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The Role of Human MSC Derived Exosomes in the Treatment of Periodontal Diseases

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THE POTENTIAL OF HUMAN MSC DERIVED EXOSOMES IN THE TREATMENT OF PERIODONTAL DISEASES

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

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LIST OF ABBREVATIONS

BM-hMSC: Bone marrow human mesenchymal stem cell

DAMP: Damage-associated molecular pattern

DMEM: Dulbecco's modified Eagle medium

ELISA: Enzyme-linked immunosorbent assay

hMSC: Human mesenchymal stem cell

Hsp70: Heat shock protein 70

IL-1: Interleukin-1

IL-6: Interleukin-6

ILV: Intraluminal vesicles

IKK: IκB kinase

LPS: Lipopolysaccharide

LPSE: *E.coli* lipopolysaccharide

miRNA: microRNA

MMP: Matrix metalloproteinase

MVB: Multivesicular body

NC-1: Negative control-1

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PAMP: Pathogen-associated molecular pattern

PMN: Polymorphonuclear leukocyte

PRR: Pattern recognition receptor

RANK: Receptor activator of nuclear factor kappa-B

RANKL: Receptor activator of nuclear factor kappa-B ligand

TEM: Transmission electron microscope

TGF-β: Transforming growth factor beta

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TSG101: Tumor susceptibility gene 101

Abstract

THE POTENTIAL OF HUMAN MSC DERIVED EXOSOMES IN THE TREATMENT OF PERIODONTAL DISEASE

By Sonia S. Talegaonkar, BSc

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

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Periodontal disease affects 47% of Americans over 30. Characterized by microbial dysbiosis and unregulated inflammation, severe periodontitis causes degradation of bone and soft tissue around teeth. Current treatments have limited regenerative outcomes and frequent reinfection by harmful bacteria. Human mesenchymal stem cells (hMSCs) have been shown to promote wound healing and tissue regeneration. Many therapeutic benefits of hMSCs are due to their secretome products, like exosomes. Our long-term goal is to develop periodontal therapies with hMSC exosomes. The objectives of this study were to determine the effect of hMSC-derived exosomes on cellular activity

of hMSCs and investigate whether hMSC exosome treatment reduces pro-inflammatory cytokine production in LPS-activated RAW264.7 cells. The specific aims of this study were: 1) Determine the characteristics of hMSC-derived exosomes, 2) Determine the biological effect of exosomes on cellular activity of hMSCs, 3) Determine whether exosomes treatment can inhibit cytokine production in activated RAW264.7 cells, and 4) Determine the role of exosomal miRNA in pro-inflammatory cytokine production of RAW264.7 cells. To investigate, exosomes were first harvested from hMSCs culture media through ultracentrifugation. Exosomes were then observed under a transmission electron microscope (TEM) and assessed for surface markers using Western Blot. A transwell migration assay was used to evaluate the chemotactic effect of exosomes. To study the effect of exosomes on stem cell proliferation, exosomes were administered to hMSCs. The immunogenicity of MSC exosome was also evaluated. After 72 hours, cells were lysed and DNA was measured. To study anti-inflammatory effects of exosomes, LPS stimulated RAW264.7 cells were treated with exosomes. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) levels of supernatant were measured by ELISA. To study exosomal miRNA, exosomal miRNAs were overexpressed in RAW264.7 cells and these cells were stimulated with LPS. IL-6 and TNFα were measured by ELISA. TEM images showed that exosomes are nano-sized vesicles (~100 nm). Western blot images showed that CD63 and CD81 are enriched in exosomes compared to total cell lysates. Exosome treatment increased cell proliferation and migration in hMSCs. At the doses that are chemotactic and mitogenic, MSC exosomes had minimal effect on the inflammatory cytokine IL-6 production. Treatment with exosomes significantly decreased IL-6 and TNFα production in RAW264.7 cells activated by LPS. Transfecting

RAW264.7 cells with exosomal miR-760 significantly decreased IL-6 production, but had minimal effect on TNFα. Our results indicate that exosomes have a pleiotropic activity, which includes stimulating stem cell migration and proliferation, and mitigating the inflammatory response. Therefore, hMSC exosome delivery is promising for the treatment of periodontal diseases.

CHAPTER 1: INTRODUCTION

1.1. SCOPE OF PERIODONTAL DISEASE

Periodontal disease affects 47% of Americans above the age of 30 and 70% of population above the age of 65 (Kolenbrander, et al., 2010). Characterized by microbial dysbiosis and unregulated inflammation, severe periodontitis can result in degradation of the gingival tissue and surrounding bone structure.

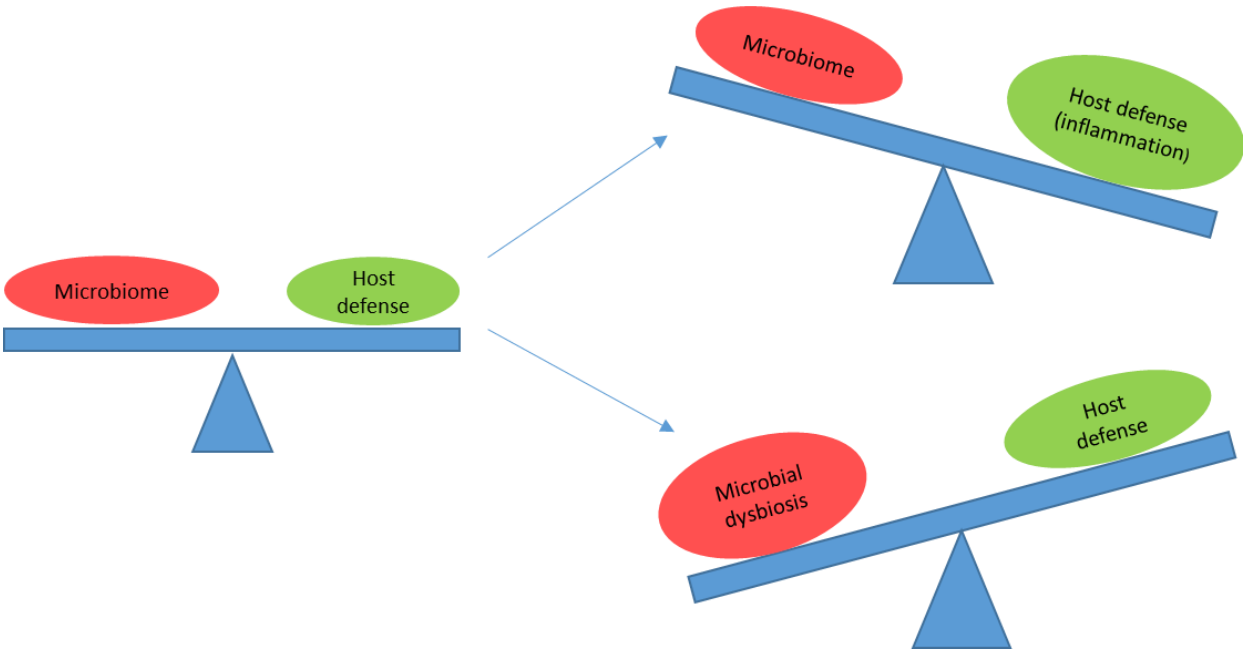


Figure 1: Normal balance of periodontal health vs. periodontal disease states caused by imbalances between the host defense and microbial systems

1.2. ORAL BIOFILM

The oral biofilm (Fig. 2) is composed of the salivary pellicle, bacterial colonizers, and coaggregators such as mucin, agglutin, and salivary amylase. This complex attaches to the tooth surface and interacts with the host to maintain the microbial balance of the oral cavity (Marsh et al., 2006). Early colonizers include *Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus mitis.* These Gram-positive bacterial strains are part of the normal oral flora, and they provide a matrix for the attachment of later colonizers, some of which are highly associated with periodontitis. *Fusobacterium nucleatum* connects the early and late colonizers. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are two late colonizers that have been implicated in periodontal disease. They are facultative or obligate anaerobes that thrive in low oxygen environments that are common in periodontal disease (Kolenbrander et al. 2010). Both strains are also Gram-negative and contain a thin peptidoglycan wall with lipolysaccharide on the outer leaflet. Lipopolysaccharide (LPS) is composed of lipid A, O-antigen (O-polysaccharide), and a core oligosaccharide (Wang et al., 2001). This component provides structural integrity and protection for the bacteria.

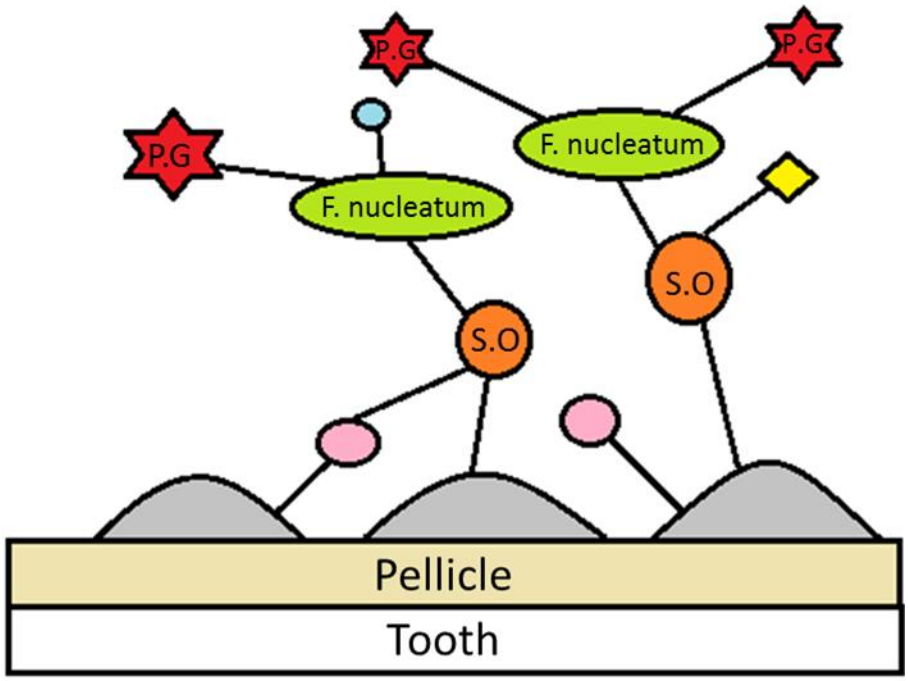


Figure 2: Simplified oral biofilm – the salivary pellicle and coaggregators (grey) attach to the tooth structure. Primary colonizers such as *S. oralis* (S.O.) attach to the pellicle, and *F. nucleatum* attaches to this. Late colonizers such as *P. gingivalis* (P.G.) are the last to attach.

1.3. PERIODONTAL PATHOGENESIS

As late bacterial colonizers accumulate at the gingiva, bacteria interact with the host's pattern recognition receptors (PRR). PRR's are immune system proteins that identify pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). They are located on the surface of immune cells. At a general level, bacterial LPS acts as a PAMP that binds to the host's PRR on a macrophage or leukocyte surface (Hayden et al., 2014). This interaction stimulates the NF-κB pathway, causing increased transcription of mRNA for inflammatory cytokine production (Hoesel et al., 2013). Pro-inflammatory cytokines interact with bone and tissue mediators, and ultimately lead to degradation of these structures. Specific processes of cytokine production and tissue degradation are examined below.

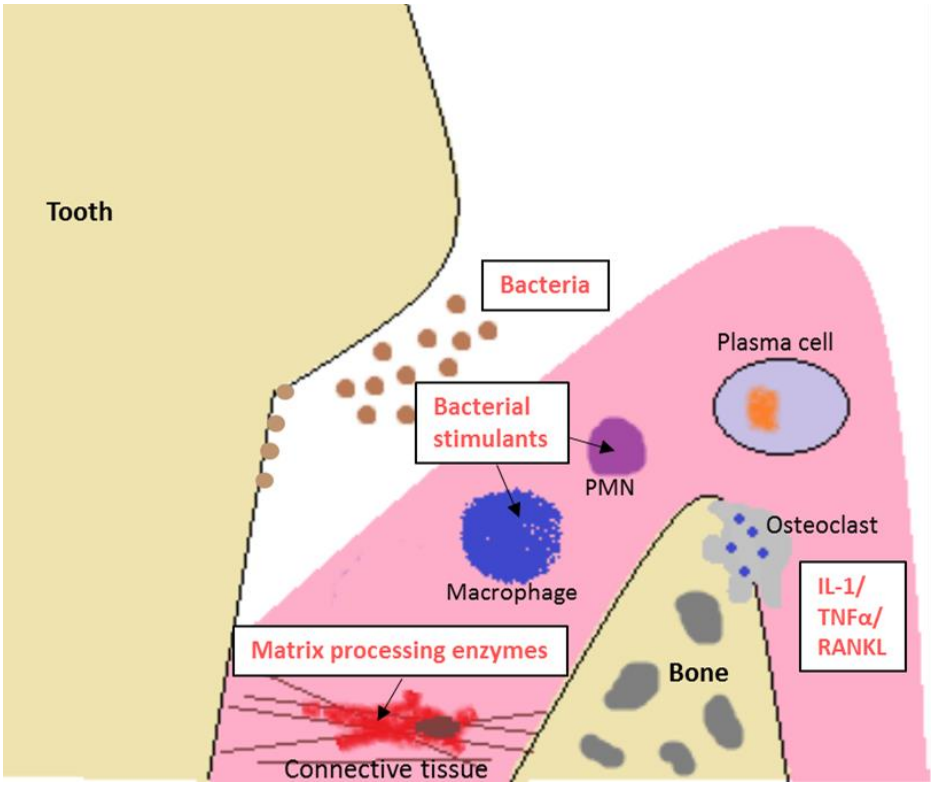


Figure 3: Process of periodontal pathogenesis– stimulants (ex: LPS, nucleases) from Gram-negative bacteria activate macrophages and leukocytes causing the production of cytokines and RANKL. Cytokines activate matrix processing enzymes (ex: collagenases, MMPs) that degrade connective tissue, while RANKL activates osteoclasts to degrade bone structure.

1.3.1 MOLECULAR MECHANISMS OF CYTOKINE PRODUCTION

The “nuclear factor kappa-light-chain-enhancer of activated B cells” (NF-κB) pathway is responsible for the intracellular production of inflammatory factors, survival factors, and growth factors (Hoesel et al., 2013). The canonical NF-κB pathway is activated when LPS binds to toll-like receptor 4 (TLR4). In addition *P.gingivalis* LPS also interacts with TLR2. TLRs are a type of PRR that are located on the surfaces of macrophages and dendritic cells. TLR4 is specifically activated by LPS, while other TLR’s are activated by bacterial lipoproteins and fimbriae (Kumar et al., 2009). TLR4 activation further activates the IκB kinase (IKK) complex, made up of IKKα and IKKβ.

When IKK complex is activated, it phosphorylates IκB (Hoesel et al., 2013). In its unphosphorylated state, IκB is complexed with RelA and p50, two components of NF-κB. Upon its phosphorylation, IκB is sent to the proteasome for degradation, leaving NF-κB free to translocate into the nucleus. NF-κB binds to various regions of DNA that result in the production of survival factors and inflammatory cytokines.

Cytokines are secreted proteins used in intercellular signaling. There are many sources of cytokines, and they can act in a pro-inflammatory or anti-inflammatory manner (Zhang, 2011). As mentioned previously, a major facet of periodontal disease is dysregulated inflammation, which is largely due to an abundance of pro-inflammatory cytokines. Some of the most common pro-inflammatory cytokines are interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) (Liberman et al., 1990). Interleukins are produced by a wide variety of cells, although they were first discovered in conjunction with leukocytes. Primarily released by activated monocytes and macrophages, interleukins aid in immune system regulation and the maturation of various cell types (e.g. B-cells and red blood cells). TNF cytokines are also released by activated macrophages, and functions to recruit other inflammatory cytokines to any wounded or infected site (Zhao et al., 2012). Both TNFα and IL-6 are involved in the acute phase reaction, and their levels are modulated in response to inflammation (Zhang et al., 2007). Within this study, IL-6 and TNFα levels were closely examined in both hMSC and macrophage experiments.

1.3.2 PERIODONTAL TISSUE DESTRUCTION

As seen in Figure 3, components of periodontal pathogens, such as LPS and nucleic acids, activate macrophages and polymorphonuclear leukocytes (PMN), causing

the production of inflammatory cytokines. This overall release leads to degradation of both tissue and bone via matrix processing enzymes (i.e. matrix metalloproteinases (MMPs) and collagenase) and receptor activator of nuclear factor kappa-B ligand (RANKL) mediated osteoclast activation (Parks et al., 2004). MMP's are not normally present at significant levels, but their expression increases in times of pathological distress (Sorsa et al., 2004). In the disease state, MMPs can be produced by a variety of cells, including macrophages, fibroblasts, and neutrophils (Sorsa et al., 2004). During times of oxidative stress, MMPs are activated and breakdown extracellular matrix. MMP-2, MMP-8, and MMP-9 in particular contribute to tissue destruction in periodontitis and peri-implantitis by degrading the connective tissue matrix (Sahingur et al., 2015). RANKL is produced by osteoblasts and activated T cells (Weitzman, 2013), and their production may be increased by pro-inflammatory cytokine signaling to osteoblasts. When uninhibited, RANKL binds to receptor activator of nuclear factor kappa-B (RANK) on the osteoclast surface. This activates osteoclasts and allows them to resorb bone structure (Boyce et al., 2008).

