T.R.
USKUDAR UNIVERSITY
INSTITUTE OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY

MASTER'S DEGREE PROGRAM OF MOLECULAR BIOLOGY

MASTER'S DEGREE THESIS

INVESTIGATION OF THE EFFECT OF DNA TOPOISOMERASE IIB ON NEURONAL SURVIVAL BY REGULATING LPS-INDUCED MICROGLIA-MEDIATED NEUROINFLAMMATION

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ABSTRACT

INVESTIGATION OF THE EFFECT OF DNA TOPOISOMERASE IIB ON NEURONAL SURVIVAL BY REGULATING LPS-INDUCED MICROGLIA-MEDIATED NEUROINFLAMMATION

Type II DNA topoisomerases are the enzymes that solve the topological problems such as supercoiling, catenation or knotting in cellular processes including DNA replication, recombination or gene transcription by creating transient double strand breaks in DNA. There are two isoforms of these enzymes in mammals called as DNA topoisomerase IIα a (topo IIα) and DNA topoisomerase IIβ (topo IIβ). While topo IIα is mainly present in proliferating cells solving the problems related to chromosome seggragation, topo IIβ in expressed in both proliferting and non proliferating cells performing more specialized functions in gene transcription. Topo IIβ generates double strand breaks to relax nucleosome structure and make DNA accessable to transcription complexes.

Topo IIβ has crucial function in processes especially in the development nervous system. It has been demonstrated that topo IIβ regulates the transcription of the genes related to neural differentiation such as axonal growth, orientation and expression of ion channels. Silencing of topo IIβ causes neurodegeneration-like effect in neurons. On the other hand, silencing of topo IIβ in neural induced mesenchymal stem cells resulted in increased expression of inflammation-related genes. In line with the data mentioned above, the hypothesis is that the topo IIβ may be one of the key regulatory proteins that control neuroinflammatory pathways and may prevent damaged caused by microglia-mediated neuroinflammation.

To study the bidirectional effects of factors secreted by microglia and neurons, the most efficient type of co-culture is transwell culture modeling. In this method, the two cell types are separated by a semipermeable membrane. Although there is no direct contact, it allows the bilateral passage of factors secreted from cell types.

In order to analyze the modulatory effect of topo IIβ on neuronal cell index in microglia – neuron relationship, HMC3 cells were transfected with the topo IIβ plasmid, followed by the application of lipopolysaccharide (LPS) to activate the microglia cells. In the Transwell co-culture system, effective secretion of factors was allowed without the cells contacting with each other. HMC3 cell line was seeded in the upper part of the Transwell culture system and neural differentiated SH-SY5Y cell lines in the lower part. Effect of the topo IIβ on cell index of SH-SY5Y cells was monitored continuously with the Real Time Cell Analysis (RTCA) device. Then, MTT assay was performed to check the viability of neurons. Annexin V assay was also performed to compare the apoptosis among groups.

The results revealed that, among the co-culture groups, while LPS caused a cell damage resulting a decrease in the cell index value approximately to 75%, TOP2B overexpression increased the cell index approximately to 120%, which exhibits that the topo IIβ protects the neuronal cells in culture against LPS induced and microglia-mediated neuroinflammation. TOP2B overexpression increased the index to 150% alone by itself, which shows that it can influence the cell index positively. MTT results revealed that TOP2B positively affects the cell viability, makes it 120% and %200 with or without LPS induction, respectively. As a conclusion, the overexpression of TOP2B in microglia might be very effective in terms of cell viability by modulating the chronic neuroinflammation. This research may enlighten the way for the neurodegenerative diseases, in which the balance between microglia – neurons is disrupted and effecting the viability in a negative way.

Keywords: DNA Topoisomerase IIbeta, Neurodegeneration, Neuroinflammation, Microglia, Co-culture, LPS

ÖZET

DNA TOPOİZOMERAZ IIB'NİN LPS İLE İNDÜKLENEN MİKROGLİA ARACILI NÖROİNFLAMASYONU DÜZENLEYEREK NÖRONAL SAĞKALIM ÜZERİNDEKİ ETKİSİNİN ARAŞTIRILMASI

Tip II DNA topoizomerazlar, DNA replikasyonu, rekombinasyonu veya gen transkripsiyonu dahil olmak üzere hücresel süreçlerdeki aşırı sarma, katenasyon veya düğümlenme gibi topolojik sorunları DNA'da geçici çift sarmal kırıklar oluşturarak çözen enzimlerdir. Memelilerde bu enzimlerin DNA topoizomeraz IIa (topo IIα) ve DNA topoizomeraz IIβ (topo IIβ) olarak adlandırılan iki izoformu vardır. Topo IIa esas olarak çoğalan hücrelerde kromozom ayrışmasıyla ilgili sorunları çözerken bulunurken, topo IIβ gen transkripsiyonunda daha özel işlevler gerçekleştiren hem çoğalan hem de çoğalmayan hücrelerde ifade edilir. Topo IIβ, nükleozom yapısını gevşetmek ve DNA'yı transkripsiyon komplekslerine erişilebilir kılmak için çift sarmalda kırıklar üretir.

