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Laboratory Study

# Effect of bone marrow-derived mononuclear cells on nerve regeneration in the transection model of the rat sciatic nerve

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ABSTRACT

Bone marrow-derived stem cells enhance the rate of regeneration and clinical improvement in nerve injury, spinal cord injury and brain infarction. Recent experiments in rat spinal cord demyelination showed that remyelination was specific to intravenous delivery of the bone marrow-derived mononuclear cell (BM-MNC) fraction, although the specific role of this fraction in peripheral nerve regeneration has not been examined. Therefore we evaluated the role of BM-MNCs in peripheral nerve regeneration in the rat sciatic nerve transection model. After anesthesia, the right sciatic nerve of 20 adult-male Wistar rats was transected under an operating microscope. In the test group, the cut ends of the nerve were approximated with two epineural microsutures, the gap was filled with rat BM-MNCs and the approximated nerve ends were covered with fibrin glue. In the control group, the transected nerve ends were repaired with two epineural microsutures and fibrin sealant only. Histological assessment of the nerve was performed 30 days and 60 days after the operation and regenerative changes were compared between the two groups. The recovery after nerve anastamosis was far better in the test group at both 30 days and 60 days. There was a statistically significant difference in axonal regeneration, remyelination and myelin thickness at sites 5 mm and 10 mm from the site of repair of the nerve. Schwann cell proliferation and degenerative changes were more prevalent in the controls. This study demonstrates that local delivery of BM-MNCs (which can be isolated easily from bone marrow aspirates) into injured peripheral nerve increases the rate and degree of nerve regeneration. The present study highlights the role of BM-MNCs in peripheral nerve regeneration.



## 1. Introduction

The mammalian peripheral nervous system has a limited ability to regenerate axons after injury. Damaged peripheral nerves in humans can be repaired either by end-to-end coaptation or by nerve-grafting procedures. However, nerve autografts are limited due to a lack of availability of donor nerves of varying lengths and diameters. Nerve allografts have not proven feasible because of immune rejection.

In the last few decades, research into peripheral nerve regeneration has shown that Schwann cells have an important role in nerve regeneration. In various experimental studies, transplanted Schwann cells were a useful substitute for nerve autografts to repair injured peripheral nerves.[1–8] However, the clinical use of Schwann cells is limited because it is difficult to obtain them in sufficiently large numbers. To overcome this difficulty, however, the ability of various types of stem cells to differentiate into Schwann cells is under evaluation.

Stem cells are multipotent, primitive cells found in most, if not all multicellular organisms. They have the ability to self-replicate and can also differentiate into various cell types of particular tissues. Neural stem cells, found in the central nervous system, can differentiate into neurons, astrocytes and oligodendrocytes *in vitro*.[9] However, the clinical utility of neural stem cells is limited because they are located deep in the brain and it is difficult to obtain them. Bone marrow stromal cells (BMSCs) are a good alternative because of their easy accessibility and large growth potential. BMSCs are multipotent stem cells that can normally differentiate into hemopoietic cells, bone cartilage and muscle;[10] however, BMSCs can also be induced to differentiate into neural cells both *in vivo* and *in vitro*.[11–15]

There have been encouraging results for BMSC transplantation in the rat sciatic nerve transection model.[16–19] BMSC transplantation facilitates nerve regeneration as well as myelination in these studies. BMSCs induce remyelination in the demyelinated spinal

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cord model of the rat. BMSCs have been induced to form Schwann cells *in vitro* and these cells have been used for transplantation.[19] BMSCs have also been directly transplanted.[16–18] However, more recent studies have focused on bone marrow mononuclear cells (BM-MNCs) for transplantation.[20–24] Recent experiments using the rat demyelinated spinal cord lesion model have shown that remyelination was specific to the intravenous delivery of BM-MNCs.[20] The specific role of BM-MNCs in peripheral nerve regeneration has not been studied. Thus, we planned to evaluate BM-MNCs in regeneration in the rat sciatic nerve transection model.

## 2. Materials and methods

### 2.1. Animals

Twenty adult-male Wistar albino rats, weighing between 250 g and 300 g, were used for this study. They were allocated into either a test group (group 1) or a control group (group 2), with 10 rats per group. The ethics committee of the All India Institute of Medical Sciences (AIIMS) gave permission for this experimental study. The study was divided into two phases: I, 30 days and II, 60 days. There were 5 rats in each test and control group in each phase.

