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A glial cell line-derived neurotrophic factor delivery system enhances nerve regeneration across acellular nerve allografts

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ABSTRACT

Acellular nerve allografts (ANAs) are used clinically to bridge nerve gaps but these grafts, lacking Schwann cells and therapeutic levels of neurotrophic factors, do not support regeneration to the same extent as autografts. Here we investigated a local drug delivery system (DDS) for glial cell line-derived neurotrophic factor (GDNF) controlled release to implanted ANAs in rats using drug-loaded polymeric microspheres (MSs) embedded in a fibrin gel. In a rat hindlimb nerve gap model, a 10 mm ANA was used to bridge a 5 mm common peroneal (CP) nerve gap. Experimental groups received DDS treatment at both suture sites of the allografts releasing GDNF for either 2 weeks or 4 weeks. In negative control groups, rats received no DDS treatment or empty DDS. Rats receiving nerve isografts served as the positive control group. The numbers of motor and sensory neurons that regenerated their axons in all the groups with GDNF MS and isograft treatment were indistinguishable and significantly higher as compared to the negative control groups. Nerve histology distal to the nerve graft demonstrated increased axon counts and a shift to larger fiber diameters due to GDNF MS treatment. The sustained delivery of GDNF to the implanted ANA achieved in this study demonstrates the promise of this DDS for the management of severe nerve injuries in which allografts are placed.

Statement of Significance

This work addresses the common clinical situation in which a nerve gap is bridged using acellular nerve allografts. However, these allografts are not as effective in supporting nerve regeneration as the gold standard method of autografting. The novel local drug delivery system used in this study provides sustained and controlled release of glial cell line-derived neurotrophic factor (GDNF), one of the most potent neurotrophic factors, which significantly improves nerve regeneration following severe nerve injuries. Results from this research will provide a mean of improving nerve allografts with locally delivered GDNF. This strategy may lead to a novel "off the shelf" alternative to the current management of severe nerve injuries.

1. Introduction

Despite substantial improvements in microsurgical techniques, patients with peripheral nerve injuries rarely recover fully [1,2]. Direct end-to-end repair of the transected peripheral nerve supports limited recovery following injury [3–5]. However, in many clinical situations, there is not enough nerve tissue to allow a tension free reconstruction [6,7]. In these cases, the current surgical standard consists of using an autograft, in which a nerve graft from the same patient is used to bridge the nerve gap. Although autografts provide tension-free repair, they require a second operative site which necessitates additional operative time, a permanent scar, donor sensory loss, and could result in persistent

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postoperative pain [8]. Moreover, due to the limitation in the available length, nerve autografts may not be feasible in cases where extensive reconstruction is required [8].

An alternative to autografting is the use of processed nerve allografts, or acellular nerve allografts (ANA)s [9]. ANAs retain the scaffold of nerve tissue but are made to be non-immunogenic to the recipient by a variety of processing methods, such as repeated freeze–thaw cycles; cold preservation; and decellularization with detergents [8,10,11]. Thus, ANAs provide a biological substrate for nerve regeneration without the requirement of immunosuppression. However, they have non-therapeutic levels of neurotrophic factors especially compared to normal denervated nerve stumps in which several growth factors are upregulated after injury [12]. Given that regenerating nerve fibers preferentially elongate toward sources of neurotrophic factors [13,14], replenishing the ANAs with key neurotrophic factors should enhance nerve gap regeneration. ANAs have been used clinically in patients for several years [15], and we questioned whether the ability of these allografts to support nerve regeneration could be improved by supplementation with key neurotrophic factors lacking in the commercially available ANA.

Delivery of neurotrophic factors holds promise in enhancing outcome following nerve injury [16]. Neurotrophic factors, such as brain-derived neurotrophic factor, nerve growth factor, and glial cell line-derived neurotrophic factor (GDNF), which are essential for peripheral nervous system development, have been shown to promote axon regeneration and enhance functional recovery [12,17,18]. However, the challenge for achieving a clinically suitable application for GDNF is its localized and sustained release to the nerve injury site [12,18]. Current investigational methods of GDNF local delivery include viral transfected Schwann cells [19,20], and catheter/mini-osmotic pump systems [21]. While viral transduction of primary cells generates local release, regulation of GDNF release is difficult to manage and can result in excess and toxic GDNF release. In addition, clinical translation may be a significant regulatory challenge and these methods are not currently approved for clinical use. Osmotic pump delivery systems, despite providing sustained and localized release, can hinder recovery due to risk of infection and even nerve compression secondary to capsular fibrosis [3]. A sustained and tunable delivery from a biodegradable and biocompatible system is therefore preferred to effectively delivery GDNF to the injured nerve.

Previously, we developed a microsphere-based biodegradable drug delivery system (DDS) supporting sustained release to the injured nerve over periods of days to weeks [22,23]. This DDS, consisting of fibrin gel containing GDNF microspheres, significantly improves axon regeneration and functional recovery after delayed nerve repair [22,23]. In this study, we combined the DDS composite system with the rat analogue of the clinically-used nerve allograft to determine the extent to which this new hybrid DDS-ANA biomaterial supported nerve regeneration.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.1. GDNF encapsulation in PLGA microsphere

Glial cell line-derived neurotrophic factor (GDNF) was encapsulated in poly(lactic-co-glycolic acid) (PLGA) microsphere (MS) using a water/oil/water double emulsion, solvent evaporation method. Briefly, an inner aqueous solution of 100 μL consisting of 250 μg GDNF (Peprotech, Rocky Hill, NJ) and 12.5 mg heparin was mixed with 230 mg PLGA 50/50 (Wako, Japan and Lactel Absorbable Polymers, Cupertino, CA, Table 1) and 12.5 mg $MgCO_3$ in 1 mL dichloromethane (DCM)/acetone (75%/25%). The mixture was sonicated for 45 s using a 3 mm probe sonicator (Vibra-Cell™ VCX 130; Sonics and Materials, CT, USA) at 30% power. The resulting emulsion was added to 25 mL of 2.5% aqueous poly(vinyl alcohol) (PVA) solution containing 10% NaCl and homogenized at 6000 rpm for 60 s. The secondary emulsion was then added to 250 mL aqueous solution of 2.5% PVA and 10% NaCl. The mixture was stirred for 3 h with venting to allow the hardening of the microspheres by complete evaporation of the organic solvent. The hardened microspheres were collected and washed by centrifugation, lyophilized, and stored at −20 °C until use.

