Comparison of Nerve, Vessel, and Cartilage Grafts in Promoting Peripheral Nerve Regeneration

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Abstract: Peripheral nerve injury primarily occurs due to trauma as well as factors such as tumors, inflammatory diseases, congenital deformities, infections, and surgical interventions. The surgical procedure to be performed as treatment depends on the etiology, type of injury, and the anatomic region. The goal of treatment is to minimize loss of function due to motor and sensory nerve loss at the distal part of the injury. Regardless of the cause of the injury, the abnormal nerve regeneration due to incomplete nerve regeneration, optimal treatment of peripheral nerve injuries should provide adequate coaptation of proximal and distal sides without tension, preserving the neurotrophic factors within the repair line. The gold standard for the treatment of nerve defects is the autograft; however, due to denervation of the donor site, scarring, and neuroma formation, many studies have aimed to develop simpler methods, better functional results, and less morbidity. In this study, a defect 1 cm in length was created on the sciatic nerve of rats. The rats were treated with the following procedures: group 1, autograft; group 2, allogeneic aorta graft; group 3, diced cartilage graft in allogeneic aorta graft; and group 4, tubularized cartilage graft in allogeneic aorta graft. Group 5 was the control group. The effects of cartilage tissue in nerve regeneration were evaluated by functional and histomorphological methods.

Group 1, for which the repair was performed with an autograft, was evaluated to be the most similar to the control group. There was not a statistically significant difference in myelination and Schwann cell rates between group 2, in which an allogeneic aorta graft was used, and group 3, in which diced cartilage in an allogeneic aorta graft was used. In group 4, myelination and Schwann cell formation were observed; however, they were scattered and irregular, likely due to increased fibrosis.

In all of the groups, nerve regeneration at various rates was observed both functionally and histomorphologically. This study demonstrates that cartilage tissue has promoting effects in nerve regeneration.

Key Words: peripheral nerve regeneration, cartilage graft, nerve graft, vessel graft, myelination

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Peripheral nerve injuries primarily occur due to trauma as well as factors such as tumors, inflammatory diseases, congenital deformities, infections, and surgical interventions. The surgical procedure chosen for treatment depends on the etiology, type of injury, and the anatomic region. The aim of the treatment was to minimize a loss of function resulting from motor and sensory nerve loss at the distal site of injury. Because complete nerve regeneration does not occur regardless of the cause of the injury, abnormal nerve regeneration usually results in functional loss and pain.

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Tension and sutures at the repair site often have negative effects on nerve regeneration.[1,2] Excessive tension increases connective tissue proliferation, which leads to scar formation, and this may inhibit axonal regeneration.[3] A sufficient blood supply is one of the most important factors for nerve regeneration.[4] Other factors include the proper coaptation of nerve edges, preservation of neurotrophic factors within the repair site, minimal foreign body reaction, and fibrosis.[5] The gold standard for treating nerve defects due to injury is the use of an autologous nerve graft. However, a major disadvantage of nerve grafts is donor-site morbidity. Loss of sensation, loss of nerve function at the donor site, and painful neuroma may occur.[6,7] Additionally, the size of the defect may be a limiting factor for autografts. Changed fascicular architecture and axonal spreading in the graft during the repair may inhibit nerve regeneration.[7] Experimental studies have shown that use of various natural and synthetic tubal structures offers a proper microenvironment for axonal regeneration and advancement, similar to an autogenous nerve graft.[8,9]

In this study, a 1-cm lesion to a rat sciatic nerve was repaired with an autologous nerve graft, an allogeneic aorta graft only, a tubularized cartilage graft harvested from the ear of the rat, or diced cartilage wrapped in an allogeneic aorta. The extremity not affected by the lesion was used as an internal control for each animal. The results were evaluated based on functional, histomorphological, and electrophysiological properties.

MATERIALS AND METHODS

This experimental study was conducted at Inonu University, Experimental Animals Research Center, with the approval of the Inonu University Medical Faculty Experimental Animals Ethics Committee. Twenty-eight rats weighing from 200 to 250 g were involved in the study. They were divided into 4 groups with 7 rats randomly assigned to each. The left sciatic nerve was lesioned in all the groups and the right sciatic nerve remained intact and was used as the control. All the surgical procedures were performed by the same surgeon under an operation microscope. The rats were housed 3 or 4 to a cage under standard laboratory conditions. Rats were carefully observed during the preoperative and postoperative periods by expert veterinarians. The animals were supplied regularly with food and water.

SURGICAL TECHNIQUE

The left sides of the rats were shaven and cleaned with povidone-iodine followed by an oblique gluteal incision. The gluteal fascia and muscular structures were passed and the sciatic nerve was exposed. The defect was created by resecting a nerve segment 1 cm in length proximal to the bifurcation of peroneal and tibial branches. In group 1, the excised nerve segment was coapted to the defect as an autograft using 10/0 nylon sutures in an epineurial fashion. In group 2, the defect was repaired using an allogeneic aorta graft with 10/0 nylon sutures. In group 3, cartilage grafts harvested from the rat's ear were diced and placed in an allogeneic aorta graft, then coapted to the defect with 10/0 nylon sutures. In group 4, the autogenous cartilage graft harvested from the rat's ear was tubularized and the defect was repaired with 10/0 nylon sutures. Group 5 was the control group.

