3D Culture of Mesenchymal Stem Cells in PEG-4MAL Hydrogels Increases Exosome Production with Bacterial Sphingomyelinase Treatment

By William Y. York

Readers: Edward A. Botchwey and Johanna Temenoff

Table of Contents:

1. Introduction..... Page 2
2. Literature Review..... Page 3-5
3. Materials and Methods .....Page 6-8
4. Results..... Page 9-10
5. Discussion..... Page 10-11
6. Conclusion..... Page 11
7. Works Cited..... Page 12-13

Faculty Advisor Signature: DocuSigned by: Edward Botchwey D66C7EF9F91F4E8...

Second Reader Signature: DocuSigned by: Johanna Temenoff BB177AAC59E8421...

1. Introduction

Mesenchymal Stem Cell (MSC) therapy, in which MSCs are used to mediate tissue regeneration and immune responses, is an attractive technique for treating injuries and diseases. However, MSC-based therapies are hindered by the risk of immune rejection of foreign cells in the host's body. Therefore, when it was found that MSC's paracrine secretion of exosomes is a major mechanism in exerting the MSC's therapeutic effects, exosome therapies began receiving attention. Exosomes are a type of extracellular vesicle that are highly biocompatible and non-immunogenic, ranging in size from 30-150nm, and that are filled with genetic material and proteins that regulate nearby cells. Potential exosome applications that are currently being investigated include their use in tissue engineering to promote wound, and as biomarkers for faster and easier prostate cancer diagnosis and prognosis. However, clinical applications for exosomes are currently hindered by a number of different factors, including a lack of methods for production and manufacturing of exosomes. Exosome production is currently limited by the growth and upkeep of MSCs stored *in vitro* in flasks, since hundreds of flasks may be required. The delivery method for exosomes is also an issue, as they are rapidly cleared out of the injection site, which greatly reduces their efficacy and is a reason why so many exosomes are required for current investigated therapies. My research is aimed at developing and improving methods for manufacturing and delivering MSC-derived exosomes.

In this project, we explore the sphingomyelin pathway as a potential method for increasing exosome production and increasing MSC immune potency. Although exosomes are vesicles secreted outward from the cell's plasma membrane, the exosome biogenesis pathway begins with the inward budding of the plasma membrane to form an endosome. This vesicle receives its contents inside the cell before fusing with the plasma membrane and being released into extracellular space as exosomes. A goal of our project is to encourage this pathway through the conversion of sphingomyelin, a common lipid found in the plasma membrane, to ceramide, a slightly smaller molecule also found on the plasma membrane, by bacterial sphingomyelinase (SMase). Because ceramide is smaller than the surrounding sphingomyelin, a portion of membrane with higher concentrations of ceramide will be forced to curve inwards, enhancing the inward budding that leads to the exosome biogenesis pathway (Figure 1A).

We also hope to combine this method for exosome production with Polyethylene Glycol-Maleimide (PEG-4MAL) hydrogels, which act as a net to encapsulate MSCs while also incorporating the SMase into the hydrogel. PEG-4MAL hydrogels are a popular delivery method and biomaterial that offer high biocompatibility and controlled degradation inside of the body. This sustained degradation of the hydrogel offers a method for the continuous release of exosomes into the surrounding tissue and may offer a solution for the rapid clearance currently seen. We hypothesize that this combination will lead to increasing the exposure of MSCs to SMase, a continual increase in exosome production, and a sustained release of exosomes into the surrounding environment.

2. Literature Review

As exosomes are a type of extracellular vesicle that is produced by inward budding of the plasma membrane before exiting the cell [8], their natural origin and lipid-based membrane creates a cell-cell communication method that has a very low risk of causing an immune response and can target specific cells [1]. In particular, exosomes from Mesenchymal Stem Cells (MSCs) have become the focus of researchers for their potential to carry out the MSC's wound healing capabilities by transporting various proteins and genetic information that mediate the MSC's immunomodulatory effects, without the risk of immune rejection due to foreign cellular presence in the wound [8]. Because of their potential in cell-free therapies and clinical applications like wound healing and as biomarkers, exosome research has recently gained attention in hopes of working around the immunogenic issues with stem cell therapies [3]. However, exosome applications remain impractical at the moment due to issues with exosome production, clinical delivery methods, and isolation and quantification methods [1, 3].

