A biological tube technique for the repair of peripheral nerve defects using ‘stuffed nerves’

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ABSTRACT

BACKGROUND: Presently described is research examining the “stuffed nerve” technique to repair peripheral nerve defects.

METHODS: Twenty-one male Wistar Albino rats were divided into 3 groups of 7, and standard 10-mm defects were created in the sciatic nerve of all subjects. Rats were treated with autogenous nerve graft (Group 1), hollow vein graft (Group 2), or vein graft stuffed with shredded nerves (Group 3). After 12 weeks, electrophysiological and histomorphological analyses were performed to evaluate axonal regeneration.

RESULTS: Rat groups were compared in terms of latency period and peak-to-peak potential. Latency period was significantly shorter and peak-to-peak potential was significantly greater in Group 1 than in Group 2. However, latency period and peak-to-peak potential did not differ significantly between Groups 1 and 3 or between Groups 2 and 3. To evaluate axonal regeneration, number of axons, axon diameter and myelin sheath thickness was compared between groups. Results indicated that axonal regeneration was similar in Groups 1 and 3, and was better than results seen in Group 2.

CONCLUSION: The stuffed nerve technique is an alternative to autogenous nerve grafting and produces similar electrophysiological and histomorphological properties.

Keywords: Biological tube; peripheral nerve defect; stuffed nerve; vein graft.
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tissue\textsuperscript{[18,19]} to prevent graft collapse, and this combined technique is effective for defects of less than 3 cm.

In the present study, a biological tube was constructed and filled with nerve particles (stuffed nerve). It demonstrated superior peripheral nerve repair to that seen following routine autogenous nerve graft. The stuffed nerve technique utilizes microenvironment of vein graft and neurotropic factors from stuffed nerve particles.

**MATERIALS AND METHODS**

This study was approved by the animal ethics committee of Dokuz Eylül University. Twenty-one male Wistar Albino rats weighing approximately 300 g each were randomly divided into 3 equal groups. Surgery was performed on the lower extremities of right side in test animals and on left side in control animals. Rats were housed in cages (7 rats per cage) with water and rat chow ad libitum at room temperature with 12-hour light/12-hour dark cycle. Nerve graft, vein graft, and stuffed nerve technique was performed on 7 rats of Groups 1, 2, and 3, respectively.

**Surgical Procedures**

All surgical procedures were performed by the same investigator using microsurgical techniques in sterile conditions with a surgical microscope (Zeiss S3; Carl Zeiss AG, Oberkochen, Germany). Anesthesia of 10 mg/kg xylazine (2% alfazine, 20 mg/mL, Rompun; Bayer AG, Leverkusen, Germany; Bayer) and 100 mg/kg ketamine (Ketalar, 50 mg/mL; Pfizer, Inc., NY, NY, USA) was administered intraperitoneally. Oblique skin incision of approximately 3 cm in length was made from the right gluteal region to the posterior thigh in all test subjects, and sciatic nerve was exposed with blunt dissection of the superficial gluteal and biceps femoris muscles and surrounding fascia junction line. From the sciatic foramen to point left of the tibial and common peroneal branches, sciatic nerve was separated and isolated from surrounding tissues (Fig. 1a). Seven mm of sciatic nerve from the proximal sciatic foramen to the point of separation from tibial and common peroneal branches was protected. Ten-mm sections from elsewhere on sciatic nerve were removed with microscissors. All anastomoses were performed using 10/0 ETHILON sutures (Ethicon, Inc., Somerville, NJ, USA). Nerve and vein grafting was performed using epineural technique with 6 stitches for every 60° angle.

**Group 1:** In nerve graft group, standard 10-mm nerve defects were created in the subjects and excised 10-mm segment was reversed and reapplied to the same site.

**Group 2:** Animals of the vein graft group received 10-mm defect in sciatic nerve and vein graft from jugularis externa was used to repair defect.