Topo IIβ, özellikle gelişim sinir sistemindeki süreçlerde çok önemli bir işleve sahiptir. Topo IIβ'nin iyon kanallarının aksonal büyümesi, oryantasyonu ve ekspresyonu gibi nöral farklılaşma ile ilgili genlerin transkripsiyonunu düzenlediği gösterilmiştir. Topo IIβ'nin susturulması, nöronlarda nörodejenerasyon benzeri bir etkiye neden olur. Öte yandan, nöral kaynaklı mezenkimal kök hücrelerde topo IIβ'nin susturulması, inflamasyon ile ilişkili genlerin ekspresyonunun artmasına neden olur. Yukarıda belirtilen veriler doğrultusunda, hipotez, topo IIβ'nin nöroinflamatuar yolları kontrol eden ve mikroglia aracılı nöroinflamasyonun neden olduğu hasarı önleyebilen anahtar düzenleyici proteinlerden biri olabileceği yönündedir.

Mikroglia ve nöronlar tarafından salgılanan faktörlerin çift yönlü etkilerini incelemek için en verimli ko-kültür türü, transwell kültür modellemesidir. Bu yöntemde, iki hücre tipi yarı geçirgen bir zarla ayrılır. Direk temas olmaksızın hücre tiplerinden salgılanan faktörlerin çift taraflı geçişine izin verir.

Mikroglia - nöron ilişkisinde topo IIβ'nin nöronal hücre indeksi üzerindeki modülatör etkisini analiz etmek için, HMC3 hücreleri topo IIβ plazmidi ile transfekte edildi, ardından mikroglia hücrelerini aktive etmek için lipopolisakarit (LPS) uygulandı.

Transwell ko-kültür sisteminde, hücrelerin teması olmadan faktörlerin etkin bir şekilde salgılanmasına izin verildi. Transwell kültür sisteminin üst kısmına HMC3 hücreleri ve alt kısmına nöral farklılaştırılmış SH-SY5Y hücreleri ekilmiştir. Topo IIβ'nin SH-SY5Y hücrelerinin hücre indeksi üzerindeki etkisi, Gerçek Zamanlı Hücre Analizi (RTCA) cihazı ile sürekli olarak takip edildi. Daha sonra, nöronların canlılığını kontrol etmek için MTT testi yapıldı. Gruplar arasında apoptozu karşılaştırmak için Annexin V testi de yapıldı.

Sonuç olarak, ko-kültür grupları arasında, LPS hücre hasarına neden olarak hücre indeksi değerinde yaklaşık %75'e varan bir düşüşe neden olurken, TOP2B aşırı ekspresyonunun hücre indeksini yaklaşık %120'ye çıkardığını ortaya koyuyor, bu da topo IIβ'nin kültürdeki nöronal hücrelerde indeksi LPS kaynaklı ve mikroglia aracılı nöroenflamasyona karşı koruduğunu göstermektedir. TOP2B aşırı ekspresyonu, indeksi %150'ye çıkarmış olup, bu da hücre indeksini kendi başına da olumlu yönde etkileyeceğini göstermektedir. MTT sonuçları, TOP2B'nin hücre canlılığını olumlu yönde etkilediğini, LPS indüksiyonu ile ve LPS indüksiyonu olmadan, sırasıyla %120 ve %200 değerlerine yükselttiğini ortaya koydu. Sonuç olarak, mikrogliada TOP2B aşırı ekspresyonu, kronik nöroinflamasyonu modüle ederek hücre canlılığı açısından büyük etki yapabilir. Bu araştırma mikroglia – nöronlar arasındaki dengenin bozulduğu ve canlılığı olumsuz yönde etkileyen nörodejeneratif hastalıklara ışık tutabilir.

Keywords: DNA topoizomeraz IIbeta, Nörodejenerasyon, Nöroinflamasyon, Mikroglia, Ko-kültür, LPS

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3R/4R TAU	: 3-repeat / 4-repeat tau
AD	: Alzheimer’s disease
βIII Tubulin	: Class III β-tubulin
BDNF	: Brain Derived Neurotrophic Factor
BV2	: Microglial cell derived from C57/BL6 murine
CHME3	: Human Microglia Clone 3
CNS	: Central nervous system
COX2	: Prostaglandin-endoperoxide synthase 2
DAP12	: Transmembrane protein present at the cell surface
DAPI	: 4′,6-diamidino-2-phenylindole
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
EDTA	: Ethylenediaminetetraacetic acid
F12	: Basal medium
FACS	: Fluorescence-activated cell sorting
FBS	: Fetal bovine serum
FITC	: Fluorescein isothiocyanate
FSC	: Forward scattering
GFAP	: Glial fibrillary acidic protein
HMC3	: Human microglia clone 3
IBA1	: Allograft inflammatory factor 1
ICRF-193	: Topoisomerase inhibitor

