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Functional recovery of sciatic nerve through inside-out vein graft in rats

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【**Abstract**】**Objective:** Present study aimed at further comprehensive functional, histomorphometrical and immunohistochemical assessment of peripheral nerve regeneration using rat sciatic nerve transection model.

Methods: The 10-mm rat sciatic nerve gap was created in rats. In control group nerve stumps were sutured to adjacent muscle and in treatment group the gap was bridged using an inside-out vein graft. In sham-operated group the nerve was manipulated and left intact. All animals underwent walking track analysis test 4, 8, and 12 weeks after surgery. Subsequently, muscle mass measurement was performed to assess reenervation, histological examination to observe the sciatic nerve regeneration morphologically and immunohistochemistry to detect Schwann cells using anti S-100. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of treatments.

Results: Functional analysis of myelinated nerve fibers showed that nerve function improved significantly in the time course in treatment group. However, quantitative morphometrical analysis of myelinated nerve fibers showed that there was no significant difference between 8 and 12 weeks in treatment group. Muscle weight ratio was bigger and weight loss of the gastrocnemius muscle was ameliorated by inside-out vein grafting. The position of positive immunohistochemical reactions further implied that regenerated axons and Schwann cell-like cells existed after vein grafting was performed, and was accompanied by the process of myelination and structural recovery of regenerated nerves.

Conclusion: Functional analysis of peripheral nerve repair is far more reliable than quantitative morphometrical analysis

Key words: ***Nerve regeneration; Sciatic nerve; Transplants***

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In case of significant damage to nerve tissue, severely-damaged nerves do not spontaneously restore their function, and their continuity has to be firstly reestablished by microsurgical intervention such as suture or interposition of a graft.[1,2] Autologus grafting is still the current surgical procedure of choice for this purpose.[3] Vein graft has been used for many years and it seems that the earliest report is from Weiss and Taylor,[4] who bridged large nerve defects in experimental animals. The advantages like no donor morbidity, simple operation of harvesting and transplanting, availability, affordability and no foreign body reactions make vein graft an attractive alternative to other standard grafts.[5] It has been reported that contact between vein graft endothelial cells and regenerating axons stimulates connective tissue development and fibrosis which causes nerve contraction, impairing axon regeneration.[6] Furthermore, the use of vein graft as a nerve conduit was criticized in the past because of their liability and tendency to collapse and it was suggested that the valves act as physical obstruction against the regenerating nerves.[7] To overcome these drawbacks, the vein graft technique is modified by pulling the vein graft inside-out before its interposition. With this technique, collagen-rich adventitial surface is exposed to the regenerating fibers. Adventitial wall of the vein promotes nerve regeneration by providing an environment rich in collagen and laminin, thereby promoting increased vascularization of new nerve.[8]

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The literature lacks a comprehensive study to assess beneficial effects of the inside-out vein graft on nerve repair. The authors found it necessary to assess nerve repair process using different methods in the same animal model simultaneously. Present study aimed at further comprehensive assessment of sciatic nerve regeneration along a 10-mm rat sciatic nerve gap 4, 8, and 12 weeks after surgery. Assessment of the nerve regeneration was based on functional (walking track analysis), histomorphometric and immuohistochemical (Schwann cell detection by S-100 expression) criteria.

METHODS

Experimental design

Fifty-four male White Albino rats weighing approximately 280 g were randomly divided into three experimental groups (*n*=18): sham-operation group as normal control (NC), transected control (TC) and inside-out vein graft (IOVG). Each group was further subdivided into three subgroups of six animals each. Eighteen male White Albino rats weighing 300-350 g were used as graft donors. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of (23±3)°C, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water.

Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.[9] The University Research Council approved all experiments.

