Hydrogel/Electrospun Fiber Composites Influence Neural Stem/Progenitor Cell Fate

by

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ABSTRACT

Cell replacement therapy with multi-potent neural/stem progenitor cell into the injured spinal cord is limited by poor survival and host tissue integration. An injectable and biocompatible polymeric cell delivery system serves as a promising strategy to facilitate cell delivery, promote cell survival and direct cell behaviour. We developed and characterized the use of a physical hydrogel blend of hyaluronan and methylcellulose for NSPC delivery, and incorporated electrospun fibers of collagen or P(CL:DLLA) to promote cell-matrix interactions and influence cell behaviour. HAMC was shown to be both cytocompatible and allow NSPC differentiation *in vitro*. Inclusion of electrospun fibers in the HAMC hydrogel further influenced cell behaviour. Composite systems of P(CL:DLLA) fibers in HAMC maintained cell survival/proliferation and enhanced neuronal and oligodendrocytic differentiation similar to HAMC. The importance of the cell delivery vehicle to NSPC survival and cell fate was demonstrated *in vitro* and will be tested in on-going studies *in vivo*.

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The two years has been a wonderful and fun experience. It is time for me to take a different route to try something new now! ☺

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BBB	Beattie, Bresnahan, and Basso 21 point score scale for rat locomotion
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
HA	Hyaluronan
HPRT	Hypoxanthine phosphoribosyltransferase 1
MC	Methylcellulose
P(CL:DLA)	Poly (ϵ -caprolactone-co-D,L-lactide)
SCI	Spinal cord injury
SEM	Scanning electron microscope
SVZ	Sub-ventricular zone

1 INTRODUCTION

Spinal cord injury (SCI) from mechanical insult results in cell death and cavity formation, disrupting signalling pathways of the central nervous system (CNS)¹. Patients often suffer lifetime sensory and motor deficits below the site of injury. Cell therapy is a promising strategy for tissue regeneration where, for example, neural stem/progenitor cells (NSPCs) have enhanced tissue repair and function after acute spinal cord injury^{2,3}. NSPCs are multi-potent CNS cells capable of self-renewal and terminal differentiation into neurons, oligodendrocytes, and astrocytes. However, poor graft survival and integration are major factors limiting the efficacy of cell transplantation therapy. After bolus injection of NSPCs into the spinal cord, significant cell death was observed^{3,4}. Moreover, selective ablation of engrafted cells in the spinal cord reversed the locomotor improvement previously observed⁵. Thus major challenges for stem cell transplantation studies are cell survival and directed cell differentiation.

To enhance stem cell survival and differentiation, we focused on engineering the stem cell microenvironment using three-dimensional culture conditions. The choice of biomaterial, and its physical and chemical properties, influence cell differentiation⁶, survival⁷ and tissue integration⁸. Hydrogels are particularly compelling for cell delivery, having highly porous and hydrated structures that permit efficient diffusion of nutrients and oxygen, and metabolic waste products and CO₂. The hydrogel can be tuned either chemically with adhesive and differentiation factors or physically through crosslinking, to yield a microenvironment that will influence cell proliferation and differentiation⁹⁻¹². We were interested in testing hyaluronan (HA)-based hydrogels for cell delivery because HA is a naturally-derived polysaccharide in the extracellular matrix and has some wound healing properties on its own¹³. While HA alone does not form a gel, a physical blend of HA and methylcellulose (MC) forms a gel due to the thermally-reversible gelation properties of

MC.¹⁴ MC itself has also been studied after traumatic brain injury¹⁵. Importantly, HAMC has been shown to have some therapeutic benefit when delivered to the intrathecal cavity after spinal cord injury and can be injected using a minimally-invasive technique¹⁵⁻¹⁸.

Here we were interested in testing a HAMC formulation for cell delivery with and without the inclusion of electrospun fibers. Our hypothesis was that the electrospun fibers would provide a site for cell adhesion and thereby influence the differentiation profile of the stem/progenitor cells cultured within the fiber/hydrogel composite. We chose to compare the influence of naturally-derived collagen fibers vs. synthetic polymers of poly(ϵ -caprolactone-*co*-DL-lactide) vs. HAMC alone. For the latter, the *in vitro* biocompatibility of NSPCs dispersed in HAMC was evaluated by measuring the number of live cells and their metabolic activity in three media conditions, compared to media controls. The differentiation profile of the NSPCs in HAMC (with and without the fibers) was studied using immunocytochemistry and quantitative RT-PCR. In these series of studies, our goal was to evaluate the cellular response *in vitro* to the different culture conditions with a view of applying this knowledge to an *in vivo* system.

1.1 Hypothesis

A cell-loaded composite of HAMC and electrospun fibers will improve NSPC distribution throughout an injected volume while supporting cell survival and differentiation in culture.

1.2 Objectives

1. Develop and characterize an injectable, thermal-reversible hydrogel for NSPC.

Hydrogel's mechanical properties and cytocompatibility with NSPC will be assessed.

2. Incorporate and evaluate effects of electrospun fibers in hydrogel on NSPC behaviour
NSPC viability/proliferation and gene expression profile will be examined.

1.3 Spinal Cord Injury

1.3.1 The Spinal Cord

The spinal cord and the brain constitute the central nervous system (CNS) that coordinates the activities of all parts of the body and controls behaviour. The nervous tissue is primarily composed of three differentiated cell types – neurons, astrocytes, oligodendrocytes. Astrocytes and oligodendrocytes provide critical support for optimal neuronal functioning, and neurons are the functional unit of the CNS, responsible for forming connections and are the communicating cells of the nervous system.

1.3.2 Spinal Cord Injury

Spinal cord injury (SCI) often results in cavity and cell death, disrupting the signalling pathway leading to loss of sensor and motor functions below the site of injury. Currently, there is no effective cure. Globally, 2.5 million people live with SCI, with more than 130,000 new cases reported every year¹⁹. Patients' suffer significant impacts on quality of life (paraplegia or quadriplegia) and economic burden (loss of income and requirement for long-term primary care).

To develop an effective strategy towards improved neuropreservation and neuroregenerative repair, understanding the neuropathophysiology of the traumatic SCI is essential. Compression injury as a result of the external mechanical insult from dislocation of spinal vertebral motion segments or displaced bone fragment is the most common injury model²⁰. Following the initial trauma to the spinal cord, a series of events including vascular changes (edema, ischemia, and hypoxia), excitotoxic biochemical alterations (formation of free radicals and NO and protease release), and cellular response (immune cells, activation of glial cells, and apoptotic death of neurons and glia) causes progressive loss of neural tissue²¹. Cavities and cysts are formed interrupting descending and ascending axonal tracts²².

