Original Article

Immunohistochemical Study of Spinal Motor Neurons Following Sciatic Nerve Repair in Adult Rat

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Abstract

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Introduction: Epineural suture and autologous graft are two routine techniques in peripheral nerve surgery. However, their efficiency can be highly limited depending on the type of lesion and the gap between two nerve stumps and because of deficient proper nerve donors. So much interest has been focused on the development of alternative instruments for bridging the nerve gaps. In the present study, we have used charged polyvinelidene fluoride (PVDF) tube filled with nerve growth factor (NGF) and collagen gel as a substitute for nerve autograft and compared the results with other current surgical techniques. We studied the changes of spinal motoneurons to evaluate the effect of repairing techniques.

Material and Methods: In this study, 30 male Wistar rats weighing 200-250 g were divided randomly in five groups: axotomy, epineural suture, autograft, nerve guidance channel, and sham operation. In all experimental groups, the left sciatic nerve was transected at mid-thigh level. The nerve was not repaired in axotomy group. In epineural suture group, it was sutured end-to-end. In autograft group, a 10 mm piece of nerve was rotated 180° and sutured again in the nerve gap. Finally, in nerve guidance channel group, a piece of PVDF tube containing NGF7s (100 ng/ml) and collagen gel (1.28 mg/ml) was replaced in the gap. After one week, one month, and two months, L4-6 segments of spinal cord were removed and 5 µm paraffin sections were prepared for bax immunohistochemical study. In all groups contralateral spinal cord was used as the control. The proportion of Bax-positive apoptotic motoneurons was studied in all groups to evaluate the efficiency of different repairing techniques.

Results: Mean percentage of Bax-positive neurons to the total number of motor neurons in left side was analyzed. One way ANOVA showed significant difference after two months. LSD post hoc test showed that mean percentage of Bax-positive neurons in axotomized group was significantly higher compared to other surgical groups ($p<0.05$).

The number of apoptotic neurons after one week, one month and two months in each type of surgical approach showed no significant difference between one week and one month and between one month and two months. Comparison of motoneuron population in left side (experimental) with right side (control) showed no significant differences after one week, but significant differences were seen ($p<0.01$) after one month and two months.

In sham group, no Bax-positive neuron was found after one week, one month, and two months.

Conclusion: A PVDF tube filled with NGF and collagen gel can be used as a proper substitute for autografts and protect motoneurons following peripheral nerve injury.

Keywords: Sciatic Nerve Repair, Nerve Guidance Channel, Spinal Motor Neurons, PVDF, NGF

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Introduction

Lesions to peripheral nerves remain a difficult and challenging problem in reconstructive surgery.

The current clinical approach for the repair of peripheral nerve defects involves the use of autologous nerve grafts. However, much interest has been focused on the development of alternative instruments for

bridging the nerve gaps and in the last two decades, various autologous or synthetic conduits have been tested in this setting (1-8).
As an alternative to nerve autografts, some guides have been shown to help direct axon sprouting, providing a conduit for diffusion of regeneration-promoting factors, and protecting the regenerating axon from interference by scar tissue (2, 5-9).
In this context, various materials have been used in experimental models of peripheral nerve repair.
These include autologous veins and muscles (2, 9, 10), collagen tubes (6), and synthetic conduits coated with laminin or fibronectin (11, 12).
Previous studies have shown that electrical charges can stimulate proliferation and differentiation of various cell types. Electromagnetic fields have been shown to play an important role in neurite sprouting and regeneration of injured nerves (13-18).
Neurite outgrowth has been demonstrated to be enhanced on polarized biodurable materials such as polyvinylidene fluoride (PVDF) and polarized polytetrafluoroethylene (PTFE) (19).
In alternative *in vitro* models, mouse neuroblastoma cells grown on PVDF and rat pc-12 cells maintained on an electrically conductive polymer-oxidized polypyrrole displayed growth and morphologic changes in response to the electrical stimuli (14).
Despite of the fact that multiple factors have been shown to promote axon regeneration, the functional recovery is still suboptimal.
Axotomy can lead to considerable changes of neurons and probably to their apoptotic death, and as Bax is a proapoptotic member of Bcl-2 family that is activated following trophic factor deprivation and cell lesion (20), it can be used as a marker to detect apoptotic motoneurons.
This investigation was concerned with the application of a synthetic material instead of nerve autograft when there is a gap in sciatic nerve repair.
We used polarized piezoelectric polyvinylidene fluoride tube filled with collagen gel and nerve growth factor (NGF) as a nerve guidance channel, and compared it with epineural suture and nerve autograft techniques.
We used anti-bax immunohistochemistry to detect apoptotic motoneurons (21), that induced by sciatic nerve transection.

