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Article *in* Journal of Reconstructive Microsurgery · November 2014

DOI: 10.1055/s-0034-1395414 · Source: PubMed

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Effects of Intraperitoneally Administered Folic Acid on the Healing of Repaired Tibial Nerves in Rats

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J Reconstr Microsurg 2015;31:191–197.

Abstract

Background Complete nerve regeneration and clinical healing remain a challenge despite considerable advances in the treatment of peripheral nerve injuries. To improve nerve regeneration, several experimental molecular procedures have been attempted. This study aimed to investigate the effects of folic acid on peripheral nerve healing after transection and end-to-end suture repair of the tibial nerve in rats.

Methods In this study, 20 adult male Wistar Albino rats weighing 225 to 250 g were used. The right tibial nerves of 20 rats were explored, transected, and sutured using the end-to-end technique. The rats were randomly allocated to either the intraperitoneally administered folic acid group (test group) or the control group. Preoperative and 6-week postoperative neurophysiological studies were performed by the same researcher. Myelin-sheathed axons were counted.

Results The results demonstrated that the folic acid–treated group exhibited improved electromyographic results compared with the control group. Histological evaluation revealed that the axons were well preserved and that the axon quantity and density were increased in the test group compared with the control group. Quantitative results also increased in the test group compared with the control group ($p = 0.001$).

Conclusion In this study, 6-week intraperitoneal administration of 80 μg/kg of folic acid significantly improved peripheral nerve healing. Histological analysis of the group that received folic acid revealed increased axon myelination with little granular tissue or fibrosis. We propose that folic acid supplementation may be an effective component of peripheral nerve injury treatment.

Keywords
- folic acid
- peripheral nerve
- nerve repair
- regeneration

Despite the momentous developments achieved in the field of peripheral nerve surgery in recent years, clinical outcomes have not reached the desired level; the necessity of further studies is widely acknowledged. Functional recovery after peripheral nerve injury remains weak, particularly in injuries located far from the target organ and close to the spinal cord.[1]

The most important factors that enhance axonal growth toward the target organ are neurotrophic factors released from the target organ and the proximal stump that forms after Wallerian degeneration.[2,3] More proximal nerve injuries and the formation of postrepair perineural fibrosis slow the regeneration process. The extension of this period may cause

received
June 11, 2014
accepted after revision
September 6, 2014
published online
November 25, 2014

DOI http://dx.doi.org/10.1055/s-0034-1395414.
ISSN 0743-684X.

irreversible functional losses in the target organ.[3,4] Folic acid, which is also called folate or vitamin B9, is a water-soluble B complex vitamin.[5] The average concentration of this vitamin in the blood is 5 to 9 g/mL (5–17 ng/mL). Folic acid plays an important role in the pathogenesis of various diseases, such as macrocytic anemia, cardiovascular diseases, thromboembolic diseases, neural tube defects, and other congenital defects, neuropsychiatric diseases and cancer.[6] Previous studies demonstrated that folic acid plays a key role in the growth and differentiation of the central nervous system. This finding may support the opinion that the effects of folic acid on the growth and differentiation of the nervous system are limited to the early embryonic period. However, Iskandar et al demonstrated that folic acid may also enhance growth and repair mechanisms in the adult central nervous system.[7]

However, no available studies addressed the effect of folic acid on peripheral nerve regeneration. In this study, we investigated the effect of folic acid on peripheral nerve regeneration.