2.2. GDNF microsphere characterization

Microsphere mean diameter and size distribution were measured via static light scattering using a Malvern Mastersizer 2000 laser diffraction particle sizer (Malvern Instruments Ltd, UK), using refractive indices of 1.33 and 1.59 for water and PLGA, respectively. Encapsulation efficiency was measured by dissolving an appropriate mass of microspheres in 1 mL dimethyl sulfoxide (DMSO) for 1 h at 37 °C followed by addition of 10 mL of 0.05 M NaOH with 0.5% w/v sodium dodecyl sulfate (SDS) and further shaking for 1 h at room temperature. The amount of GDNF was quantified by an enzyme-linked immunosorption assay (ELISA) for human GDNF according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The absorbance was read at 450 nm with an optical subtraction at 540 nm using a multi-well plate spectrophotometer, and sample concentrations were calculated from a standard curve of known GDNF concentrations. Drug loading was determined as the GDNF mass per mg of microspheres; encapsulation efficiency was the measured drug loading of the microspheres divided by the theoretical maximum drug loading.

2.3. GDNF DDS composite construction and in vitro release

Fibrin gel (80 μL total volume) was constructed by mixing equal parts fibrinogen (75–115 mg/mL, 40 μL) and thrombin (5 IU/mL, 40 μL) obtained from a Tisseel® glue kit (Baxter Healthcare, IL, USA), and then re-suspended according to the manufacturer's instructions. Fibrin gels were loaded with microspheres by incorporating 5 mg of microspheres into the thrombin solution before it was mixed with fibrinogen to form a gel. *In vitro* release of GDNF from fibrin loaded with microspheres was assessed by using 80 μL gels in 2 mL siliconized centrifuge tubes (Fisher Scientific). The time course of release was measured by incubating the fibrin gels in 1 mL of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) at 37 °C under constant gentle agitation by vortex. The PBS was collected and ELISA assays were performed

Table 1
PLGA description used in the drug delivery system synthesis.

Formulation name	PLGA inherent viscosities (dL/g)	PLGA average molecular weight (Da)	GDNF initial loading (%wt/wt in microspheres)	Encapsulation efficiency (%)
2-week release formulation	0.088–0.102	5000	250 mg (0.05%)	78 ± 3
4-week release formulation	0.15–0.25	6700	250 mg (0.05%)	78 ± 3

to measure GDNF quantity collected from the time course release studies.

2.4. Acellular nerve allograft preparation

The processed common peroneal nerve grafts from rats were provided by AxoGen, Inc (Alachua, FL, USA). Briefly, the harvested rat nerve tissue from donor rat's common peroneal nerve was decellularized using the detergent based Hudson et al. protocol [9,24]. The processed tissue was then depleted of the regeneration- inhibiting chondroitin sulfate proteoglycans with chondroitinase ABC based on the method developed by Neubauer et al. [25]. The processed grafts were sterilized with gamma irradiation and frozen at −80 °C. The grafts were stored at −80 °C until implantation.

2.5. Experimental animals

Adult female Sprague–Dawley rats (Harlan, Indianapolis, USA), each weighing 250–300 g were used in this study. All surgical procedures and perioperative care measures were performed in strict accordance with the National Institutes of Health guidelines, the Canadian Council on Animal Care (CCAC) and were approved by the Hospital for Sick Children's Laboratory Animal Services Committee.

2.6. Experimental design

Forty-eight adult female Sprague–Dawley rats were randomized into six groups ($n = 8$) (Fig. 1). Female rats gain less weight over time compared to male rats. In order to ensure consistent dosing and avoid issues associated with weight change during the experimental period, female rats were used. Animals receiving no drug delivery system (DDS) treatment or fibrin gels loaded with empty MSs served as experimental control groups. Fibrin gels loaded with microspheres releasing GDNF *in vitro* for ~2 or ~4 weeks placed at both suture sites of the nerve graft served as the primary experimental groups. Another experimental group received MSs with 2-week release formulation at the proximal suture sties and microspheres with 4-week release formulation at the distal site. Rats receiving nerve isografts (i.e., grafts taken from immunologically equivalent littermates) served as the positive control group. In additional, in each group, two *Thy*-1 transgenic rats that expressed green fluorescent protein (GFP) in their axons [26] were included for qualitative visualization of the regenerating axons within the implanted nerve grafts.

2.7. Operative procedure

All surgical procedures were performed using aseptic techniques. Isoflurane (3%) gas anesthesia was used for animal induction followed by 2.5% isoflurane gas for maintenance. The hind leg of the rat was surgically cleaned with a betadine/alcohol rub. The sciatic nerve was exposed through a dorsolateral–gluteal muscle splitting incision. Wounds were irrigated with saline, dried and closed in two layers, utilizing 5-0 Vicryl™ (Ethicon, OH, USA) sutures to close the muscle layers, and 4-0 Nylon sutures to close the skin. Experimental animals were recovered in a warm environment prior to returning to the housing facility.

During the procedure, the common peroneal (CP) nerve was dissected free (Fig. 2A) and a 5 mm nerve gap was created approximately 5 mm distal from the sciatic trifurcation (Fig. 2B). In order to restore nerve continuity without tension, and analyze nerve regeneration through a nerve gap that is considered critically large in rats, a 10 mm segment of a nerve graft was used to bridge the nerve defect using 9-0 Nylon sutures (Fig. 2C). In the groups receiving the fibrin gel based DDS, the proximal and distal nerve suture

