

A Histological and Electrophysiological Study on Axonal Regeneration Following End to Side Nerve Anastomosis with Epineural Window in Albino Rats

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ABSTRACT

Proper nerve repair is critical to the complete rehabilitation of the injured and denervated tissues. The present work was performed on the peroneal nerve of albino rats to verify the restoration of axonal regeneration through an end to side neurorrhaphy with performing an epineural window. The present work included 20 rats. The left peroneal nerves in all animals were considered as a control group. The right peroneal nerve of half the animals was sectioned and resutured in the traditional way of end-to-end anastomosis (group I). The proximal stump of the sectioned right peroneal nerve of the other half of the animals was ligated and its distal end was sutured through end-to-side anastomosis to the sciatic nerve after removal of an epineural window (group II). After 6 months of the procedures, all animals were subjected to electrophysiological and histological examinations. The electrophysiological examinations revealed successful regeneration pattern in group I and II. However, end-to-end anastomosis revealed better results. Histological examination of the distal stump of the peroneal nerve in group I depicted well myelinated nerve fibers, while some fibers showed splitting and wrinkling of myelin lamellae. The peroneal nerve in group II showed clusters of nerve fibers formed of multiple small myelinated axons, surrounded by Schwann cells. Some peroneal nerve fibers showed looping of myelin with vacuolations within its lamellae. The endoneurium was deeply infiltrated with collagen fibers. The findings of the current study indicated that nerve regeneration following end to side repair was satisfactory and this new technique could be a good option when only the distal end of the nerve is available.

INTRODUCTION

Restoration of sensory and motor functions of the transected peripheral nerve depends upon effective healing and regeneration across the repair site. Much progress has been made in delineating the relevant factors for a favorable outcome such as viable nerve endings, meticulous coaptation, tensionless repair and a well-vascularized bed [1].

Although a great number of investigators have ensured maximal functional return in peripheral nerve repairs whenever both ends could be corrected by end-to-end coaptation, surgeons still have problems in repair of peripheral nerve injuries in which the proximal stumps are not available. To overcome this problem nerve repair other than an end-to-end manner has been investigated [2,3,4].

Ballance et al. [5] were the first to report an end-to-side technique for the treatment of facial palsy. However, functional results were inadequate and the technique was abandoned. End-to-side neurorrhaphy was recently reintroduced by Viterbo [6] and Co-workers [2,3] by implanting a sectioned nerve laterally onto a healthy nerve with or without removal of the epineurium. The authors postulated that the end-to-side repair could work, allowing growth of axons laterally into the end of the attached nerve, conducting the electric stimuli and maintaining adequate trophism of the corresponding muscles [2,3,6]. The development of microsurgical techniques combined with a greater understanding of the neurobiology of nerve injury and regeneration has resulted in significant improvement in the outcome of nerve repair [7].

The purpose of the present work is to evaluate the new technique of end-to-side nerve repair in comparison to the traditional method of end-to-end anastomosis by histological and electrophysiological examination.

MATERIAL AND METHODS

The study was carried out on twenty male albino rats with average body weight of 175 gm (b. wt.

range: 150-225 gm). The rats were anaesthetized with intraperitoneal injection of thiopental sodium in a dose of 1-2 mg/kg b.wt. and were subjected to a sterile microsurgical procedures (Plate I Fig. A B,C,D). The operative procedures were done on the right peroneal nerve, while the left side was left untouched. The rats were divided into:

Control group:

Included the untouched left peroneal nerve of all rats.

Group I: (End-to-end anastomosis) (10 rats):

In which the right peroneal nerve was sectioned and both ends were sutured by the traditional technique of end-to-end (E-E) anastomosis.

Group II: (End-to-side anastomosis) (10 rats):

Which were subjected to end-to-side (E-S) neuroorrhaphy, the right peroneal nerve was sectioned 1 cm below its origin from the sciatic nerve. The distal end was sutured laterally to the main stem of the sciatic nerve after removal of a 1 mm diameter window in its epineurium. The proximal end was curved back at about 100-degree angle and ligated to prevent sprouting of the axons. Careful dissection of the sciatic nerve was done to get enough length to ensure tensionless anastomosis. When no extra length could be obtained the distal end of the peroneal nerve was sutured to the side of the tibial nerve.

As all the surgical procedures were completed, the rats were kept under the same environmental conditions as regards housing, standard food and water and were left to recover for 6 months with regular follow up.

At the end of the recovery period, the animals were anaesthetized and subjected to electrophysiological examination through application of concentric needle electromyography for the tibialis anterior and peroneus longus muscles. The examination was done to the muscles at rest and during voluntary activity (Plate II Fig. A B,C,D).

