Title: Augmenting Peripheral Nerve Regeneration Using Rat Hair Follicle Stem Cells (rHFSCs) in Rats

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Abstract

Introduction: Nowadays, cell therapy is the most advanced treatment of peripheral nerve injury. The aim of this study was to determine the effects of transplantation of hair follicle stem cells on the regeneration of the sciatic nerve injury in rats.

Methods: The bulge region of the rat whisker was isolated and cultured. Morphological and biological features of the cultured bulge cells were observed by light microscopy and immunocytochemistry methods. Percentages of CD34, K15 and Nestin cell markers expression were demonstrated by flow cytometry. Rats were randomly divided into 3 groups: Injury group, epineurium group, and epineurium-with-cell group, that rat hair follicular stem cells (rHFSCs) were injected into the site of nerve cut. HFSCs were labeled with BrdU, and double-labeling immunofluorescence was performed to study survival and differentiation of the grafted cells. After 8 weeks, electrophysiological, histological and immunocytochemical analysis assessments were performed.

Results: The results of this study show that rat hair follicle stem cells are suitable for cell culture, proliferation and differentiation. The results suggest that transplantation of rat hair follicle stem cells had the potential capability of regenerating sciatic nerve injury; moreover, evidence of electrophysiology and histology show that Epineurium with cell repair was more effective than the other experimental group (p<0.05).

Conclusion: The achieved results propose that hair follicle stem cell would improve axonal growth and functional recovery after peripheral nerve injury.

Keywords:
Peripheral nerve regeneration, Rat Hair follicle stem cell, Sciatic nerve
1. Introduction

Peripheral nerve regeneration is an important clinical problem. The peripheral nerve system (PNS) has the potential to regenerate nerve cells, and peripheral nerve injury has been successfully repaired using various procedures such as nerve auto-graft (Belkas et al., 2004). One of the effective methods of repairing peripheral nerve injury is suturing the two ends of the nerve when the resulting gap is short (Millesi, 1984). Axonal regeneration in a peripheral nerve injury needs extrinsic factors that promote growth, and supply guidance to the target. To overcome these problems, a variety of cells have been used to facilitate transplantation. The aims of cellular transplantation include bridging the gap, providing a suitable environment to induce axonal regeneration and promoting neovascularization (Li et al., 2014). Different procedures have been applied to improve the regeneration of peripheral nerves and one is seeding cells into the nerve fragments (Belkas et al., 2004, Li et al., 2014, Ishikawa et al., 2009, Fan et al., 2011, Dezawa, 2005). The overarching goal of any stem cell-based an approach to peripheral nerve injury is to establish a more favorable environment for regenerating axons and perhaps more importantly, maintain this support for an extended period of time (Fairbairn et al., 2015).

Considerable recent interest has been focused on adult stem cells for both research and clinical applications. Such cells can circumvent some of the problems associated with embryonic stem cells, such as immunologic incompatibility. However, most adult stem cells are relatively sparse in indeterminate locations and growth states (Amoh et al., 2005a). A highly promising source of relatively abundant and accessible, active, multipotent adult stem cells are obtained from hair follicles.

Hair follicle stem cells, located in the hair follicle bulge, possess stem cell characteristics, including multipotency, high proliferative potential, and ability to enter quiescence (Nobakht et al., 2011). Nestin, a protein marker for neural stem cells, is also expressed in follicle stem cells as well as their immediate differentiated progeny (Hejazian et al., 2012, Esmaeilzade et al., 2012). Nestin expressing hair follicle stem cells have the ability to differentiate different cell lines such as neurons, glial cells, keratinocytes and smooth muscle cells (Amoh et al., 2009a).

Hair follicle stem cells responded to neuregulin-1 and BMP2 (Bone Morphogenetic Protein 2) by generating Schwann cells and chondrocytes, respectively (sieber-blum et al., 2004). In vivo studies show that nestin-driven hair follicle stem cells can differentiate blood vessels and neural tissues (Amoh et al., 2005a). Hair follicle stem cells can improve vascularization leading to injured tissue repair (Aki et al., 2010, Amoh et al., 2004).