EVALUATION

For all groups, a walk test was applied 12 weeks after the left nerve surgery, followed by electromyographic analysis. All the rats were killed and samples from the left sciatic nerve were taken for histomorphologic evaluation.

Histomorphologic Evaluation

The tissue samples taken from the left sciatic nerves 12 weeks after the surgical process were fixed in 10% formalin for 24 hours for histopathological examination. After fixation and routine histologic tissue preparation procedures, the nerve tissue specimens were embedded in paraffin blocks. Sections of 6 μm thickness were prepared using a microtome and stained with hematoxylin and eosin. Images were captured using a Leica DFC 280 light microscope and analyzed using the Leica QWin Image Analysis System (Leica Microsystems Imaging Solutions, Cambridge, UK).

The 2-mm-long nerve tissue samples taken for electron microscopy were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in araldite blocks by dehydrating with acetone.

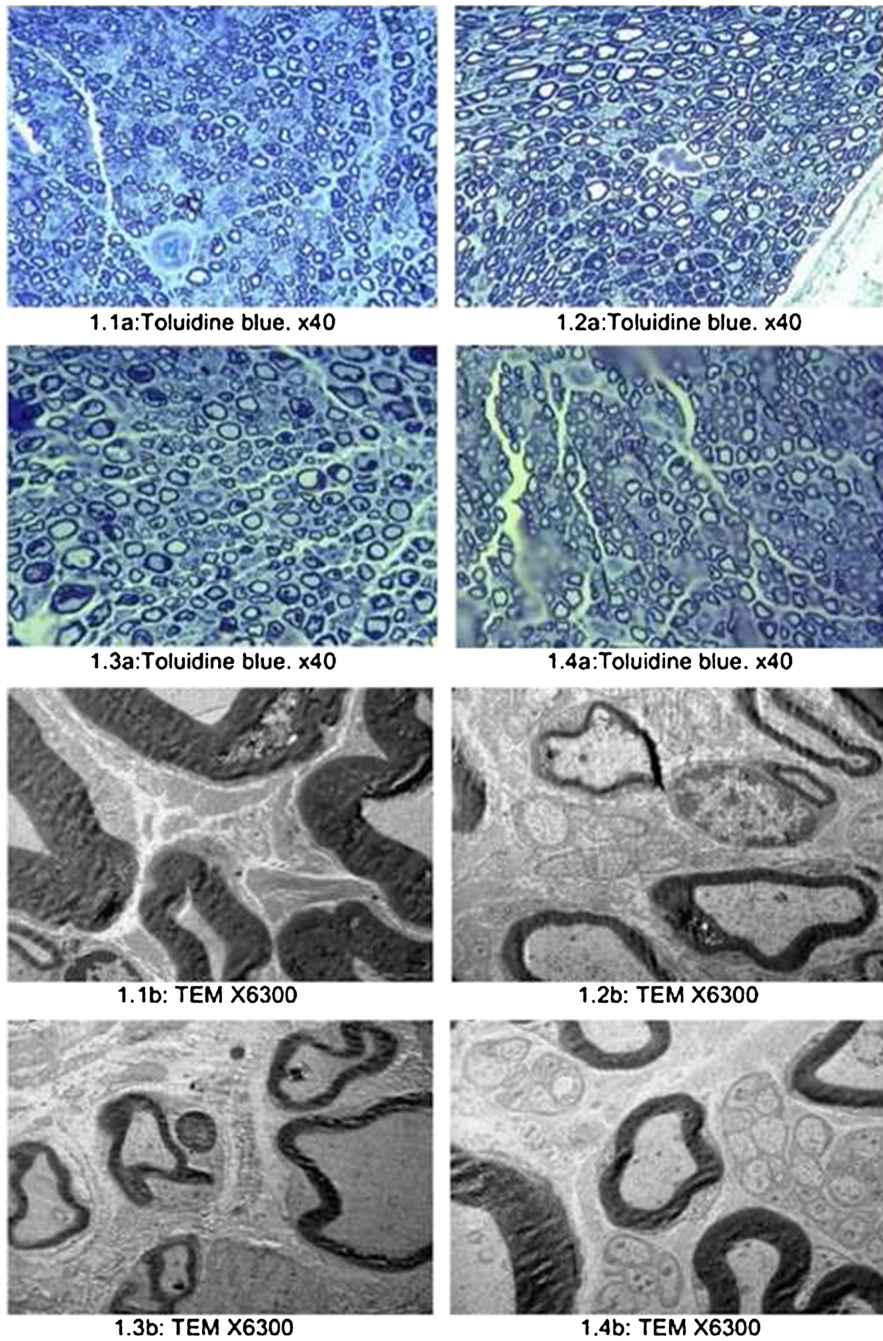

FIGURE 1. Histologic findings of proximal anastomosis. Light microscopy images: (1A) toluidine blue ×40, (2A) toluidine blue ×40, (3A) toluidine blue ×40, and (4A) toluidine blue ×40. Electron microscopy images: (1B) TEM ×6300, (2B) TEM ×6300, (3B) TEM ×6300, and (4B) TEM ×6300.