IFNy	: Interferon gamma
IL-1β	: Interleukin-1 beta
KDA	: Kilodalton
LPS	: Lipopolysaccharide
M1	: Microglia pro-inflammatory phenotype
M2	: Microglia anti-inflammatory phenotype
MAP2	: Microtubule-associated protein 2
MEM	: Minimum esential medium
MHC	: Major histocompatibility complex
MPP	: 1-methyl-4-phenylpyridinium
N2A	: Neuro2a cells
NEUN	: Hexaribonucleotide Binding Protein-3
NF-Kβ	: Nuclear Factor kappa B
NOS2	: Nitric oxide synthase 2
PBS	: Phosphate Buffered Saline
PD	: Parkinson's disease
PS	: Phosphatidylserine
RA	: Retinoic acid
ROCK	: Signaling pathway
ROS	: Reactive oxygen species
RPM	: Rounds per minute
RTCA	: Real time cell analysis
SH-SY5Y	: Neuroblastoma cell type

SSC	: Side scattering
SV40	: Simian virus 40
TDP-43	: TAR DNA-binding protein 43
TH	: Tyrosine hydroxylase
THP1	: Monocyte cell line
TLR4	: Toll like receptor 4
TNFα	: Tumor necrosis factor alpha
TOP2α	: Topoisomerase 2 alpha (Gene)
TOP2β	: Topoisomerase 2 beta (Gene)
μM	: Micromolar

1. INTRODUCTION

1.1. DNA Topoisomerase IIβ Enzyme

DNA topoisomerase II (topo II) enzyme is involved in solving DNA topology problems in basic biological mechanisms. It takes part in many vital mechanisms such as transcription and replication, chromosome condensation/segregation, and DNA damage. It acts as a catalyst in the temporary separation and unification of the two strands of the DNA structure, and regulates the DNA topology by passing the DNA double strands through each other (Chen et al., 2013; Nitiss JL, 2009). There are two isoforms of the topo II enzyme observed in mammals, topo IIα and topo IIβ. Although enzymes are structurally very similar to each other, they differ functionally. The Topo IIα enzyme is expressed only in proliferating cells. Since nuclear division could not be completed in Topo IIα knockout mice, it was observed that mouse embryos died in the early embryonic stages (Akimitsu et al., 2003).

During neural differentiation and brain development, the enzyme DNA topoisomerase II (topo II) plays a crucial function. It is 196.21 kDa in size and regulates the DNA topology by causing a temporary break on the DNA, allowing the strands to pass through each other **(Figure 1)** (Wu et al., 2013).

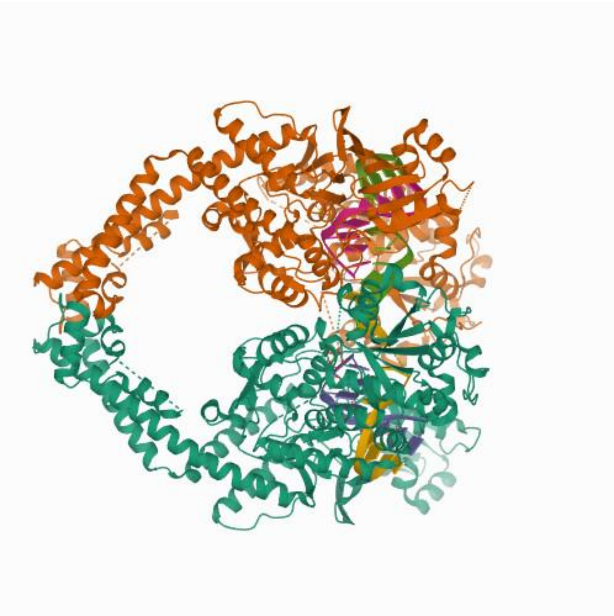


Figure 1. Human Topoisomerase IIβ – DNA Complex (Wu et al., 2013)

