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Collagen-Coated Polylactic-Glycolic Acid (PLGA) Seeded with Neural-Differentiated Human Mesenchymal Stem Cells as a Potential Nerve Conduit*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Autologous nerve grafts to bridge nerve gaps pose various drawbacks. Nerve tissue engineering to promote nerve regeneration using artificial neural conduits has emerged as a promising alternative.
Objectives. To develop an artificial nerve conduit using collagen-coated polylactic-glycolic acid (PLGA) and to analyse the survivability and propagating ability of the neuro-differentiated human mesenchymal stem cells in this conduit.
Material and Methods. The PLGA conduit was constructed by dip-molding method and coated with collagen by immersing the conduit in collagen bath. The ultra structure of the conduits were examined before they were seeded with neural-differentiated human mesenchymal stem cells (nMSC) and implanted sub-muscularly on nude mice thighs. The non-collagen-coated PLGA conduit seeded with nMSC and non-seeded non-collagen-coated PLGA conduit were also implanted for comparison purposes. The survivability and propagation ability of nMSC was studied by histological and immunohistochemical analysis.
Results. The collagen-coated conduits had a smooth inner wall and a highly porous outer wall. Conduits coated with collagen and seeded with nMSCs produced the most number of cells after 3 weeks. The best conduit based on the number of cells contained within it after 3 weeks was the collagen-coated PLGA conduit seeded with neuro-transdifferentiated cells. The collagen-coated PLGA conduit found to be suitable for attachment, survival and proliferation of the nMSC. Minimal cell infiltration was found in the implanted conduits where nearly all of the cells found in the cell seeded conduits are non-mouse origin and have neural cell markers, which exhibit the biocompatibility of the conduits.
Conclusions. The collagen-coated PLGA conduit is biocompatible, non-cytotoxic and suitable for use as artificial nerve conduits (**Adv Clin Exp Med 2014, 23, 3, 353–362**).

Key words: PLGA, collagen, mesenchymal stem cells, nerve regeneration, tissue engineering.

The difficulties in treating nerve injuries may result in patients left untreated or with little choice of treatment. In many nerve injuries, the nerve ends cannot be directly joined as the nerve gap is too big for tensionless apposition and a nerve graft is needed to bridge the defect. Most patients receiving treatment for traumatic peripheral nerve injuries do not show measurable signs of recovery

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or at least suffer from drastically reduced muscle strength [1]. Currently, a nerve autograft is the gold standard in treating nerve trunk defects when the tensionless apposition to suture the severed nerve is not possible. Nevertheless, autografts pose various drawbacks, such as the need for a secondary surgery, loss of donor site function, donor site morbidity, limited availability and structural differences between donor and recipient grafts [1, 2]. Therefore, there is an urgent and unmet need to find an alternative approach in bridging the nerve defects.

Nerve tissue engineering to promote nerve regeneration has been a promising alternative to the nerve autograft. Various synthetic biomaterials have been studied in the construction of artificial nerve conduits. The ideal artificial nerve conduit should have certain biochemical and mechanical properties such as biocompatibility, ability to regenerate the nerve along the whole length of the conduit, semi flexibility and easy handling during surgery [3, 4]. Furthermore, neurotrophic factors within the artificial nerve conduit help to align the nerve axons during regeneration as compared to the haphazard regeneration in autografts [5]. Polylactic-glycolic acid (PLGA) is one of the top biodegradable synthetic polymers used for tissue engineering due to the ease of controlling its mechanical properties and biodegradation rate [6]. PLGA hollow tubes filled with cells have been studied for use in nerve graft and showed promising results [7, 8]. However, the hydrophobicity of PLGA and absence of cell recognition sites on the PLGA scaffold may hinder the cell adhesion onto PLGA surface [9]. Thus, increasing the functionalization of the PLGA surface by coating it with various extracellular matrix protein such as collagen, laminin and fibronectin will promote cell adhesion and proliferation [10, 11]. Collagen, on the other hand, is one of the most promising materials for nerve conduit construction due to its biocompatibility and ability to support cell attachment and function [12]. Collagen-coated conduit was shown to improve nerve regeneration by promoting axonal and Schwann cell regeneration [12, 13]. The hydrophilicity of collagen, which is much greater than the synthetic polymers such as PLGA, contributes to its greater cell adhesion properties compared to that of synthetic polymers [12]. Previous studies have also shown that nerve conduits made of PLGA tube filled with mixture of collagen type-I and ectomesenchymal stem cells (isolated from neural crest of Sprague-Dawley rats) facilitated a greater nerve regeneration compared to the collagen filled PLGA conduit [14]. Hence, in this study, the effect of collagen coating to the PLGA conduit is investigated as it is proven that collagen increases cell attachment, survivability and propagation.