**Group 3:** To prepare stuffed nerves, jugular vein graft of 1 cm in length was harvested from left side of the cervical region and rinsed with saline solution (Fig. 1b). Sciatic nerve graft was cut into at least 10 parts of 1 cm each using microscissors (Fig. 1c). Initially, one end of vein graft was attached to proximal end of sciatic nerve using 10/0 ETHILON sutures (Fig. 1d) and minced nerve graft was then equally distributed in the lumen of vein graft (Fig. 1e). Free end of vein graft was joined to distal end of sciatic nerve with 10/0 ETHILON sutures (Fig. 1f). All muscle and skin incisions were sutured using 4/0 absorbable sutures (Dogsan Tibbi Malzeme San A.S., Trabzon, Turkey). All rats were observed for 12 weeks in standard cages under the conditions described earlier and then underwent further surgery.

![Figure 1. Preparation of a stuffed nerve. (a) exposure of the sciatic nerve, (b) replacement of 10-mm section from the sciatic nerve with vein graft, (c) rendering of excised nerve section, (d) proximal coaptation of vein graft and stuffing of the nerve particles from the distal end, (e) appearance of the nerve-stuffed vein graft segment, and (f) appearance of the nerve-stuffed vein graft after distal coaptation.](image-url)
to visualize the nerves. Electrophysiological function was determined using electroneuromyography (ENMG), and histological analyses were performed using light and electron microscopy after sacrifice.

**Electrophysiological Examination**

To evaluate regeneration of sciatic nerve, impulses were sent from proximal end of the nerve, and electrophysiological response of gastrocnemius muscle was recorded using ENMG. Stimuli were applied directly to the nerve for 0.1 milliseconds to avoid nonselective stimulation. Both control and treated sciatic nerves were dissected from the sciatic notch to the sciatic trifurcation using the previous incision, and impulse electrodes were positioned approximately 5-mm proximal to coaptation area of the exposed sciatic nerve, and recording electrodes were positioned on the insertion and on middle part of the gastrocnemius muscle after passing through the skin. Impulse intensity that provided maximum muscle response was determined for each animal using gradual increase from minimum stimulus amplitude of 0.3 V. Impulses were repeated at least 3 times at optimal intensity, and muscle responses were recorded. Responses of both extremities were graphed and average values were calculated using 3 action potential curves for statistical comparison using 4-channel polygraph system (BIOPAC MP35; BIOPAC Systems, Inc., Goleta, CA, USA) and computer software (BIOPAC BSL Pro, version 3.7; BIOPAC Systems, Inc., Goleta, CA, USA).

**Histological Assessment of Regenerated Nerves**

After electrophysiological measurements were recorded, 5-mm sciatic nerve biopsy samples were taken from distal coaptation line and middle of the graft from all animals. Rats were then euthanized and nerve tissue samples were incubated in Karnovsky fixative (pH 7.4) at 4°C for 48 hours. After routine electronmicroscopic procedures, tissue samples were embedded in araldite-epon mixture for polymerization, and 1-mm semi-thin sections were taken using ultramicrotome (Leica Ultracut R; Leica Microsystems GmbH, Wetzlar, Germany) and were stained with toluidine blue. Sections were placed on a grid and contrasted using uranyl acetate and lead nitrate. Ultrastructures were evaluated using Zeiss Libra 100 EFTEM (Carl Zeiss AG, Oberkochen, Germany) transmission electron microscope and images were digitally photographed. Axon diameter, myelin thickness, myelin lamellae arrangement and morphological characteristics of Schwann cells and connective tissue were analyzed. Histomorphometric analyses were performed using ImageTool version 3.0 (University of Texas Health Science Center at San Antonio, San Antonio, TX, USA) software and images were obtained using an Olympus DP 41 digital camera mounted on an Olympus CX-41 (Olympus, Corp., Tokyo, Japan) light microscope. Biopsy samples were imaged at ×4, ×10, ×20, and ×40 magnification and digital images were acquired in JPEG format with maximum resolution. Three distinct 5000-μm² areas were examined using ImageTool program at a magnification of ×40. Similarly, axon count was determined at 3 separate 5000-μm² areas on each animal. Subsequently, 10 myelinated axons were analyzed in 5000-μm² areas, diameter of myelinated axons and myelin thickness were determined at 3 different locations, and mean and standard error were calculated.

