Characterization of Engineered Dental Pulp Stem Cell Derived Exosomes

BY

APURVA KALAMKAR
B.E., University of Mumbai, India, 2015

THESIS

Submitted as partial fulfilment of the requirements
for the degree of Master of Science in Bioengineering
in the Graduate College of the
University of Illinois at Chicago, 2019

Chicago, Illinois

Defense committee:

Dr. Sriram Ravindran – Chair and Advisor, Department of Oral Biology, UI College of Dentistry
Dr. Biji Mathew, Department of Anaesthesiology, UI College of Medicine
Dr. Michael Stroscio, Richard and Loan Hill Department of Bioengineering

This thesis is dedicated to my family and mentors.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt gratitude to my advisor Dr. Sriram Ravindran for his mentorship. I am grateful for his time and effort in shaping my project, his guidance throughout my graduate studies and research, and for the opportunity to be a part of his scientific team. His patience and enthusiasm in research motivated me to work harder to better understand the field of regenerative medicine.

In addition to my advisor, I would like to thank Dr. Chun-Chieh Huang, for his meticulous training and constant support throughout my entire research experience. His wisdom and teachings were priceless and I'm grateful to have learnt the techniques from him.

I would also like to thank Dr. Biji Mathew and Dr. Steven Roth for their unwavering support and assistance. Their valuable suggestions helped me accomplish my research goals.

I would like to thank my committee member, Dr. Michael Strocio for his valuable time and support.

Most importantly, I would like to thank my parents, brother, family, friends and undergraduate mentor for their never-ending support. They have instilled confidence in me and without their constant support and faith in my success, I would have never made it this far.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

MSCs - Mesenchymal Stem Cells

DPSCs - Dental pulp stem cells

NTA - Nanoparticle Tracking Analysis

LDH – Lactate Dehydrogenase

OGD – Oxygen Glucose Deprivation

SUMMARY

Retinal Ischemia is considered as the main cause of vision impairment and degradation of retina worldwide. It results in impaired oxygen delivery to the retina. It occurs due to several disorders such as glaucoma, age-related macular degeneration, diabetes retinopathy, central retinal artery occlusion, stroke, optic neuropathies covering all age groups. Out of this diabetes retinopathy is a leading cause for vision loss among the working professionals and is expected to affect approximately 57 million by 2030. The ability of the damaged human retina to regenerate is limited. The traditional therapies use laser surgery, intra-vitreous injection using anti-angiogenic factors and eye drops. Though effective they have their own drawbacks. One of the main drawbacks is they focus on arresting the disease and not on rectifying it. Also, the efficiency depends on the extent of the disease. Therefore, alternative therapies are needed to treat the disease and rectify them. Stem cell-based therapy that uses mesenchymal stem cells (MSCs) can be a potential option. However, they have low cell integration, aberrant growth and immune response limiting their potential. Recent research has shown that MSC impacts are due to paracrine action and are intervene by MSC determined extracellular vesicles (EVs) particularly exosomes. Though the primary source of MSCs is bone-marrow they can be obtained from several other places such as adipose, placenta, dental pulp, skin, umbilical cord. This study focuses on dental pulp derived MSC exosomes.

As dental pulp stem cells (DPSCs) are extracted from the neural crest, we assumed that their exosomes will have a good efficiency for regeneration and protection of retinal neuronal cells. As a further step, we sought to enhance the anti-apoptotic and pro-proliferative properties of the DPSC exosomes by generating engineered exosomes with exosome-targeted increased expression of miR424. The effectiveness of both naïve exosomes and engineered exosomes were explored based on following experiments: 1. Endocytosis of exosomes by R28 retinal cells; 2. Binding of exosomes to ECM proteins and vitreous humor; 3. Proliferation assay; and 4. Lactate dehydrogenase assay (as a measure of dead cells). Results showed that altering the miRNA composition of the exosomes did not affect the basic properties such as size, concentration, endocytosis but enhanced their functionality in the form of

increased proliferation of R28 retinal cells and an enhanced ability to rescue them from apoptosis when subjected to simulated ischemia condition better than the naïve exosomes.

Overall these studies highlight the ability of dental-pulp-stem-cell derived exosomes in regenerative medicine and characterize the properties of engineered exosomes for function-specific tissue regeneration.

CHAPTER 1

1 INTRODUCTION

1.1 Background

Retinal ischemia is a leading cause for impaired vision and retinal degeneration. It is a pathological condition that is characterized by reduction in blood flow to the retina [1]. It occurs due to several disorders such as macular degeneration, diabetic retinopathy, stroke, central retinal artery occlusion, optic neuropathies and glaucoma [1, 2]. Diabetes retinopathy is major reason for vision loss in working adults and is expected to affect approximately 57 million by 2030. The second most common cause of impaired vision is age-related macular degeneration affecting 200 million old people worldwide[3]. The ability of the damaged human retina to regenerate is limited. The traditional therapies focus on preventing the disease advancement and use intra-vitreous medication of anti-angiogenic factors, eye drops or laser treatment. Though effective in treating the symptoms, the underlying issue remains unaddressed. Therefore, alternative therapies are needed to effectively treat these diseases. A successful therapy should be able to repair and regenerate the damaged tissue by surpassing the limitations of using growth factors [2]. A major hurdle in medicine is to develop therapeutics that support the healing of damaged tissues and organs, as the human body possesses limited ability to restore the tissues or organs to its original functionality. The emerging field of regenerative medicine and tissue engineering promises to treat variety of diseases or conditions that do not have any specific treatment. To achieve this, it uses efficient therapeutic biological strategies using stem cells, biological factors and delivery agents such as carriers or scaffolds [4, 5].

1.2 Stem cells

Stem cells are unspecified cells having the ability of proliferation, self-renewal and specializing into different cells and tissues through cell division. In general, stem cells can be of two types one being Embryonic stem cells and other being adult or somatic cells. The embryonic stem cells are considered pluripotent because of their ability to be differentiated into different cell lineages. They are extracted

from the inner cell mass and is not ethical as blastocyst is disrupted during harvesting [6]. The non-embryonic or somatic stem cells are multipotent, and they can be differentiated into specific cell types. The Non-embryonic stem cells are classified into at least two types, the haematopoietic stem cells which are responsible for haematopoiesis and the mesenchymal stem cells [7]. The stromal compartment of bone marrow has a very minute fraction of mesenchymal stem cells, 0.001- 0.01% of the overall population in the marrow [8]. MSCs can also be obtained from different adult tissues such as skin, pancreas, heart, lung, kidney, adipose tissue [9].

1.3 Mesenchymal Stem cells

Mesenchymal stem cells a type of somatic cells, belongs to the heterogenous multipotent population of cells that are isolated from various adult tissues. Adult or somatic stem cells can generally be differentiated into the tissue where they are obtained from. Morphologically they appear to be like that of fibroblast. Mesenchymal stem cells were first discovered by Friedenstein and Petrakova in 1976 and were harvested from bone marrow aspirates of the volunteers [8,10]. They are responsible for maintaining the population of haemopoietic stem cells and support in their differentiation [11].The International Society of Cellular Therapy defines MSCs as fibroblast-like adherent cells which express several markers CD73, CD105 and CD90 and lack CD34, CD19, CD14, CD45, CD11b, HLA-DR and CD79 [12]. They are induced to specialise into the cells of mesodermal lineage like adipocytes, chondrocytes and osteocytes and some other lineages [13]. The primary source of MSCs is adult bone marrow but recent research shows that they can be found at several other sites such as adipose tissue, dental pulp, liver and fetal tissues [9]. They can be easily cultured in vitro due to their property of plastic adherence while maintaining their multipotency and the yield obtained from the culture is enough to use them for therapeutic purposes. Their anti-proliferative activity is responsible for tissue homeostasis. MSCs show several cell-adhesion receptors such as ICAM, VCAM, P-selectin, L-selectin which bind to fibronectin, laminin and collagen. MSCs help in cell to cell contact which plays a very important role in ‘homing’ [14]. Easy isolation, differentiation and self-renewal capacity of MSCs have made them an attractive target for clinical application and are extensively researched.

1.4 Regenerative properties of MSCs

The ability of self-renewal is an important feature of MSCs. Although it can only be passed for limited times in vitro .The proliferation capability depends on several factors such as tissue source, donor age and culture conditions. [15]. Another remarkable property of MSCs is the wide range of differentiation. MSCS are considered as unique sub-type of adult or somatic stem cells. Other type of stem cells like neural stem cells or haematopoietic cells differentiate into specific cell type but MSCs not only differentiate into mesodermal lineages but can also differentiate into endodermal or ectodermal lineages, such as into neurons under typical experimental conditions [16]. Because of such a wide range they are accountable for growth, regeneration of cells and wound healing which are dead every day [17]. Another important phenomenon shown by MSCs is homing. Homing is a process in which the cells sense the injury and migrate to the damage tissue and differentiate into specific cell type [14]. MSCs seem to be hypoimmunogenic and are thus used for allogenic transplantation but the latest research suggests some immunosuppression once they are transplanted.