1.4. CURRENT PERIODONTAL THERAPIES

Current periodontal therapies aim to treat periodontal disease by eliminating the oral biofilm through the use of mechanical debridement techniques such as scaling and root planing. In this procedure, calculus is first removed from deep periodontal pockets along the root surface. The surface is then smoothed to remove current endotoxic components and to inhibit adhesion of future bacterial species. Mechanical debridement is sufficient to treat shallow periodontal pockets; however, it is not sufficient to completely remove the calculus in deep pockets. In more drastic cases, surgical

techniques are often needed to decrease periodontal pocket depth (Wang et al., 2001). In addition to mechanical debridement, host modulation therapy and regenerative therapy is key to restoring periodontal health. The modulation of host immune system is done through the administration of antibiotics to eliminate pathogenic bacteria. However, the use of antibiotics is only mildly effective in removing oral biofilms, and various antibiotic types can result in selective or total bacterial elimination. Furthermore, once the entire bacterial population is wiped out, there is a risk of rampant recolonization by keystone pathogens (Cunha-Cruz et al., 2008). Periodontal regeneration therapy has been developed in the last three decades. A wide range of bone graft material and biologics have been used in clinic. However, the outcome of current regenerative therapy is still limited and unpredictable, especially in large, complex defects. Many factors, such as the lack of sufficient stem cells and persistent microbial insults from the oral cavity, are associated with this compromised result. This is more prominent in the presence of systemic diseases like diabetes mellitus or cardiovascular disease (Umeda et al., 2004), when the healing capability is jeopardized with excessive inflammation.

1.5. hMSCs AND TISSUE REGENERATION

As mentioned previously, the overall tissue regenerative outcome of current periodontal therapies is unpredictable (Umeda et al., 2004). The use of hMSCs as therapeutic target is an emerging field, and one that has the potential to improve healing outcomes of patients with chronic wounds. hMSCs are multipotent cells that can differentiate into a variety of mesodermal and endodermal cell types (osteoblasts, chondrocytes, adipocytes). They are heterogeneous, non-hematopoietic, and can be

identified by CD44, CD73, and CD90 surface markers (Ullah et al., 2015). hMSCs have been previously implicated in the wound healing process, therefore many studies have tried to hone these biological mechanisms to treat specific cases. The cells can be easily harvested from a variety of sources, namely bone marrow, adipocytes, and Wharton's jelly of the umbilical cord - all these cell types are multipotent and easily expandable, making them ideal targets for chronic wound sites (Malhotra et al., 2016).

There are three stages of wound repair: inflammation, proliferation, and remodeling. Stem cells move toward the wound site in a chemotactic manner, responding to signaling molecules released during MMP activation (Shin et al., 2013). In the inflammation stage, hMSCs decrease the level of pro-inflammatory cytokines and increase the amount of anti-inflammatory cytokines present at the wound site (Fayaz et al., 2011). Regulated levels of anti-inflammatory cytokines such as IL-10, IL-4, and transforming growth factor beta (TGF-β) are beneficial to the organism and prevent excess scar formation (Maxson et al., 2012). In addition to its anti-inflammatory effects, hMSCs also promote cell proliferation, angiogenesis and extracellular matrix production in the local wound environment (Malhotra et al., 2016; Maxson et al., 2012) through multiple mechanisms including secretion of growth factors.

1.5.1 hMSC-BASED THERAPIES

In vivo results of animal studies have demonstrated the translational potential of hMSC delivery for wound healing and tissue regeneration. Wound closure trials in rabbits were assessed for re-epithelialization, cell migration and tensile strength after wound sites underwent hMSC transplantation for 21 days (Stoff et al. 2009). Histologically, wounds transplanted with hMSCs showed migration of hMSCs toward the

edges of the wound, with very little scarring after the trials were over. At higher time points in the trial, there were significant differences between tensile strength in hMSC transplant and no treatment groups. Although this experiment looked at cutaneous incisions, the increase in tensile strength and increased stem cell migration toward the wound site are facets of hMSCs that are beneficial in an intraoral setting as well. An excisional wound splint model in mice treated with bone marrow hMSCs (BM-hMSC) was completely healed in 28 days, while fibroblast-treated mice had not healed completely (Wu et al., 2007). This study confirmed that BM-hMSCs increased angiogenesis and cell infiltration at the wound site. Excisional wounds in Sprague Dawley rats treated with hMSC and fibrin glue were analyzed based on collagen production, skin hydration, and tensile strength (Mehanna et al., 2015). After histological and immunohistochemistry analysis, it was concluded that the fibrin + hMSC treatment group had a dense arrangement of collagen fibers, normal hydration levels, and significantly higher tensile strength as compared to the control. All these animal models indicated that wounds treated with hMSC had increased collagen production, increased tensile strength, and overall complete healing with minimal scar formation.

It has been mentioned that many periodontal disease patients concurrently suffer from diabetes or cardiovascular disease, two conditions that severely limit the body's ability to efficiently heal wounds. A diabetic rat model induced by streptozocin also had its wound site treated with hMSCs – results showed that wound size and healing time had significantly decreased in the experimental group (Kuo et al., 2011). Regarding the inflammatory and proliferative responses, hMSC-treated rats had decreased cytokine production from leukocytes and increased cell growth at the wound site. Growth factors

had higher levels of expression at the experimental group's wound site than in the no treatment group. Similarly, an excisional splint wound performed on a diabetic mouse model showed that hMSC grafts accelerated wound closure as compared to non-treated diabetic mice (Shin et al., 2013). The amount of angiogenic factors and endogenous cells was also increased in the hMSC graft group, similar to results in the diabetic rat model. The benefits of hMSC treatment for immunocompromised patients were clearly seen through these studies. Many diabetic patients experience tissue ischemia, the lack of blood flow to necessary tissues, causing the site to degenerate further (Falanga, 2005). Although some of these experiments dealt with cutaneous wound repair, the theory behind stem cell mechanisms is similar enough to speculate about stem cell-based gingival regeneration.

In a study to examine the differentiation of hMSCs into dental-specific stem cells, BM-hMSCs were fluorescently tagged to observe migration and differentiation patterns around an alveolar bone defect in chimeric mice. Through immunohistochemistry, GFP+ cells were observed close to the alveolar bone, which was also filled with GFP+ osteoblasts. This shows that BM-hMSCs migrated toward the alveolar bone defect, proliferated, and differentiated into osteoblasts to regenerate bone (Zhou et al., 2011). Another study placed an MSC collagen gel onto a class III furcation defect in dogs for four weeks. MSCs were also tagged with GFP in this experiment and observed using immunohistochemical methods. Transplantation of the gel resulted in significant bone and periodontal ligament growth at the root surface. Within the furcation defect, cementoblasts, fibroblasts, and osteoblasts were GFP-positive. Overall, the MSC-collagen gel transplantation resulted in both soft tissue and alveolar bone regeneration,

while collagen gel without MSCs had significantly less cementum growth and epithelial cell migration (Hasegawa et al., 2006). To test the effectiveness of an MSC “carrier”, a peptide hydrogel was combined with MSCs before being placed in a rat alveolar bone defect. MSCs were tracked *in vivo* by fluorescence in-situ hybridization (FISH) – results at 4 weeks indicated that hMSCs had migrated toward the defect, bone had partially healed, and osteoclast numbers had decreased. This study and others have demonstrated the importance of scaffolds in promoting MSC stability and viability in a hostile environment, be it due to hypoxia or a host immunological response (Tcacencu et al., 2012) and (Rios et al., 2011).

While BM-hMSCs provide a myriad of benefits, complications can arise via the stem cell source. Studies have shown immune rejection of hMSC transplants at wound sites, as well as loss of viability after a certain time point (Baglio et al., 2012). The age of stem cell donor can affect the differentiation capacity of stem cells, as well as the amount of passages that are viable. A study on stem cell capacity in murine donors of different ages demonstrated that younger donors had better adherence to surfaces. In addition, younger stem cells had higher levels of chondrogenic and osteogenic differentiation, although adipogenic differentiation was not as drastically affected (Kretlow et al., 2008) and (Zaim et al., 2012). It is interesting to note that many studies have found the opposite result – donor age has no effect on differentiation capacity; however, there were changes in proliferation and migration for both mouse (Bergman et al., 2009) and human (Mareschi et al., 2006) donors. In any case, it is an issue to be considered before making hMSC transplantation a widespread treatment for periodontal disease. When levels of MSC differentiation were assessed at wound sites, they turned

out to be very low (Shabbir et al., 2015). It was then hypothesized that many beneficial effects of hMSCs may be due to products from their secretome (Yu et al., 2014). The secretome is the spectrum of growth factors, chemokines, and immunomodulatory cytokines secreted by mesenchymal stem cells – the natural production of products is difficult to control but could have major benefits in cell-based therapies (Ranganath et al., 2012). The vast benefits of stem cells are due to the entire cell as well as the products released at the site of injury. Secretome products such as matrix vesicles and exosomes have been seen to possess some properties of their cellular origin (Sahoo et al., 2011).

1.5.2 EXOSOMES AND EXOSOME-BASED THERAPIES

Exosomes are micro-vesicles that are formed through the reverse budding of endosomes within multivesicular bodies (MVB). The resulting protrusions are termed intraluminal vesicles (ILVs). MVB that are formed contain cargo vesicles – the MVB can fuse with lysosomes to degrade internal content or fuse with a plasma membrane and then release the contained ILVs into the extracellular environment .These cargo vesicles are known as exosomes (Hirsova et al., 2016). They are 40-100 nm in diameter on average, and can contain proteins, mRNA, and microRNA (miRNA) (Yu et al., 2014). Exosomes have been identified in cell-cell communication and can have many functions depending on their cargos (Zhang et al., 2015). An important component of exosomes, miRNAs are non-coding RNA of about 22 nucleotides in length, which play important roles in a variety of cell functions such as cell proliferation and differentiation. When a miRNA binds to its complementary sequencing in mRNA,

the degradation of RNA or inhibition of translation can occur – in this manner, exosomes can alter gene expression in recipient cells.

Exosomes may have similar functions as their parent cells, as proven by a comparison between CD34+ cells and CD34+-derived exosomes (Sahoo et al., 2011). Both the cells and separated exosomes increased angiogenesis within mouse endothelial cells, with increased vascularization correlated with higher exosome doses. Hence, the angiogenic effects of hMSCs may be due to paracrine signaling molecules from secreted exosomes at the wound site. Rat wounds injected with hMSC-derived exosome exhibited increased wound closure in comparison to no treatment and hMSC medium groups (Zhang et al., 2015) – the level of healing was gauged based on collagen production, vascularization, and reduced scar width. Scratch assay results showed that fibroblasts and growth factors had migrated to the wound site, similar to the effect of hMSCs in wound sites. Exosomes from adipose-derived hMSC were shown to promote *in vitro* cell migration, fibroblast proliferation, and proliferation in a dose dependent manner (Hu et al., 2016). These exosomes also promoted wound healing within mice by increasing collagen production in the early stages of wound healing. Over time, adipose hMSC-derived exosomes were internalized by fibroblasts (Hu et al., 2016).

MSC-derived exosomes have also been seen to promote bone healing. A fracture model in male mice was treated with a local exosome injection and observed for 8 days. Bone union and fracture healing times were significantly shorter in the treatment group, while angiogenesis and vascularization at the injection site was higher (Furuta et al., 2016). In female rats, exosomal benefits were also evident. The

experimental procedure removed the ovaries of elderly female rats to create an osteoporosis model (OVX). OVX mice were treated with MSC-exosome – proliferation and differentiation into osteoblasts was observed. This resulted in increased bone formation and neovascularization proportional to exosome dosage (Qi et al., 2016). The use of exosomal treatment on an *in vivo* population that was both elderly and female – two at-risk categories for bone defects – demonstrated that exosomes truly have the capability to be used in bone regeneration. Although studies suggest that exosomes may be as effective as whole hMSCs, the mechanism and long-term immunogenicity of *in vivo* treatments is yet to be discovered.

1.6. SUMMARY

Chronic periodontitis is caused by microbial dysbiosis and severe inflammation, leading to tissue and bone degeneration in the oral cavity. Current non-surgical periodontal treatments have a risk of reinfection. Both surgical and non-surgical methods focus on ridding periodontal pockets of harmful bacteria, but have lower success rates in deeper periodontal pockets. Cellular therapy with hMSCs has been shown to speed wound healing through increasing angiogenesis, cell proliferation and matrix production and decreasing scar formation in both healthy and diabetic populations. The limitations of hMSCs are immune-mediated rejection of transplants, and limited long-term viability. Some beneficial effects of hMSCs may be due to paracrine signaling from their secretome products, such as exosomes. Exosomes are nano-vesicles that contain miRNAs, mRNA, and proteins. Wound healing studies with exosomes have shown similar results to cell therapy in terms of scar formation, angiogenesis, and decreased healing time. As these particles may have fewer

complications than whole cells, exosomes may be a novel stem cell based therapy to treat periodontitis and other sources of tissue degeneration.

CHAPTER 2: SPECIFIC AIMS

Excess periodontal inflammation results in degradation of gingival tissue and surrounding bone, which also jeopardizes the outcome of regenerative therapy. Furthermore, wound healing must proceed efficiently by increasing cell proliferation and cell migration to the wound site. The overall objective of this study is to improve the outcome of periodontal therapies by promoting tissue regeneration and reducing periodontal inflammation via exosome treatment. *The main **hypothesis** was that hMSC derived exosomes promote tissue regeneration by enhancing stem cell function and reducing inflammation.*

2.1 Specific Aims

The follow aims were developed to test our main hypothesis:

Specific Aim 1: Determine the characteristics of exosomes derived from hMSCs.

Sub-aim 1: Observe the morphology of exosomes. *The **hypothesis** of this experiment was that hMSC-derived exosomes will exhibit characteristic morphology.* Exosome samples harvested from hMSCs were viewed under a transmission electron microscope (TEM) to observe its morphology and determine the success of extraction.

Sub-aim 2: Determine the presence of exosomal surface markers. *The **hypothesis** of this experiment was that characteristic markers will be present on the*

surface of hMSC-derived exosomes. Western Blot was performed on harvested exosome and cell samples to detect the presence of characteristic exosomal surface markers.

Specific Aim 2: Determine the biological effect of hMSC-derived exosomes on the cellular activity of hMSCs. Since MSCs have been shown to promote tissue healing through enhancing cell migration and proliferation, this study sought to investigate whether hMSC exosomes, part of the MSC secretome, are chemotactic and mitogenic. We also examined whether MSC exosome treatment affects the cytokine production of recipient cells. MSCs in separate culture plates served as recipient cells for us to study the effects of exosomes in stem cells.

Sub-aim 1: Determine the effect of exosome treatment on cell migration in hMSCs. *The **hypothesis** of this experiment was that stem cells migrate towards hMSC exosomes in a dose dependent manner.* Mesenchymal stem cells were seeded in a Boyden chamber and treated with an exosome dose curve for 24 hours. The cells were counted before and after treatment to determine the number of cells that have migrated in that time.

Sub-aim 2: Determine the effect of exosome treatment on cell proliferation in hMSCs. *The **hypothesis** of this experiment was that hMSCs will have increased growth upon treatment with exosomes.* An exosome dose curve was administered to hMSCs seeded in both full medium and serum free medium. DNA quantification assays were run on harvested samples to measure cell proliferation after treatment.

Sub-aim 3: Determine the effect of exosome treatment on cytokine production in hMSCs. *The **hypothesis** of this experiment was that the exosome treatment changes the cytokine production upon recipient cells.* We measured the change in immune response of hMSCs after treatment with exosomes. An exosome dose curve was administered to hMSCs seeded in full medium. The plate was incubated for 24 hours and DNA was harvested. ELISA was performed on the harvested supernatant to detect levels of IL-6 and TNFα.

Specific Aim 3: Determine whether hMSC exosome treatment reduces proinflammatory cytokine production in LPS-activated RAW 264.7 cells. *The **hypothesis** of this experiment was MSC exosome treatment decreases the proinflammatory cytokine secretion in stimulated macrophages.* Exosomes were administered to RAW 264.7 cells activated by *E.coli* LPS. The treated cells were incubated for 6, 12 and 24 hours. Upon harvesting, ELISA was performed to detect levels of IL-6 and TNFα in harvested cells.

Specific Aim 4: Determine the role of exosomal miRNA in proinflammatory cytokine mediation of RAW 264.7 cells. *The **hypothesis** of this experiment was that certain exosomal miRNA mediates the decreased cytokine production in stimulated macrophages.* We transfected miRNA mimics into RAW 264.7 cells and incubated them for 24 hours. After transfection, we stimulated the macrophages with *E.coli* LPS for 6 hours. The harvested cells' proliferation and immunological responses were measured through DNA quantification assays and ELISA. This experiment aimed to narrow down which component of exosomal miRNA causes decreases in proliferation and proinflammatory cytokine production in activated cells. As so many types of miRNA in

the exosome work to silence different (and potentially contrasting) genes, it was important to determine which specific miRNA is responsible for the mediation of periodontal inflammation.

2.1. SIGNIFICANCE OF THE STUDY

The purpose of these studies is to develop a novel stem cell based, cell free therapy to promote tissue regeneration in periodontal disease. Current therapies focus more on eliminating bacterial strains, however, treatments targeting the overly exacerbated inflammation and enhancing regenerative capacity are still an unmet need in clinic. Cellular therapies have the potential to increase the rate of healing and regenerating bone and tissue structures. However, a variety of concerns associated with current MSC treatment, which is based on cell delivery, prevent the application of MSCs in periodontal regeneration. For example, cell delivery often results in a low engraftment rate; potential carcinogenesis is always a concern; and there will be technical and economic difficulties in the storage and transportation of cells, which is less friendly to dental practice. Therapies based on exosomes, an important component of MSC secretome, provide a cell-free solution to overcome these challenges, which has the potential to impact millions of Americans affected by periodontal diseases.