It is especially necessary for the transcriptional activation of some critical genes induced in the terminal differentiation of neuron cells (Tsutsui et al. 2001, Sano et al. 2010). Perinatal death was observed in topo IIβ knockout mice due to damage in the late stages of embryogenesis (Yang et al. 2000). In mice, in which topo IIβ was silenced in brain cells, corticogenesis damage occurred during brain development (Lyu and Wang, 2003). Microarray studies with cerebellar granular neurons have shown that topo IIβ inhibitors suppress the induction of a number of genes related to neuronal development (eg, ion channels, receptors, and signal transduction) (Lyu et al. 2006, Sano et al. 2008). Transcriptional activation of these genes occurs as a result of topo IIβ opening the chromatin structure and connecting DNA transcription factors with promoter regions (Ju et al. 2006, Lis and Kraus 2006). In addition, it has been shown that the topo IIβ enzyme is required at important stages of neural network formation, such as neurite outgrowth and axon orientation. In a study with topo IIβ knockout mice, it was observed that motor neuron axons could not enter the spinal cord (Yang et al. 2000). In a different research, it was reported that the catalytic inhibitor of topo II, ICRF-193, reduced significantly the growth cone and neurite outgrowth of cerebellar granule neurons, dorsal ganglia, and cortical neurons (Nur et al. 2007). Compared to the control group, cortical neurons from topo II mutant embryos developed shorter neurites, demonstrating the importance of topo II in neurite development (Heng and Le, 2010). In a case report, whole genome sequencing also revealed a novel mutation in the topo II gene, which was linked to autistic traits, hypotonia, progressive microcephaly, developmental delay, and intellectual disability (Lam et al., 2017). In studies on cell lines lacking the topo IIβ enzyme, it has been observed that these cells are more resistant to drugs such as mitoxantrone and amsacrine used in cancer treatments, so the cytotoxic effects of this enzyme may be an important target for the future (Errington et al., 1999). As a result of studies on knocking and knockout murine models, significant defects in proliferation, remarkable damage in the development and survival of B cells, and impair in humoral functions have been observed in patients with TOP2B mutation as a result of the inability of this gene to encode the enzyme (Broderick et al., 2019). In another study, downregulation of a gene called peroxiredoxin-2 in topo IIβ-deficient cells was reported, resulting in a significant increase in ROS levels. At the same time, the decrease in expression caused great damage to the antioxidant mechanisms and dragged the cells to apoptosis (Chikamori et al., 2006). It is known that the enzyme topo IIβ also controls motor neuron migration and connections. In the absence of the enzyme, the identity of motor neurons is damaged, resulting in damage to peripheral connections. The absence of the enzyme has also been shown to be of great importance for motor neuron connections, as it causes downregulation of Hox and Pbx genes (Edmond et al., 2017). In mouse models lacking topo IIβ, neuronal differentiation was delayed in retinal modeling, damage occurred in the plexiform layers and outer segments of photoreceptors, resulting in incredible reductions in the number of cells in the retina. In this sense, it has been shown that the enzyme directly affects the survival of postmitotic neurons (Li et al., 2013). The absence of topo IIβ is also effective in the transcription process and chromatin accessibility. It caused the death of premature postmitotic neurons and this neuronal degeneration has been shown to be directly related to a topo IIβ-dependent gene called Ngfr p75 (Tiwari et al., 2012). Further research has proven that topo II regulates the expression of certain genes that control neurite outgrowth and axonogenesis, and that its activity is critical to the proper growth and maintenance of developing post-mitotic neurons. In the previous studies it is shown that, topo IIβ in neuronal differentiation regulates the neural polarization in the process of regulating the signal transducing pathways, and its absence can contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's disease (PD) (Işık et al., 2014, Terzioğlu -Uşak et al., 2017). Recently, it is declared that silencing topo IIβ can disrupt the balance in Rho GTPases and may cause neurodegeneration and pathogenesis of neurodegenerative diseases (Zaim and Işık, 2018). Additionally, suppressing topo IIβ with its specific siRNA's, as correlated with the in vitro PD model which is created by MPP+ neurotoxin in SHSY5Y cells, causes the suppression of tyrosine hydroxylase gene expression which is very critical in dopamine synthesis and creates a neurodegeneration-like effect in cultured neurons.

Genes regulated by the TOP2B gene are known to be associated with many diseases. It can be said that developmental diseases come first among these diseases (autism, epilepsy, mental retardation). It is also known to have an effect on hemorrhagic fever, carcinoma, obesity, and cardiac diseases by interacting with genes other than nervous system functions **(Figure 2)** (Neha and Dholaniya, 2021).

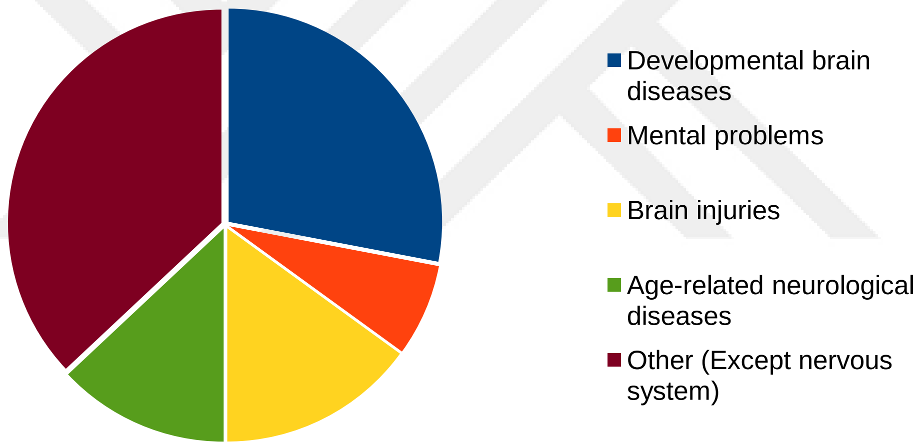


Figure 2. Association of TOP2B regulated genes with diseases (Neha and Dholaniya, 2021)