Material and Methods

Preparation of polarized piezoelectric polyvinylidene fluoride tube (PVDF)

The polyvinylidene fluoride (Harvard Apparatus Ltd) tube was polarized in the electronic laboratory of Sharif Industrial University as follows; a thin wire inserted into the lumen of the PVDF tube served as an inner electrode and a circumferential array of steel needles served as the outer electrode. The outer needle electrode was connected to the positive output of a power supply and the inner electrode was grounded. The voltage output was gradually increased to 21 KV over a 2 h period and maintained at that level for 12 h (22).
The tube was cut into 14 mm pieces, sterilized by ethanol, and filled with 1.28 mg/ml of collagen gel (Roche) and 100 ng/ml of NGF75 (Roche). It was then put in the humidified 37º C incubator for polymerization.

Animals and surgical procedure

Thirty male Wistar rats weighing 200-250 g (Pasteur institute of Iran, Tehran) were divided into a sham and four experimental groups; axotomy, epineural suture, autograft, and nerve guidance channel. Animals were housed in plastic cages with free access to food and water. Their room was maintained at constant temperature of 22-24°C under 12 hour light/ 12 hour dark cycle. Intraperitoneal ketamine (100 mg/kg) plus xylazine (10 mg/kg) was used as a general anesthetic in all surgical procedures. Under aseptic conditions, skin and muscles of the left posterior thigh were incised and the sciatic nerve was exposed between ischial spine and popliteal fossa cephalad to its bifurcation.
In axotomy group, the left sciatic nerve was transected at the mid-thigh and was not repaired. In the epineural suture group, the left sciatic nerve was transected at the mid-thigh and then sutured end to end. In autograft group, a 1-cm segment of the nerve was resected and rotated 180º; it was then sutured at proximal and distal nerve stumps as an autograft. In nerve guidance channel group, a 1-cm segment of the nerve was resected, the proximal and distal nerve stumps were inserted into a 14 mm polarized PVDF tube filled with collagen and NGF, and secured with a single 10-0 epineural suture at proximal and distal ends. In all groups, the right side of the spinal cord

was used as the control. In the sham group, the skin was sutured following incision of the skin and exposure of the sciatic nerve.

Immunohistochemistry

One, four and eight weeks after surgery 2 rats from each group were deeply anesthetized and perfused transcardially by 0.9 % heparanized saline and then 10% formal saline as fixative. Then L4, 5 and 6 segments of the spinal cord were removed and kept in the same fixative for 24 hours.Side determination of sections were signed by a longitudinal knife cut in right posterolateral side of segments (Fig. 1). Paraffin blocks were prepared and were cut serially into 5 µm sections transversally then anti-bax immunohistochemistry (21) was performed. We used rabbit anti-bax (Sigma) as the primary antibody (1:2000), peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody (1:200), H_2O_2 (3%) as endogenous peroxidase blocking, normal goat serum (1:20) (Sigma) as background blocking reagent, and diaminobenzidine tetrahydrochloride (DAB) (Sigma) as the chromogen.

Cell count and statistics

In each group, 10 transverse sections of L4-L6 level from 2 rats were randomly selected. Bax-positive (apoptotic) motor neurons that showed brown cytoplasm and Bax-negative neurons that showed clear cytoplasm (Fig. 4) were counted in the left side of spinal cord as the experimental ones and in the right side as the control.

We analyzed the data by SPSS software using one-way ANOVA, LSD (least significant difference) test, repeated measure, and paired *t* test.

Results

No contralateral motor neuron was labeled by bax-immunohistochemistry. Mean proportion of Bax-positive motor neurons in left side was analyzed. After one week, it was 7.62 ± 1.56 in axotomized group, 5.07 ± 1.1 in epineural suture group, 5.48 ± 1.2 in autograft group, and 5.84 ± 1.7 in nerve guidance channel group (Fig. 3). After one month, the mean proportion was 11.93 ± 2.3 in axotomized group, 6.64 ± 2.1 in epineural suture group, 7.56 ± 2 in autograft group, and 7.47 ± 1.9 in nerve guidance channel group (Fig. 3). After two months, it was 11.18 ± 2.4 in axotomized group, 5.02 ± 1.4 in epineural suture group, 6.01 ± 1.3 in autograft group, and 6.15 ± 1.3 in nerve guidance channel group (Fig. 3).

One way ANOVA did not show any significant mean difference between surgical groups after one week or one month, but showed significant mean difference after two months. LSD post hoc test showed that axotomized group had statistically significant increase compared to other surgical groups ($p<0.05$). Non-parametric Kruskal-Wallis test confirmed the results. In sham group, no Bax-positive neuron was found after one week, one month, or two months.

We used repeated measure test to compare the proportion of apoptotic motoneurons of each surgical approach at different time points. The results showed no significant differences between one week and one month, and between one month and two months groups (Fig. 4).