CHAPTER 3: METHODS

3.1. SPECIFIC AIM 1: DETERMINE THE CHARACTERISTICS OF EXOSOMES DERIVED FROM hMSCs

Studies performed in Aim 1 seek to successfully harvest exosomes from hMSCs and ensure that they have the appropriate morphology and surface markers. hMSCs have low immunogenicity when transplanted, potentially resulting in less inflammation when put into an animal model. The effect of hMSC-derived exosome on its "parent" cell will demonstrate its autocrine effects, namely whether exosome treatment can enhance the migratory and proliferative benefits that hMSCs already possess. The hypothesis is that harvested exosomes will be around 100 nm in diameter, and specific surface markers such as CD63 and CD81 will be present on their surface.

3.1.1. AIM 1.1: OBSERVE THE MORPHOLOGY OF EXOSOMES

Harvesting: Commercially available hMSCs derived from bone marrow (RoosterBio) were thawed in T-175 flasks containing growth medium from Roosterbio. Flasks were incubated at 37℃ until confluent, the growth medium was removed, and the cells were washed three times with PBS. DMEM with 10% exosome-depleted FBS (Thermoscientific) and 1% penstrep/fungizone was added for 2 days. The medium was then collected from the flasks, followed by centrifugation at 500g for 10 minutes in 4℃. A second centrifuge was done at 2000g for 10 minutes, and the supernatant was collected in another tube. These rounds of centrifugation removed large cells present in the medium. Collected supernatant was ultracentrifuged at 10,000g and 9,100 rotations

per minute (rpm) for 30 minutes. Once again the supernatant was collected in a new tube. Another round of supernatant ultracentrifugation was done at 100,000g (28,800 rpm) for 70 minutes. After discarding the supernatant and drying the pellet as much as possible, the pellet was mixed with sodium chloride (NaCl). The NaCl solution was ultracentrifuged once more at 100,000g and 28,800 rpm for 70 minutes. The pellet was then collected, dissolved in NaCl, and stored in a -80℃ freezer. All the ultracentrifugation was done in Beckman 50.2Ti rotor. Some exosome samples were fixed for Transmission Electron Microscope.

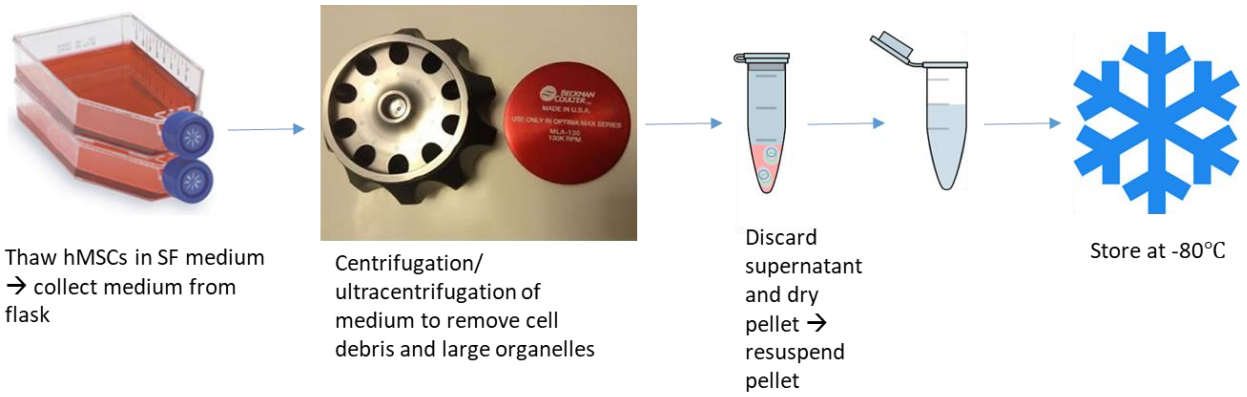


Figure 4: Harvesting hMSC-derived exosomes – hMSCs were incubated in growth medium until confluent. DMEM with 10% exosome depleted FBS and 1% penstrep/fungizone was then added for 2 days. Medium was collected and centrifuged/ultracentrifuged at 500g, 2,000g, 10,000g, and 100,000g to remove debris. An exosomal cell pellet was dried, resuspended in 0.9% NaCl, and stored at -80℃.

3.1.2. AIM 1.2: DETERMINE THE PRESENCE OF EXOSOMAL SURFACE MARKERS

A Western blot was performed to compare exosome and cell lysate protein panels. The protein content of cell and exosome samples was measured through a micro BCA assay. 1 µg of protein was injected in each lane. A gel was run and transferred to a PVDF membrane. This membrane was then incubated with primary

antibody overnight and secondary antibody for one hour. Samples were tested for: CD63, CD81, TSG101, Hsp70, and β-actin.

3.2. SPECIFIC AIM 2: DETERMINE THE BIOLOGICAL EFFECT OF hMSC DERIVED EXOSOMES ON THE CELLULAR ACTIVITY OF hMSCs

Studies in Aim 2 seek to determine whether exosomes can promote the healing capacities of hMSCs. Cell migration to a wound site and cell proliferation at the wound site ensure proper tissue regeneration, while a decrease in proinflammatory cytokines promotes faster healing of tissues. The hypothesis is that hMSC-derived exosomes can regulate cell proliferation, cell migration, and cytokine production in hMSCs.

3.2.1. AIM 2.1: DETERMINE THE EFFECT OF EXOSOME TREATMENT IN CELL MIGRATION OF hMSCs

Stem cells were seeded in the top compartment of a Boyden chamber with serum free medium at a density of 20,000 cells/well. Exosome doses of 0, 0.2, 1, and 5 µg/mL were placed in a lower chamber in a DMEM + 1% APS medium. The cells were incubated for 24 hours and stained with crystal violet before being viewed under a light microscope. Movement of cells from one field of the chamber to another was calculated by cell counting. By placing exosomes at the bottom of the well, a gradient could be created, allowing the measurement of its chemotactic effects.

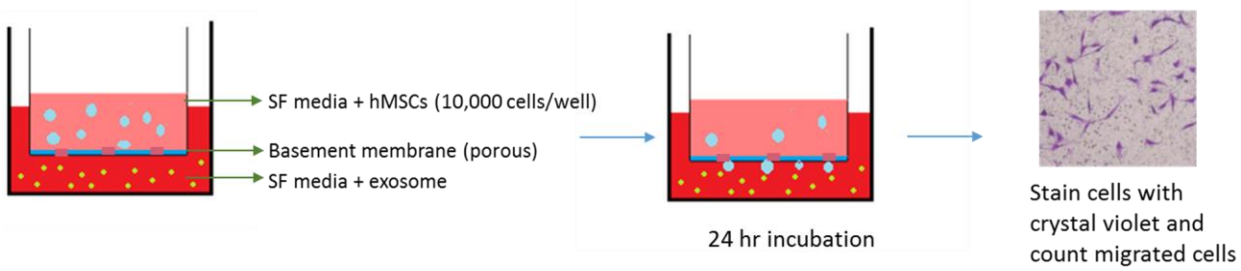


Figure 5: Aim 2.1 Research Design – hMSCs were seeded in the upper compartment of a Boyden chamber in serum-free medium. Exosome doses of 0, 0.2, 1, and 5 µg/mL were placed in the lower compartment of the chamber in serum-free medium. Cells were incubated for 24 hours and stained with crystal violet. The stained cells were counted to determine cell migration.

3.2.2 AIM 2.2: DETERMINE THE EFFECT OF EXOSOME TREATMENT ON CELL PROLIFERATION in hMSCs

Stem cells were seeded in a 48-well plate with full medium (DMEM + 10% HI-FBS + 1% APS) at a density of 10,000 cells/well. Cells were incubated at 37 °C for 24 hours until confluent. At this point, the medium was changed to either full medium or serum-free medium (DMEM + 1% APS), and the cells were treated with 0.02, 0.2, 0.5, 1, and 2 µg/mL of exosome. The exosomes were prepared in 0.9% NaCl or endotoxin-free DPBS. Treated plates were incubated at 37 °C for 72 hours. To harvest, supernatant was collected in tubes and centrifuged (4 ^{0}C, 10 minutes, 16 rcf). Empty wells were washed with DPBS, aspirated, and soaked with 0.5% Triton-X 100 (Fisher Scientific) in -80 °C. Cell proliferation was measured on Triton-X 100 samples using a fluorometric quantitation of double stranded DNA (Quantifluor dsDNA System, Promega).

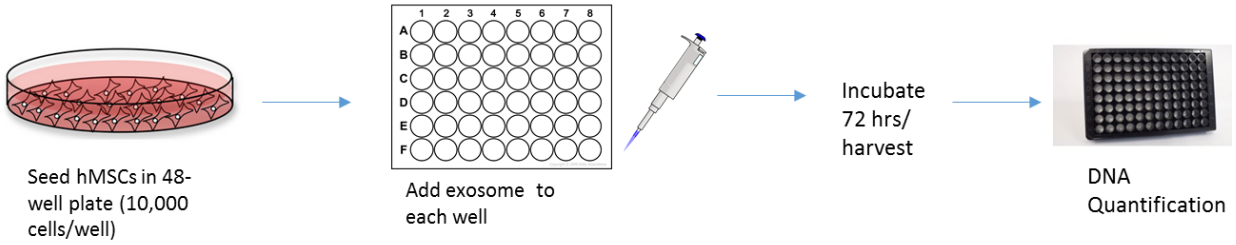


Figure 6: Aim 2.2 Research Design – hMSCs were seeded in full (DMEM + 10% HI-FBS + 1% APS) or serum-free (DMEM + 1% APS) media and treated with 0.02, 0.2, 0.5, 1, and 2 µg/mL of exosome for 72 hours. Cell proliferation was measured using a fluorometric DNA quantification assay.

3.2.3 AIM 2.3: DETERMINE THE EFFECT OF EXOSOME TREATMENT ON CYTOKINE PRODUCTION IN hMSCs

Stem cells were seeded in a 48-well plate with full medium at a density of 10,000 cells/well. Cells were incubated at 37 °C until confluent. After becoming confluent, cells were treated with 0.02, 0.2, 0.5, 1, or 2 µg/mL of exosome. Treated plates were incubated for 24 hours and harvested. IL-6 and TNFα ELISA was performed on the supernatant, and results were normalized to DNA content.

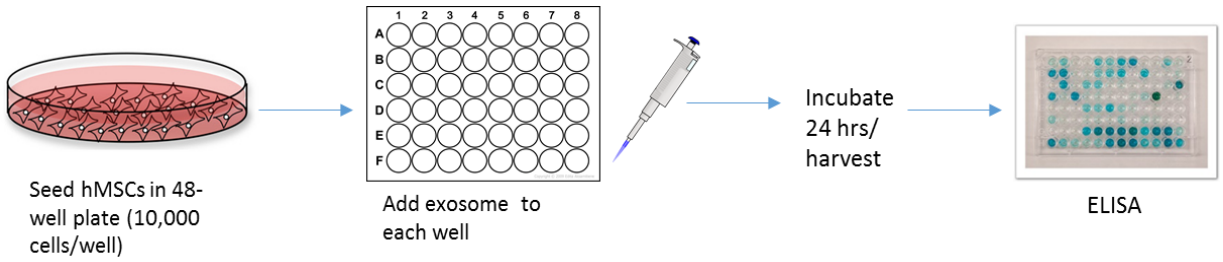


Figure 7: Aim 2.3 Research Design – hMSCs were seeded in DMEM + 10% HI-FBS + 1% APS. Cells were with 0.02, 0.2, 0.5, 1, or 2 µg/mL of exosome for 24 hours. Cytokine production was assessed using ELISA.

3.3. SPECIFIC AIM 3: DETERMINE WHETHER hMSC EXOSOME TREATMENT REDUCES PROINFLAMMATORY CYTOKINE PRODUCTION IN LPS-ACTIVATED RAW 264.7 CELLS

Studies in Aim 3 seek to determine whether exosomes can reduce the production of proinflammatory cytokines in activated macrophages. RAW 264.7 cells are male mouse macrophages from murine leukemia tissue. Macrophages, along with

neutrophils, are largely responsible for the inflammatory response. It will be beneficial to see the response of mouse macrophages to exosome treatment in *in vitro* studies before using an *in vivo* model. In addition to increasing cell proliferation and migration, decreasing inflammation is essential for adequate tissue healing. The hypothesis is that hMSC-derived exosomes can reduce proinflammatory cytokine production in RAW 264.7 cells activated by *E.coli* LPS. *E.coli* LPS is a form of LPS that is highly potent in comparison to other forms of LPS. Furthermore, *E.coli* LPS is easily attainable. It easily activates macrophages and provides a high level of initial cytokine production to work against.

Before activating RAW 264.7 cells with LPS, the sole effect of exosomes on RAW 264.7 cells was measured. Commercially available RAW 264.7 cells were seeded at a density of 25,000 cells/well in a 48-well with DMEM + 10% HI-FBS + 1% PS. Cells were incubated at 37 °C for 24 hours, and then treated with 0.5, 1, or 2 µg/mL of exosome when confluent. Treated plates were incubated for either 6, 12, or 24 hours. To harvest, supernatant was collected in tubes and centrifuged (4 °C, 10 minutes, 16.0 rcf). IL-6 and TNFα ELISA was done using the supernatant, and results were normalized to DNA content.

To look at the effects of exosome treatment on activated macrophages, commercially available RAW 264.7 cells were seeded at a density of 25,000 cells/well in a 48-well with DMEM + 10% HI-FBS + 1% PS. Cells were incubated at 37 °C for 24 hours, and then treated with 10 µg/mL *E.coli* LPS and either 0.5, 1, or 2 µg/mL of exosome when confluent. Treated plates were incubated for either 6, 12, or 24 hours. To harvest, supernatant was collected in tubes and centrifuged (4 °C, 10 minutes, 16.0

rcf). IL-6 and TNFα ELISA was done using the supernatant, and results were normalized to DNA content.

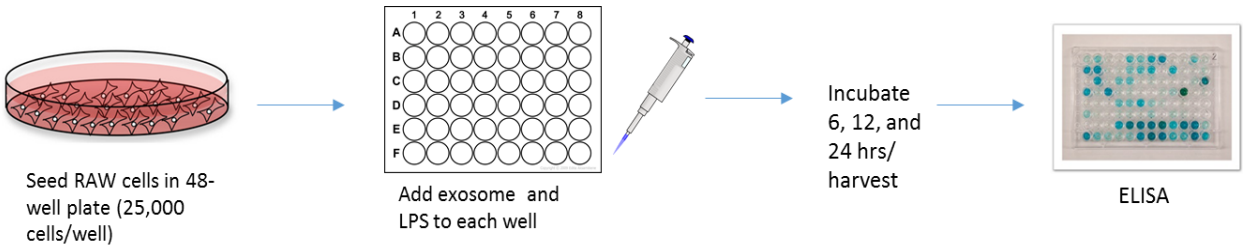


Figure 8: Aim 3 Research Design – RAW 264.7 cells were seeded in DMEM + 10% HI-FBS + 1% PS. Cells were stimulated with 10 µg/mL *E.coli* LPS and treated with 0.5, 1 and 2 µg/mL of exosome for 6, 12 and 24 hours. Cytokine production was assessed using ELISA.

3.4. SPECIFIC AIM 4: DETERMINE THE ROLE OF EXOSOMAL miRNA IN PROINFLAMMATORY CYTOKINE MEDIATION OF RAW 264.7 CELLS

Studies in Aim 4 seek to determine whether exosomal components have an effect on proinflammatory cytokine production. Several studies have shown the benefits of specific exosomal miRNA. By gathering RNA sequencing data from the hMSC-derived exosomes, it is possible to identify specific miRNA that can be administered to activated RAW 264.7 cells. The hypothesis is that some exosomal miRNAs can decrease proinflammatory cytokine production in macrophages activated by LPS.

RNA Sequencing: hMSCs were seeded in a flask with full medium and incubated at 37 °C until confluent. The flask medium was changed to exosome-free media, and the cells were subjected to osteogenic induction. Total RNA was extracted using Trizol (Invitrogen), and RNA samples were sent for sequencing.

Transfection efficiency: RAW 264.7 cells were seeded in a 48-well plate at 50,000 cells/well with DMEM + 10% HI-FBS + 1% PS. The plate was incubated at 37 °C

until confluent. To optimize transfection conditions for RAW 264.7 cells, variable amounts of Lipofectamine 2000 (Thermo-Fisher Scientific) and BLOCK-iT Fluorescent Oligo (Thermo-Fisher Scientific) were transfected in different wells. There were two trials done based on variation in Lipofectamine and Block-iT doses. The groups of Trial 1 were: 0.5 µL Lipo + 0.375 µL Block-iT, 0.5 µL Lipo + 0.75 µL Block-iT, 0.5 µL Lipo + 1.5 µL Block-iT, 1 µL Lipo + 0.375 µL Block-iT, 1 µL Lipo + 0.75 µL Block-iT, and 1 µL Lipo + 1.5 µL Block-iT. Based on Trial 1 results, a new set of doses were chosen for Trial 2. The groups of Trial 2 were: 0.25 µL Lipo + 0.375 µL Block-iT, 0.5 µL Lipo + 0.375 µL Block-iT, 1 µL Lipo + 0.375 µL Block-iT, and 1.5 µL Lipo + 0.375 µL Block-iT. At 24 hours and 48 hours, each plate was observed under a fluorescent microscope in bright field and FITC modes. By visualizing the amount of fluorescent cells in a given well, it was determined that transfecting 1µL Lipofectamine and 0.375µL miRNA per well for 24 hours was the optimum condition.

