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# Fibrin gels containing GDNF microspheres increase axonal regeneration after delayed peripheral nerve repair

**Aim:** Recovery following nerve transection declines when target reconnection is delayed for prolonged periods. GDNF has previously been shown to promote motor axon regeneration following delayed nerve repair. **Materials & methods:** We constructed delivery systems using fibrin gels containing free GDNF or poly(lactide-co-glycolide) microspheres with GDNF. The delivery systems were implanted with fluorescent fibrinogen surrounding the common fibular (CF; peroneal) nerve in transgenic Thy-1 GFP rats (whose axons express GFP) to track degradation of the system. A delayed nerve repair model was designed by transecting the rat CF nerve, where nerve regeneration was prevented by ligating the two stumps to surrounding muscle for 2 months prior to resuture. At resuture, either a delivery system with GDNF or an additional group consisting of fibrin gels with empty microspheres were implanted surrounding the repair site. In an additional positive control, the CF was transected and repaired immediately without delay. **Results:** ELISA assays demonstrated GDNF release *in vitro* for 2 weeks from fibrin gels with GDNF microspheres. Implanted delivery systems, including GDNF microspheres, remained surrounding the nerve for at least 10 days compared with 3 days for free GDNF. Four weeks after repair, histomorphometry of distal nerve cross-sections taken 20 mm from the repair site demonstrated increased fiber diameter and myelin thickness due to release of GDNF from microspheres compared with empty microspheres. Additionally, the number of motoneurons that regenerated their axons to the same site increased to comparable levels as immediate repair due to the extended delivery of GDNF from microspheres. **Conclusion:** These findings demonstrate that early measures of nerve regeneration after delayed nerve repair is improved by GDNF microspheres implanted at the coaptation site.

**KEYWORDS:** chronic axotomy ■ chronic denervation ■ common fibular nerve ■ drug delivery ■ fibrinogen ■ glial cell line-derived neurotrophic factor ■ nerve injury ■ regenerative medicine

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Peripheral nerve injury often results in a devastating loss of function and neuropathic pain [1,2]. However, nerves are capable of limited regeneration when the proximal nerve stump (which remains connected to the neuronal soma) and distal end (leading to the end-organ) of a severed nerve are sutured together [3,4]. Commonly, nerves are repaired by directly suturing the severed nerve ends together (direct repair); however, the clinical outcomes often remain unsatisfactory [1,5], especially in cases of nerve repairs that are delayed beyond 1 month [1,5,6].

Delays in nerve repair lead to states of chronic axotomy and chronic denervation, as neurons are no longer connected to their end-organ targets (chronic axotomy) and Schwann cells are no longer in contact with axonal processes (chronic denervation). In studies in which motoneurons were either chronically axotomized or distal nerve stumps chronically denervated for periods of up to 520 days prior to surgical repair, Gordon and colleagues demonstrated that chronic axotomy and Schwann cell denervation each result in progressive failure of the neurons

to regenerate their axons and to reinnervate denervated muscle [7–10]. The transient nature of the upregulation of neurotrophic factors and their receptors (including GDNF and its receptors) in axotomized neurons and in denervated Schwann cells is, at least in part, responsible for the reduced capacity for axonal regeneration after delayed nerve repair [11,12].

Delivery of growth factors to the suture site via silastic tubes connected to mini-osmotic pumps is beneficial to nerve regeneration after delayed nerve repair, but not after immediate nerve repair [13,14]. However, delivery of exogenous sources of growth factors by pumps and other permanent implantable delivery devices can fail, become infected, extrude or induce a local inflammatory response, leading to complications in a clinical situation [15]. Additionally, tubes placed around nerves to localize the delivery of the factors frequently require removal because they may lead to chronic nerve compression secondary to capsular fibrosis [1]. GDNF delivery from Schwann cells transfected with lentiviruses leading to overexpression has limitations, as excess

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and semipermanent GDNF production causes entrapment of axons in areas of concentrated GDNF and leads to neuroma formation [16]. Therefore, other more transient drug delivery strategies, such as degradable biomaterials capable of drug delivery, may be better alternatives for controlled release of growth factors at the nerve repair site.

Fibrin gels or glue have served as natural biomaterial drug-delivery systems at sites of nerve repair, as fibrin glue has delivered both GDNF and NGF for immediate nerve injury treatment [17]. As fibrin can be placed near an injury site, it can serve as a local drug-delivery system for growth factors. Moreover, fibrin has been widely considered to be a possible alternative to suture repair of nerves, as it does not affect peripheral nerve regeneration when used at the suture site [18]. However, fibrin glue and growth factor alone as an extended drug-delivery system is limited for peripheral nerve repair, as growth factor release typically occurs for only a few days *in vivo* [17], rather than the more lengthy periods required to administer growth factors (e.g., 4 weeks) [14]. Poly(lactide-co-glycolide) (PLGA) microspheres (MS) or particles have been used to encapsulate biologically active GDNF [19,20] and have been incorporated into various implantable gels for drug release [21,22]. Therefore, inclusion of MS or particles containing growth factors in a fibrin gel may be of benefit to extend the period of local growth factor release at the nerve repair site.

In this work, we implanted fibrin gels loaded with PLGA MS containing GDNF at a delayed nerve repair site. This novel delivery system may serve to provide local neurotrophic support to regenerating axons and their neurons that, without the support, was significantly reduced from that after immediate nerve repair. This study demonstrates the advantages of GDNF MS at a delayed nerve repair site. The use of MS slowed the rate of GDNF release *in vitro* and delayed the degradation of the delivery system *in vivo* compared with fibrin gels loaded with free GDNF. As a result, fibrin gels containing GDNF MS implanted at the coaptation site *in vivo* improved metrics of early nerve regeneration, as measured by histomorphometry and motoneuron regeneration.

