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ORIGINAL RESEARCH article

# Effect of *in-vitro* differentiated bone marrow mesenchymal stem cells in the treatment of peripheral nerve injury in rats

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***Keywords:*** Bone marrow mesenchymal stem cells, electro neuro myo gram, polylactic co glycolic acid, rat

**Abstract:** Peripheral nerves are more prone to damage during trauma. Though nerve grafts are used as an alternative method in treating it, the results are purely ambiguous. One such modern approach to treat peripheral nerve injury is bone marrow differentiated neuronal cells. Our present aim is to study the effect of *in-vitro* differentiated bone marrow mesenchymal stem cells (BMSCs) in the treatment of peripheral nerve injury in rats. Six-week-old rat weighing 80 gm was used for isolation and culture of BMSCs. The second passage cells were taken for neuronal differentiation. Flow cytometry and immunocytochemistry were performed with Anti goat IgG antibody indirectly conjugated with FITC to express nestin. *In vitro* differentiated BMSC along with PLGA Scaffold is injected into the site of peripheral nerve injury and the results were studied by ENMG, microdissection, and histopathology. The cells were expressed with Nestin goat polyclonal antibody. At the end of the second week, the rat reveals increased amplitude (8.3 mv) with decreased latency (0.8 ms) of the peripheral nerve. Microdissection confirms the neuronal continuity of the injured peripheral nerve. Histopathology distinctly exhibits increased myelination and decreased endoneuronal space. In conclusion, neuronal differentiated BMSC, regenerates peripheral nerve injury faster than conventional methods and can be applied as an alternate therapy in peripheral nerve repair.

## Introduction

Tissue engineering is multifaceted, and involves the isolation, characterization and differentiation of bone marrow cells. Transplantation of neuronal differentiated bone marrow mesenchymal stem cells (BMSCs) onto the site of injury results in peripheral nerve repair. This method of clinical transplantation of differentiated neuronal cells along with bioscaffolds challenges regenerative medicine for faster and complete nerve repair. Autologous nerve grafts had been the gold standard for the treatment of peripheral nerve injury that exceeded the critical gap length. Transplantation of differentiated BMSCs into Schwann cells along with a bioengineered scaffold holds promise for nerve regeneration because of the limited availability of donor nerves and donor morbidity [1]. BMSCs are supposed to be the ideal transplantable cell due to their easy accessibility, rapid expansion capacity when cultured, immune-competent and immune-privileged nature [2]. Recent transplantation studies suggest *in-vitro* differentiation of BMSCs into cells expressing Schwann cell antigen, followed by *in-vivo* transplantation, shown to have a faster regenerative effect on damaged sciatic

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nerve [3]. This finding suggests that *in-vivo* transplantation of BMSCs into a central and peripheral nervous system following *ex-vivo* differentiation has it potential to fill nerve gap repair. The main purpose of this study is *ex-vivo* differentiation of BMSCs into neuronal cells and *in-vivo* transplantation of differentiated neuronal cells with poly lactic co glycolic acid (PLGA) scaffold to aid faster regeneration of nerve gaps in trauma, gunshot and neurodegenerative diseases.

## Materials and methods

*Animals:* Randomly bred Wistar albino rats weighing 150 - 200 gm of both sexes were maintained at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC) of Saveetha University, Chennai, India. Rats were kept in polypropylene cages (three per cage) with a sterilized and dry paddy husk as breeding material. The animals were fed with commercial laboratory animal feed (TANUVAS-Chennai) and purified water ad libitum. The care and maintenance of all the animals were given as per the approved guidelines of the committee for control and supervision of experimental animals (CPCSEA, India). This study was approved by the institutional animal ethical committee (SU/SMC/RD/10/2012; DT: 21ST FEB 2022).

*Methods:* Differentiated BMSCs with PLGA are transplanted at the injured sciatic nerve. The rats were divided into two groups, six in each group (n=12). **Group I: (**injured control): was injected with saline, and **Group II: (**experimental): was trans-planted with differentiated BMSCs and PLGA. Rat was anaesthetized with ketamine (80 mg/kg) and xylozine (10 mg/kg) cocktail, intra-peritoneal. An anaesthetized animal was placed on the surgical table. The surgical area was shaved with a sterile razor and disinfected with betadine solution. A two to three-inch dorsal longitudinal incision was made over the middle and posterior aspect of the thigh in the right hind limb. 10 mm deep incision was made for the separation of muscle and the right proximal sciatic nerve was exposed in the mid-thigh region. In the exposed sciatic nerve, one cm segment was excised and removed using a sterile blade applied perpendicular to the sciatic nerve. After axotomy, differentiated BMSCs with PLGA were loaded in a microliter syringe at a concentration of 1 x $10^5$ cells/µl. In the experimental group, cells were injected into the site of injury with all aseptic precautions. In the control group, at the site of injury saline was injected. The surgical wound was about and dressed with Betadine. After two weeks of injury, electrophysiological recordings were done in both groups. At the end of the second week, the sciatic nerve was traced for its continuity by microdissection. Nervous tissue is processed for histopathology to confirm nerve regeneration.