Previously, we have successfully differentiated the human mesenchymal stem cells into neural-differentiated human mesenchymal stem cells (nMSC) [15, 16]. Phase-contrast microscopic observation showed morphological changes of human MSCs before and after induction. Undifferentiated MSCs displayed a flat fibroblast-like morphology: a spindle shape with short processes whereas differentiated cells appear in star-shape with elongated processes. Flow cytometry analysis also showed that human MSCs isolated from human bone marrow in our lab have tested positive for mesenchymal cell surface markers such as CD 90, CD105, CD 10, CD 29, and CD 44 but negative for hematopoietic cell markers, CD34 and CD 45 [15]. To examine the efficiency of MSC differentiation as an alternative source of SCs, the cells were subjected to a series of treatments with a reducing agent, retinoic acid, and a combination of trophic factors. Following induction, the differentiated cells finally look a star-shape with elongated processes. In this study, we aim to develop an artificial nerve conduit using collagen-coated PLGA, analyse the survivability and propagating ability of the nMSC in this conduit *in vitro* and *in vivo*. This study will provide insight into whether the human nMSC can survive and propagate within a collagen-coated PLGA conduit and will be a stepping stone toward its clinical trial.

Material and Methods

The study obtained approval from the institution's Research and Ethics Committee (Project approval code: 02-01-02-SF0445). All the animal works were conducted according to UKM Animal Ethics Committee guidelines.

Construction of Collagen-Coated PLGA Nerve Conduit

The dip-molding technique was utilized for the preparation. We used PLGA with a lactide-glycolide ratio of 50 : 50. One gram of PLGA was dissolved in 15 mL of methylene chloride (ratio of 0.5 g PLGA: 7.5 mL methylene chloride). To form the conduit tubes, 18-gauge needles were utilised. Cotton buds were placed with the cotton bud end into the plastic ends of needles to elongate the structure enabling better dipping later. The dip-molding process was performed within a fume hood to prevent the toxic effects of methylene chloride fumes. The needles were dipped into the solution ensuring at least three quarters or more of the needle surface was beneath the solution line. The needles were left for 1 min. They were then removed and hung

within the fume hood for 5 min. This process was repeated for 50 cycles before the needles were left to dry for 24 h. This whole cycle was repeated twice within 4 days.

After the 4th day, a clear structure was seen forming on the outer layer of the needle. The conduits were then removed from the needle scaffold by pushing the conduits off manually and were immersed in a collagen solution made from commercially available recombinant human Type-1 collagen (Coating Matrix Kit, Cascade Biologics, USA) diluted in Dilution Medium (Cascade Biologics, USA) for 16 h at 4°C. The collagen would adhere to both inner and outer walls of the conduit. Sterilization was done using 70% ethanol. Forty-two PLGA conduits of 20 mm length and about 0.5 mm diameter were obtained. Fourteen were used for *in vitro* studies while the remaining for *in vivo* studies.

Preparation of Neuro-Differentiated Mesenchymal Stem Cells

Bone marrow aspiration samples were obtained after written informed consent was granted. Bone marrow was obtained from pedicle blood during the insertion of pedicle screws in spine surgery, intramedullary nailing procedures and amputations of the limbs. These samples were cultured and differentiated as described earlier [15]. Briefly, the bone marrow was cultured in α-minimal essential medium (α-MEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA), and incubated at 37°C and 5% CO_2. After 48 h, the medium was replaced to remove the non-adherent cells. The MSCs were subcultured three times (P3) before they were induced to differentiate. The MSCs were differentiated in α-MEM containing 1 mM beta-mercaptoethanol (b-ME) without serum for 24 h before the culture media was replaced with α-MEM containing 10% FBS and 35 ng/mL all-trans-retinoic acid (ATRA) (Sigma Aldrich, USA). Three days later, the medium was replaced with α-MEM containing 10% FBS, 5 μM forskolin (Calbiochem, CA), 10 ng/mL recombinant human basic fibroblast growth factor (Peprotech, UK), 5 ng/mL platelet-derived growth factor-AA (Peprotech, UK), and 200 ng/mL heregulin-b1-EGF-domain (R&D systems, USA) and cultured for another 4–5 days. These induced MSCs were referred to as neural-differentiated mesenchymal stem cells (nMSC) hereafter.

Evaluation of Undifferentiated and Differentiated Human MSCs with Schwann Cell Surface Markers

Both undifferentiated and differentiated MSC cultures from a total of 6 patients were evaluated by a flow cytometry analysis as described previously [15].

Seeding of Conduits

A total of 3×10^6 cells were diluted in 20 μL culture medium and seeding was done through capillary technique where one end of the conduit was placed vertically in the culture media and left for 2 h to allow cells to disperse equally throughout the conduit. The cell seeded conduits were then cultured for another month before usage.

Animals

All the animal works were conducted according to UKM Animal Ethics Committee guidelines. A total of 13 female athymic mice of about 4 months of age and weighing 200 g were obtained. They were anesthetized using a mixture of zoletil, ketamine and xylazil at 0.1 mL per kg body weight before implantation.

