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POROUS PLGA SHEET AND ACELLULARIZED MUSCLE STUFFED VEIN SEEDED WITH NEURAL- DIFFERENTIATED MSCs ARE POTENTIAL SCAFFOLDS FOR NERVE REGENERATION

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ABSTRACT

Numerous studies on bridging a nerve gap without using autologous nerve graft have been reported. Our study compares two models of nerve conduit; a porous PLGA sheet and acellular muscle stuffed vein with both models seeded with mesenchymal stem cells. The micro-porous PLGA sheet was fabricated by solvent casting/salt leaching technique using methylene chloride as the solvent. Both PLGA and muscle stuffed vein was decellularized by liquid nitrogen immersion and the latter further hydrolyzed by soaking in HCl for 24 hours. Neural-differentiated mesenchymal stem cells (MSCs) were seeded and cultured on the surface of both conduits. We studied the seeded constructs for their surface morphology by scanning electron microscope and biocompatibility assessment. Both models of nerve conduit had good distribution and proliferation of seeded cells. For the PLGA sheet, pores had extensive interconnections, excellent for allowing nerve growth. For the muscle stuffed vein, cells were successfully embedded within the hydrolyzed fraction of muscle and ultra structural analysis showed healthy cells attached to the surface of acellular muscle. In conclusion, both PLGA and muscle stuffed vein seeded with neural-differentiated MSCs are suitable scaffolds for nerve tissue engineering.

1.0 Introduction

An injured nerve with segmental loss results in a nerve gap. The treatment of choice involves autologous nerve grafting using non-vital donor nerve. However, this requires a second surgical procedure of harvesting the donor nerve. Problems associated with this include donor site morbidity, neurological deficit suffered by the patients, additional surgical procedure to obtain donor nerve, limited avaibility of donor nerve and size mismatch between recipient and donor nerve[1]. To overcome these limitations, alternatives have been employed to bridge the nerve gap with either autologous or synthetic material.

Extensive research has been done to explore and fabricate the best substitute to bridge the nerve gap. It includes autologous as well as synthetic materials. Examples of autologous materials are veins or arterial grafts[2], collagen conduits[3], perineurial tubes[4], and acellular muscle graft[5]. Synthetic materials that are commonly used are silicon[6], reabsorbable polymers[7] or hyaluronic acid guides[8]. Utilizing tissue engineering techniques, these materials may possess even better functional and clinical results if supplemented with stem cells.

Fabrication of a scaffold mimicking the *in vivo* cellular microenvironment and with the ability to replace the

structure and function of extracellular matrix (ECM) coupled with strong regenerative and cell supporting capacities is of fundamental importance to the success of nerve tissue engineering[9]. Tissue engineering applications require good distribution and growth of cells on a scaffold. The scaffold serve as cell and tissue carriers and are fabricated using either natural or synthetic polymers. The fabricated scaffold should ideally be biodegradable, biocompatible and mechanically robust with large surface area and interconnected pores[10]. Porous scaffolds play an important role as templates to accommodate cells and guide new tissue formation in tissue engineering as well as enabling the exchange of nutrient and metabolic waste between the scaffold and environment.

In this study, we aim to compare synthetic porous PLGA sheet and natural muscle stuffed into vein as models of nerve conduit. Both are seeded with neural-differentiated MSCs and acts as carriers. Both conduits will be characterised to investigate its microstructure and *in vitro* biocompatibility.

2.0 Materials and Methods

We obtained approval from our Research and Ethical Committee (approval project code: 02-01-02-SF0445).

