Charles University in Prague

Faculty of science

Special chemical-biological branches

Molecular biology and biochemistry of organisms

Bachelor Thesis

# Analysis of the growth of human neural stem cells on several types of biocompatible hydrogels

Jiří Růžička

Supervisor: RNDr. Natalyia Kozubenko, Ph.D

Department of tissue cultures and stem cells

Institute of Experimental Medicine AS CR,v.v.i.

Prague, 2009

**Affirmation**

I hereby declare that I have written this bachelor thesis independently, with the use of listed literature

Prague 27.4.2009 Jiří Růžička

Signature…….

**Acknowledgments**

I wish to thanks on the first place my girlfriend, friends and family, helping me in my studies and work. I wish to thanks my supervisor Nataliya Kozubenko for patience and guidance in process of bachelor thesis preparing and writing. And also I wish to thanks my colleagues from department of tissue culture and stem cells of Institute of Experimental Medicine, for chance to work in friendly environment and for opportunity to share in interesting experiments.

## Abstract

This thesis focused on one of threading strategies of spinal cord injury models - using biocompatible hydrogels, with neural precursors as bridge and graft. Here we also summarized knowledge about spinal cord injury and about follow-up changes from natural to self-destructive and inhibitory environment. It reviews important issues like replacing, or regenerating strategies of damaged tissue. Previous result showed functional improvement after application hydrogels filed up cells as transplantation material in the rat models of spinal cord balloon compression lesion, spinal cord transsection or hemisection. The aim of practical part was to compare in vitro viability, proliferation and differentiation ability of human neural stem cells on several types of biocompatible hydrogels and to find the correlation between type of hydrogel and type of cells in process of cells growth. This work serve as a platform for future experiments using received samples for treatment spinal cord compression lesion in rat model.



**Key words**

Biocompatible hydrogels, stem cells, spinal cord injury

## Abstrakt

Tato práce je zaměřená na jednu ze strategií léčby modelu míšního poranění - použití biokompatibilních hydrogelů s neurálními prekurzory, jako lešení pro přemostění léze. Shrnuje poznatky o modelu přerušení míchy a následných změnách z přirozeného do sebedestruktivního a inhibujícího prostředí. Dále pak prochází důležité oblasti, jako jsou strategie náhrady a regenerace poškozené tkáně. Předchozí výsledky ukázaly funkční zlepšení po aplikaci hydrogelu s buňkami jakožto transplantačního materiálu v potkaních modelech míšní balónkové kompresní léze, transsekce, či hemisekce míchy. Cílem praktické části bakalářské práce bylo porovnat in vitro viabilitu, proliferaci a schopnost diferenciace lidských neurálních kmenových buněk na několika typech biokompatibilních hydrogelů a najít korelaci mezi buněčným typem a typem hydrogelu v procesu buněčného růstu. Tato práce slouží jako platforma pro budoucí využití získaných vzorků v léčbě míšní kompresní léze v potkaním modelu.



**Klíčová slova**

Biokompatibilní hydrogely, kmenové buňky, míšní léze

## Contents

## Index to abbreviations

BDNF- Brain derived neurotrophic factor

BMSCs- Bone marrow stromal cells

CNS- Central neural system

CS-PG – Chondroitin sulfate proteoglycans

DRG- Dorsal root ganglion

DRG-CM- Dorsal root ganglion conditioned medium

ECM- Extracellular matrix

ESCs-Embryonic stem cells

GDNF- Glial cell-derived neurotrophic factor

hESC-NPs- human embryonic stem cells neural precursors

hrEGF- human recombinant epidermal growth factor

hrFGF- human recombinant fibroblast growth factor

IGF-1- Insulin like growth factor-1

LIF- Leukemia inhibitory factor

-MA- Sodium methacrilate

MAG- Myelin associated glycoprotein

-MOETA- 2-(methacryloyloxy)ethyl)trimethyl ammonium chloride

MPSS- Methylprednisolone sodium succinate

NGF- Nerve growth factor

NSCs- Neural stem cells

NT-3- Neurotrophin 3

NT-4,5- Neurotrophin 4,5

OECs- Olfactory ensheating cells

PHB- poly-β-hydroxybutyrate

pHEMA- 2-hydroxy-methacrylate

pHPMA- N-2-hydroxypropyl-methacrylamid

SCI- Spinal cord injury

SCs- Schwann cells

SPC-01- fetal neural precursor's line

TF-β- Transforming factor β

# 1 Introduction

## 1.1. Spinal cord injury

### 1.1.1 Main characteristic and definition

Worldwide, an estimated 2,5 million people with spinal cord injury (SCI), and more than 130 000 new injures reported each year (S. Thuret et al., 2006). The biggest group of people with SCI, are young people around 16 to 25 years, with injury caused by motor vehicle accident (47%), followed by sport related accidents, falls and violence injury (C.E. Hulsebosch., 2002). The second big group of individuals has myelopathy or injury caused by some kind of illness, like stroke or spondylosis. The spinal cord injury is divided in five groups (the scale is made by American ASIA protocol) according to degree of damage of spinal cord tissue. The first group A include people with complete lesion: no motor or sensory function is preserved in the sacral segment S4-S5. Second group B include people with incomplete lesion: sensory but no motor function is preserved below the neurological level and includes the sacral segment S4-S5. In group C motor function is preserved below neurological level, and more than half of the key muscles below the neurological level have a muscle grade less than 3. The group D: motor function is preserved below the neurological level, and at least half of the key muscles below the neurological level have a muscle grade of 3 and more. E group include people with normal spinal cord function. Five points are assigned to the normal function of each muscle group (E. Syková et al., 2006). Another scale for describing spinal cord injury divided lesions into four groups according to pathophysiology. Injury cause significant tissue loss, cyst and cavities formation and invasion of meningeal cells, spinal tissue discontinuity of the cord. Nowadays many models of SCI are used in experimental conditions. One of the models is a laceration caused by penetration of sharp objects (open injures). In closed injures belongs the compressive injures. Three types of compression injures are massive compression, contusion and solid cord injury. Massive compression injury causes loss or destruction of spinal tissue. Another type the contusion injury is with central fluid-filled cyst and minimal invasion of connective tissue. Solid cord injury is with largely retained shape of the cord, no central hematomyelia and cyst formation, and with damaged white matter tracts. The contusion injury represents 25 to 40% of the cases and the most commonly used model in spinal cord injury (M. Oudega et al., 2005). Functional improvements after SCI can be estimated by several behavioral tests such as BBB open field locomotion score, foot print analysis, placing response, gird walk, narrow beam walking, ladder run walking test, skilled walking, which could be useful also for cortical lesions of motor cortex or for other brain and spinal cord injuries (G.A.S. Metz et

al., 2000; G.A.S. Metz and I.Q.Whisaw., 2002). We can also divide SCI in three phases according to time after lesion: acute injury, secondary injury processes and chronic phase. The acute injury is a stand of first few days, in which begins a variety of pathophysiological processes, including necrosis, or cell death of neural and other soft tissue causing by mechanical damage, ischemia, edema, electrolytic shift, loss of microcirculation by thrombosis, or hemorrhage. In the second phase in time between minutes to weeks continues the processes, as ischemic cellular death, electrolytic shift, edema, and concentration deregulation of ions, cytokines, chemokines, neuromodulators, neurotransmitters and growth inhibitors. In the chronic phase, with occurs time of days to years, apoptosis continues, many of receptors and canales have modulated levels of expression and activation. The demyelinisation of axons results in conduction deficits (C.E. Hulsebosch., 2002; J.D. Houle 2002). These problems need to be solved for spinal cord could regenerate.

### 1.1.2. Axonal growing barriers

One of the main problems during nervous tissue regeneration is a scar formation. Regenerative ability of axons in injury environment is very low, because of inhibitory environment caused by neighboring glial cells (C.E. Hulsebosch., 2002; J.W. Fawcet et al., 1999). Which cells of central nervous system could cause this growth inhibition? In central nervous system participates glial cells, including astrocytes, oligodendrocytes precursors, oligodendrocytes, meningeal cells and microglia. All of these cell types are normally permissive for axon growing, but in injured spinal cord most of them produce inhibitory factors on their surface, or diluted to the extra-cellular space.

Astrocytes in response to injury proliferating and increase in the size and complexity of their processes. Most of glial scar is made up of tightly interwonen astrocyte processes. They are huge numbers of astrocystic morphologies, and they are extreme hypothesis about their origin, such as one of them which said, that when CNS is injured, separate particular cell type of astrocytes. But this is very unlikely (J.W. Fawcet et al., 1999). There are other explanations, which said that astrocytes are able to convert from permissive to inhibitory state. It has been shown, that the astrocystic processes in certain distance from lesion gab establish ability to support axon regeneration, but the processes in the lesion site had changed to become inhibitory (S.J. Davies et al., 1996). In Guenard and colleagues experiment, when were recombined in vitro schwann cells, astrocytes and neurons, was exposed, that Schwan cells population was decreased and O1 expression and myelinization were inhibited by factors present in astrocyte-conditioned medium.

