RESEARCH ARTICLE

Effect of exogenous spastin combined with polyethylene glycol on sciatic nerve injury

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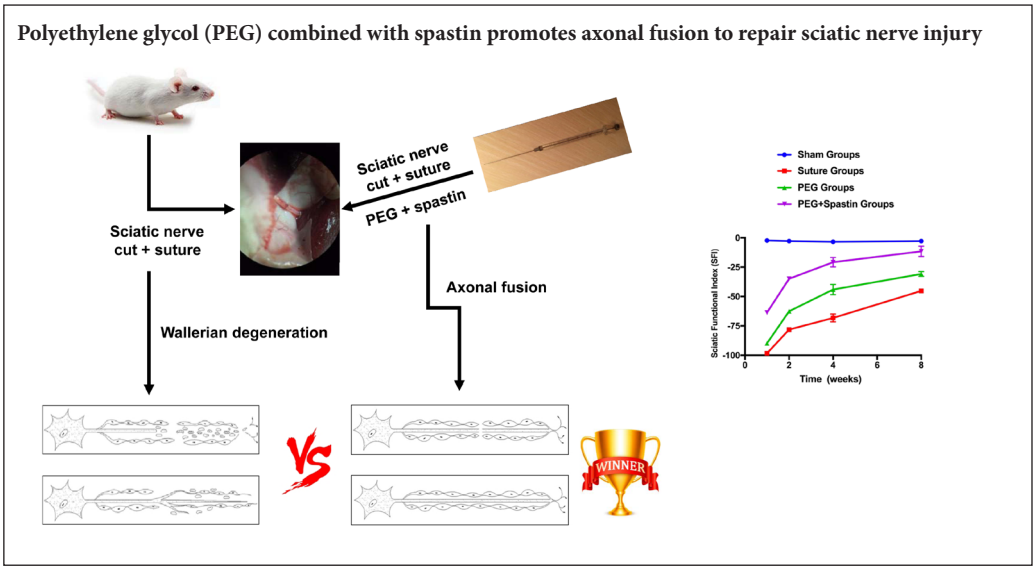
Graphical Abstract



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Abstract

Polyethylene glycol can connect the distal and proximal ends of an injured nerve at the cellular level through axonal fusion to avoid Wallerian degeneration of the injured distal nerve and promote peripheral nerve regeneration. However, this method can only prevent Wallerian degeneration in 10% of axons because the cytoskeleton is not repaired in a timely fashion. Reconstruction of the cytoskeletal trunk and microtubule network has been suggested to be the key for improving the efficiency of axonal fusion. As a microtubule-severing protein, spastin has been used to enhance cytoskeletal reconstruction. Therefore, we hypothesized that spastin combined with polyethylene glycol can more effectively promote peripheral nerve regeneration. A total of 120 male Sprague-Dawley rats were randomly divided into sham, suture, polyethylene glycol, and polyethylene glycol + spastin groups. In suture group rats, only traditional nerve anastomosis of the end-to-end suture was performed after transection of the sciatic nerve. In polyethylene glycol and polyethylene glycol + spastin groups, 50 μL of polyethylene glycol or 25 μL of polyethylene glycol + 25 μL of spastin, respectively, were injected immediately under the epineurium of the distal suture. Sensory fiber regeneration distance, which was used to assess early nerve regeneration at 1 week after surgery, was shortest in the suture group, followed by polyethylene glycol group and greatest in the polyethylene glycol + spastin group. Behavioral assessment of motor function recovery in rats showed that limb function was restored in polyethylene glycol and polyethylene glycol + spastin groups at 8 weeks after surgery. At 1, 2, 4 and 8 weeks after surgery, sciatic functional index values and percentages of gastrocnemius muscle wet weight were highest in the sham group, followed by polyethylene glycol + spastin and polyethylene glycol groups, and lowest in the suture group. Masson staining was utilized to assess the morphology of muscle tissue. Morphological changes in skeletal muscle were detectable in suture, polyethylene glycol, and polyethylene glycol + spastin groups at 1, 2, 4, and 8 weeks after surgery. Among them, muscular atrophy of the suture group was most serious, followed by polyethylene glycol and polyethylene glycol + spastin groups. Ultrastructure of distal sciatic nerve tissue, as detected by transmission electron microscopy, showed a pattern of initial destruction, subsequent disintegration, and gradual repair in suture, polyethylene glycol, and polyethylene glycol + spastin groups at 1, 2, 4, and 8 weeks after surgery. As time proceeded, axonal ultrastructure gradually recovered. Indeed, the polyethylene glycol + spastin group was similar to the sham group at 8 weeks after surgery. Our findings indicate that the combination of polyethylene glycol and spastin can promote peripheral nerve regeneration. Moreover, the effect of this combination was better than that of polyethylene glycol alone, and both were superior to the traditional neurorrhaphy. This study was approved by the Animal Ethics Committee of the Second Military Medical University, China (approval No. CZ20170216) on March 16, 2017.

