The sole effect and the combined therapy effect of the hematopoietic cytokine Erythropoeitin and mesenchymal stem cells in peripheral nerve injury in rats

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Abstract:

Injuries to peripheral nerves result in partial or total loss of motor, sensory and autonomic functions. We aimed to evaluate the neuroprotective effect of the sole or combined treatment with erythropoietin (EPO) and human umbilical cord blood mesenchymal stem cells (MSCs) transplantation in sciatic nerve injury. Sixty adult male albino rats weighing ± 200 gm were divided equally into 5 groups: control intact (sham operated) group and four other injured group: control, erythropoietin treated (5000 U/Kg) intralesional after injury, MSCs treated (3×10^5 cells/µl) grafted intralesional immediately after injury and combined EPO and MSCs with same previous doses treated group. MSCs were isolated from human umbilical cord blood by Ficoll-Hypaque density gradient centrifugation, culture of mononuclear cells and selection by CD 105+ve CD34-ve CD45-ve magnetic separation method using MACs separator. Assessment was done electophysiologically by electromyography and nerve conduction velocity (NCV) using the Biopack, MP 150 system were done at 8th week post injury . Eight weeks after the induction of injury and after completing electrophysiological Fevaluation histopathological assessment was done using both hematoxylin and eosin (H & E) and toluidine blue stains to

study the structural changes. Also, gene expression in injured nerve of synapsin I (mRNA) levels by Semi quantitative PCR technique was done 8 weeks after the injury. Injury to the sciatic nerve was done using the standard crush injury method under general anesthesia by pentobarbital sodium (50mg/kg). Complete post-operative care was performed to all groups. The study was approved by the institutional ethical committee and carried out in accordance with the current guidelines for the care of lab animals. EPO or MSCs transplantation accelerated regeneration in SFI at one month and in all other parameters at two months. We suggest that EPO could act in a synergistic way with MSCs to potentiate their neuroprotective effect following peripheral nerve injury.

Keywords:
Peripheral nerve injury, stem cells**,** erythropoietin**.**

Introduction:

Injuries to the peripheral nerves result in partial or total loss of motor, sensory and autonomic functions conveyed by the injured nerves to the denervated segments of the body, due to the interruption of axons continuity, degeneration of nerve fibers distal to the lesion and eventual death of axotomized neurons. Injuries to the peripheral nervous system may thus result in considerable disability ***(1)***. After nerve injuries, axons distal to the lesion site are disconnected from the neuronal body and degenerate. The soma of axotomized neurons undergoes a series of phenotypic changes, known as neuronal reaction and chromatolysis. Whereas Wallerian degeneration serves to create a micro- environment distal to the injury site that is favorable for the axonal regrowth of surviving neurons, neuronal reaction represents the metabolic changes necessary for regeneration and axonal elongation ***(2).***Several approaches have been proposed to have beneficial effects on peripheral nerve

regeneration, including application of an electric field, transplantation of stem cells, and administration of neurotrophic factors**.** Mesenchymal stem cells (MSCs) are emerging as an effective therapeutic approach to a wide range of neural insults since they act as a source of stem-like and progenitor cells. MSCs are known to differentiate into neurons and glial cells *in vitro*, and *in vivo* following transplantation into the brain of animal models of neurological disorders including ischemia and intracerebral hemorrhage (ICH) ***(3).*** *In-vivo* studies have shown that mesenchymal stem cells (MSCs) can improve nerve regeneration, by differentiating into Schwann-like cells, which support nerve fiber growth and myelination ***(4).*** Human umbilical cord blood (HUCB) is considered a valuable source of cells that holds special promise for the treatment of neural diseases, for which no cure is currently available. In addition, therapies based on HUCB are promising because the cells are readily available and less immunogenic as compared to other source of stem cell, such as bone marrow. The therapeutic potential of HUCB may either be attributed to the inherent ability of stem cell potential of damaged tissue outright, or alternatively, to their ability to repair damaged tissue through neural protection and secretion of neurotrophic factors by various cell types within the graft ***(5).***

This study was designed to evaluate the potential neuroprotective effect of the hematopoietic cytokine erythropoietin & human umbilical cord stem cells transplantation each of them individually as well as the combined effect of both. The study aimed to compare the role played by each strategy and reveal if both together can potentiate one another or not.

Materials and methods:

The study was carried out in the Stem Cell Unit (SCU) in the department of physiology, Faculty of Medicine, Suez Canal University. A total of 60 adult male albino rats weighing ± 200gm were used in this study. They were housed in spacious wire mesh cages at room temperature and were kept with free access to standard rat chow diet and tap water. They were left for acclimatization for one week before the start of the study. The rats were divided into five equal groups (12 rats each): the Sham surgery group, in this group the left sciatic nerve was exposed but not crushed; the injured sciatic nerve control group, in this group left sciatic nerve was exposed and crushed ; the erythropoietin treated group in this group 5,000 units per kg of body weight intra lesion given once after injury ***(6),*** the HUCB mesenchymal stem cells transplantation group, in this group sciatic nerve injury was followed by transplantation of 3 × 105 cells/rat MSCs ***(7),*** which were injected intra lesion immediately after injury; the co-treated group with erythropoietin and HUCB stem cells with the same previous doses. Procedures of all groups were followed by wound closure and postsurgical care for 8 weeks.

This experiment was approved by the Research Ethics Committee at Faculty of Medicine, Suez Canal University. Human umbilical cord blood (hUCB) was collected from full-term pregnant women after taking written consents. Thereafter, samples were prepared for tissue culture for preparation of MSCs. The cells were gated out by positive expression of CD105 and negative expression of CD34 and CD45.

Method of sciatic nerve injury:

Rats were anesthetized with pentobarbital (50 mg/kg intraperitoneal) and allowed to recover for 8 weeks after surgery ***(8).*** Sciatic nerve was exposed and crushed with 3 mm wide surgical hemostat that was maintained on the first lock for 1 minute with careful hemostasis ***(9).*** It was applied 10 mm proximal to sciatic trifurcation then the wound was closed and further antisepsis was added ***(10).***

Mesenchymal stem cells preparation

HUCB was collected from normal volunteers using strict aseptic techniques. Tissue culture plastic flasks 25 cm^2 were prepared for culture by adding a minimum essential medium supplemented with 20% fetal bovine serum, 1% antibiotic/antimycotic, and 1% glutamine. Nonadherent cells were removed and fresh medium was added to the culture flask. Cellular growth was assessed daily under inverted microscope. When the cells reached 50–60% confluence, they were harvested after trypsin/ethylenediaminetetraacetic acid (0.025%) ***(11- 15).***

Electrophysiological tests:

These were performed at the end of the 8^{th} postsurgical week.

