T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

MICROGLIA TO NEURON INTERACTION VIA EXOSOMAL MIR-34A

NURULLAH SATI

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Nurullah Satı

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LIST OF ABBREVIATIONS

CNS: Central Nervous System

miRNA: microRNA

mRNA: messenger RNA

H_2O_2: Hydrogen peroxide

LDH: Lactate Dehydrogenase

PI: Propidium Iodide

HMC3: Human Microglial Cell line 3

ROS: Reactive oxygen species

TLR: Toll-like receptor

IFN: Interferon

IL: Interleukin

TNF: Tumor necrosis factor

CCL2: C-C Motif Chemokine Ligand 2

NO: Nitric oxide

MMP12: Matrix metalloproteinase 12

MHCII: Major histocompatibility complex II

TGF: Transforming growth factor

NGF: Nerve growth factor

IGF: Insulin-like growth factor

VEGF: Vascular endothelial growth factor

NOX2: NADPH oxidase 2

UTR: Untranslated region

NSC: Neural stem cell

SIRT1: Sirtuin 1

mES: mouse Embryonic stem cell

APP: Amyloid precursor protein

EV: Extracellular vesicle

OCSCC: Oral cavity squamous cell carcinoma

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

BCL2: B-cell lymphoma 2

BD: Bipolar disorder

VPA: Valproate

GRM7: Glutamate metabotropic receptor 7

TSC: Tuberous sclerosis complex

rTBI: repetitive Traumatic brain injury

SCI: Spinal cord injury

NF-κB: Nuclear factor kappa B

mSOD1: mutant copper-zinc Superoxide dismutase 1

MN: Motor neuron

PTEN: Phosphatase and tensin homolog

Akt: Protein kinase B

mTOR: mammalian target of Rapamycin

EMEM: Eagle's Minimum Essential Medium

FBS: Fetal Bovine Serum

DMEM: Dulbecco’s Modified Eagle Medium

MPER: Mammalian Protein Extraction Reagent

PDL: Poly-D-Lysine

PI3K: Phosphatidylinositol 3-kinase

Wnt-3a: Wingless-related integration site-3a

ASD: Autism spectrum disorder

JEV: Japanese Encephalitis Virus

Csf1r: Colony stimulating factor 1 receptor

PDGFRα: Platelet-derived growth factor receptor alpha

RA: Retinoic acid

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MICROGLIA TO NEURON INTERACTION VIA EXOSOMAL MIR-34A

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ABSTRACT

Microglia are extra-embryonic yolk sac-derived myeloid cells in the Central Nervous System (CNS) playing roles in regulating brain development, repairing injuries and maintaining neuronal networks. When signaling dangers to CNS by dead cells, microbes, unnecessary synapses and soluble antigens, they remove them and maintain homeostasis. Because of being the primary source of proinflammatory cytokines, microglial cells mediate neuroinflammation and regulate cellular responses that have extensive spectrum.

Exosomes are membrane-encapsulated vesicles that are released via various cell types. They have functions in regulating cell-to-cell transmission via carrying their cargo to recipient cells containing microRNAs (miRNAs), mRNAs, proteins and DNA. Microglia-derived exosomes promote neural regeneration by stimulating neurite outgrowth and might have a role in neurological disorders. MiRNAs within exosomes have functional roles when being delivered into the recipient cells and are thought to be the key functional elements.

The aim of this thesis is to identify the impacts of microglial cells on neuronal cells via exosomal miR-34a. As a requirement of this purpose, SH-SY5Y neuronal cell line will be treated with different concentrations of hydrogen peroxide (H_2O_2) and its cytotoxicity will be evaluated via LDH (Lactate Dehydrogenase) assay and PI (Propidium Iodide) visualization. Then, the cytotoxic effect of hydrogen peroxide on neurite outgrowth will be determined by assessing the number of protrusions per cell, and neurite lengths will be analyzed and expressed as mean process length. After that, HMC3 microglial cell line will be transfected with miR-34a mimic and their exosomes will be given to SH-SY5Y cells. The impacts of miR-34a on SH-SY5Y cells in terms of neuroprotection will be determined.

Keywords: Microglia, Macrophage, CNS, Exosome, MicroRNA, Neuron, SH-SY5Y, H_2O_2, HMC3

EKZOZOMAL MİR-34A ARACILIĞIYLA MİKROGLİA – NÖRON ETKİLEŞİMİ

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ÖZET

Mikroglial hücreler Merkezi Sinir Sisteminde (MSS), beyin gelişiminin düzenlenmesinde, hasarların onarılmasında ve nöronal iletişim ağlarının devamlılığında rol alan ekstra-embryonic yolk kesesinden türeyen myeloid hücrelerdir. Merkezi sinir sistemine karşı ölü hücreler, mikroplar, gereksiz sinapslar ve çözünebilir antijenler tarafından tehlike sinyalleri aldıklarında, bunları uzaklaştırırlar ve homeostaziyi sürdürürler. Mikroglial hücreler proinflamatuar sitokinlerin birincil kaynağı oldukları için nöroinflamasyona aracılık ederler ve geniş spektrumlu hücresel cevapları düzenlerler.

Ekzozomlar birçok hücre tipi tarafından salgılanan membranla çevrili veziküllerdir. MikroRNA (miRNA), mRNA, protein ve DNA içeren kargolarını alıcı hücrelere taşıyarak hücre-hücre iletişiminin düzenlenmesinde rol alırlar. Mikrogliadan elde edilen ekzozomlar nörit fazla büyümesini tetikleyerek nöral yenilenmeyi teşvik eder ve nörolojik hastalıklarda rol alabilirler. Ekzozomlarda bulunan miRNAlar alıcı hücrelerin içine iletildiklerinde fonksiyonel role sahiplerdir ve anahtar fonksiyonel elemanlar oldukları düşünülür.

Bu tezin amacı mikroglial hücrelerin nöronal hücreler üzerindeki etkisini miR-34a aracılığıyla incelemektir. Bu amacın gerekliliği olarak, SH-SY5Y nöronal hücre hattı hidrojen peroksitin farklı konsantrasyonlarıyla muamele edilecek ve sitotoksik etkisi LDH (Laktat Dehidrojenaz) ve PI (Propidyum İyodür) görüntülemeyle değerlendirilecektir. Sonrasında, hidrojen peroksitin nörit büyümesi üzerindeki sitotoksik etkisi hücre başına düşen çıkıntı sayısının hesaplanmasıyla belirlenecek ve nörit uzunlukları üzerindeki etkisi ise analiz edilip ortalama çıkıntı uzunluğu olarak ifade edilecektir. Ardından, HMC3 mikroglial hücre hattı miR-34a mimikle transfekte edilecek ve ekzozomları SH-SY5Y hücrelerine verilecektir. miR-34a'nın SH-SY5Y hücreleri üzerindeki etkileri nöroproteksiyon yönünden belirlenecektir.

Anahtar kelimeler: Mikroglia, Makrofaj, CNS, Ekzozom, MikroRNA, Nöron, SH-SY5Y, H_2O_2, HMC3

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Under stress conditions, several types of reactive oxygen species (ROS) are produced, and exposure to these ROS may slowly lead to various types of disorders like atherosclerosis and neurodegenerative disorders (Fukui, Takatsu et al. 2011). Glia-derived exosomes have shown neuro-regenerative and neurotrophic effects in spinal cord injury and ischemic stroke. For these reasons, they can be used as therapeutic agents in neural trauma, strokes and neurodegenerative diseases (Liao, Du et al. 2019). It was found that exosomal miRNAs have regulatory roles in various pathological pathways, comprising oxidative stress response, in neurodegenerative diseases (Jiang, Gong et al. 2020). Among these miRNAs, miR-34a adjusts neuritogenesis and spinal accidence (Agostini, Tucci et al. 2011).

1.2. Aim of the Study

The goal of the study was determining the effects of microglia-derived exosomal miR-34a on SH-SY5Y neuronal cells. Aim of the study was determining the effects of microglia-derived exosomal miR-34a on changes of neurite outgrowth in neuronal cells; and to determine the effects of microglia-derived exosomal miR-34a on H_2O_2-stimulated cell death of SH-SY5Y neuronal cells.

1.3. Hypothesis of the Study

miR-34a promotes neural regeneration by stimulating neurite outgrowth reduced via H_2O_2-induced damage to SH-SY5Y neuronal cells.

2. GENERAL INFORMATION

2.1. Microglia Definition

Microglia are extra-embryonic yolk sac-derived myeloid cells in the Central Nervous System (CNS) possessing memory-like features to be able to have context-dependent

responses (Ginhoux and Prinz 2015). They are distributed throughout the brain and interact with neurons, astrocytes and oligodendrocytes. At first, they were thought to be of neuroectodermal origin (Prinz, Jung et al. 2019). However, it is now determined that they are from myeloid lineage because of expressing several genes unique for myeloid lineage (Dubbelaar, Kracht et al. 2018). Microglial cells participate in immune response and regulate homeostasis of brain through searching for both internal dangers such as damaged tissues or dying cells, and external dangers such as pathogens. When faced with these problems, they start analyzing the injury, reducing the effects of inflammation and participating in tissue repair and remodeling (Ginhoux and Prinz 2015), (Kabba, Xu et al. 2018).

2.1.1. Microglia History and Origin

Microglia were distinguished as a divergent and privatized myeloid cells of CNS with characteristic developmental source via taking for more than 150 years of research (Ginhoux and Prinz 2015). For the first time in 1932, Del Rio-Hortega described a population of phagocytic and migratory cells that are in the CNS and of mesodermal origin. He first established the term ‘microglial cell’ in 1939 and identified them as non-neuronal, non-astrocytic agents of CNS and apart from astrocytes, oligodendrocytes and macroglia (Kettenmann, Hanisch et al. 2011), (Ginhoux and Prinz 2015), (Kabba, Xu et al. 2018). Over the years with debates about the origin of microglia whether it is mesodermal or ectodermal, several researchers proved their mesodermal origin with studies coupling light/electron microscopy and immunohistochemistry (Ginhoux and Prinz 2015).

2.1.2. Microglia Activation States

Activation states of microglia is classified as M1 (classical) and M2 (alternative). M1 state is pro-inflammatory and neurotoxic that is induced via stimulating both Toll-like receptor (TLR) and interferon (IFN)-γ pathways (Colonna and Butovsky 2017). At this state, microglia generate proinflammatory cytokines and chemokines like interleukin (IL)-6, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interleukin (IL)-12 and C-C Motif Chemokine Ligand 2 (CCL2) (Dubbelaar, Kracht et al. 2018). M1 microglia also generates reactive oxygen species (ROS), nitric oxide (NO), matrix metalloproteinase 12 (MMP12), major histocompatibility complex (MHC) II, Fc receptors and integrins (Colonna and

Butovsky 2017). On the other hand, M2 state is anti-inflammatory and induces release of IL-10 and transforming growth factor (TGF)-β, and has the capacity to enable debris clearance, wound healing and recover brain tissue homeostasis (Dubbelaar, Kracht et al. 2018). M2 microglia is stimulated via IL-4, IL-13, etc., and secretes growth factors and neurotrophic factors (Colonna and Butovsky 2017).