Key Words: *nerve regeneration; peripheral nerves; Wallerian degeneration; polyethylene glycol; axonal fusion; spastin; peripheral nerve injuries; Masson staining; microtubule; neural regeneration*

Chinese Library Classification No. *R453; R364*

Introduction

Peripheral nerve injury is common in clinical settings (Cattin et al., 2015; Trehan et al., 2016; Liu et al., 2018). The classic mode of nerve regeneration includes the occurrence of Wallerian degeneration at the distal end, whereby the entire distal nerve is re-grown from the proximal end to the distal end (Wang et al., 2012). The regeneration rate after such injuries is very low (approximately 1 mm/d), and treatment of peripheral nerve injury remains a medical puzzle (Bozkurt et al., 2008; Campbell, 2008; Sadeghian et al., 2010). However, a new mode of nerve regeneration involving a process of axonal fusion that accelerates the rate of nerve regeneration has also been described (Deriemer et al., 1983; Neumann et al., 2011). As a hydrophilic high-molecular polymer, polyethylene glycol (PEG) has mainly been used to fuse cell membranes and form multinucleated cells. However, it has also been shown to promote the repair of spinal cord injury (Kohler et al., 1975; Ahkong et al., 1987) and peripheral nerve injury (Eddleman et al., 1998; Spaeth et al., 2010; Bittner et al., 2012; Riley et al., 2015; Ghergherehchi et al., 2016).

Typically, only a few distal axons can avoid Wallerian degeneration after axonal fusion, possibly as a result of unrepaired cytoskeletal structures (Spaeth et al., 2010; Riley et al., 2015; Bittner et al., 2016a, b). Thus, prompt repair of the cytoskeleton and ensuring material transport from the cell body at the distal end are key factors for nerve regeneration. Microtubules are the main structure forming the cytoskeleton (Brill et al., 2016; Gobrecht et al., 2016). In mature axons, microtubules form the path for material transport. Spastin is one of three known microtubule-severing proteins, which belong to the AAA protein family and are highly expressed in the developing nervous system. Spastin has been shown to play a prominent role in remodeling of microtubule defects and guidance of new microtubule formation (Stone et al., 2012; Rao et al., 2016). Moreover, researchers found that spastin expression changed after sciatic nerve injury, first decreasing, then increasing, and then dropping to the lowest point 7 days after surgery. This result suggested that spastin may play an important role in nerve regeneration (Lin et al., 2017).

In the present study, we intended to explore a novel neural repair strategy from a new perspective. First, the axonal fusion pathway was used to avoid Wallerian degeneration of distal nerve endings after nerve injury. Second, to regulate microtubule remodeling after peripheral nerve injury, spastin protein was used to strengthen cytoskeletal reconstruction and restore material transport in the axon, which should improve the efficiency and effect of axonal fusion. To achieve this, a model of sciatic nerve injury was established and neurological recovery was observed at different time points after injury by locally injecting PEG and spastin into the epicardium of the distal end of the anastomosis. The present study initially explored the effects of axonal fusion combined with microtubule remodeling on peripheral nerve regeneration.

Materials and Methods

Animals

A total of 120 male Sprague-Dawley rats weighing 200 ± 20 g were provided by Shanghai JieSiJie Experimental Animal Co., Ltd. [Shanghai, China; license number SCXK (Hu) 2013-0006]. The procedure was approved by the Animal Ethics Committee of the Second Military Medical University, China (approval No. CZ20170216) on March 16, 2017. Experimental rats were housed at 22°C with 14 hours of light/10 hours of dark every day.

Rats were randomly divided into sham, suture (sciatic nerve transection + nerve suture), PEG (sciatic nerve transection + PEG), and PEG + spastin (sciatic nerve transection + PEG + spastin) groups (n = 30 per group).

Surgical procedure

Experimental rats were intraperitoneally injected with 2.5% pentobarbital sodium (30 mg/kg; Shanghai XinYa Pharmaceutical Co., Ltd., Shanghai, China) and fixed in the prone position. A 2-cm posterior median incision was made on the right side. The muscle tissue was cut, and the sciatic nerve was exposed. In the sham group, no additional procedures were performed after sciatic nerve exposure. In suture, PEG, and PEG + spastin groups, sciatic nerves were cut with microsurgical scissors 5 mm below the piriformis muscle. The end-to-end suture was performed immediately with 11-0 nylon. In the suture group, only nerve suture was performed. In the PEG group, 50 μL of 50% PEG (w/v, molecular weight of 800; Sigma, St. Louis, MO, USA) was injected into the epineurium on the distal end of the anastomosis. In the PEG + spastin group, 25 μL of spastin protein [ab152700; Abcam (Shanghai) Trading Co., Ltd., Shanghai, China] combined with 25 μL of PEG (100%, w/v) was injected into the epineurium on the distal end of the anastomosis. After surgical procedures, each rat was numbered to measure additional parameters in a blinded fashion.

After rats awakened, they were judged for general behavior. The animal model was regarded as successfully established if weakness occurred in the affected limb; no autonomic activity was observed; the body could not be supported by the limb; the knee joint, ankle joint, and toe joint were difficult to flex or unable to flex and sag; and limbs were dragged forward.

General observation

At 1, 2, 4, and 8 weeks after surgery, the appearance of toes on the affected side and gait of rats were observed, including toe expansion and presence of plantar ulcers in the surgical limbs of each group. Hindlimb function at various postoperative intervals was observed by the investigators to estimate the recovery of neurological function. The better the limb function on the surgical side, the better nerve recovery was considered to be.