But O4 expression was not influenced (V. Guenard et al,. 1994). Most important astrocystic inhibitory molecules are chondroitin sulfate proteoglycans. In the astrocystic monolayer persist in the beginning permissive activity, but with time become rather inhibitory, because of trapped molecules in intracellular space. Is very different permissiveness between two and three-dimensional tissue (J.W. Fawcet et al,. 1989). The structure of astrocystic scar is very impenetrable looking, so has been postulate one of hypothesis, that the axon growing barrier through the scar has mechanical origin, but experiment in lower vertebrate nervous system speaks against that. However, in this case is less evidences for submit or disprove the hypothesis (J.W. Fawcet et al,. 1999).

The second cell type, which plays major role in axon growth inhibition are oligodendrocytes. They are minimally three molecules, which in vitro by mature oligodendrocytes and in vivo by myelin inhibit axon growth- N1250, myelin associated glycoprotein (MAG), and tenascin-R. In study of Davies and colleagues it has been shown, that transplantation of embryonic tissue in corpus calosum without trauma could demonstrate axon growing, against few cases when the operation caused more traumas, and evoked forming of glial scars without any growth of axons (S.J. Davies et al,. 1997), which proves different cells behavior in damaged or undamaged tissue and different observation of inhibitory molecules. Also oligodendrocytes precursors were examined to inhibitory abilities, because these cells are mobilized to all types of central nervous system injuries. Against mature oligodendrocytes, which have axon growth cone collapsing activity (mediated by N1250 and MAG), precursors did not show these activity (J.W. Fawcet et al,. 1999), but it has been shown, that oligodendrocytes precursors could be inhibitory in some types of injuries.

Microglia are capable to produce cytotoxic molecules like free radicals, nitric oxide, arachidonic acid derivates and other toxic molecules. In central nervous system injuries neuronal cells could changes from being neurotoxic to neuroprotective. Rabchevsky and colleagues study has shown, that microglia could have regeneration effect on axon growth and could make astrocyte more permissive to growth of axons (A.G. Rabchevsky et al., 1997). Invasion of Macrophages into lesioned nerves is very important part of repair process and microglia are moreover able to degrade myelin inhibitory molecules (S. David et al., 1990).

Meningeal cells are also capable to participate on inhibitory process. In normal central nervous system together with astrocytes create the astrocystic glia limitans and implantation of meningeal cells leads to scar-like astrocystic reaction. These cells can also produce laminin, NG2 proteoglycans or tenascin which are necessary for axon regeneration. (J.W. Fawcet et al., 1999).

The inhibitory molecules, produced by different cells are divided in two major groups- myelin associated molecules and chondroitin sulfate proteoglycans. The first group associates N1250/nogo, MAG (myelin associated glycoproteins) and tenascin-R. N1250/nogo is expressed as high-molecular weight inhibitor on myelin surface and in extract and his inhibitory activity could be blocked by antibody IN-1. Inhibition of nogo has been shown to support long tract regeneration. This treatment proved degree in axonal regeneration, with combination of neurotrophic factors (B.S.Bergman et al., 1995; E.M. Swab et al., 1990; E.M. Swab et al., 1993). Another inhibitory myelin-derived protein is myelin associated glycoprotein (MAG). It has been identified and characterized in vitro and in vivo as a protein, which is influenced by neurotrophins trough c-AMP dependent mechanism. MAG knockout has shown small increase in axon regeneration in CNS, but also in regeneration of peripheral nerves (J.W. Fawcet et al., 1999; M.T. Fitch et al., 1997; M. Schäfer et al., 1996). Tenascin-R produced by oligodendrocytes is present in CNS extracellular matrix and associated with cell surface molecule and specific proteoglycans. And in injured central nervous system is also up-regulated. Second big group are chondroitin sulfate proteoglycans. They could be expressed firstly by astrocytes, but also by oligodendrocytes precursors, or by meningeal cells. The chondroitin sulfate proteoglycans, which participate on inhibition of axonal growth, include versican, phosphocan, NG2 and neurocan (L.L.Jones et al., 2003). They are several ways of treatment including ethidium bromide injection, X-irradiation, or more considerable treatment by chondroitinase ABC. Last one looks more advantaged, because first two possibilities are influencing not only reactive cells, but also are binding on DNA and RNA structures, or directly destroying astrocytes, which are necessary in central nervous system homeostasis.

Another problems, how has been written above, are for example edema and free radical production. Furthest used treatment of edema was using of steroids and corticoids, the most successful is methylprednisolone, and/or his derivates such as methylprednisolone sodium succinate (MPSS), U-74006F and YM-14673, or nimodine. High doses of these molecules have effect on axonal growing and hind limb function, but also have several side effects. In Pointilart and colleagues, and Berhmann and colleagues studies has been shown positive effect of YM-14673 treatment, but no or small improvement was shown, using the same doses of methylprednisolone or MPSS. In different human studies findings indicate, that application of MPSS within 8 hour of their injury has improved recovery of neurological function. In one of them it has been compared with Naloxon and showed statistically significant increase of motor function (V. Pointilart et al., 2000; M.B. Bracken et al., 1992; D.L. Berhmann et al., 1994

### 1.1.3. Cellular and enzymatic ways of cure

Because of those obstacles, which were mentioned before, is necessary to develop approach that combine application of cell replacement therapies, neurotrophic factors, and using of cellular, or noncellular scaffold as bridge trough the lesion cavity . Which cell types and neurotrophic factors could be use to promote axon growing? Is many types of useful cells in central nervous system injury some of them because of their abilities to differentiate in neural or glial cell types, which can promote axon growing by blocking of inhibitory factors or by producing myelinization, and/or axonal growth promoting molecules. Cell types, which could participate on injury repairs are different type of neural progenitors, Schwann cells, olfactory ensheathing cells, macrophages, genetically modified fibroblasts, etc. Neural stem cells (NSCs) and progenitor cells could be used because of their ability to differentiate in neurons and also in oligodendrocytes in vitro and in vivo (S.A. Goldman et al., 2006). Multipotential NSCs are not only in fetal tissue but also in forebrain ventricles in adults. In adult brain are the endogenous progenitors in olfactory bulb hippocampus and both the ventricular zone and tissue parenchyma. The endogenous stem cells are able to respond to injures, but are not sufficient to result in functional improvement in cases of larger damage. From neural tissue we have examples of neurogenesis and neurodegeneration in processes of learning against drugs administration. But at most causes in central nervous system injury especially in some areas, such as spinal cord or/and striatum, where is a little evidence of ongoing neurogenesis, are endogenous multipotential cells not sufficient to replace the tissue lost (Q. Cao et al., 2002). Because of that, the attention has moved to neuronal and glial replacement by transplantation of neural stem cells or precursor cells. In studies of Cao and colleagues, and Vescovi and colleagues was shown, that more than interesting properties of grafted cells have effect the local environment. When are pluripotent neural stem cells implanted in injured spinal cord, the engrafted cells differentiated only in astrocytes, and the temporally progression of that differentiation is markedly retarded. But when the engrafted cells were more differentiated, than were both neurons and glia in lesion site found (Q. Cao et al., 2001; A.L.Vescovi et al., 1999). Other promising cell types are Schwann cells and olfactory ensheathing cells, which are using together or alone in different types of injuries. The fact, that these cell types are very similar leads to compare between them. Schwann cells would be most effective for filling up a contusion cavity promoting axon growth and myelinization in an implant. On the other side olfactory ensheathing cells are rather better in allowing axonal regeneration beyond and within a growth obstructive environment. But the mechanism of OEC effect is not fully known. Different sources had written about ability of SCs to create useful bridges trough lesion gab (Ramon-cueto et al., 1998; Ramon-

cueto et al 2000; Paul J. Reier., 2004). Using of peripheral nerves as a transplant graft also has shown some success. Also transplanted activated macrophages to the injured CNS tissue, is the process, which could accelerate injury regeneration. How has been written above the environment around engrafted cells influence their differentiation state. Problem in injured spinal cord is also post injury apoptosis of nervous tissue cells. One of solutions is caspases inhibition. Unlike other proteases, caspases are degradative in not nature environment and are thought to be important in mediating cell apoptosis. Possible way is using IGF-1, which causes increase of mitochondrial Bcl-2 formation, and thus inhibits apoptosis after SCI. In phase 1 of clinical trials are also part of bcl-2 oncogene products, which are potent death suppressors (Hung et al., 2007). Crucial issue is, that as a result of secondary injury is demyelinization and loss of conduction of survived axons. One of approaches to protect neurons and myelin sheet from massive release of transmitters, coupled with the ionic shift is use sodium channel blockers as tetradotoxins, or 4-aminopyridine. In clinical trials using of their agents looks very promising (Rosenberg et al., 1999; Rosenberg et al., 2001).

