RESEARCH ARTICLE

Two-Stage Nerve Graft in Severe Scar: A Time-Course Study in a Rat Model

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Abstract

Background: Peripheral nerve repair outcomes are suboptimal in the presence of severe soft tissue injury and excessive scarring paralleling the process in tendon reconstruction of the hand. Inspired by the advantages of the two-stage technique in tendon grafting and with encouraging preliminary results, we aimed to investigate the two-stage nerve grafting technique as an alternative method of secondary nerve repair.

Methods: Thirty female rats (~200 g) were randomly distributed into two groups (n=15). A 15 mm gap was created in the sciatic nerve of the animals and an excessive extraneural scar was induced using the "mincing" method. In this method, a thin strip of muscle was removed, minced in a petri dish and returned to the peripheral nerve. In the two-stage nerve graft group, a silicone tube was interposed in the first stage. After 4 weeks, in the second stage, the silicone tube was removed and a median nerve autograft was interposed through the newly formed vascularized sheath. In the conventional graft group, two nerve ends were protected with silicone caps in the first stage. After 4 weeks the caps were removed and the median graft was interposed. Behavioral assessments were performed at week 15 after surgery with the withdrawal reflex latency (WRL) and extensor postural thrust (EPT) and at the 3, 6 and 15-week time points with the TOA (toe out angle). Masson Trichrome staining method was used for histological assessments at week 15.

Results: According to the EPT and WRL, the two-stage nerve graft showed significant improvement (P=0.020 and P=0.017 respectively). The TOA showed no significant difference between the two groups. The total vascular index was significantly higher in the two-stage nerve graft group (P<0.001).

Conclusions: Two-stage nerve graft using a silicone tube enhances vascularity of the graft and improves functional recovery.

Key words: Graft, Peripheral nerve injuries, Rats, Scar, Sciatic nerve

Introduction

Peripheral nerve injury (PNI) in the upper limb is a significant cause of disability. Motor and sensory dysfunction and pain due to PNI result in considerable impairment and morbidity (1). Different repair and grafting techniques have been introduced since nerve grafting was initially described by Albert in 1876 (2). However, when it comes to abundant scarring and severe trauma, none of these techniques is perfect. Nerve grafting under unfavorable conditions and excessive scarring results in adhesion, tethering, and suboptimal clinical outcomes (3). A similar condition is present in reconstruction of flexor tendon injury in zone II (4). The Hunter rod technique was previously introduced as a solution for tendon reconstruction in cases with severe scarring and flexor pulley system destruction (5,6). In this two-stage approach of tendon grafting, a silicone rod is used as a temporary implant in addition to pulley reconstruction (7). A pseudosheath forms around the silicone during the 3–6 month interval between stages. In the second stage, the silicone rod is removed and the tendon graft is interposed through the

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newly formed pseudosheath (7). This technique was successfully used in multiple conditions with satisfactory results (8–12).

We hypothesized that the pseudosheath formed around the silicone tube could be used as a suitable bed for nerve graft in severely scarred soft tissue. The gliding lumen of the pseudosheath could prevent adhesion, and its stable collagenous structure with high vascularization provides a good blood supply and protects the graft from compression of the adjacent scar tissues (13).

Following the encouraging preliminary results (14), we designed an animal study to investigate the effects of the pseudosheath on peripheral nerve grafting in the presence of scar tissue.

Methods

Experiment design

Thirty female Wistar rats (180-200 gr) were divided randomly into two groups based on the different techniques of secondary repair of the nerve gap in combination with a scar induction method. Group 1 was treated with two-stage nerve grafting with a silicone tube and group 2 only received a conventional graft. Behavioral assessments were performed. After 15 weeks all rats were sacrificed for histologic assessments.

All experiments were approved by the Ethical Committee of the Tehran University of Medical Sciences (TUMS Institutional Review Board). Animals were housed one week before starting the operations for adaptation in a temperature-controlled room with 12-12 light and dark cycles and free access to food and water. All assessments were conducted by skilled observers who were blinded to the experimental groups.

