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Anti-inflammatory compound curcumin and mesenchymal stem cells in the treatment of spinal cord injury in rats

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Spinal cord injury leads to a robust inflammatory response that is an unfavorable environment for stem cell implantation. In this study, we evaluated the effect of combined therapy of curcumin and mesenchymal stem cells (MSC) on behavioral recovery and tissue sparing, glial scar formation, axonal sprouting and inflammatory responses in a rat experimental model of spinal cord injury (SCI). Balloon-induced compression lesion was performed at thoracic (Th8-9) spinal level. Out of the four groups studied, two groups received curcumin on the surface of the spinal cord immediately after SCI and then once a week for 3 weeks together with an intraperitoneal daily curcumin injection for 28 days. The other two groups received saline. Seven days after SCI, human MSC were intrathecally implanted in one curcumin and one saline group. Both curcumin and curcumin combined with MSC treatment improved locomotor ability in comparison to the saline treated animals. The combined treatment group showed additional improvement in advanced locomotor performance. The combined therapy facilitated axonal sprouting, and modulated expression of pro-regenerative factors and inflammatory responses, when compared to saline and single treatments. These results demonstrate that preconditioning with curcumin, prior to the MSC implantation could have a synergic effect in the treatment of experimental SCI.

Key words: spinal cord injury, human mesenchymal stem cells, curcumin, combined therapy, inflammatory response

INTRODUCTION

Spinal cord injury (SCI) leads to permanent deficits in sensory and locomotor functions below the injured spinal cord segment. The primary mechanical impact causes tissue necrosis and rupture of neuronal and vascular structures (LaPlaca et al. 2007). As a consequence of the primary damage, secondary injury processes, including ischemia, glutamate excitotoxicity and local immunologic responses occur. The extent of secondary processes in incomplete injuries dictates the final severity of the injury. Thus, targeting these secondary processes is a potential opportunity to reduce disability after the initial injury is sustained (Anthony and Couch 2014, Lee et al. 2010, Wang et al. 2014a). Mesenchymal stem cells have shown their pro-regenerative and neuroprotective properties in several experimental models of SCI, as well as in models of Alzheimer's disease and amyotrophic lateral sclerosis (Cocks et al. 2013, Forostyak et al. 2014, Kuroda et al. 2011, Ritfeld et al. 2012, Ruzicka et al. 2016). Their effect is attributed to paracrine action, mediated by growth factors, anti-apoptotic and anti-inflammatory molecules (Hawryluk et al. 2012, Teixeira et al. 2013). Worldwide, MSCs are already being used in clinical trials in the treatment of SCI and neurodegenerative diseases (Callera and do Nascimento 2006, Forostyak

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et al. 2013, Geffner et al. 2008). To facilitate MSC regenerative and protective potential, an appropriate combined therapy must be used to support MSC survival.

Curcumin also known as diferuloylmethane, (1*E*,6*E*)-1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) is a well-known anti-inflammatory compound with described neuroprotective properties, which has already shown a positive effect on recovery after experimental SCI (Machova Urdzikova et al. 2015, Ormond et al. 2012, Sanli et al. 2012, Wang et al. 2014b, Yuan et al. 2015). Following curcumin application, increased behavioral recovery after SCI has been observed (Machova Urdzikova et al. 2015, Ormond et al. 2012, Zu et al. 2014). Curcumin is a natural inhibitor of major inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1), IL-8 and monocyte chemoattractant protein 1 (MCP-1) (Hidaka et al. 2002, Jain et al. 2009, Yuan et al. 2015). Curcumin application after SCI or TBI resulted in a suppressed inflammatory response via a TLR4-MyD88-NFkB dependent pathway, and thus indirectly affected the microglial activation and neuronal apoptosis after injury (Ni et al. 2015, Zhu et al. 2014). The NFκB pathway has a strong correlation with TNFα and IL-1 levels after injury (Yuan et al. 2015). Additionally, curcumin affects STAT-3 and NFκB pathways by influencing NO levels and reducing astrogliosis after SCI (Gokce et al. 2016, Machova Urdzikova et al. 2015, Sanivarapu et al. 2016, Wang et al. 2014b). Moreover, curcuminoids display the potential to decrease neuropathic pain via antagonizing TRPV1 channels (Lee et al. 2013). We hypothesize that the pre-treatment of injured spinal cords with curcumin, prior to MSC implantation, might reduce the existing inflammatory environment, improving the survival of implanted cells and thus increasing the potential effect of the cell therapy.

The main objective of this study was to investigate whether the combined treatment of curcumin and MSC will have a synergistic effect in the treatment of the balloon compression model of SCI. Locomotor and sensory recovery, spinal cord tissue preservation, glial scar formation, axonal sprouting, relative expression of genes related to regenerative processes, and cytokine levels were evaluated, in both single and combined therapy, to determine the underlying mechanisms of the effect of curcumin, MSC, or their combination, on the development of SCI.

METHODS

Animals

One hundred and thirty five Wistar rats (3 months old) were used for the purpose of this study. The operation weight was set at 300 ± 15 g, in order to standardize the lesion size. All experiments were performed in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU), regarding the use of animals in research and were approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic (approved under project number 35/2012).

As an experimental model of SCI, the balloon compression lesion was used (Vanicky et al. 2001). Body temperature was maintained at 37°C during the procedure. After induction of anesthesia (Isoflurane 300 mL/min, 3% Furane, AbVie, Prague, Czech Republic) a 2-French Fogarty catheter was inserted into the epidural space (through the laminectomy of Th10), and positioned in the midline of Th8 segment. The balloon was inflated with 15 µL of saline for 5 min to induce severe compression injury. Animals received antibiotics (Gentamicine, 20 mg/mL, 50 µl i.m., Sandoz, Prague, Czech Republic) and painkillers (Rimadyl, 50 mg/mL, 50 µl i.m., Zoetis, Prague, Czech Republic) immediately after lesion induction. Animals were randomly assigned to the four treatments groups; saline (n=34), curcumin (n=27), MSC (n=28), curcumin + MSC (n=26).

Animals receiving curcumin (curcumin, Sigma-Aldrich, St. Louis, MO, USA) were given a high dose once a week (60 mg/kg diluted in olive oil) intrathecally 4 times (immediately after SCI followed by 3 subsequent weeks), and a low dose intraperitoneally (6 mg/kg diluted in olive oil) daily, starting immediately after the injury and finishing on the 28th day after SCI. One week after SCI, the animals received intrathecal (area of L4-5 segment) injections with either human bone marrow mesenchymal stem cells (MSC, 5×10^5/50 µL) or saline (50 µL). Prior to and following the SCI, the first set of treated animals; saline (n=19), curcumin (n=12), MSC (n=13), curcumin + MSC (n=11) underwent a weekly series of behavioral and sensory tests. Nine weeks after SCI, the animals were sacrificed and histological, immunohistochemical and qPCR analyses were performed on spinal cord tissue (2cm long segment, with lesion area in the middle). Additionally, the second set of animals were sacrificed at specific time points (10, 14 and 28 days after SCI) to determine the effect of the treatment on the immune response after SCI (n=5/group/time point). All treated animals were given daily i.p. doses of immunosuppression starting three days before MSC injection until the end of the study (Cyclosporine A, 10 mg/kg, Sigma-Aldrich, Germany) to prolong survival of the implanted stem cells.

The third set of animals was used to determine the survival of implanted stem cells at 3, 7, 14, 21 and 28 days after implantation (n=2 per group per time point). The last survival period was the end of the study (56 days post transplantation; Fig. 1).

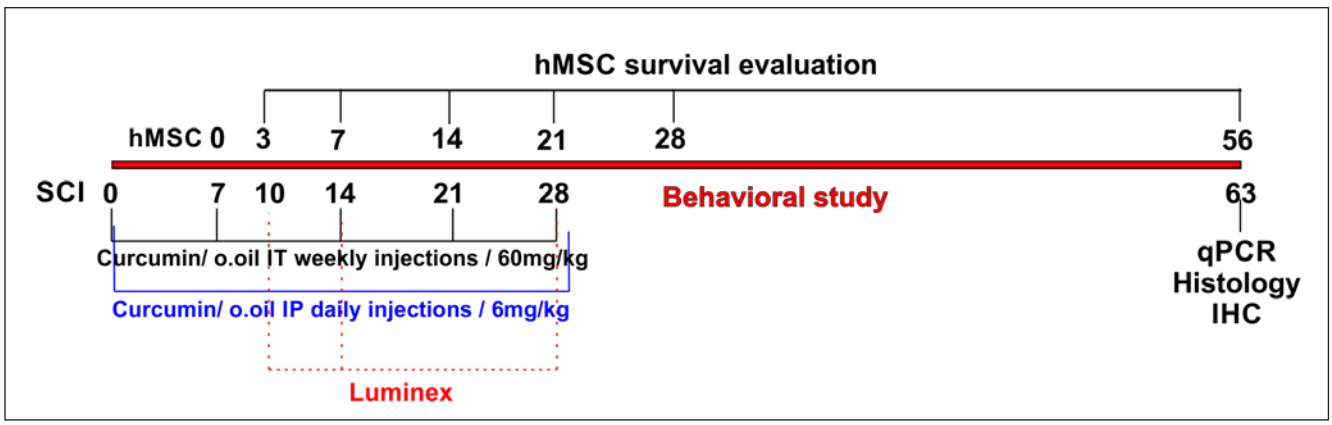

Fig. 1. The flowchart shows the days of analysis after spinal cord injury (SCI; 0-63) or human mesenchymal stem cell (hMSC; 0-56) intrathecal (IT) administration. Curcumin was injected IP for 28 days consecutively and once a week IT starting at day 0 of the SCI. The scheme indicates time points of PCR, LUMINEX, histology and the behavioral part of the study. IHC- Immunohistochemical staining, IP- intraperitoneal, IT- Intrathecal, MSC- mesenchymal stem cells, SCI- Spinal cord injury.