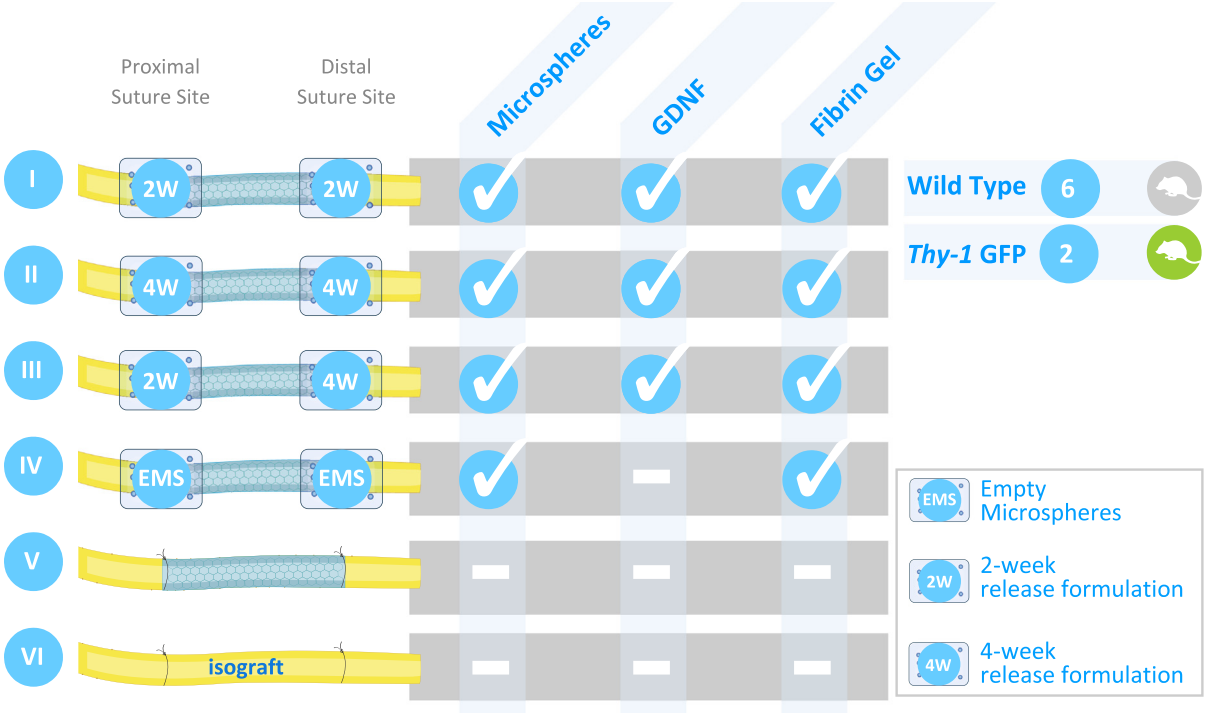

Fig. 1. *In vivo* experimental design. Experimental groups consisted of grafts receiving fibrin gels loaded with 2-week release formulation GDNF MS (I), 4-week release formulation GDNF MS (II) at both suture sites. Another experimental group received 2-week release formulation GDNF MS at the proximal suture site and 4-week release formulation GDNF MS at the distal site (III). Control groups received fibrin gel with empty MS (IV), and no DDS treatment after nerve allograft implantation (V). Animals receiving isografts served as the positive control group (VI). Each group contained six wild type rats and two *Thy*-1 GFP rats, which were included for visualization of axonal regeneration within the implanted nerve graft. MS: Microsphere. DDS: Drug Delivery System.

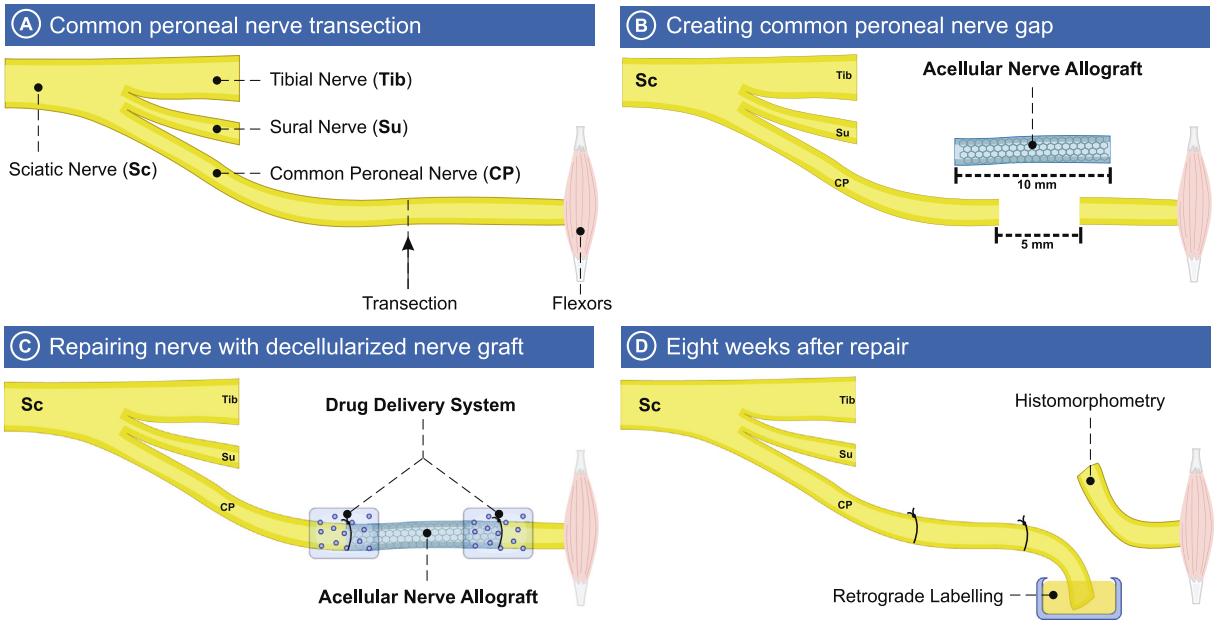

Fig. 2. Surgical procedures performed on rats. Prior to nerve repair the common peroneal nerve was transected (A) and a 5 mm nerve gap was created (B). The nerve gap was bridged with a 10 mm nerve allograft followed by placement of the drug delivery system (C). Eight weeks following nerve repair, nerve was harvested and labeled with retrograde dye 10 mm distally from the nerve graft implantation site (D).