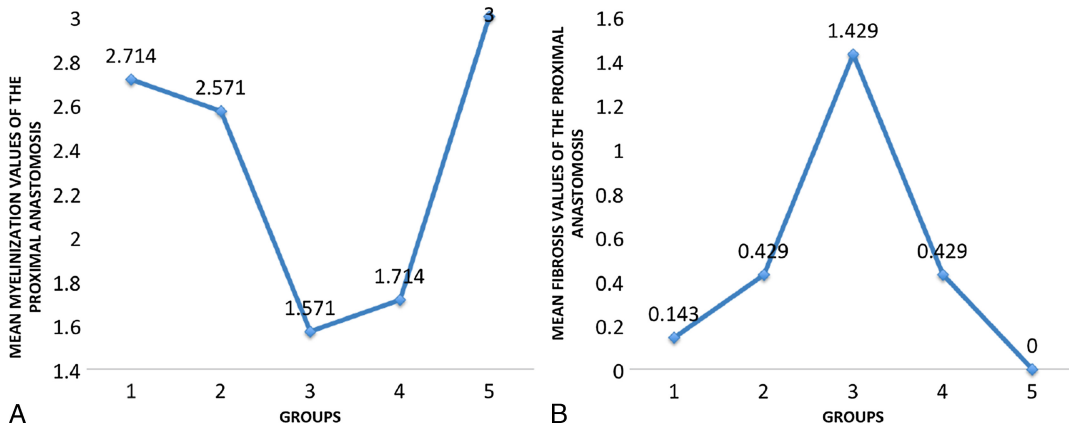

FIGURE 2. A, B, Mean myelination and fibrosis values of proximal anastomoses.

The semithin sections (1 μm thickness) taken from the araldite blocks using an ultramicrotome were placed on slides, and the thin sections (80 nm thickness) were placed on copper grids. The semithin sections were stained with toluidine blue, analyzed, and photographed with a Leica DFC 280 light microscope and the Leica QWin Image Analysis System. The thin sections were contrasted with uranyl acetate and lead citrate, then analyzed and photographed with Zeiss Libra 120 Transmission electron microscope (TEM).

The sections were scored in terms of myelination (no myelinated axons, 0; rare myelinated axons, 1; scattered, irregular, and thin myelinated axons, 2; dense, regular, and thick myelinated axons, 3), fibrosis (no fibrosis, 0; mild fibrosis, 1; moderate fibrosis, 2; and severe fibrosis, 3), edema (no edema, 0; mild edema, 1; moderate edema, 2; and severe edema, 2), and mast cell density (no mast cells, 0; 1–2 mast cells, 1; 3–4 mast cells, 2; 5 or more mast cells, 3; observed at ×40 magnification).

Statistical Methods

All the data obtained after the functional and histomorphological evaluation of the groups were exported to SPSS for Windows v.10.0 (Statistical Package for the Social Sciences) for statistical analysis. A general comparison of the differences between groups was performed using the Kruskal-Wallis test, a nonparametric test, and the double comparisons between groups were performed using the Conover test. In the analyses, $P < 0.05$ was accepted as statistically significant.

RESULTS

Walk test analysis: The sciatic function indexes (SFIs) between the groups were analyzed with the Kruskal-Wallis test. A statistically significant difference was detected between the first group, in which the autograft was used, and the other groups ($P < 0.05$).

Electromyographic evaluation: The median latencies between the groups were analyzed with the Kruskal-Wallis test, and a statistically significant difference was detected ($P < 0.0001$). The other experimental groups were analyzed with the Conover test ($P < 0.05$). Group 1 was the most similar to the control group, although a significant difference between groups was not detected.

The Histologic Findings of Proximal Anastomosis

Group 1: In the nerve sections stained with toluidine blue, irregularity, undulation, differences in thicknesses, as well as an increase in the number of the axons with thin myelin sheaths were observed (Fig. 1.1A). Examination of the sections under an electron microscope revealed local divergences of layers into thick myelin sheaths. Nonmyelinated axons wrapped in Schwann cell sheaths were present among the myelinated axons. The Schwann cells and endoneurium were assessed to have normal ultrastructural formation (see Fig. 1.1B).

Group 2: In the sections stained with toluidine blue, myelin sheaths of medium thickness were dominant. Some irregularity and undulation were detected in the myelin sheaths (see Fig. 1.2A). Examination of the sections under an electron microscope revealed axons with thin and thick myelin sheaths. In the myelin sheaths, occasional irregularities and periaxonal vacuolizations were observed. In the endoneurial tissue between the axons, a minimal level of fibrosis was detected. Nonmyelinated axons were also observed (see Fig. 1.2B).

