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Extracellular vesicles secreted by human mesenchymal stem cells affect cell viability after exposure to amyloid beta oligomers

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ABSTRACT

Alzheimer's disease is a neurodegenerative disorder that causes decreased memory and thinking, eventually to the inability to carry out simple daily tasks. This is due to the increase in destruction of cells. Extracellular vesicles (EVs) secreted by human stem cells for intercellular communications have been known to have therapeutic effects because of their delivery of cargo, which includes the biological macromolecules such as protein and microRNA. In this study, human mesenchymal stem cells (hMSCs) were cultured, and the extracellular vesicles isolated from hMSC media were characterized. Some hMSCs were then treated with amyloid beta 42 oligomers (a hallmark of ALzheimer's disease) and some contained both amyloid beta 42 oligomers and extracellular vesicles. Then live/dead assay was performed to compare and evaluate the viability of cells with amyloid beta 42 oligomers that were and were not treated with extracellular vesicles. The hypothesis was that hMSC-EVs are able to restore cell viability after exposure to Aβ42. Since there was a noticeable increase in cell viability in cells treated with extracellular vesicles, this study can potentially advance the treatment of Alzheimer's disease.

Keywords: human mesenchymal stem cells, extracellular vesicles, amyloid beta 42 oligomers, Alzheimer's disease, cell viability.

INTRODUCTION

Alzheimer's disease (AD) is a progressive mental illness characterized by memory loss, multiple cognitive impairments, changes in personality, thought, and behavior (Amakiri *et al*., 2019). This disease is prevalent in the elderly. Because the average lifespan of human beings is increasing, AD's impact on our world today is increasing as well (Amakiri *et al*., 2019). Unfortunately, there is no known cure or even an effective treatment for people suffering from this disease (Cone *et al*., 2021). Multiple cellular changes are involved in the disease process including loss of neurons, synaptic loss/damage, mitochondrial fragmentation, increased free radical production, mitochondrial DNA

damage, proliferation of astrocytes and microglia, hormonal imbalance and altered neurotransmitter levels (e.g., decreased acetylcholine) in addition to neurofibrillary tangles, and senile plaques (Amakiri *et al*., 2019). There are two types of AD. Familial AD is due to mutations in three major genes: amyloid beta precursor protein (APP) gene, presenilin1 (PSEN1) gene, and presenilin 2 (PSEN2) gene (Amakiri *et al*., 2019). Mutations in these genes induce the abnormal overproduction of amyloid-β (Aβ), leading to familial AD (Amakiri *et al*., 2019). Sporadic AD is believed to be determined by a combination of genetic factors, environmental factors, and lifestyle (Amakiri *et al*., 2019). The exact cause of sporadic AD is not as well understood (Amakiri *et al*., 2019). This study focuses mainly on familial AD with an emphasis on the effect of (Aβ). Aβ itself does not necessarily cause AD or amplify AD pathology, but it has been found to have a toxic effect on synapses in the brain, leading to cognitive impairment (Amakiri *et al*., 2019). This is amplified by the diffusion of Aβ into subcellular compartments and at neuronal terminals (Amakiri *et al*., 2019). Aβ oligomerizes to form insoluble plaques that lead to neuronal cell death (Cone *et al*., 2021). The oligomerization was found to be more pathogenic than just plaques because of the greater surface area to interact with the synaptic cells of the brain (Amakiri *et al*., 2019).

Human mesenchymal stem cells (hMSCs) are multipotent stromal cells that have been widely studied due to their therapeutic potential such as immunomodulation, anti-apoptosis, and anti-inflammatory properties (Cone *et al*., 2021). Despite stem cell transplantation being considered as one of the most promising approaches in regenerative medicine, the clinical application is facing a number of challenges (Nagelkerke *et al*., 2021). Safety concerns include risks to tumorigenicity, pro-inflammation, and rejection by the host (Nagelkerke *et al*., 2021). Additional challenges for systemic administration include maintaining cell viability, increasing stem cell permanence, in vivo stem cell differentiation, specific delivery to the desired target site, and integration into the target tissue (Nagelkerke *et al*., 2021). The initial hypothesis that hMSCs, through cellular differentiation, would replace damaged tissue was partially abandoned following observations that very few, if any, cells stably engraft in the host (Wiklander *et al*., 2019).