*Isolation and culture of BMSCs:* Six-week-old rats weighing 80 gm were used for the isolation of BMSCs [4]. The rat was sacrificed using diethyl ether and bones were collected under sterile conditions; muscles, and connective tissues attached to the bone were cleaned and rinsed in phosphate buffer solution. Marrow from each bone was collected by cutting the epiphyseal ends and flushing it with Dulbaco minimum essential medium (Sigma, USA). After filtering, cells were centrifuged at 1000 rpm for five min. Purified cells were finally dispersed in Dulbaco minimum essential medium with 15.0% fetal bovine serum.

*Cell culture and expansion***:** Isolated BMSCs were plated in T25 and T75 tissue culture flasks containing approximated stem cell nutrient medium at a density of 10x$10^5$ cells per flask. Flasks were maintained in an incubator at 37$^o$C and 05.0% carbon dioxide. The medium was replaced every third day. Cell viability was confirmed by continuous cell division and cells were sub-cultured using 3.0 ml trypsin/EDTA when the flask reached 90.0% confluence [5].

*Flow cytometry analysis:* After initial plating, primary cultures were harvested by trypsinization, and cells were fixed in neutralized 02.0% paraformaldehyde solution for 30 min. Fixed cells were washed twice with phosphate buffer saline (PBS) and incubated with the following antibodies CD44, CD90 for positive expression and CD34 for negative expression (**Figure 1**).

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**Figure 1:** Flow cytometric findings of BMSCs for surface antigen of CD 90 (30.76%), CD 34 (08.77%), CD 90 and 34 (15.29%)

