

Functional Motor Recovery Is Improved Due to Local Placement of GDNF Microspheres After Delayed Nerve Repair

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ABSTRACT: The majority of bioengineering strategies to promote peripheral nerve regeneration after injury have focused on therapies to bridge large nerve defects while fewer therapies are being developed to treat other nerve injuries, such as nerve transection. We constructed delivery systems using fibrin gels containing either free GDNF or polylactide-glycolic acid (PLGA) microspheres with GDNF to treat delayed nerve repair, where ELISA verified GDNF release. We determined the formulation of microspheres containing GDNF that optimized nerve regeneration and functional recovery in a rat model of delayed nerve repair. Experimental groups underwent delayed nerve repair and treatment with GDNF microspheres in fibrin glue at the repair site or control treatments (empty microspheres or free GDNF without microspheres). Contractile muscle force, muscle mass, and MUNE were measured 12 weeks following treatment, where GDNF microspheres (2 weeks formulation) were superior compared to either no GDNF or short-term release of free GDNF to nerve. Nerve histology distal to the repair site demonstrated increased axon counts and fiber diameters due to GDNF microspheres (2 weeks formula-

tion). GDNF microspheres partially reversed the deleterious effects of chronic nerve injury, and recovery was slightly favored with the 2 weeks formulation compared to the 4 weeks formulation.

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Introduction

Despite improved surgical techniques, recovery is generally limited after nerve injury in the peripheral nervous system. Delays in nerve repair in patients experiencing Type IV or V (complete injuries) requiring direct end-to-end nerve repair lead to poorer outcomes (Yegiyants et al., 2010). Such delays further compromise recovery because the neurons' capabilities to regenerate their axons diminishes due to the prolonged periods of disconnection with end-organ targets (chronic axotomy) and chronic denervation of Schwann cells in denervated distal nerve stumps (Fu and Gordon, 1995a,b; Gordon et al., 2011; Wood et al., 2011). Addressing the deleterious effects of chronic axotomy and denervation (as incurred when delayed nerve repairs are performed) holds promise as a means of removing one of the major barriers to improving nerve regeneration.

Growth factors are known to influence nerve regeneration and may be a source of modality-specific influence to nerve regeneration. Motoneurons (MNs) and their axons express

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receptors for different growth factors including trkB and p75 receptors for brain-derived neurotrophic factor (BDNF) and GDNFR α 1 and RET receptors for glial cell-line derived neurotrophic factor (GDNF; Boyd and Gordon, 2003b). After nerve injury, these growth factors are upregulated in Schwann cells in the distal stump while their receptors are upregulated within the neurons and proximal axons. However, the upregulation returns to normal levels of these factors after 1–2 months (Boyd and Gordon, 2003b). This transient neurotrophic support explains, at least in part, why axonal regeneration after delayed nerve repair has reduced regenerative capacity (Boyd and Gordon, 2003b; Hoke et al., 2002). Exogenous sources of the neurotrophic factors can enhance recovery following chronic axotomy; GDNF is particularly effective (Boyd and Gordon, 2003b).

Local delivery of GDNF promotes axonal regeneration of MNs following chronic axotomy (Boyd and Gordon, 2003a). Whilst this finding is promising, the delivery method of using a silicone tube and mini-osmotic ALZET pump is not ideal clinically because of risks of infection, extrusion and inducing a fibrotic foreign body response in the adjacent injured nerve (Guilhem et al., 2009). Ideally, alternative devices that localize extended drug delivery to the nerve injury site, without risk of chronic nerve compression secondary to capsular fibrosis, (Lundborg, 2000) and are biodegradable would avoid these other problems.

For clinical application to nerve repair surgery, a delivery system must supply neurotrophic support for sufficient time to optimize axon regeneration and target reinnervation. Therefore, depending on the neurotrophic factor, as brief as a week or as long as several months delivery would be required (Boyd and Gordon, 2003b). Natural biomaterials, such as fibrin gels, work well as peripheral nerve drug delivery systems due to their ease of placement (Jubran and Widenfalk, 2003) and lack of inhibitory effects on nerve regeneration (Sameem et al., 2011). Their utility as extended-release delivery devices is limited, however, because typically, drug release is limited to a few days in vivo (Jubran and Widenfalk, 2003; Wood et al., 2009, 2010, 2012). By combining materials, such as polymer microspheres, with fibrin gels or glue to construct a drug delivery device may allow tailoring for drug release profiles ranging from days, weeks, or months (Baumann et al., 2009; Garbayo et al., 2008, 2009; Wood et al., 2012). Poly(lactide-co-glycolide) (PLGA) microspheres have previously been

constructed to encapsulate biologically active GDNF (Garbayo et al., 2008, 2009; Kokai et al., 2010, 2011), have been incorporated into various implantable gels for drug release (Baumann et al., 2009; Lin et al., 2012; Wood et al., 2012), and have been used previously in peripheral nerve injuries (Kokai et al., 2010, 2011; Lin et al., 2012) making PLGA microspheres an ideal candidate to support controlled and extended drug release.