As the electrophysiological (EMG) examination was completed, the animals were sacrificed by injection of a lethal dose of thiopental sodium intraperitoneally. Then, transverse segments of peroneal nerve were taken from all groups of animals. In group I and II, specimens were harvested 1 cm below the level of anastomosis. Each specimen was divided into 2 pieces. The first piece was fixed in 1% osmium tetroxide and processed for light microscopic examination using osmium tetroxide

stain for demonstration of myelin [8]. The second piece was fixed immediately in 3% glutaraldehyde and processed for examination by Jeol 100 CX transmission electron microscope [9,10] at the Electron Microscopy Unit, Medical Research Institute, Alexandria University.

RESULTS

I- Electrophysiological results:

EMG of the studied muscles at rest, showed signs of denervation in the form of positive sharp waves and fibrillation potentials. The signs of denervation were less in samples with end-to-end anastomosis than those with end-to-side anastomosis (Fig. 1 A,B).

During voluntary activity, all samples showed signs of reinnervation in the form of small polyphasic units of early reinnervation, large polyphasic units of medium reinnervation and large biphasic units of mature reinnervation. The number of reinnervation units and the number of mature units were evident in end-to-end anastomosis (Fig. 2 A,B) than in end-to-side anastomosis (Fig. 3 A,B).

II- Histological results:

A: light microscopic results (osmium tetroxide stain).

Control group:

Cross section of control rat peroneal nerve stained with osmium tetroxide stain revealed fascicles containing regular, well myelinated nerve fibers with circular configurations embedded in the endoneurial compartment. The nerves fascicles were surrounded by perineurium (Fig. 4).

Group I (end-to-end anastomosis):

Cross sectional examination of the distal end of the rat right peroneal nerve revealed evidence of regeneration. Many nerve fibers appeared well myelinated and surrounded by moderately thickened perineurium (Fig. 5).

Group II (end-to-side anastomosis):

The distal end of the rat right peroneal nerve following end-to-side anastomosis revealed considerable degree of regeneration less than that encountered in end-to-end anastomosis as different sizes of myelinated nerve fibers were encountered. Small sized, poorly myelinated nerve fibers were also depicted separated by large amount of endoneurium. The nerves fascicles were surrounded by markedly thickened perineurium (Fig. 6).

B- Electron microscopic results:

Control group:

The peroneal nerve of the control group showed normally appearing myelinated nerve fibers. Each one consisted of a central axon surrounded by regular myelin and enveloped in a sheath of Schwann cell. The axon showed evenly dispersed neurofilaments and neurotubules and was surrounded by myelin lamellae, formed by Schwann cell membranes wrapped spirally around the axon (Fig.7).

The Schwann cell showed flattened nucleus and attenuated cytoplasm containing few mitochondria. The part of the myelin adjacent to Schwann cell nucleus appeared indented (Fig. 7). Unmyelinated nerve fibers were depicted as multiple axons enveloped in a sheath of Schwann cell. In contrast to myelinated nerve fibers, Schwann cells supporting unmyelinated fibers were depicted investing several axons (Fig. 8).

Endoneural capillary appeared with a single layer of endothelial cells linked together by tight junctions and surrounded by pericyte (Fig. 9). The endoneurium was composed of collagen surrounding myelinated and unmyelinated nerve fibers (Figs. 7,8). Around the endoneural capillaries abundant collagen and fibroblasts were also seen (Fig. 9).

Group I: (End-to-end anastomosis):

Revealed regeneration pattern, many nerve fibers appeared well myelinated but irregular. Other fibers appeared in early stage of myelination surrounded by a Schwann cell with hypertrophied nucleus (Fig. 10). Few nerve fibers showed defective myelin formation either in the form of separated myelin lamellae showing excessive wrinkling or just irregularity and looping of the myelin sheath (Fig. 11).

The endoneural capillary showed some endothelial cells with irregular nuclei. The endoneurium was seen infiltrated with excessive collagen fibers (Fig. 12).

Group II (End-to-side anastomosis):

Revealed regenerating unit of thinly myelinated axon. Small sized axons were seen surrounded by thin myelin sheath. Each myelinated axon is enveloped in a separate Schwann cell, some of the Schwann cells showed hypertrophied nuclei. Slender fibroblasts with elongated nuclei were depicted surrounding the regenerating units (Fig. 13).