MSC-derived exosomes are well known for their role in cell-cell signaling and subsequent cell regulation and have garnered attention for their ability to deliver regulatory proteins and nucleic acids in a targeted manner while also avoiding immune rejection [1]. Potential clinical applications include acting as a biomarker for prostate cancer diagnosis and prognosis [2], and various tissue engineering approaches, most commonly as therapeutics to induce wound repair and regeneration [3, 6-10, 12]. These clinical applications have been observed in laboratory settings and in numerous clinical trials, but as yet no medical treatment with exosomes has been authorized [3].

One of the main hurdles in the regular clinical use and application of exosomes is their limited supply. There is currently no established efficient exosome-production method. Since MSC-derived exosome therapies require billions of exosomes to function properly, which can require hundreds of culture flasks may be required to prepare enough exosomes[9]. Therefore, there has been recent interest in exosome manufacturing methods. One of the most common approaches to manufacturing exosomes is the regulation of MSC's exosome biogenesis pathway through key proteins and signaling pathways [9]. This method is expensive and difficult to translate to clinical applications, however. Another approach, which is less expensive, is the stimulation of MSCs with specific biomaterials, with the reasoning that such biomaterials are known to alter the paracrine function of MSCs, and exosomes are known as one of the key paracrine cell-signaling mechanisms of MSCs [9]. For example, Wu et al. [9] describe an example of this in which a Bioglass biomaterial can influence enzyme pathways that are directly involved in the exosome biogenesis process, leading simultaneously to both increased exosome production and increased exosome function. A third method is similar to the biomaterials approach, involving MSC priming through microenvironment regulation to use the MSC's surroundings to modulate both exosome production and content, the latter of which also can lead to improved functionality [7, 9]. This method has been relatively unexplored as a large-scale manufacturing method, however. Ultimately, there is as yet no widespread manufacturing method for the clinical use of exosomes despite the growing focus on the issue. Still, this is not the only problem preventing the incorporation of exosome therapies in medicine today.

The clinical uses of exosomes are also limited by the current delivery methods. The most common delivery method in clinical trials today is injection, as it provides direct access to most treatment areas in wound healing, is relatively cheap, and is very accessible [3]. The issue with this and similar methods is that exosomes introduced to the body this way will be rapidly cleared out of the injection site, which greatly reduces their efficacy and is one of the reasons why wound healing treatments require large amounts of exosomes to function [3, 6, 10, 12]. Part of the issue is that the benefit of high biocompatibility and low risk of immune rejection that is characteristic of exosomes and sought after in these therapies also means that the exosome producing MSCs cannot be injected as a method to continuously increase exosome presence, as this negates the aforementioned biocompatibility. This difficulty maintaining the reliability and stability of exosomes *in vivo* has led towards a recent surge in combining biomaterials with exosome treatments.

Particularly, hydrogels have received attention as a potential solution as they are already strongly associated with wound healing and tissue engineering, offering high biocompatibility, drug delivery capabilities, high control over drug release and material properties, and high biomimicry. Hydrogels are a biomaterial that can be made with various components depending on the goals but, generally, are gels that are largely composed of cross-linked polymers that form a net to trap water and other contents inside and that are degradable inside the body. Other biomaterials are being explored in this topic, but hydrogels offer a well-established and well-understood starting point. Once implanted, hydrogels will degrade at a controlled rate inside of the body, and this is used to release the contents that are often encapsulated inside of them. In this case, the hydrogels will slowly release exosomes into the wound site so that the microenvironment is undergoing prolonged exposure. However, this delivery method remains relatively poorly tested and requires further exploration [6].