A 15- mm segment of right external jugular vein was harvested on a tube after the rats were shaved and prepared aseptically. Grafts were washed in physiological solution and left at room temperature for 30-40 minutes. A subtle retraction of 1 mm was already expected. Each graft was inverted inside-out by pulling it down a cannula with microsurgery forceps. Following surgical preparation in the sham-operation group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. In TC group, the left sciatic nerve was transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a 10 mm gap due to retraction of nerve ends (Figure 1A). Proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture. No conduit was placed between the stumps. In IOVG group, proximal and distal stumps were inserted 2 mm each into the graft and two 10/0 nylon sutures were placed at each end of the cuff to fix the graft in place and leave a 10-mm gap between the stumps (Figures 1B and 1C). The conduit was filled with 10 μl phosphate buffered saline solution and sterile vaseline was used to seal the ends of the tubes to avoid leakage (Figure 1D).

The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH= 7.4) 4, 8 and 12 weeks after surgery (*n*=6 for each time point).

Functional assessment of nerve regeneration

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of Bain et al.[10] The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

SFI=-38.3×(EPL-NPL)/NPL+109.5×(ETS-NTS)/NTS +13.3×(EIT-NIT)/NIT-8.8

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing rats, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides. Wet weight of the muscles was recorded, using an electronic balance. All measurements were made by two independent observers unaware of the analyzed group.

Histological preparation and morphometric study

Graft middle cable in IOVG group, midpoint of normal sciatic nerve in NC group and regenerated cable in TC group were harvested and fixed with 2.5% glutaraldehyde. The grafts were then embedded in paraplast paraffin, cut into 5-μm section and stained with toluidine blue. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed-up in order to cope with sampling-related, fiber-location-related and fiber-size-related biases, respectively.[11]

Immunohistochemical analysis

In this study, anti-S-100 (1:200, Dako, USA) was used as a marker for axon and myelin sheath. Specimens prior to immunohistochemistry were post-fixed with 4% paraformaldehyde for 2 hours and embedded in paraplast paraffin. After non-specific immunoreactions were blocked, sections were incubated in S-100 protein antibody solution for 1 hour at room temperature. They were washed three times with PBS and incubated in biotynilated anti-mouse rabbit IgG solution for 1 hour. Horseradish peroxidase-labelled secondary antibody was developed by the diaminobenzidine method. The results of immunohistochemistry were examined under a light microscope.

Statistical analysis

Experimental results were expressed as mean±SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were considered significant when $P<0.05$.

RESULTS

Recovery of sciatic nerve function

Figure 2 shows SFI values in all three experimental groups. Prior to surgery, SFI values in all groups were near zero. After the nerve axotomy, the mean SFI was decreased to -100 due to the complete loss of sciatic nerve function in rats. Four weeks after surgery, mean SFI was -92.8±1.24 in IOVG group, compared with -97.3 ±0.78 in TC group. Eight weeks after surgery, the improvement in SFI was observed in IOVG, indicating that some regenerating axons passed through the vein graft and eventually into the target organ, whereas in TC group, no comparable SFI value was obtained 8 weeks after operation. At postoperative 12 weeks, the animals in IOVG group achieved a mean SFI of -64.1±2.10, i.e. an approximate improvement of 35%, whereas a mean value of -95.2±0.97, i.e. an approximate improvement of 5%, was found in TC group. Recovery of nerve function was not detected in TC group throughout 12 weeks after operation. The statistical analyses revealed that the recovery of nerve function was significantly different between IOVG and TC groups ($P<0.05$) and vein graft entubulation significantly promoted functional recovery in the time course.

Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured. There was statistically significant difference on muscle weight ratios between IOVG and TC groups ($P<0.05$). The results showed that muscle weight ratio was bigger in IOVG group than in TC group and weight loss of gastrocnemius muscle was ameliorated by inside-out vein grafting (Figure 3).

Histological and morphometric findings

In the early phase of regeneration, regenerated nerve fibers were present within the vein guide 4 weeks after operation without any foreign body reaction. In TC group, 4 rats presented the lower number of nerve fibers at distal stumps 8 weeks after operation. The other two showed degenerated distal stumps. Statistical analysis by means of a one-way ANOVA test showed that sham-operation group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared with IOVG and TC groups. Although both TC and IOVG groups presented regeneration patterns, the number of nerve fibers in IOVG group was significantly higher than that in TC group 8 and 12 weeks after operation. The mean diameter of nerve fibers in IOVG group was (7.94±0.49) μm, significantly larger than (4.11±0.22) μm in TC group. The myelin sheath thickness was (1.95±0.24) μm in IOVG group, significantly larger than in (0.83±0.02) μm TC group. Using factorial ANOVA analysis with two between-subjects factors (group×time), in IOVG group, axon diameters did not show significant difference between 8 and 12

weeks (P>0.05). Thickness of myelin sheath showed an interaction with time in both groups. Increase in mean thickness of myelin sheath did not show statistical difference between 8 and 12 weeks in IOVG group (P>0.05, Table 1).

Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross-sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in myelin sheath. The axon also showed a weak expression, indicating that Schwann cell-like phenotype existed around the myelinated axons (Figures 4, 5). In IOVG group, the structure and function of regenerated axons and myelin sheath were far more similar to those of normal nerve compared with TC group. In TC group, the expression of S-100 was dispersed and the findings resembled those of the histological evaluations.

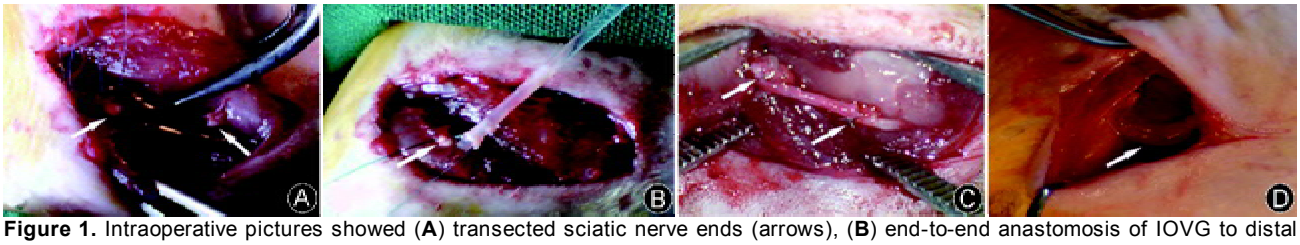

Figure 1. Intraoperative pictures showed (**A**) transected sciatic nerve ends (arrows), (**B**) end-to-end anastomosis of IOVG to distal stump of transected sciatic nerve (arrow), (**C**) proximal and distal stumps each inserted 2 mm into the graft and two 10/0 nylon sutures (arrows) placed at each end of the cuff to fix the graft in place, leaving a 10-mm gap between the stumps and (**D**) engrafted vein filled with phosphate buffered saline (arrow).

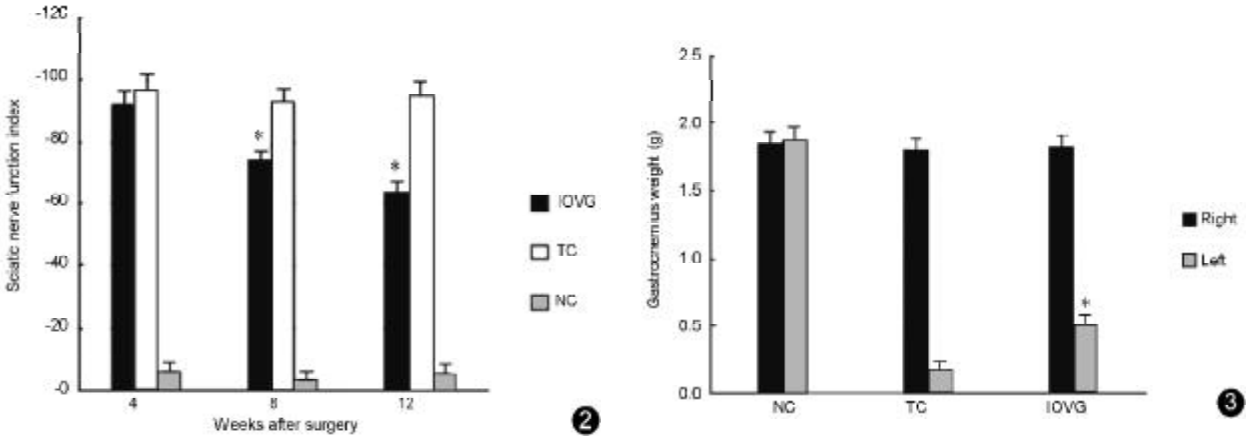

Figure 2. Diagrammatic representation of effects on SFI. Entubulation with vein graft gave better results in functional recovery of the sciatic nerve. Data were presented as mean±SD. *P < 0.05, compared with TC group. **Figure 3.** Measurement of gastrocnemius muscle. The gastrocnemius muscle of both sides (injured left and intact right) was removed and weighted in experimental groups 12 weeks after surgery. Data were presented as mean±SD. *P < 0.05, compared with TC group.