Healthy nerve (sciatic or tibial nerve) after removal of a 1 mm diameter window in its epineurium. This new technique of end-to-side neurorrhaphy was evaluated by histological and electrophysiological examinations. Light microscopic examination of the distal stump of peroneal nerve following end-to-side neurorrhaphy revealed regenerating pattern in nerve fibers. Small sized poorly myelinated nerve fibers were scattered among other myelinated fibers with variable sizes. These findings reflect that anastomosis via end-to-side manner succeeded in axonal regrowth from the healthy donor nerve to the distal stump of the sectioned peroneal nerve.

Examination of the distal stump of peroneal nerve in group I revealed better pattern of regeneration as many well myelinated axons were evenly distributed in a manner very similar to the control group denoting successful myelinated axonal regeneration.

In accordance with the results of the present work, Zhang et al. [13] also reported that end-to-side neurorrhaphy of the peroneal nerve in rats permitted axonal regeneration from the intact donor nerve and was associated with satisfactory recovery. Their histological studies with neurofilament staining revealed numerous axons at the distal end of the peroneal nerve. Their morphometric analysis also demonstrated that the presence of a window in the epineurium improved the histological picture. However, the long-term evaluation of their study after 8 and 12 months postoperatively may be a contributing factor in the successful pattern of nerve repair.

In the present work ultrastructural examination of the distal end of peroneal nerve in group II confirmed the light microscopic results.

Bundles of regenerating nerve fibers appeared thinly myelinated, each one was enclosed in a separate Schwann cell and the whole bundle was surrounded by slender fibroblasts. These bundles were described by many authors as regenerating clusters or units [14,15]. They also recorded the presence of slender encircling fibroblasts surrounding the regenerating cluster [14,15,16]. Hence, the excessive collagen deposition frequently observed around the regenerating units in group 1 and 11 of the present study is closely related to the enhanced fibroblastic activity.

Many studies attributed the regeneration of the nerve fibers to Schwann cell proliferation within

the basement membrane tube left behind by the degenerated fiber. These Schwann cells stimulate the outgrowth of axons through synthesis of nerve growth factors, hence one degenerated fiber is initially replaced by several axonal sprouts. These become individually supported by their own myelinated Schwann cell [17,18]. This finding concurs with the observed Schwann cells with hypertrophied nuclei surrounding early myelinated axons in the control group and group I.

According to Anders and Blaivas [18] the number of myelin lamellae which determine the myelin thickness is proportional to the axon caliber in normal peripheral nerves with normal axon: myelin ratio. However, in case of regenerating fibers, they recorded an increased axon: myelin ratio. These findings explain the frequently observed thinly myelinated axons in the regenerating clusters of nerve fibers.

Ultrastructural examination of group I revealed the close association of macrophages and fibroblasts to the regenerating clusters, which obviously reflects the role of these cells in the process of regeneration. Recent studies reported that macrophage response to degeneration is pivotal to the initiation of regeneration. Macrophages release interleukins that stimulate the production of nerve growth factor (NGF) and insulin-like growth factor-1 (IGF-1) by Schwann cells and probably fibroblasts, which are essential mediators for nerve fiber regeneration [19,20].

The present work revealed the presence of nerve fibers with abnormal myelin figures beside the regenerating clusters which were more pronounced in group II than those of group I. These abnormal myelin figures ranged from irregular looped myelin to splitting and vacuolations in the myelin lamellae. These findings may be attributed to demyelination

of the nerve fibers as a secondary reaction to axonal affection [18].

Examination of the endoneural capillaries of the peroneal nerve in group II revealed increased activity of the lining endothelial cells. Some cells showed irregular nuclear shape with increased pinocytotic vesicles. These findings are in accordance with the results of Nukada [21] who demonstrated an increase in endothelial activity and in the number of endoneural capillaries eight weeks after crush and transection lesions. He suggested that axonal outgrowth appears to be an important determinant of post traumatic new capillary formation.

The examination of the distal stump of peroneal nerve in group I are in agreement with other studies recorded that end-to-end anastomosis is the preferred surgical technique for peripheral nerve reconstruction after injury as long as the two nerve ends are available [22,23]. Ultrastructural examination of this group in the present work revealed successful regeneration pattern of the nerve fibers. Many well myelinated axons and axons in different stages of myelination were frequently encountered while abnormal myelin figures were infrequently depicted.

Some nerve fibers showed axons containing mitochondria with electron dense matrix and surrounded by irregular looped myelin (Fig. 14). Few nerve fibers were depicted with multiple vacuoles in the axoplasm and surrounded by myelin showing splitting and vacuolations of its lamellae (Fig. 15).