2.1.3. Microglia Function

Microglia have several functions including wiring of the brain in early development and involving in homeostasis over the course of life (Sominsky, De Luca et al. 2018). In early stages, activated microglia participate in neuroprotection, while in later stages, it participates in neuroinflammation and neurodegeneration (Petralla, De Chirico et al. 2021). Very early in development, microglia support synaptogenesis and dendritic spine generation via releasing relevant growth factors such as prostaglandin E2 and IL-10 (Lenz and Nelson 2018), and this support is important for behaviour (Sominsky, De Luca et al. 2018). In the developing central nervous system (CNS), microglia adjust brain improvement mainly by phagocytosis and releasing diffusible factors (Lenz and Nelson 2018) such as tumor necrosis factor (TNF) and nerve growth factor (NGF) (Matejuk and Ransohoff 2020). Microglia induce and promote cell formation and health through releasing insulin-like growth factor 1 (IGF1), tumor necrosis factor (TNF)-α and interferon (IFN)-γ. Living, dying or dead cells, axons and synaptic elements are phagocytosed by microglia. Several agents participate in microglia phagocytosis like vascular endothelial growth factor (VEGF), NADPH oxidase (Nox) 2 and Tyrobp/DAP12 either via identifying cells signed for scavencing or stimulating cell demise. Microglial cells also promote neurogenesis, axon twitch myelination/oligodendrogenesis, cell demise or cell survival, and excite synaptogenesis and synapse ageing (Lenz and Nelson 2018).

2.2. Hydrogen Peroxide in Neuronal Injury Model

Neurons, or the nerve cells, are the functional members in the activity of the nervous system (1997). They are more susceptible to exposure to hydrogen peroxide than other CNS cells such as astrocytes. For dopaminergic neurons, for example, hypoxia-induced metabolic

stress was hampered by hydrogen peroxide, yet, several studies indicate the role of hydrogen peroxide treatment on cultured neurons (Hohnholt and Dringen 2014).

Hydrogen peroxide is a ROS and a lipid-resolvable molecule which emanates into cytoplasm that have a role in synaptic and neuronal functionality (Ohashi, Hirano et al. 2016), and a messenger for neuron-glia signaling in the CNS (Hohnholt and Dringen 2014). Under physiological circumstances, H_2O_2 is reduced into H_2O and O_2 via some enzymes and H_2O_2 generation is equilibrated. Nonetheless, over production of H_2O_2 is shown to have a role in ischemia-reperfusion lesions of the brain (Ohashi, Hirano et al. 2016). Besides, over production of H_2O_2 promotes lipid peroxidation, DNA damage and neuronal cell demise (Liu, Feng et al. 2018). It also regulates neuronal functions such as synaptic conduction and cellular excitability in CNS (Ohashi, Hirano et al. 2016).

During cellular metabolism, H_2O_2 is generated as a byproduct and give rise to oxidative stress in high concentrations (Hohnholt and Dringen 2014). Too much oxidative stress promotes neuronal cell damage that is related with several neurodegenerative disorders. As CNS and neuronal cells demand high rate of oxygen depletion and benefication in polyunsaturated fatty acids, they are more prone to oxidative stress. Accumulation of ROS causes damage to DNA, proteins and lipid membranes, and thus, disrupts neuron functions and causes neuron demise and apoptosis (Liu, Feng et al. 2018).

2.3. microRNAs

MicroRNAs are conserved small and single stranded RNAs transcribed endogenously by RNA polymerase II or III, and discovered in 1993 by Ambros and Ruvkun group (Alural, Ozerdem et al. 2015), (Reddy, Williams et al. 2017), (O'Brien, Hayder et al. 2018). Their lengths differ from 18 to 23 nucleotides and they adjust gene expression post-transcriptionally through deadenylation, disaggregation and destabilization of target mRNAs (Bavamian, Mellios et al. 2015), (Huang 2017). MicroRNAs degrade target mRNA or inhibit its translation by directly attaching to 3’ -untranslated (3’-UTR) region of that target mRNA (Zhang, Cai et al. 2021). They have important roles in angiogenesis, apoptosis, cell cycle, differentiation and development (Alural, Ozerdem et al. 2015). They also regulate neuronal differentiation, development, dendritic spine morphology, neurite outgrowth and neuroplasticity (Bavamian, Mellios et al. 2015), (Zhang, Cai et al. 2021). Up to now, over

2000 miRNAs have been discovered that overhaul half the genes that code proteins in mammals (Reddy, Williams et al. 2017).

2.3.1. microRNA-34a

MicroRNA-34a (miR-34a) is a paralogous member of miR-34 family, together with miR-34b and miR-34c, of mRNAs that control cell-destiny decisions (Li, Huang et al. 2019), (Chua and Tang 2019). In vertebrates, miR-34a is codifyed via its own counterpart from chromosome 1p36 (Chua and Tang 2019), whereas miR-34b along with miR-34c have a familiar counterpart of chromosome 11 (Morgado, Xavier et al. 2015), (Jauhari, Singh et al. 2018). In a research with mice tissues by Bommer et al., miR-34a has been determined being expressed uttermost in brain, while miR-34b along with miR-34c greatest in lung (Fineberg, Datta et al. 2012), (Morgado, Xavier et al. 2015). According to some reports, miR-34 family is the transcriptional targets of tumor protein 53 (TP53 or p53), and overexpression of this family induces apoptosis and cell cycle interruption (Morgado, Xavier et al. 2015). P53 is recognized to mediate tumor suppression, regulate gene expression and several important events during brain development. Several studies showed entailment of miR-34a for the appropriate function of neuronal differentiation via regulating p53 action utilizing neural stem cells (NSC) from mouse. MiR-34a is also known to be upregulated mostly in motor neurons (Jauhari, Singh et al. 2018), and its overexpression in neuroblastoma cell lines regulates neuronal-specific genes (Agostini, Tucci et al. 2011).

In recent years, certain activities of miR-34a in neurodevelopment and differentiation were stated. In *Drosophila melanogaster*, neuronal survival was enhanced and neurodegeneration was decreased by up-regulating miR-34a. In a mouse model, it was shown that miR-34a adjusts neuron differentiation, neuronal development and synapse formation (Aranha, Santos et al. 2011). In a research via Chen et al. in PC12, it was shown that luteolin-mediated neurite outgrowth was regulated via miR-34a (Chen, Wu et al. 2015). Also, in a mouse neural stem cell model, overexpression of miR-34a promoted neurite outgrowth by downregulating Sirtuin 1 (SIRT1) (Aranha, Santos et al. 2011). In another study in mouse embryonic stem (mES) cells, the connectivity of cells and neurite outgrowth was decreased when expression of miR-34a was inhibited (Agostini, Tucci et al. 2011). The negative effect of amyloid precursor protein $(APP)_{L17C}$ (residue 17 in Aβ sequence) in SH-SY5Y on neuritogenesis was shown to be reversed via lentiviral-regulated miR-34a overexpression

(Luu, Ciccotosto et al. 2019). Also, transport of extracellular vesicles (EVs) containing miR-34a released from oral cavity squamous cell carcinoma (OCSCC) cells to cancer-related neurons adjusts extracellular vesicle-obtained axonogenic signals in a negative way (Amit, Takahashi et al. 2020). On the contrary to these, miR-34a increases the susceptibility of cortical neurons to damage, and its downregulation was shown to have a positive effect on neuronal survival (Santerre, Bagashev et al. 2019). In neurodegenerative diseases, miR-34a was demonstrated to be overexpressed, for instance in the hippocampi and cerebral cortex of Alzheimer's disase (AD) mouse model, in several tissues of AD patients and in peripheral tissues of patients with Amyotrophic lateral sclerosis (ALS). In AD mice model, miR-34a has been identified being upregulated and enhance pathophysiology of AD in later phases. Besides, Wang et al. have shown that aberrant miR-34a expression involves in pathogenesis of AD partly via inhibiting B-cell lymphoma 2 (BCL2) that result in abnormal apoptosis (Rostamian Delavar, Baghi et al. 2018). Also, Zhou et al. have demonstrated that, in Bipolar disorder (BD), miR-34a promoted lithium and valproate (VPA) effect via regulating glutamate metabotropic receptor 7 (GRM7) (Zhou, Yuan et al. 2009). In Tuberous Sclerosis Complex (TSC) genetic disease, miR-34a, along with miR-34b and miR-34c, was found to regulate neurogenesis and glutamate receptor signaling (Mills, Iyer et al. 2017).

2.4. Exosomal miRNAs in Neuron-Glia Interactions

Communication between neuron and glia can occur via direct contact, via the activity of released molecules, or via extracellular vesicles (Fernandes, Ribeiro et al. 2018). Exosomes are the smallest membrane-derived microvesicules ranging from 30 to 150 nm in size (Pascual, Ibanez et al. 2020), and they are released from every cell type to various body fluids (Zhang and Yang 2018), (Liao, Du et al. 2019). They contain proteins, lipids and genetic material involving great number of microRNAs (Fernandes, Ribeiro et al. 2018), and their content differs depending upon cells that they are released, and upon physiological and pathological conditions (Zhang and Yang 2018).

Some studies indicated that miRNAs can be delivered into recipient cells via exosomes (Brites and Fernandes 2015), carrying miRNAs within them, that are considered the key functional players, changing the gene expression and regulating the functional features of recipient cells (Brites and Fernandes 2015), (Zhang, Liu et al. 2019). Exosome transfer can take place from glia-to-neuron cells or from neuron-to-glia cells. The impact of microglial

exosomes on neurons was shown with several studies. Huang et al. demonstrated that transfer of microglial exosomes containing miR-124-3p inhibited neuroinflammation in repetitive traumatic brain injury (rTBI) mice, and induced neurite outgrowth (Huang, Ge et al. 2018). Also, another research indicated that microglia-derived exosomes containing miR-124-3p inhibited FIP200-regulated neuronal autophagy, resulting in reducing trauma-stimulated, autophagy-regulated nerve injury *in vitro* (Li, Huang et al. 2019). Song et al. have shown that microglial exosomal miR-124 were taken up by neurons both *in vitro* and *in vivo*, and miR-124 decreased ROCK along with USP14 expressions, inducing neuronal protection injured via ischemic stroke. Also, a recently published study was demostrated that after TBI, microglial exosomes with miR-124 enhanced neuronal renewal via inhibiting Toll-like receptor 4 (TLR4) and generating M2 microglial polarization. miR-181c was found to enhance the overexpression of tumor necrosis factor (TNF)-α in microglia causing microglia regulated neuronal injury (Petralla, De Chirico et al. 2021). Another study showed that exosomes secreted from BV2 microglia in M2 phenotype containing miR-137 enhanced the survival of ischemic neurons via Notch1 pathway (Zhang, Cai et al. 2021).