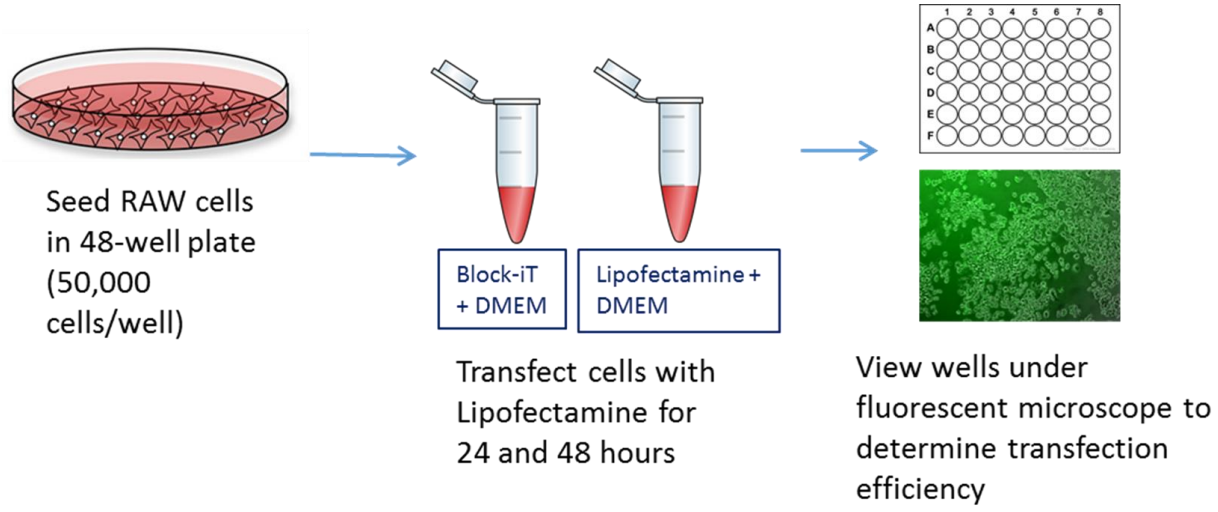


Figure 9: Aim 4 Research Design (Transfection Efficiency) – RAW 264.7 cells were seeded in DMEM + 10% HI-FBS + 1% PS. Cells were transfected with variable doses of Block-iT and Lipofectamine 2000 for 24 and 48 hours. Wells were viewed under a fluorescent microscope to visualize transfection efficiency.

Transfection: RAW 264.7 cell were seeded in a 48-well plate at 50,000 cells/well with DMEM + 10% HI-FBS + 1% PS. The plate was incubated at 37 °C until confluent. Transfection was performed using 1 µL Lipofectamine 2000 and 0.375 µL miRNA mimics per well. These mimics are artificially constructed and contain RNA fragments on the 5' end that are complementary to a target sequence in the 3' untranslated region. Referring to the exosome RNA sequencing results, miRNA mimics 122, 146, 155, 451, 486, 760, 4792, 6087, and 7641 were selected to be transfected for 24 hours. NC-1 was used as a negative control. Transfected cells were stimulated with 10 µg/mL *E.coli* LPS for 6 hours and supernatant was harvested for ELISA (IL-6 and TNFα).

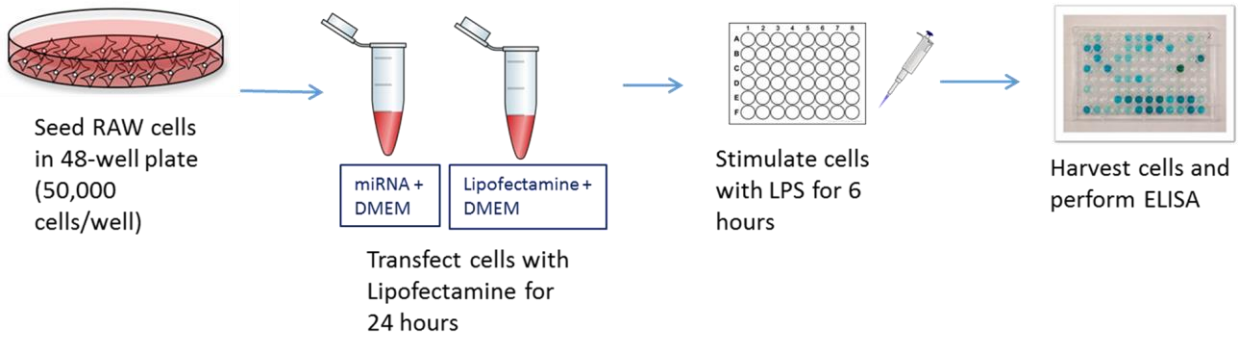


Figure 10: Aim 4 Research Design (Transfection) – RAW 264.7 cells were seeded in DMEM + 10% HI-FBS + 1% PS. Cells were transfected with 1 µL Lipofectamine 2000 and 0.375 µL miRNA per well for 24 hours. Cells were later stimulated with 10 µg/mL *E.coli* LPS for 6 hours. Cytokine production was assessed in harvested cells by performing ELISA on supernatant.

3.5. STATISTICAL ANALYSIS

ELISA and DNA quantification results were calculated in an Excel spreadsheet. The results were input into GraphPad Prism 7 software to create bar graphs and histograms. Statistical analysis was done in this software using a parametric one way ANOVA ($p<0.05$) and Turkey's multiple comparisons test. P-values were indicated using asterisks as follows: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

CHAPTER 4: RESULTS

4.1. SPECIFIC AIM 1: DETERMINE THE CHARACTERISTICS OF EXOSOMES DERIVED FROM hMSCs

After harvesting exosomes from hMSC medium, different methods have been used to validate the presence of exosomes in our samples.

4.1.1. AIM 1.1: OBSERVE THE MORPHOLOGY OF EXOSOMES

A TEM image of harvested exosome samples (Fig. 11) showed that the exosomes were heterogeneous in size, ranging from 20 to 140 nm. Exosome sizes were noted and arranged in a histogram (Fig. 12). After analyzing the descriptive statistics, it was seen that the average exosome size was 60 nm, which is in line with the current literature (Yu et al., 2014). The vesicles shown here exhibit a lipid outer membrane, indicating that the harvested samples have a characteristic exosome morphology.

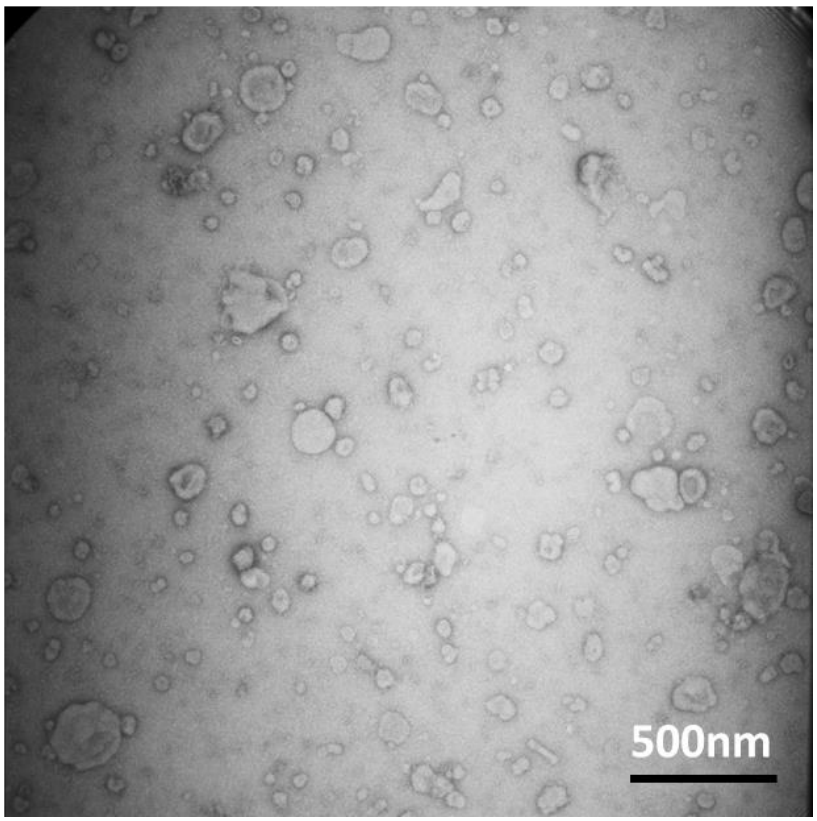


Figure 11: Transmission electron microscope image of harvested exosomes – exosome samples were fixed and viewed under a transmission electron microscope. The vesicles are encompassed by a lipid membrane and heterogeneously shaped.

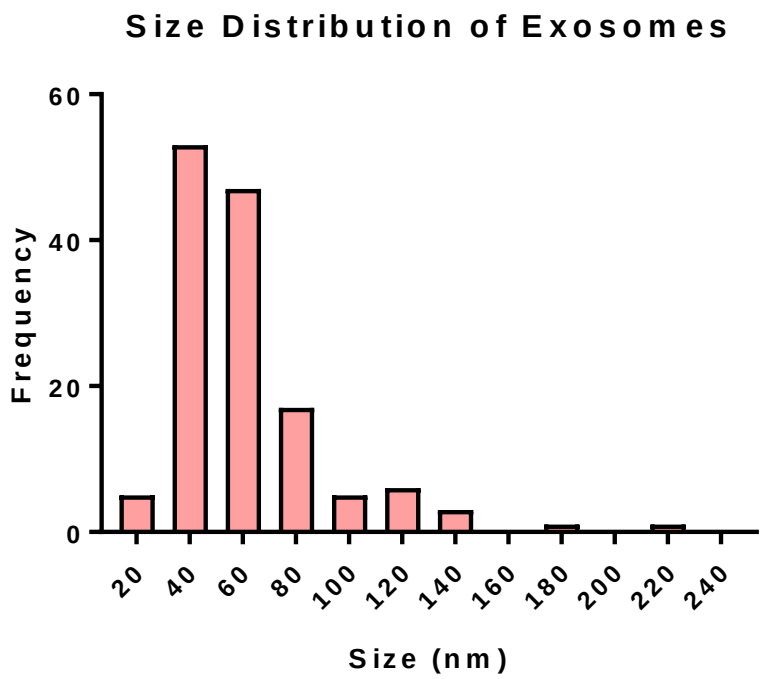


Figure 12: Histogram representation of exosome diameter – exosome sizes from Fig. 11 were counted and arranged into a histogram. They range from 20-140 nm on average.

4.1.2. AIM 1.2: DETERMINE THE PRESENCE OF EXOSOMAL SURFACE MARKERS

Exosome and cell lysate samples underwent gel electrophoresis at a concentration of 1 µg protein per lane. The samples were analyzed for CD63, CD81, tumor susceptibility gene 101 (TSG101), heat shock protein 70 (Hsp70), and β-actin. CD63 and CD81 are the main surface markers of exosomes. Western blot results (Fig.13) showed that CD81 and CD63 were dramatically enriched in the exosome, while very weakly showing up in the cell lysate. TSG101, an exosome specific protein, also demonstrated a higher signal in hMSC exosomes compared to that in cell lysate. HSP70 and β-actin were primarily seen in total cell lysate, which is consistent to previous findings. These protein panel results further indicate that exosomes were successfully harvested.

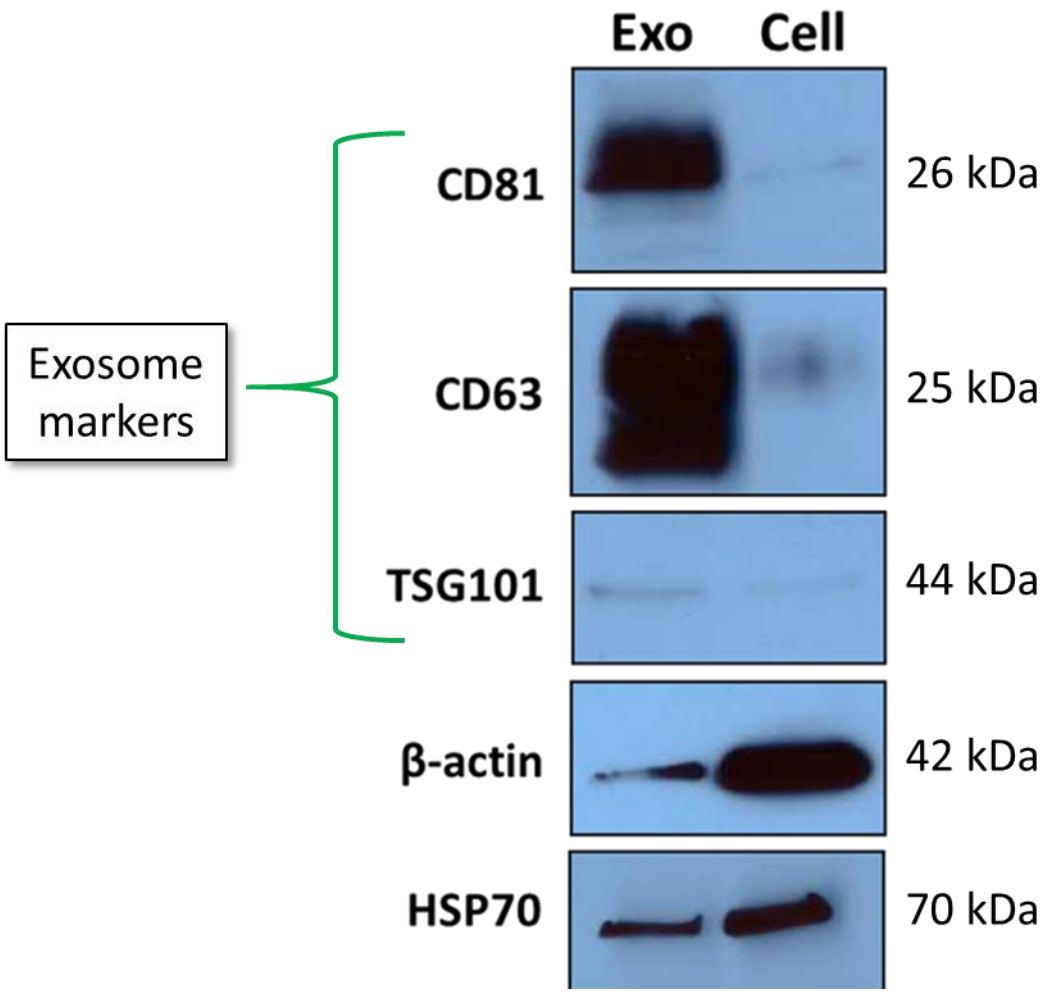


Figure 13: Western Blot panel of exosomal surface markers – 1 µg of protein was loaded onto a gel and incubated with exosome markers (CD81, CD 63, TSG101) and total cell markers (B-actin, HSP70). The strong signal of exosomal markers compared to total cell lysate indicates that exosome was successfully harvested from hMSCs.

4.2. SPECIFIC AIM 2: DETERMINE THE BIOLOGICAL EFFECT OF hMSC DERIVED EXOSOMES ON THE CELLULAR ACTIVITY OF hMSCs

Many studies have shown the benefits of hMSCs in tissue regeneration, as they increase cell proliferation and bring new cells to the area. Using hMSCs as a model, we investigated the effects of exosomes on stem cell behaviors. Specifically, we were interested in whether these exosomes are chemotactic and mitogenic. We also study

the immunogenicity of MSCs exosomes by evaluating if exosomes treatment will alter the cytokine (IL-6 and TNFα) production.

4.2.1: AIM 2.1: DETERMINE THE EFFECT OF EXOSOME TREATMENT IN CELL MIGRATION OF hMSCs

Crystal violet-stained hMSCs were counted in the Boyden chamber to determine the amount of cell migration that had occurred over 72 hours when in the vicinity of exosome. Stem cells exhibited a chemotactic effect toward hMSC-derived exosomes in a dose dependent manner. As seen in Figure 14, the cell count significantly increases from panel A to panel D, concurrent with an increase in exosome dosage (0.2 µg/mL exo: $p<0.05$, 1 µg/mL exo: $p<0.05$, 5 µg/mL exo: $p<0.01$) .

