Journal of Animal and Veterinary Advances 10 (6): 686-691, 2011
ISSN: 1680-5593

Neurobiological Observations of Bone Mesenchymal Stem Cells *in vitro* and *in vivo* of Injured Sciatic Nerve in Rabbit

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Abstract: The PKH26 is a fluorescent lipophilic dyes used for the study of Asymmetric cell Divisions (ASDs) and efficiently purifies the stem cell fraction. The aim of this study was to explore the neurobiological characteristics *in vitro* and *in vivo* and tracking fate of the transplanted rabbit Bone Marrow-Mesenchymal Stem Cells (rBM-MSCs). A fluorescent microscope was used to determine the changes in cell size, fluorescence intensity during tissue culture, track cell divisions and the distribution of PKH26 dye between daughter cells. The results showed the identification of ASDs based on fluorescence intensity of the PKH26 dye was distributed equally between daughter cells at each division *in vitro*. The labeling BMSCs with PKH26 showed within the wall of the neurons in the dorsal root ganglia *in vivo*. Labeled BMSCs which are fibroblastic-like cells in P4 showed oval shaped and less density than P2. Direct examine of the labeled BMSCs in the cryosections at 16 weeks post operation showed the BMSCs were differentiated and appeared as like Schwann cells in an anastomosed sciatic nerve in the Local Treated Group (LTG). In the Systemic Treated Group (STG) sections, the labeled BMSCs were migrated to the anastomosed sciatic nerve, ipsilateral lumber dorsal root ganglia resembling glial and stellate cells and some of the labeled cells migrated to the anterior horn of spinal cord (motor neuron). In conclusion, the biological behaviors of BMSCs *in vitro* and *in vivo* showed highly mitosis at P2, activated fibroblast-like cells, differentiated to functional myelinating Schwann-like cells in LTG. The BMSCs in STG migrated and engrafted at the dorsal root ganglia as a neuron and glial cell, glial cells and satellite in the spinal cord.

Key words: Neurobiology, MSCs, PKH-26, sciatic nerve, rabbit, intensity

INTRODUCTION

Injuries of the peripheral nerves are common and debilitating which affecting 2.8% of traumatic patients (Noble *et al*., 1998) and resulting in considerable long term disability (Kelsey *et al*., 1997). BMSCs might be capable of differentiating into nonmesenchymal cells lineages, including astrocytes (Kopen *et al*., 1999), endothelial cells (Oswald *et al*., 2004), neurons (Woodbury *et al*., 2000; Deng *et al*., 2001) and myelinating cells in the Peripheral Nervous System (PNS) (Dezawa *et al*., 2001; Mimura *et al*., 2004; Kamada *et al*., 2005). Rabbit Bone Marrow-Mesenchymal Stem Cells (rBM-MSCs) are readily accessible and overcome the ethical and immunological concerns, BMSCs are capable of supporting nerve fiber regeneration and implantation into the cut ends of peripheral nerves (Cuevas *et al*., 2002). Myelinating cells, oligodendrocytes in the Central Nervous System (CNS) and Schwann cells in the Peripheral Nerve System (PNS) played a crucial role in neurodegenerative and regenerative processes. Furthermore, BMSCs have a capacity to differentiate into myelin forming cells *in vivo* (Sasaki *et al*., 2001; Zhang *et al*., 2004) and might repair the demyelinated axons in the PNS (Dezawa *et al*., 2001; Mimura *et al*., 2004). The biological characterizations of rBm-MSCs *in vitro* and *in vivo* still not clear yet.

Therefore, the aim of this study was to explore the neurobiological characteristics *in vitro* and *in vivo* and the tracking fate of the transplanted rBM-MSCs.

MATERIALS AND METHODS

Laboratory animals: About 18 male adult New Zealand white rabbits (2-2.3 kg) were divided into 2 groups (n = 9) the first was Locally Treated Group (LTG) which injected with the labeled BMSCs into the proximal and distal nerve

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segments after neurorrhaphy. The second was Systemically Treated Group (STG), injected intravenously of the labeled BMSCs into marginal ear vein of rabbits. All animals were acclimatized for 3 weeks in individual cages, they fed on commercial rabbit pellets and given *ad libitum* water.