Behavioral testing

A series of locomotor and sensory functional tests were performed before SCI and then weekly for the entire experimental period. All animals were preconditioned and trained prior to the SCI.

BBB test

The open field basic locomotor ability was evaluated using the Basso, Beattie, Bresnahan (BBB) test scale (Basso et al. 1995). Two independent examiners studied the animal locomotor performance for 4 minutes, once a week, starting the week after SCI. Each hindlimb was evaluated separately. The average value of both hindlimbs was used for analysis.

Flat beam test

To evaluate advanced locomotor function, and, in particular, weight support and forelimb-hindlimb coordination, the flat beam test with a modified Goldstein scale (Goldstein 1997) was used. The rats were scored from 0–7, ranging from no ability to balance on the beam, to crossing the whole length of the beam properly using both hindlimbs. The test apparatus consisted of a 140 cm long device. The animals had to cross a central 1 m segment to reach a familiar darkened escape platform within 60 s intervals. This test was performed every second week for three consecutive days, with two trials per day. The average latency of performance was recorded using a video tracking system (TSE-Systems Inc., Bad Homburg, Germany)

Rotarod test

Highly advanced locomotor function including long term weight support and hindlimb coordination was evaluated on four-lane rotarod units (Carter et al. 2001) (Ugo Basile, Comercio, Italy), consisting of 7 cm diameter drums with grooves. As pre-training, a 5-minute interval of running exercise on an accelerating rod (5 rpm-10 rpm) was performed daily for five consecutive days prior to the injury. Rats with SCI underwent four trials (each max 60 s) with a constant speed of 5 rpm in every testing session. The latency to fall from the rotating rod was automatically recorded. The test was performed before surgery and 3, 5, 7 and 9 weeks after surgery.

Plantar test

A standard Ugo Basile Apparatus (Carstens and Ansley 1993) (Ugo Basile, Comercio, Italy) was used for the plantar (hot plate) test. The rats were always placed in separate semi-transparent acrylic boxes, which protected them from watching each other, and a mobile device with an infrared heating lamp was positioned below the targeted hindlimb paw, always within the same region. Latency in withdrawal response to thermal nociceptive stimulus was automatically measured. Each paw was stimulated five times, with a stable pause-measurement interval. The average value of both hindlimbs was used for analysis. Hyperalgesia was defined as a significant decrease in withdrawal latency. The latency of withdrawal of a healthy trained individual is usually from 8 to 12 s. The hyperalgesia is below 50% of the latency of a healthy individual and was defined based on the single animal time curve comparison.

Stem cell culture and preparation

Human MSCs for this experiment were prepared under good manufacturing practice (GMP) conditions and

supplied as a 1.5 mL cell suspension in Nunc tubes (Bioinova Ltd., Prague, Czech Republic; product name "Suspension of Autologous MSC 3P"). Gradient centrifugation using Gelofusine (25%, B. Braun, Melsungen, Germany) was employed to separate the mononuclear fraction containing MSCs from the bone marrow. The cell expansion was performed in media containing α Minimum Essential Medium (MEM) Eagle (without deoxyribonucleotides, ribonucleotides and UltraGlutamin (Lonza, Basel, Switzerland)), supplemented with 5% mixed allogeneic thrombocyte lysate (BioInova, Prague, Czech Republic) and 10 μg/mL gentamicin (Lek Pharmaceuticals, Ljubljanja, Slovenia). For the purpose of the transplantation, cells from the second passage were analyzed and used. The expression of specific surface markers was detected with fluorescent-activated cell sorting (FACS) analysis (FACSAria Flow Cytometer, BD Biosciences, San Diego, CA, USA). The cells were positive for CD90, CD105 and CD73 and negative for CD14, CD34, CD45 or CD79α, CD11b, and HLA-DR (Human Leukocyte Antigens locus DR).

Tissue processing and immunohistochemistry

After finishing the behavioral experiment (i.e. nine weeks after SCI), the rats were transcardially perfused with paraformaldehyde (4% in PBS) and the spinal cords were post-fixed overnight, removed from the spinal cord canals and transferred to PBS containing azide salt, from animals implanted with MSCs only, or MSC +curcumin, and several (n=2/ interval /group; 3, 7, 14, 21, 28 and 56 days after implantation) spinal cords were longitudinally sectioned for the detection of implanted stem cells (Human Nuclei, HuNu, Millipore, Billerica, MA, USA). The rest of the spinal cords were dissected, 1 cm cranially and 1 cm caudally from the center of the lesion, mounted in paraffin, and cross-sections (5 μm thickness) were cut. Sections were taken from 1-mm intervals along the cranio-caudal axis from the center of the lesion. For the purpose of the analysis, 15 cross sections, with the 8th cross section as the center of the lesion, were used. For analysis of tissue sparing, the square area of the whole cross sections was used. To distinguish the grey (GM) and white matter (WM), Cresyl violet (0.25 g of cresyl violet dissolved in 100 mL of distilled water with 1 mL of 10% acetic acid) and Luxol-fast blue (1 g of Luxol-fast blue dissolved in 100 mL of 96% ethanol with 5 mL of 10% acetic acid) staining was used (C5042 and L0294 both Sigma, St. Louis, MO, USA). The analysis of white and grey matter sparing, astrogliosis and the number of protoplasmic astrocytes after SCI, was calculated by the ZEISS AXIO Observer D1 microscope (Carl Zeiss, Weimar, Germany) and measured by Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical analysis was performed to detect microstructural changes after injury. The average μm^2 areas of GAP43 positive fibers per mm^2 were analyzed (n=5, 15 spinal cord cross sections/1mm interval). In order to detect axonal sprouting, a primary antibody against GAP43 (1:1000, AB5220, Millipore, Billerica, MA, USA) was used. As a secondary antibody, goat anti-mouse IgG conjugated with Alexa-Fluor 488 (Abcam, Bristol, UK) was applied. The effect of the treatment on axonal sprouting was evaluated by LEICACTR6500 microscope and TissueFax Software (TissueGnostics, Vienna, Austria). The graphical processing of the results was carried out in Excel and CorelX6.

For the analysis of astrogliosis, the square area of the whole cross section was used. Primary antibody against GFAP (conjugated with CY3; 1:400, C9205, Sigma-Aldrich, St. Louis, MO, USA) was used. The effect of the treatment on astrogliosis was analyzed with LEICACTR6500 microscope. The percentage of glial scar per square area of the GFAP stained section and the number of protoplasmic astrocytes was determined by ImageJ software.

qPCR

To study the expression of selected rat genes related to the process of regeneration (*Sort1/Nt3, Vegf, Bdnf, Ngf Casp3, Gfap, Gap43, Mrc1, Cd86, Irf5, CD163*), the quantitative real-time reverse transcription polymerase chain reaction (qPCR), was used [for each group n=4, five sets of paraffin sections (interstitial tissue between IHC sections; each approx. of 500 μm spinal cord length; including the central lesion]. RNA was isolated from paraformaldehyde-fixed sections using the High PureRNA Paraffin Kit (Roche, Penzberg, Germany), 9 weeks after SCI. The amount of RNA was quantified with a spectrophotometer (NanoPhotometerTM P-Class, Munchen, Germany). The isolated RNA was reverse transcribed into cDNA using Transcriptor Universal cDNA Master (Roche, Penzberg, Germany), and a thermal cycler (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA). The cDNA solution, FastStart Universal Probe Master (Roche, Penzberg, Germany) and TaqMan® Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) were used to perform the qPCR reaction. The qPCR was carried out in a final volume of 10 μl containing 25 ng of extracted RNA. StepOnePlus™ real-time PCR cycler was used for amplification (Life Technologies, Carlsbad, CA, USA). All amplifications were run under the same cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C). Samples were tested in duplicate, and a negative control was included in each array. The relative quantification of gene expression was determined using the $\Delta\Delta C_t$ meth-

od (Pfaffl, 2001). Data was analyzed with StepOnePlus® software (Life Technologies, Carlsbad, CA, USA). The gene expression levels were normalized using *Gapdh* as a reference gene, and the values obtained from SCI rats treated with vehicle were set as zero. A log2 scale was used to display the symmetric magnitude for up- and down- regulated genes.

