Title: The Effect of Muscle Graft with Nerve Growth Factor and Laminin on Sciatic Nerve Repair in Rats

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Abstract

Introduction: Peripheral nerve injury is one of the most common damages that lead to physical disability. Due to the similarity between the coatings of skeletal muscles with nerves fiber, this research conducted to determine the effect of muscle graft with nerve growth factor (NGF) and laminin (L) on nerve repair.

Methods: 10 mm sciatic nerve was transected and removed in forty-two female Wistar rats (200□250 g) and equally divided into three groups. In the muscle graft +NGF + laminin group degenerated skeletal muscle was sutured with proximal and distal ends of the transected sciatic nerve. Then, NGF (100 ng) and laminin (1.28 mg/ml) were injected into the muscle graft. In the muscle graft group, normal saline were injected into the muscle graft. In the control group the 10 mm sciatic nerve removed without any treatment. Functional recovery was assessed based on sciatic functional index (SFI), tracing and histological study was achieved to assess nerve repair. Data was analyzed by ANOVA tests.

Results: The Mean SFI significantly increased in the muscle graft +NGF + laminin (-76.6□2.9) and muscle graft (-82.1□3.5) groups in the 60 days post injury compared to the control group. The mean number of Labeled motor neuron significantly increased in the muscle graft +NGF + laminin (78.6□3.1) and muscle graft (61.3□6.1) groups compared to the control group ($P<0.001$). Mean number of myelinated axons in the distal segments of the muscle graft +NGF + laminin increased significantly compare to the muscle graft group.

Conclusion: These findings suggest that muscle graft accompanying exogenous NGF and laminin provide clear therapeutic effects on nerve repair.

Keywords: Peripheral nerve, Muscle graft, Growth factor, Laminin, Rats.

1. Introduction

Peripheral nerve injury is one of the most common damages in various communities, many of which lead to permanent disabilities and neuropathic pain (Sullivan, Dailey, Duncan, Abel, & Borlongan, 2016). The most severe of which is called neurotmesis that results in the loss of nerve trunk continuity, myelin sheath, and the connective tissues surrounding them. If there is no gap between the cut ends of the nerve, or if the

gap is short, the two nerve ends can be directly sutured, and the result of healing will depend on whether the nerve bundles at the cut ends adhere to each other correctly (Mafi, Hindocha, Dhital, & Saleh, 2012). Although some drug such as cyclosporine A and melatonin has useful effect for axonal regeneration and sprouting effect for peripheral nerve injury (Roozbehi, Joghataie, Mehdizadeh, Mirzaei, & Delaviz, 2012; Turgut & Kaplan, 2011). However, in the long gap of peripheral nerve injuries, diversion of axonal buds at the injury site is a great problem that can influence nerve repair (Panagopoulos, Megaloikonomos, & Mavrogenis, 2016). Therefore, this gap must be bridged so that the generated axons can be guided toward the distal stamp and target organ. In these cases, the material used for bridging the gap can influence the result of the repairing process (Panagopoulos et al., 2016). Polyvinylidene fluoride channel as conduit for bridging the peripheral nerves defects with nerve growth factor (NGF) and collagen gel could provide the favorable matrix for axonal regrowth (Delaviz et al., 2011). Basal membrane could stimulate and creating guidance channels for regrowth of axons toward the target tissue (Kang, Hu, Wang, & Wang, 2015). It has been shown that muscle basal lamina grafts can guidance route for the injured axons toward the distal segment of the endoneurial tubes (La Fleur, Underwood, Rappolee, & Werb, 1996). Although nerve graft considered a standard procedure, yet sacrificing a peripheral nerve is not an ideal option since it can lead to numbness in the receptor zone and may also cause formation of neuromas and visible scars (Daly, Yao, Zeugolis, Windebank, & Pandit, 2012). Therefore, it is importance to find a suitable guidance channel at the site of the nerve gap that can serve as a favorable substrate and nutritional support that cause axonal growth and, consequently, improved functional recovery. The similarity between the coatings of skeletal muscle fiber with nerve fibers makes it possible to use autologous muscle grafts as a conduit in nerve repair (Roganovic, Ilic, & Savic, 2007). Clinical recovery and histological evidence of the axonal regeneration has seen after muscle grafting of the nerve damage in leprosy (de Blaquiere, Curtis, Pereira, & Turk, 1994). Epimysial of degenerated muscle graft as a tube can guide the regenerating nerve fiber to the distal nerve defect (Yang et al., 2013).

