.

Swing duration - the time (s) that the hindpaw is not in the contact with the glass surface. This parameter did not show any significant changes between groups or in time perspectives.

Swing speed - a ratio of stride length and swing duration reflecting the speed (mm/s) of the paw during swing phase. Here we found higher values in *sci/saline* group starting 14 days after SCI and continuing up to 42th days when compared to *sci/Alg* or *sci/alg/MSCs* animals. Significant difference between *sci/saline* and *sci/Alg/MSCs* rats was observed at 28days (*sci/saline* 148 ± 24.4 ; *sci/Alg/MSCs* 95.54 ± 21.33 ; $p=0.015$) and 42 days (*sci/saline* 148 ± 24.4 ; *sci/Alg/MSCs* 94.54 ± 16.75 ; $p=0.003$) after SCI.

Stride length - the distance (cm) between two subsequent placements of the particular hindpaw. In this gait parameter *sci/saline* animals exhibited the highest values in all studied periods. At 42 days we found significant difference between *sci/saline* versus *sci/Alg/MSCs* (12.52 ± 2.63) animals ($p=0.009$). In *sci/Alg* and *sci/Alg/MSCs* animals, the values of stride length remained relative stable and unchanged during the whole experiment.

Mean intensity - the mean intensity of paw contact with glass surface in pixels. This parameter did not exhibit any significant differences between groups after SCI or after treatment. Slight non-significant increase was observed at 21, 28 and 35 day in *sci/Alg* rats.

Regularity index - the number of normal step sequence patterns (%) relative to the total number of paw placements, reflects the degree of interlimb coordination during gait. It is described as a ratio between normal step sequence patterns and the total number of paw placements during gait. In healthy animals the value of regularity index is close to the 100% (Hamers et al. 2001). All groups of animals had regularity index within the range of 96-98% before the injury (*intact control* 96 ± 10 ; *sci/saline* 98.72 ± 9.85 ; *sci/Alg* 96.81 ± 1.63 ; *sci/Alg/MSCs* 97.51 ± 2.08). Spinal cord injury caused significant decrease of interlimb coordination in all experimental groups which was obvious at first week after the impact. We found significant decrease at 7 days after injury in *sci/saline* (79.2 ± 10.7 ; $p=0.03$), *sci/Alg* (77.83 ± 12.82 ; $p=0.04$) and *sci/Alg/MSCs* (72.1 ± 10.1 ; $p=0.06$) rats. At 14 days we observed slight non significant increase suggesting regeneration. However treatment with alginate and alginate/MSCs also had an influence on this parameter. At 21 days after SCI, i. e. the first week after treatment we found significant decrease in *sci/Alg/MSCs* (73.54 ± 8.26 ; $p=0.006$) animals and at 35 days after SCI regularity index was decreased in both *sci/Alg* (78.15 ± 10.7 ; $p=0.04$) and *sci/Alg/MSCs* (74.43 ± 15.94 ; $p=0.06$).

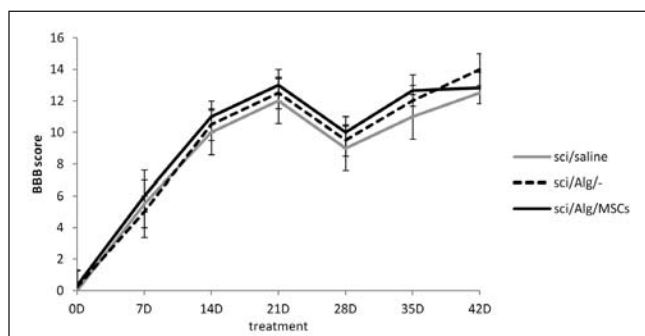


Fig. 3. BBB score. Gradual recovery of hindlimbs functions. Only small non significant improvement was observed in groups treated with alginate or alginate/MSCs. For each time point, one-way ANOVA followed by Tukey's post hoc tests was used.

Immunohistochemistry

Axonal outgrowth and orientation

For evaluation of axonal regeneration at the lesion site (L „segment“), we used GAP-43 antibody. In all an-

imal groups, with the exception of intact control rats, we observed GAP-43 positive fibers distributed in all extent of the L segment predominantly in the proximity of cavities. GAP-43 positive axons never spread through cavities with lacking spared tissue. However, with in all groups with GAP-43 positive axons, we observed

