The Role of Dental Pulp Stem Cells in Sciatic Nerve Regeneration in Rats: Functional and Histological Analysis

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J Peripher Nerve Surg 2024;8:7–15.

Abstract

Objective Trauma is the most common cause of peripheral nerve injuries. Autografts are considered the gold standard for bridging nerve defects. Allografts are often used due to limited numbers of autografts and potential donor site morbidity. Lack of adequate neurogenic growth factors, especially in long length grafts, is an important reason for poor surgical outcomes. Stem cells with their ability to secrete neurotrophic growth factors can decrease inflammation and guide regeneration and have shown good results in nerve repair. Dental pulp stem cells (DPSC) are a viable option for nerve repair. We studied regeneration in sciatic nerve injuries in rats using DPSCs.

Method Total 60 Sprague Dawley rats were included in the study. Sciatic nerve of right hindlimb was cut and repaired with allograft from Wistar rat. Depending on the type of allograft and timing of nerve repair, subjects were divided into six groups. Recovery was assessed after 12 weeks with general assessment, step length ratio, nerve conduction velocity, histopathological analysis, and plasma level of proinflammatory cytokines like tumor necrosis factor-α, interleukin-6 and interferon-γ.

Results Incidence of trophic ulcers, contractures, and autotomy was lesser in DPSC-implanted allografts. Step length ratio improved in stem cell-implanted allografts. Nerve conduction velocity improved in DPSC-implanted allografts more than in allografts without stem cells ($p < 0.05$). Myelin loss was lesser in stem cell-implanted groups. Proinflammatory cytokine levels were lesser in the stem cell group, though the difference was not statistically significant.

Conclusion DPSC-implanted allografts are viable, safe, and effective in traumatic peripheral nerve regeneration with better functional, electrophysiological, and histological outcomes

Keywords
- peripheral nerve regeneration
- stem cells
- dental pulp stem cells
- autotomy
- step length ratio
- nerve conduction velocity

DOI https://doi.org/10.1055/s-0044-1778696.
ISSN XXXX-XXXX.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Introduction

Trauma is the most common cause of peripheral nerve injury leading to functional impairment of a limb. Primary suturing of severed nerve ends without tension is the preferred method of repair. Autologous nerve grafts are commonly used to bridge the nerve gaps when required. The source of autologous graft is often limited and thus, allografts are used to make up for the deficit. Allografts may be rejected unless the antigenicity is reduced by pretreatment. Despite advances in techniques and the understanding of the pathophysiology of peripheral nerve injuries, the overall outcome following nerve repair with grafts is far from satisfactory. Among several causes of poor surgical outcome like inflammation and perineural scar formation, lack of adequate neurogenic growth factors—especially in long length grafts, is an important reason.[1] Embryonic stem cells, adult bone marrow stromal cells implanted into allografts, have been used to circumvent this and have shown modest therapeutic benefits and substantial functional recovery.[1,2]

Human adult dental pulp stem cells (DPSC) are unique as they reside within the perivascular niche of dental pulp. These are derived from cranial neural crest and express markers for both neuroectodermal stem cells and mesenchymal cells.[3,4] Unlike embryonic stem cells, DPSCs can be obtained from exfoliated deciduous and impacted adult wisdom teeth without significant ethical issues. DPSCs can differentiate into chondrocytes, osteoblasts, adipocytes and functionally active neurons in vitro, under defined conditions.[3–5] DPSCs secrete trophic factors promoting neuronal survival, proliferation, differentiation, and migration.[6–9] DPSCs express nestin, a neural progenitor marker, and glial fibrillary acidic protein.[9,10]

We hypothesized that these stem cells from dental pulp could augment peripheral nerve regeneration histologically and functionally in rats using cold preserved allografts.

Materials and Methods

This was an animal experimental study conducted at the Central Animal Research Facility of National Institute of Mental Health and Neurosciences, Bangalore between November 2013 and August 2014. Sixty male Sprague Dawley and 30 Wistar rats (3–4 months old weighing 200–250 grams) were utilized. All experiments were performed after approval from the Institute Animal Ethics Committee.

Surgical Procedure and Grouping

Sciatic nerve of the right hindlimb of Sprague Dawley rate was divided sharply and 1.5 to 2 cm of the nerve was resected. The defect was sutured with a similar length allograft (pretreated or naive) harvested from Wistar rats.

