CHARLES UNIVERSITY
Second Faculty of Medicine

Summary of the Dissertation

BIOMATERIALS AND STEM CELLS IN SPINAL CORD INJURY

BIOMATERIÁLY A KMENOVÉ BUŇKY PŘI PORANĚNÍ MÍCHY

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Contents

1. Abstract

Spinal Cord Injury is a very serious trauma which can't be effectively cured at present time. The use of ECM hydrogels as supportive and stimulatory milieu and transplantation of stem cells represent promising approaches for SCI therapy. However, current treatments are limited by inefficient delivery of stem cells into the lesion site. Therefore, the aim of this study was the development of SCI treatment using ECM hydrogels and effective stem cell delivery system. The non-invasive magnetic system was designed and used to accumulate SPION-labelled stem cells at a specific site of a SCI lesion. Decellularized porcine SC and UB tissues, synthetic P(HEMA-AEMA) hydrogel with oriented porosity and modified hyaluronic acid HA-PH-RGD were transplanted into a spinal cord lesion of rats with or without stem cells, followed by histological analysis and gene expression analysis. All types of hydrogels integrated into the lesion and stimulated neovascularization and axonal ingrowth into the lesion. There was no significant difference in the tissue infiltration between the plain hydrogels and those seeded with stem cells. However, a subacute injection HA-PH-RGD/Fibrinogen combined with Wharton's jelly-derived human mesenchymal stem cells enhanced axonal ingrowth into the lesion. Significant down-regulation of genes related to immune response and inflammation was observed in hydrogels. Therefore, combined application of injectable hydrogel scaffolds and effective delivery of stem cells are the key factors for improving survival of cells in lesion site, inhibition of systematic inflammation and *in vivo*-like neural regeneration.

Keywords

Spinal cord injury, injectable extracellular matrix derived hydrogels, hyaluronic acid, mesenchymal stem cells, magnetic field, magnetic stem cell delivery, stem cell transplantation, neuroregeneration, neovascularization, axonal ingrowth.

Abstrakt

Spinal Cord Injury is a very serious trauma which can't be effectively cured at present time. The use of ECM hydrogels as supportive and stimulatory milieu and transplantation of stem cells represent promising approaches for SCI therapy. However, current treatments are limited by inefficient delivery of stem cells into the lesion site. Therefore, the aim of this study was the development of SCI treatment using ECM hydrogels and effective stem cell delivery system. The non-invasive magnetic system was designed and used to accumulate SPION-labelled stem cells at a specific site of a SCI lesion. Decellularized porcine SC and UB tissues, synthetic P(HEMA-AEMA) hydrogel with oriented porosity and modified hyaluronic acid HA-PH-RGD were transplanted into a spinal cord lesion of rats with or without stem cells, followed by histological analysis and gene expression analysis. All types of hydrogels integrated into the lesion and stimulated neovascularization and axonal ingrowth into the lesion. There was no significant difference in the tissue infiltration between the plain hydrogels and those seeded with stem cells. However, a subacute injection HA-PH-RGD/Fibrinogen combined with Wharton's jelly-derived human mesenchymal stem cells enhanced axonal ingrowth into the lesion. Significant down-regulation of genes related to immune response and inflammation was observed in hydrogels. Therefore, combined application of injectable hydrogel scaffolds and effective delivery of stem cells are the key factors for improving survival of cells in lesion site, inhibition of systematic inflammation and *in vivo*-like neural regeneration.

Keywords

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2. Introduction

Spinal Cord Injury (SCI) is a very serious trauma that leads to extensive damage, disability and mortality and presents the global ongoing neurological problem which greatly hampers the financial and social status of patients and caregivers alike. It is estimated that 17,500 traumatic spinal cord injury cases each year in USA alone while approximately 285,000 people are living with chronic SCI (Alabama at Birmingham, 2017). In over half of the cases, the patients suffer from partial or complete paralysis of the arms, legs and torso, a condition called tetraplegia. Others may be paraplegic wherein there is partial or total paralysis of the legs. Individuals suffering from SCI not only face grave physical impairment, but also have other equally problematic vocational setbacks. Moreover, SCI patients face a greater risk of developing mental health ailments. In addition to motor and sensory impairment SCI patients are also often suffer from chronic pain, spasticity, respiratory and cardiovascular alterations, neurogenic bowel and bladder disorders, and integumentary complications, affecting overall quality of life and life expectancy.

Conventional treatments of SCI have not been efficient enough in healing the complex and deep injuries. However, discoveries of the past decades as well as developing of new promising technologies give many SCI patients new hope for at least facilitation their heavy condition. Using self-regenerating human stem cells as a treatment seems like a promising solution. They can differentiate into neurons, replacing damaged cells, or secrete many factors to accelerate growth of the existing cells (Mezey E. et al., 2000b; Ullah I. et al., 2015). In contrast to current treatment strategy stem cell therapy is considered as more promising and may significantly increase the chances for recovery. However, application of stem cells is limited by inefficient delivery strategies for targeting cells into the injured tissue. It not only has to be efficient and minimally invasive but it should also ensure proper retention and longevity of the cells for *in vivo*-like regeneration (Pluchino A. et al., 2003). Treating of SCI with stem cell-based therapies using magnetic nanoparticles for assisted magnetic targeting is an innovative approach which allows to solve these tasks. Transplantation of bone marrow cells, mesenchymal stem cells (MSCs), neural progenitor cells and induced pluripotent stem cells have been successfully performed with this technique (Landazuri N. et al., 2012, Cheng K. et al., 2014).

One of the most promising strategies may be using implants based on natural or synthetic polymers with sufficient biocompatibility and ability to reconstruct the damaged area of the spinal cord. Developed scaffold or matrix materials are made in the form of hydrogels, lyophilized sponges, fibers, powders, as well as combinations of these forms. Matrix implants

serve as scaffold, fill the defect and may have trophic functions supporting the viability of implanted stem cells (Wang W.H. et al., 2010). However, it should be noted that none of the huge number of natural and artificial matrix materials promising as components for spinal cord injury treatment fully satisfies all the requirements. Among the significant disadvantages of synthetic materials, is the fact that without additional modifications they cannot support adhesion, growth of cells and regeneration of axons, since they do not carry the appropriate ligands for cell receptors. At present time the most promising direction in the development of treatments of injuries of the nervous system such as spinal cord is to create a microstructured matrix composite implants having a three-dimensional pattern of signaling molecules that regulate cell proliferation and differentiation, stimulate adhesion and cell migration, axonal growth and recovery of brain function.

A plethora of available scientific literature describes the use of MSCs for treating traumatic SCI (Sykova E. et al., 2021). Using animal models, designed for developing rehabilitative training as well as cellular, molecular and combinatorial therapies for SCI has yielded some desirable results (Sykova E. et al., 2021). However, more research to verify the safety and efficacy of these treatments in human SCI cases is yet to be done. Therefore, the aim of our studies was developing new techniques for stem cells delivery with application of injectable extracellular matrix hydrogels as milieu for neuroregeneration *in vivo*.

3. Aims and hypotheses

The major aim of this work was improvement the results of treatment of traumatic spinal cord injury on the basis of experimental study of neuroregenerative properties of implanted biomaterials. Hydrogels based on natural extracellular matrixes, synthetic hydrogel with controlled structure and modifications of hyaluronic acid were studied to find the best substrate for implantation and support of stem cells for SCI treatment.

