Role of Adipose Tissue-Derived Stem Cells versus Differentiated Schwann-like cells Transplantation on the Regeneration of Crushed Sciatic Nerve in Rats. A Histological Study

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Abstract

Background & objectives: Despite surgical advances, recovery of peripheral nerve injuries has often been poor, leading to irreversible impairment. This study aimed to differentiate adipose-derived stem cells (ASCs) into Schwann-like cells (SCs) in vitro and assess their role versus the undifferentiated ASCs in the regeneration of crushed sciatic nerves in adult male albino rats.

Materials & Methods: We performed a simple and less costly method to differentiate ASCs into SCs. Forty rats, weighing 200-250 g, were randomly divided to 4 equal groups. Group I (control) subjected to sham operation. Group II subjected to crush injury of the sciatic nerve. Group III subjected to crush injury with local transplantation of ASCs. Group IV subjected to crush injury with local transplantation of differentiated Schwann-like cells. The rats were sacrificed 4 weeks later. We studied the nerves using H&E and Masson's Trichrome stain. Immuno-histochemical studies using S-100 and neurofilament-H (NF-H), together with morphometric and statistical studies were done.

Results: Differentiated Schwann-like cells adopted a spindle-like morphology and 87.9% of the cells became GFAP positive and 90.4% were positive to S-100. Group II showed thin discontinuous nerve fibers with proliferation of SCs. Significant increase in collagen area percentage, and significant decrease in S-100 & NF-H immune reaction was noted as well. Group IV revealed better regeneration of axons, and higher intensity of immune reaction by S-100 & NF-H, than Group III. Meanwhile, both Groups III & IV showed thicker, more packed nerve fibers with significant decrease in collagen area percentage compared to Group II.

Conclusion: Our results suggested that differentiated Schwann-like cells might have a more beneficial role than ASCs, for treatment of peripheral nerve injuries.

Keywords: ASCs; Schwann cell differentiation; Sciatic nerve crush injury; S-100; NF-H.

Introduction

There is a high incidence of peripheral nerve injuries throughout the world, and these injuries represent a major economic problem for the society. Despite surgical advances, functional recovery of these patients is relatively poor [1]

Several approaches have been proposed to exert beneficial effects on peripheral nerve regeneration including application of an electrical field, administration of neurotrophic factors, and use of autologous nerve grafts [2,3,4]

Recently, cell-based therapy has been proposed as an efficient method for regenerating injured nerves. Transplantation of Schwann cells (SCs) or stem cells of various origins which differentiate towards Schwann cell-like phenotype, stimulate peripheral nerve repair [5,6]

SCs are key regulators of the regeneration process of the injured nervous tissue by providing structural support and guidance for peripheral nerve regeneration following injury, releasing neurotrophic factors and formation of myelin sheath [1,6,7].

However, the isolation of a sufficient number of Schwann cells for clinical practice is hindered by donor morbidity and low cell yield [8,9]. Their autologous transplantation is highly traumatic, and these cells are difficult to expand in vitro [6].

That’s why alternative sources of neuronal stem cells, such as mesenchymal lineages are now proposed. Under appropriate conditions, mesenchymal stem cells (MSCs), whatever their origin, selectively differentiate not only into mesenchymal lineages but also endodermal and ectodermal cell lineages in vitro [10,11].

The adult MSCs were first discovered to be resident in the bone marrow. But bone marrow aspiration is excessively painful for patients and yield small numbers of harvested cells [11,12].
On the contrary adipose-derived stem cells (ASCs), can be easily obtained and expanded extensively in vitro for use in autologous cell therapy [6].

Currently, co-culture with SCs or addition of glial growth factors are often employed for Schwann-like cells like differentiation of ASCs. It has been reported that production of Schwann-like cells from stem cells depend on a chemical growth factor cocktail, including forskolin (FSK), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and heregulin (HRG)[13]. However, these methods are usually complicated or expensive. In the current study, we utilized trophic factors secreted from rat sciatic nerve leachate, after being cut and soaked in culture medium, to rat ASCs to enhance the differentiation of ASCs into SCs in vitro [1,4,15].

In this study, the effect of ASCs and ASCs differentiated into Schwann-like cells was assessed in a rat model of crushed sciatic nerve injury.

