Methylene blue enhances polyethylene glycol-fusion repair of completely severed rat sciatic nerves

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Abstract
Complete transection of peripheral mixed nerves immediately produces loss of sensory perception, muscle contractions and voluntary behavior mediated by the severed distal axons. In contrast to natural regeneration (~1 mm/d) of proximal axons that may eventually reinnervate denervated targets, re-innervation is restored within minutes by PEG-fusion that consists of neurorrhaphy and a sequence of well specified hypo- and isotonic calcium-free or calcium-containing solutions, the anti-oxidant methylene blue (MB) and the membrane fusogen polyethylene glycol (PEG). In this study, we examined the relative efficacy of PEG-fusion with no MB (0%), 0.5% MB, or 1% MB on the recovery of voluntary behaviors by female Sprague-Dawley rats with a complete mid-thigh severance of their sciatic nerve bathed in extracellular fluid or calcium-containing isotonic saline. The recovery of voluntary behaviors is the most relevant measure of success of any technique to repair peripheral nerve injuries. We assessed recovery by the sciatic functional index, a commonly used measure of voluntary hindlimb behaviors following complete sciatic transections. We reported that both 1% MB and 0.5% MB in sterile distilled water in our PEG-fusion protocol with neurorrhaphy significantly increased the rate and extent of behavioral recovery compared to PEG plus neurorrhaphy alone. Furthermore, 0.5% MB was as effective as 1% MB in voluntary behavioral recovery as assessed by the sciatic functional index. Since sterile 1% MB is no longer clinically available, we therefore recommend that 0.5% MB be included in upcoming human clinical trials to evaluate the safety and efficacy of PEG-fusion. All animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee (AUP-2019-00225) on September 9, 2020.

Key Words: axotomy; methylene blue; nerve repair; neurorrhaphy; peripheral nerve injury; polyethylene glycol fusion; sciatic nerve

Introduction
Peripheral nerve injuries (PNIs) that involve complete transections are often clinically or experimentally devastating due to loss of motor and sensory functions mediated by the severed nerve (Lee and Wolfe, 2000; Campbell, 2008; Fox and Kreishman, 2010; Brushart, 2011; Bittner et al., 2015). The success of any protocol or technique designed to repair PNIs is almost certainly best assessed by the rate and extent of restoration of conscious sensation and voluntary motor/sensory behaviors in experimental animal models or humans (Brushart, 2011; Wood et al., 2011; Bittner et al., 2012). Current clinically approved techniques to repair singly transected PNIs almost all involve microsuturing (neurorrhaphy) to closely appose/mechanically connect the connective tissue sheaths of the severed nerve ends (Houshyar et al., 2016). Neurorrhaphy facilitates the slow (~1 mm/d) outgrowth of surviving axons at the proximal cut end into endoneurial sheaths with surviving Schwann cells at the distal cut end whose transected axons undergo Wallerian degeneration within days (Wong et al., 2017). Transection of peripheral nerves also result in immediate loss of reflex or voluntary control of muscle contraction and

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disintegration of myelin sheaths and nerve muscle junctions (NMJs) beginning within days and continuing over months until permanent/irreversible loss of function (Coleman and Höke, 2020). Denervated muscle fibers typically exhibit significant atrophy within weeks (Brushart, 2011). No matter what type of primary surgical repair is clinically or experimentally attempted (especially of mixed nerves severed more proximally), behavioral recovery is usually slow and rather incomplete – even if repaired by the decades-old “gold standard” of neurorrhaphy (Lee and Wolfe, 2000; Campbell, 2008; Fox and Kreishman, 2010; Brushart, 2011; Bittner et al., 2015; Ghergherehchi et al., 2016).

PEG-fusion represents a unique approach to repair transection (or ablation-type) PNIs. PEG-fusion involves the local application of a sequence of bio-active reagents, one of which contains 50% w/w polyethylene glycol (PEG) and another methylene blue (MB) to a freshly performed nerve coaptation by neurorrhaphy (Lore et al., 1999; Britt et al., 2010; Bittner et al., 2012). MB presumably inhibits the formation of vesicles that reduce PEG-fusion success at closely-apposed open cut ends (Spaeth et al., 2012b; Varga and Bittner, 2019). PEG-fusion protocols, using a calcium-free hypotonic saline, induce the exposed ends of axonal membranes in the proximal and distal axonal stumps to fuse to re-establish axonal continuity. The direct application of 1% MB to the PEG-fusion site has been reported to significantly improve behavioral recovery compared to PEG without MB (Bittner et al., 2012) when axons are transected and maintained in a calcium-free hypotonic saline until PEG-fused.