Meanwhile, broad spectrum prophylactic antibiotics (Pencillin 20,000 IU, Streptomycine 20 mg kg^{-1}), antihelmintic injection of 0.2 mg kg^{-1} Ivermectin were given prior to the starting of the experiment.

The experimental al procedures performed as approved by the faculty's animal care and use committee (08 R13/Dec 08). All animals were anesthetized and inducted using intramuscular mixted injection of 35 mg kg^{-1} Ketamine hydrochloride (Bioketan, Vetoquinol Biowet, Sp. Zo.O, France), 5 mg kg^{-1} of Xylazine hydrochloride (ILIUM XYLAZIL-20, Australia), Acepromazine maleate 1 mg kg^{-1} (Calmivet. Vetoquinol. Ltd. Lure cedex, France) and maintenance was carried out by 1-2% halothane using a flow rate of 500 mL min^{-1} in a non-rebearthing circuit (modified Jackson-Reed Bain) through a face mask. About 3 animals were scarified at 2, 8 and 16 weeks Post Operative (PO).

Surgical protocol: Rabbit fur of the left hind limb was clipped from upper midline to 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). Skin was incised 2 cm long on the caudo lateral side behind the greater trochanter of the femoral bone to the stifle joint, using scalpel blade NO. 21.

Subcutanous tissue and fascia lata separated using scalpel blade NO. 15. Biceps femoralis and semitendenous muscles separated cranially and posteriorly, respectively by blunt dissection using Mayo scissors. Sciatic nerve was exposed and separated from the surrounding tissue using ophthalmic scissors. Wooden tongue depressor was placed gently under the nerve, cut it using scalpel NO. 21 and nerves ends were coapted immediately under the microscope with 6 equidistant epi and perineural sutures about 2 mm from the edge of transected using 8-0 nylon (Monofilment, ETHICON) with simple interrupted suturing. The anastomosed nerve was replaced into the normal position. Then muscles were closed using 3-0 Vicryl (Biovek, Dynek Pty Ltd) with simple continuous suturing and the skin was closed using 3-0 Vicryl subcuticular suturing.

Isolation and culture of BMSCs on modified media: Rabbits were anesthesed as above mentioned. The area from external angle of the ilium to the hip joint was clipped and disinfected and then 1.5 mL of bone marrow was collected from ilium using syringe (5 mL) with needle gauge 18. The bone marrow aspirated was immediately mixed with 3 mL of the Dublecco's Modified Eagle's Medium (DMEM) and high glucose-DMEM was supplemented with 30% Fetal Bovine Serum (FBS), 10 U mL^{-1} pencillin G, 10 U mL^{-1} streptomycin, 25 mg mL^{-1} amphotericin B, 1% nonessential amino acid (Gibco) and sodium pyruvate (100 ng mL^{-1}) all supplied by GIBCO® Invitrogen Corporation.

About 3 mL 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 removed while replacing the medium. After 12 days, the culture reached to confluence stage and the monolayer cells were washed twice with 2 mL of Phosphate Buffered Saline (PBS) (pH 7.2). The cultures were 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 till the regained their rounded shape and then discarded the trypsine. Dublecco's modified eagle's medium containing 10% FBS was added to the medium and then gently tapped to detach cells for 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 10 min, separating the precipitated pellets and 1 mL of DMEM was then added to the pellets and mixed. Cells were counted using hematocytometer to assure the minimum count 1×10^6 MSCs in 10 μL of culture medium. Bone marrow mesenchymal stem cells were identified using saturating monoclonal antibodies conjugated with Fluorescein Isothiocyanate (FITC) CD29-FITC (Ancell) and CD34-FITC (CaltagLaboratories) and analysed using Flow Cytometry (FACSCalibur) with the Cellquest software.