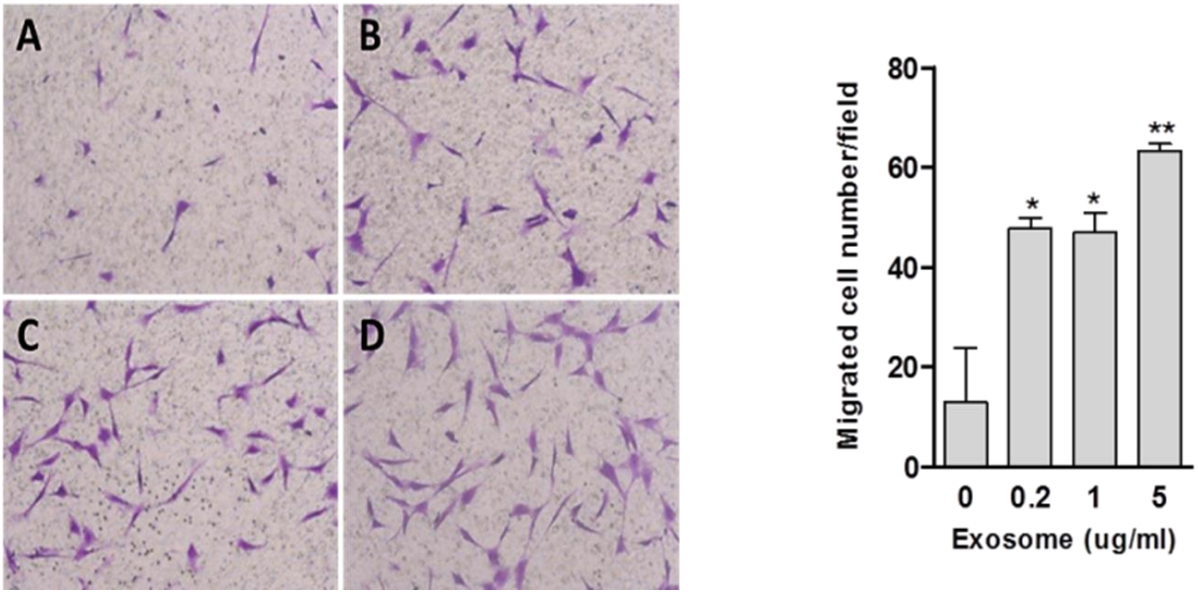


Figure 14: Effect of hMSC derived exosome on cell migration of hMSCs seeded in a Boyden chamber – hMSCs were seated at 10,000 cells/well in the top well of a Boyden chamber with serum-free medium. In the bottom of the chamber, 0, 0.2, 1, and 5 µg/mL of exosome and serum-free medium were placed. The setup was incubated for 72 hours and stained with crystal violet. Panel A is concurrent with 0 µg/mL exosome, panel B with 0.2 µg/mL exosome, etc. There is a significant increase in cell count from panel A to D, indicating that migration of hMSCs increases in a dose dependent manner.

4.2.2. AIM 2.2: DETERMINE THE EFFECT OF EXOSOME TREATMENT ON CELL PROLIFERATION in hMSCs

hMSCs in serum-free and full medium were assessed for changes in cell proliferation after exosome treatment using a fluorometric quantitation of dsDNA. In serum free condition, DNA quantification results (Fig.14) for this plate showed a significant dose-dependent (1 µg/mL exo: $p<0.001$, 2 µg/mL exo: $p<0.05$) increase in proliferation. For the full medium plate, once again, DNA quantification results showed that exosomes increased cell proliferation in a dose dependent manner, and the increase was significant (.02 µg/mL exo: $p<0.0001$, 0.2 µg/mL exo: $p<0.0001$, 0.5 µg/mL exo: $p<0.0001$, 1 µg/mL exo: $p<0.0001$) in comparison to the DPBS group. It is interesting to note that the proliferative effect seemed to decrease at a high dose (2 µg/ml).

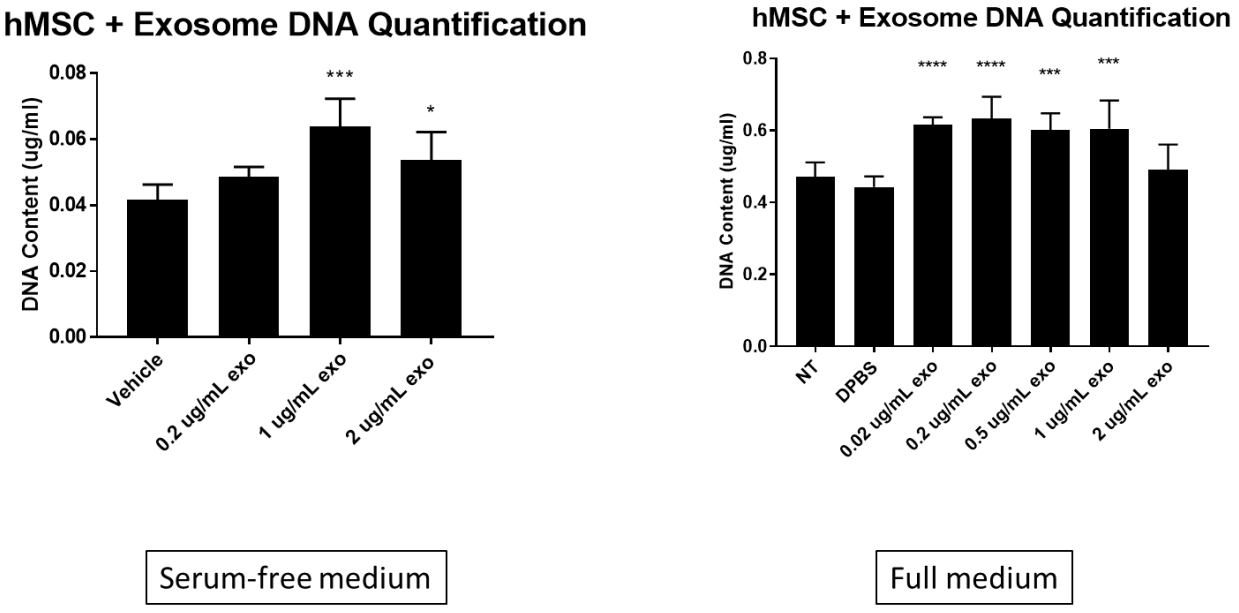


Figure 15: Effect of hMSC derived exosome on hMSC proliferation – hMSCs were seated at 10,000 cells/well in a 48-well plate (one with serum-free medium/one with full medium). The wells were treated with variable doses of exosome for 24 hours. DNA quantification results show that an increase in proliferation occurs in both full medium and serum-free medium conditions, and decreases when exosome dosage is too high. 0.9% NaCl was the vehicle in serum free experiment and is used as the control, while DPBS is the control in full medium experiment.

The increase in hMSC proliferation after treatment with exosome indicates that administration of exosome at a wound site could aid in the healing process.

4.2.3. AIM 2.3: DETERMINE THE EFFECT OF EXOSOME TREATMENT ON CYTOKINE PRODUCTION IN hMSCs

hMSCs treated with an exosome dose curve showed scattered IL-6 and TNFα ELISA results. Statistical analysis was performed in comparison to the DPBS group, as exosome doses were diluted with DPBS. Results are normalized to DNA content (Fig.16). At low dose, exosome treatment did not significantly stimulate IL-6 and TNFα production. At higher dose (2 μg/ml), mild increase of IL-6 but not TNFα was seen after exosome treatment. It is important to know that, the effect of MSC exosome itself on IL-6 production is at a much lower level compared to that induced by LPS. This is consistent to reports from other groups that IL-6 increased at after MSC exosome, but the level was much lower than other anti-inflammatory cytokines such as IL-10 (Zhang et al., 2013).

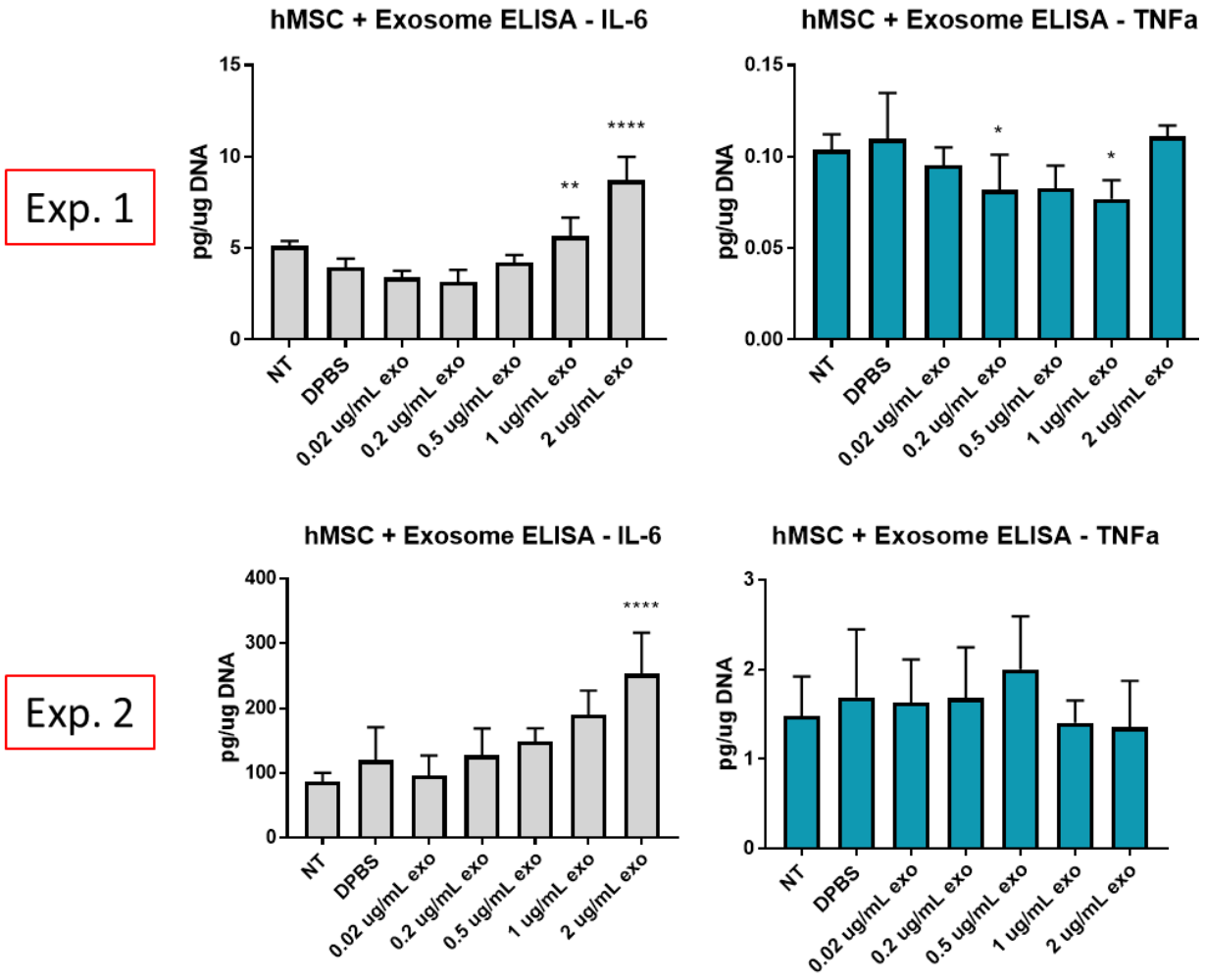


Figure 16: Effect of hMSC derived exosome on cytokine production in hMSCs – hMSCs were seated at 10,000 cells/well in a 48-well plate with full medium. Wells were treated with 0.02, 0.2, 0.5, 1 and 2 µg/mL of exosome and incubated for 24 hours. DPBS was used as a control group. Cells were harvested and supernatant was used to perform ELISA. IL-6 showed mild activation at higher exosome doses. TNFα did not exhibit activation at the exosome doses used.

4.3. SPECIFIC AIM 3: DETERMINE WHETHER hMSC EXOSOME TREATMENT REDUCES PROINFLAMMATORY CYTOKINE PRODUCTION IN LPS-ACTIVATED RAW 264.7 CELLS

Before stimulating the macrophages, the effect of exosome alone on RAW 264.7 cells was measured. RAW 264.7 cells were seated in a 48-well plate at 25,000 cells/well. Wells were treated with variable exosome doses and incubated for 24 hours. ELISA was performed using cell supernatant. At higher doses of exosome treatment, cytokine

production significantly increased in both IL-6 and TNFα compared to DPBS (Fig.17). This increased in cytokine production showed a dose-dependent effect. Results in the "cytokine/DNA" column were normalized to DNA (Fig. 18).

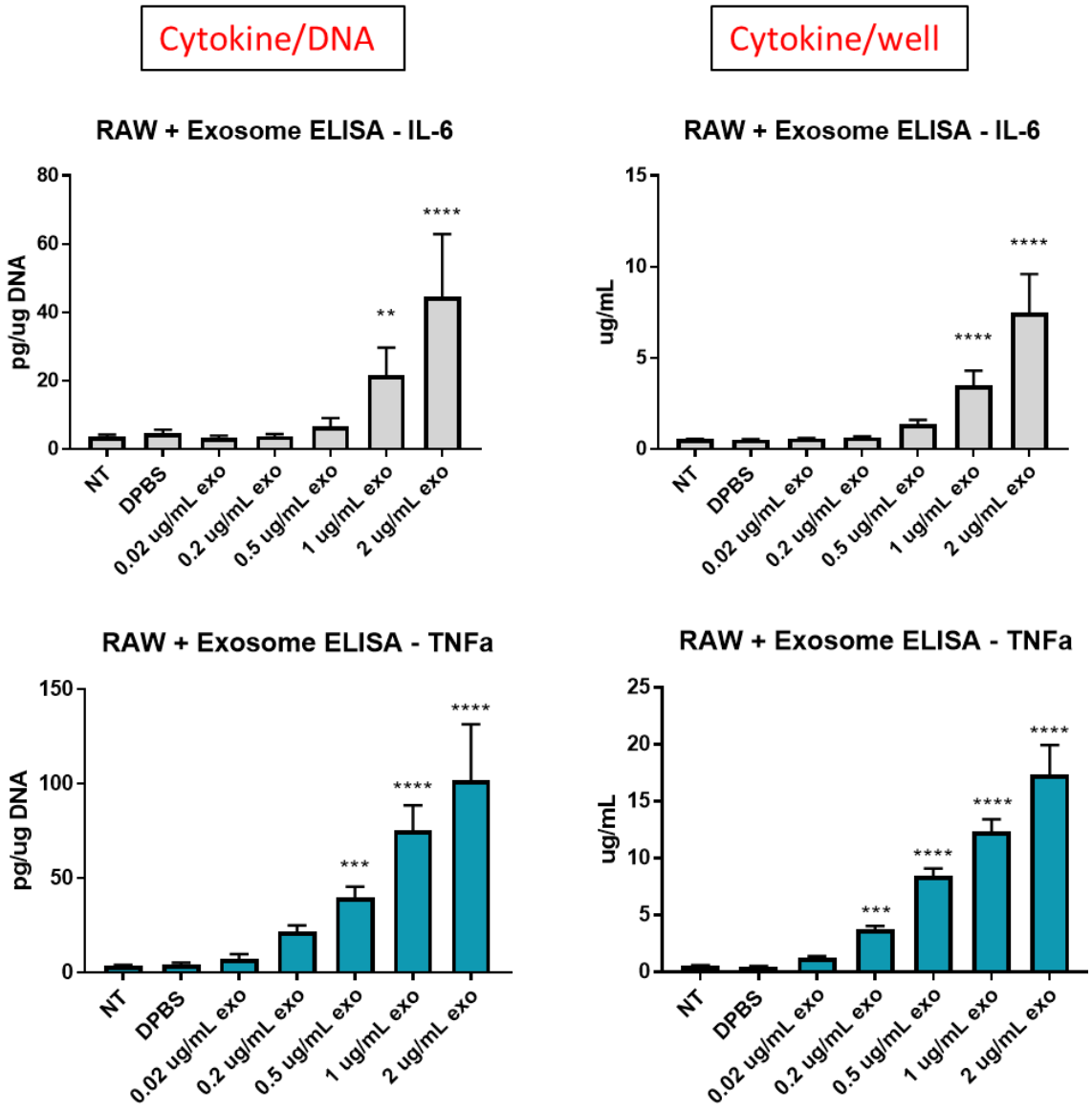


Figure 17: Effect of hMSC derived exosome on cytokine production in RAW 264.7 cells – RAW 264.7 cells were seated at 25,000 cells/well in a 48-well plate. Wells were treated with 0.02, 0.2, 0.5, 1, and 2 µg/mL of exosome and incubated for 24 hours. DPBS was used as a control. ELISA was performed using cell supernatant. Both IL-6 and TNFa showed dose-dependent activation at higher doses of exosome.

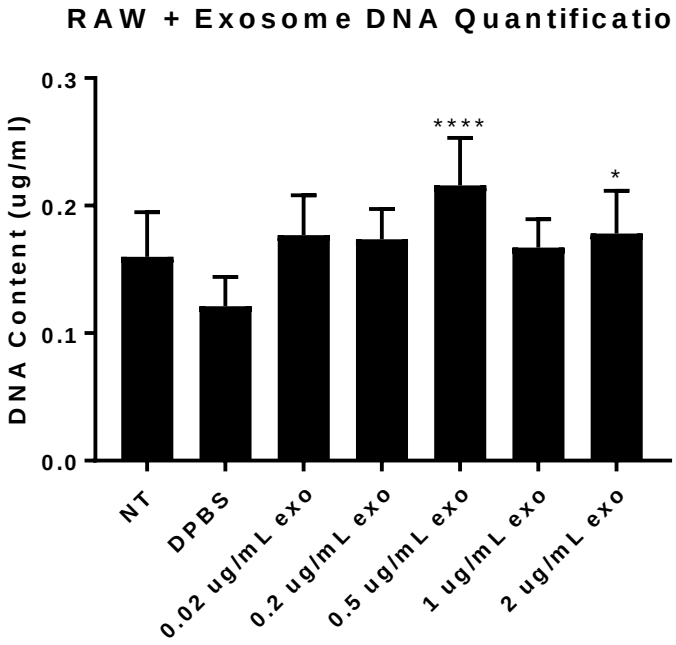


Figure 18: DNA content of exosome treatment on RAW 264.7 cells – RAW 264.7 cells were seated at 25,000 cells/well in a 48-well plate. Wells were treated with 0.02, 0.2, 0.5, 1, and 2 ug/mL of exosome and incubated for 24 hours. DPBS was used as a control group. A fluorometric DNA quantification assay was performed on harvested cells. There was no constant trend in DNA content, although some treatment groups were significantly different from the control.