Luminex analysis

To analyze the effect of treatment on inflammatory response 10, 14 and 28 days after SCI, a Luminex assay was used (each group n=5 per time point). According to our previously published method (Urdzikova et al. 2014), 2 mm long spinal cord segments, dissected from the area of the injury epicenter were incubated in media for 24 h, and medium was then collected for further analysis. A customized Milliplex inflammatory cytokine kit (Millipore, Billerica, MA, USA) and Magpix instrumentation software were used to analyze levels of the inflammatory cytokines. Rat cytokine Luminex custom eight-plex kit (for IL-2, IL-1β, IL-4, IL-6, RANTES, IL-12p70, TNFα, MIP-1α) was used for customized bead assays. According to the manufacturer's protocol, the assays were performed in 96-well filter bottom plates with antibody-conjugated beads. The concentration of 5000 beads per marker was applied. To measure the levels of cytokines on a Luminex xMAP 200 system (Luminex, Madison, WI, USA), biotinylated antibody with streptavidin-RPE (streptavidin-R-Phycoerythrin; Life Technologies, Carlsbad, CA, USA) were used. Data were analyzed using Magpix software. The raw data [mean fluorescence intensity (MFI)] were used to calculate the concentration of each cytokine, using a five-parameter logistic fit standard curve (generated for each cytokine from seven standards).

The lowest standard, which was at least three-times above the background, was used to determine the lower limit of quantification (LLOQ). The LLOQ was calculated by subtracting the background (diluent) MFI from the MFI of the lowest standard concentration, and back calculating the concentration from the standard curve (Urdzikova et al. 2014). Data is expressed as %, where 'healthy animal with no lesion' is set as 100%.

Statistical analysis

Two-way ANOVA was used to determine the level of statistical significance between treatment groups. In the case of behavioral tests (repeated measurements) or distribution of the effect (glial scar, white/grey matter sparing), two-way repeated measurement ANOVA was applied. As a *post hoc* pair-to-pair test, the Student-Newman-Keuls (SNK) test was used (All Sigmastat 3.1 Sistat Software Inc., San Jose, CA, USA). Differences were considered statistically significant when $p<0.05$. Data in the graphs are shown as mean ± standard error of mean.

RESULTS

Cell survival after implantation

The survival of intrathecally implanted MSCs was visualized by antibody staining against human nuclei (HuNu). In the MSC group, the implanted cells were found up to 21days after treatment (representative image 14 days, Fig. 2A), whereas in the case of the combined therapy, the cells were found up to 28 days after implantation (representative image 21 days, Fig. 2B; survival histogram, Fig. 2C). In both groups, the implanted cells were found randomly distributed and attached to the surface of the spinal cord, no homing into the parenchyma was observed. Many cells were trapped in the folds of arachnoid mater at the site of the injection.

White and grey matter sparing

The effect of the treatment on the sparing of the white and grey matter was evaluated using cresyl-violet luxol fast blue stained serial cross sections. No statistically significant difference in white and grey matter sparing was observed between treatment groups. There was a trend toward significance in preservation of white and grey matter (within the cranio-central region), when compared to saline treated animals. Both single treatments showed this effect prevalently in the white matter, whereas the combined curcumin + MSC treatment supported the grey matter sparing. The caudal region of interest seemed relatively unaffected by any of the treatment applications (Fig. 3A - A, B, representative image Fig. 3B, statistical values in Table I).

Structural changes in glial scar formation and GFAP+ cell morphology

The effect of the treatment on glial scar formation was measured on GFAP-CY3 stained serial cross sections. No statistically significant effect of curcumin, MSC or combined treatment on downregulation of GFAP+ signal area was observed. In the distal parts of the measured area, predominantly in the cranial di-

rection, both treatments containing curcumin slightly decreased the area of GFAP+. However, in the central region, the combined treatment, similarly to MSC application, had the opposite effect. In addition, the number of protoplasmic astrocytes per section was counted. When measured across the whole region of interest, no significant differences between groups were found. On the other hand, in the central region, MSC implanted animals showed a statistically significant increase in the number of protoplasmic astrocytes, when compared with saline or both curcumin containing treatments. Additionally, curcumin + MSC treatment showed also significantly lower numbers in the central region, when compared to saline and curcumin treatment (central region: saline 24.3±3.6, curcumin 27.8±1.4, MSC 49.3±5.1, curcumin + MSC 9±4.2 (Fig. 3A–C, D, C1/D1, statistical values in Table I).

Axonal sprouting

Axonal sprouting was measured on GAP43-stained serial cross sections. The average μm^2 areas of GAP43 positive fibers per mm^2 were analyzed. A combination of curcumin and MSC significantly increased the number of GAP43 positive fibers, when compared to saline or both single treatments. Neither curcumin nor MSC single application had any effect on axonal sprouting (saline=2.5×10^3±227, curcumin=3×10^3±114, MSC=2.1×10^3±279, curcumin + MSC=5.8×10^3±697) (Fig. 3A–E, E1, e1, 2, statistical values in Table I).

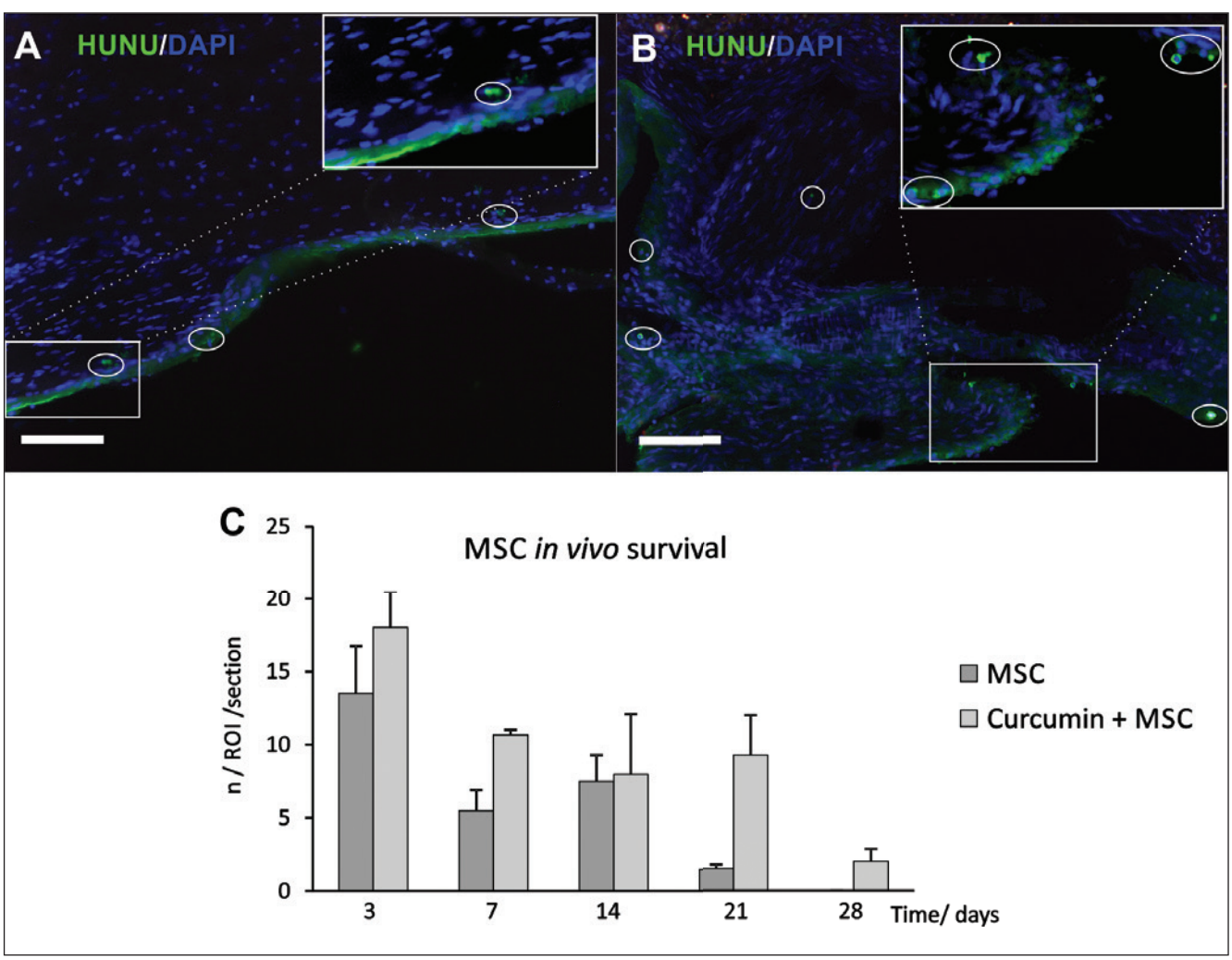

Fig. 2. The effect of curcumin application on the survival of implanted MSCs after SCI. The implantation of MSCs without curcumin led to 21 day survival of the grafted cells (representative image of spinal cord dorsal surface in close vicinity to lesion site, 14 days after SCI, (A). MSCs implanted within the period of curcumin treatment survived for 28 days after implantation (Representative image of spinal cord dorsal surface in close vicinity to lesion site, 21 days after SCI, B). The histogram showing the time dependence of MSC survival in curcumin/saline treated animals on the whole surface of 2 cm long segment of spinal cord with centralized cavity (C). Scale bar 100 μm, blue staining (DAPI), green staining (HuNu). Inserts show higher magnification. Number (n) of animals per group (n=2/time point/group). ROI –Th7-9.