1.4 Neuroregeneration Strategy

1.4.1 Cell-based therapy for SCI

Cell therapy to repair tissue and to restore function is a promising regenerative medicine modality. The main rationale for cell-based therapies following spinal cord injuries are: (1) replace degenerated spinal cord parenchyma by an axon growth supporting structure, to reconstruct the neuronal circuit as a relay bridge in the cavity; (2) remyelination of regenerating axons; and (3) produce and locally deliver neurochemically active substance to promote growth – trophic effect²³.

Transplanted cells have the potential to replace tissue lost during trauma or to support the regeneration process. Cell types delivered for SCI injury include human embryonic stem cells (hESCs), fetal stem cells, mesenchymal stem cells, neural stem cells, ES-derived oligodendrocyte progenitor cells, olfactory ensheathing cells, Schwann cells, and macrophages^{23,24}. Neural stem/progenitor cells (NSPCs) are of particular interest because of their capacity to differentiate into CNS cells, potentially becoming neurons to reconnect the signalling path or oligodendrocytes to remyelinate axons and producing growth factors.

1.4.2 Adult-derived NSPCs

The discovery of NSPCs by Reynold and Weiss²⁵ opened the door to new therapeutic avenues for cell therapy in SCI. Subsequently, NSPCs have been characterized by adherent properties, proliferation kinetics, growth factor dependence, and gene expression². In the adult mammal, NSPCs are located primarily in the sub-ventricular zone (SVZ) of the forebrain, in the sub-granular layer of the dentate gyrus of the hippocampal formation, and in the spinal cord². They are multi-potent stem cells capable of self-renewal, and terminally differentiation into neurons and glial cells²⁶. These intrinsic properties position NSPC as a promising candidate for cell-replacement therapy.

However, the repair responses from injury-induced recruitment of endogenous NSPCs are impaired or limited shown by pathology. Strategies developed to facilitate mobilization of endogenous NSPC were reviewed by Okano et al²⁷, an example including supplementation of exogenous growth factor²⁸. The repair capacity of the endogenous NSPCs is under active research. In parallel, studies of transplantation of exogenous NSPCs have shown some success with partial functional recovery².

1.4.3 Cell delivery methods

Route of cell administration is dependent on the type CNS lesion. While systemic (intravenous or intrathecal) transplantation is suitable for multifocal CNS disorder (e.g. multiple sclerosis), direct local (intralesional) cell transplantation is beneficial to focal CNS disorder (e.g. SCI, stroke)²⁶. Cell delivery for the compression SCI is either through the conventional bolus injection of cells suspended in liquid, or scaffolds/hydrogels implantation of cell seeded within.

1.4.4 Current challenges in cell delivery

NSPC survival, differentiation and integration are key requirements for an effective and efficient cell therapy. However, these conditions are generally not met. Parr and co-workers observed poor transplanted cell survival under optimized logistics, transplanting rat NSPC at subacute (9 d after compression SCI) model and 1mm rostral and caudal to the injury site⁴. Another study by Karimi et al where mice NSPCs were transplanted 2 weeks post injury 2 mm rostral and caudal to the injury site combined with growth factors (EGF, FGF, and PDGF-AA), there was significant cell death 8 weeks after transplantation with partial functional recovery³. It is further indicated by Cummings et al that transplanted cell survival in the host is critical to maintain the functional recovery, where reversed locomotor improvement was observed with selective ablation of engrafted cell⁵.

Once cell survival can be sustained post transplantation, the cells need to differentiate to become functional cells and to integrate with the host tissue. However, it has been found transplanted NSPCs tend to become astrocytes, the scar forming cells²⁹, and integration is limited at the periphery of the lesion cavity, leaving the center of the cavity disconnected. The challenges led to inefficacious cell therapy aimed to restore function. Tissue engineering, an integration to regenerative medicine, has the potential to facilitate the process to deliver cells, to present cues to guide cell behaviour and to provide the missing structural support to bridge the lesion cavity.

1.4.5 Tissue engineering approach

NSPCs are highly responsive to environmental physical and chemical cues. Designing a microenvironment using biomaterials can promote efficient delivery and modulate spatial and temporal progression of the regenerative process commenced by cell transplantation.

For cell therapy to replace the lost tissue, the structure of the tissue should be reconstructed and integrated with the host. The application of scaffold or hydrogel can act as an infrastructure to support the transplanted cell. The integration of electrospun fibers may further provide cell-matrix interactions, collectively, with the goal to promote cell survival, and to direct cell differentiation. The influences of the engineered microenvironment to stem cell responses/activities such as morphology, adhesion, proliferation, and differentiation are of interest.

A minimally invasive cell delivery vehicle and transplantation device should meet the following design criteria. The biomaterial needs to be biocompatible to the host as well to the transplanted cells, supporting appropriate cellular activities. The system needs to be injectable through a needle and provide structural support to the transplanted cell *in situ*. The biomaterial should be biodegradable to allow integration of regenerated tissue to replace the implant.

1.4.6 Cell delivery vehicle and transplant device

Among polymer scaffolds, hydrogels are attractive because of their highly porous and hydrated structure for efficient infusion of nutrients and oxygen and transfer of waste products and CO₂ for cells. Biopolymers of alginate and poly(lactic-co-glycolic acid) of natural and synthetic origin, respectively, are reviewed by Hejcl et al where both of them supported axonal regeneration across the lesion site³⁰. Other hydrogels utilized for the NSPC include Pluronic 127 (non-ionic surfactant composed of a block copolymer of propylene oxide and ethylene oxide), matrigel (basal membrane extract derived from mouse sarcoma), and Puramatrix (ionic-self-complementary oligopeptides) where some toxicity and undefined content positioned them not suitable for translation to clinical settings^{31,32}.

Composites of electrospun fiber in hydrogel have the potential to create a hospitable microenvironment for NSPCs, utilizing the well-hydrated 3D structure and sites for cell-matrix interaction, mimicking the natural ECM. Hence, we developed composites of collagen or P(CL:DLA) fibers in a hyaluronan and methylcellulose hydrogel and studied their effects with NSPCs.

1.4.7 Physical blend of HA and MC

An injectable hydrogel of hyaluronan (HA) and methylcellulose (MC) was developed that enables simple, efficient, reproducible and uniform seeding of viable NSPCs. The gel can be injected as fluid at room temperature and will gel in situ, conforming to the shape of the lesion cavity.

