The Scientific Journal of University of Benghazi

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Improvement of Motor Nerve Regeneration Using the Nanofibrils Technique in Adult Mongrel Dogs

Warda M. Abdelhafiz[1], Mahmoud A. Amer[2], Abdelwahab A. El-Ghareeb[2], Mohammad A. Abdelhameed[3], Laila R. Eljeriby[4] and Abdelraouf A. Khatal[5*]

[1] *Faculty of Dentistry, Department of Histology, University of Sirt, Sirt, Libya*
[2] *Faculty of Science, Department of Histology, Cairo University, Cairo, Egypt*
[3] *Faculty of Veterinary Medicine, Department of Histology, Cairo University, Cairo, Egypt*
[4] *Faculty of Dentistry, Department of Histology, University of Benghazi, Benghazi, Libya*
[5] *Faculty of Medicine, Department of Histology, University of Benghazi, Libya, Benghazi, Libya*

Received; 25/10/2021, accepted; 15/11/2021

الملخص

اخْتِيرَ الكلب حيواناً تجريبياً بسبب حقيقة أنه كلما كان الحيوان أكبر، زادت القوة التجديدية التي تعتمد بشكل رئيس على حجم العصب قيد الاستخدام. ضُمِّنَت تسعة كلاب هجينة بالغة في هذه الدراسة التي تهدف إلى تقويم تجديد العصب الحركي باستخدام تقنية nanofibrils الغرض من وضع الأساس المنطقي للاعتلال الحد الأدنى. عُمِلَتْ فجوة (5 /1و1سم واحد ونصف سم في العصب الثني العام comman peroneal nerve عن طريق القطع المباشر لكلا الجانبين (قطع من جانبين لتجنب الصدمة المزدوجة). واستخدمت الألياف النانوية nanofibrils لملء الفضاء وتغطية الطرفين الثابتتين في ستة من كلاب التجربة، واستخدمت ثلاثة كلاب أخرى معياراً (control) حيث قُطِعَ العصب وترك الفضاء بين الطرفين دون تثبيت الألياف النانوية (nanofibrils)) ما بين جانبين القطع . بعد أربعة أشهر من العملية الجراحية، قُوِّمت الأعصاب المتجددة عن طريق الفحص العيني والمجهري. أُخِذَت الأجزاء الطبيعية من العصب العممي العميق deep peroneal nerve (D.P.N)لمقارنة التغييرات. جميع الحيوانات المستخدمة ذات الألياف النانوية المزروعة أطرافها بنجاح عادت بعد 3-4 أسابيع طريقة المشي إلى حالتها الطبيعية دون أي أعراض ملحوظة للأعرج وأظهرت زوايا المفاصل وضعها الطبيعي. بينما ظهرت مجموعة التحكم (control))عرجاء وظهورانحناء المفاصل العليا مع ضمور ملحوظ من عضلات الأطراف. لوحظ من خلال الفحص المجهري أن عينات الأعصاب المعالجة بالألياف النانوية أفضل تنظيما من الألياف العصبية التي لم تعالج. كذلك المزيد من الألياف العصبية الملينية المتمايزة (myelinated nerve fibers) مُجْزئة بشكل جيد إلى عدة حزم عصبية صغيرة في جذوع الأعصاب المعالجة بالألياف النانوية مقارنة مع حزم أقل في مجموعة التحكم غير المعالجة. أيضا كانت أغشية النسيج الضام (الإبينيوريوم والبيرينوريوم والإندوريوم (epineurium, perineurium and endoneurum) (أكثر بروزا مع أعداد أعلى نسبيا من الأوعية الدموية الصغيرة مقارنة بمجموعة التحكم غير المعالجة. نستنتج من ذلك أن الألياف النانوية يمكن اعتبارها مادة فعالة لتعزيز التجديد في إصابات الأعصاب الطرفية.

كلمات المفتاحية: علم الأنسجة، النهاية العصبية للكلب، التجديد الذاتي.

