Bone marrow stromal cells implantation and suture repair of peripheral nerve: A comparative study of functional, histopathological, morphometric and relative gastrocnemius muscle weight in rabbits

Al-Timmemi, H. A.1*, Ibrahim, R.1, Zuki, A. Z.2, Azmi, T. I.2 and Al-Jashamy, K.3

1Department of Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.
2Department of Preclinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.
3SIGA, Medical School, Kuala Lumpur, Malaysia.

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The peripheral nervous system has the ability to regenerate after injury. Peripheral nerve injuries are caused by penetrating injury, crush, traction and ischemia compression. However, the availability of various nerve coaptation and other techniques for the attainment of functional nerve regeneration is still inadequate. The objective of this study was to compare the effectiveness of bone marrow stromal cells (BMSCs) implantation and epineural nerve suture on peripheral nerve regeneration in a rabbit model. Ten male New Zealand white rabbits were divided into two groups. In the primary epineural repair group (control group), the left sciatic nerve was skeletonized from the sciatic notch to the point of bifurcation, with the nerve been transected at the mid-shaft of the femoral bone and repaired with six epineural sutures. In the treated group, the epineural repaired nerve was implanted with BMSCs in the proximal and distal segments of the transected sciatic nerve. Assessment of the nerve regeneration was based on functional (motor and sensory), histological and morphometric criteria, including the number of myelinated nerve fibers, nerve fiber diameter, axon diameter, myelin sheath thickness, g ratio and relative gastrocnemius muscle weight. The results of the examination showed that the treated group had the best regeneration and functional recovery.

Keyword: Bone marrow stromal cells (BMSCs), histopathology, morphometric, peripheral nerve, regeneration.

INTRODUCTION

Peripheral nervous system injuries include penetrating injuries, crush, traction and ischemia compression (Robinson, 2004). Microsurgical suture repair remains the current gold standard in clinical practice (Lundborg, 2000), but obvious deficiencies remain with this technique, given that surgical repair of peripheral nerves does not result in complete functional recovery. Recent approaches have been directed towards biological factors to promote an environment conducive for growth and overcome limitations in regeneration and functional recovery.

The mesenchymal stem cells (MSCs) have become one of the most interesting targets for the study of tissue and organ regeneration because of their plasticity (Prokop, 1997). The implantation of neural stem cells, bone marrow stromal cells (BMSCs), or fibroblasts has been shown to exert a beneficial effect on peripheral nerve regeneration (Mimura et al., 2004). Thus, cell transplantation has been proposed as a method of improving peripheral nerve regeneration (Cuevas et al., 2002). The MSCs are an expression of many cytokines and cellular factors (Pitlenger et al., 1999; Bhagavati and Xu, 2004). This study was aimed to compare the effectiveness of bone MSCs implantation and epineural...
nerve suture on peripheral nerve regeneration in a rabbit model.

MATERIALS AND METHODS

Experimental animals design

Ten male New Zealand white rabbits (3 to 5 months old) weighing between 2.0 and 2.3 kg (animals unit, UKM, Malaysia) each were selected. The animals were kept in separate cages and given broad-spectrum antibiotics and antihelminic. All procedures used in this study were approved by the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Animal Care and Use Committee (08 R13/Dec '08). Rabbits were randomly divided into two groups (n = 5). The two groups comprised of animals of coaptated transected sciatic nerve with epineural nerve suture (ENS) as the control group and the bone marrow stromal cells implantation group were the second group. The animals from both groups were euthanized on day 112 post operations (PO).

Stem cells preparation, identification and surgical procedure

The BMSCs were collected from the ilium bone of rabbits, and preparation, identification, differentiation and surgical procedure were done as described by Al-Timmemi et al. (2011).

Clinical observations

Nerve functions evaluation

The motor and sensory functions of sciatic nerve clinical reflexes were evaluated daily until the end of the experiment (on day 112 PO).

Motor functions evaluation

The animals were monitored daily from the onset to assess their ability to walk from the first day to day 112 PO. They were examined for the type of walk, including crouching, crawling on heel, normal, as well as knuckling. These behaviors were classified into severe, moderate, mild and normal. The muscle contraction force was graded from weak, moderate to strong. The muscle mass atrophy was graded as severe, moderate, mild or normal.

