SCAFFOLD-FREE TISSUE ENGINEERING USING DENTAL PULP CELLS TO ENHANCE FACIAL NERVE REGENERATION.

by

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Scaffold-free Tissue Engineering using Dental Pulp Cells to Enhance Facial Nerve Regeneration.

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Introduction:

Peripheral nerve damage is a commonly encountered clinical problem caused by trauma, disease, or surgical injury. The current gold standard treatment utilizes autologous nerve grafts; however, this requires a prolonged repair time and full functional recovery is not achieved. Neurotrophic factors (NTF) are proteins known to enhance axon regeneration and growth. Dental pulp tissue contains a population of stem/progenitor cells (DPC) that secrete NTFs a characteristic likely due to their neural crest origin. Furthermore, these cells are easily accessible from autologous sources. Basic fibroblast growth factor / FGF2 is a growth factor considered as a potent mitogen and help mesenchymal stem cells maintain their stemness. The goal of this study was to develop and characterize scaffold-free DPC sheets as a NTF delivery system. We hypothesize that DPC sheets will express NTFs including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factors (GDNF) and neurotrophin-3 (NT-3), and will accelerate repair of damaged nerves and improve functional recovery.

Materials & Methods:

In this study, we fabricated scaffold-free cell sheets by culturing DPCs to super confluence with and without fibroblast growth factor 2 (FGF2). NTF gene and protein expression of DPC sheets was assessed using qRT-PCR and ELISA, respectively. DPC sheets secretome was used to culture SH-SY5Y neurons to test the functional effect of the secretome and validated its effect by using NTF inhibitors on neurite extension in vitro.

Results:

DPC sheets were formed that are robust and can be easily handled. DPC sheets expressed BDNF, GDNF, NT3 at the gene and protein level and the expressions increased with the addition of FGF2 to the culture medium. NTF present in DPC sheet secretome enhanced neurite extension in SH-SY5Y neurons indicating that DPC sheets have a positive functional effect on neurons and the addition of NTF inhibitors reversed this effect confirming the effect of NTF on neurons.

Conclusion:

DPC sheets can be formed which secrete neurotrophic factors and enhance neurite extension in neurons. Scaffold-free DPC sheets show great promise as a new therapy to accelerate the regeneration of damaged peripheral nerves and improve functional recovery.

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PREFACE

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1.0 INTRODUCTION

1.1 Anatomy of the peripheral nerve

The human nervous system is a complex and highly developed system of the body which performs intricate task of relaying and processing information and connects the human body with outside world. Structurally it can be divided into Central Nervous System (CNS) and Peripheral Nervous System (PNS). CNS consists of brain & spinal cord which processes the information received similar to the CPU of a computer. Whereas PNS consists of nerves and ganglia which help transmit information in the form of electrochemical impulse to the CNS. The peripheral nerves are complex yet delicate structures distributed throughout the body, and they are made up of bundles of axons surrounded by the protective connective tissue layers. Individual axon is surrounded by myelin sheath secreted by Schwann cells and covered by the connective tissue layer called as endoneurium. Bundles of axons collectively form the fascicle which is surrounded by another protective layer of connective tissue referred to as perineurium. The group of fascicles together enclosed by epineurium containing the blood and lymphatic supply form a peripheral nerve which transmits impulses and connect the CNS to the target end organs.

1.2 Incidence of peripheral nerve injuries and its classification

Peripheral nerve injuries (PNI) are a common occurrence worldwide caused by trauma, disease or surgical intervention such as tumor resection, and the vast and extensive distribution of nerves throughout the body make them vulnerable to injuries. PNI is a significant medical problem caused by road trauma accidents, gun-shots or stabbing incidents in military personnel and stretching or crushing injuries in athletic persons. More than one million people around the world suffer from peripheral nerve injuries each year: due to the wide distribution of peripheral nerves throughout the body, nerves are vulnerable to different kinds of traumatic injuries with an occurrence of 73% nerve injuries in patients with trauma to upper limbs. There are approximately more than 200 000 peripheral nerve repair procedures being performed annually in the United States (Grinsell and Keating 2014; Sachanandani, Pothula, and Tung 2014). Lately, 2.8% increase in nerve injuries has been reported among trauma patients and more than 50**%** of these patients failed to regain complete sensory and motor functions even after the treatment (Wang et al. 2017). Although peripheral nerves possess an intrinsic ability to regenerate and connect with their target end organs, they usually fail to achieve complete sensory and motor function recovery with critical nerve gap (Sachanandani, Pothula, and Tung 2014).

PNI can have devastating effects causing long term disability therefore affecting the patient's quality of life. Facial paralysis occurring due to the facial nerve injury caused by motor vehicle accidents, falls and gunshot wounds to the craniofacial region is one among such clinical scenarios. The facial nerve is affected in 7%-10% of the temporal bone fractures in the maxilo-facial region (Gordin et al. 2015). Injury to the facial nerve results in paralysis of muscles of facial expression including orbicularis oculi causing functional deficit and psychological distress. The consequences of facial paralysis can be agonizing due to impaired speech, masticatory difficulties,

ocular complications and difficulty conveying emotions through facial expression (Lee, Lyford-Pike, and Boahene 2013). Facial nerve dysfunction is also the most common early surgical complication of the parotid tumor resection where facial nerve is sacrificed (Marchese-Ragona et al. 2005; Kahinga, Han, and Moon 2018). Twenty percent of the parotid gland tumors have facial nerve involvement, necessitating its resection during total parotidectomy procedures (Owusu, Truong, and Kim 2016). Facial nerve injury is also reported in the otologic surgical procedures such as mastoidectomy and an incidence of 1% to 4% of otologic procedures are reported to cause iatrogenic facial nerve injury leading to facial paralysis which can have devastating effect on the patient (Ryu and Kim 2016).

Based on the clinical manifestations, Dr Seddon in 1942 was the first to develop a system to classify nerve injuries into three types (a) Neurotmesis: A nerve transection which completely divides the nerve into two separate parts and creates a total conduction block of all the impulses both sensory and motor. (b) Axonotmesis: The injury is sufficient to damage the nerve fibers and cause peripheral nerve degeneration, yet preserving the protective connective tissue layers intact such as enduneurium, perineurium and epineurium. There can be complete loss of sensory and motor conduction across the lesion. (c) Neurapraxia: It is transient loss of motor and sensory functions following trauma to the nerve causing temporary paralysis and usually it recovers completely restoring the lost functions(Seddon 1942). Additionally, with the above mentioned clinical scenarios is the crush nerve injury similar to axonotmesis which commonly occurs due to acute traumatic compression of the nerve by blunt trauma. If the external compressive force is sufficient to disrupt capillary blood flow and cause ischemia it will contribute to nerve damage and axonal degeneration resulting in loss of impulse transmission across the nerve(Ozturk 2015).

Wallerian degeneration is the disintegration of the myelin sheath and axons of the nerve distal to the nerve injury. Based on the severity of the damage to nerve structure and wallerian degeneration, peripheral nerve injuries have been further classified into five categories. (a) First degree: where the axonal continuity is maintained and there is only temporary blockade of conduction across the damaged nerve and there is no axonal or Wallerian degeneration distal to the injury. (b) Second degree: The axonal continuity is disrupted but the protective connective tissue layer endoneurium is intact. (c) Third degree: The continuity of both axon and endoneurium is interrupted but the fascicular perineurium remains intact. (d) Fourth degree: The fascicles along with the perineurium are damaged but the outermost protective tissue layer epineurium maintains continuity. (e) Fifth degree: The injury causes complete transection of the nerve including the epineurium which separates the nerve in two parts. Wallerian degeneration occurs distal to the lesion in the injuries from the second to the fifth degree, additionally due to the loss of axonal structure with disrupted endoneurium in the injuries of degree three to five the incidence of a clinical condition known as synkinesis increases which is axon misdirection during regeneration of a nerve (Humphrey and Kriet 2008).

1.3 Current treatment modalities for peripheral nerve repair

Peripheral nerve injuries are treated in different ways depending on the degree of the nerve damage. Injuries causing complete transection of the nerve but without any loss of nerve tissue are treated with neurorrhaphy. The two ends of the severed nerve are approximated and sutured together with the defined fascicular perineurium. Precaution must be exercised to avoid stretching or placing tension on the sutured nerve as it might cause cessation of epineurial blood flow leading

to ischemia and necrosis of the regenerating axons which can adversely affect the final functional recovery(Patel, Lyon, and Huang 2018).