Surgical procedures

An intraperitoneal (IP) injection of ketamine (100 mg/kg ketamine hydrochloride, Rotexmedica, Germany) was used to induce anesthesia. All procedures were performed under aseptic conditions using standard microsurgical techniques.

In stage I, the right sciatic nerve was gently exposed with the gluteal muscle-splitting approach under surgical loupe magnification. A nerve gap was created by transection of a 15 mm segment of the sciatic nerve. In group 1 (n=15), a silicone tube with an inner diameter of 1.5 mm and length of 20 mm was interposed between the nerve ends. The proximal and distal ends were fixed with two 8-0 nylon sutures while positioned 1.5 mm inside the tube. In group 2 (n=15), a 3 mm silicone cap was placed on each proximal and distal end and sutured with 8-0 nylon. In both groups, scar was induced using the "mincing" method as described elsewhere (15). Briefly, the adductor muscle and a 5 mm strip of the anterior edge of the biceps femoris were removed and minced by scalpel in a petri dish containing saline solution. After mincing, the muscle pieces were returned to the peripheral nerve and were spread along 1.5 cm of the nerve length (15). Extra care was taken to protect the nerve ends. Muscle fascia and skin were closed with nylon 4-0 sutures.

Post-surgical care was provided with administration of cefazolin (30 mg, subcutaneous injection) and normal saline (5 ml, IP injection, Sodium Chloride 0.9%) to prevent infection and dehydration. A daily application of anti-bite (Nail bite, J. Pickles and Sons, U.K) was performed over the operated limb to prevent autotomy.

In stage II, after 4 weeks, double-strand median nerve graft was used to bridge the sciatic nerve gap as described by Nabian et al. (16). Each animal was re-anesthetized and 20 mm long segments of the median nerves were harvested from both hands. The segments were preserved in sterile normal saline and the surgical site was closed with 4-0 nylon sutures. In group 1, the previous surgical site of the leg was re-opened. A sheath was formed around the silicone tube. To place the two layer graft of the median nerve through the sheath tunnel, the sutures between the nerve ends and the silicone tube were removed and one end of the double-strand nerve graft was attached to one end of the silicone tube. With gentle traction, the silicone tube was taken out of the newly formed tunnel and the graft was placed in position. Then, the nerve graft was detached from the silicone tube and the tube was discarded. The grafting procedure was completed with suturing the double-strand median graft to the proximal and distal ends of the sciatic nerve with 10-0 nylon. In group 2, silicone caps were removed from the proximal and distal ends. The double-strand median nerve graft was interposed and sutured to the proximal and distal sciatic nerve ends with 10-0 nylon. In both groups, muscles and skin were closed with nylon 4-0 sutures and post-surgical care was taken as described before.

Behavioral assessments

Extensor Postural Thrust (EPT)

At the end of week 15, the extensor muscle force was measured as described by Thalhammer et al. (17). The rat was held upright while the entire body except the hind limb was wrapped in a surgical towel. Lowering the animal toward the surface induced foot extension. The force of extension to the platform of a digital balance was measured in grams; reduction in the extensor muscle force was indicative of a deficit in the motor function (17). The percentage of this deficit between the normal and experimental leg was calculated with the following formula: (17,18)

$$Motor\ deficit\ (\%) = \frac{\text{Normal EPT} - \text{Experimental EPT}}{\text{Normal EPT}} \times 100$$

Withdrawal reflex latency (WRL)

Leg-withdrawal latency was measured at the end of week 15 with the method described by Masters et al. (19). Briefly, the rat was restrained and the hind paw was placed on a 56°C plate. The WRL was measured as the required time for the rat to withdraw the paw. An increase in the withdrawal time was considered as an indicator of reduction in nociceptive function. For animal safety, the paw was removed from the hotplate if there was no reflex after 12 seconds (to prevent tissue damage) and the maximal WRL of 12 seconds was assigned to the animal.