The major goals are summarized below:

1. Design of a magnetic system for stem cells delivery, evaluate its effectiveness on laboratory animals and use it to accumulate mesenchymal stem cells labelled with superparamagnetic iron oxide nanoparticles at a specific site of a SCI lesion.

2. Evaluation of filling the SCI lesion cavity, vessel and axonal ingrowth into CNS-derived SC-ECM hydrogel and non-CNS-derived UB-ECM hydrogel materials, in the model of SCI *in vivo*.

3. Study of the time-dependent dynamics of tissue ingrowth inside the scaffold of SIKVAV (Ser-Ile-Lys-Val-Ala-Val)-modified highly superporous poly (2-hydroxyethyl methacrylate) (HEMA) hydrogels with oriented pores.

4. Evaluation of axonal and blood vessels ingrowth in hydrogels based on modified hyaluronic acid HA-PH-RGD in the presence and absence of stem cells and fibrinogen in acute and subacute model of SCI in rats.

Hypothesis: Magnetic field allows to target and accumulate SPION-labelled stem cells specifically at the spinal cord lesion site due to focusing field in this point and superparamagnetic properties of nanoparticles. CNS-derived SC-ECM hydrogel is more compatible with spinal cord tissue and efficiently promote of neuroregeneration processes. Oriented porous structure of hydrogel better supports the neurofilaments ingrowth. Modification of hyaluronic acid by adding RGD peptide improves neurorenerative potential of scaffold hydrogels based on HA. Addition of fibrinogen enhances proliferation of stem cells.

4. Materials and methods

4.1. Preparation of extracellular matrix hydrogels

Extracellular matrix hydrogels based on porcine spinal cord and porcine urinary bladder were prepared from animal tissues as described (Medberry C.J. et al., 2013). Preparation of the P(HEMA-AEMA) hydrogel with oriented porosity was based on polymerization of monomers 2-hydroxyethyl methacrylate and ethylene dimethacrylate. Hyaluronic acid was modified by synthetic HPA-K-AHA-GRGD oligopeptide sequence.

4.2. Cell cultures

The transgenic Sprague–Dawley rats [SD-Tg(CAG-EGFP)CZ-004Osb] were kindly provided by Dr. Masaru Okabe (Osaka University, Japan). The preparation of GFP-positive MSCs and labelling with poly-*L*-lysine-coated SPION was done in accordance to published methods (Vanecek V. et al., 2012). Fresh human umbilical cord samples were collected from healthy full-term neonates after spontaneous delivery with the informed consent of the donors using the guidelines approved by the Institutional Ethics Committee at University Hospitals in Pilsen and Prague, Czech Republic. Human Wharton's jelly mesenchymal stem cells from umbilical cord were extracted and cultivated as described (Tukmachev D. et al., 2016). Rat dorsal root ganglia explant culture was prepared from Wistar rats sinal cord as described (Tukmachev D. et al. 2016).

4.3. Animal experiments

All experiments with animals were performed in accordance with the European Communities Council Directive of 22nd of September 2010 (2010/63/EU) regarding the use of animals in research, and was approved by the Ethics Committee of the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic. One week after the induction of the lesion in male Wistar rats, SPION-labelled GFP-positive MSCs were injected intrathecally at the *L5-L6* level, at a distance of 10 cm from the lesion site (Figure 1). The external magnetic system was placed around the rat (Figure 2).

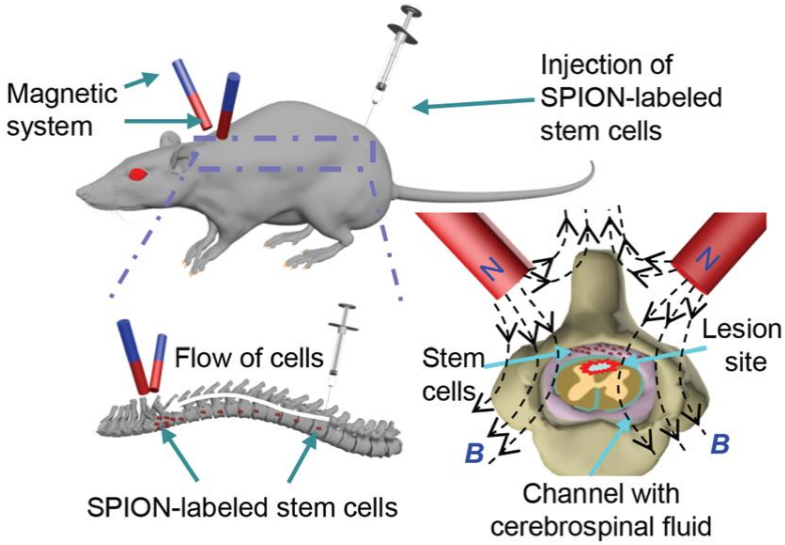

Figure 1. Schematic representation of the magnetic targeting strategy.

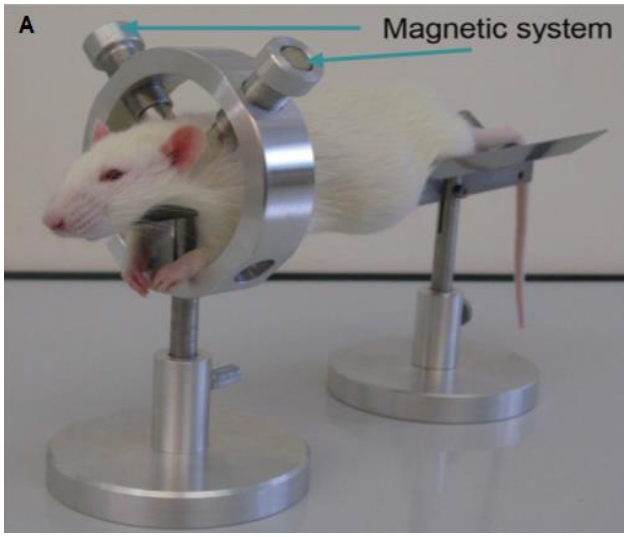

Figure 2. In vivo application of the non-invasive magnetic system for MSC targeting into SCI of a rat.

Spinal cord transection was performed in male Wistar rats (300 - 300 g, Velaz, Czech Republic) in thoracic spine Th7-9 with dissection of 2 mm segment of spinal cord, resulting a

cavity in spinal cord. One week after the transection HEMA hydrogel with or without seeded MSCs was implanted in the cavity.

Hemisection was performed on right side at the level of the 8th thoracic vertebra (Th8). Liquid pre-gel solution of SC-ECM, UB-ECM or HA-PH-RGD hydrogels were acutely injected into the spinal cord defect after hemisection in a single injection and allowed to gelate *in situ*.

