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Research Article

COMPARATIVE STUDY TO EFFECT OF NUCLEO CMP FORTE AND PLATELET RICH PLASMA ON THE REGENERATION OF SCIATIC NERVE IN DOGS

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Abstract

The objectives of this study were to evaluate the efficacy of each Cytidine Monophosphate and Platelet Rich Plasma on the regeneration of sciatic nerve injury of dogs. Eight healthy adult dogs were used to complete this study. They were randomly divided into two equal groups: Platelet Rich Plasma group and Cytidine Monophosphate group. The sciatic nerve axotomy was done on the right hind limb at the middle femoral bone and then the nerve coaptated immediately by nylon suture 0 - 5 with end-to-end anastomosis, using simple interrupted suture, and then the activated platelet rich plasma was injected immediately at the site of coaptated nerve at subepineural site but in Cytidine monophosphate group, the dogs are treated with Cytidine monophosphate 5 mg/day intramuscular for 30 day post operation. The clinical examination of animals show occurrence (onset of gait, knuckling, swelling and pain at the operated leg) that were evaluated from the start until the end of the study (sixteen week). Clinically, the cytidine group show faster improvement compared with Platelet Rich Plasma. Macroscopic examination was done for evaluating the presence of nerve stump coaptation, adhesion, thickness and neuroma. A good coaptation was observed in cytidine compared with platelet rich plasma groups. While the adhesion appeared in variable degrees in platelet rich plasma group, otherwise, it was appeared in one animal in cytidine group. Moreover, no thickness or neuroma was present in two groups. The Electrophysiological examination (conductive velocity) was carried out by ad- instrument after isolation of the sciatic nerve on 16th weeks postoperative, the results showed significant differences at ($p \leq 0.05$) in the same group between right (operated) and left sciatic nerve. Furthermore, significant differences between groups at ($p < 0.05$) are the best conductivity in Cytidine monophosphate group compared with other group. The histopathological examination of proximal, middle, and distal segments of sciatic nerve sections (three parts along each one 1cm) are isolated, and used to evaluate the sciatic nerve regeneration. The neurohistopathological results that showed increase proliferation of Schwann cells and orientation of regenerative nerve fibers are observed most clearly and improved in Cytidine monophosphate group compared with platelet rich plasma group.

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1. Introduction

The peripheral nerves injuries result in a partial or total loss of motor, sensory and autonomic functions transported by the damaged nerves to the denervated segments of the body, due to the interruption of axons continuousness, Wallerian degeneration of distal nerve fibers lesion and eventual death of axotomized neurons. Injuries of the peripheral nervous system can result in significant functional loss and decreased quality of life because of constantly reduced sensory and motor functions and secondary problems, such as neuropathic pain, and have major social sequelae in terms of health care and long periods of sick - leave (Jaquet *et al*., 2001; Rosberg *et al.,* 2005).

The moderate form of injury (neuropraxia) there is no neurohistological change in the nerve fiber and full recovery is expected. This is also the case for second-degree injuries, the mildest form of axonotmesis. In the more severe cases of injury, an active Ca^{2+} mediated process known as Wallerian degeneration, takes place distal to the lesion. The main accidents are axonal death, invasion of blood borne macrophages, breakdown of myelin sheaths together with ingestion and breakdown of the myelin material, a transient phase of Schwann cell proliferation, and a reflection of molecular expression from that special of mature myelinating and non-myelinating cells back to one that like the immature state. In the case of myelinating cells, this involves down regulation of a large number of genes related to myelination (Jessen and Mirsky, 2005; Mirsky *et al.,* 2008). This involved enzymes that provide for cholesterol synthesis, constitutional proteins such as, myelin basic protein (MBP) and membrane associated proteins such as myelin associated glycoprotein (MAG) and periaxin (Buchstaller *et al.,* 2004; Leblanc *et al*., 2005).

The important compositions of PRP contain transforming growth factor (TGF-β1), platelet derived growth factors (PDGF-AB and PDGF-BB), insulin like growth factor (IGF), vascular endothelial growth factors (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Yu *et al.,* 2011). Nucleo cytidine monophosphate (CMP) is mainly used for peripheral neurological disorders like trigeminal neuralgia, diabetic neuropathy and lumbosciaticalgia, but its central roles remain to be elucidated, Nucleo CMP improve neural growth and nerve repair, improvement, regeneration of myelinated nerve fiber, delay spinal pain transmission, enhance spinal density and acceleration of hippocampal dependent working memory in animal model study also; Nucleo CMP contains uridine monophosphate, uridine diphosphate and uridine triphosphate, which together with cysteine monophosphate induce biosynthesis of neuronal glycolipid, phospholipids, Nucleo CMP crosses the blood brain barrier and then phosphorylated into uridine triphosphate that lead to the triggering of neurotransmitter modulation (Flierl *et al.,* 2015). This study aimed to evaluate of the effect of Nucleo CMP Forter on the regeneration of peripheral nerves injury to evaluate the efficacy of PRP on the regeneration of peripheral nerves injury and Comparation between CMP and PRP in nerve regeneration.