2.1 Isolation and culture of human mesenchymal stem cells (MSCs)

Following informed consent, human bone marrow aspirates were obtained from six patients undergoing reaming procedure during orthopedic interlocking nailing of femur and tibia. The samples were obtained following informed consent. Marrow aspirates were diluted with PBS and layered onto 1.073 g/mL of Ficoll Paque. The tubes were centrifuged for 30 minutes at 3000 rpm. The middle layer was removed with a 3 mL pasture pipette, combined with 10 mL of PBS and centrifuged. The resultant pellet were resuspended in α-MEM containing 10 % FBS and cultured at 37^{O}C, 5% CO_2. After 72 hours, non-adherent cells were removed. This stage was termed as passage 0 (P0). The culture media were changed three times a week to ensure optimal growth and proliferation of cells. Once the cell was 80% confluent, they were detached from the culture dish by enzymatic reaction of Trypsin-EDTA (0.25% trypsin, 0.018% EDTA) for about 5 minutes. The supernatant was collected from the culture dish and transfered into the 50 mL centrifuge tube and centrifuged for 5 minutes at 5000 rpm. The resulting supernatant was removed and the pellet washed three times with PBS. The resultant pellet was resuspended in α-MEM media and cultured at 37^{O}C and 5% CO_2. This culture would now be termed as passage 1 and the process repeated with incremental numbering of the passage. MSCs were sub cultured three times and finally subjected to a series of induction into differentiated MSCs.

2.2 Neurogenic induction of human MSCs.

After sub culturing at a concentration of 5 - 10 X 10^3 cells/cm^2, MSCs were incubated in α-MEM containing 1 mM beta-mercaptoethanol (β-ME) without serum for 24 hours. The culture media replaced with a differentiation medium consisting of α-MEM containing 10% FBS and 35 ng/mL all-trans-retinoic acid (ATRA). Three days later, cells was finally transferred to α-MEM containing 10% FBS and trophic factors of 5μM forskolin, 10 ng/mL recombinant human basic fibroblast growth factor, 5ng/mL platelet-derived growth factor-AA and 200 ng/mL heregulin-β1-EGF-domain and cultured for a further 4-5 days before it is ready for seeding onto the construct[11].

2.3 Evaluation of Undifferentiated and differentiated human MSCs.

2.3.1 Immunocytochemistry

Both undifferentiated and differentiated human MSCs cultures were evaluated by immunocytochemistry and flow cytometry analysis. Cells were trypsinized and replated on chamber slides for immunostainning. Cells were fixed with 4% paraformaldehyde (PFA) for 1 hour. After washing with TBS buffer (3X, 5 min), non-specific antigens were blocked with 10% goat serum for 1 hour. Cells were then incubated overnight with the following primary antibodies: polycnonal anti-S100b (mouse; 1:4000; BD), monoclonal anti-NGF p75 receptor (mouse; 1:500; chemicon), monoclonal anti-nestin (mouse; 1:500; BD) and monoclonal anti-glial fibrilary acidic protein (GFAP) (rabbit; 1:100; chemicon). Cells were washed with TBS buffer (3X, 5 min) and further incubated with secondary antibody FITC anti-mouse IgG (goat; 1:50; chemicon) or FITC anti-rabbit IgG (goat; 1:50; chemicon), at 37^{o}C for 1 hour. Nuclei were counter-stained with 4', 6-diamidino-2-phenylindole (DAPI). Cultures were examined using a fluorescence microscope (eclipse Ti, Nikon)

2.3.2 Flow cytometry Analysis

For flow cytometry,undifferentiated and differentiated human MSCs cultures were trypsinized and washed three times using PBS before blocking with 10% goat serum for 1 hour. The cells are then immunolabelled with polyclonal anti-S100b (mouse; 1:4000; BD), monoclonal anti-NGF p75 Receptor (mouse; 1:500; chemicon), monoclonal anti-Nestin (mouse; 1:500; BD) and monoclonal anti-Glial Fibrilary Acidic Protein (GFAP) (rabbit; 1:100; chemicon) for 1 hour at 37^{o}C. Finally these cells were stained with secondary FITC-conjugated goat anti mouse IgG antibody or anti rabbit IgG antibody at 37^{o}C for half an hour. The total number of 100 000 events were analyzed on a FACSCalibur Flow Cytometer immediately after the staining procedure.