Methods

In this study, 20 healthy adult (i.e., average age of 6 months) male Wistar Albino rats with an average weight of 225 to 250 g were used. The study was performed with the approval of the Animal Ethics Committee. The rats were divided into test and control groups, which each contained 10 rats. The effect of folic acid (98%) (Sigma-Aldrich, Seelze, Germany) administration into the peritoneum of the rats after end-to-end primary repair of the tibial nerve in the lower extremities was investigated. After the repairs, 80 µg/kg of folic acid were administered intraperitoneally to the test groups for 6 weeks. Folic acid was not administered after the repair of the tibial nerve in the control group. The rats were fed with standard rat chow. The subjects were housed in single cages under standard laboratory conditions (i.e., 12 hours light and 12 hours darkness at 22°C with 30% humidity). In our study, histopathological and electromyographic (EMG) methods were used to evaluate nerve regeneration.[8]

The tibial nerve EMG values of all of the rats were measured using an EMG device (Dantec Cantata, Copenhagen, Denmark) before the operation. Latency and amplitude (Amp) were evaluated electromyographically in the nerves, and spontaneous activities were evaluated electromyographically in the muscles. EMG measurements were made before and after surgery by the same researcher, from the same distance (i.e., 25 mm) and on the same day.

The surgical application was made using a standard gluteal approach.[9] The rats were maintained under anesthesia with 20 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine hydrochloride administered intramuscularly (►**Fig. 1A**). The skin was passed through, and the hamstring muscle groups were subsequently passed through with blunt dissection to access the sciatic nerve and the peroneal and tibial branching area (►**Fig. 1B**). At ×16 magnification under a Zeiss microscope (Carl Zeiss Microscopy, Jena, Germany) a straight and complete section was created in a single motion using a sharp lancet, from approximately 2 to 3 mm distal to the tibial nerve branching region; immediately following the section, the nerve repair was performed epineurally using 10/0 suture (►**Fig. 1C**). All of the

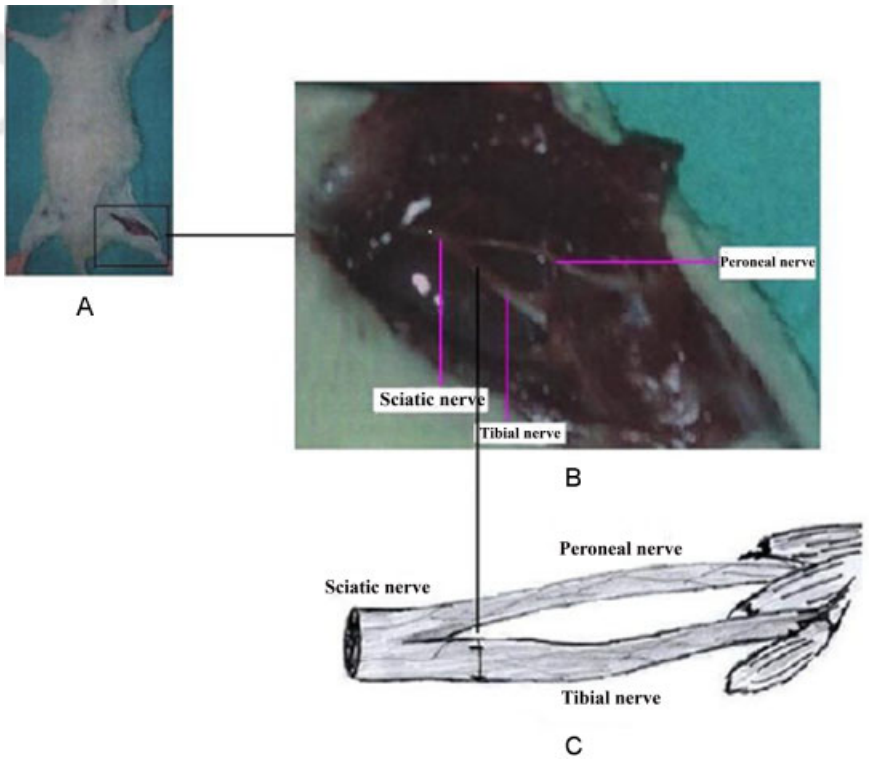

Fig. 1 (A) Standard skin incision. (B) The view of sciatic, peroneal, and tibial nerves during the operation. (C) Schematic view of the tibial nerve repair.