2. Materials and Methods

The study was conducted on twelve (12) adult dogs aged from 1 - 3 years with body weight of 20 – 30 kg. The animals were kept in cages for 15 days for acclimatization, the animals' administration of antimicrobial and anthelmintic drugs. Moreover, animals were accommodated in a same laboratory conditions by keeping them in cages (one animal per cage).

The animals were divided randomly into two equal groups; four animals were included in each group.

Platelet Rich Plasma (PRP) Group: The right sciatic nerve was transected and immediately sutured. Furthermore, the clinical signs were daily recorded post operation until the end of the experiment. The neurohistopathological examinations were done but it was injected with 1 ml of PRP at the site of operation (local on the injured nerve).

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Nucleo CMP Forter Group: A similar procedure as in PRP was used but it was injected with CMP 5 mg/daily intramuscular for 30 days. The dogs were fasted for 8 hrs and water with draw 2 hrs before operation. The site of operation was clipped and shaved carefully before operation and then given a mixture of ketamine hydrochlorid 15 mg/kg B.W and xylazine hydrochlorid 5 mg/kg B.W intramuscularly.

The operation was carried out by exposing the sciatic nerve on the right side of thigh through a caudo-lateral skin incision (5 - 7 cm in length) performed parallel and behind the femur bone and separating it bluntly from the biceps femoris muscle cranially and semitendinosus muscles caudally by curved artery forceps. The sciatic nerve was exteriorized at the wound site (Fig - 1).

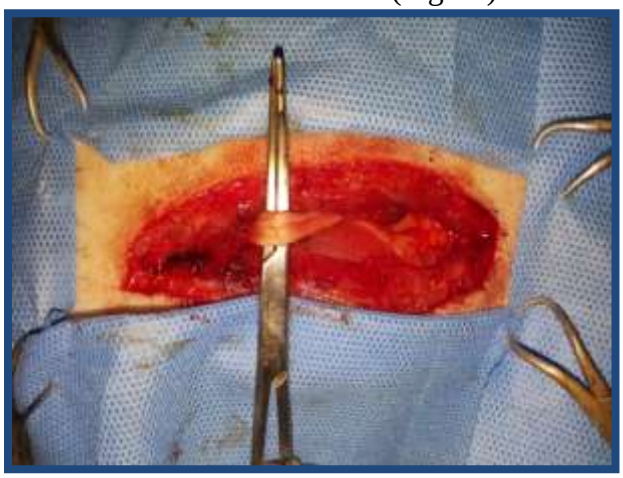

Figure - 1: Sciatic nerve exteriorization before transection

After that the nerve was transected by using a surgical blade. Moreover, the two ends of nerve suture immediately with end-to-end coaptation using four sutures by 5 - 0 nylon epineural, simple interrupted suture was placed equidistantly around the nerve in the epineurium. The first two sutures are placed at 0º and 180º of nerve to ensure the proper alignment of nerve (Simple interrupted suture) (Fig - 2).

The muscles and subcutaneous fascia was closed with No. 2 - 0 cut gut suture (simple continuous), and then the skin was closed with No. 2 - 0 silk suture. Furthermore, the animals were given systemic antibiotics 40,000 IU penicillin kg/B.W intramuscular daily for 7 days postoperative.

Figure - 2: Suture of nerve after Axotomy

PRP Preparation: Ten ml of Blood were collected from the cephalic vein using a 10 ml disposable syringe. Then, the blood sample of each animal was divided into two equal amounts of 5 ml, which were transferred to anticoagulant tubes containing 0.35 ml of 10 % sodium citrate. The blood was initially centrifuged at 160 rpm for ten minutes at room temperature. After the first centrifugation, two layers were observed in each sample. A red lower layer that consists of packed red blood cells and an upper straw-yellow layer that contains plasma component. The upper surface of packed red blood cells called Buffy coat is rich in platelets and leukocytes (Fig - 3). Plasma and buff coat were transferred to new sterile tubes. The retained component of blood samples was centrifuged again at 160 rpm for two minutes to obtain more concentrated platelets. Then, the plasma and Buffy coat was centrifuged for the second round at 400 rpm for 15 minutes. Two layers eventually appeared: the upper two thirds of the sample was designated as platelet poor plasma (PPP) and was discarded on the other hand, the lower third was PRP (Fig - 4). Moreover, the platelets were activated by 0.05 ml of 10 % calcium chloride solution to each 1 ml of PRP (Maghsoudi *et al.*,2015).