We previously designed a drug delivery system consisting of fibrin glue containing GDNF microspheres to successfully improve nerve regeneration. Motor nerve regeneration was significantly improved after delayed repair of a transection injury as measured by retrograde labeling of regenerating motor axons (Wood et al., 2012, 2013). The previous work also demonstrated the degradation rate of the delivery system (Wood et al., 2013). In this study, we designed microspheres (MSs) of variable formulations and implanted them locally in vivo to determine the effect on nerve regeneration at a much greater time point which would allow for functional recovery. Thereby we determined the optimal GDNF microsphere formulation to improve nerve regeneration and functional reinnervation of muscle in a delayed nerve repair animal model.

Materials and Methods

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

Microsphere Fabrication and Characterization

PLGA microspheres 50/50 (Wako, Japan and Lactel Absorbable Polymers, Cupertino, CA) were prepared by a W/O/W double emulsion procedure as described previously (Wood et al., 2013). Briefly, microspheres (MS) were prepared with an inner aqueous phase of 100 μ L of ddH₂O, 12.5 mg of heparin (Sigma–Aldrich product #H3393) to stabilize GDNF (Perets et al., 2003), 12.5 mg of MgCO₃, and with or without 250 or 500 μ g of GDNF (Peprotech, Rocky Hill, NJ; Table I). PLGA (230 mg) was dissolved in 1 mL of dichloromethane (DCM)/acetone (75%/25%), and the two solutions were emulsified under sonication (Vibracell VCX 130, Sonics and Materials) for

Table I. Experimental groups and PLGA (50/50) microsphere (MS) characteristics.

Group description or formulation name [group names used in rat model]	PLGA inherent viscosities (dL/g)	PLGA average molecular weight (Da)	PLGA quantity	GDNF initial loading (%wt/wt in microspheres)	Encapsulation efficiency
Empty microspheres [MS control]	0.15–0.25	6,700	100%	0 μ g (N/A)	N/A
Free GDNF [GDNF control]	N/A (no MS)	N/A (no MS)	None	250 μ g (N/A)	N/A
Formulation 1 [not tested in rat]	0.24–0.54	12,900	100%	500 μ g (0.10%)	80 \pm 5%
Formulation 2 [not tested in rat]	0.15–0.25, 0.24–0.54	6,700, 12,900	75%, 25%	500 μ g (0.10%)	88 \pm 2%
Formulation 3 [GDNF MS (4 weeks)]	0.15–0.25	6,700	100%	500 μ g (0.10%)	80 \pm 4%
Formulation 4 [GDNF MS (2 weeks)]	0.088–0.102	5,000	100%	250 μ g (0.05%)	78 \pm 3%

N/A indicates not applicable. All groups were embedded into a fibrin gel at later testing.

45 s using a 3 mm probe at 30% amplitude. The emulsion was immediately added to 25 mL of 2.5% polyvinyl alcohol (PVA) solution containing 10% NaCl and homogenized at 6,000 rpm for 60 s. The entire mixture was poured into a 250 mL bath of 0.25% PVA solution containing 10% NaCl under magnetic stirring at 125 rpm for 3 h. The hardened microspheres were collected and washed by ddH₂O using centrifugation (1,500 rpm at 5 min) for at least 5 cycles, with a total wash volume of 1,200 mL. The microspheres were collected for lyophilization in a conical tube containing the formulation of microspheres and 4 mL of ddH₂O. The microspheres were lyophilized in 4 mL of ddH₂O snap frozen in liquid nitrogen ($\sim -60^{\circ}\text{C}$) in a vented conical tube. They were stored at -20°C until use.

Microsphere mean diameter and size distribution was measured via static light scattering using a Malvern Mastersizer 2000 laser diffraction particle sizer (Malvern Instruments Ltd., Worcestershire, UK), using refractive indices of 1.33 and 1.59 for water and PLGA, respectively. The amount of GDNF incorporated into the microspheres 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.

To measure encapsulation efficiency, 5 mg of microspheres were placed in siliconized centrifuge tubes (Fisher Scientific, Toronto, ON) and 500 μL of DCM and 500 L of ddH₂O were added. The mixture was vortexed for 5 min and then spun down on a centrifuge at 1,000 rpm for 5 min. The supernatant (ddH₂O with GDNF) was removed and more volume (500 μL ddH₂O) was added. The process was repeated four times. The accumulated supernatant was measured using ELISA. The encapsulation efficiency was determined based on the experimental mass of protein in the microspheres compared to the theoretical mass (from 5 mg of microspheres).

Fibrin Gel Construction and In Vitro Release

Fibrin gels were constructed by mixing equal parts fibrinogen (75–115 mg/mL, 40 μL) and thrombin (500 IU/mL, 40 μL) obtained from a Tisseel glue kit (Tisseel[®], Baxter Healthcare, Deerfield, IL), resuspended according to the manufacturer's instructions. Fibrin gels were loaded with microspheres or GDNF by incorporating 5 mg of microspheres or 5 μg of GDNF, respectively, into the thrombin solution before it was mixed with fibrinogen to form a gel.

Release of GDNF from fibrin loaded with or without microspheres was performed by constructing 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

replaced over 15 days and collected samples were stored at -20°C . ELISA assays were performed to measure GDNF quantity collected from the time course release studies.

Experimental Animals

Adult female Sprague–Dawley rats (Harlan, Indianapolis, IN), each weighing 250–300 g were used in this study. All surgical procedures and peri-operative 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.