Autologous nerve grafting remains the current gold standard to treat peripheral neve defects with loss of nerve structure greater than 1cm (Patel, Lyon, and Huang 2018; Kolar et al. 2017). A nerve graft is harvested from a different site of the same patient and transplanted to the site of injury. The most commonly used nerve grafts are sural nerve, medial antebrachial cutaneous nerve, and posterior interosseous nerves (Patel, Lyon, and Huang 2018). Along with the possibility of above mentioned nerve grafts, facial nerve injuries are primarily treated with the great auricular nerve graft due to the similar nerve diameter and proximity to the facial nerve (Humphrey and Kriet 2008). The drawback of autologous nerve grafting is second surgical site with an increased incidence of painful neuroma formation, donor site morbidity and loss of sensory or motor supply to the area from which the nerve is harvested. The functional outcome can be poor if a sensory nerve is used to repair a motor or mixed sensory-motor nerve defects along with the possibility of size mismatch. Additionally limited availability of the healthy donor sites poses a major challenge in obtaining the suitable grafts (Patel, Lyon, and Huang 2018). Augmenting to the agonizing complications of the autologous nerve graft is the delayed and suboptimal functional recovery of the sensory and motor functions of the treated site, which necessitates the requirement for alternative therapies.

As alternatives to nerve autografts, different options have been tried such as nerve allografts, decellularized peripheral nerve allografts, autologous skeletal muscle tissue, veins and combination of vein and skeletal tissue. But the need to harvest autologous tissue from different part of the body and extended usage of immunosuppressive drugs up to 18 months for treatment

with allografts make them unfavorable for the repair of critical nerve defects (Philips, Cornelissen, and Carriel 2018).

As the search for efficient alternatives to treat nerve defect continues, tissue engineering has been promising in providing a different treatment strategy of artificial nerve conduits which act as scaffolds and guide nerve regeneration during healing or regenerating phase of the traumatic nerve. Artificial nerve guidance conduits have been extensively studied in animals to treat the nerve defects as a substitute to the use of autografts and allografts and they can be made from both synthetic and natural biomaterials such as Polyglycolic acid (PGA), Polylactic-co-glycolic acid (PLGA), chitosan, collagen, alginate etc (Patel, Lyon, and Huang 2018). The nerve guidance conduits (NGC) are required to provide mechanical support, allow transportation of the nutrients, retention of the neurotrophic factors and prevent ingrowth of the fibrous tissue which can hinder the regenerating axons. At the same time, they are also expected to increase the number and length of the regenerating axons in as less duration as possible to improve the final functional outcome (Kehoe, Zhang, and Boyd 2012). To fulfil these requirements some of the criteria which have been put forth are as follows (1) the material must be biocompatible & biodegradable and should not elicit an inflammatory reaction while maintaining a stable mechanical structure by resisting tear during suturing. (2) The NGC should be soft and flexible to avoid compression yet provide necessary cues and direct the regenerating axons to avoid misdirection. (3) The material of the NGC should be semi permeable with a desirable pore size of 5-30 microns to allow diffusion of oxygen and nutrients from the surrounding tissues as pores less than 5 microns will inhibit the proliferation of the cells and tissues and pores of greater than 30 microns will lead to excessive infiltration of the inflammatory cells hindering regeneration. (4) The NGC should meet the technical requirements of reproducibility, prolonged storage, sterilization and resist tear during

surgical suturing (Kehoe, Zhang, and Boyd 2012). Considering afore mentioned requirements, FDA has approved some NGC made from Polyglycolic acid, Type 1 collagen and porcine small intestine that are used in clinics to treat the nerve damages with loss of tissue structure. The products available in the market are, Neurotube, Neuragen, Neuroflex, Neuromatrix, Axoguard Nerve connector, Neurolac, and SaluTunnel Nerve protector from different companies such as Integra, Axoguard and synovis micro companies alliance.

In less severe trauma, such as crush nerve injury where the nerve is compressed yet the continuity is still maintained with no substantial loss of nerve tissue, another set of FDA approved devices known as nerve cuff/protectant wraps are available in the market for treatment. They are also made up of synthetic/natural biomaterials such as polyvinyl alcohol and Type 1 collagen respectively. Their main purpose is to protect the injured or crushed nerve from entrapment, prevent scar and neuroma formation and to create a favorable environment for the regeneration of damaged nerve. The nerve cuffs available to the doctors for the treatment of these kind of cases are Salubridge, Axoguard Nerve protector, NeuraWrap, NeuroMend from companies such as Integra and Stryker (Kehoe, Zhang, and Boyd 2012).

Although the clear advantage of using nerve guidance conduits or nerve cuffs over autograft is the elimination of the donor site morbidity and creation of a second surgical site which reduces the operating time and cost, the NGC are still not the ideal choice to treat the nerve injuries due to the following limitations. NGC have a variable biodegradation from 3 months to 16 months and some of them can take up to 48 months. As the device degrades the mechanical property changes and become inferior and produces degradation products which are acidic and low soluble. NGC can elicit undesirable immune response from the host and can lead to rejection if the immunosuppressive drugs are not administered. The regenerative ability of the device decreases

questionably in old nerve lesions and some of the devices may be rigid, causing compression of the regenerating nerve leading to delayed recovery and inferior outcome. Furthermore; there can be batch to batch variation during the manufacturing of the devices which can lead to variable outcome (Kehoe, Zhang, and Boyd 2012).

1.4 Neurotrophic factors and methods to enhance current therapies

During the development of CNS & PNS, neurotrophic factors namely brain derived neurotrophic factor (BDNF), neurotrophic factor- 3 (NT3), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) are vital and play an important role along with other biological cues in the development of human nervous system. Neurotrophic factors are proteins with molecular weight ranging from 14 kDa to 30.4 kDa secreted by a variety of cells such as neuronal cells, Schwann cells, neural stem cells and mesenchymal stem cells, they have also been extensively studied both in vitro and in vivo for their role during neuronal development, maintenance of adult neurons and regeneration of the injured nerves (De la Rosa, Kozik, and Sakaguchi 2018; Henderson 1996). The neurotrophic factors exert their action by binding to the Tropomyosin receptor kinases (TrK) and each neurotrophic factor has a specific high affinity receptor such as TrKA for NGF, TrKB for BDNF and TrKC for NT3 (Barbacid 1995; De la Rosa, Kozik, and Sakaguchi 2018). The neurotrophic factors produced by the target end organs help direct the extension of growth cones of nerves towards them and elevated levels of NGF and BDNF have shown to increase the density of innervation of axons from neurons (Huang and Reichardt 2001). Experiments have also shown that the presence of neurotrophic factors such as NGF to be essential for the growth of axons, and axon extension was stopped and neurons

retracted the growth cones when the effect of NGF was eliminated (Huang and Reichardt 2001). The neurotrophic factors have shown to reduce neuron apoptosis and promote axon extension by exerting cytoprotective and restorative effect on the damaged neurons (Li et al. 2018).

To enhance the regeneration potential of the nerve guidance conduits researchers have extensively studied to supplement these devices with local delivery of the neurotrophic factors. However, managing the release profile of neurotrophic factors has been very challenging due to the initial burst of these factors at the time of administration, controlled and sustained release then onwards. Additionally, adequate bioavailability and bioactivity of the administered neurotrophic factors is a noteworthy limitation. Some of the classic neurotrophic factors such as BDNF, NT3, NGF and CTNF required for nerve regeneration, have a short half-life of less than 10 minutes, making it a significant factor of consideration as the continuous infusion of these growth factors is practically impossible in traumatized nerves (Szynkaruk et al. 2013). Therefore, newer alternative and superior therapies are required to mitigate these problems yet help accelerate the regeneration and recovery of the damaged nerves.

Schwann cells are a great source for neurotrophic factors production as they have shown to enhance nerve regeneration and remyelination if infused at the site of injury. However, procuring adequate number of human Schwann cells from healthy nerve grafts causes morbidity and their slow and limited expansion in vitro makes it a debilitating and cumbersome procedure (Walsh and Midha 2009). Schwann cells have been genetically modified to overexpress some of the neurotrophic factors such as BDNF and NT3 for improved nerve regeneration, but the FDA requires the translation of cellular therapies to use minimally manipulated cells (Menei et al. 2001). Therefore, stem cell sources such as mesenchymal stem cells (MSC), neural stem cells (NSC), bone marrow derived stem cells (BMSC), adipose derived stem cells (ADSC), dental pulp stem

cells (DPSC), and induced pluripotent stem cells (iPSC) have been explored as a possibility of cellular therapy as they have shown to differentiate into Schwann cell type under specific microenvironment and also secrete neurotrophic factors which promote axon regeneration (Jiang, Jones, and Jia 2017). Stem cells are a population of cells which have the capability of self-renewal and based on the developmental stage they can be divided into embryonic stem cells and adult stem cells which possesses the ability to differentiate into multiple lineages of specialized cell type. Although, stem cells have the ability to differentiate into different cell types they promote nerve regeneration by many different mechanism such as immunomodulation, paracrine activity and fusion with Schwann cells or mitochondrial transfer to Schwann cells (De la Rosa, Kozik, and Sakaguchi 2018).