Immunostained showed positive for CD29 marker and negative for CD34 marker. The differentiation potential of rabbit BMSCs was checked for multi-linage differentiation by adipogenic and osteogenic differentiation assays as described previously.

Microscopically examination: Tissue culture cells were harvested and plated in special culture tube contains cover slip to allow the cells to grow over it after each subculture stage. When the cells reached to complete confluence, the cover slips were collected, stained with Heamatoxyline and Eosin and examined under light microscope.

Ultrastructural study: Cells from second passage were harvested and centrifuged at 2000 rpm for 10 min for pellet preparation, fixed with gluterdehyde 4% for 12 h, washing by buffer cucadylate 3 times, post fixative by osmium oxide 2% for 2 h, after that dehydrated with different concentrations of acetone (35, 50, 75, 90 and 100%) and imbedding by resin and ultra-sectioned using ultramicrotome (Leica).

Tracking fate of transplanted mesenchymal stem cells: Chemical marker PKH26 has been used to label the rBm-MSCs. The PKH26 linker kit labeled the cell membrane with a fluorescent dye which incorporates in long aliphatic tails into lipid regions of the cell membrane. Labeled rBm-MSCs were used *in vitro* and *in vivo* studies.

***In vivo* studies:** After anastomosis of the transected sciatic nerve, transplanted labeled rBMSCs (2.4×10^5) was injected at each of the proximal and distal segments in the LTG animals and another dose of 4×10^6 was injected intravenously (I/V) in the STG animals.

Exploring stem cell transplantation: The specimens with 1 cm long were collected from anastomosed area of sciatic nerve, spinal cord (5th lumber vertebrate) and dorsal root ganglia (seventh lumber vertebrate). Tissues were prepared by cryosection, the specimens embedded into Tissue-Tek OCT Compound (Tissue-tek; Miles, Laboratories, USA), sectioned at 6 microns thickness, section were mounted onto coated slides, allow to dry for 1 h at room temperature and mounted cover slip using 1-2 drops of cyanoacrylate ester glue. Finally, the tissue slides were examined using image analyzer (Olympic 51BH) under fluorescence illumination of proper filters.

RESULTS AND DISCUSSION

Adherent BMSCs were grown to 90% confluence cells colonies and irregular mononucleolar process out of their cell bodies and display morphological diverse appearances including a large size are flat, polygonal-shaped and spindle-shaped after 12 days of culture passage 0 (P0). The BMSCs from P1 were labeled using PKH26 dye (Fig. 1a and b). The most interesting finding was that BMSCs at 2nd day of subculture of 1st passage (P2) showed high confluence and most of cells grew by aligning themselves together along longitudinal axis of cell body, round and spindle-shaped (Fig. 2a). Another important finding was that labeled cells showed density and polymorphic shape >P1, the identification of ASDs based on fluorescence intensity of the PKH 26 dye was

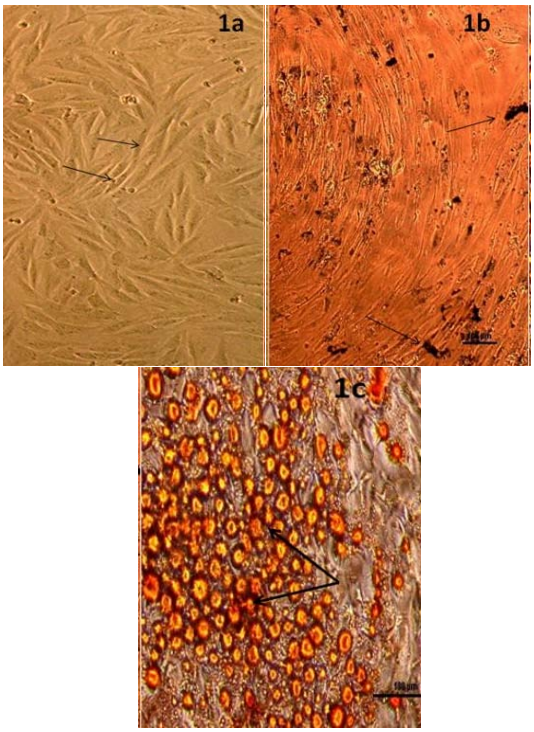

Fig. 1: BMSCs culture after 12th day (P0) showing (a) polymorphic spindle cells (arrow) as a control X100, (b) osteogenesis with mineralization of calcium deposits stained by von Kossa (arrows) and (c) adipogenesis showing an intracellular lipid droplets stained by oil red O (arrows)