**Materials and methods**

**Isolation and culture of ASCs**

The inguinal pad of fat of five male Wister rats weighing 100-150g was harvested and put in a sterile tube filled with 5ml PBS. The fat was washed extensively and cut into small pieces (1mm3). Adipose tissue was dissociated enzymatically for 80 minutes at 37°C in the incubator, using 0.1% Collagenase type I (Sigma-Aldrich, St. Louis, USA) in PBS with intermittent shaking every 10 minutes. The collagenase activity was blocked by adding an equal volume of complete medium. The suspension was centrifuged at 1800rpm for 10 minutes. The pellet cells were suspended in complete medium (DMEM with 10% FBS & 1% Penicillin/Strept). The initial cell density was 1×10^5 / cm² and seeded into 25 cm² tissue culture flasks and then incubated in a humidified incubator at 37°C, with 5% CO₂. After 24 hours, the non-adherent cells were aspirated, and fresh culture media was added. When the cells reached confluence nearly day 7 from primary culture, passing of cells was done. The rat ASCs subjected to 3-5 passages before being used [16].

**Characterization of ASCs**

The third passage ASCs were fixed by freshly prepared pre-cooled (-20°C) mixture of acetone/methanol (1:1) for 15 minutes. The cells were incubated with 10% hydrogen peroxide for 15 minutes to block the action of endogenous peroxidase. After being washed, Ultra V-block was added for 5 minutes to reduce non-specific background staining. Then the cells were incubated separately with 80-100µl of the primary antibodies (CD34, CD44 and CD105)monoclonal mouse anti-human antibodies (Thermo Fisher Scientific, Lab vision, USA) for 2 hours in a humidified chamber. The cells were washed well with PBS for 5 minutes and carefully dried. Incubation was done with Biotinylated Goat Anti-Polyvalent secondary antibody in a dilution of 1:400 for 30 minutes in a humidified chamber, then washed with PBS for 5 minutes. The cells were incubated with Streptavidin Peroxidase for 30 minutes in a humidified chamber and were washed well with PBS. Diaminobenzidine tetrahydrochloride (DAB) was added to the cells for 10 minutes and counterstained with hematoxylin for 1 minute and washed well [17].

**Differentiation of ASCs into Schwann-like cells in vitro**

The sciatic nerves were harvested from five rats weighing 100-150gm after being sacrificed, under aspetic conditions. The nerves were cut into 0.5cm fragments, washed with PBS, and incubated in complete medium at 37°C and 5% CO₂ for 48 hours. Then the conditioned medium was aspirated and filtered through an 80-micron nylon mesh to remove the fragmented sciatic nerves. The medium was then filtered through an 0.2-micron strainer to eradicate microorganism contamination. This medium was used as an induction culture medium for ASCs at P3 to differentiate into Schwann-like cells. The cells were then incubated in this medium for 5 days, at 37°C, with 5% CO₂. The induction culture medium was changed every 2-3 days [1].

**Immunohistochemistry for Schwann cell markers**

The morphology of the cells was assessed by an inverted microscope. The Schwann-like cells were also characterized by using streptavidin-biotin immunoperoxidase technique for the primary antibodies: anti-glial filament acidic protein (GFAP, mouse monoclonal, 1:400, Thermo Fisher Scientific, USA) and anti-S-100 protein (mouse monoclonal 1:500, Thermo Fisher Scientific, USA).

**Animal model of crushed sciatic nerve injury**

Forty adult male Wister rats weighing 200-250g were used in this study. Animals were kept under standard housing conditions according to the guidelines for the care and use of laboratory animals approved by the experimental animal ethical committee of Faculty of Medicine, Ain Shams University. They were randomly divided equally into 4 groups, as following:

- Group I (Sham group), rats subjected to Sham operation on their left legs. Group II (Crush group), rats were subjected to crushing of their left sciatic nerve for 2 minutes under general anesthesia. Group III (Crush group with ASCs transplantation), their left sciatic nerves were crushed under general anesthesia, as Group II, but with local transplantation of ASCs suspended in PBS (1x10⁶ cells/ml) around the crushed nerve before closure of the wound [6]. Group IV (Crush group with Schwann-like cells transplantation), the rats subjected to the same surgical procedure as group II &III, but with local transplantation of differentiated Schwann-like cells suspended in PBS (1x10⁶ cells/ml) around the crushed nerve before closure of the wound [9].