1.2. Neurodegenerative Diseases

Neurodegenerative diseases are typically defined by specific proteins' accumulation, characterized by progressive neuronal dysfunction and neuronal death. Proteotoxic stress and its abnormalities in the ubiquitin-proteasomal and autophagosomal/lysosomal systems involve important mechanisms such as oxidative stress, programmed cell death (apoptosis) and neuroinflammation (Dugger and Dickson, 2017). The amyloidoses, tauopathies, alpha synucleinopathies, and TDP-43 proteinopathies are the most prevalent neurodegenerative conditions. A neurological diagnosis can be made thanks to the abnormal protein conformation and accumulations in these disorders. Neurodegenerative diseases have specific protein accumulations and are characterized according to these deposits **(Table 1)** (Dugger and Dickson, 2017). Neurodegenerative diseases affect millions of people around the world every year, but the clinical approaches applied are about relieving the physical symptoms experienced by the patients. The risk of neurodegenerative disease increases dramatically with age. This shows that it is of great importance to examine the cause of neurodegenerative diseases and to conduct research for treatment (Alzheimer's Association, 2021). At the same time, it is known that in neurodegenerative diseases, microglia cells are activated and go into a chronic inflammation state and gradually aggravate the clinical picture of the disease (Kwon and Koh, 2020).

Table 1. The most common neurodegenerative diseases, their pathological scenario and specific protein deposits (Dugger & Dickson, 2017)

Disease	**Pathological Situation**	**Protein Accumulation**
Alzheimer’s Disease	Neurofibrillary Tangles (NFTs) Amyloid Plaques	Amyloid Beta 3R + 4R tau
Lewy Bodies Diseases	Lewy Bodies	Alpha synuclein
Amyotrophic lateral sclerosis	Motor neuron loss Bunina bodies	TDP-43
Progressive supranuclear palsy (PSP)	Circular neurofibrillary tangles Fringed-astrocytes	4R tau
Pick’s Diseases	Pick bodies Baloon-like neurons	3R tau
Multi system atrophy (MSA)	Cytoplasmic changes in the glia structure	Alpha synuclein

Neurodegenerative diseases affect millions of people worldwide each year. Considering the latest report published in the USA, it is stated that 6.2 million Americans suffer from AD. When indexed to the world population, this number reaches extremely dramatic figures. The clinical approaches applied are about relieving the physical symptoms experienced by patients, and the risk of developing the disease and progression increases significantly as age progresses (Alzheimer's Association, 2021).

1.3. Microglia-mediated neuroinflammation

Neurons and neuroglial cells make up the central nervous system (CNS), which is composed of both types of cells. Astrocytes, oligodendrocytes, and microglia cells are all included in the paradigm of neuroglial cells. The brain's main immune cells are known as microglia. Microglia emerge from the embryonic yolk sac and enter the central nervous system (CNS) through the circulatory system (Ginhoux et al., 2010). Microglia surround the neuroepithelium and move inside it during the embryonic stage. They start to adopt a branching shape during postnatal development. By promoting neuronal survival and cell death, they aid in the development of the CNS and maintain tissue homeostasis (Nayak et al., 2014). In scenarios of damage such as infection, brain trauma, stroke, and neurodegeneration, these cells can be triggered by a variety of triggers. Many cytokines, chemokines, and other immune mediators are produced and released in response (Lawson et al., 1992; Perry et al., 2004). Microglia may produce specific anti-inflammatory and neurotrophic substances to speed up the healing process in the case of neuronal damage (Liao et al., 2005). Yet, when the synergy between this advantageous condition and the interaction between tissue-damaging microglia is damaged, these cells may shift pathological and the problem could worsen (Block and Hong, 2007).

When unstimulated, microglia have a branched appearance. Cells express major histocompatibility complex (MHC) proteins and some other surface receptors (Aloisi, 2001). Meanwhile, microglia search the environment for potential signals (Nimmerjahn et al., 2005). At the same time, various neurotrophic factors are secreted by microglia. These factors help maintain neuronal cell survival. Microglia also contribute to the apoptosis of problematic neurons that occur during development and destroy remnants. It phosphorylates DAP12, which helps cytoskeletal reorganization without inducing expression of certain cytokines, such as tumor necrosis factor alpha (TNF-α) and nitric oxide synthase-2 (NOS2) (Takahashi et al., 2005). In turn, dying neurons secrete factors that promote phagocytosis, thereby accelerating the cleanliness of the environment. This process is critical for the healthy maintenance of developing neurons and neurons in the adult brain.