Experimental Groups

The athymic mice were divided into 3 surgical groups: Group 1 (5 mice) received collagen-coated PLGA nerve conduit seeded with nMSC, Group 2 (5 mice) received PLGA nerve conduit seeded with nMSC, and Group 3 (3 mice) received PLGA nerve conduit only. Figure 1A shows the nerve conduit before implantation.

Surgical Procedure for Implantation

Once the mice were anaesthetised, a longitudinal incision is made on the lateral side of the thigh. Blunt dissection through the thigh muscle down to the bone allowed visualisation of the sciatic nerve. The conduit was then rinsed using phosphate-buffered saline (PBS) and placed immediately next to the nerve. We ensured the thigh muscle would cover the conduit before suturing. The skin was closed with an absorbable suture and chloramphenicol cream (CMC) applied to protect the surgical site from infection.

Histochemical Staining

The mice were sacrificed in batches at week 1, 2 and 3 using intraperitoneal phenobarbitol. The conduits were harvested, formalin-fixed and paraffin-embedded. Cross-sectional tissues of 4 μm were taken from the central region of each conduit and stained with hemotoxylin and eosin (H & E). Sections were viewed using light microscopy. Images were captured using the software QCapture Pro 2003 (QImaging). Cell quantification was done manually with a downloadable cell grid counter under 100 × magnification view.

Immunofluorescence Staining

The histological sections (4 μm) of conduits harvested from mice were marked using a wax pen and blocked using 10% goat serum in tris buffered solution (TBS) for 30 min at 37°C. Subsequently, the specimens were incubated with 5 μg/mL mouse anti-nerve growth factor, p75 (p75NGF) monoclonal antibody (Millipore, USA) and 2.5 μg/mL rabbit anti S-100 protein antibody (Rockland Inc., USA) at 4°C overnight. On the next day, the slides were washed for 4 times with TBS containing 1% goat serum and 0.1% Tween 20 and incubated with 1 : 250 diluted Alex Fluor 488 anti-mouse IgG and Alex Fluor 594 goat anti-rabbit IgG for 2 hour at 37°C. The specimens were washed twice with TBS containing 1% goat serum and 0.1% Tween 20 and nuclear counter stained with 4',6-diamidino-2--phenylindole (DAPI) diluted 1:20000 in TBS for 20 min at 37°C. Finally the slides were washed as mentioned previously and examined using a fluorescence microscope (Eclipse Ti, Nikon Corporation, Japan).

Statistical Analysis

The data is reported as mean value ± standard deviation (mean ± SD). Student t-test was conducted to compare the groups. P value < 0.05 is considered statistically significant.

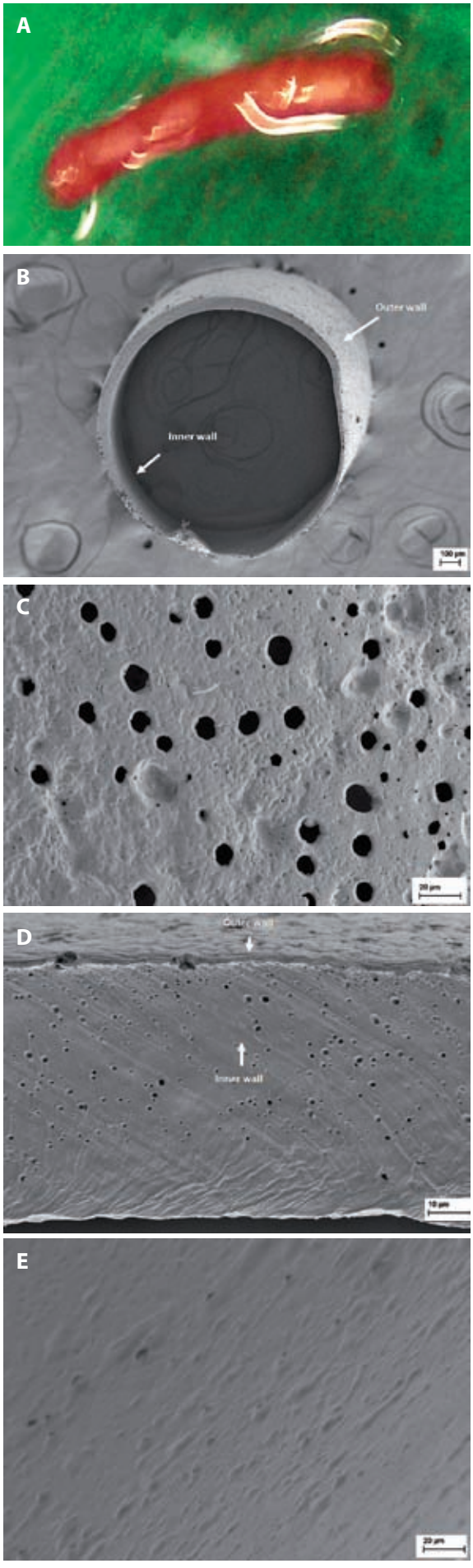

Fig. 1. (A) PLGA conduit before implantation. Electron micrograph of the conduit – (B) Tubular structure of the conduit with irregular wall thickness (magnification: ×46), (C) Cross section of the conduit showing uneven outer surface, compact built and smooth inner layer (magnification: ×1000), (D) Outer surface of the conduit containing discrete micropores (magnification: ×500), (E) Smooth inner wall (magnification: ×500)

