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Functional, Histomorphometrical and Immunohistochemical Assessment of Sciatic Nerve Regeneration through Inside-Out Vein Graft in Rat

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Abstract

Objective- Comprehensive functional, histomorphometrical and immunohistochemical assessment of sciatic nerve regeneration through an inside-out vein graft in rat.
Design- Experimental in vivo study.
Animals- Fifty- four healthy male White Albino rats.
Procedures- The rats were divided into three experimental groups (n=18), randomly: Sham-operation (NC), Transected control (TC) and Inside-out vein graft (IOVG). In NC group after anesthesia the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured. In TC group the left sciatic nerve was exposed the same way, transected proximal to the tibio-peroneal bifurcation leaving a 10 mm gap. In IOVG group the left sciatic nerve was transected the same way and proximal and distal stumps were each inserted into an inside-out vein graft. Each group was further subdivided into three subgroups of six animals each and were studied 4, 8, 12 weeks after surgery.
Results- Functional analysis showed significant improvement of nerve function in IOVG than in TC group ($P < 0.05$). Morphometric indices and immuohistochemistry indicated that there were significant differences ($P < 0.05$) between IOVG and TC groups 12 weeks after surgery.
Conclusion and Clinical Relevance- Inside-out vein graft technique has offered the hope of providing a biological method for achieving the peripheral nerve regeneration 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.
Key Words- Peripheral Nerve Regeneration, Inside-out Vein Graft, Rat.

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Introduction

Peripheral nerves have self regeneration capacity after traumatic injury. In case of significant damage to nerve tissue, severed nerves do not spontaneously restore their function, and their continuity has to be first reestablished by microsurgical intervention such as suturing or interposition of a graft.[1,2] Reconstructive surgical procedures are required following traumatic or iatrogenic damage to peripheral nerves or after excision of primitive neoplasms. Experimental studies and clinical reports indicate that insertion of a conduit could be an interesting alternative to direct end-to-end suturing of nerve stumps or interposition of an autograft.[3,4] Nowadays, conduits are mainly made of non-bioabsorbable materials including silicone or bioabsorbable materials such as aliphatic polyesters, polyurethane, collagen, chitosan and excised artery or vein.[1,5] The advantage of these conduits is the avoidance of sacrificing a segment of the donor nerve with subsequent loss of function and/or neuroma formation and also providing a microenvironment that is optimal for regeneration.[6,7] Vein grafts have been used for many years and it seems the earliest report is for Weiss and Taylor,[8] who bridged large nerve defects in experimental animals. The advantages like no donor morbidity, the ease of harvesting and transplanting, availability, affordability and no foreign reactions make vein graft an attractive alternative to other standard grafts.[9,10] Some authors have found that in standard vein grafting studies, there is minimal scar-tissue invasion inside the graft. Others reported that contact between vein graft endothelial cells and regenerating axons stimulates connective tissue development and fibrosis which causes nerve contraction and impairs axon regeneration.[11-12] Furthermore, the use of vein graft as a nerve conduit has been criticized in the past because of their liability and tendency to collapse and it has also been suggested that the valves may act as physical obstruction against the regenerating nerves.[7] To overcome these drawbacks the vein graft technique has been modified by pulling the vein graft inside out before its interposition. In this technique collagen-rich adventitial surface is exposed to the regenerating fibers.[13] Because this conduit is non-immunogenic and lined with abundant trophic and neurite-promoting factors it may provide a superior microenvironment for peripheral nerve regeneration. Adventitial wall of the vein promotes nerve regeneration by providing an environment rich in collagen and laminin thereby promoting increased vascularization of the new nerve.[14, 15]
We used well-established test systems because we aspired to examine comprehensive behavior of the inside-out vein graft in the very same animal, an effort not attempted yet to the best of our knowledge. In light of promising clinical results obtained by the inside-out vein grafting technique, the present study aimed at comprehensive functional, histomorphometrical and immunohistochemical assessments of rat sciatic nerve regeneration 4, 8, and 12 weeks after surgery.