### 2.2. Preparation of BM-MNCs

Bone marrow was aspirated with a disposable needle from the femurs and tibias of rats sacrificed for other purposes. Bone marrow from 2 rats of the same litter was pooled to obtain the optimum number of mononuclear cells. The cells were collected in heparinized syringes.

A Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ, USA) density gradient was used to isolate BM-MNCs[25] at the Stem Cell Facility of AIIMS. Briefly, bone marrow was layered over lymphocyte separation medium and centrifuged at 1500 revolutions per minute for 30 minutes. Mononuclear cells were aspirated and washed three times in heparinized normal saline to remove the traces of Ficoll. The procedure was performed under aseptic conditions. Cell viability was tested using trypan blue staining, cell morphology was examined after staining with Giemsa stain and cell number was assessed by counting the cells in a counting chamber under a microscope. For transplantation, 4 million to 8 million BM-MNCs were suspended per millilitre of normal saline.

### 2.3. Transplantation of BM-MNCs

In each phase, the animals were anesthetized with intraperitoneal sodium pentothal (40 mg/kg body weight). The dorsal aspect of the right hip and thigh was shaved and prepared. The sciatic nerve was exposed, dissected and transected under an operating microscope. In the test group, cut ends of the nerve were approximated, with two epineural microsutures (10-0 monofilament nylon 2 placed at 180° under 25 × magnification) and the gap was filled with rat BM-MNCs (2–4 million BM-MNCs suspended in 0.5 mL normal saline). Care was taken to maintain the exact orientation of the cut ends of the transected nerve with reference to the epineural vessels. The approximated nerve ends were then covered with Tisseel fibrin glue (Baxter International, Deerfield, IL, USA).

In the control group, the transected nerve ends were repaired with two epineural microsutures using 10-0 monofilament nylon and fibrin sealant only. All the nerve anastamoses were performed by a single neurosurgeon (R.K.G). The rats were allowed to recover from anesthesia and were then kept in the animal care facility. Rats were reared by trained animal care workers and checked regularly. Subsequently, nerves were harvested as per the schedule.

### 2.4. Nerve harvesting

On day 30 and day 60, phases I and II respectively, the right sciatic nerve was re-explored and transected and the distal end was labeled with a thread. It was fixed in 3% gluteraldehyde.

The segment distal to the site of repair was divided into two parts:

Site A: most distal portion, 10 mm away from the site of repair.
Site C: intermediate distal portion, 5 mm distal to the site of repair.

Similarly, the segment proximal to the site of repair was divided into two parts:

Site D: intermediate proximal portion, 5 mm proximal to the site of repair.
Site F: most proximal portion, 10 mm away (proximal) from the site of repair.

One section was taken from the site of repair and labeled "G" (Supplementary Fig. 1).

### 2.5. Nerve histopathology

The nerve segments were processed into routine paraffin-embedded blocks and transverse sections were obtained.

Routine hematoxylin and eosin stain was used to examine the nerve architecture, the general histological details, any degenerative changes and any inflammatory infiltrates.

Masson's trichrome stain was used for assessment of fibrosis and myelination. Luxol fast blue stain (modified Kluver's method) and Loyez stain were also used to assess myelination.

Immunohistochemistry was performed for various cell markers using streptavidin-biotin labeling (with diaminobenzidine as a chromogen), with positive and negative controls with the following antibodies:

- neurofilament protein (Dako, Glostrup, Denmark; 1:100 dilution) – to evaluate the number and distribution of axons.
- CD34 (Dako; 1:200 dilution) – to detect precursor cells.
- S100 (Neomarkers, Fremont, CA, USA; 1:200 dilution) – to detect Schwann cells.
- Leucocyte-common antigen (LCA) (Dako; 1:100 dilution) – to detect inflammatory infiltrates.

### 2.6. Statistical analysis

The two groups were analysed at the specified intervals (30 days and 60 days) at the above specified sites on the nerve using STATA 9.1 (StataCorp, College Station, TX, USA) software. A $p$-value $\leqslant 0.05$ was considered significant.

## 3. Results

Repaired nerve stumps remained ligated and did not adhere to surrounding structures when observed under the operating microscope at the time of harvesting nerve grafts.

### 3.1. Axonal regeneration

At A, the most distal site, axons regeneration was superior in the test group compared to the controls at both 30 days and 60 days (Supplementary Figs. 2 and 3, respectively). In the control group