Original Article

Comparative Histological Study on the Effect of Erythropoietin Versus Curcumin on the Sciatic Nerve Crush Injury in a Rat Model

Manal Ali Abdel Mohsen, Salwa Metwaly Ali, Nagla Mohamed Salama and Enas Abdallah Ali Ahmed

Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt

ABSTRACT

Background and Aim of Work: Peripheral nerve injuries are frequently confronted in clinical practice due to several reasons such as surgery or accidental trauma. They are often associated with poor nerve regeneration and inadequate functional recovery. Therefore, this study was planned to evaluate and compare the possible effect of erythropoietin (EPO) versus curcumin on crush injury of the sciatic nerve in rat model.

Material and Methods: Seventy-eight adult male albino rats were randomly allocated into 5 groups: control sham operated, sciatic nerve injury (SNI), untreated, SNI+EPO and SNI+curcumin. SNI was induced via clamping the right sciatic nerve with a sterile tissue forceps for one minute. After 24hrs, SNI+EPO group was injected intraperitoneally with EPO (5000IU/kg) daily for two weeks. SNI+curcumin group was given curcumin (40mg/kg) daily orally for two months. The compound muscle action potential (CMAP) amplitudes were recorded at the end of each experimental period. Sciatic nerve specimens were then processed for hematoxylin and eosin, osmic acid stain, toluidine blue stain and immunohistochemical staining for neurofilament (NF). Number and diameter of nerve fibers, ratio of the myelin area to the nerve fiber area as well as area percent and optical density of NF were measured and statistically analyzed.

Results: Both EPO and curcumin treated groups exhibited improvement in the histological structure of the sciatic nerve as well as significant increase in CMAP. Also, morphometric measurements including; number and diameter of nerve fibers, ratio of the myelin area to the nerve fiber area as well as area percent and optical density of NF were significantly increased compared to both SNI and untreated groups. However, all measured parameters were significantly higher with EPO as compared to curcumin.

Conclusion: EPO promotes structural and functional recovery and enhances regeneration of crushed sciatic nerve better than curcumin.

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Key Words: Crush injury, curcumin, erythropoietin, neurofilaments, sciatic nerve.

Corresponding Author: Manal Ali Abdel Mohsen, MD, Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt, **Tel.**: +20 12817 26043, **E-mail:** dr.manal14@hotmail.com

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INTRODUCTION

Peripheral nerve injury (PNI) is considered a common global illness. It negatively affects patient's life quality, as it results in partial or complete function loss in organs supplied by the damaged nerve. In recent years, many clinical reports and studies denoted that functional recovery, particularly motor function, following surgical intervention is unsatisfactory[1,2].

Crush injury is one of the most common forms of peripheral nerve injuries. Such injury occurs from sudden considerable force applied to the nerve by blunt object as surgical clamp or any other crushing object which does not cause complete transection of the nerve. It could be also induced by motor vehicle accidents or blows resulting from an assault[3-5].

Unlike central nervous system, in which injured axons are usually not capable of regeneration, peripheral nerves can regenerate. However, this ability is limited, and spontaneous neuronal regeneration is markedly slow. Thus, irreversible impairment to structure and function of the target organs occurs sooner than axonal regeneration. Functional recovery of PNI is still far from satisfactory. Searching for new options that could accelerate neural regeneration is needed in order to alleviate patients' sufferings[2].

Erythropoietin (EPO) is a hormone released from kidneys and considered the major regulator of red blood cell formation. Its receptor was found in a large variety of non-haematopoietic tissues. This suggests other functions of EPO beyond its recognized erythropoietic role. EPO have potent anti-apoptotic, anti-inflammatory, neuroprotective and neurotrophic effects[6-8].

Curcumin is a yellowish powder extracted from turmeric (Curcuma longa), one of the most effective natural herbs. Curcumin was used in traditional medicine for centuries. Also, it has been used in food, cosmetics, pharmaceuticals and medicine. Numerous studies postulated that curcumin has potent biological effects such as anti-apoptotic, anti-inflammatory, antioxidant and neuroprotective properties[9].

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The present study was conducted to evaluate and compare the possible neural regenerative effects of EPO versus curcumin on sciatic nerve crush injury in rat model; using histological, immunohistochemical, morphometric and electrophysiological methods.

MATERIAL AND METHODS

Seventy-eight adult male albino rats (± 200 grams) (6-8 weeks age) were housed in the animal house of Kasr Al-Ainy Faculty of Medicine, Cairo University. They were treated in accordance with guidelines approved by the Animal Use Committee, Cairo University. Rats were housed under standard environmental conditions in stainless steel cages with free access to standard diet and water throughout the experimental period.

Animals were allocated into 5 groups

A) Group I (control group; n. 48): rats were subdivided into two subgroups:

Subgroup IA (n. 24):

Included apparently normal rats which did not receive any medication or underwent any surgical intervention throughout life. Six of them were sacrificed with group II. The other rats were divided into 3 subgroups, six rats each, and were sacrificed with groups III, IV and V.

Subgroup IB (sham-operated group) (n. 24):

Included rats that were sham operated. They underwent the same surgical procedure as the experimental groups but without sciatic nerve compression. Six of them were sacrificed with group II while another six rats were sacrificed with group III. Another six rats received intraperitoneal (i.p.) injection of 0.2 ml phosphate buffer saline (PBS) daily for two weeks (concomitant with group IV) and were sacrificed with group IV. The other six rats received orally 1ml corn oil daily for two months (concomitant with group V) and were sacrificed with group V.

B) Experimental Groups (n. 30):

All rats in these groups were subjected to right sciatic nerve crush injury (as described below), then they were divided as follows:

Group II (Sciatic Nerve Injury; n.6) (SNI):

These rats were subjected to right sciatic nerve crush injury, left without treatment and were sacrificed one week after induction of injury to detect the degeneration following the nerve crush injury.

Group III (Untreated Group; n.12):

24 hours after induction of injury, rats were subjected to the following procedures:

Subgroup IIIA (n. 6):

Six rats received intraperitoneal injection of 0.2 ml PBS daily for two weeks (concomitant with group IV). They were sacrificed two months after initiation of PBS administration to detect spontaneous regeneration without treatment.

Subgroup IIIB (n. 6):

Six rats received orally 1ml corn oil (solvent of curcumin) daily for two months and then they were sacrificed two months after initiation of corn oil administration to exclude any benefit of corn oil for the nerve regeneration.

Group IV (SNI+EPO Treated Group; n.6):

From the next day of injury induction, rats received an intraperitoneal injection of EPO (Sedico Company for Pharmaceuticals, Egypt) at a dose of 5000 IU/kg daily for two weeks. Then they were sacrificed two months after initiation of EPO administration[10,11].

Group V (SNI+Curcumin Treated Group; n.6):

From the next day of injury induction, rats received by gastric gavage orally curcumin powder (Sigma-Aldrish) dissolved in corn oil, as it enhances curcumin absorption and bioavailability[12]. The dose was 40 mg/kg[13] daily for two months. Rats were then sacrificed two months after initiation of curcumin administration.

Surgical procedures

Induction of sciatic nerve crush injury was done according to a method modified from Yuan and Feng 2016[14] and Ramli *et al*. (2017)[15]. Under aseptic conditions, exposure of the right sciatic nerve was performed in rats anesthetized using mixture of xylazine (10mg/kg) and ketamine (100mg/kg)[15]. Two cm skin incision was done along the proximal half of the line between knee joint and the greater trochanter. Gluteal muscle splitting incision was performed and through this incision the overlying muscles were separated without being cut. The exposed right sciatic nerve was crushed one cm proximal to its division into common peroneal and tibial nerves. The crush was attained using a sterile tissue forceps applied for one minute. Next, the forceps was removed and a clear band across the nerve was seen, confirming that the nerve was completely crushed. The skin incision was sutured in 2 layers with absorbable sutures. After that, antibiotic ointment was applied on the wound 2 times daily for 3 days and the animals were returned to their cages and allowed to recover with free access to food and water. Each rat from the sham-operated group (IB) underwent the same surgical procedure, except that the clamp was not applied. This was done just to expose these animals to the same stress of the operation. The same antiseptic precautions and way of wound closure were followed as for the experimental groups.