Electromyography method

Rats were anesthetized using pentobarbital. Two stimulating hooked electrodes were placed around the sciatic nerve 5-mm proximal to the crush site. Electrical current application initiated with monophasic, single, square pulse with a duration of 1 ms and an intensity of 10 mA produced by an electric stimulator (EMG100C; Biopac Systems Inc., CA, USA). The intensity was gradually increased until the supramaximal stimulation that ensured maximal amplitude was reached (1 mA).

Thereafter, the recorded signals were digitally converted with an MP 150 (Biopac Systems Inc.). The latency period and amplitude were measured. The latency was measured from the stimulus to the takeoff of the first negative deflection and the amplitude was calculated from the baseline to the maximal negative peak ***(16).*** A heating lamp was used to keep rat's body temperature at ~37°C during the tests ***(10).***

Method of nerve conduction velocity:

Animals were anesthetized with pentobarbital, and then were sacrificed. Left sciatic nerves were dissected from the spinal emergence to the knee and stored in normal Ringer's solution. Nerve stimulation and recording was accomplished using Biopac MP 150 data acquisition system. A stimulus was applied at 50 μs duration, with intensity set at ~1.25 times, which gave the maximum height of the compound action potential. Nerve conduction velocity (NCV) was measured by dividing the distance between the stimulating and recording electrodes by the time elapsed between the initiation of the stimulus and the time when 50% of the increase of the component of compound action potential was reached.

Histopathological Assessment

Eight weeks after the induction of injury and after completing the electrophysiological evaluation the rats were deeply anesthetized with diethyl ether to be sacrificed. The sciatic nerve was dissected, including the lesion site, with surrounding tissue from the ischiadic notch to the popliteal fossa. Removed tissue was fixed with 10% formalin, dehydrated with alcohol solution, and embedded in paraffin. Transverse sections were taken 0.5 cm distal and proximal to the lesion site. Longitudinal sections were taken. Each block was serially sectioned horizontally every 5 mm. Sections were stained with hematoxylin and eosin (H & E) and

toluidine blue. The slides were viewed under a light microscope to study the structural changes ***(17).***

Detection of synapsin I (mRNA) levels in the injured sciatic nerve using Semi quantitative PCR technique:

Total RNA was isolated using Qiagen tissue extraction kit **(Qiagen, USA)** according to instructions of manufacture. The purity (*A*260/*A*280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The total RNA (0.5–2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit Fermentas, USA Amplification of synapsin I cDNA was done using Go Taq ® Flexi DNA polymerase, Promega, Madison.VVI, USA **(Catalog No.: M8305).** PCR is a test for in vitro amplification of specific DNA sequences using two primers that hybridize to opposite strands and flank target DNA region. At the end of the amplification process, the DNA product was detected using agarose gel electrophoresis. The DNA was visualized by placing the gel on an UV transilluminator. The Ethidium Bromide intercalated into DNA and gave a bright pink band on 2% agarose gel electrophoresis as recorded by the gel documentation system. GAPDH was used as an internal control and was also amplified using its specific primer for Semi-Quantitative Determination of PCR Products.

Ethical considerations:

1. The transportation, care and use of animals were harmless.
2. Procedures involving animals were designed and performed with consideration of their relevance to human health, the advancement of knowledge and the good society.
3. The animals, which were selected for the work, were of appropriate species and quality and were the minimum number required to obtain valid results.
4. Proper use of animals, including the avoidance or minimization of discomfort, distress and pain.
5. The living conditions of the animals were appropriate.
6. Animals handled gently, housed with suitable environmental and nutritional conditions, assessed for general health and body weight and anesthetized before peripheral nerve injury and before sacrifice.

Statistical Analysis Methodology*:*

The collected data was organized, tabulated and statistically analyzed using SPSS software statistical computer package version 18 (SPSS Inc, USA). For quantitative data, the mean and standard error were calculated. ANOVA (Analysis of variance) was used to test the difference about mean values of measured parameters among groups, multiple comparison between pairs of groups were performed using Tukey HSD (Post hoc range test). For interpretation of results of tests of significance, significance was adopted at $P < 0.05$. In this work, the mean ($\overline{X}$), the standard deviation (S.D), the standard error of the mean (S.E.M), the student (t) test, the F (ANOVA) test and the (P) value were calculated**.** ***(18)*.**

Results:

Electrophysiological results:

A) Nerve conduction velocity results:

Table (1) and Figure (1) show values of NCV (mm/s) in all groups at the end of the 8th postsurgical week. They show the mean value ± SEM of NCV in the injured control group (26.7 ±1.3 mm/s), which was significantly lower than the corresponding value in the sham surgery group (39.08±1.31 mm/s) (P value < 0.01). They also show statistically significant increase in NCV value (p value < 0.01) in EPO treated 34.85±1.28 mm/s, mesenchymal stem cell treated 34.87 ±1.3 mm/s combined mesenchymal stem cells and EPO 37.25 ±2.28 mm/s groups in comparison to control injured group. The values show higher ***% difference*** in the combined treatment group (39.5%) than either sole erythropoietin (30.5 %) or mesenchymal stem cells (30.6 %) treated groups. No statistical significance difference (p value > 0.05) was observed between the 3 treatment groups.

Table (1) Nerve conduction velocity (mm/s) at the end of the 8th postsurgical week

Groups / NCV(mm/s)	*G1 Normal*	*G2 Injured*	*G3 EPO*	*G4 MSC*	*G5 Combination*
Mean ±S.E	39.08 ±1.31	26.7 ±1.3	34.83 ±1.26	34.87 ±1.3	37.25 ±2.28

Data are expressed as mean ± S.E (*n* = 10); EPO: Erythropoietin;
MSC: mesenchymal stem cell; NCV: nerve conduction velocity.