For several years to attain clinical translation, our laboratories have continued to develop and refine the PEG-fusion technology, using the rat sciatic nerve as a long-standing model system to study peripheral nerve trauma (Geuna, 2015). PEG-fused sciatic nerves have axons with continuous axoplasm/axolemma from spinal cord to end organs that immediately regain much physiologic function and morphological continuity as demonstrated by: (1) conduction of compound action potentials (CAPs) and elicitation of compound muscle action potentials (CMAPs); (2) diffusion of intraaxonal dyes; (3) re-established retrograde axoplasmic transport; (4) maintained morphology of distal axonal segments, NMJs and probably sensory end organs; and, (5) much Wallerian degeneration prevented or reduced (Bittner et al., 2012, 2015; Ghergherehchi et al., 2016, 2019a, b; Smith et al., 2020). Furthermore, many conscious sensations and voluntary motor/sensory behaviors are restored within 1-6 weeks (Bittner et al., 2012, 2015, 2017a; Bamba et al., 2016a; Mikesh et al., 2018a, b; Ghergherehchi et al., 2019a, b); in humans, successful PEG-fusion has been reported for severed digital nerves in a small number of human case studies (Bamba et al., 2016b).

Clinical trials are being planned for PEG-fusion repair of severed digital and mixed peripheral nerves. Previous research has shown that 1% MB significantly improves behavioral recovery after PEG-fusion after transection in calcium-free hypotonic saline (Bittner et al., 2012). However, to further optimize the clinical translation, data need be obtained on two critical questions regarding the use of MB in the PEG-fusion protocol (Ghergherehchi et al., 2019a). The objective of this study was to answer, (1) Does 1% MB in sterile distilled water plus PEG and neurorrhaphy significantly improve behavioral recovery compared to PEG plus neurorrhaphy alone after transection in isotonic calcium-containing fluid? (2) Since 1% MB is recently no longer readily clinically available, is 0.5% MB in sterile distilled water (ProvaLyBlue™) as effective as 1% MB, as assessed by the sciatic functional index (SFI), a commonly used measure of hindlimb voluntary behavior (de Medinaeli et al., 1982; Carlton and Goldberg, 1986; Varejao et al., 2001; Bittner et al., 2012, 2015).

### Materials and Methods

#### Animals

Adult female Sprague Dawley rats (n = 80, 225–300 g; 10–16 weeks old; Envigo, Indianapolis, IN, USA) were used in this study. We have used both male and female rats in previous studies and have shown similar results for both sexes (Bitt et al., 2010; Bittner et al., 2012; Riley et al., 2015; Mikesh et al., 2018a, b; Ghergherehchi et al., 2019a, b; Smith et al., 2020). Rats were divided into seven groups; unoperated control (Unop; n = 6), sham control (Sham; n = 7), PEG-fused with no MB (PEG; n = 10), PEG-fused with 0.5% MB (PEG + 0.5% MB; n = 10), PEG-fused with 1% MB (PEG + 1% MB; n = 10), negative controls (NC; n = 18) and historical PEG-fusion data (HPEG; n = 13) for a total of 74 rats analyzed (Table 1). Animals in the PEG, PEG + 0.5% MB and PEG + 1% MB groups were analyzed prior to surgery to obtain baseline control (BC) SFI data. In addition to these 74 rats, 6 animals were euthanized due to autophagia or illness, and were not included in the analysis. All animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee (AUP-2019-00225) on September 9, 2020.

| Treatment group | Protocol description |
|-----------------|----------------------|
| Unoperated control (Unop), n = 6 | Uninjured sciatic nerves from animals tested weekly |
| Sham control (Sham), n = 7 | Sciatic nerve exposed, but not injured; incision closed |
| Baseline control (BC), n = 30 | Uninjured sciatic nerves from animals tested before any procedure |
| PEG-fused (PEG), n = 10 | Sciatic nerve transection repaired with neurorrhaphy + PEG, No MB applied |
| PEG + 0.5% MB, n = 10 | Sciatic nerve transection repaired with neurorrhaphy + PEG + 0.5% MB |
| PEG + 1% MB, n = 10 | Sciatic nerve transection repaired with neurorrhaphy + PEG + 1% MB |
| Negative control (NC), n = 18 | Sciatic nerve transection repaired with neurorrhaphy (No PEG applied) |
| *HPEG + 1% MB, n = 13 | Sciatic nerve transection repaired with neurorrhaphy + PEG + 1% MB |

*HPEG + 1% MB: Data for sciatic nerve transection repaired with neurorrhaphy + PEG + 1% MB and lactated ringers rather than normal saline + calcium otherwise used for other protocols in this study. MB: Methylene blue; PEG: polyethylene glycol.

#### Study design

The objective of this study was to examine the effects of PEG and of several concentrations (0%, 0.5%, 1%) of MB on the ability of rats to voluntarily run an inclined plane after completely transecting the left sciatic nerve in its own extracellular fluid. This voluntary behavior was assessed by the SFI from 7–42 days postoperatively (PO). Previous studies consistently showed that rats with PEG-fused sciatic nerves typically reached a plateau in SFI scores at 42 days PO (Bittner et al., 2012, 2015, 2017a; Bamba et al., 2016a; Mikesh et al., 2018a, b; Ghergherehchi et al., 2019a, b). The SFI is a well-accepted and standard" of neurorrhaphy (Lee and Wolfe, 2000; Campbell, 2003; Riley et al., 2015; Mikesh et al., 2018a, b; Ghergherehchi et al., 2019a, b; Smith et al., 2020).