Table 1 In-group comparison of the EMG results of tibial nerve in the test and control groups

		Preoperative	Postoperative	*p*
Test ($n = 10$)	Amp	11.82 ± 2.88	9.61 ± 2.94	0.231
	DL	1.14 ± 0.05	1.60 ± 0.11	< **0.001**
Control ($n = 10$)	Amp	10.72 ± 2.32	11.47 ± 4.19	0.640
	DL	1.15 ± 0.10	2.06 ± 0.15	< **0.001**

Abbreviations: Amp, amplitude; DL, distal latency; EMG, electromyographic.
Note: The bold values highlight importances of results.

rats underwent surgery on the same day. Beginning the day after the surgery, 80 µg/kg of folic acid were administered intraperitoneally to the test group daily for 6 weeks.

An EMG test was applied to the hind legs of each rat before and 6 weeks after the surgical application. The presence of spontaneous activity and motor unit potential properties were investigated in both muscle groups. The latencies and amplitudes of the responses that emerged in the relevant muscles as a result of the warnings from the proximal zone were then measured. The preoperative and postoperative EMG findings of the rats were recorded. The distal latency (DL) and amplitude (Amp) values of the test and control groups that were recorded before and after the process were evaluated statistically based on intragroup and intergroup and were then summarized in ►**Tables 1** and **2**.

The statistical tests were performed using Windows-compatible SPSS (IBM SPSS Statistics 19, SPSS Inc., IBM Co. Somers, NY). In this study, the Kolmogorov–Smirnov test was used to evaluate whether the data (i.e., DL and Amp) from both groups demonstrated a normal distribution. Because the data failed to exhibit a normal distribution, the Wilcoxon signed rank test was used for the intragroup evaluation. In the comparisons between the groups, Kruskal–Wallis variance analysis and the Mann–Whitney *U* test were used. The data for continuous variables were presented as the mean ± standard deviation. When the calculated *p* values were less than 0.05, the associated differences were considered to be statistically significant.

After the termination of the test, the repair line was accessed using a standard incision through the previous incision line. Under microscopic magnification, the tibial nerve was removed, from the bifurcation line to the neuromotor resultant. The rats were sacrificed using a high-dose ketamine injection.

For histopathological examination, sections were collected from the repair line and from the region approximately 0.5 cm distal to the repair line. An ultratome was used to collect 1-µm-thick sections. The sections were examined using an Olympus light microscope (Melville, NY). After evaluation using the light microscope, the obtained views were transferred from the slide to the computer environment via video camera and myelinated axons were counted using thebe BS 2000 DOC module. During this process, all of the myelinated axons in three different regions within an area with a breadth of 240.000 µm were counted using the classical method (i.e., manually) under ×100 magnification.[8] The statistical analysis were performed using Pearson correlation test.

The 1 mm^3 tissue samples that were collected for electron microscopic (EM) examination were fixed for 4 hours in 5% glutaraldehyde prepared with Millonig phosphate buffer at 4° C. Thin sections with a thickness of 50 nm that were obtained from the determined areas were stained with uranyl acetate and lead citrate solutions and examined using a Jeol JEM 1400 transmission EM (Carl Zeiss, Thornwood, NY).

Results

Electrophysiological Findings

The latency times were longer after the procedure in both the test and control groups. However, the expansion of the latency times in the test group was smaller than that of the control group; this difference was statistically significant ($p < 0.05$).

Similarly, the amplitude values were reduced in both the test and control groups. However, no statistically significant difference was observed between the groups. Spontaneous activities occurred in some subjects in both the test and control groups.

Histopathological Findings

During the light microscopy evaluation, irregularity in the distribution of the axons was observed in the test group.

Table 2 Intergroups comparison of the EMG results of tibial nerve in the test and control groups

	Test ($n = 10$)	Control ($n = 10$)	*p*
Preoperative Amp	1.82 ± 2.88	10.72 ± 2.32	0.710
Postoperative Amp	9.61 ± 2.94	11.47 ± 4.19	0.547
Preoperative DL	1.14 ± 0.05	1.15 ± 0.10	0.962
Postoperative DL	1.60 ± 0.11	2.06 ± 0.15	< **0.001**

Abbreviations: Amp, amplitude; DL, distal latency; EMG, electromyographic.
Note: The bold values highlight importances of results.