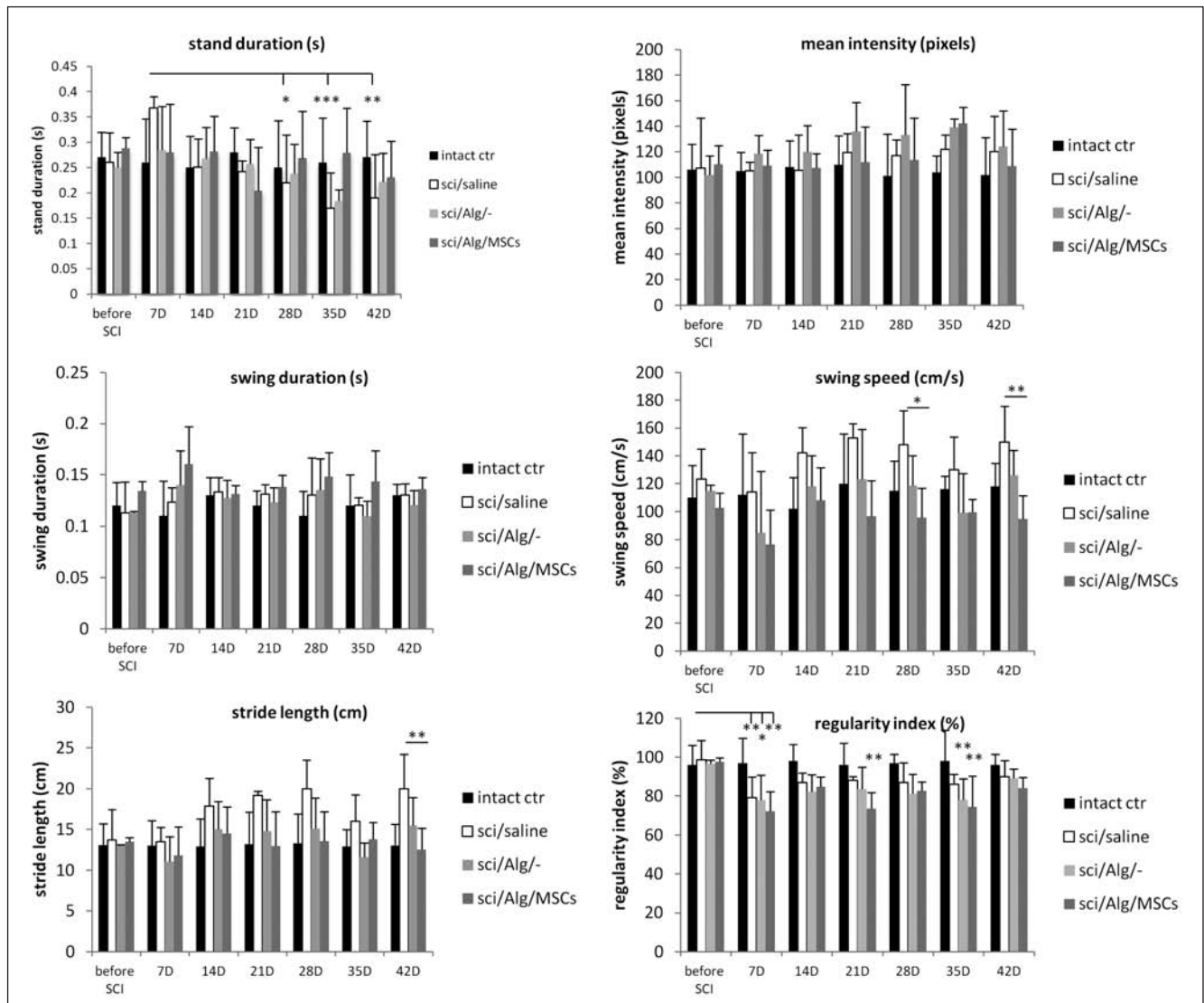


Fig. 4. Catwalk gait analysis of hindlimb locomotion. We have evaluated six gait parameters which have been previously described as relevant for spinal cord injury: *Stand duration* – the time (s) of contact of the hindpaw with the glass surface; Alginate and alginate/MSCs group did not show any significant fluctuations during the whole studied period. Only significant differences were found in sci/saline animals at 28, 35 and 42 days after injury when compared to values at 7 day which were transiently increased. *Swing duration* – the time (s) that the hindpaw is not in the contact with the glass surface; This parameter did not show any significant changes or intergroup differences in any time interval. *Stride length* – the distance (cm) between two subsequent placements of the particular hindpaw; Here we found significantly higher value at 42 days in rats injected with saline compared to alginate/MSCs treated animals. However, saline animals had this parameter higher during majority of studied intervals. *Swing speed* – a ratio of stride length and swing duration reflecting the speed (mm/s) of the paw during swing; At 28 and 42 day after SCI, rats in saline group showed significantly higher values compared to Alg/MSCs treated animals. Similarly to the stride length parameter, here saline rats also exhibited relative high values during whole experiment. *Mean intensity* – intensity of paw contact with glass surface in pixels; Here, only slight non-significant increase was observed at 21, 28 and 35 day in alginate treated rats. *Regularity index* – the number of normal step sequence patterns (%) relative to the total number of paw placements, reflects the degree of interlimb coordination during gait. Here we found significant drop in all groups with SCI on the first week after injury. After transient recovery, regularity index decreased again as a consequence of alginate or alginate/MSCs treatment – 21 and 35 days after SCI. Data are represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Tukey-Kramer test.

