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The Healing Effect of Digoxin on Peripheral Nerve Damage and Its Relation to IL-17/IL-10

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ABSTRACT

AIM: To demonstrate the curative effect of digoxin on peripheral nerve damage with its anti-inflammatory role on interleukin (IL)-17.

MATERIAL and METHODS: The study was conducted with 30 male Sprague Dawley albino mature rats, of which 10 formed the control group, 10 were surgically treated and administered saline (group S), and another 10 were surgically treated and administered digoxin (group D). Motor functions and immunohistochemical and biochemical variables of the rats were assessed after therapy.

RESULTS: The amplitude of the inclined plane test scores and the compound muscle action potential levels were greater in group D than in group S. Likewise, there were higher nerve growth factor percentages, higher axon counts, and lower fibrosis score percentages in group D than is group S. Lastly, lower tissue malondialdehyde and plasma IL-17 levels were determined in group D, while the IL-10 level was higher.

CONCLUSION: Digoxin contributes to nerve healing and neuroprotective effect by demonstrating its anti-inflammatory effect on IL-17. It can be considered an adjunctive therapy for peripheral nerve injury.

KEYWORDS: Digoxin, Peripheral nerve injuries, Sciatic nerve, IL-17, Experimental animal models

ABBREVIATIONS: CMAP: Compound muscle action potential, **NGF:** Nerve growth factor, **MDA:** Malondialdehyde, **EMG:** Electromyography, **H&E:** Hematoxylin-eosin, **DAB:** 3,3' diaminobenzidine, **RORγt:** Retinoic acid-related orphan nuclear receptor gamma thymus

INTRODUCTION

Peripheral nerves are vulnerable to traumatic injury because they extend throughout the body (8). The injury creates a heavy social burden regarding long-term disability and economic costs although it is not life-threatening (29). The gold standard treatment method, especially after mechanical trauma, is an end-to-end repair. However, nerve healing depends on many factors independent of the surgical technique since peripheral nerves have a complex structure (19,22). There are many unknown factors in recovery in addition to mechanical factors (14).

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Congestive heart failure and chronic respiratory diseases are both common causes of digoxin, which is a cardiac glycoside (16). Digoxin is proven to have anti-inflammatory effects even when used to treat bacterial pneumonia (7).

Interleukin (IL)-17 is an essential proinflammatory cytokine for neutrophil activation (23). This study aimed to identify treatments that can specifically suppress IL-17, which is implicated in the onset and progression of a wide range of autoimmune and chronic diseases, and modify its proinflammatory effects (31). Previous studies have revealed digoxin to inhibit IL-17 levels (15).

Identifying novel processes and therapeutic techniques for peripheral nerves that have been mechanically damaged is critical. Considering the anti-inflammatory activity of digoxin through interleukins, this study aimed to demonstrate its neuroprotective and healing effects in peripheral nerve damage with electrophysiological, motor function tests, and immunohistochemical analysis.

MATERIAL and METHODS

Animals

This study included 30 male Sprague Dawley albino adult rats weighing between 200 and 220 g. We fed them freely and housed them in pairs in temperature-controlled steel cages with 12-hour light/dark cycles at a constant temperature of 22 ± 2 °C. The Animal Ethics Committee authorized the experimental techniques that are used in this work (17210902).

Experimental Protocol

The rats for the experiment were given by an experimental animal facility. All research was conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals (USA).

The surgical dissection and repair of the sciatic nerve were done on twenty rats selected as experimental groups. No surgical procedure or pharmaceutical therapy was administered for the control group (n=10). The 20 remaining rats were divided into two experimental groups. Postoperatively, rats allocated to group S (surgery + saline) (n=10) orally received 1 ml/kg of 0.9% sodium chloride saline. Postoperatively, group D (surgery + digoxin) (n=10) were orally given 100 g/kg/day of digoxin (0.25 mg oral tablets, Novartis). All drugs were maintained for an additional 12 weeks. A motor function was evaluated after 12 weeks. Electromyography (EMG) recordings were taken following the motor function test. The mice were then executed and the sciatic nerve tissues were removed for immunohistochemistry analysis after the blood was collected from a tail vein (11).