Results

Electron micrograph showed a tubular structure with a smooth inner wall and a highly porous outer wall. Wall thickness was around 50 to 60 μm but did not show uniform thickness (Fig. 1B, 1C). The outer wall had unevenly distributed micropores with varied size, ranging from 1 to 20 μm (Fig. 1D). The inner wall was smooth with few pores detected on it (Fig. 1E).

The flow cytometry analysis for SC surface markers on the pre-implantation cells showed that less than 20% of the undifferentiated cells expressed SC surface markers while differentiated cells showed increased SC surface markers approximately 75% positive for GFAP, 45% for S100b, 35% for Nestin, and 30% for p75NGF receptor (Fig. 2). The increase was statistically significant with a p value < 0.0015 for all markers. The differentiated cells were subsequently used for the seeding of the conduits.

PLGA coated with collagen and seeded with nMSCs produced the most cells after 3 weeks (Fig. 3). Fig. 4A shows the mean number of cells in conduits from different groups 1 week, 2 weeks and 3 weeks after implantation. The mean number of cells here corresponded to averaging the number of cells visible under 100 × magnifications from multiple representative sections of all the conduits from each group and does not represent the total number of cell per conduit. From the findings, there was a gradual increase in the number of cells from week 1 to week 3. The higher number of cells in collagen-coated PLGA compared to that of non-collagen-coated conduit at week 1 suggested that more nMSCs adhered to the conduit and survived in it than in non-collagen-coated conduit. A small number of cells were found in non-seeded conduit at week 1 and these are most probably mouse cells that had infiltrated or migrated into the conduit. Increase in mean cell number per section at week 2 and week 3 implied that the total number of cells had multiplied. Comparatively, collagen-coated PLGA conduit yielded conduits with the most number of cells on week 3 post-implantation. The best conduit based on the number of cells it contained after 3 weeks was the collagen-coated PLGA conduit seeded with nMSCs (Fig. 4). The large standard deviation in the mean cell number per section from conduits at 3 weeks post implantation (Fig. 4A) could be caused by the variance in cell performance from different donors. The second possible factor is the difference in physiological conditions for each experimental mouse (host animal). These possibilities are drawn up from mean cell numbers at 3 weeks post implantation (n = 5) (Fig. 4A) which shows a wide-ranging cell number in the conduits across the 5 experimental mouse