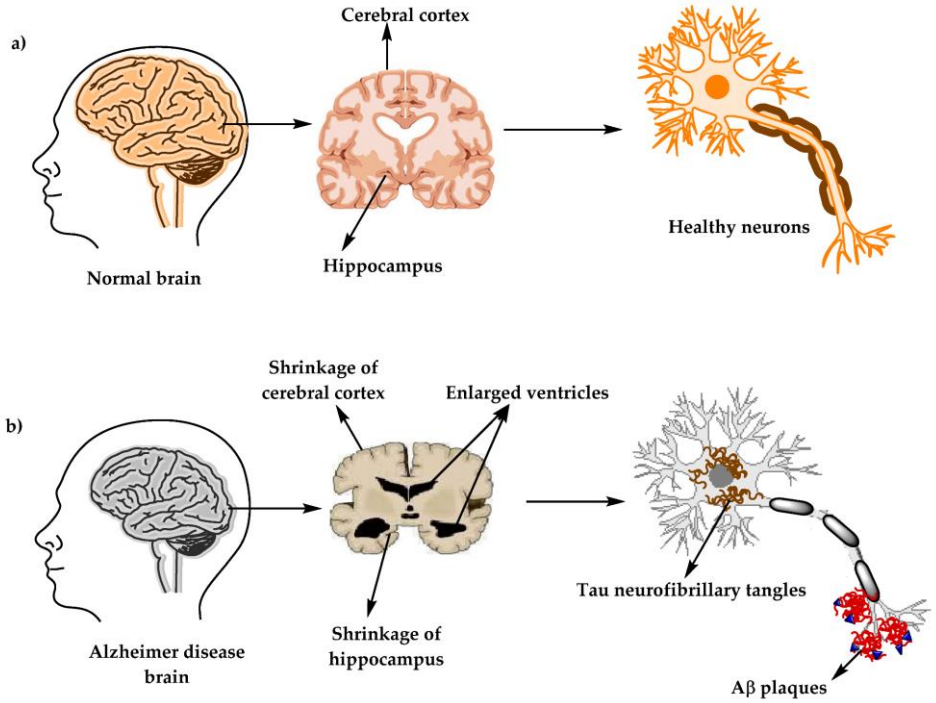


Figure 1. Healthy brain and neurons vs Alzheimer's disease and Neurons. Image from "Comprehensive Review on Alzheimer's Disease: Causes and Treatment."

Extracellular vesicles (EVs) derived from hMSCs have been studied as a therapeutic candidate in place of hMSCs, as they exhibit similar immunoprotective and immunomodulatory abilities as the host hMSCs (Cone *et al*., 2021). Communication from one cell to another, or between a cell and its microenvironment, is crucial under both physiological and pathological conditions (Chen & Yu, 2022). Such crosstalk is achieved by direct cell-cell contact and canonical secretion as well as by membrane-bound EVs (Chen & Yu, 2022). Almost all tested cells are capable of secreting various types of EVs, containing biologically active cargos such as DNA, microRNA, protein and metabolites, and other macromolecules that vary in response to the microenvironment (Chen & Yu, 2022). These vesicles resemble their parent cell origin to some extent, and reflect the real-time state of the parent cell (Chen & Yu, 2022). The underlying mechanisms attributed to the therapeutic action of hMSC-EVs by the transfer of their cargo, as well as the triggering of signaling pathways via cell surface interactions, are diverse and include mitigating or eliciting immune responses, reducing inflammation, inhibiting apoptosis, minimizing oxidative stress, stimulating wound repair, and promoting angiogenesis, which together act to ultimately ameliorate the adverse effects of diseases, promote healing, and restore function (Wiklander *et al*., 2019). EVs are also likely to have reduced potential side effects because they are less complex and better defined as compared to cell therapies (Wiklander *et al*., 2019).