Electrophysiologic evaluation

Nerve conduction test: Electrophysiological evaluation of nerve regeneration was performed in *vivo* at the end of the experiment (day of sacrifice). Conduction from regenerated right sciatic nerves to the gastrocnemius muscle was evaluated. The Compound muscle action potential (CMAP) amplitudes were recorded according to Salehi *et al*. (2017)[16] using an electromyographic recorder. This was performed at Physiology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University.

Histological evaluation

At the end of the experimental period each rat was sacrificed by i.p. injection of a lethal dose of phenobarbitone (80 mg/kg)[17]. The right sciatic nerve at and distal to the site of lesion was dissected and excised. The point of nerve crush was determined (1cm before sciatic nerve bifurcation) and the nerve from this point and distally was harvested for histological preparation[15].

Each sciatic nerve was divided into three sets of specimens, which were subjected to the following histological procedures:

Specimens of sciatic nerve were fixed in 10% buffered formalin solution for 24 hours, dehydrated and cleared then embedded in paraffin wax. Cross sections of 5-7 μm were cut and subjected to:

- Hematoxylin & eosin (H&E) stain[18].
- Immunohistochemical staining for neurofilaments[19].

Retrieval of antigen was done through boiling tissue sections within 10 mM citrate buffer, pH 6.0 for 10 minutes. Tissue sections were incubated with NF Ab-1 ready to use mouse monoclonal antibody (Thermo Scientific, CA, USA, Catalogue #: MS-359-R7) for 60 minutes. Histostain SP kit detection system (LAB-SA system, Zymed Laboratories Inc, San Francisco, USA) was used. Positive control for NF Ab-1 was a specimen of brain, which gave brown axonal cytoplasmic reaction. Negative control: the same procedure was used for processing additional sciatic nerve sections, but without addition of the primary antibody which was replaced by PBS. Primary antibody omission gave no staining reaction.

Another part of specimens was processed for osmic acid staining. Specimens were fixed in 4 % paraformaldehyde for 1-2 hours, transferred to 1% osmic acid for 3-4 days then embedded in paraffin and cut at 5-7μm[19].

Small pieces of sciatic nerve (1mm3) were fixed in 3% glutaraldehyde, post fixed in 1% osmium tetroxide, embedded in resin and cut at 1μm (semithin sections). These sections were stained with toluidine blue[19].

Morphometric studies

Using Leica Qwin 500 LTD software image analysis computer system (Cambridge, England); at Histology Department, Faculty of Medicine, Cairo University; the following parameters were measured:

- The number of myelinated axons/HPF. They were counted in osmic acid stained sections in ten randomly chosen non-overlapping fields at magnification 400.
- The diameters of myelinated nerve fibers (the smallest diameter to avoid bias of tangential sections) were measured in 50 nerve fibers for each group in semithin sections at magnification x 1000.
- The ratios of the myelin areas to the fiber areas in 50 nerve fibers for each group were measured in semithin sections x 1000.
- Area percent and optical density of NF immunoreaction (NF immune-stained sections) were measured in ten randomly chosen non-overlapping fields x 400.

Statistical Analysis

Data were presented as means and standard deviations (Means±SD) which were compared using one-way analysis of variance (ANOVA) followed by post-Hoc analysis (Tukey test). *P-values* < 0.05 were considered statistically significant. Calculations were done on SPSS version 21 software (IBM Inc., Chicago, Illinois, USA)[20].

RESULTS

No mortality was observed in the rats of all groups. Electrophysiological, histological and morphometric results of subgroups of the control group showed no differences. So, they were referred to by group I (control group). The same was observed regarding subgroups of group III (untreated group).

Histological Results

a) Hematoxylin and Eosin Stained Sections

Cross sections in the sciatic nerves obtained from control group exhibited the normal histological architecture demonstrating bundles of nerve fibers surrounded by connective tissue perineurium. The nerve fibers inside the bundles were held together by delicate connective tissue endoneurium. Each nerve fiber was formed of faint acidophilic axon surrounded by unstained area of dissolved myelin and an eosinophilic neurilemma (sheath of Schwann). Nuclei of Schwann cells were also obvious (Figures 1A,B).

Cross sections in the sciatic nerve of group II (SNI) showed marked disorganization and disruption of nerve fibers. Wide spaces in-between nerve fibers were detected. Markedly congested blood vessels as well as mononuclear cell infiltrate were also noted (Figures 2A,B,C).

Transverse sections (T.S.) in the sciatic nerve of group III (untreated group) exhibited nerve fibers disruption. Wide spaces in-between nerve fibers were also seen. Congested blood vessel was another finding (Figure 2D).

EPO treated group (group IV) illustrated apparent return to the normal histological architecture of sciatic nerve. Nerve bundles appeared compact and filled with well-organized nerve fibers. Small blood capillaries were seen in the sparse connective tissue endoneurium. Nerve bundles were separated by connective tissue perineurium and the whole nerve was covered by epineurium (Figure 3A). In curcumin treated group (group V), sciatic nerve T.S. exhibited a nearly normal histological architecture comparable to group IV except for the presence of small congested blood vessels (Figure 3B).

b) Osmic Acid Stained Sections

Sciatic nerve specimens of the control group exhibited numerous large myelinated nerve fibers. The myelin sheaths appeared as darkly stained rings (brown to black) around unstained axon. Few lightly stained nerve fibers were small unmyelinated. The nerve fibers were closely packed with little connective tissue endoneurium in between. The bundles were covered by connective tissue perineurium (Figure 4A). T.S. in the sciatic nerve of SNI group exhibited marked decline in the number of myelinated nerve fibers with wide spaces in-between the nerve fibers. Myelin sheath disruption could be also detected (Figure 4B). Untreated group showed some large myelinated nerve fibers. Most nerve fibers were with smaller diameter. Small non myelinated fibers were also seen. Wide spaces between the nerve fibers were noted (Figure 4C). EPO treated group sections demonstrated apparent marked increase in the number of large myelinated nerve fibers. The nerve fibers appeared close to each other with little connective tissue endoneurium in between (Figure 4D). Sciatic nerve cross sections of curcumin treated group showed apparent rise in the number of large myelinated nerve fibers. However, many smaller diameter nerve fibers were also present (Figure 4E).

c) Toluidine blue- Stained semithin Sections:

Cross sections in the sciatic nerve of control group showed the normal histological architecture of nerve fascicles containing many large myelinated nerve fibers, scattered groups of thin myelinated nerve fibers. Axons appeared clear with dark blue rings of intact myelin around them. Schmidt-Lanterman clefts were also detected (Figure 5). Cross sections of SNI group showed many nerve fibers with disrupted myelin sheath. Mast cell was encountered in the connective tissue endoneurium between the nerve fibers. Also, macrophages with foamy cytoplasm could be seen (Figures 6A,B,C). The untreated group exhibited relatively few number of large myelinated nerve fibers with intact myelin sheaths. Many thin myelinated nerve fibers were detected. Disrupted myelination could be seen in some nerve fibers (Figure 6D). EPO treated group showed numerous large myelinated intact nerve fibers. Few nerve fibers exhibited myelin disruption. Scattered thin myelinated nerve fibers were also seen (Figure 7A). Curcumin treated group demonstrated increased number of large myelinated intact nerve fibers, but there were numerous thin myelinated nerve fibers. Few fibers exhibited disrupted myelin sheaths (Figure 7B).

d) Neurofilament Immuno-Stained Sections:

Transverse sections in the sciatic nerve of the control group showed strong positive NF immuno-reaction in the axoplasm of the nerve fibers (Figure 8A). SNI group demonstrated many nerve fibers with faint positive NF immuno-reactivity in their axoplasm. Fragmented axons and granular disintegration of NF were also detected (Figure 8B). Untreated group illustrated many nerve fibers with small axons. They exhibited faint to moderate positive NF immuno-reactivity. However, some axons with positive NF immuno-reactivity were observed (Figure 8C). EPO treated group showed numerous nerve fibers with strong positive NF immuno-reactivity in the axoplasm (Figure 8D). Curcumin treated group exhibited many nerve fibers with variable sizes. They showed moderate to strong positive NF immuno-reactivity (Figure 8E).