Abstract

The dog was selected as the experimental animal due to the fact that the larger the animal, the higher the regenerative power which depends mainly on the size of the nerve in use. Nine adult mongrel dogs were included in this study which aimed at evaluating motor nerve regeneration using nanofibrils technique for the purpose of developing a rationale for a minimal morbidity. A one and half cm gap was made in the common peroneal nerve by the direct cutting of both sides (proximal cut and then distal to avoid the double shock). The nanofibrils were used to fill the space and cover the two fixed ends in six of the dogs and another three animals were used as control. The nerve was cut and the space in between the two fixed ends was left without nanofibrils. Four months after surgery, the regenerated nerves were evaluated by macroscopic and microscopic examination. Normal segments of the deep peroneal nerve (D.P.N.) were taken to compare the changes. All animals with implanted nanofibrils used their limbs successfully at 3-4 weeks, the animal gait returned to its normal condition without any remarkable symptoms of lameness and the joints angles displayed its normal posture. Dogs in the control group showed lameness and flexion of the upper joints all-over during the time of the experiment with marked atrophy of the limb muscles. Better organized histological structures of nanofibre in treated nerve samples than the untreated control one were noted. More compartmentation of well-differentiated myelinated nerve fibers into several small nerve bundles in nanofibre treated nerve trunks compared with fewer bundles in the untreated control group. Connective tissue stroma (epineurium, perineurium and endoneurum) were more prominent with relatively higher numbers of small blood vessels compared with the untreated control group. Thus we concluded that nanofibres can be regarded as an efficient regeneration enhancing material in peripheral nerve injuries.

Keywords: Dog motor nerve, histology, nanofibrils, regeneration, nanofibrils;

*Correspondence:
Abdelraouf A. Khatal.

Raoufkhatal2@gmail.com

 ISSN:Online **2790-1637**, Print **2790-1629**; National Library of Libya, Legal number : **154/2018**

1. INTRODUCTION

Current advances in nanotechnology had led to the development of a new field of research – nano¬science. It is the science of dealing with small particles of materials on a nanometre scale in at least one dimension (1-100 nm) [1]. Nanomaterials can interact with tissues at the molecular level with a very high degree of functional specificity and control [2]. A large group of nanomaterials includes nanotubes, nanofibres, liposomes, nanoparticles, polymeric micelles, nanogels and dendrimers. Such materials can be tailored to react with specific biological systems at a molecular or even supra-molecular level and respond to the cell environment while minimizing undesired side effects [3].

Neuron injuries lead to complex cellular and molecular interactions at the lesion site in an effort to repair the damaged tissue and to regenerate the axon for reconnection with its target organ. Strategies to enhance and stimulate regeneration use various nerve conduits and synthetic guidance devices. A promising strategy for the treatment of neuronal injuries is to support and promote axonal growth by means of nanotubes and nanofibers [4]. Damage of the peripheral nerve leads not only to the degradation of the myelin sheath, but also to the degeneration of motoneuron bodies. Knakiewicz et al. 2009, showed that after injury of the ventral branches of spinal nerves C5, C6, C7, C8 &Th1 in rabbits some neurons of spinal cord anterior horns died and this process depended on the time after the damage [5].

Unsuccessful results of neuronal regeneration after injury are influenced by various factors, such as inflammatory cell activation and production of molecules inhibiting regrowth and leading to secondary injury [6]. There are numerous barriers that must be overcome in order to achieve axonal regeneration after injury in the nervous system: scar tissue, gaps in nervous tissue formed during phagocytosis of dying cells, several factors that inhibit axon growth in the mature CNS of mammals, and a failure of many adult neurons to initiate axonal extension [7,8]. Strategies to overcome the inhibitory factors in regeneration use various nerve conduits and synthetic guidance devices. A tubular conduit, made of degradable or non-degradable compounds, can guide and facilitate peripheral nerve regeneration. Various conduits have been fabricated for bridging nerve gaps after injury, and both natural and synthetic materials have been used [9]. The main characteristic of these materials is a longitudinal organization mimicking the natural structure of the nerve pathway within the brain and spinal cord. They are designed to serve as conduits for axonal elongation and to constrain the direction of regenerative outgrowth. Moreover, they should be able to direct regenerating axons to reconnect with their target neurons and enhance functio¬nal restoration of the nerve [10]. A promising strategy for the treatment of neuronal injuries is to support and promote axonal growth by the use of nanometre-scale materials, especially nano¬tubes and nanofibres3. They serve as an extracellular scaffold to guide directed axonal growth and can regulate neurite branches [11].

Many experiments have been performed to study functional recovery after injury in animal models [12, 13]. The aim of our study is to evaluate motor nerve regeneration using the nanofibrils technique for the purpose of developing a rationale for a minimal morbidity-promising procedure with the most acceptable cosmetic and functional results for patients with peripheral nerve affections.

2. MATERIALS AND METHODS

2.1 Material

Nine healthy, mature dogs 10-12 months old and weighing about 15-20 kg each, were used in this study. All dogs were housed in separate cages, living in optimal condition. The animals were allowed unrestricted access to food and water. After exposure of the common peroneal nerve of the right hind limb in six of the dogs, a one and half cm gap was made in the nerve by the direct cutting of both sides (proximal cut and then distal to avoid the double shock). The nanofibrils were used to fill the space and cover the two fixed ends. Another three animals were used as control. The nerve was cut and the space in between the two fixed ends was left without nanofibrils(Figure 1a, b, c& d).