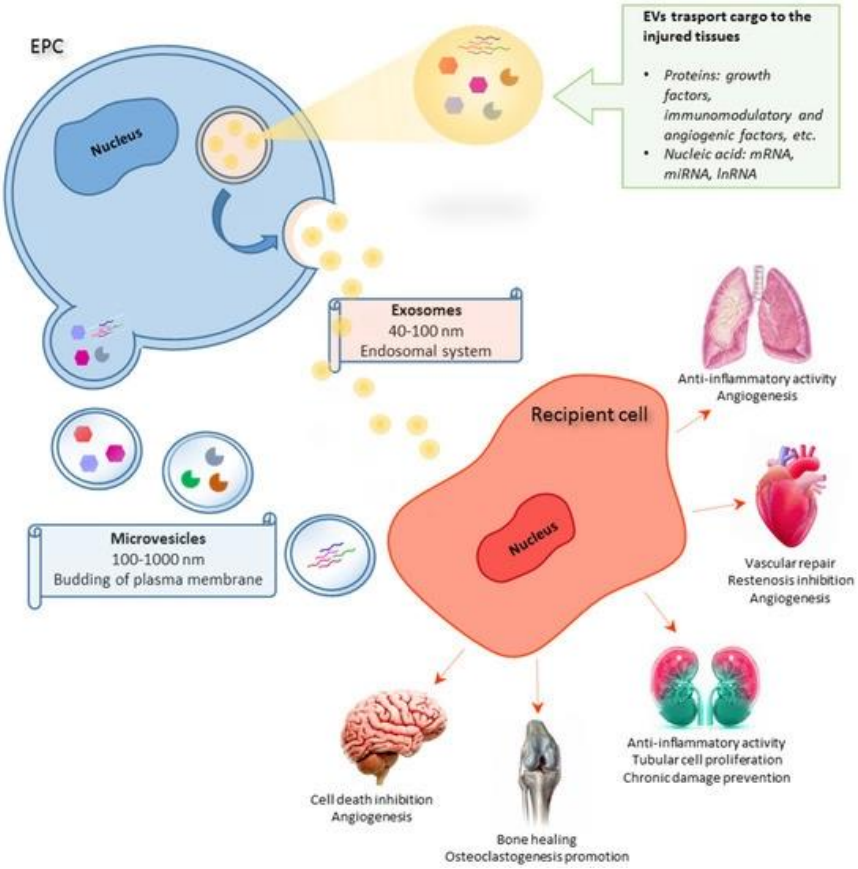


Figure 2. Therapeutic effect of EVs. Image from "Endothelial Progenitor Cell-Derived Extracellular Vesicles: Potential Therapeutic Application in Tissue Repair and Regeneration."

The first report of native hMSC-EV therapy in humans encompassed the treatment of one patient suffering from severe therapy-refractory acute graft-versus-host disease (GvHD) with EVs derived from four different bone marrow donors (Wiklander *et al*., 2019). The therapy was associated with improvement in clinical GvHD symptoms within the first week of hMSC-EV therapy that remained stable 4 months after treatment (Wiklander *et al*., 2019). Another study found that dendritic cells (DCs) secrete exosomes expressing MHC-I/II molecules and T cell co-stimulatory molecules, which suppress tumor growth depending

on T cell function (Chen & Yu, 2022). The resulting immune activation or suppression may be exploited in different disease scenarios like AD (Chen & Yu, 2022). This investigation explores the regenerative effect of hMSC-EVs in cells treated with amyloid beta oligomers. The long term goal is to develop a cell-free therapeutic platform based on hMSC-EVs to treat AD.

MATERIAL AND METHODS

2.1 hMSC culture

The hMSCs were acquired from Tulane Center for Stem Cell Research and Regenerative Medicine, which were isolated from the subcutaneous abdominal adipose tissue from de-identified healthy human donors. The thawed cells were cultured in 4 of 150mm culture dishes in culture media containing α-Minimal Essential Medium (MEM) (Life Technologies, Carlsbad, CA), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1% Penicillin/Streptomycin in a standard culture incubator at 37°C at 5% CO_2. The culture media were changed every 3-5 days in a biosafety cabinet and the cells were passaged at 2,500 cells/cm^2 when 75% confluency was reached. Images were taken using a phase contrast microscope to confirm the morphology of the hMSCs and the cells of passage 4-8 were used in this study.

2.2 mRNA Isolation

hMSCs from one dish were harvested (all operations done in biosafety cabinet). After removing the media, the cells were washed with 5 mL sterile phosphase buffered saline (PBS), then removed. 5 mL trypsin was added, and then the cells were incubated for 5 minutes or until most cells have detached (checked under microscope). Equal volume of culture media were added to neutralize the trypsin. All the liquid (which include cells) was pipetted out and put into a 15 mL sterile tube. The tube was centrifuged at 500g for 5 minutes. The liquid was removed and the cells were resuspended in 1 mL of fresh culture media. The cells were reseeded into 3 of 150 mm plates for more time to grow. The rest was saved for quantification. They were stored at 4°C in 15 mL tubes.