**Statistical Analysis**

All statistical analyses were performed using SPSS version 15.0 (IBM Corp., Armonk, NY, USA) statistical analysis program. Nonparametric differences were identified using Kruskal-Wallis and Mann-Whitney U tests and were considered significant when p<0.05.

**RESULTS**

All rats remained healthy throughout the study and no auto-mutilation or foot ulceration was observed.

**Electrophysiological Assessments**

After ENMG recordings, nerve conduction velocity (latency) values were determined by measuring time between stimuli and response. Peak-to-peak potentials were calculated from amplitude difference between positive and negative peak. Both extremities of all rats were measured at least 3 times and mean latency and peak-to-peak value of right (test group) and left (control group) sides were compared (Table 1).

Peak-to-peak and average latency values differed between test and control sides (p<0.05) and indicated that present repair procedure produced superior results. Moreover, ratio of average latency and peak-to-peak value test (right side) and control (left side) groups [\((\text{right}/\text{left}) \times 100\)] significantly differed between Groups 1 and 2 (p<0.05; Fig. 2).

**Table 1.** Electrophysiological analysis

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>1.48±0.02</td>
<td>2.15±0.09</td>
</tr>
<tr>
<td>Peak to peak (mV)</td>
<td>4.25±0.49</td>
<td>2.28±0.24</td>
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</table>
Histomorphological Assessments

**Group 1 (nerve graft group):** Unmyelinated axons were infrequently observed in sections from grafts and distal parts of grafts. Although nearly normal number and diameter of myelinated axons were observed and were well organized in graft sections, myelinated axons and some degenerated axons were found in distal sections. Similarly, electron microscopy analyses revealed that myelinated axons and myelin sheath of nerve fibers generally maintained normal structure, and Schwann cells were wrapped around myelinated and unmyelinated nerve fibers (Fig. 3a, b).

**Group 2 (vein graft group):** In sections of mid portions and distal parts of vein grafts, irregular organized myelinated axons and demyelinated axons were observed in some areas, with increased peripheral vascularity. However, small number of myelinated axons was found in sections of distal vein grafts. Moreover, diameter of myelinated axons and myelin thickness was decreased, and fibrosis and increased vascularity were observed in some areas. In addition, electron microscope analyses indicated that myelin sheaths were invaginated toward the inside of axons in significant proportion of myelinated nerve fibers, and local myelin sheath lamellae separation was evident (Fig. 3c, d).

**Group 3 (stuffed nerve group):** Examination of sections from proximal and distal parts of grafts revealed multiple regularly organized myelinated axons in sections of the graft. Moreover, myelin thickness and axon diameter were sufficient in distal sections of grafts and even greater in some areas. Electron microscope images of Groups 1 and 3 were similar (Fig. 3e, f).

Histomorphometric Analyses

Light microscopy images at ×40 magnification were analyzed using ImageTool program, and the number and diameter of axons (µm), and myelin sheath thickness (µm) were measured in cross sections from distal and middle specimens (Table 2).

Axon count was estimated in 3 different 5000-µm² central and distal coaptation areas of grafts in animals from treatment Groups 1, 2, and 3. Number of axons significantly differed between Groups 1 and 2 and between Groups 2 and 3 (p<0.05; Fig. 4).

In addition, 10 myelinated axons were selected from central areas of graft and distal sections of coaptation areas in all animals using ×40 magnification ImageTool program, selected axon diameter (µm) and myelin thickness (µm) were measured in 3 different areas for each axon and mean axon diameter was calculated for each treatment group. Mean axon diameter differed significantly between Groups 1 and 2 and between Groups 2 and 3 (p<0.05; Fig. 5).

Myelin thickness differed significantly between Groups 1 and 2 and between Groups 2 and 3 (p<0.05; Fig. 6).