Morphometric and Statistical Results

The mean values of all measured parameters were represented in the)table 1(. Statistically, treated groups (group IV and group V) showed significant increase in CMAP amplitudes, number and diameter of myelinated nerve fibers, the ratio of myelin areas to nerve fiber areas as well as in area percent and optical density of NF immunoreaction as compared to both group II (SNI) and group III (untreated). However, all values were significantly higher in group IV (EPO treated) than group V (curcumin treated).

No significant differences were recorded between group IV and the control group as regards the number of myelinated nerve fibers, the ratio of myelin areas to nerve fiber areas, the area percent and optical density of NF immunoreaction.

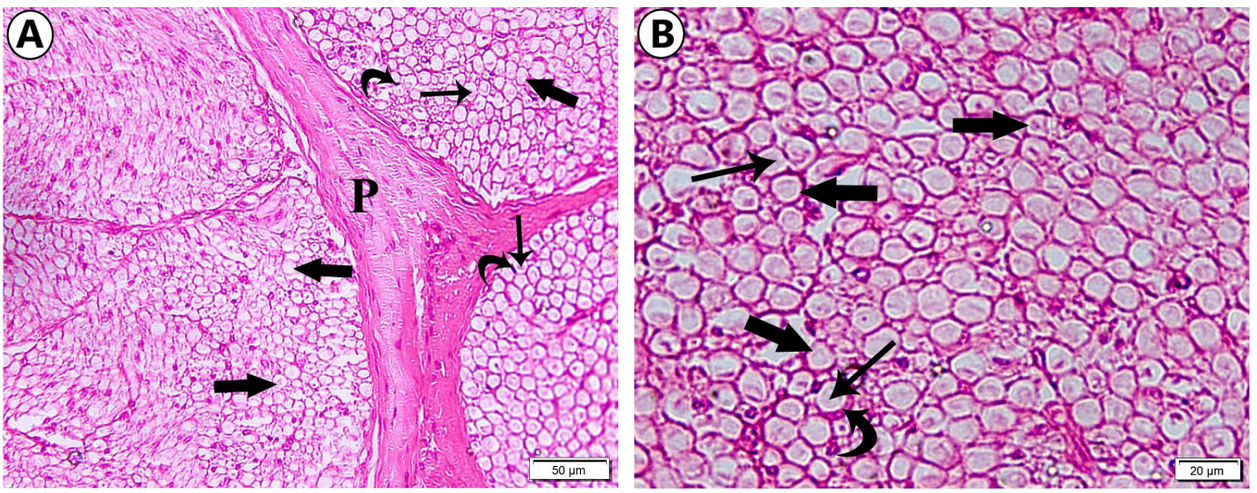

Fig. 1: (A&B) Control group showing normal histological architecture of sciatic nerve. Bundles of nerve fibers surrounded by connective tissue perineurium (P) are seen. Individual nerve fibers are held together by delicate connective tissue endoneurium. Each nerve fiber (arrows) is composed of faint acidophilic axon (thin arrows) surrounded by clear area indicating dissolved myelin; outside the myelin area the eosinophilic neurilemma or sheath of Schwann can be detected. Nuclei of Schwann cells (curved arrows) perched on the neurilemma are also noted. (A, Cross section; H&E x 200) (B, Cross section; H&E x 400)

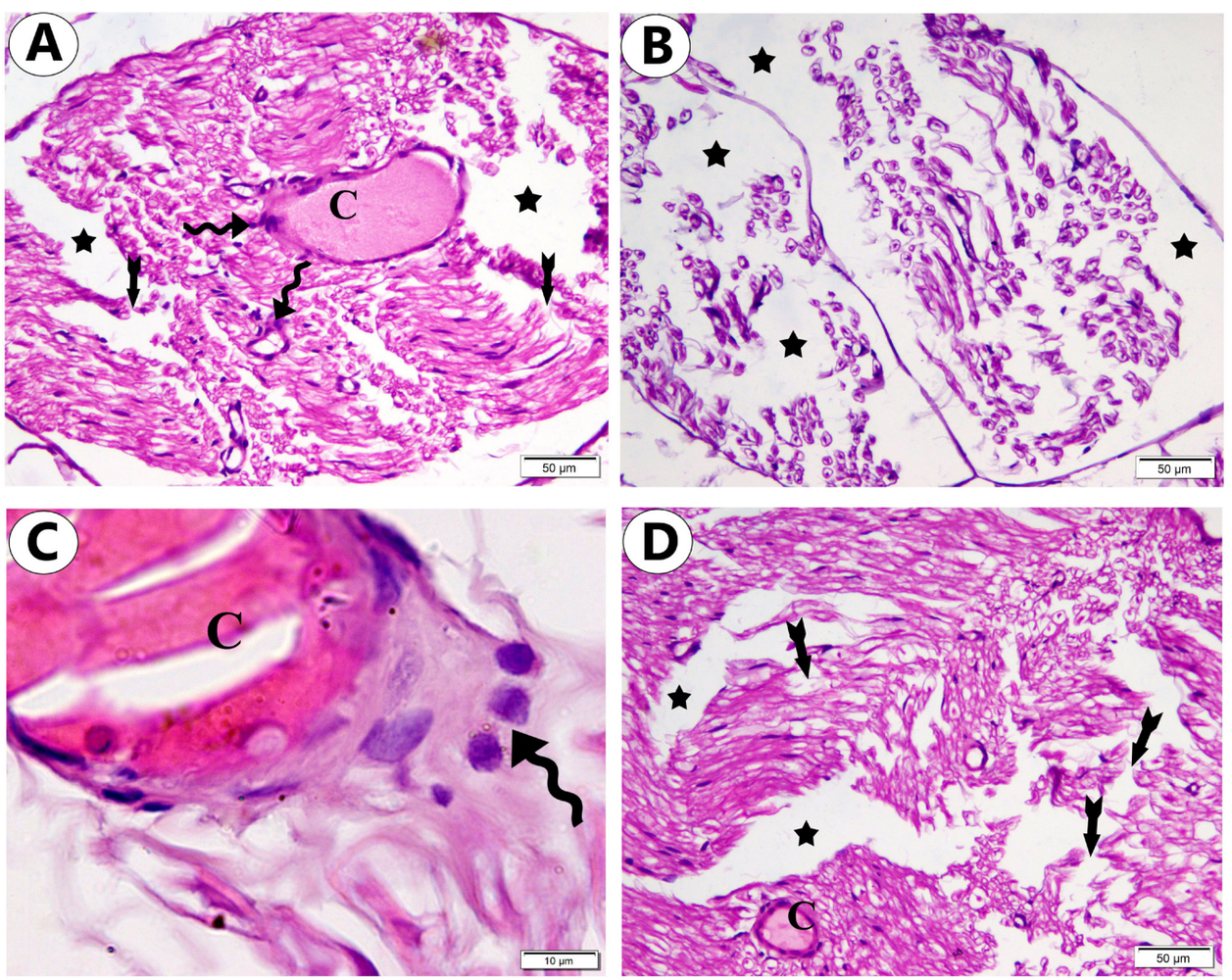

Fig. 2: (A, B &C) Group II expressing marked disorganization of nerve fibers. Nerve fibers disruption (bifid arrows) is observed. Wide spaces between nerve fibers (stars) can be detected. Note the presence of markedly congested blood vessels (C) and mononuclear cell infiltrate (wavy arrows). D) Group III exhibiting disruption of nerve fibers (bifid arrows). Wide spaces between nerve fibers (stars) could be noted. A congested blood vessel (C) is also observed. (A, B &D, Transverse sections; H&E x 200) (C, Transverse section; H&E x 1000)

