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CHARACTERIZATION OF COLLAGEN HYDROGEL FORTIFIED DERMAL FIBROBLAST CONDITIONED MEDIUM (DFCM) FOR SKIN THERAPEUTIC APPLICATION

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SUMMARY

Majority of the cell-based skin substitute requires long production time and not available for the immediate treatment of skin injury. Fibroblast is known to secrete essential factors for wound healing that can be collected from a cultured medium as dermal fibroblast conditioned medium (DFCM). Previous study shows the enhancement of keratinocytes and fibroblasts wound healing properties by DFCM. This study aims to fabricate and characterize the 3D acellular collagen hydrogel fortified with DFCM as readily available skin substitute. Fibroblasts from human skin samples (consented patients) were cultured using the serum-free keratinocyte-specific medium to collect DFCM. Subsequently, DFCM (200µg/mL) were mixed with collagen hydrogel and chondroitin-4-sulphate (C4S) to fabricate a 3D construct known as Col-DFCM. Collagen alone (Col) and collagen with C4S (Col-C4S) act as a control. All constructs were analyzed for turbidity, porosity, swelling and degradation, Fourier transform infrared spectroscopy (FTIR) analysis and compression. All groups had successfully formed a soft, semi-solid and translucent gel within 1-hour incubation at 37ºC. For turbidity, the Col-DFCM shows significantly lower absorbance compared to control groups. Besides that, Col-DFCM (35.15±9.76%) also shows a lower percentage of porosity compared to Col (105.14±11.87%) and Col-C4S (143.44±27.72%) groups. All constructs demonstrated similar pattern for swelling and degradation. The FTIR peak for all group consists of a functional group, oxygen-hydrogen bond, O-H at 3330-3340cm^{-1} and amide band I, II and III at a wavelength of 1639-1640cm^{-1}, 1500-1600cm^{-1}, and 1260-1300cm^{-1}. The mechanical strength test of the constructs was determined using bloom strength, where the higher force peaks (in Newton, N) represent higher strengths. The results showed no significant differences between all groups with strength less than 2.5N, indicated the soft collagen hydrogels in all constructs. These findings showed that the fabrication of Col-DFCM construct maintain the structure of collagen and could be an alternative for delivering essential components for skin regeneration.

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1.0 Introduction

Peripheral nerve regeneration has been gaining much attention in the field of study due to the conventional method and functional recovery has often been incomplete or unsuccessful. This in turns will affect the patients' life quality as neuropathy pain is always the complication of peripheral nerve injury (PNI) [1,2]. Nerve autograft is the gold standard in most surgical nerve cases to bridge gap between nerves in which sural nerve taken from the injured patient will be commonly used [3,4]. Allografts is an alternative for nerve regeneration however there's a major concern on immunogenic reactions in the recipient, in which requires the recipient to be immunosuppressed until the host Schwann cells regenerated on the nerve graft [5]. Hence, the usage of electrospinning technique to produce a variety of nerve conduit with various advantages such as aligned fibers, tunable porosity, and malleablility can be used as an alternative future nerve conduit [6,7].

2.0 Materials and Method

2.1 Collagen Sheet Fabrication

Collagen was obtained from Ovine tendon and soaked with 0.35M acetic acid for 3 days before blending it into a solution form. NaCl salt was added to precipitate the collagen and undergo dialysis using dialysis tubing made of cellulose membrane of 14kD MWCO to remove excess acetic acid for 3 days. The collagen solution was then freezed-dried to remove the solvent and later be dissolved back into 0.35M acetic acid in a concentration of 15mg/mL. The solution was then poured into a square casting before freeze-dry to produce a sheet of collagen and cross-linked with 0.1% genipin.

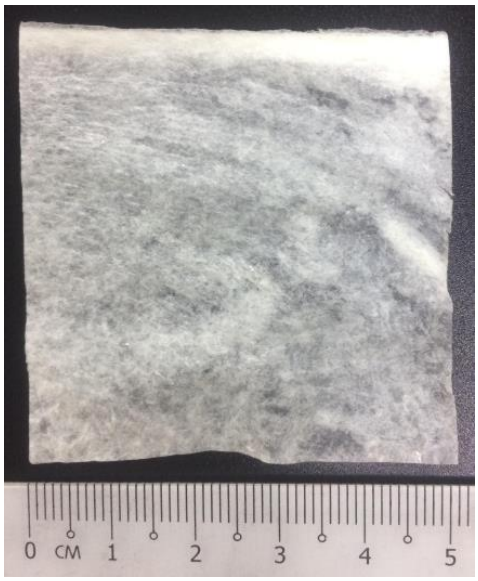

Fig. 1 Fabricated collagen matt

2.2 Electrospinning

A weight of 1.0g of PLGA 85:15 was dissolved in 5ml of DCM:DMF mixture (4:1) to produce 20% concentration of PLGA solution and mixed on a shaker overnight. The polymer solution was then electrospun on top of the collagen sheet with the following parameter. The nanofibers with the collagen sheet were then UV for 30 minutes for sterilization before cell seeding.

