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Full Length Research Paper

Clinical and histopathological observations of autologous bone marrow stromal cells implantation on the regeneration of sciatic nerve neurotmesis in rabbit

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The peripheral nerve injuries (PNI) is due to stretching and laceration. Therefore, the epineural repair technique is currently accepted as a standard method for peripheral nerve repair. Thirty-two (32) rabbits were divided equally into perineural nerve sutures as control group (CG) and bone marrow stromal cells (BMSCs) as the treatment group (TG). The aim of this study was to investigate the effect of the autologous BMSCs implantation on the regeneration of sciatic nerve neurotmesis in rabbits. There was significant improvement ($p<0.05$) of the motor and sensory functions in the TG animals as compared to the CG. Histopathological examinations of the proximal nerve stump in the CG showed mild adherence surrounding an anastomosed area, few vacuolation and plenty of collagen at the peri and epineurium. The anastomosed site showed the degeneration, disorientation and giant cell surrounding the stitches, while intraneurial scar tissues were seen in the distal segment at the 112th post operation day (POD). The histopathology of TG showed the activated Schwann cells, good myelination, minimum scar tissue, good orientation and remarkable angiogenesis at the 112th POD. BMSCs are capable of improving the motor and sensory functions and significantly promote the regeneration of sciatic nerve neurotmesis in rabbits.

Key words: Clinical, histopathological, bone marrow stromal cells (BMSCs), neurotmesis, sciatic nerve, rabbit.

INTRODUCTION

A stem cell is defined as a cell capable of self-renewal and multilineage differentiation on a single-cell basis (Weissman, 2000). This self-renewal and multipotency of the bone marrow stromal cells (BMSCs) have shown promising results regarding tissue engineering processes because they have overcome a major earlier perceived hindrance to nerve healing, regarding the inability of certain cell types, such as neurons, to regenerate, proliferate and heal (Langer and Vacanti, 1993). Bone marrow stromal cells can be induced to differentiate into cells with Schwann cell characteristics that are capable of eliciting peripheral nervous system regeneration. Moreover, some studies have shown the ability of these cells to migrate to the spinal cord, dorsal root ganglia (Corti et al., 2002), brain and differentiation into neuroectodermal and microglial cells (Josep, 2007). Therefore, the aim of this study was to investigate the effect of the autologous implantation of BMSCs on the regeneration of neurotmesis of sciatic nerve in rabbits.

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MATERIALS AND METHODS

Thirty-two (32) male adult New Zealand White rabbits weighting 2.0 to 2.3 kg were divided into two groups consisting of sixteen (16) animals each (Animals Unit, UKM, Malaysia). In the first group, the sciatic nerve was severed and anastomosed using epi-perineural sutures, which served as the control group (CG), while the treatment group (TG) was treated with BMSCs post severance and coaptation. All animals were acclimatized for 3 weeks in individual cages, fed with commercial rabbit pellets and given water *ad libitum*. Broad-spectrum prophylactic antibiotics (Pencillin 20,000 IU Streptomycine 20 mg/kg) and subcutaneous antihelminth injections of 0.2 mg/kg ivermectin (Biomectine, Vetoquinol Ltd. Lure cedex,

France, 1%) were given prior to the onset of the experiment. All procedures used in this study were approved by the Faculty's Animal Care and Use Committee number 08 R13/Dec 08. Four animals from each group were sacrificed at the 14th, 28th, 56th and 112th post operation day (POD).

Anesthetic protocol

All animals were inducted with an intramuscular injection of a combination of 40 mg/kg ketamine hydrochloride (Bioketan, Vetoquinol Biovet, Sp. Zo.O, France), 5 mg/kg of xylazine hydrochloride (lium xylazil-20, Australia) and acepromazine maleate 1 mg/kg (Calmivet. Vetoquinol. Ltd. Lure cedex, France). Anesthesia was maintained using 1 to 2% halothane using a flow rate of 500 ml/min in a non-rebearthing circuit (modified Jackson-Reed Bain) through a face mask. Similar anesthetic protocol was utilized for both study groups.

Modified surgical protocol

Rabbit fur of the left hind limb was clipped from upper midline to the stifle joint. The skin was disinfected using chlorohexidine gluconate (Hibiscrub, 4% w/v Durham, UK), isopropyl alcohol 70% (Jaya Pelita Pharma. SDN. BHD) and finally, tincture iodine 1.8% (Jaya Pelita Pharma SDN. BHD). The glove was covered with sterile skin towel and secured to the limb by towel clips. The animals were recumbent on the right lateral side and the operated limb was draped with the aperture of the fenestrated drape at the intended operation site and covered with Opsite® (Smith and Nephew-Medical Limited, England). The greater trochanter of the femur was palpated and the stifle was taken as landmarks. The skin was incised on the posterio-lateral thigh, approximately 1-cm caudo-lateral to the greater trochanter of the femur to the level of the distal one-third of the femur using scalpel blade # 21. Subcutaneous tissue and fascia lata were incised on the same line using scalpel blade # 15. The biceps femoris muscle was separated cranially and semitendenous muscle was separated posteriorly by blunt dissection using Mayo scissors to expose the sciatic nerve and separate it from the surrounding tissues using ophthalmic scissors. Plastic spatula was placed gently under the nerve to severe it using scalpel blade # 21.