The hair follicle stem cells implanted into the gap region of a severed sciatic nerve greatly enhanced the rate of nerve regeneration and the restoration of nerve function. Follicle stem cells largely transdifferentiated into Schwann cells (SCs) which are known to support neuron regrowth. Thus, Nestin-positive hair follicle stem cells can promote the regeneration of peripheral nerve injury. These cells provide an important, accessible, autologous source of adult stem cells for regenerative medicine (Hoffman, 2006, Amoh et al., 2009b, Amoh et al., 2012, Lin et al., 2009). Recently, Bhangra reported several studies on using stem cells for Peripheral Nerve Repair in an
article review (Bhangra et al., 2016). In none of the previous studies have not been used rat hair follicle stem cells. While in our previous studies (Nobakht et al., 2011, Hejazian et al., 2012, Esmaeilzade et al., 2012, Esmaeilzade et al., 2014) we could extract, culture, proliferate and differentiate rat Hfscs in vitro and in vivo.

Regarding utilization of cell therapy in peripheral nerve injury, our study differs widely in comparison to previous studies which were fully referred to especially Fairbairn et al., 2015, Bhangra et al., 2016.

In a number of former studies, the stem cells extracted from hair follicle are different in type, said that; several types of stem cells could be found in bulge area. For instance the origin of stem cells in neural crest stem cells in follicle of hair which showed different markers (Lin 2009 and Hoffman 2006). Also kind of the animal sample used is another difference (transgenic mice with fluorescent protein GFP, Amoh 2009, Amoh 2012).

Dissimilar to others, in our study there were neither other interventions e.g. nerve transplantation and neurotrophin nor other cells like Schwann cell. Consequently, in the current work, in repairment of peripheral nerve injuries without any medium, solely the potential of hair follicle cells via Nestin and CD34 markers is evaluated. Also with modified methods compared to other studies.

2. Methods

2.1. Animals and housing conditions

40 male Wistar rats (250-300g body weight) were purchased from the Animal Center of Tehran Medical University. All animal experiments were carried out according to the Guidelines of the Iranian Council for Use and Care of Animals, approved by the Animal Research Ethical Committee of Iran University of Medical Sciences (Tehran, Iran). All rats were maintained in a temperature-controlled environment of 24 ± 1°C with a 12 h dark/light cycle (Dark cycle: 8:00 P.M. to 8:00 A.M.) with free access to water and food.

Four groups of rats were formed (n= 5 in each group), (Following skin incision and muscle displacement, the sciatic nerve become apparent): A) Sham group (nerve has release a little and returns to its original condition). B) Injury group (10 mm piece was removed from the middle part of the sciatic nerve). C) Epineurium group (nerve was cut and then sutured). D) Epineurium-with-Cell group (HFSCs were injected into the region.

2.2. Hair Follicle Isolation and Cultivation

Albino Wistar rats (n = 10, weighing 250-300g, 8-10 weeks old, The Animal Center of Tehran Medical University, Tehran, Iran) were used. The rats were sacrificed with ether, and the whisker follicles were dissected as described by Sieber-Blum and Grim (Sieber-Blum and Grim, 2004). The tissues were trimmed into small pieces (4 × 8 mm2) and the samples were incubated in 2 mg/ml collagenase I/Dispase II solution (Sigma-Aldrich, USA). Most of the connective tissues and
dermis around the follicles were removed and the whisker follicles lifted out. The bulge region was then amputated from the upper follicle by making two transversal cuts at the site of enlargement spots of the outer root sheath with a fine needle. The culture procedure was performed with a slight modification, as previously described by Yang and colleagues (Yang et al., 2005). Briefly, 20 isolated bulges were cut into small pieces, plated into flask culture plates, precoated with collagen type I (Sigma-Aldrich, USA) and immersed in a 3:1 DMEM: nutrient mixture F-12 supplemented with the epidermal growth factor containing 10% fetal bovine serum. All dissection and cultivation procedures were performed under sterile conditions and incubation was at 37°C (5% CO2). Within approximately 4 days, the initiation of outgrowth of bulge cells from the bulges was observed (Figure. 1A, B). One week after the onset of the an outgrowth, the bulges were removed from the culture plates and the cells were collected by incubation with a mixture (1:1) of 0.125% trypsin (Sigma-Aldrich, USA) and 0.02% EDTA (Sigma-Aldrich, USA) at 37°C for 2 minutes. The dispersed cells were centrifuged at 259 ×g for 10 minutes, placed in other collagen coated plates and incubated for 3 days with a medium. After reaching sufficient confluence, the cells were detached by trypsin and counted by a neobar lam.