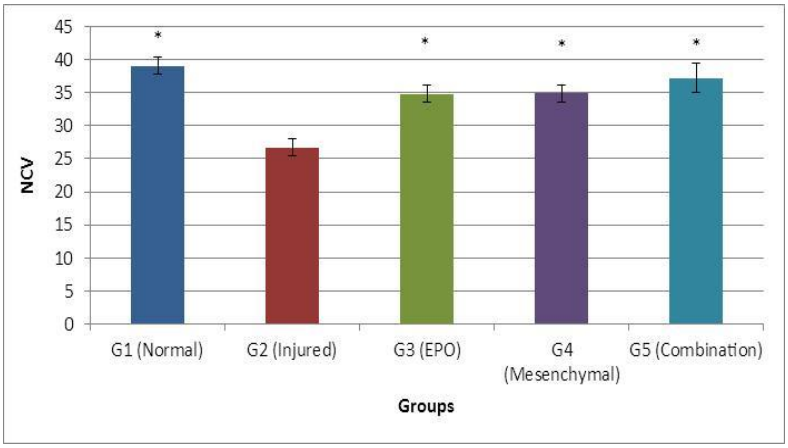
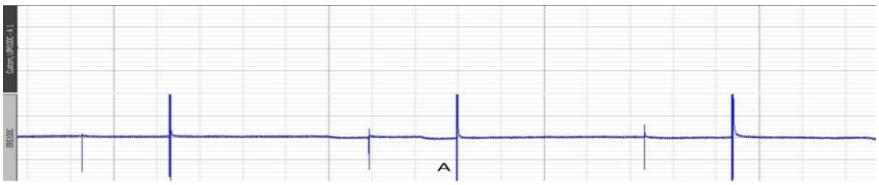
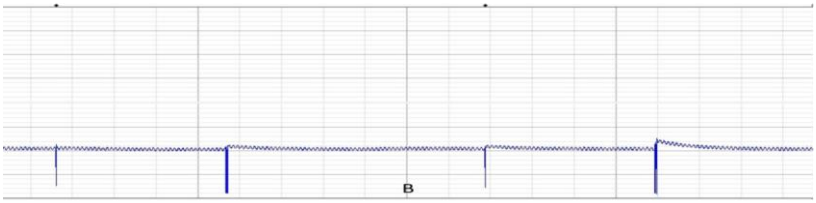


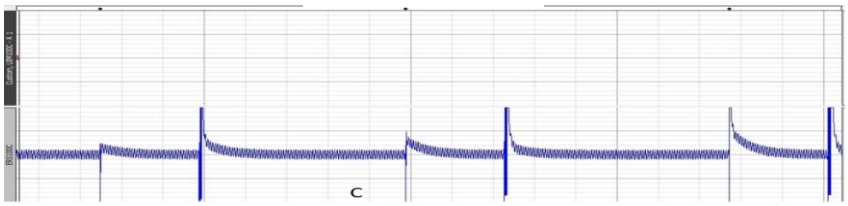
Figure (1): Nerve conduction velocity (mm/s) at the end of the 8th postsurgical week
*** Significant difference from group 2**



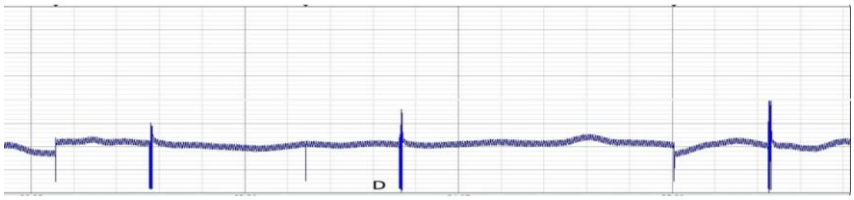
G1 Normal



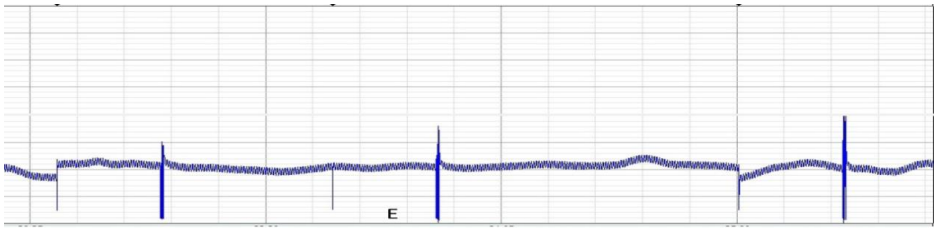
G2 Injured



G3 EPO



G4 MSC



G5 Combined ttt

Fig (2): showing individual nerve conduction velocity in each group: (A) normal, (B) injured, (C) EPO treated (D) MSC treated (E) EPO & MSC combination treated group combination treated group.

B) Electromyography results:

Table (2) Figure (3) show statistically significant (p value < 0.01) decrease in EMG amplitude values of control injured group (0.703±.049 mV) in comparison to normal non injured group (1.83± 0.065 mV) & statistically significant increase in EMG amplitude values (p value <0 .01) in EPO treated (1.453± 0.126 mV), mesenchymal stem cell treated (1.52±0.151 mV) combined mesenchymal stem cells and EPO (1.543±0.155 mV) group in comparison to control injured group. The values show higher ***% difference*** in the combined treatment group (120.48 %) than either sole erythropoietin (107.68 %) or mesenchymal stem cells (116.22%) treated groups. No statistically significant difference (p value > 0.05) was observed between the 3 treatment groups.

Table (2) Figure (4) show statistically significant (p value <0 .01) increase in EMG latency values of control injured group (1.003±0.031 S)

compared to normal non injured group (.797±.002 S) & statistically significant decrease in EMG latency values (p value < 0.01) in EPO treated (.815±0.0006 S), mesenchymal stem cell treated (.812±0.0004 S) and combined mesenchymal stem cells and EPO (.81±0.0015 S) group in comparison to control injured group. The values show ***slightly*** higher ***% difference*** in the combined treatment group (-19.3%) than either sole erythropoietin (-18.6%) or mesenchymal stem cells (-19.0%) treated groups. The comparison between the 3 treatment groups showed high statistically significant difference (p value < 0.01) between MSC treated group and EPO treated group. No statistically significant difference (p value > 0.05) observed between combined EPO & MSC treated group and MSC treated group, while high statistically significant difference (p value <0.01) observed between combined EPO & MSC treated group and EPO treated group.

Table (2) Electromyography at the end of the 8th postsurgical week

	G1 normal	***G2 Injured***	***G3 EPO***	***G4 Mesenchymal***	***G5 combination***
Amplitude of EMG in mV					
Mean	1.83	.703	1.46	1.52	1.55
±S.E.	± .065	±.049	± .127	±.151	±.156
Latency of EMG in S					
Mean	.797	1.003	.816	.812	.809
±S.E	±.002	±.031	±.0005	± .0004	±.0015

Data are expressed as mean ± S.E (*n* = 10); EPO: Erythropoietin;
MSC: mesenchymal stem cell, EMG: Electromyography