Intraperitoneal ketamine (2 mg/kg) was administered and Isoflurane at 1.0 MAC with 50% oxygen and air was used to maintain anesthesia. The animal was kept in prone position. An oropharyngeal airway was inserted to prevent tongue fall. After local shaving and painting with povidone–iodine, gluteal muscle splitting incision was taken. Sciatic nerve was identified and was exposed. The nerve (1.5–2 cm) was then resected between the sciatic notch and the point of bifurcation near the knee.

Animals were allocated to one of the six groups depending upon the type of allograft (naïve or pretreated) used and the timing of suturing the nerve defect. To decrease immunogenicity, some allografts were pretreated by storing them in Ringer lactate solution for 21 days at 5 degrees centigrade (cold preserved allografts).[11–14] Some allografts labelled as fresh allografts were used without cold storage (naive) to check immune reaction and growth potential. Depending on when the repair was done from the time of cutting the nerve, the rats were divided into Immediate Repair Group—where the graft suturing was done immediately after cutting the nerve and the Delayed Suturing Group—where the nerve was severed, wound closed, and re-explored after 6 weeks, then suturing was done with a graft after freshening the ends of the cut nerve. To check the role of DPSCs in nerve regeneration, allografts were implanted with DPSCs, incubated in a complete medium for 48hours and then used to repair nerve. They were labeled as stem cell-implanted allografts and their results were compared with allografts without stem cells in every group. Allografts were sutured with Ethilon 8-0. Incision was closed in layers after nerve anastomosis. All surgical procedures were performed under the microscope. After recovery, the rats were housed one animal per cage ($30 \times 22 \times 14$ cm). Functional and histological analysis was done after 12 weeks. So, 60 rats were equally and randomly divided in six groups as shown in ►**Table 1** (►**Fig. 1**).

Dental Pulp Stem Cells Preparation

DPSCs were collected from adults aged between 18 and 55 years who underwent third molar extraction and these were processed within 48 hours of extraction. DPSCs were labeled with PKH26 Red Fluorescent Cell Linker (Sigma Aldrich, St. Louis, Missouri, United States) according to the manufacturer's protocol for in vivo tracing and identification of the cells after migration. The PKH26 Fluorescent Cell Linker incorporates a yellow-orange fluorescent dye with long aliphatic tails into lipid regions of the cell membrane. DPSCs were harvested with 0.25% trypsin. They were suspended in a complete medium at a density of 2×10^7 cells/mL. A total of 2×10^7 DPSCs in 100 µL complete medium were injected into four

Table 1 Groups depending on types of allograft and timing of suturing

Group	
A	Cold preserved allografts with immediate suturing
B	Cold preserved allografts implanted with stem cells with immediate suturing
C	Cold preserved allografts with delayed suturing
D	Cold preserved allografts implanted with stem cells with delayed suturing
E	Fresh allografts with immediate suturing
F	Stem cells-implanted fresh allografts with immediate suturing

Fig. 1 (A) Site of incision and surgical arrangement. (B) Muscle splitting incision to expose sciatic nerve.

evenly spaced points of the nerve section. In humidified atmosphere with 5% CO_2 at 37° C, nerve grafts were incubated in the complete medium for 48 hours. Following incubation, they were collected for experiments.

Outcome Assessment

The results were assessed after 12 weeks using the following criteria:

Functional Assessment

Comparison of Wounds and Autotomy Rate

The size of wound, development of contracture, and autotomy rates were observed at regular intervals and compared at the end of 12 weeks.

Step Length Ratio

Step length ratio (SLR) was used for functional assessment. As weight bearing is not possible on the paralyzed limb, the swing phase of the normal limb is significantly reduced. As the animal starts recovering from injury, weight bearing improves and so does the step length. We calculated the SLR after 12 weeks.

Gentian violet was applied to the hindlimb soles. The animal was made to walk on a white paper straight track measuring 10×45 cm, which was darkened at one end. The step length from one middle metatarsal to that of other limb during consecutive steps while walking was measured. The ratio of right to left step length was taken for each animal. The procedure was repeated for each group. For comparison and analysis, we graded SLR as poor $= < 0.7$, good $= 0.7–0.8$, excellent $= > 0.8$.

Nerve Conduction Velocity

Nerve conduction velocity (NCV) was recorded using AD microneurography instruments. Sciatic nerve was stimulated percutaneously at its notch and conduction velocity was calculated using points of stimulation along the nerve and measuring the resultant onset latency and distance. By this method, the integrity of nerve was also evaluated. For each rat, three readings were taken, and the mean was calculated.