2.3. Flow cytometry

Hair follicle stem cells isolated from the bulge region were incubated with every 100 ml primary antibodies, mouse anti-CD34 monoclonal antibody (1:75, Sigma, USA) and mouse anti-nestin monoclonal antibody (1:200, Millipore, USA) at room temperature for 1 hour. The cells were centrifuged with 1-2 ml of PBS (0.1 M). Subsequently, they were incubated in the dark at room temperature for 1 hour following conjugate secondary antibody: goat anti-mouse FITC-conjugate IgG (1:1400, Abcam, UK). After this time, the percentage of CD34+, K15 and Nestin+ were analyzed by flow cytometry.

2.4. Immunocytochemistry

Cells seeded on collagen-coated coverslips (1 × 10^5 cells per ml) were washed 3 times with PBS for 5 minutes and fixed in 4% paraformaldehyde for 10 minutes. The fixed cells were then washed with PBS for 3 × 5 minutes and incubated in a blocking buffer (10% goat serum (Invitrogen, USA) 0.3% Triton X-100 (Fluka, USA)) at room temperature for 30 minutes. They were then incubated at 4°C overnight with the following primary antibody mouse anti-nestin monoclonal antibody (1:200, Millipore, USA). The next day, the cells were rinsed for 3 × 5 minutes to remove unbound primary antibodies. Subsequently, they were incubated at room temperature for 2 hours with the following conjugate secondary antibody: Goat anti-mouse FITC-conjugate IgG (1:1400, Abcam, UK). The cell nuclei were counterstained with 1 μg/ml 4, 6-diamidino-2- phenylindole (Sigma-Aldrich, USA) in PBS in the dark at room temperature for 1 min. After washing, the samples were mounted on a slide with mounting media for visualization using a fluorescence microscope. To examine the specificity of the nestin
antibody, 3T3 fibroblast-like cells (Pasteur Institute of Iran, Tehran) were used as a negative control. Labeled cells were identified using fluorescent microscopy (Olympus Ax70).

2.5. Bromodeoxyuridine (BrdU) labeling

48 to 72 hours before cell transplantation, BrdU (5 μmol/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the flask of cultured cells. For checking cell labeling with BrdU, 48 h after cell labeling, the labeled cells on collagen coated cover slips were washed in PBS for 3 × 5 min and fixed in 4% PFA for 10 min. Then, the fixed cells were washed in PBS for 3 × 5 min and incubated in 2 N HCl at 60°C for 45 min and were washed 2 times in 0.1 M borate buffer (pH 8.3). After being washed in blocking buffer (10% goat serum, Sigma-Aldrich, USA/0.3% Triton X-100 Fluka, USA and 1% BSA) at room temperature for 60 min, the incubated cells were again incubated with the primary antibody anti-BrdU (1:500, Sigma-Aldrich, USA) at 4°C overnight. The next day, the cells were rinsed in PBS for 3 × 5 min to remove unbound primary antibodies. Subsequently, they were incubated at room temperature for 1 h with the secondary antibody: goat anti-mouse FITC conjugate IgG (1:200, Abcam, Cambridge, UK), washed in PBS for 3 × 10 min, mounted with mounting media and visualized using a fluorescence microscope (Figure. 1C).

2.6. Transplantation procedure

The rats were anesthetized by intra peritoneal injection of a combination of ketamine (100 mg/kg), and xylazine (10 mg/kg). After skin incision, the sciatic nerve was exposed using a muscle splitting incision. Under an operating microscope, the left sciatic nerve was exposed to the mid-thigh, was cut and then sutured with 8-0 propylene sutures in the Epineurium group and in the Epineurium-with-Cell group, 5×10^5 cells/50μl were injected into the area. Finally, the skin was sutured.