Figure - 3: First step

Figure - 4: Second step

Clinical finding evaluation all animals were observed from the first week to the end of the study on 16th week postoperative. The clinical finding including the motor and sensory function of the nerve (paralysis of the leg, pain, swelling (site of operation), muscle contraction and knuckling) (Abid, 2005). Electrophysiological examinations were carried out at 16th week after nerve suture. The sciatic nerve was separated by about 3 cm, and isolated from the body, then soaked in AD instruments chamber (filled with buffer solution) attached with negative, positive (recording) electrode clamps and stimulus electrodes (Fig - 5). The conductive velocity was recorded from the multiplication of distance between recording electrodes (mm) by time interval between capacity proximal and capacity distal (ms). The stimulations were performed at a square wave of 0.2 milliseconds (ms) duration with a frequency of two pulses per second.

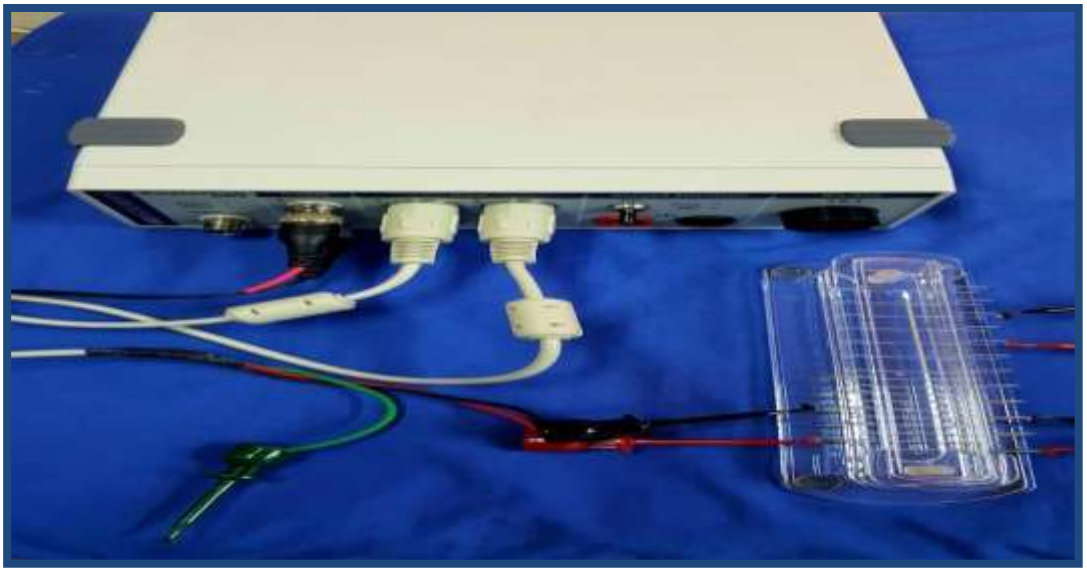

Figure - 5: Showing AD Instrument (A), isolated nerve chamber (B)

3. Result and Discussion

During the first twenty days, the muscle contraction of the affected leg was flaccid (no contraction). Moreover, the muscle had shown a mild contraction at the 30th day and then the muscle contraction became moderate at the 45th day, while during the 60th day the muscle contraction became strong, furthermore muscle contraction became near normal at the 85th day postoperative.

Platelet Rich Plasma (PRP) Group: The paralysis of the right hind limb was clear post -operation (motor and sensory loss of function) was similar to the described in control group. The pain and swelling were also recorded. The affected leg return to the right position on 7th day. Severe Knuckling had appeared at first day and then progressively disappeared on the 55th day. The muscle contraction was flaccid at the first days, then the muscle shows a mild contraction on 28th day, while the moderate muscle contraction appeared on 42th day and then progressively became normal on the 80th day.

Nucleo CMP Forter Group: The hind limb paralysis was also found in addition to the appearance of the pain and swelling at the site of operation during the first days PO. Severe knuckling also appeared during walking, knuckling became moderate and then disappeared early compared with other groups. The affected leg return to the right position at the end of day 3th PO. The muscle contraction was also flaccid during the first days and then begin to disappear progressively and became near normal early in comparism with other groups.