Neuron-microglia communication can also take place via neuron-derived exosomes. In spinal cord injury (SCI) mice model, neuron-derived exosomal miR-124-3p suppressed M1 microglia activation and decreased microglial neuroinflammation (Jiang, Gong et al. 2020). MiR-124 along with miR-9 from neuron-derived exosomes were found to inhibit CEBPα-PU.1 and NF-κB pathways, respectively, and resulted in deactivation of microglial cells in CNS (Veremeyko, Kuznetsova et al. 2019). Also, when taken up by N9 microglial cells, miR-124 from mutant copper-zinc superoxide dismutase 1 (mSOD1) neural stem cell (NSC)-34 motor neurons (MNs) exosomes polarized microglial cells into M1 phenotype early and caused promoted neuroinflammation and MN deformation (Pinto, Cunha et al. 2017). In a research via Zhou et al., α-synuclein transgenic SH-SY5Y exosomes containing miR-19a-3p was demonstrated to restrict microglial autophagy via PTEN (phosphatase and tensin homolog)/AKT (Protein Kinase B)/mTOR (mammalian target of rapamycin) signaling pathway (Zhou, Lin et al. 2019). In another research via Yin et al., exosomal miR-21-5p from neurons enhanced microglial polarization and thus, speed up the neuronal damage process via neuroinflammatory factors from microglia after TBI (Yin, Han et al. 2020). Also, when exosomal miR-21 from SH_{Swe} neuroblastoma cells (SH-SY5Y transfected via Swedish mutant

of amyloid precursor protein (APP_{695}) was taken up by CHME3 (or HMC3) microglia, it stimulated higher inflammatory response (Fernandes, Ribeiro et al. 2018).

3. MATERIALS AND METHODOLOGY

3.1. Research Type

This study is an *in vitro* type experimental study.

3.2. Time and Place of the Study

This thesis was performed at İzmir Biomedicine and Genome Institute, between the dates of February - August 2021.

3.3. Research Material

Human cells lines of HMC3 microglial clone 3 and SH-SY5Y neuroblastoma were used.

3.4. Variables of the Study

Hydrogen peroxide (H_2O_2) was the independent variable of the study. On the other hand, cell death rates and changes in neurite numbers and lengths are the dependent variables in this study.

3.5. Tools for Data Collection

Microplate reader MultiSkan Go spectrophotometer (Thermo Scientific, USA)

Fluorescent microscope Olympus IX-71 (Olympus, Japan)

Cytotoxicity Detection - Lactate Dehydrogenase (LDH) Kit (Roche, Germany)

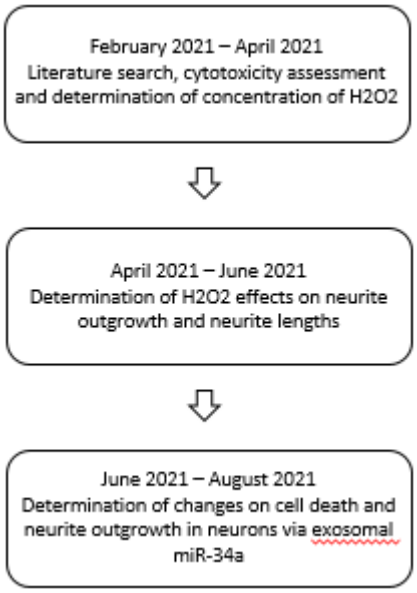
Cytotoxicity Detection - Propidium Iodide (PI) (Sigma-Aldrich, USA)

HiPerFect Transfection Reagent (Qiagen, Valencia, CA)

Total Exosome Isolation (from cell culture media) (Thermo Scientific, USA)

ImageJ 1.51n (NIH, USA)

3.6. **Study Plan and Calendar**



3.7. ***In vitro* Experiments**

3.7.1. Cell Culture

For the culturing of HMC3 cells, EMEM media (Sigma, Aldrich, Saint Louis, USA) containing 10% exosome-depleted FBS (fetal bovine serum) with 1% Pen/Strep (Penicillin-Streptomycin) (Thermo Scientific, Inc, Rockford, IL) were used, and cells were cultured at 37 °C supplemented with 5% CO_2.

For the culturing of SH-SY5Y cells, DMEM:F12 media (Gibco, Gaithersburg, MD) containing 10% exosome-depleted FBS with 1% Pen/Strep (Thermo Scientific, Inc, Rockford, IL) were used, and cells were cultured at 37 °C supplemented with 5% CO_2.

3.7.2. HMC3 Cell Transfection with miRNA Mimics

HMC3 cells were transfected with mimics via HiPerfect Transfection Reagent (Qiagen, Valencia, CA). Seeding 1.2 x 10^6 cells into T75 flask was performed, and cells were incubated overnight at 37 °C supplemented with 5% CO_2. After overnight incubation, mimics

were added into cell culture media using manufacturer's instructions and incubated for 2 days at 37 °C supplemented with 5% CO_2. For scrambled (negative control) group, same protocol was applied without adding miR-34a mimics.

3.7.3. Isolation of Total Exosomes from HMC3 Culture Media

Total Exosome Isolation (from cell culture media) (Thermo Scientific, USA) kit was used for isolation of exosomes. Culture media were harvested and centrifugation was performed at 2000 x g for 30 min. Supernatants were put into new tubes and half the volume of isolation reagent was added. The mixture was vortexed until homogenyzed and samples were incubated for a night at 4 °C. At the end of incubation, centrifugation was applied for 1 hour at 10,000 x g at 4 °C. Supernatants were removed. Pellets involving exosomes were resuspended in 50 µl 1X PBS for 1 ml started cell culture media.

3.7.4. Determination of Protein Level in Exosome Samples by BCA Assay

To determine protein concentration of exosomes, 5 µl of exosome suspension was mixed with 55 µl M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Inc., Rockford, IL) and 10 minutes vortex was applied to suspension. Protein concentrations of exosomes were evaluated with BCA Protein Assay Kit (Takara, Japan).

3.7.5. Uptake of Exosomes by SH-SY5Y Cells

Following by seeding 2 x 10^4 cells/well into 96-well plate (Sarstedt, Germany) and incubation for 24 hours, pretreatment with 5 µg/well scrambled and miR-34a mimic exosomes were performed to SH-SY5Y cells for 6 hours. After that, 25 µM Hydrogen Peroxide were added into wells and incubation were applied for 24 hours at 37 °C supplemented with 5% CO_2.

3.7.6. Determination of Cell Death by LDH Release Assay

Cytotoxicity with stimulation of Hydrogen Peroxide on SH-SY5Y was measured by LDH Kit^{PLUS} (Roche Diagnostics, Germany). Seeding of 2 x 10^4 cells/well was applied to PDL-coated (50 µg/ml) 96-well plate (Sarstedt, Germany) with 200 µl of final well volume

and incubated overnight at 37 °C supplemented with 5% CO_2. At the end of overnight incubation, treatment of cells with Hydrogen Peroxide with 25, 50 and 75 µM concentrations was performed for 24 hours. Culture media was gathered after the treatment, and LDH was measured with the given instructions. 492 nm wavelength along with 630 nm reference was applied for absorbance evaluation on microplate reader MultiSkan Go spectrophotometer (Thermo Scientific, USA). For the exosome uptake experiments, 25 µM H_2O_2 was determined to be optimal. LDH amounts from dead cells were stated as cytotoxicity and calculated by the following formula:

$$\text{Cytotoxicity} = (OD_{Sample} - OD_{Control}) / (OD_{MaximalRelease} - OD_{Control}) * 100$$

3.7.7. Determination of Cell Death by PI Staining

Seeding SH-SY5Y to PDL-coated (50 µg/ml) 96-well plate (Sarstedt, Germany) were performed with 2 x 10^4 cells/well density with 200 µl of final well volume and incubated overnight at 37 °C supplemented with 5% CO_2. At the end of incubation, treatment of cells was performed with Hydrogen Peroxide for 24 hours at 37 °C supplemented with 5% CO_2. At the end of treatment, PI (Sigma-Aldrich, USA) stain was included into the culture media for 15 minutes. Cells dyed with PI stain were observed utilizing inverted fluorescent microscope IX-71 (Olympus, Japan). For the exosome uptake experiments, 25 µM H_2O_2 was determined to be optimal. Stained cells was then numbered utilizing ImageJ 1.51n (NIH, USA) and data was stated as percentage of PI stained cells.

3.7.8. Determination of Neurite Outgrowth

SH-SY5Y was seeded into PDL-coated (50 µg/ml) 96-well plate (Sarstedt, Germany) with 2 x 10^4 cells/well density with 200 µl of final well volume and incubated overnight at 37 °C supplemented with 5% CO_2. At the end of incubation, cells were treated with Hydrogen Peroxide with 25, 50 and 75 µM concentrations for 24 hours at 37 °C supplemented with 5% CO_2. At the end of treatment, they were photographed via inverted fluorescent microscope IX-71 (Olympus, Japan) at 20X magnification. For the exosome uptake experiments, 25 µM H_2O_2 was determined to be optimal. Experiments carried out three times and samples contain minimum 30 neurites. Analysis of neurite outgrowth was done via numbering cell processes

and evaluating neurite lenghts utilizing ImageJ 1.51n program (NIH, USA). 20 μm was determined to be minimum neurite length.

4. RESULTS

4.1. Determination of Cell Death by LDH Release Assay

Varying concentrations of Hydrogen Peroxide ranging from 25 to 75 μM with an addition of non-treated group were given to SH-SY5Y cells determine the toxicity of Hydrogen Peroxide. LDH release was measured via Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics, Germany). As seen in **Figure 1**, following the treatment of cells via 25 μM H_2O_2, cell death was determined to be 37.0617 ± 5.670. Likewise, for 50 μM H_2O_2 treated group, cell death was determined to be 67.4068 ± 4.769, and for 75 μM H_2O_2 treated group, cell death was determined to be 81.162 ± 3.971 depending on Mann-Whitney test.