Thus next very important part of the repair process is using of neurotrophic factors to promote differentiation of precursor cells and support axonal elongation. The production of neurotrophic factors in injured CNS could be represented by in vitro gene therapy (genetically modified cells bridges by retroviral or adenoviral vectors), or in vivo by direct injection of neurotrophic factors, or by using of micelles with factors inside of them (Leonard L. Jones et al., 2006). These factors of neurotrophin family, consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Also other families like cytokine and transforming factor-β (TF- β) families including factors to enhance axonal growth, as such in TF- β family the glial cell-derived neurotrophic factor (GDNF), or leukemia inhibitory factor (LIF) of the neuropoetic cytokine family (L. L. Jones et al., 2006; A. Blesch et al., 2002). All of these molecules have positive effect on axon growth for example NGF stimulates sprouting of primary afferent fibers (which are unfortunately pain fibers) and also coerulospinal and ventral motor axons. NT-3 in genetically modified cells shows increase of corticospinal fibers regeneration (R. Grill et al., 1997; M.E. Schwab., 2002). BDNF promotes regeneration of sensory axons from the graft into the dorsal column white matter and also regulates interneural networks, stimulates hind limb air-stepping and co-ordinate movement. Moreover BDNF generates improvement without necessarily promoting axon regeneration and has been reported that with NT-3 could prevent the death of corticospinal neurons. GDNF is most effective neurotrophic factor in stimulating axonal growth across the dorsal root entry zone into the spinal cord white matter (L.L. Jones et al., 2006; K.M. Giehl et al., 1996).

## 1.2. Stem cells

Stem cells are cells found in most of multi-cellular organisms. They are characterized by their ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types. They are two broad types of mammalian stem cells: embryonic stem cells (ESCs), which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in adult tissues. ESCs are able to differentiate into all of the specialized tissues in vitro and in vivo. In adult organisms, stem cells and progenitor cells serve for a repair system of the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues. (James F. Battey., 2007; Ch. Cai, L. Grabel., 2007).

### 1.2.1. Origin of stem cells

ESCs have the same abilities to create different cell types, and the only thing which decided in embryo about their fate, is the position comparison others cells, which may be determine about gradient of morfogens, and so about activation, and/or inhibition of specific genes, or parts of DNA. Is very important to understand molecular mechanisms how these ESCs differentiate in specialized cell types. Molecular mechanisms, including epigenetic changes in the structure of chromatin, developmental changes in gene expression, growth factor exposure and interactions between adjacent cells. Adult stem cells are rare in organism and not only their pluripotenty, but also self renewing is retarded (James F. Battey., 2007). So stem cells are very attractive because of their abilities to create mature cell types and renew the place of degenerated cells, and because of that the ways of natural differentiation of embryonic stem cells in to the mature neuron and glia were very carefully studied. Very interesting knowledge about stem cells was the fascinating similarity to cancer cells. Stem cells and also cancer cells are thought to be capable if unlimited proliferation. And small number of cancer cells express stem cells markers, such as CD133 and ATP-biding transporter, which can serve as anti-cancer drugs pumps. These pumps could also to transport specific fluorescence dyes, like Hoechst33342. And as a third interesting issue it has been shown that undifferentiated stem cells implanted to the adult brain could be tumorigenic, because their capability to create almost any cell type of the whole body is very reactive to neighboring environment. Thus has been postulated the hypothesis that cancers contain stem cell-like cancer cells, called “Cancer stem cells” expressed stem cells markers, tumorigenic and resistant to anti-cancer drugs and irradiation (Toru Kondo., 2007). Next important view is to describe sources of pluripotent cell lines. Today are mostly using three techniques to produce embryonic stem cells.

The first and oldest method used techniques of in vitro fertilization from assisted gestation. These methods using of spared embryos to separate inner cell mass, had an ethic barrier. The second method was isolation of primordial germ cells from 5 to 7-week old embryo and placing them into culture. The third method is process called somatic cell nuclear transfer. It was also tried to dedifferentiate somatic adult stem cells to their embryonic state, but to this time unsuccessfully (James F. Battey., 2007).

### 1.2.2. Neural precursors

How to make neural derivates from embryonic stem cells? It has been postulated two approaches. Firstly by mimicking the natural environment that produces neuroectoderm trough embryonic body formation by cell-cell interactions and signals. Secondly by depriving the ESCs of both cell-cell interaction and signals in low density culture, with serum-free medium evoking a default mechanism for NSC differentiation (Chunyu Cai, Laura Grabel., 2007).

In study of Kitazava and Shimizu showed possibility to differentiate ES cells to neuron for example with adding chick dorsal root ganglion conditioned medium (DRG-CM), differentiated with help of NGF. The addition of DRG-CM and 11 days of cultivation together with DRG-CM, caused differentiation of about half of all undifferentiated ES cells on the periphery of the colon sphere into neurons. Most of them had motoneurons markers (LIM-3 and HB9), but has been observed also GABAergic (GABA), serotonergic (serotonin.5-HT) and cholinergic (ChAT) markers. And 50% of all of them was positive to βIII-tubulin and before using of DRG-CM have been 86% of them positive to BrdU (marker of undifferentiated cells) (A. Kitazava, N. Shimizu., 2007). It has been shown, that ESC derived neural stem cells (NSCs) proliferate more readily than somatic NSCs and differentiate in to neural cell types with higher efficiency and are able to produce more cell types. And also molecular findings have confirmed broader potential ESC-derived NSC to somatic one (Colombo et al., 2006). Another way how to differentiate ES cells into neural stem cells, is using of the retinoic acid protocol. The role of the retinoic acid in embryonic neurogenesis in particular regions in specific time is well known. Study of Jacobs and colleagues has suggest, that retinoic acid could be required for adult hippocampal neurogenesis. NSCs generated using the retinoic acid protocol may have restricted differentiation potential (Jacobs et al., 2006). The differentiation could be influenced by many embryonic differentiating factors, such as inhibition by bone morfogenic protein-4 (BMP-4), modulated by Wnt signaling, or activated by NOTCH genes or Sonic Hedgehog. These factors could be blocked using their antagonist, like in case of BMP4 by noggin. Presents of FGF and EGF in medium also play important role. They are

supporting proliferation of ES-derived NSCs, and their remove from medium cause terminal differentiation of NSCs. Some of growth factors could have different activity on primitive and adult form of NSCs, like vascular endothelial growth factor.

Another place where from neural precursors, neurons and glia can be derived and differentiate is a neural crest. These cells from neural crest vary widely in expressed markers and variety of cell types, they could generate. They are situated in diverse location, but sometime occupy physically proximate niches. Neural crest stem cells give rise for example to cells expressing markers characteristic of sensory, sympathetic, and parasympathetic neurons, and Schwann cells, and satellite glia (Mosher et al., 2007; M. Delfino –Machine et al., 2007), but also participate in craniofacial formation in embryonic state, and cartilage meninges, tooth papillae, stroma, cornea and many others tissues, and persist in adult as a progenitor cells in hair follicles. (M. Sieber-Blum, Y. Hu., 2008).

### 1.3. Bridges, using of polymeric scaffolds and cellular bridges

It has been shown, that implantation of porous tubes into the place of spinal cord transsection has significant increase in locomotor behavior and in hind limb function in testing with BBB score compared to group with spinal cord transaction without porous tube implant (L.F.Reynolds et al., 2008). How has been written above, one of promising approaches how to lead axons through the lesion gab is using cellular, and/or polymeric bridges, which could serve as natural-like environment and also as source of important receptors and/or activators. An ideal material for neural transplantation would have the ability, to be mixed with cells and growth factors before transplantation, and then injected to the lesion site, forming solid three-dimensional matrix, capable for cells migration and neural fibers connections (J. R. Thonhoff et al., 2008). The first cluster of noncellular polymers we can divide in few groups in respect of their origin/composition, degradability, surface charge, or additives, such as receptors etc. With regard of their composition and degradability we can use natural polymers – alginate hydrogel, type I collagen; synthetic biodegradable implant – matrigel matrix, fibronectin mats, fibrin glue, poly α-hydroxyl acids and poly ethylene glycol; nonbiodegradable hydrogels- well known neurogel (poly N-2-hydroxypropyl-methacrylamid (pHPMA)), poly 2-hydroxy-methacrylate (pHEMA), or their derivates. And last ones composite biodegradable implants and composite conduits with nonbiodegradable scaffold, which are also in the position of biocompatible materials (Novikova et al., 2003; D.R.Nisbet et al., 2007). Except hydrogels should be used nanofibers, which could have similar properties, what is concern their composition or additives, but they can easier lead the cells

growth. For cell bridges is important their ability to support axon growth not only mechanically, but also to make better the environment for example to myelinate denuded axons or to produce growth factors, if they are modified to do that. It has been shown that sometime is better to combine more molecules, scaffolds and cells to reach coveted effect, like with combination of poly-β-hydroxybutyrate (PHB), fibronectin and alginate, where were similar outcomes about rescued percents of axon like in using of BDNF or NT3. And with addition of Schwann cells in PHB, fibronectin and alginate were better (L.N. Novikov et al., 2002). Intriguing angle of view is to compare growth and differentiation of neural stem/progenitor cells in the same conditions in 2D and 3D cell systems, which support the hypothesis of using bridges in SCI. work of Brännwall and colleagues shows, that cultivation of NS/PS in type I-hyaluroan scaffold with addition of FGF and EGF, have in 3D structure up to 75% of neurons, generated from postnatal brain. Against that in 2D monolayer was only 14% of neurons (K. Brännwall et al., 2007).

