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Publication Date	2016-08-01
Publisher	American Chemical Society
Repository DOI	10.1021/acsbiomaterials.6b00167

Therapeutic Effect of Growth Factor Treatment in an Injectable Collagen Scaffold Following Rat Spinal Cord Hemisection Injury

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Word Count: 3959

Number of figures/tables: 5

Running title: Hydrogel treatment for SCI

Abstract

Background. Spinal cord injury (SCI) presents to the clinic as complete, incomplete or compressive. SCI patients also display varying quantities of spinal cord tissue damage or loss. One theory proposed to repair the injured spinal cord and regain motor control is to regenerate axons through the lesion site. Current methods proposed to increase neuroregeneration following SCI include; preserving spared neuronal tissue, increasing axonal regeneration, reducing inflammation, reducing glial scar formation and methods aimed at bridging the lesion gap to facilitate the transmittance of physical cues and provide a platform for neuronal sprouting and functional recovery. Objective. This study was designed to quantify the impact of a local injectable in situ forming hydrogel reservoir therapy following rat hemisection SCI. Method. Our group investigated the effect of hydrogel only treatment following SCI in addition to hydrogels loaded with a neuronal growth factor, Neurotrophin-3 (NT-3), immediately following SCI. Functional recovery, assessed by Basso Beattie Bresnahan (BBB), and local healing mechanism, including neuronal regeneration, neuronal survival, glial scar formation, inflammation, astrocytosis, and collagen deposition were investigated one and six weeks post-surgery. Results. Delivery of an injectable hydrogel increased functional recovery, reduced inhibitory glial scarring and reducing inflammation at the injury site. Similarly hydrogel + NT-3 delivered directly into the injury site reduced glial scarring and collagen deposition resulting in increased neuronal survival across the lesion site. Conclusion. This study represents a novel and effective therapy combining growth factor and a biomaterial based therapy following SCI.

Keywords

Spinal Cord Injury, Hemisection, Hydrogel, Collagen, Microspheres, Neurotrophin-3.

Introduction

SCI is a complex and debilitating injury which damages the structural integrity of spinal cord tissue affecting approximately 3 million people worldwide. It is estimated that there are 250,000 to 500,000 new cases of SCI per annum. [1] The severity, location and nature of SCI directly correlate to neurological deficits and axonal degeneration in motor and sensory pathways categorised as complete, incomplete and compressive.[2,3] Numerous animal models (transection, hemisection and contusion) have been adapted to replicate spinal cord injuries presenting to the clinic.

Presently no effective treatment or restorative interventions for SCI have progressed to the clinic. the inhospitable microenvironment following SCI along with the presence of a physical gap and glial scarring greatly hinders the capacity of axons to regenerate, migrate and re-connect with neurons and tracts distal to the injury site. Therefore interventions capable of bridging lesions in the injured cord, represent a promising treatment paradigm for SCIs. The pathophysiology of SCI includes as an initial acute stage, followed by a secondary phase and a chronic phase.[4] Secondary spinal cord events include apoptosis, necrosis, inflammation, ischemia, glial scar formation, demyelination, astrocyte and microglial activation and migration, neurodegeneration and wallerian degeneration within and surrounding the injury site.[5-11] Macrophage and microglial activation, migration and accumulation (resident and recruited) are pathological consequences of, injury or disease severity in humans.[12-14] Indeed inflammatory and neurotoxic events, following SCI, also occur during an initial acute (1-7 days) and delayed chronic (60-120 days) phase. Indeed secondary inflammatory events are considered a double edged sword, essential for repair but central to inflammatory mediated degeneration of remaining tissue.[15]

Neurotrophin 3 is a growth factor which promotes regeneration following SCI.[16] NT-3 affords its regenerative capability in SCI to the role in development including neuronal stem cell differentiation, survival and function.[17,18] Growth factor expression is elevated initially following SCI (48 hours) but is diminished within days.[19] Therefore replenishing growth factors levels within and surrounding injured spinal cord tissue has the potential to maintain spared tissue in addition to promoting regeneration of injured tissue. Indeed numerous groups, including our group, have demonstrated the regenerative effect of increased NT-3 expression, local delivery or via a scaffold system within the injured spinal cord.[20-26] The beneficial effect of numerous biomaterial materials, including collagen, has been reported to date in models of CNS injury *in vivo*.[27-37]

Combined treatment therapies today have proven very successful in models of SCI, Indeed recent biomaterial based studies aimed at delivering stem cell therapies, represent promising treatment strategies for SCI intervention in patients.[38-40] Our study served to focus on the effect of localised growth factor delivery within the SCI lesion site to promote regeneration of remaining nascent tissue in conjunction with an *in situ* forming collagen hydrogel scaffold. Indeed, growth factor delivery via encapsulation within an alginate hydrogel has previously been shown to promote functional recovery in a hemisection model of SCI.[33] In this study we tested the efficacy of an injectable in situ forming hydrogel and hollow microsphere system delivering a growth factor into the hemisected spinal cord. NT-3 loaded hollow microspheres were fabricated using a template method as previously described by our group.[41,42] NT-3 loaded hollow-microspheres were injected into the hemisection lesion site in an injectable collagen hydrogel. Our system facilitates localised

sustained release of NT-3 in a biodegradable, biomimetic, protein-based collagen hydrogel system.

Hydrogels represent a promising technology as they can be cast easily into various shapes (useful since there is no one defined spinal cord injury ‘shape’ that occurs following SCI) or can be injected directly into a wound for in situ gelation, adapting to the shape of the lesion site. From a translational perspective injectable hydrogels can be administered during decompression surgery to fill lesions of varying size and location. Although hydrogels have been previously used in hemisection models of spinal cord injury[34, 43], this study is the first to introduce an injectable collagen hydrogel into the hemisected rat spinal cord.

Materials and Methods

Unless otherwise stated, all reagents were obtained from Sigma Aldrich LTD, Dublin, Ireland.

Animals

Adult female Sprague-Dawley rats from Charles River UK Ltd (Charles River UK Ltd, Margate, UK) were used in this study. All housing, surgical and post-operative procedures carried out in this study were approval by the Animal Care Research Ethics Committee (ACREC) at the National University of Ireland, Galway. Surgical procedures and post-operative care was carried out by a single experienced surgeon under an animal licence from the Department of Health and Children in Ireland (B100/2591) in accordance with European Communities Regulations 2002.

Lateral hemisection surgical procedure

Female Sprague Dawley rats (*n*=78) weighing between 220-250 grams underwent spinal cord hemisection surgery. Rats were housed in a 12 hour light/dark cycle and food and water were provided ad libitum. Prior to surgery rats were weighted and pre-operative analgesia, Buprenorphine (0.1-0.025 mg/kg, FortDodge Animal Health Ltd) (>30 minutes) was delivered intraperitoneal (IP). Animals were deeply anaesthetized by IP injection of Ketamine (80 mg/kg, Vetoquinol) and Xylazine (8 mg/kg, Astra Zeneca) and maintained with Isoflurane (1-1.5%, Abbott) and oxygen for approximately one hour. All rats underwent a laminectomy procedure at thoracic level T8-T10 to expose the spinal cord. The dura mater was slit (1.5 mm) between T9-T10. the spinal cord hemisection surgery was carried out on the right side of the rat spinal cord The hemisection surgery was completed by cutting the dorsal and ventral columns on the side of the hemisection injury using the iridectomy scissors. The hemisection lesion site was inspected following surgery and any remaining tissue was removed before injecting the hydrogel or hydrogel + NT-3. Rats were randomly assigned to eight surgery groups across 2 time-points. Following surgery each rat received Enrofloxacin (Baytril) (50mg/kg, Bayer) IP as perioperative antibiotic and 3ml saline (0.9%) subcutaneously to avoid dehydration. Surgery groups analysed one week and six weeks post-surgery include as follows; Sham (*n*=10), Hemisection (*n*=10), hemisection with administration of hydrogel with hollow spheres (Hydrogel) (*n*=10), hemisection with administration of hydrogel with hollow spheres containing NT-3 (Hydrogel + NT-3) (*n*=10). Surgeries were carried out on a sterile surgery table maintained at 37°C. All surgical procedures were carried out using aseptic techniques. Rats were sacrificed one and six weeks post-surgery.