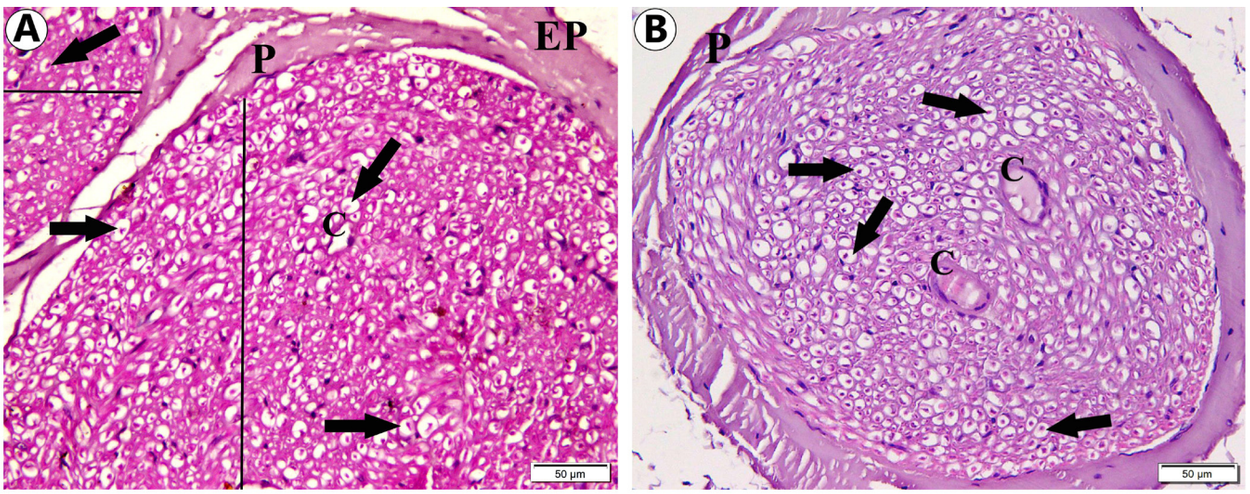

Fig. 3: (A) Group IV demonstrating parts of two nerve bundles (lines) which appear compact and full of nerve fibers (arrows). Small blood vessel (C) is seen in the connective tissue endoneurium. The nerve bundles are separated by connective tissue perineurium (P). Part of the connective tissue epineurium (EP) is seen covering the whole nerve. (B) Group V showing nerve fibers (arrows) within apparently normal compact bundle. It is surrounded by connective tissue perineurium (P). Small congested blood vessels (C) are seen within the endoneurium between the nerve fibers. (A&B, Cross sections; H&E x 200)

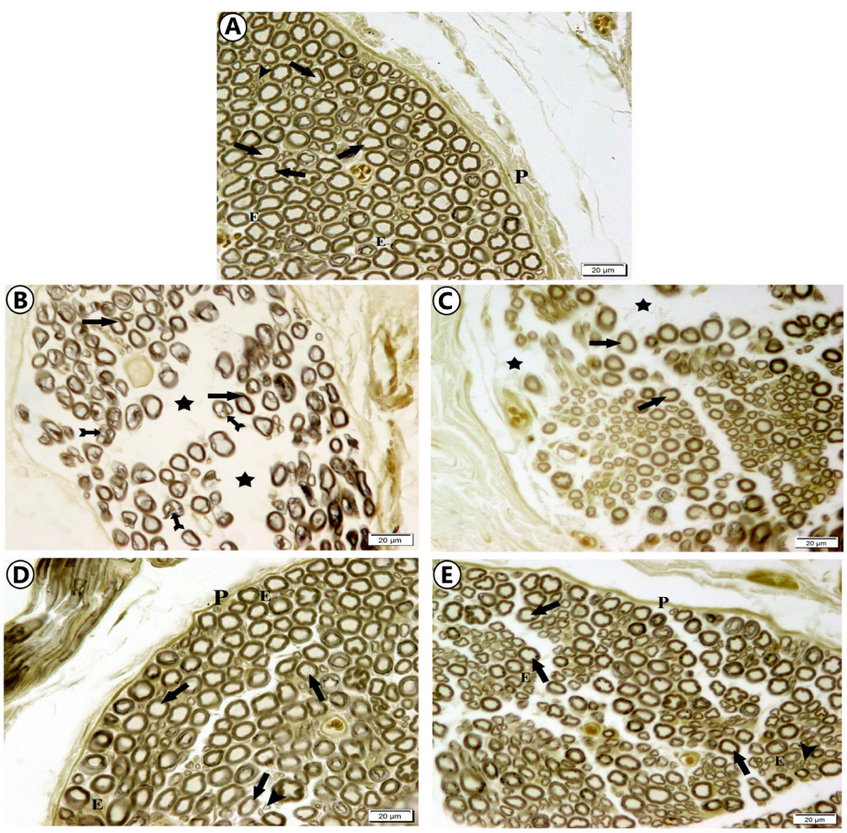

Fig. 4: (A) (Control) Nerve bundle in which most nerve fibers are large myelinated (arrows). Few nerve fibers are small unmyelinated (arrowhead). The nerve fibers are closely packed with little connective tissue endoneurium (E) in between. The bundle is covered by a connective tissue perineurium (P). (B) (SNI) Bundle of nerve fibers showing markedly decreased myelinated nerve fibers (arrows). Wide areas among nerve fibers (stars) can be seen. Note the presence of nerve fibers with myelin sheaths disruption (bifid arrows). (C) (untreated) Bundle of nerve fibers with some large myelinated nerve fibers (arrows). Most nerve fibers in the field are smaller in diameter. Wide spaces between the nerve fibers (stars) are seen. (D) (SNI+EPO treated) Nerve bundle containing large number of large myelinated nerve fibers (arrows) and little connective tissue endoneurium (E) in between. Few small unmyelinated nerve fibers (arrowhead) can be detected. The bundle is covered by connective tissue perineurium (P). (E) (SNI+Curcumin treated) Bundle of nerve fibers containing many large myelinated (arrows) nerve fibers. Smaller diameter nerve fibers can be observed. Small unmyelinated (arrowhead) nerve fibers are also noted. Little connective tissue endoneurium (E) is detected in between nerve fibers. The bundle is covered by connective tissue perineurium (P). (A, B, C, D & E, Cross sections; Osmic acid x400)