We further investigated whether hMSC exosome treatment will inhibit the inflammatory responses triggered by LPS in macrophage. *E.coli* LPS was used to stimulate RAW264.7 cells and MSC exosomes were added in some wells. LPSE (*E.coli* LPS stimulation without exosome treatment) was used as the control group, and samples were analyzed in comparison to this. ELISA results were present in normalized and non-normalized forms – non-normalized results indicate the amount of cytokine produced per well, while results normalized to DNA indicate the amount of cytokine produced per cell. The non-normalized results (Fig. 19) show that exosome treatment is not effective until at least 12 hours – at 6 hours, there is no significant difference between the LPSE and exosome dose groups, indicating that the exosomal mechanism

has not had time to work yet. At 12 and 24 hours, there is a significant difference between the various exosome groups, with cytokine production decreasing in a dose-dependent manner. This is seen most drastically in the IL-6 expression of the 24 hour plate (2 µg/mL exo: $p<0.05$). While IL-6 shows a very dramatic decrease in cytokine production, the same effect is not seen in TNFα even at higher doses. This may mean that exosomal contents are mainly targeting producers of IL-6. When ELISA results normalized to DNA content (Fig. 20) are taken into account, the potency of *E.coli* LPS is evident. Due to the potent nature of LPS, incubating it with the cells for such a long period of time may be cytotoxic. Indeed, we observed that the DNA content of RAW264.7 cells almost doubled from 6 hour to 24 hour. LPS significantly suppressed the cell proliferation (Fig. 20). The 12 hour time point seemed to be to evaluate the effect of exosomes since it provided a balance between lessened cell death and effective exosome treatment. In IL-6, the 12 hour plate (0.5 µg/mL exo: $p<0.05$, 1 µg/mL exo: $p<0.01$, 2 µg/mL exo: $p<0.001$) and 24 hour plate (0.5 µg/mL exo: $p<0.05$, 1 µg/mL exo: $p<0.05$, 2 µg/mL exo: $p<0.01$) show significant decreases according to treatment dose. Normalized to DNA, the results still indicate that a higher time point is necessary for exosome treatment to work. It is worth noting that we didn't see a large decrease in DNA content from 6 hour to 24 hour in all the LPS treatment groups. Therefore the decrease of IL-6 in exosome treatment groups (compared to LPSE group) was unlikely the result of excessive cell death. Furthermore, the effects on IL-6 and TNFα remain constant in both normalized and non-normalized results. TNFα shows some changes amongst exosome-treated groups, but the results are not significant enough to draw any

conclusion. Together, these results indicate that exosome treatment appeared to suppress IL-6 production in macrophages.

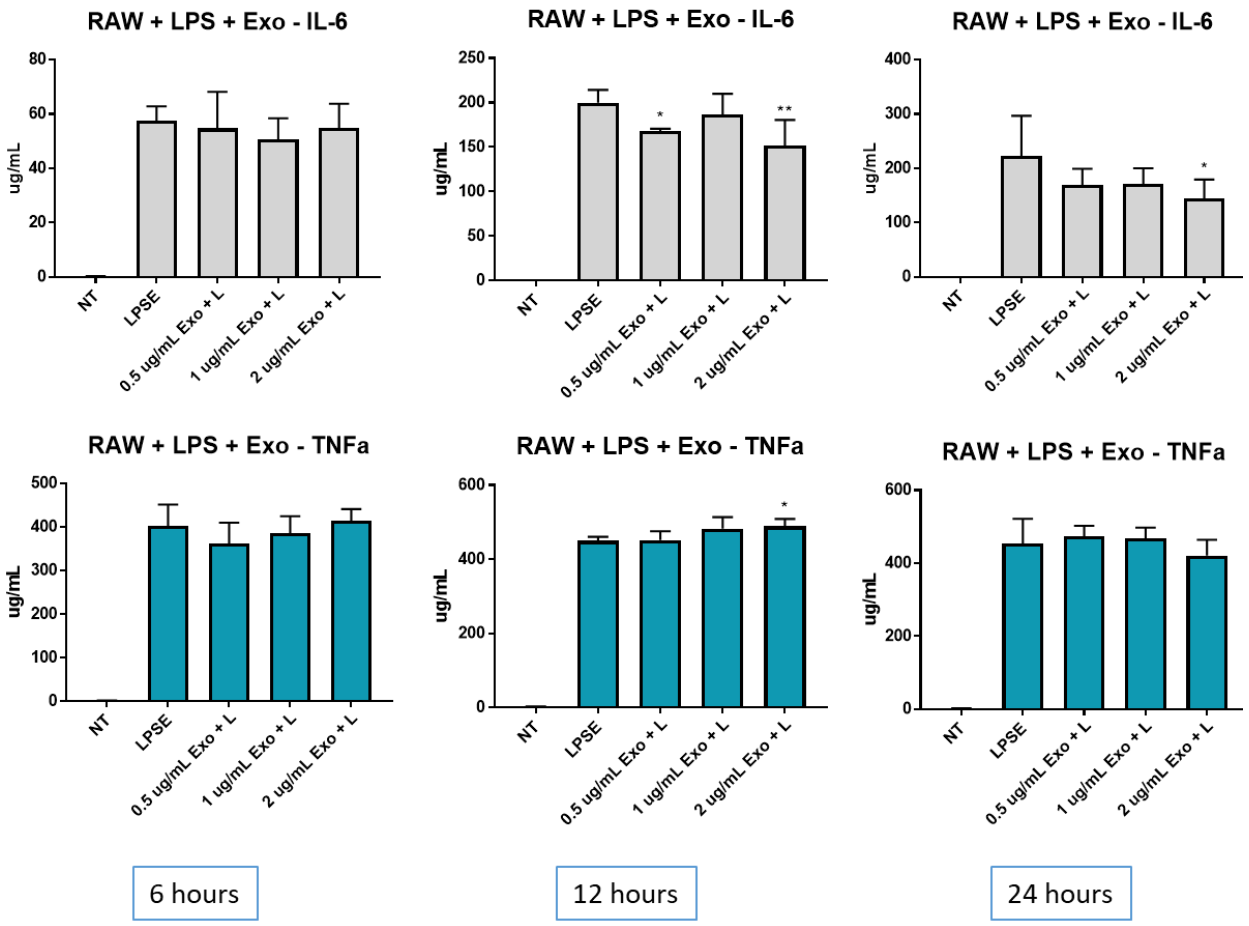


Figure 19: Non-normalized IL-6 and TNFα ELISA results – RAW 264.7 cells were seated at 25,000 cells/well in a 48-well plate. Some wells were treated with 0.5, 1, and 2 µg/mL of exosome and 10 µg/mL *E.coli* LPS (LPSE). The plate was incubated for 6, 12, and 24 hours. LPSE was used as a control. ELISA was performed using cell supernatant. The decrease in cytokine production after administration of exosome was dose-dependent and time-dependent. IL-6 production experienced a more significant decrease than TNFα.

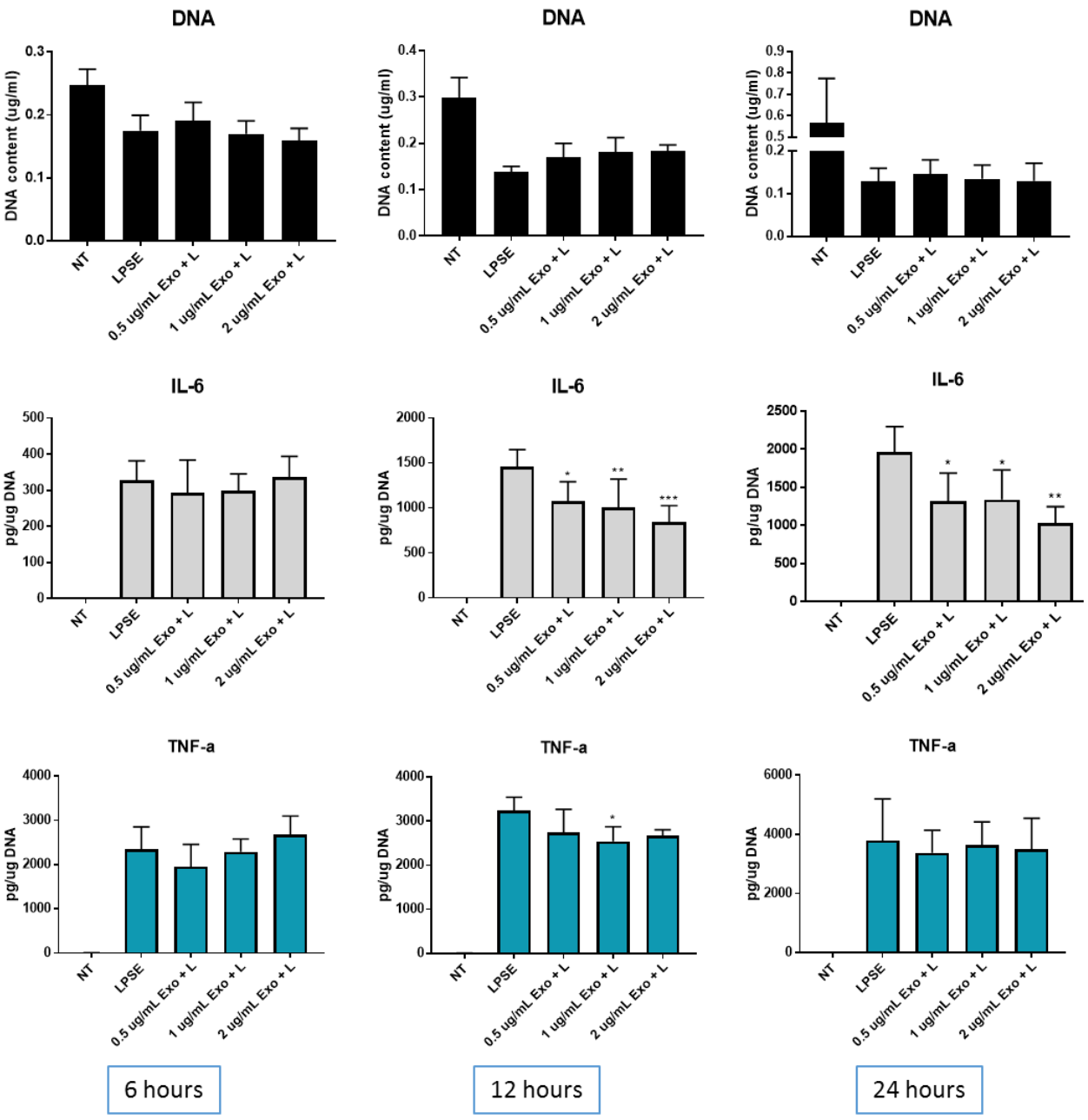


Figure 20: Normalized IL-6 and TNFα ELISA results – RAW 264.7 cells were seated at 25,000 cells/well in a 48-well plate. Some wells were treated with 0.5, 1, and 2 µg/mL of exosome and 10 µg/mL *E.coli* LPS (LPSE). The plate was incubated for 6, 12 and 24 hours. LPSE was used as a control. ELISA was performed using cell supernatant, and DNA quantification was performed on harvested cells. After normalization, IL-6 production still decreases in a dose-dependent and time dependent manner.

4.4. SPECIFIC AIM 4: DETERMINE THE ROLE OF EXOSOMAL miRNA IN PROINFLAMMATORY CYTOKINE MEDIATION OF RAW 264.7 CELLS

Since exosomes can execute their function by transferring RNA, our lab examined whether RNA existed in our exosomes. We observed a distinct RNA expression pattern in MSC-exosomes, in which small RNAs (<200nt) are highly enriched, compared to cell RNA (Figure 21A). Next generation RNA sequencing was also performed on these RNA samples. A group of miRNAs were highly enriched in MSC exosomes, including miRNA 451a, 486-5p, 122-5p, 7641, 4792, 6087, and 760 were found to have the highest relative expression levels. Therefore, it is reasonable to ask whether these miRNAs are involved in the function of exosomes. We synthesized the miRNA mimics and transfected them to RAW264.7 cell. The effect of miRNA mimics on inflammatory cytokine production was evaluated.

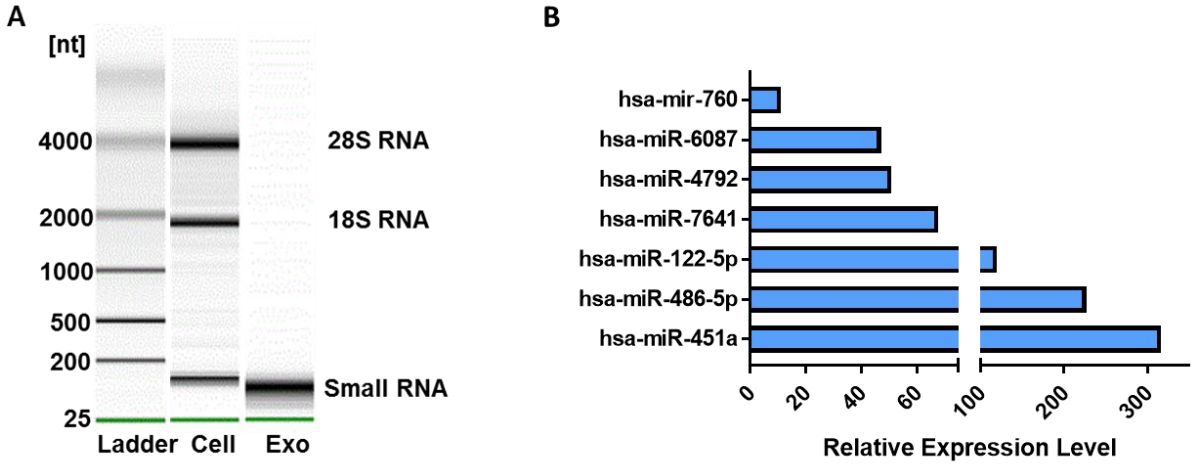
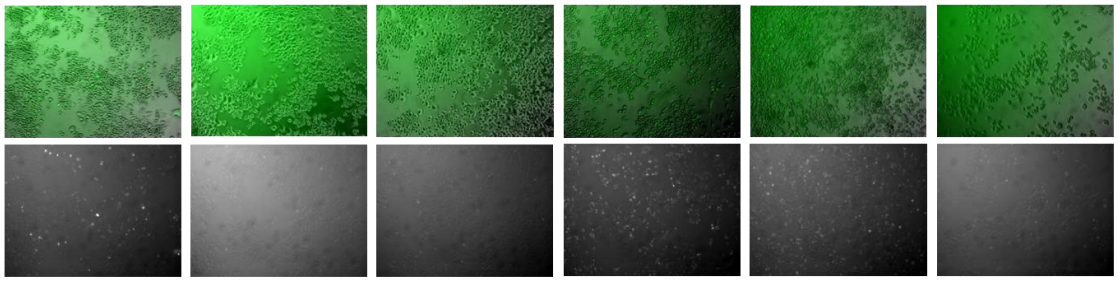


Figure 21: RNA sequencing results of exosome samples (A) and a bar graph of top 7 miRNA's with the highest expression levels (B) are shown here.

To generate a high-efficiency transfection protocol for RAW 264.7 cells, Block-iT fluorescent oligo (Thermo Fisher Scientific) and Lipofectamine 2000 (Thermo Fisher Scientific) were transfected into cells for 24 and 48 hours. To begin the transfection

efficiency trials, doses were chosen according to previous studies done on other cell types. The plates were viewed under a fluorescent microscope in bright-field and FITC, and images were merged together to create a fluorescent image of the well. Bright spots on the black and white image indicate cells that have had a successful transfection. Trial 1 (Fig. 22A and B) tested variations in both Lipofectamine and Block-iT dosage. The highest level of transfection was seen The following groups exhibited a higher transfection efficiency at 24 hours and were closely monitored at 48 hours: 0.5 µL Lipofectamine + 0.375 µL Block-iT and 1 µL Lipofectamine + 0.375 µL Block-iT. It is evident from the 24 hour results that the amount of Lipofectamine affected transfection efficiency more than the level of miRNA. A miRNA dose of 0.375 µL per well produced a good transfection result, while higher levels drastically decreased the transfection efficiency. Using these results, Trial 2 (Fig. 22C and D) was conducted. These groups had variation in Lipofectamine dosage, while Block-iT dosage stayed the same (0.375 µL/well). At 24 hours, transfection efficiency was high within the 1 µL and 1.5 µL Lipofectamine groups. At 48 hours, the results were still strong, but the fluorescent image shows a lot of cell death over a prolonged period of time. While both higher doses of Lipofectamine caused an increase in transfection efficiency, 1.5 µL Lipofectamine was considered too potent for the cells. Taking time point, cell toxicity, and transfection efficiency into account, it was decided that each well should be transfected with 1 µL Lipofectamine + 0.375 µL miRNA for 24 hours. From Aim 3 results, LPS was seen to be toxic in the cells for 24 hours. Therefore, 10 µg/mL *E.coli* LPS was added to the cells for 6 hours after the transfection was complete.