## Materials & methods

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

### ■ MS fabrication & characterization

PLGA 50/50 (Wako, Japan; 0.088–0.120 dl/g inherent viscosity in hexafluoroisopropanol) MS

were prepared by a W/O/W double-emulsion procedure. A solution of 100  $\mu$ l of ddH<sub>2</sub>O, 12.5 mg of heparin (Sigma-Aldrich product #H3393), 12.5 mg of MgCO<sub>3</sub> and 125  $\mu$ g of GDNF (Peprotech, NJ, USA; when applicable) was mixed with a solution of PLGA. The PLGA solution was made by dissolving PLGA (230 mg) in 1 ml of dichloromethane/acetone (75/25%). The two solutions were emulsified under sonication (Vibra-Cell™ VCX 130; Sonics and Materials, CT, USA) for 45 s using a 3-mm probe at 30% amplitude leading to GDNF entrapment in the MS. The emulsion was immediately added to 25 ml of 2.5% polyvinyl alcohol solution containing 10% NaCl and homogenized at 6000 rpm for 60 s. The entire mixture was poured into a 250-ml bath of 0.25% polyvinyl alcohol solution containing 10% NaCl under magnetic stirring at 125 rpm for 3 h. The hardened MS were collected and washed by ddH<sub>2</sub>O using centrifugation (1500 rpm for 5 min) for at least five cycles, with a total wash volume of 1.2 l. The MS were collected for lyophilization in a conical tube containing the batch of MS and 4 ml of ddH<sub>2</sub>O. They were stored at -20°C until use.

MS 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. The amount of GDNF incorporated into the MS was quantified by an ELISA for human GDNF according to the manufacturer's instructions (R&D Systems, MN, USA). The absorbance was read at 450 nm with an optical subtraction at 540 nm using a multiwell plate spectrophotometer, and sample concentrations were calculated from a standard curve of known GDNF concentrations. The encapsulation efficiency was determined based on the experimental mass of protein in the MS compared with the theoretical mass.

### ■ Fibrin gel construction & *in vitro* release

Fibrin gels (80  $\mu$ l total volume) were constructed by mixing equal parts fibrinogen (75–115 mg/ml, 40  $\mu$ l) and thrombin (500 IU/ml, 40  $\mu$ l) obtained from a Tisseel® glue kit (Baxter Healthcare, IL, USA), resuspended according to the manufacturer's instructions. Fibrin gels were loaded with MS or GDNF by incorporating 10 mg of MS or 5  $\mu$ g of GDNF, respectively, into the thrombin solution before it was mixed with fibrinogen to form a gel. Fibrin gels implanted

into Thy-1 GFP rats contained a 10:1 mixture of Tisseel fibrinogen/fluorescent fibrinogen and Alexa Fluor® 546 conjugate (Invitrogen), respectively. The Thy-1 GFP rat is a novel transgenic strain in which the motoneurons express GFP and appear green under ultraviolet light [23].

Release of GDNF from fibrin loaded with or without MS was accomplished by constructing 80- $\mu$ l gels with 10 mg of GDNF MS or 5  $\mu$ g of GDNF in siliconized microcentrifuge tubes (Fisher Scientific, ON, Canada). The time course of release was measured by incubating the fibrin gels in 1 ml phosphate-buffered saline (PBS) containing 1% bovine serum albumin 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 the quantity of GDNF collected from the time course release studies.

### ■ Experimental animals

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

### ■ Experimental design

Thirty-two rats were randomized into four groups ( $n = 8$ ). Fibrin gels loaded with empty MS (without GDNF; MS control) or with free GDNF without MS (GDNF control) served as experimental control groups. A dose of  $\geq 0.1$   $\mu$ g/day was desired for delivery at the coaptation site in experimental groups receiving fibrin gels, as previous research has demonstrated this dose to be effective for nerve regeneration under delayed nerve repair conditions [14]. Fibrin gels loaded with MS with GDNF served as the primary experimental group (GDNF MS). Rats without any fibrin gels, MS or GDNF and without any prior procedures (transection and tie back of the nerve stumps) served as the positive control group (immediate repair). Because the numbers of motoneurons that regenerated their axons after delayed nerve repair with or without a fibrin gel are comparable [22], a control group for delayed repair without fibrin gel was not included. An additional eight Thy-1 GFP rats [23] were included for visualization of the implanted fibrin gels using immunohistochemistry.