Animals in the PEG, PEG + 0.5% MB and PEG + 1% MB groups were analyzed prior to surgery to obtain baseline control (BC) SFI data. In addition to these 74 rats, 6 animals were euthanized due to autophagia or illness, and were not included in the analysis. All animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee (AUP-2019-00225) on September 9, 2020.
nerve transection repaired with a similar protocol in our lab (HPEG + 1% MB; n = 13; Mikeshe et al., 2018a; Ghergherehchi et al., 2019b) used in the present study (PEG + 1% MB; n = 10). The final solution (isotonic Ca²⁺-containing saline) used for HPEG animals was Lactated Ringer’s instead of Normal saline + Calcium chloride (Figure 1 and Table 2). We noted that the osmolarity as measured on a well-calibrated freezing point osmometer (Advanced Instruments, Norwood, MA, USA; model 3300) for many solutions (measured osmolality) was often significantly different from their osmolarity as calculated from their published formulations (calculated osmolarity). The calculated osmolality assumes that all ions in a solution are completely dissociated and hydrated, an assumption that can be in significant error for solutions whose osmolality approaches 300 mOsm and contains divalent cations or buffers (Tro, 2019). The calculated osmolality of Lactated Ringer’s is 273 mOsm, but the measured osmolality of this solution was 258.3 ± 5.9 mOsm (Figure 1 and Table 2). We noted that this solution was hypotonic compared to rat plasma measured at 311.1 ± 3.3 mOsm (Figure 1). Therefore, we used Normal Saline supplemented with 2 mM calcium chloride (Figure 1 and Table 2) as the final isotonic calcium-containing solution in the current study. The calculated osmolality of Normal Saline + Calcium chloride is 310 mOsm and its measured osmolality was 294 ± 3.6 mOsm (Figure 1 and Table 2).

Table 2 | Composition and osmolality of saline solutions

| Solution                | Composition (mg/100 mL) | Calculated osmolality (mOsm) | Measured osmolality (mOsm) | Note |
|-------------------------|-------------------------|------------------------------|---------------------------|------|
| Normosol-R              | 526 mg NaCl, 222 mg CH₃NaO₃, 502 mg NaC₂H₃O₂, 37 mg KCl, 30 mg MgCl₂ | 294                          | 268.6±3.0                 |      |
|                         |                         |                              |                           |      |
| Lactated Ringer’s       | 600 mg NaCl, 310 mg CH₃NaO₃, 30 mg KCl | 274                          | 258.3±5.9                 |      |
|                         |                         |                              |                           |      |
| Normal saline + calcium chloride | 900 mg NaCl, 20 mg CaCl₂ | 310                          | 294.7±3.8                 |      |

*Data are expressed as the mean ± SD.

Osmolality measurements

A freezing point osmometer was used to measure osmolality of various saline solutions in this study. Before testing each solution, the device was calibrated using a 290 mOsm/kg reference solution to ensure its accuracy. After calibration, 20 μL of each sample was loaded into the sample plunger. Measurements (Figure 1 and Table 2) were taken for rat serum (n = 7) via intracardiac puncture (Stare and Bourque, 2016). Normosol-R (n = 6) and lactated Ringer’s (n = 6) measurements were taken from two separate bags of solution (3 measures each) at room temperature. Diluted Normosol-R (n = 6) was measured from bags of Normosol-R diluted with ddH₂O to ~250 mOsm. Normal saline (n = 6) and normal saline + calcium chloride (n = 9) were made by mixing appropriate salts in ddH₂O (Table 2) until fully dissolved and measured at room temperature.

Surgical and other procedures for control and experimental groups

Rats were anesthetized using a small mammalian anesthetic device (Handelbar Anesthesia, Austin, TX, USA). Animals were initially induced to anesthesia with 4% inhaled isoflurane (Patterson Veterinary, Greeley, CO, USA), and kept at 2–2.5% for the duration of the surgery. An oxygen flow rate of 0.5–1 L/min was maintained at all times. Prior to surgery, the lateral aspect of the left hindlimb was trimmed of fur and disinfected with alternating swabs of 10% iodine/povidone and 70% ethyl alcohol. Ophthalmic ointment was applied to each eye to prevent drying. A 2–3 cm incision was then made through the skin in the mid-thigh to expose the underlying musculature. A small incision was made parallel to the muscle fibers overlying the sciatic nerve, and a blunt dissection using scissors exposed the intact sciatic nerve (Figure 2A) in extracellular fluid and kept moist with isotonic normal saline supplemented with calcium (Table 2). To confirm that the sciatic nerve and NMJs remained functional, we recorded CAPs by stimulating the nerve proximally with extracellular electrodes placed on the nerve in the proximal thigh and recorded by electrodes (ADInstruments, Sydney, Australia), placed distally on the nerve; CMAPs were recorded from recording electrodes placed in the tibialis anterior muscle. CAPs and CMAPs are bimodal (yes/no) assessments as differences in waveform are mostly a result of electrode placement (Bittner et al., 2015; Ghergherehchi et al., 2016, 2019a, b; Mikeshe et al., 2018a, b).