Histological Evaluation

The wound was re-explored after sacrificing the rats at the end of 12 weeks. The sciatic nerve proximal to the graft, the grafted portion and the distal nerve were identified and resected as a whole and stored in formalin solution for histopathological studies.

1) Hematoxylin and eosin: It was used for routine morphology, inflammation, and fiber density analysis.
2) Loyez stain for myelin: This staining was used for fiber density and myelination.

Immunohistochemistry

To demonstrate the presence of transplanted DPSCs in sutured nerves, immunohistochemistry was performed on cryosections of tissue samples.

Analysis of Levels of Proinflammatory Cytokines in Plasma by ELISA

To evaluate the anti-inflammatory effect of DPSCs, the plasma levels of proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ) in all treated groups were calculated. The values were obtained by extrapolating on a standard curve and were expressed as ng/mL.

Student's two-tailed t-test was used for comparison of the two experimental groups.

Multiple comparisons were done using one-way analysis of variance followed by Tukey's test for multiple pairwise examinations. Changes were identified as significant if *p*-value was less than 0.05. Mean values were reported together with the standard error of mean.

Results

Fifty-two out of 60 rats were included in the study as eight rats had died during the observation period. Overall comparison was done between DPSC-implanted allografts (groups B, D, F) and allografts without DPSCs. Also, respective groups, that is, group A versus B, group C versus D, and group E versus F were compared to observe the effect of DPSCs on regeneration, timing of repair, and the degree of immunogenicity of allografts.

General Assessment

After surgery, rats started walking by dragging the operated limb. Depending on the recovery, the rats used their hindlimb paws to bear weight. In those rats in whom the recovery was not good, they used to bear weight on the ankle joint leading to development of pressure sores. This repetitive trauma led to progression of wound and swelling in the hindlimb. ►**Table 2** shows the number of rats that died, had wounds contractures and autotomy after 12 weeks. The wound and contracture rate was 40 and 57.14% in group A and C, respectively (►**Table 2**). While in group E, it was 37.5 and 50%. It was 22.22, 28.57, and 22.22% in group B, D, and F, respectively, (►**Table 2**) showing less wound and contracture rate in stem cell-implanted allografts.

Rodents tend to bite their paralyzed limb. Eighteen animals had autotomy when examined at the end of 12 weeks. Autotomy rate was 40, 42.8, and 50% in group A, C, E, respectively. It was lesser in stem cell-implanted allograft groups B (22.22%), D (33.33%), and F (22.22%) (►**Fig. 2**).

Step Length Ratio

Mean SLR was best in group B who underwent immediate repair with cold preserved DPSC-implanted allografts. The difference in SLR between group E and group F was significant with *p*-value 0.042 (<0.05) (►**Table 2**). Though there was no clearly significant difference in the remaining groups, it was seen that SLR was better in rats who underwent repair with allografts that were implanted with DPSCs (►**Fig. 3**).

Table 2 Results of functional and electrophysiological analysis

Group	Death	Wound	Contracture	Autotomy	SLR	NCV in cm/sec
A	0	4	4	4	0.754 ± 0.061	176 ± 23.81
B	1	2	2	2	0.883 ± 0.07	217 ± 8.97
C	3	4	4	3	0.714 ± 0.041	134 ± 7.6
D	1	3	2	3	0.786 ± 0.079	206 ± 9.5
E	2	3	4	4	0.677 ± 0.127	130 ± 8.8
F	1	1	2	2	0.865 ± 0.037	233 ± 20

Abbreviations: NCV, nerve conduction study; SLR, step length ratio.