1.5 Paracrine actions of MSCs

The important underlying principle of MSCs favourable effects in regenerative medicine is due to its ability to induce several trophic factors that are bioactive. MSCs secrete various types of chemokines, cytokines and growth factors that rescues the damaged tissue. They are believed to secrete certain neurotrophic growth factors. Paracrine actions of these factors directly have neurotrophic or neuroprotective effect on the neurons. It induces neurogenesis, neurite growth and anti-apoptotic effects [18].The trophic factors also affect various functions such as enhancing the rate of survival, modulating the immune system, increasing proliferation, arresting cell apoptosis, promoting angiogenesis, formulation of extracellular matrix and differentiation ability [16, 19]. A single engrafted MSC has the ability to affect several cells in the vicinity by intercellular communication improving the tissue repair. MSCs show extensive immune modulatory effect that arrests the lymphocyte proliferation and inhibit apoptosis of the neutrophils [20]. They affect and inhibit both innate and adaptive immune cells.

Neovascularization is one of the major biological process that is dictated by the paracrine effects of MSCs. Along with the secretion of growth factors, chemokines and cytokines, MSCs regenerative abilities are also enhanced by the development of nanotubes in the membrane and secretion of extracellular vesicles. They affect cellular process by transporting wide range of tropic factors such as miRNAs, proteins, and messenger RNA [19]. MSCs can also guide mitochondrial transfer, cell fusion through cell-contact [21].

1.6 Exosomes

The paracrine factors are released in the extracellular environment. Additionally, number of factors are believed to be packaged into extracellular vesicles and migrate to different sites through the bloodstream [22]. Extracellular vesicle (EVs) are small vesicles secreted by almost all cell types. There are two types of EVs namely exosomes and micro vesicles/microparticles. Micro vesicles are about 150 to 1000nm in diameter and are released by the process of budding of the plasma membrane. The other type of EV is exosomes. Exosomes are nanovesicles having a diameter in the range of 40-200nm [23].They are generated in the endosomal compartment of the Multivesicular bodies(MVBs) and are released when these MVBs combine with the external boundary of the cell i.e plasma membrane. They are filled with components derived from endosome and are distinguished by a phospholipid bilayer. They contain variety of proteins and mRNAs, miRNAs that can induce a distinct cellular response. Secretion of exosomes is a common cellular process that facilitates intracellular communication [24]. When secreted, they are taken up by the cells in vicinity or they travel distantly through blood and other biological fluids. Recent research shows the presence of exosomes in number of biological fluids such as urine, plasma malignant, pleural effusion, amniotic fluid, breast milk and synovial fluid [25, 26]. Exosomes are researched for several functions such as diagnostic biomarkers [27], for Nano delivery due to their ability to cross brain-blood barrier [28]. The most important and focus here is the function of exosomes in regenerative medicine and tissue engineering specially those derived from MSCs. In published studies it seen that exosomes mitogen activated protein kinase (MAPK) pathway which increases the proliferation rate of MSCs. Various sources of MSCs and endothelial cells derived

exosomes demonstrate pro-angiogenic properties which are linked to the presence of miRNAs within the exosomes. The immunomodulatory effect of MSCs is shown to be driven by exosomes by secreting anti-inflammatory factors or by secreting M2 macrophages [24]. Additionally, the neuroprotection ability of the MSCs is believed to be the effect of paracrine actions specially exosomes. Recent studies have shown that extracellular vesicles decrease the neuronal cell death and stimulate axonal growth [2].

1.7 Dental Pulp as a source of MSCs

As mentioned earlier, MSCs can be harvested from several tissues and organs other than bone-marrow. Dental-tissue derived MSCs are among several other stem cells located in the teeth [13]. Depending on the origin, there are different types of MSC population, one of them being Dental pulp stem cell (DPSC). They were first discovered in 2000 by Gronthos et al. and was extracted from third molars [29]. DPSCs have their origin in the neural crest and can be obtained from the perivascular niche present in the dental pulp. They are considered as an excellent type of stem cells as they are harvested by simple, minimally invasive procedure and without any adverse effects. Also, they have no ethical concerns unlike embryonic stem cells [30, 31]. The innate function of DPSCs is to proliferate and migrate to damage tissue and differentiate into odontoblast thus maintaining and repairing various types of damage [29, 32]. Besides, they can also undergo adipogenic and neurogenic differentiation. Further characterization showed their osteogenic, chondrogenic and myogenic differentiation abilities [29]. Though they share similar characteristics with bone-marrow stem cells, DPSCs have shown greater proliferation rate and high frequency of colony forming cells in comparison to bone-marrow MSCs. Further studies show that they have higher osteogenic differentiation as that of bone-marrow MSCs [32]. DPSCs have markers of neuronal precursors and glial cells such as nestin and glial fibrillary acid protein (GFAP) [33]. This reflects their origin in the neural crest and have precedence in treatment of nerve injury above other sources of stem cells like MSCs obtained from bone marrow. DPSCs can also minimize neurodegeneration in the primary stage of neuronal apoptosis [34]. The trophic factors expressed by DPSC are exceptionally higher than the bone-marrow derived MSCs. Also, the cytokine expression level is greater than other types of MSC. These all benefits of DPSCs are assumed to be mediated by

secretome [35]. Published studies have shown the regeneration abilities of MSCs being associated with the secretome containing EVs or EVs themselves [31].

1.8 Research Objective

The current research study aims at accomplishing three objectives: 1. To characterize dental pulp derived exosomes. 2. To investigate the use of DPSC derived exosomes in neuroprotection. 3. To evaluate the role of engineered exosomes for the same and compare the two.

1.9 Hypothesis

This thesis is based on the hypothesis that DPSC derived exosome play an important role for neuroprotection/regeneration in retinal disorder and engineered exosomes enhancing this ability.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 DPSC and DPSC miR424

The human dental pulp derived stem cells (DPSCs) were used in the study. They were a gift from University of Pennsylvania, School of Dental Medicine by Dr. Songtao Shi. For engineering purpose, the DPSCs were transfected with miR424 that were used for all the experiments. The culture medium used for cells consisted of alpha-minimum essential medium (αMEM) along with 20% fetal bovine serum (FBS, Gibco), 1% antibiotic-antimycotic solution (anti-anti, Gibco) and 1% L-glutamine (Gibco) [36]. In order to perform experiments the cells utilized were of passage 4 or below.

2.2 Isolation of Exosomes

Both the cell types (DPSCs and DPSC miR424) were seeded and cultured until 80% confluency in T125 flask. Exosomes were purified from the culture media for a brief time approximately 3 weeks. They were purified according to the protocol published previously [24]. Before isolation, the cells were washed with PBS and cultured for 2 days in serum free media. Later the serum free media was collected and centrifuged at 1500 x g to remove any cell debris. The supernatant was then translated into a ultra-filtration tube having a filter cut-off 100-kDa and was centrifuged at 4000 x g at 4°Cfor 20min. 20% Exo Quick-TC (System Biosciences) was added to the concentrated volume of condition media to isolate the exosomes. The isolated exosomes were then suspended in PBS. The exosome concentration was normalised to number of cells obtained from culture flask and diluted to assure that 100µl of mixture contain exosomes purified from 1million number of cells as previously published [24].

2.3 Fluorescent labelling of exosomes

The fluorescent labelling of DPSC and DPSC miR424 derived exosomes was obtained by using the Exo-Glow-Green (System Bioscience) labelling kit. The exosome pellet was diluted in PBS. After adding Exo-Green the suspension was incubated for 30min at 37°C. Later Exo Quick-TC was put in

and incubated for 90min at 4°C. These labelled exosome suspensions were spin down for 3min at 13300 x g and were diluted in PBS.