Group 3: In the sections stained with toluidine blue, primarily medium-sized axons with thin, irregular myelin sheaths were observed (see Fig. 1.3A). Electron microscopy revealed axons wrapped in myelin sheaths of various sizes and thicknesses. For some axons, various sized vacuoles were noted in the space between the axolemma and the myelin sheath. Moderate edematous regions were present in the endoneurium (see Fig. 1.3B).

Group 4: In the sections stained with toluidine blue, medium-sized myelinated axons predominated, mostly wrapped in regular myelin sheaths (see Fig. 1.4A). In some myelinated axons, vacuoles were noted in the space between the axolemma and the myelin sheath. Myelin-free axons were present in addition to myelinated axon sections (see Fig. 1.4B).

Statistical Findings of Proximal Anastomosis

The median proximal myelination values between the groups were evaluated by the Kruskal-Wallis test, and a statistically significant difference was detected ($P = 0.0001$). However, there was no statistically significant difference between group 1, group 2, and group 5, or between group 3 and group 4 using the Conover test analysis ($P < 0.05$). The Kruskal-Wallis test analysis revealed a statistically significant difference in the median values of proximal anastomosis fibrosis ($P = 0.0011$). However, the Conover test analysis showed no statistically significant differences between group 1, group 2, group 4, and group 5 ($P < 0.05$) (see Fig. 2A,B).

Histologic Findings of the Graft

Group 1: In the sections stained with toluidine blue, irregularity, undulation, differences in the thickness of myelin sheaths, and small axons with thin myelin sheaths were observed most of the time (see Fig. 3.1A). Electron microscopy revealed a decrease in the density of myelinated axons and a thinning of the myelin sheaths. Additionally, periaxonal vacuolization was present in some of the myelinated axons. Moderate fibrotic changes were detected in the connective tissue between the axons. Many nonmyelinated axons were also observed in the sections (see Fig. 3.1B).