1.5 Dental pulp stem/progenitor cells

During the development of tooth from the ectomesenchyme, the soft tissue dental pulp derived from the dental papilla gets enclosed in the hard shell of the tooth that is dentin and enamel. The discrepancies in tooth and jaw dimensions and/or orthodontic treatment lead to routine extraction of the human third molars making it a less complex way of obtaining autologous dental pulp. The dental pulp along with blood vessels, nerves and fibroblasts contain a population of adult stem/progenitor cells which endogenously secrete neurotrophic factors a characteristic likely due to their neural crest origin. The ability of DPSC to secrete neurotrophic factors such as BDNF, NGF and NT3 is higher when compared to other commonly studied stem/progenitor cells such as bone marrow stem cells or adipose derived stem cells and the ease of availability of DPSC make

them a potential substitute of schwann cells in the regeneration of peripheral nerves (Mead et al. 2013).

1.6 Scaffold-free tissue engineering

Scaffold-free cell sheet based tissue engineering has enabled to transplant cells at the site of interest while maintaining their maximum viability. The transplanted cells along with replacing the lost tissue also supply growth factors and cytokines in a spatiotemporal manner for extended period of time necessary for tissue regeneration (Matsuura et al. 2014). In this approach of tissue engineering cells cultured to confluence start depositing their own extracellular matrix leading to the formation of cell sheets which could be lifted from the culture plate, manipulated and assembled into multi tissue layers (Labbé, Marceau-Fortier, and Fradette 2011). The confluent cultured cells can also be harvested as an intact cell sheet using a temperature-responsive polymer, poly(N-isoproplyacrylamide) (PIPAAm), grafted onto a polystyrene cell culture surface in which cell attachment and detachment can be done by changing the temperature across 32 °C (Haraguchi et al. 2014). This technology has been researched to regenerate tissues like cornea, esophagus, heart, periodontal ligament, bone periosteum and cartilage (Iwata et al. 2015) (Syed-Picard et al. 2014). Previously, researchers have tried to transplant cells through infusion of single cell suspension, however, it often leads to abnormal cell distribution and early loss of transplanted cells hence decreasing efficacy of the treatment (Matsuura et al. 2014). In the cell sheet technology, as cells are cultured they produce their own extracellular matrix in which they form intercellular and cell to extracellular matrix junctions acting as anchors and preventing cells from migration when transplanted.

1.7 Fibroblast growth factor 2

Fibroblastic growth factor 2 (FGF2) is a protein that binds heparin and heparan sulfate and modulate the function of a wide range of cell types and it also plays an important role in the growth and regeneration of various tissues in the human body (Nugent and Iozzo 2000). FGF-2 is a 18kDa protein which acts via an autocrine or a paracrine mechanism involving high affinity transmembrane receptors (FGFR1 to FGFR4), it is expressed mostly in tissues of mesoderm and neuroectoderm origin, and plays an important role in stimulating angiogenesis, normal wound healing and tissue development (Faitová 2004). FGF-2 has pleiotropic roles in many cell types and tissues; it is a mytogenic, angiogenic, involved in cell migration, cell differentiation and in a variety of developmental processes (Okada-Ban, Thiery, and Jouanneau 2000).

Culturing mesenchymal stem cells to confluence might alter their biological properties a feature attributed to the contact inhibition and hence culturing DPC to form cell sheets may influence their characteristics as well and their ability to secrete NTF (Abo-Aziza and A.A 2017). FGF2 is a growth factor which is considered as a potent mitogen and help mesenchymal stem cells maintain their multilineage differentiation potential through the process of cellular proliferation (Tsutsumi et al. 2001). Since FGF2 maintains multi potency of DPC following their culture to confluence, potentially it can also prevent any changes in NTF expression caused by confluent cell cultures.

2.0 HYPOTHESIS

The goal of our study was to develop a mode of neurotrophic factor delivery system using dental pulp cells via scaffold-free cell sheets to enhance regeneration of facial nerve. We hypothesize that scaffold-free DPC sheets will express neurotrophic factors including BDNF, GDNF and NT-3 and act as a sustained NTF delivery system accelerating repair of damaged nerves and enhancing functional recovery. The cell sheets can be wrapped around the injured facial nerve following current standard surgical treatment methods to accelerate repair and enhance motor recovery.

Specific aim 1: To Histologically characterize DPC sheet and assess NTF expression after in vitro culture with or without FGF2. We will histologically characterize DPC sheet for robustness and its ability to handle by detecting the presence of collagen in ECM and also characterize NTF gene expression using reverse transcription polymerase chain reaction (RT-PCR) and NTF protein expression using enzyme-linked immunosorbant assay (ELISA).

We hypothesize that DPC will form cell sheet and secrete neurotrophic factors and their expression will be enhanced by addition of FGF 2.

Specific aim 2: Determine the functional effect of DPC sheet secretome on neurite extension in SH-SY5Y neurons in vitro. We will culture human derived neuroblastoma cells SH-SY5Y neurons with DPC sheet conditioned media and quantify neurite formation and length. Additionally, we will validate that the neuritogenesis in SH-SY5Y neurons is caused by the NTF present in DPC sheet conditioned media by culturing neurons in the presence of NTF inhibitors to reverse its effect.

We hypothesize that NTF secreted by the DPC sheet will enhance neurite extension in SH-SY5Y neurons.

Specific aim 3: Evaluate the ability of DPC sheet on nerve regeneration in rat facial nerve defect model. We will create a 3mm crush defect in the buccal branch of facial nerve in rats and wrap the crushed nerve with DPC sheet in the treatment group and the control group without any treatment after crush. We will evaluate regenerated nerve histologically and functionally by electrophysiology for conduction across the damaged nerve.

We hypothesize that rats treated with DPC sheet will demonstrate faster and improved functional recovery compared to the control group.

3.0 MATERIALS AND METHODS

3.1 Dental pulp cells isolation and engineering of scaffold-free cell sheets

Dental pulp was isolated from adult human 3rd molars collected at the University of Pittsburgh, School of Dental Medicine, the teeth were free from carious lesion or other oral infections and they were collected within 24 hours of extraction and transported to lab in phosphate buffered saline with penicillin & streptomycin. A digestion cocktail containing collagenase (3mg/ml) and dispase (4mg/ml) (EMD Millipore Corporation and Worthington biochemical, USA) was used to digest dental pulp and obtain a total population of dental pulp cells. The isolated cells were expanded and cultured in growth medium (GM) made up of Dulbecco's Modified Eagle Medium (Gibco Life technologies corporation, USA), 20% fetal bovine serum (Atlanta biological, USA) and 1% penicillin & streptomycin (Gibco Life technologies corporation, USA). Further, upon reaching 80% confluence the cells were passaged and cryogenically stored in liquid nitrogen at -196^0c for future experiments. The multipotency of the isolated DPC was verified by inducing differentiation towards osteogenic lineage; the deposition of mineralized matrix was confirmed through Alizarin red staining.

Dental pulp cells from passage 2-4 were used to engineer cell sheets: DPC were plated onto 6 well plate at an initial seeding density of 200,000 cells per well in growth medium supplemented with 50 μg/ml L-Ascorbic acid (Sigma-Aldrich, USA). Cell sheets were formed with/without the effect of Fibroblastic Growth Factor 2 (Peprotech, USA) at a concentration of 5 ng/ml. The dental pulp cells were cultured with medium change once every 2-3 days for 10-12 days to form robust cell sheet that could be easily handled with forceps.

3.2 Quantification of cell number in engineered DPC sheets

Scaffold-free engineered DPC sheets cultured in growth media +/- FGF2 were rinsed with PBS twice followed by their digestion by incubating in 350 µl of TrypLE express (Gibco Life technologies corporation, USA) at 37^0c for 5-7 minutes. The detached cells were suspended in 1 ml of growth media and they were counted in hemocytometer with trypan blue stain. Cell viability of the dental pulp cells was verified with trypan blue stain; the dead cells absorbing trypan blue stain were excluded from the count.