**Surgical procedures**

The rats were anesthetized by intraperitoneal injection of Ketamine (80mg/kg) Xylazine (10mg/kg). The skin was disinfected after hair removal with Betadine (povidone iodine 10%). The left sciatic nerve was exposed using the gluteal muscle splitting incision. A suture mark was taken using non-absorbable black silk midway between the origin of the sciatic nerve and its trifurcation. Ten centimetres long straight artery forceps were applied and locked, 0.5 cm just above the level of this suture, for 2 minutes. For Group III, ASCs suspended in PBS (1x10⁶ cells/ml)
were transplanted around the crushed nerve before closure of the wound. For Group IV, differentiated Schwann-like cells suspended in PBS (1x10^6 cells/ml) were transplanted around the crushed nerve before closure of the wound. The wound was then closed in layers, by interrupted sutures, using absorbable 5/0 polyglactin 910, Vicryl. The rats received postoperative systemic antibiotics: Ceftriaxone 100 mg/kg body weight. Their postoperative pain was controlled using Declofenac Sodium ( Cataflam 75 mg/3 ml ampoule, 50 mg/kg body weight, Intramuscular). Daily dressing of the wound was applied using Betadine and Bivacyn Spray [18].

**Histological study**

After 4 weeks, the animals were sacrificed by decapitation. The left sciatic nerves of the control group and the left crushed sciatic nerves of all experimental groups were collected. The specimens were fixed in 10% buffered formalin to be processed, and 5-μm-thick sections were prepared to be stained with H&E and Masson’s trichrome stain.

**Immunohistochemical study**

Paraffin sections of left sciatic nerves were placed in 3% H₂O₂ for 10 minutes to block endogenous peroxidase. Then, boiled for 10 minutes in 0.01 M sodium citrate buffer (pH 6). Slides were then blocked for 15 minutes with anti-species serum and washed with 0.01 M phosphate-buffered saline (pH 7.4). Rabbit anti-S-100 polyclonal antibody (1:200, Sigma) and rabbit anti-neurofilament-heavy (NF-H) polyclonal antibody (1:200, Abcam) were added and incubated at 4°C overnight. Biotinylated goat anti-rabbit IgG was added for 20 minutes at 37°C. Streptavidin and biotinylated horseradish peroxidase were added for 20 minutes at 37°C. The labeled antigens were then visualized using 3,3’-diaminobenzidine. Brown staining indicated positive protein labeling [19].

**Morphometric and statistic study**

An image analyzer Leica Q win V3 program installed on a computer connected to a Leica DM2500 microscope (Wetzlar, Germany) was used. Animals from all groups were subjected to morphometric study. Measurements were taken from three different slides obtained from each animal. Five haphazardly selected non-overlapping fields were examined for each slide at objective lens x 40.

The following parameters were quantized:

1. Mean of area percentage of S-100 positive brown reaction at the crush site of sciatic nerves.
2. Mean of area percentage of NF-H positive brown reaction at the crush site of sciatic nerves.
3. Mean of area percentage of collagen fibers in Masson’s trichrome stained slides.

Mean and standard deviation (SD) of measured parameters were statistically analyzed using one-way ANOVA with post-hoc test using SPSS statistical program version 21 (IBM Inc., Chicago, Illinois, USA). Probability value of P value < 0.05 is considered significant [20].

**Results**

**Morphology of primary culture and frequent subcultures of ASCs**

Examination by phase contrast microscope on day 7 from primary culture, the attached ASCs appeared confluent (90%). The cells were different in shape, some cells appeared triangular, and others were spindle-shaped while some of them were star-shaped with multiple processes and vesicular nuclei (Fig. 1a). On day 4 after the 1st passage (P1), the ASCs reached 80% confluence, and were ready for a second subculture (Fig. 1b). On day 3 after the 2nd passage, the ASCs (P2) reached 80% confluence and were ready for subculture (Fig. 1c). On day 3 after the 3rd subculture, the ASCs (P3) reached 80% confluence and were ready to use (Fig. 1d). After frequent passing of the ASCs, the cells became homogenous in morphology, having a fibroblast-like appearance.