Hyaluronan is a high molecular weight and negatively charged glycosaminoglycan, composed of simple disaccharides of glucuronic acid and N-Acetylglucosamine with shear-thinning property¹³. It is one of the major components in the extracellular matrix (ECM) that surrounds

migrating and proliferating cells¹³, widely distributed throughout the CNS that regulate the maintenance of the stem cell population. HA has been shown to ensure the survival of the grafted stem cells while successive breakdown of the hyaluronan matrix release the contained cells to contribute to tissue regeneration³³.

Methylcellulose is an anionic polymer, derived from cellulose, with reverse-thermal gelation property¹⁴. The gelation is a kinetic process of hydrophobic interactions between methyl groups, with no involvement of chemical or photo crosslinking, minimizing inflammatory and toxicity response. It permits for microinjection of liquid form into a confined space and then gel *in situ*. It has been used as an injectable scaffold for the repair of defects in the brain, nerve gap, and SCI to promote nerve regeneration^{15,34,35}.

1.4.8 Electrospun fibers

Electropinning can be used to produce scaffolds for tissue engineering. In the process, the polymer is dissolved in a solvent then ejected through a nozzle when electrostatic repulsive forces overcome the surface tension of the droplet at the syringe tip³⁶. Fine fibers is produced at the droplet, solidified and deposited on a grounded metal collector. The nanofibrous structure of electrospun fibers with its diameters in magnitude mimics the fibrous proteins in ECM. The nano-feature of the fibers including diameter, orientation (alignment or random), nanotopography, charges, or hydrophobicity has the potential to influence cell activities^{37,38}.

Collagen is the main fibrous protein connecting and supporting bodily tissues including skin, bone, tendons, and muscles³⁹. It is inherently unstable in aqueous environment. Hence, genipin crosslinking is used to preserve structure and mechanical characteristics of the collagen fibrillar structure. NSPC progression from proliferation to neuronal differentiation and formation of functional synapses was reported for NSPC interdispersed in Type 1 collagen⁴⁰.

Poly (ϵ -caprolactone-co-D,L-lactide), P(CL:DLA), is a biodegradable and bioresorbable material with many tissue engineering applications including nerve grafts⁴¹⁻⁴³. It has also been studied for drug release⁴⁴, and biomaterial microarray to screen cell responses⁴⁵.

2. MATERIAL AND METHODS

2.1 Preparation of HAMC hydrogel

HA and MC were sterile-filtered as dilute aqueous solutions through 0.22 μm vacuum filters (Nalgene, Rochester, NY, USA) and lyophilized prior to use. . HAMC 0.5/0.5 was prepared with 0.5 wt% HA (Mw 1,500,000, Novamatrix, Norway) and 0.5 wt% MC (Mw 310,000, ShinEtsu Metolose SM-4000, Japan) dissolved in one of three cell culture media: (1) Basic media: neurobasal media (Gibco-Invitrogen, Burlington, ON, Canada) supplemented with B27 neural supplement (Invitrogen), 2mM L-glutamine (Sigma-Aldrich, Oakville, ON, Canada) and 100 $\mu\text{g/ml}$ penicillin-streptomycin (PenStrep; Sigma-Aldrich); (2) Differentiation media: basic media supplemented with 1% fetal bovine serum (FBS) (Invitrogen); (3) Proliferation media: basic media supplemented with 20 ng/ml epidermal growth factor (recombinant human EGF; Invitrogen), 20 ng/ml basic fibroblast growth factor (recombinant human bFGF; Invitrogen) and 2 $\mu\text{g/ml}$ heparin (Sigma-Aldrich).

2.2 Preparation of electrospun fiber

Electrospun fibers of genipin crosslinked collagen and poly(ϵ -caprolactone-co-D,L-lactide) (PCL:DLLA) fibers were prepared as previously described, with average diameters of 200 nm and 900 nm, respectively. The collagen fibers were disinfected by immersion in 70% ethanol for one day whereas the PCL:DLLA fibers were sterilized by gamma irradiation (Co-60, 2.5mRad). The fibers were washed (3 times, 2 h each time) in basic media and then sonicated (Sonics Vibra Cell CV18 tip sonicator) twice consecutively for 30 s at 20kHz, 25% amplitude. The resulting fiber fragments were easily incorporated into the HAMC which was still injectable through the fine 30 gauge needle. For each study, there were 5 mg/ml of electrospun fibers dispersed cell culture medium.

2.3 Mechanical testing and gelation time

Rheological evaluation: The rheological properties of HAMC were characterized by a stress-controlled steady state experiment at 25 °C and 37 °C using an AR2000 rheometer (TA Instruments, New Castle, DE, USA) equipped with a 60-mm, 0.5° cone. To allow thermal equilibration to occur, all samples were conditioned for 20 min prior to shear. Measurements were then performed at shear stresses ranging between 0.01 and 80 Pa at both 25 °C and 37 °C.

Inverted tube test: 300 µl HAMC made in basic medium was injected into the bottom of a 1.7 ml microcentrifuge tube (Fisher Scientific, Ottawa, ON, CA) and incubated at 37 °C. At 5, 10, 12, 14, 16, 18, 20 min. intervals, tubes were inverted to observe if the gel flowed. The time at which the gel did not flow was recorded as the gelation time.

2.4 Scanning electron microscopy

To prepare HAMCM for scanning electron microscopy (SEM), the hydrogel was snap frozen and freeze dried under vacuum, then sputter coated with gold (Desk II, Denton Vacuum Moorestown). Samples were imaged using XL130 (FEI company , Hillsboro Oregon) with an acceleration voltage of 20kV.

2.5 *In vitro* swelling test

100 µl of 0.5/0.5 w/w% HAMC made in media was added to a 1.7 ml eppendorf tube that was equilibrated at 37°C. On the top of the gel, 900 µl of 37°C artificial cerebrospinal fluid (aCSF): 0.5 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 0.1 mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose, pH 7.4, was added. , Post set-up, half of the media was removed from the tubes every 24 hr and exchanged with fresh incubation media. To study the trend transiently, samples were collected at each time point. At 0, 8 h, 1, 3, 6, 9 days of incubation, all the media was removed and

the weight of the wet gel was recorded ($n=4$). The polymer samples were snap frozen and dried using the freeze dryer, and the remaining polymer mass was weighed. Swelling was quantified as a function of time. The swelling value is given by the wet gel weight at each time point divided by that of time zero and multiplied by 100%.