The nerve ends were immediately coapted under dissecting microscope (Kruss, Germany). Six equidistant epineurial simple interrupted sutures were placed about 1-mm from the edge of the transected nerve using 8-0 nylon (Monofilament, ETHICON USA). The coapted nerve was carefully replaced into its anatomical site, the surgical area was flushed with physiological normal saline and the excess saline was removed with sterile gauze. The superficial fascia was sutured using 3-0 Vicryl (Biovek, Dynek Pty Ltd Australia) with simple continuous suture and the skin was closed using 3-0 vicryl subcuticular suture pattern. All animals were given 12 hourly post-operative intramuscular doses of 10 mg/kg tramadol hydrochloride (Domadol® India, 50 mg) 0.2 ml/kg IM, for three consecutive days. The paw of operated limb was protected by means of a special sock that was developed by the researchers in this study.

Control group (CG)

The left sciatic nerve was transected and immediately anastomosed using the standard method mentioned earlier. All animals were examined daily from first day to the end of the experiment at 112th POD. Animals were scarified and the anastomosed sciatic nerves were obtained for further studies.

Treatment group (TG)

Isolation and culture of BMSCs on modified media

The area from the external angle of the ilium to the hip joint was clipped and disinfected following anesthesia, and 1.5 ml of bone marrow was collected from the ilium using a syringe (5 ml) with needle gauge 18. The bone marrow aspirated was immediately mixed with 3 ml of Dublecco's modified Eagle's medium (DMEM) and high glucose-DMEM was supplemented with 30% fetal bovine serum (FBS), 10 U/ml pencillin G, 10 U/ml streptomycin, 25 mg/ml amphotericin B, 1% nonessential amino acid and sodium pyruvate (100 ng/ml), all supplied by GIBCO® Invitrogen Corporation. Three milliliters of FBS was placed in a 75 cm^2 flask for 3 min and then the mixed media with bone marrow was added to the flask. The flask was incubated at 37°C in 5% CO_2 in air for 3 days, and after 72 h, the non-adherent cells were harvested and the medium was replaced. The culture reached the confluence stage at day 12 and the monolayer cells were washed twice with 2 ml of phosphate buffered saline (pH 7.2). The cultures were then digested with 2 ml of 0.20% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA, Sigma, USA) and distributed on the surface of the layer for 2 min while checking the cells under the microscope until they regained their rounded shape and the trypsine was discarded. Dublecco's modified Eagle's medium containing 10% FBS was added to the medium and then gently tapped to detach cells for the next three sub-cultures. The cells were harvested by decanting the medium from the flask, washing with PBS, trypsine bathing and replacing with 10 ml of DMEM. The medium and cells were collected in sterile test tubes, centrifuged at 2000 rpm for ten minutes. Then, 1-ml of DMEM was added to the separated precipitated pellets and mixed. Cells were counted using a hematocytometer to ensure the minimum count of 1 x 10^6 MSCs in 10 µl of culture medium. The isolation and transplantation of BMSCs were achieved for each animal.

Characterization of mesenchymal stem cells

The stem cells have an ability to adhere, grow on the plastic surface and exhibit the fibroblast-like morphology to reach to the complete confluence. At the fourth passage, the fibroblast-like cells became morphologically homogeneous, with more than 95% purity and differentiation of mesenchymal stem cells along osteogenic and adipogenic lines. These morphological and functional changes confirm the differentiation status of the cells. Bone marrow stromal cells differentiated throughout the culture from adipogenic cell line to mature adipocytes in 21 days which appeared as intracellular accumulation of red-stained lipid vacuoles on staining with Oil Red O solution (Figure 1a). After 14 days, the osteogenic line of BMSCs were differentiated and demonstrated the mineral deposition characteristic of mature osteocytes lineage as observed under Alizarin Red S staining (Figure 1b) (Tsai et al., 2004).

In vivo

The transected sciatic nerve was immediately anastomosed and the proximal and distal segments were each injected with 10 µl of culture medium containing 1 x 10^6 MSCs using a glass capillary tube connected to a Hamilton syringe (Cuevas et al., 2004).

Clinical observations

The motor and sensory sciatic nerve reflexes were evaluated at 14th, 28th, 56th and 112th POD.