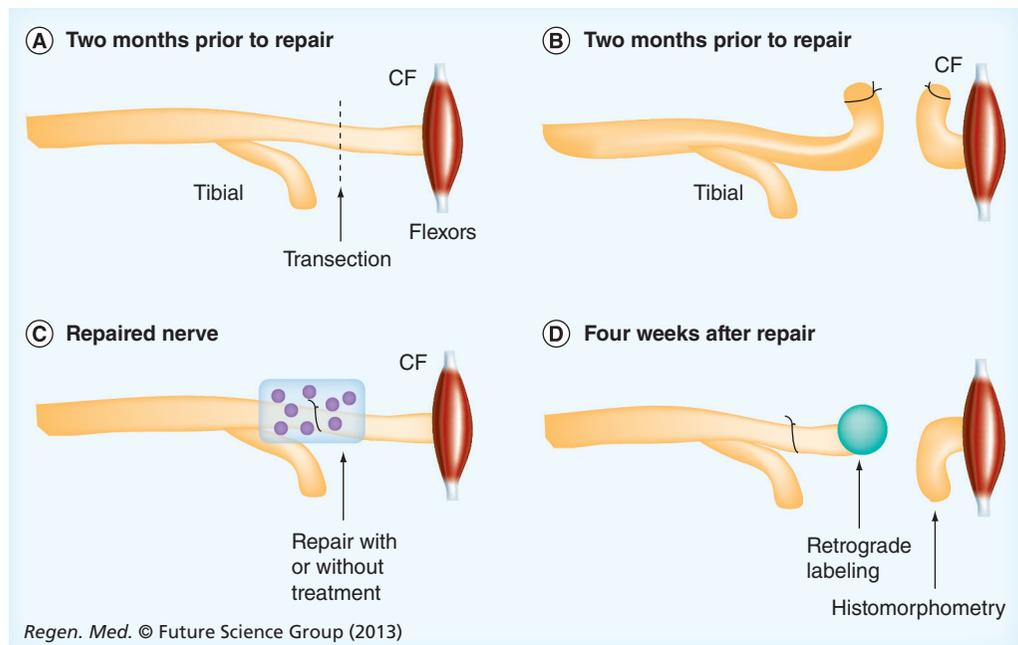
### ■ Operative procedures

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. In both procedures, 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.

In the first procedure, the common fibular (CF; peroneal) nerve was dissected free and transected approximately 5 mm distally from the sciatic trifurcation (FIGURE 1A). Experimental groups receiving delayed CF nerve repair had their CF stumps sutured back into surrounding muscle for 2 months to prevent nerve regeneration (MS control, GDNF control and GDNF MS delayed repair groups) (FIGURE 1B). Animals in the positive control group (immediate repair) and Thy-1 GFP rats did not undergo any procedures at this time. In the second procedure, CF nerves were exposed as before and the nerve stumps freed, trimmed for 1 mm on each end to remove any scar tissue and repaired using 10-0 nylon sutures (FIGURE 1C). In delayed repair groups, the nerve was surrounded by two 40- $\mu$ l fibrin gels (containing applicable components for each group) formed by pipetting the fluid fibrin mixture onto a parafilm sheet as semirectangular drops ( $\sim 5 \times 1$  mm) above and below the repair site to secure the sheet around the nerve by gently apposing the drops. Rats in the positive control group instead had their CF nerve transected approximately 5 mm distally from the sciatic trifurcation and immediately repaired with nylon sutures and no fibrin gels (immediate repair). Thy-1 GFP rats only had the fibrin gels implanted around an intact CF nerve for the procedure.

### ■ Retrograde labeling of motor neurons (ventral horn cells) & sensory neurons (dorsal root ganglia)

Four weeks postoperatively, the surgical site was reopened under general anesthesia. The CF nerve was transected 20 mm distally from the suture repair site and the proximal nerve stump was immediately placed in a silicone well containing 4% Fluoro-Gold™ in sterile saline for 1 h (FIGURE 1D). At the same time, the distal stump was harvested for histology (described below).



**Figure 1. Surgical procedures performed on rats.** Two months prior to the repair and placement of experimental groups, the CF was cut (A) and sutured back to surrounding muscle (B). After the initial injury, the nerve ends were reconnected with epineural suture repair followed by placement of experimental groups as applicable (C). Experimental groups consisted of fibrin gels loaded with either: microspheres containing GDNF; empty microspheres; or no microspheres but free GDNF. An additional experimental group did not receive any initial injury (A & B) and did not receive a fibrin gel. Four weeks following nerve repair, nerve was harvested and labeled with retrograde dye 20 mm distally from the repair site (D). CF: Common fibular nerve.

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  $\mu$ m) or DRG (20  $\mu$ m) 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 $\times$  objective (100 $\times$  overall magnification; Leica). Spinal cord counts were adjusted to account for split nuclei using the methods of Abercrombie [24].

#### ■ Histology & morphometric evaluation of nerves

For general histology and morphometric analysis, the nerve tissue taken 20 mm distally from the repair site was fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, ethanol dehydrated and embedded in Araldite<sup>®</sup> 502

(Polyscience, Inc., PA, USA). Thin (0.6- $\mu$ m) sections were made from the tissue using a LKB II ultramicrotome (LKB-Producter 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 $\times$  overall magnification, the entire nerve cross-section was captured and evaluated with image analysis software (Image-Pro<sup>®</sup> Analyzer version 7.0, Media Cybernetics, MD, USA) using a custom-designed macro, based on previous methodology [25], in order 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.

In Thy-1 GFP rats, the delivery systems and nerves were harvested at days 1 and 10 after implantation of the delivery system and fixed in cold 4% paraformaldehyde in PBS for immunohistochemistry. Cross-sections of the delivery system and nerve were cut at 10  $\mu$ m on a cryostat and stained with GDNF (Peprotech; 1:500) primary antibody followed by Alexa Fluor secondary antibody (Invitrogen; 1:500) using standard immunohistochemistry techniques.

### ■ Statistical analysis

All data are reported as the mean  $\pm$  standard error of the mean. Statistical analysis was evaluated first to confirm the normality of the data and then using analysis of variance with *post hoc* tests including Bonferroni correction for determining differences between groups, where  $p < 0.05$  was considered statistically significant.