Surgery

We intraperitoneally administered 75 mg/kg of ketamine (Alfamine, Alfasan International B.V. Holland) and 10 mg/kg of xylazine (Alfazyne, Alfasan International B.V. Holland) to rats to produce general anesthesia. The rats were then secured to the table and placed on their backs, with their heads dangling over their shoulders. The aseptic method was utilized to dissect the bilateral sciatic nerves from the sciatic notch to the nerve trifurcation, which was 1 cm away. A 3–3.5-cm long nerve section above the trifurcation was carefully removed to isolate the sciatic nerve from the neighboring soft tissue. Once the nerves had been resected, they were removed using micro cutters that are positioned 1.5 cm above their trifurcation point (i.e., starting point of the caudal sural cutaneous, tibial, and common peroneal nerves). Ethilon® 9-0 (Ethicon, USA) was utilized by the same surgeon to restore the nerves. After the incision was closed with 3-0 Vicryl®, the rats were left to recover on their own (Ethicon, USA). Rats were transferred to their cages after recovering from anesthesia and given unrestricted access to food and water (18).

Electrophysiological Recordings

A combination of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg) was used to anesthetize the rats. Electrophysiological measurements (EMG studies) were performed after the examination. We stimulated the right sciatic nerve three times to its supramaximal potential using a bipolar needle electrode for subcutaneous stimulation (BIOPAC Systems, Inc, Santa Barbara, CA) from the sciatic notch (period: 0.05 ms, sampling rate: 40 kHz/s, 1 Hz frequency, in the range of 0.55500 Hz, 10 V intensity). Compound muscle action potentials (CMAPs) were obtained using unipolar platinum electrodes from 2–3 interosseous muscles. This was done using Biopac Student Lab Pro v.3.6.7 program (BIOPAC Systems, Inc.), using CMAP distal latency and amplitude parameters as the variables. The body temperatures of each rat were kept at 36–37°C on a heating pad throughout the experiment in addition to employing a rectal sensor (HP Viridia 24-C; Hewlett-Packard Company, Palo Alto, CA) to monitor the rats' rectal temperatures throughout the EMG recordings. All trials were conducted between 10:00 a.m. and 2:00 p.m. (17).

Assessment of Motor Function

An inclined-plate test was performed by Rivlin and Tator to measure the rats' motor function (34). The rat was placed perpendicularly to the long axis of the sloped plate. The beginning angle of the slanted plate was 10°. The motor function score was computed as the maximum angle at which the rat held its position for five seconds without slipping as the inclination angle increased. The inclined-plate score is measured to obtain an average result thrice in each rat.

Quantitative Immunohistochemistry and Histology

Rats were given a 4% formaldehyde intracardiac perfusion for histology and quantitative immunohistochemistry evaluations. A microtome (Leica RM 2145, Nussloch, Germany) was used to slice the paraffin-embedded sciatic nerves into 5-m slices and mark the axons with hematoxylin and eosin (H&E). Olympus C-5050 camera module on an Olympus BX51 microscope (Tokyo, Japan) was used to measure the epineurium thickness of the sciatic nerve and view dyed tissue sections. The Image-Pro Express 4.5 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the perineural layer thickness in the grafts' core areas, the total number of axons, and the fibrosis level over these regions in the histological specimens.

Cells were counted in at least five randomly selected areas to determine the fibrosis degree. The percent fibrosis score was calculated by dividing the total number of cells in the region by the number of enumerated cells.

Eliminating the endogenous peroxidase function of the samples through 10% H_2O_2 for 30 min was necessary before blocking them with normal goat serum (Invitrogen) for 1 h at room temperature for the immunohistochemical analysis. Next, sections were incubated at 4 °C for 24 h with a specific primary antibody (Santacruz Biotechnology; 1/100) against nerve growth factor (NGF). The rabbit immunoglobulin G-specific Histostain-Plus Bulk kit (Invitrogen) was used to detect antibodies, and 3,3'-diaminobenzidine (DAB) was utilized to evaluate the result. Each slice was washed with phosphate-buffered saline and examined with an Olympus BX51 microscope. Digital photographs were shot via an Olympus C-5050 camera. Quantitative immunohistochemistry was performed on all groups and six slices from each animal. Two blinded investigators determined the total count of immune-positive Schwann cells and axons using a light microscope with different magnifications of 10× and 20×. Data were presented as the mean ± standard error of the mean.

Measurement of Plasma IL-10 and IL-17 Levels

IL-10 and IL-17 plasma levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Cusabio Biotech, Ltd., Wuhan, China). The plasma samples were diluted in 1:2 according to the manufacturer's instructions, and the IL-10 and IL-17 levels were tested twice.