What is concern the effect of surface hydrophilicity on cells adhesion and growth, it has been doing experiments, tested interaction of cortical neurons on poly L-lactic acid and poly lactic-co-glycolic acid nanofibers, with were partially hydrolyzed using potassium hydroxide. Using of potassium hydroxide decreased a little hydrophilicity of scaffolds, what caused reduction of numbers of neurons with compare to controls (D.R.Nisbet et al., 2006). These few examples represent connectivity and difficulty of solution of neurons fibers gab filing.

### 1.3.1. Nanofibers

Nanofibers were chosen for scaffold engineering first because of their ability to imitate natural extracellular matrix (ECM), to guide cells growth, and because of their good adhesion properties of surface for different molecules and cells. So the knowledge of the micro-environmental niche of cells is essential for successful creation of 3D scaffolds not only by nanofibers. The main components of ECM are glycoproteins (with the most abundant collagens), proteoglycans and hyaluronic acid. It also contains molecules depending on the specific tissue, such as fibrin, elastin, fibronectins, laminins, or hydroxilapatits. (D.R.Nisbet et al., 2008). Big different against isotropic macroporous hydrogels, and so their axon directional growth depending on exogenously delivered neurotrophic factors, is that polymeric nanofibers can interact intimately with the growth providing contact guidance cues allowing for directed neurite outgrowth. Neuronal growth direction depends on fiber alignment. And fibers diameter also seems to play important role in axon differentiation. NSCs are more likely differentiating to neurons on aligned nanofibers than microfibers (F.Yang et al., 2005). Further works showed enhanced neurite growth, with using of

modification by neuroactive peptides for example derived from tenascin-C. Of course, application of nanofibers has no limitation only on neural tissue repair, but has many possible targets, such as bones engineering, cartilage engineering, or for cardiac or vascular grafts (D.R.Nisbet., 2008). Today in clinical trials nanofibers are using mainly for treatment of diabetes feat and skin repair

### 1.3.2. Biocompatible hydrogels

Hydrogles are cross linked hydrophilic polymers with high water content and could have porous structure, mostly with pore size between 10-100μm. Pore diameter is very important factor for cell growing, because lose of contact or on the other side the block of signal connection and fluency of nutrients could have negative effect on cells survive (D.R.Nisbet et al., 2008). Benefits of hydrogels using, are come from possibility of large production, easily modifying of chemical and physical structure, similarity to neural diffusion parameters and possibility of improving by combination with stem cells, neurotrophins or signaling sequences or by possibility of creating oriented pores for directional growing etc. (A. Hejčl et al., 2008). Their function should be to calm down posttraumatic cavities formation, glial scar formation and to help to smooth tissue connection. Generally they should to create permissive environment, bypass the communication barrier and support axon regeneration trough serving as a framework and bridge for their growth (A. Hejčl et al., 2008; E. Syková et al., 2006). Using of synthetic hydrogels has opened the question of biocompatibility and fluidity, because of possible implantation and nontoxic environment. It has been tested the toxicity on hNSCs in matrigel and puramatrix in depend their gelated form (1-50%). This study has shown dynamic between gel toxicity and growth factor stimulation. Lower concentrated puramatrix hydrogel showed better conditions for cells growing, and low toxicity (J.R.Thonhoff et al., 2008). How has been written above, hydrogels are divided on several groups depend on their composition. Scientists are finding an ideal hydrogel environment for cells growth. One important question is using of biodegradable materials or not. The idea of biodegradable hydrogles is that after reconnection of axons are naturally adsorbable with no side effect for recipient. But in parameters are not stable. We have synthetic and composite biodegradable implants, which showed improvements in axons sprouting and cells survival (L.N. Novikova et al., 2003; Lev N. Novikov et al., 2002). Natural biodegradable biopolymers, such as an alginate hydrogel complemented with fibronectin, or using of matrigel bring promises. These substances were compared between olfactory ensheating cells (OECs), Schwann cells (SCs), bone marrow stromal cells (BMSCs), or neurons from dorsal root ganglium (DRG). Alginate hydrogel combination with fibronectin supported cells growth more than alginate, but matrigel had better

properties than both others possibilities. Results of Novikova and colleagues work shows similar results like L.N. Novikov and colleagues work, that combination of two well chosen materials have better properties for cells growth than each one alone (L.N. Novikova et al., 2006). Another well characterized and promised hydrogels are synthetic nonbiodegradable polymers, as such macroporous hydrogels based on HEMA or HPMA. These hydrogels were tried with many variations of precursor cells, with changed charge and with additional receptors for example for serotonin. Appealing is comparison of positively and negatively charged HEMA by addition of sodium methacrilate ($MA^-$), (2-(mathacryloyloxy) ethyl) trimethyl ammonium chloride ($MOETA^+$), or both of them HEMA-$MA^-$-$MOETA^+$ together. In Pradny and colleagues study has been shown, that mesenchymal stem cells (MSCs) had higher density on positively charged and lower on negatively charged surface. HEMA-$MA^-$-$MOETA^+$ hydrogel was between them. Most of them were present on peripheral parts of hydrogel (M. Pradny et al., 2005). Success has also using of thermoreponsive hydrogel. In study of D.R. Nishbet and colleagues cultivated primary cortical neurons and later NSCs under 2D and 3D conditions with immobilized poly-D-lysine to promote neuron adhesion and neurite outgrowth on xyloglucan hydrogel. Work from D.R. Nishbet and colleagues showed support of primary cortical neuron differentiation, survival and axonal extension of both of them (D.R.Nisbet et al., 2008). Ducoseille and colleagues work present successes of pHPMA hydrogel using in regeneration of dorsal septohippocampal neurons in rat brain. pHPMA hydrogel was used alone or with combination of n-acetyl glucosamine or glucose. In vivo showed host cells invasion by many cell types including neuronal and glial cells, and also blood vessels. All modified and unmodified pHPMA hydrogels well integrated into brain and created stable bridge between septum and hippocampus (E. Ducoseille et al., 1998). But success of these methods is also connected to corresponding type of the biocompatible material to the type of lesion, right time window of application and supporting molecules. One of them is the timing of hydrogel graft implantation with respect of secondary injury processes like edema, hemorrhage and general inflammatory environment. Because of these circumstances has been shown to be more permissive than acute transplantation, rather a week delayed transplantation, in which the graft showed smoother on ramp and of ramp borderline. Both groups of recipient showed improvement and ingrowths of neurofilaments and Schwann cells, but the delayed group has showed in MRI only a small hyperintense signal of pseudocyst cavities (A. Hejčl et al., 2008). Second issue is growing of axons in “on ramp” and “of ramp” parts of hydrogel implant. In order to growth from cut axons into the bridge, they need increasable attracting factors concentration. Firstly they need to regenerate a short distance between stumps and hydrogel. In Schwann cells bridges which produce many chemoattractants growing axons without problems. Also biocompatible hydrogels

with cells which could produce chemoattractants, or with direct injection of growth factors could be good penetrable. But problem is in the last part of implant in “of ramp” borderline, where is lower gradient of neurotrophic factors and less permissive environment then in graft, and also are there glial scars (H.M. Geller, J.W. Facet., 2001).