mRNA isolation was performed using a RNeasy mini kit (Qiagen). 10 microliters of beta-mercaptoethanol was added to 1 mL of Buffer RLT in chemical hood with 4 volumes of ethanol (96-100%) added to buffer RPE. The cell sample was centrifuged at 500 g for 5 minutes, and the liquid was pipetted out. 600 microliter buffer RLT was added to the sample in a chemical hood. One volume of 70% ethanol was added and the samples were well mixed by pipetting (in chemical hood). 700 microliters of the sample were transferred to an RNeasy Mini spin column placed in a 2 mL collection tube (supplied by kit) (in chemical hood). The column was centrifuged for 15 s at 8000 g, and the flow through was discarded. 700 microliter buffer RW1 was added to the RNeasy spin column. The column was centrifuged for 15 s at 8000 g, and the flow through was discarded. Then, 500 microliter buffer RPE was added to the RNeasy spin column. The column was centrifuged for 15 s at 8000 g, and the flow through was discarded. This step was repeated again and the 2 mL collection tube was discarded. The RNeasy spin column was placed in a new 1.5 mL collection tube (supplied by kit). 30-50 microliter RNase-free water was added directly to the spin column membrane. The tube was centrifuged for 1 minute at 8000 g to elute mRNA. The mRNA sample was tested using nanodrop for the mRNA amount and the purity (indicated by the ratio of A260/A280).

2.3 Flow cytometry

For flow cytometry analysis, the sample was spun at 300 g for 5 minutes, then resuspended in a 1 mL tube in staining buffer (SB). Equal volume of 4% paraformaldehyde (PFA) was added in a chemical hood. The cells were fixed for 15 minutes. After that, they were spun at 300 g for 5 minutes, and resuspended in 1 mL PBS. 9 volumes of cold 100% methanol (stored at -20 degrees C) was added to the tube. The tube was vortexed and then sit on ice with cover for 15-30 minutes. Equal volume of SB was added to the tube. The tube was spun at 300 g for 5 minutes, then the supernatant was aspirated. The pellet was resuspended in 100 microliter blocking buffer (BB), then transferred to two tubes and incubated at 4°C for 15 minutes. The primary Ab (antibody) was prepared by adding 1 microliter polyclonal rabbit anti-YAP (Santa Cruz) to 100 microliter SB in a 1 mL tube. 50 microliter primary Ab was added to one of the tubes. The other tube was labeled control. Primary Ab incubation was performed at 4°C for 30-60 minutes or overnight. The tubes were washed with 700 microliter SB and spun at 300 g for 5 minutes. The supernatant was decanted. The secondary Ab was prepared in the dark by adding 1 microliter Alexa-Fluor 488 Goat Anti-rabbit IgG to 100 microliter SB. 50 microliter secondary Ab was added to each tube. Secondary Ab incubation was performed

in the dark at 4°C for 30-60 minutes. The tubes were washed with 700 microliter SB. Each sample was resuspended in 150 microliters of SB. The samples were acquired using BD FACSCanto II flow cytometer. Then flow analysis was done using FlowJo software against isotype control.

2.3 EV isolation

The hMSC conditioned media were collected for EV isolation in 2 50 mL tubes. The tubes were spun at 500 g for 5 minutes. The supernatant was decanted into new tubes and the pellet was discarded. The tubes were spun at 2000 g for 10 minutes. The supernatant was decanted into new tubes and the pellet was discarded. The tubes were spun at 10,000 g for 30 minutes. The supernatant was decanted into new tubes and the pellet was discarded. PEG6000 (Sigma) was added to an 8% w/v PEG solution. The tubes were mixed well by inversion and stored overnight at 4° C. The tubes were centrifuged at 3200 rcf at 4°C for 60 minutes, and the supernatant was decanted. The tubes were air dried upside down for 10-20 minutes, with occasional tapping to remove excess liquid. The ultracentrifuge tubes were washed 3 times with bleach, 3 times with tap water, and 3 times with deionized water. The pellet was suspended in 1 mL in PBS in re-usable ultracentrifuge tubes (capless) and the tubes were balanced to two decimals. The vacuum of the ultracentrifuge was turned off. Then the lid was opened, the rotor was inserted, the lid was closed, and the vacuum was turned on. The tubes were spun at 12700 rcf at 4° C for 70 minutes, then they were removed from the ultracentrifuge and air dried for 10 minutes. The pellet was resuspended in 100 microliter PBS and spun at 1500 rpm for 5 minutes. Nanoparticle tracking analysis was performed using Nanosite LM10-HS instrument. The rest of the EV sample was saved for transmission electron microscopy (TEM), western blot assay, and in vitro treatment of cells exposed to Aβ42 oligomers.