Sensory functions evaluation

The sensory functions of the anastomosed sciatic nerves were tested daily until the end of the experiment. Toe spreading reflex, lateral aspect leg sensation, toe pinch and toe prick were evaluated as either present or absent. In addition, the foot withdrawal and vocalization tests of lateral aspect of leg sensation were also evaluated. Toe pinch and toe prick were recorded as positive responses indicating recovery and improved function.

Relative gastrocnemius muscle weight measurement (RGMW)

Following the sacrifice of the rabbit, the gastrocnemius muscle was harvested and immediately weighed. The contra lateral muscle was also harvested as negative control for weight variation between individual rabbits. Each muscle was then weighed separately using 0.0001 g weight (Sartorius Analytic Balance Model 2603, Munich, Germany) to calculate the percentage reduction in muscle mass (denervated muscle weight vs. contra lateral muscle weight). The muscles weight data were expressed as a ratio of the operated limb (left) to un-operated right limb as a negative control to calculate the RGMW.

Histopathology and histomorphometric findings

The anastomosed left sciatic nerve was exposed and harvested from each animal; three samples each of 1 cm long were collected from the proximal, middle (coaptate site) and distal segments of the coaptated sciatic nerve. Samples of the proximal and distal segment were trimmed for any excess length and divided into two parts; 5 mm long from the proximal segment each for semithin sections studies and the other for light microscopy studies. A 1 cm length nerve sample was obtained from the middle segment, which corresponded to the lesion site for longitudinal sections, for light microscopy examination. The right sciatic nerve was exposed and harvested from each animal as a negative control. The samples were fixed with 10% neutral buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 5 μm thick sections and stained with hematoxyline and eosin and Meyer’s modified trichrome stain. The specimens for semithin section were fixed with 4% glutaraldehyde overnight at 4°C. They were then dehydration, infiltrated with resin and polymerized. Following polymerization, the samples were sectioned using an ultra microtome (Leica). Semithin sections were stained with 1% toluidine blue and examined using Olympus image analysis (BX 51 TF attachment of CC 12) camera. The number of myelin nerve fibers, total fiber and axon diameters were determined using image software (Abramoff et al., 2004). The myelin thickness was derived from the differences between the fiber and axon diameter. In addition, the g ratio of each fiber was calculated as the ratio of axon diameter to the fiber diameter. The right sciatic nerve (normal right hind limb) specimen was also collected and prepared as negative control.

Statistical analysis

All data were expressed as means and standard deviations (M ± SD). Statistical comparisons between groups were performed using SPSS version 16.0 software (non-parametric tests), Kruskal Wallis and Mann-Witney tests for clinical observation and for morphometric analysis by one-way analysis of variance test (ANOVA), followed by Tukey and Duncan post-test. The p value ≤ 0.05 was considered significant.

RESULTS

Sciatic nerve functions evaluation

Motor observations

The onset and ability to walk on day 14 PO in the BMSCs group was significantly different (p ≤ 0.05) as compared to the ENS group. Crawling in the BMSC group was significantly different (p ≤ 0.05) as compared to the ENS group on day 28 PO. On day 56 PO, the type of gait became normal in the BMSCs group with a significant difference (p ≤ 0.05) as compared to ENS. On day 112 PO, the knuckling disappeared in the BMSCs group, and there was a significant difference (p ≤ 0.05) as compared to ENS. On day 28 PO, muscle force
contraction became stronger in the BMSC group with a significant difference ($p \leq 0.05$) as compared to the ENS group.

**Sensory clinical observations**

The interesting results of the sensory clinical signs included toe spread, lateral leg sensation, toe pinch and toe prick in the BMSC group which showed regained sensory reflexes with significant improvement ($p \leq 0.05$) as compared to the ENS group on day 112 PO. In addition, the foot withdrawal and vocalization tests were recorded as positive responses indicating recovery and improved function.

**Histopathological observations**

Histopathology findings on day 112 PO of the coaptated site (middle segment) of the ENS group showed presence of vacuolated and degenerative nerve fibers, granulomatous tissue surrounding the stitches and intra-neural scar tissue formation (Figure 1a). The middle segment in the BMSC sections showed less vacuolated degenerative nerve fibers, and good angiogenesis (Figure 1b).