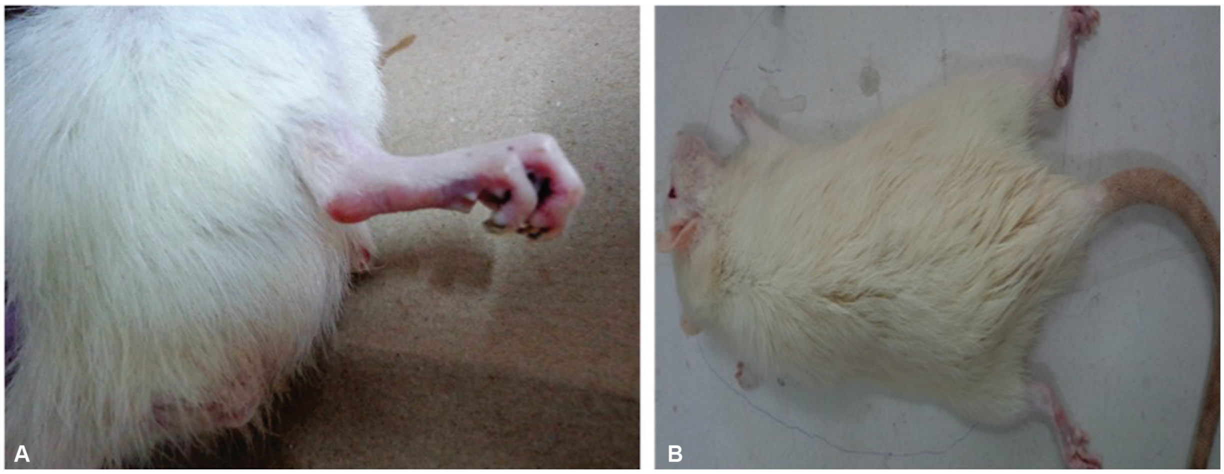

Fig. 2 (**A**) Contracture of operated limb. (**B**) Wound development on dorsal aspect of hind limb.

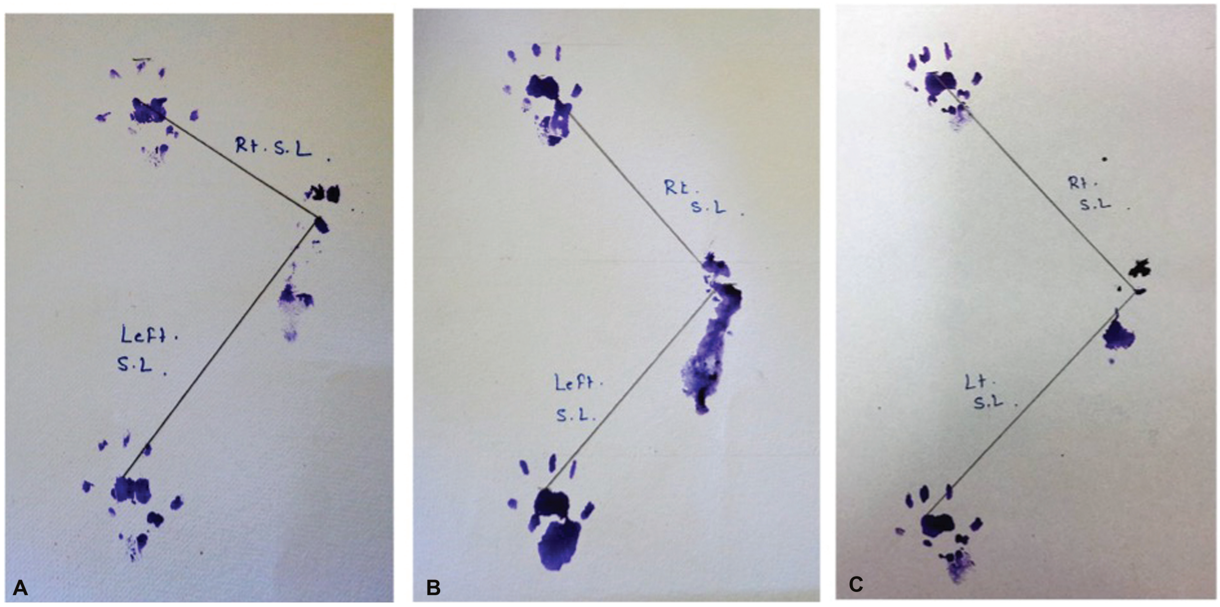

Fig. 3 Step length measurements taken from middle metatarsal head of one limb to the middle metatarsal head of another limb. Right hind limb was injured in the study. Measurements of right to left step length give a ratio. By this method, inaccuracies due to wound, contracture, and autotomy are avoided. For comparison and analysis, we graded step length ratio as poor = < 0.7 (A), good= 0.7-0.8 (B), excellent = > 0.8 (C).

Nerve Conduction Velocity

NCV measures the conduction of speed of electrical impulses through the nerve. We measured NCV at the end after 12 weeks in all groups. NCV was better ($p < 0.05$) in DPSC-implanted allografts compared to groups without stem cells. Maximum NCV was seen in rats who underwent immediate suturing of fresh DPSC-implanted allografts (group F).