remarkable variability between animals. Interestingly, quantification (Fig. 5) has revealed that the highest extent of outgrowing axons was present in *sci/Alg/MSCs* ($5967.52 \pm 1059 \mu\text{m}$) which was significantly higher ($p < 0.001$) than in rats with alginate ($1700.5 \pm 390.8 \mu\text{m}$) or *sci/saline* ($1647.6 \pm 386.75 \mu\text{m}$). GAP-43 positive axons clearly spread into the lesion site often in close proximity to transplanted cells. Analysis of axonal outgrowth orientation (Fig. 6) showed that most GAP43+ axons had tendency to grow in rostrocaudal orientation with 29% of axons angles within range of $\pm 10^\circ$ and 48% within range of $\pm 20^\circ$ in group with transplanted cells; 28% of axons angles within range of $\pm 10^\circ$ and 47% within range of $\pm 20^\circ$ in *sci/Alg* group and 28% of axons angles within range of $\pm 10^\circ$ and 46% within range of $\pm 20^\circ$ in rats treated with saline.

Quantification of astrocytes

GFAP positive cells (Fig. 7 A-H) were quantified separately in rostral (R), lesion (L) and caudal (C) segments in both the grey (Fig. 7 I) and white matter (Fig. 7 J). When comparing R, L and C segments in the grey matter, we observed slightly increasing, but not significant GFAP positivity in rostro-caudal direction in all groups of animals. Regarding individual segment differences at rostral segment, in all groups with SCI (*sci/saline* 26 ± 1.71 ; *sci/Alg/-* 29.67 ± 3.5 ; *sci/Alg/MSCs* 28.5 ± 5.3) there was highly significant ($p = 0.009$; $p = 0.0009$) increase in GFAP density compared to *intact control* (18.5 ± 1). The same pattern was found at lesion and caudal segment. At lesion segment GFAP expression significantly ($p = 0.048$; $p = 0.0009$) increased in SCI