The longitudinal section of the distal segment in the ENS sections showed mild adherence with the surrounding tissue, low vacuolated degenerative nerve fibers and the presence of collagen fibers (scar) at perineurium and epineurium and thickness of fibrous tissue at perineurium and epineurium attached with surrounding tissue (Figure 2a). The distal segment in the BMSC sections showed less vacuolated degenerated nerve fibers, minimum scar tissue and good myelination (Figure 2b).

The transverse section of distal segment of the sciatic nerve of the ENS group showed increase in the thickness of the fibrous tissue at the perineurium and epineurium which was attached to the surrounding muscles (Figure 3a). The transverse section of the distal segment of the sciatic nerve of the BMSC group showed deposition of fibrous tissue at the perineurium and endoneurium (Figure 3b). The distal sciatic nerve at 5 mm distal to the coaptated site of the ENS group showed thickness of internal perineurium, extraneurial nerve fibers and thick collagen fibers deposited in the epineurium (Figure 4a). The BMSC sections at 5 mm distal to the coaptated site showed large and small myelinated nerve fibers (Figure 4b).

**Histomorphometric and relative gastrocnemius muscle weight analysis on day 112 post operation**

Results of the negative control showed that mean values of the number of myelinated nerve fibers, diameter of fiber, myelin sheath thickness, axon diameter and g ratios

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**Figure 1.** Light micrographs on day 112 PO showing the coaptation site. (a) ENS group showed vacuolated degenerative nerve fibers (arrow heads), granulomatous tissue surrounded the stitches and fibroblast cells (arrows); H&E x100; (b) BMSCs group shows arrangement of nerve fibers, vacuolated degenerative nerve fibers (thin arrow) and good angiogenesis (thick arrow); H&E x200.
Figure 2. Light micrographs of the distal segment of the sciatic nerve on day 112 PO. (a) ENS group showing vacuolated degenerative nerve fibers (arrows), scar tissue in the epineurium and low numbers of Schwann cells (arrow head); H&E x200; (b) BMSCs group showing vacuolated degenerative nerve fibers, less scar tissue and good myelination (arrows); H&E x100.

Figure 3. Light micrograph transverse section of distal segment of sciatic nerve on day 112 PO. (a) ENS group showing thickness of fibrous tissue at perineurium and epineurium (thick arrow) attached to surrounding tissue (thin arrow), MMTS x200; (b) BMSCs group showing deposition of fibrous tissue at perineurium (arrow heads) and endoneurium (arrows), MMTS x100.

were 13292, 10.35µm, 3.47µm, 6.86µm and 0.66, respectively. Histomorphometric analysis of the proximal segment on day 112 PO showed a significant (p ≤ 0.05) decrease in the number of myelinated nerve fibers and fiber diameter in the ENS and BMSC groups as compared to the negative control group. The fiber diameter in the ENS was insignificantly different (p ≤ 0.05) as compared to the BMSC group. The axon diameter
and myelin sheath thickness in the BMSC group sections showed insigni-ficant difference (p ≤ 0.05) as compared to the negative control group, while the axon diameter and myelin sheath thickness were increased significantly (p ≤ 0.05) between the BMSC group as compared to the ENS group.

Analysis of the distal segment sections showed that the number of myelin nerve fibers was significantly different (p≤0.05) in the BMSC and ENS groups as compared to the negative control group. The fiber diameter, axon diameter and myelin sheath thickness of the ENS and BMSC groups were decreased significantly (p ≤ 0.05) as compared to that of the negative control group.

The distal segment sections showed a decrease in the number of myelin nerve fibers as compared to the proximal segment. The diameter of myelinated nerve fibers in the proximal segment sections was larger than that in the distal segment sections. The axon diameter and myelin sheath thickness in the proximal segment were greater than those in the BMSC groups as compared to the ENS group. The g ratio in the distal segment was decreased as compared to the proximal segment in the treated groups (Table 1). The transection of the sciatic nerve produced loss of neural innervation of the gastrocnemius muscle, which led to a decrease in gastrocnemius muscle mass. The statistical analysis of RGMW showed 0.57 ± 0.01 in the ENS sections as compared to 0.67 ± 0.06 in the BMSC at day 112 PO.