2.6 Neural stem/progenitor cell isolation and culture

Neural stem/progenitor cells were isolated from the subependyma of the lateral ventricles of adult female Wistar rat forebrains, as described previously¹⁹. Briefly, subependymal tissue was harvested from 8-12 week old rats and dissociated with papain (Papain Dissociation System; Worthington Biochemical Corporation, Lakewood, NJ, USA). The resultant cell suspension was centrifuged and the pelleted cells were subjected to a discontinuous density gradient to remove cell debris. Dissociated cells were re-suspended in proliferation media. The cells were incubated at 37 °C in a humidified incubator with 5% CO₂. Neurospheres appeared in 2-3 weeks, after which cells were passaged every week. All cell studies used neurospheres at passages 4 and 5.

2.7 Cell seeding

Neurospheres were centrifuged and washed once in basic media. A small sample of neurospheres were triturated into single cells and counted with a hemacytometer using trypan blue (Sigma-Aldrich) exclusion to determine the starting live cell population. Neurospheres were cultured for 4 d in the *in vitro* biocompatibility study because, after 4 d, there was significant aggregation of neurospheres in the proliferation media, which made the results difficult to interpret. Dissociated single cells were cultured for 7 d for the differentiation profile studies. 2×10^4 NSPCs were dispersed in 100 μ l of either: media alone, HAMC, electrospun collagen fiber/HAMC or electrospun PCL:DLLA/HAMC and then loaded into 1 ml syringes and injected through 30G, 1” needle into black clear-bottom 96-well plates (Greiner, Germany). 200 μ l of media was added on

day 3 to supply nutrients in the 7 d cultures. Cells were imaged on an inverted microscope (Axiovert S100, Zeiss) equipped with a camera (Cool SNAP HQ, Photometrics) on day 2 and 7.

2.8 NSPC viability/proliferation study

Quant-iT™ PicoGreen® dsDNA Kits: NSPCs were collected from cell culture wells into a 1.7 ml eppendorf tube, spun down at 3000 rpm for 5 min., and washed once in sterile PBS. NSPCs were lysed through a freeze-thaw cycle in 1X Tris-EDTA buffer and 0.2% Triton X-100 (Sigma-Aldrich). Total double stranded DNA was measured by fluorescence (Molecular Devices) by the reaction of DNA with PicoGreen (PicoGreen dsDNA Quantitation Kit; Invitrogen). An internal standard curve was prepared and used to convert the concentration of dsDNA to total number of cells. The data were expressed as fold growth relative to number of NSPCs seeded.

CellTiter-Glo Luminescent Cell Viability Assay: CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) quantifies the cytoplasmic Adenosine Triphosphate (ATP) present, an indicator of metabolically active cells. It results in cell lysis and generates a luminescent signal proportional to the total amount of ATP present in the viable cells. 100 µl of CellTiter-Glo reagent was added to the culture well and placed on a rotary shaker plate for 10 min in the dark. Luminescence was recorded immediately after using a SpectraMax Gemini EM microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The mean background luminescent signals from the media and cell-free HAMC controls were subtracted from the luminescence signals from the NSPCs in media and HAMC samples, respectively.

LIVE/DEAD® Viability/Cytotoxicity Assay: Cell viability was quantitatively assessed with a Live/Dead kit (Molecular Probes; Eugene, OR). To prepare the cells, the cells was collected in a 1.7 ml eppendorf tube, spun down at 3000 rpm for 5 min and then the supernatant removed. The

cell pellet was then resuspended in sterile PBS with 2 μ M calcein-AM (a live cell fluorescent dye, 485/530nm) or 10 μ M ethidium homodimer (a dead cell fluorescent dye, 530/645nm).

2.9 Electrospun fiber cytotoxicity study

Electrospun fibers of genipin crosslinked collagen and P(CL-*co*-DLLA) were placed in culture inserts (0.4 μ m, BD Biosciences, Fanklin Lakes, NJ, USA) and immersed in NBM, incubated at 37 °C and 5% CO₂ for 7 days. The conditioned media was then used to culture NSPCs at a density of 2 x 10⁴ single cells/100 μ l for 7 days. Cell viability was examined using PicoGreen and CellTiter-Glo assays, as described above.

2.10 NSPC immunohistochemistry

Cryostat sectioning: Single cell cultures of 2 x 10⁴ single cells/100 μ l of HAMC were grown for 7 d in the differentiation medium. Cryomatrix (Thermo Scientific, Pittsburgh, USA) was added to the well and placed at room temperature for 5 min, then snap frozen. 20 μ m sections were cut using cryotome (Leica CM3050, Bensheim, Germany), and mounted on glass slides.

Immunostaining: The following primary antibodies were used for immunohistochemistry (IHC): monoclonal mouse anti- β -III tubulin (1:1000; Abcam, Cambridge, MA, USA) for neurons; RIP monoclonal anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (1:5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for oligodendrocytes; monoclonal mouse anti-glial fibrillary acidic protein (GFAP, 1:100; Cell Sciences, Canton, MA, USA) for astrocytes; and monoclonal mouse anti-nestin (1:500; BD Biosciences, San Jose, CA, USA) for progenitor cells. Cells were fixed after 7 days of culture with PBS solution containing 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature and then washed with PBS 3 times. Cell membranes were permeabilized with 0.1% Triton X-100 in PBS for 10 min., washed 3 times, then

blocked with a solution of 10% FBS in PBS at room temperature for 1 h. Next, each primary antibody solution was added for 2.5 h at 25°C. After washing with PBS 3 times, samples were exposed to goat anti-mouse IgG Alexa-Fluor 546 (1:400; Invitrogen) for 2 h at room temperature and then washed with PBS buffer 3 times. Finally, cell nuclei were counterstained with 10 μ M Hoechst 33342 (Invitrogen) for 7 min, washed with PBS buffer and mounted/coverslipped using ProLong Gold anti-fade reagent (Invitrogen). Fluorescent signals were detected and imaged via confocal laser scanning microscopy (Olympus BX61, Ontario, Canada) equipped with a camera (Olympus DP70).

2.11 Total RNA isolation and real-time RT-PCR

To monitor the expression level of differentiation gene markers using qRT-PCR, total RNA from the cells was prepared. Cells were collected at day 7 into 2 ml screw-cap microvials with 1.0 mm Zirconia Beads (BioSpec Products, Bartlesville, OK, USA), and homogenized using mini-beadbeater-16 (BioSpec Products) for 3 min. Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method, with 1 ml Trizol reagent (Invitrogen) and 0.2 M potassium acetate to remove the polysaccharide (HAMC) from the sample before adding chloroform for RNA extraction.

