Research Report

Schwann-like mesenchymal stem cells within vein graft facilitate facial nerve regeneration and remyelination

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ABSTRACT

To compare the ability of bone marrow mesenchymal stem cells (MSCs) and transdifferentiated Schwann-like MSCs (tMSCs) in promoting transected facial nerve branches repair in a rabbit model of injury, rabbit tMSCs were induced from bone marrow MSCs, and Schwann cells markers were assessed by Western blot analysis. The left facial nerve buccal branch was transected to form a 1-cm gap in 54 rabbits, and the gaps were immediately bridged using autologous vein grafts. Animals were then randomly assigned to three groups: vein graft (VG); VG+MSCs, and VG+tMSCs (n=18/group). Saline, autologous MSCs, and Schwann-like tMSCs were injected into vein conduits. Rabbits were sacrificed at week 4, 8, and 16 post-surgery. Facial nerves regeneration and myelination were analyzed by functional, immunohistochemical, and morphological tests. In addition, myelin protein genes expression, including peripheral myelin protein 22 (PMP22), myelin protein zero (P0), and myelin basic protein (MBP), in transplanted cells in vivo were assayed using real time quantitative-reverse transcription–polymerase chain reaction (RT–PCR). Rabbit tMSCs expressed Schwann cells markers, and results demonstrated better facial nerve functional recovery in the VG+tMSCs group, with earlier horseradish peroxidase (HRP) positive neurons appearance and a greater number of MBP positive myelinated axons since 4 weeks after transplantation. Moreover, RT–PCR analysis showed transplanted tMSCs in vivo expressed higher myelin proteins at mRNA level than those of MSCs during the first 8 weeks of neural regeneration. This study suggests that rabbit transdifferentiated Schwann-like MSCs within autogenous vein graft accelerate transected axons regeneration and achieve better remyelination.

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1. Introduction

Successful peripheral nerve regeneration after injury depends on the behavior of Schwann cells, which participate in Wallerian degeneration, the formation of the Bürgner’s band guidance, and the myelination of new axon fibers (Dubey et al., 1999). However, upon axotomy, proliferation of Schwann cells originating from nerve stumps remains too limited to satisfy axonal regeneration demands, and the treatment of nerve autograft transplantation raise numerous complica-
tions to the donor-site (Yang et al., 2008). Therefore, the replacements of Schwann from different sources have been extensively investigated in supporting peripheral nerve regeneration (Ansselin et al., 1997; Amoh et al., 2005; Dezawa et al., 2001; Lin et al., 2008; Santiago et al., 2009). Bone marrow MSCs, which are multipotent stem cells, satisfy the requirement for ideal transplantable cells with regard to easy access, rapid in vitro expansion, and poor immunogenicity (Pittenger et al., 1999; Uccelli et al., 2006a). In addition, bone marrow MSCs can transdifferentiate into Schwann-like cells (tMSCs), which sheds great light on cellular transplantation strategies for repopulating Schwann cells following peripheral nerve regeneration (Dezawa et al., 2001; Keilhoff et al., 2006; Tohill et al., 2004).

The nerve tubulization technique is a surgical approach, which repairs large nerve fiber defect and recreates nerve continuity (Doolabh et al., 1996). The aim of this technology is to allow for growth of regenerated axons into the distal nerve stump, as well as remyelination in defective, long, nerve fibers. Different tube grafts have been filled with cells, extracellular matrix, and neurotrophic factors, which has confirmed constructive effects of conduits tubulization on nerve regeneration (Battiston et al., 2005). Additionally, implantation of Schwann-like MSCs is effective for repairing ruptured peripheral nerves with acellular nerve, muscle grafts, or other artificial scaffolds (Hu et al., 2007; Keilhoff et al., 2006).

The major events in the peripheral nerve regeneration are axonal extension and myelination, which requires longitudinal wrapping of Schwann cells to the axons to produce myelin sheath (Dombrowski et al., 2006; Garbay et al., 2000). Myelination determines regeneration quality and functional recovery. However, little is known about Schwann-like tMSCs as a source of myelin-forming cells in peripheral nerve regeneration and myelination by tubulization repair.