To create natural guidance channels that provided favorable conditions for axonal growth and also prevent immune responses, it is recommended the a cellular skeletal muscle grafts used in the gap region

(Roganovic et al., 2007). Cellular constituents in a the skeletal muscle tissue can be removed with using various techniques including initial treatment with alcohol, freezing and thawing (Roganovic et al., 2007). Exogenous of growth factor along with nerve conduit is playing an important role for neuronal survival and nerve regeneration (F. Ma et al., 2016). It was found that nerve guidance channels containing collagen and laminin-containing gels lead to better regeneration compared to those that contain only a saline (Verdú et al., 2002). Moreover, various isoforms of laminin, as initial compounds of basal lamina, are considered strong stimulants for axonal growth and repair (Chen & Strickland, 2003).

Therefore, in the present research the effect of a cellular skeletal muscular graft with nerve growth factor and laminin were studied on axonal regeneration and functional recovery following sciatic nerve transection.

2. Methods

2.1. Animals and groups

This experimental research was done according to the guidelines of Iranian Syndicate for application and Care of Animals and was approved by the Animal Research Ethical Committee of Yasuj University of Medical Sciences. Forty-two female albino rats of the Wistar strains (200□250 g) were purchased from the animal house of the Shiraz University of Medical Sciences. Animals were housed under conditions of controlled temperature ((22˚C □2˚C) with 12-h light/12-h dark cycle and food and water ad libitum. Animals were randomly assigned into three equal groups of fourteen: muscle graft +NGF + laminin group, muscle graft muscle graft group and control group.

2.2. Surgical procedure and muscle autografts

The rats were anesthetized via intraperitoneal injection of a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). An incision was made on the posterior surface of the thigh from the sciatic notch to the popliteal region, the muscles and fascia were pushed away and left sciatic nerve was exposed. Under aseptic conditions at mid-thigh level 10 mm of the sciatic nerve was cut and removed.

A 12 mm of narrow strip of the left gluteus superficialis muscle was removed in alignment with the lengths of the muscle fibers. The narrow strip was placed in liquid nitrogen for 5 minutes to freeze completely, then put in a normal saline for more than 5 minutes at room temperature, and was next placed in sterile distilled water for 10 minutes so that the cytoplasm and cell membrane were removed due to the osmotic phenomenon (through leakage) from the muscle (M. A. Glasby, S. E. Gschmeissner, C. L. Huang, & B. A. De Souza, 1986). Following that, a surgical blade was used to trim the thawed muscle in the form of a square block (2

2.4. Retrograde tracing of spinal motor neurons

Sixty days after treatment eight rats from each group were anesthetized and the gastrocnemius muscles on the left side were exposed. 1, 1- dioctadecyl-3, 3, 3, 3 ـtetramethylindocarbocyanin perchlorat (DiI) from Molecular Probes (Leiden, The Netherlands; cat. No, D-282) were used for labeling of the spinal motor neuron and evaluation of axonal regrowth. with using of 10-□l Hamilton syringe 8-9 □l of DiI tracer in 170 mg/ml DMSO was diluted 1:10 in saline and injected into 5 different locations in the bulk of the gastrocnemius muscle (Madison & Robinson, 2014). Ten days after application, animals perfused and spinal cord segment (L4-L6) was dissected out. Each spinal segment serially sectioned into 50 □m horizontal sections on a freezing microtome (Leica cryostat). The labeled spinal motor neurons were counted with using fluorescent microscopy (Olympus Ax70). As previously described, in each spinal segment the number of labeled motor neurons of each section was summed together to give the total number of motor neurons for each rat (Catapano et al., 2016).

2.5. Histological study

After 60 days post treatment, six rats from each group were anesthetized with using of double doses of ketamine (200 mg/kg) and xylazine (20 mg/kg), and perfused for histological studies. For counting of myelinated nerve fibers at the grafted region 12 mm of sciatic nerve with the center of the transplanted area was cut and removed. This segment (12 mm) divided into three equal of 4mm parts proximal, middle and distal segment. The proximal parts of the each segment were marked to distinguish the distal end from the proximal one. For counting and morphological study of the spinal motor neurons that participated in the sciatic nerve, 5 mm of the L4-L6 spinal cord segment was cut and removed. The spinal cord and each segment of the sciatic nerve were cut into the 50 □m and 5 □m respectively (Leica cryostat, CM 3000). The number of myelinated nerve fibers and motor neuron in the each segment of the spinal cord or sciatic nerve were counted with using of microscopes (Olympus Bx51). The images of the section were taken with a digital camera (DP 11) connected to the Microscopes. As previously described (Takami et al., 2002), the numbers of nerve fiber or neuron of each section were summed to obtain the final number of nerve fiber or neuron in a 5-mm-long spinal cord or 4 mm sciatic nerve segment in each rat.

