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DOKUZ EYLÜL UNIVERSITY
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DETERMINATION OF THE NEUROPROTECTIVE EFFECTS OF M2 POLARIZED MICROGLIA-DERIVED EXOSOMAL MIR-191-5P ON THE OXIDATIVE STRESS-MEDIATED NEURONAL DAMAGE

ÇAĞLA NİĞDE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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IZMIR INTERNATIONAL BIOMEDICINE AND GENOME ENSTITUTE

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Dokuz Eylül University İzmir International Biomedicine and Genome Enstitute Department of Genomics and Molecular Biotechnology, Molecular Biology and Genetics graduate program Master of Science student Çağla Niğde has succesfully completed her Master of Science Thesis titled "DETERMINATION OF THE NEUROPROTECTIVE EFFECTS OF M2 POLARIZED MICROGLIA-DERIVED EXOSOMAL MIR-191-5P ON THE OXIDATIVE STRESS-MEDIATED NEURONAL DAMAGE" on the date of 31.08.2022.

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İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ

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LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

ARG1: Arginase 1

BDNF: Brain-derived Neurotrophic Factor

CNS: Central Nervous System

DMEM: Dulbecco's Modified Eagle Medium

EMEM: Eagle's Minimal Essential Medium

FGF: Fibroblast Growth Factor

FIZZ1: Found in Inflammatory Zone 1

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

HMC3: Human Microglial Cell line 3

H_2O_2: Hydrogen Peroxide

IFN-γ: Interferon-γ

IGF: Insulin-like Growth Factor

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

LDH: Lactate Dehydrogenase

lncRNA: Long Non-coding RNA

LPS: Lipopolysaccharide

MHCII: Major Histocompatibility Complex II

miRNA: microRNA

mRNA: messengerRNA

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

ncRNA: Non-coding RNA

NGF: Nerve Growth Factor

PD: Parkinson's Disease

PI: Propidium Iodide

PS: Penicillin-Streptomycin

PVDF: Polyvinylidene Fluoride

RA: Retinoic Acid

ROS: Reactive Oxygen Species

TBST: Tris-buffered Saline with Tween

TEM: Transmission Electron Microscopy

TGF: Transforming Growth Factor

TNF: Tumor Necrosis Factor

YS: Yolk Sac

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DETERMINATION OF THE NEUROPROTECTIVE EFFECTS OF M2 POLARIZED MICROGLIA-DERIVED EXOSOMAL MIR-191-5P ON THE OXIDATIVE STRESS-MEDIATED NEURONAL DAMAGE

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ABSTRACT

Microglia, as resident cells of the brain, are involved in the maintenance of the neuronal environment by activating the response mechanism during brain damage. There are two types of microglial activation: "classically activated" M1 or "alternatively activated" M2 polarization. M2 polarization is described as the "curative" phenotype. M2 polarized microglial cells exert their neuroprotective roles by phagocytosis of neuron debris and secretion of trophic factors. However, the mechanism by which M2 microglia perform its neuroprotective function is largely unknown.

Exosomes are extracellular vesicles that are released into the intercellular environment by different cell types and can cross the blood-brain barrier. Exosomes play a role in cell-cell communication and that exosome cargo can contain microRNAs (miRNAs), proteins, and mRNAs (messenger RNAs), which can be stably transferred to the recipient cell. It has been revealed that exosomal miRNA may have a neuroprotective role during injury.

Hydrogen peroxide (H_2O_2) is encountered in the central nervous system as a reactive oxygen species (ROS). In case of excessive storage in the cell, it causes oxidative stress. Neuronal cells are prone to oxidative stress due to their high oxygen metabolism. Therefore, ROS-induced damage is encountered in the pathogenesis of many neurodegenerative diseases.

The aim of this thesis study is to examine the neuroprotective effects of M2 microglia-derived exosomal miR-191-5p on the H_2O_2-induced neuron damage model in SH-SY5Y cells. As a result of the study, it was determined that the neuroprotective effects of M2 microglia-derived exosomes on neuronal cells under oxidative stress were mediated by miR-191-5p.

Keywords: microglia-derived exosomes, miRNA, neuroprotection

M2 POLARIZE MIKROGLIA KÖKENLI EKSOZOMAL MIR-191-5P'NIN OKSIDATIF STRES ARACILI NÖRONAL HASAR ÜZERINDEKI NÖROPROTEKTIF ETKILERININ INCELENMESI

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ÖZET

Mikroglia, beynin yerleşik hücreleri olarak hasar durumunda tepki mekanizması aktivasyonu ile nöronal ortamın korunmasında görev alır. Mikroglia'nın "klasik olarak aktive edilmiş" M1 veya "alternatif olarak aktive edilmiş" M2 polarizasyonu olarak iki tip aktivasyonu bulunmaktadır. M2 polarizasyonu genel olarak "iyileştirici" fenotip olarak betimlenir ve M2 polarize mikroglial hücreler nöron enkazının fagositozisi ve trofik faktörlerin salgılanması nedeniyle nöroprotektiftir. Fakat M2 mikroglia'nin nöroproteksiyon görevini gerçekleştirdiği mekanizma büyük ölçüde bilinmemektedir.

Ekzozomlar, farklı hücre tipleri tarafından hücreler arası ortama salınan ve kan-beyin bariyerini geçebilen extracellular veziküllerdir. Son çalışmalarla ekzozomların hücre-hücre iletişiminde rol oynadığı ve ekzozom kargosunun, alıcı hücreye stabil bir şekilde aktarılabilen mikroRNA'lar (miRNA'lar), proteinler, mRNA'lar içerebildiği ortaya çıkmıştır. Eksozomal miRNA'nın hasar sırasında nöroprotektif rolü olabileceği ortaya çıkmıştır.

Hidrojen peroksit (H_2O_2) bir reaktif oksijen türü (ROS) olarak merkezi sinir sisteminde rastlanır. Hücrede aşırı miktarda depolanması halinde oksidatif strese yol açar. Nöronal hücreler yüksek oksijen metabolizmalarına sahip olmaları nedeniyle oksidatif strese yatkındırlar. Bu nedenle ROS kaynaklı hasar birçok nörodejeneratif hastalığın patogenezinde rastlanmaktadır.

Bu tez çalışmasının amacı M2 mikroglia kökenli eksozomal miR-191-5p'nin SH-SY5Y hücrelerinde gerçekleştirilen H_2O_2 kaynaklı nöron hasarı modelindeki nöroprotektif etkilerinin incelenmesidir. Çalışmanın sonucunda M2 mikroglia kökenli eksozomların oksidatif stres altındaki nöronal hücre üzerindeki nöroprotektif etkilerinin miR-191-5p aracılığıyla gerçekleştirdiği belirlenmiştir.

Anahtar Sözcükler: microglia kökenli eksozom, miRNA, nöroproteksiyon

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Exosomes are released from various cell types in the central nervous system and take part in intercellular communication by carrying cargo content (such as miRNAs) to target cells. Exosomal contents that reflect the physiological states of the donor cells can trigger various cellular signaling pathways and change the metabolic state in the recipient cell.

To date, limited studies have been conducted on the neuroprotection role of exosomal miRNAs released from M2 polarized microglia on neuronal cells. Although studies have shown that the expression levels of exosomal miRNAs might be altered depending on the microglial activation, the role of these specific miRNAs on microglia-to-neuron interaction can be investigated.

1.2. Aim of Study

The aim of this study is to examine the neuroprotective effects of exosomal miR-191-5p released from M2 polarized human microglia cells on H_2O_2 mediated neuron damage in SH-SY5Y cells.

1.3. Hypothesis of Study

This study hypothesizes that the neuroprotective effects of exosomes released from M2 polarized microglial cells on SH-SY5Y cells are mediated by miR-191-5p.

2. GENERAL INFORMATION

2.1. Microglia

2.1.1. Microglial Cells

Microglia cells account for approximately 10-15% of the entire population of glial cells found in the adult brain (Nayak, Roth et al. 2014). Microglia, defined as the resident immune cells of the brain, is found in the brain parenchyma and they originate from the embryonic yolk sac and migrate to the central nervous system (CNS) via the circulatory system as primitive macrophages (Ginhoux, Greter et al. 2010). During the embryonic phase, microglia cells initially surround the neuroepithelium and then enter the neuroepithelium to invade the brain parenchyma and soon acquire a rounded amoeboid morphology. During postnatal development, however, it acquires a branched morphology showing the resting physiology, and the resting-type microglia morphology is highly ramified in the adult CNS unlike macrophages and dendritic cells (Kaur, Ling et al. 1985, Kaur and Ling 1991, Nayak, Roth et al. 2014). They help maintain CNS tissue homeostasis by supporting neuronal survival, cell death and synaptogenesis, and CNS development (Nayak, Roth et al. 2014). In addition, these resting cells can be activated by pathological stimuli in the CNS in various conditions such as infection, brain trauma, stroke, and neurodegeneration (Ling, Ng et al. 2001). During activation, these cells with branched morphology transform into amoeboid reactive cells. Thus, they proliferate rapidly in response to stimuli, and their activation is characterized by the production and rapid secretion of a wide range of cytokines, chemokines, and other immune mediators (Lawson, Perry et al. 1992, Nakamura 2002, Perry 2004, Ajami, Bennett et al. 2007). Furthermore, cellular experiments, clinical studies, and models have shown that chronic activation of microglia in the CNS can have detrimental effects on the survival and normal functioning of

neurons, as well as on the neuroregeneration of stem cells (Banati, Gehrmann et al. 1993).

2.1.2. Microglia History

In the past, it was known that all tissue macrophages came from the same origin, and microglia progenitors were known to invade the brain in the early embryonic period. Microglia colonization in the brain during the early embryonic period is a process seen among vertebrate species (Ginhoux and Prinz 2015). In the first relevant studies on microglial cells, the observation that these cells were present in the early embryonic development led to the assumption that microglial cells originated from embryonic progenitors. Rio-Hortega later created a dilemma by suggesting that these embryonic progenitors are meningeal macrophages that invade the brain during early embryonic development. Concerned members of the scientific community have hypothesized that microglial cells may originate from blood monocytes (Ginhoux and Prinz 2015). Later, microglial cells were observed on 9.5 day of the embryonic development in rodent brain and therefore it was concluded that the precursors of microglia originate from the yolk sac (YS). The origin of microglia from the yolk sac is a unique feature that distinguishes these cells from other tissue macrophages. Rudolf Virchow defined glial cells as a member of the cell population in the brain other than neurons in 1856 (Wolf, Boddeke et al. 2017). In addition, it has been accepted by the scientific community that there is a specific cell type that plays an important role in brain diseases such as dementia, Multiple Sclerosis, Alzheimer's disease, and brain damage (Wolf, Boddeke et al. 2017).

The concept of microglia was widely disseminated by Pio del Rio-Hortega (Kettenmann, Hanisch et al. 2011). Rio-Hortega published four articles explaining the distinguishing features of the concepts of astrocyte, microglia, and oligodendrocyte, highlighting the concept of glia (Wolf, Boddeke et al. 2017). Thus, the first definitive classification of microglia as a specialized cellular member of the CNS and a phagocytic and migrating cell population within the CNS was made by Rio-Hortega (Kettenmann, Hanisch et al. 2011). First, Rio-Hortega named these cells “microgliocytes” and stated that these cells were of mesodermal origin (Wolf, Boddeke et al. 2017). Later, Rio-Hortega coined the term “microglia” and defined microglial cells as

components that do not belong to the neuronal cell population of the CNS and that have distinctive features from oligodendrocytes (Ginhoux and Prinz 2015). Pio del Rio-Hortega proposed many features defining microglia and are still valid today, suggesting that microglial cells are of mesodermal origin and have amoeboid morphology. He also suggested that they migrate through veins and white matter in the specific regions where they reside and move, and that they have a ramified resting state (Kettenmann, Hanisch et al. 2011).

2.1.3. Microglial Function

Microglia cells, the resident macrophages of the brain, are associated with the function and development of the CNS and the pathogenesis of several inflammatory diseases and neurodegenerative diseases in the brain (Sarlus and Heneka 2017). Under normal conditions, they self-replicate independently of circulating monocytes, but they are partially managed by circulating monocytes under disease conditions (Ginhoux, Greter et al. 2010, Sarlus and Heneka 2017). Although microglia's main function is immune surveillance and immune defense by phagocytosis, they also show trophic support for tissue repair and maintaining homeostasis in the CNS (Sarlus and Heneka 2017, Hansen, Hanson et al. 2018). Also, microglia are required during the development of the CNS, for example, they are involved in neurogenesis in conditions such as the establishment and development of neuronal circuits and maintenance of the neuronal cell pool (Czeh, Gressens et al. 2011, Pierre, Smith et al. 2017). Another function of microglia is involvement in myelination and normal vascularization of the brain and retina (Pierre, Smith et al. 2017). Microglia produce a variety of signaling molecules, including cytokines, neurotransmitters, and extracellular matrix proteins that are involved in the regulation of synaptic activity and neuronal plasticity. Therefore, microglia dysfunction can lead to interference of neuronal activity and disruption of phagocytic activity. In addition, by expressing receptors including those for sensing neurotransmitters, microglial cells are able to involve cell-to-cell communication with other macroglia, neurons, and immune cells through various signaling pathways (Kettenmann, Hanisch et al. 2011). Microglia are also associated with the formation of learning-related synapses through brain-derived neurotrophic factor (BDNF) signaling, and thus are involved in higher cognitive functions such as learning and

memory (Parkhurst, Yang et al. 2013). Microglia have two states, resting and activated, and they perform morphological and functional changes called microglia activation when induced by stimuli and changes in brain homeostasis (Choi, Aid et al. 2012). When activated, microglial cells rapidly retract, their processes shorten and accelerate, and soma growth occurs (Carta and Pisanu 2013).