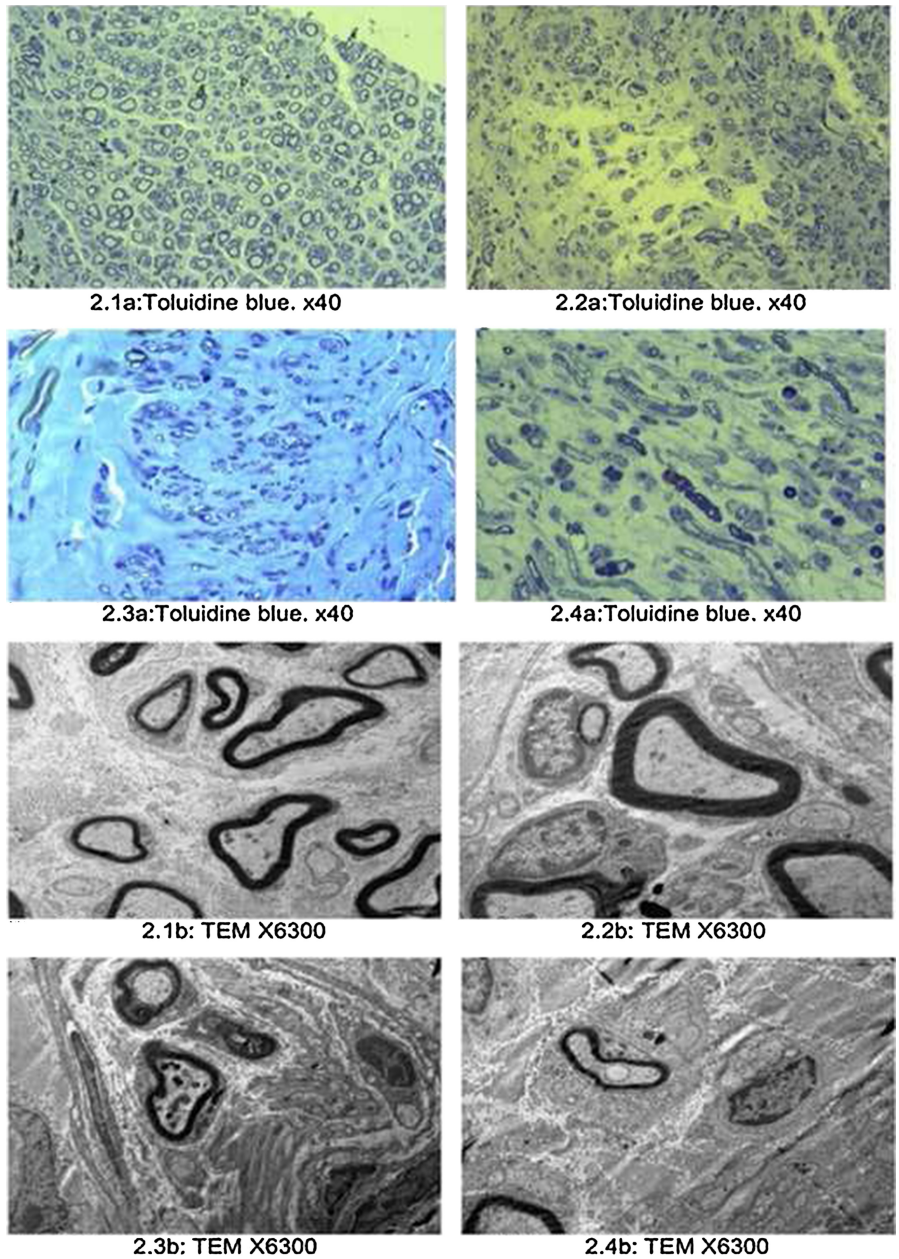

FIGURE 3. Histologic findings from grafts. Light microscopy images: (1A) toluidine blue ×40, (2A) toluidine blue ×40, (3A) toluidine blue ×40, and (4A) toluidine blue ×40. Electron microscopy images: (1B) TEM ×6300, (2B) TEM ×6300, (3B) TEM ×6300, and (4B) TEM ×6300.

Group 2: In the sections stained with toluidine blue, localized, small, thin myelinated sheaths were detected (see Fig. 3.2A). Electron microscopy of the sections revealed axons wrapped in myelinated sheaths of various sizes and thicknesses. Localized irregularities and periaxonal vacuolization were detected. Many Schwann cells were encountered in the examined sections. Fibrotic changes and edematous regions were noted in the endoneurium between axons. Nonmyelinated axons were also observed (see Fig. 3.2B).

Group 3: In the sections stained with toluidine blue, primarily small axons with thin and irregular myelinated sheaths were observed (see Fig. 3.3A). Electron microscopy revealed rare, small myelinated axons in the dense fibrotic tissue and localized, nonmyelinated axon sections. In the axoplasm of some of the myelinated axons, electron-dense granules were detected. Minimal edema was noted in all the sections (see Fig. 3.3B).

Group 4: In the sections stained with toluidine blue, localized axons with thin, irregular myelinated sheaths were observed (see Fig. 3.4A). Electron microscopy revealed dense fibrotic tissue in the whole section. Rare, small myelinated axons, Schwann cells, and nonmyelinated axons were encountered in this fibrotic tissue (see Fig. 3.4B).

Statistical Findings of the Grafts

The mean graft myelination rates between the groups were evaluated by the Kruskal-Wallis test, and a statistically significant

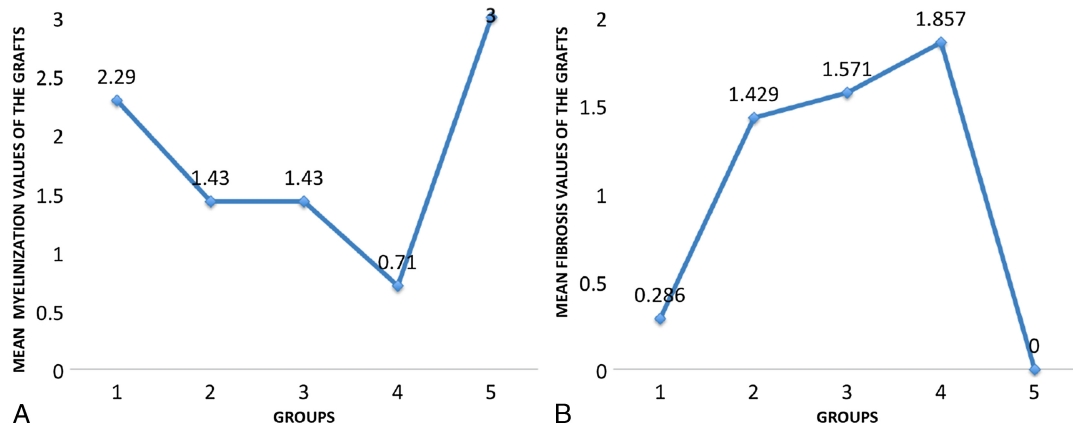

FIGURE 4. A, B: Mean myelination and fibrosis values of grafts.

difference was detected ($P = 0.0001$). However, the Conover test analysis revealed no statistically significant difference between group 2 and group 3 ($P < 0.05$). The group with the closest myelination rate to the control group was group 1. The Kruskal-Wallis test was used for the statistical evaluation of the graft fibrosis averages, and a statistically significant difference was detected ($P = 0.0001$). However, the Conover test analysis revealed a statistically significant difference between group 2 and group 3, as well as between group 1 and group 5 ($P < 0.05$) (see Fig. 4A,B).

Histologic Findings of Distal Anastomosis

Group 1: In the sections stained with toluidine blue, irregularity, undulation, and variations in the thicknesses of the myelin sheaths were noted, and small axons with thin myelin sheaths were observed most of the time (see Fig. 5.1A). The electron microscopy sections revealed axons with various thicknesses of myelin sheaths. Varying degrees of myelin irregularity and degeneration were detected in most of the thick myelin sheaths. Furthermore, in some of the axons, large vacuoles between the axolemma and the interior myelin layer were observed. Minimal fibrotic changes and edema were present in the connective tissue between axons. In addition, many nonmyelinated axons of various sizes were observed (Fig. 5.1B).