2.4 Preparation of human vein and muscle.

Human veins and muscles were harvested from below knee amputation (BKA) and above knee amputation (AKA) cases once informed consent were obtained. These veins and muscles were washed three times with PBS to remove any remaining bloods.

The vein each measuring 2 cm in length, was decellularized by immersing in liquid nitrogen (10 seconds, three times) and kept in -80^{O}C until further use. The gracilis muscle harvested measured 2 cm in length and 1 cm in width. Gracilis muscle offers a longitudinal fiber orientation, which is similar to endoneural tube structure. The muscle was decellularized by freezing in liquid nitrogen (-196^{O}C) until thermal equilibrium is reached and subsequently soaked in 8% HCl for 24 hours. The HCl-treated muscle was then neutralized with NaOH solution followed by multiple washings. Finally, the acellular muscles and vein were sterilized with 70% ethanol and washed three times with PBS and with α-MEM media before usage.

2.5 Preparation of porous PLGA sheet by solvent casting/salt leaching technique.

Thin PLGA sheets (50:50) were fabricated by the solvent casting/salt leaching method as previously describe[17,18]. PLGA crystals were dissolved in methylene chloride solvent by stirring to obtain a 5 % wt/volume solution. A ratio of 1:9 PLGA and pre-seive NaCl (<100 um) were mixed together with the solution producing porous PLGA sheets. Approximately 2 ml of the solution was casted onto the bottom of a 5 cm diameter glass petri dish to obtain a thin layer of the solution. The solvent was allowed to evaporate and after 48 hours, a thin sheet of PLGA was peeled off from the dish before soaking in miliQ water (18 Ω) to remove salt. Porous PLGA was then placed in a freeze-dryer overnight to remove any remaining solvent. The porous PLGA was cut to a dimension of 1 cm X 1 cm. The scaffold was sterilized with 70 % ethanol and washed 3 times with PBS and α-MEM media prior to cell seeding.

2.6 Fabrication of the porous PLGA and muscle stuffed vein as a scaffold

The culture-expanded differentiated MSCs was harvested by trypsinization and were seeded onto the porous PLGA sheet and pre-treated muscle with a density of 3 x 10^6 cells via static seeding. Every 30 minutes, the excessive medium was collected and seeded again onto the porous PLGA sheet and acellular muscle. This step was repeated three times followed by immersion in culture media at 37^{o}C, 5% CO_2 in the incubator. The next day, the cell-seeded muscle was stuffed into the pre-treated vein. Both scaffolds, the porous PLGA and muscle stuffed vein were incubated for 3 days before histology and SEM assessment.

2.7 Evaluation of in vitro constructs

2.7.1 Scanning electron microscopy analysis

After 3 days of culture, SEM micrographs were taken once the seeded cells had attached to both scaffolds. The PLGA and muscle stuffed vein constructs were cut in half and fixed in 4% gluteraldehyde for 12-24 hours at 4 ^{O}C. The samples was then rinsed with 1X PBS and dehydrated through a series of increasing concentration of ethanol solution , 35 % ethanol for 30 min, 50 % ethanol for 30 min, 70 % ethanol for 30 min, 85 % ethanol for 30 min, 90 % ethanol for 1 hour and 100 % ethanol for 1 hours (twice). The samples were then dried using a freeze-dryer overnight. The dried constructs were mounted on to a stub, sputter coated with gold and observed under SEM.

2.7.2 Histological analysis

The remaining half of the porous PLGA and muscle stuffed vein construct were fixed with 10% formalin for 24 hours, processed and embedded in paraffin wax for histological analysis. Four micrometers thin sections of tissues were cut using a microtome (Leica, Germany), dewaxed and dehydrated with a series of xylene and alcohol (100% & 95%) and evaluated histologically using hematoxylin and eosin staining (H&E staining).