Measurement of Lipid Peroxidation

Malondialdehyde (MDA) concentrations in tissue samples, as thiobarbituric acid reactive compounds, were used to detect the lipid peroxidation of nerve tissue. Trichloroacetic acid and the TBARS reagent were briefly added and then stirred for 60 min at 100 °C. Samples were centrifuged for 20 min at 3,000 rpm after being cooled on ice, and the supernatant absorbance was assessed at 535 nm. Tetra ethoxy propane standard calibration curves were used to measure MDA concentrations, which were then expressed as nmol/gr protein.

Statistical Analysis

IBM Statistical Package for the Social Sciences version 20 was used to conduct the statistical analysis. Each group's data was presented as the mean ±standard deviation. Multiple comparisons were subjected to the one-way analysis of the variance. A p-value of <0.05 was considered statistically significant.

RESULTS

Evaluation of Motor Function

The inclined plane test in a sciatic nerve damage model was used to assess motor function after the end of the research. We found that inclined plane test angles were statistically lower in group S than they were in group C compared to the control group. Climbing angles (44.8 ± 6.2) in group S were significantly lower than those of the control group (86.2 ± 4.3), while group D managed to climb to significantly higher angles (71.4 ± 8.3) on the inclined plane (p<0.001) (Table I).

Electrophysiological Recordings

A significant difference was seen between group S (2.2 ± 0.3) and the control group (11.9 ± 1.5) when it came to the CMAP amplitudes (p<0.001). A significant difference in CMAP latency was seen between group S (3.6 ± 0.2) and the control group (2.4 ± 0.3) (p<0.05). Additionally, CMAP amplitudes were significantly higher in group D (5.7 ± 0.6) than in group S (2.2 ± 0.3) (p<0.001). No significant difference was determined between groups D (3.5 ± 0.2) and S (3.6 ± 0.2) considering the CMAP latency (Figure 1 and Table I).

Evaluation of Histopathology and Immunohistochemistry

The axon count was determined after the sciatic nerve had undergone Wallerian degeneration. A significant decrease was determined in the axon numbers of group S (219.3 ± 35.8) than the control group (1340.4 ± 116.4) (p<0.001). There was a statistically significant difference in axonal density between groups D and S (p<0.0001), with group D having 785.2 ± 68.3 and group S having 219.3 ± 35.8. (Table II).

Sciatic nerve specimens from group S (73.5 ± 6.2) exhibited a significantly greater fibrosis score than those from the control group (1.9 ± 0.2) (p<0.001) (Table II, Figure 2). Similarly, group D (14.1 ± 4.1) exhibited a greater fibrosis level than the control group (1.9 ± 0.2). However, this increase was significantly less than that in group S (73.5 ± 6.2) (p<0.001).

The expression of NGF in Schwann cells was studied by immunostaining, and the value was statistically significantly lower in group S (8.1 ± 2.5) compared to the control group (35.4 ± 7.2) (p<0.01). A significant increase (p<0.01) was observed when group D (20.8 ± 3.7) was compared to group S (8.1 ± 2.5) (Table II, Figure 2).

Table I: The Comparison of Motor Functions and EMG Values Between the Groups

	Control Group	Group S	Group D
EMG CMAP latency (ms)	2.4 ± 0.3	3.6 ± 0.2 *	3.5 ± 0.2
EMG CMAP amplitude (mV)	11.9 ± 1.5	2.2 ± 0.3†	5.7 ± 0.6‡
Inclaned plane score (°)	86.2 ± 4.3	44.8 ± 6.2†	71.4 ± 8.3§

** p<0.05, † p<0.001 Group S compared with Control Group, ‡ p<0.001, § p<0.001 Group D compared with Group S.*
***EMG:** Electromyography, **CMAP:** Compound muscle action potential.*

Table II: The Comparison of Histological and Immunohistochemical Evaluation Between the Groups

	Control Group	Group S	Group D
NGF immunexpression on Schwann cell (%)	35.4 ± 7.2	8.1 ± 2.5*	20.8 ± 3.7‡
Total axon number	1340.4 ± 116.4	219.3 ± 35.8†	785.2 ± 68.3§
Fibrosis score (%)	1.9 ± 0.2	73.5 ± 6.2†	14.1 ± 4.1§