![Figure 1](image)

**Immunophenotyping of ASCs**

The ASCs of the 3rd passage was fixed and characterized immunohistochemically. Using CD44 antibody, most of the cells showed brown positive cytoplasmic immune reaction (Fig. 2a). Meanwhile, on using the primary antibody for CD105, most of the cells also showed positive brown cytoplasmic reactivity (Fig. 2b). In contrast, all the cells were immune negative to the surface marker, CD45, specific to the hematopoietic stem cells (Fig. 2c).

Figure 2: (a) Positive brown immune reaction of ASCs for CD44 in the cytoplasm as well as the cell processes. (b) Positive brown cytoplasmic immune reactions of ASCs for CD 105. (c) ASCs negatively immunostained for CD45. Avidin-biotin Peroxidase x 200

Morphology of Schwann-like cells in culture and their immuno-histochemical characterization

Most of the ASCs cultured in the differentiation media changed from a fibroblast-like morphology to a more elongated, slender, spindle shape, similar to that of Schwann cells in culture. Furthermore, a minority of cells displayed a bipolar cell body with two processes. In contrast to the overlapping, clustering and random pattern previously showed by the undifferentiated ASCs, the differentiated cells proliferated in a longitudinal, parallel manner typical to that observed in cultured Schwann cells (Figs. 3a & 3b). The Schwann-like cells were fixed and characterized immuno-histochemically. Upon using S-100, 90.4% of the Schwann-like cells showed positive brown reaction in their cytoplasm (Fig. 3c). Moreover, 87.9% of the cells were positive to GFAP (Fig. 3d).

Histological and morphometric results of the sciatic nerve

H&E-stained sections of group I (control group) showed well packed, parallel, thick, myelinated, acidophilic nerve fibers. The nerve fibers showed a characteristic wavy pattern. Among the nerve fibers pale, basophilic, oval nuclei belonging to Schwann cells were seen together with other nuclei belonging to the cells of the endoneurium. Nodes of Ranvier, where the ends of two adjacent Schwann cells meet, were also seen. The node appeared as a constriction of the neurilemma. The epineurium, formed of dense irregular connective tissue, was seen surrounding the nerve fascicles (Fig. 4a). Sciatic nerve of group II (spontaneous recovery group), showed thin, discontinuous nerve fibers, with many vacuolations, and many mono-nuclear inflammatory cells infiltrating the vicinity of the nerve. Traumatic degeneration

Figure 4: (a) Sciatic nerve of group I, showing the nerve fibers acidophilic, parallel, thick, wavy and well packed with Schwann cells nuclei between them (↑ T). Nodes of Ranvier appear as a constriction of the neurilemma (squares). The epineurium appears surrounding the nerve fascicles (arrowhead). (b) Sciatic nerve of group II, showing thin & discontinuous nerve fibers. Notice the extensive axoplasmic vacuolations (arrowhead), and mononuclear cells infiltration at the site of crush (squares). The proliferated SCs nuclei are forming bands surrounding discontinuous & disrupted axons (↑ T). (c) Sciatic nerve of group III, showing packed & continuous fibers, with proliferating SCs nuclei arranged in columns around them (↑ T). However, few vacuolations (arrowheads), and inflammatory cells are still obvious (squares). (d) Sciatic nerve of group IV showing most of the nerve fibers is closely packed & continuous. SCs nuclei arranged in columns (↑ T) forming an endoneurial tube (arrowhead) with large basophilic macrophages inside (↑ T). Hematoxylin & Eosin ×400

left empty spaces at the site of crush. Schwann cells nuclei were arranged in columns, forming the bands of Büngner to direct the sprouting axons (Fig. 4b). Group III (ASCs treated group) showed thicker, more packed, well oriented and continuous nerve fibers, with less vacuolations than the non-treated nerves of group II. SCs were proliferated and arranged in bands (Fig. 4c). Group IV (SCs treated group) also showed thicker, more packed, well oriented and continuous nerve fibers, with less vacuolations compared to the non-treated nerves of group II, being slightly even more continuous and thicker than group III. Some SCs were seen arranged in a cylindrical manner forming an endoneurial tube, with large macrophages removing the debris from inside the tube (Fig. 4d).