2.8 Statistical Analysis

Data was expressed as mean ± standard error of the mean (SEM) of 6 samples (n=6). Results were analyzed using Paired Student's *t*-test and the difference are considered significance when $p<0.05$.

3.0 Results

3.1 Evaluation of undifferentiated and differentiated human MSCs

Flow cytometry analysis showed the percentage of MSCs transform into differentiated MSCs before and after induction (Fig 1). About 75% of the total 100,000 cells were significantly positive for GFAP, 45% for S100b, 35% for Nestin and 30% for p75NGF receptor after induction. Immunocytochemical analysis showed that undifferentiated humans MSCs were negative for neural surface markers of S100b, GFAP, Nestin and p75NGF receptor shown by the DAPI counterstaining, but after exposure to a series of induction into neural lineage, human MSCs were positive towards all the neural surface markers tested (Fig. 2).

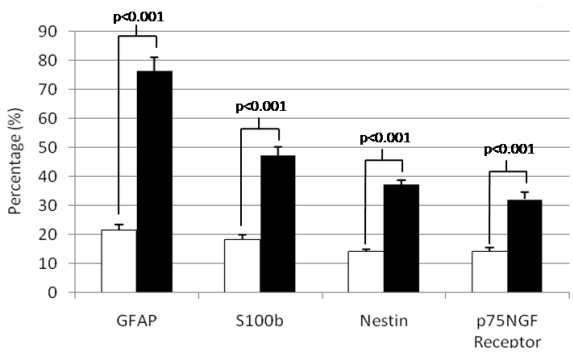

Fig. 1 Histogram showed percentage of cell before (open bar) and after (close bar) induction into differentiated MSCs. There was a significant increase of percentage before and after induction (p<0.001)

3.2 Evaluation of in vitro constructs

Ultra structural analysis demonstrate loose muscle fibres which results from the process of hydrolization during the preparation of acellular muscle. The muscle fibres mimic the structure of the nerve fibres. In contrast, the prepared PLGA sheets showed a highly porous structure each with different sizes. The pore size was 20 – 100 µm with uniform distribution and interconnections. Figure 3 showed gross picture of both constructs immediately after fabrication. At three days post-culture, the cells were seen attached to the surface of both prepared muscles and PLGA constructs. The cells were healthy and distributed evenly on the surface of the muscle and were seen to penetrate into the bigger pores of the PLGA sheets (Fig. 5). Histology analysis of muscle stuffed vein showed cells were actually embedded within the hydrolyzed fraction of the muscle (Fig. 4). Analysis of both construct showed good distribution and proliferation of seeded cells.

4.0 Discussion

MSCs derived from bone marrow have shown the potential to differentiate into glia- and Schwann cell-like cells[14]. Montzka et al.[15] demonstrated the capacity of human mesenchymal stem cells to express different neuronal and glial markers such as dopamine receptor D2, neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). Shimizu S et al.[11] produced a protocol utilizing multiple trophic and growth factors such as b-mercaptoethanol, all-trans-retinoic acid, platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), heregulin β-1 and forskolin to induce glial-like differentiation of human MSCs. Utilising Shimizu's protocol, we have successfully induced the differentiation of human MSCs into neural lineage in vitro. Quantitatively, about 30 to 75 percent of MSCs differentiated into neuro trans-differentaited MSCs by immunocytochemistry and flow cytometry analysis.