The sciatic nerve bathed in isotonic extracellular fluid or Normal saline + calcium chloride was then completely transected with sharp microscissors in mid-thigh between the two electrodes; the proximal and distal cut ends of the sciatic nerve typically separated by 2–3 mm (Figure 2B). Once the sciatic nerve was severed CAPs and CMAPs were no longer recordable (Figure 3).

As summarized in Table 3, our PEG-fusion protocol to repair a singly transected sciatic nerve consisted of the following sequential steps # (1) – (5):

1. The proximal and distal severed axonal ends were directly irrigated with a priming solution (Ca²⁺-free, hypotonic saline: Normosol-R (Hospira, Lake Forest, IL, USA; diluted with ddH₂O to ~250 mOsm) for 2–3 minutes to open the axonal ends, expel existing intra-axonal vesicles, and inhibit new vesicle formation that decrease PEG-fusion success.

2. MB (Acros Organics, Morris Plains, NJ, USA), an antioxidant, in distilled water was applied directly to the lesion site for 2–3 minutes to further open axonal ends and inhibit new vesicle formation (Figure 2C; Spaeath et al., 2012; Vargas and Bittner, 2019). We have historically used 1% MB in sterile H₂O (Figure 2D), but 1% MB is no longer readily available for clinical procedures. However, 0.5% MB is now readily available as PravoxBlue™ (American Regent, Shirley, NY, USA) (Figure 2D*). Animals in these groups that also receive PEG (see step (4) below) are referred to as PEG + 1% MB and PEG + 0.5% MB, respectively.

3. One vesicle-free donor and/or host axons and their sheaths were well trimmed at all lesion sites and all axons were closely apposed with microsutures (neurorrhaphy). The microsutures provide mechanical strength at the lesion sites to prevent PEG-fused axons from pulling apart because axonal (or other cell-type) plasmalemmas have minimal tensile strength. Microsutures also help align the two cut ends in their approximate original orientation, but do not re-appose the two cut ends of each (or possibly any) specific axon.

4. A high concentration (50% w/w solution) of the membrane fusogen PEG (Sigma Alrich, St. Louis, MO, USA; 3.35 kDa PEG in distilled water) was directly applied for 1–2 minutes to non-specifically connect/join (PEG-fuse) the closely-apposed axonal membranes (axolemmas) of open, vesicle-free, distal and proximal axons. The PEG concentration, molecular weight, and time of direct application are critical variables. Experimental animals in this group that receive PEG alone and (no MB, see step (2)) are referred to as PEG animals in this paper.

5. Several washes of Normal Saline + Calcium chloride that mimicked the host’s extracellular fluids/blood plasma was directly applied to remove all PEG, induce newly-formed vesicles to seal any remaining plasmalemmal holes, and restore axolemmal/axoplasmic continuity of rat sciatic...
we used a two-way repeated measure ANOVA followed by Tukey’s post hoc test. A value of $P < 0.05$ was considered statistically significant.

**Results**

**SFI behavioral assessments of control and experimental groups**

The SFI score of each animal tested for two trials before any procedure also served as its own BC. The mean and range ($-1.8 \pm 8.1, -11.0$ to 9.5) of the BC for $n = 30$ rats used in this study were plotted on the $Y$-axis of Figure 4. Figure 4 also plots the SFI values for Unoperated Controls (Unop) and Sham Operated Controls for 6 weeks. As shown in Table 4, the SFI curves or values at 42 days for these control protocols did not differ significantly ($P > 0.05$) from each other. Furthermore, the SFI values for Unop or Sham Control rats do not differ significantly at any times tested from 1–6 weeks or at 1 week vs. 6 weeks for any group.

**Statistical analyses**

Statistical analyses given in Table 4 showed that the curves of PEG + 1% MB vs. PEG + 0.5% MB shown in Figure 4 were not significantly different ($P > 0.05$), nor were their values at 6 weeks (42 days) PO, a PO time at which SFI curves for PEG-fused sciatic nerves plateau in previous studies (Britt et al., 2010; Bittner et al., 2012, 2019a, b; Mikesh et al., 2018a, b). However, curves at 1–6 weeks PO plotted in Figure 4 for PEG + 1% MB and PEG + 0.5% MB both showed significantly better recovery vs. PEG ($P < 0.05$; Table 4). The SFI value at 6 weeks PO for PEG + 1% MB vs. PEG was significantly better ($P < 0.05$) but not for PEG + 0.5% MB vs. PEG ($P = 0.0953$ in Table 4). We note our curve for HPEG + 1% MB using Lactated Ringer’s as the final wash solution was also significantly better ($P < 0.01$) than PEG without MB. Furthermore, our curve for HPEG + 1% MB did not differ significantly from our currently obtained curve for PEG + 1% MB ($P > 0.05$).