** p<0.01, † p<0.001 Group S compared with Control Group, ‡ p<0.01, § p<0.0001 Group D compared with Group S.*

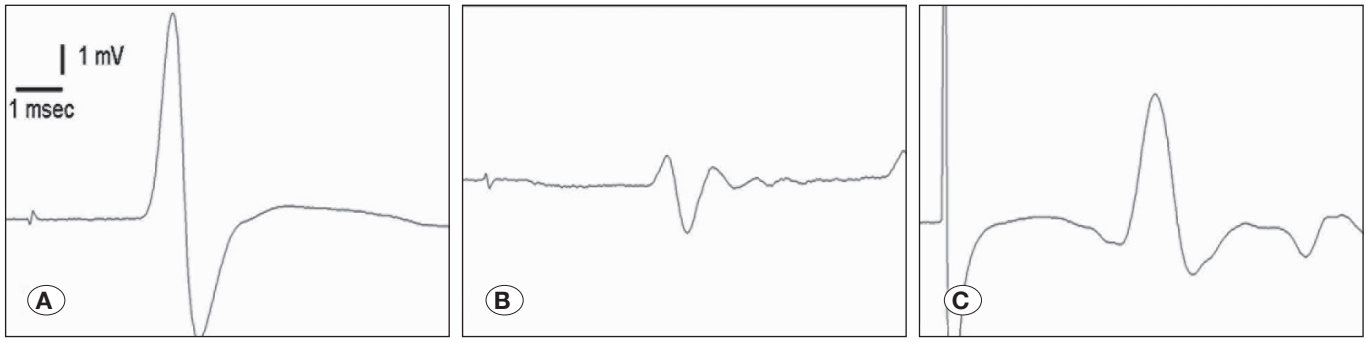

Figure 1: Electromyography of **(A)** control group, **(B)** Group S, and **(C)** Group D.