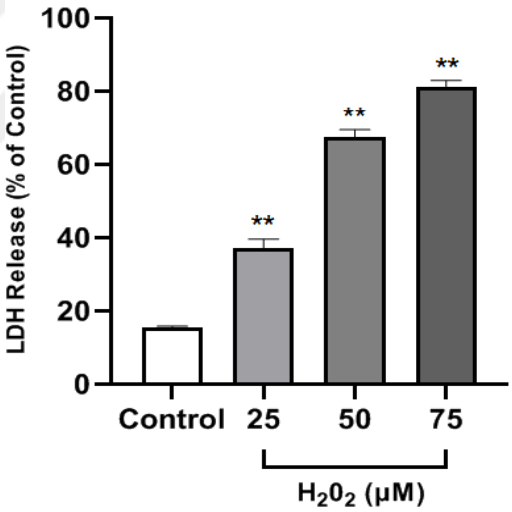


Figure 1. Impact of variable doses of H_2O_2 on cell death. Cell death with varying concentrations of H_2O_2 was investigated to determine the optimal dose. Results were given as Mean ± SEM (**p<0.01).

4.2. Determination of Cell Death by PI Assay

SH-SY5Y were stained with PI dye after treatment with varying H_2O_2 concentrations. It was shown in **Figure 2** that following cell treatments via 25 μM H_2O_2, cell death ratio was

determined to be 10.4507 ± 3.088. Likewise, for 50 µM H_2O_2 treated group, cell death ratio was determined to be 19.0946 ± 6.145, and for 75 µM H_2O_2 treated group, cell death ratio was determined to be 40.5764 ± 8.560 depending on Mann-Whitney test. It was concluded that upon treatment via H_2O_2, cell death ratio has been significantly increased with the increasing doses in SH-SY5Y.

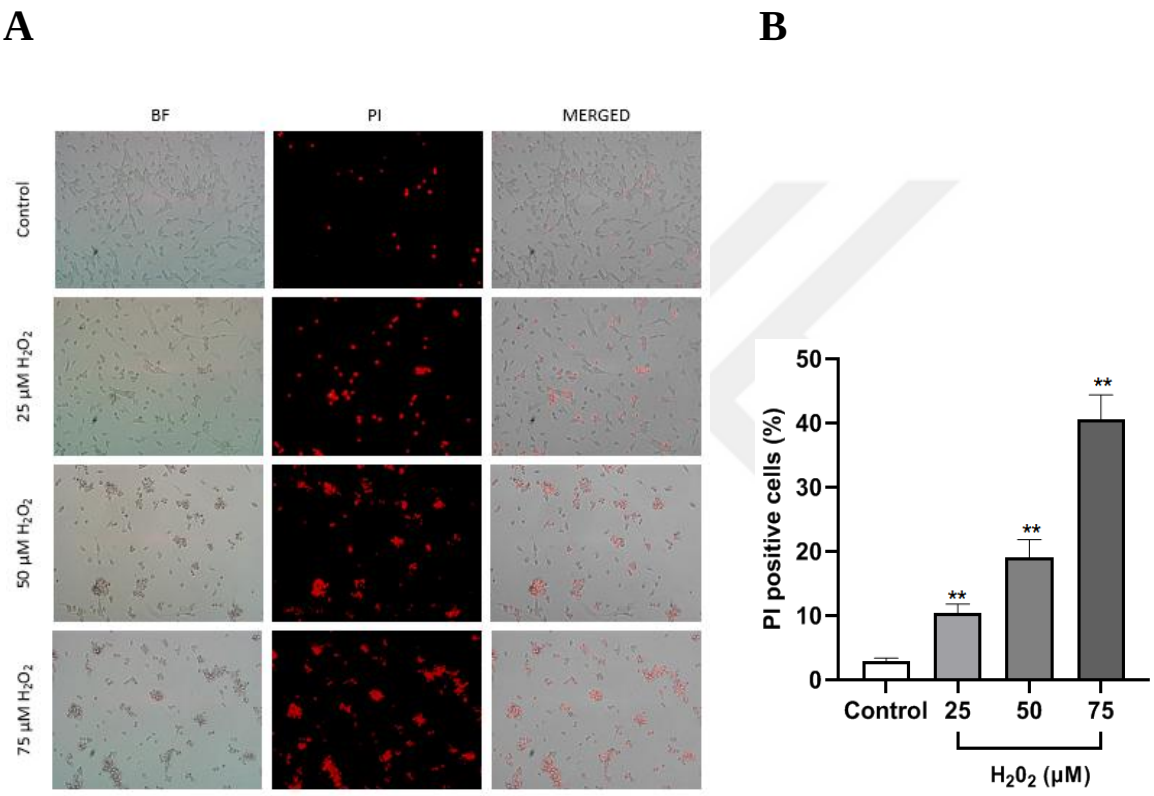


Figure 2. Impacts of varying doses of H_2O_2 on cell death via PI staining. Cell death was determined to increase significantly upon H_2O_2 treatment. Results were given as Mean ± SEM (**p<0.01).

4.3. Determination of the Effect of H_2O_2 on Neurite Outgrowth

SH-SY5Y were treated via varying doses of H_2O_2 to determine the changes on neurite outgrowth. As seen in **Figure 3**, following the treatment of cells via 25 µM H_2O_2, neurite number per cell was determined to be 2.06667 ± 0.3456, and neurite length was determined to be 41.6055 ± 6.340. Likewise, for 50 µM H_2O_2 treated group, neurite number per cell was

determined to be 1.76667 ± 0.3456, and neurite length was determined to be 37.4374 ± 8.668. For 75 µM H_2O_2 treated group, neurite number per cell was determined to be 1.46667 ± 0.1394, and neurite length was determined to be 32.9831 ± 4.833. It was concluded that upon treatment via H_2O_2, neurite outgrowth have been decreased in a dose dependent manner in SH-SY5Y cells. Significant decrease was only seen in neurite number per cell in 75 µM treated group.

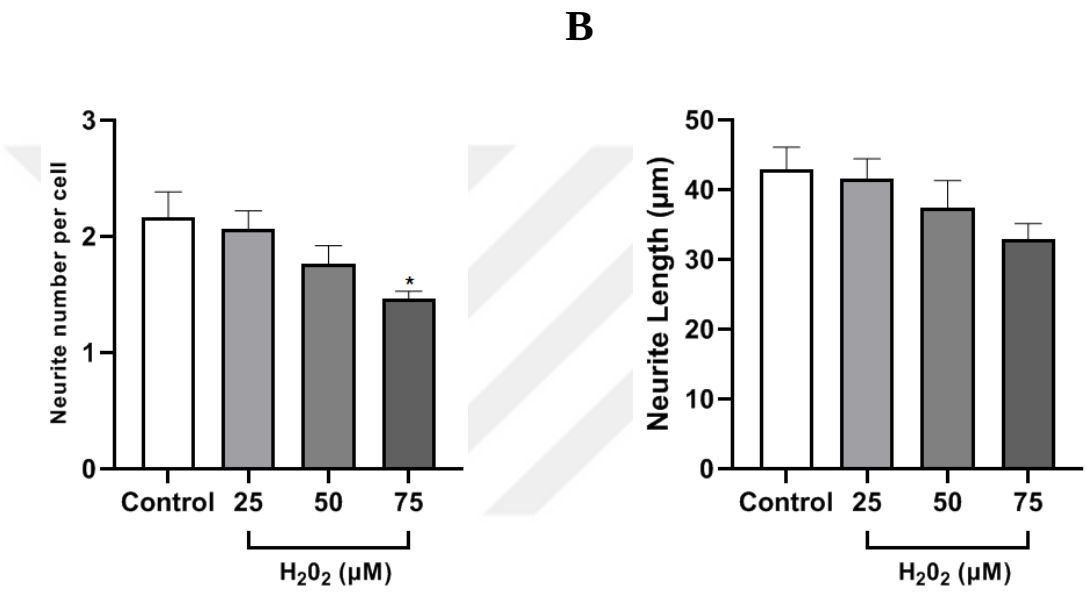


Figure 3. Impacts of varying doses of H_2O_2 on neurite outgrowth. Neurite outgrowth and neurite length were determined to decrease upon H_2O_2 treatment. Results were given as Mean ± SEM (*p<0.05).

4.4. Determination of Changes on Cell Death via miR-34a Mimic by LDH Release Assay

After seeding 2 x 10^4 SH-SY5Y cells/well to PDL-coated (50 µg/ml) 96-well plate and incubation for 24 hours, 5 µg/well of scrambled and miR-34a mimic containing exosomes along with 25 µM H_2O_2 were added into wells for 24 hours at 37 °C supplemented with 5% CO_2. As seen in **Figure 4**, following the treatment of cells via exosomes along with 25 µM H_2O_2, cell death was determined to be 59.2918 ± 4.983 for H_2O_2-only-treated group, 66.0068 ± 8.683 for scrambled group, and 51.3142 ± 12.41 for miR-34a mimic-treated group

depending on Mann-Whitney test. It was concluded that upon treatment via exosomes, miR-34a mimic exosome-treated group reduced cell death compared to scrambled group.

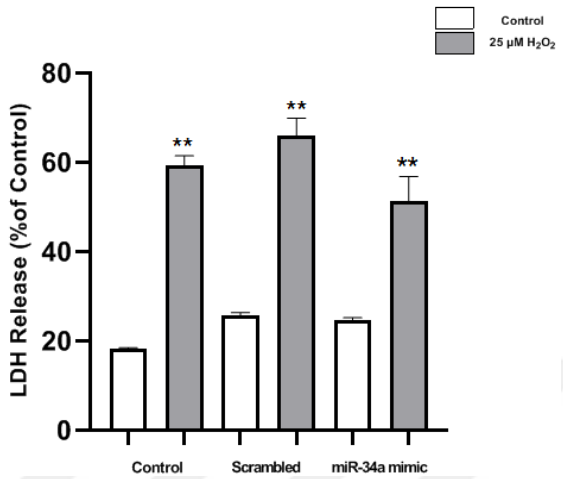


Figure 4. Impact of miR-34a mimic on cell death. Microglial scrambled and miR-34a mimic containing exosomes were given to SH-SY5Y cells to determine changes on cell death. Results were given as Mean ± SEM (**p<0.01)

4.5. Determination of Changes on Cell Death via miR-34a Mimic by PI Assay

SH-SY5Y were stained with PI dye after treatment with 5 µg/well of scrambled and miR-34a mimic containing exosomes along with 25 µM H_2O_2. As seen in **Figure 5**, following the treatment of cells via exosomes along with 25 µM H_2O_2, cell death ratio was determined to be 19.6784 ± 4.152 for H_2O_2-only-treated group, 18.5802 ± 3.523 for scrambled group, and 13.69 ± 4.447 for miR-34a mimic-treated group depending on Mann-Whitney test. It was concluded that upon treatment via exosomes, cell death ratio has been decreased in miR-34a mimic exosome-treated group compared to scrambled group.