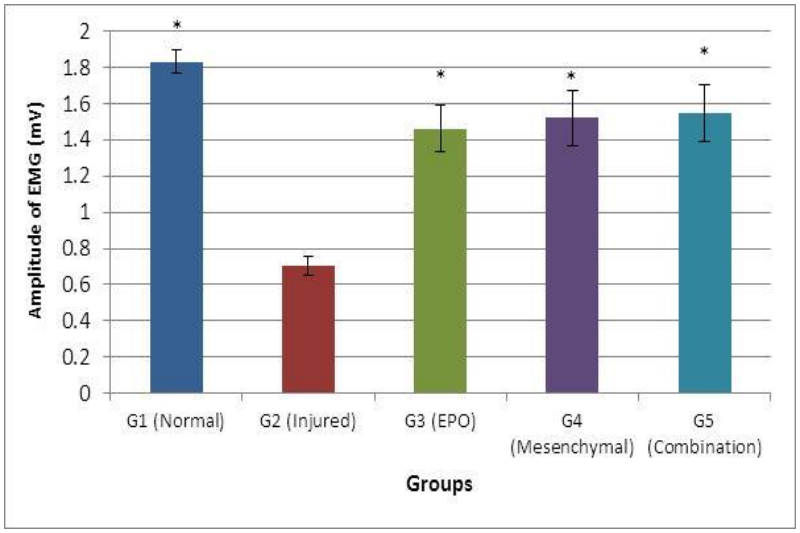


Figure (3): EMG Amplitude 8 weeks post injury

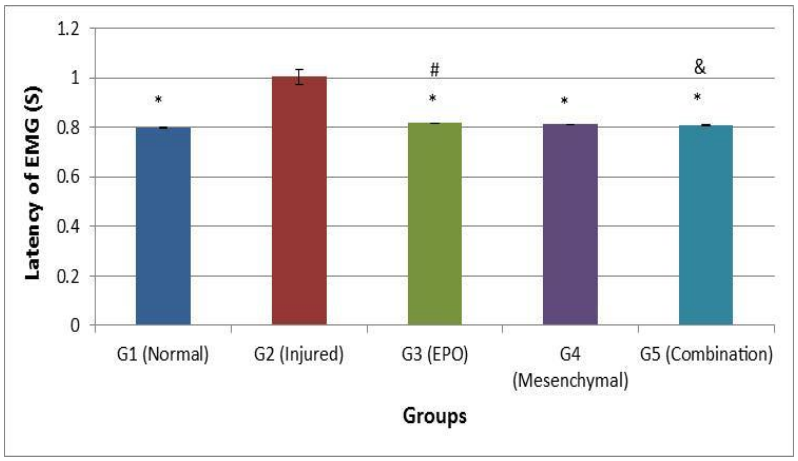
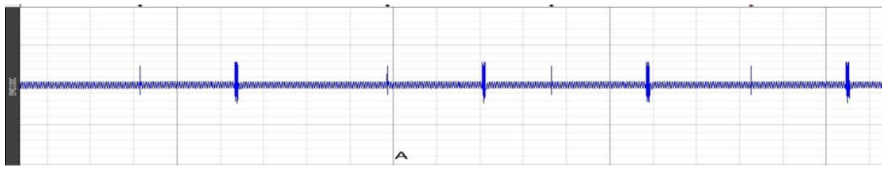


Figure (4): EMG Latency 8 weeks post injury

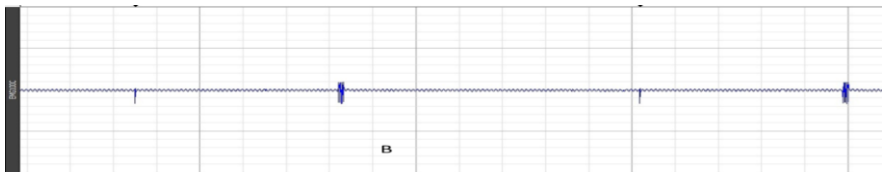
*** Significant difference from group 2**

 # Significant difference between G 3 & G 4

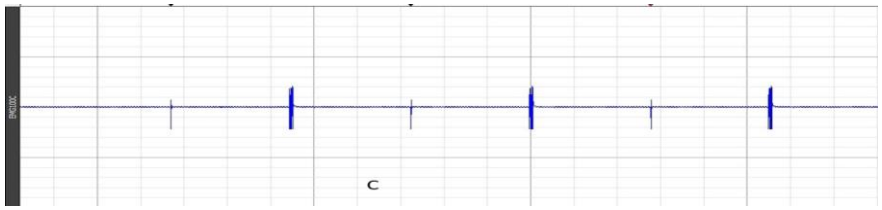
& Significant difference between G 3 & G 5