Table I. Statistical evaluation of data from histology and imunohistochemistry analysis (Fig. 3), qPCR (Fig. 4) and behavioral tests (Fig. 6). First column; type of measurement and group identifiers. Second column; type of test applied (1WAN – one way ANOVA, 2WAN – two way ANOVA, 2WRMAN – two way repeated measurement ANOVA, post-hoc tests SNK – Student-Newman-Keuls test, Dunn test). Third column; second factor of the analysis (time, section). Fourth column; group distribution test (F H or q values). Fifth column; p value.

Identifier	Test used	Factor B	F/H/*q* test	p value	Identifier	Test used	Factor B	F/H/*q* test	p value
GM	*2WRMAN*		0.529	>0.05	*Casp3*	*1WAN*		5.078	<0.01
WM	*2WRMAN*		2.627	>0.05	SNK Inter	*CMSC vs. S*		*5.078*	*=0.01*
S vs. CMSC	SNK Inter	3Ca	*3.469*	*<0.05*	*Bdnf*	*1WAN*		2.071	>0.05
		4Ca	*4.112*	*<0.01*	*Ngf*	*1WAN*		4.670	<0.05
MSC vs. CMSC	SNK Inter	4Cr	*3.948*	*<0.05*	*MSC vs. S*	SNK Inter		*4.719*	*<0.01*
		3Ca	*4.846*	*<0.01*	*MSC vs. C*	SNK Inter		*4.666*	*<0.05*
		4Ca	*4.115*	*<0.05*	*MSC vs. CMSC*	SNK Inter		*3.276*	*<0.05*
C vs. CMSC	SNK Inter	4Ca	*3.405*	*<0.05*	BBB	*2WRMAN*		5.353	<0.01
Glial scar	*2WRMAN*		1.450	>0.05	*C vs. S*	SNK Inter		*5.331*	*<0.05*
S vs. C	SNK Inter	Center	*4.047*	*<0.05*	*1w*	SNK Inter		*6.244*	*<0.001*
		1Ca	*3.745*	*<0.05*	6w	SNK Inter		*2.813*	*<0.05*
		2Ca	*3.782*	*<0.05*	FB test	*2WRMAN*		3.484	<0.05
MSC vs. CMSC	SNK Inter	2Ca	*3.323*	*<0.05*	CMSC *vs.* S	SNK Inter		*4.440*	*<0.05*
CMSC vs. C	SNK Inter	Center	*3.498*	*<0.05*			5w	*4.151*	*<0.05*
Morpho	*2WRMAN*		2.097	>0.05			6w	*4.746*	*<0.01*
MSC vs. S	SNK Inter		*5.062*	*<0.001*			7w	*5.048*	*<0.01*
MSC vs. C	SNK Inter		*5.021*	*<0.001*			8w	*4.656*	*<0.01*
MSC vs. CMSC	SNK Inter		*5.409*	*<0.01*			9w	*4.113*	*<0.05*
S vs.CMSC	SNK Inter		*4.135*	*<0.05*	C *vs.* S	SNK Inter		*3.161*	*>0.05*
C vs. CMSC	SNK Inter		*3.792*	*<0.01*			5w	*3.954*	*<0.05*
GAP43	*1WAN*		31.067	<0.001			6w	*3.630*	*<0.05*
MSC vs. S	SNK Inter		*12.277*	*<0.001*			7w	*3.468*	*<0.05*
CMSC vs. C	SNK Inter		*8.701*	*<0.001*	FB time			2.735	<0.05
CMSC vs. MSC	SNK Inter		*12.293*	*<0.001*	CMSC *vs.* S	SNK Inter		*3.542*	*<0.05*
qPCR M1/M2							3w	*3.013*	*<0.05*
Mrc1	*1WAN*		3.788	<0.05			4w	*3.071*	*<0.05*
MSC vs. S	SNK Inter		*3.518*	*=0.056*			6w	*3.880*	*<0.05*
CMSC vs. S	SNK Inter		*3.678*	*<0.05*			7w	*3.547*	*<0.05*
CD163	*1WAN*		14.438	<0.001	CMSC *vs.* C	SNK Inter		*3.035*	*<0.05*
CMSC vs. S	SNK Inter		*4.322*	*<0.01*			3w	*3.563*	*<0.05*
CMSC vs. C	SNK Inter		*8.673*	*<0.001*			5w	*3.071*	*<0.05*
CMSC vs. MSC	SNK Inter		*5.644*	*<0.01*			6w	*3.038*	*<0.05*
S vs.C	SNK Inter		*5.081*	*<0.01*	Rotarod test	*2WRMAN*		2.010	>0.05
Irf5	*1WAN*		1.364	>0.05	C *vs.* S	SNK Inter	3w	*4.255*	*<0.05*
CD86	*1WAN*		9.200	<0.05	*C vs. CMSC*	SNK Inter	3w	*4.674*	*<0.01*
MSC vs. C	Dunn Inter		*2.963*	*<0.05*	Plantar test	*2WRMAN*		1.941	>0.05
qPCR RAGs					C *vs.* S	SNK Inter	6w	*4.208*	*<0.01*
NT3	*1WAN*		0.097	>0.05	C *vs.* MSC	SNK Inter	5w	*3.790*	*<0.05*
Vegf	*1WAN*		1.465	>0.05			6w	*4.858*	*<0.01*
Gfap	*1WAN*		1.354	>0.05	C *vs.* CMSC	SNK Inter	6w	*2.809*	*<0.05*
Gap43	*1WAN*		8.304	<0.05					

Expression of genes related to macrophage phenotype

The relative expression levels of genes related to M1 (CD86, Irf5) or M2 (CD163, Mrc1) macrophage phenotypes were measured 9 weeks after SCI. Curcumin application after SCI slightly upregulated markers of both macrophage types, particularly Irf5 for M1, and CD163 for M2 phenotype. On the other hand, MSC or MSC + curcumin treatment either down-regulated or did not change any of the monitored genes (Fig. 4A). In particular, none of the markers increased in the combined treatment group (statistical values in Table I).