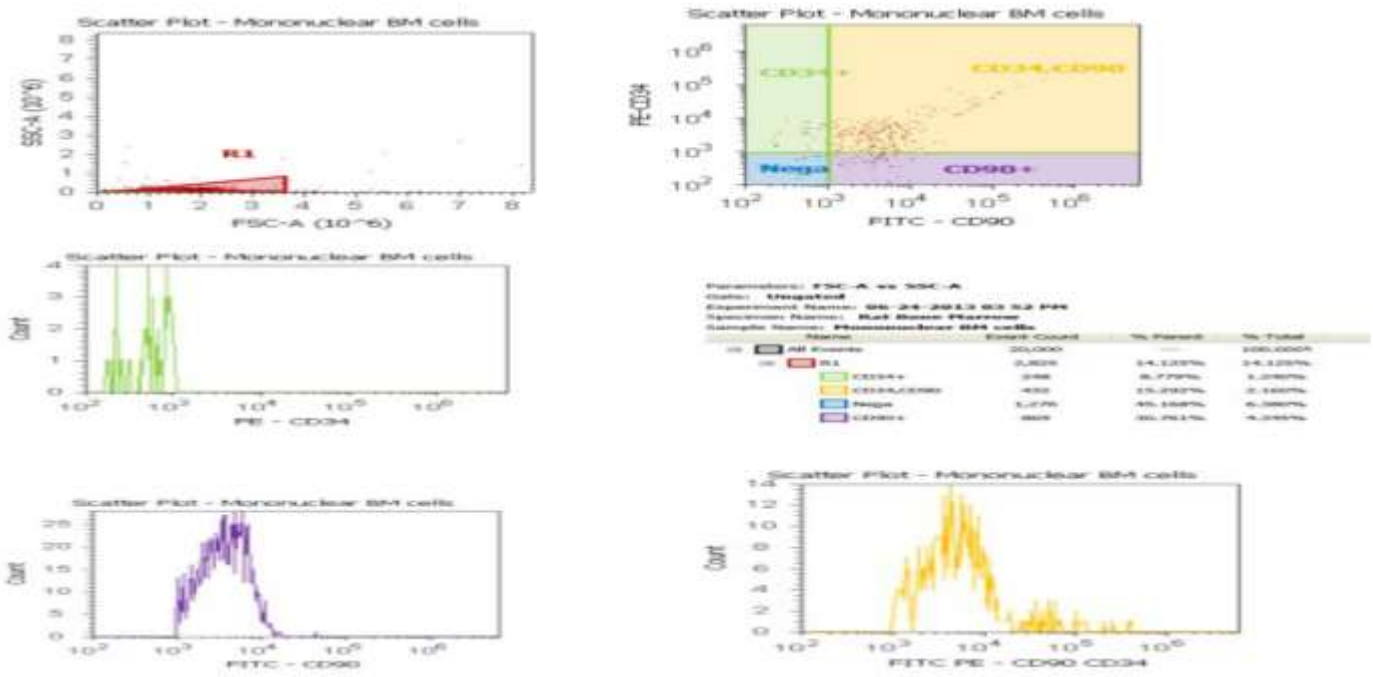



**Figure 2:** Neuronal differentiation after five hours of