2.7. Histological examination

Eight weeks after the implantation, the rats were anesthetized and sacrificed, and the regenerated nerves were harvested. The nerves were immediately fixed in a cold buffered 2.5% glutaraldehyde solution. After fixation, these nerve tissues were post-fixed in 1% osmium tetroxide, dehydrated, and embedded in resin. The semi-thin sections were then stained with 1% toluidine blue. All nerve sections were observed under a light microscope, and photographs were taken using a digital camera. Images of the histological sections were digitized and subsequently analyzed using an image analyzer system (Olysys Bioreport). The number of axons was counted in randomly selected fields (4 × 10^4 μm²) at a magnification of 400×.
2.8. Immunohistochemistry

After 8 weeks of cell transplantation for the group of Epineurium with cell, the sciatic nerve specimens were put in the medium of 10% formalin and inserted in paraffin as routine processing, subsequently 5 um nerve pieces were accumulated via a rotary microtome (Leitz, 1512, Germany). Paraffin sections were stained with Envision G12 double-stain system kit (DAKO, USA) according to the kit protocol. The following primary antibodies were used in this protocol: polyclonal anti-S100 antibody (1:400, Dako, USA), and monoclonal anti-BrdU antibody (1:500, Sigma-Aldrich, USA). The secondary antibodies in this protocol were horseradish peroxidase and alkaline phosphatase that was detected by diaminobenzidine (DAB) and permanent red staining.

2.9. Electrophysiological Measurements

Eight weeks after the transplantation, the rats were anesthetized intra peritoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg) and the sciatic nerves were exposed. Electric stimulation (duration of 0.1 ms, the intensity of 2.3 mA) was applied to the proximal side of the injured nerve. The compound muscle action potential (Electromyography) into the belly of gastrocnemius muscle, was recorded in the gastrocnemius with a needle electrode and a reference cap electrode inserted into the knee joint. A stainless steel needle used as the ground electrode was inserted into the tail skin. The area of the recorded muscle response (mV×ms), and the amplitude were calculated as these can be considered to reflect the amount of activated fiber (Chen et al., 2007, Mimura et al., 2004).

2.10. Statistical analysis

Data analysis was done by using statistical package for social science (SPSS) version 16 software (SPSS, Chicago, IL, USA). All data were analyzed by Utilising a kruskal wallis test. When statistical significance was found between groups, the mann whitney tests were performed to determine pairwise significant difference. The data were expressed as Means ± SD, and P < 0.05 was considered statistically significant.

3. Result

3.1. The primary culture of bulge cells from rat hair follicles

Cells set from the bulge. After 4 days of relocating, the bulges were pulled out and adherent cells were disconnected using trypsin treatment and finally subcultured. 72 hours before transplantation the hair follicle stem cells were tagged by BrdU (Figure 1).

3.2. Percentage and characterization of cultured HFSCs
Flow cytometry analysis showed the percentage of CD34, and Nestin of cell markers expression in cultured bulge cells (Figure 2). Immunocytochemical staining were used for showing the expression of nestin (neural stem cell Marker) in bulge cells. Our results showed that the nestin antibody staining bulge cells was seeded on coverslips (Figure 3).

3.3. Finding from the immunohistochemistry of HFSCs

Using a double-staining procedure, many cells were detected in the transplanted site which presented BrdU-S100 and it proved that transplanted marked cells differentiated to Schwann cells. Using a double-staining Envision kit (DAKO, USA), we showed that the nuclei of differentiated cells (red points), which had presented BrdU, were brown, and presentation of S100 in the cytoplasm. The results confirmed that the presence, viability and differentiation of transplanted cells in the severed sciatic nerve eight weeks after the transplantation (Figure 4).

3.4. Electrophysiological Results comprise amplitude and latency.

The electrophysiological tests results comprised both amplitude and latency measures. The time calibration bar 2 ms and the amplitude calibration bar was 10 mV. The stimulation intensity and the duration was 2.3 mA (milliampere) and 0.1 ms (Figure 5). Table 1 shows the description and comparison results of amplitude in millivolts (mV) and latency of the study groups. pairwise comparisons of groups showed that the mean of amplitude were statistically significant in two by two comparisons (0.008<p-value<0.010) except in epineurium and the epineurium with cell that were not differ significantly. There was a significant difference between groups of amplitude (p-value=0.001) and latency (p-value=0.012) (Figure 6, 7).