Post-operative care

Animal care was in accordance with institutional guidelines. bladders were manually expressed every 12 hours. Enrofloxacin (Baytril) (50mg/kg, Bayer) and Buprenorphine (0.1-0.025 mg/kg) were administered IP every 12 hours for 3 and 7 days respectively post-surgery. Deviations from normal body temperature and urinary tract infections were closely monitored. Rats were housed in isolation for the first 24 hours post-surgery and housed in pairs thereafter . Saline solution was administered subcutaneously for 3 days post surgery. Food, water and soft chow where available ad libitum.

Hydrogel formation

Hydrogels used in this study were formatted using lyophilized collagen type I (Covidien LLC, North Haven, US) was dissolved in a 0.21 M solution of BisTris Propane, pH 8.75 to its final concentration (50 mg/ml). Collagen solutions were then mixed with NT-3 (or buffer) loaded spheres (Alomone Labs, Jerusalem, Israel) (2.5 mg/µl). Gelation of collagen hydrogels is temperature and chemical dependant. Collagen solutions remain liquid at 37°C, however at room temperature they form a gel. The gelation process is accelerated by the addition of a cross-linker, pentaerythritol poly (ethylene glycol) ether octasuccinimidyl glutarate (8arm-15Ksg PEG) (Covidien, North Haven, US) was used in this study whereby the active ester groups react with the free amino groups of collagen. Immediately prior to use the cross-linker (8a15kSG PEG) was dissolved in a 0.1 M solution of BisTris Propane at pH 7.5 to its final concentration 25 mg/ml. Ten µl of hydrogel was injected per rat. Injectable collagen hydrogel scaffolds were prepared as follow; 2.5 µl of spheres (20 mg/ml) containing 25 µg of NT-3, or equal volumes of 0.21M BisTris Propoane buffer vehicle as control, the buffer was mixed with 2.5 µl of warm (37°C) collagen solution. Then, 5 µl of collagen microsphere solutions were mixed with 5 µl of 25 mg/ml cross-

linker (8a15kSG PEG) solution. Scaffolds were immediately injected into the spinal cord lesion as gelation occurs quickly after the addition of the cross-linker (40 seconds). Once injected hydrogels solidified in the lesion site *in vivo* within 20-25 seconds. To determine the stability of the collagen hydrogels used in this study, hydrogels were placed in PBS solution containing 1 mg ml^{-1} (~125 CDU mg^{-1}) collagenase, or PBS (no collagenase), *in vitro* at 37°C. Vials containing collagen hydrogels were then placed on a shaker at 37°C. Samples were monitored and the time required for completely degrade the collagen hydrogel was recorded. In the absence of collagenase the complete hydrolytic degradation of the collagen hydrogels took 9 weeks (data not shown). However, hydrogels incubated at 37°C with collagenase readily degrade within 2 days. Hence, hydrogels readily degrade upon addition of collagenase but remain intact otherwise. The degradation profile of the collagen hydrogels *in vivo* may be accelerated in the presence of enzymes and scavenger cells within the spinal cord or migrating from the exposed injury site. Scavenger cells implicated in SCI include macrophages, monocytes and microglia.

Fabrication, cross-linking and loading of hollow collagen microspheres

Hollow collagen microsphere were fabricated as described by our group previously.[41, 42] Hollow collagen microspheres using produced using commercially available 4-4.5 µm polystyrene beads (template) which were incubated with sulphuric acid to negatively charge its surface. Following sulfonation, beads were re-suspended in the positively charged collagen solution at a weight ratio of 1:7 (collagen:beads). The free amino groups of collagen were cross-linked using 8a15kSG PEG at a weight ratio of 1:2 (collagen:cross-linker). Then, the polystyrene template is removed by washing with 20% (v/v) tetrahydrofuran (THF) to produce hollow microspheres. Hollow collagen microspheres (4-4.5 µm diameter) were delivered loaded with NT-3

in the Hydrogel + NT-3 treatment group, or loaded with buffer alone (Hydrogel group), microspheres were then suspended within an injectable collagen hydrogel into the hemisected spinal cord lesion site as outlined in Supplementary Figure 3.

Mechanical testing

The compressive moduli of collagen hydrogels was measured by Covidien LLC (North Haven, US). Hydrogels used in this *in vivo* study were adapted to complement the compressive modulus of native rat spinal cord tissue (3-5kPa).[44, 45] Briefly, the unconfined compressive modules was tested by injecting collagen hydrogels into Teflon moulds, which were allowed to polymerize for 10 minutes, once the hydrogel has set it is removed using a spatula. The hydrogel was then transferred to a petri dish containing Dulbecco's PBS. Following incubation for 48 hours 14mm samples were cut from the hydrogel using a trephine punch and the compressive modulus analysed using the Dynamic Mechanical Analyser (DMA Q800) (TA instruments, USA).

Behavioural analysis

The severity of, and recovery from, an incomplete injury in rat models of SCI is quantified using the Basso, Beattie and Bresnahan (BBB) locomotor scale. Prior to and following laminectomy, rat motor function was recorded for analysis using the BBB open field gait assessment.[46] BBB functional scores were extracted by two blinded experienced examiners from 5 minute video clips. Functional scores were recorded and averaged for each limb and each time point. The hemisection surgery were deemed successful, and rats were included in the study, if they received an average post-operative (one day) BBB score of ≤ 5. Subsequent BBB scores were recorded one, four and six weeks post-surgery.

Tissue Harvesting

Rat spinal cords were extracted and cryopreserved one and six weeks post surgery. Rats were deeply anesthetized with IP Sodium Pentobarbital (50 mg/kg) and perfused transcardially when all deep reflexes were absent. Saline perfusion was followed by transcardial administration of 300 mls 4% PFA for 2 minutes 20 RPM. Following perfusion a laminectomy was performed to expose the spinal cord and spinal nerves between thoracic regions T6 to T12. The T9-T10 region was identified and carefully micro-dissected and post-fixed by immersion in ice cold 4% PFA in 1X PBS (Sigma-Aldrich, Dublin, Ireland) for 24 hours. Spinal cords were cryopreserved in 30% sucrose at 4°C for 48 hours. Spinal cords were snap frozen in liquid nitrogen before being transferred to -80°C for storage.

Cryosectioning

Rat spinal cords were cryosectioned on a Leica CM 1900 cryostat between -23°C and -26°C. Spinal cords were immersed in TissueTeck OCT (Sakura) for sectioning. Longitudinal spinal cord T9 to T10 samples were sectioned at 20 μm increments and then transferred to SuperFrost Plus® (Menzel-Gläser) charged microscope slides and stored at -80°C until use.