2.1.4. Microglia Polarization

With changing environmental conditions, microglia often can change their phenotype to maintain tissue homeostasis (Orihuela, McPherson et al. 2016). Microglial activation in the CNS is heterogeneous and can be categorized into two opposing types, the classical (M1) or alternative (M2) phenotype (Sica and Mantovani 2012, Tang and Le 2016).

M1 activation is typically induced by stimuli such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS) and is defined as classical activation (Colonna and Butovsky 2017) (Figure 1). M1-type microglia produce and secrete inflammatory cytokines and chemokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, and IL-12 (Colonna and Butovsky 2017). They contribute to neurological damage in the CNS by expressing proteins such as Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase [iNOS], major tissue compatibility complex-II (MHCII), integrins (CD11b, CD11c), costimulatory molecules (CD36, CD45, CD47) and Fc receptors (Nguyen, Blomster et al. 2017).

M2 microglia activation is induced by anti-inflammatory cytokines such as IL-4 and IL-13 and is defined as alternative activation (Colonna and Butovsky 2017) (Figure 1). As a result of M2 activation, anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β)], growth factors such as insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), and nerve-derived growth factor (NGF), neurotrophic growth factors BDNF (Colonna and Butovsky 2017). They also secrete pro-survival factor progranulin and promote the induction of mannose receptor (CD206) found in inflammatory zone 1 (FIZZ1), chitinase-3-like-3 (Chil3), arginase 1 (Arg1) (Colonna and Butovsky 2017). M2 microglia involve in the

phagocytosis of cellular and metabolic residues, unnecessary cells, and misfolded proteins, supporting extracellular matrix reconstruction and promoting neuron survival by induction of neurotrophic factors (Tang and Le 2016). Overall, M1 microglia cause inflammation and neurotoxicity, while M2 microglia have anti-inflammatory and curative functions (Colonna and Butovsky 2017).

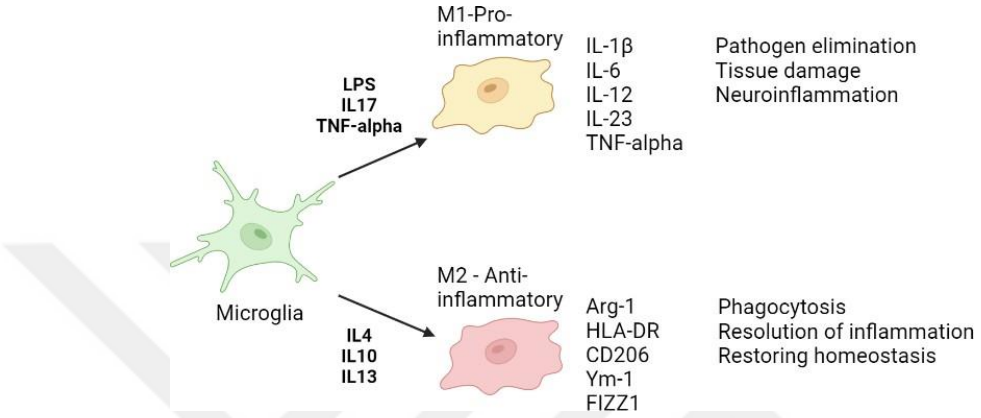


Figure 1. Microglia polarization (created in Biorender)*

2.1.5. Polarized Microglia Enriched miRNAs

Emerging studies show that miRNAs enriched in polarized microglia regulate key molecular pathways, contribute to microglia function, and influence the outcome of polarization. There are limited studies examining the regulatory role of miRNAs altered in polarized microglia (Table 1).

Table 1. The altered expression levels of miRNAs enriched in polarized microglia and targeted pathways

Polarization	miRNA	Expression	Target	References
M1	miR-155	Up-regulated	Anti-inflammatory cascades	(Freilich, Woodbury et al. 2013)
M1	miR-155	Up-regulated	Protective immunity cascades	(Cardoso, Guedes et al. 2012)
M1	miR-124, miR-689	Down-regulated	Canonical inflammatory cascades	(Freilich, Woodbury et al. 2013)
M1	miR-Let7A	Up-regulated	Autophagy-related cascades	(Song, Oh et al. 2015)
M2	miR-711, miR-124	Down-regulated	Peroxisome-proliferator-related receptor gamma pathway	(Freilich, Woodbury et al. 2013)
M2	miR-145	Up-regulated	Peripheral monocyte and macrophage differentiation	(Freilich, Woodbury et al. 2013)

2.2. Exosome

2.2.1. Exosome

Exosomes were firstly discovered by Pan and Johnstone in 1983 (Simpson, Lim et al. 2009, Hessvik and Llorente 2018). Exosomes, which are membrane-enclosed small extraceullular vesicles 30-200 nm in diameter, are found in almost all biological fluids

(Cunnane, Weinbaum et al. 2018). They are released into the extracellular space from most cell types after fusion with the plasma membrane (Hessvik and Llorente, 2018). Lipids and proteins are major components of exosome membranes, and in addition to proteins, several nucleic acids have recently been identified in exosome content, including mRNAs, miRNAs, and other non-coding RNAs (ncRNAs) (Hessvik and Llorente, 2018).

Exosomes are produced from the late endosome, during which ESCRT protein complexes coordinate cargo loading and vesicle release (Baietti, Zhang et al. 2012). Exosomes are surrounded by two lipid-containing bilayers enriched with specific membrane proteins such as tetraspanins, cholesterol, and sphingomyelin (Figure 2). The role of microglial exosomes in antigen presentation and transfer of antigens shows that microglial exosomes have a function in the immune response and are vital in managing interaction and communication in the CNS.

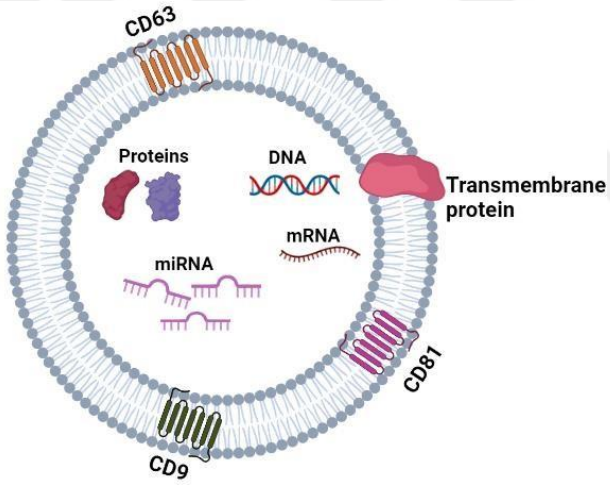


Figure 2. Exosomal structure and cargo (created in Biorender)*

2.2.2. Exosomal miRNAs in Microglia to Neuron Interactions

Cargoes in exosomes are enriched with nucleic acids such as miRNAs, and functional miRNAs are involved in cellular metabolic events, homeostasis, and functions in target cells. Moreover, miRNAs in exosome content can be an indicator of the activity status of the donor cells. There several studies investigating the functional roles of miRNAs, and their effects on donor cells during microglia-to-neuron interaction (Table 2).

Table 2. Exosomal miRNAs involved in microglia-to-neuron interaction

miRNA	**Effects**	**References**
miR-124-3p	Inhibition of neuronal inflammation, inducing neurite length	(Huang, Ge et al. 2018)
miR-124-3p	Inhibition of autophagy	(Li, Huang et al. 2019)
miR-124	Neuroprotection	(Song, Li et al. 2019)
miR-124-3p	Cognitive improvement, preventing neurodegeneration	(Ge, Guo et al. 2020)
miR-Let7a/b	Neuronal death	(Mukherjee, Akbar et al. 2019)
miR-5121	Reducing neurite length, inhibition of synaptic plasticity	(Zhao, Deng et al. 2021)
miR-137	Inhibition of neuronal apoptosis	(Zhang, Cai et al. 2021)
miR-383-3p	Necroptosis	(Wei, Xu et al. 2020)
miR-151-3p	Promoting axonal regrowth, inhibition of apoptosis	(Li, Qin et al. 2021)
miR-672-5p	Inducing axonal regeneration, inhibition of pyroptosis	(Zhou, Li et al. 2022)
miR-124-3p	Neuronal repair	(Zhao, Wang et al. 2022)

2.2.3. miR-191-5p

miR-191-5p is a 23 nucleotide-long intragenic miRNA located within intron 1 of the DALRD3 host gene on chromosome 3 (Zhang, Wu et al. 2018). Our groups investigated the exosomal miRNA expression changes released from M2 polarized HMC3 cells in 2018 within the scope of a BAP Project (Project no: 2018.KB.SAG.114), (Table 3). According to the results, miR-191-5p showed the most significant increase in expression when the data was ranked by p-value based on FC (fold change) cut-off. Based on the data, determining the effects of miR-191-5p in microglial exosomes on regulating neuronal cell death and neurite outgrowth after oxidative stress-related neuronal damage is planned. The expected results may expand the

understanding of the miR-191-5p function and its effects on neuroprotection manipulated by M2 microglial exosomes.

Table 3. The fold changes and p-values for the increase of exosomal miRNA expression levels obtained from M2 polarized HMC3 cells compared to the control group were determined in a project (2018.KB.SAG.114) conducted by our groups and supported by Dokuz Eylul University

miRNA	FC	P value
hsa-miR-191-5p -- hsa-mir-191	**3.045803**	**3.99E-13**
hsa-miR-10401-3p --hsa-mir-10401	4.810946	0.00000411
hsa-miR-451a --hsa-mir-451a	4.581337	0.000293
hsa-miR-6875-5p -- hsa-mir-6875	3.989084	0.006921
hsa-miR-3152-5p --hsa-mir-3152	3.835154	0.004453
hsa-miR-1908-5p --hsa-mir-1908	3.672245	0.0000426
hsa-miR-2110 --hsa-mir-2110	3.399664	0.0000625
hsa-miR-653-5p --hsa-mir-653	6.580935	0.005216
hsa-miR-3928-3p -- hsa-mir-3928	2.97583	0.000124
hsa-miR-92b-5p --hsa-mir-92b	2.868308	7.47E-07
hsa-miR-139-5p --hsa-mir-139	2.835613	0.000201
hsa-miR-1248 -- hsa-mir-1248	2.632891	0.000764
hsa-miR-423-5p --hsa-mir-423	2.37065	2.36E-09
hsa-miR-125a-5p --hsa-mir-125a	2.356356	2.74E-08
hsa-miR-1292-5p --hsa-mir-1292	2.296553	0.000356
hsa-miR-122-5p --hsa-mir-122	2.285146	0.00544
hsa-miR-501-5p -- hsa-mir-501 (3p)	2.101839	0.0091
hsa-miR-877-5p --hsa-mir-877	2.091119	0.00000711
hsa-miR-151a-5p -- hsa-mir-151a	2.033013	1.98E-07
hsa-miR-151b --hsa-mir-151a	2.031634	0.00000021
hsa-let-7c-5p --hsa-let-7c	2.009297	4.01E-07

2.3. H_2O_2 Induced Neuronal Injury

Oxidative damage has been causally associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotropic lateral sclerosis, ischemia, and excitotoxicity (Smith, Carney et al. 1991, Olanow 1992, Richardson 1993). Oxidative damage is caused by cell lysis, oxidative burst, or reactive oxygen species (ROS), which can be produced by the cell due to the presence of excess free transition metals (Bellavite 1988, Giulian, Vaca et al. 1993). ROS can also be produced as a byproduct of cellular respiration under normal conditions, its main task being electron transport in the mitochondria (Richter and Kass 1991). Conversion of ROS to less reactive species and inactivation of ROS by antioxidants are among the cellular defense mechanisms against oxidative damage (Yu 1994). However, imbalances in ROS production and deactivation, and activity of cellular protection mechanisms can cause excessive direct exposure of cells to ROS and thus free radical-induced damage.

H_2O_2, as one of ROS, is an important signal molecule found in the synaptic and neuronal environment of CNS (Rice 2011). It is a relatively stable and fat-soluble molecule and can diffuse into the cytoplasm (Halliwell 1992, Ward and Giles 1997). Under physiological conditions, H_2O_2 production is stabilized by enzymes such as catalase and glutathione peroxidase, which reduce H_2O_2 to H_2O and O_2 (Halliwell, 1992). Neuronal cells are more prone to oxidative stress due to their high oxygen consumption metabolism and polyunsaturated fatty acids content. Therefore, excessive ROS accumulation in the cell causes cell death andapoptosis by creating oxidative stress, causing damage to DNA, protein, and lipid membranes, and impairing neuronal cell functions (Dribben, Creeley et al. 2011, Meng, Qin et al. 2022). Thus, oxidative damage is involved in the pathogenesis of many neurodegenerative diseases.

3. MATERIALS AND METHODS

3.1. Type of Study

The thesis study is a case-control and involves *in vitro* experiments.

3.2. Time and Place of Study

The study took place at İzmir Biomedicine and Genome Institute between February 2022 and July 2022.

3.3. Materials of Study

HMC3 human microglia cells and SH-SY5Y human neuroblastoma cell lines were used as materials in this study.