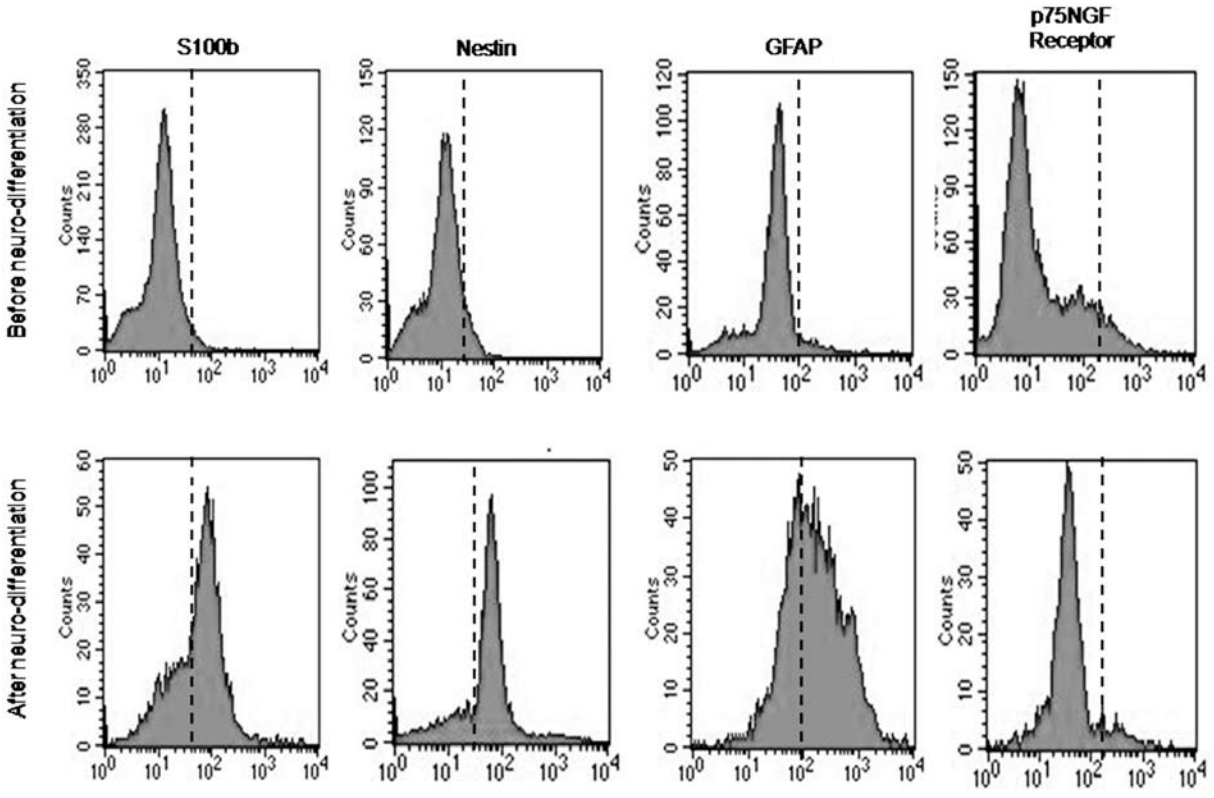

Fig. 2. Histogram of flow cytometry analysis for the presence of Schwann cell (SC) surface markers i.e. S100b, GFAP, Nestin and p75NGF receptor, before and after neuro-differentiation. Dotted lines represent the threshold level for fluorescence positivity. Level of SC surface markers expression by human nMSCs increased after neuro-differentiation