Group 2: In the sections stained with toluidine blue, rare, small axons with thin myelin sheaths were observed in the fibrotic tissue, as well as mast cells and Schwann cells (Fig. 5.2A). Electron microscopy revealed areas of scattered axons with thin myelin sheaths, as well as areas of nonmyelinated axons and Schwann cells with a medium degree of fibrotic concentration (Fig. 5.2B).

Group 3: In the sections stained with toluidine blue, small groups of small axons were observed, mainly with thin, irregular myelin sheaths (see Fig. 5.3A). Electron microscopy revealed scattered myelinated axons of various sizes that were wrapped in thin myelin sheaths and located in the dense fibrotic tissue. In addition, nonmyelinated axons were found locally, wrapped in Schwann cell sheaths (Fig. 5.3B).

Group 4: In the sections stained with hematoxylin and eosin, extensive loose fibrotic connective tissue was observed (see Fig. 5.4A). Mast cells were observed in this connective tissue. Electron microscopy revealed scattered myelinated axons, Schwann cells, and nonmyelinated axons in the irregular fibrotic tissue (Fig. 5.4B).

Statistical Findings of Distal Anastomosis

The Kruskal-Wallis test revealed a statistically significant difference in mean distal anastomosis myelination rates between the groups ($P < 0.0001$). However, the Conover test did not show a statistically significant difference between group 2 and group 3 ($P > 0.05$). A statistically significant difference was detected in the mean distal anastomosis fibrosis rates using the Kruskal-Wallis test ($P < 0.0001$). However, the Conover test showed no statistically significant difference between group 1 and group 5 or between group 3 and group 4 ($P > 0.05$) (see Fig. 6A,B).

DISCUSSION

The surgical procedure to be performed after nerve injury depends on the etiology, type, and the anatomic region of the injury. The main aim of treatment was to minimize functional loss due to the sensory and motor nerve damage distal to the injury. The gold standard for treating large defects due to peripheral nerve injuries is reconstruction with autologous nerve grafts.[6,7] However, the extent of the injury may be a limiting factor for autografts. During repair, altered fascicular architecture in the graft may hinder nerve regeneration due to a scattering of axons.[7] Another limitation is graft donor-site morbidity. Serious problems may occur such as loss of sensation, loss of donor-site nerve function, as well as painful neuroma.[6,7] Therefore, in consideration of these disadvantages, many studies have been conducted in search of alternatives to autologous nerve grafts that offer easier methods with better functional outcomes and less morbidity in peripheral nerve defect repairs.

In this study, we aimed to investigate the effects of cartilage tissue in axonal regeneration. A defect 1 cm in length was created in the left sciatic nerve of the rat, after which the cartilage graft harvested from its ear was tubularized and diced into the allogeneic aorta graft. Results of the grafted rats were compared with those of the control group and with the repairs made by allogeneic aorta grafts.

This study revealed axonal regeneration in all the groups. Similar to previously published reports, the highest amount of nerve regeneration was observed in the group where the repair was performed with autografts.[6] The outcomes of the groups treated with an allogeneic aorta graft alone or in combination with diced cartilage were similar. We compared SFI, latency, and amplitude rates for a functional evaluation of all the groups, and the autograft group exhibited the highest sciatic function. In contrast, a statistically significant difference was not detected between the other 3 groups. Gastrocnemius muscle mass was also measured, yielding similar results. In the histomorphologic evaluation, one of the most important parameters of our study, different levels (proximal anastomosis, graft, and distal anastomosis) of the rats' left sciatic nerves were scored in terms of myelination, fibrosis, edema, and mast cell density using light and electron microscopy. Prior reports have only analyzed samples taken from 1 level for histomorphology.

Myelination occurs before the axon reaches the end organ.[10] The degree of myelination is directly related to the maturation of the axon.[11] The distal sections exhibited a decrease in the number of myelinated axons and Schwann cells in all the groups, especially in groups 3 and 4,