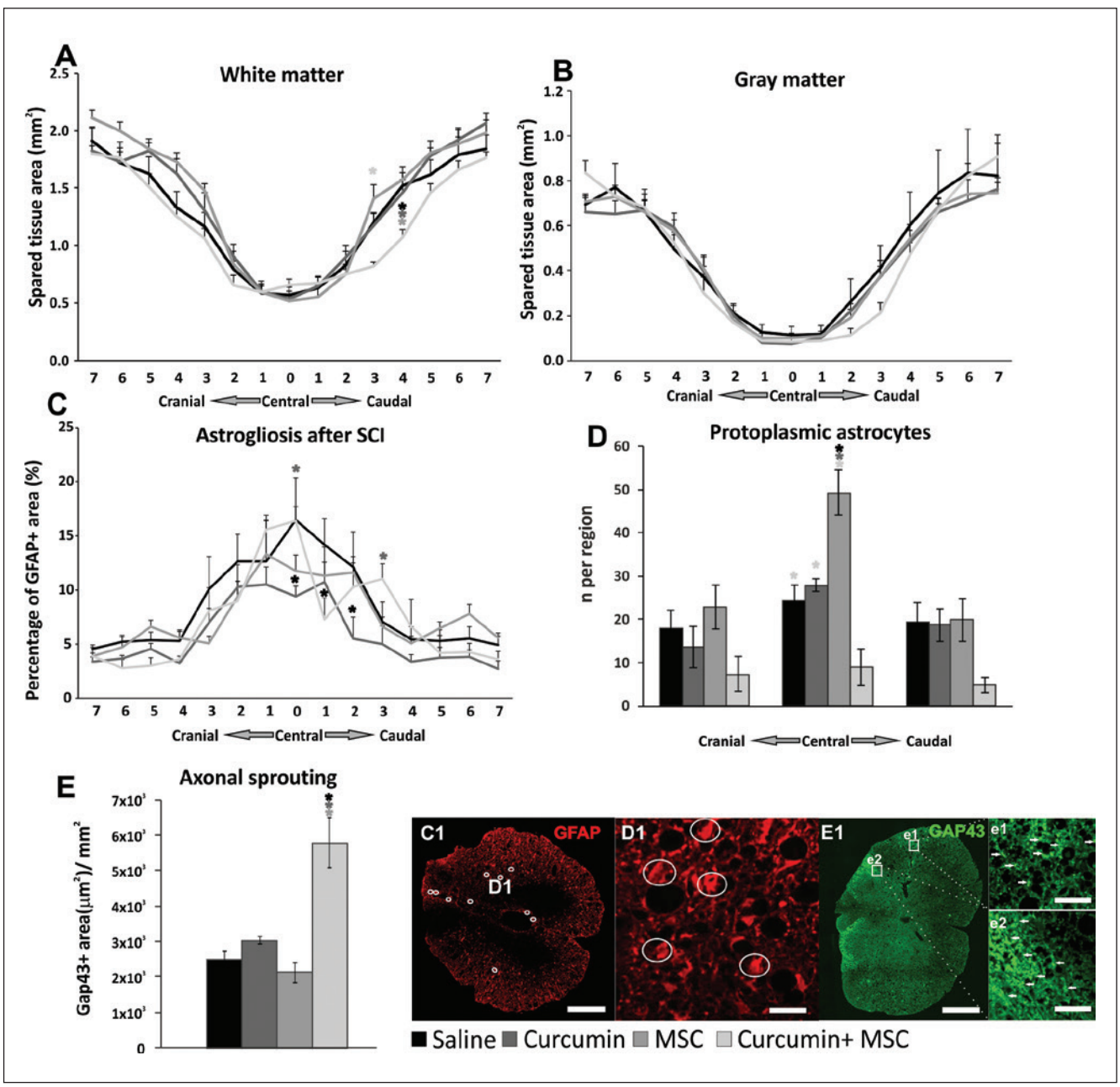

Fig. 3A. Morphometric measurements of white (A) and grey (B) matter sparing showed no significant difference between treatment groups, with the predominant effect of the treatment in the cranio-central region. Analysis of GFAP+ marker showed no significant difference in glial scaring (C), only in distribution of protoplasmic astrocytes in the MSC group (D). The effect of combined therapy on axonal sprouting, two months after SCI was observed (E). Statistical significance was marked with * when $p<0.05$. Number of animals per group (n=8/group). Representative images of IHC analysis (Astrogliosis after SCI- C1, Protoplasmic astrocytes- D1, (GFAP-CY3), Axonal sprouting -E1, e1, e2, (GAP43)). The central, cranial and caudal region of GFAP morphometry measurement consists of 5 measured sections. Scale bars 200 μm (C1, E1), 30 μm (C1, e1, e2). Caudal-X-mm caudally to the injury site, Cranial-X-mm cranially to the injury site, n- number.

Table II. Statistical evaluations of data from cytokine evaluation (Fig. 5). First column; type of measurement and groups identifiers. Second column; type of test applied (1WAN – one way ANOVA, 2WAN – two way ANOVA, 2WRMAN – two way repeated measurement ANOVA, post-hoc tests SNK – Student-Newman-Keuls test, Dunn test). Third column; second factor of the analysis (time, section). Fourth column; group distribution test (F H or q values). Fifth column; p value.

Identifier	Test used	Factor B	F/H/*q* test	p value	Identifier	Test used	Factor B	F/H/*q* test	p value
Luminex assay					C *vs.* S	SNK Inter		*3.166*	*<0.05*
MIP1a	2WAN		4.662	<0.01			28D	*3.555*	*<0.05*
CMSC vs. S	SNK Inter		*3.347*	*<0.05*	C *vs.* MSC	SNK Inter		*3.527*	*<0.05*
		14D	*5.456*	*<0.001*			28D	*3.574*	*<0.05*
CMSC vs. C	SNK Inter		*4.943*	*<0.01*	*IL12p70*	2WAN		29.062	<0.001
		14D	*6.760*	*<0.001*	*CMSC vs. S*	SNK Inter		*8.312*	*<0.001*
CMSC vs. MSC	SNK Inter		*3.686*	*<0.05*			10D	*6.281*	*<0.001*
		14D	*7.544*	*<0.001*			14D	*3.238*	*<0.05*
IL4	2WAN		27.416	<0.001			28D	*4.698*	*<0.01*
CMSC vs. S	SNK Inter		*9.952*	*<0.001*	*CMSC vs. C*	SNK Inter		*2.012*	*>0.05*
		10D	*6.884*	*<0.001*			28D	*3.184*	*<0.05*
		14D	*4.269*	*<0.01*	*CMSC vs. MSC*	SNK Inter		*10.303*	*<0.001*
		28D	*5.922*	*<0.001*			10D	*7.579*	*<0.001*
CMSC vs. C	SNK Inter		*8.850*	*<0.001*			14D	*5.064*	*<0.01*
		10D	*5.123*	*<0.001*			28D	*4.957*	*=0.001*
		14D	*4.991*	*<0.01*	C *vs.* S	SNK Inter		*8.149*	*<0.001*
		28D	*5.296*	*<0.001*			14D	*5.408*	*=0.001*
CMSC vs. MSC	SNK Inter		*11.186*	*<0.001*			28D	*7.298*	*<0.001*
		10D	*9.952*	*<0.001*	C *vs.* MSC	SNK Inter		*10.086*	*<0.001*
		14D	*5.918*	*<0.001*			14D	*7.257*	*<0.001*
		28D	5.437	*<0.001*			28D	*7.993*	*<0.001*
IL1b	2WAN		13.329	<0.001	*TNFa*	2WAN		7.045	<0.001
CMSC vs. S	SNK Inter		*7.514*	*<0.001*	*CMSC vs. S*	SNK Inter		*4.172*	*<0.01*
		10D	*4.452*	*<0.01*			10D	*5.158*	*<0.01*
		14D	*5.961*	*<0.001*	*CMSC vs. C*	SNK Inter		*5.764*	*=0.001*
CMSC vs. C	SNK Inter		*6.125*	*<0.001*			10D	*3.635*	*<0.05*
		14D	*6.275*	*<0.001*	*CMSC vs. MSC*	SNK Inter		*4.318*	*<0.05*
CMSC vs. MSC	SNK Inter		*7.301*	*<0.001*	*RANTES*	2WAN		23.346	<0.001
		10D	*5.529*	*<0.01*	*S vs. C*	SNK Inter		*7.542*	*<0.001*
		14D	*5.081*	*<0.001*			10D	*4.402*	*<0.01*
IL2	2WAN		14.385	<0.001			14D	*4.919*	*<0.01*
CMSC vs. S	SNK Inter		*4.877*	*=0.001*			28D	*3.743*	*<0.05*
		14D	*5.577*	*<0.001*	S *vs.* CMSC	SNK Inter		*4.457*	*<0.01*
CMSC vs. C	SNK Inter		*8.686*	*<0.001*			10D	*4.962*	*<0.01*
		14D	*9.277*	*<0.001*	MSC *vs.* S			*3.019*	*<0.05*
		28D	*4.824*	*<0.01*			14D	*3.162*	*<0.05*
CMSC vs. MSC	SNK Inter		*6.289*	*<0.001*	MSC *vs.* C	SNK Inter		*10.959*	*<0.001*
		14D	*6.739*	*<0.001*			10D	*5.605*	*<0.001*
IL6	2WAN		6.641	<0.001			14D	*8.571*	*<0.001*
CMSC vs. S	SNK Inter		*4.90*	*<0.01*			28D	*4.919*	*<0.01*
		14D	*7.395*	*<0.001*	MSC *vs.* CMSC	SNK Inter		*7.912*	*<0.001*
CMSC vs. C	SNK Inter		*4.88*	*<0.01*			10D	*6.164*	*<0.001*
		14D	*5.62*	*<0.001*			14D	*6.118*	*<0.001*
		28D	*3.018*	*<0.05*	CMSC *vs.* C	SNK Inter		*3.534*	*<0.05*
CMSC vs. MSC	SNK Inter		*5.347*	*<0.01*			14D	*3.390*	*<0.05*
		14D	*8.273*	*<0.001*			28D	*3.833*	*=0.01*

Expression of genes related to regenerative processes

Selected genes from the host tissue for growth factors, apoptosis and astrogliosis were assessed 9 weeks after SCI using qPCR. The application of MSCs only, or in combination with curcumin, significantly decreased the expression of *Casp3* and increased the expression of *Gap43*. Differences were detected in the expression of *Ngf*, which was downregulated in the MSC only group. The curcumin application enhanced the expression of *Nt3, Vegf, Bdnf* and *Gap43*, and downregulated the expres-