2.2 Method

All dogs (control and experiment) were euthanized after 4 months. The nerve segment was extracted and prepared for the histological examination. Normal segments of the deep peroneal nerve (D.P.N) were taken from the other limb in order to compare the changes. The collected nerve samples included in this study were run through the paraffin embedding technique to get paraffin blocks. Histological serial sections were cut from the nerve sample of each group. Serial sections 5 µm thickness were cut and mounted on glass slides, and then stained with ordinary Hematoxylin and Eosin (H and E) stain, which was used for general examination, and Masson's trichrome stains for the organization of collagen fibers stromal density along the nerve trunk [14].

3. RESULTS

3.1 Clinical observation

Throughout the experiment, the general health condition of the treated animals was satisfactory and they tolerated the surgical procedures well. The animals were apparently healthy until the time of euthanasia.

The treated animals' gait ranged from observable moderate lameness in slow motion to no weight bearing in fast motion during the first 7 days post-operatively. Complete weight-bearing occurred at 2-3 weeks after implantation of the nanofibrils. All animals used their limb successfully at 3-4 weeks and the animal gait returned to its normal condition without any remarkable symptoms of lameness and the joint angle displayed its normal posture. However, all dogs in the control group showed lameness and flexion of the upper joints all-over during the time of the experiment with marked atrophy of the limb muscles.

3.2 Histological findings

Microscopic examination of different collected samples of deep peroneal nerve (D.P.N.) trunk demonstrated the normal histological structures of nerve trunk with multiple bundles of nerve fibers separated by endoneural connective tissue, perineural connective tissue between the bundles and whole nerve trunk surrounded externally by epineural connective tissue rich in small blood vessels (Figure 3a). The deep peroneal nerve (D.P.N) is ensheathed by Schwann cells that form myelin around the trunk(Figure 3a).Well-organized collagen fibers with fine fibrils surrounding myelinated nerve fibers (endoneurium) and thicker collagen fibers in the perineurium (Figure 3b)

 ISSN:Online 2790-1637, Print 2790-1629; National Library of Libya, Legal number : 154/2018

Histopathological examination of the nerves with the implanted nanofibril revealed the normal histological structures of nerve trunk with multiple bundles of nerve fibers separated by endoneural connective tissue, perineural connective tissue between the bundles and whole nerve trunk surrounded externally by epineural connective tissue rich in small blood vessels.

Better organized histological structures of nanofibril-treated nerve samples than the untreated control one. More compartmentation of well-differentiated myelinated nerve fibers into several small nerve bundles in nanofibriltreated nerve trunks compared with fewer bundles in the untreated control group. Connective tissue stroma (epineurium, perineurium and endoneurum) were more prominent with relatively higher numbers of small blood vessels compared with the untreated control group in addition to mild inflammatory cells infiltrations were detected.

In nanofibril treated nerve trunk were showing thicker nerve trunk with nearly complete bridging of nerve fibers across several bundles and separated by thick perineural connective tissue(Figure 4a). There was homogenous basophilic material representing scaffold remnants (Figure 4b). These scaffold remnants were surrounded with fibrous connective tissue rich in thick collagen fibers, dilated blood vessels (Figure 5a).

In higher magnification were showing well-differentiated myelinated nerve fibers with many Schwann cells and fine thin collagen fibers of the endoneurium (Figure 5b).

Non-treated nerve trunk showed several nerve bundles with incomplete bridging of nerve fibers separated by moderate thick highly fibrous perineural connective tissue carrying few small blood vessels(Figure 6). Schwann cells densities were fewer in number compared with nanofibriltreated group and also they showed less differentiated nerve axons (Figure 7 a, b)

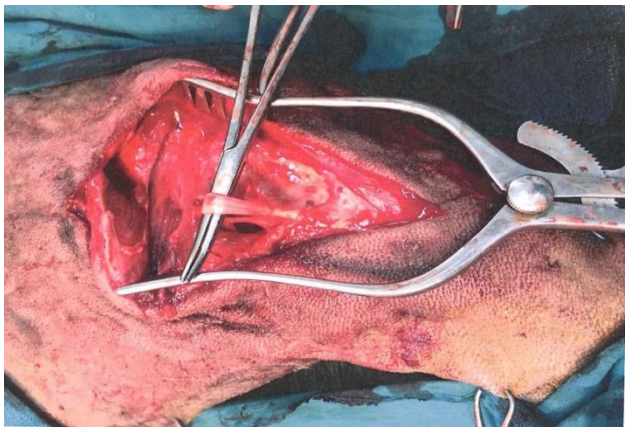

Figure 1a: A photograph showing the deep peroneal nerve over the artery forceps.

