Turk Neurosurg, 2020

Original Investigation

DOI: 10.5137/1019-5149.JTN.30784-20.3

Received: 19.05.2020
Accepted: 20.07.2020

Published Online: 10.12.2020

Alpha-Tocopherol and Cyanocobalamin Combination Accelerates Peripheral Nerve Healing: An Experimental Animal Study

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ABSTRACT

AIM: To evaluate the functional and histopathological results of alpha-tocopherol (vitamin E) and vitamin B_{12} on an experimental rat model of peripheral nerve injury.

MATERIAL and METHODS: This research included 32 Wistar Hannover rats. The sciatic nerves of the animals were crushed using an aneurysm clamp. The rats were divided into 4 groups, as group 0 (the controls; no treatment), and groups B_{12}, E, and B_{12}+E, respectively. The rats were analyzed functionally, using the sciatic functional index (SFI), and histopathologically.

RESULTS: In the functional analysis, it was determined that vitamin E was as influential as B_{12}. Concomitant use of these 2 vitamins was found to be more beneficial. The SFI was significantly higher in the B_{12}+E group when compared with that of the B12 group, which indicated that vitamin E improved the healing effects of vitamin B_{12}. In the histopathological evaluation, vitamin E was not effective in the treatment of axonal degeneration (AxD) or edema/inflammation (EI) by itself. Although vitamin B_{12} was effective in the treatment of EI, it was ineffective in the treatment of AxD. However, the combination of these vitamins decreased both AxD and EI, which showed that the additive effects of these vitamins could reverse neurological injury when used together.

CONCLUSION: Vitamins B_{12} and E were effective in the functional recovery of peripheral nerve injury (PNI). Neither vitamin B_{12} nor E was effective in the treatment AxD; however, their combination was effective in the treatment of AxD. The results suggested that vitamin E was effective in the treatment of PNI, especially when combined with vitamin B_{12}. It is our belief that the combination of these vitamins could be used in the treatment of PNI, especially after future studies have been conducted on humans.

KEYWORDS: Alpha-tocopherol, Axonal degeneration, Cyanocobalamin, Peripheral nerve injury, Sciatic nerve, Rats

ABBREVIATIONS: PN: peripheral nerve, **PNI:** PN injury, **AxD:** Axonal degeneration, **AL:** Axonolysis (vacuolization), **EI:** Edema/inflammation, **PNR:** PN regeneration, **PNS:** Peripheral nervous system, **CNS:** Central nervous system, **B12:** Cyanocobalamin, **E:** Alpha-tocopherol, **SN:** Sciatic nerve, **SFI:** Sciatic functional index, **WTA:** Walking tract analysis, **ROS:** Reactive oxygen species, **NGF:** Nerve growth factors.

INTRODUCTION

Crushing, cutting, or stretching injuries of the peripheral nerve (PN) are the main mechanisms that can cause PN injury (PNI) (17). As PNI is seen mostly in younger patients with a mean age of 38.1 years old, it is an important cause of functional, social, and psychological problems and economic loss (12,24). PNI can induce complex pathophysiological processes, including edema/inflammation (EI), demyelination, axonolysis (AL) and/or axonal degeneration (AxD) of the nerve, which may result in exacerbated damage

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mechanisms or repair mechanisms of the nerve tissue (15). Although exacerbated damage of the PN can cause severe sensorial and/or motor dysfunction, PN regeneration (PNR) is possible because of the repair mechanisms in the peripheral nervous system (PNS).

Surgical techniques have been improved over recent decades. Even though the outcomes have not been sufficiently good, surgical intervention is still accepted as the main treatment for complete transections of the PN (3). However, PNR is believed to be possible for incomplete PNI, which is usually treated with medicines such as thymoquinone, alpha lipoid acid, omega 3-6, and several vitamins (1,6).