2.4 Characterization of Exosomes

The exosomes isolated from DPSC and DPSC miR424 were identified for their size, distribution and exosomal surface markers. A Nanosight (LM10-HS, Malvern, Westborough, MA) instrument was used to perform Nanoparticle Tracking Analysis (NTA). It recorded the exosome size distribution and concentration to maintain the configuration and uniformity during all the experiments. The presence of surface markers CD 63 and CD9 were confirmed using Western Blot. We lysed the exosome pellets in RIPA buffer (1X) and diluted with SDS sample buffer. The lysate was then boiled for 5min and incubated at -80°C overnight. Before loading the samples, they were boiled for 5min and equal amount of each were loaded onto the gel. Proteins were electroblotted to nitrocellulose membrane (0.2µm) overnight at 30V. Nonspecific binding was blocked with LICOR Odyssey blocking buffer. The primary antibodies were added, and the nitrocellulose membrane was incubated in the freezer overnight. The primary antibody used are : CD63 (rabbit monoclonal, Abcam, 1/500), CD 9 (mouse monoclonal, Abcam, 1/100) mixed with blocking buffer [2]. The membrane was then washed 3times with PBST (PBS mixed with 0.1% Tween 20) for 5minutes per wash and was incubated for 60min with secondary antibodies: Goat anti-rabbit (LI-COR, 1/15000) and Donkey anti-mouse (LI-COR, 1/10000). After the wash with PBST, the membrane was air-dried and the immunoblot was scanned using an instrument LICOR Odyssey (Lincoln, NE)[2]. To cross verify expression of miR424 in the exosomes, the total miRNA from the cells and exosomes was extracted by using miRNeasy® Mini Kit (QIAGEN). Purified miRNA was reverse transcribed using miScript II RT kit (QIAGEN) according to the manufacturer's guidelines. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was conducted in quadruplets using SYBR® Green PCR kit (QIAGEN) to analyse the expression of miR424 in engineered exosomes. The primers used for PCR experiments are as follows: miR424 3p (5' GGC AGC AGC AAT TCA TGT TTT GAA 3'), miR424 5p (5' GCA AAA CGT GAG GCG CTG CTA T 3') and Hs_RNU6-2_11 miScript Primer Assay (QIAGEN).

2.5 R28 retinal cell culture

R28 neuronal-like cells were bought from Kerafast (Boston, MA) and seeded as per manufacturer's guidelines. R28 neuronal-like cells are the precursor for retinal cell-line and are extracted from postnatal 6 Sprague-Dawley rat retina and was immortalized with the 12S E1A gene [2]. The cells have been passaged more than 20times and show no morphological difference while maintaining its heterogenous nature. Cells were cultured in DMEM low glucose (1g/L) with 10% fetal bovine serum (420ml DMEM 1g/L, 15ml 7.5% sodium bicarbonate, 50ml fetal bovine serum, 5ml MEM non-essential amino acid, 5ml MEM vitamins, 5ml L-glutamine and 0.625ml anti-anti), with pH being 7.4 [2].

2.6 Endocytosis experiments

The in vitro endocytosis study was conducted in 96 well ELISA plates. Briefly, 20K R28 retinal cells were seeded into each well. 24h after culture, DPSC and DPSC miR424 derived labelled exosomes were added in increasing amount to each well. The samples were incubated for 2h at 37°C. For blocking studies, 10µl of exosomes were added to each well containing 20K R28 cells. Methyl-β-cyclodextrin (MBCD, 0,2.5 and 5mM) , Arg-Gly-Asp peptide (RGD, 0 and 2.5mM) were added to the cells in increasing amounts before the endocytosis experiment and exosomes were treated with heparin (0, 2.5, 5 and 10µM) at the start of the experiment for 1h at 37°C. Also, the cells were treated at 4°C for 1h post that they were incubated by adding labelled DPSC and DPSC miR424 derived exosomes. The experiments were performed in repeat of six. The wells were cleaned with PBS 3 times and were fixed with the help of 4% neutral formalin buffer. A suitable band pass filter was utilized to record the amount of fluorescence and the 96 well plate reader (BioTek) was used for the same.

For imaging purpose, the coverslip was put inside 6 well ELISA plates and 50K R28 retinal cells were cultured onto it. After 24h of seeding, the cells were treated with 50µl of fluorescently labelled DPSC and DPSC miR424 exosomes and was incubated for 2h at 37°C. The coverslips were cleaned 3 times with PBS and fixed with the help of 4% neutral formalin buffer [2]. To observe the microtubules, cells

were immuno-stained with anti-tubulin. The coverslips were fixed on a glass slide and confocal images were obtained using a Zeiss LSM 710 microscope.

2.7 MTS cell proliferation assay

The MTS cell proliferation assay was carried out on R28 retinal cells using DPSC and DPSC miR424 derived exosomes. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) kit was used as per manufacturer's instructions. For the cell proliferation assay, 2000 cells were seeded per well and were cultured for 1, 3, 5 and 7days. At the end of each time points, 20µL of proliferation assay reagent was mixed to 100µl of culture media and added to each sample well. The samples were then incubated at 37°C for 60min, 90mins, 120mins and absorbance was measured at 490nm using BioTek 96 well plate reader and results were plotted to compare the difference in rate of proliferation between cells treated and non-treated with exosomes.

2.8 Binding experiments

The peptides DGEA, GFPGR, Collagen, Fibronectin and RGD were coated onto the 96well plate at 5µg/well in 0.2M carbonate buffer overnight at 4°C. The vitreous humor was obtained from rat eyes was coated at 50µg/well using 0.2M carbonate buffer. The Plates were cleaned and incubated at 37°C for 2h with increasing amounts of fluorescently labelled DPSC and DPSC miR424 derived exosomes. The plates were cleaned 3 times using PBS and fixed with the help of 4% neutral formalin buffer. The fluorescence of bound exosomes was recorded with the help BioTek 96 well plate reader and results obtained were graphically represented.

2.9 In vitro model of low oxygen and glucose

The R28 retinal cells were subjected to simulated oxygen-glucose deprivation (OGD) condition. 2000 cells/well were seeded in 96 well ELISA plate. To represent OGD condition, cells were grown in no glucose medium and treated with hypoxic condition i.e 1% O2, 5% CO2 for 6h. Equal amounts of DPSC and DPSC miR424 derived exosomes (3µl) were added and cells were reoxygenated at 37°C overnight and lactate dehydrogenase (LDH) was measured using LDH kit (Promega, Madison, WI).

Briefly, the supernatant from normal and OGD treated cells were transferred to a new 96 well plate and each sample well was treated with 20µl of CytoTox 96® reagent. The plate was then incubated for 30min at room temperature. The absorbance from the samples was measured at 492nm after adding the stop solution. Cytotoxicity was estimated based on the amount of LDH present in the supernatant of samples. The results were plotted, and percentage cell death was obtained with and without DPSC and DPSC miR424 derived exosomes.

2.10 Statistical Analysis

All the data is represented as mean ± standard deviation (SD), and the significance was calculated by student's t-test where appropriate.

CHAPTER 3

3 RESULTS

3.1 Characterization of Exosomes

The isolated DPSC and DPSC miR424 derived exosomes were characterized by NTA and western blot. In general, EVs are small vesicles released by most cell types and depending on the size they are segregated as microvesicles 150 to 1000nm, exosomes 40 to 200nm and larger apoptotic bodies. The purified EVs were identified as exosomes based on the size and composition. Using NTA the size and concentration of exosomes derived from DPSC were analysed. The curve obtained represented a gaussian distribution and maximum area under the bell-shaped curve denoted the size range of 40-200nm which confirmed the presence of exosomes in the suspension. The highest peak was seen at 107nm. Another peak was observed at 147nm, that's likely being the mixture of exosomes and microvesicles. Similarly, for DPSC miR424 exosomes highest peak was observed at 110nm and another at 142nm (Figure 1). Thus, the results obtained shows no significant difference in size and morphology between the DPSC and DPSC miR424 derived exosomes.

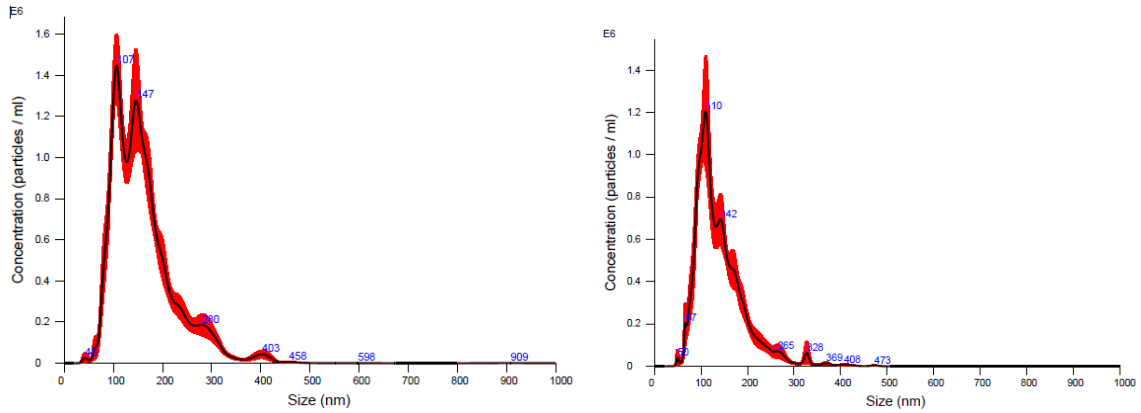

Figure 1. Nanoparticle Tracking Analysis (NTA) histogram of DPSC and DPSC miR424 exosomes

Proteins from DPSC and DPSC miR424 derived exosomes lysates was treated with SDS PAGE and western immunoblotting was performed. The blots showed the presence of exosomal surface markers CD63 and CD9 in the lysate of both DPSC and DPSC miR424 derived exosomes (Figure 2).