Platelet Rich Plasma (PRP) Group: The animals in this group had shown a good coaptation of the proximal and distal ends of nerve stump in the operated site, while moderate adhesion was observed in one animal. Thickness and neuroma were not present.

Nucleo CMP Forter Group: A good coaptation is observed, adhesion was present in one animal, neuroma does not appeared, and thickness was not present.

Conductive velocity at 16 week: The conductive velocity of right limb (operative) on 16 weeks presented significant differences between control, PRP and CMP groups at $P<0.05$. However, significant differences were observed in the same group between left sciatic nerve and right sciatic nerve as in Table -1. The best conductivity was observed in CMP group compared with PRP group (Fig - 6 & 7).

Table - 1: Statistical analysis of conductive velocity of control, PRP and CMP group at 16 weeks PO.RSN: Right Sciatic Nerve. LSN: Left Sciatic Nerve

Groups		At 16 weeks
PRP	RSN	25.47±2.61*
	LSN	34.12±3.56
CMP	RSN	26.47±2.41*
	LSN	39.90±4.36

Platelet Rich Plasma (PRP) Group: The histopathological examination of longitudinal section of the proximal part of sciatic nerve at 16th week PO showedfew vacuolated degenerated nerve fibers (Fig - 14). The middle transverse part of nerve showed increase number of Schwann cells, mild vacuolated degenerated nerve fibers and mild wallerian degeneration (Fig - 8). The longitudinal section of the distal part of sciatic nerve showed moderate remylination of nerve fibers (Fig - 9).

Nucleo CMP Forter Group: The histopathological examination of longitudinal section of the proximal part of sciatic nerve at 16th week showed complete remylination of nerve fibers (Fig -10). The middle transverse part of the nerve showed highly increase number of Schwann cells and noticed regeneration of neural tube (Fig - 11). The distal longitudinal section of the nerve showed regular and remylination of nerve fibers (Fig - 12).

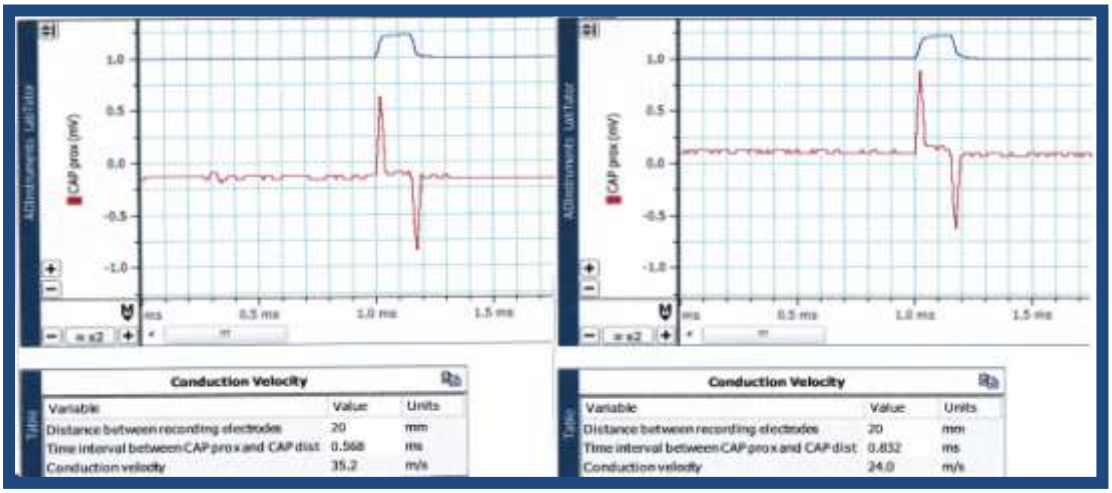

Figure - 6: Shows the sciatic nerves waves (A) left (B) right, and conductive velocity for PRP group at 16 weeks, the C.V measured by multiplucated the distance between recording electrodes on time interval