Plasma Level of Proinflammatory Cytokine TNF-α, IL-6, IFN-γ

TNF-α is one of the main cytokines involved in acute phase reaction and expression of other proinflammatory factors like IL-1 and IL-6. TNF-α levels vary depending on time since injury, extent of insult, and individual immunogenic reaction to injury. The level of proinflammatory cytokine levels at 12 weeks after surgery was analyzed. Blood was collected via retro-orbital puncture before sacrificing rats. In our study, the level of all three proinflammatory markers was significantly higher in group A as compared to group B. In stem cell-implanted allograft groups, proinflammatory marker levels were lesser as compared to those without stem cells but the difference was not significant (►**Fig. 4**).

Histopathological Study

Visual subjective grading of recovery, regeneration, and inflammation was done by our pathologist who was blinded to the groups. In group A, there was reduction in myelinated nerve fibers (10%) along with neuroma formation. In group B, there was mild endoneural fibrosis with mild reduction in myelination (5%). There was marginal improvement in DPSC-implanted group as compared with the allografts only group. In group C, there was severe endoneural fibrosis due to severe

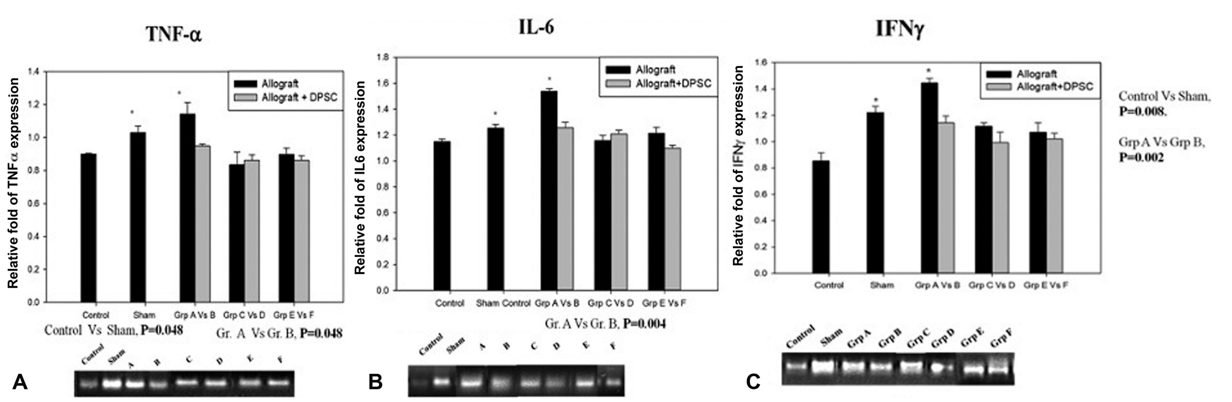

Fig. 4 Serum proinflammatory level. Tumor necrosis factor-α (**A**), interleukin-6 (**B**), and interferon-γ (**C**) levels are significantly lower in group B, though the difference in other groups is not significant.