After RNA isolation, DNase I treatment was performed followed by the measurement of total RNA concentration and purity (NanoDrop ND-1000). For the reverse transcription (RT) reaction, 15.7 μ L of RNA (at 5 ng total RNA/ μ L) was incubated with oligo(dT) primers and random hexamers at 65 °C for 5 min. After cooling to room temperature, the RNA-oligo(dT)/hexamer mix, buffer, dNTPs, RNase inhibitor, Stratagene AffinityScript RT enzyme (La Jolla, CA, USA) for 60 min at 42 °C. The reaction was terminated by 70 °C for 15 min. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) amplification was

performed with a Roche LightCycler® 480 (Laval, Quebec, Canada) using SYBR Green I (Invitrogen) detection chemistry. The 5' to 3' sequences for the forward primers and reverse primers were previously designed in our lab from rat mRNA sequences from the National Center for Biotechnology Information (NCBI) given in Table 1. For qRT-PCR analysis of each sample, the following were prepared in a 11 µL reaction buffer: 1 µL of DNA sample, buffer, 3-4 mM MgCl₂, 0.2 mM DNTPs, 200-400 nM of each forward and reverse primer, 0.5X SYBR Green I, 1X ROX reference dye (Sigma-Aldrich), and 0.055 U HotStarTaq (Qiagen, Valencia, CA, USA). Quantitative RT-PCR amplification was achieved with a 15 min activation step at 95 °C, followed by 50 cycles of 15 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a fluorescence measurement (excitation/emission – 494/521nm). All qRT-PCR reactions were performed in triplicate. A standard curve (cycle threshold value versus relative template concentration) was prepared for each target gene and for the endogenous reference (HPRT). Gene expression for each target gene was compared across experimental groups, and the relative fold change was calculated by normalizing against the endogenous reference genes using Pfaffl's efficiency corrected calculation model for multiple reference genes. Primer sets were used shown in Table 1.

Table 1. Primer and probe designs for quantitative RT-PCR

Gene*	Forward Primer Reverse Primer
Hypoxanthine phosphoribosyltransferase 1 (HPRT)	CTCATGGACTGATTATGGACAGGAC GCAGGTCAGCAAAGAACTTATAGCC
βIII tubulin	ACTTTATCTTCGGTCAGAGTG CTCACGACATCCAGGACTGA
2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase)	CAACAGGATGTGGTGAGGA CTGTCTTGGGTGTCACAAAG
Glial fibrillary acidic protein (GFAP)	GAGAGAGATTCGCACTCAGTA TGAGGTCTGCAAAGCTTGGAC
Nestin	TGGCACACCTCAAGATGTCCCTTA AGAAAGCCAAGAGAAGCCTGGGAA

*All sequences are 5' - 3'

2.12 Statistical analysis

All statistical analyses were performed using JMP IN 7.1 (SAS Institute, Cary, NC, USA). Differences among groups were assessed by ANOVA with Tukey's *post hoc* analysis to identify statistical differences among three or more treatments. All errors are given as standard deviation.

3. RESULTS

3.1 HAMC as a NSPC delivery vehicle

3.1.1 HAMC Characterization

The 0.5/0.5 w/w% HAMC hydrogel was characterized by SEM, time to gelation, viscosity, rheology and swelling (Figure 1). As shown by SEM of the freeze-dried sample, HAMC is a porous structure with pores on the order of 20 to 50 μm (Figure 1a). By the inverted tube test, HAMC gells after 18 min at 37 $^{\circ}\text{C}$.

Figure 1: HAMC characterization. (a) SEM of HAMC (250% magnification); (b) swelling; (c) viscosity vs. shear rate, demonstrating shear-thinning (d) shear stress vs. shear rate, demonstrating a yield stress at 37 $^{\circ}\text{C}$

The shear stress (τ) vs. shear rate ($\dot{\gamma}$) data in the limit of low shear rate for HAMC at both 25 and 37 °C are illustrated in Figure 1d. At 25°C, HAMC displays nearly-Newtonian behaviour - that is a linear shear stress vs. shear rate relationship without a yield stress, where ($\tau=\eta\dot{\gamma}$), reflecting the nearly constant viscosity in the low-shear portion of the flow curve. At 37°C, however, a yield stress of 0.5 Pa is observed. Thus, the blend exhibits nearly perfect Bingham plastic behaviour, according to $\tau=\mu_p\dot{\gamma}+\tau_y$, where μ_p is the plastic viscosity of approximately 0.6 Pa•s and a yield stress of 0.5 Pa, which corresponds to the formation of a very weak gel.

To gain greater insight into the injectability of HAMC at the 0.5/0.5 w/w% composition, its viscosity at room temperature was characterized over a broader range of shear rates. At room temperature (25°C), HAMC is a moderately-viscous solution with shear thinning properties (Figure 1c). This rheological signature is characteristic for polymeric blends, where the viscosity, η , remains nearly constant ($\eta = 1.2$ Pa•s) at low shear rates and decreases with a power law relationship at high shear rates, and in this case, with a scaling exponent of ~ 0.5 .

Since HAMC is designed to be injected into a tissue cavity, the degree of gel swelling was investigated to ensure minimal tissue damage such as compression of the soft tissue at the injured site. The swelling of HAMC reached a maximum swollen volume of approximately 115% by 1 day and gradually decrease to 50% by day 9 (Figure 1b).

3.1.2 NSPCs cultured in HAMC vs. Media: Cell Distribution

NSPCs cultured in media were compared to those cultured in HAMC in terms of cell distribution over time. As shown in bright-field images (Figure 2), NSPC neurospheres cultured in differentiation media of EGF/FGF2/heparin alone aggregated (Fig 2a) whereas those cultured in differentiation media in HAMC remained dispersed in the gel (Fig 2b). The aggregated neurospheres cultured in media had darkened necrotic cores unlike those cultured in media in

HAMC. In differentiation media of 1% FBS, NSPC neurospheres displayed different morphologies when cultured in media vs. HAMC. Interestingly, there was little evidence of differentiation after 48 h of NSPCs cultured in differentiation media, where the cell body remained spherical (Fig 2c). In contrast, NSPCs cultured in differentiation media in HAMC extended processes and formed networks (Fig 2d).

Figure 2: Bright-field microscopic images of NSPC neurosphere culture at day 2. HAMC's (a) cells cultured in proliferation media aggregated whereas those cultured in (b) proliferation media in HAMC remained dispersed throughout the gel. (c) NSPC neurospheres cultured in differentiation media remained spherical whereas (d) NSPC neurospheres cultured in differentiation media in HAMC extended processes and formed networks with other neurospheres. Arrows highlight processes and morphological changes of NSPCs in (d). The scale bar is 100 µm.

