Effect of folic acid administration on In vivo motor nerves regeneration.

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Abstract: Peripheral nerves injury may occur due to trauma or surgical procedures. It can result in loss of muscle function, impaired sensation and/or painful neuropathies. Successful nerve regeneration requires the concerted interplay of non neuronal cells, growth factors and regenerating axons. Foliates are vitamins essential to the development of the central nervous system. Furthermore, it was shown that parenteral folic acid produces up to 10-fold dose-dependent improvement in axon re-growth and functional recovery after injury to the adult CNS. The aim of the current study is to investigate the effect of folic acid administration on the regeneration of motor nerves after its severance with 15 mm gap between nerve endings. Rabbits were evaluated by clinical examination, nerve conduction velocity, and electron microscopic examination of the regenerate. Results showed that folic acid administration improves neural regeneration and help in its rapid maturation.
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1. Introduction:

Despite extensive research and technical improvements in repairing a peripheral nerve, injury remains a very serious lesion. Return of function although at times is surprisingly good, is more often suspicious. This clearly suggests that there are basic factors underlying peripheral fibre re-growth that are not understood, De Medinaceli et al., (1983).

Nerve regeneration is a complex biological phenomenon. In the peripheral nervous system, nerves can regenerate without any treatment if nerve continuity is maintained whereas more severe type of injuries must be surgically treated, Matsuyama et al., (2000); Siemionow and Brzezicki (2009).

A commonly observed clinical problem following nerve injury is the incomplete recovery of function associated with the formation of a neuroma in continuity, Beveridge and Politis (1988).Injury to motor peripheral nerves result in demyelination or axonal degeneration and, eventually, loss of motor function. Recovery of function occurs with remyelination, axonal regeneration, and re-innervation of the muscle end plates, Mackinnon et al.,(2010); Sanders and Jones(2006).

Many factors in addition to the type of repair are known to affect return of nerve function. These factors include; age, nature of injury, level of injury, size of the gap between nerve ends, and time lapse after injury, Young et al.,(1981).

It is accepted that the obstacles within the connective tissue of the anastomotic zone determine the paths along which the new nerve fibres grow, Lehman and Hayes (1967). After nerve injury, fibrin is deposited at the nerve, and its deposition exacerbates nerve damage, Akassoglou et al., (2000). Fibrin deposited in the nerve after injury changes the composition of extracellular matrix, inhibits Schwann cell migration, and induces pro-inflammatory cytokine expression, Akassoglou et al., (2002) and (2003).

Successful nerve regeneration requires the concerted interplay of non neuronal cells , growth factors and their receptors; cell adhesion molecules , extracellular matrix materials and regenerating axons , Madison and Archibald (1994).

Following nerve injury, the traditional surgical method of repair is by nerve suturing, and in some cases when the gap is large, nerve grafting is mandatory, Trezis et al.,(1975). Although autografts offer the best results in nerve reconstruction, their disadvantages include donor site morbidity, sacrifice of a functional nerve, formation of potential painful neuromas, structural differences between donor and recipient grafts, and insufficient graft material, Panseri et al.,(2008);. Siemionow and Brzezicki (2009).

Microscopically; three segments of the injured nerve may be distinguished; the proximal portion, the distal portion, and the region of repair between these two, Lehman and Hayes (1967);Haftek and Thomas(1968). Post traumatic regeneration in the peripheral nerves depend to a large extent on appropriate technique for the surgical apposition of the injured nerves, Kuljis et al.,(1983), significant

reduction of the gap, and better approximation of more health stumps that result in a decrease in misrouted neuritis, De Medinaceli (1988).

Recent studies have implied that the problem of poor recovery of function can be directly attributed not only to the anatomical but also to the physiological changes that occur in the regenerated nerve and in its target muscle, Gilmour et al.,(1995).