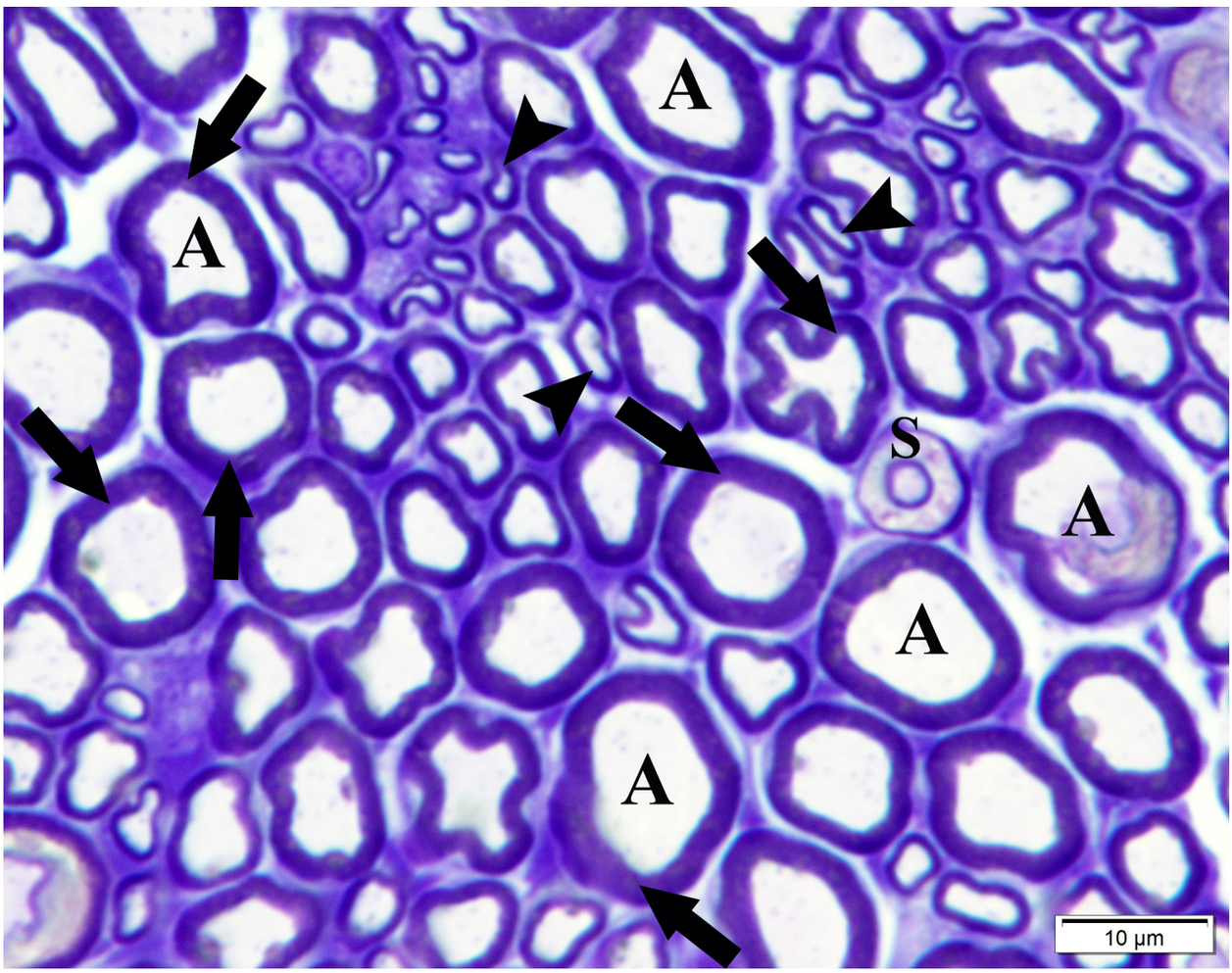

Fig. 5: Cross section in the sciatic nerve of control group showing its normal histological architecture. A nerve fascicle illustrates numerous large myelinated nerve fibers (arrows). These fibers are formed of large pale stained axons (A) encircled with thick darkly stained blue rings of intact myelin sheath. Scattered groups of thin myelinated nerve fibers (arrowheads) could be detected. Note the presence of nerve fibers with double separate rings denoting Schmidt-Lanterman clefts (S). This feature indicates the presence of residual cytoplasmic lakes separating inner and outer rims of darkly stained myelin. (Toluidine blue x1000)

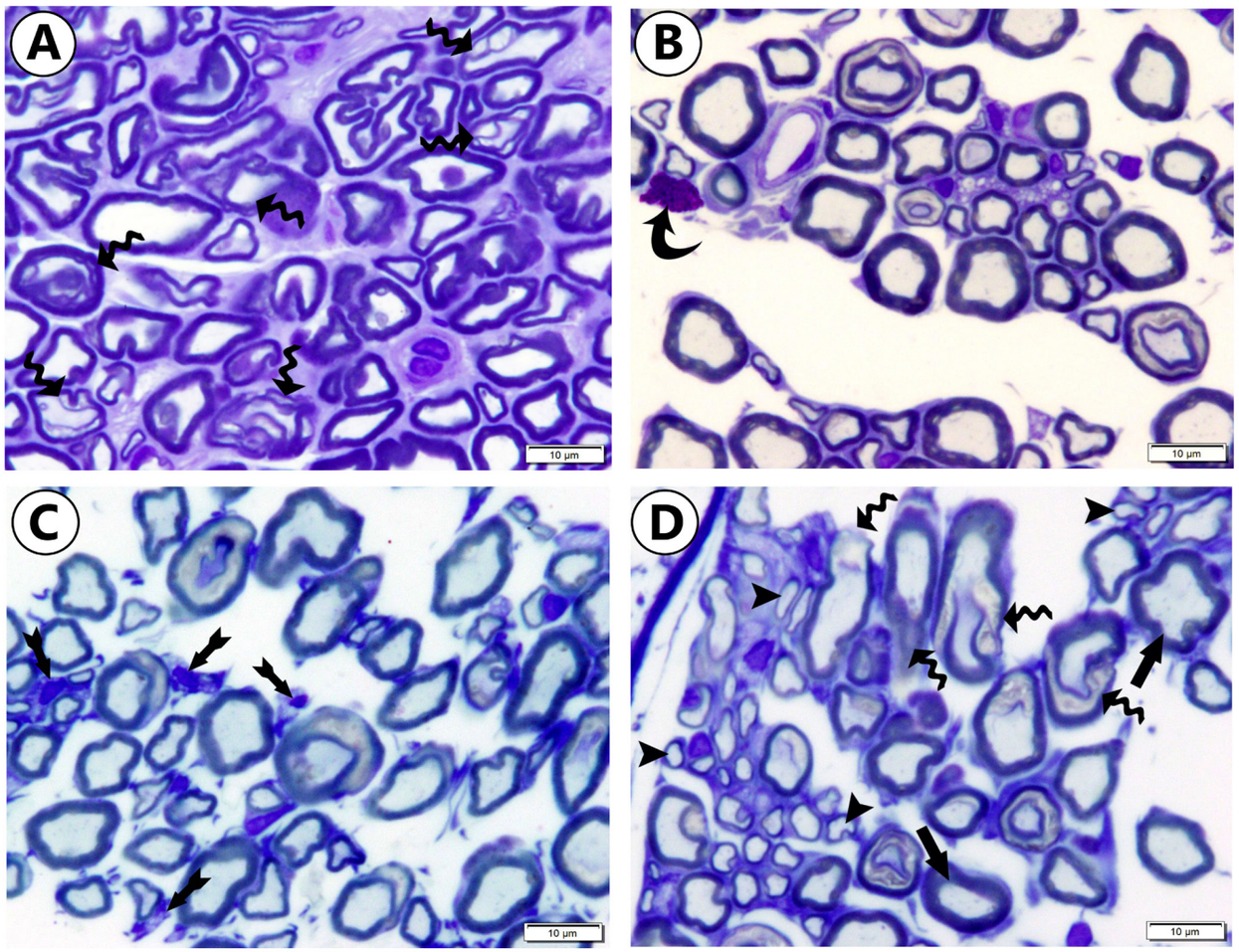

Fig. 6: (A, B &C) SNI group demonstrating many nerve fibers with disrupted myelination (wavy arrows). Note the presence of mast cell (curved arrow) with its characteristic metachromatically stained granules. Plump foamy macrophages (bifid arrows) with its cytoplasm containing residual particles is also detected. (D) Untreated group showing relatively few number of large myelinated nerve fibers (arrows) with intact myelin sheaths. Many small nerve fibers (arrowheads) are observed. Some fibers with disrupted myelin sheath (wavy arrows) can be seen. (A, B, C &D, Transverse sections; Toluidine blue x1000)