Functional assessment

The sciatic functional index (SFI) for rats in each group was

tested at 1, 2, 4, and 8 weeks after surgery to evaluate recovery of hindlimb function. For these evaluations, rat hindlimbs were dipped in black Chinese ink and their footprints on white paper were analyzed, in accordance with the method described by de Medinaceli et al. (1982) and modified by Bain et al. (1989). The inter-toe distance (IT), width between the first and fifth toes (TW), and podogram length (PL) of footprints on affected (E) and uninjured (N) sides of each rat were assessed in accordance with the schematic diagram in **Figure 1**, and included in the Bain formula (Bain et al., 1989):

SFI = −38.3(EPL − NPL)/PL + 109.5(ETW − NTW)/NTW + 13.3(EIT−NIT)/NIT − 8.8.

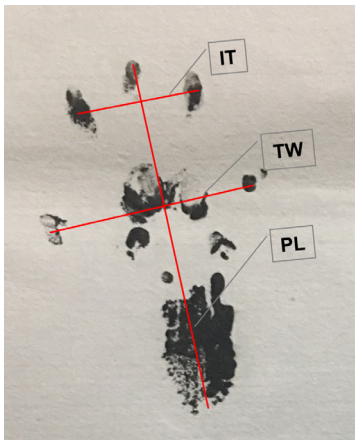

Figure 1 Rat footprint.
IT: Inter-toe distance; TW: width between the first and fifth toes; PL: podogram length.

Sensory fiber regeneration distance

The extent of sensory fiber regeneration was assessed using the pinch test in six rats from each group 1 week after surgery. After intraperitoneal injection with 2.5% sodium pentobarbital and anesthesia, the right sciatic nerve was exposed and the nerve anastomosis was identified. At 2 cm from the anastomosis, the sciatic nerve was clamped with microscopic tweezers in a distoproximal direction. When the tips of the fastest growing sensory axons were clamped, a reflex response was elicited that could be observed as movement of the leg and contractions of the muscles of the back. The distance between this site on the nerve and the nerve coaptation site was measured under magnification with vernier calipers. The value obtained was regarded as the regeneration distance.

Muscle mass assessment

At 1, 2, 4, and 8 weeks after surgery, rats were anesthetized at the abdominal cavity, and the gastrocnemius muscles were excised along the calf. After removing connective tissue from the muscle surface, the muscle was dried with filter paper and then promptly weighed using an analytical balance (with an accuracy scale interval of 1 mg; R200D, Sartorius, Hamburg, Germany). The muscle mass preservation ratio was recorded by dividing the wet weight of the gastrocnemius muscle on affected and uninjured sides.

Muscle fiber diameter measurement

At 1, 2, 4, and 8 weeks after surgery, specimens of the middle gastrocnemius muscle were obtained and fixed in 4% polyformaldehyde, embedded in paraffin, and used to create 5-mm-thick transverse sections. Five sections acquired from every specimen were subjected to Masson staining before photography with a DFC 300FX color digital microscope (Shanghai Leica Instrument Co., Ltd., Shanghai, China). For each section, images were captured from five random fields and analyzed with Image Pro Plus software to measure changes in the diameter of muscle fibers.

Ultrastructural observation

At 1, 2, 4, and 8 weeks after surgery, a 1-mm-long sample of nerve tissue was obtained from a point 5 mm from the distal end of the nerve anastomosis and quickly fixed in fixative for 4 hours at 4°C. The sample was then rinsed with 0.1 M phosphate-buffered saline (pH 7.4) and fixed in 1% osmium acid. Tissues were then dehydrated in a series of ethanol solutions [concentrations ranging from 50% to 70% (24 hours), then 80% to 90% and 95% to 100% (15 minutes each)], followed by osmosis with a mixed solution (1:1 solution of acetone and 812 embedding agent) and pure 812 embedding agent solution. After pruning samples to a surface area of less than 0.2 mm × 0.2 mm, 70-nm ultrathin longitudinal sections were obtained and subjected to uranium-lead double staining with 2% uranyl acetate saturated aqueous solution and lead citrate. After drying at room temperature overnight, stained sections were observed by transmission electron microscopy (Hitachi, Tokyo, Japan) and obtained images were collected and analyzed.

Statistical analysis

Data are expressed as the mean ± SD. SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was used to analyze data. Paired *t*-test or repeated measures analysis of variance was used for comparisons across different time points in each experimental group. Experimental groups (suture, PEG, and PEG + spastin) were compared with the sham group using one-way analysis of variance followed by Dunnett's *post hoc* test, with a test level $\alpha = 0.05$.

Results

Effect of exogenous spastin and PEG on hindlimb function of rats after sciatic nerve injury

Rats in the sham group showed normal hindlimb function after surgery. One week after surgery, rats in suture, PEG, and PEG + spastin groups presented limited toe opening and different degrees of dysfunction of the affected limbs. The degree of paralysis in PEG and PEG + spastin groups was lower than observed in the suture group (**Figure 2**). Four weeks after surgery, rats in the suture group showed toe defects and local plantar ulcers, and were still unable to flex the foot at the operated side or completely open their toes. Damaged toes and localized ulcers were visible in PEG and PEG + spastin group rats. However, the severity of these defects was less in PEG and PEG + spastin groups compared with the suture group. The degree of toe damage and local ulcers was lower in the PEG + spastin group than in suture

and PEG groups. Eight weeks after surgery, obvious recovery of hindlimb function was detectable on the operated side and rats could walk in PEG and PEG + spastin groups. However, the foot on the operated side was unable to fully participate in walking because of the plantar flexion dysfunction, and the walking movement was not coordinated in the suture group.