Therefore, although acute neuroinflammation plays a protective role on neurons, chronic neuroinflammation is considered harmful (Russo et al. 2018). The two functional polarization states of activated microglia cells are known as M1 type (classical/pro-inflammatory activation) and M2 type (alternative/anti-inflammatory activation) (Aguzzi et al. 2013, Jha et al. 2016). The M1 phenotype negatively impacts neurons and is essential in mediating the inflammatory and neurotoxic functions of microglia (Kigerl et al. 2009, Liao et al. 2012). Research demonstrate that M2 microglia produce anti-inflammatory cytokines and tissue-repairing cytoactive substances, which help to maintain homeostasis, decrease neuroinflammation, and protect nerve tissues (Colton, 2009). Thus, it is understood that microglia express mediators including IL-1, TNF-, ROS, and NO and are active in many neurodegenerative conditions like Parkinson's disease (PD), Huntington's disease, and ALS disease (Subhramanyam et al., 2019). These mediators can act to take care of the maintenance of neurons and clear apoptosis cells, but in neurodegenerative diseases chronically activated microglia can exacerbate the scenario by releasing excessive amounts of these cytotoxic factors into the environment. Microglia can be activated in different phenotypes depending on the stimulus. The M1 phenotype generally expresses cytokine/chemokines and neurotoxic factors, while the M2 phenotype expresses anti-inflammatory and neuroprotective factors **(Figure 3)** (Subhramanyam et al., 2019). The M2 phenotype may indeed be neuroprotective against disorders of the nervous system.

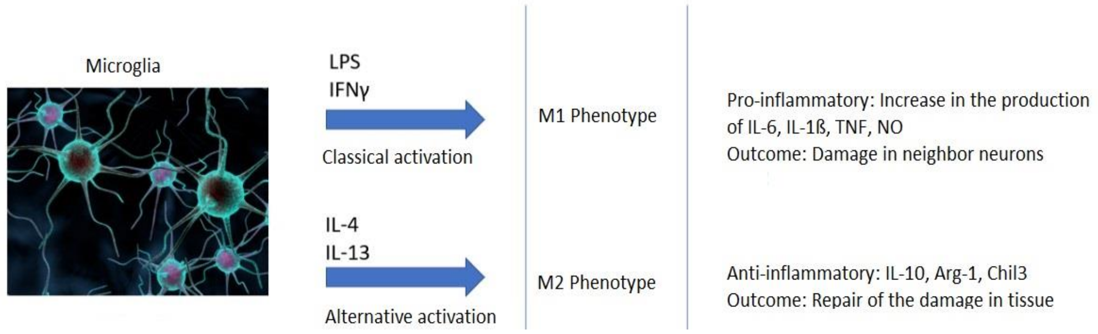


Figure 3. Types of microglia activation, resulting phenotypes and secreted inflammatory molecules depending on the stimulus (Subhramanyam et al., 2019)

1.4. Activation of Microglia Cells

Different activators are used in the literature to trigger microglia activation. The most commonly used stimulant is lipopolysaccharide (LPS). It is found in the outer membrane of gram-negative bacteria and is an endotoxin that initiates the immune response (Raetz and Whitfield, 2008). LPS acts by binding to TLR4, which is located on microglia and activates intracellular proteins for gene transcription of inflammatory molecules. Therefore, LPS is often used to represent a threat **(Figure 4)** (Machado, 2011). However, in many diseases such as stroke and neurodegenerative diseases, microglia are activated by cell damage and by stimulants released into the environment such as proinflammatory cytokines IFN-y and TNF-α, and therefore they are frequently used in microglia-mediated neuroinflammation models (Brown and Vilalta, 2015). It is an extremely reliable agent for bringing microglia into a neurotoxic state. Apart from exogenous threats, microglia can also be activated by endogenous threats such as cell damage and stress. They respond to cytokines secreted by other cells. One of the most commonly used endogenous activators is IFN-γ. Microglia exposed to IFN-γ produce pro-inflammatory cytokines. Similarly, tumor necrosis factor alpha (TNF-α) is frequently utilized. It is found at high levels in neurodegenerative diseases and attracts microglia to an active inflammatory state, leading to high levels of glutamate secretion and toxicity (Takeuchi et al., 2005; Puzi and Vidyadaran, 2020). These activators can be applied alone or in combination (Peng et al., 2016).

Since LPS is an agonist of the M1 phenotype, cells become active after administration, increasing the expression levels of M1-level markers such as TNF-α, IL-1β, COX-2, Nitric Oxide (NO). Markers such as CD206, IL-10, arginase 1 are used to monitor the transition to the M2 level (Wang et al., 2022).