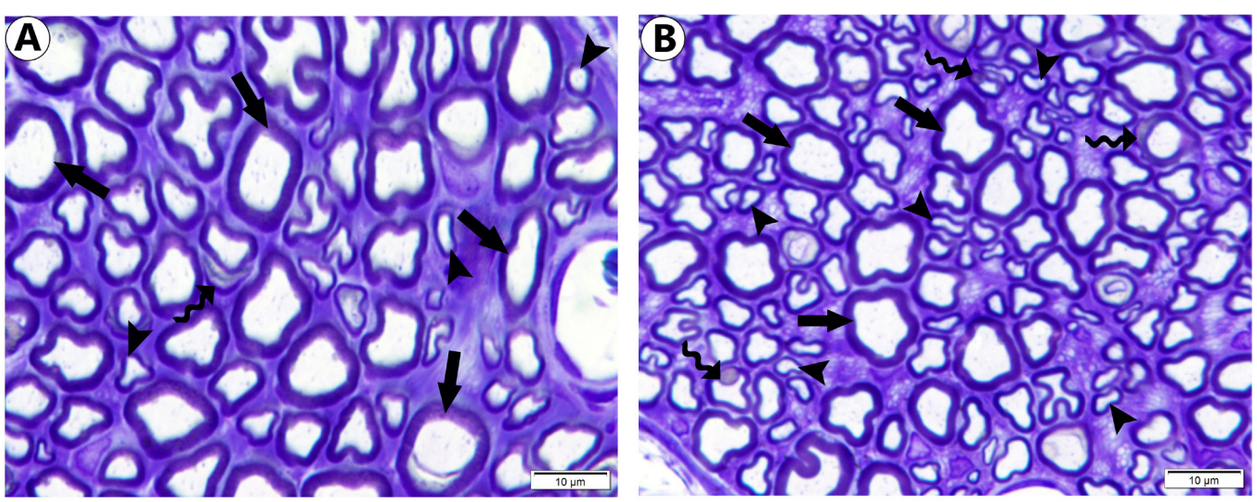

Fig. 7: (A) SNI plus EPO exhibits many large myelinated nerve fibers (arrows) with intact myelin sheaths. Few fibers with myelin disruption (wavy arrow) can be detected. Scattered thin myelinated nerve fibers (arrowheads) are also observed. (B) SNI plus curcumin showing myelinated nerve fibers of variable diameters. Increased number of intact large myelinated nerve fibers (arrows) can be seen, however, thin myelinated nerve fibers (arrowheads) are numerous. In addition, myelin disruption (wavy arrows) could be detected in few fibers. (A & B, Cross sections; Toluidine blue x1000)

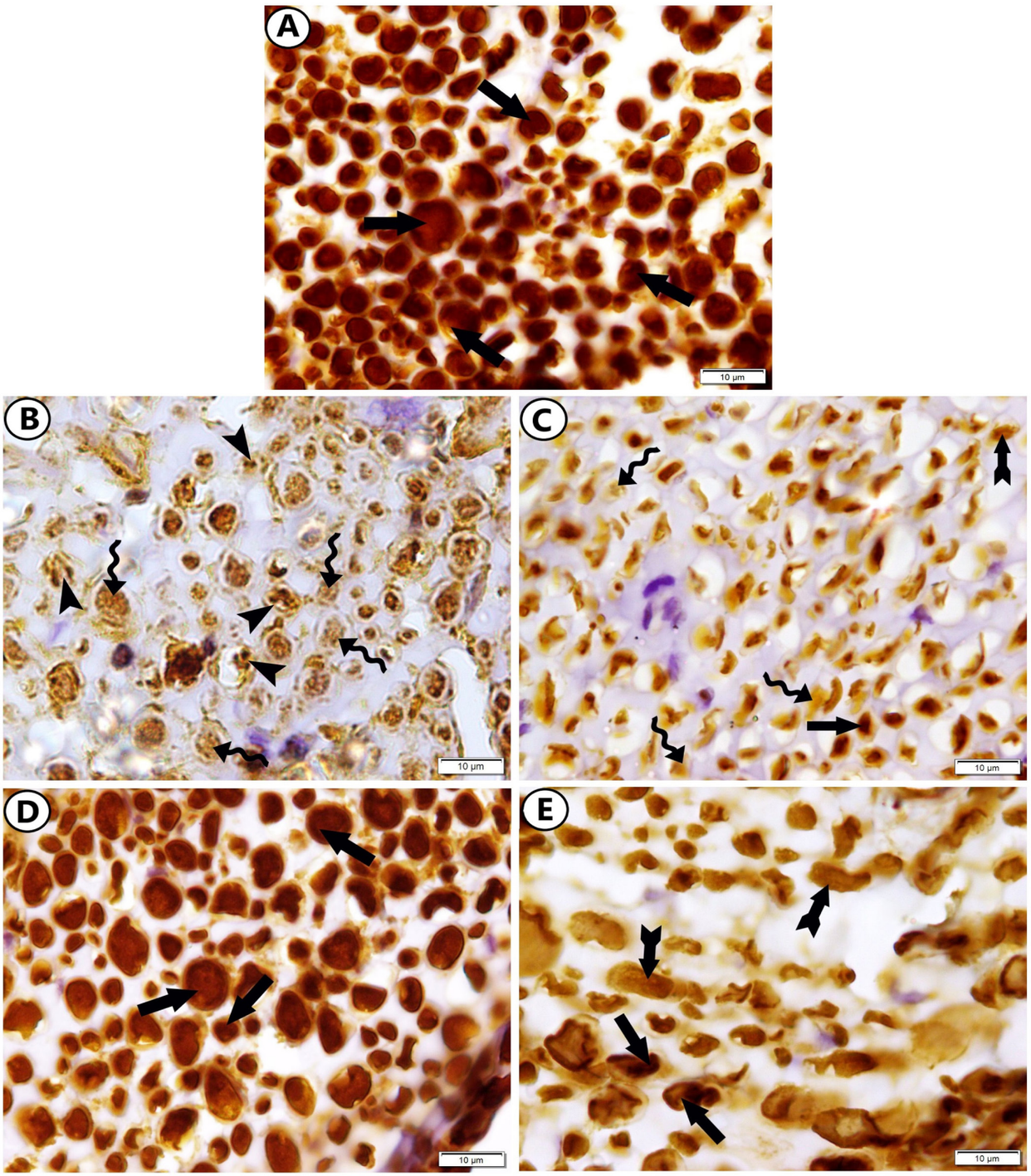

Fig. 8: (A) Nerve fibers of the control group exhibit strong positive NF immuno-reactivity in the axoplasm (arrows). (B) SNI group illustrates that most nerve fibers show faint positive NF immuno-reactivity (wavy arrows) in their axoplasm with granular disintegration of NF. Many nerve fibers with fragmented axons (arrow heads) are also noted. (C) Untreated group showing many nerve fibers with small axons that exhibit faint (wavy arrows) and moderate (bifid arrows) positive NF immuno-reactivity. Some axons showing strong (arrows) positive NF immuno-reactivity could be detected. (D) EPO treated group demonstrating that most nerve fibers exhibit strong positive NF immuno-reactivity in the axoplasm (arrows). (E) Curcumin treated group showing many nerve fibers of variable sizes that exhibit moderate (bifid arrows) to strong (arrows) positive NF immuno-reactivity in the axoplasm. (A, B, C, D &E, Transverse sections; NF x1000)

Table 1: Mean values (±SD) of CMAP amplitudes and morphometric results in the studied groups

Groups	The mean values (±SD) of CMAP amplitudes	The mean count (±SD) of myelinated axons	The mean diameter (±SD) of myelinated nerve fibers	The mean ratio (±SD) of the myelin area to the fiber area	The mean area percent (±SD) of NF immune reaction	The mean optical density (±SD) of NF immune reaction
Group I	33.40 ± 1.42	103 ± 22.92	12.30 ± 1.30	0.69 ± 0.08	75.44 ± 6.02	0.64 ± 0.09
Group II	9.10 ± 1.66*	31 ± 8.70*	4.57 ± 0.74*	0.32 ± 0.06*	20.30 ± 6.11*	0.22 ± 0.04*
Group III	18.30 ± 1.15*	36 ± 9.66*	6.17 ± 1.28*	0.43 ± 0.05*	40.33 ± 6.29*⌂	0.24 ± 0.04*
Group IV	29.60 ± 1.26*#•	96 ± 11.50#•	10.99 ± 1.19*#•	0.67 ± 0.05#•	71.31 ± 8.00#•	0.57 ± 0.04#•
Group V	23.20 ± 1.75*#	71 ± 17.09*#	9.55 ± 0.96*#	0.55 ± 0.05*#	58.03 ± 7.38*#	0.45 ± 0.01*#

* Significantly different as compared to the control group ($P < 0.05$).
⌂ Significantly different as compared to group II ($P < 0.05$).
Significantly different as compared to groups II and III ($P < 0.05$).
• Significantly different as compared to group V ($P < 0.05$).