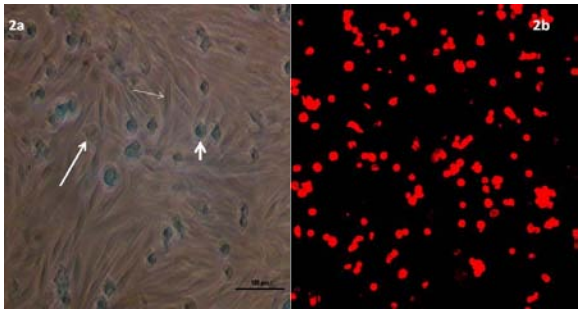

Fig. 2: BMSCs culture after 12th day (P1) showing (a) polymorphic cells spindle (arrow), round (head arrow) and polyglonal (thick arrow), (b) BMSCs labeled with PKH26 (P1)×100

distributed equally between daughter cells at each division *in vitro* (Fig. 2b). The BMSCs after 5 days at P3 of subculture showed large, flat, spindle, round and polygonal-shaped cells and the labeled cells showed less density. BMSCs showed fibroblastic-like cells and spindle shaped with central nucleus at P4 (Fig. 3a) and the labeled cells characterized by oval-shaped and less density than

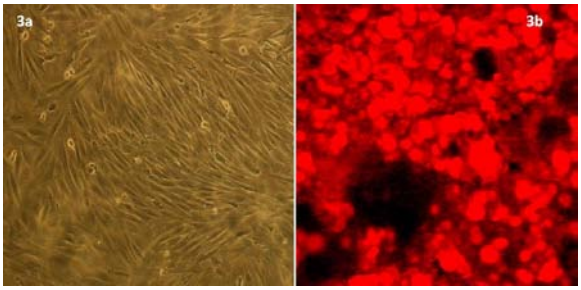

Fig. 3: First-passage culture (P2) BMSCs showing (a) the fibroblastic-like BMSCs colonies grew to confluence; b) BMSCs labeled with PKH26 with density and polymorphic shape

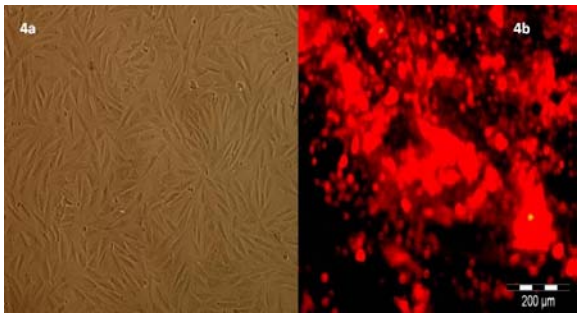

Fig. 4: BMSCs (P4) showed (a) fibroblastic-like cell and spindle shaped with central nucleus; b) BMSCs labeled with PKH26 characterized by oval-shaped and less density

P2 (Fig. 3b). The cells appeared spindle-shaped like fibroblast with central oval nucleus and sometimes at mitosis stage in P3 (Fig. 4a). Ultrastructural examination of BMSCs showed a cell body contains a large, irregular nucleus surrounded by clear nuclear membrane, active golgi apparatus, endoplasmic reticulum, mitochondria and polyribosomes. The cells appeared long, thin, widely dispersed and present of many vesicles on the outer cell membrane with long and thin processes (Fig. 4b).