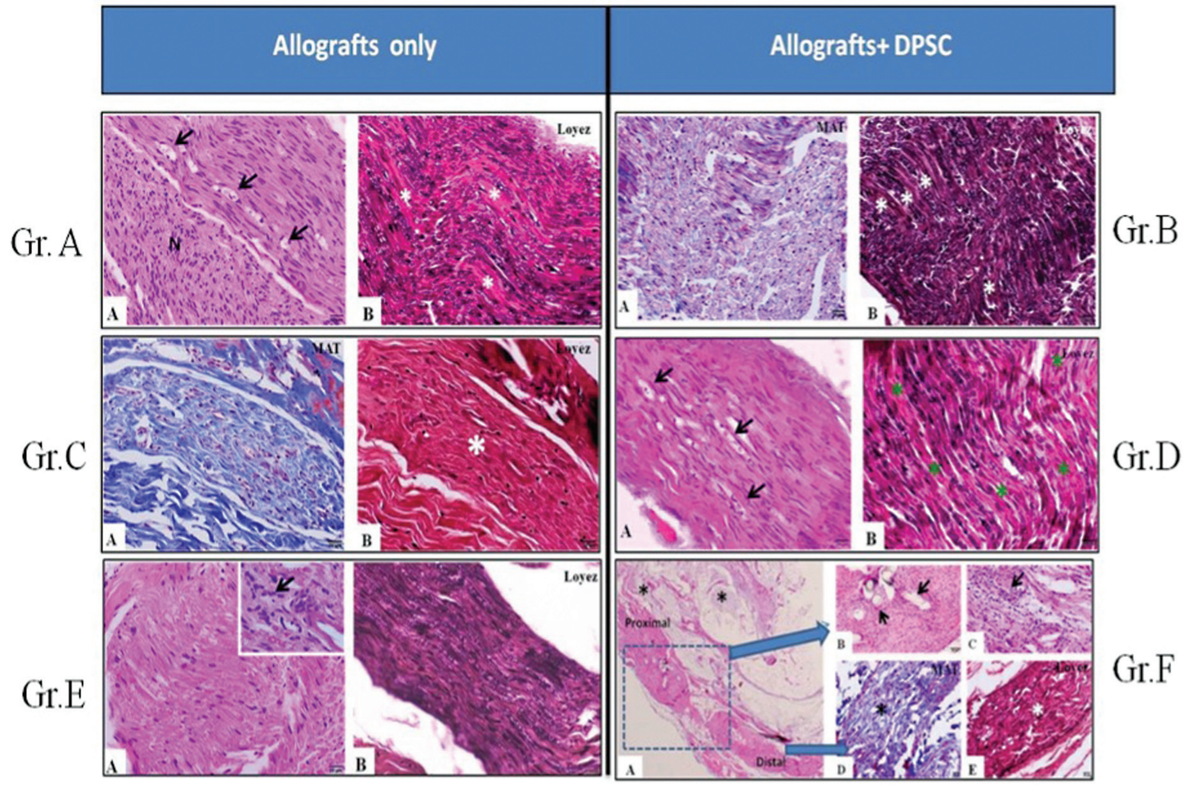

Fig. 5 Group: (**A**) Neuroma formation (N), focal acute Wallerian degeneration (arrow). (**B**) Mild reduction in myelinated fiber density (∼10%), seen in pockets (*). Group (B) Mild endoneural fibrosis (A) with mild reduction in myelinated fiber density (∼5%), seen in pockets (*). Group (C) Endoneural fibrosis (A) and in B severe reduction (> 20%) in myelinated fiber density (*). Group (D). Presence of Wallerian degeneration with myelin ovoids (arrow). (B) Moderate reduction in myelinated fiber density Group: E) B: Preserved myelinated fiber density with focal endoneural inflammation (A, inset arrow) Group F) Low magnification view of nerve with prominent myxoid change in soft tissue (*) (**B, C**): Proximal end showing suture granuloma and inflammation (arrows) (*) (**D, E**): Distal end showing fibrosis (D, *) with reduction in myelinated fiber density (E, *).

reduction in myelinated fiber density. In group D, there was moderate myelination loss (20%), which showed better histological outcome as compared with group C. In group E, myelinated fiber density was preserved with focal endoneural inflammation and in group F, there was prominent inflammation with presence of myxoid changes in soft tissues (►**Fig. 5**).

Presence of Transplanted DPSCs in Sciatic Nerve

Three months after DPSCs transplantation through sciatic nerve allografts, red fluorescence of PKH26-labeled DPSCs was observed in cryosections of sciatic nerves. The cell nuclei were further stained with 4',6-diamidino-2-phenylindole. This showed that the DPSCs were still viable in the graft (►**Fig. 6**).

Discussion

The aim of our study was to evaluate the role of timing of surgery from injury and DPSCs in peripheral nerve regeneration. Different mechanisms have been postulated regarding the role of stem cells in peripheral nerve regeneration such as reduction in inflammatory response, promotion of growth factors, neuronal circuitry restoration, replacement of myelinating cells, remyelination of spare axons, and endogenous precursor cell stimulation.[6–8,15] mesenchymal stem cells (MSCs) like expression of cell surface molecules CD90, CD73, CD105 and are negative for MHCII marker HLA-DR. They are also suggested to possess immunomodulation properties and locally secrete large amounts of angiogenic cytokines and neurotrophic factors like Vascular endothelial growth factor (VEGF), nerve growth factor (NGF), glial derived nerve factor (GDNF), brain derived neurotrophic factor (BDNF); bone morphogenetic protein 2 (BMP2), like that of bone marrow mesenchymal stem cells (BM-MSCs).