## 2. Summary

This work has summarized barriers and concluded approaches of spinal cord injury (SCI) treatment. In theoretical part of this work was observed which obstacles stop the regeneration after injury. Central nervous system injuries are studied nearly because of their actuality in our life. The number of patients with complete or incomplete injury is increasing each year, and up to this time ways of successful treatment was not developed. SCI is divided up to the cause and range of signal damage. For measuring of locomotor improvement are serving behavioral tests such as BBB open field locomotion score, foot print analysis, placing response, gird walk, narrow beam walking, ladder run walking test, skilled walking. Adult central nervous system (CNS) has stable structure and low capacity for regeneration, and in cases of injuries become in opposition to peripheral nerves to the inhibitory environment stage. SCI includes dynamic processes from acute injury through secondary injury processes into the chronic phase. The secondary injury processes have large range from edema and electrolytic shift to glial scar forming and demyelinisation. This inhibition is mediated by glial cells produced myelin associated glycoproteins and chondroitin sulphate proteoglycans (CS-PGs) as a two major agents. Processes of secondary damage are obstacles, which need to be solved for cells transplantation strategies. The neighboring tissue need to be allayed and mechanically and physiologically reconnected. Decreasing of caspases activity after injury with using of Leukemia inhibitory factor and Bcl2 gene, and using of chondroitinase ABC against CS-PGs are several successful methods how to prepare injured tissue for further transplantation. The endogenous neurogenesis is inconvenient and in larger injuries can not be sufficient. So the injured neural system needs exogenous neural and glial precursors derived from embryonic or adult stem cells. But these precursors require being well characterized and fit to the type of injury and their differentiation state. Because of obstacles in inhibitory environment is necessary to combine application of cell replacement therapy, neurotrophic factors and using of cellular or noncellular scaffolds, as a bridge trough the lesion cavity. In the first stages of treatment scientists were founding how to allay the injured tissue and to stop the glial scar forming in pharmacological way, what can be performed by application of methylprednisolone or other corticoids and steroids. But using of biocompatible material shows, that one week delayed transplantation of HEMA based hydrogel in transsectioned tissue indicates smooth connection, less edema and glial scar forming between hydrogel and spinal cord stumps. 3D constructs of biocompatible materials, which remain extracellular matrix, also show better conditions for neurons growth than 2D monolayer materials. Such polymeric bridges are very useful. Their using in combination with growth factors and stem cells promise to be optimal way for tissue

engineering. In cases of bone, cartilage or skin engineering positive effect of nanofibers application is well known. New compounds of biocompatible materials (biodegradable or not), which have better abilities to support growth and proliferation of neural precursors are still testing. Commonly used nonbiodegradable biocompatible materials are N-2-hydroxypropyl-methacrylamid (HPMA), 2-hydroxy-methacrylate (HEMA) and their derivate. From biodegradable materials is well known alginate hydrogel, matrigel, or pura matrix. Biocompatible materials have an ideal adhesive surface, which could by performed by addition of specific molecules, or serotonin and acetylcholine receptors for better permissivity and adhesivity. Biomaterials can lead cell growth in case of nanofibers by their diameter and direction of fibers and in case of hydrogels by directional pores and doses of neurotrophic factors. Neurotrophic growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), the glial cell-derived neurotrophic factor (GDNF), or leukemia inhibitory factor (LIF) are well known, by their positive results on injuries repair and cells growth/differentiation. Growth factors are important in process of on ramp and of ramp growth through the hydrogel environment, where serve for applicated cells as a chemoatracting guidance. The cell replacement therapy is important part of repair process, and includes not only neural precursors, but also glial cells, such as Schwann cells, or olfactory ensheating cells which are helpful in myelin associated repair processes. Sources of neural precursors could be not only ESCs, but also adult stem cells, or neural crest derived stem cells. Neural precursors are surviving, and are able to growth and differentiate in well chosen hydrogel in vitro and in vivo. That is important step for axon regeneration. Because of these results is clear that every part of repair process, such as hydrogel using, cell replacement therapies, or neurotrophic factors alone, is not sufficient for complete treatment of SCI, and further experimental part is situated as a logically out line of modern findings.

## 3 Materials and methods

*Hydrogels materials*

Five types of macroporous hydrogels based on N-2-hydroxypropyl-methacrylamid (HPMA), 2-hydroxy-methacrylate (HEMA) were used in this study. These used hydrogels are HEMA-MA; HPMA; E4,5; 32,5; and W(neurogel). The first of them is poly 2-hydroxy-methacrylate- sodium methacrilate (HEMA-$MA^-$), which has pores origin from crystals, washed away. Then poly N-2-hydroxypropyl-methacrylamid (pHPMA), of which structure is from small blobs. The E 4,5 is HPMA hydrogel with structure based on HEMA crystal pores. The 32,5 hydrogel is another name for HEMA-MOETA+ (2-hydroxy-methacrylate -2-(mathacryloyloxy)ethyl)trimethyl ammonium chloride). And the last one W (neurogel) is HPMA from France.

*Cell culture*

In our work two lines of neural precursors: human embryonic stem cell derived neural precursors (hESC-NPs) and fetal neural precursors SPC-01 were used. hESC derived NPs were cultivated in neural precursor medium consisted if DMEM/F12 (1:1), B27 supplement (1:50), 2mM L-glutamine and penicillin/streptomycin at 50 U/ml (GIBCO), supplemented with 20 ng/ml human recombinant epidermal growth factor (hrEGF) and 20ng/ml human recombinant fibroblast growth factor (hrFGF). SPC-01 line was derived from human somatic stem cells and modified with conditional immortalizing gene, c-myc$ER^{TAM}$. In presence of 4-hydroxy-tamoxifen this fusion protein generated by c-myc$ER^{TAM}$ stimulates cell proliferation, and in absence of this drug growth arrest and cells differentiated in to neurons and astrocytes (K.Pollock et al., 2006). SPC-01 line was cultured in DMEM/F12 with 20% human albumin solution, 50mg/ml apo-transferin, 8,1mg/ml putrescune DiHCL, 10mg/ml human recombinant insulin, 20µg/ml progesteron, 200mM L-glutamin, 20µg/ml sodium selenit, 10µg/ml hrFGF, 10µg/ml hrEGF, penicillin/streptomycin at 50 U/ml (GIBCO) and 1mM 4-hydroxy-tamoxifen as an anti-apoptotic factor.

*Cells in hydrogel environment*

The small pieces of hydrogels were put in the two 24-wells plate, with the aim to separate them in respect of their material (first line E4,5; second HPMA; third 32,5; fourth HEMA-MA and last fifth W(neurogel)) and to two plates up to their medium and different neural precursors(hESC-NPs, SPC-01). Suspension of cells (hESC-NPs or SPC-01) in concentration of 100.000 cells/ml was injected in wells of 24-wells plate contained samples of gels, 500ul per well. The medium was

changed daily. After 24 hours, 7, 14 and 28 days samples were taken away, and then washed, fixed and stained for neural specific markers, DAPI and Phaloidin.

*Fixation and immunohistochemistry staining*

Gel samples with cells were washed with PBS and fixed by 4% paraformaldehyd during 15 minute. Fixed samples were washed with PBS twice, prior to imunostaining. To permeabilization and blocking buffer consisting 0,1% Triton,5% goat serum and 1mg/ml bovine serum albumin in tris buffer has been used for 20 minute. To identify neural precursors and differentiated neurons antibodies against NF70 (neurofilaments), nestin and βIII-tubulin were used. For visualization primary labeling secondary antibodies were used. Nucleuses were colored by DAPI. After immunostaining cover slips were mounted using Vectashield mounting medium and examined using a confocal microscope equipped with an Ar/HeNe laser.

## 4 Results

In this work we compared the growth of two neural precursor lines (hESC-NPs and SPC-01) on several types of biocompatible hydrogels (32,5; E4,5; HEMA-MA; HPMA; W) with different charges and structures. Estimation has been made on the basis of the confocal microscopy outline. The scale of rating was from one to ten; 1,2- none or only a few cells; 3,4- small groups of cells without markers of spreading out; 5,6- bigger neurospheres with continual spreading out; 7,8- neural precursors networks with positive staining for markers of neural precursors (βIII-tubulin, NF70, nestin) in almost whole surface of hydrogel; 9,10- three dimensional network of neural precursors with specific markers and high density in entire hydrogel. In some cases cells were in the same degree of scale but in higher density. In the table the hydrogels with higher cell-density were marked with higher number. Main differences between tested hydrogels were in their structure, their solidity and their surface charge. All tested hydrogels displayed ability to support the neural precursor growth. From 24 hours to the 14 days samples showed remarkable growth and then came little drop to the steady state caused by living space, limited by size of hydrogel. This limitation was most clear in case of HEMA based hydrogels, which were fill up completely. Comparing behavior of cells type on in HPMA and W (neurogel) came out in correspondence with presumption, because both hydrogels have similar structure, only were produced in different laboratories. Their behavior was almost the same – cells formed of both tested line neurospheres which were positive for neuronal markers. Minimal survival of cells was detected on 4,5 hydrogel: only SPC-01 formed several neurospheres during 4 weeks; hESC-NPs were not formed there in the end of experiment. Against that 32,5 (Fig.1 A, B, C and D) and HEMA-MA (Fig.2 A, B, C and D) hydrogels showed most remarkable ability to support growth of cells. There were small differences between cells density on surface of HEMA-MA and 32,5 hydrogel: 32,5 hydrogel had better conditions for cells growth. hESC-NPs and SPC-01 cells on HEMA based hydrogels showed the ability to connect in three-dimensional network, and were positive for markers of neuronal differentiation (nestin, NF70, βIII-tubulin). It was very interesting to compare hESC-NPs and SPC-01 growth. In 24 hours samples showed hESC-NPs better growth than SPC-01 cells, but in 7 days and 14 days the SPC-01 samples have shown better growth and showed better markers of neural differentiation, such as βIII-tubulin (Fig.3 E), nestin (Fig.3 A, B and D), or neurofilaments 70 (Fig.3 C) against hESC-NPs. Another important finding is that higher concentration of cells was on the edge of hydrogels. Summarizing presented results we can say that from 4 type of hydrogels used in our study 32,5 support growth and differentiation both type of NPs the most and the HEMA-MA, HPMA, W and E 4,5, consequently with decreasing supportive properties. It is

important to note as well, that SPC-01 survive, proliferate and differentiate better than hESC-NPs (table 1.)