Table 1. Electrospinning parameters

Distance (cm)	*Voltage (kV)*	*Flow rate (ml h^{-1})*	*Rotating Mandrel Speed (rpm)*
33	12-14	0.15	1400

2.3 Cell Culture and Seeding

Bone marrow mesenchymal stem cell (BM-MSC) was cultured using alpha minimum essential medium (α-MEM) with 10% FBS and 1% antibiotic antibiotic until P3. A series of concoction with the following chemicals and growth factors in the α-MEM was then used to induce the BM-MSCs into neural-like cells. Neural protein markers (p75 NGF, S100ß, Nestin and GFAP) were then detected via immunocytochemistry.

Table 2 Neural induction medium for the BM-MSCs

Days of incubation	*Chemical/Growth Factor*	*Concentration in medium*
1	ß-Mercaptoethanol	1mM
3	FBS	10%
	All Trans Retinoic Acid (ATRA)	35ng/mL
	FBS	10%
5	bFGF	10ng/mL
	PDGF-AA	5ng/mL
	Heregulin	200ng/mL
	Forskolin	5μM
	B_{27}	2%

The induced MSCs were then seeded onto the PLGA nanofibers at a seeding density of 3000 cells/cm^2 in a 12-well plate. The seeded cells were cultured for another 7 days followed by detection of neural protein markers expression via immunocytochemistry.

2.4 Fiber Characterization

The electrospun nanofibers were evaluated using Scanning Electron Microscope (FEI, USA). Diameter of the fibers and the alignment of the fibers were determined using Image J software (NIH, USA).

3.0 Results

3.1 Fiber Characterization

Most of the nanofibers appeared smooth and aligned. When sampled from 200 fibers were measured, approximately 80% were aligned in one direction and has a diameter ranging from 700-1000nm. Figure 2 shows the morphology of the fibers spun on a collagen sheet seen under the SEM.

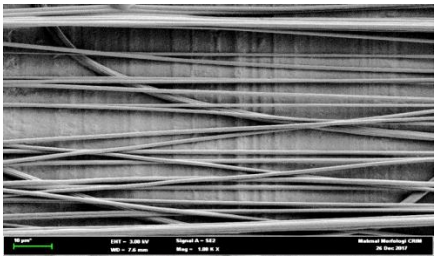

Fig. 2 Nanofibers under SEM imaging (1000x magnification)

3.2 Neural Marker Expression

The induced MSCs were subjected to immunocytochemical staining to detect the protein expression of GFAP, S100ß, Nestin and p75 NGF.

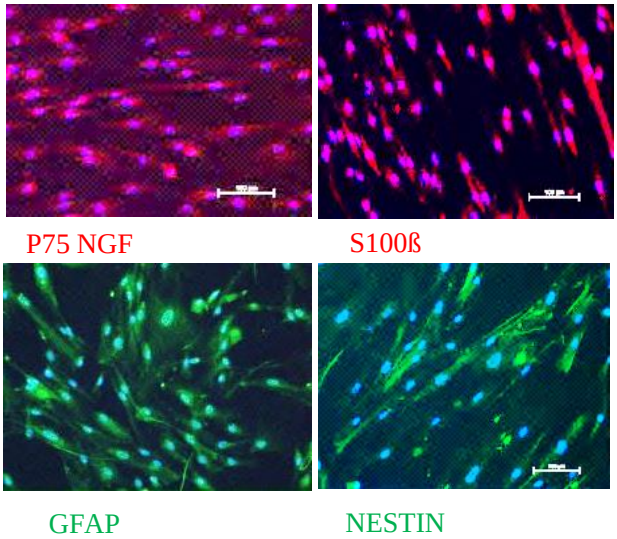

Fig. 3 Protein expression of p75 NGF (red), S100ß (red), GFAP (green) and Nestin (green) by neural-differentiated MSCs seeded for 7 days on PLGA nanofibers.Cell nucleus were counterstained with DAPI (blue). Magnification: 40X (Scale bar: 100µm)

4.0 Discussion & Conclusion

We have successfully electrospun PLGA nanofibers with unidirectional alignment. This is deemed crucial as nerve fibers in peripheral nerves are also unidirectional. BM-MSCs were successfully induced into neural-like cells as demonstrated by expression of neural protein markers such as nestin, S100ß, GFAP, and p75 NGF. The expression of the neural markers persisted after the seeding of these cells on the PLGA nanofibers for 7 days. In our observation, the cells attached preferentially on the nanofibers compared to the collagen mat, and they migrated along the nanofibers in a unidirectional manner. This attribute greatly supports the use of these nanofibers as nerve conduit to facilitate the migration of neural cells from the proximal stump of the peripheral nerve during axonal regeneration. The collagen mat used to support the nanofibers can be rolled into a cylindrical conduit to bridge a peripheral nerve gap. Furthermore, the collagen mat provided the mechanical strength required for the conduit to be sutured onto the peripheral nerve tissue. Following this success, proof of concept in animal models will be performed.

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