Materials and Methods

Experimental Design
Fifty-four male White Albino rats weighing approximately 280g were divided into three experimental groups (n=18), randomly: Sham-operation (NC), Transected control (TC) and Inside-out vein graft (IOVG). Each group was further subdivided into three subgroups of six animals each (Fig. 1). Eighteen male White Albino rats weighing 300-350 g were used as graft donors. The rat was chosen for this study due to the fact that this species and especially the male, is one of the most frequently species used for the investigation of peripheral nerve repair.[16] An experimental period of 12 weeks was used because in rats functional recovery

after repair of a transected peripheral nerve occurs during this timeline.[17-19] Two weeks before and during the entire experiments, the animals were housed in individual plastic cages (50 × 40 × 20 cm) with an ambient temperature of 23 ± 3º C, stable air humidity, and a natural day/night cycle. The animals were handled on a regular daily basis for 2 weeks prior to the study in order to acclimatize them with testing area and experiments. This could minimize anxiety related testing inaccuracies.[20] The rats had free access to standard rodent laboratory food and tap water.

Grafting procedure

Animals were anesthetized by intraperitoneal administration of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). All procedures were carried out in accordance with the guidelines of the Ethics Committee of the International Association for the Study of pain.[21] The University Research Council approved all experiments.

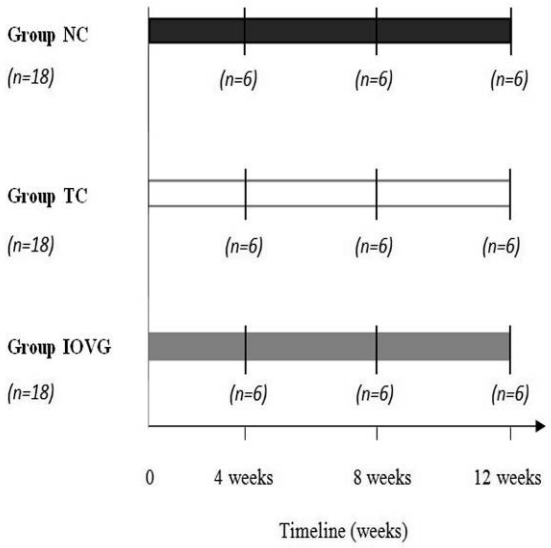

Figure 1. Schematic representation of the experimental design. Group sizes are indicated in *italics*. Animals were sacrificed 4, 8 and 12 weeks after surgery

The right external jugular vein was exposed through a paramedian neck incision and cannulated, and the length of a 15- mm segment was harvested on the tube after the animals had been shaved and prepared aseptically. The diameters were 1.6 ± 0.2 mm at the proximal end and 1.4 ± 0.5 mm at the distal end. There were four to five branches on the vein graft that would need to be positioned inside-out so as to prevent any potential branching of the axons through them during regeneration. Donor animals were euthanized with high dose anesthetic after grafts had been harvested. Grafts were washed in physiological solution and left at room temperature for 30-40 min.[22] A subtle retraction of 1mm was already expected. Allografts did not receive preliminary treatment to reduce their antigenicity. Each graft was inverted inside-out by pulling it down the cannula with microsurgery forceps. Following surgical preparation in the sham-operation group (NC), 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. The rats were observed on a heating pad during recovery. In transected control group (TC) the left sciatic nerve was exposed the same way, transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a gap about 10 mm due to retraction of the nerve ends. The proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture. No conduit was placed between the stumps. In inside-out vein group (IOVG), proximal and distal stumps were each inserted 2 mm 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 (Fig. 2). 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.[23] All surgical procedures were carried out by the same surgeon, using a sterile microsurgical technique. After the surgery the animals were housed in groups of six

per cage under the same conditions mentioned above. No drugs were administered during post operative period.
The animals of each group were anesthetized by intraperitoneal administration of ketamine-xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH = 7.4) at 4 (n = 6), 8 (n = 6) and 12 weeks (n = 6) after surgery.