2.6. Statistical analysis

All statistical analyses were performed by SPSS 18.0 (SPSS Inc., USA). A one-way ANOVA, followed by post hoc Tukey's was used for data analysis. All data are expressed as mean □SD. $P < 0.05$ was considered to be statistically significant.

3. Result

3.1. Gait analysis

Assessment of the functional recovery was conducted by a researcher who is not known about animal grouping. On the day after the operation, the rats drag of their foot with foot drop and toe adduction in all three groups. As shown in the (table 1), gait analysis on the 1, 8 days in the different groups indicated that functional recovery of the sciatic nerve was lost, and one-way ANOVA analyses demonstrated that no significant differences were observed between the three groups on the injured foot ($P>0.05$). The ability to walk on the operated legs in the muscle graft +NGF + laminin and muscle graft groups improved on 29 days and the mean of SFI increased significantly in these two groups compared to the control group ($P<0.05$) (figure 1). Sixty days after the surgery, the treated group with muscle graft +NGF + laminin on the left hind limb showed toe-spreading and better footprints walking tracks compared to the other two groups. Statistical analysis on the 60 day indicated the mean SFI increased significantly in the muscle graft +NGF + laminin (-76.6□2.9) and muscle graft (-82.1□3.5) groups compared to the control group (-96.9□2.1). Although improvement of functional motor movement in the muscle graft +NGF + laminin group enhanced compared to the muscle graft group, but this difference was not statistically significant. It must be mentioned that one days prior to surgery normal gait was recorded in all three groups and hind-limb toes fully spread and there were no significant differences between the groups.

3.2. Retrograde DiI labeled neuron

DiI positive nerve cells were observed in the perikaryon of the spinal cells through the axonal regrowth seventy days after treatment (Figure 2). DiI tracer in the perikaryon of the motor neuron in the muscle graft +NGF + laminin and muscle graft groups were seen more and more specific than control group (Figure 2). Mean number of DiI labeled motor neuron in the spinal segment of the L4-L6 in the muscle

graft +NGF + laminin (78.6□3.1) and muscle graft (61.3□6.1) groups were respectively, that one-way ANOVA indicated that there were significant differences compare to the control group (25.2□2.2) (<0.001) (Figure 3).

3.3. Histological outcome

Histological assessment of the distal segment in the transplanted groups showed axons could grow and reached the distal part (Figure 4). The thicknesses of myelin of the regeneration axon were increased in the muscle graft +NGF + laminin and muscle graft compares to the control rats (Figure 4).

Mean number of myelinated nerve fiber in the muscle graft +NGF + laminin (3353â34.2) and muscle graft (3382â42.6) groups increased in the proximal segment compare to the control group (3216â53.1) (Table 2). The One- way ANOVA analysis showed no difference is found at proximal segment between the groups (P>0.05). The Mean number of myelinated nerve fiber in the muscle graft +NGF + laminin(343â11.1) and muscle graft (312â15.9) groups in the middle segments enhanced significantly compared to the control group (200â18.3) (P<0.05). The Mean number of myelinated nerve fiber in the muscle graft +NGF + laminin (18.2â1.6) and muscle graft (14.6â2.1) groups in the distal segments enhanced significantly compared to the control group (6.3â3.4).

Sciatic nerve transection was reduced the number of motor neurons in the anterior horn of the injured side compared to the healthy side (figure 5). In each group, the mean number of spinal motor neuron decreased significantly in the left side (The fiber of these neurons were transected in the left sciatic nerve) of the 5 mm of the L4-L6 spinal cord segment compared to the right side (P>0.05). Mean number of the motor neurons in the left side of the spinal segment in the muscle graft +NGF + laminin (475.5□6.1) and muscle graft (403.2□3.8) groups enhanced significantly compared to the control rats (219.6□2.2) (Table 3). Although the number of motor neuron in left side of the spinal segment increased in the muscle graft +NGF + laminin group compared to the muscle graft group but this difference was not significant (P>0.05).