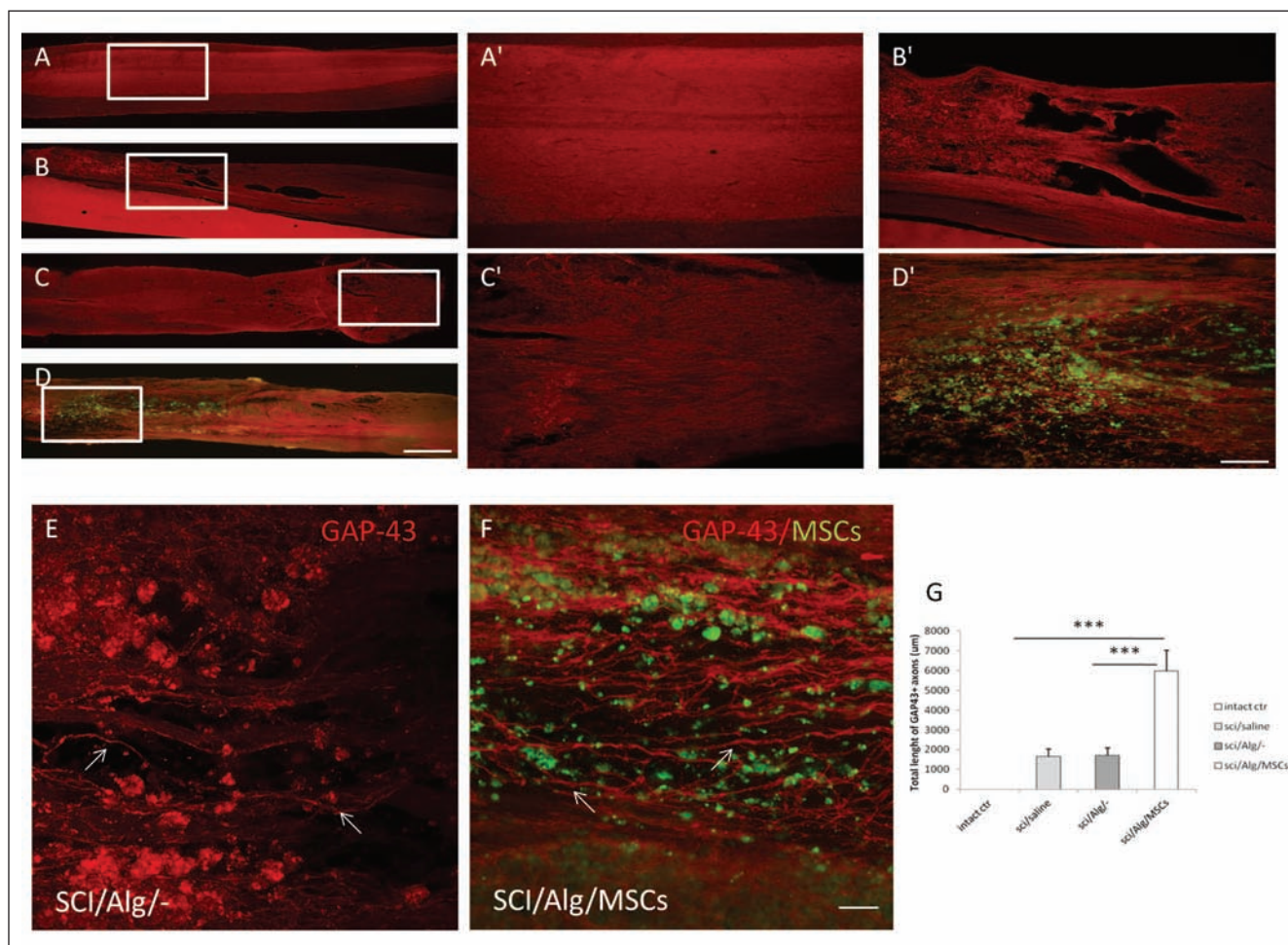


Fig. 5. Quantification of GAP-43 positive axons at the lesion site in three experimental groups with SCI and intact control. Intact animals showed no GAP-43 positivity within the whole extent of the spinal cord (A-A'). The highest axonal sprouting was found in the group treated with both alginate and MSCs (D-D', F). The similar expression of GAP-43 was found in the group treated with saline and in the group with alginate (B-B',C-C', E). (G) total length of GAP-43 positive axons among experimental groups. Data are represented as mean \pm SEM. *** $P < 0.001$, one-way ANOVA followed by Tukey-Kramer test. Figs (A-D) (whole spinal cord sections) scale bar 1000 μm , (A'-D') (magnified areas from A-D) scale bar 200 μm . (E-F) representative pictures of GAP-43 positive axons in two experimental groups, scale bar 25 μm .

animals (*sci/saline* 28.1 ± 1 ; *sci/Alg/-* 35.5 ± 6.02 ; *sci/Alg/MSCs* 34.6 ± 5.74) comparing to *intact control* (19.3 ± 2.44) which was similar to caudal segment (*intact control* 20.3 ± 2.97 ; *sci/saline* 29.1 ± 2.1 ; *sci/Alg/-* 35.4 ± 8.1 ; *sci/Alg/MSCs* 39.7 ± 7.63 ; $p=0.046$). In the white mater we did not find any significant change in GFAP expression after SCI or treatment except of *sci/saline* group (36.6 ± 4.7) versus *intact control* group (17.7 ± 4.01 ; $p=0.006$) in the lesion segment and *sci/saline* vs. *sci/Alg/-* (21.3 ± 6.3 ; $p=0.009$).

Microglia quantification

Similarly to astrocytes quantification, number of Iba-1 positive microglia was evaluated in R, L and C segments of the spinal cord in both grey and white matter. In all three segments, it was obvious that injury caused increase in the number of microglia cells in the grey matter (Fig. 8 H) in each group with trauma. At rostral segment we observed significant ($p=0.03$) increase between *intact control* (3 ± 1) and *sci/Alg/-* (14.14 ± 2.11). Lesion segment exhibited the highest number of Iba-1 microglia compared to rostral and caudal segments. Here, we found significant increase in all three groups

with trauma (*sci/saline* 25 ± 7.44 ; $p=0.0007$), (*sci/Alg/-* 21.8 ± 3.78 ; $p=0.0059$), *sci/Alg/MSCs* (17.8 ± 8.42 ; $p=0.018$) compared to *intact controls* (5.6 ± 1.15). Similar pattern was observed at the caudal segment, where significant increase was detected in *sci/saline* (17.25 ± 4.79 ; $p=0.014$) and *sci/Alg/-* (21.6 ± 7.77 ; $p=0.0008$) in comparison to *intact control* (4 ± 1). In the white mater (Fig. 8 I) we found significant differences between *intact control* (3.3 ± 1.53) and *sci/Alg/-* (12.67 ± 3.83 ; $p=0.0007$) at the lesion segment and between the same groups at the caudal segment (*intact control* 4 ± 1 ; *sci/Alg/-* 15.83 ± 4.3 ; $p=0.0007$).