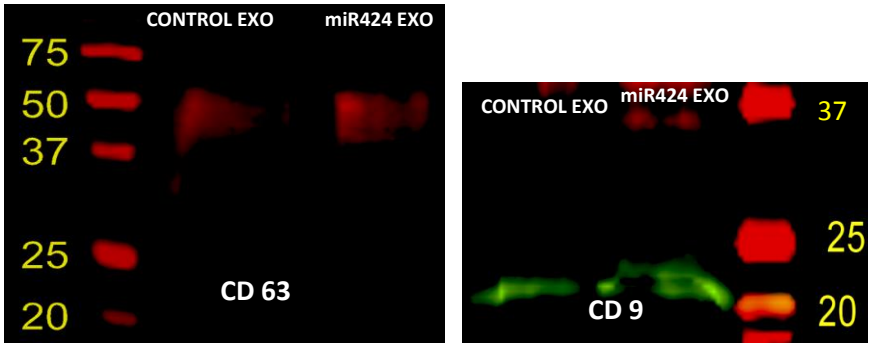

Figure 2. Western Blot for surface markers CD63 and CD9

miRNA location	**Fold change**	**P-value**
Intracellular	5.23	0.0002
Exosomes	115.17	2.37E-5

Table 1. Expression of gene miR424 5p in exosomes and cells

3.2 Endocytosis of exosomes by R28 retinal cells

Exosomes possess the required mechanism to enter and transfer complex molecular signals to the target cells [37]. These experiments were performed to demonstrate that the exosomes are internalised efficiently by the cells. The exosomes isolated from DPSC and DPSC miR424 were tagged green and R28 retinal cells along with labelled exosomes were incubated at 37°C and 4°C. As a standard, the R28 cells were subjected to same conditions but contained no fluorescently labelled exosomes in it. They were used for all endocytosis experiments and no green fluorescence was detected from such samples. To verify if the exosomes are internalised by the cells, they were stained with tubulin and the nucleus being stained using DAPI. Figures 3 and 4 demonstrate exosomes being endocytosed by the cells. Quantitatively the same results were observed, and Figure 5 represents a dose-dependent and saturable endocytosis of DPSC exosomes. Figure 6 show a similar saturable curve of DPSC miR424 derived exosomes being endocytosed by R28 retinal cells. It was also seen that the internalization was significantly affected at 4°C, denoting the dependence of temperature. When compared, both the DPSC

and DPSC miR424 derived exosomes are endocytosed in a similar manner and are energy dependent. This confirms that endocytosis is a guided process predominantly receptor mediated and does not occur randomly process.

Previous research demonstrate that exosome endocytosis is mediated via integrins. To identify the involvement of integrins, R28 retinal cell membrane was blocked by increasing concentration of Arg-Gly-Asp peptide (RGD 2.5mM and 5mM). The results obtained didn't show any significant difference in the endocytosis of exosomes by the R28 cells demonstrating the absence of integrin-mediated endocytosis (Figure 7). In order to simulate the binding of HSPG on R28 retinal cell membrane, the DPSC exosomes were blocked with heparin. A significant and dose-dependent decreasing profile was observed in terms exosome endocytosis. The results eliminated integrin-mediated endocytosis but did show involvement for cell surface Heparan Sulfate Proteoglycans (HSPG) (Figure 8).

According to published studies, lipid rafts/caveolae play a role in endocytosis of exosomes. To analyse the same, R28 retinal cells were blocked by means of an inhibitor named MBCD. It eliminates the cholesterol present on the plasma membrane and is efficient in blocking the caveolae mediated endocytosis pathway. The aftermath showed endocytosis being affected and blocked in dose-dependent manner (Figure 9). In general, all the endocytosis experiments showed a saturable response and occur by means of lipid raft/caveolae and HSPG pathway.

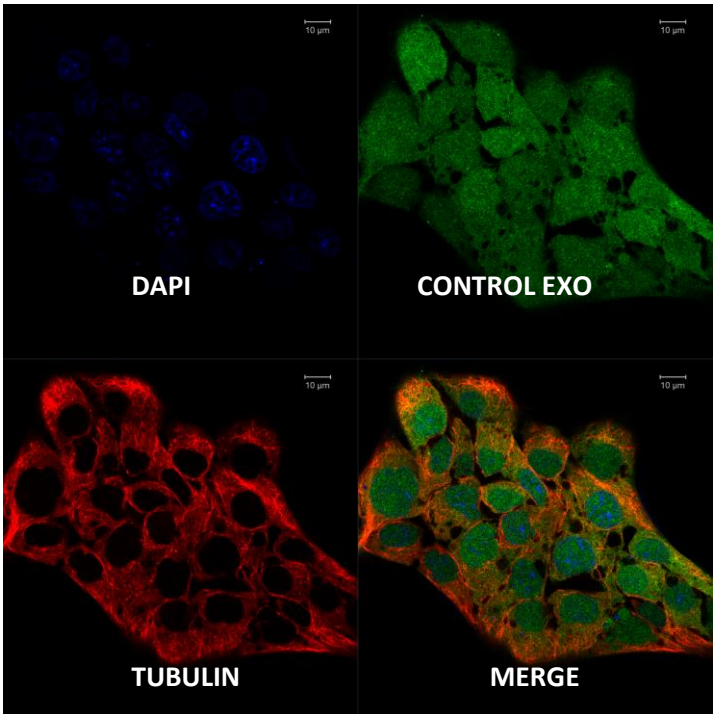

Figure 3 . Confocal image of DPSC exosome endocytosed by R28 retinal cells

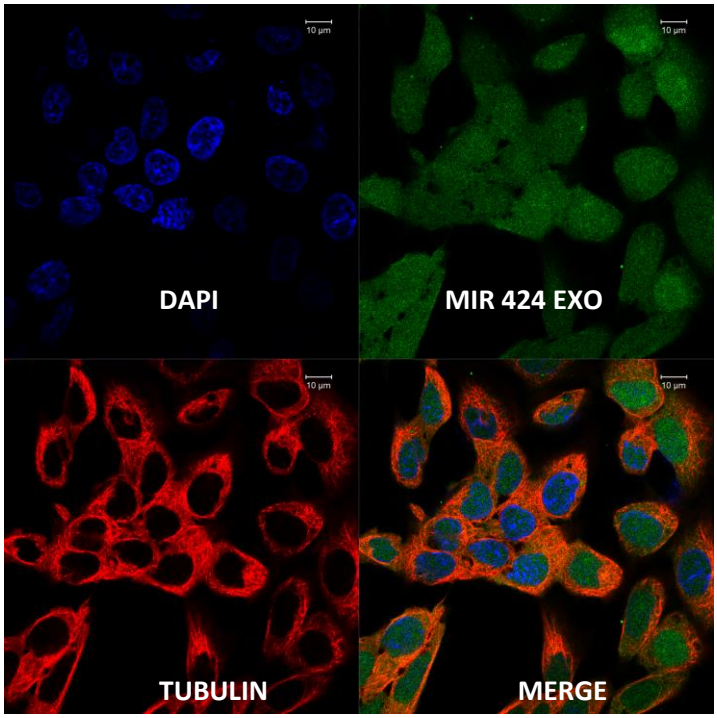

Figure 4. Confocal image of DPSC miR424 exosomes endocytosed by R28 retinal cells