3.1.3 NSPC viability and differentiation in HAMC vs. Media Controls

The number of NSPCs cultured in HAMC was compared to those in media after 4 days of culture where dsDNA content by the Picogreen assay was used as a proxy for cell number. Importantly, cells cultured in HAMC were injected via a 30-gauge needle into the culture well, thereby simulating the in vivo injection procedure. The day 4 data was normalized to day 0 to account for any variability in the number of NSPCs seeded. The number of NSPCs cultured in media was comparable to those dispersed in HAMC (Fig 3a). In basic culture conditions, there were more live cells in HAMC relative to the media control. While there were no other differences observed among cells grown in media vs. HAMC for the other media conditions, NSPCs grew more rapidly when cultured in the presence of mitogens (EGF/FGF2) than the basal and differentiation media conditions.

To assess the metabolic activity of NSPCs in different culture conditions, total cellular plasma ATP was measured using the luminescence assay (Fig 3b). No difference was found of cells cultured in basal or differentiation media conditions; however, cells cultured in the EGF/FGF2 proliferation media had higher ATP content per live cell. Interestingly, cells cultured in this proliferation media in HAMC had a lower luminescent signal, and thus less ATP/live cell, than cells cultured in just the proliferation media. We further conducted a small study, culturing NSPCs in the three media and measuring their ATP content per live cell. The same trend was verified, with higher ATP content of proliferating cells in EGF/FGF supplemented media (data not shown).

To gain a thorough understanding of how the culture environment affected cell viability, the percentage of live cells, by calcein AM staining, and the percentage of dead cells, by ethidium bromide homodimer, were measured (Fig 3c, 3d). The percentage of live cells was similar for NSPCs cultured in neurobasal or differentiation media with and without HAMC. However, the

percentage of live cells was lower in the proliferation media, whether cultured in HAMC or media, relative to the basal and differentiation media. Interestingly, there were more live cells in HAMC than media controls. Thus, while the PicoGreen data for dsDNA (Fig 3a) showed greater numbers of cells in the proliferation media, the percentage of live cells was lower in the proliferation media than the other media conditions and HAMC, which is consistent with the brightfield microscopic images of Figure 2. The percentage of dead cells data was consistent with the percentage of live cells data. There was no difference in the percentage of dead cells in the basal or differentiation media for cells cultured in media or HAMC; however, there was a higher percentage of dead cells cultured in the proliferation media relative to the basal and differentiation media conditions. Moreover, there were more dead cells cultured in proliferation media than proliferation media in HAMC. These data are consistent with having necrotic cores in the neurospheres cultured in proliferation media alone vs. those in HAMC.

Figure 3: NSPC neurospheres cultured in different media alone vs. different media in HAMC after 4 days; (a) The PicoGreen assay for dsDNA shows greater number of cells in the proliferation, EGF/FGF2, media and comparable numbers for cells cultured in media or HAMC; (b) CellTiter Glo luminescence assay for ATP shows greater ATP content per cell for cells cultured in the proliferation media than in basal and differentiation media conditions. Within the same media condition, cellular metabolic activity in HAMC was comparable to media controls in basal and differentiation conditions; however, the ATP content per live cell was higher in the proliferation media control vs. HAMC. (c) By calcein AM, the percentage of live cells was similar for basal and 1% FBS differentiation media, yet lower in the proliferation media, where there was a lower percentage of live cells in media vs. HAMC. (d) By ethidium homodimer, the percentage of dead cells was lower in HAMC than media controls for all media conditions. Of the media conditions, the percentage of dead cells was greater in the proliferation media. (mean $\pm$ standard deviation are shown for $n = 3$; different letters indicate significant differences at $p < 0.05$).

NSPCs have been previously shown to differentiate to neurons, oligodendrocytes and astrocytes when cultured in 1% FBS-supplemented media. To ensure that NSPCs maintain this differentiation capacity when cultured in HAMC, the cells were characterized by immunohistochemistry after 7 d of culture. Fluorescent microscopy showed cells stained positive for: BIII-tubulin for neurons (Fig 4a); GFAP for astrocytes (Fig 4b); RIP for oligodendrocytes (Fig 4c); and nestin for neural precursor cells (Fig 4d). These data demonstrate that HAMC impacts neither the differentiation capacity of NSPCs to neurons, astrocytes, and oligodendrocytes nor the presence of the precursor population.

Figure 4: NSPCs cultured in HAMC in 1% FBS media for 7 d differentiated to: (a) β III-tubulin-positive neurons; (b) GFAP-positive astrocytes; (c) RIP-positive oligodendrocytes; and (d) nestin-positive progenitor cells. Cell nuclei were stained with Hoechst shown in blue. Scale bar is 200 μ m.

3.2 NSPC viability and differentiation in electrospun fiber/HAMC composites

3.2.1 Fiber cytotoxicity study

Prior to testing NSPCs in the composite system, the P(CL-co-DLLA) and collagen electrospun fibers were independently screened for cytotoxicity. The fibers were separately immersed for 7 d in the basic media that was used to then culture NSPCs for 4 d. Cell viability (using the PicoGreen assay for dsDNA) and metabolic activity per live cell (using the luminescence assay) were measured, and no differences were observed when compared to the media controls (Figure 5).

Figure 5: Electrospun fiber cytotoxicity study. NSPCs were cultured in fiber-conditioned media for 7 d. Both (a) PicoGreen: number of live cells, and (b) CellTiter Glo: cellular metabolic activity showed that the fibers were not cytotoxic to NSPCs.

3.2.2 NSPC viability in fiber/HAMC composite

Electrospun fibers were sonicated into small fragments and then dispersed in HAMC with NSPCs prior to injection through a 30G needle used for cell delivery. As shown in Figure 6, NSPCs were distributed throughout the HAMC and fiber/HAMC composite gels at both 2 and 7 days. At day 2, there was little difference between media controls (Fig 6a) and HAMC systems (Fig 6b, c,

d); however, the NSPCs appeared to be in close contact with the electrospun collagen fibers (Fig 6c, inset) and to extend their processes toward and adhere to the P(CL-co-DLLA) electrospun fibers (Fig 6d, inset). At day 7, cells suspended in media formed larger clusters due to cell aggregation and proliferation (Figure 6e) whereas NSPCs in the HAMC and fiber/HAMC composites remained more evenly distributed, forming smaller spheres and interacting with the fibers as at day 2.

Figure 6: Bright-field microscopy of NSPCs seeded as single cells for 2 days (a-d) and 7 days (e-f) in: (a) and (e) media; (b) and (f) HAMC; (c) and (g) collagen/HAMC composite; and (d) and (h) P(CL-co-DLLA)/HAMC composite. Scale bar is 100 μ m.