## Results

### ■ *In vitro* characterization of MS & GDNF release from fibrin gels

MS constructed with heparin (5% wt/wt) and  $\text{MgCO}_3$  (3% wt/wt) demonstrated a high encapsulation efficiency ( $78 \pm 3\%$ ) and a size of  $45 \pm 5 \mu\text{m}$ . The dynamic release of GDNF from fibrin gels with or without MS loaded during the polymerization into PBS (pH 7.4, containing 0.1% bovine serum albumin) was followed for 15 days at  $37^\circ\text{C}$ . FIGURE 2 demonstrates this release of GDNF into PBS, where the data are normalized to the recovered GDNF (free GDNF,  $4.5 \pm 0.1 \mu\text{g}$ ) or the successfully encapsulated GDNF in MS (GDNF MS,  $4.2 \pm 0.4 \mu\text{g}$ ). A statistically equivalent total amount of GDNF was released from the GDNF MS ( $4.2 \pm 0.4 \mu\text{g}$ ) and GDNF control ( $4.5 \pm 0.1 \mu\text{g}$ ) groups. Neither group demonstrated a large initial burst of GDNF release from the fibrin gels, probably due to the high concentration of fibrinogen within the fibrin gels ( $\sim 50 \text{ mg/ml}$ ; FIGURE 2A). GDNF release from fibrin gels loaded with MS containing GDNF (GDNF MS) was slower than from the fibrin gels loaded with free GDNF (GDNF control). All the GDNF was released by 15 days in the former group and by 11 days in the latter group. Additionally, the ability to release at least  $0.1 \mu\text{g/day}$  of GDNF to the nerve repair site is necessary to improve motor nerve regeneration in chronic axotomy nerve models [14]. Both delivery systems, with or without MS, delivered GDNF at levels  $\geq 0.1 \mu\text{g/day}$  for at least 11 days *in vitro* (data not shown).

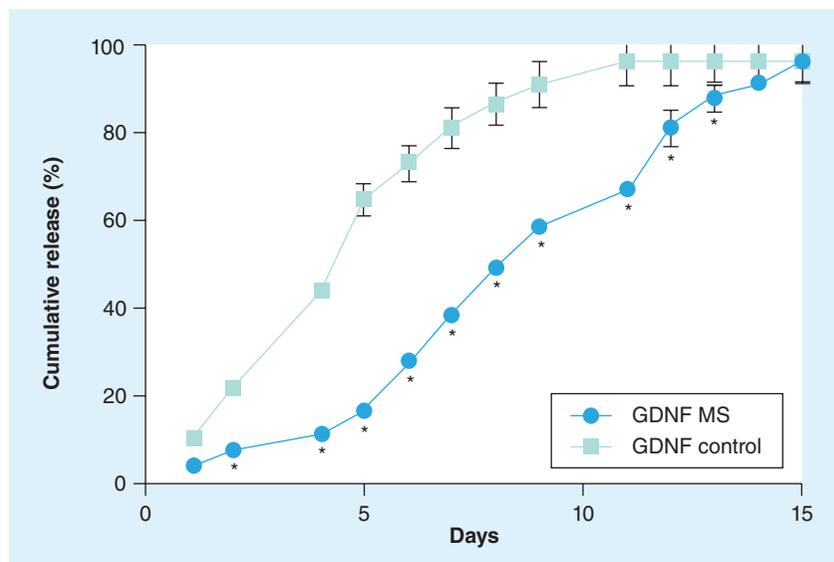
### ■ Immunohistochemistry of the implanted fibrin gels *in vivo*

The CF nerve repair site was completely surrounded by fibrin gel containing free GDNF or GDNF within MS; the gel remained in place at day 1 following implantation *in vivo* (FIGURE 3A & 3B). Fibrin gels containing GDNF MS remained around the nerve at the repair site for at least 10 days (FIGURE 3C). By contrast, fibrin gels with free GDNF were visibly degraded after

3 days; the complete degradation by 10 days contrasted with the sustained gels that contained GDNF MS (FIGURE 3C & 3D).

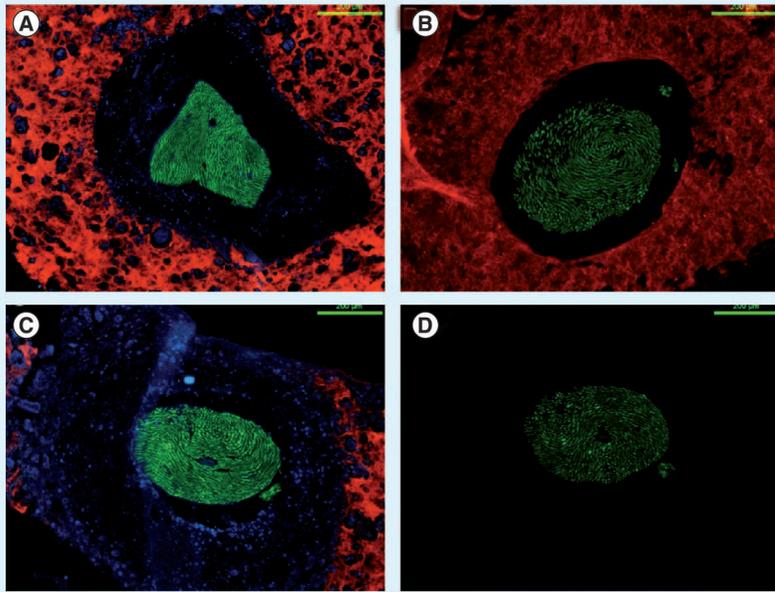
### ■ *In vivo* retrograde labeling of neurons following nerve repair