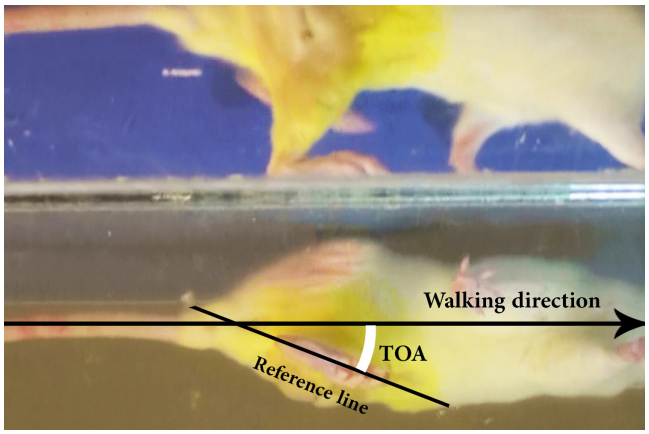

Figure 1. Toe out angle analysis on a mid-stance frame of the right foot. The Toe out angle is the angle between the direction of the walking and the reference line from the calcaneus to the tip of the third digit.

Toe out angle (TOA)

At the 3, 6 and 15-week time-points, the TOA was measured to evaluate the overall function of the hind limb. Varejão et al. defined the TOA as the degree of the foot rotation during normal walking (20). The test was performed on a transparent Perspex walking track that was 1 meter in length and 15 cm in width. A flat mirror was placed beneath the pathway with 45° angle to reflect the view of the plantar surface of the rat's hind paw. A digital high-speed camera (Sony, HDR-SR12 high definition, Japan) was used to record the walking process. Mid-stance frames of the right foot (just before the swinging left leg passes the right leg) were analyzed for TOA. The TOA was defined as the angle (degree) between the direction of walking and a reference line on the foot (the line from the calcaneus to the tip of the third digit) [Figure 1].

Histological assessments

At the end of week 15, all animals were sacrificed with intracardiac perfusion by 4% paraformaldehyde in 0.1M phosphate buffer (PH= 7.4). Anesthesia was induced with ketamine (100mg/kg, IP, ketamine hydrochloride, Rotexmedica, Germany).

The entire sciatic nerve and surrounding tissue were removed en bloc and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and cross-sectioned at 6 µm. Sections were stained with Masson Trichrome and photographed under a light microscope. Image-J software was used to measure the vascular and scar indexes (21). The vascular index was calculated by dividing the cross-sectional area of the vessels by the nerve area (22). The scar index was calculated by dividing the cross-sectional area of the scar tissue (epineural area) by the whole area of the nerve (epineurium and nerve) (23).

Statistical analysis

Behavioral and histological data was analyzed with the student t-test and ANOVA using SPSS version 19 (Armonk, NY: IBM Corp). A *P*-value <0.05 was considered statistically significant.

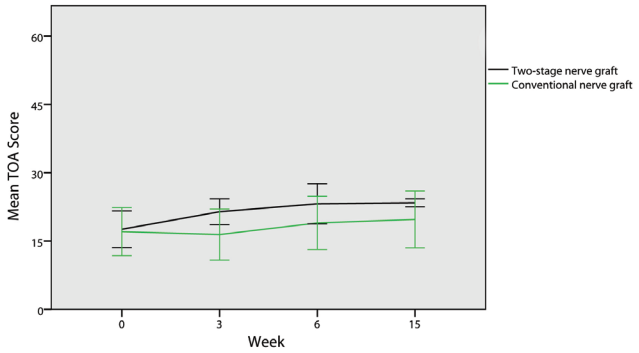

Figure 2. Toe out angle at the 3, 6 and 15-week time points. The time-course Toe out angle assessment showed no significant difference between the two groups.

Results

Extensor Postural Thrust (EPT)

After 15 weeks, group 1 showed lower deficiency compared with group 2 (43.8±12.9 vs. 57.3±10.4). In other words, the extensor force was significantly higher in group 1 (t-test, *P*=0.020).

Withdrawal reflex latency (WRL)

The mean WRL at the end of week 15 was 2.18 (±1.1) seconds in group 1 and 5.24 (±3.4) seconds in group 2. Reflex latency was significantly lower in the first group (t-test, *P*=0.017).