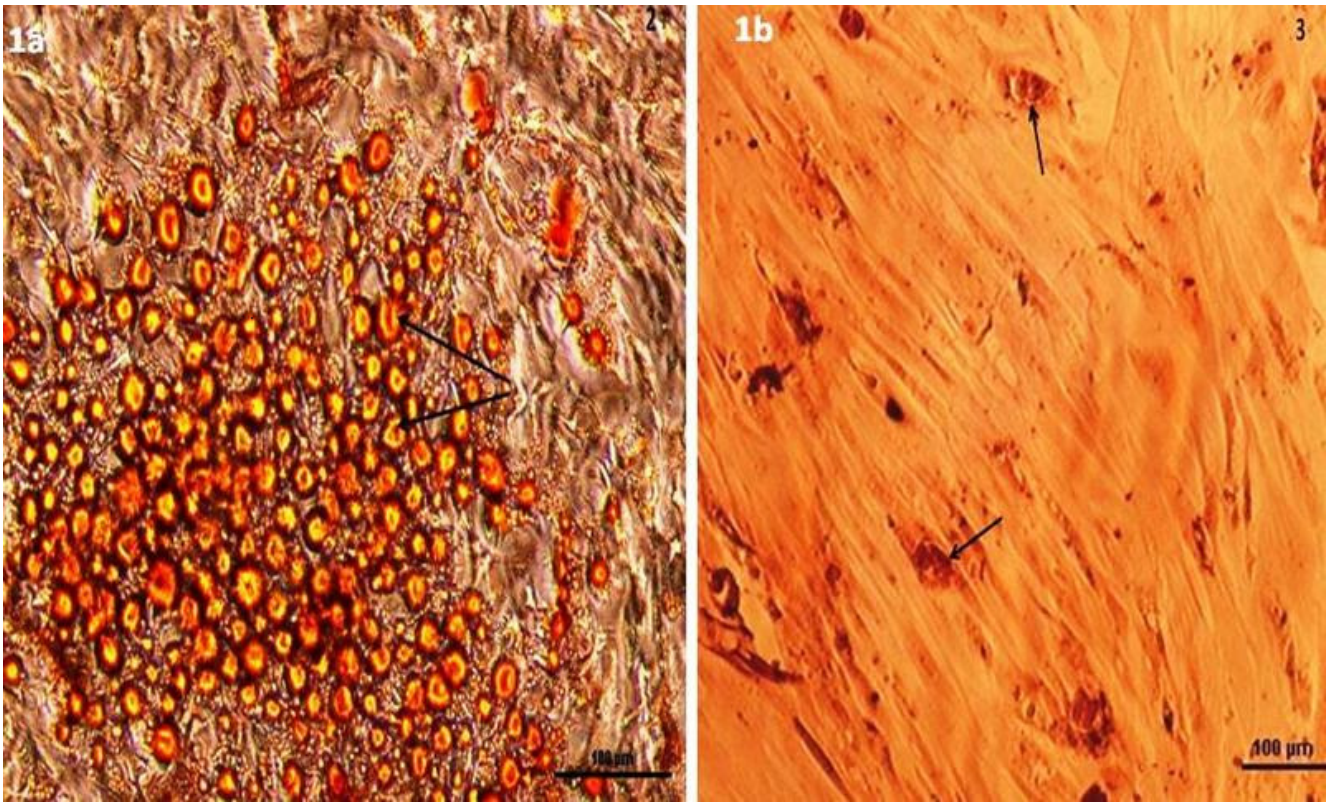

Figure 1. (a) Cultured BMSCs exhibited the accumulation of intracellular lipid droplets detected by Oil Red O staining (arrows). Scale bar = 100 µm. (b) Cultured BMSCs exhibiting the deposition of calcium (arrows) was determined by Alizarin Red S staining. Scale bar = 100 µm.

Sciatic nerve motor functions evaluation

Animals were monitored daily for onset and ability to walk up to the 112th POD. The animals were examined for type of walking including crouching, crawling on heel and normal, as well as knuckling, which was classified into severe, moderate, mild and normal. The muscle contraction force was graded as weak, moderate or strong, while muscle mass atrophy was graded as severe, moderate, mild or normal.

Sciatic nerve sensory functions evaluation

Sensory functions of the anastomosed sciatic nerves were tested daily for 112 days. Toe spreading reflex, lateral aspect leg sensation, toe pinch and toe prick were evaluated as either present or absent. In addition, the foot withdrawal and vocalization tests of lateral aspect leg sensation, toe pinch and toe prick were recorded as positive responses indicating recovery and improved function.

Neurohistopathological changes

Four animals from each group were euthanized at the 14th, 28th, 56th and 112th POD using intracardial injection of pentobarbitone (Dolethal 180mg/ml) 1 ml/kg under ketamine/xylazine anesthesia. The anastomosed left sciatic nerve was exposed, examined grossly and then harvested for histopathological studies. Three specimens of 1-cm length were collected from the proximal, middle (coaptate site) and distal segments of the coapted sciatic nerve. The specimens were fixed in 10% neutral buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 5 µm thick sections and stained with Hematoxyline and Eosine stain and Meyer's modified trichrome stains. All stained slides were viewed under an Olympus image analysis (BX 51 TF, with attached CC 12 camera).

Statistical analysis

All data were expressed in means and standard deviation of the mean (M ± SD). Statistical comparisons between groups were performed using Statistical Package for the Social Sciences (SPSS) 16.0 software (non-parametric tests), Kruskal Wallis and Mann-Witney tests. A p value of ≤0.05 was considered significant.

RESULTS

Sciatic nerve motor functions evaluation

All animals showed obvious motor hind limb dysfunction at the first three days of operation. The onset and ability to walk progressed significantly earlier ($p<0.05$) in the TG animals as compared to the CG at 14th POD. The animals in the TG showed the crawl improvement with $p\leq0.05$ significance as compared to the CG animals at the end of the 14th and 28th POD. The knuckling in the TG showed