The number of studies about the healing effects of vitamin supplementations on PNIs has increased over recent years. Most of these studies have been about cyanocobalamin (vitamin B_{12}), which is the most widely known and recommended medical supplementation for positive regeneration effects with neurotrophic factors, which stimulate the regeneration of both the myelin and axons (1,2). Alpha-tocopherol (vitamin E) is a chain-breaking antioxidant. It ensures membranous solidity (23). Vitamin E has been shown to be neuroprotective in patients with diabetes, alcohol abuse, and cisplatin usege (9). In addition, vitamin E deficiency by itself is known to be a cause of peripheral neuropathy. However, the effects of vitamin E on nerve regeneration after PNI is not yet as clear as those of vitamin B_{12}.

The purpose herein was to present gait analysis outcomes and histological results of vitamins E and B_{12} alone and in combination.

MATERIAL and METHODS

Subjects

The study animals comprised 32 Wistar Hannover rats, weighing 200 to 250 g, at the Experimental Research Center of the . The study began after it was determined to be feasible through a preliminary study using 2 rats. The animals were randomly divided into 4 groups with 8 rats in each group, as determined using standard power analysis. The rats were weighed daily for dose evaluation. The temperature of the room and humidity were maintained at 22 °C and 50%, respectively. The study was conducted for 28 days.

Surgery

It was decided that damage the sciatic nerve (SN) would be performed as the PNI, as it has both motor and sensorial functions. An intraperitoneal mixture of 70 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine hydrochloride was used for the anesthetics. Surgery was performed via a 1-inch incision following subcutaneous tissue dissection, just below the left iliac crest, 1-cm lateral to the middle line, and extending to the popliteal fossa. The left SN and embranchment were completely exposed by dissecting the superficial gluteal muscle and biceps femoris, and the fascia surrounding the junction line of the surface. Next, an area of the nerve 10 mm above the embranchment was damaged using a clamp. The same clamp was used for all of the rats and maintained for 15 min. Subcutaneous 0.1 cc/kg carprofen was used for pain control of the rats immediately after closing the incision. All of the procedures were standardized and applied by the first author.

Medication was started just after completion of the crush injury. The vitamin doses were determined using those of previous studies in corporation with veterinarians (26,28). The treatments were applied to all of the rats at the same time each day for 4 weeks. The crush injury severity and period of damage were homogenized by applying the same 50-g closing pressure clamp to all of the rats for the same period of time. All of the rats were observed individually until the incision closure. Next, the rats were combined and 8 rats were placed into each cage. They were not subjected to postoperative restrictions for movement or diet. No local or systemic complications were observed.

Groups of the Subjects and Treatment Doses

According to the sample size determination using the standard power analysis, 4 groups were established, with 8 rats per group, as given below.

Group 0: control (no treatment)

Group B_{12}: 1 mg/kg intraperitoneal cyanocobalamin for 28 days

Group E: 100 mg/kg intraperitoneal Alpha-tocopherol for 28 days

Group B_{12}+E: 1 mg/kg intraperitoneal cyanocobalamin and 100 mg/kg Alpha-tocopherol for 28 days

The treatment dose was calculated according to the weights of the rats each day during the study protocol.

Functional Evaluation

Following the completion of medication process at the end of the 28 days, walking tract analysis (WTA) was performed. Functional outcomes were evaluated using sciatic functional index (SFI) measurements.

The most important measurement of nerve healing is functional recovery. Electromyography (EMG) may not show a connection between functional improvement and the healing date (15).

An axon can grow approximately 1–3 mm per day (7). Therefore, the SFI, which is measured by the WTA, is one of the most widely used and valuable assessment methods.

The SFI was first presented in 1982 (7). It is used to calculate the percentage of loss of function in the sciatic nerve of the damaged side when compared to the uninjured side. Both hind legs were pressed with a black ink impregnated stamp on absorbent papers and the SFI, which is a mathematical formula that measures SN malfunction, was measured from the obtained footprints in a 50-cm-long walking corridor (Figure 1). During the placement of the rats in the walking corridor, the walking was repeated until a proper footprint was obtained in the event of distribution of the ink because of urination or walking.