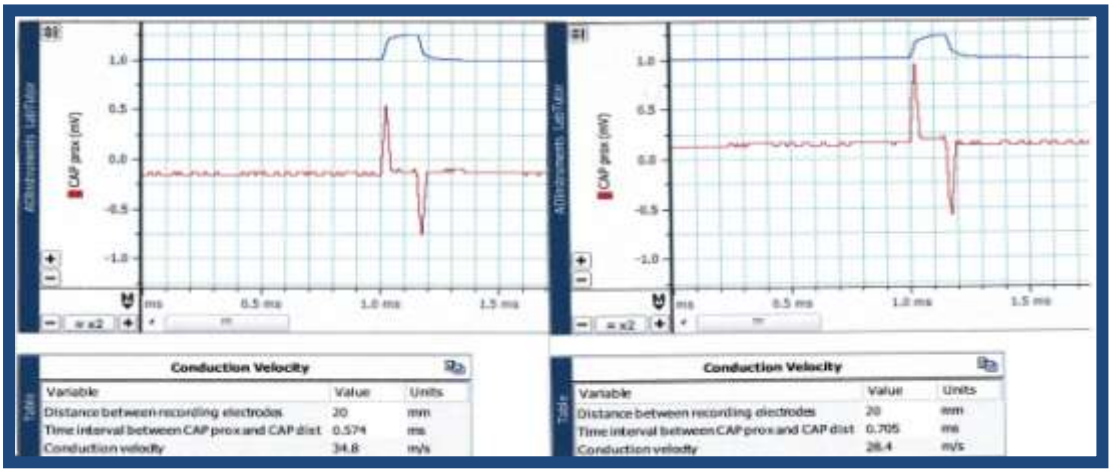

Figure - 7: Shows the sciatic nerves waves (A) left (B) right, and conductive velocity for CMP group at 16 weeks PO, the C.V measured by multiplucated the distance between recording electrodes on time interval

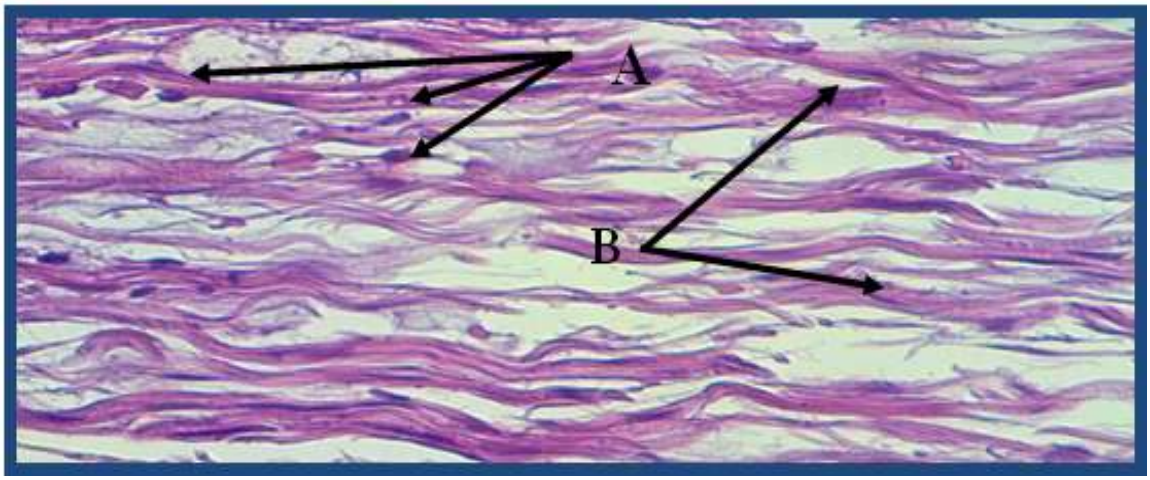

Figure - 8: The transverse section of middle part of sciatic nerve of PRP group (A) Increase number of Schwann cell (B) Moderate remylination of neural sheath with mild WD (H&E40X)