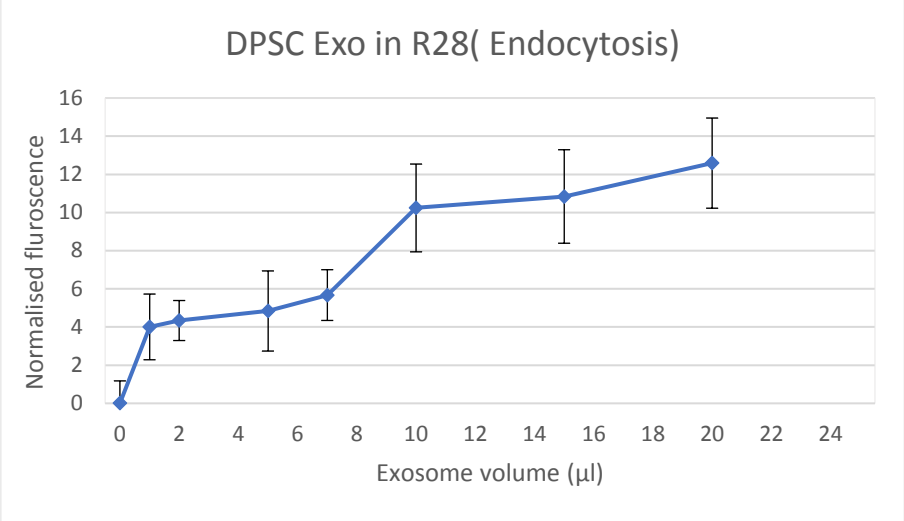

Figure 5. Dose-dependent endocytosis of DPSC exosomes

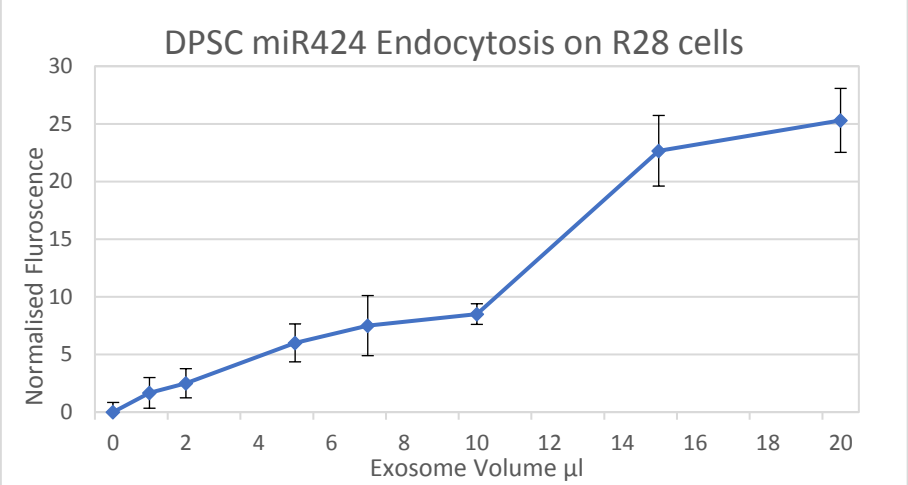

Figure 6. Dose-dependent endocytosis of DPSC miR424 exosomes

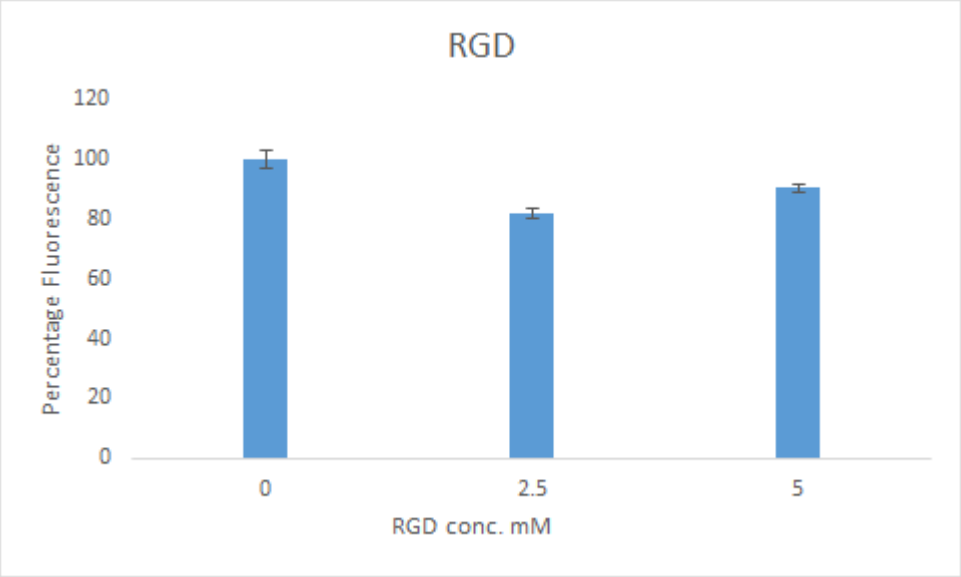

Figure 7. Blocking by RGD

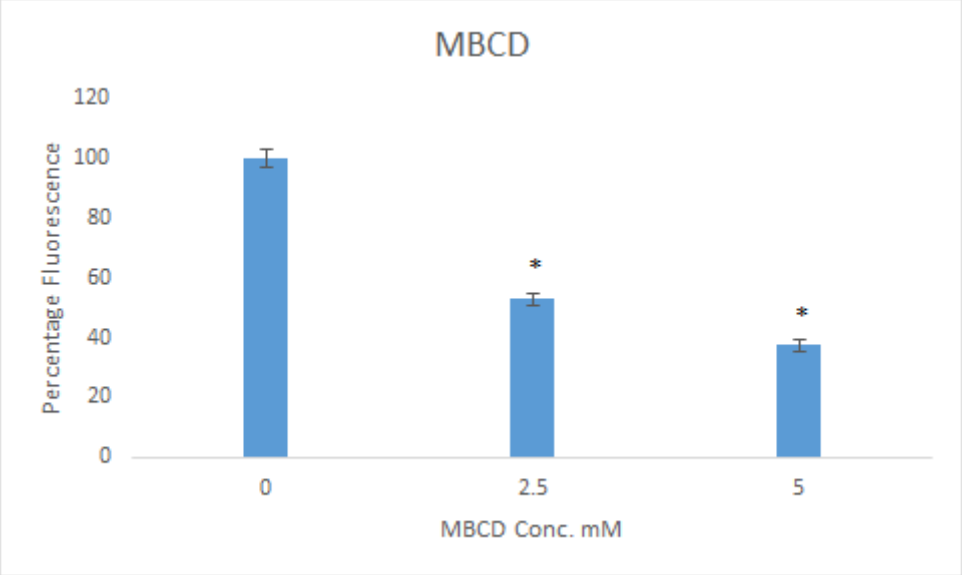

Figure 8. Blocking by MBCD

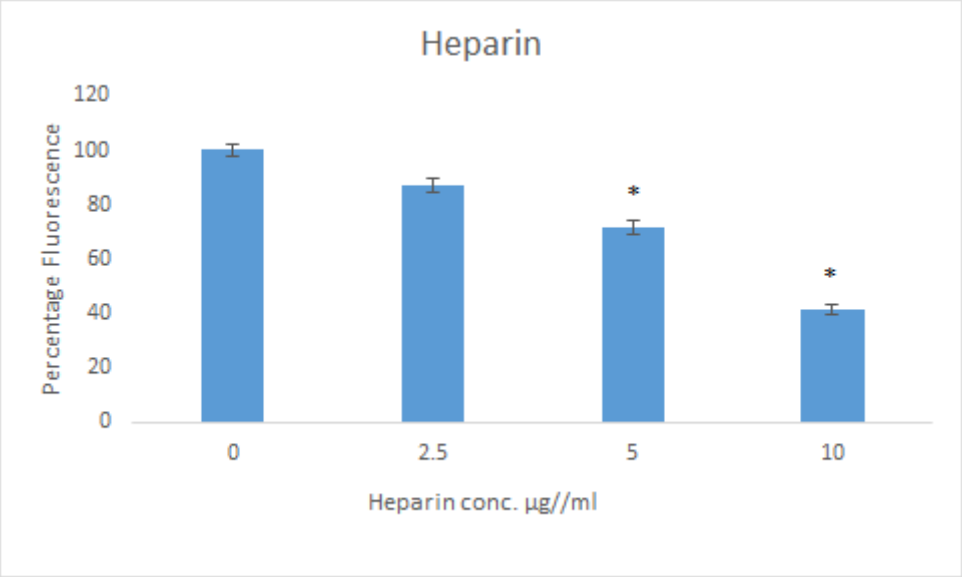

Figure 9. Blocking by Heparin

3.3 Proliferation of R28 retinal cells

After confirming the presence of exosomes in the conditioned media and its internalisation by R28 retinal cells, we wanted to know the effect of exosomes on the proliferation of R28 retinal cells. Hence 2000 cells per well were seeded into 96well plates. The cells were treated with exosomes and MTS proliferation assay was conducted. As a control, R28 cells were cultured under similar conditions but were not treated with exosomes. The number of metabolically active cells were measured at 1,3 and 5days post exosome treatment. The results did show increased proliferation rate of R28 cells when treated with DPSC and DPSC miR424 exosomes with respect to the R28 cells not treated with exosomes. The data shows higher % increase in the number of R28 cells when treated with DPSC miR424 exosomes as compared to the cells treated with naive DPSC exosomes (Figure 10).