Effect of exogenous spastin and PEG on SFI in rats after sciatic nerve injury

There were no significant changes in imprints or SFI in the sham group. By the seventh postoperative day, none of the rats in suture, PEG, or PEG + spastin groups showed longer and narrower imprints compared with preoperative imprints. At 2, 4, and 8 weeks after surgery, imprints were shorter and wider than those obtained the first week. In addition, absolute SFI values gradually decreased in suture, PEG, and PEG + spastin groups ($P < 0.05$). At different postoperative intervals, the order of SFI values was sham group < PEG + spastin group < PEG group < suture group, and intergroup differences were statistically significant ($P < 0.05$; **Table 1**).

Effect of exogenous spastin and PEG on sensory fiber regeneration distance in rats after sciatic nerve injury

Distances measured by the pinch test at 1 week after surgery were 9.33 ± 0.02 mm, 11.63 ± 0.18 mm, and 13.93 ± 0.12 mm in suture, PEG, and PEG + spastin groups, respectively. Sensory fiber regeneration distances in PEG and PEG + spastin groups at 1 week after surgery were significantly greater than in the suture group ($P < 0.05$). Distances in the PEG + spastin group were greater than observed in the PEG group ($P < 0.05$).

Effect of exogenous spastin and PEG on gastrocnemius wet weight in rats after sciatic nerve injury

The order of gastrocnemius wet weight percentage values at different postoperative intervals was sham group > PEG + spastin group > PEG group > suture group ($P < 0.05$, except for PEG and suture groups at 1 week). Except for the sham group, all groups presented a decreasing trend for wet weight of the gastrocnemius muscle. Intragroup differences in values obtained at different time points were also statistically significant ($P < 0.05$; **Table 2**).

Effect of exogenous spastin and PEG on muscle fiber diameter in rats after sciatic nerve injury

Changes in muscle fiber diameter at different time points are shown in **Table 3**. In the sham group, no significant change was detected in muscle fiber diameter at 1, 2, 4, or 8 weeks after surgery ($P > 0.05$). However, muscle fiber diameter showed a gradual downward trend in suture, PEG, and PEG + spastin groups, with the most significant decline in the suture group. Muscle fiber diameter was significantly different between the suture group and PEG and PEG + spastin groups at 2, 4, and 8 weeks after surgery ($P < 0.05$). In addition, muscle fiber diameter was different at various time points in PEG and PEG + spastin groups. Although muscle fiber diameter was larger in the PEG + spastin group compared with the PEG group ($P > 0.05$), intragroup differences between these two groups were not significant at 4 and 8 weeks after surgery ($P > 0.05$) (**Figure 3** and **Table 3**).

Effect of exogenous spastin and PEG on axon ultrastructure and myelin sheath of rats after sciatic nerve injury

As shown in **Figure 4**, axonal ultrastructure was normal in the sham group at 1, 2, 4, and 8 weeks after surgery. Ultrastructure analysis of suture, PEG, and PEG + spastin groups showed a pattern of initial destruction, subsequent disintegration, and gradual repair over 1, 2, 4, and 8 weeks after surgery. One week after surgery, suture, PEG, and PEG + spastin groups showed obvious destruction of the myelin sheath, disintegration of microtubule and microfilament structures, blurred myelin lamellar structures, loose gaps, and irregular shape. Ultrastructural damages in the suture group were most significant and complete, while PEG and PEG + spastin groups showed slightly better residual partial structures. Two weeks after surgery, new nerve fibers were observed in suture, PEG, and PEG + spastin groups. In addition, Schwann cells began to proliferate, microtubules and microfilaments gradually became clear, and the myelin structure began to appear, but the sheaths were small, the lamellar structure was unclear, and the shape remained irregular. At this point, a clearer and complete new myelin structure, thicker myelin sheath, and increased proliferation of Schwann cells were detectable in PEG and PEG + spastin groups compared with the suture group. At 4 and 8 weeks after surgery, the number of nerve fibers was remarkably increased in the PEG + spastin group. At 8 weeks after surgery, the number, morphology, and size of myelin and nerve fibers in the PEG + spastin group resembled the sham group. In contrast, in suture and PEG groups, the number of nerve fibers decreased and the myelin sheath was thinner than observed in PEG + spastin and sham groups, but the recovery of axon structure was better in the PEG group than in the suture group.

Because a complete myelin sheath was rarely observed and the myelin sheath disintegrated within 1 to 2 weeks after surgery, the myelin sheath thickness could not be accurately measured at later points. Thus, only complete myelin sheaths observed at 4 and 8 weeks after the operation were examined in each group. **Table 4** exhibits changes in myelin sheath thickness of each group after 4 and 8 weeks. At 4 to 8 weeks after surgery, myelin sheath thickness gradually increased, with thicknesses in the order of sham group > PEG + spastin group > PEG group > suture group. Myelin sheath thickness was significantly different among sham, suture, PEG, and PEG + spastin groups ($P < 0.05$). At 8 weeks after surgery, myelin sheath thickness in the PEG + spastin group was similar to that observed in the sham group ($P > 0.05$). Myelin sheath thickness in the PEG + spastin group was not significantly different at 4 and 8 weeks after surgery. However, myelin sheath thickness was significantly different between suture and PEG groups ($P < 0.05$; **Table 4**).