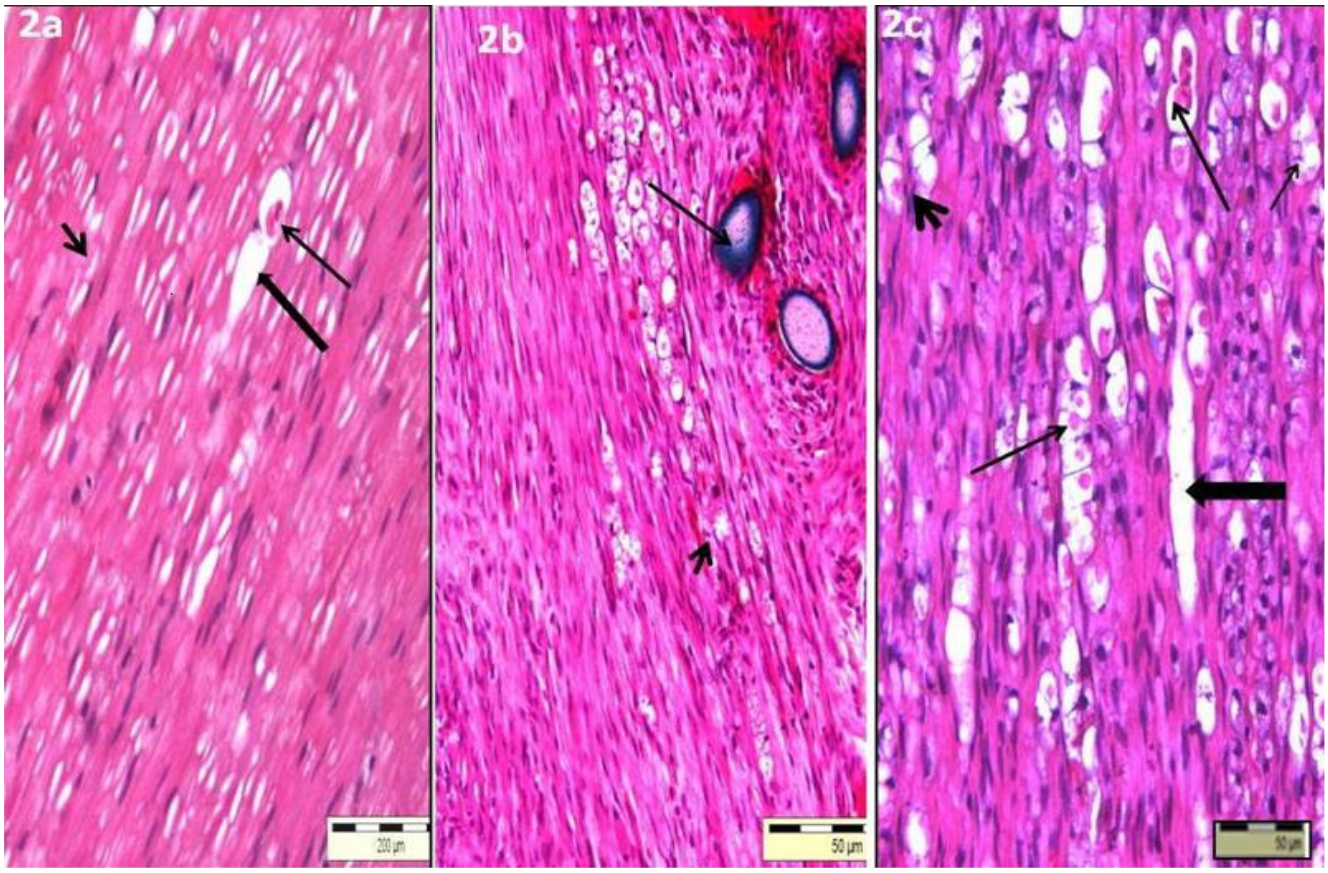

Figure 2. Photomicrographs of the sciatic nerve in CG at the 14th POD. a) Proximal stump demonstrates the degeneration, vacuolation (arrow head), ovoid (thin arrow) and digestive chambers of collagen (thick arrow); b) Mid-portion shows granulation tissue surrounding the stitches (arrow) and deposit of collagen fiber (arrow head); c) Distal stump shows degeneration and vacuolation (arrow head), ovoid (thin arrow) and digestive chambers of collagen (thick arrow), (H and E).

moderate, mild and normal at the end of the 14th, 28th, 56th and 112th POD, respectively, with p≤0.05 significance as compared to the CG group. The type of gait showed normal in TG with p≤0.05 significance as compared to the CG at 56th POD, while animals in both groups showed normal gait at the end of the 112th POD.

The muscle force contraction at 14th POD appeared moderate in all groups. In the 28th POD, it became strong in the TG with p≤0.05 significance as compared to the CG group. The muscle force contraction became strong in all animals with no significant differences at end of the 56th and 112th POD. Animals in the TG showed moderate muscle mass atrophied with p≤0.05 significance as compared to the CG at the end of 14th POD. All animals in both groups appeared with severe muscle mass atrophy at the end of the 28th and 56th POD and moderate at the end of the 112th POD with no significant differences.

Sensory functions evaluation

The sensory clinical signs tested for, including toe spread, lateral leg sensation, toe pinch and toe prick did not show sensory reflexes at the 14th, 28th and 56th POD. Interestingly, the TG animals showed significant (p<0.05) sensory functions at the 112th POD in comparison with the CG animals.