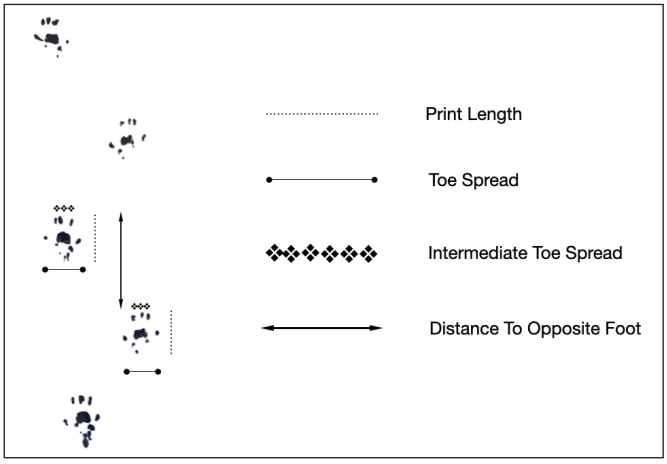

Figure 1: SFI parameters from rat footprints obtained from the walking corridor.

Below is the SFI formula:

SFI = [(ETOF – NTOF)/NTOF + (NPL – EPL)/EPL + (ETS – NTS)/NTS + (EIT 2 NIT)/NIT)] 220/4.

Here, TOF is the measurement from the most extreme point of a foot to the other end of the other foot; PL is the footprint length; TS is the toe spread, which is the measurement from first to the fifth toe); and IT is the intermediate toe spread, which is the length from second to the forth toe (7). E refers to the experimental side and N refers to the normal (uninjured) side. The largest values of the footprint measurements were used for the SFI parameters. The SFI gives a numerical value between –100 and 0, in which 0 represents normal function of the SN, while –100 represents the full-thickness cut of the SN (Figure 1) (13).

The SFI was measured by the first author for all of the subjects. The rats were numbered and mixed randomly and the person who measured SFI was blind to the treatment procedure that the rats were given. All of the measurements were made blindly to avoid bias. Obtained values were analyzed statistically.

Histopathological Investigation

After completion of the WTA, SN biopsies were taken from both the injured left SN and uninjured right SN. SN segments between 10-mm distal and 20-mm proximal to the bifurcation were excised non-traumatically. Damaged and undamaged sciatic nerve tissue samples of the 2 sides were used for the histopathological examinations for AxD, AL (vacuolization), and EI. For the histopathological examinations, the hematoxylin-eosin and toluidine blue staining methods were utilized. AxD, AL, and EI (between 0% and 100%) were analyzed under light microscope with comparison to the uninjured SN segments and the damage levels were scored by dividing them into 10% intervals.

The second author performed the pathological evaluations for all of the rats. Biopsy materials were numbered and mixed randomly, and the pathologist was blind to the groups and treatment procedures that the rats were given to avoid bias.

Ethics

Approval for this study was obtained from the Local Ethics Committee of the Experimental Animals Laboratory of the Hospital, (2015-08). All of the rules of law on the protection of experimental animals of The Ministry of Agriculture and Rural Affairs and ethical standards of the 1964 Helsinki declaration were followed.

Statistical Analysis

The NCSS 2007 software package (Utah, USA) was used. One-way analysis of variance, the Tukey multiple comparison test, Friedman test, and Dunn multiple comparison test were used for the normally distributed variables, subgroups, non-normally distributed data, and subgroups, respectively. $p<0.05$ and $p<0.001$ were accepted as statistically significant and very significant, respectively.

RESULTS

A comparison of the mean SFI, AxD, and EI levels among the groups was conducted. The SFI ($p<0.0001$), AxD ($p=0.02$), and EI ($p=0.016$) levels showed statistically significant differences among all of the groups (Table I).

Table II presents the comparison of the SFI, AxD, and EI levels among the groups.