The endoneurium showed macrophages with multiple cell process, irregular nucleus and electron dense cytoplasm containing vacuoles (Fig. 16). The endoneurium was also infiltrated with collagen fibers. The endoneural capillaries showed endothelial cells with irregular nuclei and increased number of pinocytotic vesicles (Figs. 14,15,16).

Plate (I): The microsurgical technique of end to end and end to side anastomosis.



Fig. (A): The rat is anesthetized and fixed in prone position.



Fig. (B): Exposure of the right sciatic nerve after division of the right gluteus maximus muscle.

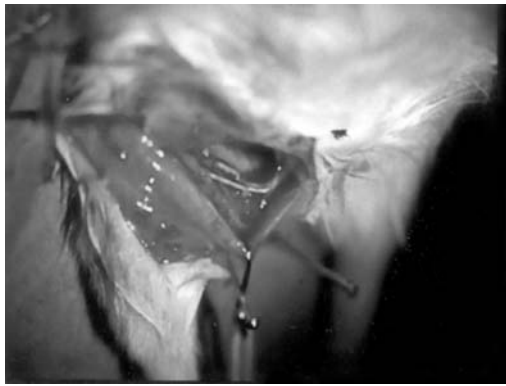


Fig. (C): End to end anastomosis of the transected right peroneal nerve.

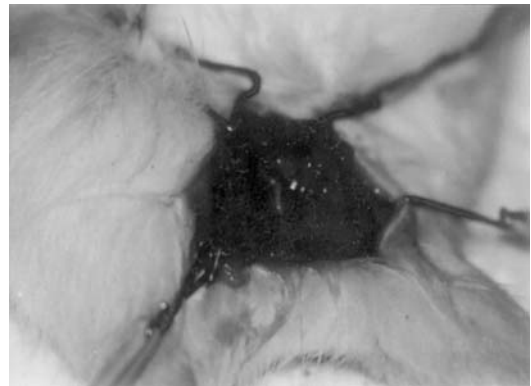


Fig. (D): End to end anastomosis of the distal stump of the transected right peroneal nerve to the side of the right sciatic nerve.

Plate (II)

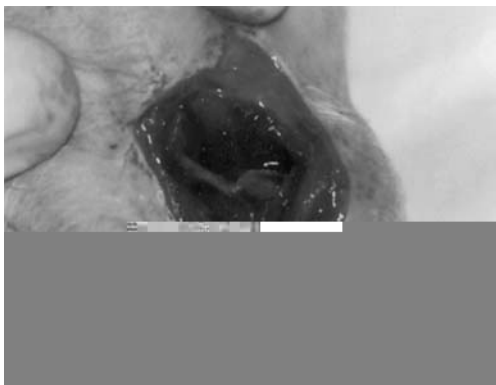


Fig. (A): Exposure of the anastomotic site in end to end anastomosis.



Fig. (B): Exposure of the anastomotic site in end to side anastomosis.

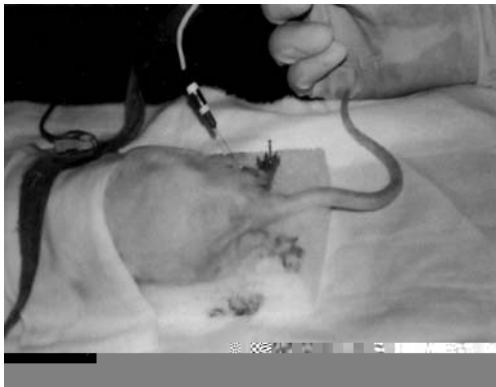


Fig. (C): Concentric EMG study with inserted needle in the right peroneal nerve distal the anastomotic site.



Fig. (D): The needle inserted in the right leg muscle of the rat.

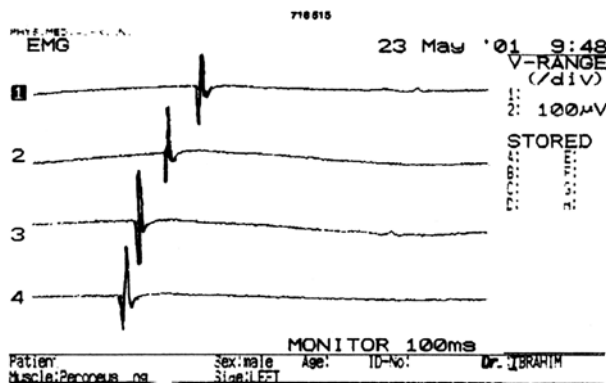


Fig. (1-A): EMG showing signs of denervation (fibrillation potentials) in group I.