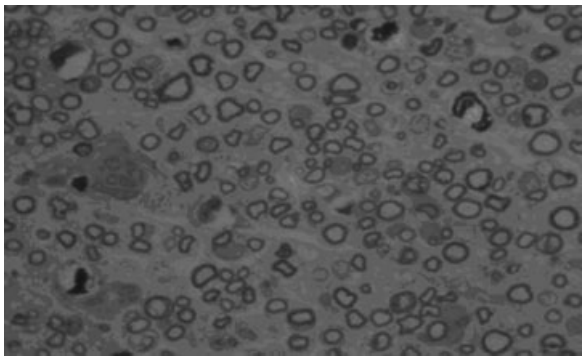

Fig. 2 Light microscope view of the nerve in the test group (×1,000, toluidine blue).

However, the axons in that group were better protected than those in the control group. The significant increase in the number and density of the axons compared with those in the control group attracted our attention (►**Fig. 2**).

Irregularities in the axon distribution, regenerated axon clusters, a significant increase in the endoneurium, and thickening of the perineurium were observed in control group. A small number of degenerated axon clusters were observed (►**Fig. 3**). The suture material and suture granulomas were detected in the section passing through the lower repair line in both examples from the first and second groups.

In the counts made in the sections collected from the distal part of the nerve repair line using thebe BS 2000 DOC module, averages of 219 ± 44.59 and 191.5 ± 16.29 myelinated axons were counted in the test and control groups, respectively. This increase was statistically significant ($p < 0.05$).

In the EM evaluation, expansions were observed in myelinated nerves and in endoplasmic reticulum cisternae in the cytoplasm of the Schwann cells in the test group; the mitochondria and the nucleus exhibited a normal morphologic appearance similar to that of the normal nerve structure. Again, we observed that the axons and myelin sheath structures had characteristics similar to those of the normal neural structures. Unmyelinated nerve fibers and the Schwann cells surrounding them exhibited morphological characteristics

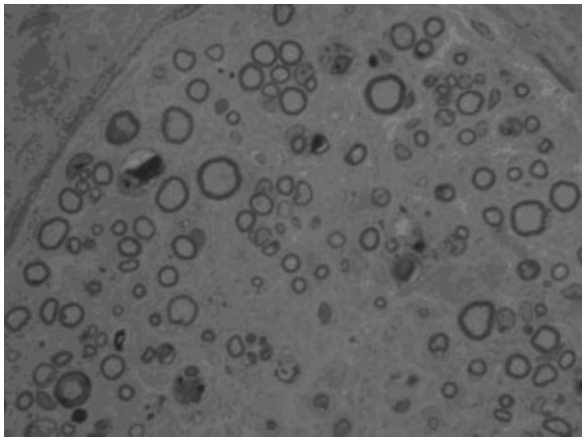

Fig. 3 Light microscope view of the nerve in the control group (×1,000, toluidine blue).

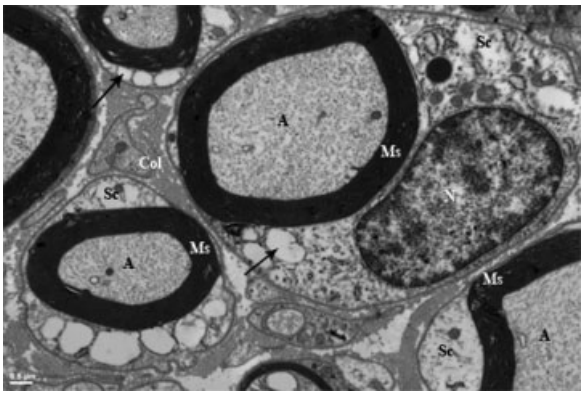

Fig. 4 Test group. Axon (A) and myelin sheath (Ms) structures of the myelinated nerve fibers are seen to be in normal morphological appearance. It is observed that there exists expansions in the endoplasmic reticulum cisternae (arrow) in the cytoplasms of the Schwann cells (Sc) surrounding the myelinated nerves. Collagen (Col) fibers are observed in the endoneurium. Nucleus (N). Bar 0.5 µm.

similar to those of the normal nerve structures of the collagen fibers in the endoneurium (►**Fig. 4**).