DISCUSSION

PNI represents a complex challenge because of its various etiologies and degrees of severity. The patients following PNI are left with a wide range of disability. It ranges from mild dysfunction to complete loss of motor and sensory functions[21]. Even with microsurgical techniques advances and rehabilitation facilities improvements, reaching a desired functional regeneration of peripheral nerves is still insufficient[22]. Additionally, the delayed recovery will lead to permanent atrophy of the supplied muscles[23].

Crush nerve injury is among the most common etiologies of peripheral nerves lesions. For this reason, crush injury experimental model was chosen in the present study. This lesion results in axonal disruption followed by Wallerian degeneration then slow regeneration[24,25].

Also, this study was conducted on rat sciatic nerve which is the most commonly used and well-established model investigating nerve regeneration after crush injury. Rat sciatic nerve is large, easily accessible and its structure is similar to human. Also, many electrophysiological tests are available for studying its functional recovery[26,27,23].

The present work evaluated and compared the usefulness of EPO and curcumin in accelerating the process of neural regeneration. Various histological techniques, in addition to morphometric parameters and electrophysiological measurements were used.

Immunostaining for neurofilaments (NFs) was performed in the current work to evaluate the regenerative capacity of injured axons. NFs are the major constituent of the cytoskeleton in mature neurons and their expression is closely related to growth of axons. NFs have a key role in regeneration of axons. They are easily detectable and are one of the most common immunohistochemical markers for nerve regeneration[28,29].

In this study, group I (control) showed normal histological architecture of sciatic nerve. Electrophysiological study went parallel with histomorphometric examination and revealed normal nerve function for the control group.

In the present study, the SNI group revealed that crush injury induction method was successful as it elicited the morphological changes of the degeneration phase. These degenerative changes were in the form of marked disorganization and disruption of nerve fibers together with axonal fragmentation and disruption of myelin sheaths. Wide spaces in between nerve fibers denoting missed nerve fibers and/or endoneurial edema were evident. In addition, marked vascular congestion and infiltration by mononuclear inflammatory cells, macrophages and mast cells were detected. Disintegration of the NF was observed.

Further approvement to the degenerative changes occurring in the sciatic nerve of this group was achieved by the statistical morphometric measurements. It showed a significant decrease in the number and diameter of myelinated nerve fibers, significant decrease in the ratio of the myelin area to the fiber area, as well as significant decrease in the mean area percent and the optical density of NF immunoreactivity, when compared to the control group.

Functionally, a statistically significant impairment of CMAP was recorded in this group. This confirms the occurrence of nerve dysfunction and was in accordance with the previously mentioned histological changes.

The present electrophysiological results are similar to the findings of Wood *et al*. (2018)[30] who demonstrated that crush injury of the sciatic nerve caused sharp decline of CMAP one week after injury compared to control rats.

Meanwhile, the morphological alterations and morphometric results detected in SNI group of the current study go in hand with previous study of Burnett and Zager (2004)[31] who mentioned that crush nerve injury consequently results in Wallerian degeneration distal to injury site. They reported axonal fragmentation, myelin disintegration, inflammatory cells infiltration including mast cells and macrophages, increased vascular permeability and oedema. Also, Turedi *et al*. 2018[32] detected degenerated nerve fibers, disrupted myelin sheaths and mast cell infiltration 7 days post crush injury. Moreover, they reported significant decline in number and diameter of myelinated nerve fibers and myelin sheath thickness when compared to control.

Faint NF immunostaining which is the result of granular disintegration of NF was detected in SNI group of this study. Menorca *et al*. (2013)[33] described this sign to be the hallmark of degenerative stage during nerve injury. In addition, Omura *et al*. (2004)[34] stated dramatic decrease in NF positive immunoreactivity in axoplasm 7 days post nerve injury. Coinciding with these results, Fowler *et al*. (2015)[35] mentioned that injured nerves expressed less NF immune-staining intensity.

Wallerian degeneration affects distal stump of nerve in response to injury. It occurs within week postinjury with most intense degeneration 3 and 7 days after injury. This process provides the proper microenvironment for the process of regeneration as it clears growth inhibitors and myelin debris[36-38].

The previously mentioned findings could be explained by Gao *et al*. (2013)[39] who stated that peripheral nerve crush injury leads to inflammatory reaction. This promotes an increase in the local endoneurial vascular permeability and subsequent endoneurial oedema occurs. The endoneurial oedema greatly increases local pressure leading to reduction in blood flow. This ischemic phenomenon causes axonal degeneration and myelin sheath decomposition that finally results in neurovascular deficits.

This explanation was consistent with Turedi *et al*. (2018)[32] who reported that impairment of the vasa nervosum occurs following sciatic nerve crush injury. In addition to ischemia, free radical production can lead to oxidative stress which can trigger cell death through apoptosis or necrosis.

Gaudet *et al*. (2011)[40] reported that macrophage has a crucial role in clearing up myelin debris and growth inhibitor molecules within it, facilitating the process of regeneration. Lopez-Vales *et al*. (2008)[41] documented that the foamy morphology of Macrophages (which results from the presence of end-products of myelin/lipid degradation) is widely used for their identification in toluidine blue stained semithin cross sections. Esposito *et al*. 2002[42] and Ellis and Bennett, (2013)[43] revealed that mast cells play an important role in nerve degeneration and regeneration. Mast cells release inflammatory mediators that contribute to the recruitment of neutrophils which in turn attract macrophages to site of injury. Also, these mediators increase the endoneurial vascular permeability providing access for mast cell precursors and macrophages.

In the current work, group III (untreated group) showed poor improvement as indicated by the persistence of most histological alterations. It also exhibited non-significant increase of CMAP amplitudes, number and diameter of myelinated nerve fibers as well as the ratio of the myelin area to the fiber area as compared with group II. This denotes the importance of treatment to accelerate nerve regeneration. However, a significant increase in the mean area percent of NF immunoreactivity in this group was detected as compared to group II. This might indicates starting the regeneration process.

In accordance with these findings Takemura *et al*. (2012)[44] reported gradual rise in CMAP amplitude, but full recovery could not be achieved at 8weeks after injury. Also, Malysz *et al*. (2010)[45] documented that morphological and morphometric alterations were detected in sciatic nerve distal segment at 13 weeks after crush injury. Moreover, the predominance of small thin myelinated nerve fibers with augmented endoneurium indicated incomplete spontaneous regenerative process after distal segment Wallerian degeneration. Alvites *et al*. (2018)[25] reported that the chief obstacles to competent regeneration could be the establishment of uncontrolled vigorous inflammatory process, failure in stimulation of axon regrowth and remyelination.

In the present work, EPO treated group (group IV) sections almost regained the normal histological architecture of the sciatic nerve. Most morphometric measurements were in line with this. There was no statistically significant difference between EPO treated group as compared to control regarding mean number of nerve fibers, myelin to nerve fiber ratio, mean area percent and optical density of NF. A significant increase in; the number and diameter of myelinated nerve fibers, ratio of the myelin area to nerve fiber area as well as area percent and optical density of NF were detected when compared to groups II and III. Also, it exhibited a significant improvement of CMAP amplitude when compared to both groups II and III. On the other hand, and despite the increase in the values of both nerve fiber diameter and CMAP amplitudes, there was significant decline as compared to control. It could be hypothesized that more time or dosage are required to increase the degree of maturity of the regenerated nerve fibers, providing more axonal enlargement and thicker myelin and hence, returning to before injury state.