Histological examination of Masson’s Trichrome stained sections of group I (control group), showed packed, parallel, thick & wavy pink nerve fibers, surrounded and separated by the endoneurium. The endoneurium consisted of delicate loose connective tissue. The green collagen fibrils were apparent, running parallel to and around the nerve fibers. SCs nuclei were also evident between the nerve fibers (Fig. 5a). Group II (spontaneous recovery group), showed thin discontinuous nerve fibers, and separated by thick green fibrous tissue scarring, with many vacuolations (Fig. 5b). Fibrous tissue invasion was minimal in group III (ASCs treated group). However, the endoneurium was not well recognized (Fig. 5c). Fibrous tissue invasion was also minimal in group IV (SCs treated group) (Fig. 5d). Masson’s Trichrome area percentage was significantly higher (P<0.05) at the crush site of sciatic nerves of group II compared to group I. On the other hand, there was a highly significant decrease (P<0.05) in the mean surface area percentage stained by Masson’s Trichrome at the crush site of sciatic nerves of group III & Group IV compared to Group I. Group IV also showed no significant change (P>0.05) compared to group III (Table 1, Histogram 1).

**Figure 5:** (a) Longitudinal section of the sciatic nerve of group I, showing the nerve fibers are surrounded and separated by the delicate green collagen fibrils of the endoneurium. (b) While, the sciatic nerve of group II, showing thick dense fibrous tissue scarring (↑) replacing the nerve fibers. (c) The sciatic nerve of group III, showing minimal fibrous tissue between nerve fibers (↑). However, the endoneurium is not well recognized. (d) The sciatic nerve of group IV, showing minimal fibrous tissue between nerve fibers (↑). The endoneurium is also not well recognized. Masson’s Trichrome × 400

**Figure 6:** (a) Group I showing positive brown immune reaction for S-100 in the cytoplasm as well as the nuclei of Schwann cells that were observed in between the sciatic nerve fibers (Fig. 6a). Minimal positive brown immune reaction was detected in SCs of group II, with thinner, disoriented, and

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**Immuno-histochemical analysis of sciatic nerve**

Sections from the group I demonstrated positive brown immune reaction for S-100 in the cytoplasm as well as the nuclei of Schwann cells that were observed in between the sciatic nerve fibers (Fig. 6a). Minimal positive brown immune reaction was detected in SCs of group II, with thinner, disoriented, and
discontinuous nerve fibers (Fig. 6b). The positive brown reaction of S-100 was significantly increased in group III compared to group II (Fig. 6c). Intense brown positive immune reaction was seen in group IV, with more packed and thicker nerve fibers (Fig. 6d). The mean area percentage of S-100 positive reaction at the crush site of sciatic nerves of group II significantly decreased (P<0.05) when compared to group I. On the other hand, there was a highly significant increase (P<0.05) in the mean surface area percentage of S-100 positive reaction at the crush site of sciatic nerves of group III & group IV compared to group I. Group IV also showed a significant increase (P<0.05) in the mean area percentage of S-100 positive reaction at the crush site of its sciatic nerves compared to group III (Table 1, Histogram 2).

We further examined the regenerating nerve by immunohistochemistry against NF-H, an axonal cytoskeleton protein.

Group I revealed positive brown immune reaction for NF-H that were observed within the axoplasm of sciatic nerve fibers (Fig. 7a). While, minimal positive brown immune reaction was detected in the discontinuous nerve axons of group II (Fig. 7b). The positive brown reaction of NF-H was significantly increased in group III compared to group II(Fig. 7c). More intense brown positive immune reaction was detected in group IV, within the more packed regenerated nerve axons(Fig. 7d). The mean area percentage of NF-H positive reaction at the crush site of sciatic nerves of group II significantly decreased (P<0.05) when compared to group I. On the other hand, there was a highly significant increase (P<0.05) in the mean area percentage of NF-H positive reaction at the crush site of sciatic nerves of group III & group IV compared to group I. Meanwhile, group IV also showed a significant increase (P<0.05) in the mean area percentage of NF-H positive reaction compared to group III (Table 1, Histogram 3).

We further examined the regenerating nerve by immunohistochemistry against NF-H, an axonal cytoskeleton protein.

Group I revealed positive brown immune reaction for NF-H that were observed within the axoplasm of sciatic nerve fibers. The positive brown reaction of S-100 was significantly increased in group III compared to group II (Fig. 6c). Intense brown positive immune reaction was seen in group IV, with more packed and thicker nerve fibers (Fig. 6d). The mean area percentage of S-100 positive reaction at the crush site of sciatic nerves of group II significantly decreased (P<0.05) when compared to group I. On the other hand, there was a highly significant increase (P<0.05) in the mean surface area percentage of S-100 positive reaction at the crush site of sciatic nerves of group III & group IV compared to group I. Group IV also showed a significant increase (P<0.05) in the mean area percentage of S-100 positive reaction at the crush site of its sciatic nerves compared to group III (Table 1, Histogram 2).