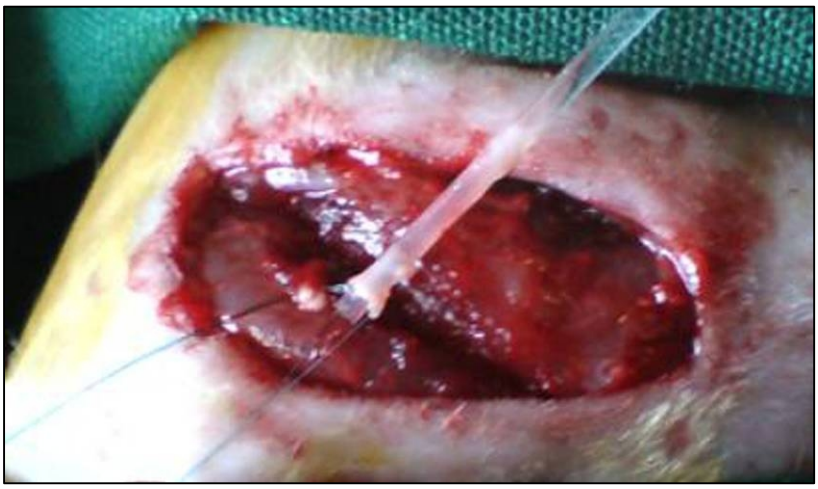

Figure 2. End-to- end anastomosis of IOVG to distal stump of transected sciatic nerve. Proximal and distal stumps were each inserted 2 mm into the graft and two 10/0 nylon sutures were placed at each end of the cuff to fix the graft in place and leave a 10-mm gap between the stumps.

Functional assessment of nerve regeneration
Walking track analysis was performed 4, 8 and 12 weeks after surgery for the last subgroup (n=6) of each group that was scheduled to be sacrificed 12 weeks after surgery based on Bain et al.,[24] 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) in each animal was calculated by the following formula:

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

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

Histological preparation and morphometric studies
Graft mid-substance (IOVG), midpoint of normal sciatic nerve (NC) and regenerated mid-substance (TC) were harvested and fixed with glutaraldehyde 2.5%. The grafts were then embedded in paraplast paraffin, cut in 5 μm 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, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases.[25]

Immunohistochemical analysis
In this study, anti-S-100 (1:200, DAKO) was used as marker for myelin sheath. Specimens prior to immunohistochemistry were post fixed with 4% paraformaldehyde for 2h and embedded in paraffin. After non-specific immunoreactions were blocked, sections were incubated in S-100 protein antibody solution for 1h at room temperature. They were washed

three times with PBS and incubated in biotynilated anti-mouse rabbit IgG solution for 1h. 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 means ± SD. All data were analyzed by one-way analysis of variance (ANOVA) to assess statistical significance between experimental groups (SPSS 17.0 for Windows). The differences were considered significant when $P < 0.05$.

Results

Recovery of sciatic nerve function

Figure 3 shows sciatic function index (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 decreased to -100 due to the complete loss of sciatic nerve function in all animals.

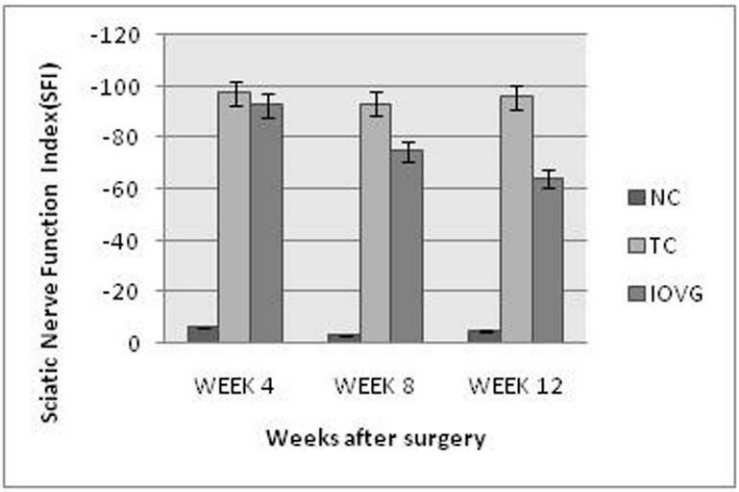

Figure 3. Diagrammatic representation of effects on the sciatic nerve function index (SFI). Better functional recovery was observed in IOVG group compared to TC group ($P<0.05$).