**DISCUSSION**

Autogenous nerve grafting is currently ideal method for re-

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**Table 2.** Histomorphometric analysis

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td></td>
<td>Middle</td>
<td>Distal</td>
<td>Middle</td>
</tr>
<tr>
<td>Number of axons</td>
<td>97.4±6.3</td>
<td>101.6±6.6</td>
<td>62.1±4.4</td>
</tr>
<tr>
<td>Diameter of axons (µm)</td>
<td>7.20±0.7</td>
<td>7.44±0.4</td>
<td>5.44±0.1</td>
</tr>
<tr>
<td>Myelin sheath thickness (µm)</td>
<td>1.36±0.1</td>
<td>1.37±0.1</td>
<td>0.85±0.01</td>
</tr>
</tbody>
</table>
pairing peripheral nerve defects. However, nerve grafting from donor site requires anesthesia and may lead to paresthesia, neuroma formation, pain, and additional scarring. Moreover, long surgery duration and mismatches of diameter may occur. Thus, development of simpler and more functional procedures that reduce morbidity are eagerly awaited. Recent studies have examined various conduits using biological fillings and synthetic tubes to repair nerve defects. In particular, biological and synthetic conduits with similar qualities to autogenous nerve grafts reportedly provide suitable microenvironments for axonal regeneration. Vein grafts are the most frequently used biological conduits, and autogenous tissue grafts do not need to be removed from the surgical field after nerve repair. Furthermore, all 3 layers of vein graft are rich in laminin and share similarities with the basal lamina that surrounds normal and traumatized nerve fibers. Laminin plays role in the adhesion, multiplication, and differentiation of nerve cells. The most commonly ob-

Figure 3. Electron and light microscopy analyses. (a) and (b), Group 1; (c) and (d), Group 2; (e) and (f), Group 3. Sections (a), (c), and (e) were obtained from middle part of the nerve, whereas sections (b), (d), and (f) were obtained from distal part of the nerve. All images are of semi-thin sections stained with toluidine blue (×40 magnification) and small frames represent electron microscopic micrographs stained with lead citrate-uranyl acetate; (a-c) and (d), ×10000; (e) and (f) sections, ×8000. Myelinated nerve axons (a), myelin sheath (Msh) and Schwann cells (Sh) were observed. Changes in myelinated nerve fibers are indicated with a star (★). Myelin sheath lamellae degeneration is visible in (c) and (d).
The served disadvantage of vein grafts is fibrotic contraction and associated risks of curling and collapse.\textsuperscript{4,16,27} Hence, the use of vein graft with other materials that benefit axonal growth will likely produce significant improvements in outcomes.

To reduce complications of vein grafts, Keskin et al. enhanced graft microenvironment by forming biological conduit with nerve graft inside vein graft, and functional, electrophysiological and histomorphometric evaluations revealed no significant differences with nerve grafts.\textsuperscript{17} Alternatively, Sahin et al. created 1-cm defect on rat’s tibial nerve and repaired it with minced nerve tissue in vein graft\textsuperscript{23} and then placed 1 of the 3 equal-sized pieces of nerve graft into vein graft. Although they did not report details of their mincing process, subsequent outcomes were similar to those achieved with autogenous nerve grafts. Similarly, we divided nerve graft into as many small pieces as possible prior to placement in vein graft, which was then tightly stuffed into the defect area (Fig. 1f). In subsequent analyses, no fibrosis was observed, and axonal regeneration was almost identical to that following autogenous

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Statistical analyses of axon count in central graft areas and distal coaptation sections of grafts.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Comparison of axon diameter from central area of graft and distal coaptation area between animals of Groups 1, 2, and 3.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Comparison of mean myelin thickness from central area of graft and distal coaptation area from animals in Groups 1, 2, and 3.}
\end{figure}
nerve grafts. In histomorphometric analyses, Sahin et al. only
determined myelinated axon counts, whereas we also deter-
mined myelin sheath thickness and axon diameter (Table 2).
However, further gait analyses are required to confirm the
efficacy of stuffed nerve grafts.