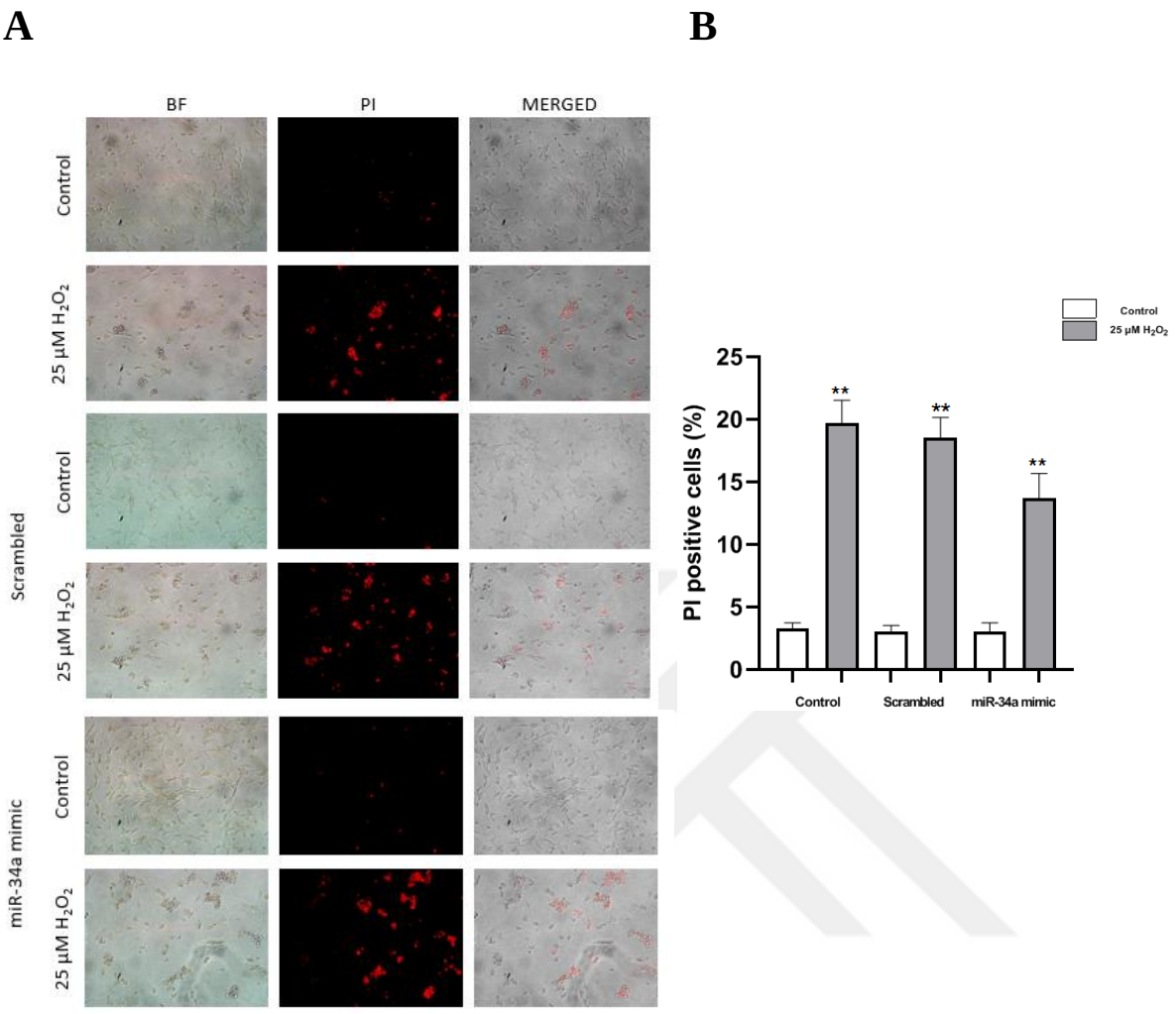


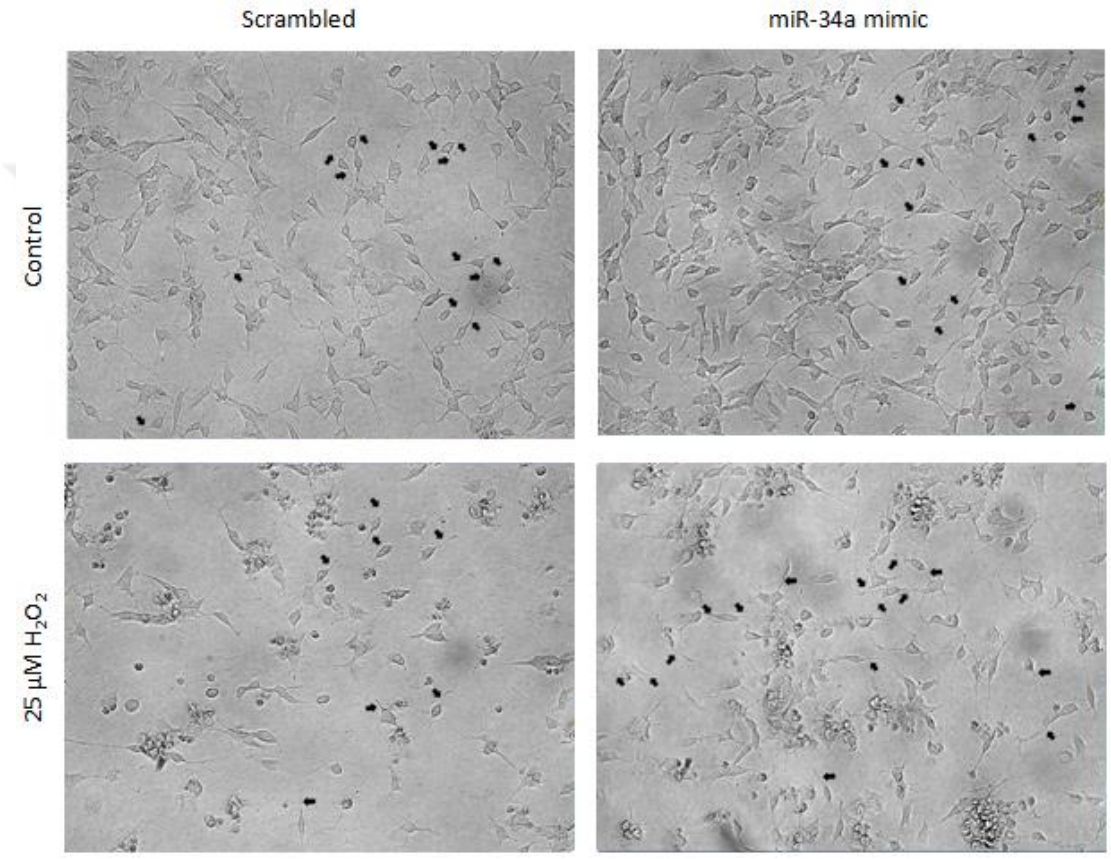
Figure 5. Impact of miR-34a mimic on cell death via PI staining. Cell death was determined to decrease upon miR34-a mimic treatment compared to scrambled exosome treatment. Results were given as Mean ± SEM (**p<0.01).

4.6. Determination of Changes on Neurite Outgrowth and Neurite Length via miR-34a Mimic

SH-SY5Y cells were treated via scrambled and miR-34a mimic exosomes to determine the changes on neurite outgrowth and neurite length. As seen in **Figure 6**, following the treatment of cells via scrambled and miR-34a mimic exosomes, neurite number per cell was determined to be 1.7 ± 0.1394, and neurite length was determined to be 29.6705 ± 2.388 for H_2O_2-only treated group. Likewise, for scrambled exosome treated group, neurite number per cell was determined to be 1.56667 ± 0.1900, and neurite length was determined to be 34.7833

± 4.924, and for miR-34a mimic exosome treated group, neurite number per cell was determined to be 1.9 ± 0.4183, and neurite length was determined to be 41.7109 ± 2.154. It was concluded that upon treatment via exosomes, miR-34a mimic exosome application increased neurite outgrowth compared to scrambled exosome application.

A



B **C**

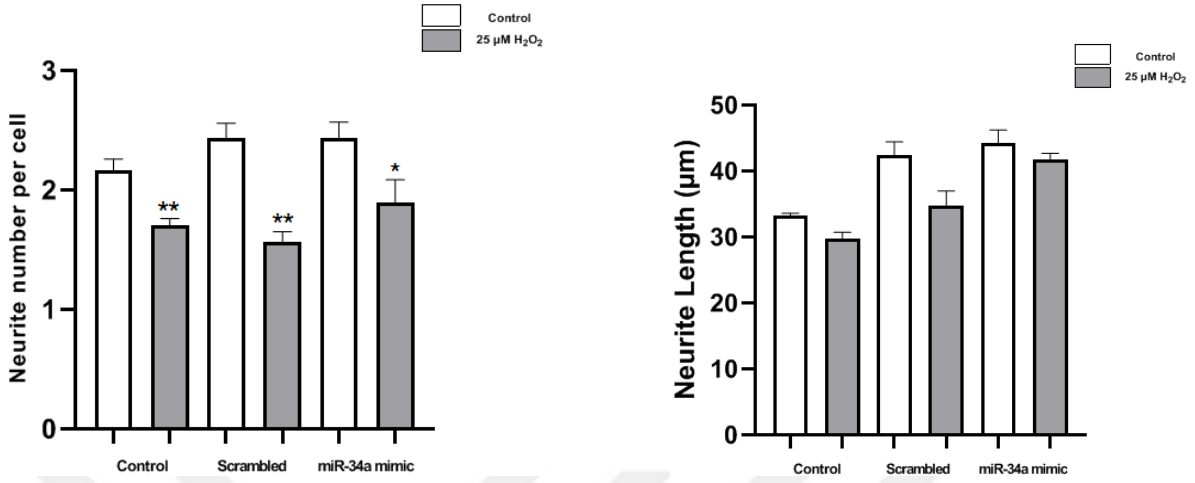


Figure 6. Impact of miR-34a mimic on neurite outgrowth. Neurite outgrowth and neurite length were determined to increase upon miR34-a mimic treatment compared to scrambled exosome treatment. Arrowheads indicate the neurites arising from cells. Results were given as Mean ± SEM (*p<0.05, **p<0.01).