Immunohistochemistry

Frozen spinal cord sections were rehydrated by immersion in PBS / 0.1% Triton X-100, three changes 5 minutes each. Sections were immersed in 10% normal goat serum (NGS) in PBS for 30 minutes to prevent non-specific background staining. Primary antibodies used in this study included; Glial Fibrillary Acidic Protein (GFAP) (Dako, Z0334) raised in rabbit (1:400), βIII-Tubulin (Millipore, MAB1637) raised in mouse (1:200), Ionizing calcium-Binding Adaptor molecule 1 (Iba1) (Wako, 019-

19741), raised in rabbit (1:200) and NG2 (Invitrogen, 37-2700) raised in mouse (1:100). Primary antibodies were incubated on the sections diluted in PBS with 5% NGS, 0.3% Triton X 100, overnight at 4°C. Following primary antibody incubation spinal cords sections were washed in PBS / 0.1% Triton X-100 (3 times). Secondary antibodies were then added in PBS with 5% NGS, 0.3% Triton X 100 for 1 hour at room temperature and were protected from the light. Secondary antibodies used in this study included; Alexa Fluor® 488 goat anti-mouse IgM (H+L), Alexa Fluor® 546 goat anti-rabbit. Following incubation spinal cords were rinsed in PBS / 0.1% Triton X-100 (2 times, 5 minutes) and counterstained with Hoechst 33342 (Life Technologies, H3570) diluted at 1:2000 in PBS and incubated for 10 minutes in the dark. Excess Hoechst was removed by PBS washes (2 times, 5 minutes) and slides were covered with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, F4680) mounting media and a glass cover slide.

Histology

Collagen-rich scar tissue was evaluated with Masson's Trichrome staining on frozen spinal cord sections from each treatment group. Following rehydration, sections were incubated in potassium permanganate with sulphuric acid (0.5% each in distilled water) for 2 minutes, sections were briefly rinsed in water then transferred to sodium metabisulphate solution. After 30 seconds in water sections were incubated in 70% alcohol for 1 minute then for a further 1 minute in Gomori's aldehyde fuchsin. Following a brief rinse in water, sections were transferred to 95% ethanol for 10 seconds, water for 10 seconds after which sections were incubated in Celestine blue solution for 4 minutes. Excess stain was removed by rinsing in running water for 30 seconds before incubation in Mayer's haemalum for 4 minutes. Before the next stain sections were placed in water for 20 seconds, then in acid alcohol for 20 seconds

and then left in running water for a further 4 minutes. Sections were incubated in Masson's cytoplasmic stain for 1 minute, briefly rinsed in water and then placed in a solution of 1% dodeca-molybdophosphoric acid for 1 minute. Briefly rinsed off before incubation in fast green in acetic acid for 1 minute and 1% acetic acid for 1 minute. Excess stain and acetic acid was removed in running water before dehydrating sections through a series of alcohols (50%, 70%, 95%, 100% and 100%) for 2 minutes each and clearing by two 10 minute xylene washes xylene. Sections were mounted with DPX mounting medium.

Image Analysis

All images were acquired using the Olympus VS120 digital slide scanner with VS-ASW software. Images were viewed using OlyVIA software (version 2.6) and Image J was used for image analysis. Quantification of specific spinal cord regions (lesion site and cranially and caudal to the injury site) is outlined in Supplementary Figure 2.

Statistics

All statistical analysis was performed using GraphPad Prism™ Version 5 (GraphPad Software, Inc.). Data were compared using paired t-tests or one-way analysis of variance (ANOVA) followed by multiple comparison procedures (Dunnett's test). Values were considered as significantly different with a $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$.

Results

This study aimed to determine the efficacy of an injectable hydrogel system embedded with hollow collagen microspheres in an *in vivo* model of rat hemisection

SCI. A previous study by our group demonstrated the efficacy of this injectable reservoir technology *in vitro*.[42]

Treatment with an injectable hydrogel promotes functional recovery following SCI

Significant increases in functional recovery were observed in the hydrogel only treated groups at four weeks post-surgery which was maintained up to six weeks post-surgery (Fig. 1). No significant difference was observed between the hemisection and hydrogel + NT-3 treatment groups post-surgery. Locomotor performance was performed prior to surgery to ensure all animals were capable of reaching the cut-off point (BBB score of 21) prior to inclusion in the study (data not shown). Spinal cord hemisection surgery was deemed successful if animals registered a BBB score <5 one day post-surgery.

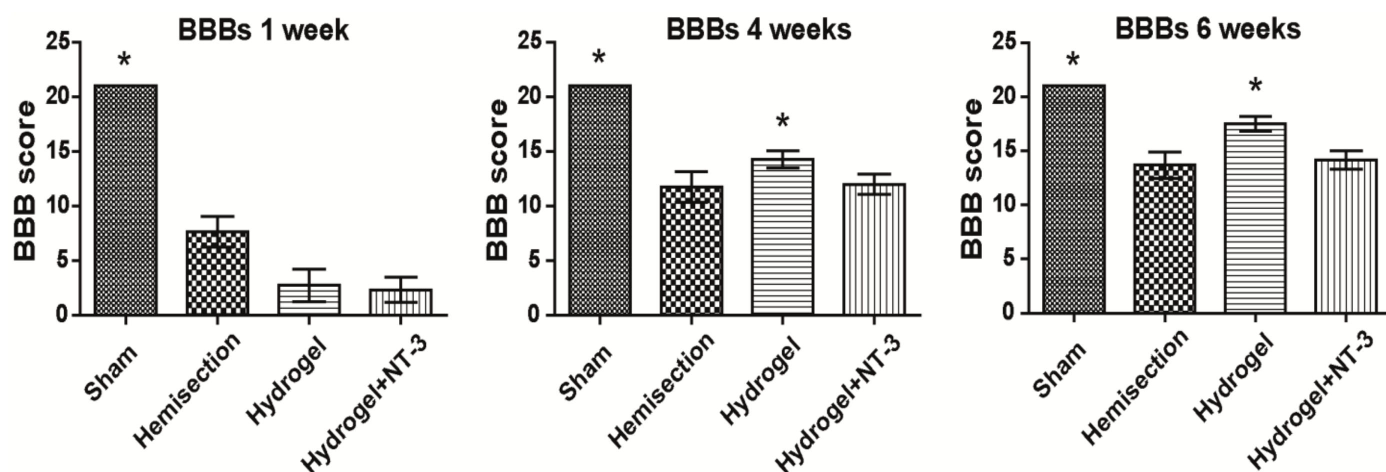

Figure 1. Improved functional recovery following injectable hydrogel treatment

Locomotor assessment via BBB analysis of hindlimb motor function following rat spinal cord hemisection injury one week (*n*=40), four weeks (*n*=38) and six weeks (*n*=38) post-surgery. Average right and left hindlimb BBB recordings demonstrate a

significant improvement in locomotor function following hydrogel treatment at four weeks post-surgery which is maintained until six weeks post-surgery *$p<0.05$, (n=9-10).

Reduced glial scar formation following injectable hydrogel treatment *in vivo*

The effect of an injectable hydrogel reservoir system on glial scar formation was determined using NG-2 immunohistochemistry on hemisected rat spinal cord tissue one and six weeks post-surgery in sham, hemisection, hydrogel only and hydrogel + NT-3 treatment groups (Fig. 2A-E). NG2 is a chondroitin sulfate proteoglycan which is highly inhibitory in the growth cone, thus its production inhibits axonal sprouting and regrowth. Following SCI NG2 is expressed by microglia, astrocytes and oligodendrocyte precursor cells within the lesion area and its expression peaks one week post-surgery (Jones, Yamaguchi et al. 2002). The area of NG-2 positive cells was significantly reduced in the hydrogel only and hydrogel + NT-3 groups at each time point when compared to the hemisection only group one and six weeks post injury. Immunohistochemical staining for the astrocyte marker GFAP did not show any significant difference between hemisection groups and the hydrogel treatment groups (see Supplementary Fig 1).