To quantify the number of neurons regenerating their axons and to compare the extent of motor versus sensory regeneration, retrograde dyes were applied to regenerated axons 20 mm distally from the repair site 4 weeks after the nerve repair. Following the 2-month period of delayed nerve repair, only approximately 51% of motoneurons (based on the normal number of motoneurons) regenerated their axons in the CF nerve, as demonstrated by the MS control group. The number for the delayed nerve repair group with free GDNF (GDNF control) was significantly lower than the immediate repair group ( $\sim 83\%$  of the normal number of motoneurons) and equal to the number that regenerated their axons with fibrin gels with empty MS (MS control;  $\sim 51\%$  of the normal number of motoneurons). However, just as many motoneurons regenerated their axons in the delayed nerve repair group in which fibrin gels with GDNF MS were implanted as in the immediate repair groups ( $\sim 78$  and  $\sim 83\%$  of the uninjured control number of  $400 \pm 20$  motoneurons) (FIGURE 4A). The GDNF MS group was the only experimental group that



**Figure 2. *In vitro* release of GDNF from fibrin gels.** The release of GDNF from fibrin gels loaded with MS containing GDNF (GDNF MS) was slower than fibrin gels loaded with free GDNF (GDNF control). Data are normalized to the recovered GDNF (free GDNF) or the successfully encapsulated GDNF MS. Data ( $n = 4$ ) represent mean  $\pm$  standard error of the mean.

\*Statistical significance between GDNF MS and GDNF control at each individual time point ( $p < 0.05$ ).  
MS: Microsphere.



**Figure 3. Immunofluorescent micrographs of fibrin gels with and without implanted microspheres containing GDNF surrounding the nerve at days 1 and 10 following placement.** The experimental delivery systems were visualized by implanting Thy-1 GFP rats (nerves are green) with fluorescently labeled fibrin (red) gels containing GDNF microspheres (MS; blue) or free GDNF, which was stained after harvesting using standard immunohistochemistry. Fibrin gels loaded with microspheres containing GDNF (**A**; GDNF MS) were visibly more porous compared with fibrin gels loaded with free GDNF (**B**; GDNF control). By day 10, (**C**) only GDNF MS groups contained visible fibrin, while (**D**) fibrin localization was completely absent in GDNF control groups. Scale bars: 200  $\mu\text{m}$ .

was statistically the same as the immediate repair group ( $p > 0.05$ ) in the number of motoneurons that regenerated their axons.

The immediate repair group was the only group with appreciably improved sensory regeneration, as the number of sensory neurons that regenerated their axons compared with the MS control group was significantly increased ( $\sim 81$  vs  $\sim 50\%$  of the uninjured normal number of  $1000 \pm 50$  sensory neurons) (FIGURE 4B). Both of the numbers of DRG sensory neurons that regenerated their axons in the GDNF control and GDNF MS groups were not significantly different from the immediate repair group ( $p > 0.05$ ) or from the MS control group ( $p > 0.05$ ). Overall, sensory neurons within the DRG were unaffected by placement of GDNF delivery systems at the repair site.

#### ■ *In vivo* nerve histology & morphometric measures of regeneration following nerve repair

Four weeks following nerve repair with or without implantation of fibrin gels, nerve was harvested 20 mm distally from the repair site for histological analysis by light microscopy. Qualitative

examination of the nerve revealed differences in nerve regeneration (FIGURE 5). Axons were clustered within portions of the nerve rather than being uniformly distributed throughout the nerve in the GDNF MS group, where GDNF was contained within MS (FIGURE 5C), compared with the MS control group with empty MS (FIGURE 5A) and the GDNF control group with free GDNF in the fibrin gel (FIGURE 5B). The immediate repair group (positive control) (FIGURE 5D), with visibly larger myelinated fibers, more closely resembled the GDNF MS group (FIGURE 5C) than the MS control (FIGURE 5A) or GDNF control (FIGURE 5B) groups. Uninjured normal nerve was distinctly different from all experimental groups as the intact axons were more tightly packed within the nerve with a completely uniform distribution of axons throughout the nerve, and individual units were not visible compared with regenerating nerve tissue (FIGURE 5E).

Histomorphometric measures of the entire nerve cross-section were evaluated to quantify the extent of nerve regeneration at this early time point (TABLE 1). No significant differences in myelinated axon counts were found between any experimental groups. However, both the diameter and myelin thickness were significantly higher in the GDNF MS and immediate repair groups compared with the MS control group, indicating a more rapid maturation of the regenerating axons. The GDNF control group was not statistically different in either of these metrics compared with the MS control group ( $p > 0.05$ ) and was inferior to the immediate repair group ( $p < 0.05$ ).

A shift towards larger-diameter fibers in both the GDNF MS and immediate repair groups is demonstrated in frequency distributions plotted for the fiber diameters (TABLE 2). The most apparent shift in fiber distribution was seen between the MS control and the immediate repair groups, as all fiber sizes were different ( $p < 0.05$ ). This shift indicated that the largest fibers ( $>4 \mu\text{m}$ ) were more frequent than the smallest fibers (1–2 and 2–3  $\mu\text{m}$ ) due to immediate repair versus a delayed repair. Conversely, the distribution of nerve fibers was similar ( $p > 0.05$ ) between the MS control and the GDNF control groups, indicating a lack of effect due to placement of fibrin gels with free GDNF next to the repaired nerve. However, the GDNF MS group resulted in a statistical increase ( $p < 0.05$ ) in the largest nerve fibers ( $>4 \mu\text{m}$ ) compared with the MS control and GDNF control groups. The GDNF control group did have some effect on the size of fibers present, as it matched ( $p > 0.05$ ) the medium fibers (3–4  $\mu\text{m}$ ) found with the immediate repair group. Only

the GDNF MS group matched ( $p > 0.05$ ) the immediate repair group in frequency of the largest nerve fibers ( $>4 \mu\text{m}$ ).