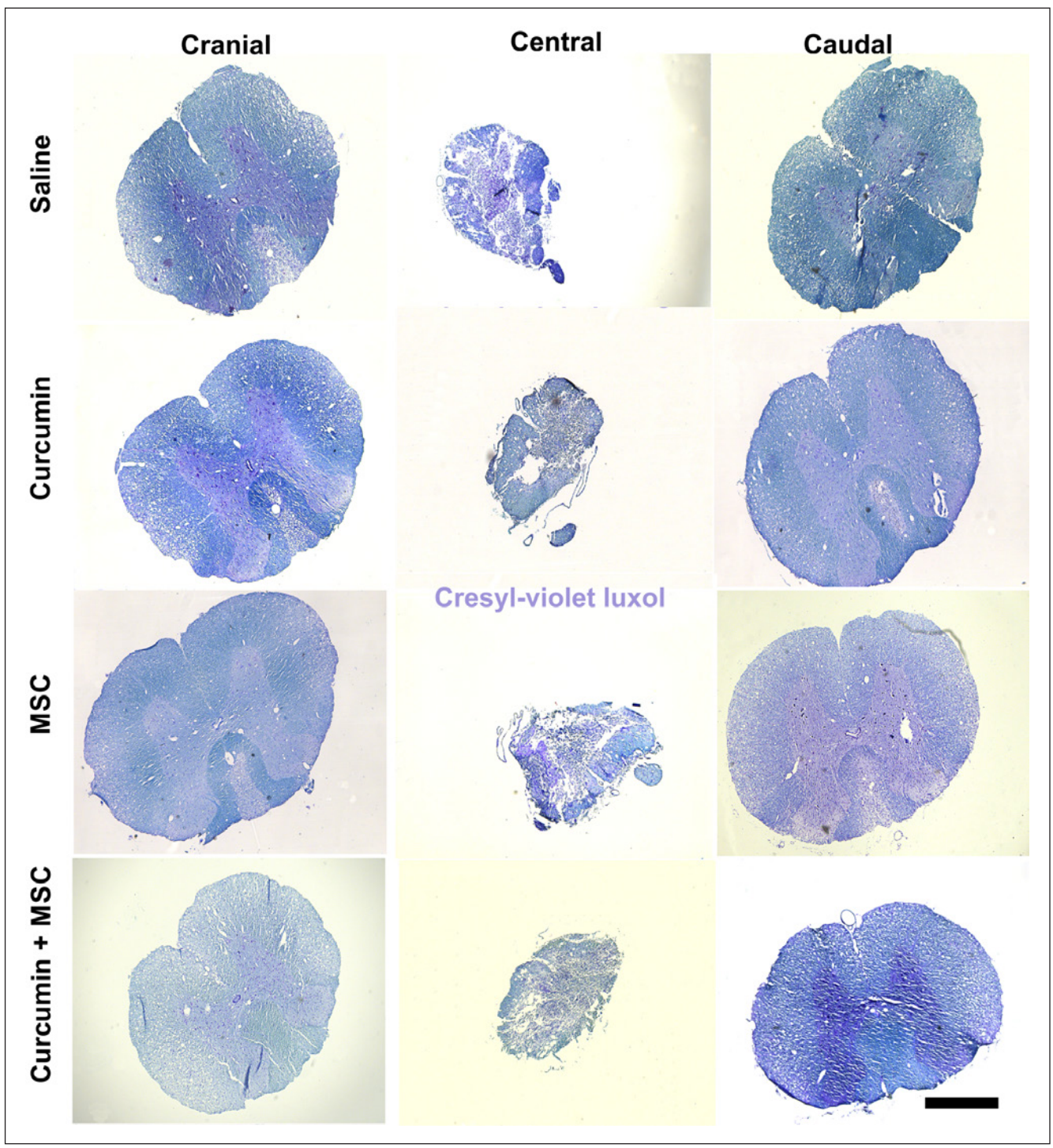

Fig. 3B. The representative images of white and grey matter sparing analysis in the cranial, central and caudal sections. Distal images are 7 mm from the lesion center. CVL – cresyl-violet luxol. Scale bar 500 μm.

sion of *Casp3*, however, these changes were not statistically significant (Fig. 4B, statistical values in Table I).

Levels of proinflammatory cytokines

The effect of curcumin, MSC, or their combination on levels of proinflammatory cytokines in the center of the lesion was evaluated 10, 14 and 28 days after SCI, using a Luminex assay (Fig. 5A-C). All three treatments

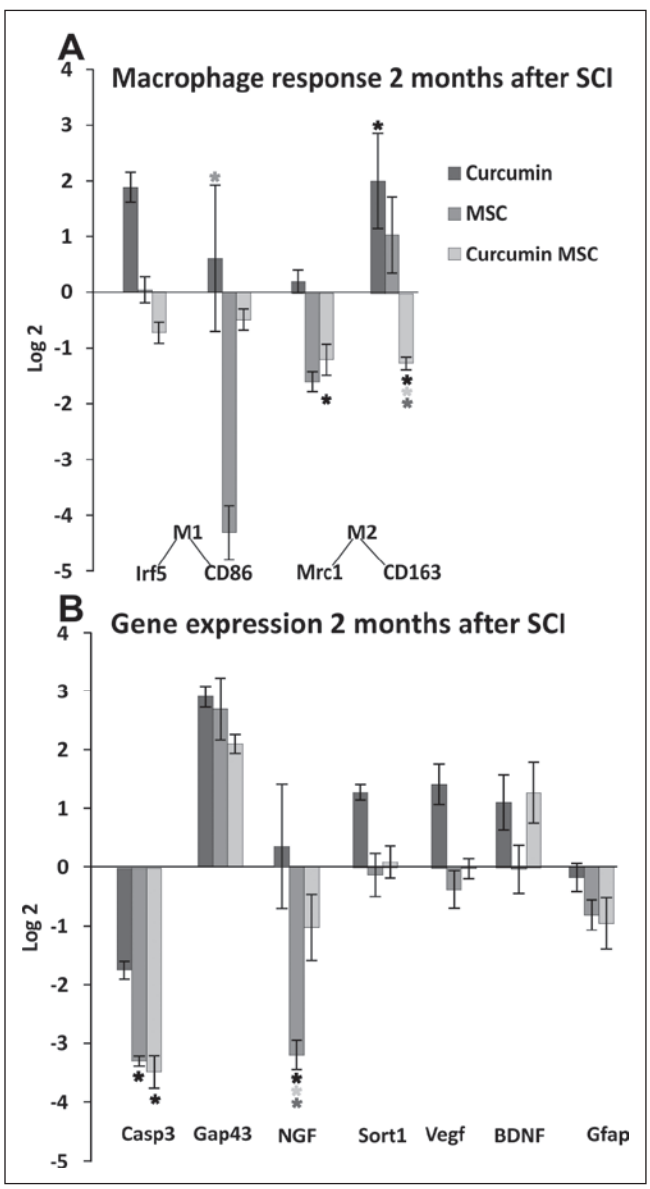

Fig. 4. Profiling of relative expression of host genes related to macrophage response (A) and regenerative processes (B) in the lesion center affected by treatment, 2 months after SCI. Statistical significance was marked with * when p<0.05. Number of animals per group (n=5/group). M1 indicate pro-inflammatory macrophage markers, while M2 are pro-regenerative macrophage markers.

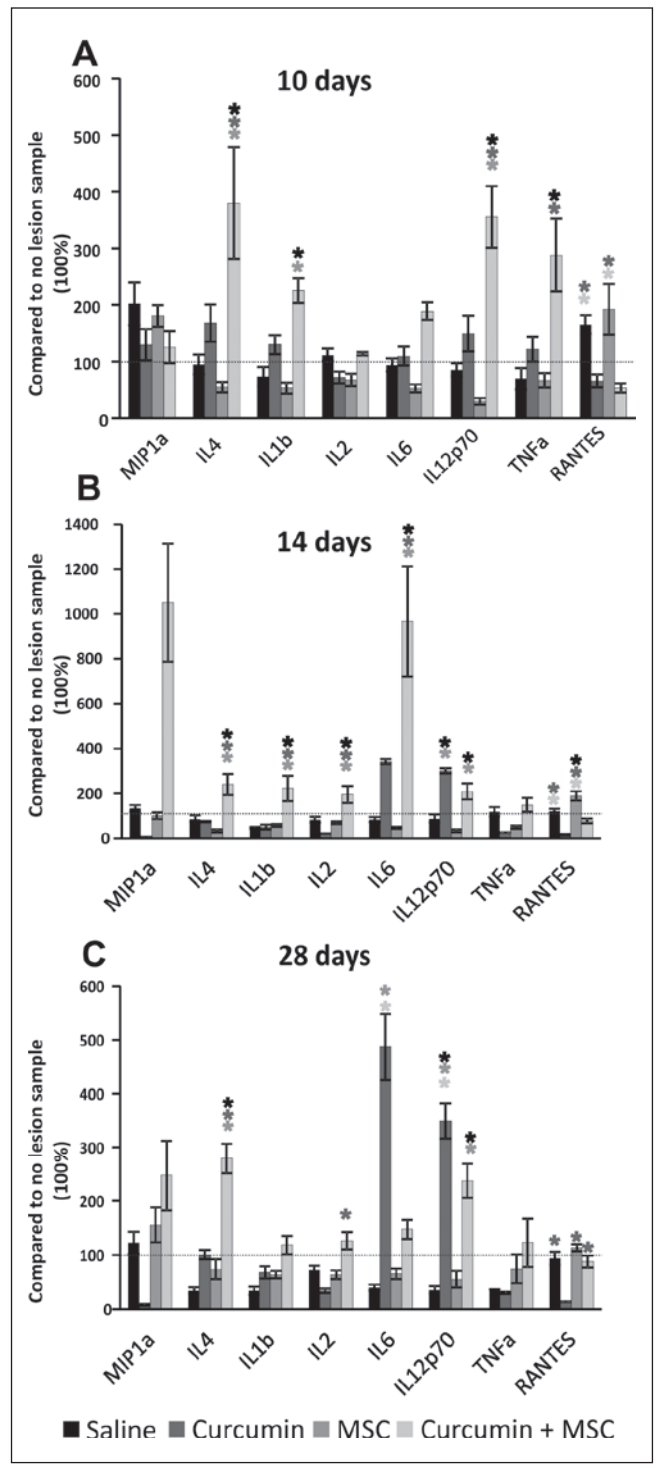

Fig. 5. Immune response to applied treatment 10 (A), 14 (B) and 28 (C) days after SCI. Ten days after SCI, a significant increase in levels of IL4, IL1β, IL12p70 and TNFα, and a significant decrease in RANTES were observed in the curcumin MSC group, when compared to saline as well as single treatment groups. Fourteen days after SCI, all cytokine levels, except for TNFα and RANTES, remained elevated in comparison with saline treated animals. Statistical significance was marked with * when p<0.05. Number of animals per group (n=5/time point/group). No lesion ~ healthy animal with no lesion.

have shown a different impact on the levels of intrinsic immune molecules (MIP1α, IL4, IL1β, IL2, IL6, IL12p70, TNFα and RANTES). On the 10th day after SCI, the combined treatment greatly increased levels of IL4, IL1β, IL12p70 and TNFα, and decreased levels of RANTES. On the other hand, MSC-only treated animals showed low levels of cytokines, except for RANTES, which increased. These values obtained from MSC treated animals remained unchanged for all the time points. On the 14th day after SCI, most of the inflammatory proteins (except TNFα and RANTES) remained elevated after combined treatment, when compared to saline treated animals as well as to both single treatment groups. The increase of IL4, IL2 and IL12p70 also persisted until day 28. In curcumin treated animals, increased levels of IL6, IL12p70 and decreased levels of RANTES were detected on day 14, and remained the same until the end of the experiment (day 28) (statistical values in Table I).