2.4 Transmission Electron Microscopy (TEM)

Electron microscopy imaging was used to confirm the morphology and size of EVs. Briefly, EV isolates were resuspended in 30 µL of filtered PBS. For each sample preparation, intact EVs (15 µL) were dropped onto Parafilm. A carbon coated 400 Hex Mesh Copper grid (Electron Microscopy Sciences, EMS) was positioned using forceps with coating side down on top of each drop for 1 h. Grids were rinsed three times with 30 µL filtered PBS before being fixed in 2% PFA for 10 minutes (EMS, EM Grade). The grids were then transferred on top of a 20 µL drop of 2.5% glutaraldehyde (EMS, EM Grade) and incubated for 10 min. Samples were stained for 10 min with 2% uranyl acetate (EMS grade). Then the samples were embedded for 10 min with a mixture of 0.13% methyl cellulose and 0.4% uranyl acetate. The coated side of the grids were left to dry before imaging on the Transmission Electron Microscope HT7800.

2.5 Western blot assay

EV samples were lysed in radio-immunoprecipitation assay buffer (150 mM sodium chloride, 1.0% Trition X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8) with addition of Halt Protease Inhibitor Cocktail. Samples were incubated on ice for 20 min and centrifuged at 14,000 g for 20 min. The supernatant was collected and a Bradford assay was carried out to determine the protein concentration. Protein lysate concentration was normalized to the lowest sample concentration and then denatured at 100°C in 2 x Laemmli Sample buffer for 5 min under reducing conditions (2-Mercaptoethanol). Proteins were separated by 12% BIS-Tris-SDS gels and transferred onto a nitrocellulose membrane. The membranes were blocked for 60 min in 5% nonfat dry milk (w/v) in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) with 0.1% Tween 20 (v/v) (TBST) and incubated overnight in the presence of the primary antibody (CD81) diluted in blocking buffer (1:100) at 4°C. Next, the membranes were washed three times with TBST and incubated with IR secondary antibodies (LI-COR, Lincoln, NE) at 1:5,000 for three hours at room temperature. Membranes were washed three times in TBST and processed using the LI-COR Odyssey (LI-COR) system.

2.6 Culture and treatment of hMSCs

The cells were passaged from the 150 mm culture plates to 4 wells of a 6-well plate (all operations were done in a biosafety cabinet). After removing the media, the cells were washed with sterile PBS (5 mL), then removed. 5 mL trypsin was added, and then the cells were incubated for 5 minutes or until most cells have detached (checked under microscope). Equal volume of culture media was added to neutralize the trypsin. The cell suspension was pipetted out and put into a 15 mL sterile tube. The tube was centrifuged at 500g for 5 minutes. The cell were resuspended in 1 mL of culture media and reseeded into 4 wells of a 6-well plate in 2

mL of culture media. The cells were maintained in a standard culture incubator at 37°C at 5% CO_2.

10 microliter DMSO diluted with 1 mL PBS was added to 2 tubes of Aβ42. The tubes were incubated overnight at 4°C. Then they were added to two of the wells (and the wells were labeled) at 5 μM. After 3 days, the media was changed and hMSC-EVs were added to one of the wells that was treated with Aβ42 oligomers, and one of the wells without Aβ42 oligomers.

2,7 Live/dead assay to evaluate cell viability

Three days after the hMSC-EVs were added, live dead assay was performed on all of the cells according to the manufacturer's protocol (ThermoFisher). 1 microliter calcein and 1 microliter EthD-1 was added to each well in the dark. The cells were incubated for 30-45 min at room temperature. Fluroescent microscope (Olympus IX70) was used to take pictures of live and dead cells. Dead cells were dyed red, and live cells were dyed green. The cells were bleached and then disposed of down the sink. ImageJ software (http://rsb.info.nih.gov/ij)was used to analyze images and determine the cell viability.

3. RESULTS AND DISCUSSION

Images of hMSCs were taken to confirm cell morphology and make sure that there is nothing wrong with the cells before characterizations.