3.4. Variables of Study

Dependent variable: IL-4

Independent variable: M2 polarization on HMC3 cell line

3.5. Tools for Data Collection

3.5.1. HMC3 Cell Culture

The human microglia cell line HMC3 was purchased from the ATCC (Manassas, VA). Briefly, HMC3 cells were cultured in Eagle's minimal essential medium (EMEM) (Sigma Aldrich, USA) supplied with 10% exosome-depleted fetal bovine serum, and 100 µg/ml Penicillin-Streptomycin (PS) (Thermo Scientific, Inc., Rockford, IL) in an incubator with the humidified atmosphere at 5% CO_2 at 37 °C. The complete EMEM medium was changed every two days, and HMC3 cells were passed every week once cells were grown to 80% confluency. For stimulation of M2-activated microglia, HMC3 cells were treated with 10 ng/ml interleukin

4 (IL-4) for 24 hours.

3.5.2. Validation of M2 polarization in HMC3 cells by qPCR

HMC3 microglia were seeded into 100mm cell culture dish with 1 x 10^6 cells density and cells were incubated overnight with 37 ℃ and 5% conditions. After incubation, HMC3 microglial cells were pretreated with 10 ng/ml IL-4 concentrations for 24 hours. After the treatment, cells were collected from flasks by using cell scrapers. Total RNA was isolated from microglia cells using Trizol reagent (Invitrogen, USA, catalog no:15596026) according to manufacturer's instructions. RNA purity and concentrations within the isolated sample were determined via Nanodrop. cDNAs were synthesized by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo, USA). Total RNA (2 µg) and random hexamer primer were used for amplification by SYBER green technology in GoTaq qPCR Master Mix (Promega, USA). Human primers for CD206 and HLA-DR M2 polarization markers were used. For normalization of data, endogenous housekeeping gene of Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was used. Primer sequences of CD206 are: Forward- 5'TCT TTG CCT TTC CCA GTC TCC 3', Reverse: 5' TGA CAC CCA GCG GAA TTT C 3'.
Primer sequences of HLA-DR are: Forward- 5'AGT CCC TGT GCT AGG ATT TTT CA 3', Reverse: 5'ACA TAA ACT CGC CTG ATT GGT C 3'.

3.5.3. Isolation of Microglia-derived Exosomes

After IL-4 treatment, 8ml cell media was collected for exosome isolation by differential centrifugation and ultracentrifugation. Cell media was centrifuged at 1,000 rpm for 5 minutes at 4°C, and the pellet was discarded. The supernatant was transferred to a new tube and centrifuged at 3,000 rpm for 10 minutes at 4 ℃ and the pellet was discarded. The supernatant was centrifuged at 4,000 rpm for 30 minutes at 4 ℃ and the supernatant was transferred to an ultracentrifuge tube. Ultracentrifuge was performed at 22,000 rpm for 140 minutes at 4 ℃, then the pellet was resuspended in 8ml sterile PBS. PBS suspension was ultracentrifuged at 22,000 rpm for 140 minutes at 4 ℃, and the pellet was resuspended in 500 µl PBS. Exosome suspension was stored at -80 ℃ until further experiments were performed.

3.5.4. Determination of Protein Level of Exosome by BCA Assay

To measure protein concentration in exosomes, 5 µl of exosome suspension was lysed in 55 µl M-PER Mammalian Protein Extraction Reagent (Thermo Scientfic, Inc., Rockford, IL) by vortexing for 10 minutes. Then, protein concentration was determined by using BCA Protein Assay Kit (Takara, Japan).

3.5.5. Exosome Characterization by Western Blotting

Exosomes were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientfic, Inc., Rockford, IL) for 10 minutes of vortexing. Exosomal proteins (10 µg) were separated by 10% SDS-PAGE and then proteins were transferred to 0.45 µm polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, USA). PVDF membranes were blocked with 3% milk powder (Bioshop, Canada) in tris-buffered saline with tween (TBST) for 1 hour at room temperature. Membranes were incubated with CD63 and Calnexin primary antibodies at 4 °C overnight. Incubation with secondary antibodies was performed for 1 hour at room temperature. After washing steps with TBST, membranes were imaged using chemiluminescence via using Horseradish Peroxidase Substrate reagent (Millipore, USA). CD63 (Santa Cruz-5275, anti-mouse, MX.49.129.5) and Calnexin (SC-11397, anti-rabbit) primary antibodies were taken from from Santa Cruz (California, USA), (1:1000); HRP-conjugated secondary antibody was taken from Cell Signaling (Massachusetts, USA), (1:2000).

3.5.6. Transmission Electron Microscopy (TEM)

Exosome morphologies were determined by TEM. Exosome suspension (5 µl) was dropped onto the parafilm. 100 mesh Formwar-coated copper grid (Electron Microscopy Sciences, USA) was left onto that exosome suspension. After incubation for 1 hour at room temperature, the grid was washed on 30 µl of PBS for 2 minutes long and this washing step was repeated three times in total. Then, grid was fixed with a drop of 2% paraformaldehyde by incubation for 10 minutes at room temperature and the washing steps were repeated as before. The grid was contrasted with a drop of 2% uranyl acetate for 15 minutes at room temperature

and in the dark. The grid was left to be dried for 5 minutes. Exosome suspension was diluted 1:5 before adsorption to avoid aggregation. Dried grids were examined with a Zeiss Sigma500 transmission electron microscope in the electron microscopy core at the Izmir Biomedicine and Genome Center of Dokuz Eylül University.

3.5.7. NTA

The size distribution of exosome particles derived from microglia was measured with the NanoParticle Tracking analysis by using NanoSight (NS300) according to the manufacturer's instructions in HÜNİTEK – Hacettepe University.

3.5.8. qRT-PCR Validation of miRNA Expression in HMC3 Microglia-derived Exosomes

To validate the increase of miR-191-5p expression in M2 polarized HMC3 cells derived exosomes compared to control, total exosomal RNA (35 ng) from exosomes was used as a template for cDNAs using miScript II RT Kit (Qiagen, Hilden, Germany). cDNA synthesis was conducted according to the manufacturer's protocol. The cDNA synthesis program was performed as 1 cycle at 37 °C for 60 minutes and 1 cycle at 95 °C for 5 minutes. For the normalization of data 10 pmol syn-cel-miR-39 miRNA (Qiagen, Hilden, Germany, Lot:274797028) was added to RNA mixture.

To determine the level of miR-191-5p, miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used. 2.5 µl of cDNA was added to each reaction performed at 25 µl reaction volume. Amplification steps involved 1 cycle at 95 °C for 15 minutes, 50 cycles at 95 °C for 15 seconds, and 1 cycle at 60 °C for 1 minute, and all steps were performed on LightCycler 480 II (Roche, Switzerland). hs-miR-191-5p miscript primer assay 10x (Qiagen, Hilden, Germany, Lot: 2019032701111) and ce-miR-39 miscript primer assay 10x (Qiagen, Hilden, Germany, Lot: 200358401) were used.

3.5.9. SH-SY5Y Cell Culture

The human neuroblastoma cell line SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM) (Gibco, Gaithersburg) enriched with 10% fetal bovine serum (FBS), and 100 µg/ml Penicillin Streptomycin (PS) (Thermo Scientific, Inc., Rockford, IL) in

an incubator at 5% CO_2 at 37 °C with 50 µg/ml PDL-coating (Sarstedt, Germany). The complete DMEM medium was refreshed twice a week, and SH-SY5Y cells were passed every week once cells were grown to 80% confluency. For neuronal differentiation, SH-SY5Y cells were treated with 10 µM retinoic acid (RA) for 7 days. The cell medium was replaced every two days. Then, SH-SY5Y cells were used for further experiments. To induce neuronal damage, the SH-SY5Y cell line was treated with 25 µM H_2O_2 for 24 hours.

3.5.10. Labeling and Uptake of Exosomes by HMC3 Microglia Cells by PKH67 Staining

Exosomes were labeled by using PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma Aldrich, USA). Protein concentration (100 µg) equivalents of exosomes suspended in 250 µl PBS were added to 250 µl of Diluent C and suspension was mixed with 500 µl of PKH67 dye diluted in Diluent C for 5 minutes in dark. The dye reaction was inhibited by 1000 µl of exosome depleted FBS (Thermo Scientific, Inc., Rockford, IL) addition to the mixture and incubated for 1 minute. The exosome dye mixture was centrifuged at 100,000 x g for 70 minutes. Then, the exosome pellet was washed with PBS at 100,000 x g for 30 minutes twice. Finally, the exosome pellet was resuspended in 100 µl of PBS and stored at -80 °C.

SH-SY5Y cells were seeded into a 96-well plate (Sarstedt, Germany) containing 10% exosome-depleted FBS and 1% penicillin-streptomycin and incubated for 24 hours. After retinoic acid differentiation was performed, SH-SY5Y cells were treated with 10 µg/ml exosomes for 24 hours.

3.5.11. LDH Release Assay

Exosomal miR-191-5p-induced cytotoxicity on differentiated SH-SY5Y cells was measured by using Cytotoxicity Detection KitPLUS (Roche Diagnostics, Germany). SH-SY5Y cells (2 x 10^4 cells/well) were seeded in a 96-well plate (Sarstedt, Germany) with 200 µl of final media volume and incubated overnight at 37 °C with 5% CO_2 condition. After incubation, cells were differentiated for 7 days and treated with 25 µM H_2O_2 at the end of the differentiation. After the treatment, 10 µg exosomes were added to culture media and incubate for 24 hours. For the maximal group, cells were treated with LDH cell lysis buffer. Then, 200 µl cell culture

media was collected, and LDH activity in sample was quantified according to descriptions in manufacturer's protocol. Absorbance was measured at 492 nm and 630nm (reference wavelength) via MultiSkan Go spectrophotometer (Thermo Scientific, USA) as a microplate reader. Cytotoxicity data was presented as a percentage of the total LDH amount released from lysed cells, and calculation was performed by the given formula:

Cytotoxicity = (OD of Sample – OD of Control) / (OD of Maximal Release – OD of Control) ∗100

3.5.12. PI Staining

Propidium iodide (PI) (Sigma-Aldrich, USA) dye was used to stain dead cells. SH-SY5Y cels (10,000 cells/well) were seeded into a 96-well plate and a 200 µl of fresh medium was added to each well. Cells were incubated overnight at 37 ℃ under a 5% CO_2 condition. After incubation, cells were treated with RA for 7 days and with H_2O_2 for 24 hours. Then, cells were incubated with 10 µg exosomes for 24 hours. After incubation was completed, PI dye (50 µg/ml) was added to each well and incubated for 15 minutes in dark at room temperature. PI stained (positive) cells were observed using a fluorescent microscope (Olympus IX-71, Japan). PI positive and negative cells were counted using ImageJ 1.51n software.

3.5.13. Neurite Outgrowth Assay

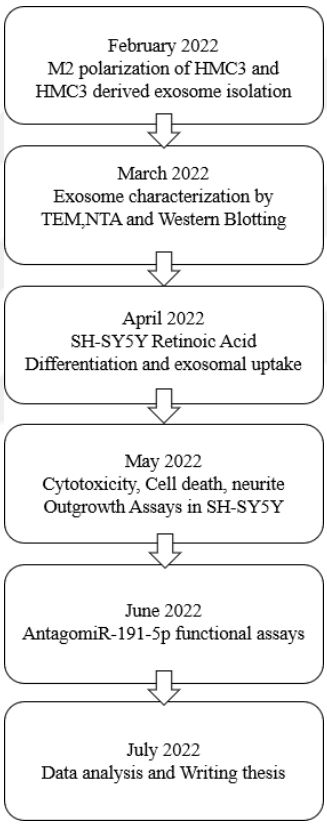
SH-SY5Y cells were seeded in 96-well plates at a density of 15 x 10^3 cells/well, and retinoic acid differentiation for 7 days was performed before treatments and imaging. The brightness, sharpness, and contrast of each image were standardized. For all the experiments, cell count, neurite count, and neurite length were performed. Three different field from each well were taken, and wells were seeded into five wells for each group. A neurite was defined as greater than 20 µm in length, and only the cells with neurites were counted. The neurite length results have presented as the ratio of total neurite length/ neurite number, and the average neurite number results are presented as the ratio of total neurite number/ cell number.

3.5.14. Exosome Transfection with miR-191-5p Inhibitor

For loading of exosomes with miR-191-5p inhibitor (IDT, Integrated DNA Technologies,

USA) and negative control (IDT, Integrated DNA Technologies, USA), inhibitor/control was mixed with Lipofectamine RNAimax (Invitrogen, USA) at the final concentration of 2 µmol /ml in 100 µl of siRNA buffer, and the mixture was incubated at room temperature for 10 minutes. Then 200 µl exosome suspension was added to the mixture and 30 minutes incubation was performed at room temperature. To decrease the activity of the Lipofectamine reagent, the final solution was kept at +4 ℃. Loaded exosomes were incubated with SH-SY5Y cells in a 96-well plate for 24 hours.

3.6. Study Plan and Calendar



3.7.Data Evaluation

Prism 8.0 (Graphpad, USA) software was used to visualize the data as graphs and the Mann-Whitney U test was applied for comparison between data groups. The significance level was accepted as $p<0.05$ in all tests. Also, all data are given as mean ± S.E.M with $*p < 0.05$,

**p < 0.01 and ****p < 0.0001.

3.8. Limitations of Study

The HMC3 cell line was used as the microglial cell and had a low response to stimuli. The level of isolated exosomes secreted by HMC3 microglia and the level of miRNAs in exosome content were highly low.

Since the study carried out is an *in vitro* experiment, the results need to be confirmed by *in vivo* studies by primary cell culture experiments.

3.9. Ethics Committee Approval

Ethics committee approval has the 2022-004 protocol number under the date of 04.03.2022.