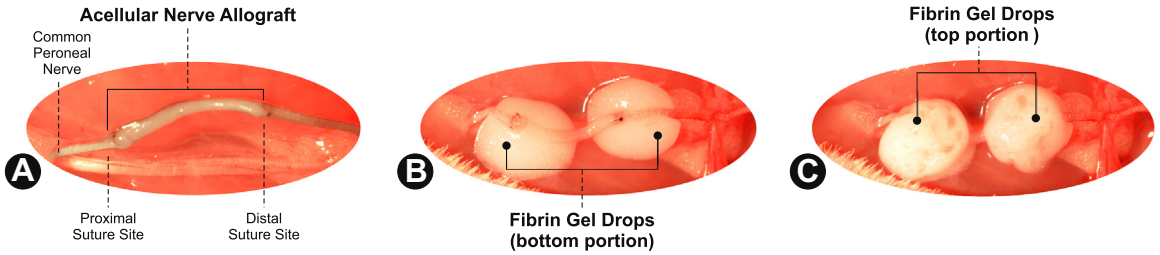

Fig. 3. Fibrin gel based drug delivery system implantation. (A) Intraoperative image of common peroneal nerve with a 5 mm nerve gap bridged with a nerve 10 mm acellular nerve allograft. (B) 40 μm fibrin gel drops were placed at the bottom of the distal and proximal coaptation sites of the nerve allograft. (C) The final drug delivery systems were formed by placing 40 μm fibrin gel drops at the top of each coaptations sites. The fibrin gel drops at each coaptation sites were adhered to each other instantly and were secured around the nerve suture sites.

sites were surrounded by two 40 μL gels (Fig. 2C), formed by pipetting the fibrin mixture, before setting as a gel, onto Parafilm as semirectangular drops (~5 mm × 5 mm). The gel drops were placed centered above and below the repair sites and secured by gently opposing the gel drops on one another (Fig. 3). The fibrin gel drops instantly adhered to each other around the nerve suture site and the fibrin gel mixture did not show any signs of migration upon implantation, as shown previously by our research group [23].

2.8. Retrograde labeling of motoneurons (in ventral horn) and sensory neurons (in dorsal root ganglia)

Eight weeks after nerve graft implantation, the surgical site was reopened under general anesthesia. The CP nerve was transected 10 mm distally from the distal repair site and the proximal nerve stump was immediately placed in a silicone well containing 4% Fluoro-Gold™ in sterile saline for 1 h (Fig. 2D). At the same time, the distal stump was harvested for histology (described below). The silicone wells and Fluoro-Gold solutions were removed, incisions were closed and rats were allowed to recover as described previously. Seven days following the procedure, the rats were euthanized and perfused with 0.9% NaCl saline and cold 4% paraformaldehyde in PBS. The lumbar region (L3–L6) of the spinal cord and L4–L5 of the dorsal root ganglia (DRG) were dissected free for frozen sectioning. Axial sections of the lumbar spinal cord (50 μm) or DRG (20 μm) were sectioned on a cryostat (Leica, ON, Canada). The number of labeled cell bodies within the ventral horn of each spinal cord section or within every fifth DRG section was counted using a fluorescent microscope with a 10× objective (100× overall magnification; Leica). Spinal cord counts were adjusted to account for split nuclei using the methods of Abercrombie [27].

2.9. Histology and morphometric evaluation of nerves

At the time of the retrograde labeling surgery, the nerve tissue taken 10 mm distally from the nerve graft distal suture site was collected (Fig. 2D), fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, ethanol dehydrated and embedded in Araldite® 502 (Polyscience, Inc., PA, USA). Thin (0.6 μm) sections were made from the tissue using a LKB II ultramicrotome (LKB-Produckter AB, Sweden) and then stained with 1% toluidine blue for examination by light microscopy. The slides were evaluated for overall nerve architecture and quality of regenerated fibers. At 1000× overall magnification, the entire nerve cross-section was captured to

count regenerated axons, measure myelin thickness and fiber diameter, and calculate G-ratio using a semi-automated MATLAB program [28].

In *Thy*-1 GFP rats, the nerve graft was harvested nine weeks after implantation at the time of spinal cord tissue dissection and fixed in cold 4% paraformaldehyde in PBS. Longitudinal sections of the nerve graft were cut at 30 μm on a cryostat. To qualitatively analyze the axon morphology and density with the graft, the obtained sections were imaged using a fluorescent microscope with a 10× objective (100× overall magnification; Leica).

2.10. Statistical analysis

The *in vitro* dynamic release data in Section 3.1 are reported as mean ± standard deviation. The *in vivo* retrograde labeling data in Section 3.2 and quantitative histology data in Section 3.4 are reported as mean ± standard error. Statistical testing was performed in GraphPad Prism 6 to confirm the normality of the data and then differences between groups were assessed by analysis of variance (ANOVA) with Bonferroni's post-hoc at 95% confidence intervals.

3. Results

3.1. In vitro microsphere characterization and GDNF release from DDS

Microspheres (MS) constructed with poly(lactic-co-glycolic acid) (PLGA), heparin and $MgCO_3$ demonstrated a glial cell line-derived neurotrophic factor (GDNF) encapsulation efficiency of 78 ± 3% and GDNF loading of 0.72 ± 0.08 μg per mg of microspheres. The microspheres had diameters of 45 ± 5 μm. The molecular weight of the PLGA used for the synthesis of MS was adjusted to modulate the release kinetics of GDNF from the drug delivery system (DDS) containing the MS, into the phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) at 37 °C. Consistent with previous studies [22], the dynamic release of GDNF was 15 days from MS synthesized with PLGA that had an inherent viscosity of 0.088–0.102 dL/g and a molecular weight of 5000 Da (Fig. 4A).

This type of DDS was termed the "2-week release" formulation of DDS due to the 15-day GDNF release period from the system. By increasing the inherent viscosity and the molecular weight of PLGA to 0.15–0.25 dL/g and 6700 Da, respectively, the encapsulated GDNF release period from the MS was extended to 28 days (Fig. 4A). Thus, this type of DDS was called the "4-week release" formulation of DDS. There was no initial GDNF burst release from both DDS during the first 24 h (Fig. 4B). The daily mass release indicated that there was a sustained release of GDNF over 15 days with the mean of 287 ± 25 ng/day for the 2-week release formulation of DDS and over 28 days with the mean of 220 ± 30 ng/day for the 4-week release formulation of DDS.