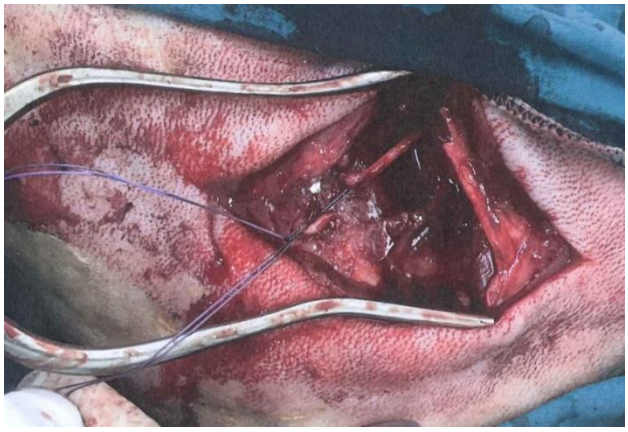

Figure 1b: A photograph showing the two ends of the nerve with relaxed stitch.

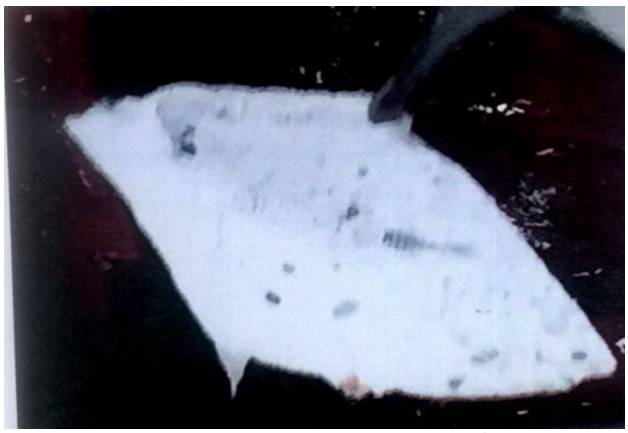

Figure 1c: Photograph showing the modulated nanofibrils material.

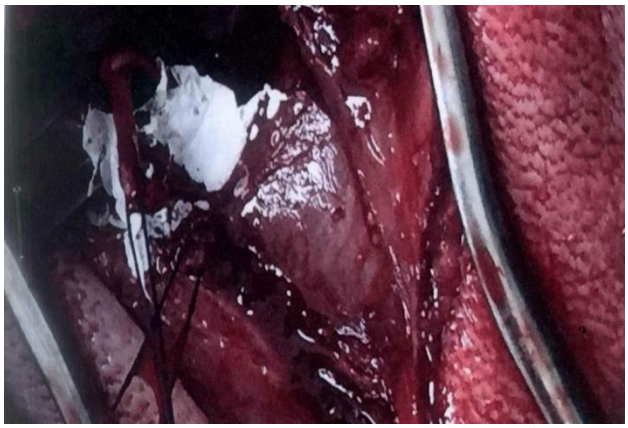

Figure 1d: A photograph showing the nanofibrils after implantation.

 ISSN:Online 2790-1637, Print 2790-1629; National Library of Libya, Legal number : 154/2018

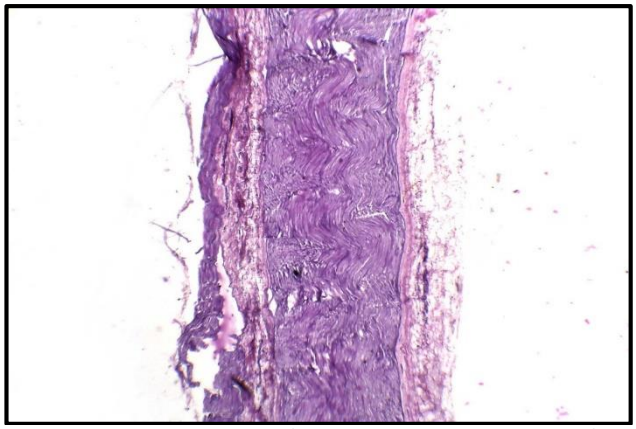

Figure 2a: photomicrogrph from longitudinal tissue section of normal DPN trunk showing well-organized, densely packed nerve fibers arranged in packed bundles and surrounded with connective tissue stroma in the form of endoneurium, perineurium and epineurium. (H&E 40X)