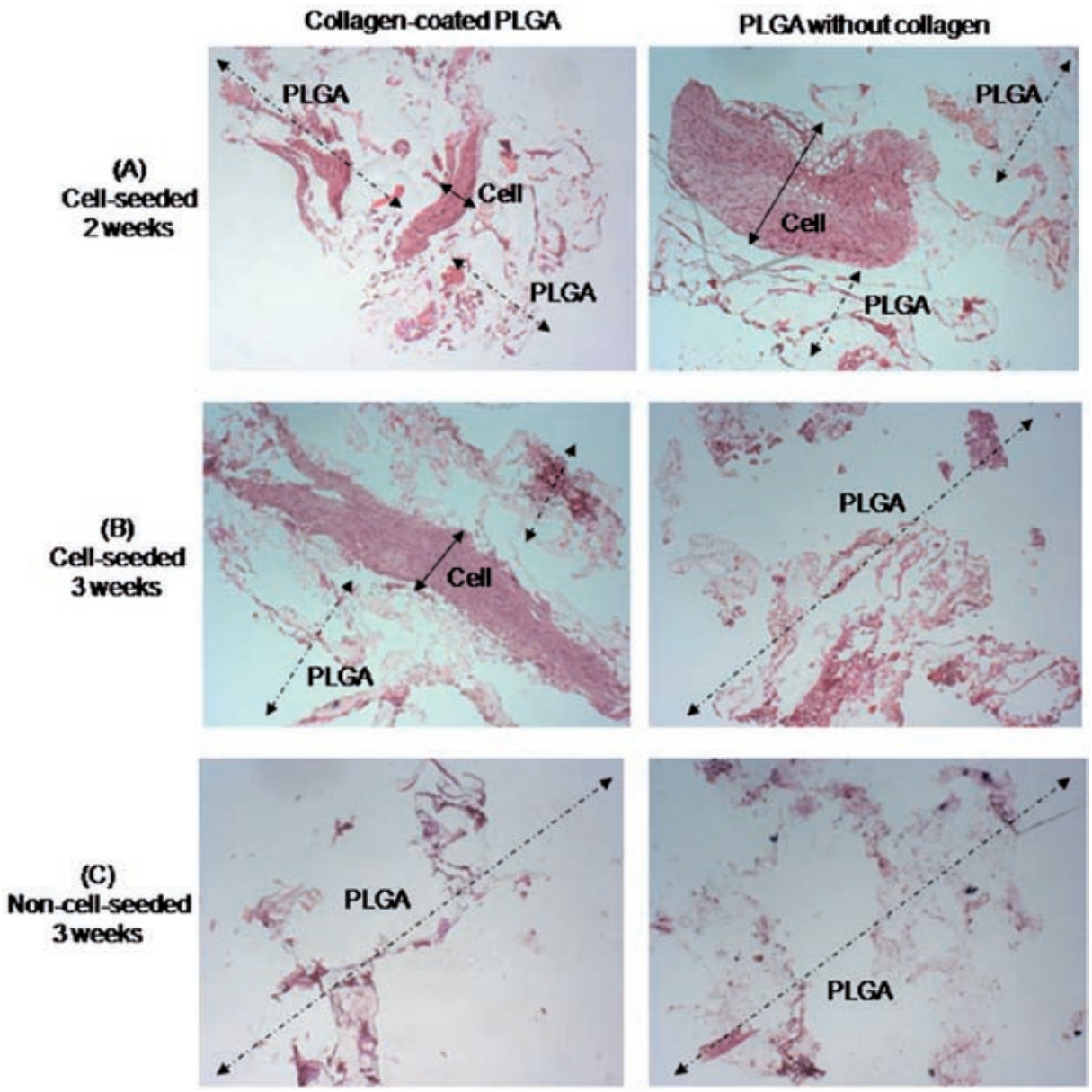

Fig. 3. Histochemical staining of implanted conduits' cross section. (A) PLGA conduit seeded with nMSCs 2 weeks post-intramuscular implantation. (B) PLGA conduit seeded with nMSCs 3 weeks post-intramuscular implantation. (C) PLGA conduit without any seeded cells 2 weeks post intramuscular implantation (×40 magnification). Arrow () shows the area populated with cells surrounded by PLGA scaffold. In (C), PLGA scaffold appeared degraded

where PLGA-collagen conduits consistently scored higher. However, the results of these preliminary studies consistently and clearly have indicated that the collagen-coated PLGA conduit is not only suitable for attachment and survival of the nMSCs but also suitable for its cellular proliferation.

The immunofluorescence staining was conducted to identify and distinguish the nMSCs from the infiltrated host cells. Logically, neural cell markers will only be present in the seeded human nMSC and not on the infiltrated host cells as the conduit has been implanted subcutaneously. Furthermore, the p75NGF monoclonal antibody that was used in this study was specific for human antigens and did not cross-react with rat or mouse antigens. Thus, the observed fluorescence corresponding to S100 and p75NGF proteins (Fig. 5a, 5b) is inferred to have emitted from the seeded human nMSC. The survival and propagation of seeded cells in the conduit shows that both the PLGA conduit and collagen-coated PLGA conduit have good biocompatibility. We found minimal or no host cell infiltration in the non-seeded conduit.

Discussion

Since the 1980s a multitude of research has been done and is still ongoing on the topic of synthetic nerve conduits. Previous research using

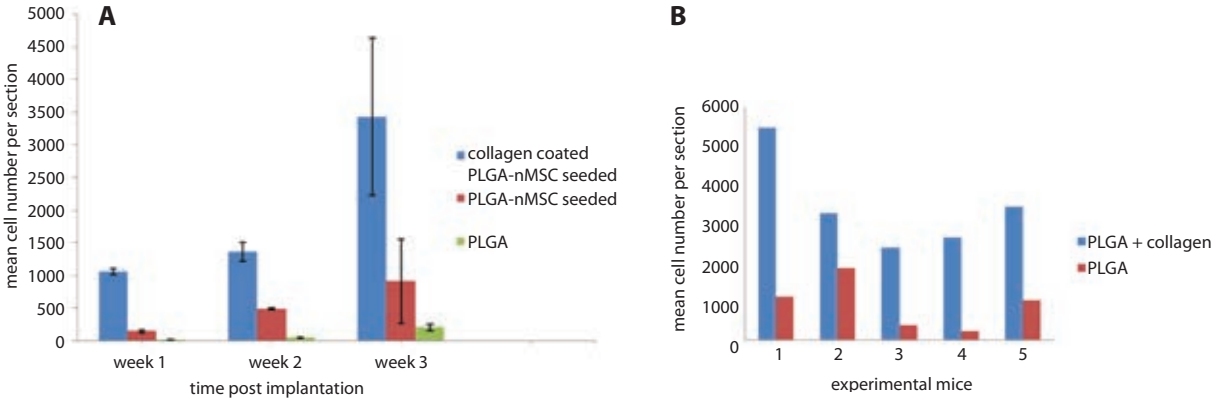

Fig. 4. A. Mean and standard deviation of cell number per the H & E stained representative sections from conduits after 1 week, 2 weeks and 3 weeks post-implantation. * The difference in the cell number between the collagen-coated PLGA-nMSC seeded and PLGA-nMSC seeded at week 3 post implantation is statistically significant ($p < 0.01$) ($n = 5$ for the collagen-coated PLGA-nMSC seeded and PLGA-nMSC seeded group, and $n = 2$ for the PLGA group). B. Mean cell number per the H & E stained representative sections of paired conduits harvested from each experimental mouse at 3 weeks post-implantation