DISCUSSION

Axonal outgrowth

In this study we investigated both behaviorally and immunohistochemically the effects of alginate or alginate/MSCs treatment on injured spinal cord in rats. One of the key parameters studied here was the outgrowth of regenerating axons, which we evaluated in the lesion segment using the GAP-43 antibody. In accord with previous reports (Curtis et al. 1993, Novotna et al. 2011) we did not

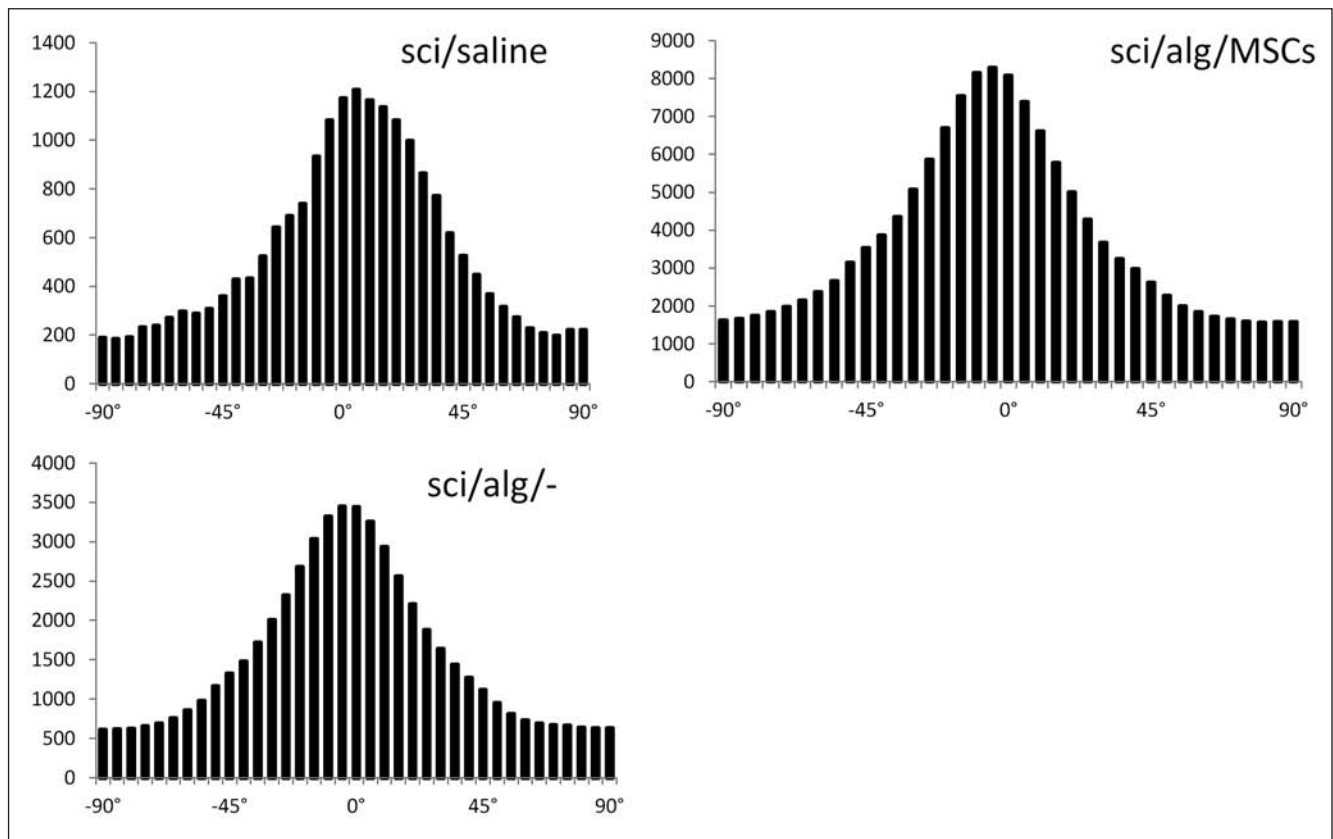


Fig. 6. Orientation of regenerating GAP-43 positive axons at the lesion site. Axons in all three groups of animals exhibiting GAP-43 positivity are oriented mostly in longitudinal (rostrocaudal) direction.

detect any GAP-43 positive axons in the group of intact rats. On the other hand, in all groups with SCI we found relatively high levels of GAP-43 positivity. This finding confirms that the expression of this protein is closely related to damage of the neural tissue, or possibly with endogenous regenerative processes (Linda et al. 1992). The highest presence of axonal outgrowth was found in animals in which the combination of biomaterial and MSCs was applied. Numbers of GAP-43 positive axons in groups with alginate alone or saline were approximately at the same level. The common sign in all animal groups with the presence of regenerating axons was that GAP-43 positive fibers were exclusively present in those parts of the

lesion where at least a small amount of tissue remained. In other words, axons did not exhibit any signs of outgrowth through the hollow cavity, but were present only along its margins. In the case of spinal cords treated with MSCs, we found regenerating axons also in the proximity of these cells, suggesting a permissive environment at least partially caused by these cells. This is supported by the findings of other authors reporting potential positive effects of MSCs on axonal regeneration (van Velthoven et al. 2010). A common phenomenon however, was relatively high variability between individual animals within groups. Considering biomaterials, other studies (Teng et al. 2002, Park et al. 2010) demonstrated that treatment