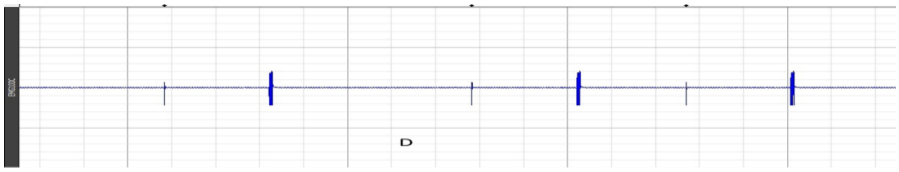
Group (1) Normal



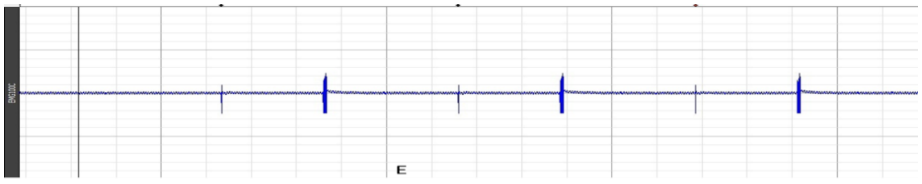
Group (2) Injured



Group (3) EPO



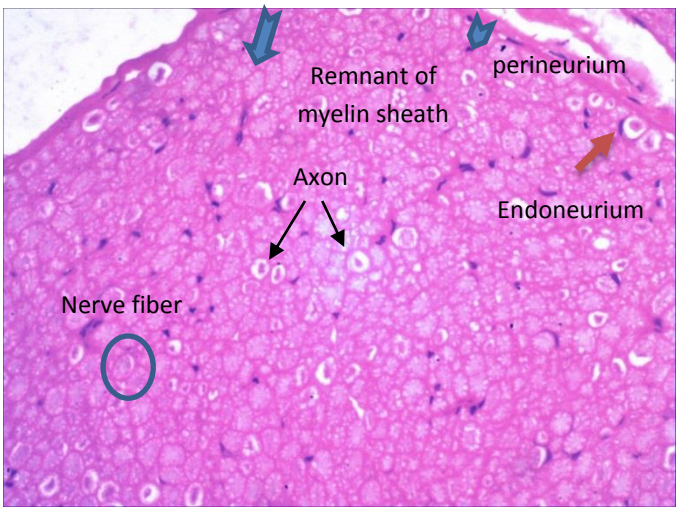
Group (4) MSC



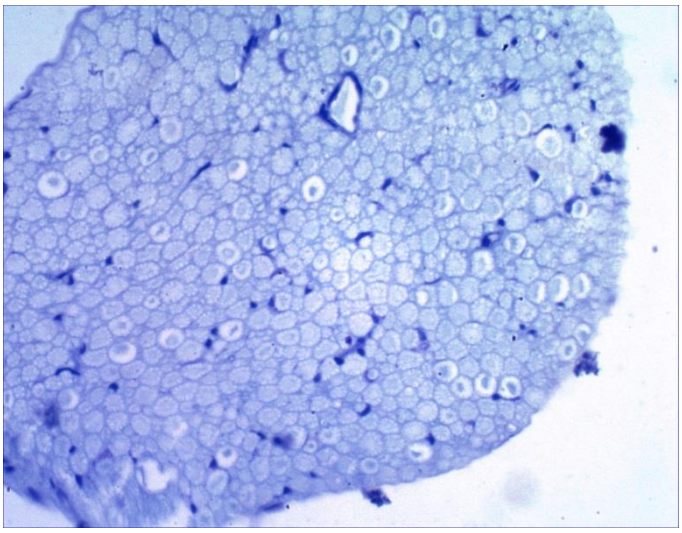
Group (5) Combined ttt

Fig (5): showing individual electromyography in each group: (A) normal, (B) injured, (C) EPO treated (D) MSC treated& (E) EPO & MSC combination treated group.

Histopathological results:

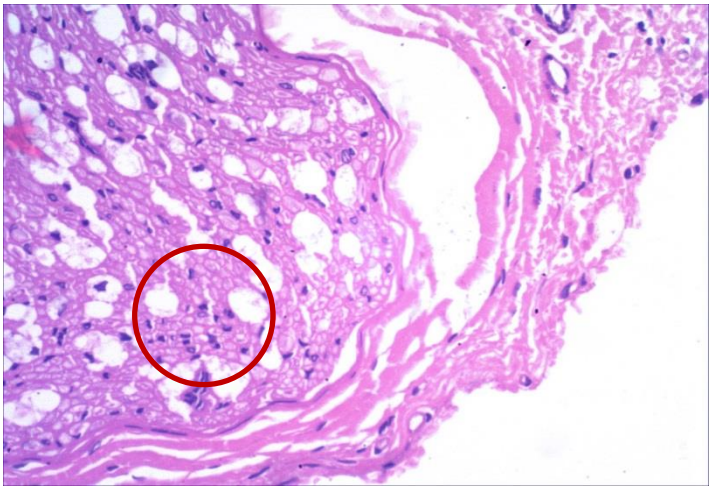


(H&E, 400)

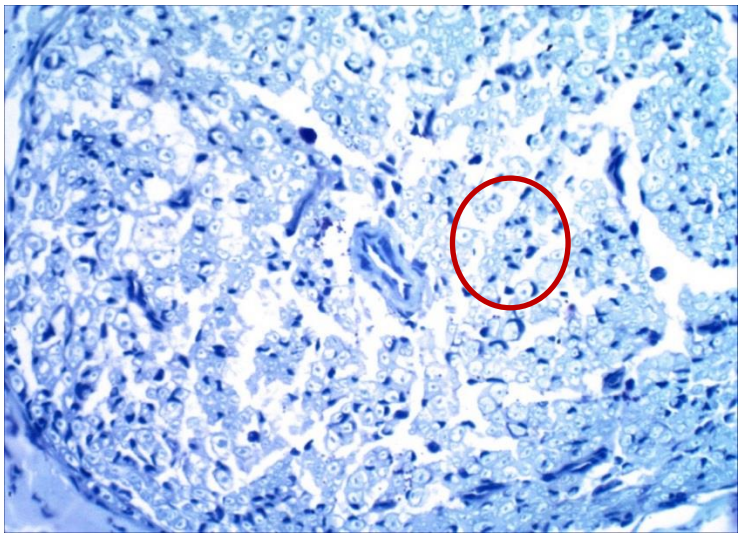


(Toluidine blue, 400)

Photomicrographs of sections in the sciatic nerve of the normal sham operated side of rats show: the myelinated nerve fibers (circles), composed of axon (black arrow), remnants of myelin sheath (thick arrow), endoneurim (red arrow) and part of of nerve (perineurium) (arrow head).



(H&E, 400)

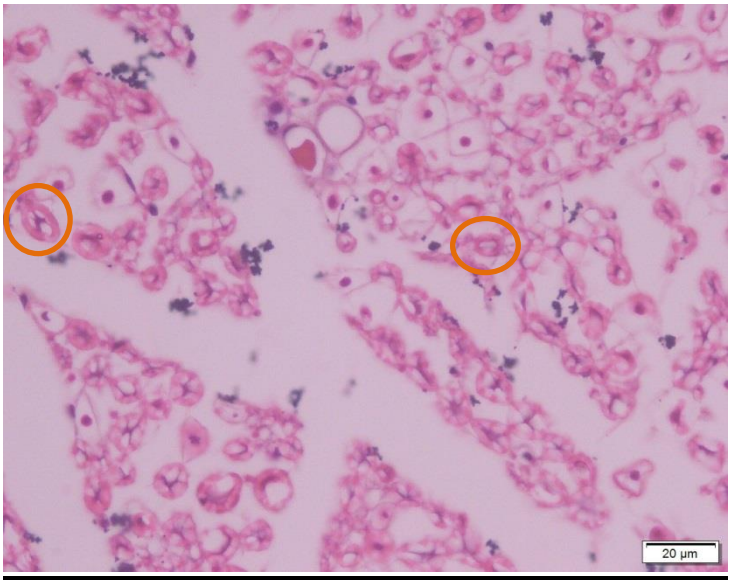


(Toluidine blue, 400)

Photomicrographs of sections in the injured sciatic nerve :

H &E stained sections show *Wallerian degeneration*; Fragmentation and loss of myelin and axons. Schwan cell hyperplasia and marked thickening of epineurium and perineurium

Cross section of plastic-embedded nerve stained with Toluidine Blue also shows infiltrating macrophages (circle) removing myelin and axon debris