Figure 4 and Table 4 both show that the 1–6 week PO curves and 6 week PO values for both PEG + MB groups are all very significantly better ($P < 0.0001$) than the NC group. The PEG curve is also significantly ($P < 0.05$) better than the NC curve and the PEG alone protocol is significantly better than the NC protocol at 6 weeks PO ($P < 0.001$).

**Figure 5** shows the SFI scores for individual animals in all treatment groups. Unoperated and Sham Control animals (Figure 5A) showed no general trend in scores from 1–6 weeks post-operatively. Negative Control animals (each individual animal designated as “NC” followed by assigned number, Figure 5B) all showed loss of function and little, if any, recovery. Historical PEG + 1% MB animals (each individual animal designated as “HP” followed by assigned number, Figure 5C) showed a general trend of increased SFI scores over time. SFI scores for most individual animals in the PEG, PEG + 0.5% MB and PEG + 1% MB experimental groups (each

Table 3 | PEG-fusion protocols and rationale to immediately repair (join) cut axonal ends

| Preparation | Completely sever and trim nerve ends | Prepare nerve ends for neurorrhaphy and PEG repair/fusion |
|-------------|--------------------------------------|------------------------------------------------------|
| 1. Priming  | Irrigation of surgical field with hypotonic Ca$^{2+}$-free saline for 1–2 min | Increase axoplasmic volume. Open cut axonal ends |
| 2. Protection | Administration of 0.5% methylene blue (MB; an antioxidant) in distilled water for 1–2 min to open cut ends | Expel intracellular membrane-bound vesicles/organelles |
| 3. Coaptation of cut nerve ends | Perform neurorrhaphy | Prevent formation of intracellular vesicles/organelles that interfere with PEG-fusion of cut ends and can seal-off each apposed cut ends rather joining/fusing them |
| 4. PEG-fuse many axons | Apply 50% w/w 3.53 kDa PEG in H$_2$O for 1–2 min to the coaptation site | Provide mechanical strength to epineurium to prevent PEG-fused axons pulling apart. Closely appose cut axon ends |
| 5 Complete membrane repair | Irrigation of coaptation site with isotonic Ca$^{2+}$-containing saline | Remove bound cell water to induce closely apposed, open, axonal membranes to non-specifically fuse |

PEG: Polyethylene glycol.

nerves in the upper thigh (Figure 2E). We have historically used Lactated Ringer’s (Hospira, Lake Forest, IL, USA) that calculated as 273 mOsm. However, Lactated Ringer’s measured 258.3 ± 5.9 mOsm when recently assayed with a well-calibrated osmometer, i.e., some ionic particles are not completely dissociated (hydrated). Hence, in this study we used a normal saline with physiological levels of calcium but a different formulation, i.e. 0.9% NaCl + 0.02% CaCl$_2$. This solution calculates as 310 mOsm, but measures 294.7 ± 3.8 mOsm, i.e., isotonic calcium-containing saline. We confirmed that PEG-fusion was initially successful by recording CAPs and CMAPs from PEG, PEG + 0.5% MB, and PEG + 1% MB animals (Figure 3).

The PEG-containing solution in step #4 above was omitted for “NC animals” that never exhibited rapid re-innervation, immediate axonal restoration of through conduction of action potentials (Figure 3) and behavioral recovery within several weeks (Figure 4).

**SFI**

We used the SFI to measure recovery of hindlimb functions mediated by the sciatic nerve (de Medinaceli et al., 1982; Carlton and Goldberg, 1986; Varejao et al., 2001; Bittner et al., 2012, 2015). A week prior to surgery, animals were handled and allowed to run up the board lined with paper strips. Tapes with red (uninjured) or blue (injured) ink, and the rat was induced annealing of the open cut axonal ends

Explain how to fuse axonal ends

1. Priming Irrigation of surgical field with hypotonic Ca$^{2+}$-free saline for 1–2 min to open cut ends
2. Protection Administration of 0.5% methylene blue (MB; an antioxidant) in distilled water for 1–2 min to open cut ends
3. Coaptation of cut nerve ends Perform neurorrhaphy
4. PEG-fuse many axons Apply 50% w/w 3.53 kDa PEG in H$_2$O for 1–2 min to the coaptation site
5 Complete membrane repair Irrigation of coaptation site with isotonic Ca$^{2+}$-containing saline

PEG: Polyethylene glycol.
individual animal designated as “O” followed by assigned number. Figure 5D–F showed a general trend of increased SFI scores over time. However, a few individual animals in the different PEG groups (Figure 5D–F) showed little or no recovery of their SFI scores by 42 days PO. The lowest SFI scores for individual PEG treated animals at 42 days PO in Figure 5D–F (O1; SFI = –87), PEG + 0.5% MB (O11; SFI = –90) and PEG + 1% MB (O22; SFI = –82) were similar to SFI scores at any PO time for the NC group (Figure 5B). Individual PEG treated animals with intermediate SFI recovery at 42 days PO (O32; SFI = –66), PEG + 0.5% MB (O15; SFI = –53) and PEG + 1% MB (O35; SFI = –50) all had higher SFI scores than NC animals at 42 days PO (Figure 5B). Individual PEG-treated animals with the greatest SFI recovery (O4; SFI = –45), PEG + 0.5% MB (O17; SFI = –34) and PEG + 1% MB (O26; SFI = –32) achieved SFI scores never observed for any individual NC animal at any PO time.