The present study filled autologous Schwann-like tMSCs into autologous vein conduits to bridge 1 cm defects in the rabbit facial nerve. The function of these cells in promoting new axonal fibers extension and myelination were analyzed.

2. Results

Generally, all experimental animals tolerated surgery well and the wounds healed without inflammatory or discomfort symptoms.

2.1. Cell characteristics

Flow cytometry analysis showed that rabbit bone marrow MSCs were positive for surface markers CD29 and CD44 but negative for CD11b and CD31 which were recognized as the hematopoietic stem cell surface markers (Fig. 1). Rabbit bone marrow MSCs exhibited a fibroblast-like shape, and Schwann-like tMSCs exhibited a spindle-like morphology (Fig. 2A). Western blot analysis revealed positive expression of Schwann cell proteins, GFAP, S100, and p75NTR in tMSCs, while bone marrow MSCs were negative for these proteins (Fig. 2C). Approximately 90% cells illustrated GFP expression at 48 hours after retroviral vector transfection (Fig. 2B ex vivo). Obvious green fluorescence signals were observed in vein conduit frozen sections at 2 weeks after in vivo transplantation (Fig. 2B in vivo).

![Fig. 1](image1)

Phenotypic characterization of cultured rabbit bone marrow mesenchymal stem cells (MSCs). Flow cytometry analysis showing the third passage of rabbit MSCs to be positive for CD29, CD44, and negative for CD11b, CD31.
2.2. Facial nerve functional assessment

Whisker movement recovery in this study indicated muscles reinnervation through 16 weeks post-surgery. Left facial nerve transection resulted in scores of 0, which indicated the complete absence of whisker movement. The whisker movements in the right side were recorded as 3. At each time point, scores were greater in the VG+tMSCs group compared with VG+MSCs group (P<0.05), and the VG+MSCs group achieved significantly higher scores than those of VG group. (P<0.05) (Fig. 3A).

Stimulation of the un-operated facial nerve evoked CMAPs with a latency of 1.4±0.2 ms and an amplitude of 6.3±0.4 mV. CMAP recovery ratios at the three time points in three groups are shown in Fig. 3B. CMAP recovery in all groups dropped to ≤25% during the first 4 weeks, but the difference among groups was not significant. At 8 weeks after surgery, ratios in the VG+tMSC and VG+MSC groups were restored to above 40% of normal levels, which were significantly greater than the VG group (26.75%) (P<0.05, respectively). Ratios in the VG+tMSC group were 62% at

Fig. 2 – A: The morphological changes between rabbit bone marrow mesenchymal stem cells (MSCs) at passage 3 and transdifferentiated Schwann-like mesenchymal stem cells (tMSCs). Scale bar=100 μm. B: GFP expression in transfected MSCs ex vivo (scale bar=100 μm) and GFP-positive transplanted cells in the vein conduit at two weeks post-surgery in vivo (scale bar=50 μm). White arrows indicate GFP-positive transplanted MSCs, and larger arrowheads point to the auto-fluorescence of vein wall. C: Western blot analysis of glial fibrillary acidic protein (GFAP), S100, and p75NTR expression in primary MSCs and Schwann-like tMSCs. GAPDH served as the internal reference.
16 weeks, which were significantly greater than the VG (43.25%) and VG+MSC (52.75%) group \((P<0.05)\), respectively.

2.3. Myelin protein mRNA expressions in transplanted cells in vivo

The mRNA expression of PMP22, P0, and MBP suggested that bone marrow MSCs and Schwann-like tMSCs had different responses to axonal myelination in vivo \((P<0.05)\). The mRNA expression patterns in the VG and VG+MSCs groups were increased during the whole experimental period. However, in the VG+tMSCs group, PMP22 expression was increased, MBP was reduced, and P0 was increased then dropped to the normal level. Although PMP22 expression was low in groups at the early period, its expression increased and beyond the normal levels at 16 weeks after surgery \((VG: 1.06\text{-fold}; \ VG+MSCs: 5.66\text{-fold}; \ VG+tMSCs: 32\text{-fold})\). As for P0, the VG+tMSCs group exhibited maximum mRNA expression, which was 2.25-fold higher than the normal levels, at 8 weeks, but it was reduced to normal baseline by the last time point. All of the groups showed extraordinarily low MBP mRNA expression during the entire experimental period. The maximum expression level \((4\text{ weeks post-surgery in VG+tMSCs group})\) was also less than 10% of normal level. Therefore, the mRNA expressions of the three genes were significantly different between the VG+MSCs and VG+tMSCs groups \((P<0.05)\), respectively.