Experimental Design

Thirty rats were randomized into five groups ($n = 6$). The loading or drug dose of GDNF for groups is described in Table I. Fibrin gels loaded without GDNF but with empty microspheres (MS control) or free GDNF without microspheres (GDNF control, 250 μg of GDNF) served as experimental control groups. Fibrin gels loaded with microspheres releasing GDNF in vitro for ~ 2 or ~ 4 weeks served as the primary experimental groups (GDNF MS [2 weeks] and GDNF MS [4 weeks]; 250 and 500 μg of GDNF, respectively; Fig. 1). Rats without any fibrin gels, microspheres, or GDNF and without any prior procedures (transection and tie back of the nerve stumps) served as the positive control group (immediate repair). Previous studies demonstrated no difference in regeneration following delayed nerve repair with or without a fibrin gel and microspheres (Wood et al., 2012), therefore, no control group for delayed repair without a fibrin gel and microspheres was used. Additionally, the empty microsphere group was made from the PLGA used in constructing the GDNF MS (4 weeks) group.

Operative Procedure

All surgical procedures were performed using aseptic technique. Three percent isoflurane gas anesthesia was used for animal induction followed by 2.5% isoflurane gas for maintenance. The hind leg of the rat was prepared and the sciatic nerve was exposed through a dorsolateral–gluteal muscle splitting incision. In both procedures, wounds were irrigated with saline, dried, and closed in two layers, utilizing 5-0 Vicryl suture to close the muscle layers, and 4-0 nylon suture to close the skin. Experimental animals were recovered in a warm environment prior to returning to the housing facility.

In the first procedure, the common fibular (CF) nerve was dissected free and transected ~ 5 mm from the sciatic trifurcation. Experimental groups receiving delayed CF nerve repair had their CF stumps sutured back to surrounding muscle for 2 months (MS control, GDNF control, GDNF MS groups). Animals in the positive control group (Immediate repair) did not undergo any procedures

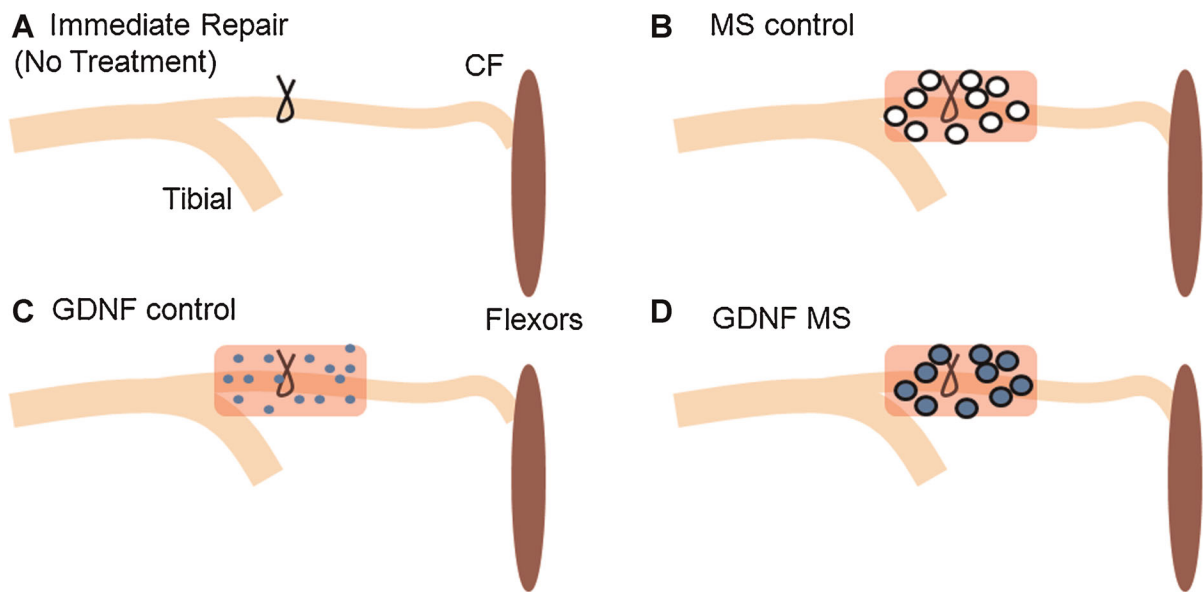


Figure 1. Experimental treatments implanted following initial nerve injury. The common fibular (CF) nerve was cut and sutured back to surrounding muscle 2 months prior to the above shown repair and placement of experimental groups. After the initial injury, the nerve ends were reconnected with epineural suture repair followed by placement of experimental groups as applicable. A positive control group did not receive any initial injury and did not receive a fibrin gel (immediate repair; **A**). Experimental groups consisted of fibrin gels loaded with either: empty microspheres (MS control; **B**), no microspheres but free GDNF (GDNF control; **C**), or microspheres containing GDNF (GDNF MS [2 weeks] or GDNF MS [4 weeks]; **D**). Twelve weeks following nerve repair muscles were analyzed for contractile force and nerve was harvested for histology.

at this time. In the second procedure, CF nerves were exposed as before and the nerve stumps were repaired using 10-0 nylon sutures. In applicable groups, the nerve was surrounded by two 40 μ L gels, formed by pipetting the fibrin mixture, before setting as a gel, onto Parafilm as semi-rectangular drops ($\sim 5 \text{ mm} \times 1 \text{ mm}$). The gel drops were placed centered above and below the repair site and secured by gently pressing the gel drops toward one another. Animals in the positive control group instead had their CF nerve transected $\sim 5 \text{ mm}$ from the sciatic trifurcation and immediately repaired with nylon sutures and no fibrin gels.