It seems evident that the alternatives that exist today in handling large nerve defects, suture under tension or nerve grafting are unsatisfactory. The nerve graft also suffers from additional drawbacks, such as, sacrifice of a healthy functional tissue, an additional surgery time, and size mis-match, Mahesh and Ravi(2008).

Peripheral nerves can regenerate across nerve gaps when guided by an appropriate nerve conduit, it has been documented in many animal studies that nerves will regenerate across various tubes, Cheng and Chen (2002).

Entubation repair has been used as a nerve repair method for almost a century. Many materials have been utilized with greater or less success to ensheath-injured nerve, Liu (1992), as polyglactin Cheng and Chen (2002). , polyethylene Stevenson et al.,(1994), polylactic acid, Chiu and Sidman (1985);Xie et al.,(2008); andpolytetrafluroethylene, Zetti et al.,(1991); Williams et al.,(1993); Smith and Robinson (1995);Lanzetta et al.,(2003); Dodla and Bellamkonda(2006) .

The effect of exogenous biochemical agents on nerve regeneration such as laminin, Cao et al.,(1991); Ding et al.,(2011); Homma et al.,(2011), steroid hormones, English et al.,(2005), and nerve growth factor, Dodla and Bellamkonda(2006) have been applied to the injured peripheral nerves either in systemic or local ways with variable results.

Therefore, therapies with relevant growth factors increasing attention in recent years, although growth factors therapy is a difficult task because of the high biological activity (in pico- to nanomolar range), pleiotrophic effects (acting on a variety of targets), and short biological half-life (few minutes to hours). Thus, growth factors should be administered locally to achieve an adequate therapeutic effect, Schmidt and Leach (2003); Sandra et al., (2010).

Since during regeneration axons require neurotrophic support, they could benefit from their presence during axonal regeneration. Folates are vitamins essential to the development of the central nervous system. Insufficient folate activity at the time of conception and early pregnancy can result in congenital neural tube defect, Malouf et al.,(2003).

The folate pathway plays a crucial role in the regeneration and repair of the adult CNS after injury. Folic acid and possibly other nontoxic dietary methyl donors may therefore be useful in clinical interventions to promote brain and spinal cord healing, Iskandar et al.,(2010). Furthermore, it was shown that parenteral folic acid produces up to a 10-fold, dose-dependent improvement in axon regrowth and functional recovery after injury to the adult CNS, an effect well in excess of other interventions, Iskandar et al.,(2004). The aim of the current study is to investigate the effect of folic acid administration on the regeneration of entubated motor nerves after its severance with long gap in between nerve endings.

2.Materials and methods:

Prior to this work a trial surgical procedure was performed on the buccal branch of the facial nerve, several cuts were done ,size of tube tested and degree of magnification selected. Myelinated axons counted and neural structure were recorded, also, nerve conduction velocity for the healthy buccal branch of facial nerve was recorded for comparison.

2.1. Materials

2.1.1. Samples:

The current study included 20 newzealand rabbits of both sexes previously vaccinated and free from diseases or congenital anomalies, weighing approximately 2.5 to 3 kg body weight.. Rabbits were randomized divided into two groups:

Group(A):

It included 10 rabbits in which Gore-Tex tubes implanted for nerve guided regeneration and acted as control.

Group (B):

It included 10 rabbits in which Gore-Tex tubes were implanted and received parenteral folic acid injection.

2.2. Methods:

2.2.1. Surgical procedure:

i-The rabbits prevented from water and food four hours pre-operatively, then rabbits were anesthetized with xylazine hydrochloride (0.15 mg/kg) (Rompun 2% solution equivalent to 20 mg active ingredient, Bayer Leverkusen-Germany) plus Ketamine hydrochloride (50 mg /kg) (Ketalar 50 mg per ml Park-Davis Co USA) by intramuscular injection in the rabbit's gluteus maximus muscle.