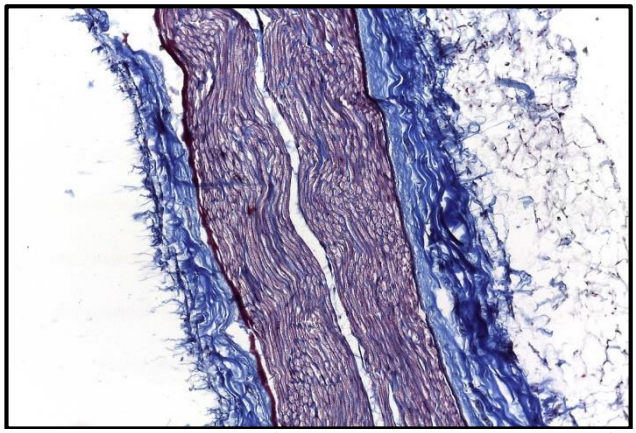

Figure 2b: photomicrogrph from the longitudinal tissue section of normal DPN trunk showing organization of collagen fibers stromal density along the nerve trunk. (Masson's trichromestain 40X)

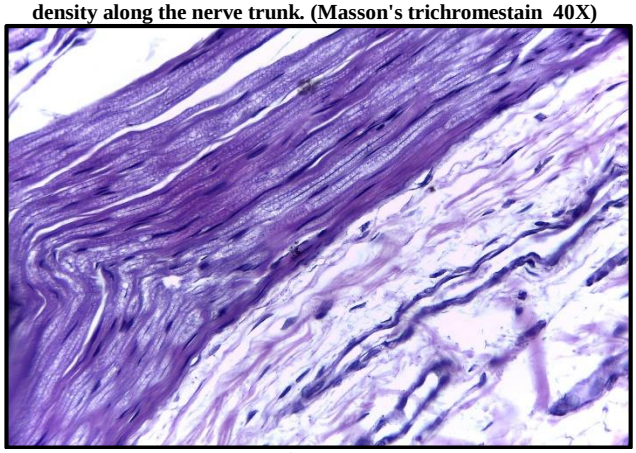

Figure 3a: photomicrogrph of normal DPN trunk showing intact myelinated nerve fibers, Schwann cells (yellow arrow) and perineural connective tissue housing many blood capillaries (red arrow) (H&E 100X)

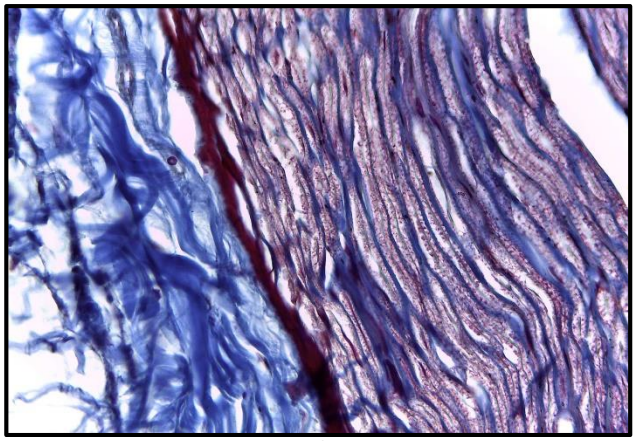

Figure 3b: A photomicrogrph of normal DPN trunk illustrates organized collagen fibers with fine fibrils surrounding myelinated nerve fibers (endoneurium) (yellow arrow) and perineural thicker fibers (red arrow)

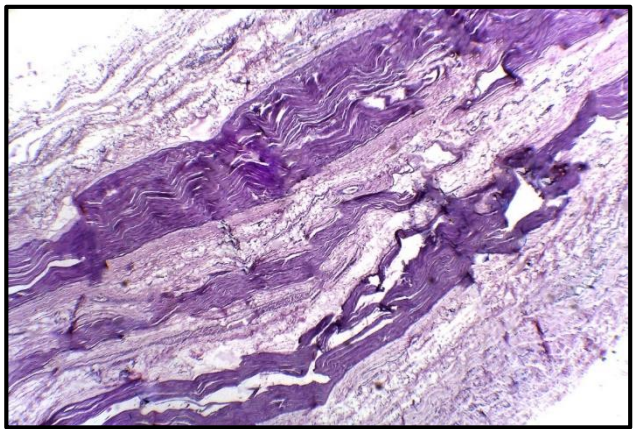

Figure 4a: A photomicrogrph of treated trunk illustrates thick nerve trunk with nearly complete bridging (H&E 40X)