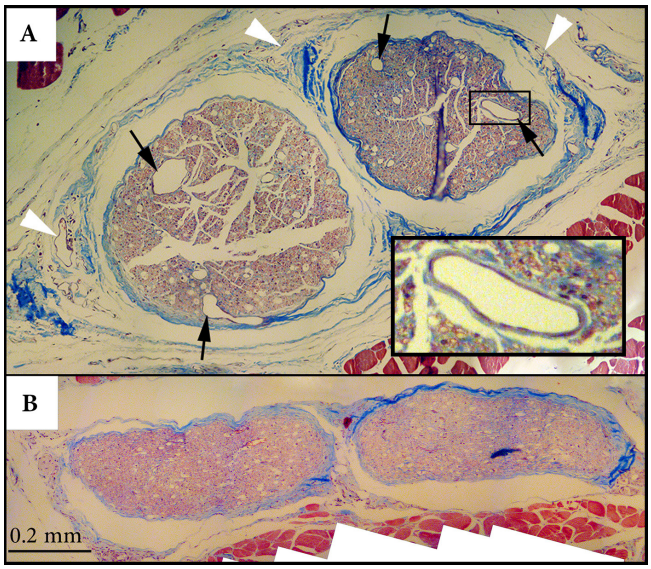

Figure 3. Masson Trichrome-stained slides. A) Increased vascularity of the endoneurial (black arrows) and epineurial (white arrow heads) compartments in the two-stage nerve graft technique. B) A nerve treated with conventional nerve graft. Original magnification X 200.

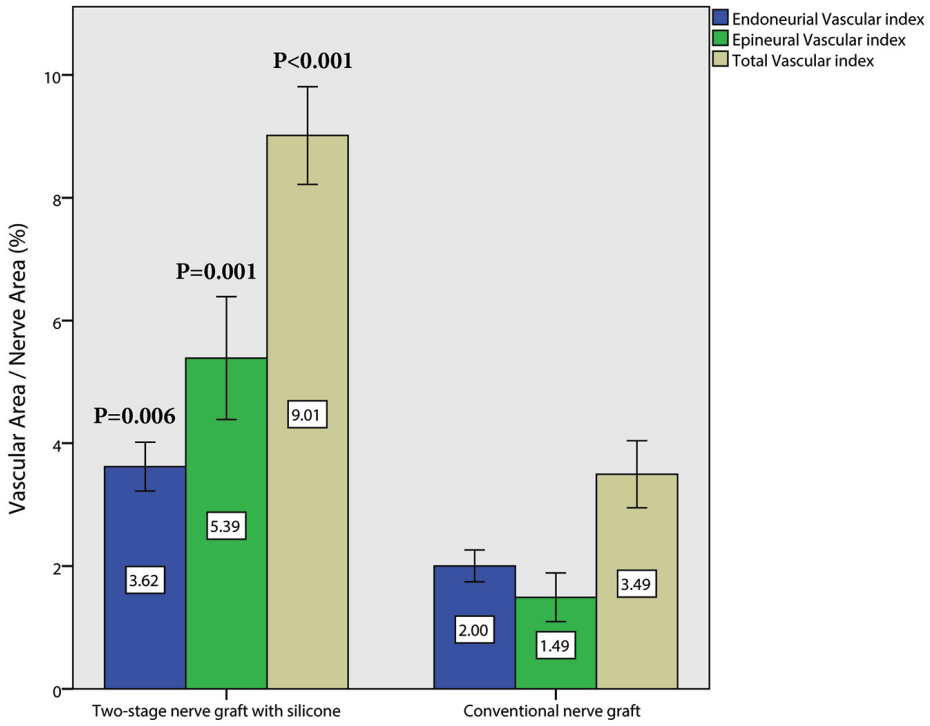

Figure 4. Vascular index. Endoneurial, epineurial and total vascular indexes were significantly higher in the two-stage nerve graft silicone group.

Toe out angle (TOA)

At the 3, 6 and 15-week time points the TOA showed no significant difference between the two groups (ANOVA, *P*>0.05). The detailed time course of the TOA assessment is shown in Figure 2.