4. Discussion

Peripheral nerve injury is a major problem that annually affects millions of people in the world and in most cases requiring surgical nerve repair (Perretta & Green, 2017). A graft procedure is necessary to improve the functional recovery when a long-distance of the nerve is lost (Mohammadi, Delaviz, Mohammadi, Delaviz, & Rad, 2016). When there is a large gap between the nerve ends, a suitable anatomical conduit is necessary between them so that the axons in the proximal segment can guided into the endoneurial tubes of the distal segment to restore the function of the target organ (Iijima, Ajiki, Murayama, & Takeshita, 2016). To improve and accelerate nerve regrowth different conduit including olfactory mucosa, adult Schwann cells, amniotic membrane with betamethasone were used for nerve conduit in experimental study (Delaviz et al., 2008; Hedayatpour et al., 2007; Sadraie et al., 2016; Zarinfard, Tadjalli, Razavi, & Kazemi, 2016) . Among the various attempts, degenerated skeletal muscle autografts could recover the foot and hand sensation in leprosy patients (Pereira, Bowden, Narayanakumar, & Gschmeissner, 1996). This study confirm other study result that showed the repair of sciatic nerve could enhance the survival of the spinal ganglion cells (Atlasi, Mehdizadeh, Bahadori, & Joghataei, 2009).

The similarity between the tubular matrix of skeletal muscles (the basal membrane) and the endoneurial tubes of a damaged nerve makes it possible to use autologous muscle grafts as a favorable biological conduit for nerve repair (Kang et al., 2015). Therefore, the sheaths of skeletal muscle were employed as the guidance channel in repairing of nerve injury in the clinical and experimental research (Meek, Varejão, & Geuna, 2004; Pereira et al., 1996; Sanes, 2003). Gait analysis of this study indicated that motor function improved in the groups with degenerated muscle grafts compared to the control group. It has been shown that axonal regeneration could grow from the skeletal muscle grafts and reach the target muscle, and re innervation of the muscle fiber to improve the motor function (M. Glasby, S. Gschmeissner, C.-H. Huang, & B. De Souza, 1986). Result of this study conform the Glasby et al. study that demonstrated the use of non-neural autografts and skeletal muscle fibers with parallel array of the nerve fiber created a matrix of basal membrane tubes that were both anatomically and chemically similar to peripheral nerves sheet (M.

Glasby, S. Gschmeissner, C.-H. Huang, et al., 1986). Moreover, Norris et al. showed that the basal membrane tubes derived from skeletal muscles was sufficient diameter to match the largest nerve fibers (Norris, Glasby, Gattuso, & Bowden, 1988). Basal membranes of muscle fibers were in the form of long cylinders lying parallel to each other and providing a suitable substrate for the growing axons (Houštava, Dubový, Haninec, & Grim, 1999). The axon regrowth faster when the conduit matrix of the degenerating muscle is coaxial with the nerve fiber (Glasby, Gschmeissner, Hitchcock, & Huang, 1986; M. Glasby, S. Gschmeissner, C.-H. Huang, et al., 1986). Degenerated muscle grafts for nerve repair in primates leads the myelination of the nerve in the graft region and axonal regeneration to the distal nerve segment with normal electrophysiological result (M. Glasby, S. Gschmeissner, R. Hitchcock, et al., 1986).

In the present research, functional recovery and axonal regeneration was improved in the muscle graft +NGF + laminin compare to the group that only received muscle grafts or control group. NGF is important for survival neuron, synaptic development, axonal regrowth and functional recovery for central and peripheral nervous system (Mesentier-Louro et al., 2017). The enriching of the conduit nerve tube with NGF has more potential to axonal regeneration a 10-mm long gap of the rat sciatic nerve (Mesentier-Louro et al., 2017). Nerve growth factor by up-regulating $p75^{NTR}$ expression in Schwann cells could stimulate the nerve regrowth in the Wistar rats (S. Ma, Peng, Wu, Wu, & Gao, 2013). Further, the favorable of microenvironment such as laminin and collagen can provide an appropriate matrix for regrowth axon (Cao et al., 2011). Laminin has biological activity such as neurite promoting activity and could create favorable microenvironment for neurotic regeneration in culture (Manthorpe et al., 1983). Nerve conduction tube filling with a substrate such as laminin, fibronectin and NGF provides a favorable condition for nerve regrowth and cell migration (Glasgow et al., 2016). Suitable concentrations of laminin and growth factors are important of the nerve repair (Valentini, Aebischer, Winn, & Galletti, 1987). Thus, the high density of laminin inhibits the diffusion of growth factors and act as a barrier for axonal regeneration (Valentini et al., 1987). Our result confirmed the Labrador . study that showed low-density of laminin or collagen could provide the better axonal regrowth (Labrador et al., 1998). Laminin-8 and Laminin-2 plays an important role in the development of nerve growth and nerve regeneration after