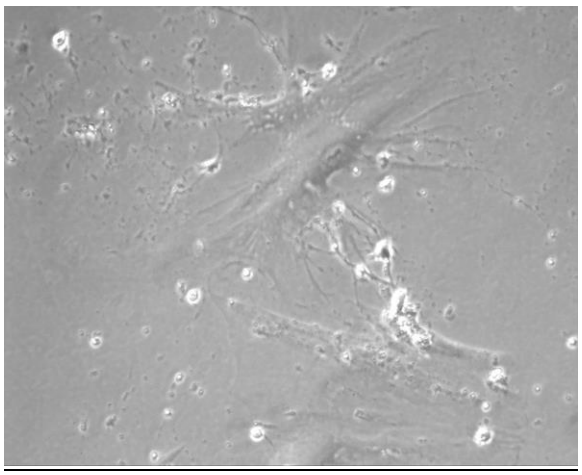


Figure 3. Image of hMSCs.

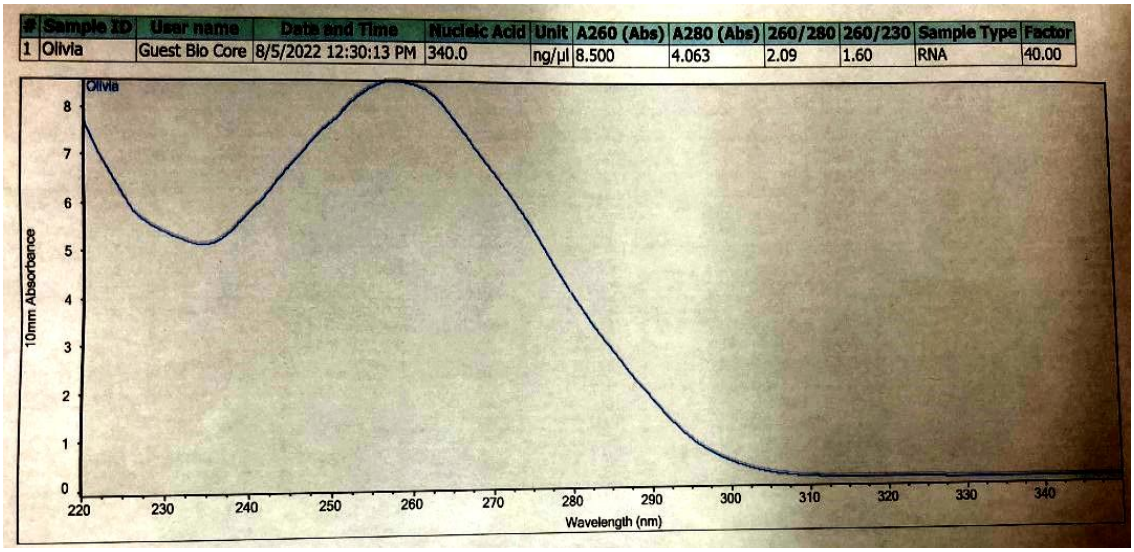


Figure 4. mRNA isolation results. mRNA absorbance wavelength: 260-280 nm.

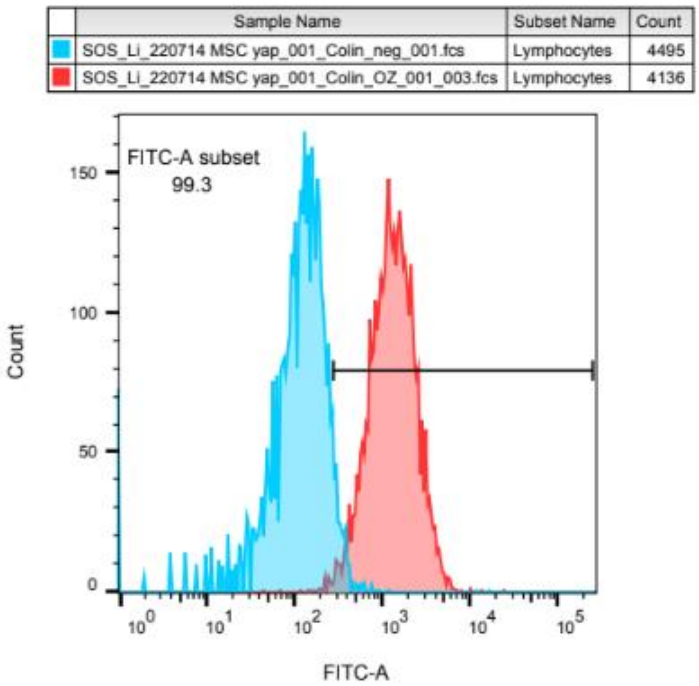


Figure 5. Flow cytometry results.