Force and Motor Unit Number Estimation (MUNE) Analysis and Muscle Harvesting

Twelve weeks postoperatively, CF motor nerve function was assessed by examining the motor response in reinnervated EDL muscle upon stimulation of the CF nerve, as described previously (Wood et al., 2010). Briefly, all animals were re-anesthetized and nerve branches isolated. The distal portion of the EDL muscle and tendons were attached to a 5 N thin film load cell (S100, Strain Measurement Devices, Inc., Meriden, CT) using a steel S-hook. A custom-designed force measurement jig (Red Rock Laboratories, Inc., St. Louis, MO) was used to immobilize the leg and deliver cathodic, monophasic electrical impulses (duration = 200 μ s, frequency = 0–200 Hz, amplitude = 0–3 V) using a single-channel isolated pulse stimulator (Model 2100, A-M Systems, Inc., Carlsborg, WA) to the proximal CF nerve

via bipolar silver wire electrodes. Resulting active force output was recorded on PC with custom Matlab software (The Mathworks, Inc., Natick, MA). Twitch contractions measured using the custom force recording system were utilized to determine the optimal stimulus amplitude and muscle length (L_0) for use in all recordings of isometric force production in the EDL muscle. Tetanic contractions were recorded by delivering 300 μ s bursts of increasing frequency (5–200 Hz) to the CF nerve and maximum isometric tetanic force was calculated from the active force plateau. MUNE was calculated by running a protocol adapted from Major et al. whereby twitch contractions were recorded over a range of stimulation amplitudes to measure the MUNE value for the muscle (Major and Jones, 2005; Major et al., 2007). Healthy, uninjured CF nerves and EDL muscles were similarly tested and evaluated. The muscle mass of both the injured and uninjured EDL muscles were harvested after testing and weighed. The injured muscle mass was normalized to the uninjured muscle mass to determine the relative muscle mass and level of muscle atrophy following injury.

Histology and Morphometric Evaluation of Nerves

For general histology and morphometric analysis, nerve tissue was taken 20 mm distal to the repair site immediately following functional assessment described above and fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, ethanol dehydrated, and embedded in Araldite 502

(Polyscience, Inc., Warrington, PA). Thin (0.6 μm) sections were made from the tissue using a LKB II Ultramicrotome (LKB-Produkter A.B., Bromma, 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 1,000 \times overall magnification, the entire nerve cross-section was captured and evaluated with image analysis software (Image-Pro Analyzer version 7.0, Media Cybernetics, Bethesda, MD) using a custom designed macro, based on previous methods (Hunter et al., 2007; Wood et al., 2013), to measure nerve morphometry. From the entire imaged nerve cross-section, the number of myelinated axons, fiber size and distribution, and myelination thickness were measured.

Statistical Analysis

Means with standard error of the mean are reported. Statistical analysis was evaluated first to confirm the normality of data and then using Analysis of Variance (ANOVA) with post hoc tests including Bonferroni correction for determining differences between groups, where $P < 0.05$ was considered statistically significant. MUNE cumulative distributions were compared pair-wise using the Kolmogorov–Smirnov test, where $P < 0.05$ was considered statistically significant.

Results and Discussion

In Vitro Characterization of Microspheres and GDNF Release From Fibrin Gels

MSs in this study were designed to release in vitro for relatively short duration (~ 2 –4 weeks), as others have found that long term release of GDNF in vivo can be problematic due to biological degradation and, in addition, can lead to entrapment of motor axons in the region of GDNF (Eggers et al., 2008; Tannemaat et al., 2008). MS formulations were constructed with heparin (5% wt/wt), MgCO_3 (3% wt/wt), GDNF (0.05–0.1% wt/wt), and varying molecular weight PLGA (Table I). All microsphere formulations demonstrated high encapsulation efficiencies (78–88%) and were of an average size between 40 and 60 μm , comparable to other PLGA double emulsion systems (Garbayo et al., 2008, 2009). Fibrin gels with or without microspheres were loaded during fibrin polymerization into PBS (pH 7.4, containing 0.1% BSA). The dynamic release of GDNF from these fibrin gels was followed in vitro for 28 days at 37°C. None of the gels with microspheres demonstrated a large initial burst (1st 24 h of release) of GDNF release (Fig. 2). This is likely due to the high concentration of fibrinogen within the fibrin gels (~ 50 mg/mL), as the decreased porosity of the fibrin network would limit diffusion (Blombäck et al., 1984; Carr and Hardin, 1987). GDNF release from fibrin gels loaded with any of the constructed microspheres containing GDNF (formulations 1–4) was slower than from the fibrin gels