3.3 Histological characterization of engineered DPC sheets

Engineered DPC sheets were washed with PBS twice and fixed with 10% formalin for 20 minutes and stored in 70% ethanol at 4^0c overnight. The cell sheets were processed in tissue processor (LEICA ASP300S, Leica Instruments GmbH Germany) for standard paraffin embedding. Processed cell sheets were sectioned at 5 microns thickness in microtome (LEICA RM2135 style, Leica Instruments GmbH Germany) and incubated at 60^0c for 15 minutes. The cell sheet sections were processed for Hematoxylin and Eosin (H&E) and immunostaining by deparaffinizing and rehydrating the sectioned samples by series of washes in xylene, ethanol and water.

Hematoxylin and Eosin stain: The hydrated samples were stained with H&E (Richard-Allan scientific) by a series of washes in hematoxylin, water, Nu-clear (acid/alcohol), bluing, water, ethanol, eosin, ethanol, xylene and mounted with xylene based mounting media (Thermo scientific, USA). The images were captured using ZEISS Scope.A1 AXIO microscope.

Immunostaining: The hydrated sample slides were incubated in 10mM citrate + 0.05% triton X at 60^0c overnight for heat induced epitope retrieval. Further, sample sections were permeabilized with 0.1% triton X and incubated with 5% goat serum for 1 hour. Sections were incubated with either type 1 collagen antibody (Anti-collagen I antibody ab34710, abcam) at 1:200 concentration overnight at 4^0c or in blocking solution as negative control. The sections were stained with secondary antibody Alexa Fluor 488 (ThermoFischer, USA) at 1:500 concentration for one hour followed by counterstaining with 4′,6-diamidino-2-phenylindole DAPI (Sigma-Aldrich, USA) stain for nuclei at 1:500 concentration. The sections were mounted with aqueous mounting media (Shandon Immu-mount USA). The images were captured using Nikon ECLIPSE Ti microscope and processed in ImageJ software.

3.4 Analysis of neurotrophic factor gene expression using reverse transcription polymerase chain reaction (RT-PCR)

Scaffold free engineered DPC sheets were harvested after 10-12 days in culture and RNA was extracted using QIAGEN RNeasy Mini Kit following the manufacturer's protocol. Briefly, the DPC sheet was lysed and homogenized using RLT buffer and mixed with 1 volume of 70% ethanol. This was followed with a series of washes and centrifugation with RW1 and RPE buffer before collecting RNA in 30 µl of RNase-free water. The quantity and quality of the RNA was measured using nanophotometer (NanoDrop One Thermo Fisher, USA). RNA was collected from cell sheets cultured in media containing +/- FGF2. RNA was also isolated from DPC at the time of plating and human embryonic kidney fibroblasts (hek 293t) as control samples.

RT-PCR was performed with TaqMan PCR kit (Applied Biosystems) to analyze the expression of neurotrophic factor genes using primers for human BDNF, GDNF & NT3 (Taqman Gene Expression Assays) and GAPDH was used as housekeeping gene. The assay was conducted in QuantStudio 6 Flex (Applied Biosystems Life technologies). The obtained data was analyzed and Ct values greater than 35 were considered as negative readings, the fold change for each of the sample was calculated using the ΔΔCt method. First, the ΔCt value was calculated by normalizing the Ct value of each sample gene to that of the housekeeping gene. Then, the difference in the ΔCt values between the experimental and control group was calculated as the (ΔΔCt). The fold change for each sample was calculated as 2(-ΔΔCt).

3.5 Detection of neurotrophic factor protein secretion by DPC sheets using enzyme linked immunosorbent assay (ELISA)

Conditioned medium (CM) was obtained from DPC sheets cultured in growth medium with/without FGF2, the CM was spun down at 2000 rpm for 5 minutes to remove any cellular debris and stored at -80^{0}c for future experiments. The uncultured growth media +/- FGF2 was aliquoted and incubated at 37^{0}c for 48 hours and stored at -80^{0}c for future use as control samples. The amount of BDNF, GDNF and NT3 proteins secreted by DPC in the cell sheet was measured by ELISA using Human BDNF PicoKine™ ELISA Kit EK0307, Human GDNF PicoKine™ ELISA Kit EK0362 (Boster Biological technology CA, USA) and Human NT-3 ELISA Kit (RayBiotech, USA) respectively. The ELISA assay was performed following manufacturer's protocol, briefly conditioned medium, growth medium and reconstituted proteins were added to the antibody pre coated 96-well plates and incubated at 37^{0}c for 90 minutes followed by incubation

with biotinylated antibody at 37^0c for 60 minutes. The plates were washed with 0.01M PBS for three times and then incubated with Avidin-Biotin-Peroxidase Complex (ABC) at 37^0c for 30 minutes. The plate was washed with 0.01M PBS for five times and incubated with TMB (3,3',5,5'-tetramethylbenzidine) solution in dark at 37^0c for 20-25 minutes which developed blue color. Further TMB stop solution was added which changed the color to yellow immediately and the plate was read at optical density (O.D) absorbance value of 450 nm in a spectrophotometer (SYNERGY H1 microplate reader, BIOTEK USA) within 30 minutes of adding the TMB stop solution. The quantity of secreted proteins (pg/ml) were calculated against standard curves produced using recombinant protein concentrations provided by the manufacturer using linear regression. The experiment was repeated 3 times using cell sheet formed from cells isolated from 3 different individuals. Mean value ± standard deviation represent the biological triplicate; average across 3 experiments.

3.6 In vitro neurite outgrowth assay using SH-SY5Y neuroblastoma cells

Conditioned media (CM) collected from DPC sheets was used to assess the functional effect of the neurotrophic factors secreted by DPC on SH-SY5Y neuroblastoma cells (ATCC CRL-2266). SH-SY5Y neuronal cells were plated on 8-well Poly-L-Lysine coated chamber slide (11,500 cells/well) with culture media containing DMEM/F12 with 10% fetal bovine serum for 24 hours followed by neuronal induction with 10uM retinoic acid (ACROS organics, USA) for 48-72 hours of incubation (Kolar et al. 2017). Further, neurite formation and extension was induced by treating the neurons with 400ul of conditioned media +/- FGF2 obtained from DPC sheet or growth media +/- FGF2 as control. The SH-SY5Y neurons were further cultured for 6 days with

conditioned/growth media change once every 2-3 days. After 8-9 days of treatment the cells were washed with PBS twice and fixed for 20 minutes with 4% paraformaldehyde (Sigma-Aldrich, USA) prepared fresh. The SH-SY5Y cells were washed with PBS twice and stained for immunostaining with anti-tubulin βIII antibody (Biolegend, USA) and DAPI at concentrations of 1:250. The images were captured with Nikon ECLIPSE Ti microscope.

To validate the potential axon enhancement effect seen by culturing neurons with DPC conditioned media was due to NTFs, TrK B receptor blocker for BDNF, TrK C receptor blocker for NT3 (R&D systems, USA) and neutralizing antibody against GDNF (R&D systems, USA) were added to the conditioned media at a concentration of 5μg/ml.

Neurite extensions of SH-SY5Y neurons were manually quantified with ImageJ software. Cell extensions twice the length of their cell body were considered as neurite and the longest neurite was measured for each neurite bearing cell. The percentage of neurite positive cells, the longest neurite outgrowth and range of neurite extensions were quantified and reported from conditioned media from three different human dental pulp cells.

3.7 Animal surgery and DPC sheet implantation

Immunocompromised rats aged 4-6 weeks were purchased from Charles River Laboratories (USA) and were housed under standard conditions of alternate light and dark cycle. The surgical procedure was approved by the Institutional Animal Care and Use Committee (IACUC) University of Pittsburgh. Before the surgical procedure, rats were anesthetized by intraperitoneal injection of Ketamine hydrochloride (40mg/kg) plus xylazine hydrochloride (5mg/kg). The surgical site was prepared by trimming the hairs and cleaning with 10% povidone-

Iodine solution swabsticks. Skin incision of 1cm - 1.5 cm was placed over the buccal surface anterior to preauricular region and the buccal branch of the facial nerve was exposed. Using microscissors the nerve was separated and released from its underlying fascia and using a 3mm flat edge non-serrated forceps, 3mm of buccal branch of facial nerve was crushed for 20 seconds. The crushed nerve was wrapped with the DPC sheet, the transplanted DPC sheet was secured with 9-0 nylon suture at the proximal and distal end of the crush. The skin incision was closed with 4-0 vicryl suture. After the surgery the rats were placed under the warm bag, allowed to recover consciousness and housed with access to food and water. Post-operative analgesia was administered by mixing acetaminophen (1.5mg/ml) in the drinking water and the rats were observed for normal eating and drinking with active movement. The control animals underwent the same surgical procedure but no DPC sheet was implanted and to mark the crushed site a 9-0 suture was placed around the nerve loosely. The undamaged contralateral side served as the positive control for this experiment and the experiment was carried out for 4 weeks with each group having 6 animals.