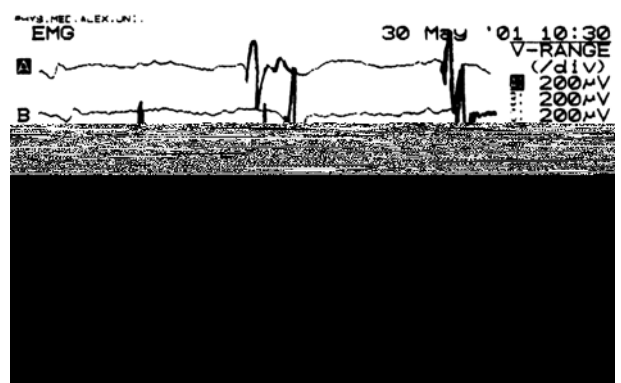


Fig. (1-B): EMG showing signs of denervation (positive sharp waves) in group II.

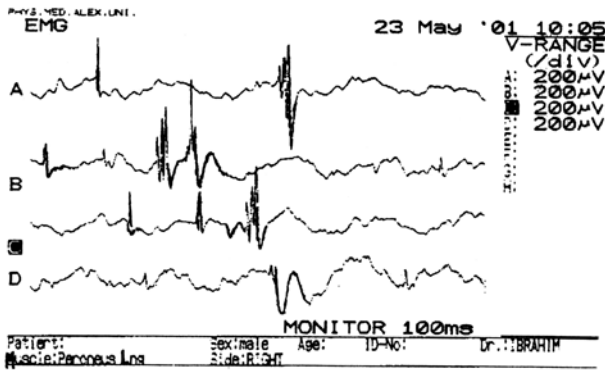


Fig. (2-A): EMG showing multiple small polyphasic motor unit action potentials indicative of partial reinnervation in group I.



Fig. (2-B): EMG showing small polyphasic motor unit action potentials indicative of partial reinnervation process in group II.

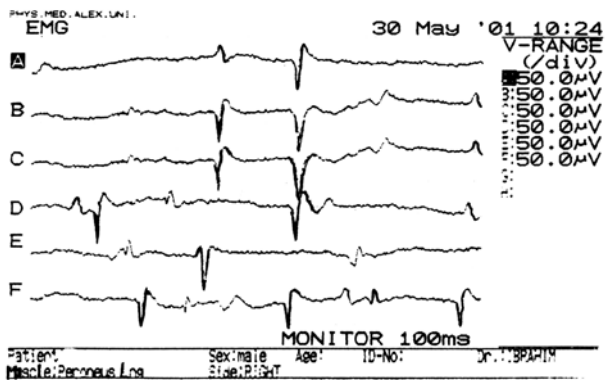


Fig. (3-A): EMG showing multiple large biphasic units indicating mature reinnervation in group I.

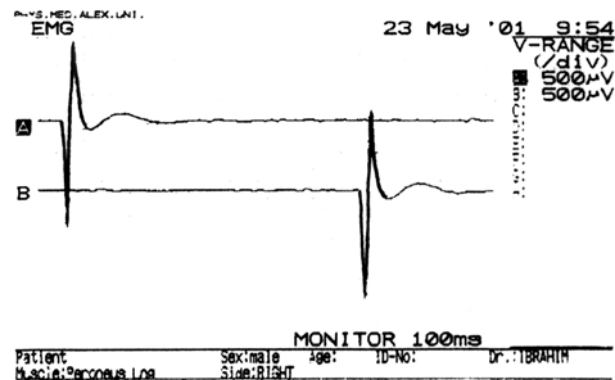


Fig. (3-B): EMG showing few large biphasic units indicating mature reinnervation in group II.

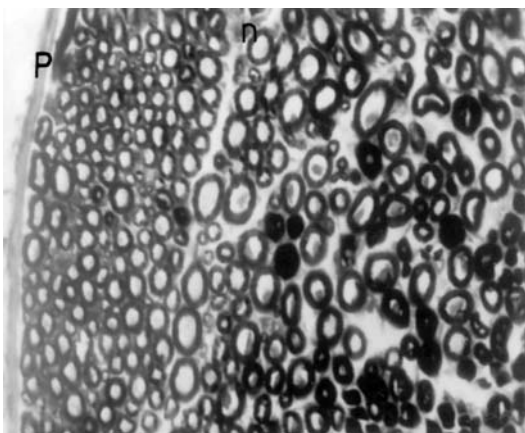


Fig. (4): Micrograph of a control rat peroneal nerve showing well myelinated and regular nerve fibers (n) present in the endoneurial compartment (En) and surrounded by perineurium (P). Osmium tetroxide stain. Mic. Mag. X400.