In terms of the SFI, groups B_{12}, E, and B_{12}+E were significantly higher than group 0 ($p=0.011$, $p=0.035$, $p=0.0001$, respectively). However, groups B_{12} and E were not statistically different. ($p=0.961$). Group B_{12}+E was statistically significantly higher than groups B_{12} and E ($p=0.048$, $p=0.015$, respectively).

A comparison of the AxD levels among the groups is seen in Figure 2A-D, where it can be seen that the only significant difference was between groups 0 and B_{12}+E ($p=0.016$).

Figure 3A-D shows the AxD and EI levels in the different rats. Groups B_{12} and B_{12}+E exhibited statistically significantly decreased EI levels when compared to group 0 ($p=0.019$ and $p=0.048$, respectively); however, the mean EI levels were not significantly different among the groups ($p>0.05$).

DISCUSSION

PNI is a challenging health problem with effects that can range from mild discomfort to life-long impairment according to the level of neural involvement (10). PNI can be caused by compression, crush, or transection injuries (17). It can either be acute or chronic, and complete or incomplete (17). Approximately 1.8% of patients who have had lower extremity trauma suffer from PNI (12). It can cause motor and/or sensorial dysfunction, social and psychological problems, and loss of work-power (18).

Although repair is slow and often insufficient, the destroyed axons can be regenerated in the PNS, which is known as PNR. PNI causes endothelial damage and inflammation, which leads to an increase in the permeability of the vessels and edema; thus, neutrophils release chemical mediators, and reactive oxygen species (ROS) and cytokines are produced (14,16,19).

Table I: Comparison of Mean SFI Levels, AD and EI Among Groups

Mean	Group 0	Group B12	Group E	Group B12+E	p
SFI	-36.05 ± 9.04	-26.88 ± 5.3	-28.21 ± 2.6	-19.43 ± 1.3	**0.0001**
AxD (%)	36.25 ± 7.44	25 ± 9.26	26.88 ± 11	21.88 ± 7.53	**0.02**
EI (%)	19.38 ± 13.21	8.13 ± 0.35	15 ± 5.35	9.88 ± 0.35	**0.016**

SFI: *Sciatic functional index,* ***AxD:*** *Axonal degeneration,* ***EI:*** *Edema/inflammation.*

Table II: Tukey Multiple Comparisons of SFI, AxD and EI

	SFI	AxD (%)	EI (%)
Group 0/B12	0.011	0.078	0.019
Group 0/E	0.035	0.178	0.615
Group 0/B12+E	0.0001	0.016	0.048
Group B12/E	0.961	0.975	0.239
Group B12/ B12+E	0.048	0.896	0.96
Group E/ B12+E	0.015	0.68	0.487

SFI: *Sciatic functional index,* ***AxD:*** *Axonal degeneration,* ***EI:*** *Edema/inflammation.*