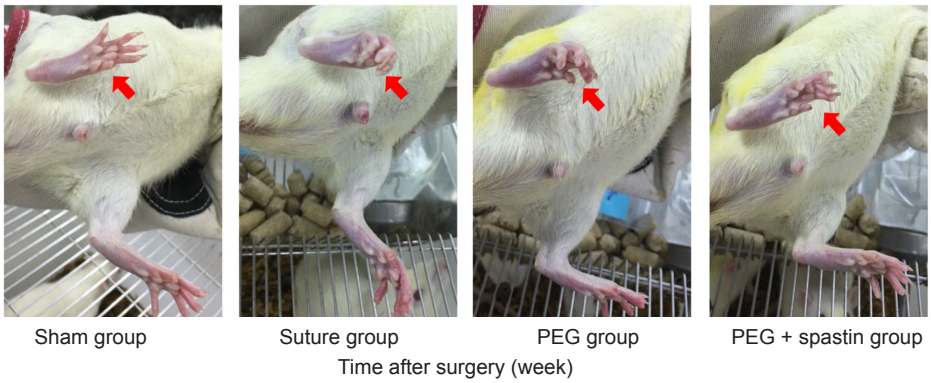

Figure 2 Conditions of rat hindlimbs 1 week after surgery.
Red arrows indicate the hindlimb on the surgical side. Hindlimb function in the sham group was normal, with no paralysis or malformation. In suture, PEG, and PEG + spastin groups, varying degrees of paralysis were observed. Especially in the suture group, typical hindlimb paralysis was found after sciatic nerve injury, with the toes curled up and closed. In PEG and PEG + spastin groups, rats also showed paralysis, but the severity of paralysis in these groups was reduced compared with the suture group. The PEG + spastin group presented the lowest degree of paralysis among suture, PEG, and PEG + spastin groups. PEG: Polyethylene glycol.

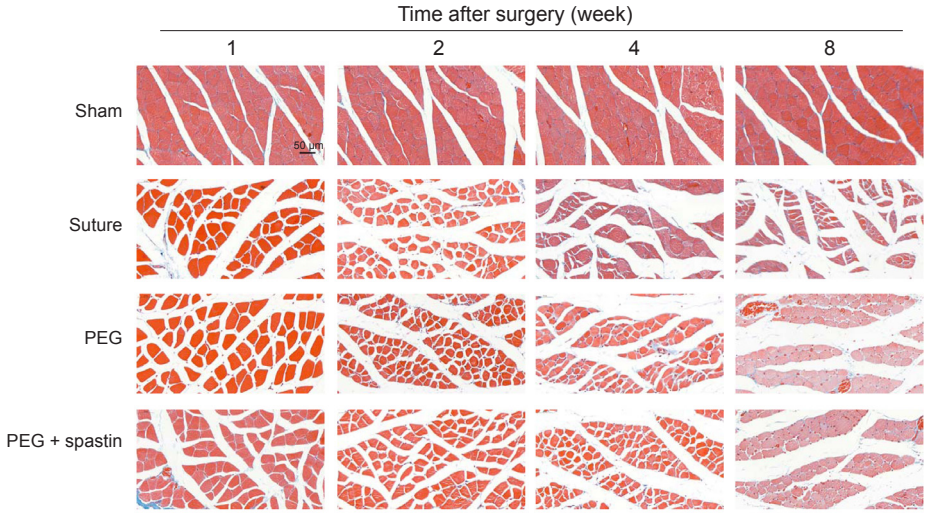

Figure 3 Masson staining of gastrocnemius muscle of rats in each group at different time points.
Images were captured with a DFC 300FX color digital microscope. Cellular structures were normal in the sham group after surgery. In suture, PEG, and PEG + spastin groups, skeletal muscle morphology changed, muscle fiber gaps widened, muscle cells decreased, and extracellular collagen gradually increased with time. Muscular atrophy was most serious in the suture group, followed by PEG and PEG + spastin groups. Scale bar: 50 μm. PEG: Polyethylene glycol.