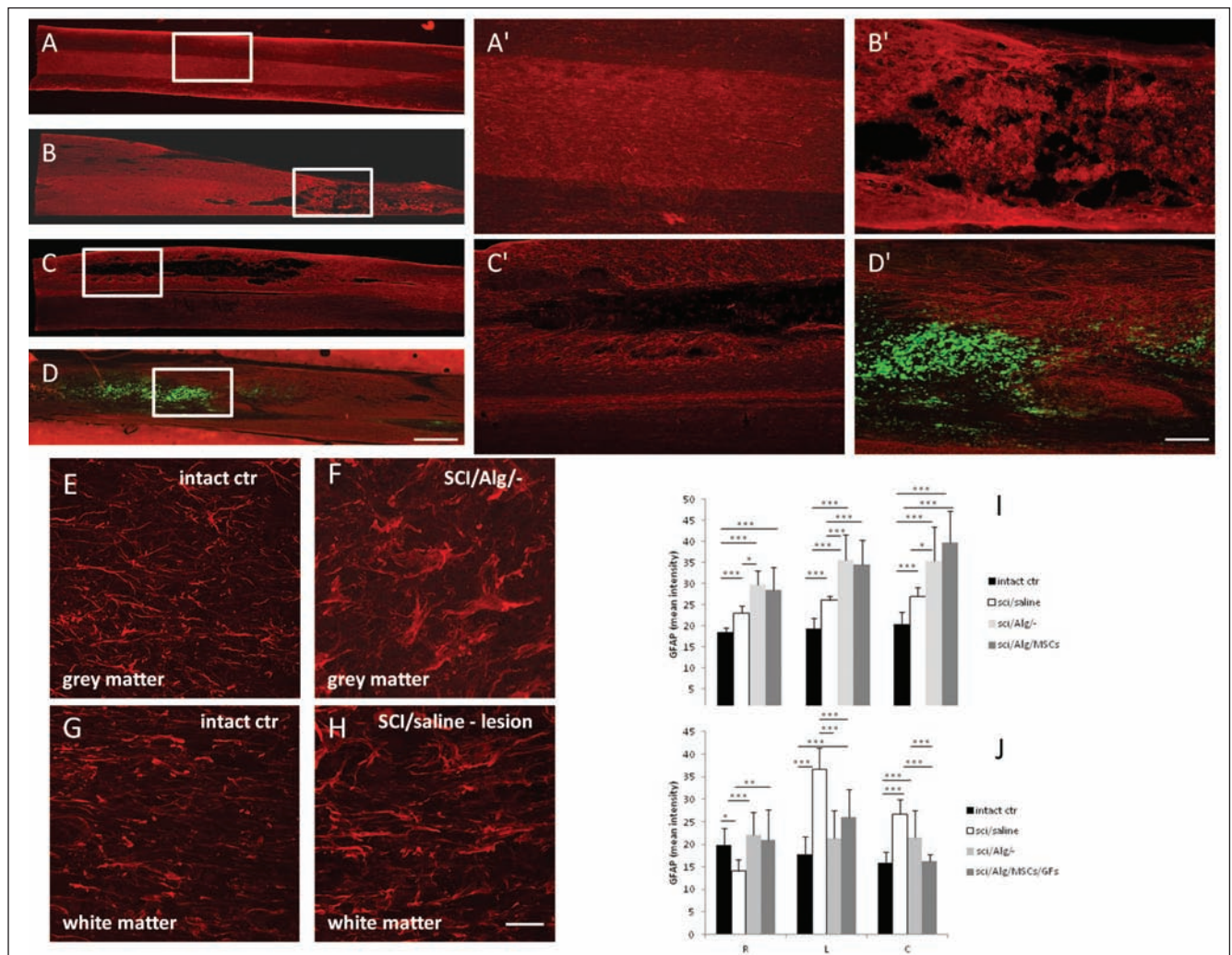


Fig. 7. Quantification of astrocytes in the rostral, lesion and caudal segments of the spinal cord in the grey (I) and in the white matter (J) in intact (A-A'), saline (B-B'), alginate (C-C') and alginate/MSCs group (D-D'). When comparing R, L and C segments in the grey matter, we observed gradually increasing, although not exclusively significant GFAP positivity in rostro-caudal direction in all groups of animals. In the white matter, the reaction of astrocytes appeared as less prominent after SCI in R and C segments. Significant increase in GFAP expression was observed only in the L segment of saline treated group when compared to control values. Data are represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Tukey-Kramer test. (E-H) representative pictures of GFAP positive cells in the grey and white matter of intact and injured animals (scale bar 25 μ m). Figs (A-D) (whole spinal cord sections) scale bar 1000 μ m, (A'-D') (magnified areas from A-D) scale bar 200 μ m.