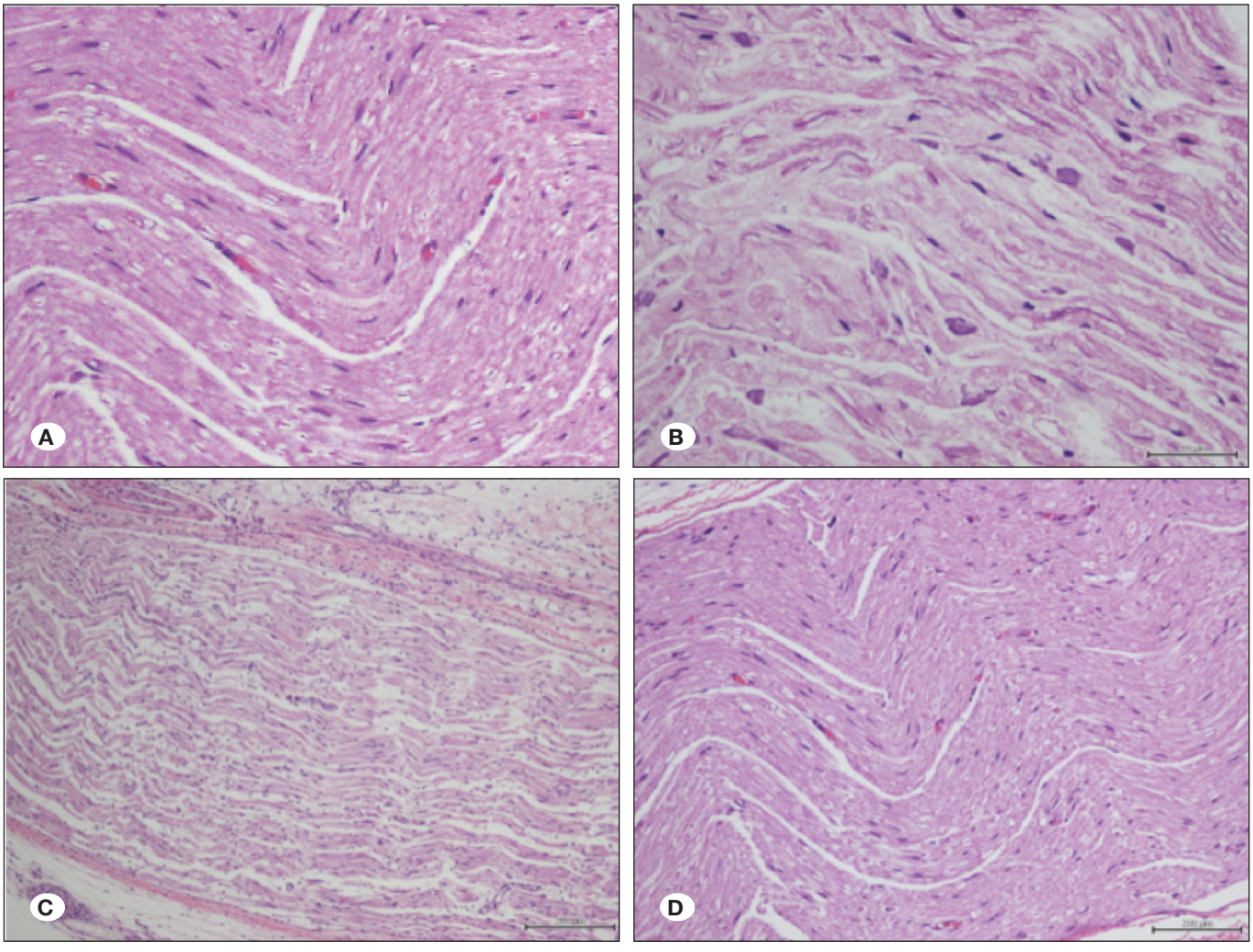

Figure 2: A) Unclamped (normal) sciatic nerve (H&E, x110); **B)** AxD, 30% degeneration, B_{12} group, H&E, x440; **C)** AL and AxD, 30% degeneration, E group, H&E, x440; and **D)** minimally damaged nerve preparation, B_{12}+E group, H&E, x220. (**H&E:** hematoxylin and eosin).