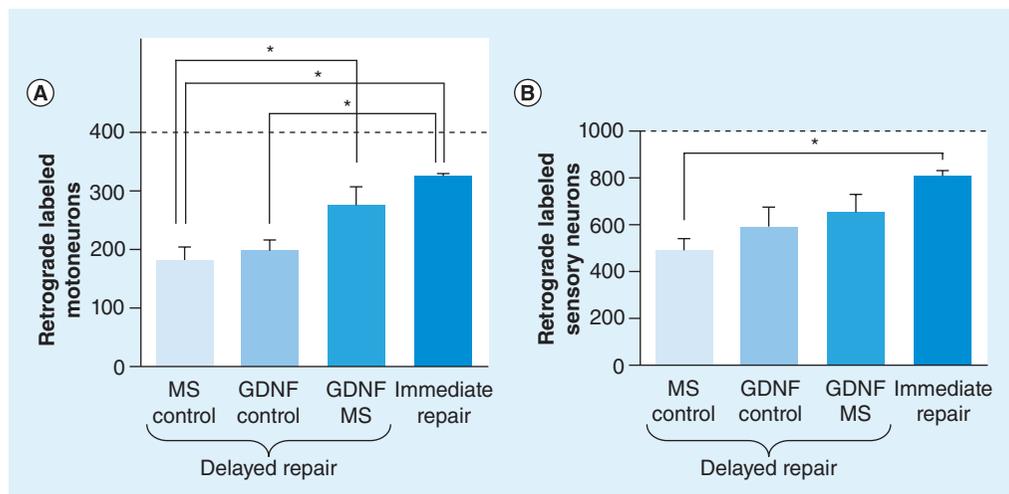
## Discussion

We sought to determine whether fibrin gels with GDNF MS could improve axon regeneration following delayed nerve repair. In both experimental and control groups, the fibrin gel facilitated placement of either free GDNF or GDNF MS near regenerating axons at the nerve repair site. Free GDNF did not enhance nerve regeneration with regards to either the number of neurons regenerating their axons or the maturity of the regenerating axons. GDNF MS encased in fibrin gel promoted the regeneration of axons that, in turn, showed morphological evidence of greater maturity.

The incorporation of MS containing GDNF into fibrin gels allowed a slower release of GDNF from fibrin gels *in vitro* than with free GDNF in a fibrin gel alone, as has been previously noted by others [26]. These MS thereby extended GDNF release compared with free GDNF in fibrin gels. While our release was short compared with typical controlled release systems with MS [20,27], it was anticipated based on the low-molecular-weight polymer chosen for our MS [28].

However, this result does not indicate that GDNF from fibrin gels containing GDNF MS at the repair site is delivered with perfect efficiency, as biological degradation and the distance to diffusion to the tissue are limitations of the system *in vivo*. In addition, GDNF released and encapsulated from the *in vitro* release results would likely be significantly lower in the *in vivo* release scenario due to biological degradation, which was not measured in this current work. This scenario is particularly true as the *in vivo* half-life of GDNF is approximately 37 h within tissue [29]. Therefore, not all ELISA-measured protein will be biologically active, and so an overestimation of the incorporated biologically active growth factor may be present. Nevertheless, our results indicate that the dose of GDNF placed at the nerve repair site had an effect on early nerve regeneration.

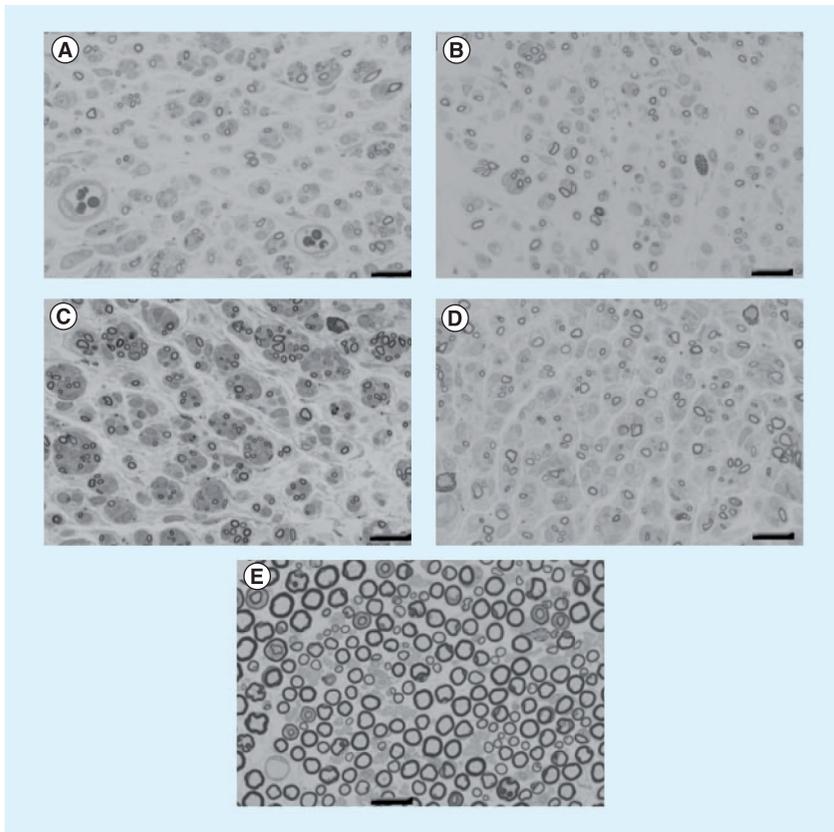
Micrographs of the different delivery systems *in vivo* demonstrated that fibrin gels with only free GDNF are quickly degraded from the implantation site, while fibrin gels with GDNF MS were degraded more slowly over time comparatively (FIGURE 3). Effectively, the MS may act to stabilize the fibrin network, which in turn keeps the GDNF better localized to the injury site, as has been demonstrated with other