**Table 1:** Mean ± SD of area percentage of collagen fibers, S-100 and NF-H immune expression at site of crush in all groups.

<table>
<thead>
<tr>
<th>Masson’s Trichrome collagen area %</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>5.822 ± 0.803</td>
<td>19.514 ± 3.035</td>
<td>3.178 ± 1.101</td>
<td>2.762 ± 0.441</td>
</tr>
<tr>
<td><strong>S100 Positive area %</strong></td>
<td>3.796 ± 0.641</td>
<td>1.578 ± 0.331</td>
<td>10.928 ± 1.086</td>
<td>12.758 ± 1.355</td>
</tr>
<tr>
<td><strong>NF-H positive area %</strong></td>
<td>8.108 ± 1.958</td>
<td>3.177 ± 0.881</td>
<td>6.586 ± 1.003</td>
<td>7.957 ± 1.585</td>
</tr>
</tbody>
</table>

*a= highly significant change compared to Group I. b= highly significant change compared to Group I. c= highly significant change compared to Group I. d= highly significant change compared to Group II. e= highly significant change compared to Group II. f= non-significant change compared to Group III.*

**Figure 7:** (a) Group I showing positive brown immune reaction for NF-H (↑) in the axons of nerve fibers. (b) Group II showing minimal positive brown immune reaction for NF-H (↑), in the thin, disoriented axonal sprouts. (c) Relative increase in the positive brown immune reaction for NF-H in group III compared to group II (↑). (d) Group IV showing strong positive brown immune reaction for NF-H in the regenerated closely packed axons, compared to group II & III (↑). Streptavidine Peroxidase for NF-H×400.

**Histogram 2:** Mean area% of S-100 immune expression in sciatic nerve fibers in different groups.

**Histogram 3:** Mean area% of NF-H immune expression in sciatic nerve fibers in different groups.

Discussion

The functional recovery of patients with peripheral nerve injury never reached the pre-injury level following nerve anastomosis, so the need for neurotrophic factors or cell guidance is necessary. Stem cells can solve this problem, by self-proliferating and differentiating to replace cells lost[215]. Candidate cells for transplantation should be easy to obtain by ethically acceptable methods, besides, they should be able to differentiate into the target cells and/or secrete essential factors to stimulate regeneration.

In this study, adipose tissue was our preference as a potent and easy to harvest source of stem cells. We did not only target using ASCs, but we also aimed to assess a new method to trans-differentiate those ASCs into a Schwann-like phenotype in-vitro. And then we compared the effect of those Schwann-like cells to the effect of ASCs in the regeneration of crushed sciatic nerves.

As part of our observation during tissue culture, passages of cells was performed twice a week, indicating the high growth rate of cells isolated from adipose tissue, this was consistent with [22], who described an average population doubling time of 45.2 hours for ASCs, in contrast to 61.2 hours for BMSCs. If translated to human studies, this could mean a reduction in the time required to generate a therapeutically useful stock of cells.

Moreover, many studies declared that ASCs were able to improve nerve regeneration in vivo and in vitro [2,3,24]. Following nerve injury, adult Schwann cells present in the sciatic nerve stumps can produce several neurotrophic factors, such as brain derived neurotrophic factor (BDNF), nerve growth factor NGF and neurotrphin-3 (NT-3), that make it possible for stem cells to differentiate into neuron-like cells[1,25].

The present study tried the method described by Liu et al. ASCs were differentiated into Schwann-like cells by using the leachate of the sciatic nerve. We cut the sciatic nerve into small pieces, simulating a state like nerve injury, and incubate them in culture medium for two days. Many growth factors were secreted by the sciatic nerve and elaborated in the culture medium. Then, the sciatic nerve leachate was used as an induction medium for ASCs trans-differentiation into Schwann-like cells [1].