4.4. Tissue processing and histology

In corresponding time after induction of spinal cord lesion, the animals were deeply anesthetized, perfused with 4 % paraformaldehyde in 0.1 M PBS. The spinal cord was left in the bone overnight, then removed and postfixed. GFP-positive MSCs quantification was performed in longitudinal sections (20 μm) using a fluorescent microscope (Carl Zeiss, Rochester, NY). Hematoxylin-eosin (H&E) and Masson´s trichrome staining was performed using the standard protocol and the slides were specifically evaluated using an Axio Observer D1 microscope (Carl Zeiss Microimaging GmbH). For immunohistological staining, the corresponding primarily and secondary antibodies were used. The morphology of the cells on the hydrogels was examined by immunofluorescent staining for actin filaments. The nuclei were visualized by using 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye (1:1000; Invitrogen, UK). For axonal and vessel ingrowth analysis NF160 staining and RECA staining correspondingly were used.

4.5. Gene expression analysis

The specific genes expression was studied using quantitative real-time polymerase chain reaction (qRT-PCR).

4.6. Statistical methods

The statistical significance of differences in cell counts in the spinal cord lesions between the groups of animals was determined using ANOVA Fisher's LSD and Newman-Keuls tests and a Student's two-sample t-test (probability values <0.05 and <0.01 were considered statistically significant). The mean values are reported as mean ± SEM to plot all the data in the graphs.

5. Results

5.1. An effective strategy of magnetic stem cell delivery for spinal cord injury therapy

To enhance the efficacy of stem cell delivery in rat model of SCI we proposed a new magnetic system consisting of two cylindrical NdFeB magnets placed on a ring-shaped holder with the alike poles facing toward each other (Figure 2). A specific feature of the proposed

magnetic system is the existence of a focusing zone – trapping area – where both the horizontal and vertical magnetic force components (X- and Z-components) are almost zero (Figure 3). The magnetically labelled cells have to be focused namely in this trapping area (Figure 4).

First, we tested whether the MSCs labelled with SPIONs can be efficiently attracted by a magnet *in vitro* (Figure 4A). As follows from Figure 4A SPION-labelled cells effectively concentrated in magnetic field focusing area. Then distribution of SPION-labelled MSCs under magnetic field was evaluated *in vivo*. One week after the induction of the lesion, 5×10^5 cells were injected intrathecally at the L5–L6 level, at a distance of 10 cm from the lesion site (Figure 1) and external magnetic system was placed around the rat (Figure 2).

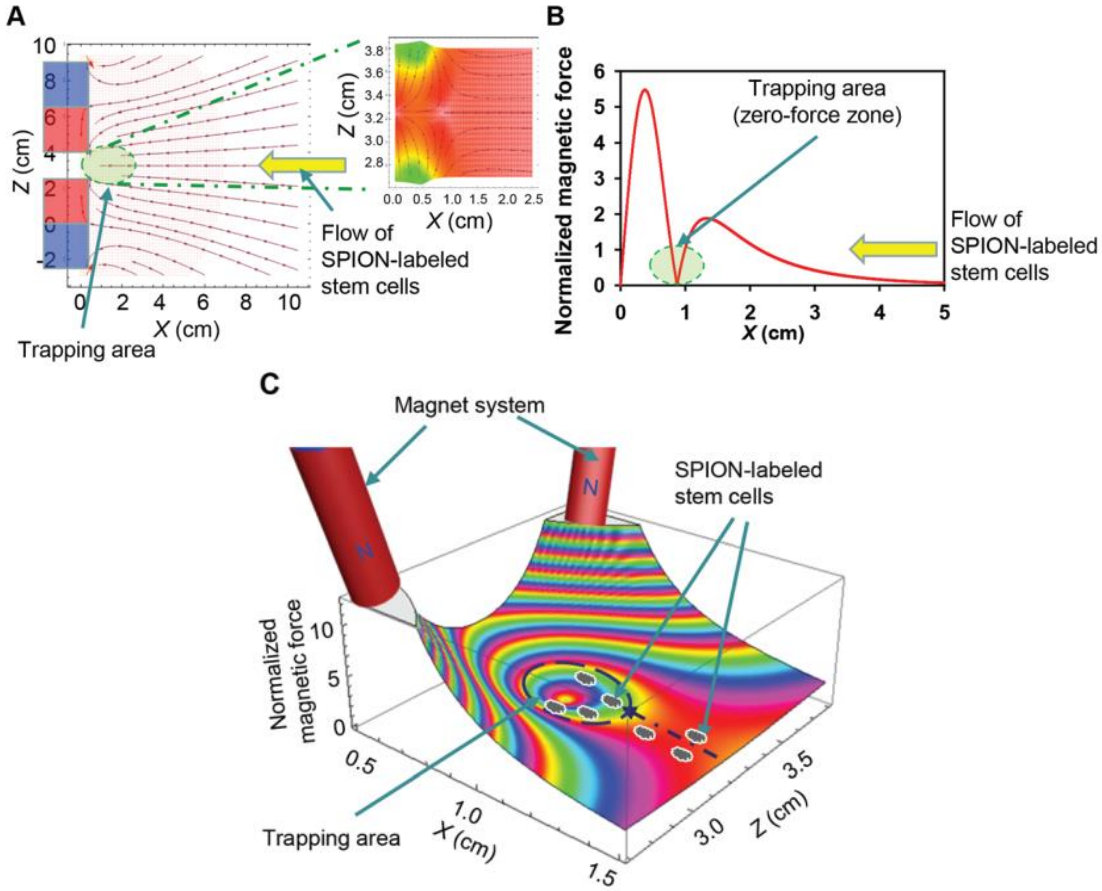

Figure 3. Spatial distribution of the magnetic gradient forces between the magnets of the designed magnetic system. (A) Calculated vector field plot of the magnetic gradient force (X–Z-plane – the vertical cross-section of the spinal cord). The insert represents an enlarged region of the trapping area (zero-force zone). The arrows show the directions of the magnetic gradient forces (f_m α ∇B^2) applied to a cell (where B is the magnetic induction). (B) Modulus of magnetic gradient force, $\left|(\vec{B}\vec{\nabla})\vec{B}\right|$, normalized to $(\mu_0 M_r)^2 r^{-1}$, as a function of the X-coordinate which is along the cerebrospinal channel (M_r represents the remnant magnetization and $\mu_0 M_r$ = 1.2 T for the used magnets, magnet radius r = 0.5 cm). In (A) and (B) the focusing area is shown by the green ellipses. (C) 3D plot of the normalized magnetic gradient force (X–Z-plane).

We can observe dramatic increase of SPION-labelled stem cells retention at the lesion site in the group of animals injected with SPION-labelled MSCs and exposed to MF. On the other hand, in animal groups injected by cells without SPIONs or cells loaded with SPIONs but without magnets exhibited, practically homogenous distribution through the spinal cord channel was observed (Figure 4B). SPION-labelled cells accumulated maximally in the trapping area of the magnetic system.