A separate issue that the exosome therapeutics field faces is that the methods for isolation and characterization of exosomes are expensive, inefficient, and non-standardized [11]. This issue is more relevant to the diagnostic applications of exosomes and current exosome research, whereas increasing exosome production is more relevant to tissue engineering. Methods currently used for exosome characterization and isolation include ultrafiltration, density gradients, and ultracentrifugation, which is the gold standard as used in 56% of researchers in the field [11]. These methods particularly face issues related to efficiency which limits their applicability for routine clinical diagnostic measures. Other common exosome identification methods involve imaging, in which the gold standards are Cryo-Transmission Electron Microscopy (TEM), Scanning Electron Microscope (SEM), and Atomic Force Microscopy (AFM). These methods have been used for over 30 years and are well-accepted for their ability to analyze exosomal structure but fail to image protein content within the exosome, which becomes one of the limitations on using exosomes as biomarkers currently [5]. TEM with negative staining has been proposed as a solution to this issue by Jung et al. [5] but further studies are still needed. There are also other issues that have yet to be addressed, including a focus on *in vitro* studies which leads to an inability to deal with various body fluids and a lack of standardized characterization of exosomes between research labs which leads to further variation in protocols [11]. An example of this is that the reported definition of exosome diameters can change from 20-150nm to 30-300nm. This lack of efficient and standardized separation and characterization processes in exosome research limits the field’s progress and also what is possible for patients.

In summary, MSC-derived exosome research has a lot of attention from the biomedical engineering research community for its potential clinical applications but is currently held back by a number of limitations. There is yet to be a method to consistently and reliably produce exosomes for their wide-spread use in medicine; delivery methods are still being explored; and isolation and characterization standards are yet to be established. Even amongst the cited works, there were some contradictions and uncertainties in characterizing exosomes and no solutions clearly offered a transition into the real-world applications. Notably, all but one of the references sources date from 2018 or later, demonstrating the current attention and excitement in this field.

In our project, we plan to address production and delivery method issues by working with a relatively unexplored mechanism of the exosome biogenesis pathway in which sphingomyelinase turns the cell membrane's sphingolipids into ceramides, a smaller molecule, to induce membrane curvature and provoke exosome production while utilizing hydrogels as an *in vitro* 3D model and potential delivery method. Our work will build off projects that have explored the exosome biogenesis pathway, hydrogel manufacturing and applications, and exosome isolation and characterization, with the goal of building on this fast-growing body of work in order to offer insight into exosomes, their production pathway, and possible translational steps towards clinical application.

3. Materials and Methods

This project has been broken down into two major components: assessing whether SMase along with PEG-4MAL hydrogels can increase MSC exosome production and characterizing the exosomes that are produced. The first half expanded upon prior work in the lab assessing the effect SMase has on MSC exosome production in 2D, while the second half will begin to assess real world applicability and clinical possibilities of the exosomes produced. Chronologically, this project focused on PEG-4MAL hydrogel production proper for MSC encapsulation, culturing and treating MSCs, collecting and isolating exosomes, quantifying the number of exosomes produced, and evaluating their immunomodulatory potential.

3.1 PEG-4MAL Hydrogel Fabrication

Hydrogels were prepared by mixing 20 kDa PEG-4MAL (Laysan Bio), VPM Crosslinker peptide with 2 thiols, adhesive peptide RGD (arginylglycylaspartic acid) with 1 thiol, and either Phosphate Buffer Solution (PBS) or MSCs in a 2:1:1:1 volume ratio to create the hydrogels. Each component was diluted to the appropriate concentration in HEPES buffer (Thermo Fischer Scientific) to prepare a 4.5% weight/volume solution, and RGD and VPM were adjusted to a pH of 6.5 before combining. Components were then added together to create a 25µL hydrogel inside of a 24-well well plate.

3.2 Cell Culture

220,000 human MSCs from Rooster Bio's 177 cell line were thawed from liquid nitrogen and diluted in 10mL of house-made media (88% DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fischer, 10% FBS (Fetal Bovine Serum), 1% L-glutamine, and 1% pen strep). Cells were then centrifuged for 10 minutes at 200 g, resuspended in 5 mL media, and then moved to a T-75 flask (Thermo Fischer) with another 5mL of media. Cells were grown for 5 days before being either encapsulated inside of PEG-4MAL hydrogels or plated onto tissue culture plastic (TCP) in the 24-well well plates. 8,000 cells were either plated in each well or encapsulated in hydrogels in 500µL of media with 10 replicates of both PEG-4MAL hydrogels and TCP.

3 days after plating or encapsulation, all cells were switched to exosome-depleted media as created before but using exosome-depleted FBS. Half of the cells grown in 2D and 3D were also exposed to 1.42µL of 0.14U/L SMase. 48 hours later, media was then extracted for subsequent exosome isolation. The cells were removed with incubation of Accutase for 2D culture or incubation with 2mg/mL collagenase type II for MSCs encapsulated in PEG-4MAL hydrogels. Collected cells were centrifuged at 200 g for 10 minutes, resuspended in PBS, and frozen for downstream analysis.