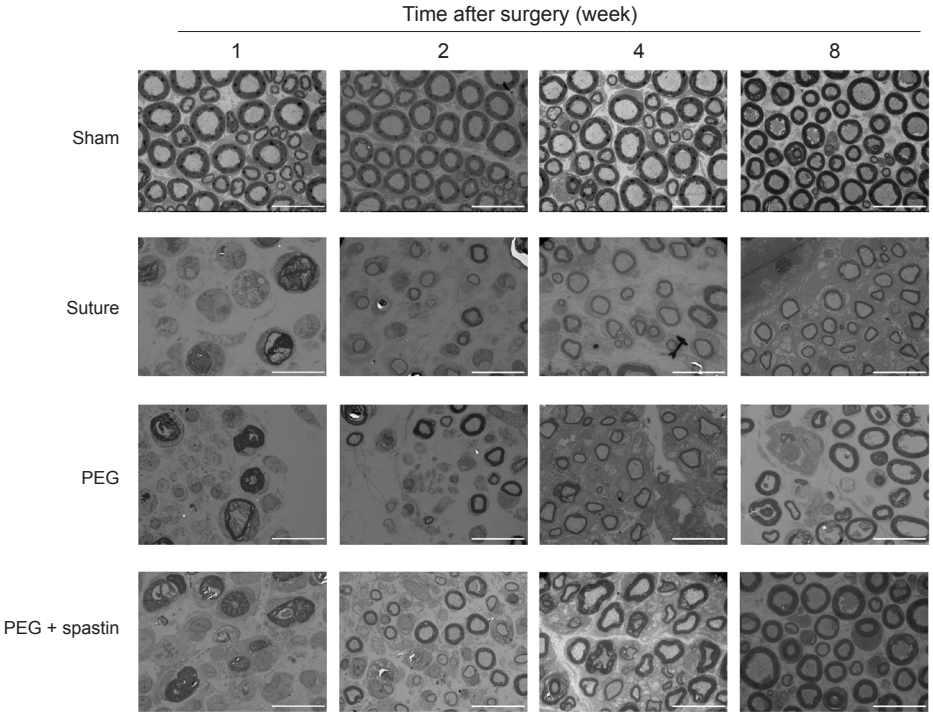

Figure 4 Ultrastructural changes in axons after sciatic nerve injury in each group.
Axon ultrastructure was normal in the sham group after surgery, whereas suture, PEG, and PEG + spastin groups showed a pattern of initial destruction, subsequent disintegration, and gradual repair over 1, 2, 4, and 8 weeks after surgery. One week after surgery in suture, PEG, and PEG + spastin groups, there was obvious destruction of myelin sheaths and disintegration of microtubule and microfilament structures. Two weeks after surgery, regenerated nerve fibers, microtubules and microfilaments gradually became clear, and myelin structures began to appear. As time proceeded, the ultrastructure of axons gradually recovered. The PEG + spastin group was similar to the sham group at 8 weeks after surgery. Scale bars: 20 μm. PEG: Polyethylene glycol.

Table 1 Sciatic functional index changes at different time points in rats after sciatic nerve transection

Group	1 week after surgery	2 weeks after surgery	4 weeks after surgery	8 weeks after surgery
Sham	–2.29±0.25	–2.81±0.71	–3.36±0.66	–2.82±1.36
Suture	–98.29±0.68*	–78.15±1.03*	–68.17±3.35*	–45.21±0.16*
PEG	–89.83±0.73$^{*\#}$	–62.55±0.38$^{*\#}$	–44.01±4.40$^{*\#}$	–30.81±1.99$^{*\#}$
PEG + spastin	–63.64±1.42$^{*\#\dagger}$	–34.87±0.01$^{*\#\dagger}$	–20.77±4.00$^{*\#\dagger}$	–11.53±4.35$^{*\#\dagger}$

$*P < 0.05$, *vs.* sham group; $\#P < 0.05$, *vs.* suture group, $\dagger P < 0.05$, *vs.* PEG group (one-way analysis of variance followed by Dunnett's *post hoc* test). Data are expressed as the mean ± SD ($n = 30$). Differences were compared among suture (sciatic nerve transection + nerve suture), PEG (sciatic nerve transection + PGE), and PEG + spastin (sciatic nerve transection + PEG + spastin) groups at different time points using paired *t*-test. PEG: Polyethylene glycol.

Table 2 Percentage (%) of gastrocnemius muscle wet weight of rats after sciatic nerve transection

Group	1 week after surgery	2 weeks after surgery	4 weeks after surgery	8 weeks after surgery
Sham	99.47±2.37	99.87±1.29	99.34±0.12	99.58±0.04
Suture	74.02±3.17*	55.68±0.27*	44.51±1.10*	30.29±0.49*
PEG	78.90±4.20*	65.41±2.06$^{*\#}$	52.35±0.17$^{*\#}$	42.52±0.62$^{*\#}$
PEG + spastin	92.41±0.10$^{*\#\dagger}$	79.01±1.97$^{*\#\dagger}$	66.63±0.52$^{*\#\dagger}$	57.87±0.67$^{*\#\dagger}$

$*P < 0.05$, *vs.* sham group; $\#P < 0.05$, *vs.* suture group, $\dagger P < 0.05$, *vs.* PEG group (one-way analysis of variance followed by Dunnett's *post hoc* test). Data are expressed as the mean ± SD ($n = 30$). Differences were compared among suture (sciatic nerve transection + nerve suture), PEG (sciatic nerve transection + PGE), and PEG + spastin (sciatic nerve transection + PEG + spastin) groups at different time points using paired *t*-test. PEG: Polyethylene glycol.

Table 3 Muscle fiber diameter (μm) at different time points in rats after sciatic nerve transection

Group	1 week after surgery	2 weeks after surgery	4 weeks after surgery	8 weeks after surgery
Sham	28.82±1.78	28.41±1.49	28.98±1.67	28.23±1.61
Suture	22.24±0.29*	18.41±1.95*	14.29±3.05*	12.96±1.73*
PEG	23.74±0.16*	21.02±0.25$^{*\#}$	18.46±1.04$^{*\#}$	17.40±0.20$^{*\#}$
PEG + spastin	25.09±0.15*	22.00±1.46$^{*\#}$	19.08±0.22$^{*\#}$	18.00±0.41$^{*\#}$

$*P < 0.05$, *vs.* sham group; $\#P < 0.05$, *vs.* suture group, Data are expressed as the mean ± SD ($n = 30$). Differences were compared among suture (sciatic nerve transection + nerve suture), PEG (sciatic nerve transection + PGE), and PEG + spastin (sciatic nerve transection + PEG + spastin) groups at different time points using paired *t*-test. PEG: Polyethylene glycol.