Five days after soaking ASCs in this induction medium, nearly all cells showed morphological changes in the form bi- or tripolar and elongated spindle shapes. Differentiated ASCs also displayed thin cytoplasmic extensions and large nuclei similar to that of Schwann cells; they were elongated, slender and growing in a longitudinal parallel manner. Furthermore, immuno-histochemical analysis showed that 90.2% of the cells were positive for S-100 and 87.9% of the cells were positive to GFAP.

All the characteristics of differentiated ASCs were similar to genuine SCs. These results agreed with those of Liu et al.[1].

Although the method performed by the present study was effective and practical for trans-differentiation of ASCs into Schwann-like cells in vitro, further investigation is needed to detect which factor(s) played a key role in this differentiation.

In the current study, the tool and duration, in addition to the site of crushing were all the same in Group II, III & IV. For Group III & IV, ASCs & differentiated Schwann-like cells were locally transplanted around the crushed sciatic nerve, on table, before closing the wound in layers, directly after the crushing. Unlike other studies, where systemic injection of the stem cells was performed[26,27].

We used longitudinal sections of left sciatic nerves of all groups to evaluate the nerve continuity four weeks postoperative. Light microscopic examination of sciatic nerves of Group II (spontaneous recovery) showed degeneration of most of the sciatic nerve fibers with degenerative axoplasmic vacuolations at the site of crush resulting from Wallerian degeneration. Axons were thin, less compact, irregular, discontinuous, and heavy inflammatory cell infiltrate was also seen. These findings were in accordance with reports of other authors[26,27,28,29].

We also detected proliferation of SCs, forming columns around discontinuous sciatic nerve fibers with some nerve sprouts inside. This could be explained that in case of peripheral nerve injury, SCs undergo dedifferentiation (non-myelinating phenotype), which results in decreased expression of myelin specific proteins and increased secretion of several glial growth factors. Under the influence of glial growth factors, Schwann cells divide, proliferate, and arrange themselves into cellular bands or columns along their external laminae called the bands of Büngner to guide the growth of new nerve sprouts of regenerating axons[30].

In contrast, sciatic nerves of Group III (ASCs treated) showed that most of the Schwann cell columns were invaded by sprouts of regenerating nerve fibers. The axons were more continuous and packed.

In addition, Group IV (SCs treated) showed that most of the Schwann cell columns were invaded by sprouts of regenerating nerve fibers. The axons were even more continuous and more packed compared to Group III. Endoneurial tubes were detected with macrophages inside their lumen. This could be attributed to the fact that SCs together with macrophages are directed to remove the myelin debris and degenerated axons from those tubes, after nerve injury, to promote regeneration [31].

Moreover, on examination of Masson’s Trichrome stained sections of the sciatic nerves. Group II lacked the normal delicate loose connective tissue endoneurium. Instead, it showed extensive deposition of green collagen fibers, replacing nerve fibers and filling the spaces between the disrupted axons. This can be explained by the heavy immune cell infiltrate seen in H&E sections of this group. As explained by Lemke et al.; following nerve injury and breakdown of the blood-nerve barrier, immune cells such as T-cells and macrophages, were recruited around the epineurium and entered the nerve, causing peri- and intraneurial fibrous tissue deposition and scarring. This has been one of the major challenges following injury and surgical procedures of peripheral nerves. Scarring often resulted in pain and nerve dysfunction[29].

On the other hand, no fibrous tissue invasion was seen at the...


... crush site of Group III & IV supporting better tissue healing. But, surprisingly, the amount of stained collagen fibers seen at the crush site, was even significantly lower (P<0.05) than that seen in the endoneurium of the control group. The area percentage of Masson’s Trichrome staining was also significantly lower than that of Group II (P<0.05). That’s why; we recommend longer studies that may assess the nerve regeneration later than 4 weeks after injury, to assess the exact timing and mechanisms needed for the establishment of a proper endoneurium. We assumed Schwann cells were preoccupied directing the nerve sprouts and secreting the necessary growth factors rather than forming the supporting endoneurium during those first four weeks after injury.

Concerning immuno-histochemical analysis of S-100, and consistent with Di Summa et al. and Abd El Samad et al., Group II showed a significant decrease (P<0.05) in the immune reaction of S-100 compared to Group I (control). S-100 is positive in actively myelin-forming SCs, supporting very weak myelin sheath formation in sciatic nerves in this group, that received no treatment [23, 28].