ii-After the buccal area was shaved and prepared with 70% ethanol with betadine, A horizontal curvilinear incision about 2.5 cm was done below the

lower eye lid. The superficial facial muscles were sharply divided and the facial branches distal to the parotid gland were exposed. The buccal branch of the facial nerve was freed from the surrounding structures.

iii-Following the identification of the buccal division of the facial nerve that verified with muscle twitching, About 15 mm segment was removed .The proximal and distal cut nerve endings were sutured into the Gore-tex tube with 8-0 vicryl sutures (poly glactin 910 braided coating glycolide lactide calcium stearate Ethicon synthetic absorbable suture).

iv-The wound was closed in one layer with 000 black silk sutures (Mersilk Ethicon LTD UK. Slim blade cutting 15mm needle). The rabbits were covered with towels and observed during recovery. After recovery the rabbits were housed individually with free access to food and water.

In group (B) parenteral folic acid ["Folvite" N-(p(((2-Amino-4-hydroxy-6-pteridinyl)-methyl)Amino) benzoyl)] each ml of folic acid solution contains sodium folate equivalent to 5 mg of folic acid] were injected in the gluteus maximus muscle (80 µg / kg per day), starting from the day of operation and continued to the end of follow up period.

Five rabbits from each group were sacrificed at three, and six weeks post –operatively.

2.2.2. Evaluation of regenerated nerve:

The parameters

I-clinical observation:

The rabbits were observed for spontaneous and /or induced movements of the nose, whiskers and upper lip, also symmetry of the face was taken into consideration.

II-Nerve conduction velocity

At the end of each follow up period (three and six weeks post operative) buccal nerve conduction velocity (NCV) was performed. Surgical exposure of buccal nerves of both sides and a shock-emitting electrode is placed directly over the nerve, and a recording electrode is placed over the nasal muscles controlled by that nerve. Several quick electrical pulses are given to the nerve, and the time it takes for the muscle to contract in response to the electrical pulse is recorded. The speed of the response is called the conduction velocity. This value is called the latency and is measured in milliseconds (ms). NCV of the same nerve on the other side of the same animal with the same electrode distance was recorded and the value subtracted from that of surgically treated side. Data collected and statistically analyzed using paired Student's "T" test and the results recorded in tables.

III microscopic examination:

Tissue harvesting and processing:

- ***Fixation:***

The area of nerve surgery was infiltrated with the same fixing mixture by injection of 2 ml in the site of previous nerve surgery before tube removal. The tube is disconnected from the nerve by cutting the nerve at each end of the tube and then a longitudinal cut was made into the tube carefully. The regenerated segment carefully examined and was transferred from the inside of the tube to the collecting solution that formed of 4 % glutaraldehyde. One millimeter from the mid portion of each specimen was selected for preparation and examination.

- ***Specimen preparation:***

The regenerated part was transferred to a wide Petri dish, fragmented into very small pieces and stained by using 0.5% toluidine blue stain in 2.5 % $NaCo_3$ to ensure proper orientation of the specimen within block. The blocks then cut with diamond knife until thickness of the ultra thin samples ranges between 60 to 100 nm. Ultra thin sections picked on cupper grids and were contrasted with 7% uranyl acetate in absolute methyl alcohol, then with lead citrate, followed by rinsing with soda solution. The grids were examined with electron microscope

3. Results:

3.1. Clinical observations:

All rabbits showed moderate postoperative swelling at the site of operation. It reaches the maximum at the second post operative day and disappeared at the fifth post operative day.

Function of eating and mastication were not affected in all rabbits. Neither of rabbits showed complete nor partial return of motor function after three weeks postoperatively.

Group (A):

Two cases showed partial return of motor function (whiskers and nasal movements only) at the 39^{th}, 41^{st}, day postoperatively without return of upper lip movements until the end of follow up period. Three cases showed neither partial nor complete return of motor function.