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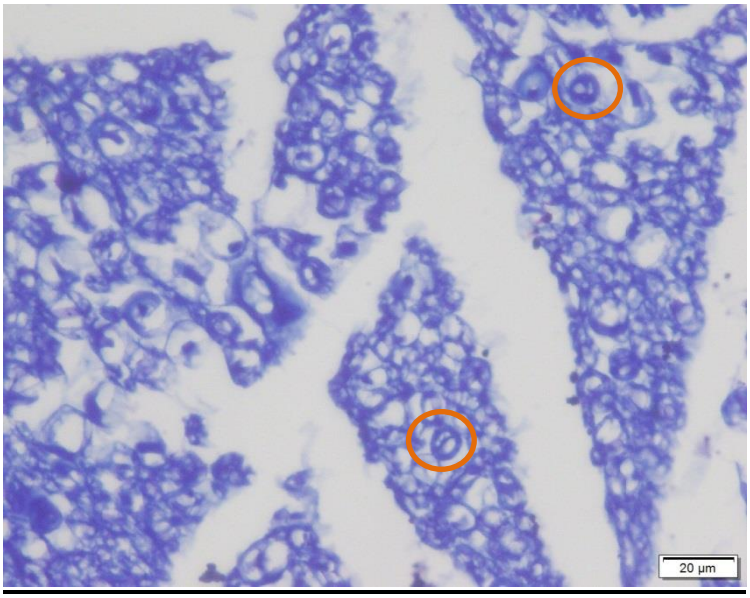
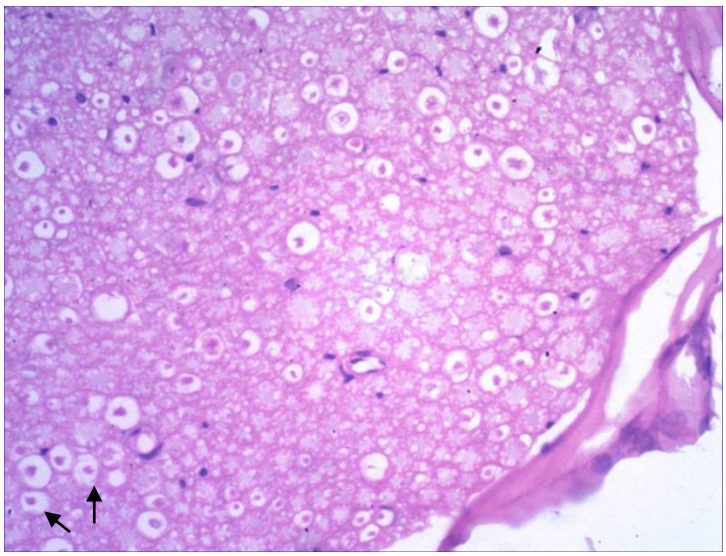
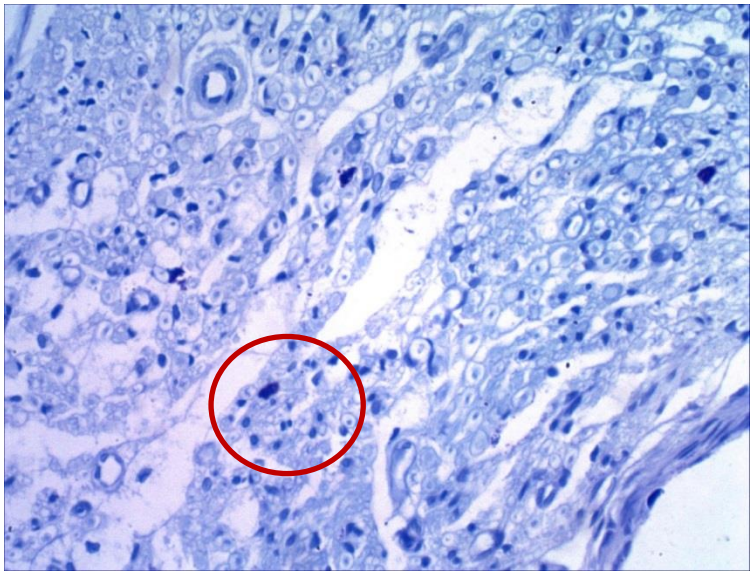


(Toluidine blue, 400)

Photomicrographs of sections in the injured sciatic nerve treated with EPO show "Onion bulb" formations (circle) which are concentric layers of Schwann cell processes and collagen around an axon. This proliferation is caused by repetitive segmental demyelination and regeneration of myelin and can cause gross thickening of peripheral nerves (hypertrophic neuropathy) with moderate degree of *Wallerian degeneration*; Fragmentation and loss of myelin and axons.

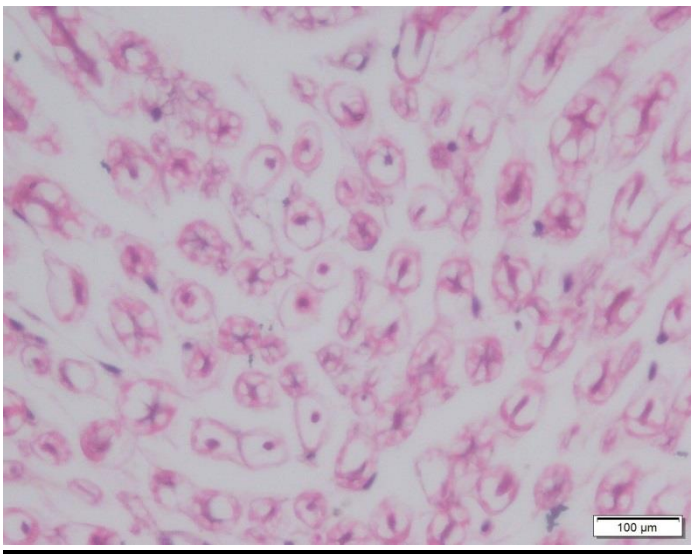


(H&E, 400)

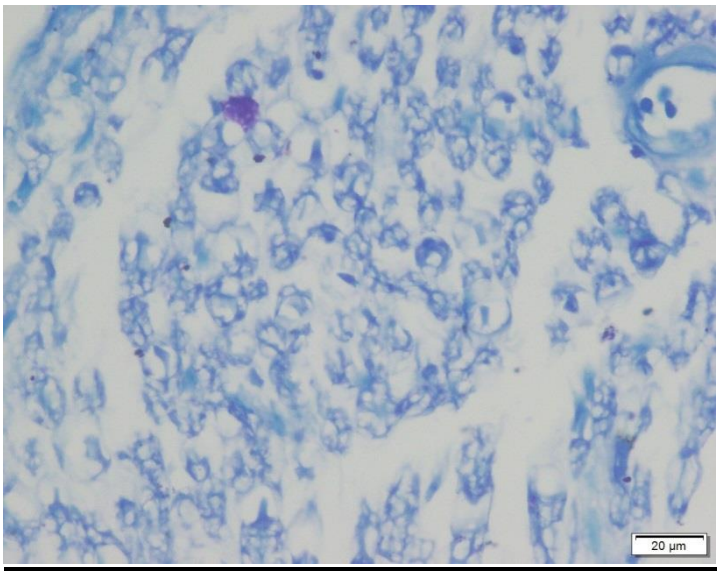


(Toluidine blue, 400)

Photomicrographs of sections in the injured sciatic nerve treated with MSC:
H & E stained sections show lesser degree of *Wallerian degeneration*; Fragmentation and loss of myelin and axons with evidence of axonal regeneration (black arrows), surrounded by thin layer of perineurium, and normal Schwan cells umber.
Toluidine blue stained sections show the both hyperchromatically stained degenerated fibers and the macrophage like cells (circle) removing myelin and axon debris.