The incidence of wounds, contractures, and autotomy was more in rats who underwent nerve repair with allografts without stem cells. Due to contracture, toe spread was inadequate. Also, it was observed that when rats were awaiting delayed suturing for 6 weeks, they developed wounds on ventral aspect of their hindlimb. Repeated trauma in the same position while walking leads to increase in size of the wound and a tendency for rats to bear minimal weight on the wounded limb. So, in these rats, though electrophysiological studies show good results, functional recovery was far from satisfactory. Cuevas et al, compared the rate of autotomy in rats and found less autotomy, edema, and infection in rats who underwent allograft transplant implanted with MSC.[16] Sporel-Özakat et al found a 40% decrease in automutilation after using antinail biting lotion.[17] Weber et al observed less automutilation with good functional nerve recovery.[18] Autotomy, wound rate, and contractures are independent factors to measure the functional outcome. Though the Sciatic Function Index (SFI) is

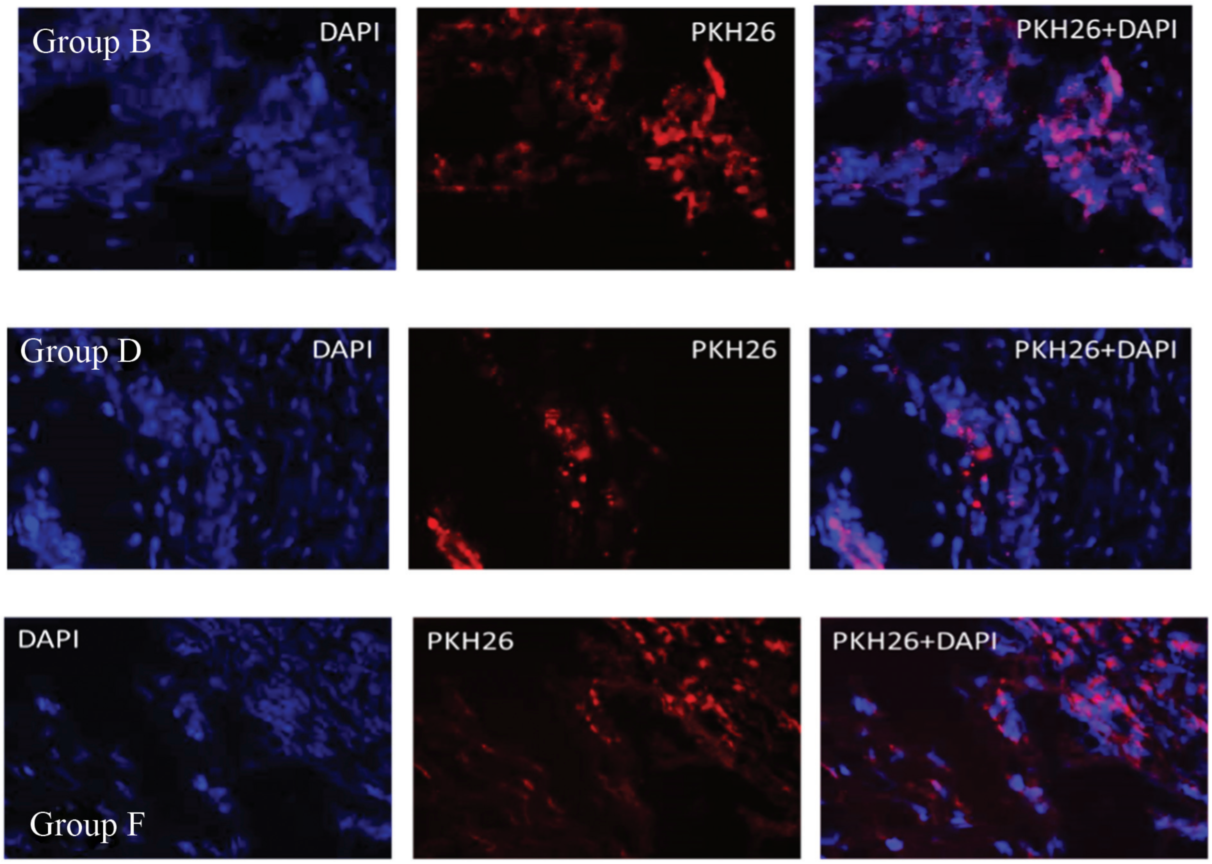

Fig. 6 Red fluorescence of PKH26-labeled dental pulp stem cells showing viable stem cells. DAPI, 4',6-diamidino-2-phenylindole.