***Group (B)*:**

Two cases showed complete return of motor function with symmetry of the face was clearly noticed; one case showed nasal movements (partial

return) started at 23rd post operative day, then followed by upper lip and whiskers at the 31st day post operative(complete return). In the other case, nasal movements started at 38th day post operative, and complete return of function was at the end of 6th week post operatively.

Two cases showed partial return of motor function (whiskers and nasal movements); one of them showed return of nasal movements at the 39th day postoperatively without return of upper lip movements or whiskers until the end of follow up period. The other case showed nasal movement at the 31st postoperative day without complete return of function. One case showed neither partial nor complete return of motor function. Summary of the data are shown in Table (1).

Table (1): Showing the number of animals that showed return of motor function at the end of six weeks clinical observation.

Group A		Group B	
Complete return of motor function	0	Complete return of motor function	2
Partial return of motor function	2	Partial return of motor function	2
No return of motor function	3	No return of motor function	1

3.2.-Nerve Conduction Velocity:

The speed of nerve impulses is slower compared with the normal other side of the same animal. The difference in nerve conduction velocity (NCV) for Group (A) animals was higher than animals of Group (B), i.e.: there is significant decrease in latency when compared to the group (A) animals Measured at both follow up periods. Latency is related to the diameter of the nerve and the degree of its myelination. Increasing in nerve diameter and proper myelination resulted in increasing in NCV and decrease in latency according to the equation " NCV = 2.5 D" (D=diameter of the nerve). Data summary are shown in Table (2).

Table (2): showing the differences in measurements between both sides of the same animal in NCV for test groups through the follow up period, and the statistical analysis of data using paired Student's T test

GROUP A		GROUP B		GROUP A		GROUP B	
Case No	NCV 3W	Case NO	NCV 3W	Case No	NCV 6W	Case No	NCV 6W
1A	5.1 m/s	1B	3.5 m/s	6A	3.3 m/s	6B	1.3 m/s
2A	4.5 m/s	2B	4.1 m/s	7A	3.6 m/s	7B	1.9 m/s
3A	4.9 m/s	3B	3.7 m/s	8A	2.4 m/s	8B	1.6 m/s
4A	4.2 m/s	4B	3.4 m/s	9A	2.6 m/s	9B	0.4 m/s
5A	5.9 m/s	5B	4.1 m/s	10A	4.1 m/s	10B	1.3 m/s
Total	24.6 m/s		18.8 m/s	Total	16 m/s		6.5 m/s
Mean	4.92 m/s		3.76 m/s	Mean	3.2 m/s		1.3 m/s
SD	±0.64		±0.32	SD	±0.70		±0.56
P = 0.010*				P = 0.004*			

*Significant if P ≤ 0.05

3.3. Electron microscopic evaluation:

The stages of regeneration of rabbit's buccal nerve over 15 mm transactional gap were examined using transmitted electron microscopy. Examination revealed that the regeneration processes showed four stages; establishment of a cellular intergap matrix, the ingrowth of mesodermally derived cells (macrophages and fibroblasts, and endothelial cells) the ingrowth of ectodermally derived cells (Schwann cells) and lastly neural maturation by progressive axonal enlargement myelination and compartmentalization.

The first two stages occur during the first three weeks regeneration, the third and fourth stages occur next to the third week of neural regeneration on wards. The regenerated tissues grew inside the guide channels without significant inflammatory response.

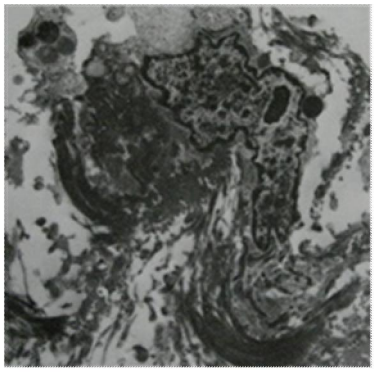
No significant cavities or cysts have been detected in the regenerated tissues.