4. RESULTS

4.1. HMC3 Microglia M2 Polarization

IL-4 was employed as potent M2 polarizing stimuli. CD206 and HLA-DR are phenotypic markers useful to identify HMC3 microglial cells which were M2-polarized. HMC3 microglial cells were exposed to IL-4 for 24 h, and mRNA expression levels were assessed. Stimulation with IL-4 increased mRNA expression of studied M2 microglia marker CD206 (1.464 ± 0.1423, p=0.0317) (Figure 3A). Also, the mRNA expression level of M2 microglia marker HLA-DR enhanced (1.410 ± 0.09127, p=0.0079) (Figure 3B). Thus, IL-4 stimulation switched the HMC3 cells to an M2 polarization.

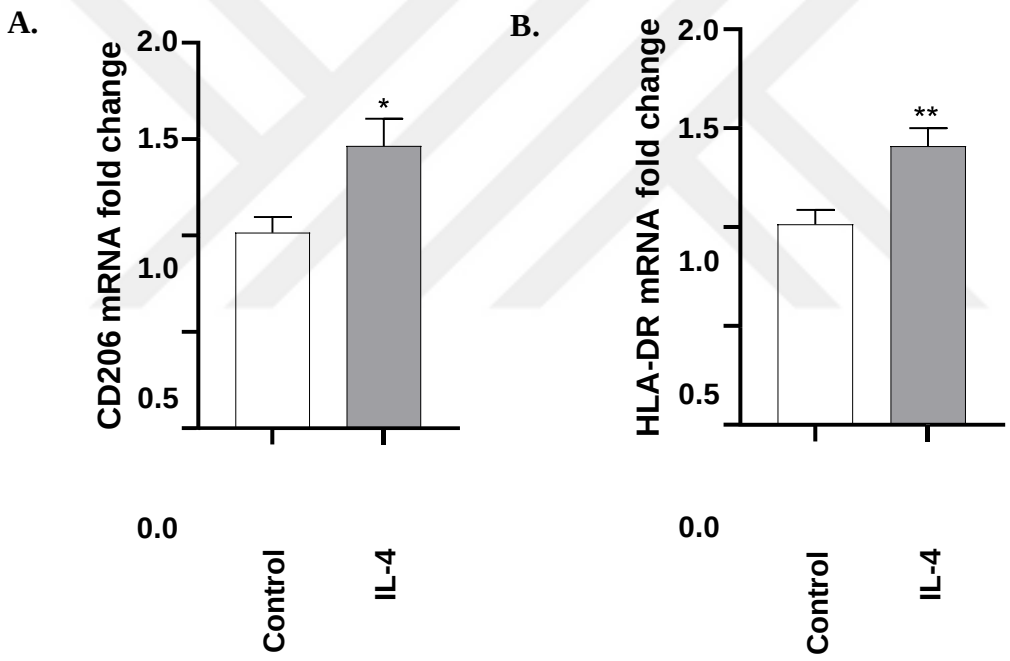


Figure 3. mRNA levels of M2 markers including CD206 and HLA-DR in IL-4 treated HMC3 cell line

4.2. Exosome Characterization by Western Blotting

Exosomes isolated from M0 and M2 HMC3 cells were characterized using Western blotting by detecting the presence of the exosome-specific positive surface marker CD63 and

the absence of endoplasmic reticulum protein as a negative exosome marker. Figure 4 shows the Western blot results showed that CD63 protein was enriched in both M0 and M2 polarized

HMC3 microglia derived exosome samples and Calnexin protein was not detected in these samples.

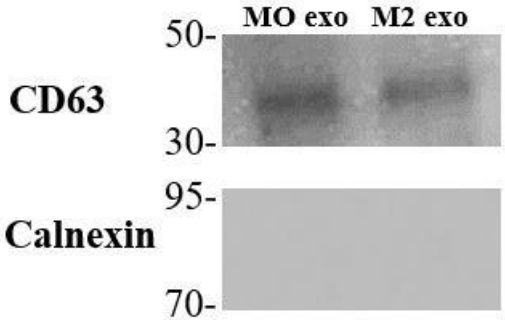


Figure 4. Expression of positive exosomal marker "CD63" and negative exosomal marker "Calnexin" in M0 and M2 Exo.

4.3. Exosome Characterization by NTA

The particle size of isolated exosomes was examined by Nanoparticle Tracking Analysis using Nanosight NS300. Results showed that M0 HMC3 cells released exosomes with a peak of 176 nm size in diameter (Figure 5A) and M2 HMC3 cells released exosomes with a peak of 190 nm size in diameter (Figure 5B).

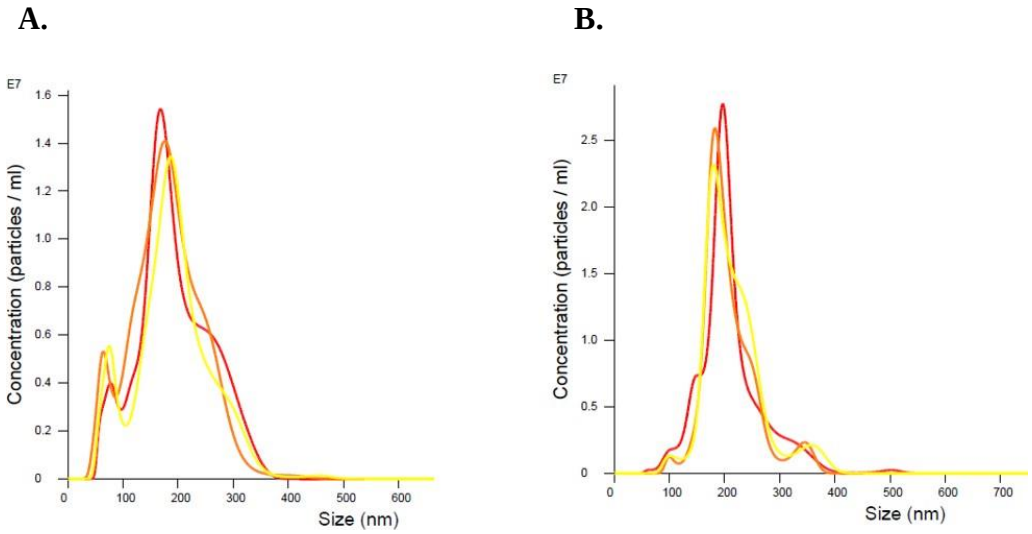


Figure 5. NTA results of A) M0 HMC3 and B) M2 HMC3 microglia-derived exosomes

4.4. Exosome Characterization by TEM

For the characterization of the exosomes isolated from the HMC3 cell line, TEM visualization was performed in which exosome size and morphology could be determined. TEM photographs revealed that the exosomes were as expected in size and morphology. As seen in Figure 6, the dimensions of the exosomes were determined, and their shapes were observed as spherical and double-membrane structures.

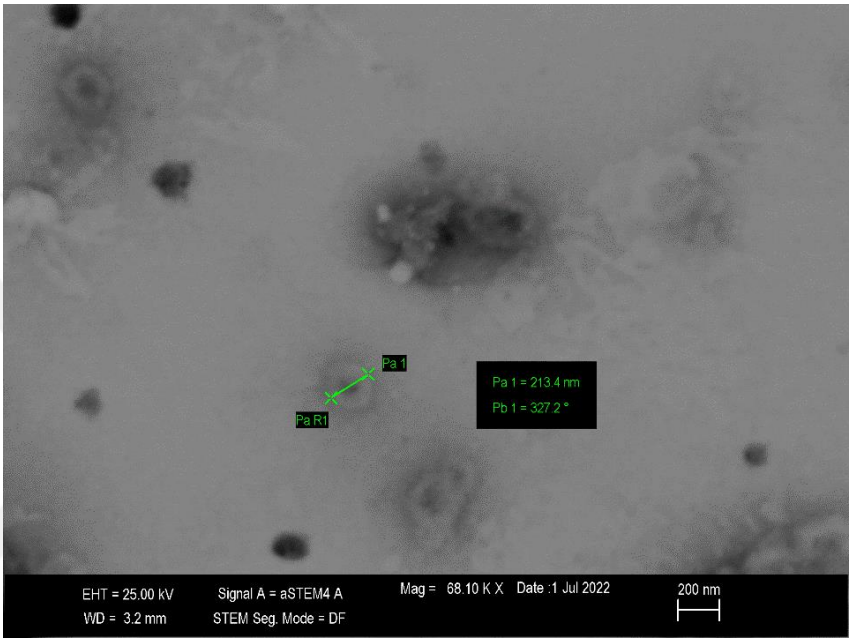


Figure 6. TEM imaging of HMC3 microglia-derived exosomes

4.5.Validation of Increased miR-191-5p Expression in M2 HMC3 Microglia-derived Exosomes by qPCR

The expression change of miR-191-5p was detected through RT-qPCR in “M0 exo” (M0 exosome) and “M2 exo” (M2 exosome) derived from an HMC3 cell line. According to the result shown in Figure 7, the miR-191-5p expression level in exosomes was enhanced (2.882 ± 0.6163, p=0.0260) after M2 polarization by IL-4 treatment in HMC3 cells.

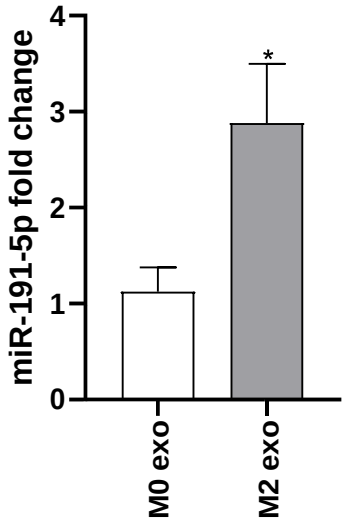
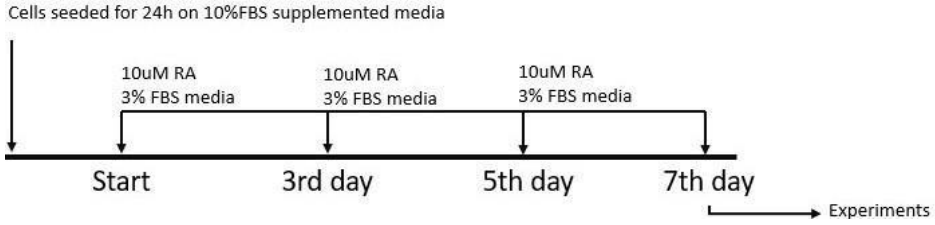


Figure 7. miR-191-5p expression levels in HMC3 microglia-derived M0 Exo and M2 Exo

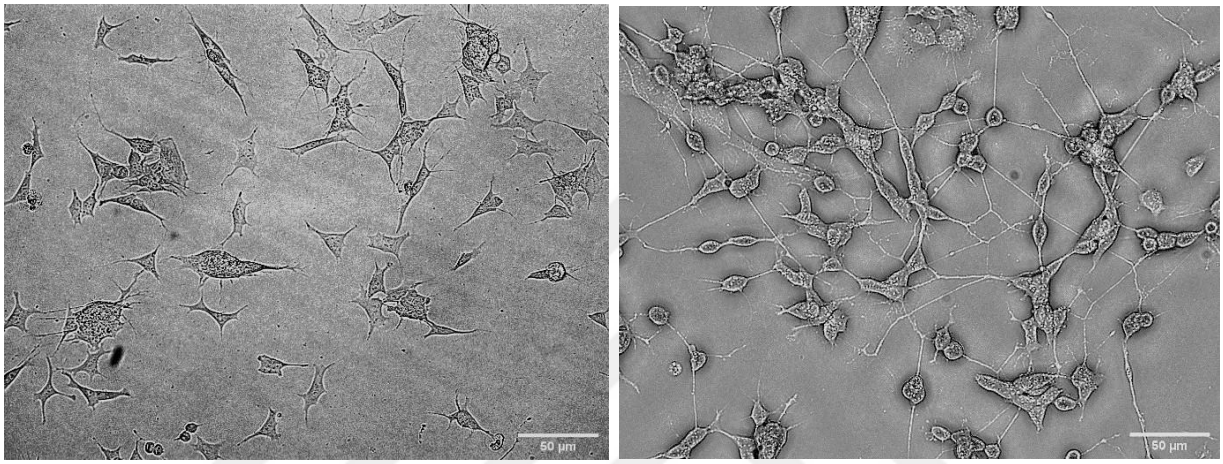
4.6. Retinoic Acid Differentiation Imaging of SH-SY5Y cells by Bright Field Microscope

Retinoic acid treatment on SH-SY5Y cells was performed for seven days to induce neuronal phenotype and increase neurite outgrowth in SH-SY5Y cells. Cells were seeded using DMEM complete medium with 3% FBS with 10 µM retinoic acid, and the medium was refreshed every 48 hours, presented as diagram in Figure 8A. Bright-field images showed that retinoic acid treatment induced neuronal differentiation in SH-SY5Y cells, which was observed as enhanced neurite outgrowth (Figure 8B). Also, neurite outgrowth analysis was performed for the quantification of average neurite length of SH-SY5Y cells on Day 0 and Day 7 of retinoicacid treatment (Figure 8C). After 7 days of retinoic acid treatment, average neurite length increased (58.90 ± 1.188, p= 0.0079).

A.



B. **Day 0** **Day 7**



C.