Representative footprints for individual animals in Unop and Sham control groups (Figure 6) showed no obvious difference between the two groups or at any PO time. All animals in these control groups were able to lift their heel and spread toes during locomotion required for the SFI test. In contrast, all NC animals (each individual animal designated as “NC” followed by assigned number) lost the ability to completely lift their heel off the ground, and total toe spread and intermediate toe spread only modestly recovered for individual NC animals with the highest SFI scores (NC7; SFI = –72). Individual NC animals with low SFI scores (NC9; SFI = –103) completely lost sciatic all nerve mediated behaviors. For individual HPEG animals (each individual animal designated as “HPEG” followed by assigned number) with the lowest (worst) SFI score at 42 days PO (HPEG11; SFI = –84) had footprints similar to the average individual NC animal at 42 days PO (NC1; SFI = –84). Footprints for individual HPEG animals with average to excellent SFI scores showed intermediate and total toe spread similar to those of individual Unop or treated animals with intermediate SFI recovery at 42 days PO (HPEG + 1% MB, PEG + 1% MB, PEG + 0.5% MB) all showed significantly (P < 0.01) improved SFI scores compared to Negative Controls. PEG-fusion without MB also showed significantly (P < 0.05) improved SFI scores compared to negative controls. Two-way analysis of variance followed by Tukey’s post hoc test was used. Range of baseline data (–11 to +9.5) indicated by shaded gray region, with a dotted line indicating the mean (–1.8) baseline control score. Data are expressed as the mean ± SD; HPEG: historical PEG-fusion data; MB: methylene blue; PEG: polyethylene glycol; PO: postoperatively; SFI: sciatic functional index.

Sham control animals but lacked an ability to completely lift their heel off the ground during locomotion in the SFI test. Individual animals in the PEG, PEG + 0.5% MB and PEG + 1% MB experimental groups (Figure 7) with the worst (lowest) SFI scores exhibited footprints associated with footprints of individual NC animals with similar SFI scores. Individual animals in the PEG-treated groups with average to excellent SFI scores also had well-spread toes similar to those observed for individual Unop and Sham controls and were also unable to completely lift their heel off the ground.

Discussion

Summary of results

Our SFI data show that maintaining rats in cages for 6 weeks does not affect their SFI scores, nor does surgery to expose (but not damage) their sciatic nerves. Furthermore, 1% and 0.5% MB as part of PEG-fusion protocol significantly improves voluntary behavioral recovery as measured by SFI compared to a PEG-fusion protocol that does not directly apply MB to the lesion site. The SFI scores for PEG without 0.5% MB eventually recover to a similar extent as PEG with 0.5% MB at six weeks, but recovery with the addition of 0.5% MB is more rapid and complete when all time points (1–6 weeks) are considered.
All PEG-fusion protocols tested herein are significantly better than our NC protocol, i.e., a “gold standard” neurorrhaphy that does not apply a high concentration of PEG directly to the lesion site (Campbell, 2008; Brushart, 2011). Given these data and the fact that 1% MB in sterile distilled water is no longer readily clinically available, we will now use 0.5% MB in future studies that provide data/protocols to translate successful PEG-fusion in animal model systems (rats, swine) to clinical case studies/trials.

Our data also document that there are obvious differences in the way individual animals in any given control or experimental group behave and/or recover from a complete cut of the sciatic nerve in mid-thigh, as measured by the SFI. While PEG-fusion rapidly fuses membranes in close apposition, the recovery of animals might depend upon specificity of axons that are fused, or peripheral and central plasticity to compensate for altered connections over time (Mikesh et al., 2018a, b; Ghergherehchi et al., 2019b). Therefore, while the fusion of severed axonal membranes occurs within minutes, the return of behavioral function may take days or weeks to occur. Our data additionally confirm that there is an occasional surgical failure for individual animals in any PEG-fusion protocol. Finally, except for surgical failures, our data show that individual animals with PEG-fused sciatic nerves have consistently better SFI scores than NC “gold standard” animals.