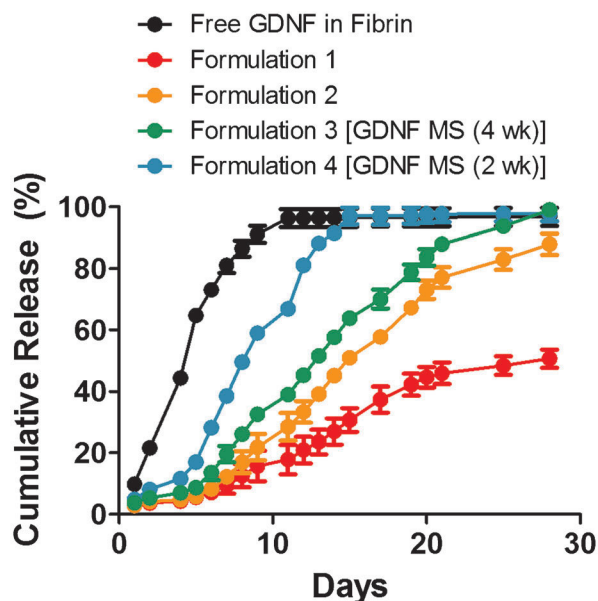


Figure 2. GDNF release in vitro from various formulations of microspheres. The release of GDNF from fibrin gels loaded with microspheres containing GDNF was generally decreased by constructing microspheres with higher PLGA inherent viscosities or molecular weights (formulations 1 > 2 > 3 > 4; also see Table I). Microspheres used as experimental groups to be tested in rats were chosen (formulations 4 and 3) based upon their release of GDNF for 2–4 weeks in vitro, respectively. Data ($n = 4$) represent mean \pm SEM and the composition of the microsphere formulations can be found in Table I.

loaded with free GDNF (free GDNF in fibrin—control; $P < 0.05$) except at Day 1. The release duration of GDNF from the fibrin gels loaded with microspheres containing GDNF was generally decreased by constructing microspheres with lower PLGA inherent viscosities or molecular weights (formulations 1 > 2 > 3 > 4) based upon their release of GDNF for 2–4 weeks (Fig. 2). This effect has been demonstrated by others using different encapsulated proteins in microspheres (Yeo and Park, 2004).

Of the formulations constructed, two formulations (formulations 3 and 4) fitted in vitro release profiles that would lead to 2 and 4 weeks release in vitro (Fig. 2). Formulations 1 and 2 were not chosen to be tested in our rat injury model as the release may be too slow to avoid in vivo biological degradation and were not considered for further analysis. Additionally, we determined using ELISA that formulations 3 and 4 would deliver ≥ 0.1 μg GDNF/day (in vitro), thus the initial loading differences between the formulations (data not shown). At least 0.1 μg GDNF/day, measured in vitro by ELISA, is necessary to promote improved nerve regeneration, determined previously using mini-osmotic pumps (Boyd and Gordon, 2003a).

Functional Muscle Assessment

Functional recovery was assessed by isometric contractile muscle force in the EDL 12 weeks after delayed nerve

repair with or without the implantation of fibrin gels. MUNE was performed to determine the extent and quantity of EDL reinnervation by MNs. For the immediate nerve repair the mean MUNE was 38 ± 3 and not significantly different from MUNE for the unoperated EDL muscle (48 ± 5 ; Fig. 3A). MUNE was significantly reduced as anticipated for the experimental conditions of chronic nerve axotomy and denervation when the CF nerve was cut and the proximal and distal nerve stumps ligated 8 weeks prior to delayed resuture of the nerve stumps (Fu and Gordon, 1995a,b). Both the MS control and GDNF control groups had substantially diminished MUNEs, 11 ± 1 for MS control and 17 ± 2 for GDNF control, which were not different amongst themselves ($P > 0.05$). The GDNF MS groups both had greater MUNE values than the MS control, 24 ± 2 for GDNF MS (2 weeks) and 22 ± 1 for GDNF MS

(4 weeks), but were not different directly from one another or the GDNF control ($P > 0.05$), corresponding well with previous MN retrograde labeling data (Wood et al., 2013).

The average motor unit twitch production was the same in all of the experimental groups in which microspheres were implanted with or without GDNF within the gel; it was not different from the immediate repair control group either (~ 9 – 12 mN; $P > 0.05$). These findings indicate that the differences in contractile muscle force and in the MUNE values could be attributed only to changes in the numbers of MNs that reinnervated the denervated EDL rather than a compensatory enlargement of reinnervated motor units within the 12 week period of nerve regeneration and muscle reinnervation. Cumulative distribution of the individual motor unit twitch forces revealed all experimental groups'