with cells or other components seeded on an alginate scaffold increased GAP-43 expression as well as producing significant functional improvement. According to our observations, despite evidently increased axonal sprouting in the group treated with both alginate and MSCs, increased GAP-43 expression as a positively accepted indicator was not reflected in the behavioral improvement. In our future efforts, the question of axonal guidance through the modification of biomaterial physical properties should be taken into account (Gunther et al. 2015b).

Reaction of astrocytes

Reactive astrogliosis, i.e. the reaction of astrocytes to SCI, is well documented (Fitch and Silver 2008).

Besides its protective effect in the early stages of injury, it is believed that glial scar formation prevents later tissue regeneration (Silver and Miller 2004). It has been reported that MSCs therapy after SCI leads to reduction of astrocytes proliferation and inhibition of glial scar formation (Abrams et al. 2009; Voulgari-Kokota et al. 2012). Here, we performed quantification of astrocytes not only in the lesion segment, but also in the segments above (rostral) and below (caudal) the central injury. Similarly to previous findings, we found that animals treated with alginate or alginate/MSCs showed slightly increased GFAP positivity in all three studied segments in the grey matter, but decreased positivity in the white matter of the lesion segment. Thus the therapy used in this study had only small influence on glial scar formation reflected in

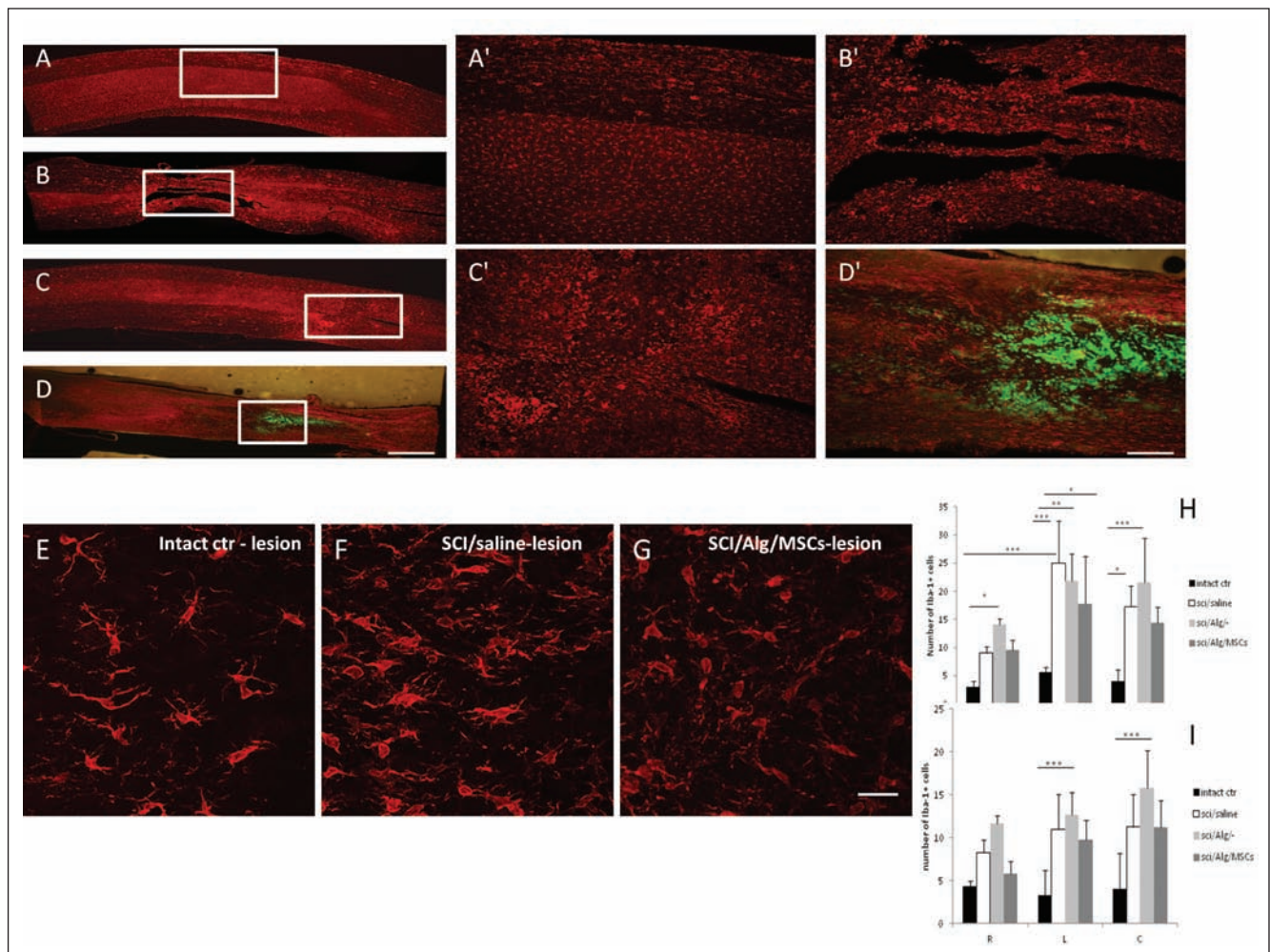


Fig. 8. Quantification of microglia in the rostral, lesion and caudal segments of the spinal cord in the grey matter. SCI caused increase in the number of microglia cells in the grey matter in each group with spinal trauma (B-B', C-C', D-D') compared to intact control animals (A-A'), however without significant difference between these groups in both grey and white matter (H, I). (E-G) Representative pictures of Iba-1 positive microglia showing different morphology and density of cells after injury. Data are represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Tukey-Kramer test. Scale bar (A-D) 1000 μ m, scale bar for (A'-D') 200 μ m. Scale bar (E-G) 25 μ m.

GFAP expression. It is however questionable whether the suppression of glial scar is “good or bad”, since recent studies suggest that the role of astrocyte scar formation may be important in the process of axonal regeneration (Anderson et al. 2016).

Reaction of microglia

The microglia, as a key player in CNS immunity, plays a crucial role in the inflammation process by releasing inflammatory cytokines, nitric oxide and other molecules (Aloisi 2001, Block and Hong 2005). There is some evidence that another positive effect of MSCs therapy lies in its capacity to down-regulate microglia activity (Kim et al. 2009, Yan et al. 2013). Our results show that SCI clearly causes increase in the number of microglia cells and change in their morphology. This was observed in both the grey and white matter of all three studied segments. Kim (2009) found that after co-cultivation of LPS-activated microglia with MSCs, the number of microglia cells was significantly reduced after a short time period (6–24 hours). On the other hand, *in vivo* experiments with longer survival time have shown that MSCs could increase Iba-1 positive cell expression (Lee et al. 2012). In our study we could not see any significant effect of alginate/MS therapy on the number of microglia cells. This could probably be because of single and not repeated application of cells or a different experimental model of SCI.