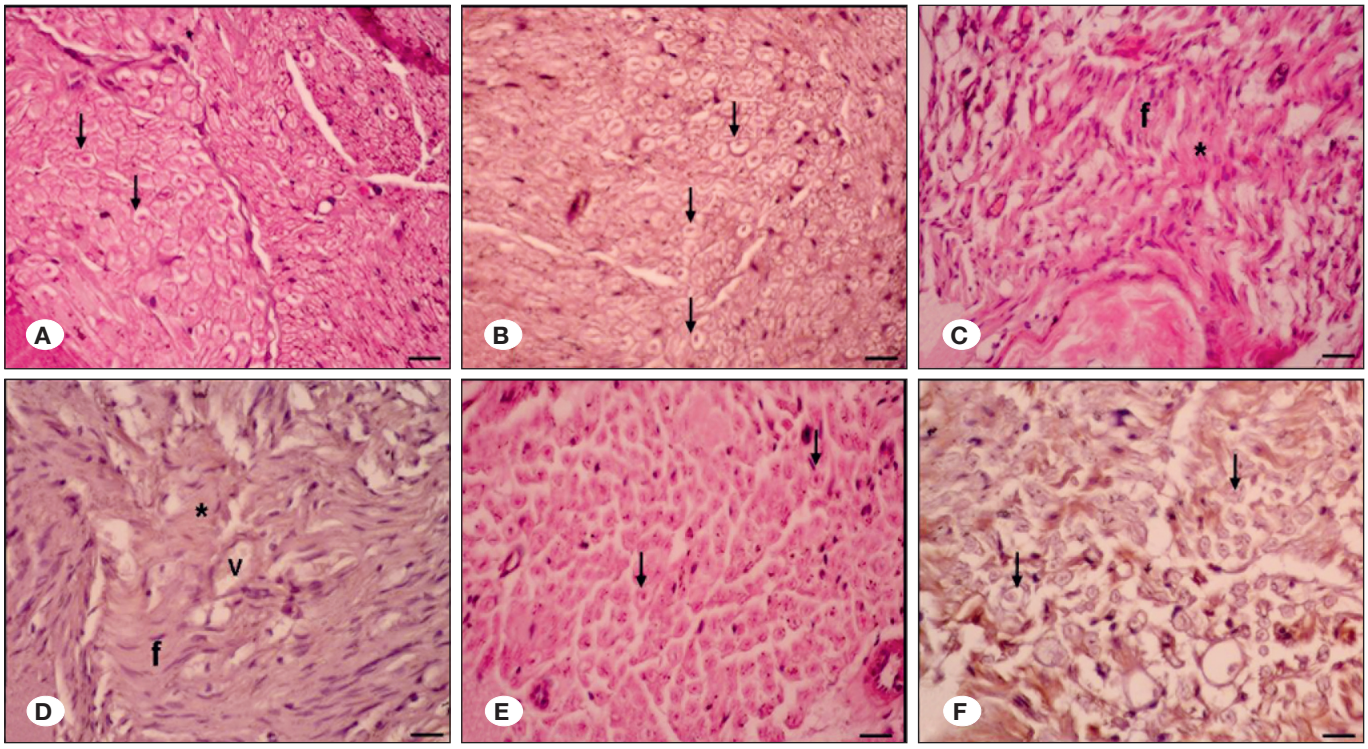

Figure 2: x20 Magnification. Hematoxylin & Eosin and NGF immunostaining. **A, B)** Control Group. Normal axon and schwann cell (arrow). **B-D)** Group S. Increased fibrosis **(F)** and vascularization (v) were indicated. Very diminished axon, Schwann cell, and NGF immunoexpression (asterisk). **E,F)** Group D. Increased axon, Schwann cell, and NGF immunoexpression (arrow).

Table III: The Comparison of Biochemical Evaluation Between the Groups

	Control Group	Group S	Group D
MDA (nmol/μgr)	112.7 ± 8.6	205.4 ± 18.4†	155.2 ± 11.9§
Plasma IL-17 (pg/ml)	14.2 ± 0.8	24.6 ± 1.5*	17.9 ± 0.4‡
Plasma IL-10 (pg/ml)	218.6 ± 10.1	165.4 ± 7.7*	381.2 ± 16.3§

** p<0.01, † p<0.001 Group S compared with Control Group, ‡ p<0.01, § p<0.001 Group D compared with Group S.*

Evaluation of Lipid Peroxidation

MDA is a measure of oxidative stress that was significantly greater in group S (205.4 ± 18.4) than in the control group (112.7 ± 8.6) (p<0.001). MDA levels in group D (155.2 ± 11.9) were significantly lesser than in group S (205.4 ± 18.4) (p<0.001) (Table III).

Evaluation of Inflammatory Markers

Our study indicated a statistically significant increase in plasma IL-17 levels (24.6 ± 1.5) in group S compared to the control group (14.2 ± 0.8). These levels were significantly lower in group D (17.9 ± 0.4) than in group S (24.6 ± 1.5). IL-10 had a similar pattern of behavior. IL-10 levels in group D (381.2 ± 16.3) were significantly higher than in group S while plasma IL-10 levels in group S (165.4 ± 7.7) were lower than in the control group (218.6 ± 10.1).

DISCUSSION

The neural tissue and the microvascular structure of this tissue degrade following the mechanical deterioration in peripheral nerve damage. The microcirculation is interrupted after endothelial injury to the microvascular structure, resulting in the release of free oxygen radicals. A lack of antioxidative enzymes and impaired microcirculation make it harder to remove free oxygen radicals. Hence, the establishment of a favorable environment for nerve repair is prolonged (2,8).

Preliminary studies have shown that digoxin inhibits the function of the retinoic acid-related orphan nuclear receptor (RORt) gamma thymus and reduces Th17 cell growth and IL-17 production (9). Th17 cells are effective inflammation inducers, with a greater role in autoimmune and chronic inflammatory disease pathogenesis (21). Prophylactically, digoxin has been demonstrated to decrease IL-17 synthesis *in vivo* and have a role in the inflammatory response (25).

IL-17 is a proinflammatory cytokine that is vital for neutrophil activation (23). Therapeutic drugs that precisely inhibit IL-17 production, which is a cytokine that has a proinflammatory function implicated in the etiology of autoimmune and chronic inflammatory illnesses, and inhibit its proinflammatory effects have been the topic of investigations. The transcription factor that regulates the IL-17 synthesis is known as retinoid-related orphan receptor γt (RORγt) (4,24,31). Digoxin effectively reduces the IL-17 synthesis in the bloodstream because it antagonizes RORγt function, and digoxin treatment is proven specific to the IL-17 pathway (15).

McGill et al. revealed that digoxin administration reduces the IL-17 synthesis *in vivo*, and the anti-inflammatory activity was demonstrated through IL-17 (25). Vieira et al. compared digoxin derivatives in their study according to their anti-inflammatory activities, and IL-17 suppression was determined (35). Tani et al. revealed that digoxin administration increased IL-10 levels in rat colonic mucosa while decreasing mRNAs for Th17-related cytokines (32). In our study in which we created peripheral nerve damage, obtained findings support other studies. There was a significantly increased IL-10 level in group D, but a significant decrease in IL-17 levels in group S. These data have confirmed digoxin's anti-inflammatory effect.