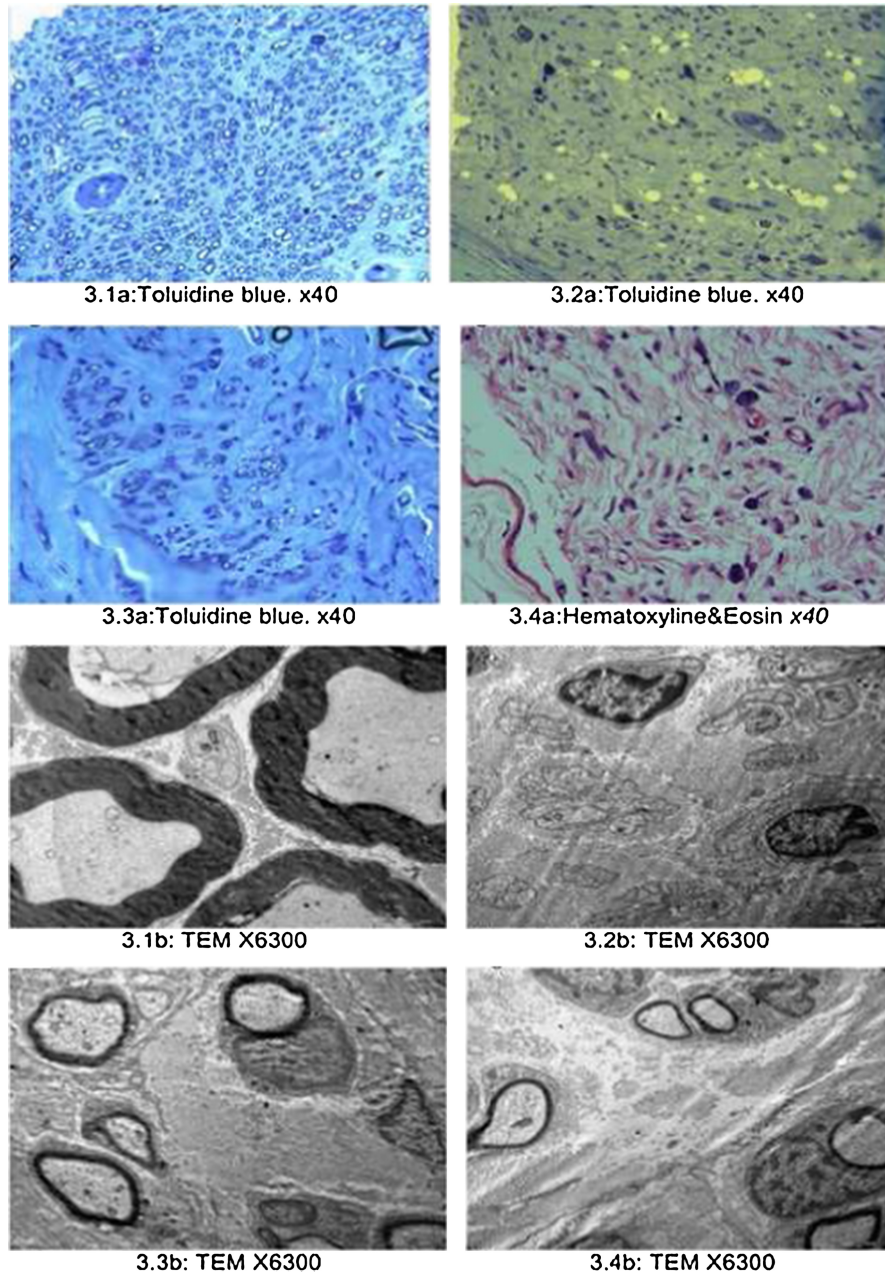

FIGURE 5. Histologic findings of distal anastomosis. Light microscopy images: (1A) toluidine blue ×40, (2A) toluidine blue ×40, (3A) toluidine blue ×40, and (4A) hematoxylin and eosin ×40. Electron microscopy images: (1B) TEM ×6300, (2B) TEM ×6300, (3B) TEM ×6300, and (4B) TEM ×6300.

although an increase in the amount of fibrosis and edema was noted. This was supposed to be due to the graft reaction that the cartilage created.

We think that the reason for why there was not a statistically significant difference in terms of SFI and latency values between the groups except the control and autograft groups was that the numerical superiority of axonal regeneration between the groups did not meet the adequate amount to affect these 2 parameters.

In the evaluation for proximal anastomosis, a statistically significant difference in myelination was not detected between the control group and group 1, in which an autograft was used, and group 2, which involved an allogeneic vessel graft. There was also no statistically significant difference between group 3, in which diced cartilage was placed into the allogeneic vessel graft, and group 4, in which the cartilage was tubularized; however, the degree of myelination was found to be lower in both of these groups compared to the other groups. There was no statistically significant difference in terms of myelination and Schwann cell detection rates between the sections taken from the graft where we expected to find the most particular histomorphologic data and group 2, with an allogeneic vessel graft, or group 3, with diced cartilage placed in the vessel graft. Myelination and Schwann cells were present in group 4, but these were rarer and more scattered than in the

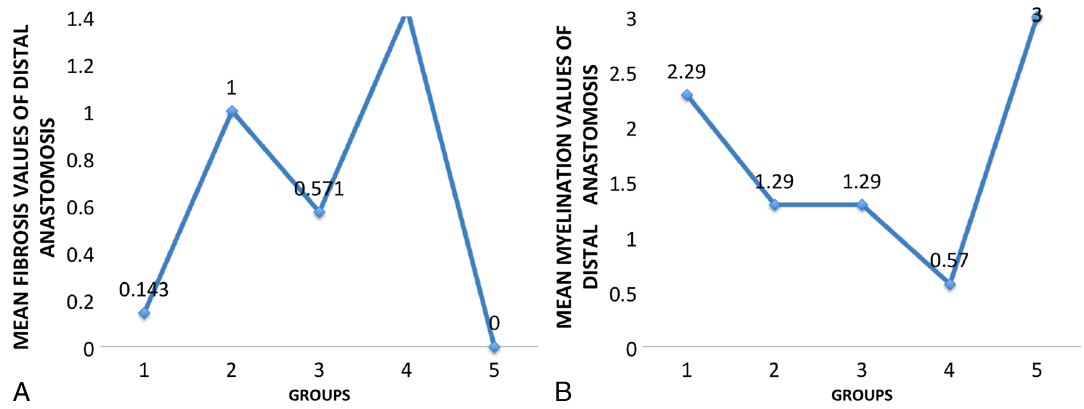

FIGURE 6. A, B, Mean myelination and fibrosis values of distal anastomoses.