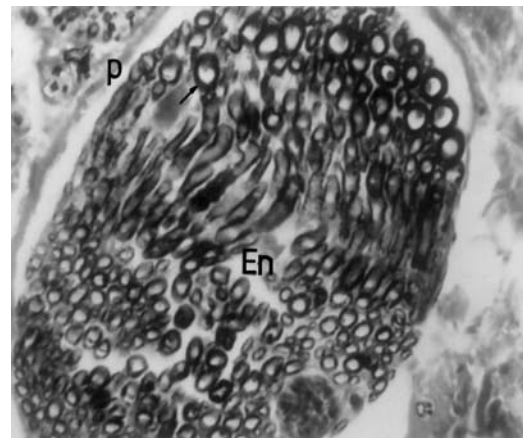


Fig. (5): Micrograph of rat peroneal nerve in group I showing well myelinated nerve fibers (arrow) surrounded by moderately thickened perineurium (P). En; endoneurium. Osmium tetroxide stain. Mic. Mag. X400.

Fig. (6): Micrograph of rat peroneal nerve in group II revealing scattered small and medium sized myelinated nerve fibers (arrow) and many small sized poorly myelinated fibers (double arrow) separated by endoneurium (En). Note the markedly thickened perineurium (P). Osmium tetroxide stain. Mic. Mag. X400.

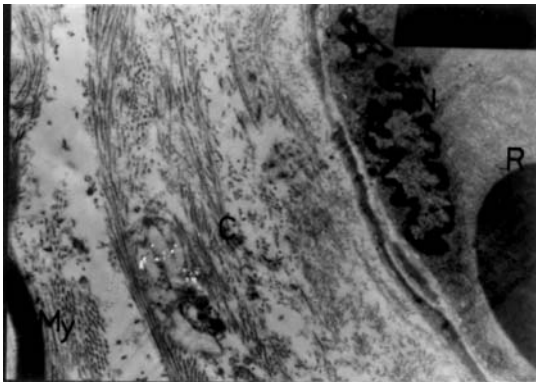


Fig. (12): Electron photomicrograph of endoneurial capillary of rat peroneal nerve of group I showing an endothelial cell with irregular nucleus (N). The endoneurium is deeply infiltrated with collagen fibers (C). R; red blood cell, My; myelin. Mic. Mag. X6,700.

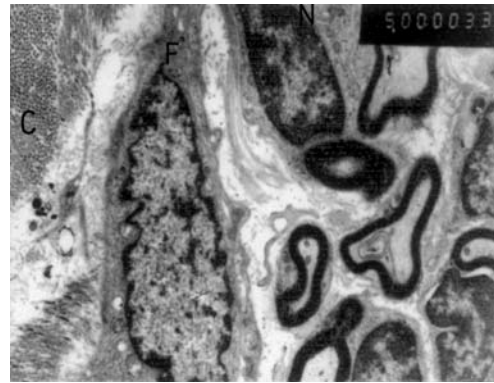


Fig. (13): Electron photomicrograph of rat peroneal nerve of group II showing regenerating unit of irregular nerve fibers formed of multiple small sized, thinly myelinated axons (Ax), each one is surrounded by Schwann cell, some of them shows hypertrophied nucleus (N). A fibroblast is seen embarrassing the regenerating unit. C; collagen. Mic. Mag. X5,000.

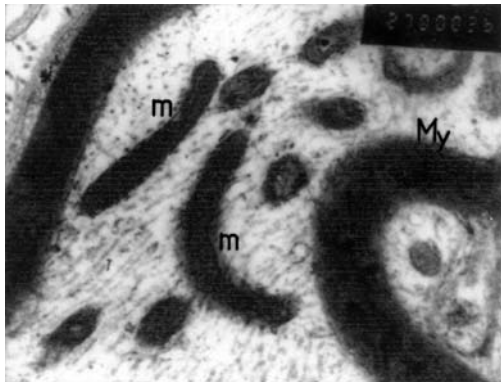


Fig. (14): Electron photomicrograph of group II rat peroneal nerve showing a myelinated nerve fiber. The axoplasm shows mitochondria (m) with electron dense matrix. Myelin (My) shows excessive looping (arrows) and is surrounded by Schwann cell cytoplasm (*). Mic. Mag. X27,000.