3.2. In vivo retrograde labeling of neurons following nerve repair

In order to quantify the number of common peroneal (CP) neurons regenerating their axons through the nerve graft and to compare the extend of motor versus sensory regeneration, retrograde labeling was performed 8 weeks after nerve graft implantation (Fig. 2D): Fluoro-Gold was applied to the regenerated axons 10 mm distally from the distal suture site of the nerve graft. The empty MS and no DDS treatment control groups had similar number of neurons that regenerated their axons ($p = 0.8121$, Fig. 5), indicating fibrin gel with MS did not inhibit nerve regeneration. Following nerve gap surgical repair, approximately 50% of the CP motoneurons (compared to the uninjured normal number of 400 ± 20 motoneurons) regenerated their axons within the 8 weeks under conditions of no DDS treatment (200 ± 42) or with

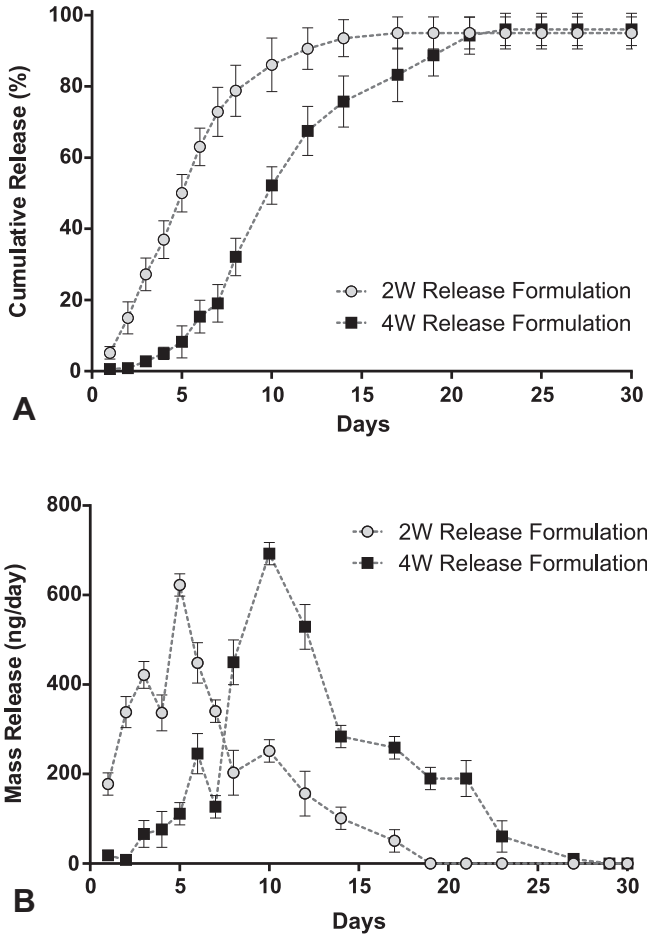

Fig. 4. *In vitro* release of GDNF from fibrin gels loaded with microspheres. (A) Cumulative mass release of GDNF from "2 week release" formulation of DDS (○), and "4 week release" formulation of DDS (■). The GDNF encapsulated within the microspheres were completely released during 15 days from the "2-week release" formulation. The period of GDNF release was extended to 28 days with the 4-week release formation of DDS. The data were normalized to the successfully encapsulated GDNF in microspheres. (B) The daily mass release profile of GDNF from both formulations of microspheres confirms sustained release over 15 days for the 2-week release formulation of DDS and over 28 days for the 4-week release formulation of DDS. GDNF content in the release samples was determined using ELISA. (Mean ± standard deviation, $n = 3$ per release study). DDS: Drug Delivery System.

microspheres that were empty (247 ± 50). There was a significant increase in these numbers when GDNF was included within the microspheres (Fig. 5A). Indeed, the acellular nerve allografts (ANAs) with GDNF MS treatment were statistically indistinguishable from the isograft (Fig. 5A) with 397 ± 46 and 443 ± 48 motoneurons regenerating their axons, respectively ($p = 0.0842$). The group that received the 2-week release formulation of DDS at the proximal suture site and the 4-week release formulation of DDS at the distal site was different from the isograft treated group (365 ± 47; $p = 0.0079$) but not different from the other groups with delivery of GDNF MSs at both ends of the grafts with either 2 or 4 weeks release duration.

Sensory neuron regeneration mirrored that of the motoneurons. After the nerve graft implantation, the control groups with no DDS treatment and empty MS containing 566 ± 74 and 605 ± 140 retrogradely labeled sensory neurons had only approximately 60% sensory neuron regeneration (compared to the normal uninjured number of 1000 ± 22 sensory neurons, represented by the dashed line in Fig. 5B). These numbers were significantly increased in the isograft and all the GDNF MS treated groups ($p < 0.0001$). The