The degenerated myelin sheath exhibited a structure with characteristic concentric lamellae in myelinated nerve fibers; there were occasional separations in the myelin sheath. The neurotubulus, the neurofilament, and the mitochondria in the axon exhibited a normal structure. In addition, an expansion of the plain endoplasmic reticulum cisternae was observed in the control group. It is worth noting that increased heterochromatin was observed in the nucleus of the Schwann cells. Expansions of endoplasmic reticulum cisternae and mitochondria were observed in the Schwann cell cytoplasm, and the formation of vacuoles occurred occasionally. The structure of the myelinated nerve fibers was protected, the arrangement of fibroblasts and collagen fibers was normal and these components exhibited characteristics similar to those of the normal nerve structure (►**Figs. 5** and **6**).

Discussion

Peripheral nerve injuries often cause permanent and significant function losses. Trauma and iatrogenic injuries constitute the majority of peripheral nerve injuries.[10–12] A properly applied nerve repair is critically important for the rehabilitation of damaged and denervated tissues. The development of microsurgical techniques led to an improved understanding of nerve damage and regeneration neurobiology and enabled significant improvements in nerve repair outcomes.[11,13,14]

Despite these advancements in microsurgical techniques, functional recovery after peripheral nerve injury remains quite weak.[15] Additional adverse factors that affect this process include neuronal loss, loss of regeneration capacity, scar formation in the repair area, and loss of proper sensory stimulation induced by weak regeneration.[16] Various hormones and pharmacological agents have been used in attempts to shorten the repair time and minimize scar formation.[3,7,17] Scar formation in the repair area cannot be prevented completely in patients who undergo peripheral

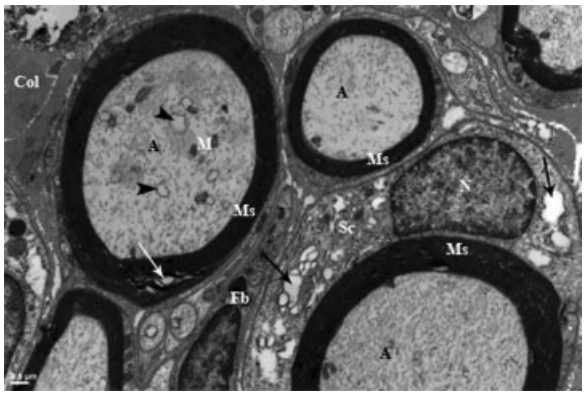

Fig. 5 Control group. It is observed that there exists separations from place to place in myelin sheath (Ms) of the myelinated nerve fibers; concentric lamellar structures are degenerated (white arrow); neurotubulus, neurofilament, and mitochondria (M) in the axon (A) were with normal structure; and there was expansion in plain endoplasmic reticulum cisternae (arrow head). It is observed that there is a heterochromatin increase in the nucleus (N) of the Schwann cells (Sc) surrounding the myelinated nerves; and there are expansions in endoplasmic reticulum cisternae in the Sc cytoplasms as well as formation of vacuoles (black arrow). Existence of fibroblasts (Fb) and collagen (Col) fibers are observed in the endoneurium. Bar 0.5 µm.