(**H&E, 400)**



(Toluidine blue, 400)

Photomicrographs of sections in the injured sciatic nerve co- treated with EPO and MSC show more axon regeneration, minimal wallerian degeneration and no macrophages.

Results of Synapsin I detection by Semi quantitative-PCR technique:

Detection of Synapsin I mRNA levels in all group using Semi quantitative RT-PCR technique was done 8 weeks post injury.

0.01) < Table (3) Figure (6) showed statistically significant (p value decrease in Synapsin I of control injured group (0.232 ± 055) in comparison to normal non injured group (1.117±.075) & statistically 0.01) in EPO treated group <significant increase in Synapsin I (p value (.748±.042), mesenchymal stem cell treated group (.772± .058) and combined mesenchymal stem cells and EPO group (.947 ±.032) in comparison to control injured group. The values show higher *% difference* in the combined treatment group (308.2 %) than either sole erythropoietin (222.4 %) or mesenchymal stem cells (232.8 %) treated groups. The comparison between the 3 treatment groups showed no statistically significant difference (p value > 0.05) between MSC treated group and EPO treated group, statistically significant difference (p value < 0.05) between combined EPO and MSC treated group and MSC treated 0.01) <group, and highly statistically significant difference (p value between combined EPO and MSC treated group and EPO treated group.

Table (3): Synapsin I mRNA levels 8 weeks post injury

	G1 normal	***G2 Injured***	***G3 EPO***	***G4 MSC***	***G5 combination***
Synapsin I					
Mean	1.117	0.232	.748	.772	.947
±S.E	±.075	±.055	±.042	± .058	±.032

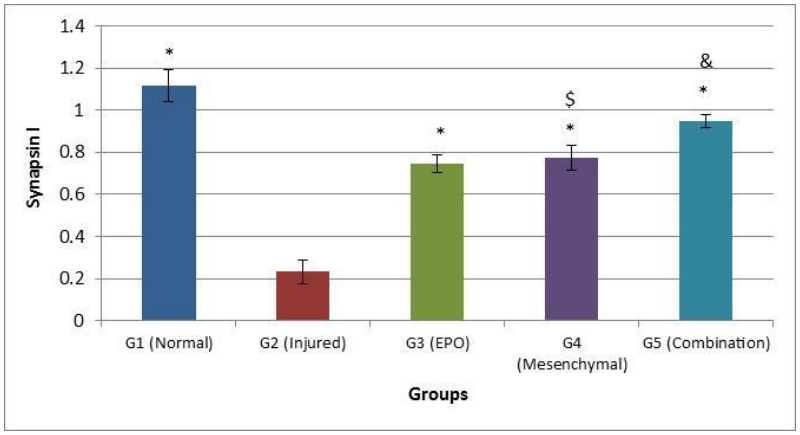


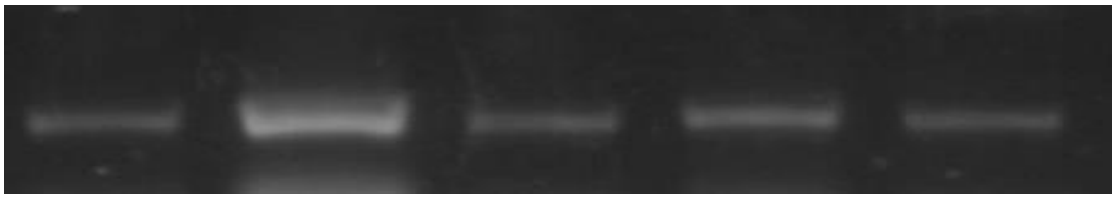
Figure (6): Synapsin I mRNA levels 8 weeks post injury

*** Significant difference from group 2**

$ Significant difference between G 4 & G 5

& Significant difference between G 3 & G 5

Synapsin I



GAPDH

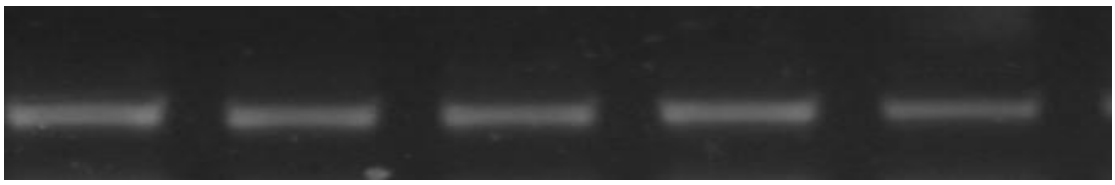


Figure (7): An agarose gel electrophoresis show PCR products of synapsin 1 in different studied groups

Lane 1: control group

Lane 2: injured group.

Lane 3: EPO treated group

Lane 4: Stem cell treated group

Lane 5: EPO + Stem cell treated group

Discussion:

In the present study, the crush nerve injury model produced an axonotmetic peripheral nerve injury. The electrophysiological studies in the injured control group after 8 weeks of injury revealed that NCV and EMG amplitudes were significantly low, whereas EMG latency showed significant prolongation when compared with corresponding values in the sham surgery group. Those results may be explained by direct effect of trauma ***(19),*** crush-related ischemia or reperfusion injury, which increases neural damage, or due to activation of reactive oxygen molecules ***(20).*** Electrophysiological studies in EPO treated group showed marked improvement when compared with the injured control group at the end of the eighth postsurgical week revealed by higher NCV and EMG amplitude and lower EMG latency, which were significant when compared with corresponding values in the injured control group. ***Zhang et al, 2015*** reported after administration of EPO encapsulated in poly lactide-co-glycolide (PLGA) microspheres that allowed sustained release of EPO within a period of two weeks, the peripheral nerve injured rats had significantly better recovery evidenced by significantly higher motor nerve conduction velocity (MNCV) compared with those which received daily intraperitoneal injection of EPO at a dose of 5000 U/kg EPOin 0.5 mL saline for 2 weeks, empty PLGA microspheres, or saline treatments ***(21). Orhan et al.*** also concluded that recombinant human erythropoietin (rhuEPO) prevented Cisplatin (potent anticancer drug) induced motor neuropathy by sparing the number of normal nerve fibers and by protecting the amplitude and area of the compound muscle action potential (CMAP). They also concluded that rhuEPO may also play a role in active myelination which is an active agent in protection against Cisplatin - induced peripheral neuropathy ***(22).***