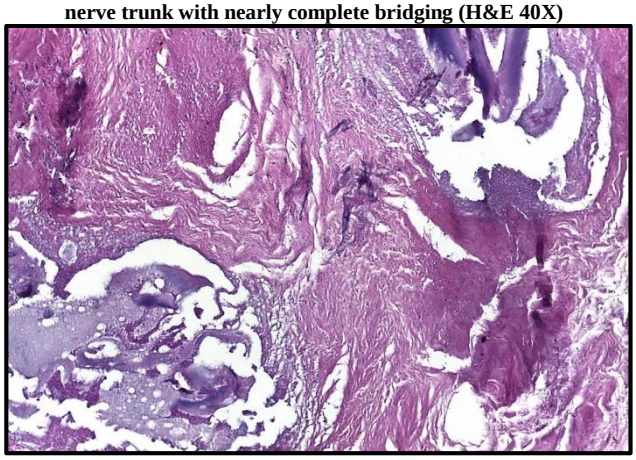

Figure 4b: Photomicrogrph of treated trunk illustrates homogenous basophilic scaffold remnants (arrow). (H&E 100X)

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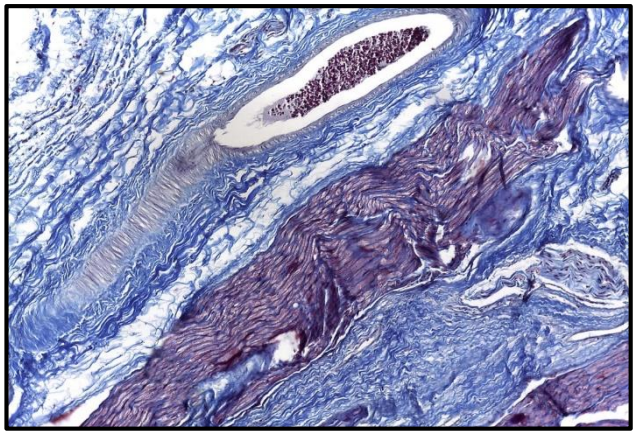

Figure 5a: A photomicrogrph illustrates densely backed thick collagen fibers (perineurium& epineurium) surrounding well-organized myelinated axons of nerve bundles with dilated blood vessels (arrow). (Masson's trichrome stain, 40X).

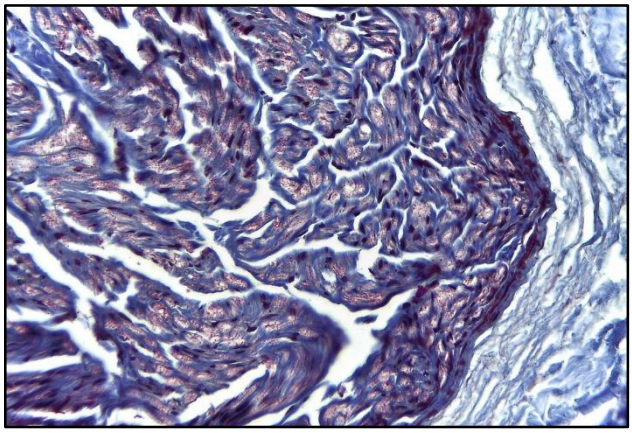

Figure 5b: A photomicrogrph of higher magnification from figure 5a showing well-differentiated myelinated nerve fibers with many Schwann cells and fine thin collagen fibers of endoneurium . (Masson's trichromestain 100X).

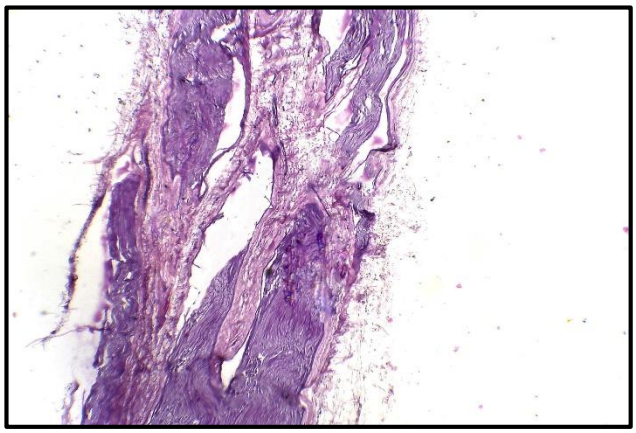

Figure 6: A photomicrogrph of non-treated nerve trunk showing several nerve bundles with incomplete bridging of nerve fibers and separated by moderate thick highly fibrous perineural connective tissue with minimal vasculature (H&E 40X)