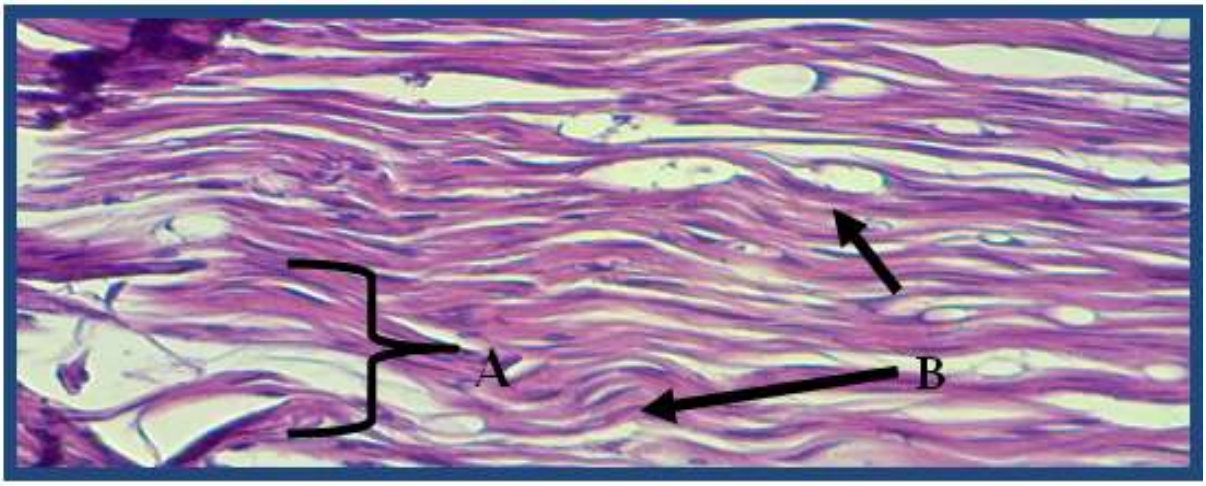

Figure - 9: The distal longitudinal section of sciatic nerve of PRP group (A) Irregular arrangement of nerve fibers (B) Mild vacuolated degenerative nerve fiber and moderate regeneration (H&E40X)

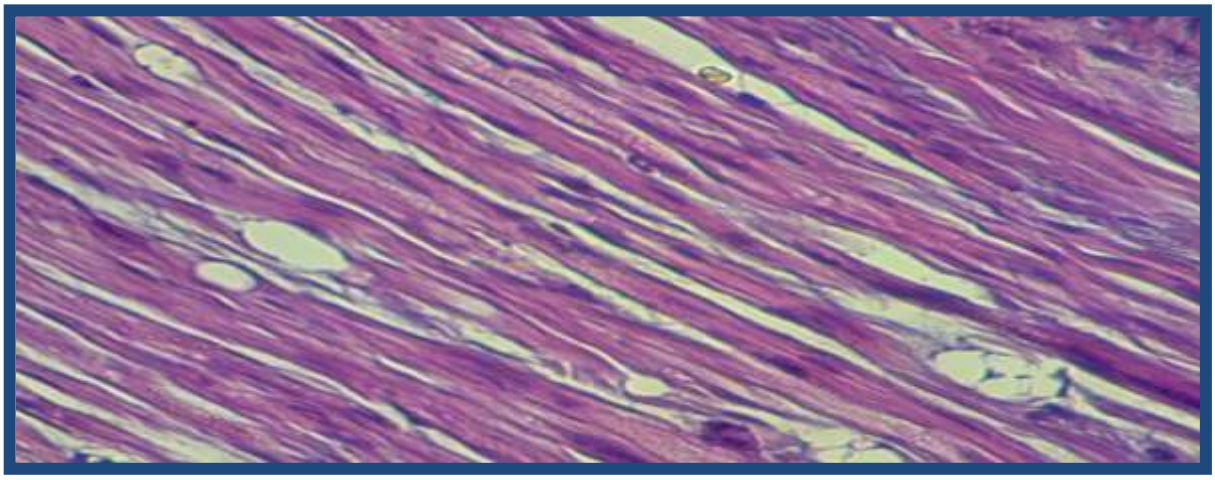

Figure - 10: The proximal longitudinal section of sciatic nerve of CMP group. (A) Regular arrangement of nerve fibers. (B)Complete remylination and regeneration of neural tube (H&E40X)