3.5. Histological findings.

To evaluate the regeneration efficacy of myelinated and unmyelinated fibers, we examined semi-thin sections of the nerves. Light microscopic examination of toluidine blue-stained cross-sections of these nerves revealed a clear qualitative difference between the experimental and Injury groups (Figure 8). Statistical analysis showed significant difference in myelin thickness of groups (p-value=0.022, Table 1). The only statistically significant pairwise comparison (Figure 9) was referred to injury and sham group (p-value=0.045). The number of nerve fibers density were significantly different between groups (p-value=0.027, Table 1). Pairwise comparisons (Figure 10) showed significant difference between injury group with others (both p-values=0.045).
4. Discussion

Injuries of peripheral nerves described as one of the most challenging microsurgical problems. These damages are associated with considerable disability due to loss of both motor and sensory functions. Development of a high-quality replacement for autografts is needed because the autograft procedure entails multiple surgeries, loss of function, and loss of sensation at the donor site (Belkas et al., 2004, Li et al., 2014, Fan et al., 2011).

The great hope in the field of regenerative medicine for nerve repair is the exploitation of the regenerative potential of cell-based therapies. This is of particular relevance especially for long gaps, where the use of nerve guides alone has failed to provide successful regeneration (Faronia et al., 2015). Nerve regeneration by nerve guidance scaffolds improved through a limitation of myofibroblast infiltration, accumulation of neurotrophic factors in high concentrations and reduction of scar formation (G.R.D. Evans, 2001).

Because stem cells are important seeding cells for peripheral nerve regeneration, special attention has been paid to the development of a rich and accessible cellular reservoir for this cell type (Amoh et al., 2005a). In this study, we evaluated the effect of transplantation of rHFSCs on recovery of rat sciatic nerve injury. Several investigators have shown that HFSCs can repair the injury on mice sciatic nerve (Amoh et al., 2005a, Amoh et al., 2012, Amoh et al., 2010). But there are differences between the methods and results and they have not evaluated the results under similar conditions on the rat in this study. Since the rats are the most common and the most accessible laboratory animals our research is done on this animal. In addition, our team in previous studies succeeded to isolate, culture and differentiate these cells in the rat. Recently, the other researcher has done study on rHFSC characteristics (Quan et al., 2016). This shows that HFSCs in rats have also taken into consideration.

The present study confirmed the presence of HFSCs by using anti Nestin antibody and anti CD34 in vitro. This finding is consistent with previous reports (Hejazian et al., 2012, Amoh et al., 2012). We cultured and labeled the HFSCs with BrdU. The immunohistochemistry double-stain process showed that BrdU-positive HFSCs can survive in vivo and transdifferentiate largely into SCs (S100 positive cells) at the sciatic nerve injury after 8 weeks.

During peripheral nerve regeneration, trophic factors and supporting substances are essential molecules that play important roles (Chen et al., 2000, Lee et al., 2003). Researchers have indicated several mechanisms that may result in the promotion of functional improvement by HFSCs (Amoh et al., 2005a, Amoh et al., 2012, Amoh et al., 2010).

Hair follicle stem cells were implanted into the gap region of mice severed sciatic nerve greatly enhanced the rate of nerve regeneration and the restoration of nerve function. HFSCs can promote axonal regeneration in PNS (Amoh et al., 2005a, Amoh et al., 2010). Neural stem cells are marked by the expression of an intermediate filament of nestin. The expression of the unique protein, nestin, in both neural stem cells and hair follicle stem cells suggests their possible relation (Hejazian et al., 2012, Esmaeilzade et al., 2012, Amoh et al., 2005b). These cells transdifferentiated largely into Schwann cells (SCs) which are known to support neuron regrowth (Amoh et al., 2005a, Amoh et al., 2009, Amoh et al., 2012, Amoh and Hoffman, 2010b). Schwann
cells secrete many factors such as neurotrophic factors that induce tissue plasticity, and neuroprotective factors (Rodriguez et al., 2000). SCs have the ability to release neurotrophic factors such as NGF, BDNF, GDNF, CNTF and VEGF, as well as to produce extracellular matrix proteins such as collagen I, collagen IV, fibronectin and laminin (Moradi et al., 2012, Feneley et al., 1991). Therefore, there is good evidence to support the hypothesis that SCs differentiated of transplant-dated HFSCs may repair peripheral nerve injuries.