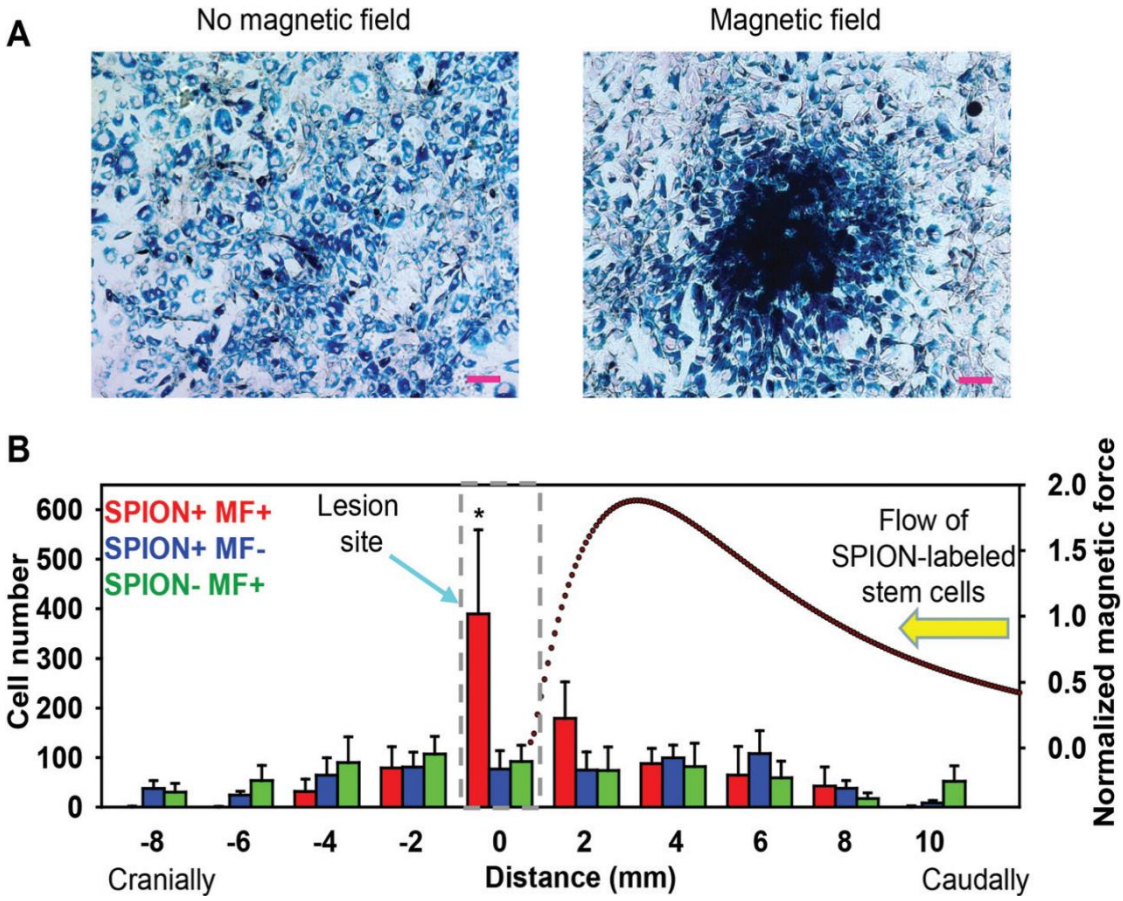

Figure 4. Distributions of SPION-labelled cells and non-labelled cells with and without the magnetic field. (A) Attraction of SPION-labelled cells to a cylindrical magnet in vitro. MSCs were labelled with SPIONs at a concentration of 15.4 μg mL^{-1} and exposed to an external magnetic field for 48 h. Cells were stained for intracellular iron using Prussian blue. Scale bar: 100 μm. (B) Numbers of the captured SPION-labelled cells and non-labelled cells in the rat model as a function of the distance from the lesion site of the SCI. After the induction of the lesion, SPION-labelled MSCs were injected intrathecally at the L5–L6 level, at a distance of 10 cm from the lesion site. Thereafter, animals were subjected to the magnetic system exposure for 2 h in longitudinal spinal cord segments. The dotted curves represent the respective magnetic gradient force distribution taken from Figure 3. Data are expressed as mean ± SEM, *P < 0.05.

5.2. Injectable extracellular matrix hydrogels as scaffolds for spinal cord injury repair

Both SC- and UB-ECM hydrogels showed comparable ability to support *in vitro* hWJ-MSCs in the 2D and 3D cell cultures proliferation, which did not significantly differ from the

control cell culture seeded on standard plastic tissues. In 3D culture hWJ-MSC extended their lamellipodia within the hydrogels and formed a 3D network. Both hydrogels have neurotrophic properties supporting neurite ingrowth in dorsal root ganglion (DRG) explant culture.

UB-ECM and SC-ECM hydrogels were injected into the cavity of the spinal cord hemisection and the tissue response to the scaffolds was histologically evaluated by analyzing axonal ingrowth, vascularization, and infiltration of macrophages/microglia, astrocytes and oligodendrocytes within the injury site. Both hydrogel types were biocompatible with the surrounding host tissue and entirely filled the lesion cavity. The hydrogels were mostly degraded but still remained detectable after 4 weeks in the lesion area and were densely populated by the host cells. Macrophages massively infiltrated the periphery of the lesion where several small cysts developed due to the rapid degradation of the graft. At 8 weeks the hydrogels had fully degraded, which was followed by further progression of cyst formation.

To evaluate axonal ingrowth into the hydrogels, a neurofilament marker (NF160) was used (Figure 5). The ingrowth of NF was maximal at 2 weeks in both hydrogel groups and did not further increase at later time points. Astrocytes did not migrate inside the lesion.

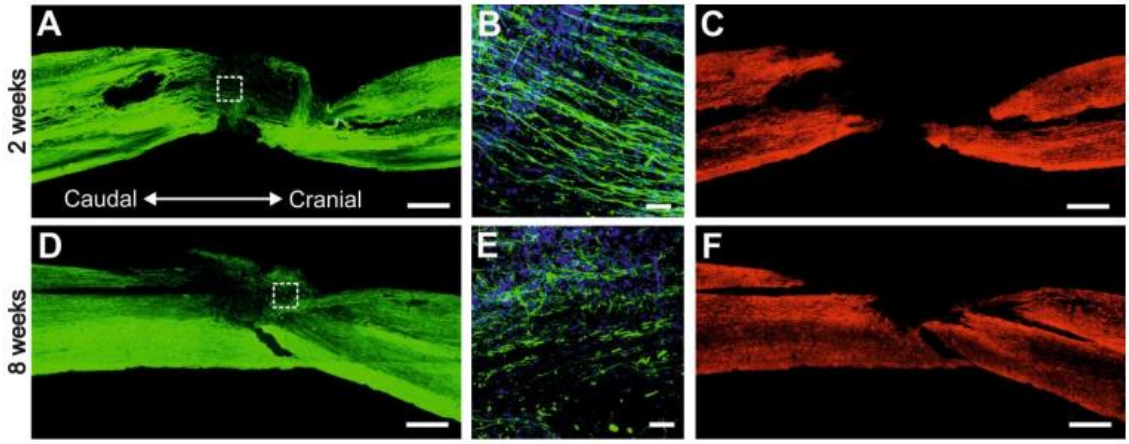

Figure 5. Representative images of the spinal cord lesion (A-C) 2 and (D-F) 8 weeks after injection of SC-ECM hydrogels. Immunofluorescent staining for (A, B, D, E) neurofilaments (NF160), (C, F) astrocytes (GFAP) and (B, E) cell nuclei (DAPI, blue). Squares (A, D) are also shown under the higher magnification insets (B and E).