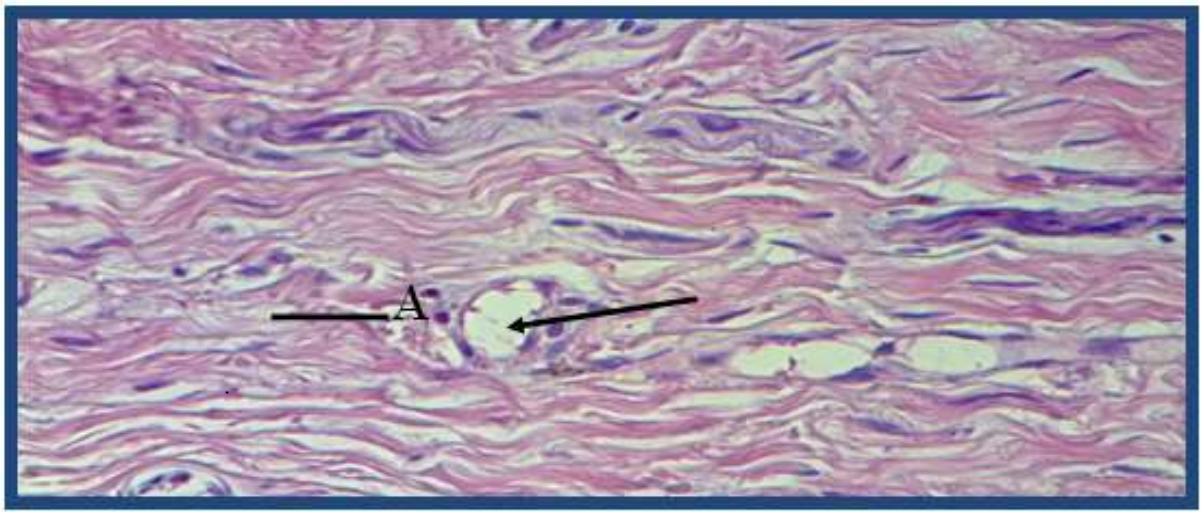

Figure - 11: The transverse section of middle part of sciatic nerve of CMP group (A) Regeneration of neural tube (B) High number of Schwann cells (H&E40X)

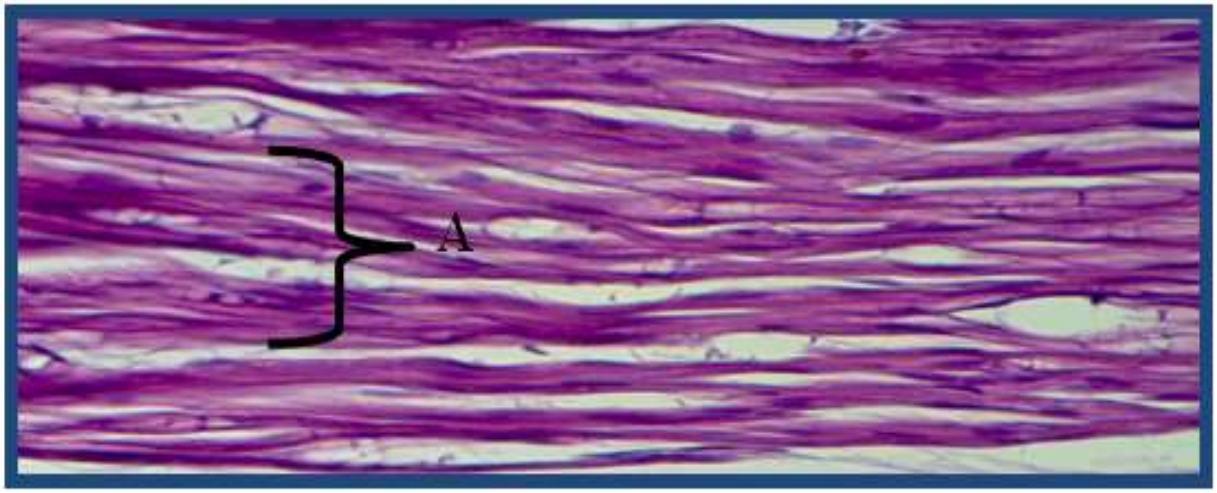

Figure - 12: The distal longitudinal section of sciatic nerve of CMP group (A) Good orientation of regenerated nerve fibers (H&E40X)

The sciatic nerve, acts as a mixed function nerve, is made of both sensory and motor nerve fibers; autonomic nerve is involved in skeletal muscle movement of hind limbs (McLean *et al*., 2002). In the present study, after transecting of sciatic nerve, the animal show several signs such as paralysis (sensory and motor dysfunction), since this signs disappeared gradually. Furthermore, the paralysis disappeared earlier in the Nucleo CMP forter compared with PRP group. Moreover, PRP may be used in the treatment of injuries to nerve, tendon, ligament, muscle, bone, and joint with success at pain reduction and return to desired level of activity (Kristin and David, 2010).

On the other hand, the paralysis disappeared faster in the Nucleo CMP forter group compared with PRP group. Nucleo CMP forter is mainly used for peripheral neurological disorders, improves neural growth and never repair, improve regeneration of myelinated nerve fiber, Nucleo CMP forter was contains uridine monophosphate, which together with cytidine monophosphate induce biosynthesis of neuronal glycolipid, phospholipid, RNA and DNA, Nucleo CMP forter crosses the blood brain barrier and then phosphorylated into uridine triphosphate that leads to triggering of neurotransmitter modulation (Flierl *et al*., 2015).