Four weeks after surgery mean SFI was -92.8 ±1.24 in group IOVG, compared to -97.3 ±0.78 in group TC, with insignificant change in the NC. Eight weeks after surgery the improvement in SFI was observed in IOVG, indicating that some regenerating axons have passed through the vein graft and eventually into the target organ, whereas in group TC, no comparable SFI value was obtained after 8 weeks. After 12 weeks, animals of group IOVG achieved a mean value for SFI of -64.1 ± 2.10, i.e. an approximate improvement of 35%, whereas in group TC, a mean value of -95.2 ± 0.97, i.e. an approximate improvement of 5%, was found. Recovery of nerve function was not detected in the TC throughout 12 weeks post operation. The statistical analyses revealed that the recovery of nerve function was significantly ($P<0.05$) different between IOVG and TC and interposition of the vein graft significantly promoted functional recovery in the course of time.

Histological and Morphometric findings

All inside-out vein grafted animals showed nerve fibers at distal stumps 4 weeks after surgery. In the early phases of regeneration (at four weeks post operation) regenerated nerve fibers were present within the vein guide and regenerated nerve fibers could be confirmed after 4

weeks without any foreign body reaction. In TC group, with no conduit between the proximal and distal stumps, four animals presented lower number of nerve fibers at distal stumps after 8 weeks. The other two showed degenerated distal stumps. Sham-operation group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to IOVG and TC animals. Although both TC and IOVG presented regeneration patterns, the number of nerve fibers in IOVG both after 8 and 12 weeks was significantly higher than TC (Fig. 4). The mean diameter of the nerve fibers in the IOVG (7.94 ± 0.49) was significantly larger than that of TC (4.11 ± 0.22). The myelin sheath thickness in IOVG (1.95 ± 0.24) was significantly larger than in TC (0.83 ± 0.02) (Table. 1).

Table 1. Morphometric analyses of regenerative nerves for each of the experimental groups: values are given as mean ± SD

	NC			TC			IOVG		
Weeks	**4**	**8**	**12**	**4**	**8**	**12**	**4**	**8**	**12**
N	8124 ± 385	8379 ± 446	8028 ± 404	0‡	1003 ± 295†	1131 ± 219†	1849 ± 297†	3217 ± 307†	3584 ± 264†
D	12.01 ± 0.01	11.93 ± 0.17	12.06 ± 0.23	0‡	3.98 ± 0.55†	4.11 ± 0.22†	3.24 ± 0.69†	7.49 ± 0.37†	7.94 ± 0.49†
d	7.03 ± 0.02	6.97 ± 0.39	7.06 ± 0.46	0‡	2.38 ± 0.36†	2.44 ± 0.63†	2.22 ± 0.47†	3.87 ± 0.25†	4.05 ± 0.02†
T	2.56 ± 0.01	2.48 ± 0.02	2.53 ± 0.01	0‡	0.81 ± 0.13†	0.83 ± 0.02†	0.51 ± 0.03 †	1.82 ± 0.34†	1.9 5± 0.24

N: Number of fibers D: Diameter of fibers (µm) d: Diameter of axon (µm) T: Thickness of myelin sheath (µm)

†Results were significantly different from those of sham-operated (NC) animals ($p < 0.05$)

‡ Results were significantly different from those of sham-operated (NC) animals ($p < 0.001$)

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 the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 5). In the IOVG group, the structure and function of regenerated axons and myelin sheath were far more similar than TC group to those of normal nerve. In TC, the expression of S-100 was dispersed and the findings resembled those of the histological evaluations.