The area of blood vessels gradually increased with time. Remodeling response marker CD68+ cells populated the hydrogels at all time points, and remained in the lesion site after the hydrogel had degraded. Macrophages at the interface of the ECM hydrogel and the host tissue were predominantly of the M1 phenotype, while M2 phenotype macrophages were mostly present within the hydrogel area. Infiltration of oligodendrocytes within the lesion site indicated that myelination occurred in some of the regenerated axons. Numerous endogenous Schwann cells were detected within the lesion site as well as in the surrounding tissue.

SC-ECM hydrogels seeded with hWJ-MSCs were densely infiltrated by endogenous tissues. Transplanted cells did not further promote the ingrowth of NF-positive fibres or blood vessels. However, an increase in NF positive fibres was found in those animal groups which received immunosuppression.

Downregulation in the mRNA expression of genes related to inflammation (Ccl5), M1 macrophages (Irf5), M2 macrophages (CD163), growth factors (Fgf2), axonal sprouting (Gap43), astrogliosis (Gfap) was observed 2 weeks after injury. At 4 weeks, no significant changes were detected between both hydrogel groups and the control group. Expression of pro-inflammatory cytokines IL-2, IL-6, Il12b and Nos2 was undetectable in all groups.

5.3. Dynamics of tissue ingrowth in SIKVAV-modified highly superporous PHEMA scaffolds with oriented pores after bridging a spinal cord transection

The transection cavity was satisfactorily bridged by P(HEMA-AEMA) hydrogel. Negligible pseudocystic cavities were observed and no foreign body reactions were noted in or around the hydrogels. Blood vessels dominantly infiltrated the hydrogels two days post its implantation (Figure 6A). Connective tissue elements also appeared in the complete volume of the hydrogels one week after the implantation (Figure 6B). A week after the implantation, there were a few axons specifically in the peripheral parts of the scaffold (Figure 6C). Examination half a month post infiltration revealed a notable increase in the number of axons in the peripheral parts and the neural sprouts grew into the central parts of the scaffold. No statistically notable difference in neurite and blood vessels ingrowth was seen between seeded with MSCs and those without MSCs groups at any point of time.

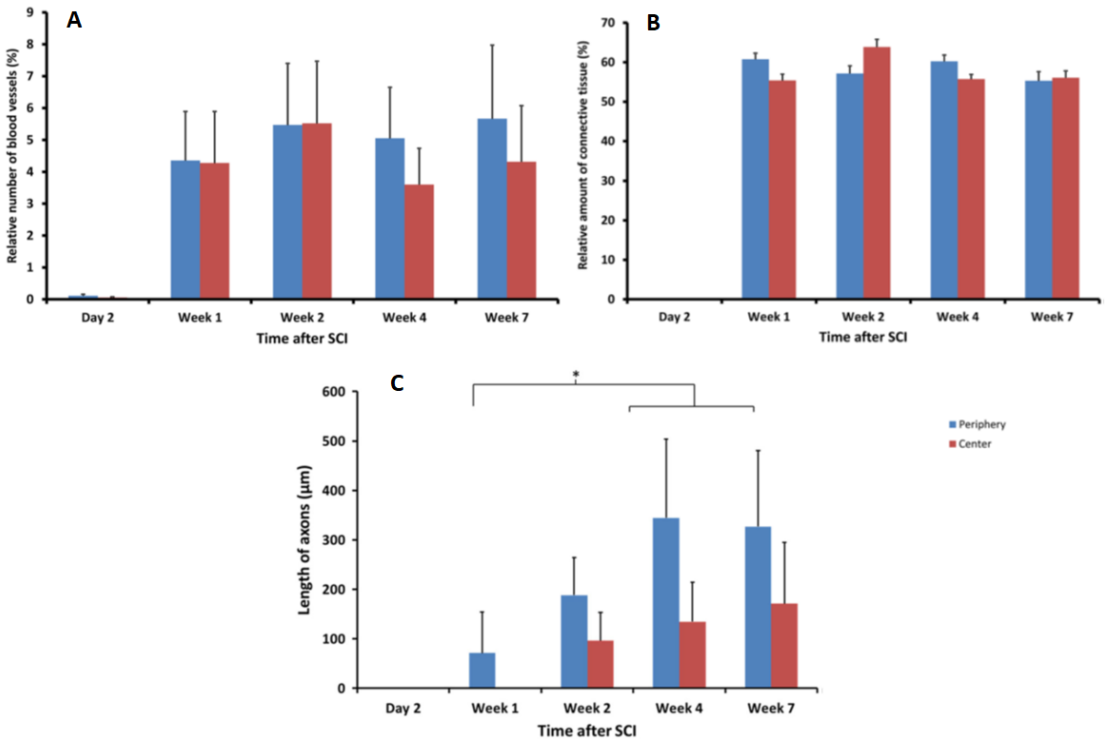

Figure 6. Dynamics of blood vessel ingrowth (A), connective tissue infiltration (B) and axonal ingrowth (C) in the SIKVAV-HEMA scaffold. A statistically significant increase in the number of axons is apparent between Day 7 and Weeks 4 and 7 after hydrogel implantation (*p <0.05). There is a gradual increase in the number of axons infiltrating the scaffold.

5.4. Injectable hydroxyphenyl derivative of hyaluronic acid hydrogel modified with RGD as scaffold for spinal cord injury repair

Both the implanted as well as the injected HA-PH-RGD hydrogels occupied the lesion cavity along with a high population of endogenous cells in acute SCI lesions (Figure 7). The dense ingrowth of neurofilaments, and blood vessels into the hydrogel-treated lesion was observed throughout the whole implant after half a month, and persisted with no considerable changes 2 months post application (Figure 7).

Both HA-PH-RGD and HA-PH-RGD/F hydrogels enhanced neurofilament infiltration density in the subacute SCI lesion. This increase in neurofilament density was magnified by the introduction of hWJ-MSCs. The extent of blood vessel infiltration into the hydrogel treated lesion was higher as compared to the control lesion. The migration of the M1 and M2 macrophages into the hydrogel treated lesion was verified. The oligodendrocytes were absent in the control lesion; however, they were presented in the area of hydrogel treated lesions. After 2 months post cell application, the spinal cord tissue did not have any hWJ-MSCs. Decreased expression of genes related to macrophages (Irf5, Cd86), inflammation (Ccl3) and glial scar formation (Gfap, Ptprz1) were noted in the lesions with injected HA-PH-RGD and HA-PH-

RGD/F hydrogels (not significant). Significant downregulation was then found for the expression of Gap43 when compared to the untreated control lesion. Contrarily, the expression of Gap43 was significantly increased when the HA-PH-RGD/F was combined with hWJ-MSCs. Along these lines, a combination with hWJ-MSCs led to significant upregulation of both M1 (Irf5, Cd86) and M2 macrophages markers (Mrc1). No significant differences in the average hindlimb locomotor score were observed between the tested groups.