The inclined plane test was utilized in our study to assess the motor functions of experimental animals. Results revealed that the obtained data were higher in group D than in group S.

Electrophysiological parameters were evaluated with EMG during nerve recovery. Electrophysiological tests are the most often utilized assessment tool in peripheral nerve damage models (28). Amplitude is the value that gives information about the sensory-motor fibers and the size of the innervated motor units and is measured by CMAP values (5). Our study revealed a slightly lower CMAP latency in group D than in group S. However, CMAP amplitude was significantly higher when the same groups were compared. The increased number of motor units innervated by regenerated axons is reflected in the elevated CMAP amplitudes.

Several studies have demonstrated the growth factor values in the healing and regeneration of damaged nerve tissue (27). One of these growth factors that play a critical function in developing and regenerating the peripheral nervous system is NGF (12). NGF is a signaling molecule that belongs to the family of neurotrophins and is essential for the formation, function, and survival of brain cell to have neurotrophins (3). The loss of neuronal cells in Schwann cells, which contribute to axonal extension and myelination, is linked to the NGF immunoexpression percentage (10). The literature revealed no evidence that digoxin affects NGF immunoexpression. Therefore, we histologically and immunohistochemically examined the percentages of fibrosis, NGF, and axon counts in Schwann cells to determine nerve repair. This study revealed that group D had a greater NGF%, a greater axon count, and a decreased fibrosis score% compared to group S. This suggests that group D had superior neuron recovery.

The nerve healing process has been influenced by microenvironmental factors as well as cellular and molecular activity

(30). Ischemia-reperfusion injury leads to microenvironmental disruption through neutrophil and macrophage activation and increased mitochondrial oxidative stress. Accordingly, the nerve healing process after nerve damage is adversely affected (6). Lipid peroxidation (LPO) is a crucial indicator of free oxygen radical-induced oxidative stress. Meanwhile, MDA is an LPO marker, and a rise in MDA levels suggests greater oxidative stress and microenvironmental deterioration (20). Our investigation found that group D had significantly lower MDA levels than group S. This result indicates that digoxin contributes to nerve healing by regulating the microenvironment with its anti-inflammatory effect and reducing oxidative stress.

The anti-inflammatory factor IL-10 is crucial in the inflammation that arises after peripheral nerve injury (13). A peripheral nerve injury results in an inflammatory response because of the fast proinflammatory cytokine production by macrophages and Schwann cells (26). IL-10's role is to progressively diminish and eliminate inflammation while promoting a favorable microenvironment for peripheral nerve healing (33). Atkins et al. observed that IL-10 reduces scar formation after nerve damage and enhances axonal regeneration in their studies that demonstrated the effects of IL-10 on sciatic nerve recovery (1). Huang et al. revealed that saikosaponin reduces scar formation over IL-10 after sciatic nerve injury (13). Group D had significantly greater IL-10 levels than group S in our investigation. This condition corroborates previous research as well as the neuroprotective impact of digoxin.

Our study has several limitations. Currently, there is no evidence to support the use of digoxin to treat sciatic nerve damage. However, studies proved digoxin's anti-inflammatory efficacy over IL-17 or examined IL-17 levels in peripheral nerve injury. In our study, we had difficulty comparing our study findings with the literature since we intended to examine the neuroprotective and healing properties of digoxin over IL-17 in peripheral nerve damage. This is the first limitation of our study. Another limitation is the limited number of utilized animals due to ethical issues because the study was conducted on animals. Therefore, additional research that includes more subjects and tries to compare digoxin to alternative therapy options for peripheral nerve damage is necessary.

CONCLUSION

In conclusion, our study demonstrated the beneficial effect of digoxin's anti-inflammatory properties on nerve repair in peripheral nerve injury. Group D recovered much faster than the placebo group. Simultaneously, inflammatory cytokine level alterations were statistically significant. This research suggests that digoxin might be used to help treat peripheral nerve injuries. However, additional comparative research is required on this issue.

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AUTHORSHIP CONTRIBUTION

Study conception and design: OE
Data collection: MAE
Analysis and interpretation of results: MAE
Draft manuscript preparation: MAE, GG
Critical revision of the article: GG
Other (study supervision, fundings, materials, etc...): OE, HK, GY, CK, GK
All authors (GG, MAE, GK, HK, GY, CK, OE) reviewed the results and approved the final version of the manuscript.

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