Histological assessments

Masson Trichrome-stained slides at week 15 showed an increased vascularization in group 1 [Figure 3]. The mean endoneurial, epineurial, and total vascular indexes were significantly higher in group 1 (t-test, *P*<0.001). The mean total vascular index was 9.01% (±1.59%) in group 1 and 3.49% (±1.54%) in group 2. The details of the vascular index is summarized in Figure 4.

In the scar assessments, the normal leg served as the control for the experimental leg. The mean scar index was 0.33±0.03 in the two-stage nerve graft group, 0.34±0.09 in the conventional grafting group, and 0.08±0.01 in the control group (normal leg). Scar formation in the experimental groups was significantly higher than the control group (ANOVA, *P*=0.001), confirming the quality of scar induction.

Discussion

In this study, we aimed to compare a new technique with conventional secondary repair. We proposed a two-stage nerve graft with silicone tube interposition at the first stage to reduce the negative effects of severe scarring. According to our experiment, the two-stage nerve graft technique was superior in some aspects compared with conventional grafting in a secondary repair.

Withdrawal reflex latency as a sensory test and EPT as an assessment of motor recovery showed advantages of the two-stage technique. Normal withdrawal time in other studies is 4.3 seconds or less (18). This time was 5.24 seconds for the conventional group in our study, which means the mean reflex time of the conventional group was impaired. The relevance of the EPT for measuring the magnitude of the motor function was previously demonstrated (17,18). In our study the EPT showed a better recovery in the two-stage group; however, another functional test (TOA) showed no significant difference between the groups.

Histological analysis confirmed better vascularization of the two-stage technique. Higher vascularity of the sheath forming around the silicone tube was previously reported in the studies of two-stage tendon graft (13). Afterwards, Lundborg and Mackinnon in separate studies attempted to utilize this vascularized pseudosheath as a nerve conduit (24–28). To produce a vascularized pseudosheath, Lundburg introduced a silicone rod surrounded by a thin stainless steel spiral subcutaneously in the back of the rats (24). After three weeks, a vascularized pseudosheath with spiral metal framework was used as a nerve conduit. Mackinnon, in a similar procedure, evaluated this sheath in a primate model (27). Although the primary results were encouraging, the idea of using pseudosheath in nerve regeneration studies was suspended - perhaps because the use of this sheath as a conduit had no advantage over

other gradually introduced biodegradable conduits such as polyglycolic acid (PGA), caprolactone, collagen, etc. Also, using the vascularized sheath as a conduit needed an unnecessary second surgery. Thus, we propose to use this vascularized sheath as a bed for a nerve graft in severe trauma where the primary repair by graft is unsuccessful or impossible - not as a nerve conduit.

A suitable scar induction was essential for this study. There are different chemical (tetracycline, silver nitrate, etc.) and physical (crushing, abrading, lacerating, mincing, etc.) scarring models in rats, but studies on comparison of such scarring models are not sufficient to demonstrate a standard for experimental scar induction (15). Scar induction quality in our study was confirmed with the scar index.

The 4-week interval between the two stages was decided based on previous studies (29–32). Selecting the optimal time is important not only to preserve the capacity of nerve regeneration, but also to have a mature vascularized sheath. According to previous studies, the capacity of nerve repair in the rat models starts to decrease after 1-2 month of denervation (29-31). On the other hand, the time for maturation of the sheath around the silicone is 3-4 weeks in the rat (32). Thus, we selected the 4-week interval to have a mature sheath and to save the optimal capacity of the nerve for regeneration.

This study had some limitations. In our study, it was not possible to calculate the SFI (Sciatic Functional Index) due to automutilation. Thus, we used TOA for functional analysis, which has good correlation with SFI and is less affected by automutilation (20). In this study, we did not use electrophysiology and axon counting that can be considered in future studies.

In conclusion, two-stage nerve grafting with a silicone tube could bypass the acute inflammatory phase of severe trauma and reduce the negative effects of excessive scarring (compression, adhesion and low vascularity).

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