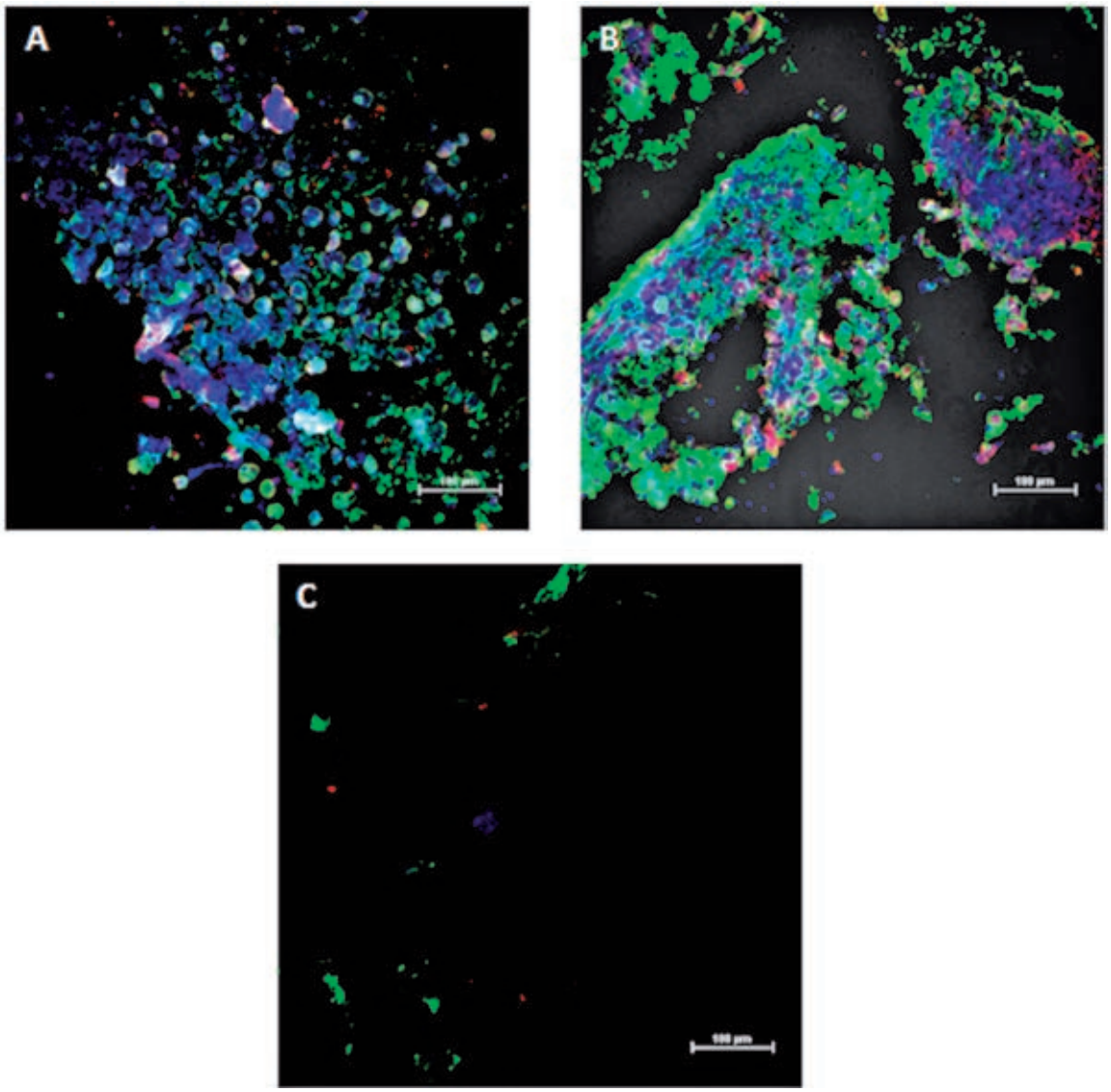

Fig. 5. Immunofluorescence staining of the implanted conduits' cross section. Cells with DAPI-counterstained nucleus (blue) are expressing S-100 (red fluorescence) and p75NGF (green fluorescence) (A) collagen-coated PLGA conduit seeded with nMSCs, (B) non-collagen-coated PLGA conduits seeded with nMSCs 2 weeks post-implantation. (C) Control – empty non-collagen-coated PLGA conduits 2 weeks post-implantation emitting background fluorescence. (×40 magnification)

a simple PGA conduit has evolved into a veritable number of types and combinations to produce the best synthetic nerve conduit [17]. Researchers are currently studying the benefits of filling these conduits with biological material such as collagen or with neurotrophic factors to further enhance the potential of nerve regeneration [18, 19]. This vast variability in producing a synthetic nerve conduit has resulted in no clear consensus on the best product available.

The collagen-coated PLGA nerve conduit seeded with human nMSCs is an improvement in the development of a suitable conduit system for nerve regeneration. Previous studies have shown that PLGA-coated collagen is superior to vein graft in nerve regeneration [20, 21]. Furthermore, the ability of human MSCs to differentiate into neuronal cells and facilitate the neuronal growth that has been studied previously [15, 22]. Thus, we undertook the study to evaluate the survival and propagation of human nMSC in collagen-coated PLGA tube. We chose PLGA as our core polymer as its biomechanical property could be tightly controlled by adjusting the ratio of its two monomers, namely, lactic and glycolic acid. Differing its ratio would affect its strength, flexibility and biodegradability. Degradation, in turn, controls the rate of release of growth-promoting substances, as they are released primarily in association with degradation of the polymer walls [23]. PLGA itself provides good scaffolding for collagen and is a known complete biodegradable product. Although collagen tubes promote nerve regeneration and have been approved for human use [13], collagen tube can break and its lumen could collapse due to movement. Furthermore, the fast resorption of collagen may cause failure of nerve regeneration when the conduit is used to bridge a extended nerve gap defect [24]. In this study, we constructed the conduits using PLGA and coated it with collagen to enhance its tensile strength and provide a surface conducive for cell adherence, propagation and also infiltration. Such an approach has not been studied previously.