HUCB mesenchymal stem cells treated group showed also a significant increase in both NCV values and EMG amplitude values and significant decrease in EMG latency values done 8 weeks post injury in comparison to control injured group. ***Ashour et al.*** reported the ameliorative effect of MSCs transplantation on oxidative stress of the crush nerve injury, revealed by significantly decreased serum malondialdehyde (MDA) levels and elevated serum total antioxidant capacity (TAC) in the MSCs transplantation group when compared with the injured control group after 48 h of injury and improved neurotrophic support in addition to reported ability of undifferentiated stem cells to differentiate into Schwann cells *in vivo* ***(23).***The Co-administration group (EPO and MSCs treated group) showed a highly significant increase in both NCV values and EMG amplitude values and a highly significant decrease in EMG latency values when compared to the corresponding mean values of the injured non treated group. ***Min et al.*** chose EPO as an adjunct to UCB stem cells therapy for cerebral palsy because EPO has neuroprotective and neural-repair properties. According to them EPO stimulates Jak2-PI3K-Akt to exert its neuroprotective action, which is similar to the pathway stimulated by UCB stem cells so EPO acts in a synergistic way with MSC to potentiate neurogenesis ***(24).Ikeda and Oka*** analyzed the relationship between motor nerve conduction velocity (MNCV) and morphological changes in individual fibers. They concluded that the mean fiber diameter (axon plus myelin sheath) increased with time after nerve transection and recovery of mean fiber diameter was well correlated with MNCV, even though regenerating nerves contained many small nonconducting fibers. However, they concluded that mean myelin thickness, axonal diameter, and *g*-ratio (quotient axon diameter/fiber diameter, a measure of relative myelin thickness) decreased after transection and were not well correlated with time or MCV recovery ***(25).***

The photomicrograph of the cross section of the injured non treated sciatic nerve stained with Hematoxylin and eosin (H & E) 8 weeks after the injury showed aspects of Wallerian degeneration process, Schwan cell hyperplasia and marked thickening of epineurium and perineurium. Toluidine blue stained sections showed distorted myelin sheath around the degenerated axons. EPO treated group sections stained with (H&E) showed Onion bulb" formations which are concentric layers of Schwann cell processes and collagen around an axon caused by repetitive segmental demyelination and regeneration of myelin with moderate degree of *Wallerian degeneration*; Fragmentation and loss of myelin and axons. ***Zhang et al, 2015*** demonstrated the most significant improvement in recovery of injured circular bundles, lots of mature bundles with circular morphologies could be observed, which were more similar to normal nerves in the group treated with sustained delivering of EPO by poly (lactide-co-glycolide) (PLGA) microspheres compared to saline treated and daily intra-peritoneal EPO treated groups ***(21). Wang et al, 2015*** also observed that morphologically, by electron microscopy using dual staining with uranium acetate and lead citrate compared to saline treatment, EPO treatment led to the growth of myelin sheath, the recovery of normal morphology of axons and Schwann cells, and higher density of myelinated nerve fibers. They conclude that EPO promotes the regeneration and functional recovery of the injured sciatic nerves at least partially via the upregulation of IGF-1 expression ***(6)***. Mesenchymal stem cell treated group sections stained with (H&E) showed good improvement of most of nerve fibers surrounded by thin layer of perineurium, and normal Schwan cells number. Toluidine blue stained sections showed the both hyperchromatically stained degenerated fibers and macrophage like cells. This result is compatible with ***Abo El-soud et al.*** who concluded that the histopathological examination with H&E

stained sections and toludine blue sections from the sciatic nerve mesenchymal stem cells-treated group showed a picture nearly similar to the normal sciatic nerve control group ***(26).*** The Co-administration group (EPO and MSCs treated group) showed more axon regeneration, minimal wallerian degeneration with no macrophages. ***Yan et al.*** proved that neural stem cells (NSCs) transplantation combined with intraperitoneal injection of EPO could promote the recovery of the neurological function and benefit the survival and regeneration of injured axons after spinal cord injury in the adult rats ***(27).***

Syapsin I (SyI) mRNA levels showed a significant decrease in the control injured group 8 weeks post injury in comparison to normal non injured group. *Kwon et al.* investigated the time-dependent translocational changes of Synapsin I in the regenerating axonal sprouts following sciatic nerve crush injury by ligation for 24 hs. At various times after release, they performed immunocytochemistry and found that SyI accumulated in the proximal region at the early phases after release and was translocated from the proximal to the distal site of ligation (anterograde transport) and also involved in the sprouting of regenerating axons.Their results suggest that SyI may be related to the translocation of vesicles to the elongated membranes and contribute to the insertion of the regenerating sprout of glycoprotein into the axolemma by a fast axonal transport in the regenerating sprouts ***(28).*** EPO treated group showed a statistically significant increase in Synapsin I mRNA levels compared to control injured group***. Emery et al***. mentioned that recent evidence is reviewed that the injured adult CNS exhibits events and patterns of gene expression that are also observed during development and during regeneration following damage to the mature peripheral nervous system concerning the coordinated expression of growth-related proteins which leads to the

neuronal growth during development or regeneration ***(29).*** Based on the previous finding, ***Sargin et al***. concluded that EPO treatment right after the lesion performed on the right parietal cortex of juvenile mice, for 2 weeks every other day prevented lesion-induced brain atrophy and cognitive decline. EPO treatment increased the number of oligodendrocytes and prevented the reduction in myelin protein expression. They finally proved that the decrease in the expression of synapsin 1 protein upon lesion was also prevented by early EPO treatment ***(30).*** MSCs treated group showed a significant increase In Synapsin I mRNA levels compared to control injured group. According to ***Chen et al.***, intravenously transplanted bone marrow mesenchymal stem cells can migrate to the injured spinal cord, promote expression of synapsins and improve the neurological functions of rats with spinal cord injury which may result from bone marrow mesenchymal stem cell differentiation into functional neurons in the injured spinal cord ***(31).*** The group of combined therapy of EPO and MSCs showed a significant increase in Synapsin I mRNA in comparison to control injured group. ***Offen et al***. used mouse induced pluripotent cells (iPS) to generate panneural proliferating progenitor cells (pNPCs) *in vitro* which further differentiated into mature neurons and oligodendrocytes. They investigated the effect of erythropoietin (EPO) on their survival, proliferation and neurodifferentiation. They demonstrated that after 14 days of differentiation the presynaptic marker synapsin became visible and after 22 days in culture, synapsin expression was pronounced ***(32).***

Conclusion:

We concluded that both EPO treatment and MSCs transplantation improved peripheral nerve functional individually and the combined therapy had a superior impact over the sole role of either EPO or MSCs. This can be revealed by the functional and morphological improvement as shown by the electrophysiological and the histopathological results. Our results suggested the synergistic effects of EPO and MSCs leading to more functional recovery that is greater than either strategy alone.

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