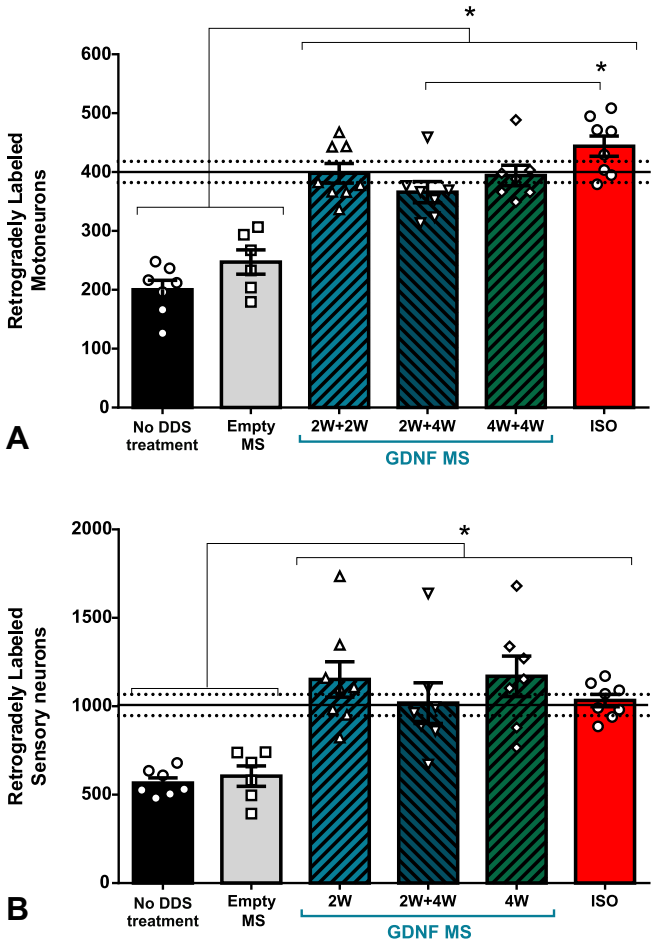

Fig. 5. *In vivo* GDNF release from microspheres embedded in fibril gels made the acellular nerve allografts as effective as the isografts in supporting nerve regeneration. To assess motor and sensory neuron regeneration, retrograde labeling of neurons was performed 10 mm distally from the distal repair site 8 weeks following experimental treatment. The numbers of the fluorescently labeled motoneurons were counted in the spinal cord's ventral horn sections (A; 50 μm sections, all sections counted, correction factor = 0.6 [27]) and sensory neurons were counted in the dorsal root ganglia sections (B; 20 μm sections, every fifth section counted). The experimental groups receiving fibrin gels loaded with MS containing GDNF had comparable nerve regeneration to the isograft group and showed significantly higher motor (A) and sensory (B) neurons regeneration through nerve allografts compared with the empty MS and no DDS treatment control groups. The period of GDNF release from the drug delivery system did not influence the extent of nerve regeneration. The control groups receiving no DDS treatment and fibrin gels with empty MS had similar number of regenerated neurons, indicating the drug delivery system did not diminish nerve growth. Data represent the mean ± standard error of the mean. Normal uninjured values ± standard error are represented by the dashed line. $^{*}p < 0.05$. MS: Microsphere. DDS: Drug Delivery System.

numbers of sensory neurons regenerating their axons through the ANA with GDNF MS treatment were statistically indistinguishable from the numbers through the isograft (1151 ± 282 and 1032 ± 98, respectively; $p = 0.2796$; Fig. 5B).

Overall, the numbers of motor and sensory neurons that regenerated their axons through the implanted ANAs were significantly higher in all the primary experimental groups treated with GDNF MS compared with the control groups receiving empty MS in their fibrin gels or with no DDS treatment after the ANA implantation. Despite a decrease in the neurons' mean number for the group that received DDS with 2 weeks GDNF release at the proximal suture site and 4 weeks GDNF release at the distal site, all the experimental groups with GDNF MS treatment demonstrated similar success of nerve regeneration through the allografts. These findings indicate that the total amount of GDNF available over time played a significant role in promoting the growth of the regenerating axons through the ANA. Thus, for the histomorphometric analysis, the GDNF MS treated group with the 2-week release formulation DDS was used as the representative experimental group.

3.3. Axon morphology through nerve graft

The implanted nerve grafts were harvested from the *Thy*-1 GFP rats at the time of spinal cord tissue dissection to perform morphometric analysis on axon regeneration through the nerve graft. A qualitative analysis of the 30 μm longitudinal nerve graft sections revealed the axon alignment and axon density within the nerve graft (Fig. 6A–C). There was a uniform axon distribution within the isografts and GDNF MS treated ANAs (Fig. 6B and C). GDNF treatment using the microspheres (Fig. 6B) did not influence the axon alignment within ANAs and they were similar to that of the isografts and no DDS treatment groups (Fig. 6). An autofluorescence in all fluorescent channels was observed within the acellular nerve allografts regardless of the DDS type treatment. This could be related to the byproducts of the grafts extracellular matrix remodeling during axon regeneration through the nerve grafts.

3.4. In vivo nerve histology and morphometric measures of regeneration

Eight weeks following nerve repair with graft implantation, nerve samples were harvested at 10 mm distal from the nerve graft for histology analysis by light microscopy. Qualitative analysis of nerve samples revealed similar nerve morphology for the groups receiving empty MS and no DDS treatment (Fig. 7A and B). All the GDNF MS groups had similar nerve morphology to the group receiving isografts (Fig. 7C and D) with significantly higher myelinated axons present in the nerve cross sections compared with the empty MS and no DDS treatment control groups (Fig. 7A and B). In all the groups, regardless of the treatment, axons were uniformly distributed throughout the nerve. Quantitative fiber frequency distribution analysis on the entire nerve cross section revealed that the GDNF MS and isograft treated groups had similar fiber distribution (Fig. 7E). These two groups demonstrated greater numbers of larger diameter fibers (4–6 μm) and fewer numbers of smaller diameter fibers (2–4 μm) compared with the no DDS treatment and empty MS treated groups (Fig. 7E). Larger fibers are more mature. The strong shift toward larger diameter fibers with GDNF MS treatment ($p = 0.0049$) demonstrates that the GDNF local release to the allografts enhanced axonal regeneration.

Quantitative histomorphometric analysis of the entire nerve cross sections confirmed the significant increase in the number of myelinated axons in the GDNF MS treated groups compared with the no DDS treatment control group (3173 ± 204 and 2028 ± 524, respectively; $p = 0.0038$; Fig. 7F). The number of myelinated axons that regenerated in the GDNF MS treated groups was similar to that of the isografts treated group with 2028 ± 695 axons ($p = 0.5375$). As a measure of nerve maturity, nerve fiber diameter, and myelin thickness was determined. There were no significant differences in myelin thickness and fiber diameter in all the groups (Fig. 7G and H). The G-ratio, calculated as the ratio of the axon diameter to the total fiber diameter, was statistically equivalent for all groups (Fig. 7I).

4. Discussion

Acellular nerve allografts (ANA) have the potential to support nerve regeneration by providing a cell free scaffold and maintaining much of the internal structural and molecular composition of