induction with 10 mM BME

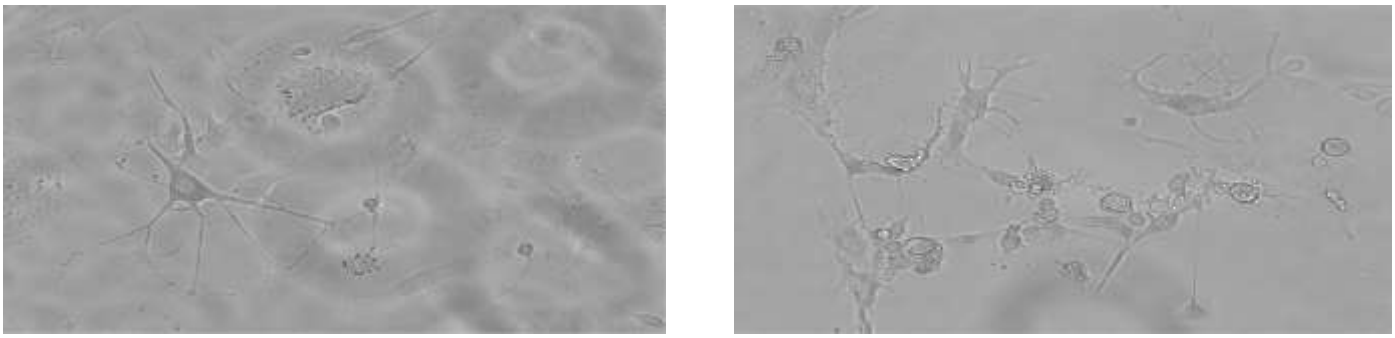
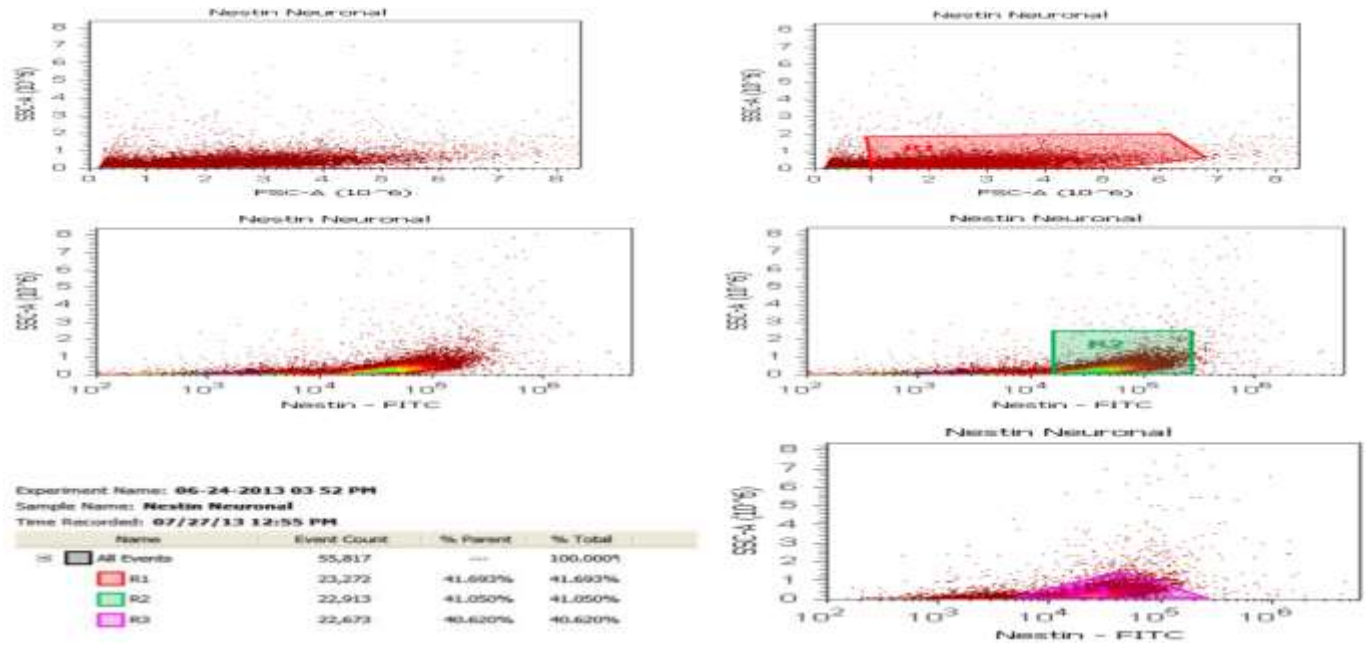