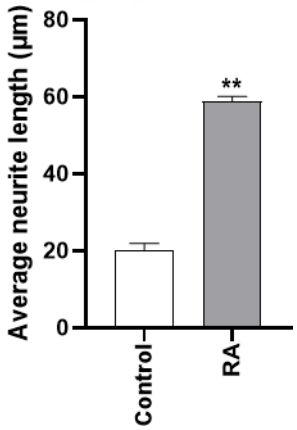


Figure 8. Retinoic acid differentiation in SH-SY5Y cells. A) Experiment design of Retinoic Acid treatment in SH-SY5Y cells, B) 40x imaging of Retinoic Acid differentiation in SH-SY5Y cells by bright field microscope on Day 0 and Day 7 C) Quantification of neurite outgrowth analysis performed on Day 0 and Day 7

4.7. Validation of Exosomal Uptake by SH-SY5Y cells with PKH67 Staining

Exosomes isolated from the HMC3 cell line were labeled with PKH67 dye and incubated with SH-SY5Y cells for 24 hours to visualize their uptake by neuronal cells. 20X images of cells were taken and bright field and fluorescence images were merged to show the entry of exosomes into the cell. Also, the quantification for PKH67 positive cells after 24 hours is given (88.80 ± 1.281, p=0.0079), and data is presented as a percentage of total cells (Figure 9). "Exo treated" group represents PKH67 labelled exosome treated SH-SY5Y cells, while untreated group represents no exosome treatment in SH-SY5Y cells.

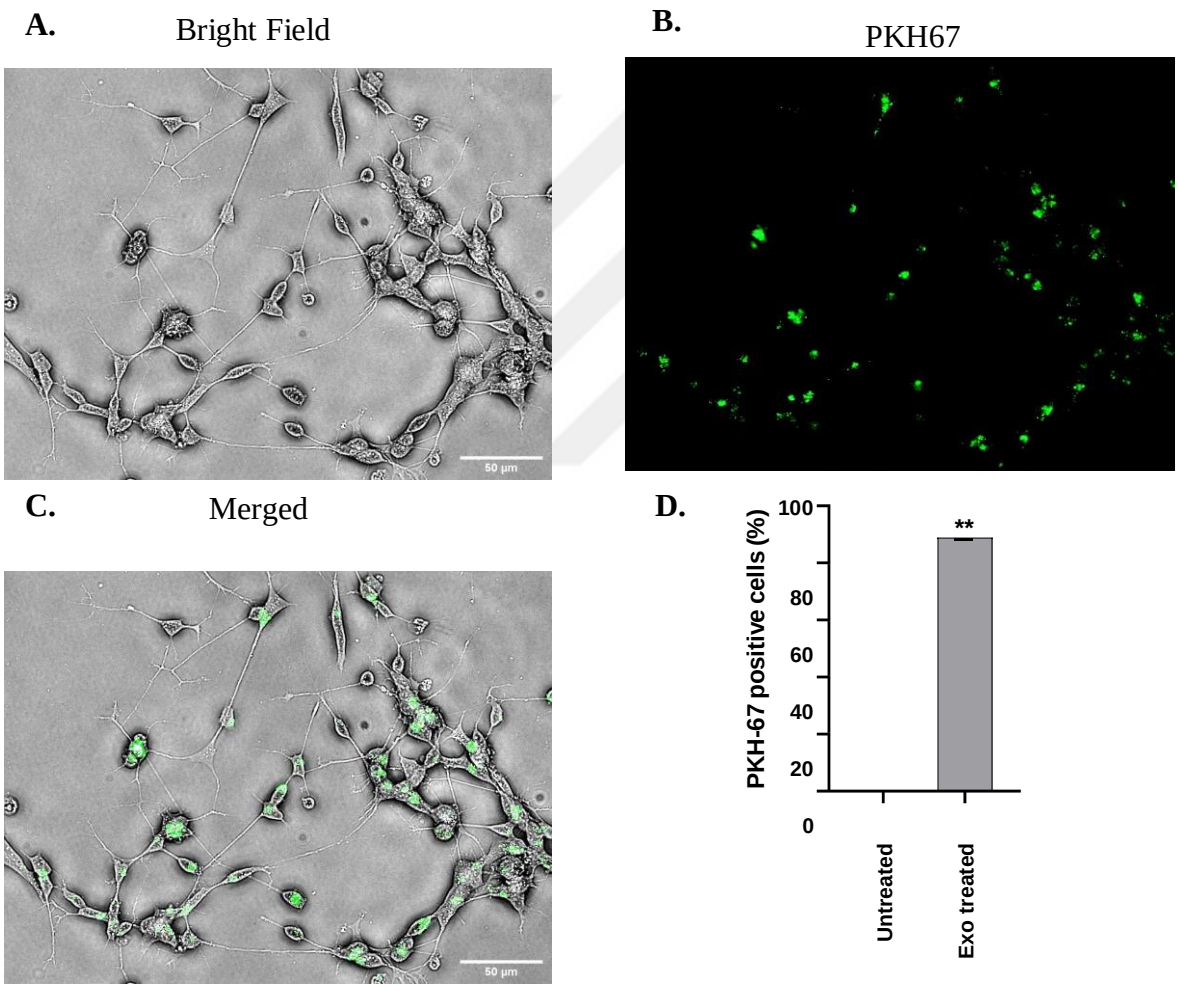


Figure 9. PKH67 labeled exosomal uptake. 40x imaging of PKH67 labeled exosomal uptake by SH-SY5Y cells in A) bright field imaging of SH-SY5Y cells B) fluorescence imaging of PKH67 labelled exosomes C) merged of bright field and fluorescence images D) the

quantification of PKH67 positive cells (%)

4.8. Determination of Exosome Cytotoxicity in SH-SY5Y cells by PI Staining

SH-SY5Y cells were stained with propidium iodine dye for 15 minutes after treatment with varying doses (2 µg/ml to 30 µg/ml) of HMC3 microglia-derived exosomes to examine percentage of PI positive cells. As seen in Figure 10, after 2 µg/ml, 5 µg/ml, and 10 µg/ml of exosome treatment of SH-SY5Y cells, PI positive cell percentage was not altered statistically. However, following the treatment of 20 µg/ml (9.973 ± 0.6816, $p= 0.0079$) and 30 µg/ml (12.65 ± 0.7439, $p=0.0079$) exosomes, PI positive percentage of SH-SY5Y cells were significantly elevated. These results showed that 20 µg/ml and 30 µg/ml doses of exosomes significantly enhanced cell death, and these doses are cytotoxic for SH-SY5Y cells.

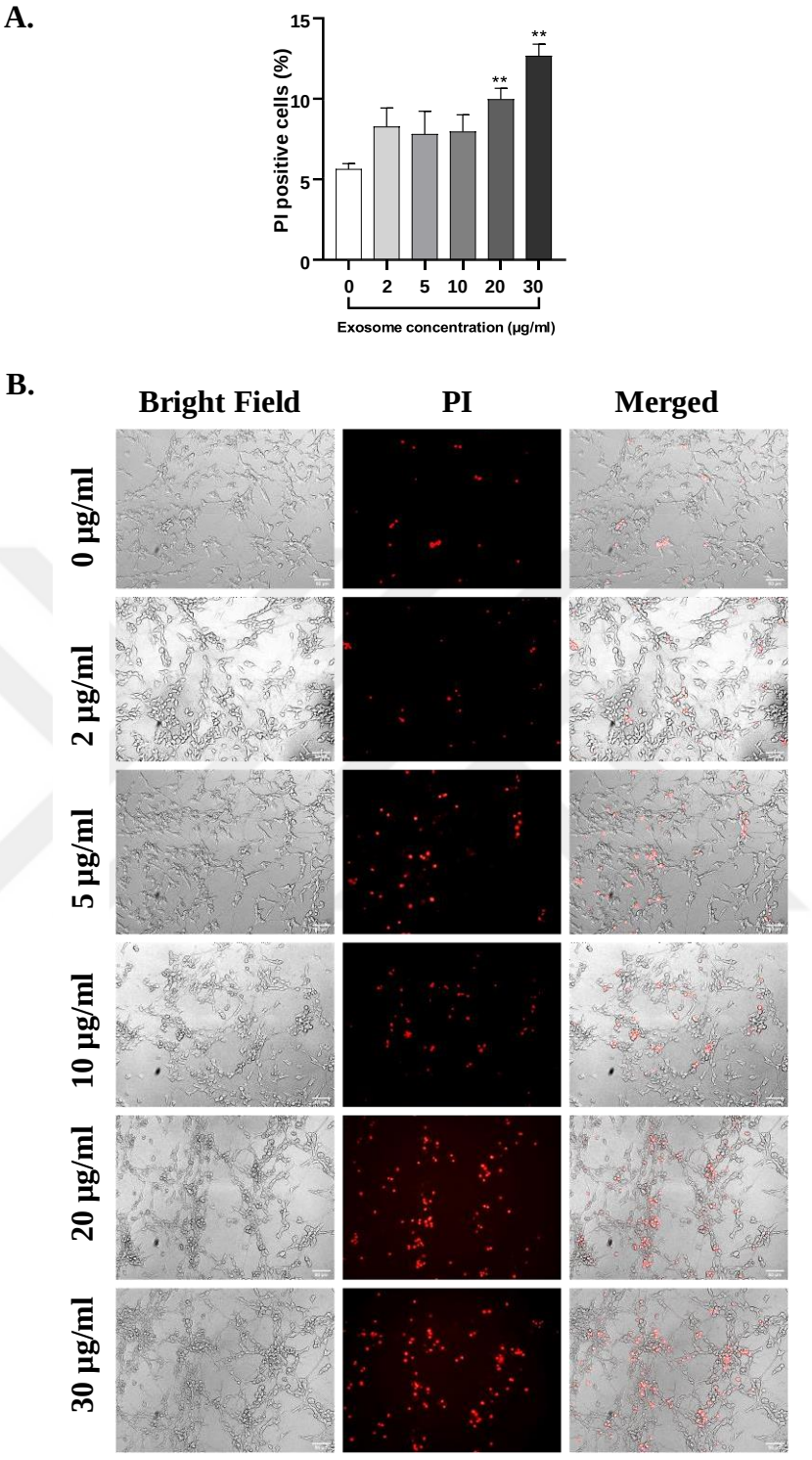


Figure 10. Cytotoxicity of different doses os exosomes in SH-SY5Y cells by PI staining A) The quantification of PI positive cells (%) and B) 20x fluorescence imaging of PI-stained cells

4.9. Determination of Exosome Cytotoxicity in SH-SY5Y cells by LDH Release Assay

Varying doses of exosomes ranging from 2 µg/ml to 30 µg/ml were given to SH-SY5Y cells to determine the cytotoxicity of exosomes. LDH release was measured by Cytotoxicity Detection KitPLUS (Roche Diagnostics, Germany). As seen in Figure 11, following the treatment of SH-SY5Y cells with 2 µg/ml, 5 µg/ml, and 10 µg/ml exosomes were not increased cell death significantly. However, cell death was increased significantly after 20 µg/ml (2.302 ± 0.1710, p= 0.0159) and 30 µg/ml (5.069 ± 0.3062, p=0.0159) exosome treatment, and these doses were determined to be cytotoxic for SH-SY5Y cells.

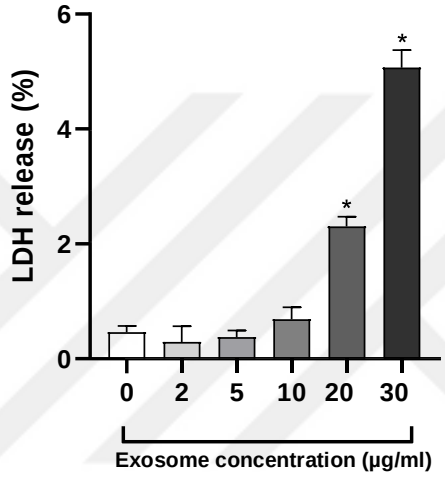


Figure 11. Cytotoxicity of different doses of exosomes in SH-SY5Y cells by LDH release assay

4.10. Determination of Neuroprotective Effects of Microglial Exosomes on SH-SY5Y cells by PI Staining

To investigate whether M0 and M2 HMC3 microglia-derived exosomes affect neuronal cell death, neuronal damage in SH-SY5Y Cells was performed by 25 µM H_2O_2 treatment for 24 hours after exosome treatment. To see the effects of M0 and M2 HMC3 microglia exosomes on cell death, SH-SY5Y cells were stained by PI after treatments were performed. As shown in Figure 12, H_2O_2 treatment significantly induced cell death (21.81 ± 1.164, p=0.0022), and the result showed that, compared to the "H_2O_2" group, cell death did not alter significantly in "M0 exo + H_2O_2" group. However, SH-SY5Y cells death significantly decreased in the "M2 exo +

H_2O_2" treatment group contrary to "H_2O_2" group and "M0 exo + H_2O_2" group (10.64 ± 0.7811, p=0.0022).