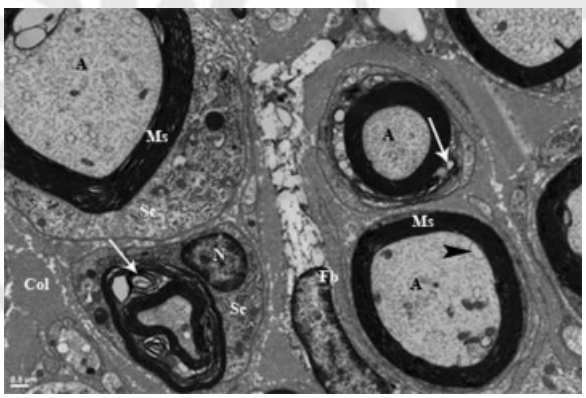

Fig. 6 Control group. It is observed that there exists separations from place to place in myelin sheath (Ms) of the myelinated nerve fibers; concentric lamellar structures are degenerated (white arrow); and there was expansion in plain endoplasmic reticulum cisternae (arrow head). It is observed that there is a heterochromatin increase in the nucleus (N) of the Schwann cells (Sc) surrounding the myelinated nerves. Existence of fibroblasts (Fb) and collagen (Col) fibers are observed in the endoneurium. Bar 0.5 µm.

nerve surgery.[3] This process occurs due to ischemia and results in irreversible nerve damage. In a study conducted using adult rats, Özgenel[3] demonstrated that he could prevent perineural scar formation by applying hyaluronic acid topically on the nerve repair line. Madura et al[18] demonstrated that ibuprofen positively affects nerve healing in an experimental model that employs the peripheral nervous system of rats. In a study performed in zebrafish, Rieger and Sagasti[19] demonstrated that hydrogen peroxide administered externally increases regeneration in axonal injuries that accompany damaged skin. Yin et al[20] demonstrated that erythropoietin enhances nerve regeneration and functional recovery. Ayan et al[21] demonstrated experimentally that a human placenta suspension makes a positive contribution to peripheral nerve regeneration. Saceda et al[22] showed that human growth hormone enhances nerve regeneration. Luria[23] et al demonstrated that glatiramer acetate, a substance which enhances cellular immunity, increases peripheral nerve regeneration. Tacrolimus (FK506) as an immunosuppressive drug has been proven to have neuroprotective and neurotrophic actions in experimental models, increasing neurite elongation and accelerating the rate of nerve regeneration.[24]

Due to studies of the critical effects of folic acid on the development and differentiation of the embryonic central nervous system, the application of dietary folic acid support has become widespread and significant reductions in the formation of neural tube defects and other developmental diseases have occurred.[7,11,12,15,17,25] To prevent neural tube defects, folic acid should be administered before and during pregnancy.[7,11,12,15,17,25] This recommendation suggests that the effect of folic acid on growth and differentiation is limited to the early embryonic period. In an experimental study conducted using a model that involves the formation of a lesion in the central nervous system of rats, Iskandar et al[7] demonstrated that the effect of folic acid on growth, repair, and healing mechanisms in the central nervous system is not limited to the embryonic period; this vitamin is also effective for the central nervous systems of rats. In another study performed by Iskandar et al,[26] folate was demonstrated to have a critical importance in the repair and regeneration of the adult central nervous system; the observed effects were biphasic and dose dependent.

In this experimental study, we demonstrated that the administration of 80 µg/kg of folic acid intraperitoneally for 6 weeks significantly enhances peripheral nerve regeneration. A statistically significant shortening of the latency values was observed in the test group in comparison to the control group. Similarly, we identified a statistically significant increase in nerve regeneration in the test group in comparison to the control group, according to the EMG results.

EM evaluation provides important information about the number of axons, axon diameter, and the cell structure within the endoneurium. Nevertheless, the evaluation of the number of axons, myelin thickness, and axon diameter requires the collection and evaluation of standard pathologic findings; thus, this type of evaluation is a highly difficult and delicate process.[8] Therefore, we counted axons and myelinated axons using the classical manual method. This method is technically more difficult and time consuming but provides more accurate results than the automatic method.[8]

A direct correlation exists between both the number of axons and myelin sheath thickness and axon regeneration.[27] In our study, we counted all of the myelinated axons using the method preferred by Almquist et al due to difficulties with standardization and a lack of equipment.[28]

The number of axons in the samples collected from the proximal and distal portions of the repair area provides more valuable information.[8] Nevertheless, we obtained sections from the repair line and counted the myelinated axons, with

the intent to investigate the effect of folic acid on regeneration. The number of myelinated axons was significantly higher ($p = 0.001$) in the test group than in the control group.