**Figure 3**: Level of nestin expression with Anti-goat IgG-FITC



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*Differentiation of cells:* Second passage cells were taken for neuronal differentiation. *In-vitro* nerve cell differentiation has taken 21 days. Under appropriate conditions BMSCs were induced to differentiate into progenitor cells by adding three ml of one mM neuronal (pre-induction) differentiation medium (DMEM) low glucose with 20.0% FBS, 01.0% PEN-STREP and 10 mM beta mercapto ethanol (BME) three micro-liters. The cells are stained with 1: 100 nestin goat polyclonal antibody (Santacruz) in a blocking buffer. After five hours cells are checked for neuronal differentiation (**Figure 2**). Primary antibodies were directly conjugated with FITC to perform immune-cytochemical and flow cytometric analysis (**Figures 3** and **4**).

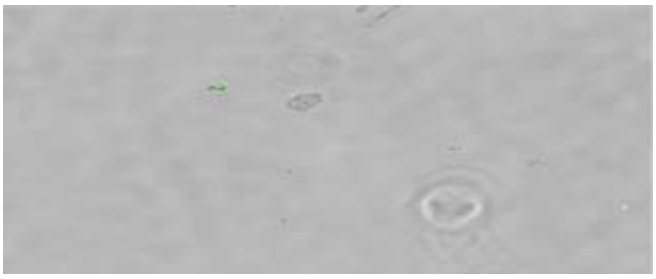


**Figure 4**: immunofluorescence staining for nestin noted at perikaryon

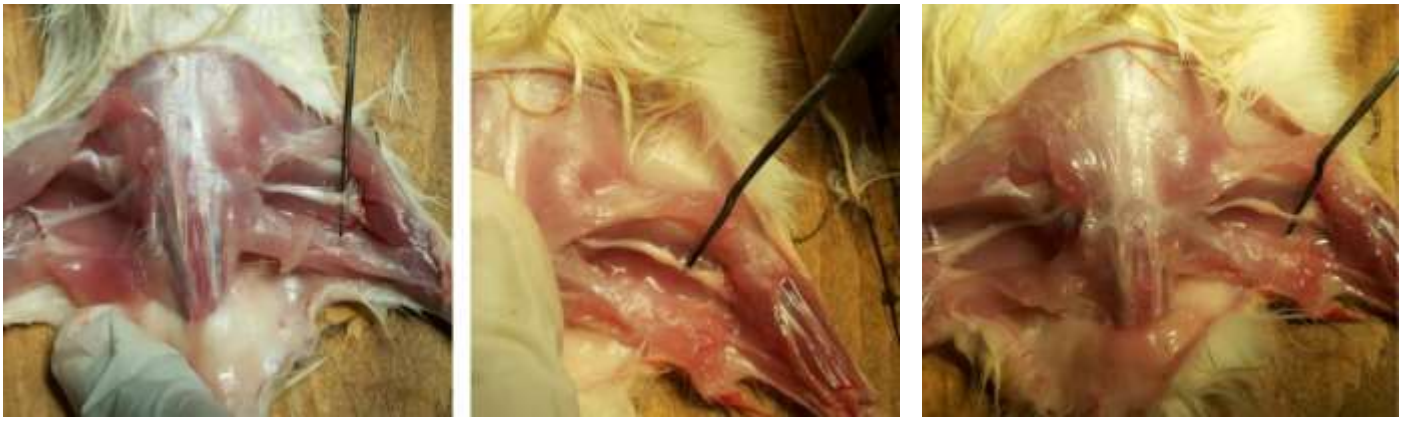
## Results

BMSCs are grossly characterized by cell adherence to the base of the culture flask. Cells were analyzed by flow cytometry. Characterization of BMSCs by FACS Analysis reveals positive expression for CD 44, CD90 and negative for CD 34. In-vitro nerve differentiation has taken 21 days, and under correct conditions BMSCs were induced to differentiate into progenitor cells, neuroblasts and neurons. Nestin present in perikarya conjugated with IgG-FITC exposed direct immunofluorescence.

*Observation chart:* Axotomy is confirmed by right foot drop. End of the second week, the rats were subjected to electrophysiological study for nerve conduction and results were subjected to analyses.

*Microdissection:* At the end of 2$^{nd}$ week, dissection was performed to confirm neuronal continuity of the sciatic nerve between the experimental and control groups. Thin hair-like axonal continuity was caused to be visible in the injured control group. In the experimental group, axonal continuity and axonal fiber regrowth are observed in four animals. The width of the nerve is slightly bigger than the proximal and distal parts of the nerve. One animal shows thin hair-like axonal continuity and another on tracing revealed dumble-shaped axonal continuity (**Figures 5A, 5B, 5C**).

**Figure 5:** Microdissection of the experimental group showing axonal sprout continuity reaching a distal segment (A) complete axon sprouts, (B) partial axonal sprouts (c) very thin axonal sprouts.



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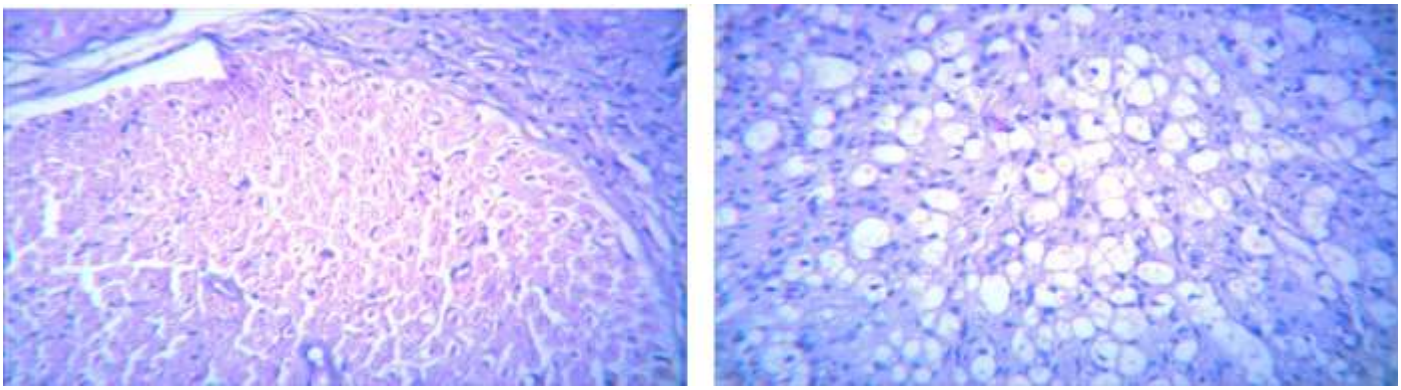
*Histopathology:* Histopathology observations were compared between control and experimental groups for nerve regeneration. The experimental group reports increased Schwann cells and myelin sheath with less axonal degeneration, vacuolization and endoneural space. Whereas the control group depicts decreased Schwann cells, myelin sheath and vacuolization with increased axonal degeneration and endo-neural space. This marked difference between the control and experimental groups confirms EMG findings and microdissection observations (**Figures 6A, 6B**).

**Figure 6:** Histopathological findings of sciatic nerve in experimental and control group



(Hematoxylin and Eosin x 400) (A) less axonal degeneration and vacuolization (arrow) than the control group (B) Decreased myelin sheath, vacuolization (arrow) and increased endoneuronal space.