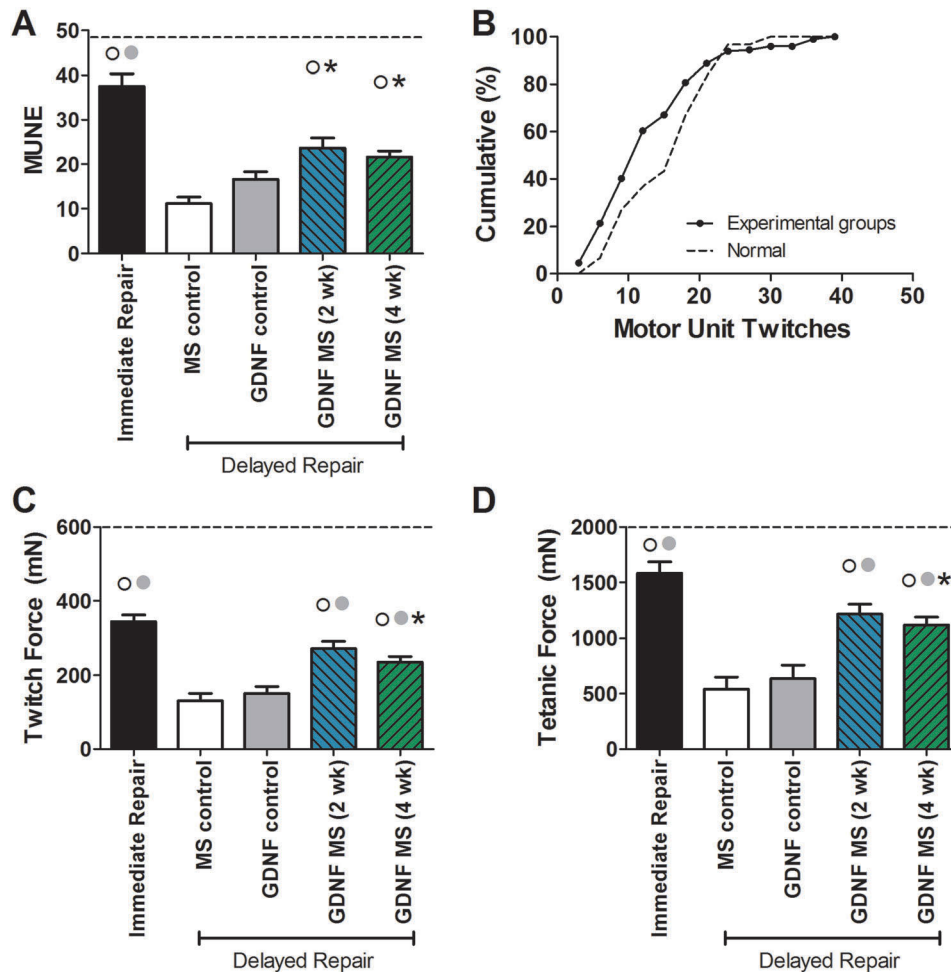


Figure 3. Contractile force measurements and MUNE of EDL muscle 12 weeks following experimental treatment. EDL muscles were stimulated proximal to the repair site to produce maximal twitch and tetanic muscle force and to measure MUNE. Experimental groups with GDNF MSs increased the estimated number of motor units (MUNE) (A) compared to no GDNF treatment (MS control) while not affecting the motor unit twitch force (B), which indicates an increase in the number of motor neurons that regenerated their axons and made functional connections to their targets. This experimental treatment also increased twitch (C) and tetanic force (D) compared to both control groups (MS control and GDNF control). Data ($n=6$) represent mean \pm SEM and * indicates statistical significance compared to Immediate Repair, \circ compared to MS control, and \bullet compared to GDNF control ($P < 0.05$). Normal, uninjured values are represented by the dashed line.

cumulative motor unit forces (data shown as pooled since all groups were statistically similar) had a marked statistically significant shift to the left compared to the normal distribution in the muscle demonstrating early reinnervation of the muscle and immaturity to full muscle recovery ($P < 0.05$; Fig. 3B). Overall, the MUNE data for the experimental groups generally agree with previous counts of retrograde labeled MNs that regenerated their axons 20 mm distal to the delayed repair site in the presence of GDNF microspheres contained within fibrin gels (Wood et al., 2013).

Supramaximal stimulation of the normal, uninjured CF nerve elicited maximal twitch and tetanic forces in the EDL muscle of 600 ± 20 and $2,000 \pm 100$ mN, respectively. The reinnervated EDL muscle after immediate nerve repair and 12 weeks regeneration, recovered twitch and tetanic forces of 340 ± 20 mN ($\sim 60\%$ of uninjured force production) and $1,600 \pm 100$ mN ($\sim 80\%$ of uninjured force production), respectively (Fig. 3C and D). Delayed nerve repair groups (all other experimental groups) experienced substantially reduced muscle force production. Both the MS control and GDNF control groups had substantially diminished twitch and tetanic forces, 130–150 and 540–640 mN, respectively, compared to the immediate repair group. There were no differences between the controls ($P > 0.05$). The placement of GDNF microspheres (GDNF MS [2 weeks] and [4 weeks] groups) improved muscle force production, as twitch and tetanic forces were greater than both controls ($P < 0.05$). However, the GDNF MS (4 weeks) group did not match the immediate repair group in muscle force production ($P < 0.05$), twitch (240 ± 20 mN) and tetanic ($1,100 \pm 70$ mN). Only the GDNF MS (2 weeks)

group was able to match the immediate repair group in both force metrics ($P > 0.05$), twitch (270 ± 20 mN) and tetanic ($1,200 \pm 90$ mN), but the GDNF MS groups were not directly different from one another ($P > 0.05$). Taken together with previous observed increases in MN regeneration (Wood et al., 2013) and current observations of increased MUNE, GDNF microspheres were anticipated to increase contractile muscle force.