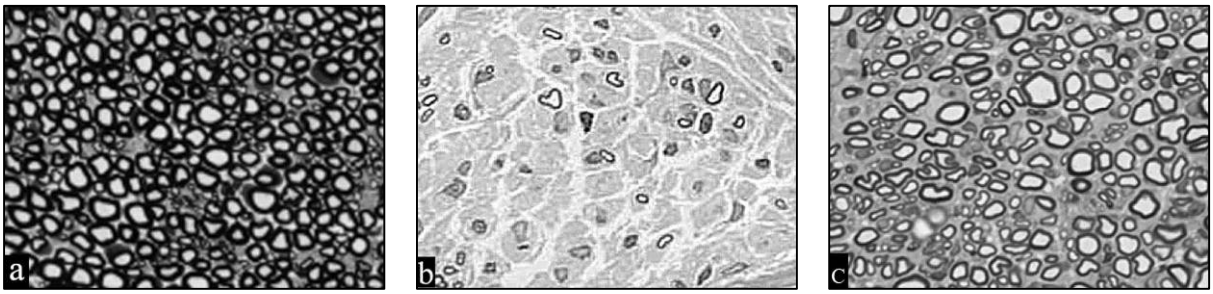

Figure 4. Light micrograph of representative cross section taken from (a) midpoint of normal sciatic nerve (NC), (b) regenerated cable (TC) and (c) middle cable (IOVG) 12 weeks after surgery. (Toluidine blue, ×400).

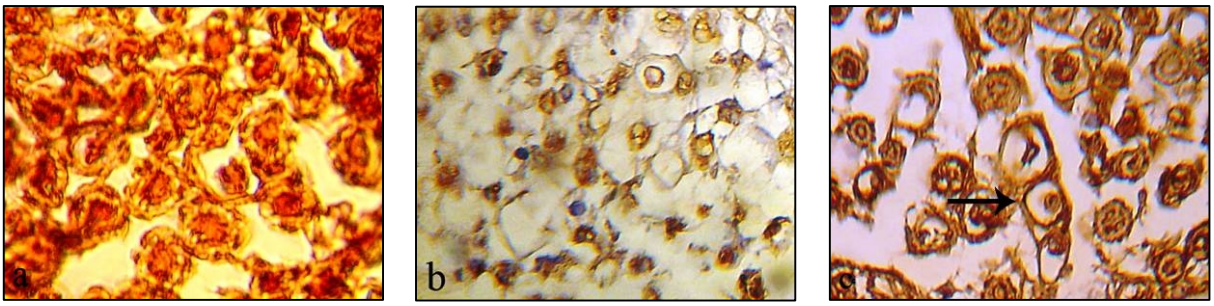

Figure 5. Immunohistochemical analysis of the regenerated nerve 12 weeks after surgery from (a) midpoint of normal sciatic nerve (NC), (b) regenerated cable (TC) and (c) middle cable (IOVG).There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well-organized structural nerve reconstruction in entubulated nerve compared to transected control. (×1000).

Discussion

The vein as a conduit has been utilized to repair segmental nerve tissue loss which proved to be supportive conduit for peripheral nerve axonal regeneration and maturity irrespective of the resilience of the wall.[7,15,21,26-29] The functional, histological and immunohistochemical examinations of this study demonstrated that entubulation of transected nerve ends enhanced rat sciatic nerve regeneration in that its resilient flexible wall did not have a potential for deformation and, therefore subsequent regeneration of nerve segment occurred.

We have demonstrated in this study that the tube can support axonal regrowth across a 1 cm gap in adult rat sciatic nerve. We used well-established test systems because we aspired to examine comprehensive behavior of the conduit, an effort not attempted yet to the best of our knowledge. Both stumps of the severed nerve were fixed and sealed into the ends of the vein conduit and created a medium stimulating growth of axons from the proximal nerve stumps. This offered an advantage of frequent use of the graft because there are no other influences restricting regenerating axons in their growth. The newly regenerated axons are probably "navigated" to the appropriate peripheral stump fascicles by neurotropic factor.[30]

Results of this study supported other reports[7, 9,22,31,32] and further emphasized that the usage of inside-out vein graft was associated with improved morphometric indices and functional recovery. Previous work by Wang ***et al*** suggested that it conferred not only a structural but also a functional benefit.[13,14] They postulated that this benefit was due to the change in the microenvironment of the regenerating nerve. The adventitia of the 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.[33] This may also contribute to the increased myelination in the IOVG group in our study.