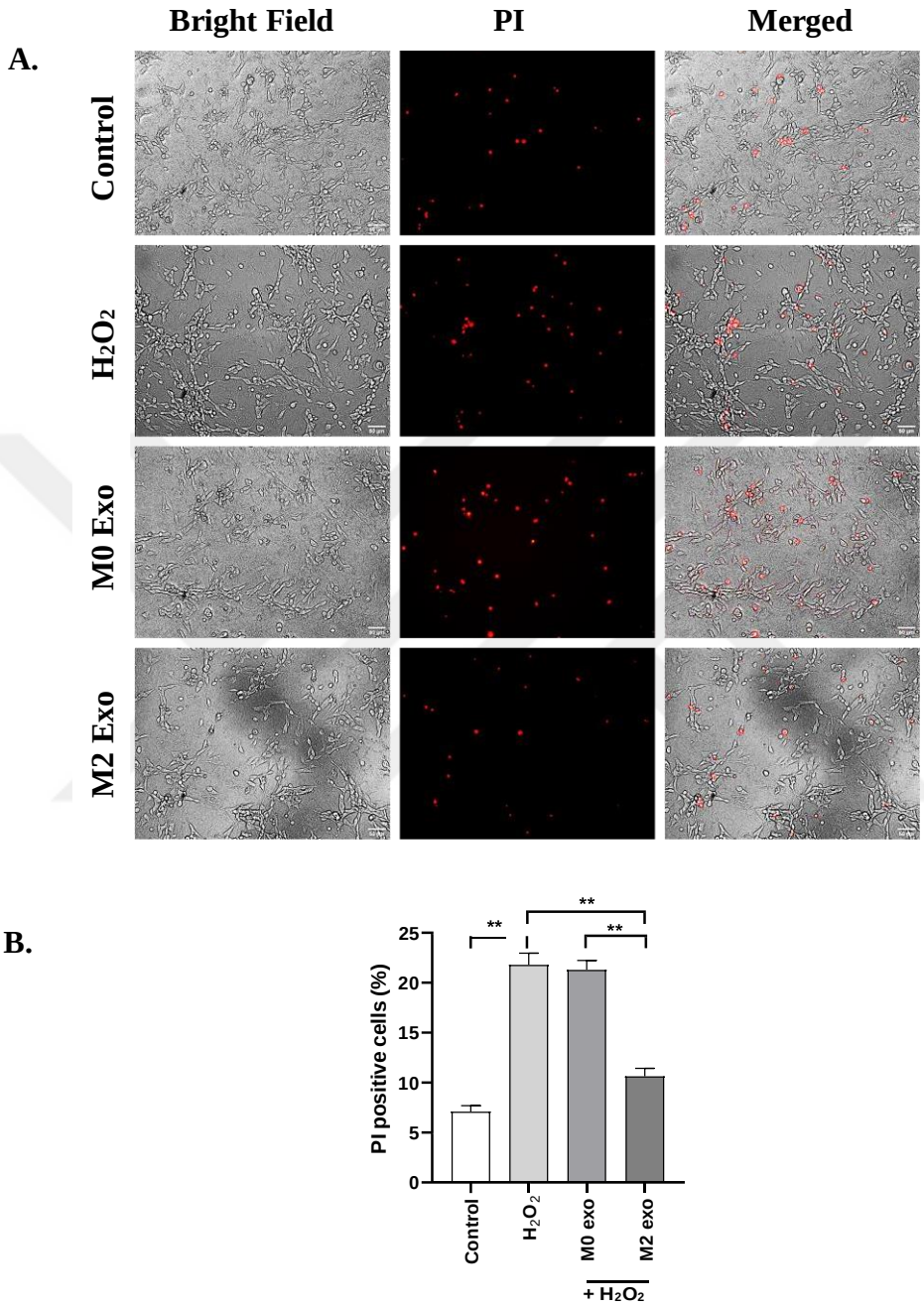


Figure 12. Neuroprotective effect of M0 and M2 exosome treatment on H_2O_2 treated SH-SY5Y cells by PI staining. A) 20x fluorescence imaging of PI-stained cells B) The quantification of PI positive cells (%)

4.11. Determination of Neuroprotective Effects of Microglial Exosomes on SH-SY5Y cells by LDH Release Assay

To determine the effect of M0 and M2 HMC3 microglia-derived exosomes on neuronal cell death, exosome treatment was performed before neuronal damage. To investigate the effects of M0 and M2 HMC3 microglia exosomes on cell death, an LDH release assay was done. As shown in Figure 13, H_2O_2 treatment significantly enhanced cell death (10.76 ± 0.3154, p=0.0079), and the result showed cell death did not increase significantly in "M0 exo + H_2O_2" group contrary to the "H_2O_2" group. On the other hand, SH-SY5Y cell death significantly decreased in the "M2 exo + H_2O_2" treatment group (6.777 ± 0.1491, p=0.0079) compared to "H_2O_2" group and "M0 exo + H_2O_2" group.

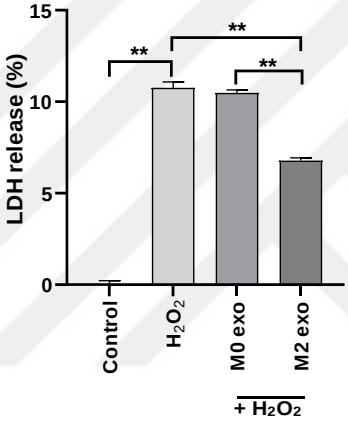


Figure 13. Neuroprotective effect of M0 and M2 exosome treatment on H_2O_2 treated SH-SY5Y cells by LDH release assay

4.12. Neurite Outgrowth Analysis in SH-SY5Y cells Treated with Microglial Exosome

To examine the neuroprotective role of exosomes released from HMC3 cells on oxidative stress induced in SH-SY5Y cells, neurite outgrowth analysis was performed. As seen in Figure 14A, H_2O_2 treatment decreased average neurite length (40.54 ± 0.8137, p=0.0079). In "M0 Exo + H_2O_2" groups, average neurite length did not change significantly compared to the H_2O_2 group, and SH-SY5Y cells send out neurites with 38.75 ± 1.621 µm. On the other hand, "M2 exo" treatment increased the average neurite length of SH-SY5Y cells (48.97 ± 2.065, p= 0.0079) compared to "H_2O_2" group and "M0 Exo" groups.

As seen in Figure 14B, in the "H_2O_2" treatment group, neurite number per cell decreased (1.216 ± 0.02676, p=0.0317). In the "M0 Exo + H_2O_2" group, there was no significant change in neurite number per cell compared to the "H_2O_2" group. However, in the "M2 Exo + H_2O_2" group, neurite number per cell increased significantly (1.400 ± 0.07969, p=0.0397) compared to the "H_2O_2" group. These results indicate that "M2 exo" treatment in SH-SY5Y cells is neuroprotective on H_2O_2-related oxidative stress by increasing the neurite length and neurite number per cell. Bright field images of these SH-SY5Y cells in 1) control, 2) H_2O_2 3) M0 exo 4) M2 exo groups are given in Figure 14C.

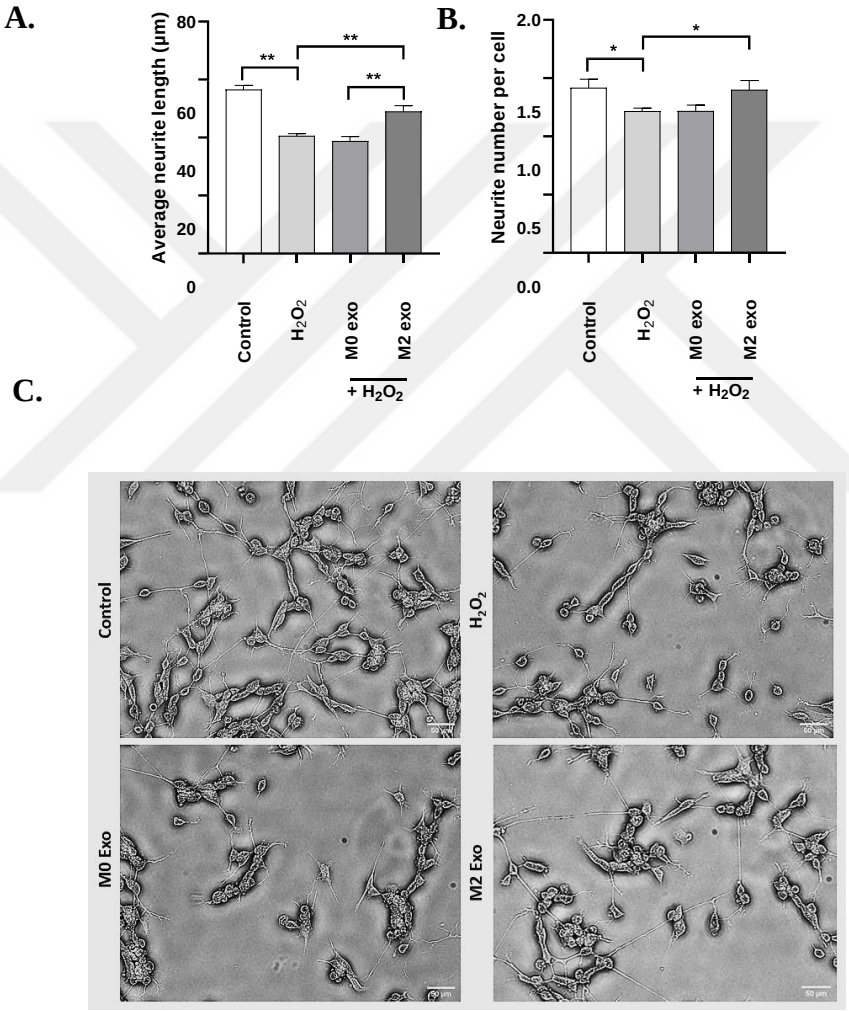


Figure 14. Neuroprotective effect of M0 and M2 exosome treatment on H_2O_2 treated SH-SY5Y cells by neurite outgrowth assay A) average neurite length (µm), B) neurite number per cell C) 40x bright field images of SH-SY5Y cells in 1) Control, 2) H_2O_2 3) M0 exo 4) M2 exo

groups

4.13. Validation of Exosome Transfection with AntagomiR-191-5p by qPCR

The expression level of mir-191-5p was measured by qPCR for confirmation of exosome transfection after loading exosomes with mir-191-5p inhibitor or control by Lipofectamine RNAiMax transfection reagent. As seen in Figure 15, mir-191-5p expression in M2 HMC3 microglia-derived exosome content was suppressed (1.134 ± 0.2463, p=0.0079) after exosome transfection with mir-191-5p inhibitor.

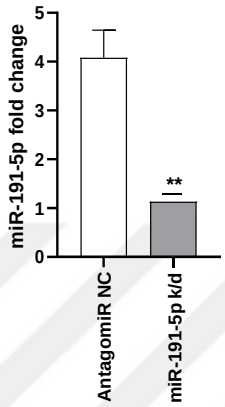


Figure 15. miR-191-5p expression level in M2 exosomes treated with A) antagomiR negative control, B) miR-191-5p inhibitor

4.14. Determination of Neuroprotective Effects of M2 HMC3 Microglia-derived Exosomal miR-191-5p on SH-SY5Y cells PI Staining

To evaluate whether the M2 HMC3 microglia-derived exosome exerts its neuroprotective effect through mir-191-5p during neuronal damage, functional experiments were performed using the mir-191-5p inhibitor. SH-SY5Y cells were stained with PI dye to show the effect of mir-191-5p on neuronal cell death during neuronal damage. SH-SY5Y cells were treated with "M2 exo" or M2 exo transfected with "miR-191-5p k/d" or M2 exo transfected with "AntagomiR NC (Antagomir negative control)" for 24 h before H_2O_2 treatment. According to the results in Figure 16, cell death increased after H_2O_2 treatment (26.75 ± 1.963, p= 0.0079) and decreased in the "M2 exo + H_2O_2" group (14.90 ± 1.188, p=0.0079). There was no significant difference between the level of cell death in the "AntagomiR NC + H_2O_2" group and the "M2 Exo + H_2O_2" group. Also, cell death was significantly enhanced in the "miR-191-5p k/d + H_2O_2" group (20.17 ± 1.310, p=0.0152) compared to "AntagomiR NC + H_2O_2" group.

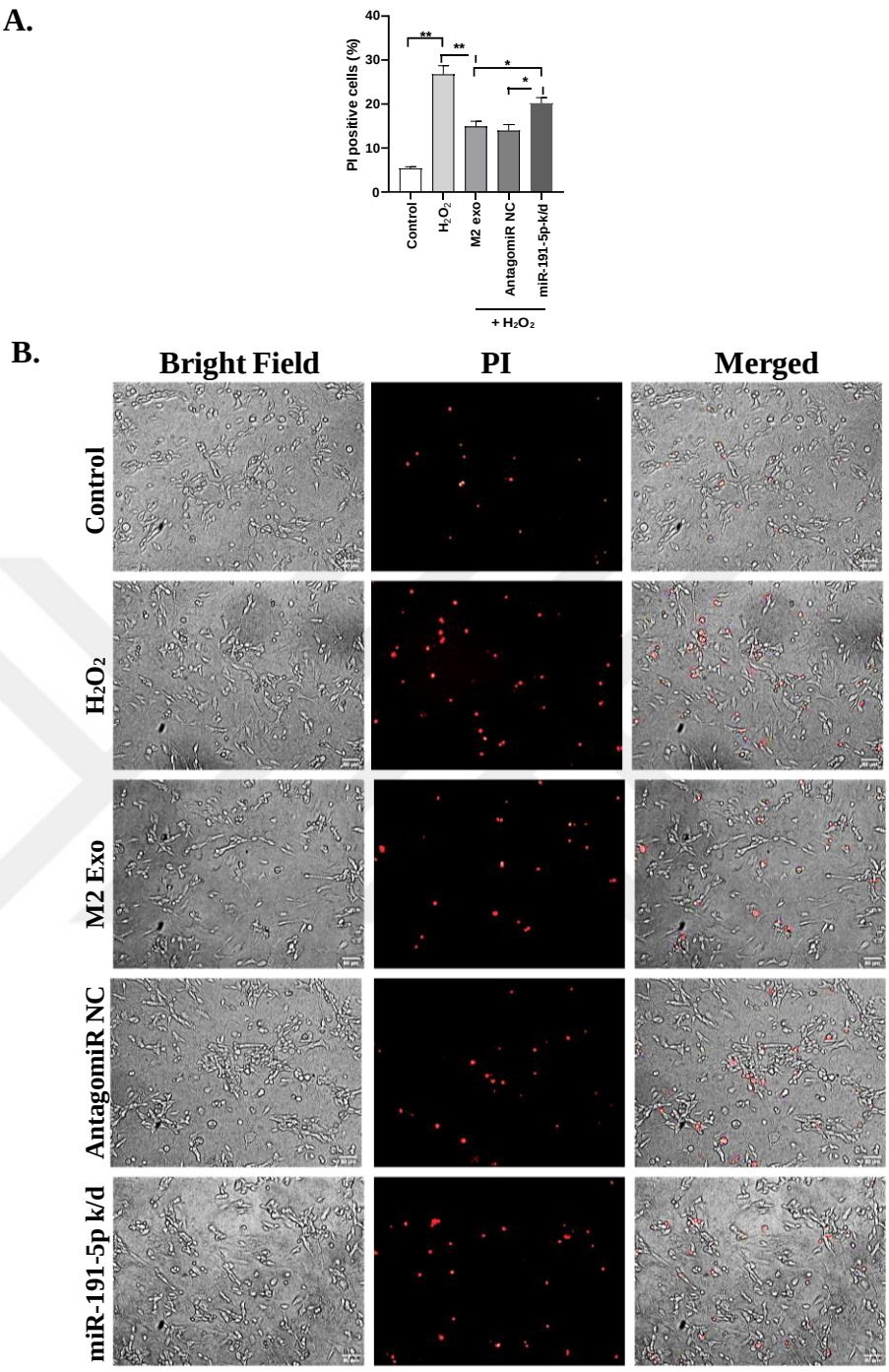


Figure 16. Neuroprotective effect of M2 HMC3 microglia-derived exosomal miR-191-5p on H_2O_2 treated SH-SY5Y cells by PI staining. A) The quantification of PI positive cells (%) and B) 20x fluorescence imaging of PI-stained cells