Enhancement of PEG-fusion by MB
Application of antioxidants such as MB before applying PEG reduces vesicle formation at cut axonal ends and reduces the probability that such ends partially collapse (Spaeth et al., 2012; Vargas and Bittner, 2019), consistent with the hypothesis that mechanisms to seal plasmalemmal holes, including complete axoplasmic transactions, are similar in all eukaryotic cells (Spaeth et al., 2010; Vargas and Bittner, 2019).
Vesicles at cut ends decrease the ability of the axolemma at two closely apposed cut ends to PEG-fuse and thereby increase their ability to PEG-seal (Vargas and Bittner, 2019). Hence, application of MB in hypotonic solutions (e.g., distilled water) applied before applying PEG should decrease the ability of the plasmalemma to seal the complete transection, keep axonal ends open and free of vesicles, and enhance PEG-fusion. Our data in this and other papers are consistent with all these hypotheses (Bittner et al., 2012, 2015; Spaeth et al., 2012). The ability of MB to reduce the formation of intracellular membranous structure presumably depends on the MB concentration in a conventional dose-response curve in which 0.5% MB and 1% MB in distilled water both have near-maximum effect (Spaeth et al., 2012a). We also note that 1% MB had faster rates of recovery than melatonin after PEG-fusion of sciatic nerves in calcium-free saline (Bittner et al., 2012).

After applying PEG, adding an oxidizing agent to the calcium-containing isotonic saline might increase PEG-fusion success by further enhancing the sealing of any remaining axolemmal holes in PEG-fused axons (Spaeth et al., 2012b). Neuroprotective effects of MB or PEG (Vargas and Bittner, 2019) might enhance the survival of axons repaired by PEG-fusion increasing by the number of PEG-fused axons and/or improving survival of proximal segments of axons not PEG-fused. Axons that are not PEG-fused might regenerate by outgrowths at ~1 mm/day to re-innervate distal target tissues and eventually further increase behavioral recoveries observed for PEG-fused axons at 4–6 weeks PO after severing rat sciatic nerves. We also note that application of 0.5% MB and 1% MB stain nerve axons, making them easier to visualize in microsurgical procedures for better alignment of fascicles during repair.

Comparisons of SFI data in this and previous papers

The SFI scores for control and PEG-fused experimental groups at 0–42 days PO for rat sciatic nerves singly transected in calcium-containing isotonic saline/extracellular fluid in this study are similar to scores published in previous in previous studies (Bittner et al., 2015; Bamba et al., 2016a; Ghergherehchi et al., 2016, 2019a, b; Mikes et al., 2018a, b; Smith et al., 2020). The SFI scores for control and PEG-fused experimental groups at 0–42 days PO for rat sciatic nerves singly transected in calcium-free hypotonic saline/extracellular fluid in a previous study (Bittner et al., 2012) are slightly better than scores obtained in this paper or previous papers (Bittner et al., 2015; Ghergherehchi et al., 2016, 2019a, b; Mikes et al., 2018a; Smith et al., 2020). We hypothesize that the cut ends of axons severed and immediately PEG-fused in calcium-free hypotonic saline have even-fewer vesicles at open cut ends and hence are more likely to PEG-fuse than PEG-seal (Spaeth et al., 2012a; Vargas and Bittner, 2019).

Limitations

Limitations of the current study include the use of only female adult rats, and the exclusive use of the SFI to measure functional recovery. However, we note that behavioral assays such as the SFI are the most valid and appropriate measure of recovery following a traumatic lesion to a peripheral nerve. That is, the number of sciatic axons, innervated NMJs, and CAP/CMAP amplitudes are often not highly predictive of recovery of voluntary behaviors following PNIs (Brushart, 2011; Bittner et al., 2012, 2015; Ghergherehchi et al., 2016, 2019a, b; Mikes et al., 2018a, b; Smith et al., 2020). We have also previously shown the efficacy of PEG-fusion in both male and female rats (Britt et al., 2010; Bittner et al., 2012; Riley et al., 2015; Mikes et al., 2018a, b; Ghergherehchi et al., 2019a, b; Smith et al., 2020).

Clinical translation

PNIs often result in severe long-term disability (Lee and Wolfe, 2000; Campbell, 2008; Fox and Kreishman, 2010; Brushart, 2011). Depending on the site of nerve transection, the recovery can be extremely prolonged due to the time specific nature of axonal regeneration following Wallerian degeneration. The more proximal the PNI, the longer the distance needed for axons to regenerate before re-innervating sensory organelles and NMJs. In fact, very proximal PNIs often result in minimal or no significant recovery of distal limb.