injury (Wallquist et al., 2002). Laminin as substrate has influence on Schwann cell migration during nerve repair and have effective role for axonal regeneration in a cellular nerve allografts (Heermann & Schwab, 2013). Further laminin with growth factors have a significant role for axonal regeneration in long gap (20mm) in experimental study and NGF increase nerve regrowth and myelin formation (Barbon et al., 2016; Dodla & Bellamkonda, 2008). Our result confirmed the Pu et al study that showed enriching of the conduit nerve tube with NGF has more potential to axonal regeneration a 10-mm long gap of the rat sciatic nerve (Meek, 2000). Biological conduit such as muscle-vein combined could reconstruct the digital nerve defect up to 4 cm in patient s (Battiston, Geuna, Ferrero, & Tos, 2005). Although, in many studies, they do not use of intact group we can say that one limitations of this study was the lack of an intact group that results can compared with them.

Conclusion

There is no drug or special approach with robust effects favorable for treatment of peripheral nerve injury. When the long nerve continuity is lost, a one technique alone cannot provide satisfactory treatment for nerve repair and a combined treatment with conduit tube might be able to provide a comprehensive treatment. Result of this study demonstrated the use of biological tubulization such degenerated skeletal muscle autografts with NGF and laminin could provide a suitable scaffold for bridging a nerve defect. This method is easy to use, less expensive, without inflammatory response and complications.

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Conflict of Interests

The authors declared no conflict of interest

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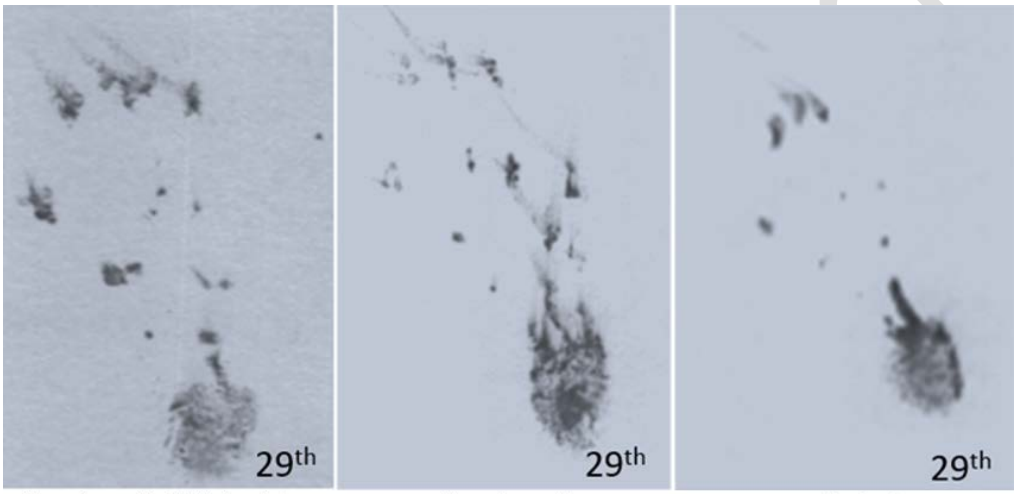

Figure 1. Footprint recorded during 29th on the injured left foot.

Table1. The mean sciatic functional index (SFI) in different group

Days GROUP	1	8	15	22	29	60
muscle graft +NGF + laminin	-103.3□3.8	-96.6□2.9	-96.4□3.9	-95.6□3.6	-82.8□3.4*	-76.6□2.9*
muscle graft	-102.4□3.2	-98.8□3.1	-97.9□3.4	-95.8□3.7	-86.01□2.2*	-82.1□3.5*
control	-104.2□2.3	-99.8□2.9	-99.3□2.9	-99.3□2.7	-98.6□3.4	-96.9□2.1

*A significant difference compared with the control group (<0.01), n=14.