2.4. Re-connection and remyelination of the transected nerves

The vein grafts filled with transplanted cells remained similar in diameter with the nerve stump \((P<0.05)\). The HRP retrograde staining of facial nucleus was to determine whether the axonal regeneration resulted in anatomical reconnection of the transected nerve. Following the first 4 week regeneration, positive staining nucleuses were observed in the VG+tMSCs group \((P<0.05)\), whereas no staining was detected in animals of the VG and VG+MSCs groups \(n=5/group\). This finding suggested regenerated axons extended from the proximal stump into the distal stump to supply pathway for HRP retrograde passage in rabbits treated with VG+tMSCs.

Facial nerve sections distal to the vein conduits exhibited MBP protein expression during regeneration \((P<0.05)\). At 4 weeks post-surgery, there was no positive MBP in the VG group \((P<0.05)\), and little MBP was detected in transplanted cell groups at the early time point \((P<0.05)\). A significant increase in MBP expression was observed in each group at 8 weeks after surgery. At this time point, the VG group exhibited irregular positive spots \((P<0.05)\), while
the VG+MSCs group showed ring-like positive expression shape (Fig. 5E) and the VG+tMSCs group had greater MBP expression (Fig. 5F). At 16 weeks, robust MBP ring-like expression was distributed in the two cell translation groups, respectively. Furthermore, in the VG+tMSCs group, the shape of the MBP positive area was more similar to

Fig. 4 – (A and B): The gross anatomy of the vein grafts after they were transplanted in vivo. A: The vein conduit, filled with transplanted cells, remained similar in diameter with the transected facial nerve 16 weeks after transplantation. B: A significant decrease in the diameter of the vein graft that only received saline injection. C: Retrograde labeling of facial nerve neurons HRP positive labeling in the facial nucleus 4 weeks after repair surgery in the VG+tMSCs group. The yellow arrows point to the HRP positive neurons (green). Scale bar=40 μm.

Fig. 5 – MBP immunohistochemistry of regenerated facial nerves, 5 mm distal to the vein grafts, in all groups at three time points. The brown spots indicated MBP positive area. Scale bar=15 μm.
myelinated axons with larger axonal diameter and thicker myelin sheaths. The VG group, however, exhibited scattered and dot-like MBP expression (Fig. 5G–I).

Details of regenerated axons and myelin sheaths from semi-thin and ultrathin sections at 16 weeks are illustrated in Fig. 6. The VG group presented with a greater amount of fibrous tissue, a larger number of non-myelinated axons, few myelinated axons, and disordered nerve fibers with hypertrophic Schwann cells surrounding immature myelin (Fig. 6Aa and Ba). In the VG+MSCs group, newly formed axons presented with irregular diameters (Fig. 6Ab and Bb). The VG+tMSCs group exhibited regenerated nerve fibers with larger axonal diameter and thicker myelin sheaths, which were more similar to normal nerve fibers (Fig. 6Ac and Bc).

In 16 weeks post-surgery, the number of myelinated axons pre area in VG group was significantly lower than the other two groups (\( P < 0.05 \), respectively). However, there was no significant difference between VG+MSCs and VG+tMSCs groups (Fig. 7A). The \( g \) ratio in the VG group was significantly higher than those in the cells transplantation groups (\( P < 0.05 \), respectively). There was also no significant difference existed between the VG+MSCs and VG+tMSCs group (Fig. 7B). On the other hand, myelin area in the VG+MSCs group was significantly smaller than VG+tMSCs group and significantly larger than VG group (\( P < 0.05 \), respectively) (Fig. 7C). The decreased \( g \) ratio and enlarged myelin area reflects the increase of myelin thickness.