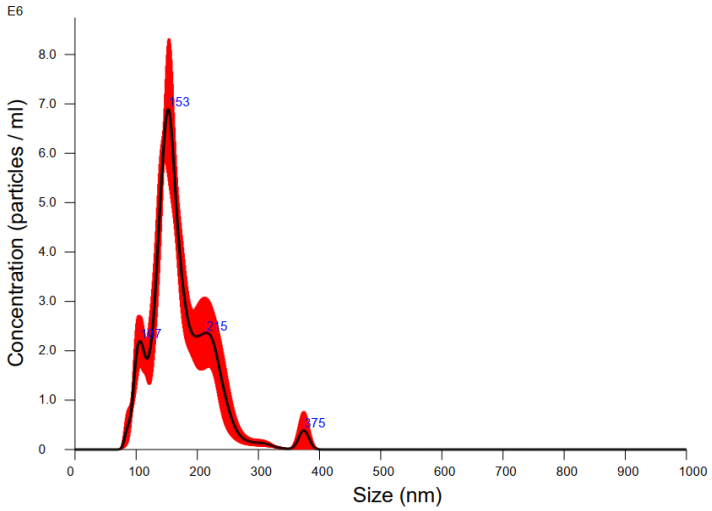


Figure 6. Nanoparticle tracking analysis results. EV size: 100-200 nm

Figure 4 indicates that there is a large amount of mRNA (340 ng/µL with A260/A280 ratio of 2.09) in the hMSC samples, which is important for facilitating the secretion of EVs. Potentially, the mRNA can be used for determining EV biogenesis marker expression (e.g., RAB27A, RAB27B etc.) using reverse transcription polymerase chain reactor (RT-PCR).

Figure 5 shows that hMSCs are positive for the YAP protein expression (>95%), which helps in transcription activities, leading to more mRNAs which include EV biogenesis marker, which in turn leads to EV secretion. The correlation of YAP localization (indicating the biophysical properties and the mecho-transduction from extracellular microenvironment to the cells) and the EV biogenesis needs further investigation in future.

Figure 6 shows that there is a sufficient amount of hMSC-EVs in the sample and the size of majority EVs is in the range of 100-200 nm, which is the typical range for exosomes. This is to make sure there are enough EVs to use for treatment.

TEM confirmed the morphology of cup-shaped EVs, indicating the presence of EVs in the isolated samples

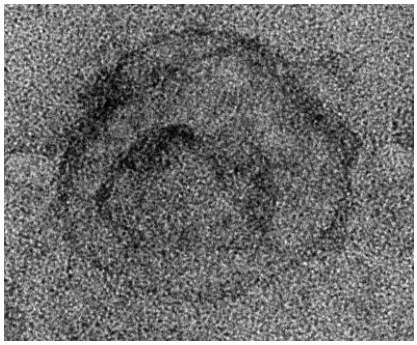


Figure 7. Image of EV.

Western blot confirmed the presence of CD81 (a typical exosomal marker) in the EV samples.

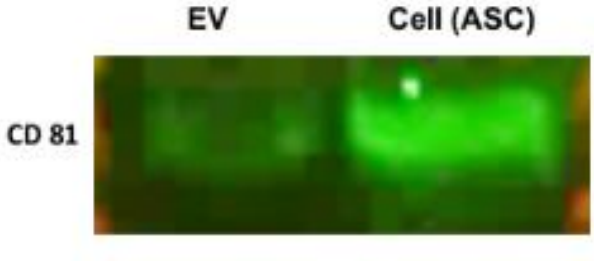


Figure 8. Western Blot Results.

Live/dead assay was performed to determine the percentage of live cells for each of the four treatment conditions (EV+AB42+, EV+AB42-, EV-AB42+, and EV-AB42-). Both the qualitative and quantitative analysis below show a noticeable increase in cell viability in cells treated with both Aβ42 and EVs, compared to cells treated with only Aβ42. The cell viability in cells treated with both Aβ 42 and EVs are similar to the cells without Aβ 42

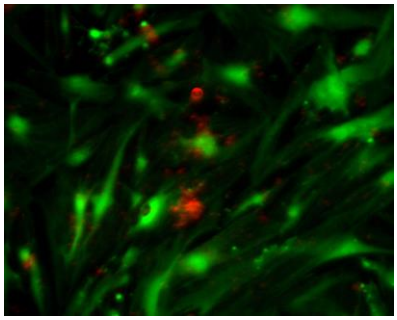


Figure 9. Image of live and dead cells treated with both Aβ 42 and EVs.