3.8 Histology and immunofluorescence analysis of the regenerated nerve

Four weeks after the surgery, rats were sacrificed by CO_2 inhalation and the regenerated nerves were explanted and fixed in 4% paraformaldehyde. The nerve tissue was flash frozen in OCT media and 5 micron sections were cut in cryostat. Routine hematoxylin and eosin staining was done to analyze the general architecture of the nerve.

Further sections were processed for immunofluorescence to detect the regenerated axons. Primary antibody for anti-beta tubulin (1:100) was incubated overnight at 4^0c and Alexa fluor 488

(1:500) was used as secondary antibody. Nuclei was counterstained by DAPI and the images were captured by NIKON eclipse TE2000-E microscope and processed with imageJ software.

4.0 STATISTICAL ANALYSIS

The data is presented as means ± standard deviations. Statistical comparison of the quantified number of cells in cell sheets cultured in +/- FGF2 media was done using Student's t-test. qRT-PCR data was analyzed by comparing the fold change results between treatment and control group using one-way ANOVA with post-hoc Tukey test. ELISA data was analyzed using the final picogram concentrations between treatment and control group by one-way ANOVA with post-hoc Tukey correction. All the statistical tests were done in Graphpad Prism software and statistical difference at *p* value less than 0.05 was considered significant.

5.0 RESULTS

5.1 Engineering DPC sheets.

We were able to engineer cell sheets from the dental pulp cells isolated from pulp of human third molars as shown in Figure 1.

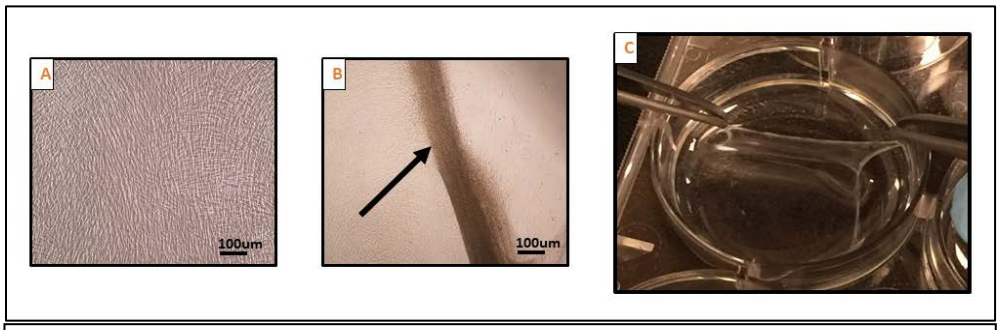

Figure 1: Fabrication of Dental pulp cell (DPC) sheet. (A) Microscopic image showing confluent DPC and (B) DPC forming cell sheet, arrow pointing to the cell sheet that is detaching from the base of a well from 6 well plate dish. (C) Photograph of DPC sheet being handled by forceps in a 6 well plate dish.

DPCs when plated at a seeding density of 200,000 cells/well reached to confluence in 2-4 days, and with a further culture for 8-10 days they formed cell sheet which started to detach from the base of the well (Figure 1A&B). The formed DPC sheets were robust solid tissue structure and able to handle with the forceps (Figure 1C). The results were consistent with DPC sheets being formed from dental pulp cells isolated from three different patients.

5.2 Histological characterization of DPC sheets.

Cell sheets engineered from culturing in the media with/without FGF2 were histologically characterized, the hematoxylin and eosin stain confirmed that the formed cell sheets in both the groups were solid multicellular tissue structure as shown in figure 2A&D.

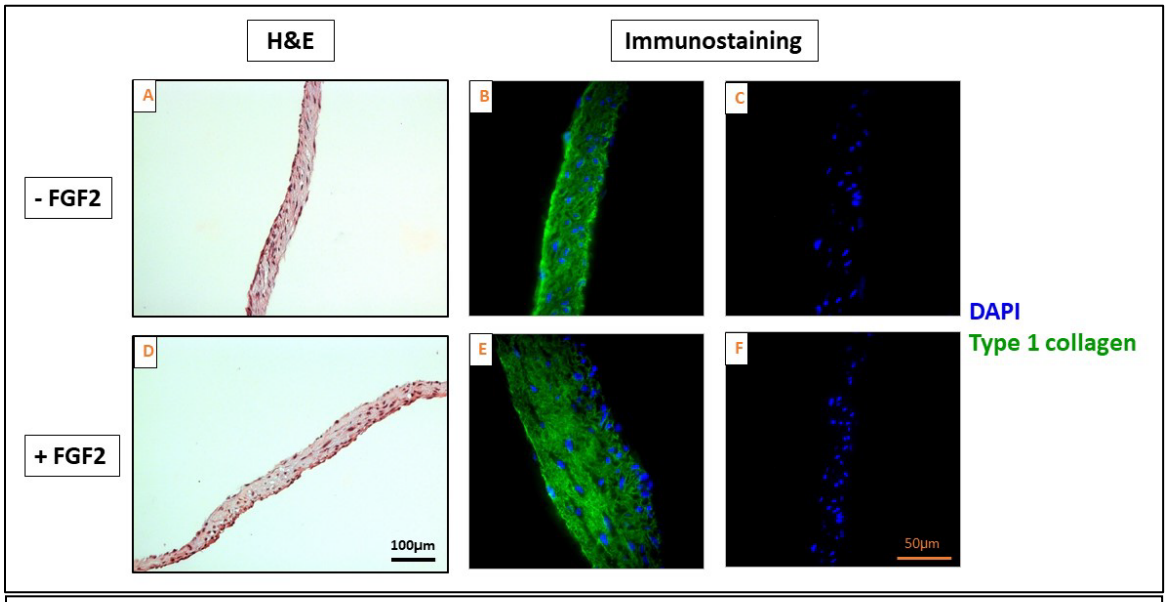

Figure 2: Histological characterization of DPC sheets. (A) & (B) H&E and immunofluorescent staining of DPC sheets cultured in media without Fibroblastic growth factor (FGF2) respectively. (D) & (E) DPC sheets cultured in media with FGF2. (C) & (F) Negative controls for immunofluorescent staining of type 1 collagen for cell sheets cultured in without & with FGF2 respectively. H&E staining of DPC sheet sections show that the cell sheets are solid and multicellular structures. Immunofluorescent staining shows that the extracellular matrix of DPC sheet contains type 1 collagen (green). Cell nuclei was detected by DAPI stain (blue).

Immunostaining for type 1 collagen confirmed the presence of extracellular matrix in the cell sheets along with the presence of nuclei stained for DAPI as shown in figure 2B&E. An intriguing difference in the number of DAPI stained nuclei was observed between the cell sheets formed with FGF2 when compared with the ones without FGF2 in their culture media.

5.3 Quantification of cell number in DPC sheet.

The cell number in the DPC sheet cultured with FGF2 contained approximately 2 million cells which was twice the number compared to DPC sheets cultured without FGF2 which had close to 1 million cells as shown in figure 3.

The results were consistent for cell quantification procedure which was repeated three times with cells from three different patients. The cell viability verified by the trypan blue stain showed that the cells were almost 100% alive across the 3 experiments. Students t-test showed that the difference noticed was statistically significant with *p* value less than 0.05.

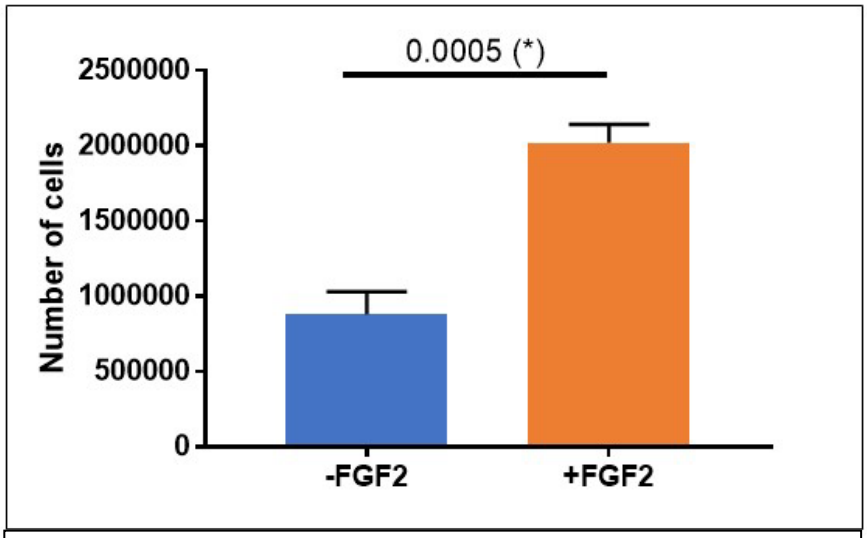

Figure 3: Quantification of cell number in DPC sheet. Bar graph shows that number of cells in DPC sheets cultured in media with FGF2 have approximately twice the number of cells when compared with DPC sheets cultured in media without FGF2. *P* value <0.05 was considered significant.