3.3 Exosome Isolation

Extracted media was centrifuged at 2000 g for 30 minutes. The supernatant was then extracted and Total Exosome Isolation (TEI) reagent was added in accordance with the providers' instructions. Following an overnight incubation, the media was then ultracentrifuged at 10,000 g

for 1 hour at 4 degrees C before being resuspended in 100µL of PBS and stored in -80 degrees C until analyzed by Nanoparticle Tracking Analysis (NTA).

3.4 Nanoparticle Tracking Analysis

NTA was performed using a Malvern NanoSight NS300 (Malvern). 5-minute-long videos were captured of each sample using the standard measurement feature with the camera level set between 10 and 16 and the detection threshold set between 5 and 45 and automated settings were used for blur, minimum tracking length, and focus. Samples were prepared before running by diluting to 1mL with PBS. 1mL of PBS was run before each sample and was measured in the same way as the samples. After running, the numbers of particles in each capture of PBS were subtracted from the number of particles of each sample's captures. Particles were identified as exosomes if their size was between 30.5 and 150.5nm as identified by the NTA.

3.4 Immunomodulatory Potential Assay

TNF-α levels produced after Lipopolysaccharides (LPS) stimulation of RAW 264.7 macrophages were used as a metric for measuring the immunomodulatory potential of exosomes. Exosomes were produced following the same procedures as mentioned above. RAW 264.7 Macrophages were cultured in 18 wells of a 24-well well plate at 150,000 cells per well with 0.5mL of house-made media and incubated for 48 hours. MSCs were given one of the four treatments for 48 hours before the media is collected, and the exosomes were isolated the same way as before. The exosome solutions were then pooled together based on treatment group totaling 720µL for the 2D groups and 800µL for the 3D groups before 50µL was extracted for future NTA from each. 12mL of media were combined with 670µL of the pooled 2D exosome solutions, 750µL of the pooled 3D exosome solutions, 750µL of PBS, or 2.49µL of SMase with 750µL of PBS for a total of 6 different treatment groups. From these, 2mL were added to each well of RAW 264.7 macrophages before being incubated for 1 hour. Then, the media was aspirated, and the cells were washed with PBS before half of the wells were treated with 20µL of 50ng/mL LPS along with 200µL of Brefeldin-A in 2mL of media and incubated for 2 hours. The cells were then prepared for flow cytometry analysis.

3.5 Flow Cytometry

The macrophages were removed from the wells with Accutase and fixed with 100uL of 4% paraformaldehyde for 20 minutes. They were then permeabilized with 2 washes of 1x permeabilization buffer (BioLegend) and centrifuged at 400 g for 5 minutes. The cells were then incubated with BV510 anti-mouse TNF-α antibody (BioLegend) in the permeabilization buffer for 20 minutes. Cells were resuspended in FACS buffer and the staining was detected using flow cytometry, conducted with a FACS-AriaIIIu flow cytometer (BD Biosciences). Samples were analyzed based on mean fluorescent intensity (MFI) with FlowJo software and graphed on PRISM.

3.6 *Statistical Analysis*

Each sample ran through NTA had 5 technical replicates that were averaged. Each treatment group had 3 samples and the average of those were compared using a two-way ANOVA with multiple comparisons and Tukey's post-hoc test in PRISM. The MFI between groups were compared using a two-way ANOVA with multiple comparisons and $p < 0.05$ was used to determine significance.

4. Results

In this study, we evaluate both the changes in the quantity and quality of exosomes produced by MSCs under different conditions.

4.1 Exosome Production

Exosome production was evaluated by quantification and size measurement through NTA. Exosomes were present in all treatment conditions with particles of approximately 100nm detected (Figure 1A). Significant increases were noted when MSCs were cultured in PEG-4MAL hydrogels with SMase stimulation relative to all groups at the 100nm size range (Figure 1B). MSCs stimulated with SMase while in PEG-4MAL hydrogels were also found to significantly increase particles between 30-150nm, characterized as exosomes here, produced relative to all groups (Figure 1C).