**Figure 4. Retrograde labeling of neurons regenerating their axons performed 20 mm distally from the repair site 4 weeks following experimental treatment.** Ventral horn cells in the spinal cord (A; 50- $\mu\text{m}$  sections, all sections counted) and sensory neurons in the dorsal root ganglia (B; 20- $\mu\text{m}$  sections, every fifth section counted) were counted to assess motor and sensory neuron regeneration, respectively. The experimental group receiving fibrin gels loaded with MS containing GDNF (GDNF MS) were comparable to the immediate repair group and improved motor nerve regeneration, as more motor neurons regenerated their axons compared with fibrin gels loaded with empty MS (MS control). Experimental groups that delivered any amount of GDNF (GDNF MS and GDNF control) had similar numbers of sensory neurons that regenerated their axons compared with the MS control group, indicating no improved sensory regeneration. Data ( $n = 8$ ) represent the mean  $\pm$  standard error of the mean. Normal, uninjured values are represented by the dashed line.

\* $p < 0.05$ .

MS: Microsphere.



**Figure 5. Representative light micrographs of semi-thin nerve cross-sections taken 20 mm distally from the repair site 4 weeks following experimental treatment.** Regenerated nerve cross-sections of the following experimental groups are displayed: **(A)** fibrin gels loaded with empty microspheres (MS; MS control); **(B)** fibrin gels loaded with free GDNF (GDNF control); **(C)** fibrin gels loaded with MS containing GDNF (GDNF MS); **(D)** nerve repaired without prior injury or fibrin gels (immediate repair); and **(E)** normal uninjured nerve. The GDNF MS group **(C)** and immediate repair group **(D)** were qualitatively more similar to one another, as regenerating units were larger compared with the MS control **(A)** and GDNF control **(B)** groups. Scale bars: 20  $\mu\text{m}$ .

drug-delivery systems incorporating both MS and a carrier matrix for implantation [30].

Our delivery system was designed for future ease of use. We designed this delivery system for future clinical application as a fibrin glue that could be applied during nerve repair in the operating room. The use of fibrin glue for peripheral nerve repair is widely practiced by surgeons [18]. The ability to apply fibrin glue to deliver growth factors for direct nerve repair may be advantageous over guidance channels, since a conduit is not necessary to achieve repair. Furthermore, a conduit utilized simply to act as a drug-delivery system may have adverse side effects that negatively affect nerve regeneration [31,32].

Placement of GDNF MS promoted the regeneration of axons from more motoneurons after delayed nerve repair as compared with a lack of GDNF in delayed nerve repair (FIGURE 4).

In light of previous studies demonstrating staggered axon regeneration of axons across a suture site and the regeneration of all motoneurons by 4 weeks after immediate nerve repair, evaluation of axon regeneration after 4 weeks in this study permitted the comparison of the administration of fibrin gels with either free GDNF or GDNF contained within MS [13,14,33]. We saw improved histomorphometric outcomes with the placement of GDNF MS (compared with either empty MS or a lack of GDNF). In both retrograde labeling counts of motoneurons regenerating their axons and histomorphometric measures, only GDNF MS improved these nerve regeneration metrics to match the level of regeneration achieved by immediate repair. Therefore, GDNF MS are needed to improve nerve regeneration compared with no GDNF following delayed nerve repair, as shown previously [14]. Similarly, others have shown that motoneurons regenerated more axons through acellular polymer conduits that contained GDNF embedded in the wall of the conduit and through polymer conduits in which GDNF was noncovalently sequestered in a fibrin gel [34–37].

Nerve fiber diameter and myelin thickness are measures of regenerated nerve maturity and quality of regeneration [38,39]. Larger axon diameters and thicker myelination result in greater function compared with smaller, more thinly myelinated fibers [40]. The placement of GDNF MS loaded into fibrin gels at the nerve repair site increased fiber maturity. Although the differences between the GDNF MS and immediate repair groups compared with the MS control group were small, these small differences in fiber diameter and myelination indicate a potential for improved nerve regeneration, as early regeneration metrics such as these are correlated with better final nerve regeneration [10,38]. Myelination and nerve fiber diameter correlate with each other, and the increased maturity of the regenerated axons corresponded with the effectiveness of GDNF MS in promoting the regeneration of axons, increasing the number of motoneurons that regenerated their axons into the distal nerve stump. Indeed, the GDNF MS effectively overcame the decline in numbers of neurons that regenerate their axons after delayed nerve repair, with the numbers of regenerating motoneurons reaching the number after immediate nerve repair. These findings demonstrate the effectiveness of MS containing neurotrophic factors placed at a nerve injury site to compensate for the decline

**Table 1. Histomorphometric analysis of nerve cross-sections taken 20 mm distally from the repair site with or without treatment.**