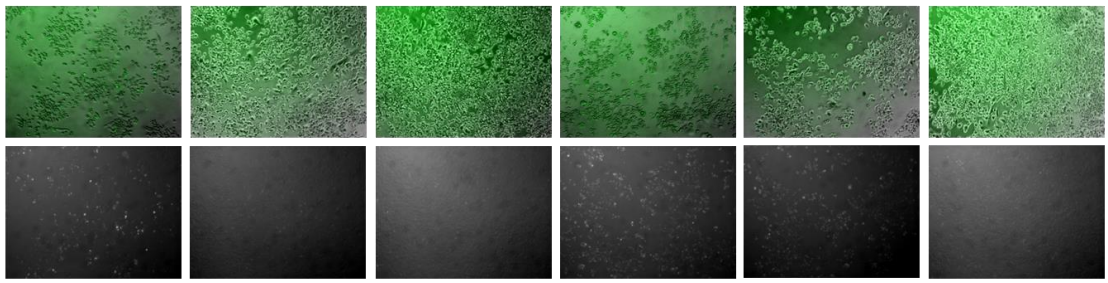
A)



0.5 µL 1 µL **Lipo**

0.375 µL 0.75 µL 1.5 µL 0.375 µL 0.75 µL 1.5 µL **Block-iT**

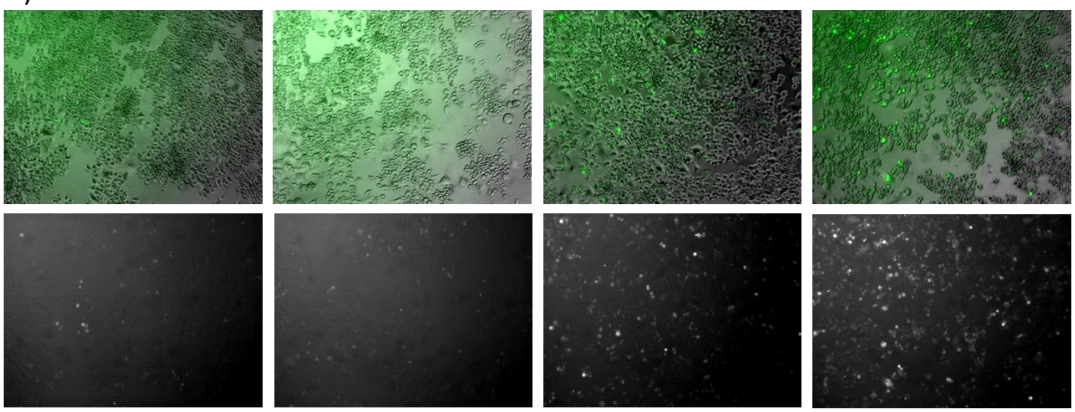
B)



0.5 µL 1 µL **Lipo**

0.375 µL 0.75 µL 1.5 µL 0.375 µL 0.75 µL 1.5 µL **Block-iT**

C)



0.25 µL 0.5 µL 1 µL 1.5 µL **Lipo**

0.375 µL **Block-iT**

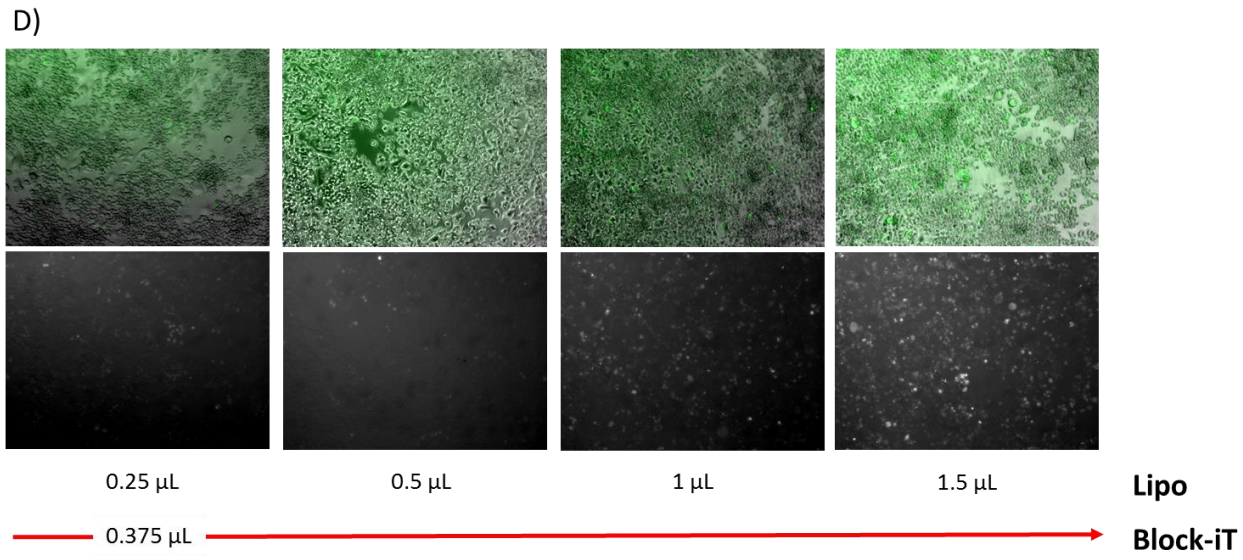


Figure 22: Fluorescent microscope images of transfection efficiency at Trial 1: 24 hours (A) and 48 hours (B) and Trial 2: 24 hours (C) and 48 hours (D) – RAW 264.7 cells were seated at 50,000 cells/well in a 48-well plate. Different doses of Block-iT and Lipofectamine 2000 were transfected in RAW 264.7 cells and incubated for 24 and 48 hours. According to the images, the best protocol was to transfect 1 µL of Lipofectamine and 0.375 µL of Block-iT per well for 24 hours.

A panel of miRNA 122, 451a, 486-5p, 760, 4792, 6087, and 7641 was transfected in RAW 264.7 cells with Lipofectamine for 24 hours. The entire plate, including the NT group, was treated with 10 µg/mL of E.coli LPS. NC-1 was used as a negative control, and cytokine production was analyzed in comparison to this group. 10 µg/mL of LPS was added to the wells for 6 hours and then harvested. The ELISA results (Fig. 23) were varied, but showed that miR-486 and miR-760 had decreased IL-6 and TNFα production in all trials. While many other miRNA's exhibited a decrease in one trial or another, their results weren't constant. Results were normalized to DNA content (Fig. 24).

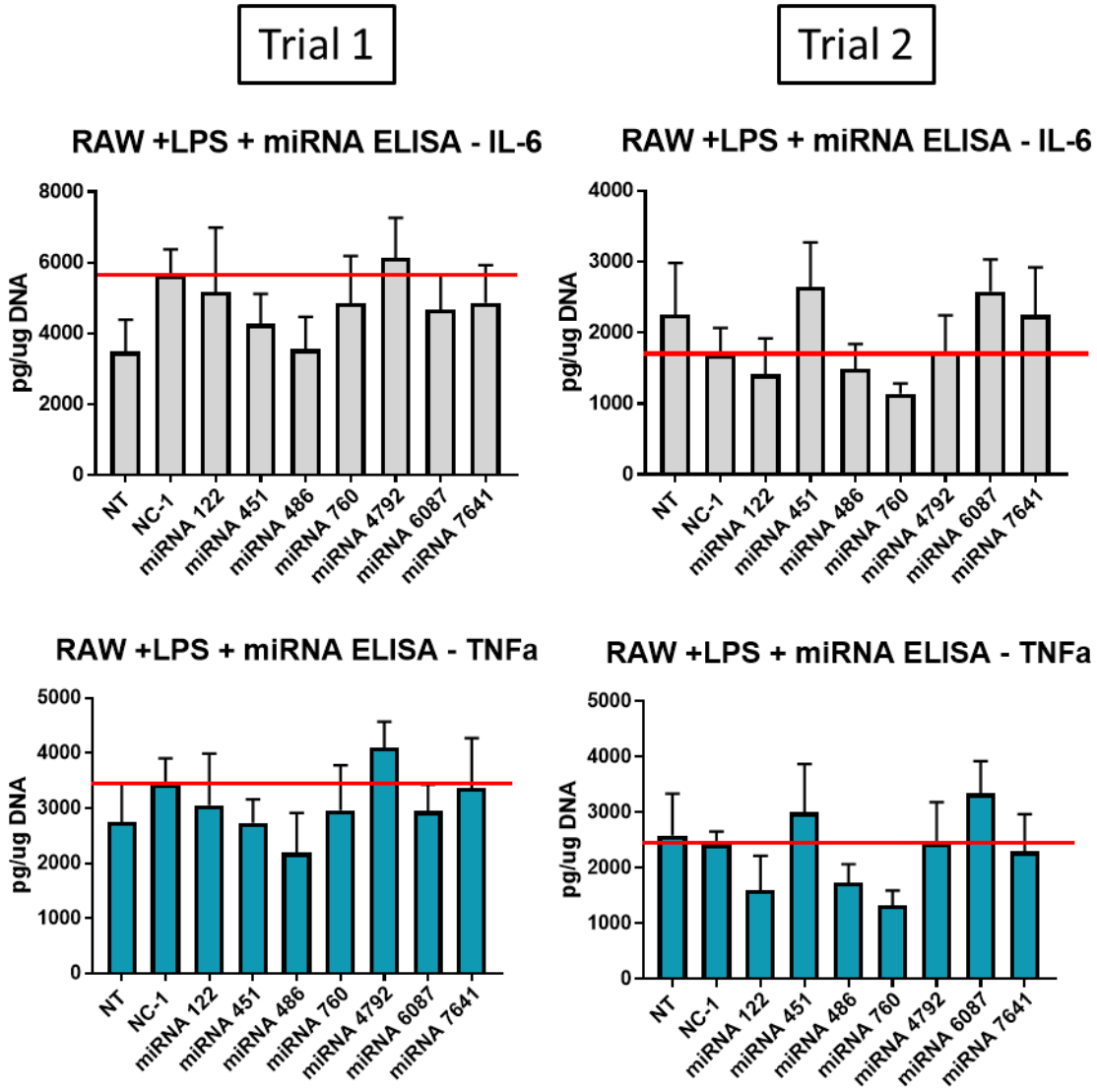


Figure 23: IL-6 and TNFα ELISA results of exosomal miRNA panel – RAW 264.7 cells were seated at 50,000 cells/well in a 48-well plate. Cells were transfected with exosomal miRNA using Lipofectamine 2000 for 24 hours. The entire plate was treated with 10 µg/mL LPS for 6 hours and cell supernatant was harvested for ELISA. Results were normalized to DNA content. NC-1 was used as a negative control and compared to exosomal miRNA's. In all trials, miR-486-5p and miR-760 exhibited a decrease in cytokine production.

Future transfection trials were performed using only miR-486-5p and miR-760, with LPSE as an overall control and NC-1 as a negative control. Once again, changes in cytokine production were analyzed relative to NC-1 results.

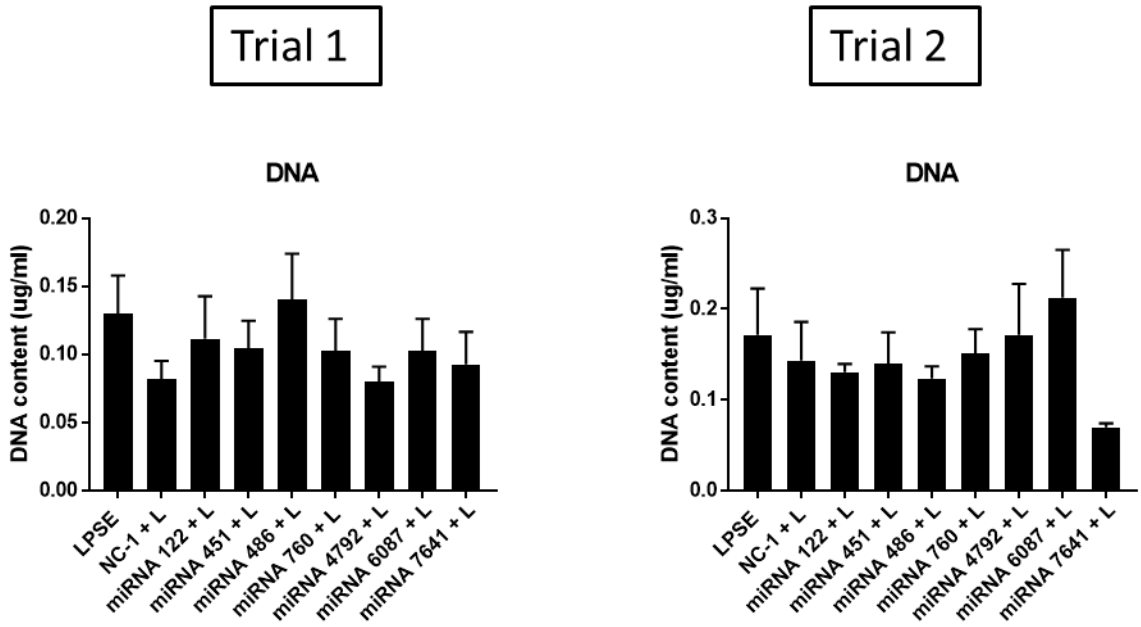


Figure 24: DNA content of exosomal miRNA panel

IL-6 results (Fig. 25) showed that there was a significant decrease in cytokine production within the miR-760 group ($p<0.01$), but not in miR-486-5p. Figure 25B shows ELISA results normalized to DNA in order to account for cell death, but this practice is not common in immunological analyses. In both normalized and non-normalized results, there is a significant decrease in miR-760 ($p<0.05$), although the change is not as pronounced in normalized results.

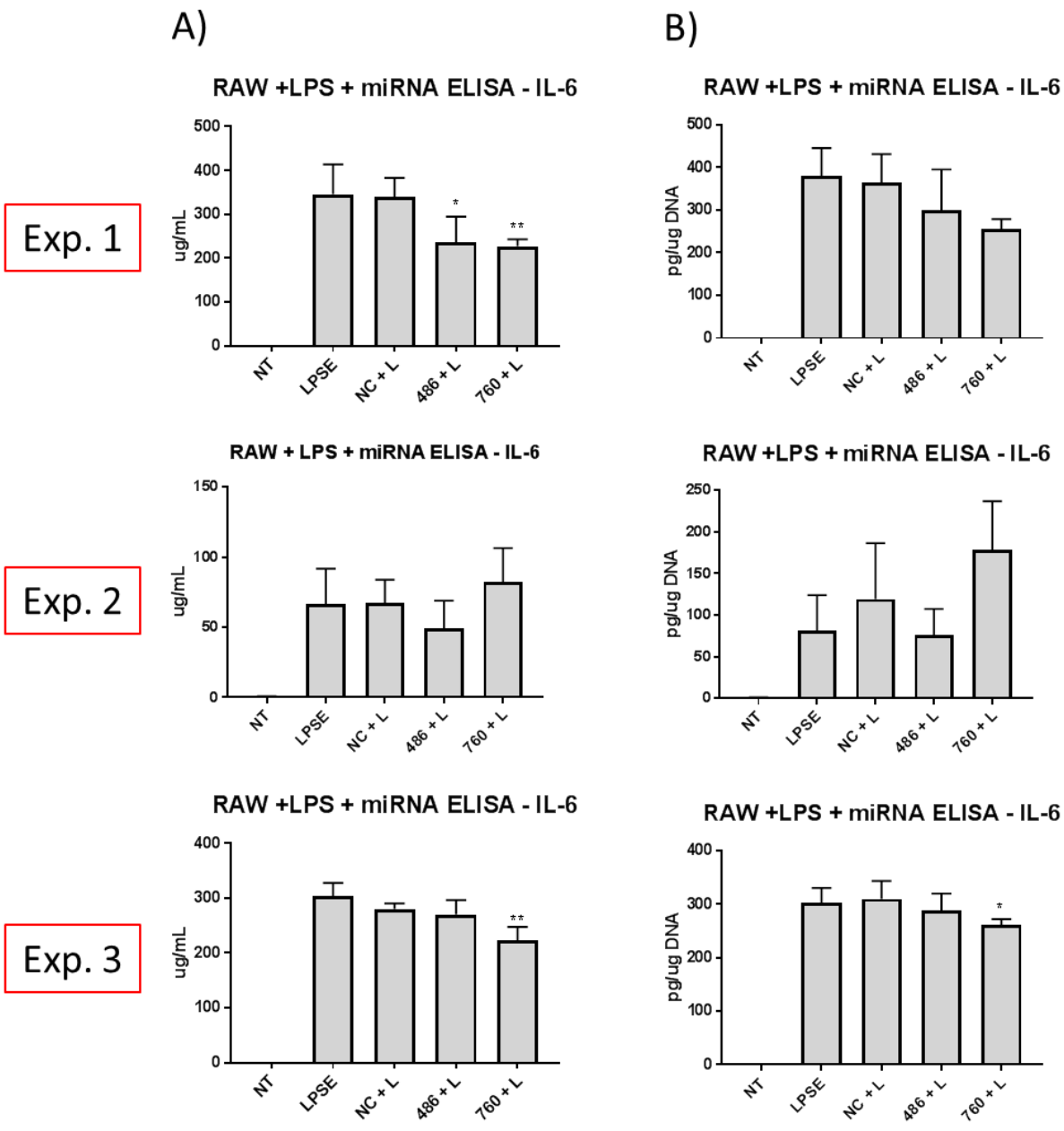


Figure 25: IL-6 ELISA Results – Non-normalized (A) and normalized to DNA (B) – RAW 264.7 cells were seated at 50,000 cells/well in a 48-well plate. Cells were transfected with exosomal miRNA using Lipofectamine 2000 for 24 hours. The plate was then treated with 10 µg/mL LPS for 6 hours, and cell supernatant was harvested for ELISA. Results were normalized to DNA content. NC+L was used as a negative control and compared to exosomal miRNA's. There was a significant decrease in IL-6 production within miRNA 760, both in normalized and non-normalized results.