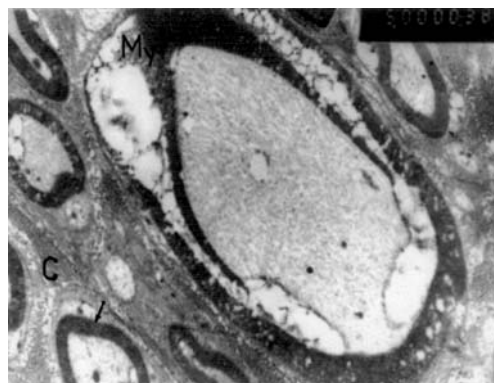


Fig. (15): Electron photomicrograph of rat peroneal nerve of group II showing myelinated nerve fiber with splitting and vacuolations of its myelin lamellae (My). The axoplasm shows vacuoles (v). Small adjacent better myelinated nerve fibers are also depicted (arrows). C; collagen. Mic. Mag. X5,000.

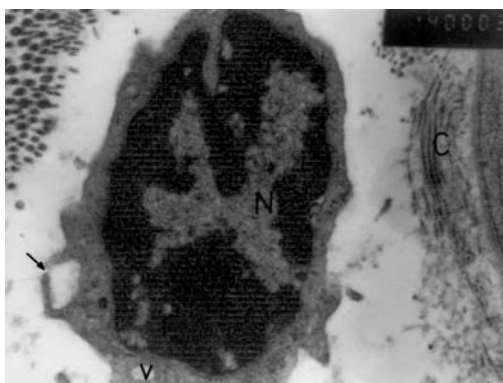


Fig. (16): Electron photomicrograph of rat peroneal nerve of group II showing endoneurial macrophage with irregular nucleus (N), electron dense cytoplasm (*), vacuoles (v) and multiple cell processes (arrows). C; collagen. Mic. Mag. X14,000.

DISCUSSION

Proper nerve repair is critical to the complete rehabilitation of injured and denervated tissues. Although, it is well documented that end-to-end nerve repair is the obvious choice when both ends of the injured nerve are available, still there are problems in repair when proximal stumps are not available. A new treatment modality through end-to-side nerve repair has been investigated to overcome this problem [11,12].

The present work tried to assess nerve repair following anastomosis of the distal stump of the sectioned peroneal nerve to the lateral side of a healthy nerve (sciatic or tibial nerve) after removal of a 1 mm. diameter window in its epineurium. This

new technique of end-to-side neurorrhaphy was evaluated by histological and electrophysiological examinations.

Light microscopic examination of the distal stump of peroneal nerve following end-to-side neurorrhaphy revealed regenerating pattern in nerve fibers. Small sized poorly myelinated nerve fibers were scattered among other myelinated fibers with variable sizes. These findings reflect that anastomosis via end-to-side manner succeeded in axonal regrowth from the healthy donor nerve to the distal stump of the sectioned peroneal nerve.

Examination of the distal stump of peroneal nerve in group I revealed better pattern of regeneration as many well myelinated axons were evenly distributed in a manner very similar to the control group denoting successful myelinated axonal regeneration.

In accordance with the results of the present work, Zhang et al. [13] also reported that end-to-side neurorrhaphy of the peroneal nerve in rats permitted axonal regeneration from the intact donor nerve and was associated with satisfactory recovery. Their histological studies with neurofilament staining revealed numerous axons at the distal end of the peroneal nerve. Their morphometric analysis also demonstrated that the presence of a window in the epineurium improved the histological picture. However, the long-term evaluation of their study after 8 and 12 months postoperatively may be a contributing factor in the successful pattern of nerve repair.

In the present work ultrastructural examination of the distal end of peroneal nerve in group II confirmed the light microscopic results. Bundles of regenerating nerve fibers appeared thinly myelinated, each one was enclosed in a separate Schwann cell and the whole bundle was surrounded by slender fibroblasts. These bundles were described by many authors as regenerating clusters or units [14,15].

They also recorded the presence of slender encircling fibroblasts surrounding the regenerating cluster [14-16]. Hence, the excessive collagen deposition frequently observed around the regenerating units in group I and II of the present study is closely related to the enhanced fibroblastic activity.

Many studies attributed the regeneration of the nerve fibers to Schwann cell proliferation within the basement membrane tube left behind by the degenerated fiber. These Schwann cells stimulate the

outgrowth of axons through synthesis of nerve growth factors, hence one degenerated fiber is initially replaced by several axonal sprouts. These become individually supported by their own myelinated Schwann cell [17,8]. This finding concurs with the observed Schwann cells with hypertrophied nuclei surrounding early myelinated axons in the control group and group I.