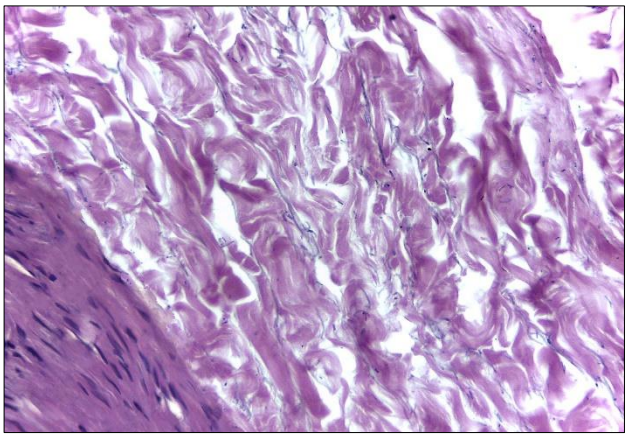

Figure 7a: Photomicrogrph of non treated nerve trunk illustrates the density of Schwann cells (arrow) were fewer in number compared with Nano treated group in figure (7b) Note: Poorly vascularized perineurum. (H&E 100X)

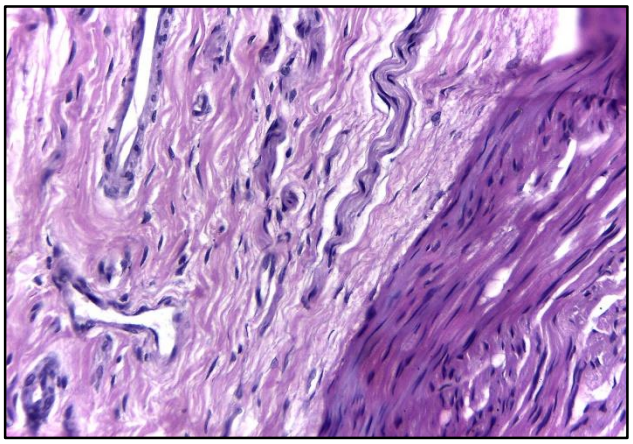

Figure 7b: A photomicrogrph of nano-treated nerve trunkillustrates density of Schwann cells (arrow). Note: Highly vascularized perineural connective tissue with many blood vessels. H&E 100X. trichrome stain,

4. DISCUSSION

Neuron injuries lead to complex cellular and molecular reactions at the lesion site in an effort to repair the damaged tissue and to regenerate the axon for reconnection with its target organ. Damage of the peripheral nerve leads not only to the degradation of the myelin sheath but also to degeneration of motoneuronbodies [15]. Under the right conditions, however, axon extensions can regenerate over gaps caused by injury, reconnecting with the distal stump and eventually reestablishing functional contacts. Peripheral-nerve injuries that result in long gaps require surgical implantation of a bridge or guidance channel between the proximal nerve end and the distal stump in order to restore full function and organ re-innervation [16].

The development of techniques to improve nerve repair in both the peripheral and central nervous systems have been the object of a tremendous amount of scientific and medical investigation and has recently also come under study by biotechnologists, biochemical engineers and materials scientists. The typical graft of choice is the autograft, which is a segment of nerve removed from another part of the body. Disadvantages of the nerve autograft include a second surgical procedure, limited availability and permanent denervation at the donor site. Allografts have also been used, but these are accompanied by the

 ISSN:Online 2790-1637, Print 2790-1629; National Library of Libya, Legal number : 154/2018

usual need for immune-suppression and have very poor success rates [17]. Autologous and autogenous are biological guidance tubes including blood vessels18and muscle fibers [19] had also been used as conduits for nerve regeneration with varying levels of success, but these still suffer from some of the same disadvantages as auto- and allografts. Conduits have also been formed from other biological materials, with collagen showing the greatest potential for success [20]. Avoiding the problems of availability and immune-rejection, a promising alternative for extending the length over which nerves can successfully regenerate is the artificial nerve graft (also known as a nerve guidance channel).

Current advances in nanotechnology have led to the development of a new field of research– nano¬science. The main goal of nanotechnology is the development and application of nanomaterials that display unique physical, chemical and functional properties not shown by bulk materials. Nanomaterials can interact with tissues at the mole¬cular level with a very high degree of functional specificity and control [1]. Nanotechnology has significant potential for future clinical application in the diagnosis and treatment of various disturbances of the central and peripheral nervous systems [21]. A variety of biodegradable materials have been processed into nanofibrous scaffolds using the electrospinning technique for PNI repair [22]. In an early study, a bilayer chitosan conduit with an inner layer of nano/microfibrous structure modified with oligopeptide was generated to repair a 15mm sciatic nerve gap in rats [23]. This novel integrative chitosan conduit effectively promoted axonal regeneration that was comparable to that of autologous nerve grafting on histological assessment.