Muscle Mass and Atrophy

After completion of functional muscle assessments, the EDL and tibialis anterior (TA) innervated by the CF nerve were harvested and weighed to determine the level of muscle atrophy or recovery experienced due to loss of axons innervating the muscle originally. The experimental side was compared to the uninjured side to determine the relative muscle mass ratio. Immediate repair significantly promoted muscle mass recovery of the EDL muscle mass as it was $94 \pm 5\%$ of the contralateral mass (Fig. 4A). No other experimental group could match this result ($P < 0.05$). The control groups (MS control and GDNF control) lost significant muscle mass compared to the immediate repair group as they were now at $55 \pm 5\%$ and $65 \pm 4\%$ muscle mass. The GDNF MS (2 weeks) group recovered muscle mass as $74 \pm 3\%$ of mass remained making it greater than the MS control ($P < 0.05$). GDNF MS (4 weeks) did not recover more muscle mass than either control at $70 \pm 2\%$ of relative mass. The TA muscle mass recovered similar to the EDL muscle mass in all experimental groups. The immediate repair group recovered to $73 \pm 2\%$ of relative muscle mass

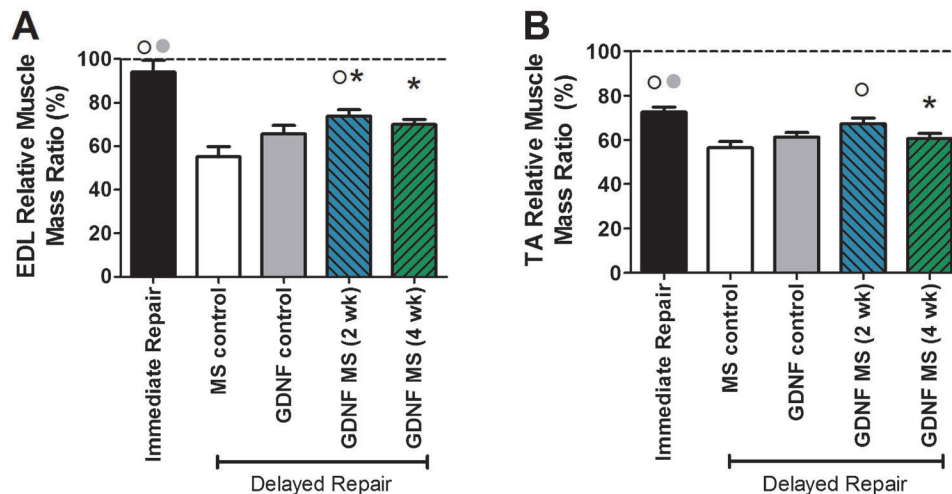


Figure 4. Relative muscle mass of EDL and TA 12 weeks following experimental treatment. EDL and TA muscles (experimental and contralateral sides) were harvested, weighed after force testing, and the experimental mass normalized to the contralateral mass. Experimental groups with GDNF MS delivering GDNF for 2 weeks (GDNF MS (2 weeks)) demonstrated slight improvement in muscle mass, as the GDNF MS (2 weeks) group was greater than the MS control group in relative mass for both the EDL (A) and TA (B) muscles. The GDNF MS (4 weeks) group demonstrated no improvement over the controls. Data ($n = 6$) represent mean \pm SEM and * indicates statistical significance compared to Immediate Repair, ○ compared to MS control, and ● compared to GDNF control ($P < 0.05$). Normal, uninjured values are represented by the dashed line.

(Fig. 4B). The immediate repair group was again greatly recovered from atrophy compared to most other experimental groups, except the GDNF MS (2 weeks) group which was statistically equivalent at $67 \pm 3\%$ of muscle mass. Both control groups were substantially decreased at $56 \pm 3\%$ and $61 \pm 2\%$ muscle mass. Again, the GDNF MS (2 weeks) group was greater than the MS control, while the GDNF MS (4 weeks) group ($61 \pm 2\%$) was similar to the control groups in muscle mass. The similar muscle masses between the GDNF MS (4 weeks) and control groups may be attributed to the reduced myelinated axon counts in the GDNF MS (4 weeks) group (Fig. 5), as greater axon numbers innervating a muscle would provide a protective and recovery effect to the muscle, decreasing muscle atrophy.

In Vivo Nerve Histology and Morphometric Measures of Regeneration

Twelve weeks following nerve repair with or without implantation of fibrin gels and after force analysis described above, nerve was harvested 20 mm distal to the repair site for histological analysis by light microscopy. Axons were uniformly distributed throughout the nerve in all groups, regardless of treatment. The entire nerve cross-section was quantitatively evaluated using histomorphometric measures of nerve regeneration. There was a significant increase in the number of myelinated axons in the GDNF-MS (2 weeks) and immediate repair groups compared to the MS control group ($\sim 3,000$ vs. 1,500; Fig. 5A). This approximate twofold