5. DISCUSSION

Oxidative trauma resulted from ROS stimulation is important in regulating brain damage (Chetsawang, Govitrapong et al. 2010). Among different ROS, hydrogen peroxide (H_2O_2) is a non-radical form and is turned into highly reactive hydroxyl radicals that give rise to injury to many cellular constituents (Chetsawang, Govitrapong et al. 2010), (Feng, Luo et al. 2016), and neuronal and glial cell death as great level of polyunsaturated fatty acids present in brain that are more prone to ROS attack (Guesmi, Bellamine et al. 2018). Comprehending the molecular mechanism behind H_2O_2-stimulated neuronal cell death is really important for creating interference strategies. Thus, H_2O_2 is extensively used in *in vitro* studies resembling oxidative damage (Sun, Zheng et al. 2019). For example, in a human SH-SY5Y neuroblastoma cell model, H_2O_2 was utilized to determine the activation of Ras-dependent death signaling cascade (Chetsawang, Govitrapong et al. 2010). Also, in a research via Feng et al., H_2O_2-induced death of SH-SY5Y cells was shown to be reversed via lycopene (Feng, Luo et al. 2016). In our thesis study, H_2O_2 was used to induce oxidative trauma on human SH-SY5Y neuroblastoma cells. In several studies, different amounts of H_2O_2 were

used on SH-SY5Y cells to determine its toxic effect between the range of 0 and 400 μM for 24h (Jia, Zhu et al. 2008), (Morelli, Piscioneri et al. 2014), (Chung, Ahmed et al. 2021), (Feng, Luo et al. 2016). Based on these studies, concentrations from 0 to 75 μM H_2O_2 for 24h were used in our study, and 25 μM H_2O_2 for 24h was selected for further use as higher concentrations caused too much cell death, leaving insufficient amount of cells not suitable to study.

Neurodegenerative disorders are correlated with neuronal cell demise and neurite outgrowth deterioration generally resulted from oxidative stress (Duangjan, Rangsinth et al. 2021). Neurite outgrowth is described as the buds of cell protrusions that will then transform into axons and dendrites, and is an important process of neural differentiation and regeneration, creating appropriate connection in a neuronal network (Kim, Yoon et al. 2011), (Duangjan, Rangsinth et al. 2021). Its regulation can enhance neuronal renewal from nerve damage and neurological diseases, featuring significant roles in improving therapies for neurodegenerative diseases (Duangjan, Rangsinth et al. 2021), (Chen, He et al. 2020). As indicated in some studies, increasing concentrations of H_2O_2 were used to determine SH-SY5Y cell architecture, resulted in alleviated neurite numbers and aggregation of clumping cells such as 250 μM for 2h (Chen, He et al. 2020), 300 μM for 0 to 4h (Ashabi, Ahmadiani et al. 2013) and 150 μM for 24h (Chung, Ahmed et al. 2021). Based on these studies, concentrations from 0 to 75 μM H_2O_2 for 24h were used in our study, and it was found that, increasing concentrations of H_2O_2 affected neurite numbers per cell and neurite lengths.

In our thesis study, human microglia was used to investigate its effects via exosomes on neuronal cells since they can carry information through releasing high amounts of exosomes to target cells (Fan, Li et al. 2020). In the CNS, an injury or disease drives microglia to active state to fight pathology and provide neuroprotection. However, if the activating signals become chronic, microglia-derived agents become destructive to neuronal survival (Hooper, Sainz-Fuertes et al. 2012). In our study, protective role of microglia via exosomes was demonstrated as this is also supported by some studies. For example, IL-4-treated microglia-derived exosomes were shown to ameliorate damage resulted from ischemic stroke via enhancing angiogenesis through miRNA-26a contained in the exosomes (Tian, Zhu et al. 2019). In another study with SCI rat model, since resveratrol stimulates autophagy in several neurological disorders, resveratrol-loaded microglial exosomes were reported to promote neuronal survival and raise autophagy proportions during reducing apoptosis degrees through

phosphatidylinositol 3-kinase (PI3K) signaling pathway (Fan, Li et al. 2020). Also, in a research via Hooper et al., wingless-related integration site (Wnt)-3a-treated microglial exosomes was shown to be taken up by neurons (Hooper, Sainz-Fuertes et al. 2012), exhibiting important roles in neurodevelopmental stages and neurological disorders such as synapse generation and plasticity, and autism spectrum disorder (ASD) (Mulligan and Cheyette 2017), (Lizarraga-Valderrama and Sheridan 2021). Besides neuroprotective activities, there is a study that shows neurotoxic activities of microglial exosomes on neurons. According to that study by Mukherjee et al., *let-7a/b* expression can be stimulated by Japanese Encephalitis Virus (JEV) in microglial cells, and it was shown that, when *let-7a/b* was released to the extracellular space via exosomes, they are taken up by neurons and causing neurotoxicity via activating caspases (Mukherjee, Akbar et al. 2019). Therefore, this study should not be ignored in studies investigating virus-stimulated pathogenesis mediated through microglial exosomes. As a result, to our knowledge, there is no such a study in the literature that created oxidative stress with H_2O_2 on neuronal cells and tried to observe the effects of microglial exosomes. Therefore, our thesis is the first study that created that model.

Microglial exosomes, like other cells-derived exosomes, contain miRNAs that have different effects in biological processes such as oxidative stress, angiogenesis and neurogenesis (Petralla, De Chirico et al. 2021). Among them, miR-34a was shown to have important functions in brain (Wang, Tang et al. 2020), and microglial exosomal miR-34a was shown to have protective effects on neuronal cells in our thesis research. In previous studies, miR-34a was demonstrated to have neuroprotective effects. For example, in Schwann cells, miR-34a is expressed constantly, and was demonstrated to regulate proliferation and myelination after peripheral nerve injury, showing regenerative response (Viader, Chang et al. 2011). In another research, in SH-SY5Y damaged via paraquat, miR-34a expression was shown to be decreased. However, with the addition of lithium, this effect was reversed (Alural, Ozerdem et al. 2015). Other than neuroprotective activities of miR-34a shown in these studies, some studies report different roles of miR-34a. For example, in SCI rats, miR-34a has been shown to involve in glial scar generation, cell apoptosis and inflammatory answer via mediating Notch1, colony stimulating factor 1 receptor (Csf1r) and platelet-derived growth factor receptor α (PDGFRα) (Chen, Cao et al. 2016). In addition, in traumatic brain injury (TBI) model, miR-34a has shown to be overexpressed, inducing elevated stress and fragility of transfected cultured neurons (Truettner, Motti et al. 2013). All in all, since

miR-34a has a dual role, the impacts of microglial exosomal miR-34a in neuroprotection are not exactly known. Therefore, researches are required to understand the exact mechanisms behind these roles. In our study, overexpression of miR-34a was accomplished via microglial transfection with miR-34a mimic (Wang, Tang et al. 2020), following the transfer of microglial exosomal miR-34a to SH-SY5Y cells, resulting in alleviated cell death and enhanced neurite outgrowth and length.

There are some limitations in our study. First, human microglial HMC3 cell line was used as exosome source. Since they were immortalized via SV40-dependent viral mechanism (Janabi, Peudenier et al. 1995), their phenotype might not reflect the primary microglial phenotype. They show genetic and functional differences from primary microglia (Timmerman, Burm et al. 2018). Second, SH-SY5Y cell line was used. These cells are neuroblastoma cells that are primarily derived from metastatic bone tumor biopsy (Biedler, Roffler-Tarlov et al. 1978). Because of that, they are not mature enough and their appearances are different from the phenotype of mature neurons (Kovalevich and Langford 2013). Third, undifferentiated SH-SY5Y has been utilized that have a tendency to grow in clusters and create clumps, seeming to grow on top of each other and showing truncated processes. Differentiation process can be performed with retinoic acid (RA), and has some advantages. For example, differentiated cells can generate and extend neuritic processes, and they are less prone to toxin-mediated cell death than undifferentiated cells. That's why, in further studies, differentiated SH-SY5Y cells should be used and differences should be observed (Kovalevich and Langford 2013). Fourth, to our knowledge, there is no *in vivo* delivery experiments of microglial exosomal miR-34a in the literature. Since *in vivo* experiments were considered to be expensive and time consuming, it stimulates real body conditions and gives clinically relevant results (Omidi, Fatehinya et al. 2017). Therefore, *in vivo* experiments should be performed to get more reliable results. Fifth, overexpression of miR-34a was established via microglial transfection with miR-34a mimics, and exosomes containing miR-34a mimics were transferred to neuronal cells. Even though being an effective mechanism, impacts of miRNA mimics are temporary and reside in transfection efficiency. In addition, it is challenging and expensive for miRNA mimics to be used for *in vivo* studies. Therefore, polymerase II promoters like CMV or SV40, and polymerase III promoters can be used instead of mimics to induce priMIR expression because of the powerful transcriptional action of polymerase II promoter (Fan, Feng et al. 2020). Sixth, in our study, exosome uptake took

place from microglia to neuronal cells. Because of being a selective process, mechanisms of exosome uptake differ with respect to cell types. According to previous reports, neurons uptake exosomes via clathrin and dynamin-dependent endocytosis, whereas microglia uptake exosomes derived from oligodendrocytes via micropinocytosis (Zhang and Yang 2018). Considering this, efficient transfer of exosomes between different cells should be taken into account in studies.

6. CONCLUSION

In conclusion, this study demonstrated that exosomal miR-34a treatment ameliorates H_2O_2-stimulated cell death and enhances neurite outgrowth in neuroblastoma cells, attributing some clues about how miR-34a involve in neuroprotection through microglial exosomes. Moreover, since this is the first study that shows the transfer of miR-34a to neurons through microglial exosomes, further studies are needed to comprehend the exact mechanisms.

7. REFERENCES

1. (1997). "The principles of nerve cell communication." Alcohol Health Res World **21**(2): 107-108.
2. Agostini, M., P. Tucci, J. R. Steinert, R. Shalom-Feuerstein, M. Rouleau, D. Aberdam, I. D. Forsythe, K. W. Young, A. Ventura, C. P. Concepcion, Y. C. Han, E. Candi, R. A. Knight, T. W. Mak and G. Melino (2011). "microRNA-34a regulates neurite outgrowth, spinal morphology, and function." Proc Natl Acad Sci U S A **108**(52): 21099-21104.
3. Alural, B., A. Ozerdem, J. Allmer, K. Genc and S. Genc (2015). "Lithium protects against paraquat neurotoxicity by NRF2 activation and miR-34a inhibition in SH-SY5Y cells." Front Cell Neurosci **9**: 209.
4. Amit, M., H. Takahashi, M. P. Dragomir, A. Lindemann, F. O. Gleber-Netto, C. R. Pickering, S. Anfossi, A. A. Osman, Y. Cai, R. Wang, E. Knutsen, M. Shimizu, C. Ivan, X. Rao, J. Wang, D. A. Silverman, S. Tam, M. Zhao, C. Caulin, A. Zinger, E. Tasciotti, P. M. Dougherty, A. El-Naggar, G. A. Calin and J. N. Myers (2020). "Loss of p53 drives neuron reprogramming in head and neck cancer." Nature **578**(7795): 449-454.
5. Aranha, M. M., D. M. Santos, S. Sola, C. J. Steer and C. M. Rodrigues (2011). "miR-34a regulates mouse neural stem cell differentiation." PLoS One **6**(8): e21396.
6. Ashabi, G., A. Ahmadiani, A. Abdi, S. B. Abraki and F. Khodagholi (2013). "Time course study of Abeta formation and neurite outgrowth disruption in differentiated human neuroblastoma cells exposed to H2O2: protective role of autophagy." Toxicol In Vitro **27**(6): 1780-1788.
7. Bavamian, S., N. Mellios, J. Lalonde, D. M. Fass, J. Wang, S. D. Sheridan, J. M. Madison, F. Zhou, E. H. Rueckert, D. Barker, R. H. Perlis, M. Sur and S. J. Haggarty (2015). "Dysregulation of miR-34a links neuronal development to genetic risk factors for bipolar disorder." Mol Psychiatry **20**(5): 573-584.