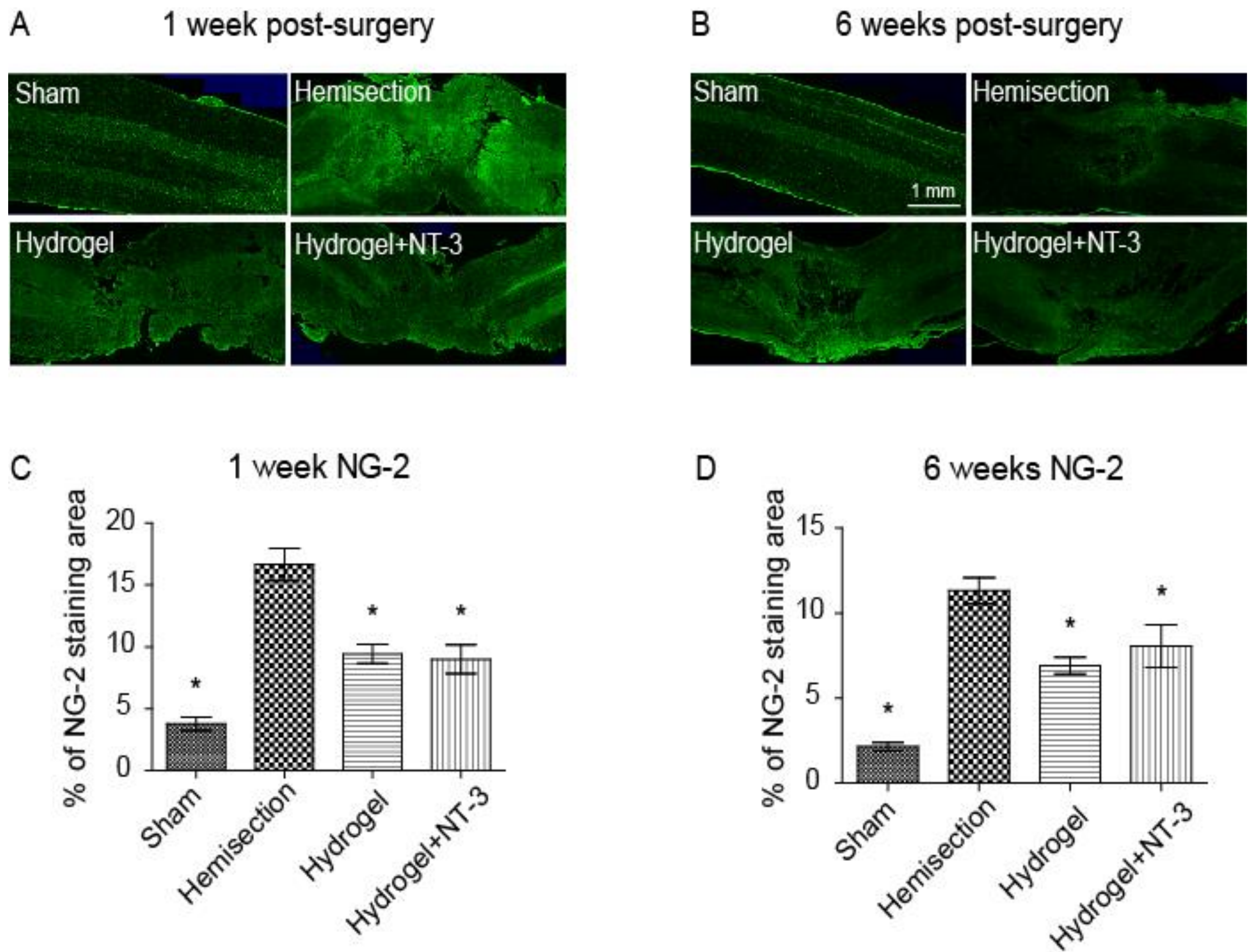

Figure 2. Injectable hydrogel system reduces glial scar formation following SCI

Longitudinal sections of injured spinal cord tissue stained with NG-2 (green) one (**A**) and six (**B**) weeks post-surgery. Quantification of NG-2 positively stained glial scar tissue in the lesioned spinal cord one (**C**) week and six (**D**) weeks post-surgery, *$p<0.05$, ($n=4$-7). Scale bar A, B = 1mm.

Reduced inflammation following injectable hydrogel treatment *in vivo*

To determine the effect of injectable hydrogel treatment on inflammatory macrophage/microglial activation following SCI, spinal cord sections were stained with Iba-1 one and six weeks post-surgery. The amount of Iba-1 staining, and resultant area fraction of positively stained cells surrounding the lesion site, is directly proportional to macrophage/microglial specific activation in response to injury in the CNS. Reduced macrophage/microglial activation, during the initial and acute stages of inflammatory mediated neurodegeneration, concur with a reduction in NG2 staining (Fig. 2). Injectable hydrogel treatment significantly reduced macrophage/microglial activation following SCI. The area fraction of macrophage/microglial cells was significantly reduced in all hydrogel (hydrogel only and hydrogel + NT-3) treatment groups one and six weeks post-surgery (Fig 3A, B, C and D).