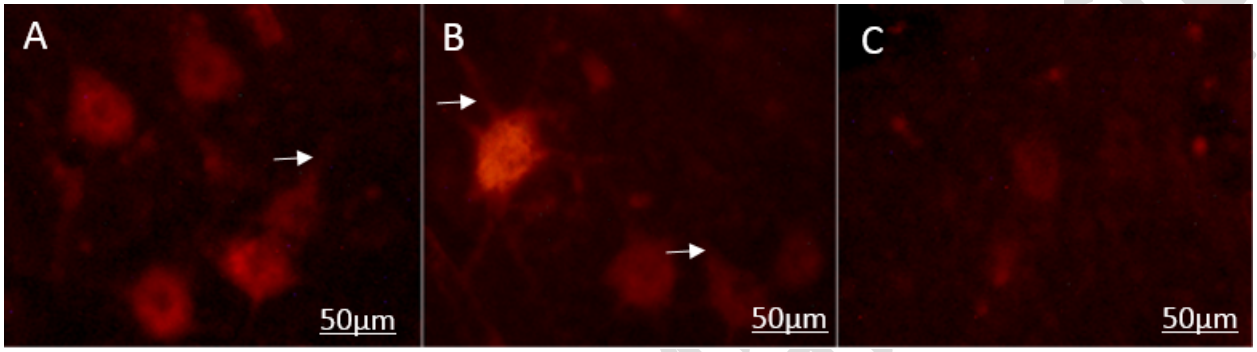

Figure 2. Photomicrographs showing retrogradely-labeled motor neurons in transverse sections of the sciatic nerve 10 days after application of DiI: 1,1□-Dioctadecyl-3,3,3□,3□-tetrame thylindocarbocyanine perchlorate. Number of DiI motor neurons has seen more in the muscle graft +NGF + laminin (A) compare to the muscle graft (B) or control (C) groups. Nucleus of the neurons appear darker and arrow exhibits cytoplasmic process. Magnification □400 (A, B, C) Scale bar: 50 □m (A, B, C).

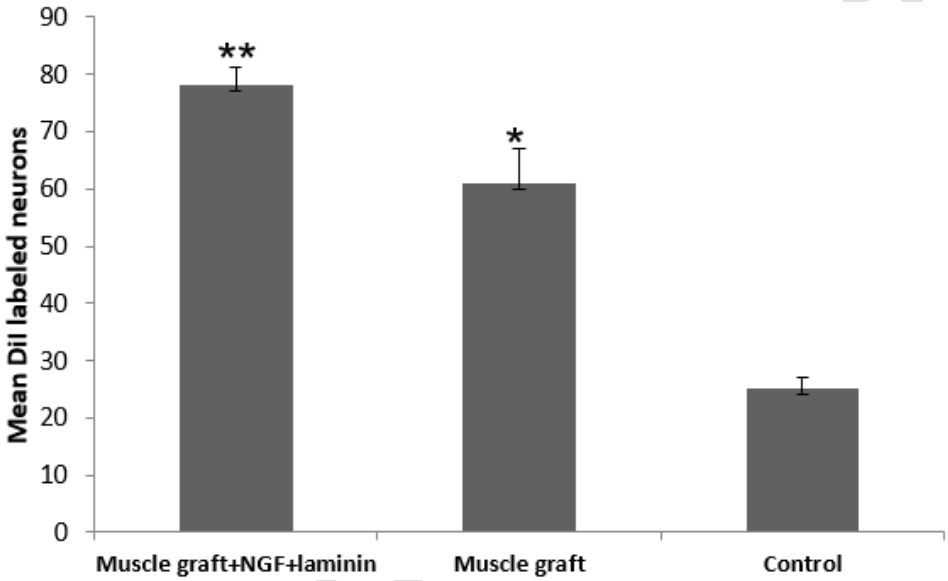

Figure 3. Spinal DiI labeled motorneurons in the spinal cord segment (L4-L6) in each group. **P<0.05 compare to the muscle graft group and **P<0.001 compare to the control rats, n=8. *P<0.01 compare to the control.