8. Biedler, J. L., S. Roffler-Tarlov, M. Schachner and L. S. Freedman (1978). "Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones." Cancer Res **38**(11 Pt 1): 3751-3757.
9. Brites, D. and A. Fernandes (2015). "Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation." Front Cell Neurosci **9**: 476.
10. Chen, P. Y., M. J. Wu, H. Y. Chang, M. H. Tai, C. T. Ho and J. H. Yen (2015). "Up-Regulation of miR-34a Expression in Response to the Luteolin-Induced Neurite Outgrowth of PC12 Cells." J Agric Food Chem **63**(16): 4148-4159.
11. Chen, S. X., J. H. He, Y. J. Mi, H. F. Shen, M. Schachner and W. J. Zhao (2020). "A mimetic peptide of alpha2,6-sialyllactose promotes neuritogenesis." Neural Regen Res **15**(6): 1058-1065.
12. Chen, Y., S. Cao, P. Xu, W. Han, T. Shan, J. Pan, W. Lin, X. Chen and X. Wang (2016). "Changes in the Expression of miR-34a and its Target Genes Following Spinal Cord Injury In Rats." Med Sci Monit **22**: 3981-3993.
13. Chetsawang, J., P. Govitrapong and B. Chetsawang (2010). "Hydrogen peroxide toxicity induces Ras signaling in human neuroblastoma SH-SY5Y cultured cells." J Biomed Biotechnol **2010**.
14. Chua, C. E. L. and B. L. Tang (2019). "miR-34a in Neurophysiology and Neuropathology." J Mol Neurosci **67**(2): 235-246.
15. Chung, Y. S., P. K. Ahmed, I. Othman and M. F. Shaikh (2021). "Orthosiphon stamineus Proteins Alleviate Hydrogen Peroxide Stress in SH-SY5Y Cells." Life (Basel) **11**(6).
16. Colonna, M. and O. Butovsky (2017). "Microglia Function in the Central Nervous System During Health and Neurodegeneration." Annu Rev Immunol **35**: 441-468.
17. Duangjan, C., P. Rangsinth, S. Zhang, M. Wink and T. Tencomnao (2021). "Anacardium Occidentale L. Leaf Extracts Protect Against Glutamate/H2O2-Induced Oxidative Toxicity and Induce Neurite Outgrowth: The Involvement of SIRT1/Nrf2 Signaling Pathway and Teneurin 4 Transmembrane Protein." Front Pharmacol **12**: 627738.
18. Dubbelaar, M. L., L. Kracht, B. J. L. Eggen and E. Boddeke (2018). "The Kaleidoscope of Microglial Phenotypes." Front Immunol **9**: 1753.
19. Fan, J., Y. Feng, R. Zhang, W. Zhang, Y. Shu, Z. Zeng, S. Huang, L. Zhang, B. Huang, D. Wu, B. Zhang, X. Wang, Y. Lei, Z. Ye, L. Zhao, D. Cao, L. Yang, X. Chen, B. Liu, W. Wagstaff, F. He, X. Wu, J. Zhang, J. Moriatis Wolf, M. J. Lee, R. C. Haydon, H. H. Luu, A. Huang, T. C. He and S. Yan (2020). "A simplified system for the effective expression and delivery of functional mature microRNAs in mammalian cells." Cancer Gene Ther **27**(6): 424-437.
20. Fan, Y., Y. Li, S. Huang, H. Xu, H. Li and B. Liu (2020). "Resveratrol-primed exosomes strongly promote the recovery of motor function in SCI rats by activating autophagy and inhibiting apoptosis via the PI3K signaling pathway." Neurosci Lett **736**: 135262.
21. Feng, C., T. Luo, S. Zhang, K. Liu, Y. Zhang, Y. Luo and P. Ge (2016). "Lycopene protects human SHSY5Y neuroblastoma cells against hydrogen peroxideinduced death via inhibition of oxidative stress and mitochondriaassociated apoptotic pathways." Mol Med Rep **13**(5): 4205-4214.
22. Fernandes, A., A. R. Ribeiro, M. Monteiro, G. Garcia, A. R. Vaz and D. Brites (2018). "Secretome from SH-SY5Y APPSwe cells trigger time-dependent CHME3 microglia activation phenotypes, ultimately leading to miR-21 exosome shuttling." Biochimie **155**: 67-82.

23. Fineberg, S. K., P. Datta, C. S. Stein and B. L. Davidson (2012). "MiR-34a represses Numbl in murine neural progenitor cells and antagonizes neuronal differentiation." PLoS One **7**(6): e38562.
24. Fukui, K., H. Takatsu, T. Koike and S. Urano (2011). "Hydrogen peroxide induces neurite degeneration: Prevention by tocotrienols." Free Radic Res **45**(6): 681-691.
25. Ginhoux, F. and M. Prinz (2015). "Origin of microglia: current concepts and past controversies." Cold Spring Harb Perspect Biol **7**(8): a020537.
26. Guesmi, F., H. Bellamine and A. Landoulsi (2018). "Hydrogen peroxide-induced oxidative stress, acetylcholinesterase inhibition, and mediated brain injury attenuated by Thymus algeriensis." Appl Physiol Nutr Metab **43**(12): 1275-1281.
27. Hohnholt, M. C. and R. Dringen (2014). "Short time exposure to hydrogen peroxide induces sustained glutathione export from cultured neurons." Free Radic Biol Med **70**: 33-44.
28. Hooper, C., R. Sainz-Fuertes, S. Lynham, A. Hye, R. Killick, A. Warley, C. Bolondi, J. Pocock and S. Lovestone (2012). "Wnt3a induces exosome secretion from primary cultured rat microglia." BMC Neurosci **13**: 144.
29. Huang, S., X. Ge, J. Yu, Z. Han, Z. Yin, Y. Li, F. Chen, H. Wang, J. Zhang and P. Lei (2018). "Increased miR-124-3p in microglial exosomes following traumatic brain injury inhibits neuronal inflammation and contributes to neurite outgrowth via their transfer into neurons." FASEB J **32**(1): 512-528.
30. Huang, W. (2017). "MicroRNAs: Biomarkers, Diagnostics, and Therapeutics." Methods Mol Biol **1617**: 57-67.
31. Janabi, N., S. Peudenier, B. Heron, K. H. Ng and M. Tardieu (1995). "Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen." Neurosci Lett **195**(2): 105-108.
32. Jauhari, A., T. Singh, P. Singh, D. Parmar and S. Yadav (2018). "Regulation of miR-34 Family in Neuronal Development." Mol Neurobiol **55**(2): 936-945.
33. Jia, Z., H. Zhu, H. P. Misra and Y. Li (2008). "Potent induction of total cellular GSH and NQO1 as well as mitochondrial GSH by 3H-1,2-dithiole-3-thione in SH-SY5Y neuroblastoma cells and primary human neurons: protection against neurocytotoxicity elicited by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or hydrogen peroxide." Brain Res **1197**: 159-169.
34. Jiang, D., F. Gong, X. Ge, C. Lv, C. Huang, S. Feng, Z. Zhou, Y. Rong, J. Wang, C. Ji, J. Chen, W. Zhao, J. Fan, W. Liu and W. Cai (2020). "Neuron-derived exosomes-transmitted miR-124-3p protect traumatically injured spinal cord by suppressing the activation of neurotoxic microglia and astrocytes." J Nanobiotechnology **18**(1): 105.
35. Kabba, J. A., Y. Xu, H. Christian, W. Ruan, K. Chenai, Y. Xiang, L. Zhang, J. M. Saavedra and T. Pang (2018). "Microglia: Housekeeper of the Central Nervous System." Cell Mol Neurobiol **38**(1): 53-71.
36. Kettenmann, H., U. K. Hanisch, M. Noda and A. Verkhratsky (2011). "Physiology of microglia." Physiol Rev **91**(2): 461-553.
37. Kim, T. H., S. J. Yoon, W. C. Lee, J. K. Kim, J. Shin, S. Lee and S. M. Lee (2011). "Protective effect of GCSB-5, an herbal preparation, against peripheral nerve injury in rats." J Ethnopharmacol **136**(2): 297-304.
38. Kovalevich, J. and D. Langford (2013). "Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology." Methods Mol Biol **1078**: 9-21.
39. Lenz, K. M. and L. H. Nelson (2018). "Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function." Front Immunol **9**: 698.