one of the most widely used outcome scales, the validity of the SFI has been questioned because of autotomy, print smearing, contracture, tail dragging, and contamination leading to difficulty in obtaining clear print marks.[19,20] To assess SLR, measurements are taken from middle metatarsal head of one limb to the middle metatarsal head of another limb. It avoids inaccuracy when measurements are taken from sole to sole, heel to heel, or from toe tip to toe tip. These reference points become imprecise after developing wounds, contractures, and autotomy. Also measuring only SL is not useful as it gets affected by walking speed. The use of the right: left SL eliminates the variation caused by speed and is therefore an independent parameter. As the regeneration proceeds, the power of the operated limb and SL improves, and so does the SLR. In our study, though the DPSC-implanted allograft did better, the difference was only significant between group E and F ($p = 0.042$). Chen et al, in his study on peripheral nerve injury, analyzed walking pattern in rats by injecting BMSC in allografts and observed similar results of improvement in stem cells-implanted allografts compared to those without stem cells.[21] Cuevas et al, who used MSCs for peripheral nerve repair, demonstrated that implantation of MSCs improves functional recovery by at least 36% ($p < 0.05$) and 78% ($p < 0.001$) at day 18 and 33, respectively, when compared with culture medium-treated transected nerves.[16] Failure to improve may be due to misdirected regenerating axons in a polyfascicular sciatic nerve. Research mainly focusing on functional outcome should evaluate walking tract analysis, as proper walking needs coordinated functions involving sensory input, cortical integration, and motor response.

NCV was analyzed based on compound muscle action potential (CMAP) latencies and distance between electrodes. In this study, the amplitude of CMAP was not considered to assess the functional recovery. After nerve transection, the conduction pathway was completely interrupted and depending upon the course of regeneration, NCV improved.

On comparing individual groups, there was a significant difference (p-value ≤ 0.05) suggesting stem cell-implanted allografts improve the NCV. The highest NCV was in rats who underwent immediate suturing by DPSC-implanted fresh allografts (233 ± 20.95 cm/sec). Mimura et al observed similar results of more mean NCV in rats in whom BMSCs were used. They also observed that though results of the electrophysiological study and walking track analysis showed a better results in the BMSC group than in the control group 6 months after transplantation, ankle contractures were observed in both the BMSC and control groups and concluded that musculoskeletal sequelae of complete denervation cannot be prevented by the strong regenerative effect of BMSC.[22] Liu et al studied adipose-derived stem cells (ADSC) to promote peripheral nerve repair and observed similar result of higher NCVs and peak amplitude in the ADSC-treated groups with the difference being statistically significant.[23] Similarly, in a study by Orbay et al, NCV was better in ADSC-implanted allografts as compared with control but it was not statistically significant.[24] Our study shows that like BMSC and ADSC, DPSCs help in improving electrophysiological outcome. Cui used embryonic stem cells for peripheral nerve regeneration in rats and had a similar result of improved electrophysiological outcome in stem cell transplanted animals.[25] DPSC-implanted allografts in group B and D showed less endoneural fibrosis and myelin loss as compared with group A and C (without DPSC). There was no difference between thickness of myelination between any group. Cui demonstrated substantial axonal regrowth and nerve repair in stem cell-implanted allografts as compared to control groups. Also, stem cell-implanted group had near normal diameter with longitudinally oriented and densely packed Schwann cell like phenotype.[25] Fu et al observed thicker and more deeply colored myelin sheath with greater number of myelinated axons in conduits seeded with stem cells.[26] DPSCs inhibit the proliferation of mononuclear cells that involve indolamine 2, 3-dioxygenase.[27] They also inhibit autologous and allogenic T cell proliferation through an apoptosis independent mechanism. In our study, TNF-α, IL-6, and IFN-γ level was significantly higher in group A rats sutured with allografts without stem cells as compared with group B rats in whom stem cells-implanted allografts were used. In group D, a cold preserved DPSC-implanted allografts with delayed suturing, TNF-α and IL-6 level was insignificantly higher than group C. In rest of the groups, inflammatory marker levels were lower in DPSC-implanted allograft groups as compared with those without DPSC, but the difference was not significant.

We studied the presence of DPSCs and their survival in allografts by initially staining DPSCs with PKH26. The stem cells were viable at the end of study as seen by red fluorescence of PKH26-labeled DPSCs in cryosections of sciatic nerves. Also, this was the first study to show effectiveness of stem cells in delayed nerve repair.

Summary

Microsurgical techniques have improved the functional recovery in peripheral nerve injuries, even though recovery is incomplete and associated with lifelong disability. DPSCs have shown promising results in neuronal regeneration by decreasing wound rates, contractures, autotomy and improving NCV significantly. SLR and weight bearing on operated limb were better in DPSC-implanted rats. Inflammatory markers and histological analysis favored results to DPSC. This is the first study to show the effect of stem cells in delayed repair in nerve injury.

To conclude, DPSCs can be further explored for potentiating the repair of peripheral nerve injuries.

Conflict of Interest
None declared.

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