According to Anders and Blaivas [18] the number of myelin lamellae which determine the myelin thickness is proportional to the axon caliber in normal peripheral nerves with normal axon: myelin ratio. However, in case of regenerating fibers, they recorded an increased axon: myelin ratio. These findings explain the frequently observed thinly myelinated axons in the regenerating clusters of nerve fibers.

Ultrastructural examination of group I revealed the close association of macrophages and fibroblasts to the regeneration. Recent studies reported that macrophage response to degeneration is pivotal to the initiation of regeneration. Macrophages release interleukins that stimulate the production of nerve growth factor (NGF) and insulin-like growth factor-1 (IGF-1) by Schwann cells and probably fibroblasts, which are essential mediators for nerve fiber regeneration [19,20].

The present work revealed the presence of nerve fibers with abnormal myelin figures beside the regenerating clusters which were more pronounced in group II than those of Group I. These abnormal myelin figures ranged from irregular looped myelin to splitting and vacuolation in the myelin lamellae. These findings may be attributed to demyelination of the nerve fibers as a secondary reaction to axonal affection [18].

Examination of the endoneural capillaries of the peroneal nerve in group II revealed increased activity of the lining endothelial cells. Some cells showed irregular nuclear shape with increased pinocytotic vesicles. These findings are in accordance with the results of Nukada [21] who demonstrated an increase in endothelial activity and in the number of endoneural capillaries eight weeks after crush and transection lesions. He suggested that axonal outgrowth appears to be an important determinant of post-traumatic new capillary formation.

The examination of the distal stump of peroneal nerve in group I are in agreement with other studies recorded that end-to-end anastomosis is the

preferred surgical technique for peripheral nerve reconstruction after injury as long as the two nerve ends are available [22,23]. Ultrastructural examination of the group in the present work revealed successful regeneration pattern of the nerve fibers. Many well myelinated axons and axons in different stages of myelination were frequently encountered while abnormal myelin figures were infrequently depicted.

The results of electromyographic study in the present work go hand in hand with the histological study. The electromyographic examination revealed signs of reinnervation during voluntary activity in both groups of muscles supplied by peroneal nerve in both groups (I & II). However, the results reflected better reinnervation with the traditional method of nerve anastomosis via end-to-end technique.

In agreement with the EMG results of the present work, several studies have demonstrated the effectiveness of end-to-side neurorrhaphy for muscle reinnervation. Caderna et al. [24] studied the chronic effect for six months of end-to-side neurorrhaphy between the end of tibial nerve and the side of peroneal nerve on the donor muscle structure. They concluded that end-to-side neurorrhaphy did not affect the long term structure or function of muscles innervated by the donor nerve. Moreover, Lutz et al. [25] reported that end-to-side neurorrhaphy of the median nerve as a recipient nerve and the ulnar nerve as a donor nerve resulted in motor recovery of 70% muscle power with well coordinated muscle function as compared to end-to-end neurorrhaphy which reflects effective axonal sprouting through end-to-side anastomosis.

There is a great controversy about the novel approach of removal of an epineural window in the healthy nerve. Lundborg et al. [26] noted that the rate of axonal regeneration was higher with epineural windows than with no windows. Al-Qattan and Al-Thunyan [16] reported that with using nerve grafts perineural sutures are more likely to induce collateral sprouting than epineural sutures. They explained this finding by producing more injury to the parent nerve through perineural sutures than with epineural sutures and hence induce more axonal sprouting. In disagreement with this point of view, Hermanns et al. [27] suggested that the scar formed after injury is an obstacle for successful axonal regeneration and a decrease in the amount of scar tissue is a prerequisite for regrowing axons to cross the lesion site.

However, the results of satisfactory nerve regen-

eration in the present work following end-to-side technique with epineural window are in agreement with several studies [28,29,30] which demonstrated that end-to-side neurorrhaphy is comparable to well-performed end-to-end neurorrhaphy and the formation of epineural window in the healthy nerve is a reliable technique and helps in growth of axons laterally from the healthy donor nerve to the distal stump of the sectioned nerve.

The present work concluded that end-to-side neurorrhaphy is a reliable choice for surgeons when end-to-end anastomosis is not applicable as in cases of avulsion nerve injuries, long segment loss of peripheral nerve or whenever the proximal end of the injured nerve is unavailable. Also, in the treatment of facial palsy especially Moebius syndrome, prevention and treatment of painful neuroma as well as its prospective use in the creation of a neosphincter in patients suffering from foecal incontinence.

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