Figure 7: Bright-field images Figure 6 a, b, c, d zoomed in. Red arrows point to cells and blue arrows point to fibers. (a) Cell body stayed spherical. (b) Cell body enlargement, process extension,

and network formation. (c) Collagen fibers surrounded NSPCs, and process extension was limited. (d) Cell processes interacted with P(CL:DLLA) fibers and adhered to fiber surface. Scale bar is 20 μ m.

In the presence of collagen electrospun fibers, cells close in contact with the fibers had processes entangled with the fine fibers (Figure 7c). Although cell bodies were enlarged, processes were not able to extrude out. With P(CL:DLLA), NSPC had either extended their processes out to interact with fibers, or they migrated towards the fibers and adhered to the fiber surface if the cell and the fiber were in close proximity (Figure 7d). By day 7, NSPCs proliferated and migrated to junction points.

Cell viability was studied over 7 days using the PicoGreen assay for dsDNA (Figure 8). Importantly, cell viability was similar for all samples in each of the media conditions studied except the electrospun collagen/HAMC composite system where there were fewer viable cells in all media conditions than the other systems tested – media, HAMC and electrospun P(CL-co-DLLA)/HAMC composite. The data shown in Figure 5 suggested that neither collagen nor P(CL-co-DLLA) electrospun fibers released cytotoxic factors; thus, the decreased number of viable cells in the presence of collagen fibers suggests either a contact mediated mechanism or a high the local concentration of cytotoxic released factors.

3.2.3 NSPC differentiation profile in fiber/HAMC composite

The NSPC differentiation profile in the three media (NBM, 1% FBS and EGF/FGF2) and the different matrices (Media, HAMC, electrospun collagen/HAMC, electrospun P(CL-co-DLLA)/HAMC) was assessed using quantitative RT-PCR. The expression level of the gene of interest was evaluated against the house keeping gene, hypoxanthine phosphoribosyl transferase (HPRT) (Figure 8). The genes selected were: nestin for progenitor cells (Fig. 8a); β III-tubulin for neurons (Fig. 8b), CNPase for oligodendrocytes (Fig. 8c), and GFAP for astrocytes (Fig. 8d).

As shown in Figure 8a, NSPCs grown in all media and all matrices expressed the gene for nestin, with the highest expression found for cells in the proliferation media with EGF/FGF2. This is consistent with previous results where EGF/FGF2 mitogens promote proliferation of precursor cells vs. differentiation. In Figure 8b, the β III-tubulin gene expression was similar across matrices in each media group and greatest for NSPCs cultured in NBM and 1% FBS-supplemented media. Interestingly, the greatest β III-tubulin gene expression was found for NSPCs cultured in NBM in P(CL-co-DLLA)/HAMC composite gels, suggesting that these materials have a greater influence on gene expression than the media. In contrast, the NSPCs cultured in NBM and 1%FBS in collagen fiber/HAMC composite gels had the lowest β III-tubulin gene expression, confirming the importance of the extracellular environment. The β III-tubulin gene expression was lowest for cells cultured in EGF/FGF2, as may have been expected for the proliferation medium. A similar trend was observed for CNPase gene expression (Figure 8c). NSPCs cultured in NBM and 1% FBS in P(CL-co-DLLA) fiber/HAMC composite gels had the greatest CNPase gene expression whereas those cultured in collagen fiber/HAMC composite gels had the least. Again the EGF/FGF2 medium overwhelmed the CNPase gene expression and there was no difference across groups. The GFAP gene expression was influenced primarily by the medium (and not the matrix) as shown in Figure 8d, where the only differences observed, within one medium, were for the collagen fiber/HAMC composite system. Otherwise, the differences in GFAP gene expression were as expected, greatest in the differentiation, 1% FBS medium and NBM and least in the proliferation EGF/FGF2 medium.

Figure 8: The gene expression profile at 7 d for NSPCs cultured in one of three media - serum-free neural basal media (NBM), differentiation media of NBM supplemented with 1% FBS, proliferation media of NBM supplemented with EGF/FGF2 - and one of four matrices: media (control), HAMC, collagen fiber/HAMC composite, P(CL-co-DLLA) fiber/HAMC composite. The NSPCs were studied in terms of their gene expression profiles of (a) nestin for progenitor cells; (b) β III-tubulin for neurons; (c) CNPase for oligodendrocytes; and (d) GFAP for astrocytes.

4. DISCUSSION

With the promises from NSPC therapy to spinal cord injuries, the limitation impeding an effective cell therapy is the survival and integration of the transplanted cell. The need for a microenvironment providing chemical or physical cues to direct cell behaviour has been recognized. Hence, it is our goal in this study to develop an injectable cell delivery vehicle and a microenvironment that would promote cell survival/proliferation and modulate cell behaviour, evaluated by delivering a viable cell population and a desirable cell differentiation profile with enhanced neuronal and oligodendrocytic differentiation.

An injectable hydrogel allows for a minimally invasive cell delivery. Fast gelation of the thermal-reversible gel is defined in the condition that cells remain homogeneously dispersed in the volume through the process. HAMC's shear-thinning and reverse-thermal gelation properties allow it to be injected through a fine needle at room temperature and to become a weak gel at physiological temperature at 37°C. Taking advantage of its minimal polymer weight percent required for hydrogel formation, the extent of HAMC swelling is greatly reduced, minimizing the risk of secondary compression to the injury site.

Implant materials considered to be biocompatible may not be suitable for cell delivery. Hence, cell compatible design criteria must be included and tested in developing a cell culture system. In addition to its high oxygen and nutrient permeability as a hydrogel, HAMC obtains the osmolarity of the media that makes up 99% of the volume, creating a good cell culture microenvironment. The concept of biocompatibility concerns not only the material but also the encapsulation process. HAMC's spontaneous gel formation/fabrication mechanism is gentle to NSPCs, with no involvement of organic solvents/cross-linkers or UV for chemical or photo-initiating crosslinking methods, respectively. Conventional cell seeding methodology including gravitational force, perfusion, 3D printing, or infiltration often require an incubation period and result in heterogeneous distribution and cell damage⁴⁷. Therefore, mixing cells in HAMC is favourable for its simplicity and mild formulation conditions. The uniform viable cell dispersion in HAMC with high cell-seeding density and cell-survival efficiencies allows cell therapies to maximize its efficacy, especially when implanting micro volumes.