Neurohistopathology

Longitudinal sections of the proximal nerve stumps revealed congested blood vessels at histopathology by the 14th POD. Degeneration, vacuolation, ovoid digestive chambers characteristic of Wallerian degeneration, low concentration of Schwann cells, segmental demyelination and collagen deposition in the epineual region were also observed on the 14th POD (Figure 2a). The mid-portion of the anastomosed line revealed giant cell surrounding the stitches, conspicuous presence of inflammatory cells with many macrophages and lymphocytes and disorientation of nerve fibers (Figure 2b). The distal nerve stump showed highly vacuolated and degenerated nerve fibers, digestive chambers and moderately scattered collagen between nerve fibers (Figure 2c). However, on the 28th

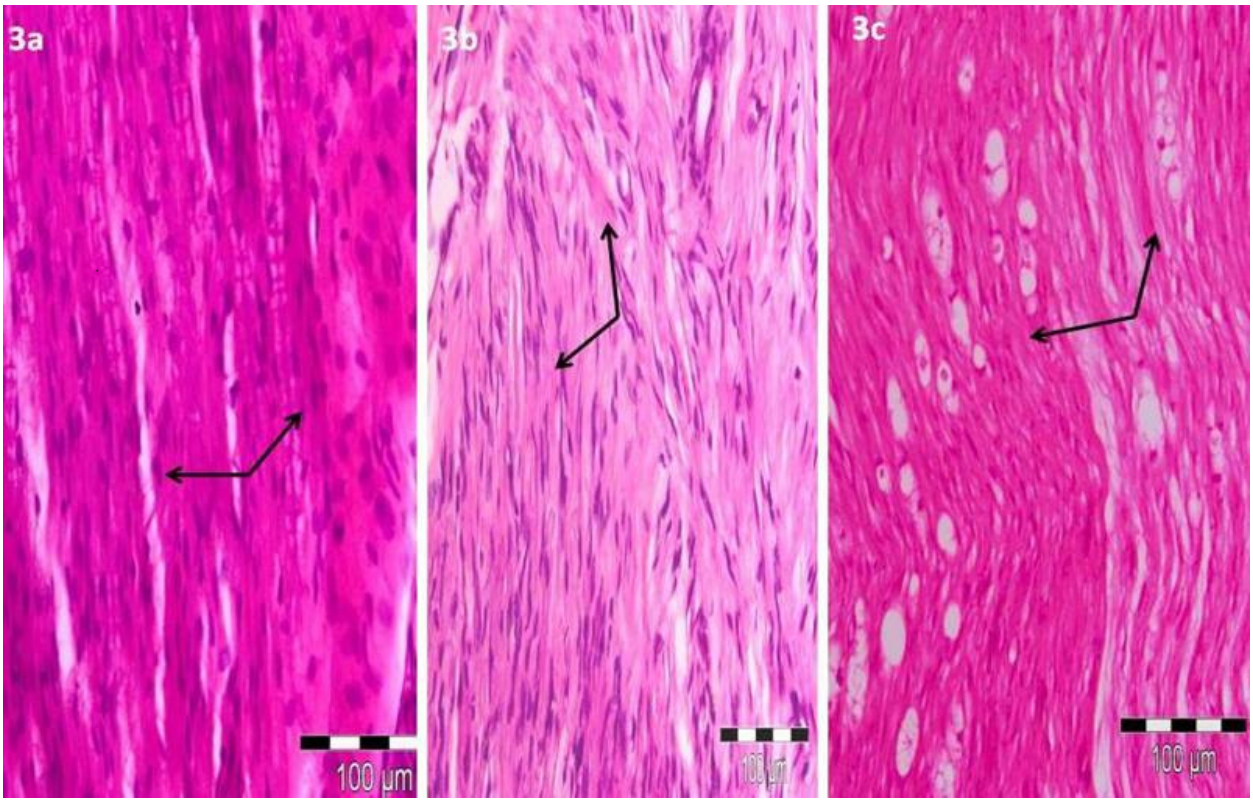

Figure 3. Photomicrographs of the sciatic nerve in CG on the 112th POD: a) Proximal stump demonstrates little degeneration and vacuolation of the nerve fibers (arrows), and present collagen fibers (scare) at peri and epineurium; b and c) mid-portion and distal stump shows respectively, a little degeneration and vacuolation of nerve fibers (arrows), H and E.

POD, there was lesser degeneration and decreased inflammatory response. On the 56th POD, the proximal segment histopathology showed less vacuolation, ovoid, digestive chambers and slight improvement in the orientation of nerve fibers with thickening of the epineurium and perineurium. Sections of the stitches had giant and other inflammatory cells surrounding the stitches as well as scar formation, adherence of nerve to muscle, disarray and vacuolation of nerve fibers. The distal segment showed vacuolated and degenerated nerve fibers, thickening epineural connective tissue and internal perineurium, as well as ovoid digestive chamber.

Sections of the proximal stump at the end of the 112th POD showed mild adherence of the anastomosed line with adjacent tissues, few vacuolation and degeneration of nerve fiber, abundant collagen fibers (scar) at peri and epineurium and a small number of Schwann cells in the nerve fibers (Figure 3a and b). Giant cells surrounding stitches and intraneurial scar tissues were noted in the distal segment (Figure 3c). The transverse section of sciatic nerve showed thick fibrous tissue proliferation at the perineurium and epineurium, and adhesion of the nerve with surrounding muscles (Figure 6a).