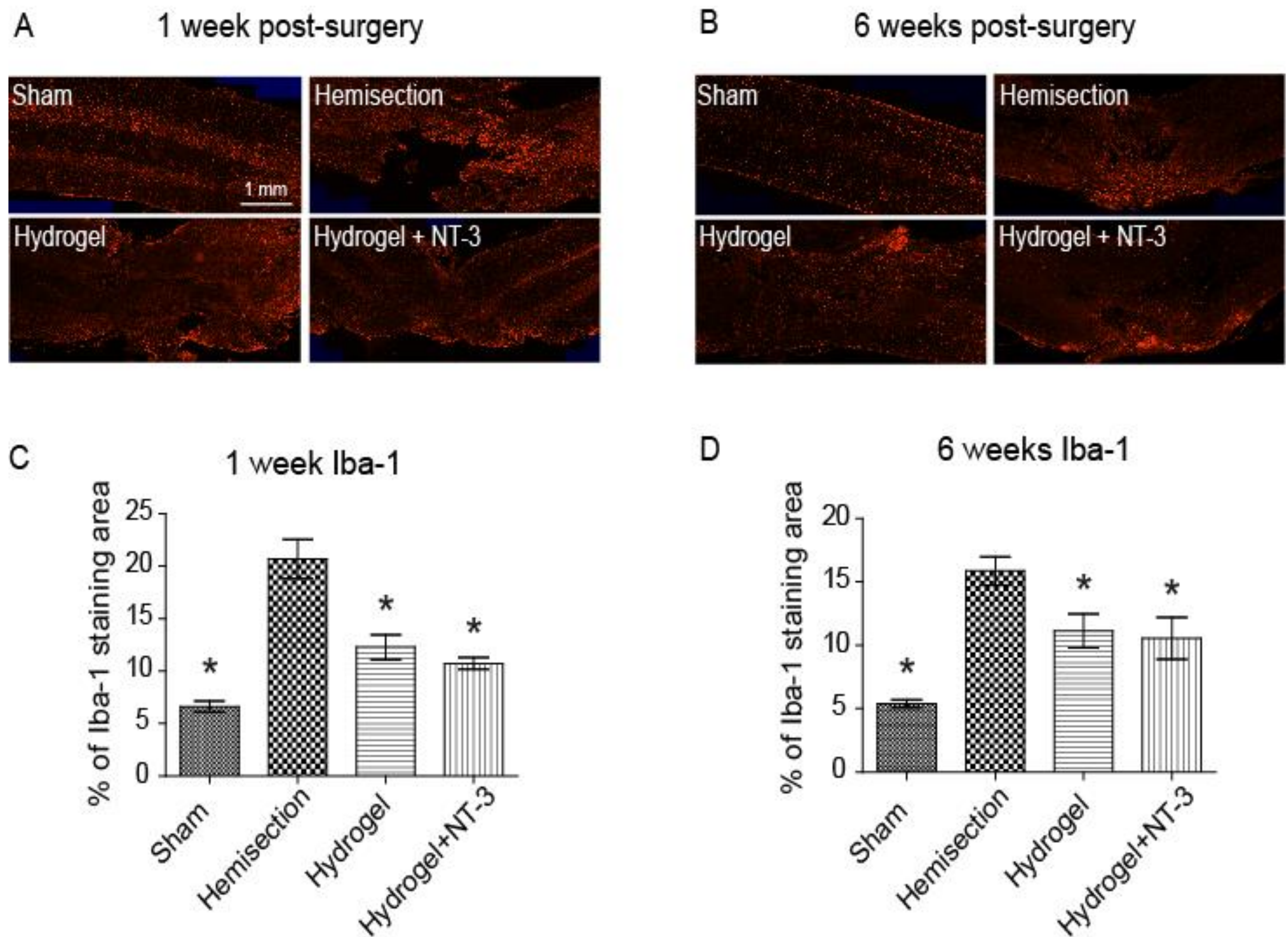

Figure 3. Injectable hydrogel reduces macrophage/microglial activation following SCI

Iba-1 stained spinal cord sections demonstrating reduced macrophage/microglial activation during the primary peak of microglial activation (~7 days) compared to hemisection only (**A, C**) and prior to the secondary inflammatory peak (**B, D**). *$p<0.05$, (n=4-7). Scale bar A, B = 1mm.

Hydrogel + NT-3 treatment promotes neuronal survival *in vivo*

A trend towards an increase in neuronal survival was observed in hydrogel and hydrogel + NT-3 treatment groups at one week, yet only significantly in the hydrogel + NT-3 group (Fig 4A, B, C, D)). Hydrogel treated rats also displayed increased locomotor function which might be correlated with increased βIII Tubulin positive staining, however increased neuronal survival is evident following treatment with hydrogel + NT-3 loaded spheres. Local neuronal survival did not translate to significant increases in locomotor function. Indeed the beneficial effect of combined hydrogel + NT-3 is preserved up to six weeks post-surgery, at which point a significant increase in neuronal survival is evident in the hydrogel + NT-3 treatment group (Fig 4D).

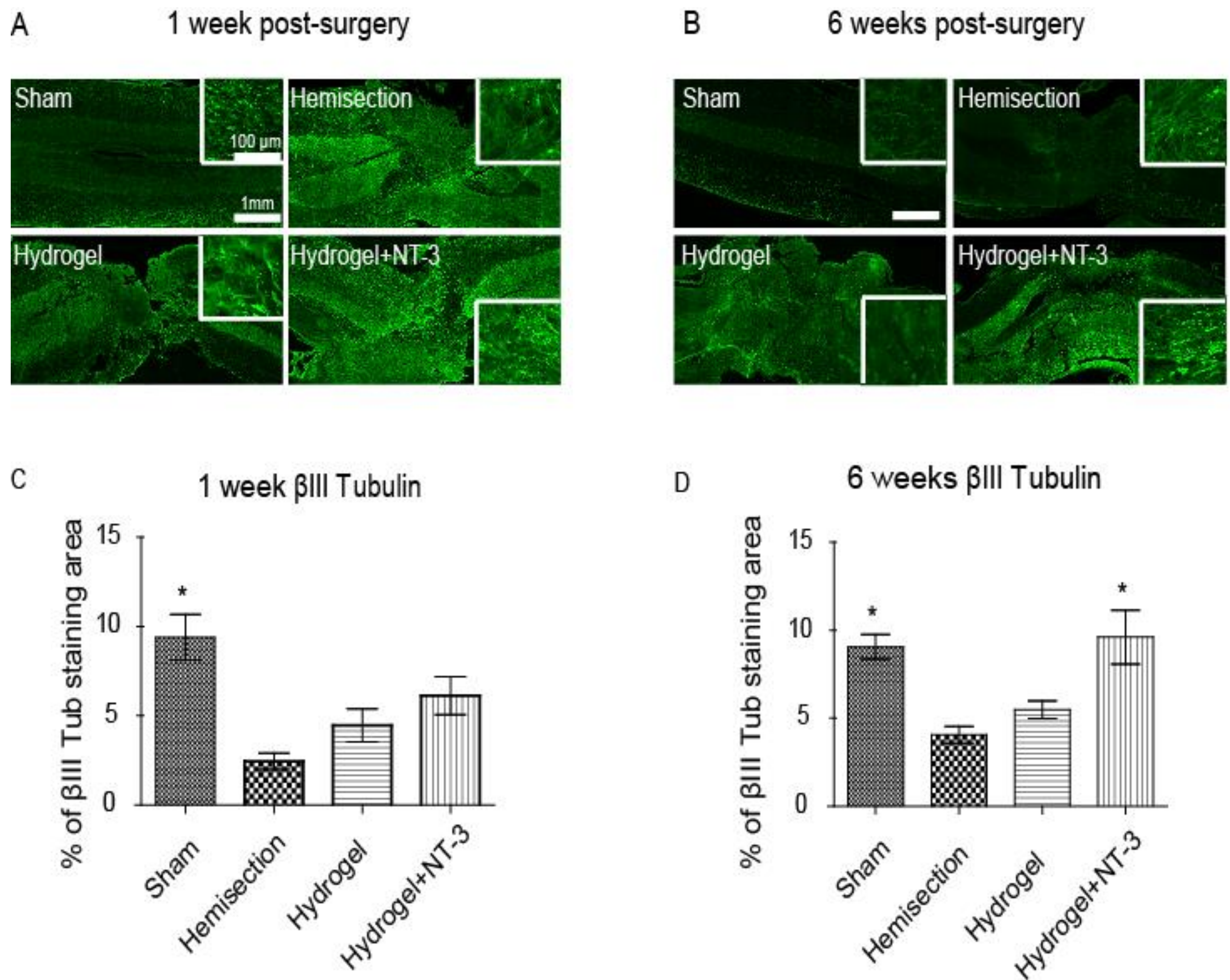

Figure 4. Combined Hydrogel + NT-3 treatment promotes neuronal survival following SCI

Spinal cord sections were stained with βIII tubulin (green) (**A, B**) to determine neuronal survival following hemisection injury and subsequent injectable hydrogel treatment *in vivo*. Higher magnification images showing individually stained βIII tubulin cells. Quantification of βIII Tubulin area fraction shows increased neuronal survival/sparing in hemisected spinal cords treated with hydrogel + NT-3 one week and six weeks post-injury, *$p<0.05$, (n= 4-7). Scale bar A, B = 1mm, higher magnification images Scale Bar = 100µm.