Treatment with ASCs also showed a significantly (P<0.05) higher S-100 expression than the untreated nerves of Group II, proving better myelin formation. Moreover, S-100 expression of this group was significantly higher (P<0.05) than the control group. This agreed with the findings of other authors who suggested that, during nerve regeneration, the myelination of nerve fibers shown by the intensity of the immune reaction of S-100, was a valuable marker to determine the intensity of nerve regeneration [32], as well as the Schwann cell integrity [25]. The reaction of S-100 appeared to be predominantly found in active myelin-forming SCs and correlated directly with the thickness of the myelin sheath formed by these SCs. Thus, the expression of S-100 was considered a marker to evaluate the intensity of nerve regeneration [3].

In addition, nerves treated with SCs in Group IV showed the highest S-100 expression among the four groups, being significantly higher than Group I & II, and higher than Group III (P<0.05), proving very good myelin formation and indicating the best nerve regeneration intensity [3].

Furthermore, we studied the axonal intermediate filament protein subunit neurofilament heavy (NF-H). Group II showed a significant decrease (P<0.05) in the immune reaction of NF-H compared to Group I (control). NF-H is abundant in axoplasm of axons and is crucial for radial growth of axons during development, axonal regeneration after injury, maintenance of axon diameter and nerve conduction velocity [33].

Transplantation of ASCs at the site of crush (Group III) lead to a significantly (P<0.05) higher NF-H positive regenerating nerve fibers expression than the untreated nerves of Group II. Meanwhile, treatment with SCs in Group IV also showed a significant increase (P<0.05) in the mean area percentage of NF-H positive reaction compared to Group III, indicating more NF-H positive regenerating nerve fibers. These results were coincident with other authors who suggested that BM-MSCs were capable to differentiate into myelinating cells and could be applied to induce nerve regeneration [34,35]. In addition, Carriel et al demonstrated that the degree of intensity of NF expression could be directly related to the structural stabilization and maturation of the axonal cytoskeleton [36].

Researchers have studied many mechanisms through which ASCs can be used to repair and regenerate tissues. ASCs delivered into an injured or diseased tissue secrete cytokines and growth factors that stimulate recovery in a paracrine manner. The ASCs also modulate the “stem cell niche” of the host by stimulating the recruitment of endogenous stem cells to the site of injury and promoting their differentiation into the required lineage pathway [37].

ASCs might also provide antioxidants, and free radical scavengers. As a result, toxic substances released into the local environment would be removed, thereby promoting recovery of the surviving cells [38].

Our results demonstrated that differentiated Schwann-like cells might have a more beneficial role than ASCs, for treatment of peripheral nerve injuries. This could be due to the crucial role of SCs in nerve regeneration. SCs could also up-regulate extracellular matrix molecules such as laminin and fibronectin [39].

On the other hand, the beneficial effects of undifferentiated ASCs showed in our study could not be ignored and was also confirmed by former studies indicating that undifferentiated BMSCs could also enhance regeneration and improve motor function. This improvement could be attributed to some form of trans-differentiation that have occurred in vivo, after micro-environmental changes caused by local signals from injured Schwann cells and axons [39,40].

However, some studies believed that only differentiated bone marrow MSCs, rather than untreated MSCs, could stimulate nerve regeneration [41,42]. Similarly, Kingham et al., proposed that only differentiated ASCs could enhance nerve regeneration [13]. In contrast, Orbay et al., demonstrated that ASCs and differentiated ASCs could equally promote nerve healing in case of nerve gaps [25].

Conclusion

The results of the present study indicated that differentiated Schwann-like cells had a more beneficial therapeutic role than undifferentiated ASCs, in peripheral nerve regeneration of a rat model of nerve crush injury. However, the relevance of the therapeutic potential of undifferentiated ASCs could not be ignored. So, both might represent a valuable tool for stem cell-based therapy in traumatic, inflammatory and degenerative diseases of the PNS.

Recommendations

We recommend using adipose tissue as a source of MSCs, as the ASCs were found to be more abundant and easier to harvest. We do appreciate using the filtered leachate of sciatic nerve in culture medium, as an induction medium for differentiation of ASCs into Schwann-like cells in vitro. This is a less expensive and simpler method compared to conventional ones, yet successful.
Further studies trying longer duration are needed, to assess the exact time needed to establish proper endoneurium.

Conflicts of interest
The authors have no conflicts of interest.

References
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