The technique of nerve conduit fabrication in this study allows the production of a relatively acceptable conduit at marginal costs using apparatus readily available anywhere. However, there are a few limitations in this technique. Firstly, production of the conduits manually is time consuming and labour intensive. A machine which can aid in the repetitive dipping step can significantly decrease production time. We also noticed the conduits become slightly brittle after sterilization with ethanol; a possible alternative, such as gamma irradiation, could be explored in future studies.

Researchers observed that wall thickness plays an important factor in axon re-growth within the conduit; with very thick walls (> 0.81 mm) the regeneration process is stunted due to loss of implant permeability [25]. Our constructs had a wall thickness of 0.05 to 0.06 mm under the scanning electron microscope (SEM). Wall permeability also plays an important role in determining the performance of the implanted material [26]. However, it has been shown that smaller pore size actually fares better than a larger one, due to better diffusion through smaller pores [27]. Our conduits had smooth inner surfaces but a highly porous outer layer. The inner wall was smooth due to the close contact with the smooth needle surface used during its construction.

The *in vivo* study was performed using athymic mice to reduce the possibility of immune reaction towards the human cells introduced. We had shown our conduits were biocompatible as all mice survived following implantation up to 3 weeks. We also decided to place the implant intramuscularly as an initial phase to ensure its biocompatibility and its ability to maintain and promote cellular growth. The next logical step would be end-to-end anastomoses with cut nerve in a larger athymic animal model using the same conduits. At harvesting time, all conduits were still present although those at 3 weeks showed significant loss of structure. This shows that the stability of the conduits *in vivo* needs to be improved to enable the nerve regeneration in a reasonable time frame. A higher ratio of lactide in the polymer e.g. 60 : 40 or 70 : 30 lactide: glycolide would be able to enhance the stability of the conduit.

Through histological examination, three main features were evaluated. Firstly, the cellular density within the conduit at week 1 post-implantation was evaluated. Abundant cells were found within the conduits at week 1. The highest number of cells in collagen-coated PLGA tube seeded with human nMSCs (Fig. 4) suggests that collagen supports cell adherence and proliferation. The seeded nMSCs are expected to function similarly to Schwann cells (SC) and support axonal regrowth by increasing their synthesis of surface cell adhesion molecules (CAMs) and secreting neurotrophic factors [28]. There were also minimal inflammation cells surrounding the conduit throughout the study (Fig. 3). This shows that the conduit has good biocompatibility and allows the proliferation of cells within it, making it a good candidate for nerve reconstruction. This is in concordance with previous studies [20, 23]. Secondly, we compared cell proliferation in collagen-coated conduits with non-collagen-coated conduits in week 2 and 3. There was a steady increase of cellular proliferation with corresponding degeneration of PLGA structure in both cell seeded conduits. In the future study, the determination of cell number

seeded in the conduit prior to implantation is necessary as it can provide better and accurate indication on the efficiency of cell seeding.

Thirdly, conduit stability between the cell seeded and empty conduits were compared. Neurotrophic factors such as nerve growth factor (NGF) and bone marrow stromal cells (BMSc) have been studied extensively and have been shown to promote nerve regeneration within a conduit [29]. Provided that a controlled release of these growth factors is allowed through gradual degradation of the conduit, the nerve regeneration would also align properly within the conduit [30]. In this study, minimal cell infiltration into the conduits was shown. The immunofluorescence staining has shown that nearly all of the cells found in the cell seeded conduits are non-mouse origin and have neural cell markers. The non-seeded conduit only emitted background fluorescence which shows nonexistence of cells in the conduit. Empty conduits showed minimal cellular activity and also loss of structural integrity. While cell seeded conduits at week 2 and 3 showed abundant cell proliferation and cellular organization, and maintained its structure to certain extend.

However, these results only indicate the preliminary outcome as the project could not be conducted with a larger sample size and extended beyond the planned duration due to constrain of resources. Additional studies with larger sample size, longer time period and also involving sciatic nerve grafting need to be conducted to ascertain functional outcomes of treatment with collagen-coated PLGA nerve conduit seeded with human nMSCs in comparison with the gold standard.

The authors concluded that the collagen-coated PLGA conduit had the suitable properties as a synthetic nerve conduit. It proved to be biocompatible allowing cell proliferation and collagen coating further enhanced cell adherence.

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