Locomotor and sensory recovery after SCI

Basic locomotor recovery was measured according to the BBB scale. Animals treated with curcumin, MSC or their combination showed increased locomotor recovery after SCI, when compared to saline treated animals.

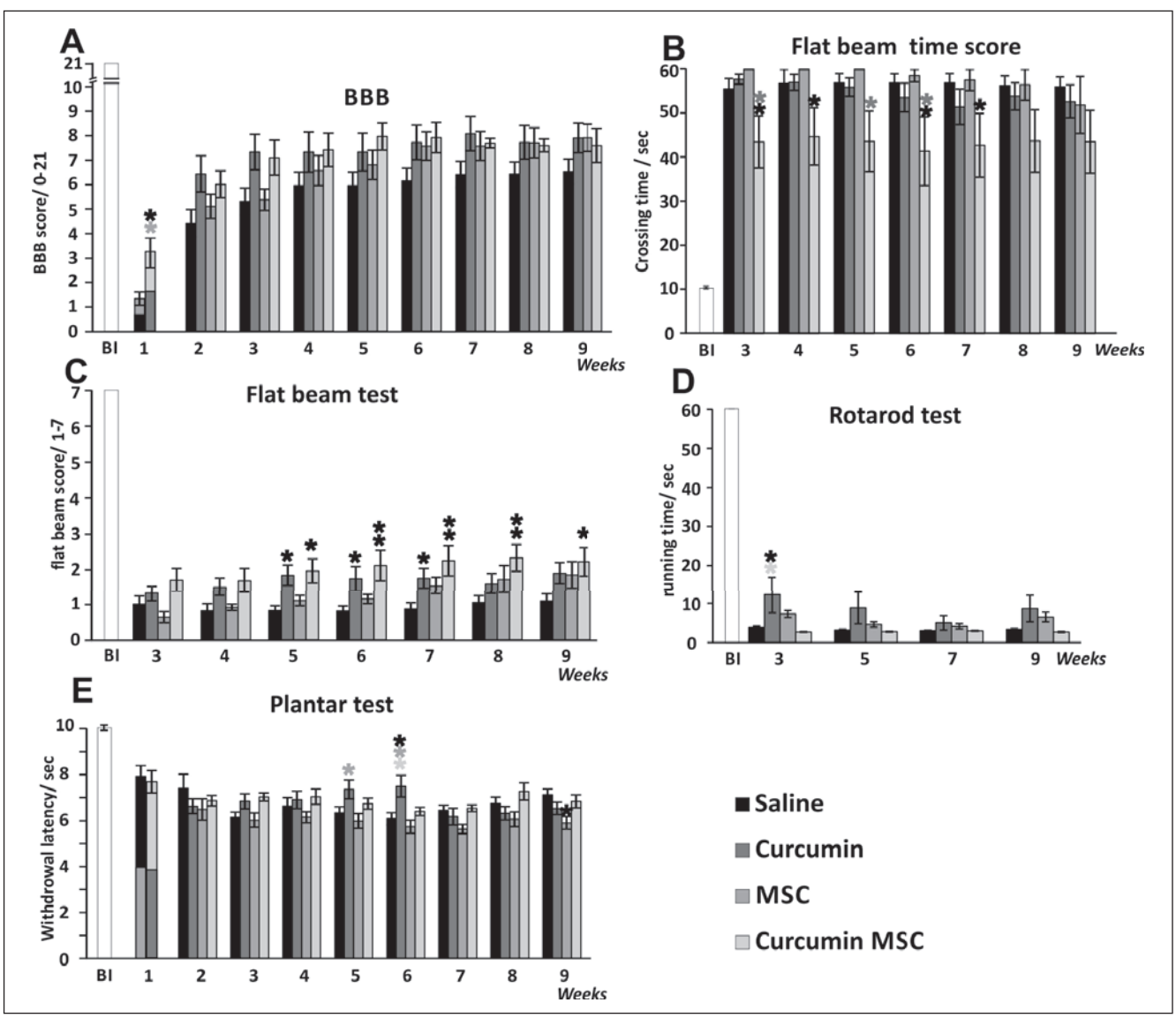

Fig. 6. Functional recovery after SCI. The locomotor performance of saline, MSC, curcumin or curcumin + MSC treated animals evaluated on BBB (A), Flat beam test (B, C) and Rotarod (D), 9 weeks after SCI. All treatments had a positive impact on locomotor recovery (A) after SCI. Combined therapy showed significantly enhanced advanced locomotor skills (B, C). No hyperalgesia in response to nociceptive thermal stimulus on plantar test apparatus (E) was observed. Statistical significance was marked with * when $p<0.05$. Number of animals per group (saline n=19, curcumin n=12, MSC n=13, curcumin + MSC n=11).

Statistical significance was observed only in the case of curcumin treated animals, in which a significant improvement was already observed from the first week after SCI. Only the curcumin + MSC group showed a strong trend towards significance (Fig. 6A, statistical values in Table I).

Advanced locomotor skills including weight support and forelimb-hindlimb coordination were evaluated using the flat beam test. The flat beam test gives a more accurate view on the movement, coordination ability and balance so it can reveal more subtle differences than the BBB test. All treatment groups demonstrated at least moderately faster recovery when compared to saline treated animals. However, only the curcumin + MSC combined therapy reached a statistically significant improvement in comparison with the saline group, when compared to all the data sets. The animals treated with curcumin scored significantly higher but only at weeks 5, 6 and 7, even though the curcumin showed a strong trend towards increased scores in the flat beam test as well as the time scores (Fig. 6C). Apart from the score, the time needed for crossing the selected region of the beam was measured. The shortest time in this task was achieved by animals from the curcumin + MSC group (Fig. 6B, statistical values in Table I).

To determine the effectiveness of treatment on the recovery of the high-level locomotor score, long-term weight support, endurance and hindlimb coordination, the rotarod test was used. As expected, taking into consideration the severity of the injury model, none of the treatment groups significantly improved their performance. Only curcumin treated animals at week 1 scored significantly better than controls and those which received combined treatment (Fig. 6D, statistical values in Table I).

SCI leads to hyperalgesia. A plantar test apparatus was used to measure latency in response to painful thermal stimulus (Ugo Basile, Comercio, Italy). No statistically significant change in the latency of response was observed in any of the treatment groups. In contrast, the tendency of curcumin and combined treatment to increase the latency of response, and thus attenuate hyperalgesia has been observed. This tendency has reached, at several time points, statistical significance (Fig. 6E, statistical values in Table I).

DISCUSSION

Our study has demonstrated that the combination of the anti-inflammatory compound curcumin and mesenchymal stem cells provides a mild synergistic effect in the treatment of spinal cord injury when compared to both single treatments, and shows enhanced recovery when compared to vehicle treated animals. The combined treatment affected MSC survival, endogenous inflammatory response, and axonal sprouting, which in turn led to a significant recovery in locomotor skills.

Both curcumin (Ormond et al. 2012, Zhang et al. 2017) and MSCs (Morita et al. 2016, Mukhamedshina et al. 2018, Nakajima et al. 2012, Urdzikova et al. 2006) were previously reported to show a positive effect on behavioral recovery after SCI. In our study, both single treatments and their combination, had a positive impact on locomotor recovery after SCI. Curcumin had a major effect during the initial phase of the study, on both basic and advanced locomotor performance measurements. Its immediate and continuous application after SCI, slowed down the secondary injury processes and thus accelerated and facilitated spontaneous recovery. Mesenchymal stem cell treated animals reached the same levels of BBB score, but over a longer period of time, showing only a mild impact on advanced locomotor skills. A combination of curcumin and MSCs displayed no additional effect on the basic BBB test, however it showed the best flat beam test score, which assesses the advanced locomotor skills of the animals. The short-term effect of MSC treatment may therefore correspond to their lower numbers at the end of the study, when the paracrine effect was not further prolonged. The effect of combined therapies on recovery after SCI is not always predictable, and described cases in the literature indicate that these therapies may not overcome the potential of single treatments, and may even bring undesired effects, such as delayed recovery or additional hypersensitivity of handicapped paws (Bretzner et al. 2008, Park et al. 2013).