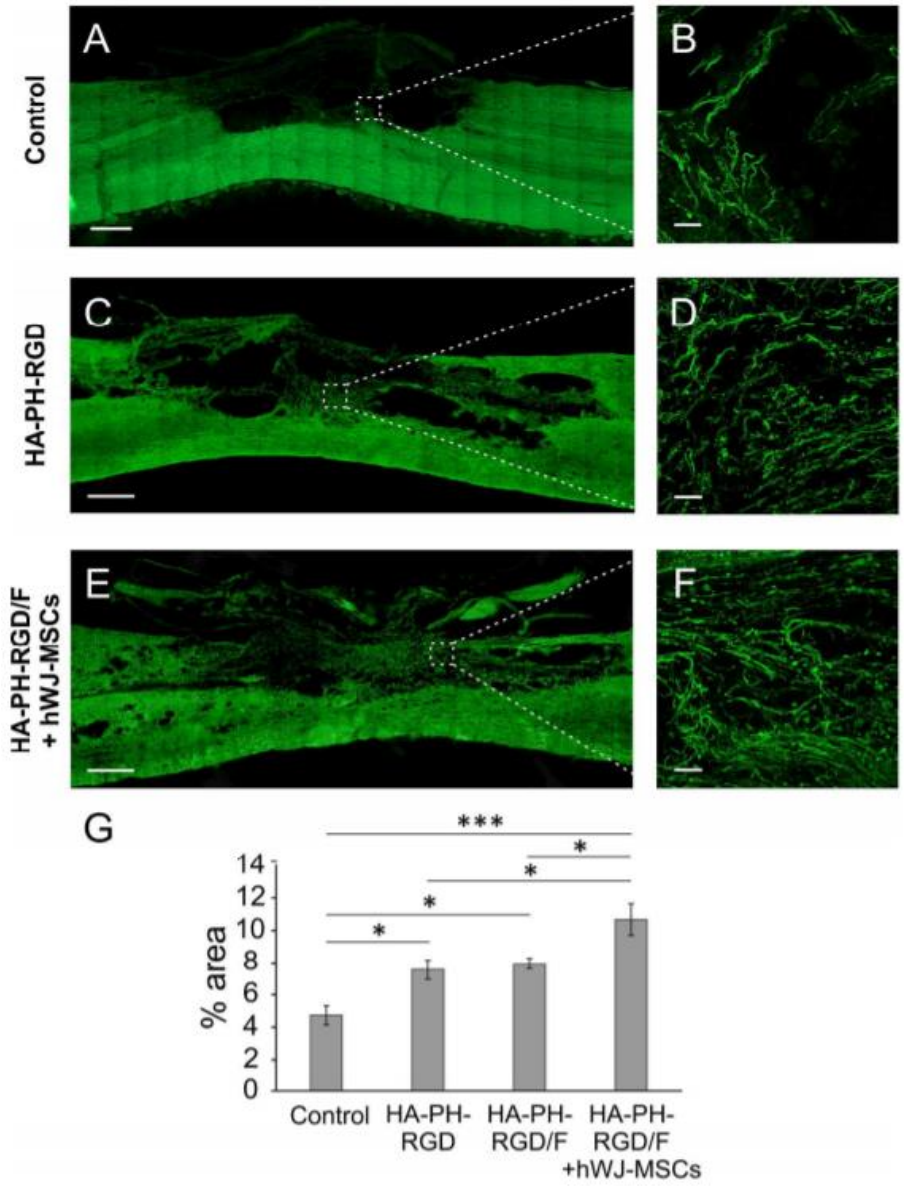

Figure 7. Representative images of the longitudinal sections of the spinal cord lesion in (A, B) controls and at 8 weeks after the subacute injection of (C, D) HA-PH-RGD and (E, F) HA-PH-RGD/F hydrogels combined with hWJ-MSCs, stained for neurofilaments (NF-160). Squares (A, C, E) are also shown under the higher magnification insets (B, D, F). (G) Quantitative analysis of axonal ingrowth is expressed as the percentage of NF160 positive area from a total lesion area (n = 6). *p < 0.05, ***p < 0.001. Scale bar: 500 µm (A, C, E), 50 µm (B, D, F). 194x261mm (300 x 300 DPI).

6. Discussion

Using stem cells for SCI treatment is modern promising method with high potential in the future. However, the major factor determining the success of the stem cells transplantation is delivery technique. Therefore, development and improvement of targeting cell delivery techniques are necessary for enhancing the efficacy of SCI treatment. Combination of magnetic nanomaterials conjugated with stem cells and focused magnetic field to guide cells migration and trap them in the lesion site has high potential for further development and introduction in practical therapy. Retention of cells at lesion site, engraftment efficacy and functional recovery were significantly enhanced when SPION-labelled stem cells delivery was magnetically guided to target organ/tissue/location (Landazuri N. et al., 2013; Vandergrif A.C. et al., 2014; Cheng K. et al., 2014, Pislaru S.V. et al., 2006, Polyak B. et al., 2008, Riegler J. et al., 2013, Panseri S. et al., 2012, Kamei G. et al., 2013, Yanai A. et al., 2012). However, only few attempts were performed for magnetic delivery of stem cells to SCI lesion site (Nishida K. et al., 2006; Vanecek V. et al., 2012; Hamasaki T. et al., 2007).

The main drawback in the application of magnetic delivery is poor focusing ability. In this study we designed new magnetic system which allows to focus magnetic field strongly in the spinal cord lesion site (Figure 12, 14) – trapping area. MSCs labelled by the poly-L-lysine-coated SPIONs were efficiently attracted by a magnet and concentrated in magnetic field focusing area *in vitro* as well as at the lesion site *in vivo* after injection intrathecally at the L5–L6 level after only 2 hours of exposition. Therefore, the application of our magnetic system demonstrates the potential benefits of fast and efficient stem cells delivery into SCI lesion. The proposed strategy can be used for stem cells-based treatments of not only traumatic SCIs, but also for non-traumatic SCI or neurodegenerative diseases (Sykova E. at al., 2006, Forostyak S. at al., 2013).

Other significant task which has to be solved in SCI treatment is replacement of the lesion cavity by implantation of matrix to support further tissue regeneration. This matrix can represent syntethic polymer or native extracellular matrix (ECM) and must provide supportive substrates for replacing lost tissue and re-establishing damaged connections (Kubinova S. and Sykova E., 2012, Assuncao-Silva R.C. at al., 2015). Scaffolds composed of native ECM represent structures very similar to uninjured host tissue with natural three-dimensional structure, biological activity promoting cell adhesion and proliferation, and biodegradability (Crapo P.M. at al., 2012). Currently, ECM scaffolds are being widely used for various tissue reconstructions, including heart valves, blood vessels, etc, but only few studies addressing for the repair of SCI (Zhang X.Y. at al., 2011, Li C. at al., 2012, Liu J. at al., 2012).