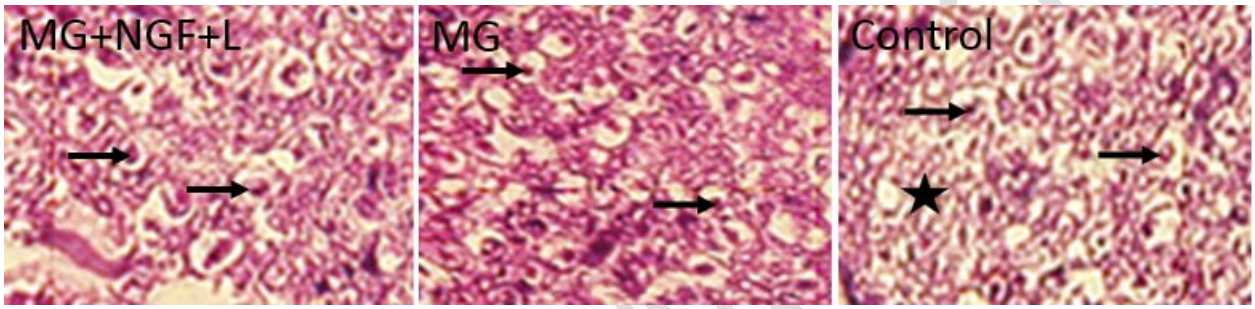

Figure 4. Transverse section of the distal segment in the muscle graft +NGF + laminin rats showed some axons (arrow) covered by a thin layer of myelin membrane. The larger diameter of axons (arrow) has seen in the muscle graft +NGF + laminin and MG groups compare to the control rats. Axonal degeneration and myelin break was seen (star) in the control rats 60 days after sciatic nerve transection. Haematoxylin and eosin staining, = □m for all images.

Table 2. Mean number of myelinated nerve fiber in different groups

Group	No.	Proximal	Middle	Distal	Total
laminin + muscle graft +NGF	6	3353â34.2	343â11.1*	18.2â1.6**	4314.2â47.4*
muscle graft	6	3382â42.6	312â15.9*	14.6â2.1**	3708â39.7
control	6	3216â53.1	200â18.3	6.3â3.4	3422.3â34.6

*A significant difference compared to control group (<0.05).

**A significant difference compared with the control group (<0.01).

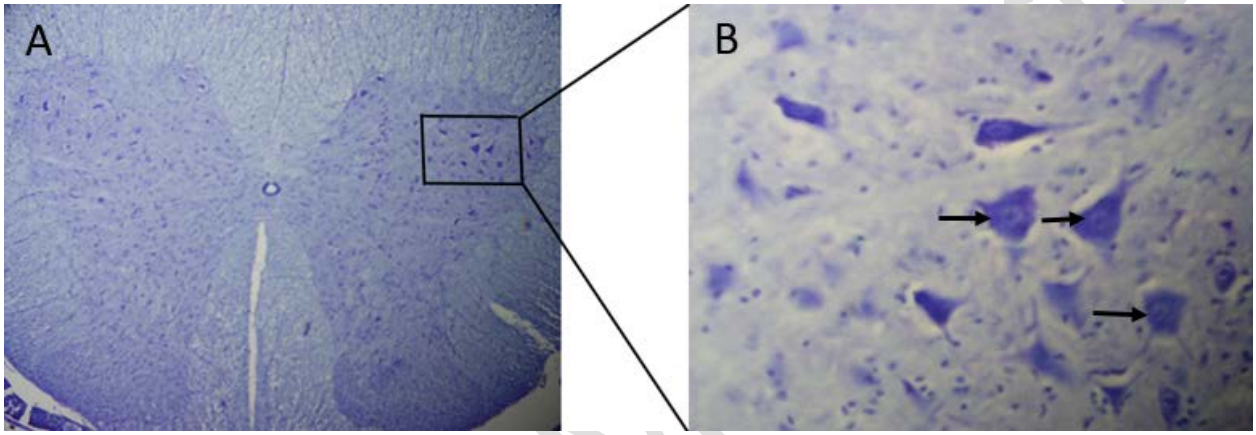

Figure 5. Transverse section of the L4-L6 spinal cord segment (A) 60 days post treatment. High power photomicrographs (B) from the frame show (arrow) these cells are large, multipolar with many dendrites and have a coarsely granular cytoplasm. Cresyl violet staining, Scale bars= 50 □m in A, 20 □m in B.

Table 3. The number of motor neurons in the right and left side of anterior horn of the L4-L6 spinal cord segment

Group	number	Right side motor neuron	Left side motor neuron
muscle graft +NGF + laminin	6	793.4□3.4*	475.5□6.1**
muscle graft	6	828.5□2.9*	403.2□3.8**
control	6	786.6□3.2*	219.6□2.2

*A significant difference compared with the Left side motor neuron of the same group (<0.001).

**A significant difference compared with the control group (<0.05).