Table1.-in this table is measured the growth of cells of hESC-NPs and SPC-01 lines, and their capability to present neural markers on five types of hydrogels.

| Type of cells | Time, days | 32,5 | E4,5 | HPMA | HEMA-MA | W |
|---|---|---|---|---|---|---|
| hESC-NP | 1 | 5 | 1 | 3 | 6 | 3 |
| | 7 | 8 | 1 | 4 | 7 | 3 |
| | 14 | 9 | 2 | 5 | 8 | 4 |
| | 28 | 9 | 2 | 5 | 8 | 4 |
| SPC-01 | 1 | 5 | 1 | 3 | 6 | 3 |
| | 7 | 8 | 1 | 4 | 8 | 4 |
| | 14 | 10 | 3 | 6 | 9 | 5 |
| | 28 | 10 | 3 | 6 | 9 | 5 |

The measuring scale is from 1 to 10 up to cells growth behavior and cells density 1,2- none or only a few cells; 3,4- small groups of cells without markers of spreading out; 5,6- bigger neurospheres with continual spreading out; 7,8- neural precursors networks with visible markers of neural precursors (βIII-tubulin, NF70, nestin..) in almost whole surface of hydrogel; 9,10- three dimensional network of neural precursors with specific markers and really high density in entire hydrogel. In some cases were cells in the same degree of scale but higher density these ones were in the table marked with higher number.

Fig.1

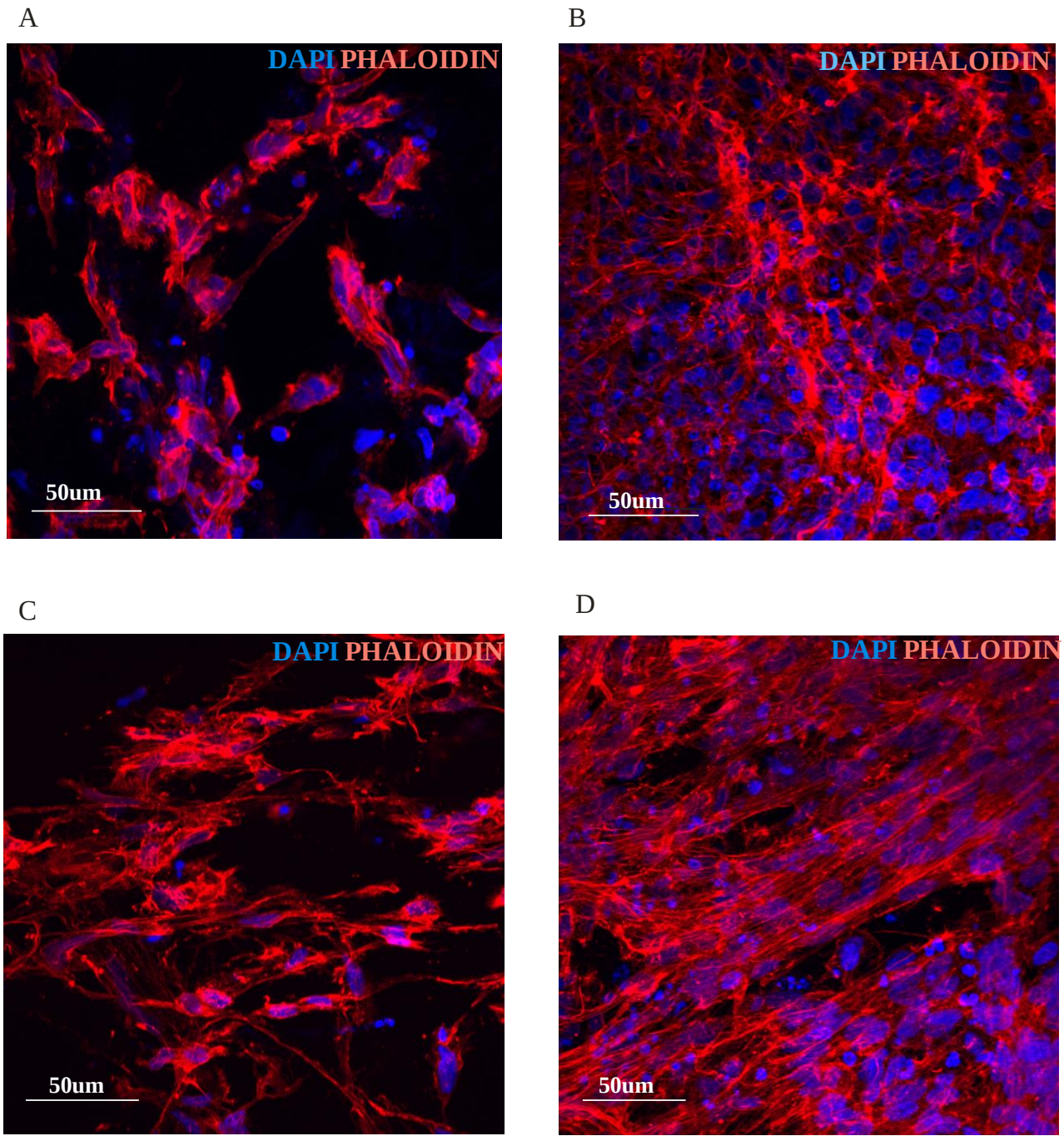



Fig.1. Imunohistochemical staining of hESC-NPs and SPC-01growing on 32,5 hydrogel. hESC-NPs staned for Phaloidin and DAPI (A) 24 hour and (B) 14 days after seeding. SPC-01 staned for Phaloidin and DAPI (C ) 24 hours and (D) 14 days after seeding.

Fig.2

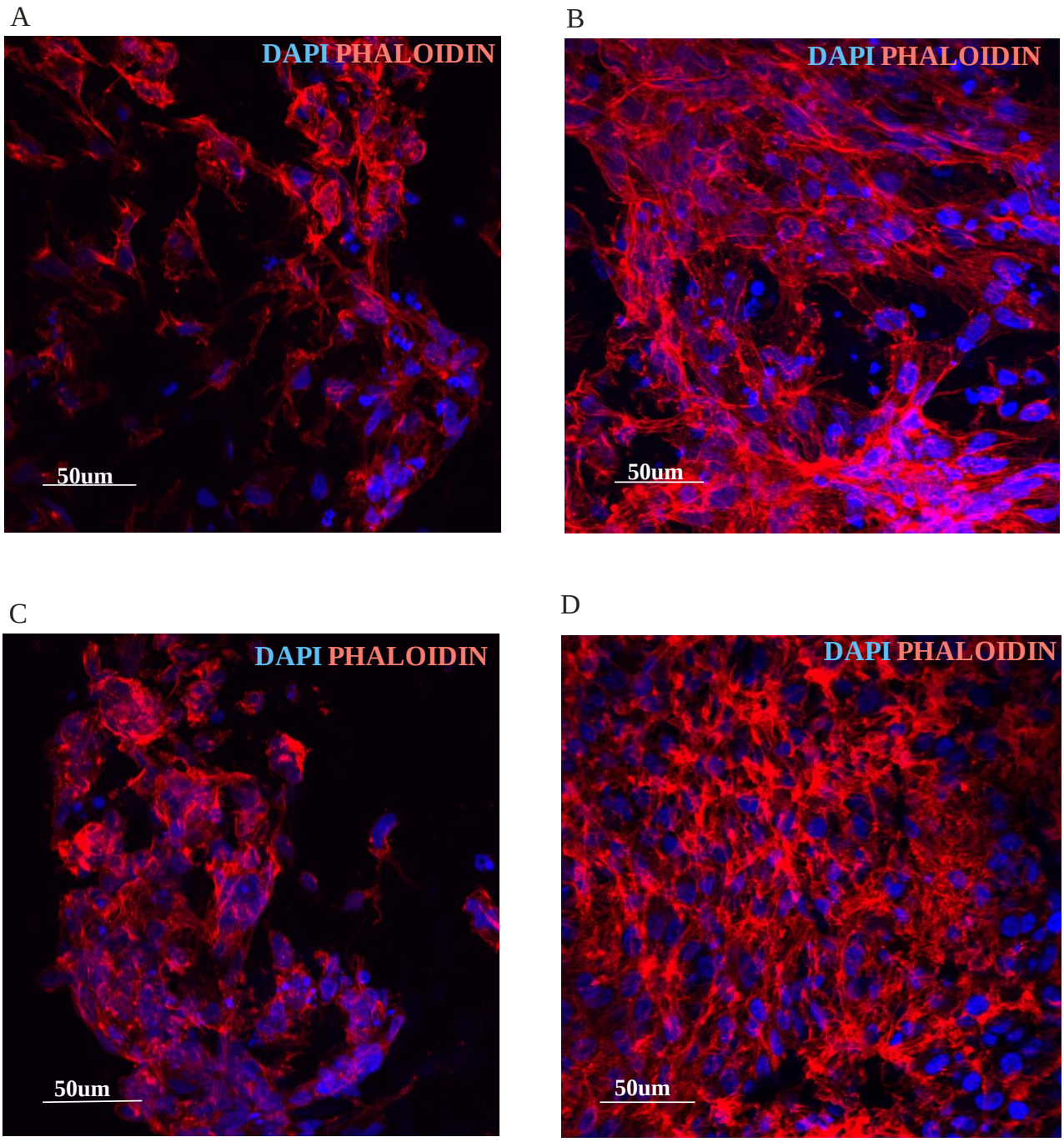
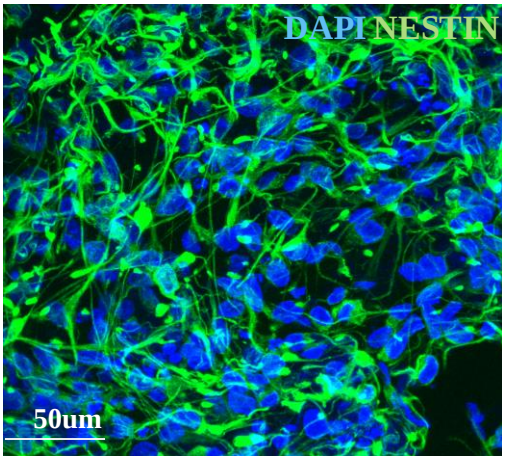