Recently, a blend of biodegradable polymers PLGA/PCL was used to produce electrospun tubes to bridge a 10mm long sciatic nerve lesion gap in rats. Four months after surgery, most of the electrospin conduit-treated animals showed neural regeneration and functional restoration on immunohistochemial studies and electrophysiological assessment [24]. Schnell et al., 2007, demonstrate that electrospun fibers composed of a collagen and PCL blend represent suitable substrate nanofibers on enhancing glial cell growth in vitro, guiding for supporting cell proliferation, process outgrowth and migration and as such would be a good material for artificial nerve implants [25].

Kim et al., 2008, reported that the neurite outgrowth and Schwann cell migration were in the same direction as the aligned fibers and extended significantly longer than those cultured on random fibers. The peripheral nerve regeneration was also significantly improved by the aligned fiber construct [26]. Wang et al.,2009, discovered that crossed fibers in the aligned fiber scaffold could be detrimental in axonal outgrowth, and that cell attachment and growth could depend on fiber density [27]. In addition, the diameter of electrospun fibers could also influence cell attachment, proliferation and migration [28]. Zhu et al., 2011, demonstrated that the nanofibrous nerve conduit significantly improved the regeneration of injured peripheral axons and motor functional recovery at 2 and 12 months' post-surgery [29]. Jiang et al., 2012, proved that the nanofiber-treated rats showed a significantly greater total number of myelinated fibers and thicker myelin sheaths when compared with a group of rats that received microfiber and film conduits at three months' post-treatment. These positive observations provide useful insights for the applications of electrospun nanofibrous nerve conduits with designed nanostructure in the development of peripheral nerve guide conduits [30]. Yang et al., 2004, were showed that the scaffold enhanced cell adhesion and facilitated cell differentiation and neuron outgrowth [31].

Jenny et al., 2012, conducted an experiment where ten-millimeter segments of the sciatic nerve were resected in 44 Lewis rats. The gaps were either left unrepaired, repaired with nerve autograft or repaired with conduit. After 12 weeks, nerve conduction latency, compound muscle action potential amplitude, muscle force and muscle mass were measured. The experiment proved that muscle recovery for the animals treated with this aligned nanofiber conduit approached that of autograft, suggesting the importance of internal conduit structure for nerve repair [32]. Biazar et al., 2013, used a chitosan–cross-linked nanofibrous biodegradable poly (3-hydroxybutyrate-co-3-hydroxyvalerate) nerve conduit. These polymeric conduits were implanted into a 10 mm gap in the sciatic nerves of the rats. Four months after surgery, the regenerated nerves were evaluated by macroscopic assessments and histology. Cellular experiments showed a better cell adhesion, growth, and proliferation inside the cross-linked nanofibrous scaffolds compared to uncross-linked ones, also Schwann cells were well attached on chitosan–cross-linked nanofibrous surface [33].

Abd El Azeem et al., 2015, used silicone tubes embedded with collagen type I sponge seeded with stem cells and neurogenic media "containing nerve growth factors" were sutured, a gap of 3 cm was made on the right side of the dogs' facial nerve of both ends, while in the left side as a control side empty silicone tubes were sutured, healing was evaluated at 6,8 and 10 weeks. Histological examination of the dogs' facial nerves illustrated the massive proliferation of undifferentiated cells at 6 weeks then multiple blood vessels formation and nerve-like structures with partial absorption of the scaffold at 8 weeks then well-organized nerve-like tissues with dilated blood vessels at 10 weeks. This proves that collagen scaffolds seeded with the mononuclear layer containing stem cells and nerve growth factors showed efficacy in regenerating facial nerve [34]. As demonstrated above, our work is in concordance with other authors who worked in the same research field but on different animal models and different types of biomaterial scaffolds.

5. CONCLUSION

Our study points out the efficacy of Chitosan-based nanofibrils in bridging the gap inflicted on the chosen deep peroneal nerve of dogs and the success of the procedure of nerve regeneration. Histopathological evaluation also showed the more organized histological structures of nanofibrils treated nerve samples than the untreated control one. More importantly, the recovery of injured sensory and locomotor function has been shown to occur in a number of peripheral and central injury models using different animal species

6. RECOMMENDATION

Future research in the field of nerve regeneration by nanofibres requires further study due to the fact that it will replace autologous grafting, negating its discussed drawbacks and opening new horizons for rehabilitation and decreasing the previously inevitable effects of traumatic nerve injury in humans.

 ISSN:Online 2790-1637, Print 2790-1629; National Library of Libya, Legal number : 154/2018

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