Pain and swelling were also recorded in the operated leg in two groups, they disappeared earlier in PRP compared with Nucleo CMP forter groups while they disappeared rapidly in Nucleo CMP forter group compared with PRP group. Similar results were recorded by Negrao *et al*. (2014) who mentioned that Nucleo CMP forter was more effective on pain redaction, decreasing the number of effective areas and the extent of pain. Furthermore, nucleotide receptor agonists have been shown to have a potent antinociceptive effect in neuropathic pain models (Ando *et al*., 2010).

Sever knuckling appeared in the operated leg in two groups, these results are constituent with Ammar (2009). Furthermore, this sign disappeared during a short period in Nucleo CMP forter and PRP groups compared with control group. On other hand in Nucleo CMP forter group, the knuckling disappeared in a shorter period than PRP group. Nucleotides is used for speeding up and reinforcing neuromuscular recovery in animals with experimental lesions of the sciatic nerve and increasing of innervation (Wattig *et al*., 1992).

During the week sixteen PO, significant differences in conductive velocity were observed between PRP, Nucleo CMP forter and control groups at $P \leq 0.05$. On other hand, significant differences were also observed in the same group between left and right sciatic nerve. Moreover, a good conductivity was in PRP group compared with controls, this result agreed with Ding *et al*. (2009) who mentioned that the use of PRP that may be increased regenerating axons that increase the conductivity of nerve fibers. Furthermore, the best conductivity was in the Nucleo CMP forter group compared with

PRP and control group. This result is consistent with (Cansev *et al*., 2005) who mentioned that using Nucleo CMP forter increasing the synthesis of neuronal membrane phospholipid and neurogenesis that improve the transmission of nerve impulses.The regeneration of peripheral nerves axons requires neurotrophic support, it could benefit from the presence of a growth factors delivery cell system capable of responding to stimuli of the local environment during axonal regeneration (Amado *et al*., 2010).

In a normal integrated peripheral nerve, trophic factors, which affect nerve tissue are generated in the target organ and transported in a retrograde fashion along the cell body. Schwann cells (SCs) are not only the actors of Wallerian degeneration that occurs following the nerve damage but are also responsible for the production of trophic factors that regulate regeneration. The growth factors generated by Schwann cells, such as nerve growth factor, brain-derived neurotropic factor, ciliary neurotropic factor and glial cell line-derived neurotropic factor are involved in the modulation of recovery. Neurotrophins is diffusely distributed around the damaged axons after releasing from the Schwann cells. Regenerating axons tend to extend towards the distal segment with a high concentration of neurotropic (Yu *et al*., 2011).

In the present study, the results of the neurohistopathological examination of the longitudinal and transfers section of sciatic nerve demonstrated presence of vacuolated degeneration nerve fibers in two groups but in variable degrees. Stoll and Muller (1999) demonstrate that axotomy or crush of a peripheral nerves leads to degeneration of the distal nerve stump referred to as Wallerian degeneration.

The decrease number of the vacuolated degenerative nerve fibers and prominent presence of Schwann cells proliferation and increase number of regenerative nerve fibers in the middle and distal part of nerve stump and absent of fibrous tissue in the PRP and Nucleo CMP forter groups. On other hand, this histopathological changes such as an increase in Schwann cells and nerve fibers, decrease swollen vacuolated degenerative nerve fiber that were better in Nucleo CMP forter group compared with PRP group. Schwann cells down - regulate their normal proteins such as myelin basic protein (MBP), peripheral myelin protein - 22 (PMP-22), myelin associated glycoprotein (MAG), P0 and connexin - 32 (Trapp *et al*., 1988) in order to convert the pre-myelinating cells phenotype (Hall, 2005).

The differentiated Schwann cells up regulate nerve growth factor (NGF) expression, cytokines, neurotrophic factors, and The latter are important in preventing neuronal apoptosis in response to injury and potentiate the migration and adhesion of Schwann cells to axonal projections (Boyd and Gordon, 2003). Nucleo CMP forter is useful in the regeneration of nerve cells by stimulating the synthesis of phospholipids and sphingolipids (the major components of neuronal cell membranes and myelin sheath). Furthermore, Nucleo CMP forter has an essential role in the activation of Schwann cells (Martianez *et al*., 2012). Moreover, a successful regeneration of peripheral nerve after nerve injury depends on activated Schwann cells and their supportive role in the production of neurotrophic and neurotropic factors (such as nerve growth factors) that enhance neural recovery (Oyama *et al*., 2004). The role of PRP in the tissue repair and regeneration has already been studied (Farrag *et al*., 2007). PRP is a concentration of fundamental growth factors known to be actively secreted by platelets to initiate wound healing.

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