The migration of MSC through cerebrospinal fluid into the injured spinal cord tissue was studied in Lewis rats. Penetration to spinal cord parenchyma and perivascular spaces, and MSC integration has been described (Satake et al. 2004). Conversely, in our study, the MSCs persisted at the injection site in between the roots or were attached to the spinal surface. This might be due to the different model of injury, in which the dura mater is less affected in the region of the injury. However, in our study we observed the prolonged survival of MSC in combination with the curcumin application. It was also noted that the cells were present in the spinal channel and since they did not home into parenchyma, their final numbers could have been be affected by tissue processing.

According to published studies, MSCs (Mukhamedshina et al. 2018, Ritfeld et al. 2012,) or curcumin (Ormond et al. 2012) showed a significant impact on white/grey matter sparing after SCI. In our study, a positive effect of co-treatment was observed, but was not statistically significant. Their combination showed no

additional impact on total volume sparing, but a small positive effect in the area of the lesion epicenter was detected in the combined treatment. Both curcumin (Wang et al. 2014b, Yuan et al. 2015) and mesenchymal stem cells (Nakajima et al. 2012) have been described to attenuate the process of glial scar formation. In agreement with previous findings, curcumin application showed a mild decrease of the total amount of GFAP+ area, similar to that effect observed in MSC treatment, but with a different pattern of signal distribution. In the case of the combination of these two treatments, no significant synergic decrease of GFAP+ area was detected. However, in the curcumin group, and particularly in the combined therapy, the strongest effect on decreasing the number of protoplasmic astrocytes in injured spinal cord was observed. This could correspond with findings that curcumin can have a positive effect on reactive astrocytes in various models of neurodegenerative diseases or injured CNS (Daverey and Agrawal 2016, Jiang et al. 2011, Seyedzadeh et al. 2014, Wang et al. 2005). In contrast, in our study the MSCs led to the opposite effect, which is rather contradictory to what was shown in other studies using the application of MSCs. This might be related to the lack of MSC penetration into the parenchyma in our study and thus a limited paracrine effect, when compared to the studies where the cells were directly implanted into the spinal tissue or migrated into the parenchyma (Liu et al. 2018, Park et al. 2015). The effect on astrocyte morphology and function after SCI is one of the tools used to modulate the secondary processes after SCI, and mitigate the impact of the injury. Reactive astrocytes (A1 type) can produce cytotoxic factors and secrete cytokines to communicate with other glial cells; this then leads to decreased neuronal survival and plasticity. The decreasing numbers of protoplasmic astrocytes after SCI can then promote axonal sprouting and support tissue sparing (Gaudet and Fonken 2018). In our study, a strong synergic effect on axonal sprouting was observed. Curcumin has a neuroprotective and anti-inflammatory effect, which can increase the survival of neurons as well as glial cells leading to the preservation or recovery of myelin sheets (Naeimi et al. 2018, Zhao et al. 2017). Additionally, curcumin could have more impact on neurite outgrowth and axon protection (Lu et al. 2014, Ruzicka et al. 2018, Tegenge et al. 2014). In multiple studies, MSCs have been shown to support the axonal sprouting after SCI or TBI, mostly by paracrine action (Kocsis and Honmou 2012, Morita et al. 2016). The effect of curcumin application might be potentiated by the higher survival rate of MSCs in the combined therapy. In single MSC treatment, this effect might be transient and could slowly diminish at the time the tissue is processed (i.e. two months after SCI).

The expression of selected genes related to endogenous tissue regenerative processes in combined therapy exhibited an effect similar to MSC treatment alone. We observed downregulation of all macrophage related genes and caspase 3. All treatments increased the expression of the GAP43 gene, however, a significant increase in the expression of GAP43 protein (increased axonal sprouting) was only observed in the combined treatment. This could be connected to MSC prolonged survival by co-implantation with curcumin, and thus a longer impact of therapy on tissue reconstruction.

Despite this, curcumin only slightly upregulated all macrophage and several growth factor (*Nt3*, *Vegf* and *Bdnf*) genes. Our findings partially correspond to previous studies (Machova Urdzikova et al. 2015, Ormond et al. 2012, Urdzikova et al. 2014). On the other hand, both MSC and the combined group downregulated Casp3 expression, and combined therapy had a strong effect on the downregulation of macrophage related genes. In our previous studies, a single treatment with MSC (Ruzicka et al. 2017) or curcumin (Machova Urdzikova et al. 2016) has been shown to have a positive impact on increasing the M1/M2 ratio over time (10 and 28 days) following SCI. In our present study, we expected a similar or more pronounced effect by the combined treatment, which did not happen. We also performed a qPCR study 10 days after injury (3 days after cell application), however, we did not observe any differences between any of the animal groups (data not shown).

One of the mechanisms of secondary injury processes, leading to progressive damage on the cellular and spinal tissue levels, is inflammation. Several mediators including those tested in this study (MIP1α, IL4, IL1β, IL2, IL6, IL12p70, TNFα and RANTES) have been identified as playing a crucial role in this process. Curcumin has been shown to be a potent anti-inflammatory compound suppressing levels of IL1, IL8, MCP-1 and TNFα across various disease models (Hidaka et al. 2002, Jain et al. 2009, Yuan et al. 2015). The application of curcumin after SCI has been reported to show similar anti-inflammatory potential, by downregulating levels of inflammatory cytokines such as IL1-β, RANTES, MIP-1α and TNF-α, and inhibiting several molecular pathways, including JAK/STAT, MAPK and nuclear factor-κB activation (Chen et al. 2015, Dong et al. 2014, Lin et al. 2011, Zu et al. 2014). The application of MSCs displayed immunomodulatory potential by increasing levels of IL4 or IL13 and reducing TNF-α, IL-1 or IL6 levels (Nakajima et al. 2012, Tsumuraya et al. 2015). These findings are in agreement with the single therapies of MSC or curcumin in our previous studies (Machova Urdzikova et al. 2015, Urdzikova et al. 2014). However, in contrast, the combination of MSC and curcumin application showed not only an increase in levels of IL4, but also IL-2, IL1-β,

TNF-α, and somewhat increased IL6 and IL12p70 levels, when compared to saline treated animals and both single treatments. The reason behind the high IL4 levels in the combined treatment could be an effect of curcumin, potentiated by MSC application (Machova Urdzikova et al. 2015, Ormond et al. 2012). On the other hand, proinflammatory markers such as IL-2, IL1-β, TNF-α, and to some extent IL6 and IL12p70, are usually described to be suppressed by curcumin or MSC single application (Nakajima et al. 2012, Wang et al. 2014b). IL4 has been described as a pure anti-inflammatory factor. Increased levels of IL4 after SCI have been shown to shift the microglia and macrophages response to the (M2) anti-inflammatory and pro-regenerative form (Francos-Quijorna et al. 2016). IL6 was originally described as a contributor to secondary pathological and inflammatory responses after SCI. However, recently it has been shown that increased doses of IL6 in myelin protein treated DRG cultures or an *in vivo* model of SCI can enhance axonal sprouting and decrease the levels of Nogo-A or NgR. Moreover, treatment by IL6 can lead to functional recovery after SCI (Yang and Tang 2017). However, this imbalanced immune response during the combined treatment might be one of the reasons for the low synergy of curcumin and MSC implantation.

One of the major drawbacks of in vivo curcumin application is its solubility in lipophilic substances. In our study, unmodified curcumin dissolved in oil was administered at the injury site in addition to systemic intraperitoneal application, in order to maintain bioavailability for a period of at least 28 days. However, to fully support curcumin potential, a specific pH sensitive carrier (Requejo-Aguilar et al. 2017) or modifications to increase solubility in water are needed.

CONCLUSIONS

The results of our recent study have confirmed only partial synergy in the treatment of experimental SCI with the combination of the anti-inflammatory compound curcumin and MSCs. The combined therapy led to significant preservation of advanced locomotor skills, and increased axonal sprouting, most likely due to the longer survival of the graft, modulation of the immune response, and changes in the expression of intrinsic genes related to regenerative processes after SCI. To potentiate the effect of preconditioning prior to stem cell implantation, the curcumin compound should be modified to fully release its potential in the injured spine region.

AUTHOR CONTRIBUTIONS

Pavla Jendelova, Lucia Machova Urdzikova and Jiri Ruzicka conceived the concept of the study, designed the experiments and wrote the manuscript. Anubhav Amin performed the cytokine analysis; Sarka Kubinova and Jana Dubisova performed the gene expression analysis and interpreted the data. All animal experiments including SCI, behavioral testing, histology and immunohistochemistry were carried out by Jiri Ruzicka, Anna Kloudova and Lucia Machova Urdzikova. Sarka Kubinova and Pavla Jendelova interpreted the data in relation to spinal SCI, while Meena Jhanwar-Uniyal and Meic Schmidt interpreted the data in relation to cytokine analysis.

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