FGF2: Fibroblastic growth factor 2.

5.4 Reverse transcription-polymerase chain reaction to detect NTF gene expression.

RT-PCR done with RNA extracted from DPC sheets cultured +/- FGF2, sub confluent DPC and human embryonic kidney fibroblasts showed the expression of NTF genes BDNF, GDNF and NT3.

The expression of BDNF gene in the cell sheets cultured in media with FGF2 was

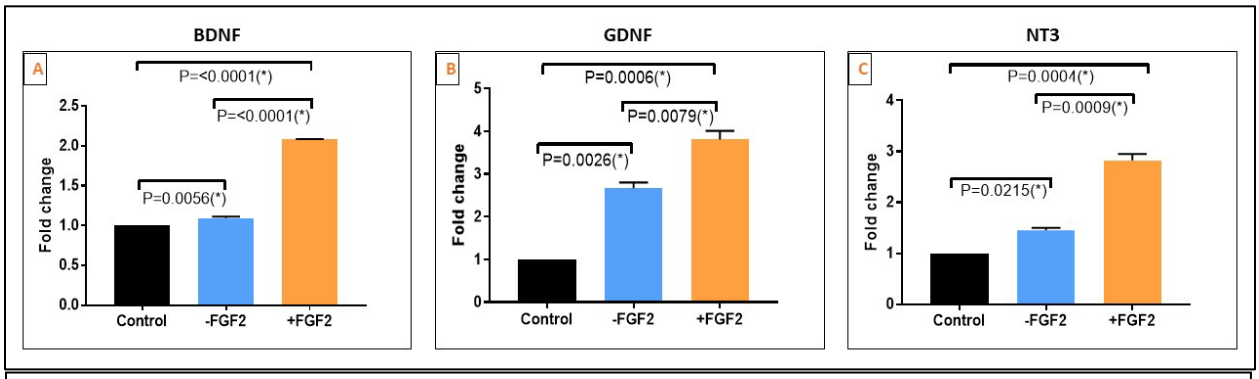

Figure 4: Reverse transcription-polymerase chain reaction (RT-PCR) results show that dental pulp cells cultured as cell sheets express genes for (A) Brain derived neurotrophic factor(BDNF), (B) Glial-cell derived neurotrophic factor(GDNF) and (C) Neurotrophic factor 3 (NT3), their expression was increased in the group treated with FGF2. RNA extracted from the DPC used at the time of plating wells was used as control. Fold change expressed as mean +/- standard deviation with *P* value <0.05 considered as significant. FGF2: Fibroblastic growth factor 2.

upregulated significantly when compared to the other groups without FGF2 and controls as seen in figure 4A. A similar trend of upregulation for the genes GDNF and NT3 was also noticed as shown in figure 4B&C. One-way ANOVA analysis with tukey's post hoc test showed the difference to be statistically significant between the groups at a *P* value less than 0.05.

5.5 Enzyme linked immunosorbent assay (ELISA) to detect the concentration of proteins.

ELISA analysis done from the conditioned media collected from DPC sheet and growth media as negative control showed that the DPCs secrete BDNF, GDNF and NT3 proteins as shown

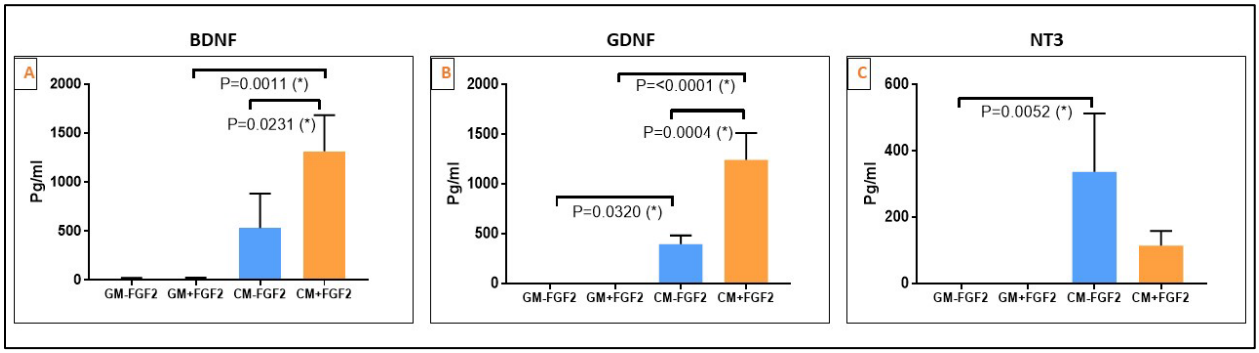

Figure 5: Enzyme linked immunosorbent assay (ELISA) was done to detect the concentrations of Brain derived neurotrophic factor (BDNF), Glial-cell derived neurotrophic factor (GDNF) and Neurotrophic factor 3 (NT3) proteins in conditioned media collected from DPC sheets cultured in +/-FGF2 and uncultured growth media +/-FGF2 was used as negative control. (A)& (B) shows that the amount of proteins BDNF & GDNF secreted was high in CM+FGF2 compared to CM–FGF2. (C) Shows that NT3 protein secretion was increased in CM–FGF2 compared to CM+FGF2. Concentrations expressed as means +/- standard deviations with *P* value <0.05 considered as significant.

GM+/-FGF2: Growth media +/- fibroblastic growth factor 2

CM+/-FGF2: Conditioned media +/- fibroblastic growth factor 2

in figure 5. The concentration of proteins BDNF and GDNF was increased in the conditioned media with FGF2 (CM+FGF2) when compared to conditioned media without FGF2 (CM-FGF2) and the difference was statistically significant as seen in figure 5A&B. The concentration of NT3 protein was found to be more in CM-FGF2 group in comparison with CM+FGF2 as seen in figure 5C. The presence of proteins was not detected in control samples that is growth media with/without FGF2.

5.6 Functional evaluation of DPC sheet conditioned media on SH-SY5Y neuroblastoma cells.

Culturing SH-SY5Y neurons with growth media +/- FGF2 and conditioned media +/- FGF2, we found that the neurite extensions were evident when cultured with the conditioned media from DPC as shown in figure 6C&D.

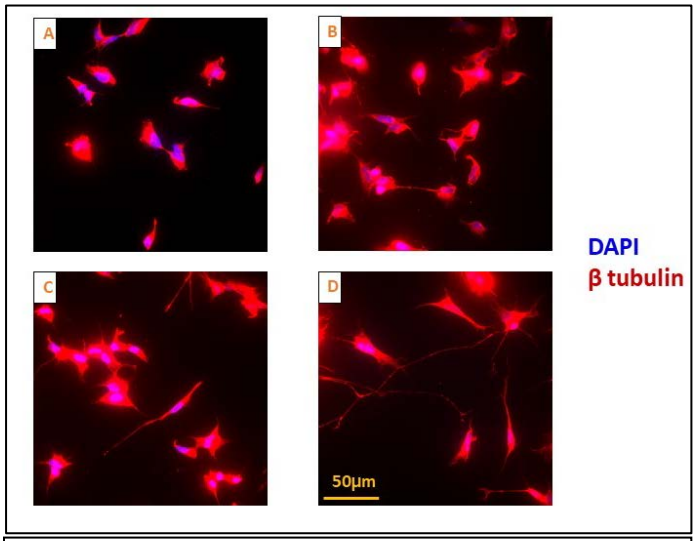

Figure 6: Neurite extension in SH-SY5Y neurons was detected by beta-3 tubulin immunostaining (red) and counterstained with DAPI (blue) for the nuclei. Neurite extension in SH-SY5Y neurons was induced by culturing in conditioned media +/-FGF2 and growth media +/-FGF2. (A) & (B) SH-SY5Y neurons cultured in growth media without and with FGF2, (C) & (D) SH-SY5Y neurons cultured in conditioned media without and with FGF2 respectively.