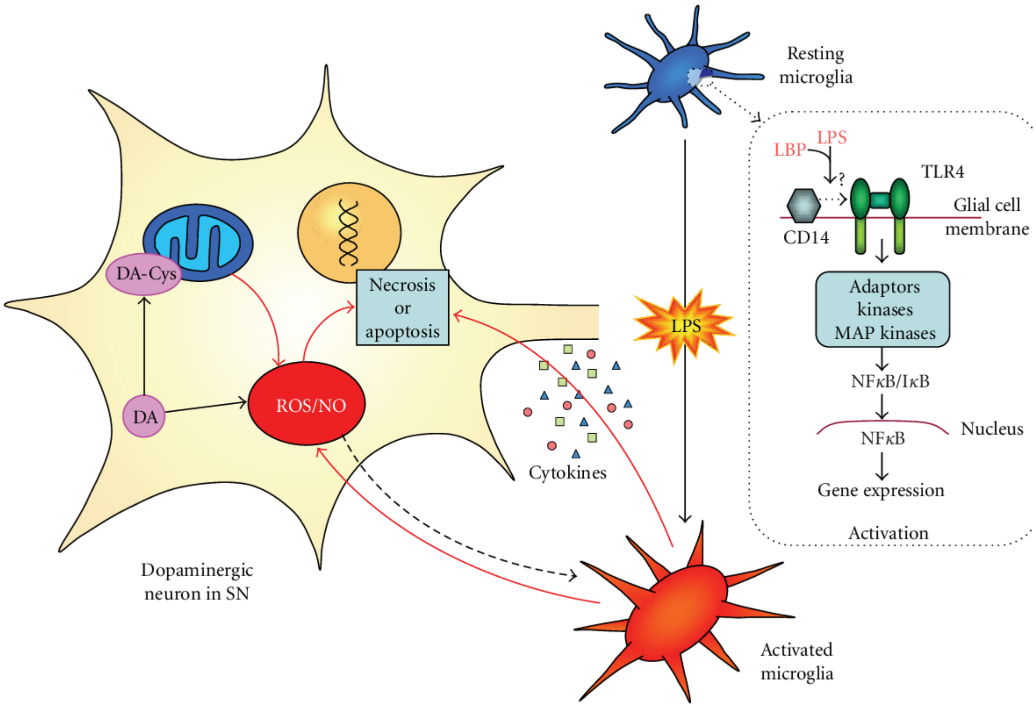


Figure 4. LPS-induced neuroinflammation model on dopaminergic neuron (Machado, 2011)

1.5. HMC3 and SH-SY5Y Cell Lines

In addition to direct neuroprotective therapy research, modulating the inflammatory responses of microglia cells is an important potential method for modulating neurotoxicity (Skaper et al., 2014). Different in vitro models are used to perform analyses of microglia and neurotoxic effects. Accordingly, co-culture models are extremely valuable to observe the effects of various substances on the responses of microglia to neurons.

HMC3 cells are a cell line that retains primary microglial cell characteristics generated by SV40-dependent immortalization of embryonic microglial cells. They contain a homogeneous cell population and can be grown indefinitely. Different names such as The nomenclature CHME3 has also been used in the literature. While this cell line is negative for the astrocyte marker GFAP, it is positive for CD68 and CD11b. The endotoxin receptor CD14 and the microglia/macrophage marker IBA1 are also present on HMC3 cells. Incubation with interferon gamma increased the expression of microglial activation markers as MHCII, CD68, and CD11b. Moreover, HMC3 cells have polarized phenotypes with CD40 and CD86 positivity for M1 and CD163 and CD206 positivity for M2 (Chrysanthou et al., 2022).

In microglia-mediated neuroinflammation models, there are studies on different neuronal cell lines in the literature. The SH-SY5Y cell line used in this study is extremely suitable for modelling. The SH-SY5Y cell line is neuroblastoma cells originally derived from bone tumors. They are catecholaminergic cells that express tyrosine hydroxylase. It is suitable for modeling neurodegenerative diseases such as Parkinson's disease. The SH-SY5Y cell line can be used in differentiated or undifferentiated form. The undifferentiated cell line is a more suitable model for developing neurons, while the differentiated cell lines are used to model adult neurons. Retinoic Acid (RA) and brain-derived neurotrophic factor are two substances that are widely employed in literature to initiate neuronal development (BDNF). RA stops cell division and pulls cells into the resting phase, known as G0. BDNF, on the other hand, stimulates the growth of neurons and βIII-tubulin, MAP2, NeuN, etc. Induces the expression of neuronal markers (Gordon et al., 2013).

Before triggering neuronal differentiation, chemicals such as collagen, laminin, fibronectin can be used to increase the adhesion strength of cells. Fibronectin is a master in matrix organization. The storage and assembly of structures such as collagen, fibrillin, and tenascin are associated with fibronectin junctions (Kaur and Reinhardt, 2015). At the same time, the relationship between fibronectin and proteins such as integrin and syndecan plays an important role in the adhesion, migration and differentiation of cells. It is a very valuable protein that is frequently used in extracellular matrix biology. In real-time cell analysis sheets, it was observed that cell adhesion increased significantly when cells were coated with fibronectin before seeding, and significant data were obtained in cell index value (Serra et al., 2014).

1.6. RhoA-ROCK Pathway

Rho GTPases are members of the Ras GTPase superfamily. RhoA and RhoB are seen as the most important among these members (Humphries et al., 2020). ROCK (Rho-associated kinase) is the most studied effector molecule for RhoA in the literature. It has been demonstrated that activation of this pathway triggers many risk factors in Alzheimer's disease, such as accumulation of amyloid-beta plaques, damage to synapses, and neuroinflammation (Koch et al., 2018). In line with these findings, it is thought that deactivation of the pathway may be an important first step in the treatment of neurodegenerative diseases. ROCK is an important effector molecule in many aspects such as cell migration, attachment, growth and resistance to apoptosis. Its isoforms are widely expressed in mammals. Although many members of the Rho family are known to function in mammals, RhoA is mainly observed in neurons (Shimokawa, 2016). In most scenarios, although ROCK is present in the cytoplasm in an inactive form, various diseases can occur as a result of their activation or increased expression. They can regulate morphogenesis, regulate synaptic plasticity in the brain by acting on the actin cytoskeleton. Cognitive disorders are observed in Alzheimer's patients as a result of synapse damage in brain regions closely related to memory. It is known that amyloid-beta deposition regulates actin cytoskeleton dynamics by activating RhoA and ROCK pathways, the severe side effect of which manifests itself as cognitive impairments in connection with synapse losses (Sellers et al., 2018). It has been stated that inhibiting ROCKs will positively affect neuron migration and axon growth, and improve cognitive disorders observed in diseases (Gentry et al., 2016).