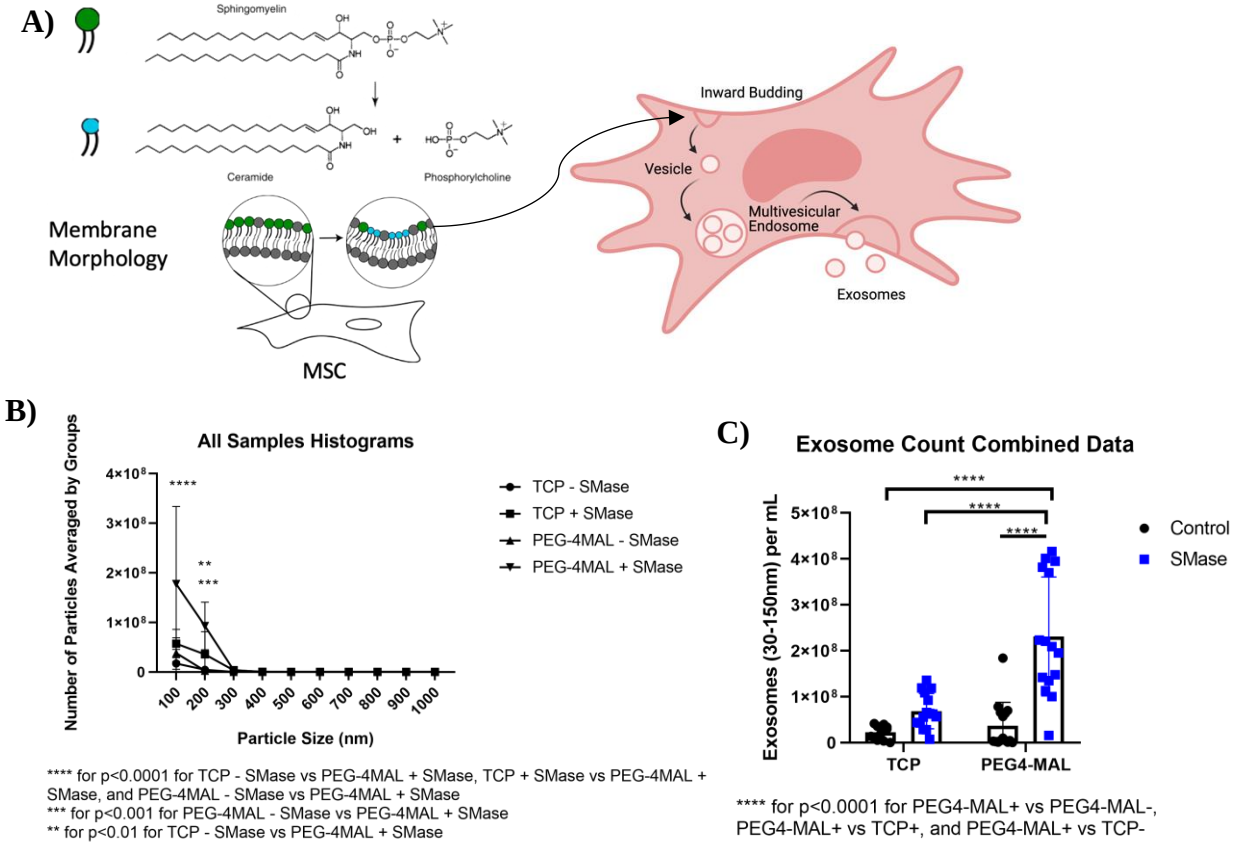

Figure 1. Exosome Identification and Quantification. (A) Proposed mechanism for how SMase is used to increase exosome production. (B) Size distribution of particles tracked averaged between samples within treatment groups. (B) Count of exosomes produced by MSCs tracked by NTA averaged within treatment groups. Each sample of exosomes that were isolated from the media of MSCs were diluted to 1mL with PBS. For each sample, 5 1-minute-long

captures were taken to represent the sample in which both the number of particles and the sizes of the particles were tracked. Particles from size ranges 30-150nm were characterized as exosomes. Statistical analyses were conducted using two-way ANOVAs with Tukey's post-hoc test.