ECM hydrogels prepared by decellularization of porcine spinal cord (SC-ECM) and porcine urinary bladder (UB-ECM) provide a supportive environment for the *in vitro* neural cell growth (Medberry C.J. at al., 2013, DeQuach J.A. at al., 2011). However, experimentally, it is unknown whether these materials can be successfully used for SCI repair, either alone or in combination with various types of cells. We examined the effects of injectable SC-ECM and UB-ECM hydrogels in the acute model of SCI. Despite the lack of a native three-dimensional ultrastructure intrinsic for source tissue, ECM hydrogels retain their biological activity and possess mechanical properties similar to that of soft neural tissue, with the advantage of injectability and *in situ* polymerization, which offer minimally invasive delivery techniques and facilitate the possibility of clinical translation. When injected into the SCI, both hydrogel types were well integrated into the surrounding tissue, and supported massive cell infiltration and neovascularization. Macrophages were the predominantly infiltrating cells within the grafts that participated in the ECM degradation. Degradation of ECM scaffolds is essential for the constructive tissue remodelling process by which a degradable biomaterial serves as a temporary inductive niche, which is gradually replaced by functional tissue as opposed to scar tissue (Badylak S.F. at al., 2009, Tottey S. at al., 2011, Valentin J.E. at al., 2009). Moreover, degradation of ECM scaffolds stimulates the release of matricryptic molecules which possess a variety of bioactive properties such as antimicrobial activity, angiogenic effects, as well as the recruitment of endogenous stem and progenitor cells (Valentin J.E. at al., 2009). Implantation of ECM hydrogels with seeded stem cells may partly prevent the massive scaffold contraction within the lesion cavity. However, the inflammatory milieu of the acute lesion together with the massive infiltration of macrophages did not support cell survival. Higher *in vivo* cell survival rate could be achieved by increasing the number of implanted cells.

Some synthetic hydrogels (SIKVAV (Ser-Ile-Lys-Val-Ala-Val)-modified highly superporous poly(2-hydroxyethyl methacrylate) hydrogel with oriented pores) possess a moderate modulus of elasticity, which has been shown to promote good bridging, tissue infiltration and abundant axonal ingrowth (Kubinova S. et al., 2010). SIKVAV sequence is a synthetic peptide from active regions of the chain A of basement membrane laminin, which promotes cell adhesion and neural outgrowth by the binding of transmembrane integrin receptors (Tashiro K. et al., 1989). Moreover, synthetic nature of implant attenuates fast scaffold degradation and provide stable matrix for cells filling. Other significant findings include the observation that the oriented pores of the hydrogel directed axonal growth in the cranio-caudal and caudo-cranial direction. Such aligned ingrowth enabled easier evaluation of the amount and the length of axons into the scaffold.

According to our experience with hydrogel bridging in experimental SCI, we noticed that new axons do grow inside most scaffolds during the first weeks but, when evaluated at later time points, the number of axons seems to be rather inadequate with respect to the earlier results. Only few studies throw light on the time-related dynamics of the tissue infiltration of these scaffolds. Therefore, we estimated the time-dependent dynamics of ingrowth of connective tissue, axons and blood vessels inside the implanted scaffold hydrogel with or without seeded stem cells. SIKVAV-HEMA-based hydrogel scaffold environment supported the ingrowth of connective tissue, blood vessels and axons. Axonal infiltration into the scaffold was a slow and gradual process as compared to the much faster connective tissue ingrowth. Axons slowly and gradually infiltrated the hydrogel within the first month, after which the numbers became stable. One reason could be the spatial limitation in the hydrogel pores, due to the connective tissue infiltration. Deficiency of nutrients and growth factors can however be another plausible cause.

Progressive infiltration of the connective tissue into the hydrogel pores continues only for the first week, after which it plateaus for the next 2 months. On the contrary, the axons continue to grow into the hydrogel pores even at a time of one month after the implantation. This is not facilitated by the presence of MSCs inside the hydrogel pores. However, using genetically engineered MSCs, that over-express some of the growth factors like BDNF and VEGF, instead of simple MSCs, it may be possible to stimulate axonal ingrowth and improve tissue repair (Stewart A.N. et al., 2017). Inclusion of some other supportive cells such as Schwann cells may also improve neuroregenerative effects alongside the MSCs (Yang E.Z. et al., 2017). Thus, it is safe to assume that modified MSCs may promote their positive effect on the neuronal repair after SCI.

The physical and chemical properties of the scaffold dictate its inner architecture and structure, which is vital part of the micro-environmental milieu. In one of previous studies, was found that the web-like architecture of the hydrogel, together with the HPMA-based backbone promotes the ingrowth of new axons despite promoting the adhesion of fewer MSCs compared to HEMA-based hydrogels (Hejcl A. et al., 2013). The results of these studies yielded points to the fact that the effect of MSCs seeded on hydrogels is less important than the inner milieu of the hydrogel, influenced by the chemical backbone and the architecture of the scaffold.

Despite benefits of HEMA-SIKVAV hydrogel on axonal regeneration and functional outcome (Tysseling-Mattiace V.M. et al., 2008; Tysseling V.M. et al., 2010) it is still not the solution to the problem how to bring for long-term survival and promotion of axons in the hydrogel scaffolds.

Hyaluronic acid (HA) is a vital structural component of the ECM and plays an important role in regulating tissue regeneration because it acts as a signaling molecule via some specific HA receptors (Litwiniuk M. at al., 2016, Knopf-Marques H. at al., 2016). HA is very commonly used as biomaterial in the clinical settings as it possesses significant biocompatibility, non-immunogenicity, and biodegradability. HA based substrates have previously been used *in vitro* for neural stem cell cultures and also *in vivo* in neural tissue engineering activities (Seidlits S.K. at al., 2010). They can be used either by themselves or as carriers for cell delivery to enhance cell retention and integration (Mothe A.J. at al., 2013, Raynald, Li Y., 2016, Liang Y., 2013, Li L.M., 2017). Injectable HA-based hydrogels were also developed for localized intrathecal delivery of bioactive molecules into the SCI (Fuhrmann T., 2015, Gupta D., 2006). The most significant benefit of using this material is that it can be manufactured in a reproducible manner under GMP (Good Manufacture Practice) conditions, which are required to allow the transfer of its production from bench-to-bedside in clinical practice.

Since native HA can't form a gel or support cell adhesion, it has to be modified chemically by addition of hydroxyphenyl (PH) groups. An enzymatic crosslink reaction initiated by horseradish peroxidase and hydrogen peroxide was used to form the hydrogel and set mechanical properties comparable to the native spinal cord tissue under physiological conditions (Kučera L., 2015). In our study, the HA-PH derivative bearing the newly developed 3-(4-hydroxyphenyl) propionic acid - L-lysine – aminohexanoic acid - L-glycine - L-arginine - L-glycine - L-aspartic acid (HA-PH-RGD) sequence was used to improve cells adhesion and migration into implants. RGD increased affinity for several integrin receptors and also supported cell spreading, cytoskeletal organization and cell proliferation (Mackova H., 2016, Karoubi G., 2009, Zapotocky V., 2017).

Fibrinogen offers cellular adhesive domains and has been previously proved as a natural additive to enhance cell survival, growth and proliferation (Karoubi G., 2009, Zapotocky V., 2017). Therefore, a combination of HA-PH-RGD with fibrinogen (HA-PH-RGD/F) enhanced the adhesive abilities of the hWJ-MSCs. Contrarily, the addition of fibrinogen had no effect on the assessed tissue repair parameters in subacute SCI that may be explained by overlapping endogenous integrins (i.e. fibronectin) from extracellular fluid entered the hydrogel. Injection of both HA-PHRGD and HA-PH-RGD/F hydrogels similarly promoted axonal ingrowth into the lesion and this effect was further enhanced when the HA-PH-RGD/F was combined with hWJ-MSCs. On the other hand, no effect was found on locomotor recovery or the blood vessel ingrowth and density of glial scar around the lesion.