The observed increase in latency values indicates axonal degeneration and slowed nerve conduction.[28] The speed of nerve regeneration in the rats was 3.6 ± 0.5–5.1 ± 0.5 mm/d, which is slightly faster than the speed observed in humans.[11] Reduced amplitude values indicate axonal loss and degeneration.[18] Normally, spontaneous activity does not exist in muscles. The existence of spontaneous activity indicates degeneration and fibrosis in the target muscle.[27,29] According to Frykman et al,[8] a direct correlation exists between EMG values and improvements in motor function. The application and reproducibility of EMG studies in rats are low because the observed amplitude changes may differ based on differences in the areas where the electrodes are placed into muscle.[8] Frykman et al report that to ensure that evaluations of amplitude are significant, they should be repeated before and after the test, as in our study, or they should be compared with the corresponding intact part.[8] We ensured that all measurements were made by the same researcher and from the same distance to enhance the reliability of the values. The latency and spontaneous activity values obtained from EMG tests are more reliable than the amplitude values.[30] In our study, we found that the latency values were significantly lower in the test group than in the control group. This finding is consistent with the literature.[3,27]

The histopathological evaluation revealed that the axons were well protected, and a significant increase in the number and intensity of axons was observed in the group subjected to repair followed by the administration of folic acid, when compared with the control group. The histopathological findings demonstrate that folic acid has a positive effect on nerve healing, although this effect was observed to a lesser extent when compared with the EMG results.

We used the intraperitoneal method for standardizing the dose of folic acid administered to each subject in the study. We did not perform a dose-response relationship of folic acid on regeneration of peripheral nerve; however, we used the study performed by Iskandar et al. In this study, 0 to 800 µg/kg of folic acid was administered to the rats intraperitoneally and 80 µg/kg was determined to be the most effective dose.[7] No toxicity related to excessive use of folic acid has been reported in the literature.

In earlier studies on nerve regeneration, the application time varied depending on the type of subject used and the peripheral nerve subjected to surgery; however, this time ranged between 1 week and 6 months.[7,11,31] We determined the duration of this study to be 6 weeks. In the literature, experimental animals such as rats, cats, rabbits, monkeys were used. Rats were preferred because they are easy to obtain and feed.

The end-to-end epineural repair technique was used for nerve repairs. Traditionally, this is the most commonly used technique and the first choice in injuries. If the surgeon can separate the fascicles at the proximal and distal stumps according to their motor and sensory properties, the fascicles may be more suitable for repair.[13]

A literature search revealed that the effect of folic acid on the regeneration of the adult central nervous system has been studied by only Iskandar et al.[7] But we found no studies of the adult peripheral nervous system. According to the electrophysiological and histological results of our study, folic acid significantly enhances the healing of peripheral nerves.

Conclusion

The total duration of folic acid support after injury and the time range required for the maximum effect is unknown. We believe that folic acid, which is widely used in clinics for protection against neural tube defects and other congenital defects, can also be used to accelerate recovery after peripheral nerve injuries and to shorten the time required for extremities to regain function. Thus, significant financial and medical benefits will be provided, enabling the patient return to work sooner. We believe that new prospective studies of humans and experimental animals should be performed to ensure that folic acid is used routinely in the clinic after nerve injuries. We propose that folic acid supplementation may be an effective component of peripheral nerve injury treatment.

Acknowledgment

We thank Salim Yologlu, MD, from the Department of Biostatistics for his great effort in the statistical analysis of our experimental study.

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