## Discussion

Peripheral nerve injuries have always been a critical clinical problem among defense personnel and civilians in accidents and nerve crush injuries. The nerve regeneration is often blocked by scar formation and the direction of axons [6, 7]. Faster regeneration of axons was adopted and achieved in this study to serve humanity because of the high incidence level of crush injury leading to sectioning of nerves with impaired function. Six-week-old rats used for isolation of BMSCs for differentiation 1$^{st}$-2$^{nd}$ passage is ideal. Axonal survival factors are secreted by neurons for the survival and development of Schwann cell precursors [4]. The study firmly establishes the plasticity of BMSCs by their differentiation into Schwann-like cells with typical spindle morphology [8]. BMSC expresses mesenchymal stem cell characteristics on flow cytometry with surface markers CD44 and CD90 for positive expressions and CD45 for negative expressions. BMSCs can differentiate into a variety of connective tissue cells; bone, cartilage, tendons, muscle, adipose tissue and nerve [7]. Study ingrains BMSC induced to differentiate into progenitor cells, neuroblasts and neurons. Nestin-treated IgG-conjugated FITC confirms neuronal differentiation with immunofluorescence [8]. This result indicates the capability of BMSCs differentiating into neuroblast can be used as a therapy in neurodegenerative diseases like Parkinson's, Huntington's, and Alzheimer's, amyotrophic lateral sclerosis, multiple sclerosis and spinal cord lesions [9]. Differentiated progenitor nerve cells with PLGA at the traumatized nerve reveal regeneration of axons much faster (2 weeks) when compared with injured control. Electroneuromyography study waves increased amplitude (8.3 mv) and decreased latency (0.8 ms) among the experimental group. This could be due to the regeneration of axons with myelin. Findings indicate the regeneration of axons; this probably could be due to the differentiation of progenitor cells into Schwann cells [1, 3]. Decreased latency in the experimental group is due to intact axons and conductivity. Decreased latencies and increased amplitude are the sign of axonal regeneration. The control group decreased amplitude (6.9 mv) and increased latency (2.5 ms) showing axonal degeneration and the non-availability. This is due to non-availability of progenitor cells and PLGA to increase regeneration and vascularity. At the end of 2$^{nd}$ week, a microdissection of the

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experimental group expressed a one cm nerve gap repaired with axonal continuity. In four animals, there was a slight bulge seen at the site of injury which is continuous with the proximal and distal parts of the nerve, this might be due to the differentiation of progenitor cells into Schwann cells and vascularity facilitated by PLGA porosity. Bulge at the site of injury probably could be, due to inflammation which may subside in want of time. One rat unveils a dumble-shaped axon, while the other with thin hair-like continuity. This may be due to the absence of cell signaling or the non-homing of differentiated cells by a scaffold in place. In the experimental animals, a one cm gap is closed either by thick or thin continuity of axon. The injured control group shows thin axonal continuation; probably due to the beginning of Wallerian regeneration. This also confirms ENMG study where the amplitude (6.9 mv) and latency (2.5 ms) portray the poor conduction velocity of the nerve [10]. Histopathological findings in the control group showed increased vacuolizations and endoneural space. Whereas, in the experimental group, few vacuolization observed, endoneuronal space decreased and axons with myelin [11, 12]. In the present study, the control group increased endoneuronal space due to the absence of neuro-vasculogenesis. Experimental group axonal angiogenesis was achieved with BMSCs differentiated progenitor, which resulted in increased myelination, decreased vacuolation and decreased endoneural pockets. Decreased vacuolation and increased myelination is probably due to the neuron protein matrix. This is due to the multifaceted nature of bone marrow mesenchymal stem cells.

*Conclusion:* Findings of microdissection, ENMG and histopathology of experimental groups strengthen axonal regeneration by differentiated BMSCs faster than the control. Neuronal phenotypes of BMSCs may help in the treatment of neurodegenerative diseases such as Parkinson's, Huntington's, Alzheimer amyotrophic lateral sclerosis, multiple sclerosis and spinal cord lesions. Assessment of the neurogenic differentiation potential of BMSCs *in vitro* may help in the development of autologous-based regenerative therapy.

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**Author contribution:** AT collected data, SP conceived and designed the study, BK contributed in data analysis, SS performed the analysis and interpretation of the data, NK & DSS contributing in data analysis and drafting the manuscript. All the authors approved the final version of the manuscript and agreed to be accountable for its contents
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**Ethical issues:** Including plagiarism, informed consent, data fabrication or falsification, and double publication or submission were completely observed by the authors.
**Data availability statement:** The raw data that support the findings of this article are available from the corresponding author upon reasonable request.
**Author declarations:** The authors confirm that all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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