3. Discussion

Results from the present study demonstrated the possibility of rabbit bone marrow MSCs to transdifferentiate into Schwann-like cells, which were more effective in enhancing facial nerve regeneration and myelination when combined with autologous vein conduits.

In this study, applying autologous bone marrow MSCs was to avoid immunological rejection. MSCs, even undergoing neuronal differentiation in vitro, still have low immunogenicity and immunomodulatory function, which result in an immune response that inhibits neural regeneration in peripheral nerve repair (Liu et al., 2006; Sanders and Jones, 2006). Similar to rat or monkey MSCs (Dezawa et al., 2001; Keilhoff et al., 2006; Tohill et al., 2004), the rabbit tMSCs altered cellular morphology to glial characteristics, expressed Schwann cell markers, and enhanced their ability of repairing nerve fiber injury. The modification in Schwann-like MSCs transdifferentiation protocol was the dosage of ATRA. Previous researches reported the dosage of ATRA used in MSCs transdifferentiation was 35 ng/ml. However, we observed that the MSCs proliferation capacity was enhanced stronger than their differentiation ability under 35 ng/ml ATRA. As soon as the process of ATRA induction was finished, the cultured cells demonstrated excessive cells density which is adverse to the following transdifferentiation process. After a

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Fig. 6 – A: Toluidine blue staining of semi-thin cross sections, 5 mm distal to the vein graft, in all groups at 16 weeks after repair surgery. Scale bar=20 \( \mu \)m. B: Transmission electron microscopy reveals regenerated myelinated nerve fibers in each group at 16 weeks post surgery. Scale bar=1 \( \mu \)m. (a: VG group; b: VG+MSCs group; c: VG+tMSCs group; MA: myelinated axon; UMA: unmyelinated axon; SC: Schwann cell).
Fig. 7 – At 16 weeks post-surgery, quantification of myelinated axon counts, average g ratios, and myelin areas in the regenerated nerve fibers, where 5 mm distal to the vein graft in each group (n=3/group). Data are expressed as mean ± SEM. In each chart, different lowercase letters above bars showed statistical groupings among three treatments (P<0.05).

continuous dosage of ATRA preparing experiment, the most optimal ATRA dosage was adjusted to 350 ng/ml, which could regulate MSCs proliferation and differentiation properly. GFP expression was observed in the conduits at 2 weeks after transplantation, which suggested that the transplanted cells survived in the vein tubes. As a result, facial nerve regeneration was clearly influenced by the existence of the transplanted cells.

Facial nerve functional recovery is dependent on the new axons outgrowth, myelination, and their correct reinnervation of the target organ. The HRP positive labeling neurons at 4 weeks after surgery illustrated that VG+tMSCs has comparative advantages on protecting injured neuronal survival on one hand and providing connection of the transected axons on the other at the early stage of regeneration. Additionally, functional outcomes, as measured by recovery of whisker movement and CMAP, indicated successful reinnervation of the facial nerves.

It is known that the local environment influences the transdifferentiation fate of the MSC populations (Sanchez-Ramos et al., 2000). As for vein grafts, they supply rich extracellular matrix, collagen, and the basal lamina membrane maintenance which are necessary for myelin-forming cells (Chernousov et al., 2008). Meanwhile, the vein conduit microenvironment, which is similar to that of silicone tube, could induce MSCs to differentiate into Schwann-like cells in vivo (Chen et al., 2006). It was reported that almost 5% of transplanted MSCs spontaneously differentiate into Schwann cells in vivo without any artificial intervention (Cuevas et al., 2002). These transdifferentiated Schwann cells included myelinating and non-myelinating Schwann cells (Aquino et al., 2006). However, only the myelinating Schwann cells contribute to myelin formation. Myelination in the peripheral nerve system takes place when Schwann cells ensheath axons in layers, which ensures for rapid conduction velocity. MBP mRNA level is probably due to the effect of transcription factors, such as Erg-2, Sox-10, and Oct-6 (Svaren and Meijer, 2000). Therefore, the disparity between MBP protein level and gene expression in myelin-forming cells reflects the myelination ability of these cells. During the regeneration, if regenerated axonal diameter is ≤0.7 μm or the interaction between axons and myelin-forming cells is not yet established, the myelination process will not occur (Windebank et al., 1985).