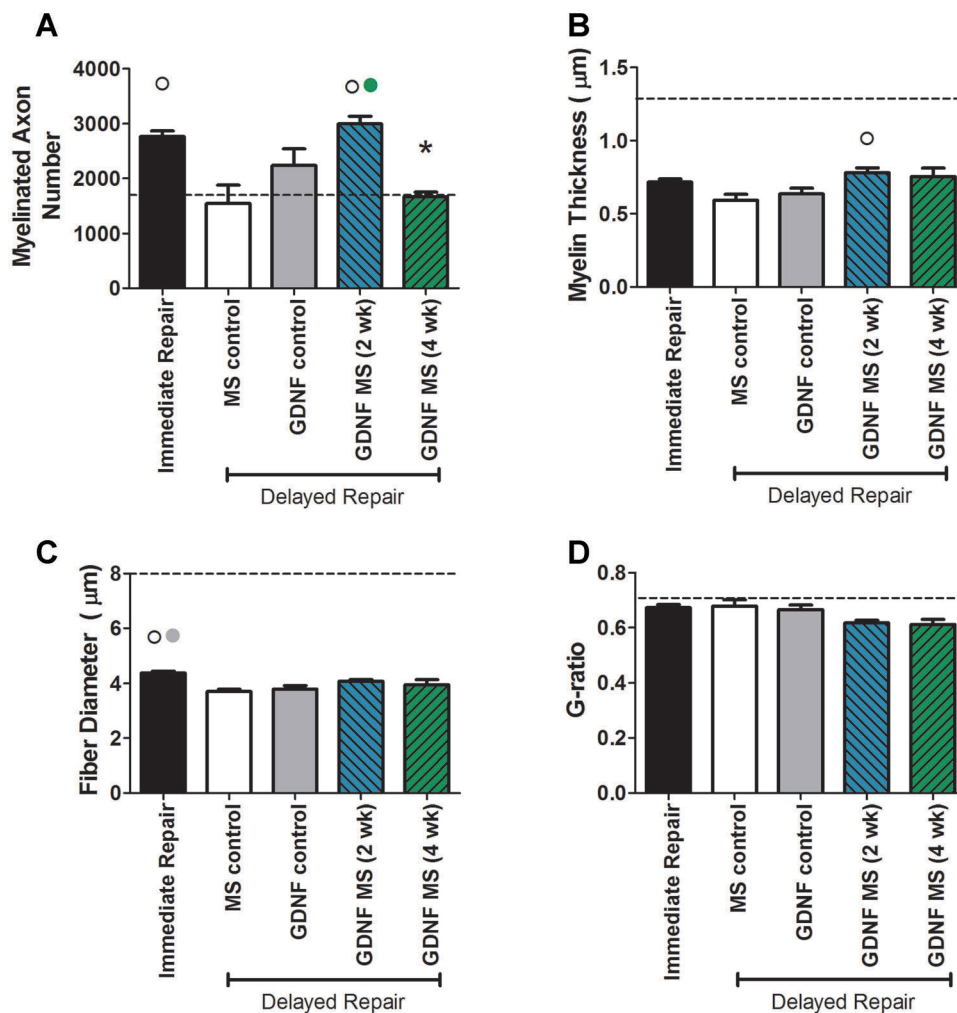


Figure 5. Histomorphometric analysis of nerves 20 mm distal to the repair site 12 weeks following experimental treatment. Total number of myelinated nerve fibers (A), myelin thickness (B), fiber diameter (C), and G-ratio (D) were measured by quantitative histomorphometry immediately following functional motor assessment. The immediate repair and GDNF MS (2 weeks) groups had increased axon counts compared to the MS control and GDNF MS (4 weeks) groups (A). Only the GDNF MS (2 weeks) group had increased myelination compared to the MS control group (B), while both the GDNF MS (2 weeks) and (4 weeks) had fiber diameters that matched the immediate repair group ($P > 0.05$) (C). No groups contained differences in G-ratios (D). Data ($n = 6$) represent mean \pm SEM and * indicates statistical significance compared to Immediate Repair, \circ compared to MS control, \bullet compared to GDNF control, and \bullet compared to GDNF MS (4 weeks; $P < 0.05$). Normal, uninjured values are represented by the dashed line.

increase in axon counts for both the GDNF–MS (2 weeks) and immediate repair groups was also statistically significant compared to the GDNF–MS (4 weeks) group. The GDNF–MS (2 weeks) group also statistically increased the degree of myelination of regenerating axons compared to the MS control group, suggesting a more rapid maturation of the regenerating axons (Aitken, 1949; Aitken et al., 1947) due to GDNF delivery (Fig. 5B). This degree of increased myelination over the control group was not seen in the immediate repair or GDNF MS (4 weeks) groups, suggesting an effect due to GDNF microsphere formulation. Additionally, overall nerve fiber diameters were statistically equivalent to the immediate repair group in both GDNF MS groups but not in either control groups ($P > 0.05$; Fig. 5C). However, the overall size of regenerating nerve fibers was greatest in the immediate repair group, as overall nerve fiber diameters were statistically increased compared to both the MS control and GDNF control groups. The GDNF control was not statistically different in any of these metrics compared to the MS control group ($P > 0.05$) and was inferior to the immediate repair group in nerve fiber diameter ($P < 0.05$). The axon diameter to degree of overall nerve fiber size (represented by the G-ratio) was equal in all groups ($P > 0.05$) and approaching the normal range between 0.6 and 0.7, suggesting all groups were regenerating nerve fibers that would properly conduct signals to their end-organ targets (Fig. 5D).

Conclusions

In summary, the goal of this study was to evaluate the effects of GDNF microspheres formulation on nerve regeneration and functional motor recovery in a delayed repair nerve injury model. We found that GDNF microspheres improved axonal regeneration and hastened the maturation of the regenerated axons. It also improved functional motor outcomes, such as improved contractile muscle force and preservation of muscle mass. While there was a slight advantage to GDNF microsphere formulations of 2 weeks in vitro, ultimately, GDNF microspheres, promoted improved nerve regeneration compared to a lack of GDNF for the treatment of delayed nerve repair. The different GDNF microsphere formulations affected nerve regeneration, which may be due to differences in release duration in vivo or in biological activity or degradation of the GDNF released in vivo (the dose of GDNF). However, we can only speculate, as we did not specifically study in vivo delivery of GDNF from our microspheres to the nerve tissue. Overall, the findings demonstrate the effectiveness of microsphere technology to encapsulate neurotrophic factors for placement at a nerve injury site following chronic axotomy and chronic denervation (delayed nerve repair).

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