3.4. Results after third week entubation:

In both groups there were establishment of inter gap matrix interlaced within the matrix are fibroblasts whose orientation were parallel to the long axis of the tubes as confirmed by serial cuts along the regenerate. Macrophages were dispersed in the matrix, it had prominent vesicles which contained phagocytosed crystals. Figure(1).

Three cases of group (A) showed fibroblasts devoid of cytoplasmic processes and had pale nucleolus and endoplasmic reticulum. Collagen bundles were abnormally arranged while blood cells as P.N.L. and RBCs were dispersed in the matrix. Two cases showed thin regenerate with moderate fibroblastic and collagen bundles condensation. The mid chamber contents of all cases were devoid of any neural elements (axons and schwann cells).

All cases of group (B) showed fibroblasts were associated with fibrin and collagen production and the collagen was deposited in the matrix in lamellar pattern, on the other hand the fibroblasts showed tendency to aggregate while they deposit collagen at a perpendicular angle to the long axis of the chamber. These fibroblasts were elongated and had the perineurial cell characteristics (i.e elongated nucleolus, prominent enlarged rough endoplasmic reticulum, presence of cytoplasmic vesicles and presence of prominent basement membrane on outer and inner surfaces of cell processes).Fig(1)



Figure(1):Macrophages were dispersed in the matrix, it had prominent vesicles which contained phagocytosed crystals.

3.5. Results after 6 weeks entubation

Three cases from group (A) showed that the outer fibroblastic layer surrounded a central core containing premyelinated and unmyelinated axons but the unmyelinated axons were the predominant neuroregenerates. premyelinated axons were also present which were enstheased by schwann cells and had no myelin sheath . The axons were in close association with the perineurial fibroblasts most of axons were of small diameter and grouped together into small unites by perineurial fibroblast cellular processes. Each unit surrounded with fibroblastic layer represented fascicles and contained approximately 3 and 12 axons. Fig (3).

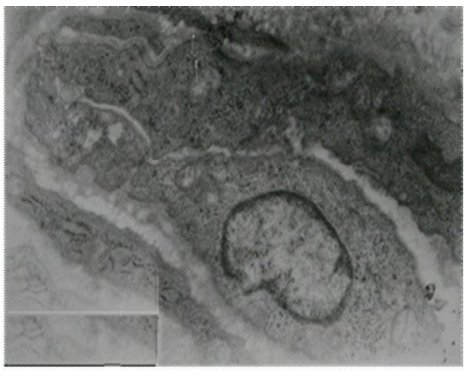


Figure (2) : Elongated fibroblasts with perineurial cell characteristics i.e elongated nucleolus(A), prominent enlarged rough endoplasmic reticulum(B), presence of cytoplasmic vesicles(C), and presence of prominent basement membrane on outer and inner surfaces of cell processes(D).

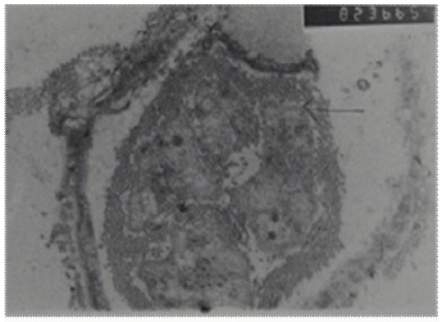


Figure (3): Showed outer fibroblastic layer (A)surrounded a central core containing premyelinated axons(B) The axons were in close association with the perineurial fibroblasts most of axons were of small diameter and grouped together into small unites by perineurial fibroblast cellular processes. Each unit surrounded with fibroblastic layer(C) represented fascicles and contained approximately 3 and 12 axons.