The findings of direct examine of the labeled BMSCs in the cryosections of sciatic nerve at 2, 8, 16 weeks PO, enhance the understanding of BMSCs in the LTG were differentiated and appeared as like Schwann cells in an anastomosed sciatic nerve (Fig. 5a and b). The 2nd major finding was that in STG sections where the labeled BMSCs migrated to the anastomosed sciatic nerve, ipsilateral lumber dorsal root ganglia that striking to the perineurasl, resembling glial and satellite cells (Fig. 6a and b) and the labeled cells migrated to the anterior horn of spinal cord (motor neuron) (Fig. 7a and b). The efficiency of mesenchymal stem cell purification approach can be seen from the results of reconstitution *in vitro* in which

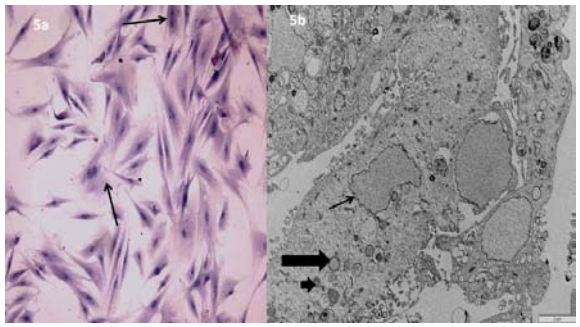

Fig. 5: (P2) BMSCs showing (a) showing spindle-shaped like fibroblast with central oval nucleus and sometimes at mitosis stage in mitotic cell division (arrows) stained×100 H and E; b) Ultra photography showing with irregular nuclear membrane (arrow), many vesicular bodies (arrow head) and ER (thick arrow); lead citrate and uranyl acetate

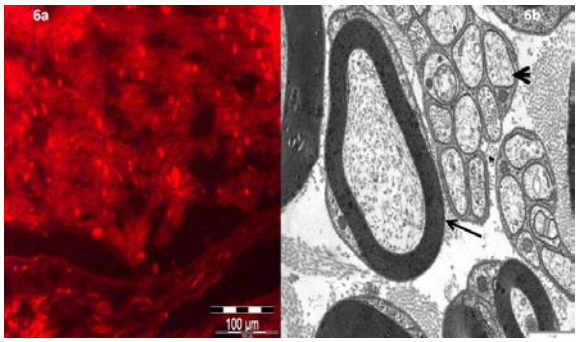

Fig. 6: (a) Micrograph of a transverse section of sciatic nerve (16 weeks PO) treated with BMSCs labeled with PKH26 survived and differentiated to Schwann-like cells; b) Ultra photography showing good myelin axon (arrow) with Schwann cells and nonmyelinated axon (head arrow) Uranyl acetate and lead stain, lead citrate and uranyl acetate

to able to reconstitute the mesenchymal stem to differentiate into neuroepithelial cells *in vivo* study. This study demonstrated that the bone marrow stroma contains a heterogeneous cell population consisting of hematopoietic cells and non-hematopoietic cells (BMSCs) that can be isolated through plastic-adherent cells, a morphology or cell shape (fibroblast like spindle shape) and growth rates were determined and plating efficiency which is depended on cells forming colonies of the BMSCs were highly expanded rapidly in culture. The proliferation of the BMSCs at P1-P3 (2-5 days) was higher more than that at P0 (12 days) with a maximum of proliferation at P2 (2 days) but the morphology cells was

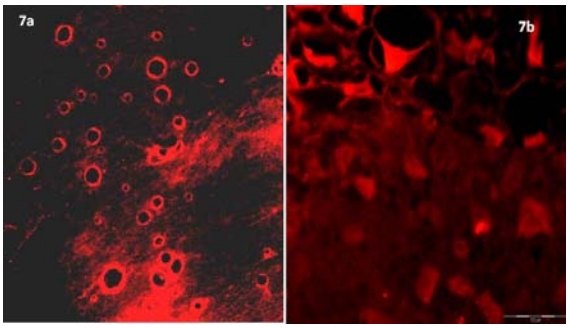

Fig. 7: (a) Dorsal root ganglia (16 weeks post operation) treated with PKH26 showing the stain at wall of glia 100; b) Spinal cord at L5 (16 weeks PO) treated with PKH26 showing the dye concentrated around neurons