** represents statistical significance (student's t-test). NS represents not significant.*

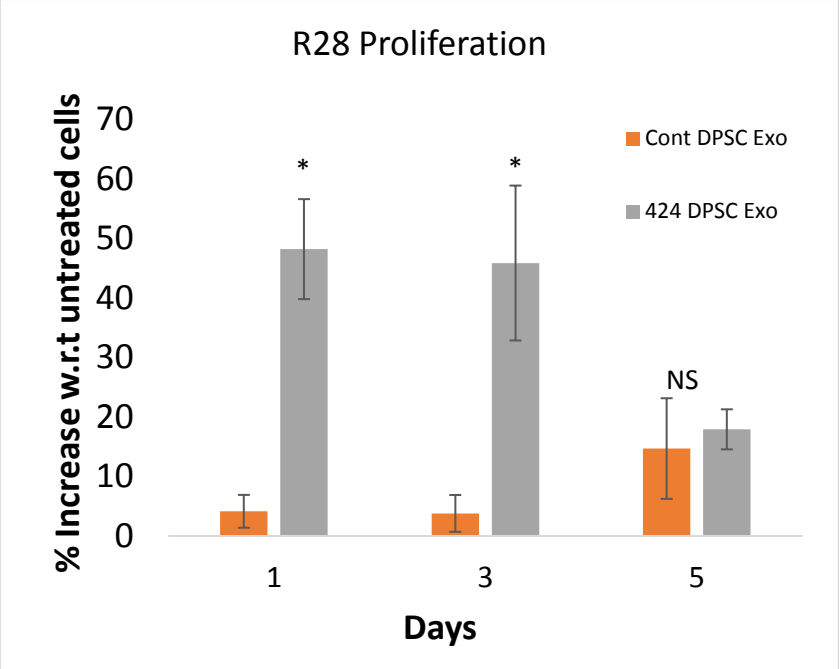

Figure 10. Proliferation assay

3.4 Exosome binding to ECM

Exosomes bud out from plasma membrane implying that the exosomal membrane is composed of the plasma membrane. It is important to understand adherence capabilities of exosomes to ECM proteins and vitreous humor so as to assess the ability of exosomes to be adhere to biomaterials. So far, the results obtained show the ability of exosomes to be internalized by the R28 cells. In order to incorporate them in 3D scaffolds and assess their capabilities, we verified whether exosomes can bind to ECM proteins and vitreous humor. Quantitative analysis of exosomes binding to ECM proteins was performed. Results demonstrated in Figure 11 show a dose-dependent response implying DPSC exosomes binding to ECM proteins (Fibronectin, type I collagen, RGD and vitreous humor). The type I collage, fibronectin binding is saturable but not RGD and vitreous. RGD was used at 5µg/well and hence not all was used up. Vitreous probably contains several exosomes binding proteins. A similar graph (Figure 12) was obtained when DPSC miR424 exosomes were tested for its binding capacity. When compared both DPSC and DPSC miR424 derived exosomes show a similar dose-dependence response for binding to ECM proteins and vitreous humor.

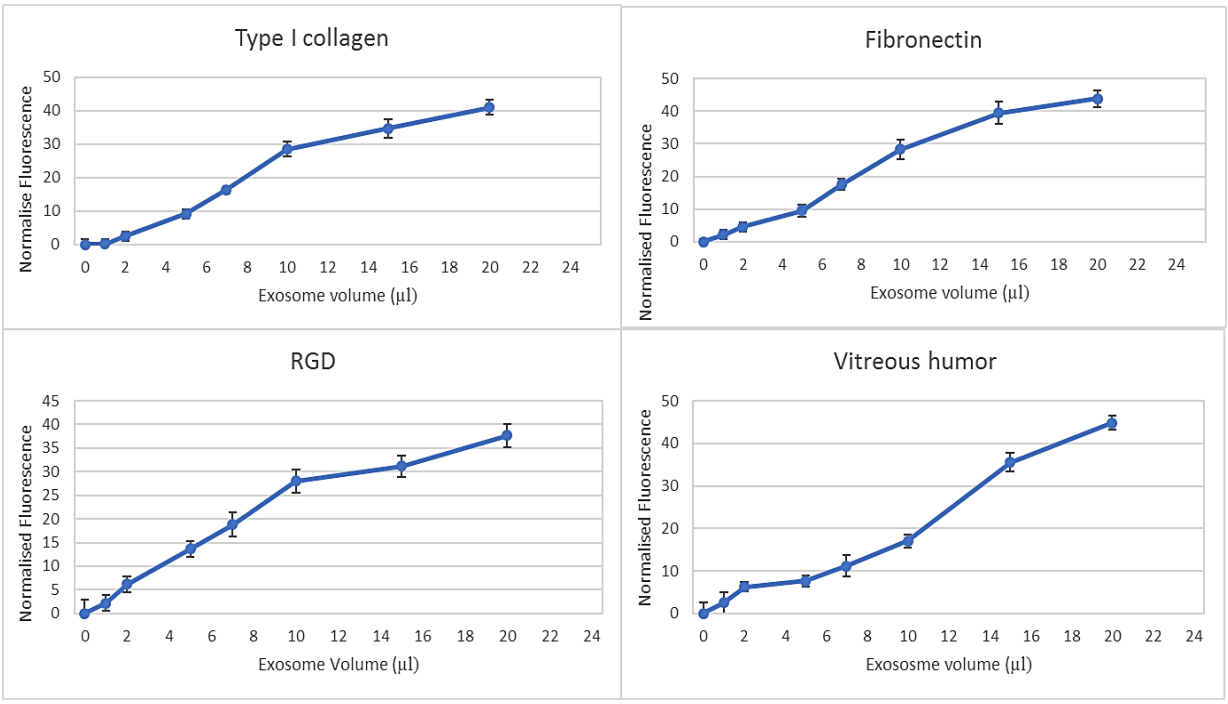

Figure 11. Binding of DPSC exosomes

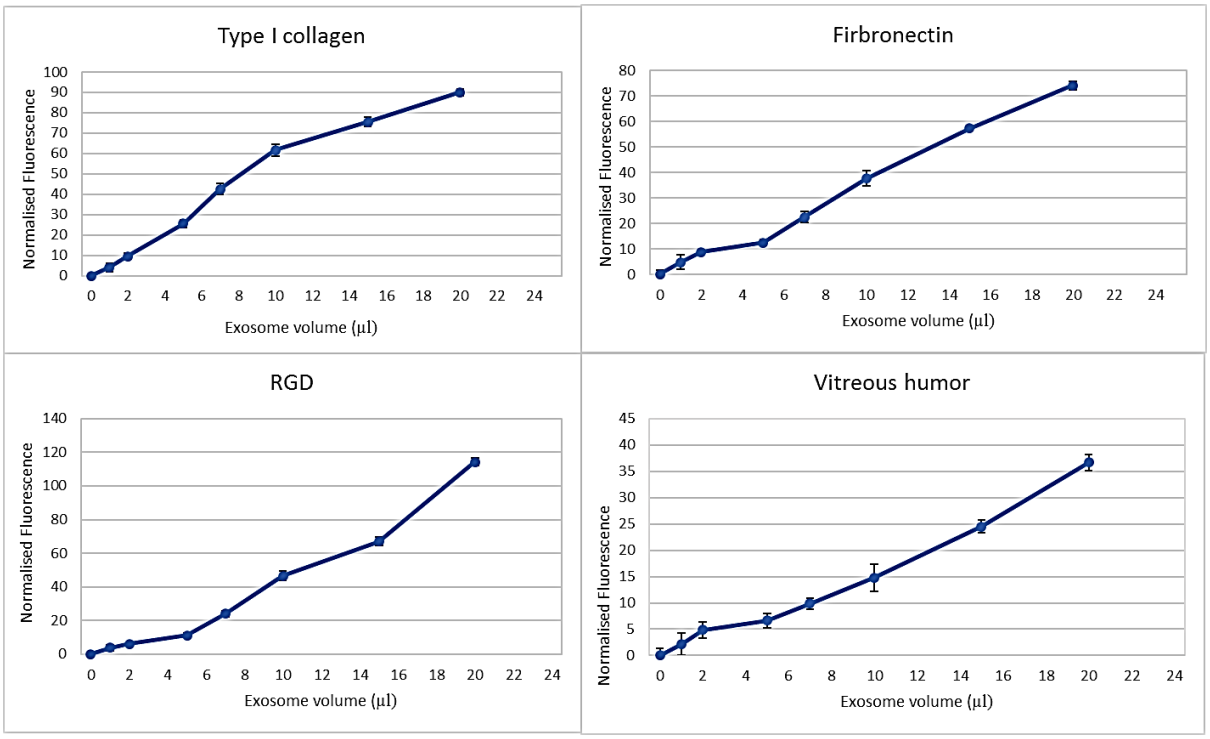

Figure 12. Binding of DPSC miR424 exosomes

3.5 In vitro model of low oxygen and glucose

To mimic ischemic condition, the R28 cells were subjected to OGD conditions. To test the hypothesis if exosomes can save R28 retinal cells from ischemic cell death, the R28 retinal cells were subjected to OGD conditions for 6h and later were treated with exosomes for approx. 16h. The cytotoxicity was measure from LDH released by the cells. As seen in figure 4A, OGD conditions caused more than 50% of cell death. Conversely when same were treated with 3µl of DPSC exosomes (corresponding to exosomes from 300k cells) a significant reduction in %cell death as compared to cells with absence of exosomes was observed. The same experiment was performed using DPSC miR424 derived exosomes. When compared, DPSC miR424 derived exosomes proved more effective than DPSC exosomes at lower concentrations (Figure 13). Also, condition media depleted of exosomes were tested and fewer protective effects were seen implying that the protective effects are due to the presence of exosomes.