4.15. Determination of Neuroprotective Effects of M2 HMC3 Microglia-derived Exosomal miR-191-5p on SH-SY5Y cells by LDH Release Assay

To determine the effect of M2 HMC3 microglia- derived exosomal miR-191-5p on cell death, an LDH release assay was conducted. According to the results in Figure 17, H_2O_2 treatment induced cell death (28.80 ± 1.780, p=0.0022), and M2 exosome treatment decreased cell death (15.27 ± 0.8011, p= 0.0022) compared to "H_2O_2" group. There was no significant difference between the level of cell death in the "AntagomiR NC + H_2O_2" group and the "M2 exo + H_2O_2" group. In addition, cell death was significantly enhanced in the "miR-191-5p k/d + H_2O_2" group (21.57 ± 1.157, p=0.0260) compared to "M2 Exo + H_2O_2" and "AntagomiR NC + H_2O_2" groups. Overall, these results showed that M2 microglia exosomes exerted their neuroprotective effects on cell death via miR-191-5p.

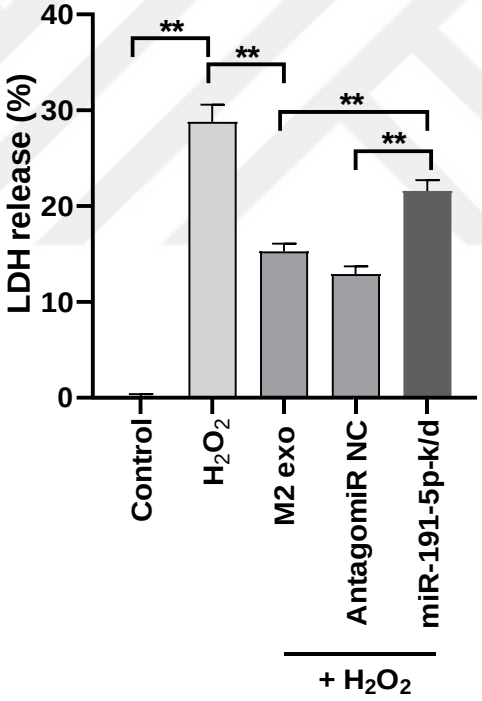


Figure 17. Neuroprotective effect of M2 HMC3 microglia-derived exosomal miR-191-5p on in H_2O_2 treated SH-SY5Y cells by LDH release assay

4.16. Determination of Neuroprotective Effects of M2 HMC3 Microglia-derived Exosomal miR-191-5p on Neurite Outgrowth in SH-SY5Y cells

To examine whether the neuroprotective effect of M2 microglia-derived exosomes on neurite outgrowth is mediated by miR-191-5p, exosome transfections with miR-191-5p k/d, and AntagomiR NC were performed before H_2O_2 treatment was induced. According to the results sin Figure 18A, the average neurite length was significantly reduced in the "H_2O_2" group (41.05 ± 0.4096, p=0.0079). M2 exosome treatment significantly increased average neurite length (50.70 ± 1.264, p= 0.0079) compared to the "H_2O_2" group. There was no significant change between the average neurite length of the "M2 exo + H_2O_2" and "AntagomiR NC + H_2O_2" group. However, the average neurite length in "miR-191-5p k/d + H_2O_2" deteriorated (43.74 ± 0.8880, p=0.0317) compared to the "AntagomiR NC + H_2O_2" group and "M2 Exo + H_2O_2" group.

As shown in Figure 18B, the neurite number per cell significantly decreased in the "H_2O_2" group (1.181 ± 0.05485 p=0.0079). Also, M2 exosome treatment significantly improved the neurite number per cell (1.624 ± 0.05411, p= 0.0079) compared to the "H_2O_2" group. There was no significant change between the neurite number per cell of the "M2 exo + H_2O_2" and "AntagomiR NC + H_2O_2" group. However, the neurite number per cell in "miR-191-5p k/d + H_2O_2" decreased (1.313 ± 0.04135, p=0.0159) compared to "antagomiR NC" group. Bright field images of these SH-SY5Y cells in 1) control, 2) H_2O_2 3) M2 exo 4) AntagomiR NC 5) miR-191-5p k/d groups are given in Figure 18C.

Overall, according to these results, the neuroprotective effect of M2 exosomes on neurite outgrowth is partially mediated through miR-191-5p, and knockdown of miR-191-5p with miRNA inhibitor reduces neurite length and neurite number in SH-SY5Y cells.

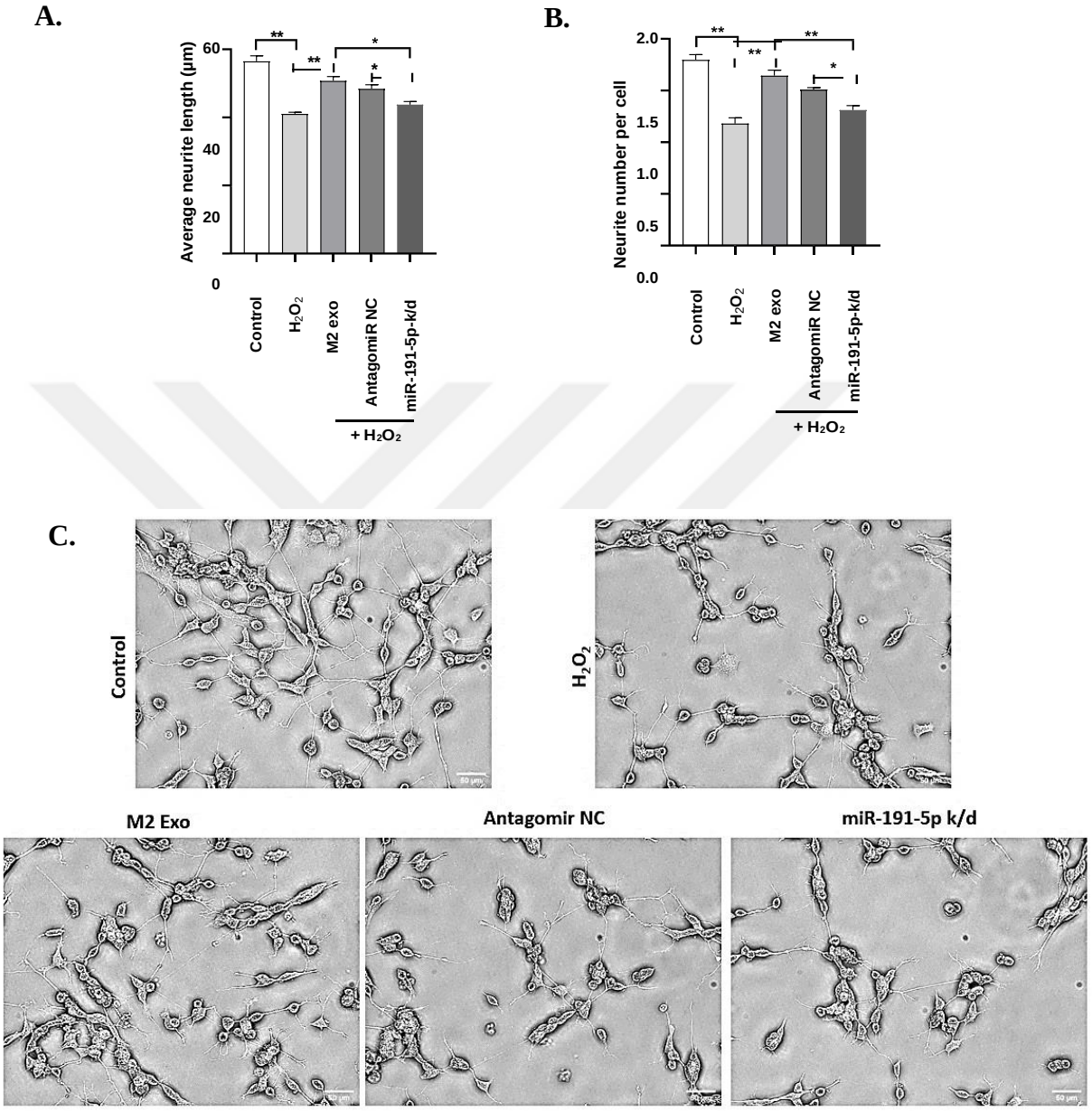


Figure 18. Neuroprotective effect of M2 HMC3 microglia-derived exosomal miR-191-5p on H_2O_2 treated SH-SY5Y cells by neurite outgrowth assay A) average neurite length (µm), B) neurite number per cell C) 40x bright field images of SH-SY5Y cells in 1) control, 2) H_2O_2 3) M2 exo 4) AntagomiR NC 5) miR-191-5p k/d groups.

5. DISCUSSION

In the present study, for the first time, it was demonstrated that the level of miR-191-5p in microglia-derived exosomes increased as a result of M2 polarization. The present study also investigated that exosomes derived from M2 microglial cells could be taken up by neurons. Furthermore, the increased miR-191-5p in M2 microglial exosomes significantly inhibited neuronal cell death and promoted neurite outgrowth in neurons under H_2O_2-related oxidative stress.

5.1. Microglia and M2 polarization

Microglia colonize in CNS and support brain development and maintenance of CNS (Chen and Trapp 2016). Microglia cells have mainly 1) housekeeping function of the neuronal environment to maintain health and physiological function; 2) seizure function for sensing environmental changes and homeostasis; and 3) a defensive function for neuroprotection (Salter and Stevens 2017). Microglial cell populations have been shown to predominate in areas of CNS damage, as they promote both injury and repair (Jin and Yamashita 2016). Microglia exert their defense responses by forming different subsets of microglial activation, i.e., they can induce either "classically activated" pro-inflammatory M1 polarization or "alternatively activated" anti-inflammatory M2 polarization (Orihuela, McPherson et al. 2016, Tang and Le 2016). Accumulating evidence has shown that M2 polarized microglia exert remarkable neuroprotective effects on the progression of many brain diseases, such as cerebral ischemia (Liu, Wen et al. 2018), intracerebral hemorrhage (Lin, Yihao et al. 2017), and Parkinson's disease (Subramaniam and Federoff 2017). Therefore, examining the neuroprotective effect of M2-polarized microglia and its underlying mechanism will make an important contribution to the treatment of brain diseases and injuries. In this thesis, it was reported that exosomes secreted by M2 polarized microglia HMC3 cells are internalized by differentiated SH-SY5Y cells exposed to oxidative stress-related damage, thereby promoting neuron survival and neurite outgrowth via exosomal miRNA-191-5p.

5.2. The altered expression of exosomal miRNAs

Exosomes exert their functions via carrying exosomal cargo such as miRNAs, mRNAs, DNA, and proteins to the recipient cells (Feng, Chen et al. 2021). miRNAs represent a significant proportion of the exosomal cargo, so exosomal miRNAs have attracted substantial attention. miRNAs are a single-stranded non-coding RNAs and can induce mRNA degradation by binding to the 3' untranslated region on their target mRNA. There are studies investigating the altered miRNA expression levels in polarized microglia-derived exosomes. For example, the expression level of miR-124 significantly increased in M2 microglial exosomes compared to M0 microglial exosomes (Song, Li et al. 2019). It was determined that decreased level of exosomal miR-5121 was detected in M2 polarized microglia after LPS stimulation (Zhao, Deng et al. 2021) . Also, up-regulated miR-672-5p was derived from M2 polarized microglia (Zhou, Li et al. 2022). In a study conducted by our groups in 2018 within the scope of BAP project (Project no: 2018.KB.SAG.114), the miRNA content of exosomes released from M2 polarized HMC3 microglia was examined, and it was shown that miR-191-5p expression level was upregulated in M2 HMC3 microglial exosomes. Also, in this study, the increase in expression of miR-191-5p in exosomes isolated from the M2 polarized HMC3 cell line was confirmed by RT-qPCR assay. This result suggests that miRNA-191-5p might be involved in the role of M2 microglial exosomes.