Hydrogel + NT-3 treatment reduces collagen deposition and glial scaring following SCI

Glial scarring and collagen deposition was quantified within and surrounding the lesion site one and six weeks post injury. Positively stained glial scar tissue was quantified as total area fraction of tissue remaining post-injury. Reduced glial scarring, indicating the transition from an inhibitory, non-permeable, environment to a permissive growth promoting environment, is observed one week and maintained up to six weeks post injury following Hydrogel (Fig 5B) and Hydrogel +NT-3 treatment (Fig 5A & B).

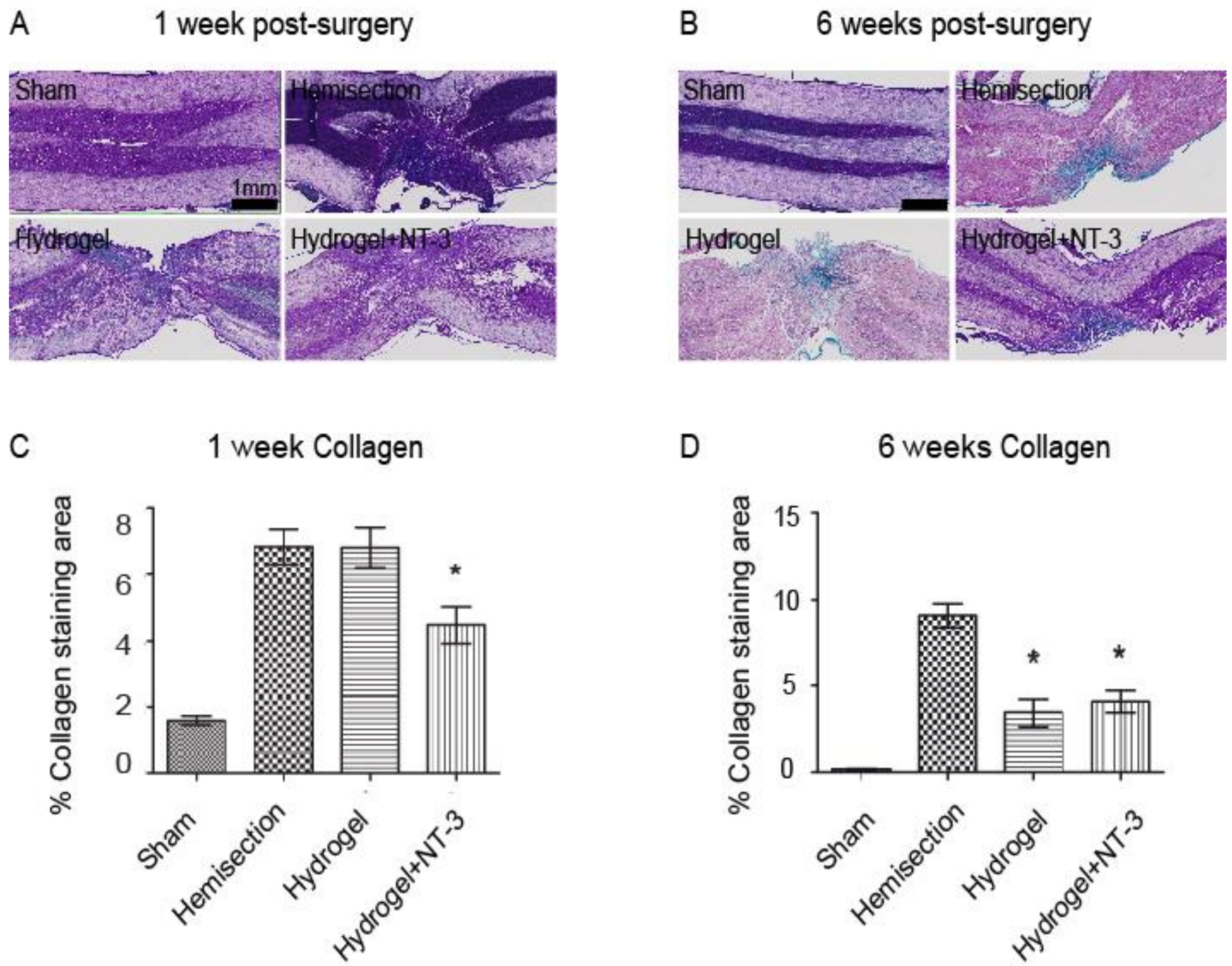

Figure 5. Hydrogel + NT-3 treatment reduces scar formation following SCI *in vivo*.

Positively stained Masson's Trichrome scar tissue percentage area was quantified across treatment groups one week (**A**) and six weeks (**B**) post SCI. Total area fraction of collagen rich scar tissue within the total tissue area was reduced as a result of hydrogel + NT-3 treatment one week post-surgery. Indeed a reduction in scar formation was maintained up to six weeks post-surgery in hydrogel + NT3 and also in hydrogel only treatments when compared to the hemisection animal group. Representative Masson's Trichrome stained scar tissue within, and surrounding, the

lesion sight in all treatment groups one (**C**) and six (**D**) week's post-surgery. *p<0.05 (n=4-7). Scale bar C, D = 1mm,

Discussion

Collagen is a widely used, biocompatible, biodegradable and flexible biomaterial suitable for use in *in vivo* models of SCI and repair.[47] Rigid collagen scaffolds have been used in SCI with varying success.[48] Indeed these scaffold systems have proven beneficial in comparison to no treatment controls and have undergone numerous modifications to facilitate increased and guided neuronal regeneration. Including the incorporation of internal and external modifications including; channels, pores, aligned fibres, altering porosity, physical cues, chemical cues and stem cells.[49-51] However these scaffolds remain rigid and require surgical manipulation to implant and may not be compatible in instances of irregular and misshapen lesions. Additionally these scaffold systems have the capacity to become dislodged and increase the distance between spinal cord tissue and scaffold. Following on from *in vitro* studies completed by our group we decided to test the efficacy of our injectable collagen hydrogel system in an *in vivo* SCI paradigm.[42] The injectable, in-situ forming, collagen hydrogel utilised in the study was specifically designed to complement the compressive modulus of nascent rat spinal cord tissue to provide structural support and to withstand external pressure from the vertebral column and surrounding muscle.[44, 45] Injectable hydrogel systems have the capacity to fill lesion voids, regardless of the shape, while also remaining juxtaposed to all edges of the lesion site while also being minimally invasive. Injectable hydrogel systems have proven beneficial following SCI supporting axonal growth, improving cell attachment and proliferation.[31] Indeed hydrogel only treatment improved functional recovery in

additional to promoting a hospitable environment for neuronal regeneration by reducing glial scaring, inflammation and collagen deposition. Hydrogel only treatment used in our study increased functional recovery four and six weeks post-surgery. In addition hydrogel treatment reduced local inflammation and the formation of a glial scar in the injury site which promotes a more hospitable environment to promote neuronal regeneration and hence functional recovery.