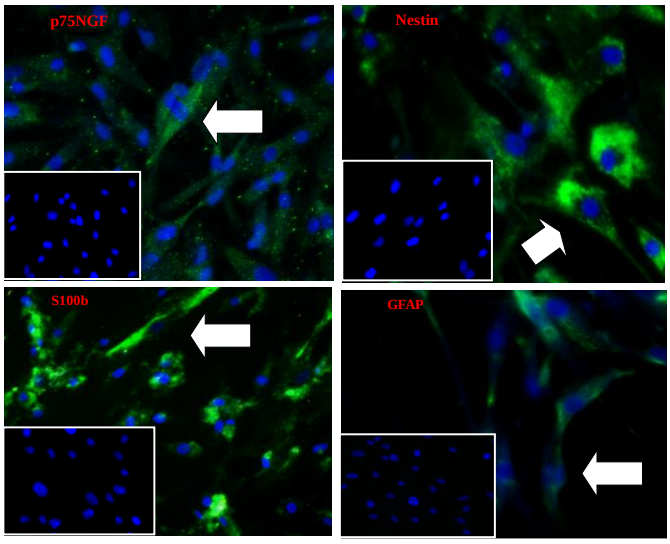

Fig.2 Immunocytochemistry of S100b, GFAP, p75NGF receptor, and Nestin after differentiation. Insert showed undifferentiated MSCs were negative towards SCs surface markers shown by the DAPI counterstaining. Arrows indicate some of the positive cells. (40X)

The process of hydrolization in the preparation of the acellularized muscle is important to loosen the fiber bundles of the muscle and allow resemblance of the structure of nerve fibers. Skeletal muscles have proven effective in peripheral nerve repair. Once the sarcoplasm and plasma membrane have been eliminated by macrophages, the basal lamina of the muscle acts as a guide for the regenerating axon[5]. The nature

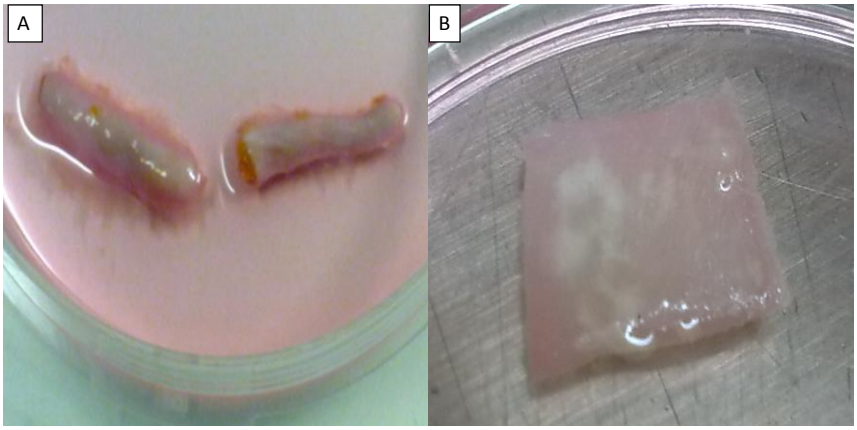

Fig. 3 Gross picture of muscle stuffed vein construct (A) and PLGA sheet (B).

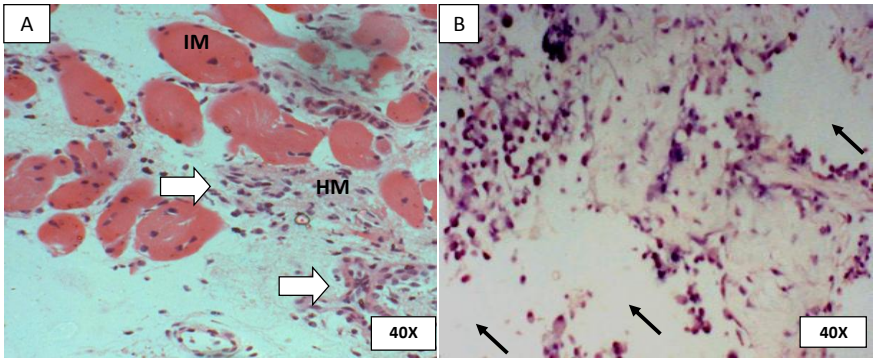

Fig. 4 H&E staining of hydrolyzed (HM) and intact muscle (IM) of muscle stuffed vein constructs (A) and pores within the PLGA sheet (B) marked by black arrows. White arrows shows seeded cells embedded within the hydrolyzed fraction of the muscle matrix.