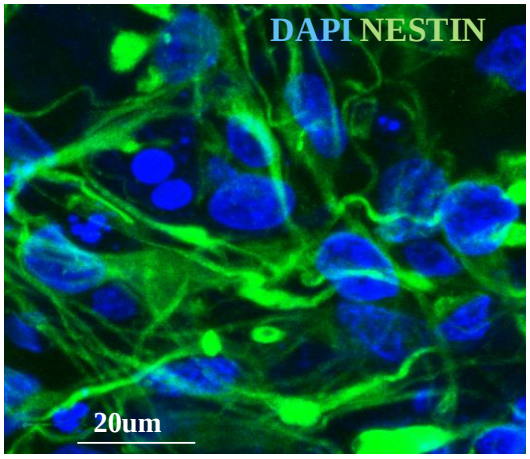

Fig.2. Imunohistochemical staining of hESC-NPs and SPC-01 growing on HEMA-MA hydrogel. hESC-NPs staned for Phaloidin and DAPI (A) 24 hours and (B) 14 days after seeding. SPC-01 staned for Phaloidin and DAPI (C ) 24 hours and (D) 14 days after seeding

Fig.3

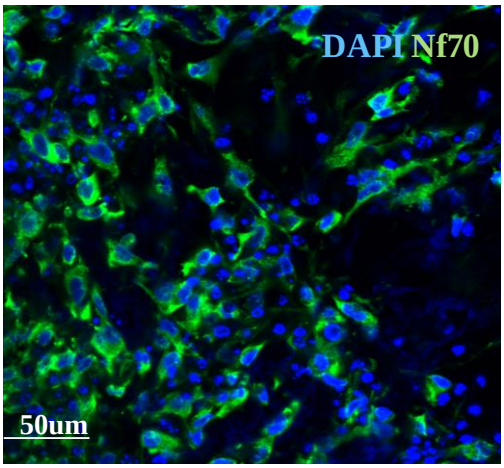
A



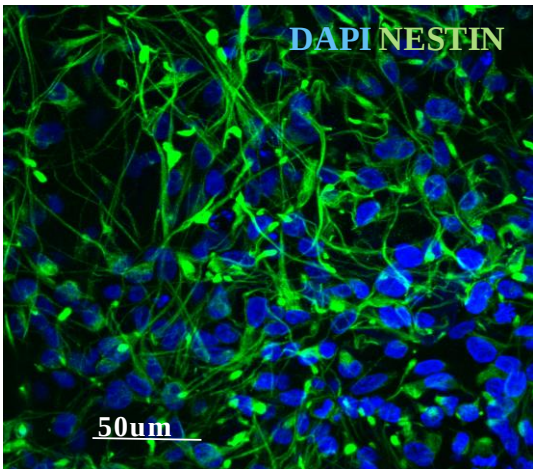

B




C




D




E

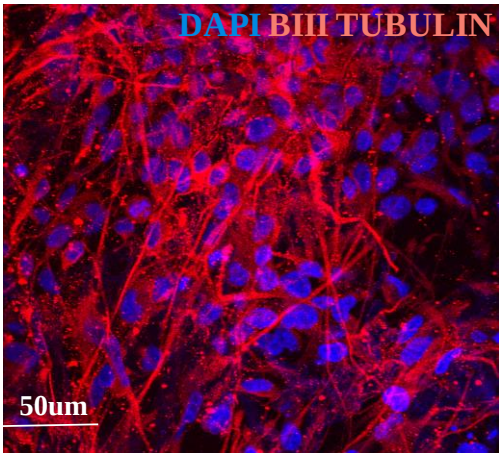



Fig.3. Immunohistochemical characterisation of difenetiation SPC-01.
A: Immunostaining for Nestin 28 days after starting experiment on 32,5 hydrogel.
B: higher magnification view of the staining shown in A.
C: Immunostaining for NF70 28 days after starting experiment on 32,5 hydrogel.
D: Immunostaining for Nestin 28 days after starting experiment on HEMA-MA hydrogel.
E: Immunostaining for BIII-Tubulin 28 days after starting experiment on HEMA-MA hydrogel

## 5 Discussion

Here we demonstrate the growth, proliferation and differentiation of cells of hESC-NPs and SPC-01 lines on five types of hydrogels. How has been written above important role in this experiment played similarity of hydrogels origin. Two different compounds HEMA and HPMA based hydrogels have different ways of polymerization and their structure and pores are very different. In case of E4,5 hydrogel came failure of in vitro cultivation of both hESC-NPs and SPCs-01, because of HEMA based pores in HPMA constitution. Only SPC-01 showed after 14 days of four weeks study growth in small neurospheres. Comparing of growth in HPMA and W hydrogles bring small differences explainable by different laboratory. In spite of that, HPMA hydrogel showed in all study a little bit better growth ability. This study also confirm the fact, in consonance with of M. Přádný and colleagues study, that positively charged quarternary ammonium group on HEMA-MOETA$^{+}$ (32,5) hydrogel surface shows a little better supporting ability of cells growth, than negatively charged metaacril acid group on HEMA-MA$^{-}$. An important fact is that, the concentration of cells was different on the edge and inside of hydrogel. This different cells concentration on the edge of gel showed not absolute ability for accessibility of nutrients. But these facts are depending on used cell types, because not for all cell types or lines is the same structured environment convenient. Both used precursor lines hESC-NPs and SPCs-01 growth very well. hESC-NPs had faster start of growing, but from neurospheres started SPC-01 cells, expanded after 24 hours measuring. On 7 days samples had SPC-01cells higher concentration and markers of neural differentiation, which shows, that they have better properties to growth and differentiate in hydrogel conditions.

# 6 Conclusions

In this work we compared several biocompatible materials in vitro - nonbiodegradable biocompatible hydrogels, such as HEMA, HPMA in their different forms, with two neural precursor lines hESC-NPs and SPC-01. This experiment confirmed the fact, that surface charge and molecules playing important role in permissiveness and adhesivity for grafted cells, and opened space for new compounds testing. The role in density high and proliferation ability plays choice of suitable neural precursors.

## 7 References


Battey JF. (2007). Stem cells: current challenges and future promise. Dev Dyn 236, 3193-3198.-rev.

Behrmann DL, Bresnahan JC & Beattie MS. (1994). Modeling of acute spinal cord injury in the rat: neuroprotection and enhanced recovery with methylprednisolone, U-74006F and YM-14673. Exp Neurol 126, 61-75.

Belegu V, Oudega M, Gary DS & McDonald JW. (2007). Restoring function after spinal cord injury: promoting spontaneous regeneration with stem cells and activity-based therapies. Neurosurg Clin N Am 18, 143-168, xi.

Blesch A, Lu P & Tuszynski MH. (2002). Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. Brain Res Bull 57, 833-838.-rev.

Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon J & et al. (1990). A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. N Engl J Med 322, 1405-1411.

Bracken MB, Shepard MJ, Collins WF, Jr., Holford TR, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon JC, Marshall LF & et al. (1992). Methylprednisolone or naloxone treatment after acute spinal cord injury: 1-year follow-up data. Results of the second National Acute Spinal Cord Injury Study. J Neurosurg 76, 23-31.

Brannvall K, Bergman K, Wallenquist U, Svahn S, Bowden T, Hilborn J & Forsberg-Nilsson K. (2007). Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. J Neurosci Res 85, 2138-2146.