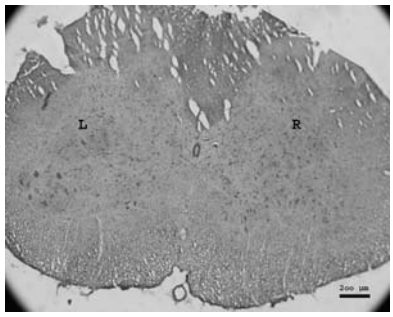

Fig 1: Spinal cord transverse section. Bax-positive neurons (arrow-heads) in left anterior horn of axotomized group after one month in upper right corner cut are shown (L = left, R = right, bar = 50 µm, and Mag. = 50x).

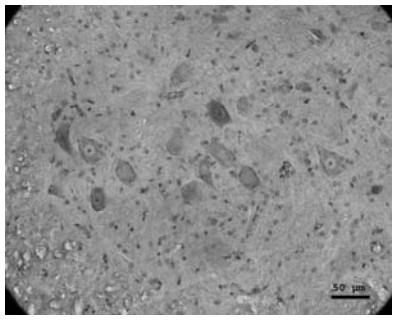

Fig 2: Three Bax-positive neurons (arrow-heads) are seen in left anterior horn of axotomized group after two months (bar = 50 µm, Mag = 200x).

In order to compare the number of motor neurons in left side (experimental) and right side (control), paired *t* test was used, which showed no significant differences after one week. However, they decreased significantly ($p<0.01$) in the left side after one month and two months (Fig. 5 and 6).

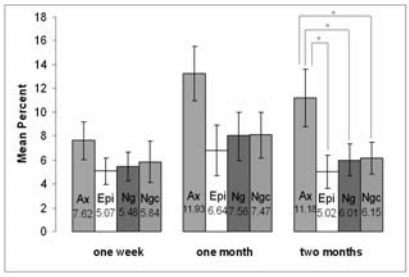

*Fig 3: Mean proportion of Bax-positive neurons in four surgical groups after one week, one month, and two months. Axotomized (Ax) group showed statistically significant increase compared to other groups after two months (*p<0.05). Epineural suture (Epi), nerve graft (Ng), and nerve guidance channel (Ngc). Bars show means ±SEM.*

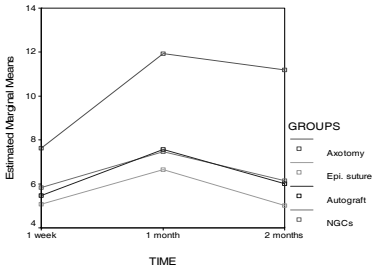

Fig. 4: The proportion of Bax-positive neurons showed no significant differences between one week and one month, and between one month and two months in different groups.

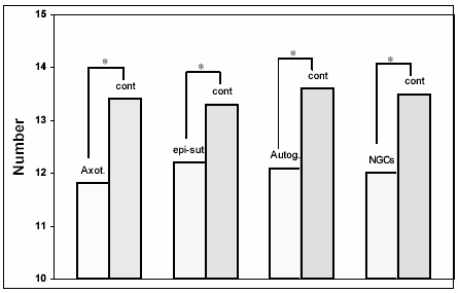

Fig 5: Comparison of means of motoneurons in left and right halves of L4-L6 segments in different groups one month after surgery. It showed significant differences in all groups (p<0.01).*

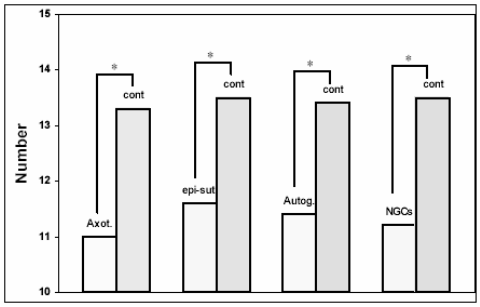

Fig. 6: Comparison of means of motoneurons in left and right halves of L4-L6 segments in different groups two months after surgery. Significant differences were seen in all groups (p<0.01).*

Discussion

Our study showed that apoptosis was detected following sciatic nerve transection whether or not nerve repair was done. The direct epineurial repair of injured peripheral nerves is the current standard approach when nerve ends can be joined without undue tension at the repair site (23). If a gap exists between the nerve ends, direct joining may cause tension at the repair site, a condition that has been shown to compromise functional outcome (23). When this situation occurs, a graft is required to bridge the gap between the nerve ends. Although many substances have been used, including vein conduits, muscles, and synthetic nerve guide tubes, the current standard material for bridging a nerve gap is an autologous nerve graft. Bridging a nerve gap with a tubular prosthesis ("entubulation repair") offers an alternative to repair transected peripheral nerves. The use of such "nerve guide conduits" is especially pertinent for clinical situations where direct realignment of nerve fascicles is impossible. Such situations include nerve injuries with severe laceration or injuries where a segment of nerve has been damaged or removed and the proximal and distal nerve stumps have been separated by a distance that must be bridged. In such a more severe nerve injuries, the standard approach is to use a nerve autograft to bridge the deficit. However, there may be insufficient donor nerve to be sacrificed in severe injuries.