**represents significance w.r.t control and # represents significance w.r.t naïve DPSC exosomes.*

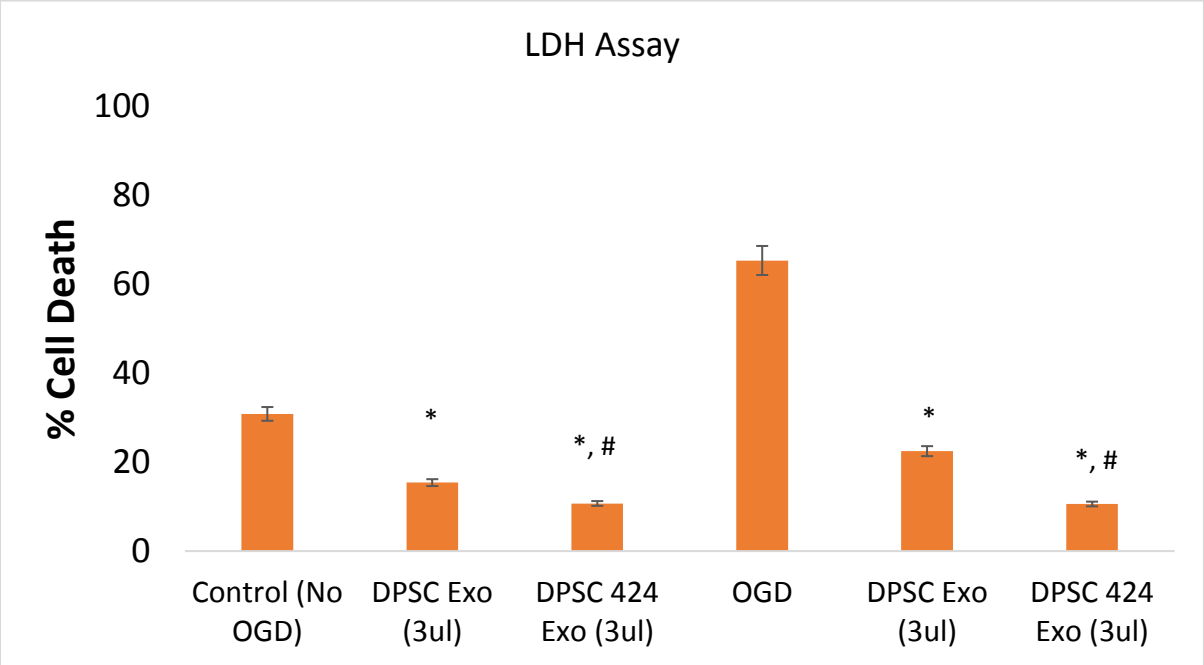

Figure 13. Cytotoxicity assay

CHAPTER 4

4 DISCUSSION

In this study, we have characterized DPSC exosomes containing increased levels of miR424. We show by qPCR that the cells and exosomes contain increased levels of miR424. After this, NTA analysis was performed to see if engineering the exosomes changed their basic properties. NTA results showed similar size distribution around 110nm for both DPSC and DPSC miR424 exosomes demonstrating no significant difference when engineered. Similarly, when checked for exosomal markers, the western blot showed presence of both CD63 and CD9 in DPSC miR424 exosomes. Exosomes are the mediator of cell-cell communication. They are loaded with genetic materials such as mRNA, miRNA, pre-miRNA and proteins that can have significant impact on target cell function[38]. Exosomes can fuse with the target cells or can be endocytosed to transfer or deliver the content to have the effect on target cells and their cellular processes[39]. For therapeutic benefits, the viability of exosomes is based on the productivity of endocytosis by the cells of interest. The specificity of the target increases with increase in endocytic effectiveness reducing the ectopic impacts. Hence it is important to understand the uptake of exosomes by the target cells. The results obtained demonstrate that exosomes are internalised by R28 retinal cells and is dose-dependent, saturable and dependent on temperature indicating that endocytosis is mediated by the receptor. Previous research demonstrate that exosomes are subjected endocytosis via variety of mechanisms depending on the composition of exosomal membrane and the source[40, 41]. Cells can internalise exosomes by variety of mechanisms such as phagocytosis, macro-pinocytosis receptor-mediated [2, 39]. A dose-dependent blocking by heparin on the binding sites of exosomes or by lipid rafts/caveolae was seen from the quantitative data obtained. As seen in results section engineering of exosomes by altering its miRNA composition did not change the uptake of exosomes indicating no change in basic exosomal properties.

To realize the potential of exosomes for therapeutic applications, they should be suitable with already available scaffold materials or other type of materials designed for tissue engineering. Previous studies suggest that exosomes adhere to ECM indicating a potential pool that can be delivered if ECM

undergoes degradation[42]. One way to evaluate this is to assess the adherence capacity of exosomes with the ECM proteins. Many of the scaffold materials are functionalised by RGD peptide or are made up of type I collagen. We therefore hypothesized that the engineered exosomes should bind to ECM proteins. Results in this study demonstrate a dose-dependent and saturable response when DPSC miR424 derived exosomes adhere to type I collagen and RGD. Also, exosomes binding to vitreous humor from the eye demonstrates a dose-dependent and saturable response indicating vitreous humor to be the store for treating the retina with exosomes. This feature can be beneficial when using for therapeutic purpose as the major challenge is site specificity. Binding to vitreous humor can open avenues for use of several biomaterials.

Exosomes play a role in several processes such as cell-cell communication, immunomodulation recycling of membrane proteins and lipids, cell proliferation, apoptosis and angiogenesis. Several studies have shown these functions with respect to individual tissue or organ[42]. For example, Bruno et al showed the renoprotective effects in kidney model. The exosomes increased the proliferation of tubular cells and improvised the injury caused to the tubule and kidney. Also, the results, associated activation of proliferative pathways by means of mRNA transfer by exosomes[38]. Specifically, miR424 is known to regulate cell proliferation. To evaluate this the cultured cells were treated with DPSC miR424 derived exosomes. The results indicated a significant increase in cell proliferation as compared to DPSC exosomes during the initial days of culture. Published studies have shown miRNA from the exosomes as an important contributor in cardio-protection, liver protection[38, 39]. Yu et al. showed exosomes overexpressed with specific miRNA demonstrated anti-apoptotic effects when subjected to ischemic conditions[38]. Interestingly, one of the studies showed hypoxia upregulates the expression of miR424 in endothelial cells[43]. To verify the anti-apoptotic property of miR424 an in vitro ischemic model was created mimicking the retinal ischemia. Our results showed neuroprotection effects of miR242 by rescuing the R28 retinal cells with a higher efficiency compared to naïve exosomes. Overall the experimental data denotes precedence of miR424 in functional assays. Other properties of miR424 include anti-angiogenetic and anti-inflammatory. In the context of retina these properties are beneficial. Retina is one of the organs where higher vascularisation can have detrimental

effects and can eventually lead to vision loss. Apart from retina, this anti-apoptotic function of miR424 can be beneficial for regeneration of avascular tissues such as cartilage. Also, one of the studies demonstrate anti-apoptotic function of miR424 in recovering the cerebral ischemia[44]. It would be interesting to study the function of miR424 in different ischemic tissues. Hence, we predict that future studies using engineered exosomes can help in repair and regeneration of various types of tissues along with neuronal tissues.

CHAPTER 5

5 CONCLUSION

The experimental data indicate that DPSC exosomes engineered to possess increased levels of miR424 are internalised by R28 cells. The endocytosis takes place in a dose-dependent, saturable and receptor-mediated manner. By internalising the exosomes, the R28 retinal cells exposed to simulated ischemic conditions can be protected or rescued. Also, the engineered exosomes increase proliferation of the retinal cells and can significantly reduce the recovery time. The binding of exosomes to ECM proteins and vitreous humor serves as a starting point in development of scaffolds or biomaterials for enhanced and site-specific delivery.