| Groups | 0–42 d PO | 42 d PO |
|--------|----------|---------|
| | Significance | P value | Significance | P value |
| Unoperated (n = 3) vs. Sham | ns | 0.9999 | ns | 0.9883 |
| Unoperated (n = 3) vs. HPEG + 1% MB (n = 13) | **** | < 0.0001 | **** | < 0.0001 |
| Unoperated (n = 3) vs. Negative Control (n = 18) | **** | < 0.0001 | **** | < 0.0001 |
| Unoperated (n = 3) vs. PEG + 1% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Unoperated (n = 3) vs. PEG + 0.5% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Sham vs. HPEG + 1% MB (n = 13) | **** | < 0.0001 | **** | < 0.0001 |
| Sham vs. Negative Control (n = 18) | **** | < 0.0001 | **** | < 0.0001 |
| Sham vs. PEG + 1% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Sham vs. PEG + 0.5% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Sham vs. PEG (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| HPEG + 1% MB (n = 13) vs. Negative Control (n = 18) | **** | < 0.0001 | **** | < 0.0001 |
| HPEG + 1% MB (n = 13) vs. PEG + 1% MB (n = 10) | ns | 0.9037 | ns | 0.9999 |
| HPEG + 1% MB (n = 13) vs. PEG + 0.5% MB (n = 10) | ns | 0.8658 | ns | 0.9795 |
| HPEG + 1% MB (n = 13) vs. PEG (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Negative Control (n = 18) vs. PEG + 1% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Negative Control (n = 18) vs. PEG + 0.5% MB (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| Negative Control (n = 18) vs. PEG (n = 10) | **** | < 0.0001 | **** | < 0.0001 |
| PEG + 1% MB (n = 10) vs. PEG + 0.5% MB (n = 10) | ns | 0.9999 | ns | 0.9999 |
| PEG + 1% MB (n = 10) vs. PEG (n = 10) | ns | 0.9999 | ns | 0.9999 |
| PEG + 0.5% MB (n = 10) vs. PEG (n = 10) | ns | 0.9999 | ns | 0.9999 |

ns: Not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, respectively according to two-way analysis of variance with multiple comparisons from SFI data from 0–42 days PO, or SFI data from 42 days PO only.
function or voluntary behavioral use due to degeneration of distal synapses/sensory end organs and atrophy of denervated muscles. The lack of protective sensation also adds additional risk/liability to the limb as it is constantly in danger of injury and/or infection without the patient’s realization (Lee and Wolfe, 2000; Campbell, 2008; Fox and Kreishman, 2010).

PEG-fusion of single transection PNs has an obvious potential to greatly benefit patients by dramatically increasing the speed and amount of functional recovery, including much restoration of protective sensation (Bamba et al., 2016b). Theoretically, PEG-fusion has even greater potential if combined with nerve transfers, autograft reconstruction, or even allograft nerve reconstruction with viable axons for segmental (ablation) defects (Roballo and Bushman, 2019). The field of vascularized composite allotransplantation (VCA) or “reconstructive transplantation” could be significantly altered if PEG-fusion greatly increased the probability of restoring motor and sensory function of a proximally transplanted limb. Hence, optimizing the solutions, such as MB, used to induce successful PEG-fusion is of great importance for upcoming and future clinical trials. MB has been used safely in humans for many years for multiple purposes unrelated to PEG fusion including methemoglobinemia, urinary tract dysfunction, cyanide poisoning, as well as a topical dye for tissue marking and/or debridement (Bradberry, 2003; Dorafshar et al., 2010; Cheung et al., 2018; Huang et al., 2018). When used intravenously in doses < 2 mg/kg, it is considered very safe.

Conclusion
Our current and previous data (Bittner et al., 2012) show that application of 0.5–1% MB does significantly enhance behavioral recovery of PEG-fusion repair of PNSs and we therefore recommend its inclusion in human clinical trials evaluating the efficacy of PEG-fusion.

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Conflicts of interest: Dr. Bittner has assigned all of his economic interests in a licensed PEG-fusion patent estate to a third party that affects in no way any data analyses or text in this manuscript.

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References
Bamba R, Riley DC, Kelm ND, Does MD, Dortch RD, Thayer WP (2016a) A novel technique using hydrophilic polymers to promote axonal fusion. Neural Regen Res 11:525-528.
Bamba R, Waltayawin F, Vizakula R, Riley DC, Boyer B, Sexton KW, Booyzziakiel C, Niempoog S, Kelm ND, Does MD, Dortch RD, Shack RB, Thayer WP (2016b) A novel therapy to promote axonal fusion in digital nerves. J Trauma Acute Care Surg 81:S177-86.
Bittner GD, Keating CP, Kane BJ, Britt IR, Spach ES, Fan JD, Zuick A, Wilcock RW, Thayer WP, Winograd JM, Gonzalez-Lima F, Schallert T (2012) Rapid, effective, and long-lasting behavioral recovery produced by microsutures, methylene blue and polyethylene glycol as “fused” repair. J Neurosci Res 90:367-380.
Bittner GD, Sengelaub DR, Ghergherehchi CL (2017a) Conundrums and confusions regarding how PEG-fusion produces excellent behavioral recovery after peripheral nerve injuries. J Neurosurg Res 95:863-866.
Bittner GD, Sengelaub DR, Trevino RC, Ghergherehchi CL, Mikesh M (2017b) Robinson and Madison have published no data on whether polyethylene glycol fusion repair prevents reinnervation accuracy in rat peripheral nerve. J Neurosci Res 95:863-866.