According to our results, any kind of injury to the sciatic nerve can cause cell changes and consequently cell death in some of the spinal motor neurons. The Bax is a proapoptotic molecule and so Bax-immunohistochemistry brings apoptotic motoneuron in primary stages into view (20). We observed Bax-positive neurons in all time series; one week, one month, two months, and also in all surgical groups. Statistical analysis did not reveal significant differences among surgical groups after one week and one month. However, significant difference was observed after two months between axotomy group and other groups. Using charged PVDF with NGF and collagen gel as nerve guidance channel reduced cell death rate at the level of autograft. Hollowell *et al.* in 1990 used silicone tube as nerve autologous in sciatic nerve repair and nearly no cell death was observed in dorsal root ganglia after 10 weeks (24). Silva *et al.* reported in 1985 that following sciatic nerve repair by absorbable nerve guidance channel, two-thirds of dorsal root ganglia cells were

reduced after 6 weeks (25). Our results lie between those of Hollowell and Saliva.

The concept that electrical fields can influence cell behavior is long-standing. The effect of direct current stimulation appears to be related to the natural electric properties of many biological materials. For instance, direct current stimulation has been observed to enhance bone repair (26), promote spinal fusion (27, 28), accelerate ligament reconstruction (29), and enhance nerve regeneration (30). Given that electrical stimulation is shown to be of benefit in tissue repair, it is interesting to consider the potential for rendering the nerve scaffold repair material electroactive. Studies by Schmidt *et al.* in 2003 have revealed that the median neurite length associated with PC12 cells grown on electrically stimulated oxidized polypyrrole was nearly twice that recorded in PC12 cells maintained on unstimulated polypyrrole controls (31). It has also been shown that neurite outgrowth is enhanced on charged polyvinylidene fluoride and charged polytetralluroethylene and that electromagnetic fields can influence neurite extension and regeneration of transected nerve ends *in vivo* (13, 14). We used charged PVDF tube filled with collagen gel as a matrix inside it. Although the application of exogenous electromagnetic fields allows the control of stimulus administered, it is still difficult to localize it completely. It is hypothesized that the charged polymer construct generates a transient electrical field or surface charge, which enhances neurite outgrowth by delivering the stimulus to the localized site of interest (19).

Bax-immunohistochemistry showed obvious cell-death reduction in repairing groups compared to axotomized group after two months. Bax has an important role in cell death in the absence of nerve trophic factors. Baba *et al.* reported in 1999 that Bax protein increased before cell death, while Bcl-2 decreased (32). We can assume that these reports confirm our reports.

Our tracing results proved that regenerating axons had crossed the injury side and reinnervated gastrocnemius muscle. In addition, electron micrographs confirmed immunohistochemical findings (unpublished data).

Comparison of the motor neuron number in the left (experimental) side and right (control) showed no significant differences after one week (Fig. 10), but significant differences ($P<0.01$) after one and two months (Fig. 11, 12). On the other hand, in all surgical groups the mean percent of Bax-positive neurons were increased from one week to one month while decreased from one month to two months; however, this process did not shos significant changes by repeated measure test (time series test). These results suggest that repairing techniques are more effective in preventing cell death after two months.

Axotomy causes disturbance in intra-axonal and retrograde transportation of nerve growth factors from target organ to somata of motor neurons. Absence of neurotrophic factor induces some degenerative changes and perhaps neuronal death of motor neurons (33). Ma and *et al.* in 2003 reported that 16 weeks postoperatively, the nerve repair had significantly reduced the loss of C7 motor neurons. Most strikingly, a 30% motor neuronal loss in the control was almost eliminated by early nerve repair (34).

It seems that during/after sciatic nerve repair, growth molecules from Schwan cells transport to somata retrogradely and prevent atrophy and death of motor neurons. By more regeneration and connecting to target muscles retrograde current of trophic molecules increased. This might be why the rate of Bax-positive neurons decreased from one month to two months.

Conclusion

Guide tubes offer several potential advantages over conventional repair techniques: (a) minimal dissection and fewer sutures cause less surgical trauma to the nerve ends; (b) less tension is applied at the repair site; and (c) growing axons may be able to respond to "guidance cues" in the distal stump and direct themselves to the appropriate target organs. An alternative nerve repair method with as good or better results compared to standard autograft would promisingly contribute to the field of clinical peripheral nerve repair.

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