CITED LITERATURE

1. Minhas, G., R. Morishita, and A. Anand, *Preclinical models to investigate retinal ischemia: advances and drawbacks.* Front Neurol, 2012. **3**: p. 75.
2. Mathew, B., et al., *Mesenchymal stem cell-derived extracellular vesicles and retinal ischemia-reperfusion.* Biomaterials, 2019. **197**: p. 146-160.
3. Rivera, J.C., et al., *Ischemic Retinopathies: Oxidative Stress and Inflammation %J Oxidative Medicine and Cellular Longevity.* 2017. **2017**: p. 16.
4. Samaneh Hosseini, L.T., Fatemeh Safari, Mohamadreza Baghaban Eslaminejad, *Regenerative Medicine Applications of Mesenchymal Stem Cells*, in *Cell Biology and Translational Medicine*. 17 May 2018, Springer, Cham. p. 115-141.
5. Moustapha Kassem, B.M.A., *Human bone-marrow-derived mesenchymal stem cells: biological characteristics and potential role in therapy of degenerative diseases*, in *Cell and Tissue Research*. 2007, Springer-Verlag.
6. Al Jumah, M.A. and M.H. Abumaree, *The immunomodulatory and neuroprotective effects of mesenchymal stem cells (MSCs) in experimental autoimmune encephalomyelitis (EAE): a model of multiple sclerosis (MS).* Int J Mol Sci, 2012. **13**(7): p. 9298-331.
7. Tuch, B.E., *Stem cells--a clinical update.* Aust Fam Physician, 2006. **35**(9): p. 719-21.
8. Barry, F.P. and J.M. Murphy, *Mesenchymal stem cells: clinical applications and biological characterization.* Int J Biochem Cell Biol, 2004. **36**(4): p. 568-84.
9. Musial-Wysocka, A., M. Kot, and M. Majka, *The Pros and Cons of Mesenchymal Stem Cell-Based Therapies.* Cell Transplant, 2019: p. 963689719837897.
10. Nancarrow-Lei, R., et al., *A Systemic Review of Adult Mesenchymal Stem Cell Sources and their Multilineage Differentiation Potential Relevant to Musculoskeletal Tissue Repair and Regeneration.* Curr Stem Cell Res Ther, 2017. **12**(8): p. 601-610.
11. Uccelli, A., et al., *Neuroprotective features of mesenchymal stem cells.* Best Pract Res Clin Haematol, 2011. **24**(1): p. 59-64.

12. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.

13. Huang, G.T., S. Gronthos, and S. Shi, *Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine.* J Dent Res, 2009. **88**(9): p. 792-806.

14. Kolf, C.M., E. Cho, and R.S. Tuan, *Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation.* Arthritis Res Ther, 2007. **9**(1): p. 204.

15. Via, A.G., A. Frizziero, and F. Oliva, *Biological properties of mesenchymal Stem Cells from different sources.* Muscles Ligaments Tendons J, 2012. **2**(3): p. 154-62.

16. Wakao, S., et al., *Regenerative Effects of Mesenchymal Stem Cells: Contribution of Muse Cells, a Novel Pluripotent Stem Cell Type that Resides in Mesenchymal Cells.* Cells, 2012. **1**(4): p. 1045-60.

17. Wei, X., et al., *Mesenchymal stem cells: a new trend for cell therapy.* Acta Pharmacol Sin, 2013. **34**(6): p. 747-54.

18. Wakabayashi, K., et al., *Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model.* J Neurosci Res, 2010. **88**(5): p. 1017-25.

19. Yao Fu, L.K., Ling Wu, Jeroen Leijten, Sanne K. Both, and Marcel Karperien., *Trophic Effects of Mesenchymal Stem Cells in Tissue Regeneration*, in *Tissue Engineering Part B: Reviews*. 2017.

20. Bifari, F., L. Pacelli, and M. Krampera, *Immunological properties of embryonic and adult stem cells.* World J Stem Cells, 2010. **2**(3): p. 50-60.

21. Hofer, H.R. and R.S. Tuan, *Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies.* Stem Cell Res Ther, 2016. **7**(1): p. 131.

22. Maumus, M., C. Jorgensen, and D. Noel, *Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes.* Biochimie, 2013. **95**(12): p. 2229-34.

23. Cosenza, S., et al., *Mesenchymal stem cells-derived exosomes are more immunosuppressive than microparticles in inflammatory arthritis.* Theranostics, 2018. **8**(5): p. 1399-1410.

24. Narayanan, R., C.C. Huang, and S. Ravindran, *Hijacking the Cellular Mail: Exosome Mediated Differentiation of Mesenchymal Stem Cells.* Stem Cells Int, 2016. **2016**: p. 3808674.

25. Marote, A., et al., *MSCs-Derived Exosomes: Cell-Secreted Nanovesicles with Regenerative Potential.* Front Pharmacol, 2016. **7**: p. 231.

26. Chang, Y.H., et al., *Exosomes and Stem Cells in Degenerative Disease Diagnosis and Therapy.* Cell Transplant, 2018. **27**(3): p. 349-363.

27. Muller, G., *Microvesicles/exosomes as potential novel biomarkers of metabolic diseases.* Diabetes Metab Syndr Obes, 2012. **5**: p. 247-82.

28. Alvarez-Erviti, L., et al., *Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes.* Nat Biotechnol, 2011. **29**(4): p. 341-5.

29. Ledesma-Martinez, E., V.M. Mendoza-Nunez, and E. Santiago-Osorio, *Mesenchymal Stem Cells Derived from Dental Pulp: A Review.* Stem Cells Int, 2016. **2016**: p. 4709572.

30. Song, M., et al., *Human Dental Pulp Stem Cells Are More Effective Than Human Bone Marrow-Derived Mesenchymal Stem Cells in Cerebral Ischemic Injury.* Cell Transplant, 2017. **26**(6): p. 1001-1016.

31. Kichenbrand, C., et al., *Dental Pulp Stem Cell-Derived Conditioned Medium: An Attractive Alternative for Regenerative Therapy.* Tissue Eng Part B Rev, 2019. **25**(1): p. 78-88.

32. Tatullo, M., et al., *Dental pulp stem cells: function, isolation and applications in regenerative medicine.* J Tissue Eng Regen Med, 2015. **9**(11): p. 1205-16.

33. Wislet-Gendebien, S., et al., *Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells.* J Cell Sci, 2003. **116**(Pt 16): p. 3295-302.

34. Kolar, M.K., et al., *The neurotrophic effects of different human dental mesenchymal stem cells.* Sci Rep, 2017. **7**(1): p. 12605.

35. Luo, L., et al., *Potential Roles of Dental Pulp Stem Cells in Neural Regeneration and Repair*. Stem Cells Int, 2018. **2018**: p. 1731289.

36. Banizs, A.B., et al., *Endocytosis Pathways of Endothelial Cell Derived Exosomes.* Mol Pharm, 2018. **15**(12): p. 5585-5590.

37. Keshtkar, S., N. Azarpira, and M.H. Ghahremani, *Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine.* Stem Cell Res Ther, 2018. **9**(1): p. 63.

38. Mulcahy, L.A., R.C. Pink, and D.R. Carter, *Routes and mechanisms of extracellular vesicle uptake.* J Extracell Vesicles, 2014. **3**.

39. Li, C., et al., *CD97 promotes gastric cancer cell proliferation and invasion through exosome-mediated MAPK signaling pathway*. World J Gastroenterol, 2015. **21**(20): p. 6215-28.

40. Alcayaga-Miranda, F., M. Varas-Godoy, and M. Khoury, *Harnessing the Angiogenic Potential of Stem Cell-Derived Exosomes for Vascular Regeneration.* Stem Cells Int, 2016. **2016**: p. 3409169.

41. De Jong, O.G., et al., *Extracellular vesicles: potential roles in regenerative medicine.* Front Immunol, 2014. **5**: p. 608.

VITA

APURVA KALAMKAR

EDUCATION

University of Illinois at Chicago (UIC) – Master of Science, 2019.

Major: Bioengineering

University of Mumbai, India – Bachelor of Engineering, 2015.

Major: Biomedical Engineering

PROJECTS

JAMA SOFTWARE – University of Illinois at Chicago

- Risk analysis of electrocautery device.
- Worked on Product design, Risk management and performed FMEA for the medical device using JAMA software according FDA standards**.**

Optimization of Instrumentation for analysis of dynamic properties of liposomes

- Synthesized liposomes using different phospholipids like DPPC and SOPC.
- Characterized the micro pump to use as pressure manipulating and adjusting device.
- Surface properties were studied using Bartel's micro pump rather than the conventional water column.
- Designed a piezo electric pump like Bartels mp6. We started with the layouts using CAD software**.**

ACHIEVEMENTS AND AWARDS

- Awarded second prize for the poster presentation "Intracranial *aneurysm clips*" in the Inter Departmental Poster presentation.