Group	Myelinated axon count	Fiber diameter ( $\mu\text{m}$ )	Myelin thickness ( $\mu\text{m}$ )
MS control	1300 $\pm$ 130	2.98 $\pm$ 0.07	0.50 $\pm$ 0.01
GDNF control	1290 $\pm$ 110	3.03 $\pm$ 0.07	0.51 $\pm$ 0.02
GDNF MS	1680 $\pm$ 260	3.27 $\pm$ 0.06*	0.61 $\pm$ 0.03*
Immediate repair	1600 $\pm$ 190	3.35 $\pm$ 0.06*,**	0.63 $\pm$ 0.03*,**
Normal, uninjured nerve	1600 $\pm$ 50	8.00 $\pm$ 0.05	1.20 $\pm$ 0.05

Mean  $\pm$  standard error of the mean ( $n = 8$ ) values are shown. Normal nerve was not included in the statistical analysis.  
\* $p < 0.05$  compared with MS control; \*\* $p < 0.05$  compared with GDNF control.  
MS: Microsphere.

in the expression of these factors after chronic axotomy and chronic denervation.

Sensory nerve regeneration did not appear to be greatly affected by GDNF from either MS or fibrin. Placement of GDNF by fibrin gels containing free GDNF was not different from the GDNF MS group, and both groups were similar to the MS control. This result is not surprising since previous studies have shown that GDNF release for a short period (<2 weeks) had no effect on sensory nerve regeneration [37]. This result may indicate that release for >2 weeks may be necessary to stimulate improved sensory regeneration due to GDNF delivery or that sensory neurons have already been lost due to chronic axotomy [41]. Additionally, as many as 35–40% of sensory neurons in the DRG may die 2 months following peripheral nerve injury [42], and the type of sensory neuron that dies, such as cutaneous innervating versus sensory motor, varies due to injury type [41]. We saw a 20% reduction in sensory neurons that regenerated their axons due to immediate nerve injury and a 50% reduction due to chronic axotomy and denervation for 2 months. It is unclear in our current studies how many sensory neurons died versus did not regenerate their axons due to chronic axotomy. As there are only 40–50%

of lumbar DRG in the adult rat that express GDNF receptor  $\alpha$ -1 and Ret, both of which are needed for GDNF signaling [43,44], if a large percentage of these died, the effects of growth factor delivery such as by GDNF would be diluted, as we observed.

## Conclusion

In summary, the goal of this study was to evaluate the effects of GDNF release on early nerve regeneration in a delayed repair nerve injury model. We found that the placement of fibrin gels with GDNF MS at the nerve repair site improved motoneuronal regeneration of axons and hastened the maturation of the regenerated axons, as indicated by increased myelination and fiber diameters. We believe that fibrin gels incorporating GDNF MS offer insights into potential future alternatives for the treatment of chronic peripheral nerve injuries.

## Future perspective

Addressing the deleterious effects of chronic axotomy holds promise as a means of removing one of the major barriers to improving nerve regeneration. Our use of chronically axotomized neurons that have undergone subsequent repair models the common clinical scenario in which

**Table 2. Histomorphometric analysis of nerve fiber distributions from nerve cross-sections taken 20 mm distally from the repair site with or without treatment.**

Group	Fiber diameter 1–2 $\mu\text{m}$	Fiber diameter 2–3 $\mu\text{m}$	Fiber diameter 3–4 $\mu\text{m}$	Fiber diameter >4 $\mu\text{m}$
MS control	12 $\pm$ 2*	45 $\pm$ 2*	31 $\pm$ 1*	12 $\pm$ 2*
GDNF control	11 $\pm$ 2*	43 $\pm$ 2*	33 $\pm$ 1	14 $\pm$ 2*
GDNF MS	8 $\pm$ 1*	40 $\pm$ 2*	31 $\pm$ 1*	21 $\pm$ 2**,***
Immediate repair	4 $\pm$ 1	31 $\pm$ 2	38 $\pm$ 1	27 $\pm$ 2

Mean  $\pm$  standard error of the mean ( $n = 8$ ) values are shown.  
\* $p < 0.05$  compared with immediate repair; \*\* $p < 0.05$  compared with MS control; \*\*\* $p < 0.05$  compared with GDNF control.  
MS: Microsphere.

patients present with a transected nerve many months after injury. A combination of the surgical repair with drug-delivery systems is likely to provide a suitable solution to severe nerve injuries.

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### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

### Executive summary

- This study demonstrates the advantages of GDNF microspheres at a delayed nerve repair site.
- Microspheres constructed with heparin (5% wt/wt) and MgCO<sub>3</sub> (3% wt/wt) demonstrated a high encapsulation efficiency (78 ± 3%), a size of 45 ± 5 μm and a dynamic release for at least 2 weeks *in vitro*.
- Fibrin gels containing GDNF microspheres remained around the nerve at the repair site for at least 10 days, while fibrin gels with free GDNF were visibly degraded after 3 days.
- The number of motoneurons that regenerated their axons increased to comparable levels as those observed after immediate repair due to the placement of GDNF microspheres.
- Histomorphometry of distal nerve cross-sections taken 20 mm from the repair site 4 weeks following repair demonstrated increased fiber diameter and myelin thickness due to GDNF microspheres compared with empty microspheres.
- Frequency distribution plots of fiber diameters revealed a shift towards larger-diameter fibers in both the GDNF microsphere and immediate repair groups.
- We believe GDNF microspheres offer insights into potential future alternatives for the treatment of chronic peripheral nerve injuries.

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