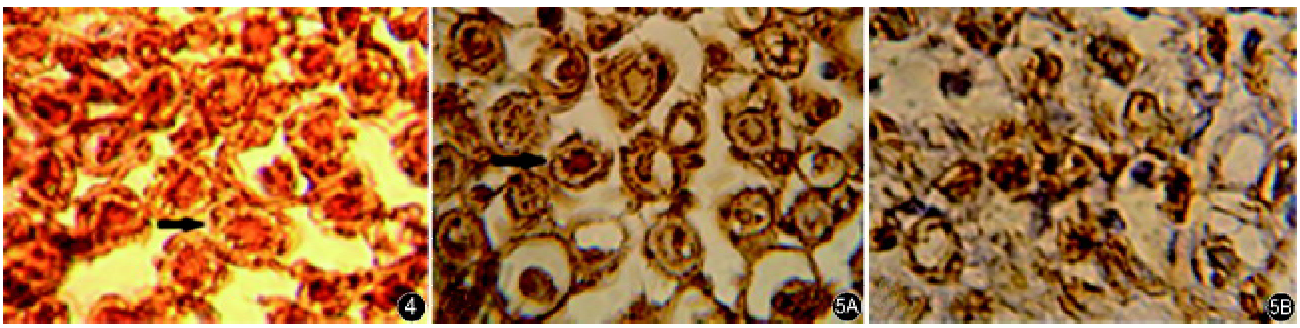

Figure 4. Immunohistochemical analysis of the regenerated nerve 4 weeks after surgery from midpoint of normal sciatic nerve (NC group). There was positive staining of the myelin sheath-associated protein S-100 (arrow) within the periphery of nerve, indicating normal histological structure in normal nerve (×400). **Figure 5.** Immunohistochemical analysis of the regenerated nerve 12 weeks after surgery from **A:** middle cable (IOVG group); **B:** regenerated cable (TC group).There was clearly more positive staining of the myelin sheath-associated protein S-100 (arrow) within the periphery of nerve, indicating well-organized structural nerve reconstruction entubulated nerve (×1000).

Table 1. Morphometric analyses of regenerative nerves in each experimental group (mean±SD)

Groups	Number of fibers			Diameter of fibers (μm)		
	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
NC	8124±385	8379±446	8028±404	12.01±0.01	11.93±0.17	12.06±0.23
TC	0**	1003±295*	1131±219*	0**	3.98±0.55*	4.11±0.22*
IVOG	1849±297*	3217±307*	3584±264*	3.24±0.69*	7.49±0.37*	7.94±0.49*
Groups	Diameter of axon (μm)			Thickness of myelin sheath (μm)		
	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
NC	7.03±0.02	6.97±0.39	7.06±0.46	2.56±0.01	2.48±0.02	2.53±0.01
TC	0**	2.38±0.36*	2.44±0.63*	0	0.81±0.13*	0.83±0.02*
IVOG	2.22±0.47*	3.87±0.25*	4.05±0.02*	0.51±0.03*	1.82±0.34*	1.95±0.24

*P<0.05, **P<0.01, compared with NC group.

DISCUSSION

Peripheral nerve tubulation repair has been studied experimentally and clinically for many years by different techniques, and peripheral nerve repair using vein graft with varying degrees of success has a long history.[12-14] The vein as a conduit has been utilized to repair segmental nerve tissue loss, and it is proved to be supportive conduit for peripheral nerve axonal regeneration and maturity in short gaps irrespective of the resilience of the wall.[15,16] Necessity to assess beneficial effects of inside-out vein graft on nerve repair process across a 1 cm gap in an adult rat sciatic nerve using functional, histomorphometrical and immunohistochemical methods in the same animal was encouraging and ended up this finding that histomorphometric analysis did not match functional findings at least in a time period during the study.