**DISCUSSION**

In this study, we injected equal doses of BMSCs in both the proximal and distal segments of the transected sciatic nerve, on the assumption that the inflammatory reaction at the site of coaptation might interact with axonal transported to avoid the pathological processes that might interact with the regeneration through implantation. However, this is the first study, applying stem cells implantation in both sites of transected sciatic nerve. Previously, Taniuchi et al. (1986) reported that the transection of the sciatic nerve might induce the Schwann cells and increase levels of nerve growth factor receptors in the distal segment but not in the proximal segment.

Functional results of this study showed more progress in animals of the BMSC group as compared to the ENS group. The roles of the BMSCs in this respect could be to promote the recovery function of the transected sciatic nerve due to interleukines (IL), growth factors and chemokines. Eaves et al. (1991), Ji et al. (2004) and Liu et al. (2005) reported that cytokines act as survival, growth or differentiation factors and may modulate primary sensory neurons response to injury, and thus influence pain behavior. Knuckling disappeared in all animals in the BMSC group due to functional recovery of transected sciatic nerve that was treated with MSCs on day 112 PO, which innervated the extensor and flexor muscles that control normal locomotion of the limb. The effects of BMSCs in peripheral nerve injuries are theorized.
to be supportive in nature and are of two folds. BMSCs are believed to act as Schwann cells, in that, they function to prevent neuronal cell death and promote directional axonal growth. Also, BMSCs synthesizes and secrete neurotrophic and neurotrophic factors which enhance the regeneration of the transected sciatic nerve.

Muscle contraction force and muscle mass atrophied were related to muscular denervation and muscle disuse, and increased muscle mass indicated progress of the motor function of the sciatic nerve. Neurometesis of the sciatic nerve reduced muscle force contraction and muscle mass early; therefore, the BMSC animals regained their muscle force contraction and muscle mass more rapidly than the ENS group due to the promotion of reinnervation. Burnett and Zager (2004) reported that innervation of the muscle protected the muscle mass and muscle contraction force.

The degree of sensory reflex including the spreading of toe increased from day to day. Gradually regaining function of the second, third and fourth toes collaborates with the report of Gutmann et al. (1942) and Schmitz and Beer (2001) who described the gradual return of peroneal nerve function. Therefore, the study of the recovery of the sciatic nerve in rabbits was evaluated as a sign of functional recovery alongside concurrent muscle weights. Progress of functional recovery of the transected sciatic nerve in the BMSC group was due to the implantation of the fibroblast-like BMSCs. Previous studies indicated that the MSCs expressed trophic factors and supporting substances, including nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, collagen, fibronectin and laminin (Chen et al., 2007).

In this study, the histopathological observations in the BMSC group showed a significant improvement as compared to the ENS group. The histopathological results of BMSC implantation showed the acceleration of nerve tissue regeneration. The Schwann cells might egress in the proximal and distal segments; these cells were provisions of trophic (feeding) and tropic (guidance) factors for regenerating axons (Osbourne, 2007). Histopathological observations in this study showed that the BMSC group was more progressive in healing than the ENS group due to the accuracy of reinnervation, which depended largely on the nerve reconnection with end-organs. Regenerating motor growth cones will enter their original fascicles in the distal nerve stump and connect with the target organ (Brushart, 1993; Yun et al., 2010).

The distal segment of the BMSC group showed an increase in the number of Schwann cells in the distal stump to form the myelin sheath when the new axons entered the distal stump. This result was consistent with the study by Pellegrino and Spencer (1985) who demonstrated an increase in the number of Schwann cells in the distal stump for remyelination of regenerated axon. In the ENS group, the histology of the transection injured sciatic nerve fibers challenges both histopathology and regeneration because of the disruption of the endoneurial sheath with the loss of the axon alignment. The continuous Wallerian degeneration and deposition of collagen retarded the myelination process. This influences the functional recovery and these histological findings are supported by the results of function observation, which showed delayed progress of motor and sensory functions in the ENS group. This result is consistent with the findings of Dilley et al. (2003) who reported that extra-neural fibrosis and wound-bed adhesions may tether the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
<th>ENS Group</th>
<th>BMSC Group</th>
</tr>
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<tbody>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of fibers</td>
<td>13292±1091(^a)</td>
<td>8435±406(^b)</td>
<td>10841±537(^d)</td>
</tr>
<tr>
<td>Diameter of the fiber (µm)</td>
<td>10.35±0.73(^a)</td>
<td>6.14±1.90(^b)</td>
<td>6.58±2.38(^c)</td>
</tr>
<tr>
<td>Thickness of the sheath (µm)</td>
<td>3.47±0.41(^a)</td>
<td>2.28±0.85(^b)</td>
<td>2.02±1.23(^c)</td>
</tr>
<tr>
<td>Diameter of the axon (µm)</td>
<td>6.86±0.28(^a)</td>
<td>3.86±1.69(^b)</td>
<td>4.56±1.74(^ab)</td>
</tr>
<tr>
<td>g ratio</td>
<td>0.66±0.07(^a)</td>
<td>0.62±0.08(^a)</td>
<td>0.69±0.01(^a)</td>
</tr>
</tbody>
</table>