Neurons in the central nervous system, and indeed the injured spinal cord, have the potential to regenerate, within a supportive environment. However for the most part are hindered by multiple pathophysiological and physical complications following SCI. Indeed the SCI microenvironment is extremely inhospitable while also lacking sufficient neurotrophic support to facilitate neuronal recovery. However, the provision of trophic support in isolation is insufficient to achieve neuroregeneration. Similarly, the provision of a structured support in the form of a scaffold will not address the pathophysiological changes in the microenvironment in the defect site. Therefore, combined therapeutic and physical support mechanisms are required.[52] NT-3 facilitates sprouting of corticospinal axons and ascending sensory axons following SCI *in vivo*.[53,54] Controlled local delivery of NT-3 following SCI provides sufficient trophic support to facilitate neuronal fiber sprouting when delivered immediately following SCI or as a delayed therapy.[28] Local, sustained delivery of NT-3 also avoids the limitations of systemic delivery and bioavailability of growth factors to treat SCI.[55] Armed with this information we decided to further functionalise our hydrogel system with the addition of NT-3 encapsulated spheres within a collagen hydrogel reservoir system for SCI treatment. Indeed Hydrogel + NT-3 treatment increased neuronal survival, reduced glial scarring and reduced inflammation however the beneficial effects of the addition of NT-3 did not translate to functional

recovery as assessed by BBB in this study. Reduced functional recovery may not be indicative of reduced neuronal survival or sprouting but may indicate rewiring, or miswiring, issues in the absence of guidance cues within the injury site.

Future strategies to further functionalise and maximise recovery following SCI using this system may include the incorporation of alignment in the hydrogel system. Alignment will facilitate directed neuronal sprouting across the injury void. Additionally the inclusion of optimal differentiated stem cells committed to a specific neuron lineage embedded within hydrogel + NT-3 may aid functional recovery across lesion sites. Delivery of additional growth factors in a spatio-temporal manner may also facilitate recovery over time as the hydrogel degrades and surrounding neuronal tissue regenerates. Delayed hydrogel delivery techniques may be work investigation to accurately recapitulate the human situation where treatment might be delayed for hours, days or weeks. These strategies, in isolation or combination, may lead to further advancements in the research of SCI.

Acknowledgements

The authors acknowledge the facilities, scientific and technical assistance (Mr Mark Canney and Dr Kerry Thompson) of the Centre for Microscopy and Imaging at the National University of Ireland, Galway (www.imaging.nuigalway.ie), a facility which is co-funded by the Irish Government's Programme for Research in Third Level Institutions, Cycles 4 and 5, National Development Plan 2007-2013. The authors acknowledge the facilities and technical assistance provided at the National Centre Biomedical Engineering Sciences (NCBES), National University of Ireland, Galway.

Funding

This study was funded by Covidien LLC and the Industrial Development Agency (IDA) grant.

Authors' note

Bridget Breen and Honorata Kraskiewicz contributed equally to the work.

Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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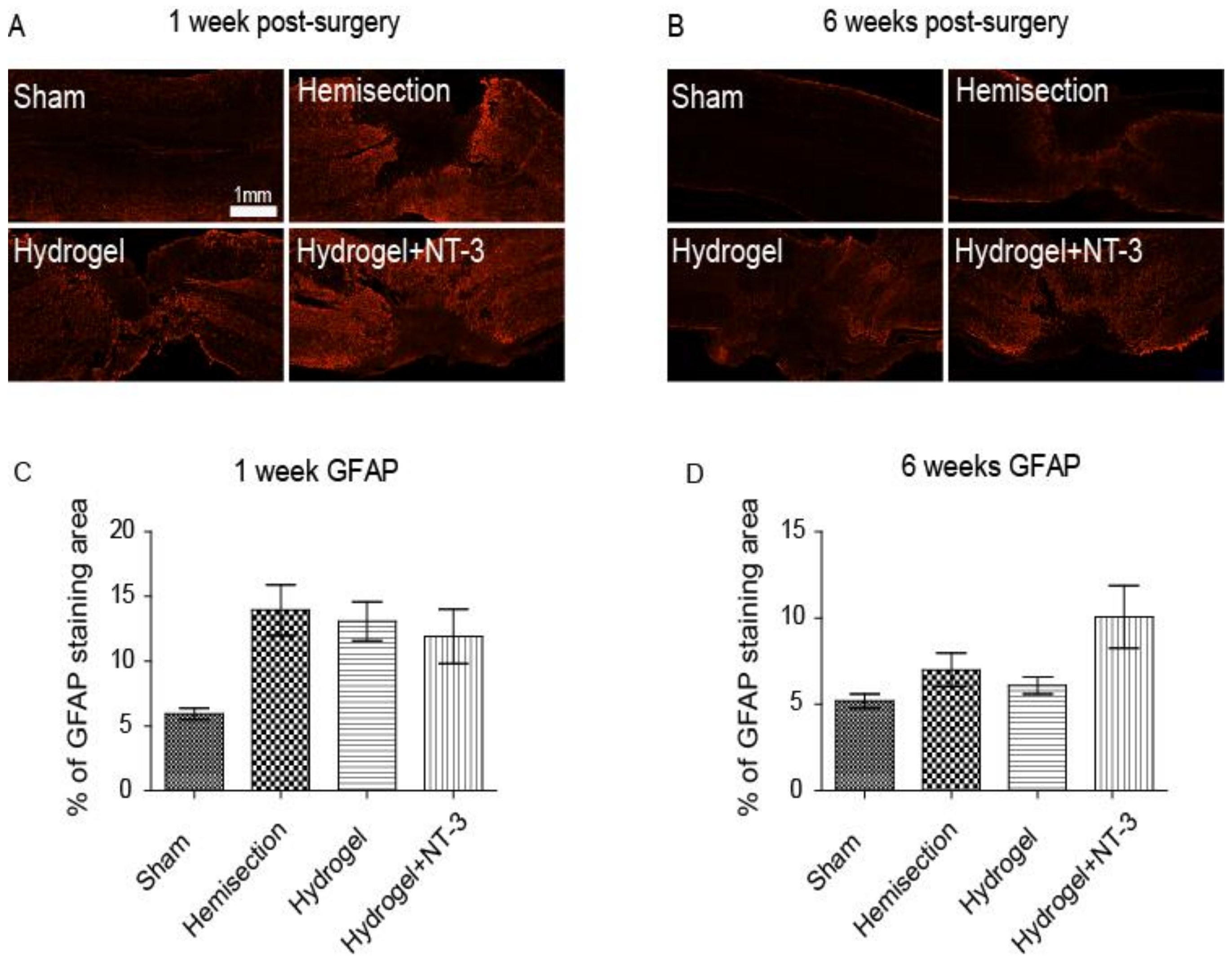
Supplementary Figure 1



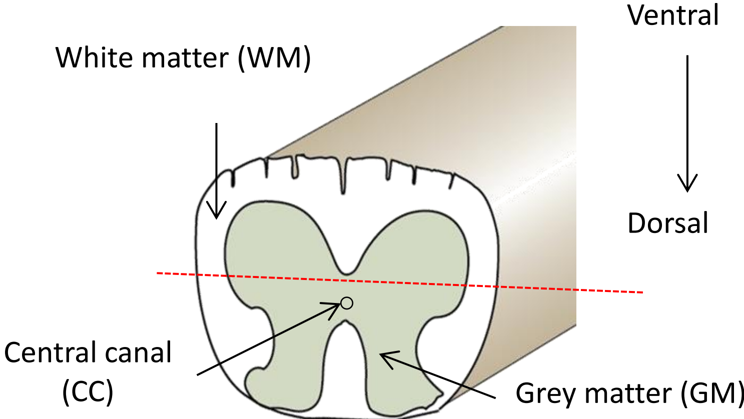
Injectable hydrogel treatment does not alter astrocytosis following SCI *in vivo.*

Spinal cord sections (20μm) were stained with GFAP (red). The area of positively stained GFAP spinal cord was compared between treatment times (one week and six weeks) and treatment paradigms (n=4-7). A slight yet non-significant difference in reactive astrocytes was observed following hydrogel + NT-3 treatment one week post-surgery. However the reverse was observed six week post-surgery where

hydrogel alone significantly reduced reactive astrocytosis at this time point. No significant difference in the area of GFAP staining $p<0.05$, (n=4-7). Scalebar = 1mm.