The effect was more pronounced and enhanced with CM +FGF2 where SH-SY5Y neuronal cells had increased neurite positive cells with majority of the neurites in the range of 100-200 microns and few more than 200 microns when compared with CM-FGF2 where the neurite length extensions were approximately equally distributed in the range of either 50-100 microns or 100-200 microns and some neurite extensions were less than 50 microns as shown in figure 7A&B. When SH-SY5Y cells were cultured with the growth media majority of the neurite extensions were in the range of 50-100 microns with some less than 50 microns. The neurite forming effect of SH-SY5Y neuronal cells was validated with the addition of inhibitory blockers to the conditioned media or growth media. The neurite formation and

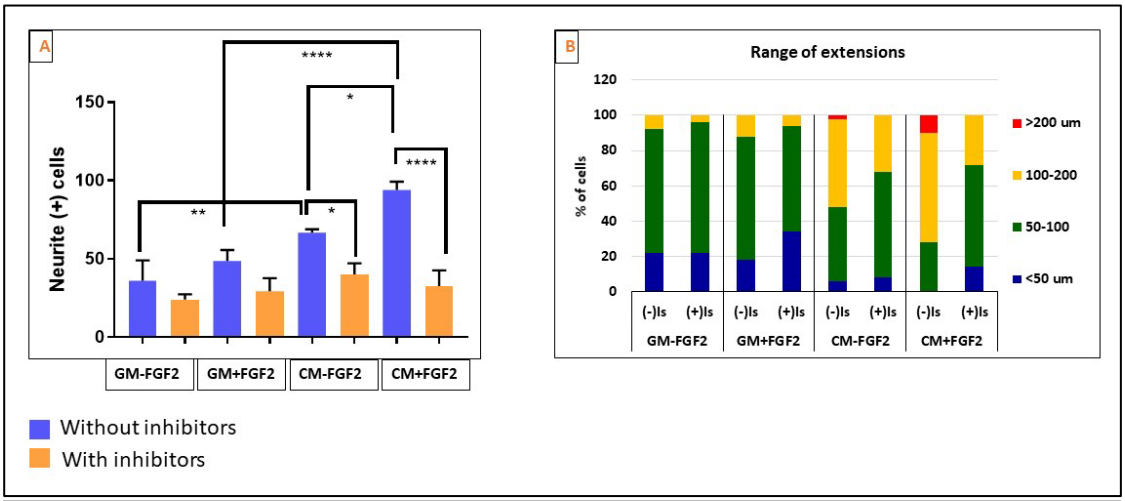

Figure 7: Neurite inhibitory assay. To validate the neurite extension effect induced by the NTF in conditioned media, SH-SY5Y neurons were cultured with conditioned media supplemented with inhibitors for NTF. TrKB, TrKC receptor blockers & antibody to GDNF were added to the conditioned media and growth media and neurite quantification was done. (A) Bar graph shows percentage of neurite positive SH-SY5Y cells and (B) range of neurite length extensions of SH-SY5Y neurons after culturing in media without and with inhibitors.

Note: Extensions of SH-SY5Y cells twice the length of cell body were considered as neurite and length of longest neurite per neuron was measured.

GM+/-FGF2: Growth media +/- fibroblastic growth factor 2

CM+/-FGF2: Conditioned media +/- fibroblastic growth factor 2

Is (+)/(-): Inhibitors with/without

extension effect was reversed as there was significant decrease in the neurite positive cells and length of neurite extensions formed as shown in figure 7A&B.

5.7 Evaluation of nerve regeneration in rats.

To evaluate if DPC sheets were able to enhance nerve regeneration, in vivo study was performed as described previously in section 2.7. The buccal branch of the facial nerve in rats was exposed and crushed for 20 seconds (figure 8A) and the DPC sheet was implanted in the treatment

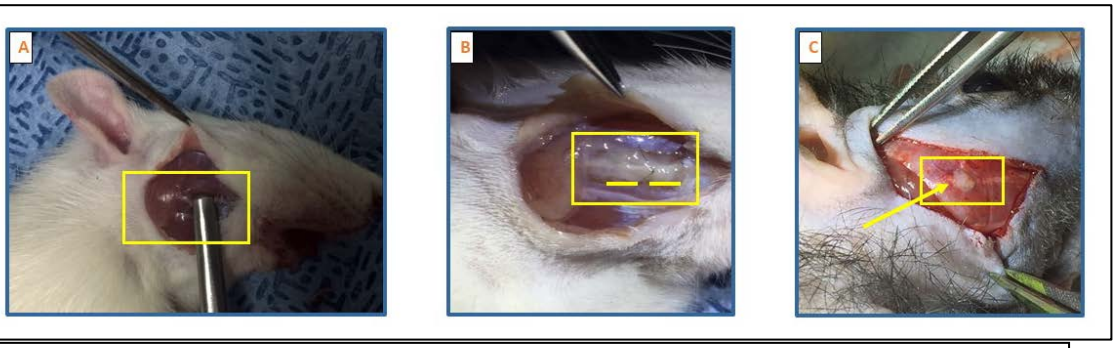

Figure 8: Crush nerve defect and DPC sheet implantation in rats. (A) Crush defect of 3mm was induced in the buccal branch of the facial nerve in immunocompromised rats with a non-serrated forceps for 20 seconds. (B) DPC sheet was implanted at the site of crush defect and secured with 9-0 nylon suture. (C) 4 weeks post-surgery harvesting the regenerated nerve, arrow points to DPC sheet that can be seen around the regenerated nerve.

group (figure 8B). After 4 weeks, the surgical site was revisited to explant the regenerated nerve and the implanted DPC sheet was seen to be present and integrated around the nerve as shown in figure 8C in the animals of treatment group.

The histological analysis of the regenerated nerve in the injured untreated animals showed discontinuous regeneration of the crushed nerve and the injured area was densely infiltrated with cells, the beta tubulin stained axons at the proximal and distal end of the nerve appeared to be disconnected and broken at the injured site (figure 9E&G). The crushed nerve treated with DPC sheet showed the presence of more continuous axons across the defect and the regenerated area showed cellular infiltration but not as dense as compared to the untreated group (figure 9F&H). The presence of DPC sheet around the nerve was also noted.