Neovascularization throughout regenerate with blood vessels were both longitudinal and radial in distribution. The blood vessels were found outside the neural compartments and circulation documented by the presence of RBCs in the vessel lumina. Two

cases showed thin regenerate inside the tube and EM examination revealed prominent outer fibroblastic layer surrounding a central core containing axon schwann cell interaction that appeared random and was not organized. There was incomplete and rudimentary compartmentalization between the neural elements. The core structure was enclosed within perineural fibroblast cellular process.In group (B) cases the overall findings were better than group (A). Four cases from group (B) showed collagen IV, one of the main components of the basal lamina in nervous tissue, was found in noticeable amounts and evenly distributed throughout the implant lumens in treated animals. Higher fibroblast concentrations were detected both nearby and within the conduit inner walls. Myelinated axons were noticed throughout the tissue regenerated inside the conduits. premyelinated axons were also present in few numbers which were enthused by Schwann cells and had thin myelin sheath . The axons were in close association with the perineurial fibroblasts. Most of axons were of moderate diameter and grouped together into small unites by perineurial fibroblast cellular processes each unit surrounded with fibroblastic layer represented fascicles and contained approximately 7 and 18 axons . neovascularization throughout the regenerate with blood vessels were both longitudinal and radial in distribution. One case showed thin regenerate inside tube and EM examination revealed prominent outer fibroblastic layer surrounding a central core containing axon Schwann cell interaction with incomplete compartmentalization between the neural elements. The core structure was enclosed within perineural fibroblast cellular process.

4. Discussion:

Regenerating axons require neurotrophic support, they could benefit from their presence during axonal regeneration. During embryonic development of the nervous system, the developing axons use a variety of chemotactic agents to find their target organs, Song and Poo (2001). Many agents have been used in several studies in order to promote nerve regeneration in vivo and direct the axons towards their target tissues, Aebischeret al., (1989); Ahmed et al., (1999) ; Chen et al., (2000). Folic acid is a vitamin essential to the development of the central nervous system. Insufficient folate activity at the time of conception and early pregnancy can result in congenital neural tube defect, Maloufet al., (2003). Folic acid proved that it plays a role in the regeneration of the adult CNS after injury, Iskandaret al., (2004; 2010).

Based on this hypothesis; testing the effect of folic acid administration to enhance the regeneration of motor peripheral nerves is the aim of the current study. This provides possible avenues for new pharmacologic approaches to treating neural injuries. The buccal branch of the rabbit's facial nerve after its severance with long gap in between nerve endings was selected as an experimental model for testing regeneration of motor nerves.

Experimental work, Radtke and Vogt (2009) indicates that motor peripheral nerves has a capacity to regenerate after injury if proper environment available. When appropriate alignment of proximal and distal stumps of cut nerve occurs, the probability of correct nerve-target reestablishment is increased. When regenerating peripheral nerves are blocked from reaching target and neuroma formation occurs, the regenerating axonal sprouts become maladaptive.

In the current study entubation repair facilitates the regenerating axons in the proper way to reach their muscles without neuroma formation. If regenerating axons do not grow within the tubes, but through the connective tissue, they stop regenerating and do not reach target. Thus, the tubes provide an important environment for axonal regeneration.

Nerve entubation repair has been used as a nerve repair method for almost a century. however most of them make use of rigid channel guides that may cause cell loss due to local stresses exerted over the nervous tissue during tube movement. Gore-tex guidance channels characterized by negligible inflammatory response with satisfactory biocompatibility that proved by little inflammatory cells detected in the field by microscopic examination and thin fibrous capsule surrounding the guiding tube.

Spontaneous recovery of motor function in nerve crush injury model was reported several decades ago and again recently, it could be argued that in these studies there was a tendency toward spontaneous recovery even without any treatment. In the present study, the transaction with 15 mm gap which considered serious nerve damage, impossible to heal without treatment.

The beneficial effects of folate supplementation and optimal regeneration after CNS injury reported at a dose of 80 µg/kg body weight. As a water-soluble vitamin, folate cannot be stored in large amounts in the body, even in very high amounts folic acid is non-toxic, Iskandar et al., (2010) In the current study parenteral administration selected to avoid

malabsorption possibilities and selected previously tested dose applied.