shown heterogeneously. Ultramicrography of mesenchymal stem cell was shown as similar of fibroblast and activated at mitotic stage. Labeled BMSCs appeared, survived and activated into the site of anastomosed sciatic nerve were at 16th weeks which expressed into Schwann cell-like phenotype. The results of this study consisted with previous report that MSCs expressed into Schwann cell-like phenotytic in the injected area of the transected sciatic nerve but survived 33 days after implantation (Cuevas *et al*., 2002, 2004).

The relevance of the delivery method in differentiation cells status on experimental outcomes clarifies that stem cells were survived and differentiated in the injured nerve to convey a therapeutic effect >16 weeks and this outcome of present study was contrasted with (Chen *et al*., 2003).

Biological features of MSCs are capable of self-maintenance divided for new progeny cells and differentiation into specialized tissue-forming cells. This finding has important implications for developing of BMSCs in the dorsal root ganglia and spinal cord at the end of 112 days POD. This finding corroborates the ideas of cells ability migration to the spinal cord, dorsal root ganglia (Corti *et al*., 2002) and differentiation into neuroectodermal and microglial cells (Marshak *et al*., 2001). Ultrastructural characteristics of MSCs were appeared considerable the proliferative potential are shown as a fibroblast-like cells with some mitotic figures to maintain cells population, these aspects were attributed to Musina *et al*. (2004).

Stem cells for supplementing peripheral nerve repair it has a capacity for self-renewal such that it is possible to deliver large numbers progeny stem cells into injured nerve site (Tohill and Terenghi, 2004). By delivering stem cells in a naive state of the injured nerve had a capacity of proliferative, maintains and it is expected to prompt the microenvironment to differentiate into the required cell type (Humphreys *et al*., 2007).

Previous studies showed that transplantation of stem cells increase the endogenous neurogenesis putatively through the expression and release of certain growth factors. An angiogenic environment is also essential for tissue repair and functional recovery after an ischemic insult. It is well known that ischemic insults can activate the angiogenesis (Wei *et al*., 2001). The BMSC transplantation was improved functional recovery of spinal cord injury in rats (Chopp *et al*., 2000; Wu *et al*., 2003).

Remyelination and subsequent restoration of neuronal function can be achieved by either promoting endogenous repair mechanisms or providing an exogenous source of myelinating cells via transplantation. A number of cell types like Schwann cells, neural stem cells or stem cell derived oligodendrocytes were transplanted into the injured areas which enhance remyelinated of the demyelinated axons and encourage axonal regeneration (Ide, 1996).

CONCLUSION

In the study, the biological behaviors of BMSCs *in vitro* and *in vivo* were shown highly mitosis at P2, activated fibroblast-like cell, differentiated to functional myelinating Schwann-like cells in LTG. The BMSCs in STG migrated and engraftment at the dorsal root ganglia as a neuron and glial cell and glial cells and satellite in the spinal cord.

ACKNOWLEDGEMENT

Researchers would like to take this opportunity to thank all staff of virology and histology laboratories for helping to get to this point.

REFERENCES

Chen, J., Z.G. Zhang, Y. Li, L. Wang and Y.X. Xu *et al*., 2003. Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after in rates. Circ. Res., 92: 692-699.

Chopp, M., X.H. Zhang, Y. Li, L. Wang and J. Chen *et al*., 2000. Spinal cord injury in rat treatment with bone marrow stromal cell transplantation. Neuroreport, 11: 3001-3005.

Corti, S., F. Locatelli, C. Donadoni, S. Strazzer and S. Salani *et al*., 2002. Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sesory ganglia. J. Neurosci. Res., 70: 721-733.

Cuevas, P., F. Carceller, I. Garcia-Gomez, M. Yan and M. Dujovny, 2004. Bone marrow stromal cell implantation for peripheral nerve repair. Neurol. Res., 26: 230-232.