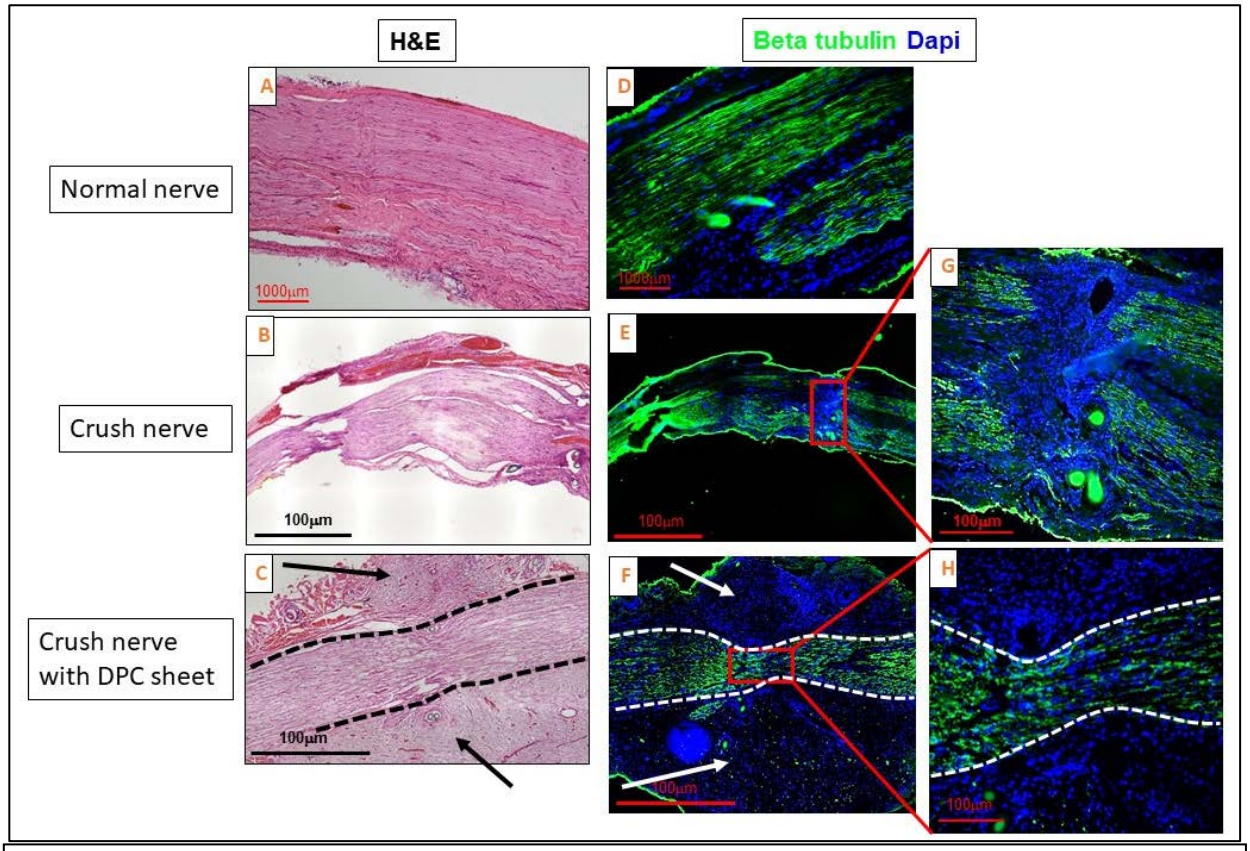

Figure 9: Histological evaluation of the regenerated nerve. About 3mm of the buccal branch of the facial nerve was crushed in rats and treated with DPC sheet implantation in the experimental group and without any treatment in the control group. (A) Hematoxylin and eosin stain and (D) immunostaining for axons (green) and DAPI for nuclei (blue) of a normal nerve as a positive control. B&C are the H&E stained images of the regenerated crush nerve and crush nerve with DPC sheet implantation. Immunostaining done with anti-beta tubulin stain (green) and counterstained with DAPI (blue) for nuclei in the crush nerve alone showed cellular infiltration at the injured site and the axon extension to be discontinuous (E&G). Whereas crush nerve implanted with DPC sheets shows axon extensions to be more continuous across the defect. The black and white arrows point to the presence of DPC sheet around the nerve in the H&E and immunostained images respectively. The delineation shows the nerve trunk surrounded by DPC cell sheet in the animals treated with DPC sheet implantation.

6.0 DISCUSSION

In our study we evaluated the ability of human DPC to form cell sheets and their potential to be used as a method of localized and sustained delivery of neurotrophic factors to enhance the current therapies for peripheral nerve regeneration. Although many researchers have demonstrated the use of cell sheet engineering for regeneration of periosteum, periodontium, cornea and myocardium (Iwata et al. 2015; Syed-Picard et al. 2014), it is the first time that we have shown usage of DPC sheets in regeneration of the peripheral nerves. Due to the neural crest origin of DPSC, it is well established that the use of these cells to differentiate into neuronal cell lineage representing to be a valid tool for peripheral nerve repairs. In addition to their neural crest origin, the ease of procuring and isolating autologous DPC and delivering them localized in the form of cell sheets to the damaged or degenerated sites might provide an excellent candidate for regenerative medicine purposes especially in the field of neural tissue engineering and in the treatment of degenerative disorders of nervous system.

In accordance with the previous studies our findings also demonstrated that DPC isolated from human third molars expressed neurotrophic factor genes BDNF, GDNF and NT3 (Mead et al. 2013; Kolar et al. 2017). Furthermore, their culture to confluence to form cell sheets did not decrease their potential to express NTF genes. Additionally, we also noticed an increase in the expression of NTF genes when cultured with FGF2 which could be attributed to the fact that FGF2 helps mesenchymal stem cells maintain their differentiation potential in different culture system (Zhang et al. 2017). As the cells reach confluence to form cell sheet, it may alter the stem cell characteristics and decrease the proliferation rate due to cell-cell contact inhibition (Polyak et al. 1994). In our experiment with the addition of the FGF2, we noticed that the number of DPC in cell

sheets were approximately double the number of cells when cultured without FGF2 which was in accordance with the previous studies where they found FGF2 to increase the proliferation rate of mesenchymal stem cells (Solchaga et al. 2005).

Our study showed that with simple culture of DPCs to form cell sheets they secrete neurotrophic factor proteins such as BDNF, GDNF and NT3. The ELISA detection for the concentration of NTF proteins in the conditioned media were found to be of considerable amount, which if delivered locally to the damaged nerves can enhance nerve regeneration. Dr. Osam Mazda in his study has demonstrated that ELISA test done from the conditioned media obtained by culturing Schwann cells at a seeding density of 200 000 cells showed the concentration of the NTF proteins BDNF and GDNF to be in the range of approximately 60 and 40 pg/ml respectively (Sowa et al. 2017). In our study DPC sheets cultured with FGF2 contained approximately 2 million cells and the ELISA test from the conditioned media detected the concentration of NTF proteins BDNF and GDNF in the range of approximately 1200 pg/ml each, which is more than the level secreted by Schwann cells. Hence, DPC sheets could act as a potential source of localized and sustained delivery of NTF proteins. The DPC sheets cultured without FGF2 also secreted NTF proteins but their level of proteins BDNF and GDNF was significantly less when compared to the DPC sheets cultured with FGF2 except for the protein NT3. This can be explained by the fact that compared with transcription, post-transcriptional modulators e.g. microRNAs and translational or post-translational events also play an important role in determining overall accumulation and release of the mature proteins. Additionally, binding of the proteins in the extracellular matrix may also be a contributing factor to the decreased concentration in the conditioned media. A further analysis for protein detection such as western blot analysis to detect the concentration of the proteins in extracellular matrix or from cell lysate may be required for better understanding of the secretion

of these NTF proteins. Previous studies have shown that the dental pulp stem cells secrete various different growth factors such as angiopoietin-1, nerve growth factor, vascular endothelial growth factor which we have not studied in our present study (Kolar et al. 2017). We anticipate that DPC sheets might be secreting these various growth factors which may have a potential positive effect on nerve regeneration in vivo which may widen the scope of this study in future.

Based on the above findings, a goal of our study was also to evaluate the functional effect of the conditioned media from DPC sheet on SH-SY5Y neurons. Our findings demonstrated that the conditioned media from DPC sheet formed more SH-SY5Y neurite positive cells and enhance their neurite extensions when compared to SH-SY5Y neurons cultured in control growth media. This effect can be attributed to the presence of neurotrophic factor proteins in conditioned media secreted by DPC sheet which was in line with the previous studies showing the neurite formation and extension in SH-SY5Y neurons (Kolar et al. 2017). Moreover, the use of neurotrophic factor inhibitors enabled us to identify that DPC sheet derived BDNF, GDNF and NT3 to be important neurotrophic factor proteins responsible for neurogenic and neurite extending effect in SH-SY5Y neuronal cells.

The model of crush nerve injury in the buccal branch of facial nerve is one of the commonly used model to evaluate peripheral nerve regeneration in the craniofacial region. In our study we found that animals treated with DPC sheet implantation, the cell sheet was integrated around the damaged nerve and promoted axon extension as revealed by the histological analysis. This effect could be due to the secretion of NTF proteins by the implanted DPC sheet and also it may help recruit the host cells to differentiate into Schwann cells which have shown to be necessary for the alignment of the nerve fibers and further NTFs secretion (Carnevale et al. 2018). The Dio stained human DPCs detected around the regenerated nerve (data not shown) intrigued us to think that the

DPC remained in the defect area and assisted in the axon extension and nerve regeneration throughout the process of healing. In the control animals without the DPC sheet minimal and discontinuous axon extension was observed, taken together these findings DPC sheet might contribute to facial nerve regeneration. To claim nerve regeneration, functional evaluation of the regenerated nerve has to be tested with the electrophysiological testing which we plan to do in our future experiment.

Cell reprogramming technologies have made it possible to convert the adult differentiated cells such as fibroblast into Schwann like cells, and use them as a means of delivering neurotrophic factors in the regeneration of nerves (Sowa et al. 2017). The FDA regulations for the cellular therapies requiring translation into the clinical area stern to use minimally manipulated cells. In our study we have used total population of dental pulp cells, in future this study can be done more specifically with the pure population of dental pulp stem cells. These dental pulp stem cell sheets can be used in conjunction with the existing therapies to enhance the treatment outcome. It could be used to augment regenerating capacity of nerve autografts, acellular nerve grafts and can also be integrated with nerve cuffs. Research has shown that dental pulp stem cells can differentiate into neurons and also integrate into the nervous tissue, the dental pulp stem cell sheets could be used potentially to treat degenerating central or peripheral nervous system disorders.

7.0 CONCLUSION

In conclusion, our study demonstrated that robust DPC sheets containing type 1 collagen in the extracellular matrix can be engineered that can be easily handled. FGF2 along with increasing the number of DPC in cell sheets, enhanced the expression of NTF genes and protein secretion. DPC sheet conditioned media induced neurite extension in SH-SY5Y neurons in vitro due to NTFs expression. DPC sheet enhanced axon regeneration and extension in crush nerve defect in buccal branch of the facial nerve in rats. Scaffold-free DPC sheets show great promise as a new therapy to enhance and accelerate the regeneration of damaged peripheral nerves and improve functional outcome.

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