Results showed that administration of folic acid significantly improved peripheral motor nerves growth and maturation regarding time and quality of the regenerates which agreed with previous studies, Iskandar et al., (2004) that tested its effect on regeneration of CNS.

NCV revealed re-establishment of functional neuronal connections of the target muscles in all of the treated animals. The interpretation of nerve conduction studies is complex, but in general, different pathological processes result in changes in latencies. For example, slowing of the NCV for Group (A) animals usually indicates that there is incomplete myelination; because myelination and intergap formation converts the conduction from slow nonsaltatory conduction to more rapid saltatory conduction.

Another explanation for slowing of nerve conduction is generalized peripheral neuropathy i.e deficient axon organization and proper compartmentalization. In contrast to animals of Group (B) in which regenerated and re-myelinated axons have myelin sheath, and proper axonal organization and compartmentalization, so they achieve good conduction.

When the axon is severed by nerve injury, the axon die backs a millimeter or two from the injury site and the distal segment degenerates, a feature known as Wallerian degeneration, Waller (1850) The myelin debris is phagocytized by macrophages. While the axon segment distal to the injury site degenerates, the Schwann cells proliferate typically within the basal lamina and form a column of Schwann cells or band of Büngner. This Schwann cell column is act as pathway for directed axonal regeneration. Axons regenerate from the proximal healthy side within these basal lamina tubes to reach motor or sensory targets, Ramon and Cajal (1928).

A critical first step in the sequence of endogenous nerve repair across a gap is the proper formation of an aligned, regenerative fibrin matrix between the nerve stumps. The fibrin matrix provides physical support to the initial influx of migrating fibroblasts and Schwann cells, Liu (1992) . Early detection of the matrix and properly oriented fibroblasts in specimens after three weeks of entubation is considered a mark of good regeneration within the tube, furthermore the difference in maturation of the fibrin matrix and neurofibroblasts confirmed by difference in conduction velocity.

The initial formation of the fibrin cable thus predetermines at an early time the final morphology of the regenerating nerve, Williams and Varon (1985); Zhao (1993).

The regenerating cells in turn form the framework of the regeneration cable, replacing the fibrin matrix with a more permanent network of collagens and start of premyelination and then myelination clearly detected by EM examination at the six weeks post operatively. Myelination and neurofilament expression of nerve fibers indicated the early evidence of nerve regeneration potential and was considered a marker to determine the quality of nerve regeneration. NCV appeared to correlate directly with the thickness of the myelin sheath formed by Schwann cells.

Even though group (B) showed improvement in the rate and quality of neural maturation, they were still with low percentage of return of motor function. It is possible that the regenerating axons take longer than six weeks to traverse the 15 mm nerve gap and the distal nerve stump, to form new neuromuscular junctions regeneration. A study with longer duration of regeneration may result in higher percentage of full muscular recovery.

Folic acid significantly improved all stages of nerve regeneration which confirmed by EM findings at three and six weeks postoperatively. The animals received parenteral folic acid showed faster regeneration and better quality in cellular structure and organization. Conduction velocities of the regenerated nerves correlate directly with the stage of regeneration. Furthermore and based on these findings Group (A) animals display lesser number for neuromuscular recovery than group (B) at both follow up intervals.

In conclusion, we report the novel design, implementation and evaluation of chemical agents for motor peripheral nerve regeneration. Data demonstrates that nerve regeneration across 15 mm nerve gap could be possible.

The safety and simplicity of folate supplementation call for clinical investigations of folate supplementation in patients with neurotrauma. Although clinical evidence is still largely lacking, folate may also have a role not only in the treatment of but also in the primary prevention of nerve dysfunctions. The results of this study gives room for further motor nerve regeneration improvements, like tuning the properties of the tubular structure or providing biomimetic condition. Moreover, these guidance conduits can be loaded with various fillers

like collagen loaded with folic acid gel or combination of folic acid and neurotrophic factors.

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