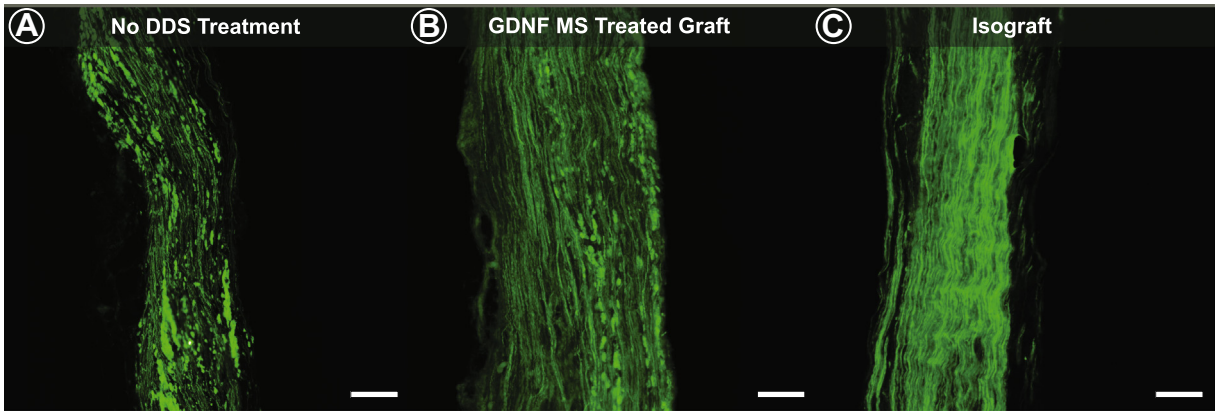

Fig. 6. Axon density within the acellular nerve allograft increased after treatment with fibrin gels loaded with GDNF microspheres. Representative segments of (A) acellular nerve graft with no drug delivery system treatment, (B) with GDNF delivery system treatment, and (C) isograft, 8 weeks post implantation were obtained from the Thy-1 GFP rats (nerves are green). Longitudinal nerve graft sections (30 μm each) indicated that GDNF treatment using the microspheres enhanced allografts' axons alignments and increased the axon density, to the same extent as the isografts. Scale bar: 300 μm. DDS: Drug Delivery System. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the normal nerve extracellular matrix. While these allografts do not induce immunogenic responses after implantation, they lack Schwann cells and therapeutic levels of neurotrophic factors important for peripheral nerve regeneration. Therefore, the addition of neurotrophic factor support may lead to a clinically superior ANA [29–31].

In this study, we sought to determine the effect of localized and sustained release of glial cell line-derived neurotrophic factor (GDNF) on axon regeneration through ANAs. Natural hydrogels, such as fibrin, have been commonly used to obtain controlled neurotrophic factor delivery to peripheral nerves [32]. In order to avoid the high burst release associated with hydrogels due to simple diffusion and non-covalent interactions as well as to prolong drug release [33], GDNF was incorporated within the fibrin gel in poly (lactic-co-glycolic acid) (PLGA) microspheres (MS). Based on the PLGA molecular weight and inherent viscosity, the PLGA/fibrin gel drug delivery system (DDS) was designed to provide *in vitro* GDNF release for up to either 15 days (the "2-week release" formulation) or 28 days (the "4-week release" formulation). The *in vitro* release profile showed a sustained release without an initial burst within the first day, suggesting the formation of electrostatic or hydrophobic interactions between GDNF, fibrinogen and PLGA microspheres within the fibrin gel. One limitation of the current study was that GDNF dynamic release was investigated only through *in vitro* analysis. Future work will focus on *in vivo* local dose detection of GDNF. Both the 2-week release formulation and the 4-week release formulation of DDS were able to deliver GDNF at levels of $\geqslant$100 ng/day for the entire period of release for the 2-week release formulation of DDS, and for at least 23 days for the 4-week release formulation of DDS (Fig. 4B). The ability to deliver more than 100 ng/day of GDNF to the nerve repair site is necessary for improved motor nerve regeneration [18].

In order to make this study clinically relevant and facilitate clinical translation, recombinant human GDNF was used in this work. Based on previous evidence, recombinant human GDNF can significantly enhance nerve regeneration in rat models of nerve injury [18,22,23,34,35]. Previously, we have shown GDNF release for the periods of 2 weeks and 4 weeks is effective in enhancing nerve regeneration in chronic axotomy models [22]. In this study, in order to try to provide maximum stimulation of axonal growth, a combination of the 2-week release formulation of DDS and 4-week release formulation of DDS were placed at the two suture sites of implanted ANAs. In two experimental groups, the DDS provided GDNF release up to either 2 weeks or 4 weeks at both suture sites of the implanted grafts. In an additional experimental group, in order to provide steady GDNF concentration available to the axons' growth cone through the graft and at both suture sites, the 2-week release formulation of DDS was placed at the proximal suture site and the 4-week release formulation of DDS was placed at the distal suture site.

Our findings demonstrated that the GDNF delivered locally to the implanted ANA from a biodegradable DDS could enhance significant axon growth in a clinically relevant model. Implantation of the GDNF MS-containing DDS, regardless of the MS formulation, significantly improved motor and sensory neuron regeneration compared to the control groups without GDNF (Fig. 5). Even though the numbers of motoneurons that regenerated their axons in the experimental group that received the combination of 2-week release formulation of DDS and 4-week release formulation of DDS did not match the isograft treated group, the GDNF MS-treated groups were not statistically different from one another or the isograft treated group. Such observations can be further analyzed by investigating the effect of DDS placement at specific locations along the nerve allograft, for example at only the proximal, central, or distal parts of the allograft. In addition, based on the results of this study, we can now perform experiments in which the effects of other factors of this modular engineered system, such as the drug loading and hydrogel volume, can be determined.

The nerve histomorphometric findings paralleled the counts of the motor and sensory neurons that regenerated their axons. With the GDNF MS treatment, axon density (Fig. 6), the total number of myelinated axons and the frequency of the larger diameter fibers (Fig. 7) matched the isograft group, which was statistically distinguishable from the control groups without GDNF treatment. Nerve fiber diameter is a measure of nerve maturity and quality [34,35]; more fibers with larger diameter indicates better functional recovery compared with smaller diameter fibers [36–38]. In the future, functional studies, such as assessment of muscle force, electrodiagnostic studies and behavioral analysis will allow us to assess the functional benefits of treating the ANAs with GDNF MS containing DDS [36].