4.2 Exosome Immunomodulatory Potential

The exosomes produced by the differently treated MSCs had their bioactivity evaluated by measuring their ability to lower TNF-α levels. All groups pre-treated with exosomes were found to significantly lower TNF-α levels relative to the control (Figure 2.). Both exosome groups that were formed without SMase did show significantly higher TNF-α levels than the exosomes produced in 2D with SMase. Even the SMase alone control group demonstrated significantly lower TNF-α levels relative to the PBS control and TCP group without SMase.

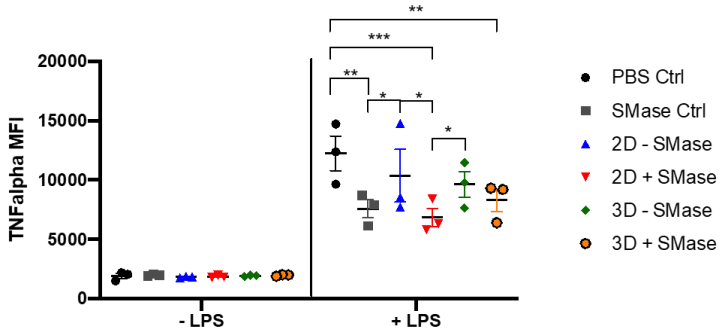

Figure 2. Exosomes lower TNF-α levels in LPS-stimulated RAW 264.7 Macrophages. Macrophages were pre-treated with the exosomes produced by each method for 1 hour before being stimulated with LPS and Brefeldin-A for 2 hours. Flow cytometry analysis demonstrates significant decreases in TNF-α levels in all groups treated with exosomes compared to control. Two-way ANOVA was conducted with multiple comparisons and Tukey's post-hoc test.

5. Discussion

Exosome production is a bottleneck in research and clinical applications due to inefficiencies in cell culturing and the large number of exosomes required in research. Here, we demonstrate that SMase stimulation of MSCs inside of PEG-4MAL hydrogels offers a new, more efficient production method that can be used to facilitate future research.

5.1 Exosome Production

Our lab had previously demonstrated that SMase stimulation of MSCs in standard TCP conditions significantly increased exosome production. Here, we build upon that by culturing the MSCs in a 3D scaffold using PEG-4MAL hydrogels, which better simulates their intended and optimal environment, allowing for improved bioactivity. Trajkovic et al. has also demonstrated

that increasing ceramide in the lipid membrane in endosomes makes them more likely to become exosomes instead of following other pathways inside of the cell, and that inhibition of SMase reduced exosome production [8]. This, along with ceramide-induced membrane curvature, may offer possible explanations why SMase stimulation of MSCs significantly increases exosome production. However, SMase stimulation was not explored as a possible method for exosome production. Various hydrogels have been explored as a potential delivery method for exosomes, but PEG-4MAL hydrogels remain untested and hydrogels, in general, have yet to be used as a cell culturing factor [14]. Future studies are required to test the release mechanics of exosomes from PEG-4MAL hydrogels.

5.2 Exosome Immunomodulatory Potential

Ultimately, exosome research is commonly focused on their cell-cell communication abilities and potential to increase tissue regeneration. To evaluate their effectiveness and gain insight into some of the potential clinical applications that the exosomes produced via this method may be used for, we evaluated their ability to lower TNF-α in macrophages. This metric serves as a representation for their ability to reduce inflammation, which is important for tissue regeneration. All the treatment groups used to produce exosomes significantly reduced TNF-α levels, including the control with MSCs in TCP without SMase. This demonstrates that this production methods to increase exosomes does not significantly alter the functionality of the exosomes related to reducing inflammatory cytokines in the immune response. However, there were also significant differences in TNF-α levels between exosomes derived from MSCs cultured in TCP with SMase and TCP or PEG-4MAL without SMase, which will require further studies to explore possible explanations. The macrophages pre-treated with only SMase also had a significant decrease in TNF-α levels, which goes against current literature that demonstrates that disruption of neutral sphingomyelinase will disrupt the TNF-α pathway in macrophages [13]. Further studies are required to explore this relationship.