Histopathological findings of the TG group specimens at the proximal nerve stump sections at the end of 14th POD showed degeneration, vacuolation and axons infiltrated with inflammatory cells, remarkable angiogenesis, demyelination, and good orientated nerve fibers (Figure 4a). Giant cell were observed around the stitches with scattered inflammatory cells. There was also degeneration, vacuolation, increased number of Schwann cells and slight disorientation of nerve fibers observed in the mid-portion (Figure 4b), while the distal nerve stump portion showed an increased number of Schwann cells, infiltrated inflammatory cells, ovoid and digestive chambers and demyelination of the nerve fibers (Figure 4c). Histopathological observations on day 28 showed similar findings with slightly lower degeneration, vacuolation and increased angiogenesis. On the 56th POD, there was a further increase in the number of Schwann cells, less degeneration, vacuolation and less collagen deposition, as well as an increase in the angiogenesis and good parallel orientation of the nerve fibers.

Interestingly, the sections of the proximal segment in this group at the end of 112th POD showed the absence of degeneration and vacuolation, increased number of Schwann cells, good orientation and scanty scar tissue in the epineurium of nerve fibers (Figure 5a). The mid and distal segments showed remarkable angiogenesis, good orientation and myelination of nerve fibers (Figure 5b band c). The transverse section of distal stump of sciatic

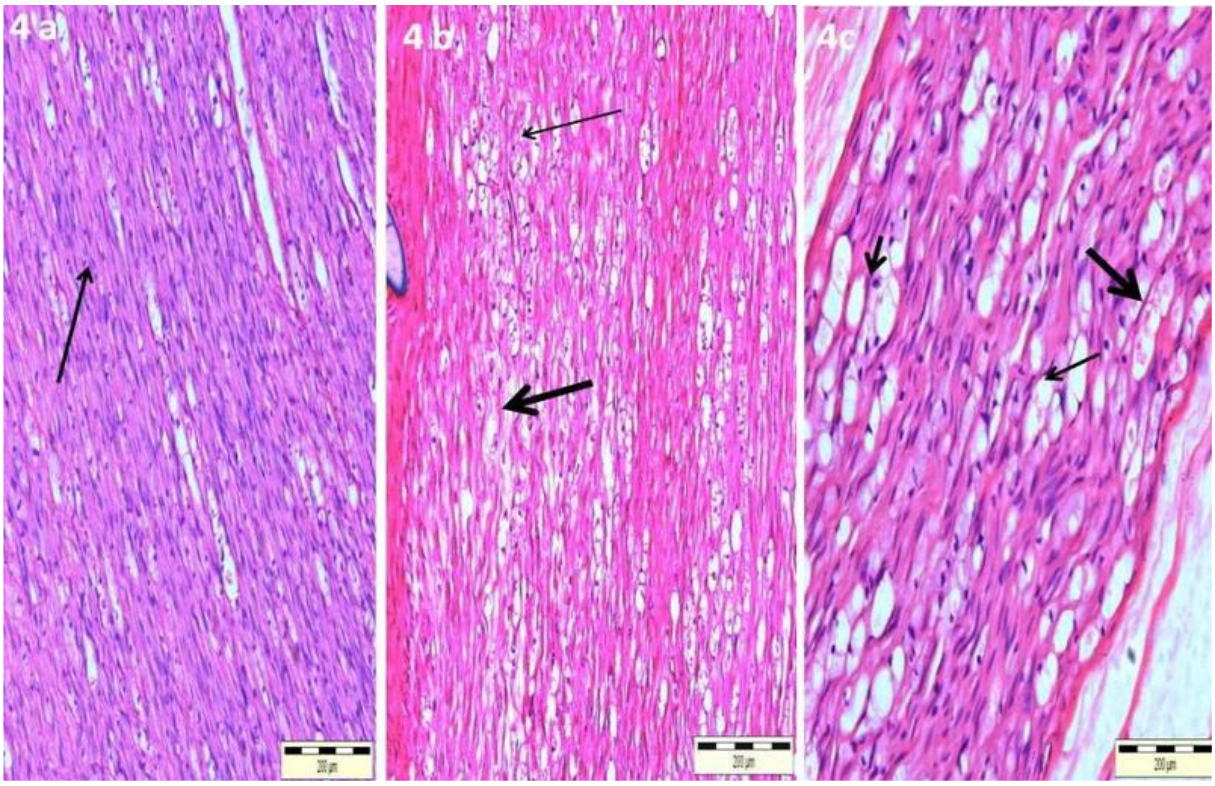

Figure 4. Photomicrographs of the sciatic nerve in TG on 14th POD; a) Proximal stump demonstrates degeneration and vacuolation (arrow head), ovoid (thin arrow) and digestive chambers of collagen (thick arrow); b) mid-portion shows ovoid (thin arrow) and digestive chambers of collagen (thick arrow), increasing in the number of Schwann cells (long arrow); c) distal stump shows increased number of Schwann cells (long arrow), H and E.