The previous experiments have tested that nerve repair using the inside-out vein graft conferred a benefit on the regenerating nerve which was associated with improved morphometric indices of recovery.[7, 17-20] The adventitia of rat jugular vein receives sympathetic and parasympathetic nerve fibers, both of which have Schwann cells. Inversion of the vein brings these Schwann cells directly in contact with the regenerating neurites.[21] That is why our study was designed based on inside-out vein grafting technique.

It is known from previous studies that regeneration process in rats can not be completed by 12 weeks, a phenomenon which has been reported in a variety of experimental models since the introduction of vein graft entubulation as a research tool.[22, 23] Quantitatively, our results are consistent with these findings. Reportedly a 12-week experimental period is sufficient for evaluation of regeneration process because functional recovery after repair of a transected peripheral nerve in rats occurs during this period of time.[24]

Castaneda et al[24] suggested that arrival of sprouts from proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function so they did not perform quantitative analysis in their work. Walking track analysis is frequently used to reliably determine functional recovery following nerve repair in rat models.[10, 25] Statistical analyses and Bonferroni test in our study indicated that in IOVG group axon diameter and myelin thickness did not improve significantly 8 weeks after surgery. In contrary, in IOVG group, functional recovery occurred from week 8 to week 12 and SFI values showed significant improvement 8 weeks after surgery. This again supports the idea that selection of an appropriate method to evaluate nerve repair process is crucial. Others have suggested that walking track analysis is more comprehensive than electrophysiological or histomopphometrical methods alone. This study also supports the idea that the walking track analysis is more comprehensive and reliable than histomorphometric methods in peripheral nerve repair studies.[24, 26, 27] Nonetheless we believe that the combination of SFI with electrophysiological assessment could lead us to more definitive judgment about the superiority of SFI over morphometrical analyses and electrophysiological examinations in nerve repair process which could be taken into consideration in future studies.

Improved functional recovery after vein graft bridging in present study is similar to those SFI values in other researches.[19, 28] Our possible explanation for the improvement is that regenerating nerve fibers easily grow out throughout the vein graft.

As the posterior tibial branch of sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation.[29,30] In the present study, 12 weeks after surgery, the muscle mass was found in IOVG group. IOVG group showed significantly greater ratios of mean gastrocnemius muscle weight than TC group, indicating indirect evidence of successful end organ reinnervation in IOVG group.

In our histological studies, the number of nerve fibers regenerated after transection appeared to be significantly higher than TC group when vein graft was used. A lower number of myelinated fibers were counted 4 weeks after surgery in IOVG group compared with NC group. Nerve fiber diameter and myelin thickness were also lower in IOVG and TC groups than in NC group. Regenerating axonal sprouts tended to be smaller than those from uninjured axons. It could be assumed that the microenvironment provided for nerve regeneration by vein graft contributed higher morphometrical indices in IOVG group than in TC group.

In immunohistochemistry, the expression of axon and myelin sheath special proteins was evident in NC group which indicates the normal histological structure. The location of positive reactions to S-100 further implied that both regenerated axon and Schwann cell-like cells existed when the vein grafting was performed, and was accompanied by the process of myelination and the structural recovery of regenerated nerve.

In addition to the above findings, a vein graft seems to have several distinct advantages for the treatment of transected peripheral nerves:(1) it can be used as autogenous transplantation;(2) it does not provoke any noticeable foreign body reaction;(3) it can be harvested by minor surgeries without complications;(4) no functional deficit and injury occurs at the donor site in contrast to nerve and artery grafts.

In conclusion, functional analysis of peripheral nerve repair is far more reliable than quantitative morphometrical analysis. Inside-out vein graft technique has offered the hope of providing a biological method for achieving the peripheral nerve repair in the least harmful way that is available, easily performed and affordable. It also averts the need for foreign materials that are likely to provoke a foreign body reaction.

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