In addition, SCs mediated by immunoglobulin superfamily molecules, like the nerve cell adhesion molecule, protein 0 (P0), cadherin, and the protocadherins, are important for axonal elongation and organized sprouting. SCs also produce basal lamina components, like collagen IV, and laminin that play an essential role in nerve regeneration. Among the adhesion molecules, laminin is the most potent factor for promoting axonal outgrowth. It thus seems likely that transplantation of SCs may also repair peripheral nerve injuries (Chew et al., 2008, Ide, 1996, Walsh et al., 2009).

Time is one of the several aspects require specific attention in the clinical treatment of peripheral nerve injury. Because delay of nerve injury treatment may cause neurobiological alterations in neurons and Schwann cells, impairing nerve functional recovery and affect neuron survival (Egle De Stefano et al., 2013). In this study we would transplant nestin expressing cells after two weeks. In the other study cells were cultured for 1–2 months before transplantation to the injured nerve which would not be optimal for clinical application, because the patient should be treated soon after injury.

The condition velocity is an quantitative and valid index in evaluating of action potential conduction in peripheral nerves (Ao Q et al, 2011). Solely after growth of sufficiently regenerated fibers across the nerve bridge, the target muscle can construct muscle action potential and then it might be measurable. Additionally the condition velocity relies directly on different factors such as the diameter of axons, the thickness of the myelin sheath, and the length of internodes (Matsumoto K et al, 2000).

Magnitude of EMG correlates directly to the number of nerve fibers that innervate the muscle, thus the conduction velocity of the motor nerve could be calculated. EMG examinations gives a vital index for the function of conduction in the peripheral nerve (Wang X et al.2005). In this paper, the recovery index of EMG magnitude and the medium percentage of conduction velocity illustrated that animals in Epineurium and Epineurium with cell groups experience a considerable improvement in nerve regeneration.

In fact the recovery index was related to the numbers of factors including the diameter of the regenerating axons, thinner myelin sheaths with shorter internodes, and immaturity of myelinated nerve fibers as a whole (Matsumoto K et al, 2000, Wang X et al.2005) that has complete accordance with morphometric findings).

In this study, we showed the benefit of repairing nerve defects by using HFSCs, as evidenced by electromyography tests in the gastrocnemius muscle, and histological examination measured by myelinated and unmyelinated axonal number analysis. The results of the histological examination showed that there were statistically significant differences between the Injury group and the experimental groups (Epineurium and Epineurium with cells). Our results showed that fewer
axons, less myelinated fibers, reduced nerve fiber density, increased axonal degeneration, some axonal atrophy, and reduction of the mean thickness of the myelin sheath in injury group compared with other groups. In the epineurium and the epineurium with cell groups were showed an increased number of distinct axons, increased nerve fiber density, increased number of regenerating axons, and reduced axonal degeneration. Some previously reported data support our findings (Amoh et al 2005a). Although there aren’t significantly higher thickening of myelinated fibers in the experimental groups.

The number of nerve fibers density was higher in the experimental groups compared to the Injury group (Figure 10). However, the axonal regeneration seen in Epineurium with cells is much greater than that those in other groups.

The latency diagram shows the delay in the conduction of the electric wave, which indicates the velocity of the conduction of the electric wave in the neuromuscular synapse. So, whatever the level of latency is lower, the function of the synapse is better and the nerve is repaired better. These findings confirm those documented previously (Fan, 2011, Ide, 1996).

**In conclusion:** The current work revealed that the injection of hair follicle cells caused the recovery of severely injured sciatic nerves, which could result in development of gastrocnemius muscle via electric stimulation. 8 weeks after transplantation of hair follicle stem cells, histological evaluation revealed higher concentration of myelinated and unmyelinated fibers within the nerve in the epineurium with cells. The achieved results propose that hair follicle stem cell would improve axonal growth and functional recovery after peripheral nerve injury.