Cai C & Grabel L. (2007). Directing the differentiation of embryonic stem cells to neural stem cells. Dev Dyn 236, 3255-3266.-rev.

Cao Q, Benton RL & Whittemore SR. (2002). Stem cell repair of central nervous system injury. J Neurosci Res 68, 501-510.-rev.

David S, Bouchard C, Tsatas O & Giftochristos N. (1990). Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. Neuron 5, 463-469.

Davies SJ, Field PM & Raisman G. (1994). Long interfascicular axon growth from embryonic neurons transplanted into adult myelinated tracts. J Neurosci 14, 1596-1612.

Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G & Silver J. (1997). Regeneration of adult axons in white matter tracts of the central nervous system. Nature 390, 680-683.

Delfino-Machin M, Chipperfield TR, Rodrigues FS & Kelsh RN. (2007). The proliferating field of neural crest stem cells. Dev Dyn 236, 3242-3254.

Duconseille E, Woerly S, Kelche C, Will B & Cassel JC. (1998). Polymeric hydrogels placed into a fimbria-fornix lesion cavity promote fiber (re)growth: a morphological study in the rat. Restor Neurol Neurosci 13, 193-203.

Fawcett JW & Asher RA. (1999). The glial scar and central nervous system repair. Brain Res Bull 49, 377-391.-rev.

Fitch MT & Silver J. (1997). Glial cell extracellular matrix: boundaries for axon growth in development and regeneration. Cell Tissue Res 290, 379-384.

Fitch MT & Silver J. (2008). CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp Neurol 209, 294-301.-rev.

Fouad K, Metz GA, Merkler D, Dietz V & Schwab ME. (2000). Treadmill training in incomplete spinal cord injured rats. Behav Brain Res 115, 107-113.

Geller HM & Fawcett JW. (2002). Building a bridge: engineering spinal cord repair. Exp Neurol 174, 125-136.

Giehl KM & Tetzlaff W. (1996). BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. Eur J Neurosci 8, 1167-1175.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V & Fouad K. (2007). Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain 130, 2993-3003.

Goldman SA & Windrem MS. (2006). Cell replacement therapy in neurological disease. Philos Trans R Soc Lond B Biol Sci 361, 1463-1475.-rev:

Grill R, Murai K, Blesch A, Gage FH & Tuszynski MH. (1997). Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. J Neurosci 17, 5560-5572.

Guenard V, Gwynn LA & Wood PM. (1994). Astrocytes inhibit Schwann cell proliferation and myelination of dorsal root ganglion neurons in vitro. J Neurosci 14, 2980-2992.

Harel NY & Strittmatter SM. (2006). Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? Nat Rev Neurosci 7, 603-616.-rev.

Hejcl A, Lesny P, Pradny M, Michalek J, Jendelova P, Stulik J & Sykova E. (2008). Biocompatible hydrogels in spinal cord injury repair. Physiol Res 57 Suppl 3, S121-132.-rev.

Hejcl A, Urdzikova L, Sedy J, Lesny P, Pradny M, Michalek J, Burian M, Hajek M, Zamecnik J, Jendelova P & Sykova E. (2008). Acute and delayed implantation of positively charged 2-hydroxyethyl methacrylate scaffolds in spinal cord injury in the rat. J Neurosurg Spine 8, 67-73.

Houle JD & Tessler A. (2003). Repair of chronic spinal cord injury. Exp Neurol 182, 247-260.-rev.

Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH & Evans RM. (2006). Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proc Natl Acad Sci U S A 103, 3902-3907.

Jones LL, Oudega M, Bunge MB & Tuszynski MH. (2001). Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. J Physiol 533, 83-89.-rev.

Kitazawa A & Shimizu N. (2007). Characterization of neurons differentiated from mouse embryonic stem cells using conditioned medium of dorsal root ganglia. J Biosci Bioeng 104, 257-262.

Kondo T. (2007). Stem cell-like cancer cells in cancer cell lines. Cancer Biomark 3, 245-250.

Metz GA & Whishaw IQ. (2002). Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 115, 169-179.

Metz GA, Merkler D, Dietz V, Schwab ME & Fouad K. (2000). Efficient testing of motor function in spinal cord injured rats. Brain Res 883, 165-177.

Nisbet DR, Forsythe JS, Shen W, Finkelstein DI & Horne MK. (2008). Review Paper: A Review of the Cellular Response on Electrospun Nanofibers for Tissue Engineering. J Biomater Appl.0, 1-23.-rev.

Nisbet DR, Pattanawong S, Nunan J, Shen W, Horne MK, Finkelstein DI & Forsythe JS. (2006). The effect of surface hydrophilicity on the behavior of embryonic cortical neurons. J Colloid Interface Sci 299, 647-655.

Nisbet DR, Pattanawong S, Ritchie NE, Shen W, Finkelstein DI, Horne MK & Forsythe JS. (2007). Interaction of embryonic cortical neurons on nanofibrous scaffolds for neural tissue engineering. J Neural Eng 4, 35-41.

Novikov LN, Novikova LN, Mosahebi A, Wiberg M, Terenghi G & Kellerth JO. (2002). A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury. Biomaterials 23, 3369-3376.

Novikova LN, Mosahebi A, Wiberg M, Terenghi G, Kellerth JO & Novikov LN. (2006). Alginate hydrogel and matrigel as potential cell carriers for neurotransplantation. J Biomed Mater Res A 77, 242-252.

Novikova LN, Novikov LN & Kellerth JO. (2003). Biopolymers and biodegradable smart implants for tissue regeneration after spinal cord injury. Curr Opin Neurol 16, 711-715.-rev.

Oudega M, Moon LD & de Almeida Leme RJ. (2005). Schwann cells for spinal cord repair. Braz J Med Biol Res 38, 825-835.

Oudega M. (2007). Schwann cell and olfactory ensheathing cell implantation for repair of the contused spinal cord. Acta Physiol (Oxf) 189, 181-189.

Pointillart V, Petitjean ME, Wiart L, Vital JM, Lassie P, Thicoipe M & Dabadie P. (2000). Pharmacological therapy of spinal cord injury during the acute phase. Spinal Cord 38, 71-76.

Pollock K, Stroemer P, Patel S, Stevanato L, Hope A, Miljan E, Dong Z, Hodges H, Price J & Sinden JD. (2006). A conditionally immortal clonal stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke. Exp Neurol 199, 143-155.

Pradny M, Lesny P, Smetana K, Jr., Vacik J, Slouf M, Michalek J & Sykova E. (2005). Macroporous hydrogels based on 2-hydroxyethyl methacrylate. Part II. Copolymers with positive and negative charges, polyelectrolyte complexes. J Mater Sci Mater Med 16, 767-773.

Rabchevsky AG & Streit WJ. (1997). Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. J Neurosci Res 47, 34-48.

Reier PJ. (2004). Cellular transplantation strategies for spinal cord injury and translational neurobiology. NeuroRx 1, 424-451.-rev.

Reynolds LF, Bren MC, Wilson BC, Gibson GD, Shoichet MS & Murphy RJ. (2008). Transplantation of porous tubes following spinal cord transection improves hindlimb function in the rat. Spinal Cord 46, 58-64.

Schafer M, Fruttiger M, Montag D, Schachner M & Martini R. (1996). Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. Neuron 16, 1107-1113.

Schwab ME, Kapfhammer JP & Bandtlow CE. (1993). Inhibitors of neurite growth. Annu Rev Neurosci 16, 565-595.-rev.

Schwab ME. (1990). Myelin-associated inhibitors of neurite growth and regeneration in the CNS. Trends Neurosci 13, 452-456.-rev.

Sieber-Blum M & Hu Y. (2008). Epidermal neural crest stem cells (EPI-NCSC) and pluripotency. Stem Cell Rev 4, 256-260.-rev.

Sykova E, Homola A, Mazanec R, Lachmann H, Konradova SL, Kobylka P, Padr R, Neuwirth J, Komrska V, Vavra V, Stulik J & Bojar M. (2006). Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury. Cell Transplant 15, 675-687.-rev.

Sykova E, Jendelova P, Urdzikova L, Lesny P & Hejcl A. (2006). Bone marrow stem cells and polymer hydrogels--two strategies for spinal cord injury repair. Cell Mol Neurobiol 26, 1113-1129.-rev.

Thonhoff JR, Lou DI, Jordan PM, Zhao X & Wu P. (2008). Compatibility of human fetal neural stem cells with hydrogel biomaterials in vitro. Brain Res 1187, 42-51.

Thuret S, Moon LD & Gage FH. (2006). Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 7, 628-643.-rev.

Yang F, Murugan R, Wang S & Ramakrishna S. (2005). Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering. Biomaterials 26, 2603-2610.

Z'Graggen WJ, Metz GA, Kartje GL, Thallmair M & Schwab ME. (1998). Functional recovery and enhanced corticofugal plasticity after unilateral pyramidal tract lesion and blockade of myelin-associated neurite growth inhibitors in adult rats. J Neurosci 18, 4744-4757.