5.3. The role of exosomal miRNAs in microglia-to-neuron interaction

In many studies carried out in recent years, it has been shown that the role of exosomes in intercellular communication and regulating physiological processes in recipient cells is the result of a basic mechanism via the intercellular transport of cargoes such as proteins, DNA, mRNAs, long non-coding RNAs (lncRNAs) and miRNAs (Schorey, Cheng et al. 2015, Meldolesi 2018). Recent studies have indicated that microglial exosomes have a critical role in microglia-to-neuron interaction in both healthy and pathological CNS. In physiological

condition, microglial exosomes can perform a significant interaction with neurites and induce neurite outgrowth (Chen, Chen et al. 2015). On the other hand, in neurodegenerative disease such as Alzheimer's disease (AD) and Parkinson's disease (PD), activated microglia-derived exosomes transfer misfolded proteins to recipient neurons causing neuronal dysfunction and cell death (Ratajczak, Miekus et al. 2006, Caruso Bavisotto, Cappello et al. 2017). Therefore, exosome-mediated therapeutic strategies for the treatment of diseases are receiving increasing attention, and studies are being carried out on the development and progression of CNS diseases (Paolicelli, Bergamini et al. 2019).

Currently, there are limited studies on the effects of microglia-derived exosomal miRNAs on neurons. The studies revealed that miRNA content delivered from microglia to neurons via exosomes might be beneficial or detrimental. Among the studies examining the harmful effects of microglia-derived exosomal miRNA on neurons, it was found that exosomal let7a/b miRNA released from microglia induces neuronal death via caspase activation (Mukherjee, Akbar et al. 2019). Also, in a study performed in an intracerebral hemorrhage model, it was revealed that miR-383-3p in microglial exosomes causes necroptosis (Wei, Xu et al. 2020). In addition, down-regulated exosomal miR-5121 obtained from scratch-injured activated microglia cells reduces neurite length and inhibits synaptic plasticity after traumatic brain injury *in vivo* and *in vitro* (Zhao, Deng et al. 2021).

There are also studies investigating the beneficial effects of microglia-derived exosomal miRNA on neuron. For example, miR-124-3p upregulation in microglial exosomes was shown to prevent neuronal inflammation and increase neurite length in the case of traumatic brain injury *in vitro* and *in vivo* (Huang, Ge et al. 2018). Also, it was found that miR-124-3p in microglial exosomes exerts neuroprotective effects as the inhibition of autophagy against traumatic brain injury (Li, Huang et al. 2019). It has been revealed that increased expression of miR-124-3p in microglial exosomes supports cognitive improvements and prevents neurodegeneration after traumatic brain injury (Ge, Guo et al. 2020). Moreover, it was demonstrated that abundant miR-151-3p in microglia-derived exosomes might promote axonal regrowth and reduces the level of neuronal apoptosis after spinal cord injury (Li, Qin et al.

2021). It was indicated that induced miR-124-3p expression in microglia-derived exosomes can promote neuronal repair after traumatic brain injury (TBI) (Zhao, Wang et al. 2022). In addition, there are studies involving M2 microglia-derived exosomes miRNAs to investigate the effect of the altered miRNA content in M2 polarized microglial exosomes on neuroprotection. Initially, the protective effects of exosomal miR-124 released from M2polarized microglia on primary neuron cells were demonstrated in mice with ischemia- reperfusion injury (Song, Li et al. 2019). Moreover, it was suggested that neuronal apoptosis during ischemic injury can be inhibited by exosomal miR-137 derived from M2 polarized microglia (Zhang, Cai et al. 2021). M2 microglia-derived exosomal miR-672-5p induces axonregeneration and inhibits pyroptosis after traumatic spinal cord injury (TSCI) (Zhou, Li et al. 2022). In this study, it was focused on the effect on miR-191-5p in exosomes released from M2HMC3 microglia in order to investigate the neuroprotection effect of this miRNA on neuron. The results supported that altered expression changed of miR-191-5p in M2-microglia derived exosomes can induce neuroprotection.

5.4. miR-191-5p

miRNAs are small non-coding RNAs that can bind to target mRNA to inhibit translation, and each miRNA can affect hundreds of mRNAs and thousands of genes. miR-191-5p is 23 nucleotide-long intragenic miRNA located on chromosome 3 (Zhang, Wu et al. 2018). In recent studies, it was found that miR-191-5p is downregulated in AD and is considered a serum biomarker in AD (Tan, Yu et al. 2014). Furthermore, it was shown that miR-191-5p attenuates microglia cell injury (Bigeard and Hirt 2018). Also, miR-191-5p has been reported as an important regulator in multiple sclerosis (MS) (Vistbakka, Sumelahti et al. 2018), and attention-deficit/hyperactivity disorder (Sanchez-Mora, Soler Artigas et al. 2019). In addition, the over-expression of miR-191-5p induced cell viability and decreased apoptosis rate of Aβ1-42 treated microglial cells (Zhao, Deng et al. 2021). Also, there are studies determining the harmful effect of miR-191 up-regulation on neuronal outcome. For example, it was shown that the down regulation of miR-191a and miR-191b promotes the function and plasticity of synapses in neurons by increasing dendritic area and decreasing spinal loss (Hu, Yu et al. 2014). Li et al. investigated that miR-191 is up-regulated in hippocampal tissue of rats upon isoflurane

inhalation, and down-regulation of miR-191 can ameliorate isoflurane-related impairment by increasing neurological score by targeting BDNF (Feng, Chen et al. 2021). However, the effects of up-regulated miR-191-5p on neuroprotection are still poorly investigated. To further investigate the neuroprotective roles of M2 microglia exosomal miRNA-191-5p in response to oxidative stress-related injury, miR-191-5p inhibitor transfection to M2 exosome was performed. After transfection, the functional assays such as PI staining, LDH release assay, and neurite outgrowth analysis were carried out. It was determined that up-regulated miRNA expression in M2 microglial exosomes induces neurite outgrowth and inhibits cell death in neurons during neuronal damage. However, miR-191-5p knock down due to inhibitor transfection caused enhanced cell death and deterioration in neurite outgrowth compared to antagomir negative control group. Taken together, neuroprotective response of M2 phenotype microglial exosomes on oxidative-stress related neuronal damage was partially exerted by miR-191-5p.

5.5. Limitations

Regarding the limitations of this study, the first thing to consider is the microglia cell line used in our study. HMC3 cells, human microglial clone 3 cell line, is a cell line SV40-dependent immortalized in 1995 from human embryonic microglial cells. It has been validated for use in studies by the American Type Culture Collection (ATCC®) and is distributed as the designation HMC3 (ATCC®CRL-3304) (Dello Russo, Cappoli et al. 2018). HMC3 cells have a different gene profile than primary microglia and respond differently to various stimuli. Therefore, the results obtained in this study with HMC3 cells cannot be fully applied to primary microglia. In addition, SH-SY5Y cell line is thrice cloned subline of human neuroblastoma cells (ATCC HTB11) (Kovalevich and Langford 2013). Thus, gene profile, function, and morphology of these cells as neuroblastoma cancer cells differ from primary neuronal cells. Also, to further investigate how microglia-derived exosomal miR-191-5p exerts its neuroprotective role on neurons, target analysis for miRNA-mRNA interaction should be performed. There is a list involving potential target genes in CNS (Table 4). There are target genes that can be involved in neuronal function and neuronal cell death cascades such as WSB1, TMOD2 and OXSR1. In

a study showing that miR-25-35 suppressed oxidative stress and apoptosis by targeting OXSR1(Li, Wen et al. 2020), thus OXSR1 could be potent target for miR-191-5p to exert its neuroprotective effect via inhibition of oxidative stress.

Table 4. Potential targets of miR-191-5p in CNS

Targets	Function	References
PLCD1	Tumor suppressor	(Xiang, Li et al. 2010)
ZBTB34	Transcripton repressor in cancer	(Jutooru, Guthrie et al. 2014)
NEURL4	Transcriptonal activity of tumor suppressor	(Cubillos-Rojas, Schneider et al. 2017)
BDNF	Angiogenesis during cerebral infarction	(Wu, Yang et al. 2021)
SAT1	Cell migration and proliferation	(Zhou, Zhang et al. 2020)
WSB1	Modification and aggregation of neurotoxic proteins	(Haque, Kendal et al. 2016)
TMOD2	Neurite growth	(Fath, Fischer et al. 2011)
OXSR1	Oxidative stress and apoptosis in neurons	(Li, Wen et al. 2020)

5.6. Conclusion

In conclusion, the results obtained in this thesis study suggest that M2 phenotype microglial exosomes can alleviate the oxidative stress damage in neurons *in vitro* through exosomal miRNA-191-5p by suppressing neuron death and promoting neurite outgrowth. This study provides a new perspective for the development of therapeutic strategies based on M2 polarized exosomal miRNAs for oxidative stress injury.

6. CONCLUSION AND SUGGESTIONS

In this study, the neuroprotective effects of M2 microglia-derived exosomal miR-191-5p on oxidative-stress-induced SH-SY5Y have been investigated. In accordance with this purpose, exosomes were isolated from the IL4-induced M2 polarized HMC3 cell line, and their neuroprotective effects have been determined by PI staning and LDH release analysis and neurite outgrowth analysis. Following the determination of the neuroprotective effect of M2 microglia-derived exosomes, it was determined whether exosomes exert their neuroprotective response via miR-191-5p. Consequently, it was shown that miR-191-5p knockdown partially reversed neuroprotection exerted by M2 microglia exosomes. miR-191-5p inhibitor treatment significantly enhanced cell death and inhibits neurite outgrowth in SH-SY5Y cells after H_2O_2 treatment for oxidative stress induction. Therefore, miR-191-5ptransferred via M2 microglia exosomes to neurons is a promising psrotective molecule againstoxidative stress in neuronal cells. Regarding the continuation of the study, miR-191-5p targetanalysis and the mechanism of action will be investigated.

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8. APPENDIX

8.1. Ethics Committee Approval

İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI

Toplantı Tarihi	: 04/03/2022	**Toplantı Günü**	: Cuma
Toplantı Sayısı	: 4	**Toplantı Saati**	: 10:30

Sayın Prof. Dr. Şermin GENÇ,

2022-004 Protokol No'lu; sorumlusu olduğunuz "M2 polarize mikroglia kökenli eksozomal miR-191-5p'nin oksidatif stres aracılı nöronal hasar üzerindeki nöroprotektif etkilerinin incelenmesi" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. H. Alper BAĞRIYANIK **Başkan**	**Dr. Serap ERKEK ÖZHAN** **Başkan Yardımcısı**
Prof. Dr. Sedef AKGÜNGÖR **Üye**	**Prof. Dr. İnci ALACACIOĞLU** **Üye** (Katılmadı)
Prof. Dr. Gülgün OKTAY **Üye**	**Prof. Dr. Hilmi ORHAN** **Üye** (Katılmadı)
(Proje Yürütücüsü) **Prof. Dr. Şermin GENÇ** **Üye** (Katılmadı)	**Doç. Dr. Canan Aslı YILDIRIM** **Üye**
Doç. Dr. Can KÜÇÜK **Üye**	**Dr. Öğr. Üyesi Yavuz OKTAY** **Üye**

8.2. Curriculum Vitae

ÇAĞLA NİĞDE

MOLECULAR BIOLOGIST

RELEVANT EXPERIENCE

MSc Student in İzmir Biomedicine and Genome Center

Prof. Dr. Şermin Genç | 09/2020 - TODAY

MASTER'S THESIS:

Determination of the neuroprotective effects of M2 polarized microglia-derived exosomal miR-191-5p on the oxidative stress-mediated neuronal damage

PROJECTS:

1) The role of Long noncoding RNA NEAT1 in the regulation of microglial NLRP3 inflammasome activation
2) Microglia to neuron interaction via exosomal miR-34a
3) Could autophagy in cord blood and placenta be a new marker to predict morbidities associated with prematurity?

Special Project in Middle East Technical University

Prof. Dr. Çağdaş Devrim Son | 10/2019 - 01/2020

PROJECT: K-Ras protein tagging with fluorescence proteins and visualization by confocal microscope

Summer Internship in İzmir Biomedicine and Genome Center

Prof. Dr. Neşe Atabey | 07/2019 - 08/2019

Effects of C-Met protein on liver cancer cell line
Blood sheer stress and extravasation
Lab on a chip (LOC) system

EDUCATION

IZMIR BIOMEDICINE AND GENOME INSTITUTE

Years Attended: 2020 - Today

Master of Science in Genome Sciences and Molecular Biotechnology Department

MIDDLE EAST TECHNICAL UNIVERSITY

Years Attended: 2015 - 2020

Bachelor of Science in Molecular Biology and Genetics Department

TECHNICAL SKILLS

- Fluorescence in situ hybridization (FISH)
- Immunofluorescence staining (IF)
- Neurite Outgrowth Analysis
- LDH and WST-8 assay / PI staining
- PBMC isolation
- Cell culture techniques
- Western Blot
- Agarose gel electrophoresis / SDS-PAGE
- Plasmid, DNA, RNA and protein isolation
- PCR / qRT-PCR / Primer Design
- Transformation, restriction, transfection
- R programming / Data analysis
- Handling Drosophila melanogaster
- Spectrophotometry / Nano drop
- Chromatography
- Preparation of media, staining, inocculation techniques in microbiology
- Recombinant DNA Techniques
- Bright field, fluorescence, and confocal microscope
- RNA immunoprecipitation (RIP)

LANGUAGE

Turkish - Native

English - Advanced / Fluent

Germany - Beginner

COMMUNICATION

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