Behavioral outcomes

Several studies have produced evidence that treatment with MSCs causes locomotory improvement which is a crucial criterion in the evaluation of therapy efficacy (reviewed in (Oliveri et al. 2014)). On the contrary, according to more recent research, functional effect of MSCs is very limited or lacking (Brock et al. 2015, Sandner et al. 2016). In any case, it is important to mention that there is not always a correlation between histological and behavioral outcomes. Some studies have yielded evidence that better locomotory scores were accompanied by improved histological outcomes such as axonal outgrowth (Cizkova et al. 2006, Hu et al. 2010). On the other hand, it has also been demonstrated that increased expression of markers for axonal outgrowth, in the case of GAP-43, is not always linked with functional recovery, and *vice versa* (Oestreicher et al. 1997). In our study we combined classical BBB-score open field testing with the more recently developed computer-assisted Catwalk gait analysis system (Hamers et al. 2001). Based on

previous studies (Joosten et al. 2004, Hendricks et al. 2006, Koopmans et al. 2007), we chose six gait parameters which could be relevant for evaluation of locomotory behavior after SCI. Reflecting our histological data, neither the BBB-score nor the Catwalk analysis showed relevant significant differences between experimental groups. In fact, the high variability between individual animals did not allow any conclusion about the positive effect of therapy at a behavioral level. Spinal cord injury is a very complex pathological condition, which is to a large extent given by the complexity of the nervous system itself. One of the future therapeutic approaches could be based on the utilization of CNS endogenous regenerative potential by supporting axonal regeneration and overall recovery of lost cell populations. However, an important step for such a challenge is to provide a hospitable environment in which these changes could take place. From this point of view, biomaterials such as alginate represent an interesting tool with high potential. In this study we have shown that the application of mesenchymal stem cells seeded on alginate hydrogel produces a supportive environment for endogenous regenerative processes which are reflected in increased axonal outgrowth.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

This article does not contain any studies with human participants performed by any of the authors.

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AUTHOR CONTRIBUTIONS

DC have got the funding for the project; JB, DC have written the paper; JB, JK have done *in vivo* experiments; ES, LS have done *in vitro* experiments.

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