other groups. The degree of fibrosis was highest in group 4, but similar results were found in groups 2 and 3. The distal anastomosis sections revealed similar results in groups 2 and 3. Although the degree of myelination was low in group 4, that of fibrosis was greater (but similar between groups 3 and 4).

Most studies have underscored the importance of tension-free repair and have reported a high incidence of fibrosis in the repairs performed under tension.[1] Using a rat model of sciatic nerve injury, studies have compared the primary repair and the 2-mm-long repair models and reported equivalent nerve transmission outcomes. However, the repair of a 6-mm defect without a nerve graft significantly decreased transmission.[12] A study in which 3.6- and 9-mm defects were created in the sciatic nerves of rats, primary repair outcomes revealed that the amount of tension in the repair zone increased proportionally with respect to the size of the defect and scar formation, and that the functional outcomes of nerve healing significantly decreased.[13]

Because the cartilage conduit that we used did not collapse, we suggest that it did not cause nerve compression and furthermore, that it contributed to axonal regeneration by providing a favorable microenvironment of extracellular matrix. One of the important disadvantages of cartilage graft use is a delay in the advancement of regenerated axonal bundles in Schwann cells, likely due to fibrosis and edema formation around the lesion and the decrease in myelination density and thickness.

Repair with vein grafts, one of the natural tubularization techniques used in peripheral nerve repair, has been tested in many studies.[14–16] Chiu et al[17] created a 1-cm nerve defect in the sciatic nerve of a rat and repaired it by a vein graft and demonstrated nerve regeneration both histologically and electrophysiologically. They intended to repair the defect by placing a nerve graft in the vein tube graft to minimize the drawbacks of vein grafts alone and to benefit from the vein graft microenvironment. However, no statistically significant difference from the nerve autograft alone was detected.[18] Additionally, when compared with conventional nerve grafts, no additional benefits were observed in the outcomes of studies in which denatured or fresh muscle tissues were used to prevent vein graft collapse.[19–22] Similarly, no preference was observed for the use of diced cartilage when compared with vessel grafts alone, possibly due to a delay and a reduction in axonal bundle advancement, because of the increase in the fibrosis of cartilage tissue expected to contribute to Schwann cell proliferation. In another study, Benedetto et al investigated the outcomes of autograft, vein graft, and strip-shaped perineurium placed in a vein graft for nerve regeneration. They reported that the most favorable outcomes were obtained with an autograft. However, the group in which perineurium was placed into the vein graft yielded better outcomes than the group in which the vein graft was used alone, suggesting that the perineurium constituted a favorable microenvironment for axonal regeneration.[23] Studies on the repair of nerve defects with the use of synthetic tubes have also been conducted. The outcomes of the nerve repair studies performed with vein grafts and polyglycolic acid conduits revealed similar results.[16]

Braga-Silva et al[24] demonstrated that nerve repair with bone marrow mononuclear cells placed into a silicone tube yielded better results than the repair performed with only the silicone tube. In the experimental studies where nerve repairs were performed with laminin-injected conduits, laminin had positive effects on nerve regeneration.[25]

The success of nerve regeneration after tubularization depends on angiogenesis, reformation of fibroblasts, Schwann cells, and a new extracellular matrix.[26] Rodríguez et al[27] used high-permeability polytubes, obtaining better nerve regeneration than with nonpermeability tubes, which suggested an easy transfer of molecules that increased nerve regeneration from the permeable tubes. In our study, the cartilage autograft harvested from the rat's ear was tubularized and used as a conduit, and nerve regeneration was observed. The use of a tubularized cartilage graft has a conduit effect that may promote the advancement of Schwann cells from the proximal stump to the distal stump; the cartilage extracellular matrix provides a favorable microenvironment for nerve regeneration and contributes to Schwann cell proliferation. Nerve regeneration was positively affected by imitating pluripotent (mesenchymal) stem cells.

This study shows that cartilage tissue has positive effects on nerve healing. The use of cartilage tissue, which has low immunity, can provide axonal regeneration by Schwann cell proliferation and preservation of neurotrophic factors. It was evident that the cartilage tissue had positive effects on nerve regeneration that was analyzed histomorphologically, offering similar functional outcomes despite the increased fibrosis rates when compared to other reconstruction methods. The additional procedures to decrease fibrosis rates on the equilibrium of regeneration and fibrosis will make more contribution to the nerve regeneration. Further research on this topic may enable cartilage conduits for clinical use.

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