Small axonal diameters and a shortage of axon-myelinating cell contact could be responsible for the low mRNA levels for PMP22, P0, and MBP at 4 weeks in the present study. The lack of stem cells resulted in low levels of gene products in the VG group. In addition, it was hypothesized that gene expression differences between VG+MSCs and VG+tMSCs groups were due to cell transdifferentiation process in vitro. Previous results showed agents, such as forskolin, have been used during Schwann-like cells transdifferentiation process could elevate the level of intracellular cyclic adenosine monophosphate (cAMP). On one hand, elevated cAMP level could further stimulate myelin-related gene expression; on the other hand, cultured Schwann cells increasing their myelin-related gene expression via the CAMP pathway (Afshari et al., 2001). The transplanted cells played important roles in myelination, which was also dependent on the regulation of transcriptional factors, such as Erg-2, Sox-10, and Oct-6 (Svaren and Meijer, 2008). Therefore, the disparity between MBP protein level and MBP mRNA level is probably due to the effect of transcription process.

In summary, using autogenous vein grafts filled with stem cells, rabbit facial nerve defects were bridged and regeneration was promoted. The electrophysiology, morphometry and functional results showed that the Schwann-like tMSCs possess a significant advantage in promoting peripheral nerve regeneration with faster axonal extension rate and...
better myelin sheath formation quality. Although producing Schwann-like MSCs requires additional induction process, satisfied neural regeneration outcomes suggest that Schwann-like tMSCs combined with vein graft would be a potential therapy for severely injured peripheral nerves.

4. Experimental procedures

These experiments were performed in 54 adult female New Zealand white rabbits (mean weight 2.5–3.2 kg), which were randomly assigned to three groups: empty autogenous vein conduit bridging nerve gap without cells transplantation (VG group, n=18), autogenous bone marrow MSCs transplantation in autogenous vein graft (VG+MSCs group, n=18), and transdifferentiated Schwann-like MSCs transplantation in autogenous vein graft (VG+tMSCs group, n=18). All experimental procedures involving animals were authorized by the Ethics Committee at Sichuan University.

4.1. Bone marrow MSCs isolation and culture

Rabbits were anesthetized by intravenous injection of 3% pentobarbital sodium 1.2–1.5 ml/kg. Briefly, marrow blood was collected by aspiration from the iliac crest of each rabbit via a sterile bone marrow aspiration needle. Bone marrow MSCs were isolated by density gradient centrifugation, and the cells were suspended in growth medium (α-MEM supplemented with 20% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin all obtained from Sigma, St Louis, MO, USA) in 75-cm² flasks. The cultures were incubated in 95% humidified atmosphere at 37 °C and 5% CO₂. Culture medium was replaced every 3–4 days.passaging was performed as soon as adherent cells were 90% confluent. The third passage cells were used for in vitro transdifferentiation and in vivo transplantation. To identify the third passage cell phenotype, flow cytometry analysis was performed. The adherent cells were treated with trypsin and stained with primary antibodies against rabbit CD11b, CD29, CD31, and CD44 (Abcam, Cambridge, UK), anti-GFAP, and anti-p75NTR (Milipore, Billerica, MA, USA). The latter reaction was developed with Dylight488-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (lgG) secondary antibody (Jackson ImmunoResearch Laboratories, USA). Between steps, cells were washed twice with phosphate-buffered saline fetal bovine serum (4%, vol./vol.). As negative control, cells were incubated with Dylight488-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (lgG) secondary antibody (Jackson ImmunoResearch Laboratories, USA).