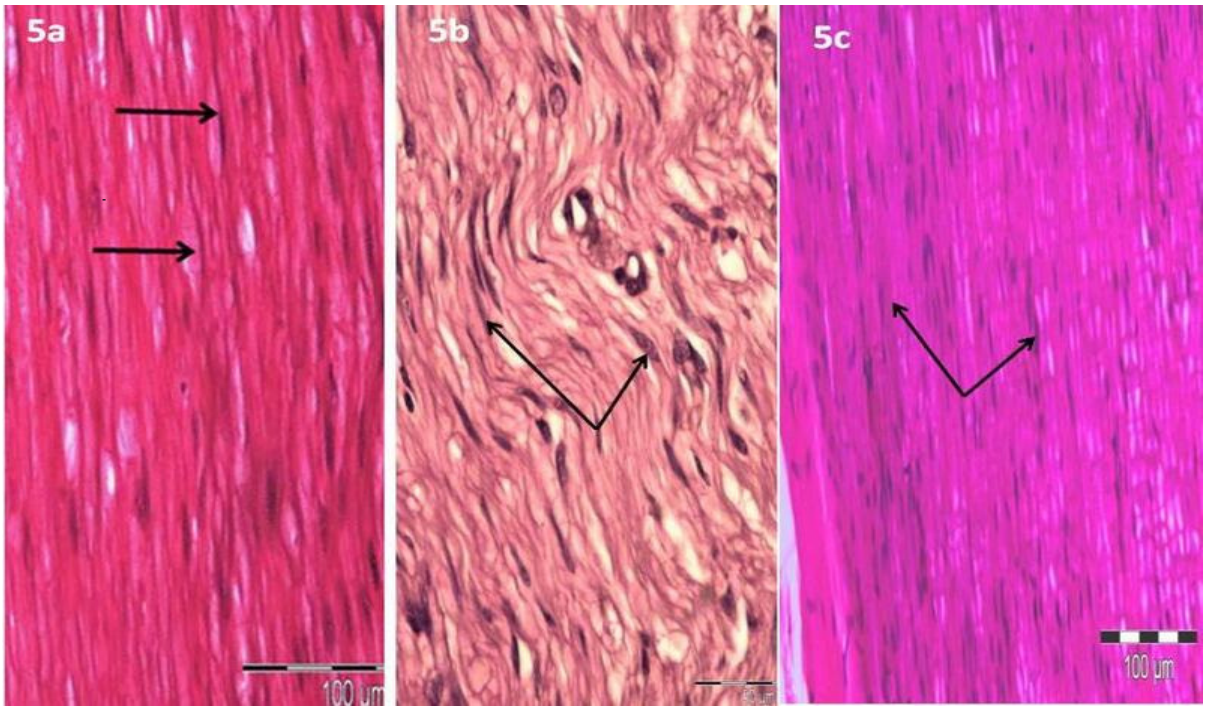

Figure 5. Photomicrographs of the sciatic nerve in TG on the 112th POD; a, b and c) proximal, mid and distal stumps show respectively, increased number of Schwann cells and good orientation of the nerve fibers (arrow), H and E.

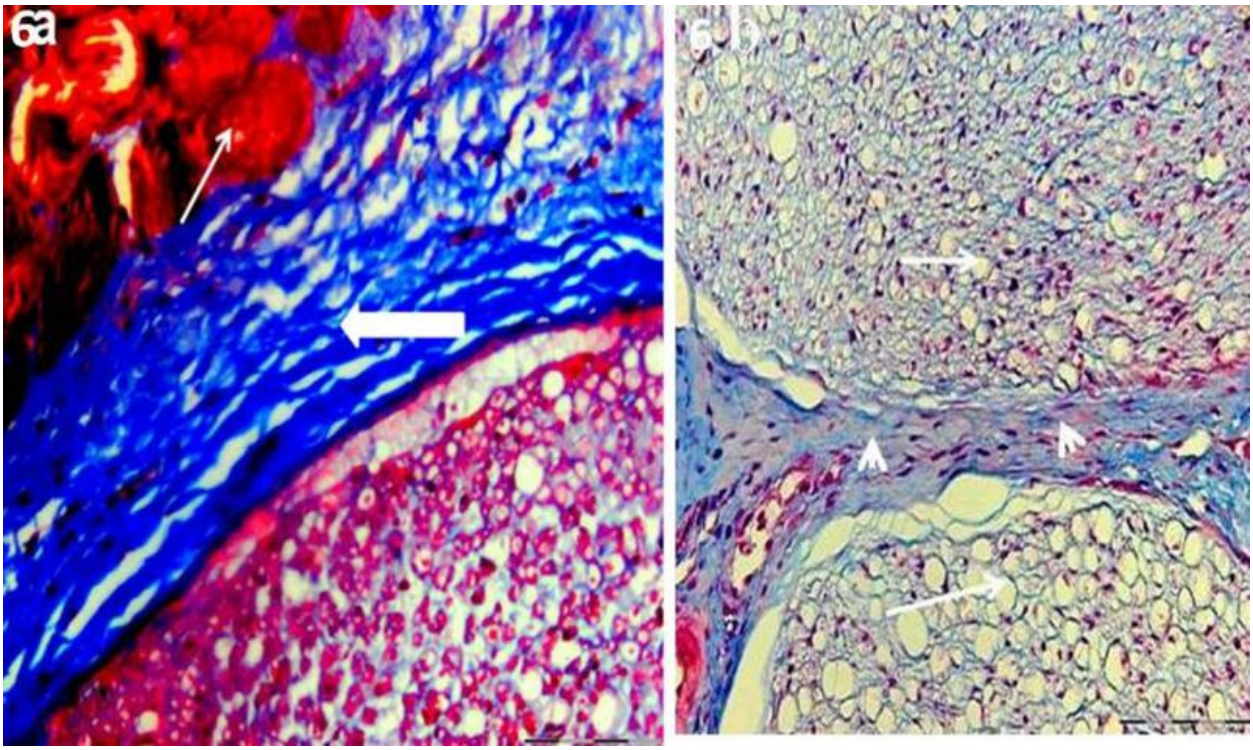

Figure 6. Photomicrograph of transverse section of distal segment of sciatic nerve of control group at 112th day showed high thickness of fibrous tissue at perineurium and epineurium (thick arrow) and adhesion with surrounding muscles (thin arrow) x200. (b) Micrograph of distal segment of sciatic nerve of treatment group at 112th day showed deposit of thin layer of fibrous tissue at perineurium (arrow head) and endoneurium (arrow), Meyer's modified trichrome stains x100.