Thus, although there are several scaffolds that can support axonal growth and spouting after SCI, there are few of those which are able to achieve significant functional recovery, due to the reduced regeneration of long-tract axons through sites of SCI. Designing and developing materials suitable for bridging the lesion must be accompanied with efforts to modify the extrinsic and intrinsic factors limiting regrowth after injury which represents the current challenge of how to overcome the inhibitory properties of the axon–scar environment. Combinatorial therapies will probably be essential to achieve such functional connectivity (Chew D.J., 2012).

In conclusion, we have developed and characterized injectable HA-PH-RGD based hydrogel, which represents a suitable material for further combinatorial therapies in neural tissue engineering. The injected HA-PH-RGD hydrogels bridge the SCI lesion cavity, support vascularization and also increase axonal sprouting into the lesion. Combining the hydrogels with hWJ-MSCs assisted in these activities. In spite of the significant improvements of neuroregeneration processes, it is still imperative to find additional treatments that would further stimulate axonal reconnection to best functional SCI restoration.

7. Conclusions

1. The non-invasive magnetic system was designed and used to accumulate mesenchymal stem cells labelled with superparamagnetic iron oxide nanoparticles precisely at a specific site of a SCI lesion. This system allowed to reach a significantly higher concentration of SPION-labelled stem cells into the vicinity of a lesion site.

2. Two types of ECM hydrogels derived from decellularized porcine spinal cord and urinary bladder tissues were evaluated as scaffolds for SCI repair. Both ECM hydrogels showed significant immunomodulatory and neuroregenerative effects and provided the substrate for tissue bridging after SCI.

3. Connective tissue and blood vessels quickly infiltrate the SIKVAV-HEMA scaffold within the first week, however, axons show a rather gradual infiltration over the first month, and this is not facilitated by the presence of MSCs inside the hydrogel pores.

4. Injectable HA-PH-RGD derivative hydrogel was developed and characterized. Injection of HA-PH-RGD hydrogels bridged the lesion cavity, supported vascularization and increased axonal sprouting into the lesion, which was further improved by its combination with hWJ-MSC.

8. Summary

Restoration of lost neuronal function after spinal cord injury (SCI) still remains a big challenge for current medicine. One important repair strategy is bridging the SCI lesion with a supportive and stimulatory milieu that would enable axonal rewiring. Extracellular matrix (ECM)-derived hydrogels may serve as scaffold and provide infiltration of blood vessels and neurites. Treatment of SCI utilizing stem cell transplantation also represents a promising therapy. Hydrogel scaffolds which bridge the lesion, together with stem cell therapy represent a promising approach for spinal cord injury (SCI) repair. However, current conventional treatments are limited by inefficient delivery strategies of cells into the injured tissue. Therefore, the aim of this study was to develop new techniques for SCI treatment on the basis of addressing stem cell delivery via magnetic field or transplantation with injectable extracellular matrix hydrogels as a milieu for neuroregeneration *in vivo*.

We designed a magnetic system and used it to accumulate stem cells labelled with superparamagnetic iron oxide nanoparticles (SPION) at a specific site of a SCI lesion. The magnetic system allowed rapid guidance of the SPION-labelled cells precisely to the lesion location. Histological analysis of cell distribution throughout the cerebrospinal channel showed a good correlation with the calculated distribution of magnetic forces exerted onto the transplanted cells.

Natural and synthetic ECM: decellularized porcine spinal cord (SC) and urinary bladder (UB), superporous hydrogel, modification of hydroxyphenyl derivative of hyaluronic acid (HA-PH) with the integrin binding peptide RGD were evaluated for their neuroregenerative properties in a rat model of SCI. Hydrogels were injected or transplanted into the spinal cord hemisection cavity with or without stem cells. Histological analysis and gene expression analysis were performed after implantation.

All hydrogels supported neovascularization and axonal ingrowth into the lesion. No significant differences in tissue infiltration were found between effects of SC-ECM and UB-ECM, as well as between the plain hydrogels and seeded with stem cells hydrogels. However, injection of HA-PH-RGD/Fibrinogen with Wharton's jelly-derived human mesenchymal stem cells (hWJ-MSCs) enhanced only axonal ingrowth.

In conclusion, the combination of injectable hydrogel scaffolds and technique of noninvasive magnetic delivery of labelled stem cells seems to be promising strategy for SCI treatment due to improved cell survival, inhibition of systematic inflammation, enhanced engraftment and providing *in vivo*-like neural regeneration.

Souhrn

Poranění míchy (SCI) je velice vážné trauma, které vede k významné mortalitě a morbiditě. Léčba SCI byla vždy léčbou nejobtížnější, nicméně využití transplantace kmenových buněk představuje slibnou terapii. Další důležitou reparační strategií je přemostění léze SCI podpůrným a stimulačním prostředím, které by umožnilo axonální přepojení. Nedávno bylo zjištěno, že injikovatelná extracelulární matrix (ECM) odvozená z hydrogelů má neurotrofický potenciál in vitro. Cílem této studie bylo proto vytvořit nové techniky pro léčbu SCI s využitím řešení, které spočívá v transportu kmenových buněk magnetickým polem nebo transplantaci využívající injikovatelné hydrogely na bázi extracelulární matrix jako prostředí pro neuroregeneraci *in vivo*.

Byl navržen neinvazivní magnetický systém, který byl následně použit k akumulaci kmenových buněk značených superparamagnetickými nanočásticemi oxidu železa (SPION) na specifickém místě léze SCI. Magnetický systém navíc umožnil rychlé navádění buněk značených SPION přesně na místo léze. Histologická analýza distribuce buněk v celém mozkomíšním kanálu prokázala dobrou korelaci s vypočítanou distribucí magnetických sil působících na transplantované buňky.

Decelularizované tkáně prasečí míchy (SC) a močového měchýře (UB) a superporézní hydrogel s orientovanou porozitou a modifikací hydroxyfenylderivátu kyseliny hyaluronové (HA-PH) s peptidem RGD vázajícím se na integrin jako skafoldové hydrogely byly hodnoceny z hlediska jejich neuroregenerativních vlastností na potkaním modelu SCI. Hydrogely s kmenovými buňkami nebo bez kmenových buněk byly injikovány nebo transplantovány do dutiny míšní hemisekce. Po injekci byla provedena histologická analýza a analýza genové exprese.

Všechny typy hydrogelů se integrovaly do léze a stimulovaly neovaskularizaci a axonální vrůstání do léze. Mezi SC-ECM a UB-ECM nebyly zjištěny žádné významné rozdíly. Nebyl zjištěn žádný rozdíl v infiltraci tkání mezi prostými hydrogely a hydrogely s nasazenými kmenovými buňkami. Subakutní injekce HA-PH-RGD/fibrinogenu v kombinaci s lidskými mezenchymálními kmenovými buňkami získanými z Whartonova želé (hWJ-MSC) však zesílila pouze axonální vrůstání do lézeю

Závěrem lze konstatovat, že kombinovaná aplikace injikovatelných hydrogelových skafoldů a technika neinvazivního magnetického transportu jsou klíčovými faktory pro zlepšení přežití buněk, jejich přihojení, neurální regeneraci podobnou *in vivo* a inhibici systematického zánětu.

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10. List of author's publications

Original scientific works in extenso, which are the basis of the dissertation (with impact factor):

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