4.2. Schwann-like MSCs transdifferentiation and identification

Schwann-like cell transdifferentiation was performed as previously described with modification (Dezawa et al., 2001; Keilhoff et al., 2006). Briefly, rabbit MSCs were incubated in growth medium at 500/cm² for 24 h and a further 24 h with 1 mM β-mercaptoethanol (Sigma,USA) addition. Then medium was replaced by growth medium with 350 ng/ml all-trans retinoic acid (Sigma,USA) for 48 h. The Schwann cell induction medium consisted of α-MEM, 10% fetal bovine serum (Hyclone,USA), 5 μM forskolin (Sigma,USA), 10 ng/ml b-FGF (Peprotech,USA), 200 ng/ml Her-β (Sigma,USA) and 5 ng/ml PDGF-AA (Peprotech, USA) for a week. Each time of changing induction medium, cells were washed by phosphate-buffered saline (pH 7.4). As soon as the transdifferentiation process was finished, Western blot analysis was performed to determine protein expression levels of S100, glial fibrillary acidic protein (GFAP) and p75NTR in MSCs and Schwann-like tMSC, respectively. Cell samples were lysed in ice-cold lysis buffer containing protease inhibitors. Following centrifugation, protein concentration was determined with a BCA assay (Pierce, Rockford, IL, USA). An aliquot corresponding to 5 mg of total protein from each sample was separated on 4–20% gradient SDS/polyacrylamide gel and transferred them to a PVDF membrane (0.2 μm and 0.45 μm, Millipore, Billerica, MA, USA) through electrophoresis. After blocking the binding with 5% nonfat milk, the membranes were incubated overnight at 4 °C with primary antibodies: anti-S100 (Abcam, Cambridge, UK), anti-GFAP, and anti-p75NTR (Milipore, Billerica, MA, USA). Then the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody. Reactions were detected by chemiluminescence (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control.

To label cells prior to their transplantation, the green fluorescent protein (GFP) gene was transfected into MSCs and Schwann-like tMSCs by using a retroviral vector (gift from Dr. Man Cheng, Sichuan University, China). GFP expression was observed under fluorescent microscopy (Leica, Solms, Germany) to determine the transfection efficiency in cells at day 2 after transfection.

4.3. Surgical procedure

Rabbits were anesthetized by intravenous injection of 3% pentobarbital sodium 1.2–1.5 ml/kg. Under sterile conditions, a 10-mm segment from the left buccal divisions of facial nerve was transected. Simultaneously, a left facial vein graft (14 mm) was harvested to bridge the facial nerve gaps. The epineurium of the proximal and distal nerve stumps was coated and anchored to the vein conduit wall with 9-0 nylon sutures under microscopic conditions. Schwann-like tMSCs and MSCs were supplemented in 15 μl saline at a density of 2×10⁶/μl in a runny form and were transplanted into the lumen of vein tubes in the VG+MSCs group and VG+tMSCs groups, respectively. At the same time, the VG group received a vein tube filled with 15 μl physiological saline. Subsequently, the skin incisions were sutured with 3-0 nylon sutures. All of the rabbits were separately housed and feeding food and water ad libitum. At 2 weeks after surgery, three animals from each group were randomly sacrificed and the vein graft samples were harvested immediately. The frozen sections (15 μm thick) were cut with a cryostat microtome (Leica, Solms, Germany) and observed under a fluorescence microscope (Leica, Solms, Germany).

4.4. Functional assessment

Facial nerve functional assessment was performed in two parts: vibrissal whisking evaluation without anesthesia and electrophysiology examination under general anesthesia. As
previously described, whisker movements were described into four different categories with scores ranging between 0 and 3 (Kobayashi et al., 2003). Ipsilateral whisker movement evaluation was performed in every 2 weeks ever since the 4th week following surgery. To avoid subjective bias, two independent observers assessed the animals.

To perform electrophysiological tests, repaired facial nerves were re-exposed under general anesthesia at weeks 4, 8, and 16 post-surgery when whisker movement scores were recorded. A heating lamp maintained the rabbit body temperature at 37 °C. To make sure the stimulant impulses actually passed through the regenerated axons, other ipsilateral facial nerve branches were resected from the muscle tissue. The electrophysiological test was performed by an electromyography system (MEB-2200 Nihon Konhden, Tokyo, Japan). Analysis time of 3–5 ms and 10–10,000 Hz filters were applied. Consequently, compound muscle action potentials (CMAPs) in both sides were measured. The ratio of CMAPs in the operated sides compared with normal sides was used to evaluate the facial nerve functional recovery.