It is known from previous studies that regeneration process in rats would have not 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.[34,35] Quantitatively, our results are consistent with those findings. However, a 12-week experimental period is sufficient for evaluation of regeneration process because in rats functional recovery after repair of a transected peripheral nerve occurs during this timeline.[17-19]

Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models.[36] In our study as observed histologically, morphometrical values did not differ significantly between 8 and 12 weeks in entubulated defects. However, recovery of nerve function improved significantly in the course of time in IOVG group. This study again supports the idea that the walking track analysis (SFI) is more

comprehensive than histomorphometrical methods.[19,37] Improved functional recovery after vein graft bridging in present study was similar to those SFI values of other authors .[31,38] Our possible explanation for improvement in function is that regenerating nerve fibers easily grow out throughout the vein graft.

In the histological studies, the number of nerve fibers regenerated after transaction, appeared to be higher when vein graft was used. In this study a lower number of myelinated fibers were counted by the week four after surgery in IOVG group. Nerve fiber diameter and myelin thickness were also lower in IOVG groups and TC group than in NC group. Regenerating axonal sprouts tended to be smaller than those from uninjured axons.

The expression of axon and myelin sheath special proteins was evident in NC group which indicated 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 vein grafting was performed, and were accompanied by the process of myelination and the structural recovery of regenerated nerve.

In addition to the findings already mentioned 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.

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. The technique, if combined with local delivery of other biomaterials, facilitates and maximizes nerve regeneration, restoring its function as much as and as soon as possible. It also averts the need for foreign materials that are likely to provoke a foreign body reaction.

Acknowledgements

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چکیده

ارزیابی فانکشنال ، هیستومورفومتریک و ایمنوهیستوشیمیایی ترمیم عصب سیاتیک با استفاده از گرافت وریدی در رت

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هدف- ارزیابی جامع فانکشنال ، هیستومورفومتریک و ایمنوهیستوشیمیایی ترمیم عصب سیاتیک با استفاده از گرافت وریدی در رت.

طرح مطالعه- مطالعه تجربی در حیوان زنده.

حیوانات- 54 راس رت آلبینو نر سفید و سالم.

روش کار- رت‌ها بطور تصادفی به سه گروه 18 تایی تقسیم شدند. در گروه کنترل نرمال عصب سیاتیک سمت چپ پس از برش پوست و عضله سرینی دستکاری شده و پس از خونبندی موضع بخیه گردید.درگروه شاهد پس از دستیابی به عصب سیاتیک با قطع عصب، نقیصه‌ای به طول 10 میلیمتر ایجاد گردید . در گروه درمان پس از ایجاد نقیصه 10 میلیمتری، انتهاهای قطع شده پروکزیمال و دیستال عصب با استفاده از گرافت وریدی به هم مرتبط شدند.هر گروه متعاقبا به سه زیر گروه 6 تایی تقسیم گردیده و در مقاطع زمانی 4، 8 و 12 هفته بعد از جراحی مورد مطالعه قرار گرفتند.

نتایج- تست فانکشنال نشان داد که عملکرد عصب سیاتیک در رت های دریافت‌کننده گرافت وریدی بهبودی قابل ملاحظه‌ای پیدا کرده بود ($P < 0.05$). شاخص های مورفومتریک و ارزیابی های ایمنوهیستوشیمیایی بیانگر ترمیم قابل ملاحظه ای در گروه درمان در مقایسه با گروه شاهد بودند ($P < 0.05$).

نتیجه گیری و کاربرد بالینی- از نظر بالینی گرافت وریدی به عنوان یک روش بیولوژیک امیدهای تازه ای را در ترمیم بهتر اعصاب محیطی ایجاد کرده است. این روش نسبت به سایر روشهای ترمیمی ساده تر، کم خطر تر، در دسترس و ارزانتر بوده و خطر ایجاد واکنشهای اجسام خارجی را نیز ایجاد نمیکند.

کلید واژگان- ترمیم عصب محیطی، گرافت وریدی، رت.