6. Conclusion

Exosomes from stem cells, as extracellular vesicle cell-cell communicators, offer great potential to regulate immune response for therapeutic purposes as a stem cell replacement or supplement. However, production efficacy is far too low to realistically get the number of exosomes required for research and clinical applications. Therefore, methods to increase production efficiency by stem cells are needed for clinical applications to become feasible treatments for patients. By encapsulating MSCs inside of PEG-4MAL hydrogels along with SMase, we have identified a novel production method that produces exosomes at a significantly increase rate while maintaining their anti-inflammatory properties. Future research is required to further characterize the exosomes produced, identify their therapeutic effects *in vivo*, and further optimize the production method.

Works Cited:

1. Baglio, Serena Rubina et al. “Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy.” Frontiers in physiology vol. 3 359. 6 Sep. 2012, doi:10.3389/fphys.2012.00359
2. Brzozowski, Joshua S., et al. “Lipidomic Profiling of Extracellular Vesicles Derived from Prostate and Prostate Cancer Cell Lines.” *Lipids in Health and Disease*, vol. 17, no. 1, 2018, doi:10.1186/s12944-018-0854-x.
3. Colao IL, Corteling R, Bracewell D, Wall I. Manufacturing Exosomes: A Promising Therapeutic Platform. Trends Mol Med. 2018 Mar;24(3):242-256. doi: 10.1016/j.molmed.2018.01.006. Epub 2018 Feb 12. PMID: 29449149.
4. Elkhoury, Kamil, et al. “Engineering Smart Targeting Nanovesicles and Their Combination with Hydrogels for Controlled Drug Delivery.” *Pharmaceutics*, vol. 12, no. 9, 2020, p. 849., doi:10.3390/pharmaceutics12090849.
5. Jung, Min Kyo, and Ji Young Mun. “Sample Preparation and Imaging of Exosomes by Transmission Electron Microscopy.” *Journal of Visualized Experiments*, no. 131, 2018, doi:10.3791/56482.
6. Li, Liming, et al. “Transplantation of Human Mesenchymal Stem-Cell-Derived Exosomes Immobilized in an Adhesive Hydrogel for Effective Treatment of Spinal Cord Injury.” *Nano Letters*, 7 May 2020, doi:10.1021/acs.nanolett.0c00929.s001.
7. Showalter, Megan R., et al. “Primed Mesenchymal Stem Cells Package Exosomes with Metabolites Associated with Immunomodulation.” *Biochemical and Biophysical Research Communications*, vol. 512, no. 4, 2019, pp. 729–735., doi:10.1016/j.bbrc.2019.03.119.
8. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brügger B, Simons M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science. 2008 Feb 29;319(5867):1244-7. doi: 10.1126/science.1153124. PMID: 18309083.
9. Wu, Zhi, et al. “Bioglass Enhances the Production of Exosomes and Improves Their Capability of Promoting Vascularization.” *Bioactive Materials*, vol. 6, no. 3, 2021, pp. 823–835., doi:10.1016/j.bioactmat.2020.09.011.
10. Yang, Jiayi, et al. “Umbilical Cord-Derived Mesenchymal Stem Cell-Derived Exosomes Combined Pluronic F127 Hydrogel Promote Chronic Diabetic Wound Healing and Complete Skin Regeneration.” *International Journal of Nanomedicine*, Volume 15, 2020, pp. 5911–5926., doi:10.2147/ijn.s249129.
11. Zarovni, Natasa, et al. “Integrated Isolation and Quantitative Analysis of Exosome Shuttled Proteins and Nucleic Acids Using Immunocapture Approaches.” *Methods*, vol. 87, 2015, pp. 46–58., doi:10.1016/j.ymeth.2015.05.028.
12. Zhang, Kaiyue, et al. “Enhanced Therapeutic Effects of Mesenchymal Stem Cell-Derived Exosomes with an Injectable Hydrogel for Hindlimb Ischemia Treatment.” *Applied Materials and Interfaces*, 17 Aug. 2018, doi:10.1021/acsami.8b08449.s001.
13. Al-Rashed F, Ahmad Z, Thomas R, et al. Neutral sphingomyelinase 2 regulates inflammatory responses in monocytes/macrophages induced by TNF-α. *Scientific Reports.* 2020;10(1):16802.

14. Khayambashi, Parisa, et al. “Hydrogel Encapsulation of Mesenchymal Stem Cells and Their Derived Exosomes for Tissue Engineering.” *International Journal of Molecular Sciences*, vol. 22, no. 2, 2021, p. 684., https://doi.org/10.3390/ijms22020684.