Cuevas, P., F. Carceller, M. Dujovny, I. Garcia-Gomez and B. Cuervas *et al*., 2002. Peripheral nerve regeneration by bone marrow stromal cells. Neurol. Res., 24: 634-638.

Deng, W., M. Obrocka, I. Fischer and D.J. Prockop, 2001. *In vitro* differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem. Biophys. Res. Commun., 282: 148-152.

Dezawa, M., I. Takahashi, M. Esaka, M. Takano and H. Sawada, 2001. Sciatic nerve regeneration in rats induced by transplantation of *in vitro* differentiated bone-marrowstromal cells. Eur. J. Neurosci., 14: 1771-1776.

Humphreys, D.B., C.B. Novak and S.E. Mackinnon, 2007. Patient outcome after common peroneal nerve decompression. J. Neurosurg., 107: 314-318.

Ide, C., 1996. Peripheral nerve regeneration. Neurosci. Res., 25: 101-121.

Kamada, T., M. Koda, M. Dezawa, K. Yoshinaga and M. Hishimoto *et al*., 2005. Transplantation of bone marrow stromal cell-derived Schwann cells promotes axonal regeneration and functional recovery after complete transection of adult rat spinal cord. J. Neuropathol. Exp. Neurol., 64: 37-45.

Kelsey, J.L., A. Praemer, L.M. Nelson, A. Felberg and D.P. Rice, 1997. Upper Extremity Disorders: Frequency, Impact and Cost. 1st Edn., Churchill Livingstone Inc., New York, pp: 86.

Kopen, G.C., D.J. Prockop and D.G. Phinney, 1999. Marrow stromal cells migrate throughout forebrain and cerebellum and they differentiate into astrocytes after injection into neonatal mouse brains. Proc. Natl. Acad. Sci. USA., 96: 10711-10716.

Marshak, D.R., R.I. Gardner and D. Gottlieb, 2001. Stem Cells Biology. Cold Spring Harbor Laborary Press, New York, USA.

Mimura, T., M. Dezawa, H. Kanno, H. Sawada and I. Yamamoto, 2004. Peripheral nerve regeneration by transplantation of bone marrow stromal cell-derived Schwann cells in adult rats. J. Neurosurg., 101: 806-812.

Musina, R.A., Y.Y. Yegorov and A.V. Belyavsky, 2004. Stem cells: Properties and prospective medical a pplications. Mol. Biol., 38: 469-481.

Noble, J., C.A. Munro, V.S. Prasad and R. Midha, 1998. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. J. Trauma, 45: 116-122.

Oswald, J., S. Boxberger, B. Jorgensen, S. Feldmann, G. Ehninger, M. Bornhauser and C. Werner, 2004. Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. Stem Cells, 22: 377-384.

Sasaki, M., O. Honmou, Y. Akiyama, T. Uede, K. Hashi and J. Kocsis, 2001. Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. Glia, 35: 26-34.

Tohill, M. and G. Terenghi, 2004. Stem-cell plasticity and therapy for injuries of the peripheral nervous system. Biotechnol. Appl. Biochem., 40: 17-24.

Wei, L., J.P. Erinjeri, C.M. Rovainen and T.A. Woolsey, 2001. Collateral growth and angiogenes is around cortical stroke. Stroke, 32: 2179-2184.

Woodbury, D., E.J. Schwarz, D.J. Prockop and I.B. Black, 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res., 61: 364-370.

Wu, S., Y. Suzuki, Y. Ejiri, T. Noda and H. Bai *et al*., 2003. Bone marrow stromal cells enhance differentiation of coculture neurosophere cells and promote regeneration of injured spinal cord. J. Neurosci. Res., 72: 343-351.

Zhang, P., X. He, K. Liu, F. Zhao and Z. Fu *et al*., 2004. Bone marrow stromal cells differentiated into functional Schwann cells in injured rat's sciatic nerve. Artif Cells Blood Substit. Immobil. Biotechnol., 32: 509-518.