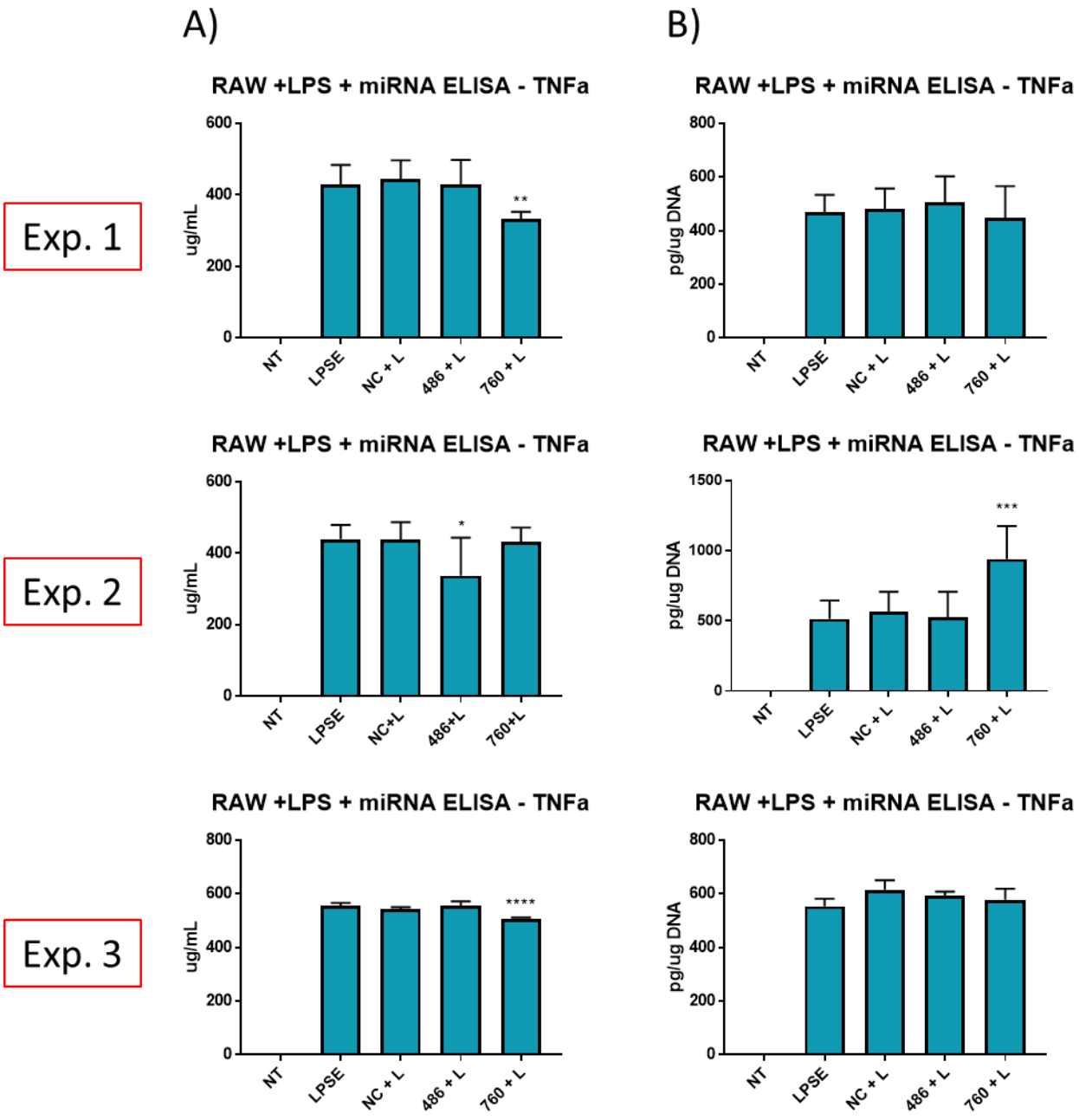


Figure 26: TNFα ELISA Results – Non-normalized (A) and normalized to DNA (B) – RAW 264.7 cells were seated at 50,000 cells/well in a 48-well plate. Cells were transfected with exosomal miRNA using Lipofectamine 2000 for 24 hours. The plate was then treated with 10 µg/mL LPS for 6 hours, and cell supernatant was harvested for ELISA. NC + L was used as a negative control and compared to exosomal miRNA's. The results were less consistent than IL-6, but still showed a decrease in cytokine production within the miRNA 760 group.

TNFα (Fig. 26) results do not show a decrease in cytokine production that is as pronounced as IL-6 results. Two of three trials show a significant decrease within the

miR-760 group (p<0.01), but normalized results are inconclusive. DNA quantification results are included (Fig. 27), and show the changes in cell survival amongst transfection groups. Most of the trials did not exhibit a significant difference in DNA content between NC-1 and the other miRNAs. However, in trial 2, there was a significant difference between miR-760 and NC-1 (***: p<0.001). From these results, we can infer that exosomal miR-760 regulates IL-6 production, but its effect on TNFα is not as strong.

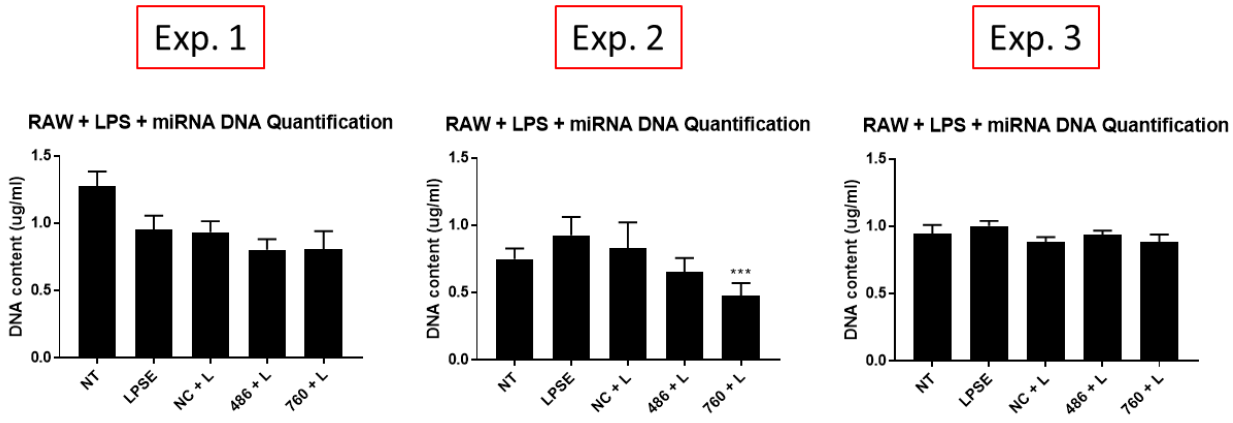


Figure 27: DNA Quantification Results – effect of exosomal miRNA and 10 µg/mL LPS on DNA quantification in RAW 264.7 cells

CHAPTER 5: DISCUSSION

The findings presented in this work showcase the proliferative, chemotatic, and anti-inflammatory effects of hMSC-derived exosomes. Exosomes increased the proliferation and migration of stem cells in a dose dependent manner. In murine macrophage RAW264.7 cells, exosomal treatment resulted in significantly lower proinflammatory cytokine IL-6 production. Through a screening, we also found that the effect of MSC exosomes may be due to the exosomal miR-760.

In Aim 1, our TEM image results showed that exosomes harvested by ultracentrifugation were heterogeneous vesicles that are about 100 nm in diameter. Consistent to previous reports, our exosomes highly express exosome markers CD81 and CD63, indicating that our exosomal harvest was successful. Interestingly, CD9 was not found in our sample, which differed from other studies (Yu et al., 2014).

In Aim 2, we showed that MSC exosome treatment stimulated cell migration and proliferation, which is consistent to previous observations, as seen in Chapter 1.5.2. (Hu et al., 2016). The mechanism behind exosome's effect on cell proliferation and migration is unknown. Studies have found that Wnt proteins are secreted onto the surface of exosomes (Gross et al., 2012). The Wnt signaling pathway may be responsible for exosomal effects on hMSCs. Wnt proteins have been shown to play important roles in cell proliferation, migration and differentiation. Canonical Wnt signaling increases the amount of β-catenin in the cell nucleus, which then activates the transcription of cell cycle genes responsible for mitosis (Kaldis, 2009). It is already known that Wnt is

responsible for the migration of cells to form various tissues during embryogenesis, and this process may have been responsible for cell migration in hMSCs (Gross et al., 2012). In MSC studies, it was found that activation of Wnt signaling inhibits cementoblast differentiation and promotes cells proliferation (Nemoto et al., 2009). Another *in vivo* study showed MSCs that expressed Wnt-4 had a better rate of osteogenic differentiation and bone regeneration. The periodontal defect model in rats had enhanced mineralized tissue formation and better healing in critical bone defects (Chang et al., 2007). So it is possible that Wnt proteins may exist in MSC exosomes and mediate part of their cellular functions.

We observed that MSC exosomes do not stimulate the inflammatory cytokine production at lose dose, however, slight increase of IL-6 was seen in high dose. This is not very surprising because some of the components in the lipid bilayer of exosomes may bind to TLR (Bretz et al, 2013). Interestingly, if the exosome sample was boiled before treating cells, the immunogenicity significantly decreased. Using RT-PCR, Zhang et al. also reported a similar finding that MSC exosome can mildly increase IL-6 level in human macrophage THP-1. However, at the same time, anti-inflammatory cytokine IL-10 and TGF-β increases at much higher level. This may explain the net effect of MSC exosome on immune modulation. Our efforts to study the production of IL-10 were hindered by low signal. In the future, we should further study whether MSC exosomes can stimulate the expression of anti-inflammatory cytokines in stem cells. It also suggests us that a relatively low dose may be more effective when MSC exosomes are used for therapy.

Indeed, we observed that our MSC exosomes are anti-inflammatory to some degree. Exosome treatment suppressed the inflammatory cytokine production triggered by LPS. Other studies have also shown that hMSC-derived exosomes prevent proinflammatory cytokine production *in vivo* in rats with burn injuries – levels of TNFα and IL-1β were significantly lower compared to the NT group (Xiao et al., 2016). We didn't observe similar effect on TNFα. It is possible that the anti-inflammatory nature of exosomes is affected by multiple factors such as cell culture condition, different cell maturation status, donor variance, etc. Taking results from Figure 17, it is important to note that exosomes did cause a dose dependent increase in cytokine production. Therefore, it may be possible that the cytokine decrease seen in further studies could be due in part to saturation of the system rather than only the anti-inflammatory effect of exosomes. Better characterization of the components responsible for the anti-inflammatory effect, such as miRNAs, will help in the optimization of exosome therapy in the future.

In Aim 4, the transfection protocol caused the data to be initially skewed. While certain dosages of Lipofectamine 2000 were sufficient in one cell type, the same is not necessarily true for other cell types. Our ultimate transfection efficiency was less than 40%, while the ideal transfection efficiency in these cells should be above 70%. This may also be attributed to the use of Lipofectamine as a transfection reagent. For this reason, siRNA transfection reagents (INTERFERin, VWR™) and other options should be considered as well. If exosomal miRNA were to be used clinically *in vivo,* administering the RNA without causing any cytotoxicity would be of the utmost importance. Experiments within this aim showed that miRNA 760 was responsible for a

decrease in cytokine production, especially IL-6. As IL-6 was more strongly affected with both exosome and exosomal miRNA treatment, it was hypothesized that miRNA 760 was present in higher amounts in certain batches of exosomes. Furthermore, miRNA 760 may target genes present in IL-6 production. After searching for possible targets on MiRDB, it was found that miR-760 has the highest complementarity with Ankyrin repeat domain 54 (ANKRD54), SCY1-like 1 (SCYL1), proteasome inhibitor subunit 1 (PSMF1), and ataxin 7 (ATXN7). The sequence of miR-760 is CGGCUCUGGGUCUGUGGGGA. This sequence specifically had matches with the -CAGAGCC- sequence of aforementioned genes. After checking microRNA.org to cross-reference the targets, miRNA-760 also targeted ANKRD11 and ATXN7L1 according to this database. While the specific genes or parts of the gene are not identical between the two databases, these types of genes still had the highest target scores. IL-6 was also shown to be targeted by miR-760, although the target score was lower than that of the aforementioned genes. This area of gene study is rather murky - while the genes may be associated with certain diseases, their pathways are not wholly known. ATXN7 gives rise to the ataxin 7 protein, which is associated with neuronal degenerative disorders in the cerebrospinal region. Ankyrin repeat domains are implicated in protein-protein interactions. They are associated with diverse functions such as signal transducing, cell cycle regulation, and cytoskeletal regulation. Ankyrin repeat domains have been seen in p16, a cell cycle inhibitor linked to cancer, and the neuronal protein Notch. As of now, these genes have not been directly linked to wound healing or immunomodulatory behavior.

Normalization of ELISA results to DNA content was done to gauge the amount of cytokine production per cell – however, the fluorometric DNA quantification assay used did not allow us to differentiate between live and dead cells. To normalize our results more accurately, it may be better to use cell counting kit-8, a cytotoxicity assay. Our cell proliferation assay can also be measured using this kit for the same reasons – although the Aim 2.2 results did show an increase in DNA content, we could not be sure what the state of the cells was without either an apoptosis assay or live cell assay.

Exosomes are not yet used as a clinical therapy, but have potential in intracellular signaling, decreasing inflammation, and recruiting growth and mediatory factors to a particular wound site. In harvesting exosomes through ultracentrifugation, our process was time consuming and used many materials. Therefore the practicality of harvesting exosomes on a large scale comes into question in terms of cost, supply, and use of materials. The mechanism of miRNA is normally posttranscriptional gene regulation within the cell that is transcribing it - miRNA's silence specific genes by targeting mRNA for degradation (Chen et al., 2012). However, the mechanism of exosomal miRNA is not yet known. Several studies have shown that exosomal transfer of miRNA to different cell types is possible. For example, dendritic cells and T cells can secrete exosomes and take them up from different sources - this is considered a novel mechanism in intracellular communication (Alexander et al., 2015). In terms of immune response and inflammatory mediation, miRNA 155 and miRNA 146 are now known modulators. These miRNA are released by exosomes and can be taken up by other cells to perform opposing functions: miR-155 strengthens the inflammatory response,

while miR-146a inhibits it (Valadi et al., 2007). The miRNA types have been found in human cells, and have also been tested for functionality in vivo in a mouse model.

Some limitations of this study were the use of only *in vitro* experiments. While these demonstrated the anti-inflammatory, proliferative, and migratory capabilities of hMSC-derived exosomes, *in vivo* studies should also be performed to see the microbial and inflammatory systems interact. While results were successful *in vitro*, *in vivo* studies in a mouse or rat model may have different results due to confounding factors. For instance, many of the cytokine factors produced through the NF-kB pathway suppress further cytokine production through negative feedback. Also, the products and interactions of entire bacterial cells will differ as opposed to only using LPS. While human mesenchymal stem cells were used for proliferative and migratory studies, mouse macrophages were used for experiments on cytokine production. Such results may differ between different cell species. Lastly, these experiments used *E.coli* LPS in lieu of *P.gingivalis* LPS, as the former is more potent and produced more consistent dose curve results. *E.coli* does not naturally occur in human or mouse oral cavities, while *P.gingivalis* is the keystone species of periodontal disease. This should be taken into consideration, as using *P.gingivalis* LPS would have more similarities to an *in vivo* mouse or human oral cavity.

In future studies, the effect of hMSC-derived exosomes will be tested on THP-1 human macrophages or human gingival fibroblasts, as these cell types naturally occur within the gingiva and subgingiva. Furthermore, we will use *P.gingivalis* and try to improve its potency so ELISA results fall within the standard curve. Eventually, we will move exosome therapies to an *in vivo* rat model. Periodontal disease can be induced by

an LPS or ligature model, so that the effect of exosomes with varied bacterial-host interactions can be studied.

CHAPTER 6: CONCLUSION

Periodontal disease therapies currently focus on clearing periodontal bacteria, which increases in difficulty with deeper periodontal pockets. Current rates of bone regeneration are still compromised and unpredictable, and bacterial reinfection is a frequent risk. Exosomal therapy may provide alternative cell therapy that aids in wound healing for immunocompromised patients.

Our study aimed to observe the effect of hMSC-derived exosomes on cell migration, cell proliferation, and cytokine production. As the exact mechanism of exosomes is unknown, another objective was to observe the effect of exosomal miRNA on cytokine production. Exosomes increased the rate of cell migration and cell proliferation within hMSCs in a dose dependent manner. However, exosomes did not have any significant effect on inflammatory cytokine production at an effective treatment dose in stem cells. In activated RAW264.7 cells, exosomes inhibited cytokine production in a dose dependent manner. IL-6 had a more significant decrease in production, while the impact on TNFα was at a much smaller extend. Exosomes were seen to have mainly small RNAs, with miR-486, 760, 6087, 7641, and 4792 in highest relative abundance compared to total cell lysate. A panel of miRNAs that are enriched in exosomes were chosen to be transfected into RAW 264.7 cells for 24 hours. These cells were then activated with *E.coli* LPS for 6 hours. miR-760 and miR-486 consistently had an inhibitory effect on cytokine production, although it was not statistically significant due to the large group number. Further experiments validated that miR-760 significantly decreased the IL-6 production. These results indicate that miR-760 may be

involved in the anti-inflammatory effect of exosomes. The experiments of this study were only performed *in vivo*, and need to be performed in rodent and human models before being used as a therapy. Overall, we can conclude that exosome treatment has dual effects: increasing cell proliferation and migration in hMSCs, and decreasing IL-6 production in active macrophages.

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