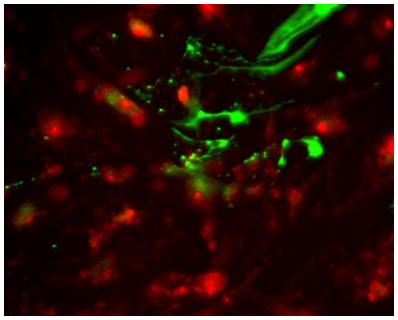


Figure 10. Image of live and dead cells treated with only Aβ 42.

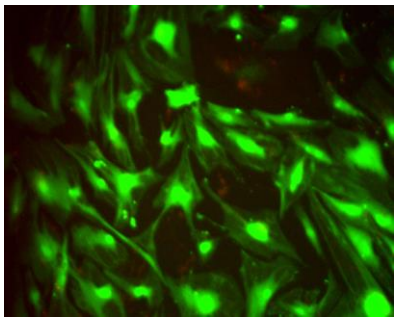


Figure 11. Image of live and dead cells with no treatment.

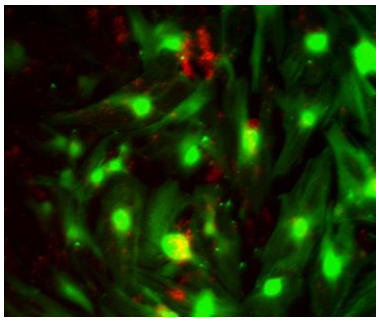


Figure 12. Image of live and dead cells treated with only EVs.

Striked through values on the tables are considered outliers and are taken out of percentages of live cells calculations. In the pie charts, red indicates dead cells and blue indicates live cells.

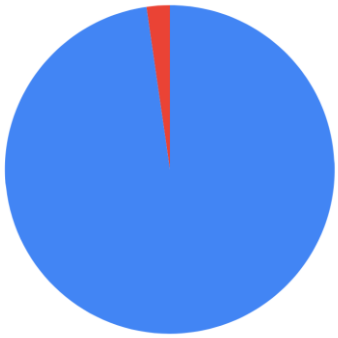
Treatment

Tables 1-4. Amount of live and dead cells in each image, percentage of live cells in each image, along with average and standard deviation.

Figures 13-16. Average percentage of live cells.

Both Aβ 42 and EVs

	Live	Dead	%Live
Image1	16	1	94.12%
Image2	30	0	100.00%
Image3	30	1	96.77%
Image4	16	0	100.00%
Average	23	1	97.72%
S_x	8.08	0.58	2.84%



Aβ 42 only

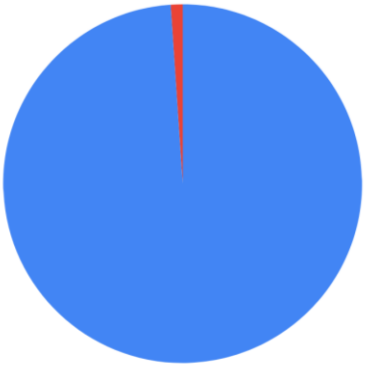
	Live	Dead	%Live
Image1	4	26	13.33%
Image2	4	14	22.22%
~~Image3~~	~~32~~	~~0~~	
Image4	7	11	38.89%
Average	12	13	24.81%
S_x	13.57	10.69	12.97%

EVs only

	Live	Dead	%Live
~~Image1~~	~~1~~	~~0~~	
Image2	35	4	89.74%
Image3	25	4	86.21%
Image4	33	0	100.00%
Average	31	3	91.98%
S_x	5.29	2.31	7.16%

Neither Aβ 42 nor EVs

	Live	Dead	%Live
Image1	30	1	96.77%
Image2	8	0	100.00%
~~Image3~~	~~2~~	~~2~~	
Image4	29	0	100.00%
Average	17	1	98.92%
S_x	14.36	0.96	1.86%



CONCLUSION

This study investigated whether or not hMSC-EVs were able to increase cell viability after the cells were exposed to Aβ42 oligomers. The results shown above support the initial hypothesis that EVs are able to restore cell viability after the cell exposure to Aβ42 oligomers. This is shown by the significant increase in live cells in the samples with both Aβ42 and EVs, EVs only, and neither Aβ42 nor EVs compared to the samples with just Aβ42oligomers. This is likely because EVs carry cargoes that assist in cell function and have an anti-apoptosis effect, which in turn helps cells survive. This experiment provides reason for further research and promise of advancement of hMSC-EV treatments for neural degeneration.

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Conflict of interest: The authors declare that they have no conflict of interest.

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