Table 4 Changes in the thickness (μm) of myelin sheaths of rats after sciatic nerve transection

Group	4 weeks after surgery	8 weeks after surgery
Sham	1.63±0.01	1.70±0.00
Suture	0.72±0.00*	1.02±0.01*
PEG	1.22±0.00$^{*\#}$	1.49±0.00$^{*\#}$
PEG + spastin	1.51±0.00$^{*\#\dagger}$	1.61±0.00$^{\#\dagger}$

$*P < 0.05$, *vs.* sham group; $\#P < 0.05$, *vs.* suture group, $\dagger P < 0.05$, *vs.* PEG group (one-way analysis of variance followed by Dunnett's *post hoc* test). Data are expressed as the mean ± SD ($n = 30$). Differences were compared among suture (sciatic nerve transection + nerve suture), PEG (sciatic nerve transection + PGE), and PEG + spastin (sciatic nerve transection + PEG + spastin) groups at different time points using paired *t* tests. PEG: Polyethylene glycol.

Discussion

The traditional repair model for peripheral nerve injury is based on the Wallerian degeneration process, which has multiple drawbacks such as non-directional nerve regeneration, tendency to stagger growth, slow nerve growth, and long-term target muscle denervation. These drawbacks may lead to fibrosis, degeneration, and atrophy of sensory corpuscles, and degeneration or even disappearance of motor endplates (Ma et al., 2011). At present, the use of microsurgical sutures, adhesives, and neurotrophic drugs for nerve injury repair is based on a traditional approach, which inevitably leads to the problems mentioned above (Ascano et al., 2012; Shakhbazau et al., 2012; Yang et al., 2012; de Luca et al., 2014). However, an alternative axonal regeneration mechanism identified in freshwater crayfish, leech, and nematodes, involving axonal fusion (Deriemer et al.,1983; Neumann et al., 2011; McGill et al., 2016), poses a challenge to the traditional Wallerian degeneration model. Indeed, Mokarizadeh et al. (2016) demonstrated that PEG solution loaded in a chitosan tube improved functional recovery of transected sciatic nerves in rats. Evangelista et al. (2015) verified that novel PEG single-lumen conduits manufactured using stereolithography can facilitate nerve regeneration with a 3-cm gap. Sexton et al. (2015) showed that PEG delivery *via* a conduit may provide a simple and effective way to fuse severed axons and regain early nerve function.

PEG is a high-molecular-weight polymer that can facilitate cell membrane fusion (Luo and Shi, 2007; Time et al., 2018). Spaeth et al. (2010) showed that PEG could effectively close

the axilemma and reconnect the transected axon in isolated rat B104 hippocampal cells. Bittner et al. (2012) used the same method to repair peripheral nerve injury and achieved a better therapeutic effect than obtained with traditional microsurgical sutures.

The mechanism underlying PEG-mediated repair of axonal injury mainly involves early fusion of the damaged axon membrane, reduction of mitochondrial membrane permeability and cytochrome C release, and regulation of Ca^{2+} concentration (Borges., 2001; Koob et al., 2006). Many scholars have confirmed that use of PEG in traumatized neurons can effectively seal the axon membrane and delay Wallerian degeneration (Krause et al., 1990; Donaldson et al., 2002; Britt et al., 2010; Bittner et al., 2016a, b). Therefore, PEG is currently being used (with good effect) as a membrane fusion agent in neural repair and regeneration (Sexton et al., 2012; Rodriguez-Feo et al., 2013). However, use of PEG for axonal fusion alone cannot completely avoid Wallerian denaturation, which may be associated with the unrepaired cytoskeleton. Thus, prompt reconstruction of the cytoskeleton is important to restore axon transport.

Spastin, which consists of 616 amino acids, encodes a member of the ATPase associated with various cellular activities (AAA) protein family (Hazan et al., 1999; Errico et al., 2002; Reid et al., 2005). Binding of spastin to microtubules, an important part of the cytoskeleton and primary structure for transport of materials in axons (Sakakibara et al., 2013), is associated with the microtubule-interacting and -trafficking domain (Yu et al., 2008). Spastin is involved in microtubule movement and can transiently bind microtubules to regulate their development through ATPase activity in the N-terminal AAA region (Errico et al., 2002). Knocking out spastin in Drosophila neurons resulted in the microtubule skeleton of these cells becoming more stable. However, growth of synapses and neurotransmitter release were limited, and the extent of synaptic stretch was reduced. Overexpression of spastin leads to a decrease in microtubule stability, demonstrating that spastin can regulate the strength of neuronal presynaptic neurotransmitters and stability of presynaptic microtubules (Trotta et al., 2004). In rat neurons cultured *in vitro*, spastin selectively promoted the formation of lateral buds and regulated axonal morphology (Yu et al., 2008). Thus, spastin may be responsible for modifying microtubules into short-segment mobile fragments, thereby participating in the formation of microtubules in new buds. Indeed, short-segment microtubule fragments observed at the axon branch site strongly support this hypothesis.