| Distal                     |                  |           |            |
|----------------------------|                  |           |            |
| Number of fibers           | 13292±1091\(^a\) | 8072±52\(^b\) | 10355±698\(^c\) |
| Diameter of the fiber (µm) | 10.35±0.73\(^a\) | 4.63±1.94\(^b\) | 5.81±2.13\(^d\) |
| Thickness of the sheath (µm)| 3.47±0.41\(^b\) | 1.60±0.73\(^a\) | 2.11±0.54\(^a\) |
| Diameter of the axon (µm)  | 6.86±0.28\(^b\) | 3.03±1.30\(^b\) | 3.70±1.66\(^b\) |
| g ratio                    | 0.66±0.07\(^b\) | 0.65±0.02\(^b\) | 0.63±0.07\(^b\) |

\(^a\), \(^b\), \(^c\), \(^d\) Means (n=5) with different superscript within same row are significantly different at (p < 0.05) compared to negative control (right hind limb).
suture site and adjacent nerve bed.

The histomorphometric analysis showed the decrease in the number of myelinated nerve fibers, fibers diameter, axon diameter and myelin sheath thickness in the proximal and distal segments of the sciatic nerve on day 112 PO of the ENS and BMSC groups when compared with the negative control. However, the number of myelinated nerve fibers in the BMSC group was more than that in the ENS group, this increase in the fiber number is attributed to the promotion of angiogenesis which produces a favorable environment for axon growth and increases the speed and quality of nerve regeneration (Fernandes et al., 2008).

The diameter of the myelinated regenerative nerve fibers of the transected sciatic nerve in the BMSCs group was thicker and larger than that in the ENS group and this could be due to the connection with target organs to re-establish correctly as well as the growth factor secreted from target organs to activate the sensory and motor neurons. Previous studies have found that the regenerative axons that reached the target, were enlarged, matured and regained close-to-normal diameters as a result of the neurotrophic supply from the target organs (Gold et al., 1991). The ENS group showed increased collagen deposit around the distal stump endoneurium, which reduced the caliber of endoneurium correlate with smaller fibril diameters. These results are in agreement with those of previous studies (Roytta and Salonen, 1988), which reported that after nerves injury, the amount of collagen is increased in the distal stump endoneurium and reduced their caliber.

As for the g ratio, it is the ratio of the axonal diameter to the total fiber diameter (axon diameter/fiber diameter) which is thus reflective of the myelin sheath thickness and the progress of maturation (Perrot et al., 2007). In this study, both the proximal and distal stumps of all the groups exhibited a similar normal g ratio approximation on day 112 PO. However, the BMSC group was better in the healing process using this parameter when compared to the ENS group due to the high value of the g ratio proximally and low value distally which indicated reconnection with target organs. On the basis of the g ratio, an initially low value was seen during the normal healing process and then tended towards a higher value as healing progressed till the g ratio approached and reached normal (Fraher and Dockery, 1998).

Comparative relative gastrocnemius muscle weights assessment indicates the regenerative status of the sciatic nerve. Since atrophy ensues immediately after post-nerve injury, thereby decreasing the weight of the muscle target organ, a comparison between the study groups showed that the BMSC group had a better relative muscle weight ratio than the ENS group. Therefore, on day 112 PO, there was more remarkable weight gain recorded, indicating a large proportion of the muscle innervated by the regenerated nerve fiber as confirmed by the histopathology and histomorphometric findings. In conclusion, this study show that the motor and sensory functions and acceleration of sciatic nerve regeneration in the BMSC group improved more rapidly when compared with the ENS group.

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