4.5. Retrograde labeling of facial motor neurons

Three animals in each group were anesthetized 7 days before sacrificed at 4 weeks post-surgery to receive 15 μl 4% horseradish–peroxidase subcutaneous injections in the left whisker pads. After further 7 days, these rabbits were perfused transcardially by paraformaldehyde. The blocks of pons, which contain the facial nucleus, dehydrated in gradient sucrose. Frozen sections were cut at 40 μm and were stained with tetramethyl-benzidine for light microscopy observation.

4.6. Quantitative real-time reverse transcription–polymerase chain reaction

Vein grafts and normal facial nerve tissue from each animal were removed at different time points, immediately frozen in liquid nitrogen, and stored at –80 °C until they were homogenated. Total RNA was extracted using the Trizol (Invitrogen, Carlsbad, CA, USA), and cDNA was reverse transcribed using Reverse Transcriptase kit (Takara, Dalian, China) according to manufacturer instructions. Primer pairs of myelin proteins were used as follows: peripheral myelin protein 22 kDa (PMP22) forward primer 5′-CTGTTCTCAGAACTGTA-3′, reverse primer 5′-CATGTGGGCCTGAGACGA-3′; protein zero (P0) forward primer, 5′-ACAGAGATGCTGCAAGAGGCCT-3′, reverse primer, 5′-AGAGCAACAGCAGCAACAGCAC -3′; myelin basic protein (MBP) forward primer 5′-GACTCCATCGGCGCATT-3′, reverse primer 5′-CAATGTCTTGAGAAGTGGGACT-3′; GAPDH forward primer 5′-GCCATGCTGCAACACCA-3′, reverse primer, 5′-CAGTGACCTGACCCGCTTC-3′. GAPDH served as the internal control. Quantitative real-time PCR was performed using the SYBR green master mix (Takara, Dalian, China) on an ABI prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). Cycle number (Ct) of the each reaction was calculated from the amplification curve to determine the relative gene expression by using the comparative cycle threshold method (Livak and Schmittgen, 2001). Sample values were normalized to the Ct value of GAPDH for each time point. The fold changes of target genes were calculated by using mRNA expressions in normal facial nerve tissue as references.

4.7. Assessment of myelination

Repaired left facial nerve samples, 5 mm distal to the vein graft, were collected from three groups at weeks 4, 8, and 16 post-surgery and further fixed in 4% paraformaldehyde immediately. Then, the specimens were embedded in paraffin and sectioned into 10 μm thick sections. Subsequently, deparaffinization, antigen retrieval, and hydrogen peroxidase incubation were performed one by one. The primary antibody was bovine anti-rabbit myelin basic protein (Chemicon, Billerica, MA, USA), which diluted to 1:200 in PBS. All sections were incubated at 4 °C overnight, followed by incubation with secondary antibody, and were visualized through DAB (3,3′-diaminobenzidine tetrahydrochloride) system.

Three distal facial nerve specimens of week 16 were fixed in cold 2.5% glutaraldehyde in 0.1 M PBS for 24 hours, followed by post-fixation in 2% Na-cacodylate-buffered osmium tetroxide, ethanol dehydration and Epon embedding. Semi-thin and ultrathin sections were examined under light microscopy and transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV, respectively. The captured images were analyzed with OpenLab software (Improvement, Conventry, UK). Under the qualitative observation, parameters of number of nerve fibers, diameter of axons, outer diameter of myelinated fibers, axon area as well as myelin area were obtained from 100 randomly selected nerve fibers on digitized images.

4.8. Statistical analysis

All the data were expressed as means ± standard error of the mean for independent sample in three groups. Data analyses were performed using the general linear ANOVA model procedure of SPSS 16.0 (SPSS Inc. Chicago, IL, USA). Comparisons were tested using the Student–Newman–Keuls (SNK) test, with a significant level of P<0.05.

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