nerve demonstrated deposition of thin layer of fibrous tissue at the perineurium and endoneurium (Figure 6b).

DISCUSSION

This study shows an improvement in the motor functions recovery in animals treated with BMSCs of coaption sciatic nerve earlier than control group. This improvement might be due to the BMSCs' ability to accelerate transected sciatic nerve regeneration and decrease neuropathic pain generation. This finding is congruent with that of Musolino et al. (2007) who reported that the BMSCs prevented the generation of mechanical allodynia and declination of the number of cold allodynia responses. Moreover, MSCs constitutively secretes a diverse spectrum of neurotrophic growth factors, which have ability to activate neurotic outgrowth, guide regeneration of axon, play roles in survival, growth or differentiation and may modulate primary sensory neuronal response to injury and thus influence pain conception (Eaves et al., 1991; Ji et al., 2004).

Gait in the TG normalized at the 84th POD, while the CG continued crawling until the end of the study. It has been proposed that the therapeutic benefits after BMSCs implantation in the damaged nerve tissue may be due to two different but synergistic mechanisms. The first being functional integration and cells morphology; secondly, the production of neurotrophic, extracellular matrix molecule synthesis and anti-apoptotic molecules by the BMSCs, promoted the sciatic nerve healing. This result is in agreement with previous studies (Cuevas et al., 2002; Hudson et al., 2004) that reported that BMSCs might integrate and provide neuroprotection in the spinal cord and peripheral nerve injury.

Although, the levels of muscle force contractions and muscle mass atrophy were significantly different ($p \leq 0.05$) between the two groups at the 14th and 28th POD, this became non significant after the 56th day in both groups. This finding corroborates those of Zhang et al. (2005) who suggested that the BMSCs might induce the immune processes of limited local infiltration of blood-borne inflammatory cells and neuroprotection, resulting in the promotion of the peripheral nerve regeneration and functional improvement of the nerve after neurotmesis.

One of the interesting findings emerging from this study is that the sensory reflex in the TG was significantly improved ($p \leq 0.05$) as compared to CG group by the 112th POD. In this study, motor functions were regained earlier than the sensory functions, which took a longer period in TG animals. This finding is consistent with previous works on different nerves (Brushart et al., 1998). Chen et al. (2006) and Brohlin et al. (2009) observed that some of the differentiated BMSCs turned into Schwann cell-like

cells, and were accompanied by greater number and good myelination of the regenerated nerve fibers.

The macroscopic examinations of the coapted nerve segments did not cause any dehiscence and neuroma formations, indicating the accuracy of the surgical technique such as good alignment of the sciatic nerve in TG animals. Moreover, the regeneration of anastomosed sciatic nerve in the TG group showed earlier recovery as compared to the CG group, this finding supports previous researches in this area (Cuevas et al., 2002; Henery et al., 2009). Microscopically, TG sections showed robust nerve fibers orientation and angiogenesis initiated by transplanted stem cells, thereby possibly playing important roles as a therapeutic regimen. These findings are in consistent with those of Hamano et al. (2000) and Shen et al. (2006), who reported the effects of stem cell therapy on neovascularization, which might be explained by multiple nonexclusive pathways. First, transplanted stem cells differentiate into endothelial cells to support new blood vessel components. Secondly, transplanted cells might release the angiogenic and/or trophic factors, which facilitate the angiogenesis. These findings further support the idea of human bone marrow stromal cells being able to promote the angiogenesis by increasing the endogenous levels of the vascular endothelial growth factor (VEGF) (Chen et al., 2003). The VEGF is considered the most endothelial cell-specific growth factor, which has direct effects on the Schwann cells, microglial cells, neural stem cells and astrocytes. The VEGF might protect the motorneuron cells from hypoxia and oxidative stress-induced apoptosis (Oosthuyse et al., 2001; Byts and Siren, 2009), stimulate axonal outgrowth and promote the survival of neuron and satellite cells in dorsal root ganglia (Sondell et al., 1999).

Also, histopathological results showed the mild intraneural scarring in TG, while in CG there was severe collagen deposit. The success of axon regeneration can be viewed as a balance between regeneration and scar formation, and relationship was found between scar tissue formation and axonal regeneration (Atkins et al., 2006). According to Zou et al. (2006) there was an increase in the axonal regeneration and myelination in mice following the application of tissue plasminogen activator (tPA) to repair sites. This might provide further evidence for the potential role of scar inhibition in promoting axonal regeneration in the peripheral nervous system. In conclusion, the clinical observations and histopathological analysis of this experimental study suggests that the BMSCs might be capable of promoting the regeneration of nerve after neurotmesis of the sciatic nerve.

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