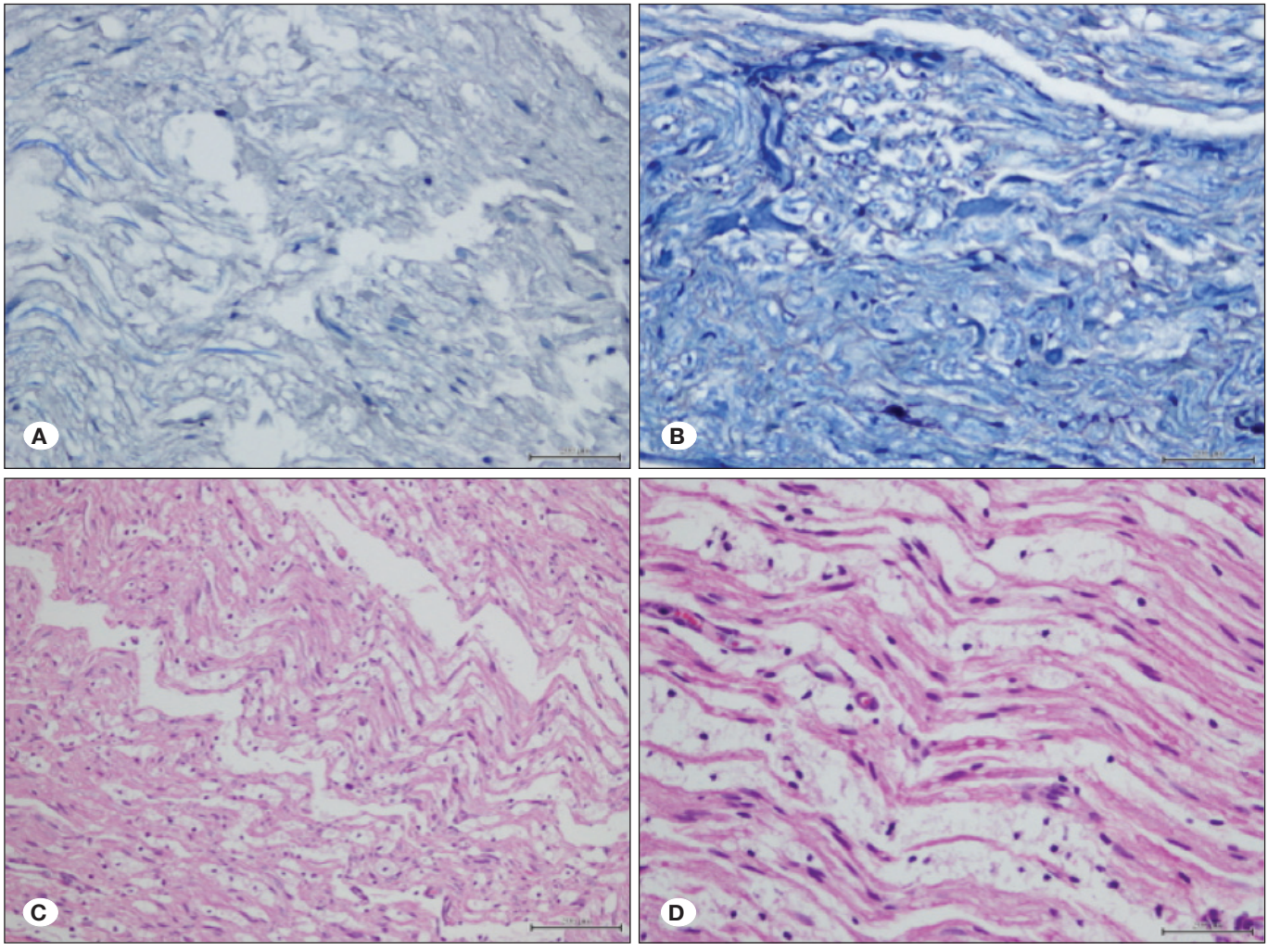

Figure 3: A) AxD and edema, control group, toluidine-blue x440; **B)** significant AxD and AL in group 0, toluidine-blue x440; **C)** axon vacuolization and edema H&E, x220; and **D)** significant edema and axon vacuolization and inflammation H&E, x440. (**H&E:** hematoxylin and eosin).

As the PN is ensheathed by Schwann cells, which provide trophic support by nerve growth factors (NGFs), the PNS, unlike the central nervous system, has the ability to regenerate (4). The PNR starts from the demyelinated site where contact occurs between the Schwann cells and the axons. The PNR is ensured by the migration of macrophages into the injured area, where contact occurs between the Schwann cells and the axons, and elimination of the demyelinated area takes place. The capability of the PNR is directly related to neurotrophic factors, such as NGFs, which are located on the cell membrane (10).