From the previous findings, it could be assumed that administration of EPO resulted in improvement of nerve injury both morphologically and functionally. It markedly accelerated the regeneration process and it was about to achieve the full recovery like normal.

This assumption was consistent with Grasso *et al*. 2007[46] who postulated that EPO was effective in improving neurological impairment and CMAP amplitudes after experimental sciatic nerve crush injury in rats. The present findings were also consistent with Yin *et al*. (2010)[10] who reported an enhanced functional recovery going together with significant increase in the total count of nerve fibers and myelin sheath thickness in rats treated with EPO after sciatic nerve injury in comparison with untreated group. Govindappa *et al*. (2020)[47] reported similar findings with intraperitoneal EPO administration after sciatic nerve injury. Coinciding with the present immunohistochemical results, Geary *et al*. (2017)[48] observed that treatment with EPO resulted in significant increases in NF positive immunoreactivity following nerve crush.

EPO seems to exert its neuroprotective effect through many mechanisms. It was clarified that EPO receptors

(EPO-R) are expressed on Schwann cells surface and this expression is upregulated in response to adjacent nerve injury. Consequently, EPO promotes Schwann cell migration and proliferation at injury site. It directly promotes Schwann cell remyelination. EPO possess anti-apoptotic activity, preventing neuronal apoptosis through inactivation of pro-apoptotic pathways. Moreover, it prevents Schwann cells apoptosis via secreting anti-apoptotic proteins and preservation of DNA integrity[49-51].

Suppression of inflammatory cytokines and antioxidative stress activities are other involved mechanisms. Furthermore, direct neurogenic effects of EPO were suggested as it enhances mesenchymal and endothelial progenitor stem cells recruitment to site of injury, hence promoting angiogenesis and neuronal regeneration[49,50,52,53].

EPO has been considered a great candidate for therapeutic enhancement of neural regeneration in peripheral nerve injuries[50]. The present results support this consideration and indicate that early administration of EPO (one day following injury) can accelerate functional recovery and improve morphologic appearance in crush injury of peripheral nerves.

In the current work, Curcumin treated group (group V) revealed a nearly normal histological architecture. This was accompanied with significant increase in CMAP amplitudes when compared to both groups II and III. Additionally, morphometric measurements including: the mean number and diameters of myelinated nerve fibers, the ratio of the myelin area to the nerve fiber area and the area percent and optical density of NF, exhibited significant increase when compared to both groups II and III. However, all morphometric measurements and CMAP amplitudes in this group were significantly lower when compared to both control and EPO treated group. This indicates that curcumin has a less neuroprotective effect than EPO. Longer time course than EPO may be needed to complete the process of regeneration and to achieve optimal functional and structural recovery.

The histomorphometric findings went in hand with Liu *et al*. (2016)[54] and Ma *et al*. (2016)[55], They reported that curcumin therapy was effective in enhancing nerve regeneration following sciatic nerve injury. Curcumin significantly increased the number and diameter of myelinated nerve fibers and myelin thickness compared to untreated rats. Going parallel with these findings, Yuce *et al*. (2015)[56] stated that curcumin administration improved CMAP impairment in injured rats after sciatic nerve crush injury.

The mechanism by which curcumin protects against nerve degeneration and promotes nerve regeneration after injury was described by Mohammadi and Mahmoodi 2013[57] who reported anti-inflammatory effect of curcumin as it can modulate the expression of inflammatory cytokines e.g. TNF and IL-6. Also, curcumin have antioxidant capacity and is a potent scavenger of reactive oxygen species. Moreover, Sang *et al*. (2018)[58] clarified that the neuroprotective effect of curcumin can be related to its ability to stimulate secretion of nerve growth factor (NGF) which is considered to be a key element in nerve regeneration and functional recovery.

Other mechanism was described by Babu *et al*. (2015)[59] who reported that curcumin significantly decreased calcium ion influx inside injured sciatic nerve. Hence, it prevents cytoskeleton disintegration and axonal degeneration during Wallerian degeneration. Furthermore, curcumin has been found to stimulate Schwann cells proliferation and ability of myelination. It also reduces Schwann cells apoptosis[60,61].

CONCLUSION

Data from the present study showed that EPO enhances functional recovery and augments nerve regeneration of the experimentally crushed sciatic nerve in rats better than curcumin.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوچية مقارنة على تأثيرعقار الايريثروبيوتين مقابل الكركم علي الاصابة الهرسية للعصب الوركي في نموذج الجرذ

منال على عبدالمحسن، سلوي متولي على، نجلاء محمد سلامة و إيناس عبدالله علي أحمد

قسم علم الأنسجة، كلية الطب، جامعة القاهرة، مصر

الخلفيه والهدف من العمل: كثيرًا ما يتم مواجهة إصابات الأعصاب الطرفية في الممارسة السريرية لأسباب عديدة مثل الجراحة أو الحوادث. ترتبط هذه الاصابات غالبًا بسوء تجدد الأعصاب وعدم كفاية التعافي الوظيفي. وبناء على ذلك, تم تصميم هذه الدراسة لتقييم ومقارنة التأثير المحتمل لعقار الايريثروبيوتين مقابل الكركم على الاصابة الهرسية للعصب الوركي في نموذج الجرذ.

مواد و طرق البحث: تم تقسيم ٧٨ من ذكور الجرذان البيضاء الى خمس مجموعات: المجموعة الضابطه, مجموعة اصابة العصب الوركى, المجموعة الغير معالجة, مجموعة اصابة العصب الوركى + الايريثروبيوتين و مجموعة اصابة العصب الوركى + الكركم. وقد تم عمل الإصابة عن طريق الضغط على العصب الوركى بملقط لمدة دقيقة. تلقت مجموعة اصابة العصب الوركى + الايريثروبيوتين بعد ٢٤ ساعة من إحداث الاصابة حقن فى البريتون من الايريثروبيوتين بجرعة ٥٠٠٠ وحدة دولية / كجم يومياً لمدة أسبوعين. أما مجموعة اصابة العصب الوركى + الكركم فقد تم علاجها بالكركم عن طريق الفم بجرعة ٤٠ ملغم / كغم لمدة شهرين وذلك بعد ٢٤ ساعة من إحداث الاصابة. تم تسجيل سعة الجهد العضلي المركب لجميع الجرذان في نهاية التجربة. تم تجهيز عينات العصب الوركى للهيماتوكسيلين والإيوسين, صبغة حامض الاوزميك, صبغة التولويدين الأزرق و الصبغ الهستوكيميائى المناعى للجسم المضاد للخيوط العصبية. تم القياس و التحليل الاحصائى لكل من عدد و قطر الألياف العصبية, نسبة مساحة الغلاف النخاعى الى مساحة المحور العصبى وايضا نسبة مساحة و الكثافة البصرية للتفاعل المناعي للخيوط العصبية.

النتائج: أظهرت كل من المجموعه المعالجه بالايريثروبيوتين والكركم تحسنا فى التركيب الهستولوجى للعصب الوركى, كما اظهرت زياده ملحوظه فى سعة الجهد العضلي المركب. ايضا, زادت بشكل ملحوظ القياسات المورفوميتريه و التى تشمل; عدد و قطر الألياف العصبية, نسبة مساحة الغلاف النخاعى الى مساحة المحور العصبى وايضا نسبة مساحة و الكثافة البصرية للتفاعل المناعي للخيوط العصبية مقارنة بكل من مجموعة اصابة العصب الوركى و المجموعة الغير معالجة. ومع ذلك, كانت جميع القياسات اعلى بكثير فى المجموعه المعالجه بالايريثروبيوتين مقارنة بالكركم

الاستنتاج: يعزز الايريثروبيوتين التعافى التركيبى و الوظيفى و يحسن تجدد العصب الوركى المعرض للهرس افضل من الكركم.