40. Li, D., S. Huang, Z. Yin, J. Zhu, X. Ge, Z. Han, J. Tan, S. Zhang, J. Zhao, F. Chen, H. Wang and P. Lei (2019). "Increases in miR-124-3p in Microglial Exosomes Confer Neuroprotective Effects by Targeting FIP200-Mediated Neuronal Autophagy Following Traumatic Brain Injury." Neurochem Res **44**(8): 1903-1923.
41. Liao, W., Y. Du, C. Zhang, F. Pan, Y. Yao, T. Zhang and Q. Peng (2019). "Exosomes: The next generation of endogenous nanomaterials for advanced drug delivery and therapy." Acta Biomater **86**: 1-14.
42. Liu, H., Y. Feng, M. Xu, J. Yang, Z. Wang and G. Di (2018). "Four-octyl itaconate activates Keap1-Nrf2 signaling to protect neuronal cells from hydrogen peroxide." Cell Commun Signal **16**(1): 81.
43. Lizarraga-Valderrama, L. R. and G. K. Sheridan (2021). "Extracellular vesicles and intercellular communication in the central nervous system." FEBS Lett **595**(10): 1391-1410.
44. Luu, L., G. D. Ciccotosto, L. J. Vella, L. Cheng, L. C. Roisman, G. Multhaup, A. F. Hill, L. M. Munter and R. Cappai (2019). "Amyloid Precursor Protein Dimerisation Reduces Neurite Outgrowth." Mol Neurobiol **56**(1): 13-28.
45. Matejuk, A. and R. M. Ransohoff (2020). "Crosstalk Between Astrocytes and Microglia: An Overview." Front Immunol **11**: 1416.
46. Mills, J. D., A. M. Iyer, J. van Scheppingen, A. Bongaarts, J. J. Anink, B. Janssen, T. S. Zimmer, W. G. Spliet, P. C. van Rijen, F. E. Jansen, M. Feucht, J. A. Hainfellner, P. Krsek, J. Zamecnik, K. Kotulska, S. Jozwiak, A. Jansen, L. Lagae, P. Curatolo, D. J. Kwiatkowski, R. J. Pasterkamp, K. Senthilkumar, L. von Oerthel, M. F. Hoekman, J. A. Gorter, P. B. Crino, A. Muhlebner, B. P. Scicluna and E. Aronica (2017). "Coding and small non-coding transcriptional landscape of tuberous sclerosis complex cortical tubers: implications for pathophysiology and treatment." Sci Rep **7**(1): 8089.
47. Morelli, S., A. Piscioneri, S. Salerno, M. B. Al-Fageeh, E. Drioli and L. De Bartolo (2014). "Neuroprotective effect of didymin on hydrogen peroxide-induced injury in the neuronal membrane system." Cells Tissues Organs **199**(2-3): 184-200.
48. Morgado, A. L., J. M. Xavier, P. A. Dionisio, M. F. Ribeiro, R. B. Dias, A. M. Sebastiao, S. Sola and C. M. Rodrigues (2015). "MicroRNA-34a Modulates Neural Stem Cell Differentiation by Regulating Expression of Synaptic and Autophagic Proteins." Mol Neurobiol **51**(3): 1168-1183.
49. Mukherjee, S., I. Akbar, B. Kumari, S. Vrati, A. Basu and A. Banerjee (2019). "Japanese Encephalitis Virus-induced let-7a/b interacted with the NOTCH-TLR7 pathway in microglia and facilitated neuronal death via caspase activation." J Neurochem **149**(4): 518-534.
50. Mulligan, K. A. and B. N. Cheyette (2017). "Neurodevelopmental Perspectives on Wnt Signaling in Psychiatry." Mol Neuropsychiatry **2**(4): 219-246.
51. O'Brien, J., H. Hayder, Y. Zayed and C. Peng (2018). "Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation." Front Endocrinol (Lausanne) **9**: 402.
52. Ohashi, M., T. Hirano, K. Watanabe, H. Shoji, N. Ohashi, H. Baba, N. Endo and T. Kohno (2016). "Hydrogen peroxide modulates neuronal excitability and membrane properties in ventral horn neurons of the rat spinal cord." Neuroscience **331**: 206-220.
53. Pascual, M., F. Ibanez and C. Guerri (2020). "Exosomes as mediators of neuron-glia communication in neuroinflammation." Neural Regen Res **15**(5): 796-801.

54. Petralla, S., F. De Chirico, A. Miti, O. Tartagni, F. Massenzio, E. Poeta, M. Virgili, G. Zuccheri and B. Monti (2021). "Epigenetics and Communication Mechanisms in Microglia Activation with a View on Technological Approaches." Biomolecules **11**(2).
55. Pinto, S., C. Cunha, M. Barbosa, A. R. Vaz and D. Brites (2017). "Exosomes from NSC-34 Cells Transfected with hSOD1-G93A Are Enriched in miR-124 and Drive Alterations in Microglia Phenotype." Front Neurosci **11**: 273.
56. Prinz, M., S. Jung and J. Priller (2019). "Microglia Biology: One Century of Evolving Concepts." Cell **179**(2): 292-311.
57. Reddy, P. H., J. Williams, F. Smith, J. S. Bhatti, S. Kumar, M. Vijayan, R. Kandimalla, C. S. Kuruva, R. Wang, M. Manczak, X. Yin and A. P. Reddy (2017). "MicroRNAs, Aging, Cellular Senescence, and Alzheimer's Disease." Prog Mol Biol Transl Sci **146**: 127-171.
58. Rostamian Delavar, M., M. Baghi, Z. Safaeinejad, A. Kiani-Esfahani, K. Ghaedi and M. H. Nasr-Esfahani (2018). "Differential expression of miR-34a, miR-141, and miR-9 in MPP+-treated differentiated PC12 cells as a model of Parkinson's disease." Gene **662**: 54-65.
59. Santerre, M., A. Bagashev, L. Gorecki, K. Z. Lysek, Y. Wang, J. Shrestha, F. Del Carpio-Cano, R. Mukerjee and B. E. Sawaya (2019). "HIV-1 Tat protein promotes neuronal dysregulation by inhibiting E2F transcription factor 3 (E2F3)." J Biol Chem **294**(10): 3618-3633.
60. Sominsky, L., S. De Luca and S. J. Spencer (2018). "Microglia: Key players in neurodevelopment and neuronal plasticity." Int J Biochem Cell Biol **94**: 56-60.
61. Song, Y., Z. Li, T. He, M. Qu, L. Jiang, W. Li, X. Shi, J. Pan, L. Zhang, Y. Wang, Z. Zhang, Y. Tang and G. Y. Yang (2019). "M2 microglia-derived exosomes protect the mouse brain from ischemia-reperfusion injury via exosomal miR-124." Theranostics **9**(10): 2910-2923.
62. Sun, J., J. Zheng, Y. Li, M. Yan, P. Li and L. Ma (2019). "LncRNA EPIC1 downregulation mediates hydrogen peroxide-induced neuronal cell injury." Aging (Albany NY) **11**(23): 11463-11473.
63. Tian, Y., P. Zhu, S. Liu, Z. Jin, D. Li, H. Zhao, X. Zhu, C. Shu, D. Yan and Z. Dong (2019). "IL-4-polarized BV2 microglia cells promote angiogenesis by secreting exosomes." Adv Clin Exp Med **28**(4): 421-430.
64. Timmerman, R., S. M. Burm and J. J. Bajramovic (2018). "An Overview of in vitro Methods to Study Microglia." Front Cell Neurosci **12**: 242.
65. Truettner, J. S., D. Motti and W. D. Dietrich (2013). "MicroRNA overexpression increases cortical neuronal vulnerability to injury." Brain Res **1533**: 122-130.
66. Veremeyko, T., I. S. Kuznetsova, M. Dukhinova, W. Y. Y. A, E. Kopeikina, N. S. Barteneva and E. D. Ponomarev (2019). "Neuronal extracellular microRNAs miR-124 and miR-9 mediate cell-cell communication between neurons and microglia." J Neurosci Res **97**(2): 162-184.
67. Viader, A., L. W. Chang, T. Fahrner, R. Nagarajan and J. Milbrandt (2011). "MicroRNAs modulate Schwann cell response to nerve injury by reinforcing transcriptional silencing of dedifferentiation-related genes." J Neurosci **31**(48): 17358-17369.
68. Wang, S., Q. Tang, F. Ge and Q. Guo (2020). "Typhae pollen polysaccharides protect hypoxia-induced PC12 cell injury via regulation of miR-34a/SIRT1." Int J Immunopathol Pharmacol **34**: 2058738420910005.
69. Yin, Z., Z. Han, T. Hu, S. Zhang, X. Ge, S. Huang, L. Wang, J. Yu, W. Li, Y. Wang, D. Li, J. Zhao, Y. Wang, Y. Zuo, Y. Li, X. Kong, F. Chen and P. Lei (2020). "Neuron-

derived exosomes with high miR-21-5p expression promoted polarization of M1 microglia in culture." Brain Behav Immun **83**: 270-282.
70. Zhang, D., G. Cai, K. Liu, Z. Zhuang, K. Jia, S. Pei, X. Wang, H. Wang, S. Xu, C. Cui, M. Sun, S. Guo, W. Song and G. Cai (2021). "Microglia exosomal miRNA-137 attenuates ischemic brain injury through targeting Notch1." Aging (Albany NY) **13**(3): 4079-4095.
71. Zhang, G. and P. Yang (2018). "A novel cell-cell communication mechanism in the nervous system: exosomes." J Neurosci Res **96**(1): 45-52.
72. Zhang, J., S. Li, L. Li, M. Li, C. Guo, J. Yao and S. Mi (2015). "Exosome and exosomal microRNA: trafficking, sorting, and function." Genomics Proteomics Bioinformatics **13**(1): 17-24.
73. Zhang, Y., Y. Liu, H. Liu and W. H. Tang (2019). "Exosomes: biogenesis, biologic function and clinical potential." Cell Biosci **9**: 19.
74. Zhou, R., P. Yuan, Y. Wang, J. G. Hunsberger, A. Elkahloun, Y. Wei, P. Damschroder-Williams, J. Du, G. Chen and H. K. Manji (2009). "Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers." Neuropsychopharmacology **34**(6): 1395-1405.
75. Zhou, T., D. Lin, Y. Chen, S. Peng, X. Jing, M. Lei, E. Tao and Y. Liang (2019). "alpha-synuclein accumulation in SH-SY5Y cell impairs autophagy in microglia by exosomes overloading miR-19a-3p." Epigenomics **11**(15): 1661-1677.

8. APPENDIX

8.1. Ethics Committee Approval

T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASI
BİYOTIP VE GENOM ENSTİTÜSÜ

Sayı : 96350690-302.14.01- 154
Konu : Tez Önerisi

02/04/2021

Sayın Nurullah SATI

30/03/2021 tarih ve 6/12 sayılı Yönetim Kurulu Kararı ile "*Microglia to Neuron Interaction via Exosomal miR-34a*" başlıklı tez öneriniz kabul edilmiştir.

Bilgilerinizi rica ederim.

Prof. Dr. İbrahim Mehmet Ali ÖKTEM
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8.2. Currriculum Vitae

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Türkiye	Dokuz Eylül	İzmir Uluslararası Biyotıp ve Genom Enstitüsü	Moleküler Biyoloji ve Genetik	Yüksek Lisans	2021
Türkiye	İYTE	Fen Fakültesi	Moleküler Biyoloji ve Genetik	Lisans	2020

UZMANLIK ALANLARI

Ekzozom, MikroRNA, Moleküler Biyoloji ve Genetik

GÖREV ALDIĞI PROJELER

Proje Adı	Kurum	Proje Dönemi	Projedeki Görevi

ULUSAL ve ULUSLARARASI KONGRE/KONFERANS/SEMPOZYUM BİLDİRİLERİ
ULUSAL

EĞİTİM, KURS ve DİĞER ETKİNLİKLER