The creation of microtubule fragments by spastin shearing is a necessary step for the formation of new axons and branches. This process is particularly important during remodeling of the nervous system of adult animals (Vietri et al., 2015). During regeneration of damaged axon stumps and the formation of new collaterals, spastin expression noticeably increased, indicating that spastin is indispensable in the formation and growth of new neuronal processes (Stone et al., 2012). Therefore, spastin may be a key molecule for repairing the cytoskeleton in axonal anastomosis after peripheral nerve injury (Lee et al., 2009; Diaz-Valencia et al., 2013).

In the current study, the effect of combining exogenous spastin and PEG on nerve regeneration was investigated in a rat model. The results were analyzed by general observation, SFI measurement, pinch test, muscle histological assessment, and evaluation of axon ultrastructure. Amongst suture, PEG, and PEG + spastin groups, the severity of hindlimb paralysis was lowest, and local ulcers were relatively reduced in the PEG + spastin group. At 8 weeks after surgery, rats in the PEG + spastin group showed good hindlimb functional recovery and could walk with no obvious uncoordinated movement. Although the hindlimb function of rats in the PEG group largely recovered 8 weeks after surgery, animals in the suture group still showed plantar flexor disorders in the injured foot that led to uncoordinated walking.

The fundamental purpose of nerve repair is to improve the rate of functional recovery. Thus, an ideal index for functional recovery includes behavioral indicators, such as SFI, to evaluate sciatic nerve regeneration. SFI could reflect the recovery of limb muscle strength, as well as muscle coordination. At each time point after surgery, SFI was as follows: PEG + spastin group > PEG group > suture group, indicating that recovery of sciatic nerve occurred in PEG + spastin group > PEG group > suture group. At 8 weeks after surgery, SFI in the PEG + spastin group was similar to that in the sham group, indicating good recovery of sciatic nerve function. However, the sensitivity of SFI was not sufficient and easily affected by other factors. Therefore, multiple indicators were necessary.

Histological examination was utilized to observe changes of axons and corresponding target muscles during nerve regeneration from a microscopic point of view. To this end, Masson staining of the gastrocnemius muscle and transmission electron microscopy of the nerve were applied to evaluate changes in axons and corresponding target muscles. After nerve injury, the target muscle increased in length with time and atrophied. However, amongst suture, PEG, and PEG + spastin groups, muscle atrophy rate was lowest in the PEG + spastin group, followed by PEG and suture groups. Muscle atrophy was most obvious at the early stage in the suture group. Collectively, these findings indicate that PEG and spastin can improve the recovery of neurological function and delay muscle atrophy.

Observations of axon ultrastructure with transmission electron microscopy indicated ultrastructural damage, microtubule and microfilament disintegration, and myelin degeneration after surgery in suture, PEG, and PEG + spastin groups. Ultrastructural recovery was most obvious in the PEG + spastin group, followed by the PEG group. At 4 and 8 weeks after surgery, thickness of the myelin sheath was measured. The results showed that myelin sheath recovery was most obvious in the PEG + spastin group, which was similar to that of the sham group 8 weeks after surgery. Thus, the combination of PEG and spastin could improve the regeneration of peripheral nerves.

Detection of sensory fiber regeneration distance during the early stage of nerve regeneration has value for indicating

the ultimate outcome. With the passage of time, sensory fibers grow to a certain extent and the index becomes inaccurate. Therefore, in this study, regeneration distance was only measured 1 week after surgery. At this time, sensory fiber regeneration distances were greater in the PEG + spastin group than in PEG and suture groups, indicating that the effect of local PEG and spastin application was better than that obtained by applying PEG alone. Thus, our findings suggest that although application of PEG and spastin cannot completely avoid Wallerian degeneration, it can improve the regeneration of peripheral nerves and yield a better effect than obtained by applying PEG alone.

Although the mechanism underlying the combined effect of PEG and spastin in improving peripheral nerve regeneration requires further study, we hypothesize that the main reason for promotion of nerve repair by PEG and spastin involved increased fusion of axonal membranes and reduced Wallerian degeneration. Notably, this process plays an important role only in the early and middle stages of repair. Thus, with regard to long-term effects, there would likely be no obvious difference compared with simple nerve suture. As such, long-term observations were not made. Early nerve repair, earlier functional exercise, and slowing of target muscle atrophy and loss of function may be the most remarkable features of this repair mode. We plan to conduct *in vitro* experiments to amplify the spastin gene to further validate our conclusions. In addition, we are preparing to further study the effects of PEG and spastin injection methods on nerve repair.

The innovation of this study is the use of a new neural regeneration model to repair peripheral nerve injury. PEG was employed to fuse the axon. Spastin protein was applied to promote material and neurotrophic transport, and improve the efficiency and effect of axonal fusion to repair nerves. Of course, there were some limitations of this study. Notably, after spastin application, its role in the corresponding spinal cord was not observed.

After sciatic nerve injury in rats, simple nerve end-to-end anastomosis, application of PEG, and application of PEG + spastin were helpful in the recovery of neurological function. The effect of PEG combined with spastin protein was better than that of PEG alone, and both were superior to traditional end-to-end anastomosis. Thus, our findings provide an experimental basis for the repair of peripheral nerve injury using exogenous spastin and PEG.

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