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**Conflict of Interest**

The authors declared no conflicts of interest.
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Figure 1. Characteristics of hair follicle stem cells. The primary culture of bulge cells from rat hair follicles. (A) The growth of bulge cells after 4 days and (B) 7 days. (C) The hair follicle stem cells were labeled with BrdU 72 hours prior to transplantation Scale bars=50µm (A, B).

Figure 2. Flowcytometry determine that HFSCs are primarily stemming cells. Results show that the percentage of CD34 and Nestin-positive cells. No positive reaction was seen with the K15 antibody. This demonstrates that bulge-derived neural stem cells are K15 negative.
Figure 3. Probing bulge-derived cells with specific antibodies is done before differentiation. Staining with the Nestin antibody and DAPI nuclear stain represents neural stem cells that originated from the neural crest. A) Immunocytochemically, cultured hair follicle stem cells (HFSCs) were Nestin positive, B) 4, 6-diamidino-2-phenylindole (DAPI) staining image for nuclei, C) HFSCs were Nestin positive with their nuclei after merge. Scale bars =20 µm (A, B, C).

Figure 4. Differentiation of hair follicle stem cells (HFSCs) to Schwann cells, showed 8 weeks after cell transplantation in Epineurium with cell group by using EnVision kit. Microscopic immunodetection of S-100 (pink) and BrdU (brown) in the site of nerve cut and HFSCs injected in. The cells incubated with bromodeoxyuridine (BrdU) 72 hours before transplantation, then paraffin-embedded sections immunostained with antibodies against BrdU (diaminobenzidine (DAB), brown) and S-100 (permanent red, red). Scale bars = 40 µm.
**Figure 5.** Electrophysiological waves for Injury, Epineurium, and Epineurium with Cells groups 8 weeks after surgery. Amplitude and latency are shown for each group. The time calibration bar was 2 ms and the amplitude calibration bar was 10 mV. The stimulation intensity was 2.3 mA and the duration was 0.1 ms.

**Figure 6.** Graphs showing the electrophysiology tests of latency; the results of electrophysiology tests of latency showed there were statistically significant differences between the Injury group and the experimental groups (Epineurium and Epineurium with Cells). Error bars represent means ± SD (n=5).
Figure 7. Graphs showing the electrophysiology tests of amplitude: the results of electrophysiology tests of amplitude. The amplitude were statistically significant in two by two comparisons (P< 0.05). Error bars represent means ± SD (n=5).
Figure 8: Histology assessment of the sciatic nerve 8 weeks after the operation. The sciatic nerve distal to the nerve transaction repair site was harvested. Semi-thin (500 nm) sections were stained with Toluidine Blue and were seen by light microscopy. 

A) Injury 
B) Epineurium 
C) Epineurium with cells (arrows show more myelination). Scale bars =20 µm (A), 50 µm (B, C).
Figure 9. Graphs showing the density of myelinated and unmyelinated fibers; results of histological examination. Statistical analysis showed significant difference in myelin thickness of groups (P< 0.05). The only statistically significant pairwise comparison was referred to injury and sham group. Error bars represent means ± SD (n=5).
Figure 10. Graphs showing the number of myelinated and unmyelinated fibers. The number of nerve fibers density were significantly different between groups. Pairwise comparisons showed significant difference between injury group with others (P< 0.05). Error bars represent means ± SD (n=5).

Table 1: comparison of amplitude, latency, myelination, number of axons between four groups of study.
| Group          | Value   | p-Value | F Value | df1 | df2 | p-Value | 
|----------------|---------|---------|---------|-----|-----|---------| 
| Sham injury    | 32.58(1.40) | 0.001   | 16.27/3 | 1.30(0.28) | 10.94/3 | 1.93(0.06) | 49.33(3.22) | 7.22/2  | 0.027 |
| Epineurium     | 7.96(2.42)  | 0.012   | 1.66(0.64) | 1.33(0.07) | 645.33(28.02) |  |
| Epineurium+Cell| 12.20(3.45) | 0.022   | 1.60(0.23) | 1.46(0.12) | 806.67(66.58) |  |