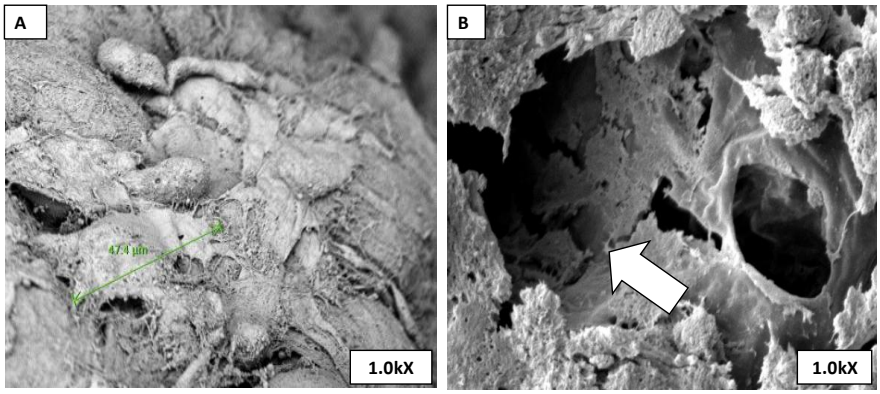

Fig. 5 Scanning electron micrograph of differentiated MSCs seeded (A) on acellular muscle and (B) on porous PLGA sheet. Sizes of the seeded cells are 20 - 60 µm. The seeded cells have penetrated deeper into the bigger pores of the PLGA (white arrow).

of muscle graft itself facilitates diffusion of nutrients resulting in a rapid revascularization time of 5 to 7 days and a highsurvival rate of implanted Schwann cell as reported by Keilhoff et al.[12]

The intact acellular vein can be easily sutured to adjacent nerve tissue. Additionally, the vein has the necessary barrier to prevent infiltration of unwanted tissue into the conduit from the outside [13]. Also the muscle stuffed vein conduit is strong enough to remain without tearing at the sutured site. Benedetto GD et al.[16]combine muscle and vein but this was not seeded with cells, thus our study is unique by presenting data on seeded muscle stuffed vein conduits.

Our prepared porous PLGA sheets had numerous pores of various sizes. Each pore interconnected with each other

forming a network of tunnels within the sheet. The highly porous structure of PLGA sheet renders it highly permeable, appropriate for nerve regeneration where it is essential for entry of nutrients into the inner part of the conduit to promote nerve regeneration. However, the prepared porous PLGA sheets showed weak mechanical properties as a result of brittleness and stiffness. The PLGA sheets were not able to be rolled into cylindrical tube like-structures due to its brittleness.

We evaluated the biocompatibility of the prepared nerve conduit by seeding differentiated cell in a tissue culture flask. Both PLGA sheet and muscle stuffed into vein did not induce any morphological changes or cell death and SEM observation demonstrated good attachment of the cells. There was good biocompatibility of muscle stuffed vein and PLGA materials. However, we noted that the degradation products of PLGA are more acidic when compared to muscle, which may cause some damage to the surrounding tissue. Using a static seeding procedure, cells were found attached to the surface of the acellular muscle in a desired position allowing further proliferation, because the hydrolyzation process loosened the fiber bundles.

H&E staining showed good distribution and growth of the seeded cells on the porous PLGA sheet. We believe interconnecting pores enhances cell proliferation throughout the outer and inner surface of the porous PLGA sheet.

5.0 Conclusion

Both PLGA biodegradable polymer and biological muscle stuffed vein conduit seeded with neural-differentiated MSCs are suitable conduits for nerve tissue engineering. This study provides promising results for further investigation of these conduits implanted into an animal host or model.

Acknowledgement

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