At high cell density, NSPCs have the tendency to aggregate which creates concentration gradient from the outer edge to the center, resulting in varying growth rate and nutrient consumption rates. Hence, when the size of neurospheres or cell aggregates exceed the critical

mass, it leads to cell death due to limitation of nutrient, oxygen, and accumulation of metabolic waste, forming necrotic centers^{48,49}. HAMC maintained even dispersion of NSPCs in 3D, minimized cell sedimentation and aggregation. This is further exemplified in proliferation media where cell density increased substantially over time, forming large cell aggregates in media leading to some cell death. Hence, HAMC minimizes cell death while keeping the cells in close proximity, maintaining a large percentage of viable cells within a population at high cell density.

The ability of the transplanted cell to interact with its surrounding and to become functional within an appropriate time frame is essential to an effective cell therapy. NSPCs responded quickly with morphological changes when dispersed in HAMC in contrast to suspended in media. The phenomenon could be potentially attributed to HAMC's mechanical and chemical properties mimicking CNS which minimized the lag time cells required to adjust to its new seeding environment. With processes extension and network formation, the functional NSPCs may be able to act to the hostile injured environment quickly. Cell grafts *in vivo* often experience heterogeneous cell responses from the core to the periphery. The graft close to the injured tissue would respond and integrate to the surrounding tissue, while the cells in the core of the graft remain quiescent⁵⁰. The simultaneous commence of cell development throughout the graft encourage cells to navigate and interact with its niche collectively as a whole, with the potential to improve and to accelerate the bridging process.

HAMC is not toxic and it is cytocompatible to NSPCs. Conventional ATP assays were originally selected to assess cell viability; however, it was found that cellular metabolic activity depends on whether cells are proliferating or differentiating. Hence, the bulk ATP signal intensity cannot be used to compare cell viability across different media conditions. This indirect measurement is only effective and reproducible when used to determine numbers of viable cells in

the same culture condition. A standard curve will need to be determined for each cell culture condition, with linearity and the slope of the curve to be tested and measured. Normalizing bulk ATP readings to the number of cells or dsDNA content yields information of how metabolic active each live cell is through the treatment. The inattentive use of the indirect measurement of viable cells by bulk ATP quantification could potentially give false positive results to cells at hypermetabolic state.

There is controversy in the literature as to whether cells have higher or lower metabolic activity at different cell states. Cho et al and Lonergan et al reported an increase of ATP content in human embryonic stem cells and stromal stem cells, respectively, when cells undergo greater differentiation^{51,52}; whereas, Park et al found higher mitochondria metabolic activity when human corneal endothelial cells proliferate⁵³. Since metabolic activity for different cell types/lines varies, this is the first report to examine ATP content of NSPCs at different states. It is found that NSPC has higher total plasma ATP content when proliferating. The intermediate ATP content per live cell between differentiation and proliferation states, obtained in NSPC dispersed in HAMC with proliferation media, (Figure 3b), may potentially represent a bulk average of a mixed population of NSPC at different states. Among these, the majority might be proliferating while some were induced to differentiate, as shown in by bright-field microscopic images and the differentiation profile.

In CNS regeneration by stem cell transplantation, the polymeric microenvironment acts as a template to promote cell adhesion, survival and to modulate cellular differentiation³². Electrospun fibers were incorporated in HAMC to provide substrates for direct cell-matrix interactions. Collagen scaffolds have been reported in literature to facilitate neural stem/progenitor cell transplantation and to promote recovery in the injured spinal cord⁵⁴. And the biodegradable

synthetic polymer, P(CL:DLA), have been widely used as tissue-engineering scaffolds and shown biocompatible to NSPC⁵⁵. Direct contact of cells with the fiber can reveal the effect of the cell-matrix interaction to cellular behaviour. Fine collagen fibers surrounding NSPCs have deleterious effects on cell survival/proliferation and functions. Whereas, P(CL:DLA) sustained NSPC survival and guided cellular differentiation to the neuronal (functional unit in the CNS) and oligodendrocytic (supporting cell) lineages. It has been shown in literature that nanotopographical features including fiber diameter, surface morphology, as well as mechanical strength can alter cell behaviour⁵⁶⁻⁵⁸. Hence, the distinct effects of collagen and P(CL:DLA) to NSPCs may be attributed to several possible speculations: the fine and fragmented collagen fibers may be unable to sustain mechanical strength and to provide structural support when cells extended processes and apply tension, or the physical entrapping of NSPC in collagen fibers blocking processes extension. Nevertheless, P(CL:DLA)'s beneficial effect to efficiently modulate NSPC differentiation profile towards desirable lineages without addition of growth factors should be further investigated and utilized.

In addition to cell-matrix interactions, soluble factors can also influence cell behaviour. In media containing 1%FBS, CNPase and GFAP gene expression were higher in contrast to basic media. The effect of serum to promote glial differentiation coincided with common practice in literature, where serum is used to sustain survival and to promote glial cell lineage^{59,60}.

5. CONCLUSION

Overall, we found that the cellular microenvironment is defined by the media and the hydrogel composite and each of these can influence the differentiation and proliferation profiles of NSPCs. The HAMC 0.5/0.5 w/w% provided a facile hydrogel for stable cell distribution and delivery through an injectable delivery strategy. The inclusion of electrospun fibers in the hydrogel composite promoted cell survival and guided cell differentiation relative to media controls, demonstrating the importance of the microenvironment to cell behaviour. Of the two electrospun fibers examined, the P(CL-co-DLLA)/HAMC composite was most suitable for NSPC differentiation towards neuronal and oligodendrocytic phenotypes, the latter of which is important to myelination to either limit further degeneration or of regenerated axons. Future studies will evaluate these systems *in vivo* for NSPC survival and differentiation in established rodent SCI models.

6. RECOMMENDATIONS AND FUTURE DIRECTIONS

Rodents' functional behaviour will be assessed to examine cell therapy efficacy with the help from a designed microenvironment. Further work can extend to include more integrative bioactive electrospun fibers for concurrent drug delivery or cell adhesive molecule immobilized sites. The versatility of HAMC hydrogel could serve as a platform for combinatory strategies, with the potential to lead to a clinically relevant and effective cell therapy.

The fast induction of NSPC response in HAMC in contrast to media controls observed at day 2 was not extended and translated to the gene expression profile at day 7. BIII-tubulin, CNPase, and GFAP expression in HAMC were comparable to media controls. At longer cell culture, NSPCs suspended in media had also started to differentiate, levelling out the initial burst of the number of cells that underwent differentiation. Further studies can look at the genes that are selectively expressed in more mature CNS cell types at day 7, or conduct the differentiation profile study at day 3 to reveal the differential effect.

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