In a previous study, silicon conduits containing minced nerves
were placed into 12-mm defects in rat sciatic nerve. [28] How-
ever, no significant difference in motor or sensory variables
was observed between animals with differing nerve frag-
ments, potentially reflecting use of non-biological synthetic
conduit that lacks the selective permeability and flexibility of
vein graft. In addition, those investigators did not perform
electrophysiological assessments to confirm histological and
morphometric observations.

In another study, Zhang created 4-cm defect in rabbit tibial
nerve and repaired it with autologous vein graft containing
transplanted Schwann cells or with conventional vein graft.
Subsequent comparisons revealed superior axon regenera-
tion in veins with transplanted Schwann cells. [29] However,
Schwann cells are difficult to isolate and are expensive, mak-
ing the use of minced nerve parts as shown in the present
study a practical option.

Although numerous biological and synthetic nerve conduits
continue to be investigated, none have clinical advantages
over conventional nerve grafts. However, present vein grafts
are biological tubes, and suitable microenvironment was pro-
duced by stuffing shredded nerves into the graft, providing
neurotrophic and neurotropic factors for axon regeneration.
Subsequent ENMG analyses showed shorter latency in nerve
graft group (Group 1) than in vein graft group (Group 2), sug-
gesting more rapid transmission of growth signals in the pres-
ence of nerve tissue. Peak-to-peak potential was significantly
higher in nerve graft group than in vein graft group, reflect-
ing more efficient axonal progress. However, latencies and
peak-to-peak potentials did not significantly differ between
Groups 1 and 3, indicating similar properties of autogenous
and stuffed nerve grafts.

The concept of regeneration unit is that axonal sprouting
occurs, involving movement of myelinated axons toward the
endoneurial tube. Numerous axons have been observed pre-
viously in histological analyses of nerve repair areas, although
these axons were not fully functional until they gained access
to appropriate receptors. [28] Functional recovery has been ob-
served following the access of sufficient numbers of axons to
correct sensory/motor destinations. [31] However, number of
axons may be excessive in experimental models, and inappro-
priate axons that cannot locate appropriate end-organs are
removed from fascicles and eliminated by atrophy over some
years. In a rat model of nerve repair, distal axon numbers
were significantly increased in the first few months and were
present in numbers twice normal at 3 months and 2 years. [32]
Axon diameter is dependent on the source and maturity
of the nerve, and degree of myelination has been associated
with axon maturity. [33] Because myelination occurs before
contact of axons with target organs, it may not be associ-
ated with function. However, rate of axon myelination may
indicate progress of regeneration. [34,35]

Accordingly, number and diameter of myelinated axons as well
as myelin sheath thickness were determined in the present
histomorphological analyses, and there were significant differ-
ences between Groups 1 and 2 and between Groups 2 and 3
(p<0.05). Number, diameter and myelin thickness of axons in
Group 2 were smaller than in the other groups, and similari-
ties between Groups 1 and 3 indicated equivalent regenera-
tion following nerve and stuffed nerve grafts (Figs. 4, 5 and 6).

In summary, the effects of autogenous nerve, conventional
vein, and stuffed nerve grafts on axonal regeneration were
compared using series of electrophysiological and histomor-
phological analyses. These experiments demonstrated that
autogenous nerve and stuffed nerve grafts were more suc-
cessful than conventional vein grafts.

**Conclusion**

In the present study, repair of sciatic nerve defects using
stuffed nerve tissue was superior to conventional vein graft
technique. The present technique offers advantages of avoid-
ing collapse often seen in vein grafts to repair defects of >3
cm. Defect size and nerve diameter were limited to rat sci-
atic nerves in the present study, precluding comparisons with
previous studies. Nonetheless, the present data indicate that
stuffed nerve grafts offer convenient alternative to nerve
grafts, which have disadvantage of donor site morbidity. Ad-
ditional basic and clinical studies of stuffed nerve graft appli-
cations are warranted.

**Conflict of interest:** None declared.

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AMAÇ: Periferik sinir defektlerinin onarımında ‘sinir dolması’ tekniği araştırılması.

DENYESEL ÇALIŞMA - ÖZET

Periferik sinir defektlerinin onarımında biyolojik tüp kullanımı ‘sinir dolması’