The engineered biomaterial in this study was designed to function as a biocompatible drug delivery system for both the injured nerve and also the acellularized nerve allograft. Because the numbers of motor and sensory neurons that regenerated their axons and the numbers of regenerated axons were similar in the empty MS treated and the no DDS treatment groups, we conclude that the placement of the drug delivery system around the graft suture

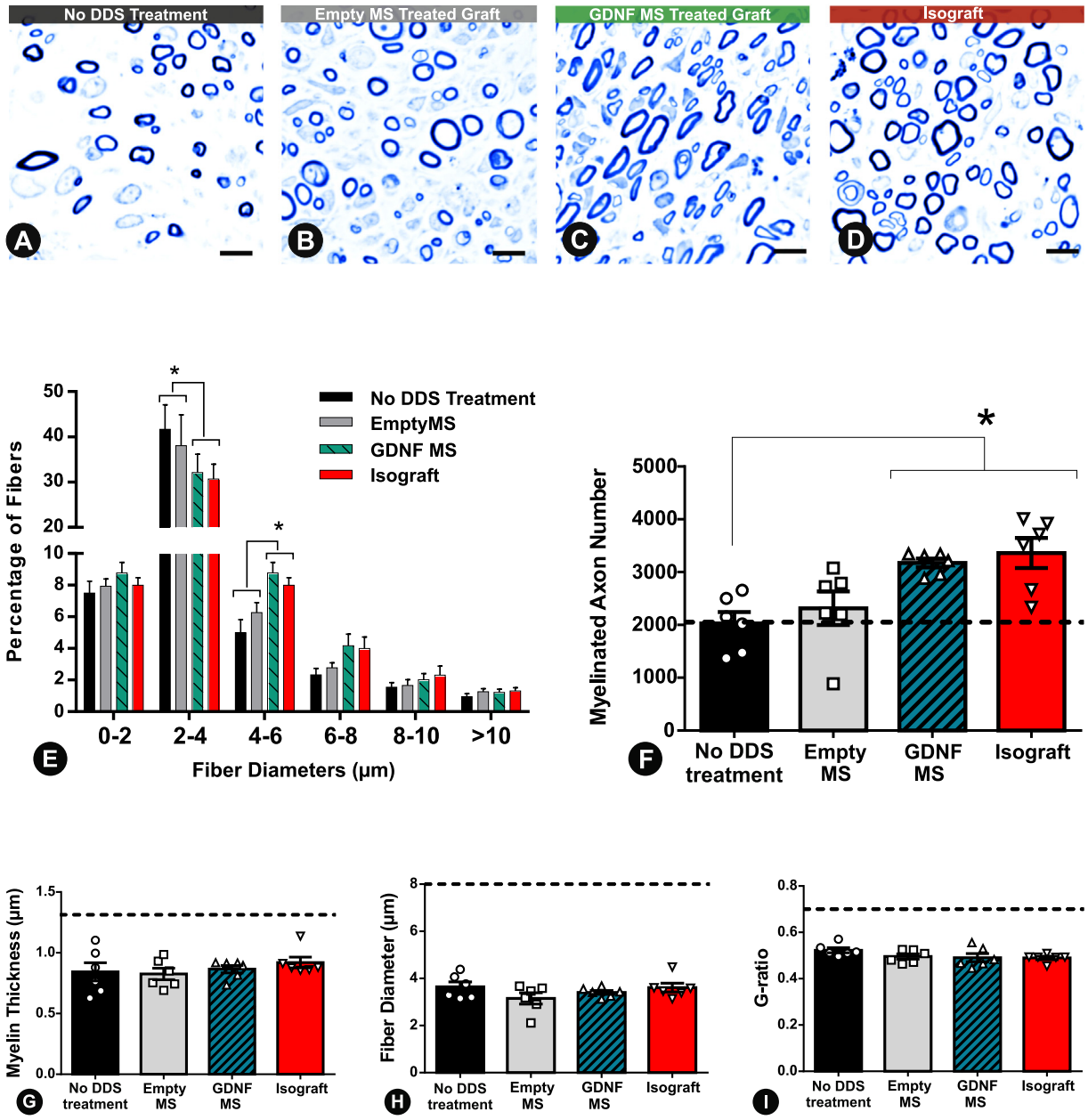

Fig. 7. Treatment of acellular nerve allografts with GDNF delivery system increased myelinated axon regeneration and number of fibers with larger diameter. Light micrographs of nerve cross sections were analyzed in (A) no DDS treatment group, (B) empty microspheres treated group, (C) GDNF MS treated groups, and (D) isograft treated group. Fiber frequency distribution (E) revealed similar fiber distribution for the GDNF MS and the isograft treated groups. There was a shift to the larger diameter nerve fibers (4–6 μm) for the GDNF MS and isograft groups compared with the no DDS treatment and empty MS treated groups, which had more of the smaller fibers (2–4 μm). Histomorphometric analysis of the nerve cross-sections indicated significantly higher number of myelinated axons (F) in GDNF MS and isograft treated groups compared with the no DDS treatment group. No groups exhibited significant differences in myelin thickness (G), fiber diameter (H), and G-ratio (I), but all were below the values of normal uninjured nerves (demonstrated by the horizontal dashed lines). Data represent the mean ± standard error of the mean for 6 animals per group. $^{*}p < 0.05$. Sections A–D are representative light micrographs of nerve cross sections for each group. Scale bars: 10 μm. MS: Microsphere. DDS: Drug Delivery System.

sites did not diminish nerve regeneration. The biocompatible DDS was engineered with clinical ease of use in mind. Surgeons could readily use such a system at the time of nerve reconstruction to enhance nerve regeneration. Fabricating the DDS entirely from biodegradable polymers had eliminated the need of a secondary surgery for the system removal. The degradation of the DDS *in vivo* prevented foreign body formation and chronic nerve compression over time; therefore, the system is likely to be of great clinical value for nerve repair.

Our group has previously shown that the PLGA/fibrin gel composite provides a biodegradable and biocompatible DDS with the potential to significantly enhance nerve regeneration and functional outcome after a delayed nerve repair model [22]. Importantly, we build on previous knowledge and show, for the first time, the local and controlled release of a neurotrophic factor to the acellular nerve allograft suture sites from this fibrin gel based DDS and the consequent axonal growth benefits.

5. Conclusion

A polymeric biocompatible drug delivery system (DDS) was investigated for sustained and controlled release of glial cell line-derived neurotrophic factor (GDNF) to the implanted acellular

nerve allograft (ANA) for bridging a clinically relevant gap model of peripheral nerve injury. Based on the degradation rate of the DDS, GDNF was released locally *in vitro* over periods of 2 weeks or 4 weeks. Implantation of the DDS *in vivo* around the suture sites of ANAs did not induce any adverse side effects on nerve regeneration. Importantly, GDNF local administration from the DDS enhanced nerve regeneration and made the ANAs as effective as isografts in supporting nerve regeneration. The combination of the allograft biomaterial and the GDNF MS delivery system in this study has the potential to provide an "off the shelf" alternative in the current management of severe nerve injuries.

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