Supplementary Figure 2

A



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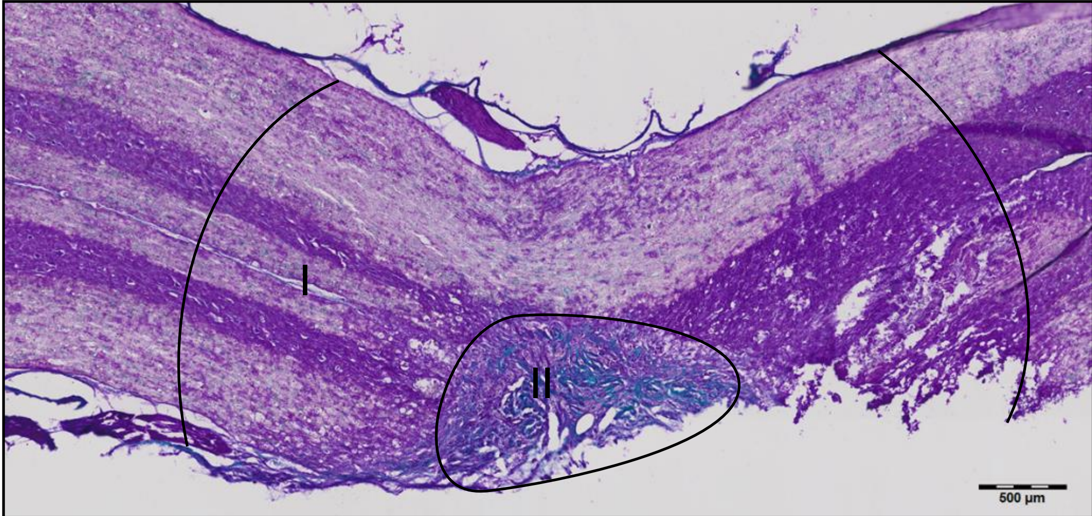
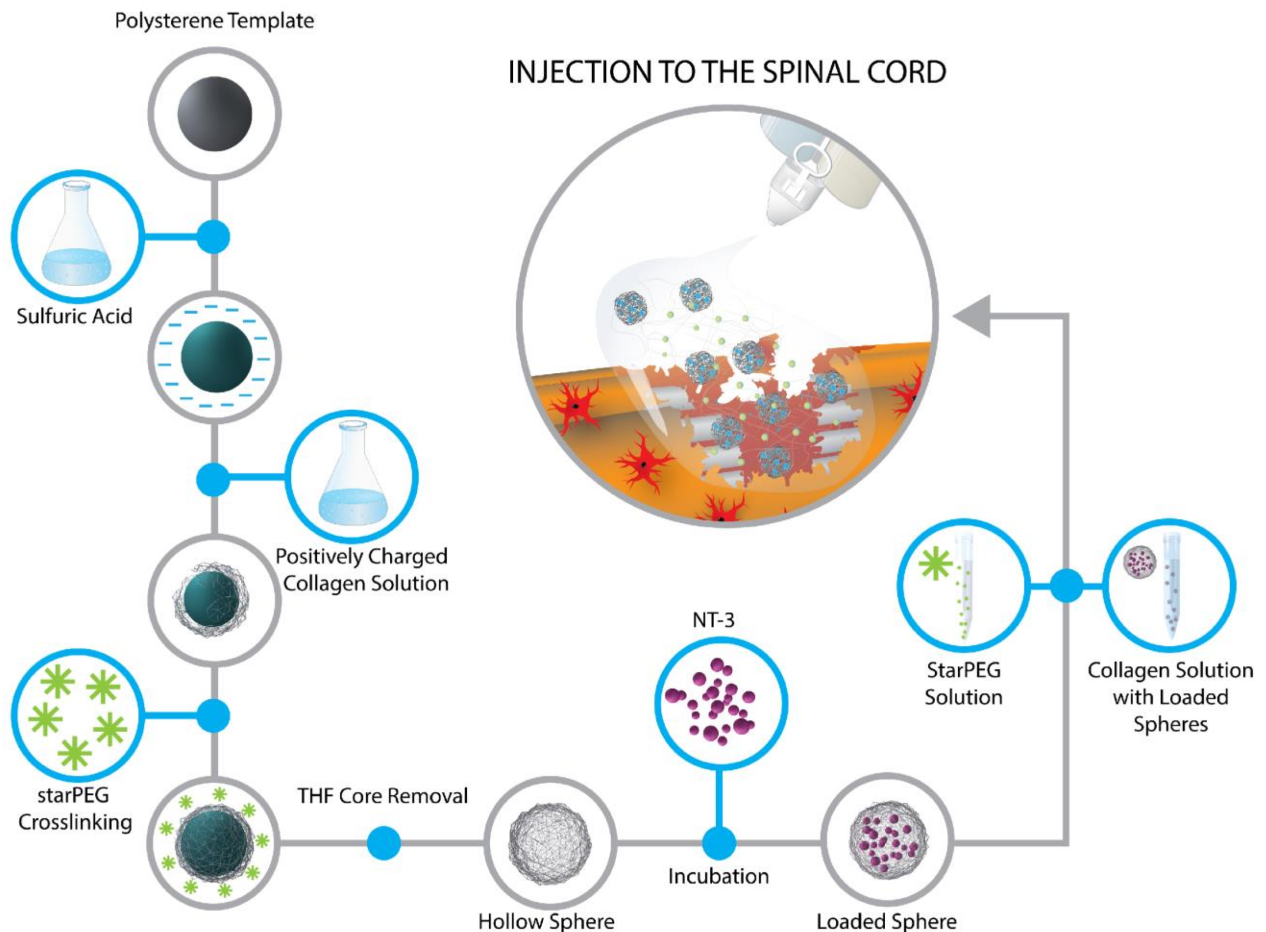


Supplementary Figure 2: Schematic illustrating staining analysis of spinal cord sections.

(A) Spinal cord tissue was cryo-sectioned from ventral to dorsal direction. Sections at central level (yellow line) were analysed immunohistochemically. **(B)** A representative image analysed using Image J software. Within the area of approximately 1 mm from the lesion border tissue was analysed for GFAP staining and Masson's Trichrome staining (region I), axonal re-grow was assessed by βIII Tubulin in the immediate lesion region (region II). Iba-1 and NG-2 staining were analysed within region I and II.

Supplementary Figure 3



Supplementary Figure 3: Schematics of sphere fabrication, sphere loading and scaffold preparation.

Commercially available 4.5 µm **polystyrene beads (template)** are incubated with **sulphuric acid** to negatively charge its surface. Following sulfonation, beads are re-suspended in the positively charged collagen solution at a weight ratio of 1:7 (collagen: beads). Free amino groups of collagen are cross-linked using **8a15kSG**

PEG at a weight ratio 1:2 (collagen: cross-linker). Then, the polystyrene template is removed by washes with 20% (v/v) tetrahydrofuran (THF) to produce hollow spheres. Sphere are incubation with **NT-3** solution (1 mg of spheres /250 µl of PBS-0.05% Tween / 10 µg of NT-3) to allow proteins to translocate into spheres. NT-3 loaded spheres are mixed with warm (37°C) 50 mg/ml collagen solution and just before **injection** collagen-sphere solution is mixed with the same volume of PEG solution.