If the injury causes 20%–30% AxD, the main PNR mechanism is undamaged axon collaterals by the NGFs is; however, if the injury causes more than 90% AxD, Wallerian degeneration, axonal restoration, and target organ innervation are the 3 main processes of the PNR, and the lack of success in 1 of the 3 processes contributes to inadequate clinical healing (17). The degree of nerve injury, severity of the lesion, length and size of damaged nerve segment, and duration of exposure are the factors that play role in progressive nerve healing. In addition, oxidative stress and the massive production of ROS play a major role in neuronal cell death after PNI (5,13).

Although the mechanisms of PNI and PNR are well-known, fully functional recovery is still limited. There is no complete agreement regarding the treatment protocol for PNI. The main goals of PNI treatment are to accelerate healing and ensure that the healing is nearly complete and to prevent complications and other possible sequelae. PNI can be classified as neuropraxia, axonothymesis, neurotomies, or complete nerve cut (22). Microsurgery is main standard course of therapy in full-thickness nerve cuts; however, the success rate is mostly very limited. As a result, medical supplementations have been attempted in incomplete PNI. As PNR occurs in the first weeks of injury, surgery or medication should be initiated at the earliest possible time (11).

Vitamins have significant roles in nerve transmission (25). Since vitamin B_{12} has antioxidant, antiapoptotic and antinecrotic effects, it has been shown to protect deoxyribonucleic acid, increase muscle cells, and provide nervous tissue formation.

Vitamin B_{12} induces growth factors and supplies myelin structure and axonal growth (25,28).

Vitamin E is presumed to have a principal role in protecting membrane lipids from lipid peroxidation. Vitamin E derivative supplementation treatment in PNI in diabetic rats was observed to have decreased oxidative-nithodative stress markers and improved demyelination and nerve blood flow (17). Cisplatin neurotoxicity and diabetic and surgical neuropathies were found to be prevented by vitamin E supplements (27). In addition, vitamin E was found to decrease inflammatory mediators and nociceptive signaling molecules in PNI (17). The combined use of vitamin E and other antioxidant vitamins, such as vitamins C and A, was shown to have decreased the oxidative markers of rodents (20). Local vitamin E administration has shown significant improvement and acceleration of PNR, and facilitated the growth and regeneration of injured neurons (21).

In the current study, vitamins B_{12} and E, and both together, were found to be effective in the functional recovery of the PNI. The efficacy of vitamin E was found to be as effective as that of vitamin B_{12} in functional recovery, which was in agreement with the literature. According to the SFI, the combination of vitamins B_{12} and E was more effective than that of vitamins E and B_{12} separately. The SFI was higher in the combination treatment when compared with that of vitamin B_{12} alone, which indicated that vitamin E improved the healing effects of vitamin B_{12}. In the histopathological evaluation; vitamin E was not effective in the treatment of AxD or EI by itself. Although vitamin B_{12} was effective in the treatment of EI, it was ineffective in the treatment of AxD by itself. However, the combination of these 2 vitamins decreased both AxD and EI. This supported the additive effects of these vitamins, wherein they can reverse neurological injury together.

This study had some limitations, as it did not include an electrophysiological evaluation, such as EMG. However, functional evaluation of the PN is much more important than electrophysiological evaluations. In literature, it was reported that the use of electrophysiological test was not correlated with functional outcomes, as assessed by the WTA, in rats (8).

CONCLUSION

Vitamins B_{12} and E were both effective in the functional recovery of PNI. The antiinflammatory healing effects were mainly the result of vitamin B_{12}. Neither vitamins B_{12} nor E were effective in axonal regeneration by themselves; however, the combination of these 2 vitamins was effective. The results suggested that vitamin E was effective in PNI treatment, especially when it was combined with vitamin B_{12}. The current study is the only research that has compared the functional and histological recovery outcomes of the separate and combined use of cyanocobalamin and alpha-tocopherol on a nerve injury model. These 2 vitamins are cheap and easy to find, and may be helpful in the treatment of PNI. It is our belief that the combination of these 2 vitamins can be used in the treatment of PNI, especially after future studies have been conducted on humans.

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