In many neurodegenerative diseases, including Alzheimer's, the responsibility should not be limited only to the accumulation of various proteins. On the contrary, it is the neuroinflammation mechanism in the brain that is most responsible for aggravating the scenario in these diseases. Microglia cells are also known to be responsible for triggering neuroinflammation (Baik et al., 2019). Neuroinflammation will reveal the toxic microglia phenotype in this direction, microglia will no longer lose their neuroprotective properties and will lose their positive effects on homeostasis and cause neuronal death (Kwon and Koh, 2020). It is known that the ROCK pathway triggers a neurotoxic microglia phenotype and increases the expression of important inflammation molecules such as

TNF-α and nitric oxide synthase (Shinozaki et al., 2019). In addition, it is known that ROCK activation can also up-regulate microglial expression via the NF-Kβ pathway and thus increase microglial movement and support the release of inflammatory cytokines (Rodriguez-Perez et al., 2015). *In vivo* animal studies have shown that inhibiting the ROCK pathway reduces neuroinflammation by reducing microglial polarization (Zhu et al., 2020). Since RhoA/ROCK signaling pathways also induce microglia to enter a chronic activation state, analysis of the pathway is thought to be extremely important in terms of treating neurodegenerative diseases in the future. Overexpression of topo IIβ causes an increase in neuronal differentiation and blocks the RhoA/ROCK2 pathway (Zaim and Işık, 2018). From recent papers it is declared that blocking RhoA can decrease the level of ROS and inflammatory cytokines, therefore topo IIβ may act as an inhibitor in this context.

1.7. Co-culture Models

In the microglia-neuron relationship, different co-culture models are used to observe the effect of substances on the response of microglia to neurons. The models created vary according to the desired effects to be observed in cell relations. As the most used models; cell-to-cell direct culture, medium/supernatant transfer, and transwell culture modeling (Puzi and Vidyadaran, 2020). In cell-to-cell direct culture modeling, the cell populations to be studied are cultured individually and then seeded on top of each other. Then, the toxicity process can be initiated by adding LPS and/or other activators and the effect of microglia on neurons can be examined. In the medium/supernatant transfer modeling, after the microglia culture is again activated with LPS and a similar agent, the cellular debris is precipitated with the help of a centrifuge and the supernatant is transferred onto a neuronal culture. However, since there is a risk of loss of some factors produced by microglia in this method, it cannot be said that the efficiency is very high (Carroll et al., 2014). To study the bidirectional effects of factors secreted by microglia and neurons, the most efficient type of co-culture is transwell culture modeling. In this method, the two cell types are separated by a semipermeable membrane. Although there is no direct contact, it allows the bilateral passage of factors secreted from cell types.

It is very important to examine the neuroinflammation effect and ultimately neurodegeneration effect of activated microglia as a result of the two-way communication between microglia and neuron. While microglia cells affect neurons by secreting pro-inflammatory factors and oxidative stress factors, neurons respond to this situation through neuropeptides (Szepezi et al., 2018). In the model called Transwell, a paracrine communication is allowed by providing compartmentalization thanks to porous membranes. In the relationship between microglia and neurons, there are different cell models such as BV2 and SH-SY5Y, THP1, N2a, and it is known that tri-culture-like systems including astrocytes can be established (Chrysanthou et al., 2022).

1.8. Purpose and Goals of the Study

Silencing of topo IIβ in neurally induced MSCs led to a significant increase in the expression of genes associated with inflammation (Işık et al., 2015).

Topo IIβ has been shown to act on axons by regulating Rho GTPases. Silencing topo IIβ causes neuronal axon degeneration in human bone marrow mesenchymal stem cells (MSCs), and its overexpression inhibits the RhoA/ROCK2 pathway, protecting axons and increasing the neural differentiation potential of MSCs (Zaim and Işık, 2018).

In line with the data mentioned above, we think that the topo IIβ enzyme may be one of the key regulatory proteins that may modulate neuroinflammatory pathways and may prevent neurodegeneration caused by microglia-mediated neuroinflammation.

Modulating the inflammatory responses of microglia cells to neurons is an important target, so that the damage caused by chronic inflammation can be prevented or reduced. In line with the literature review, there is no study investigating the effect of topoisomerase IIβ enzyme on neuroinflammation in the microglia-neuron relationship.

The purpose of the study, is to investigate the relation between topo IIβ overexpression on HMC3 microglial cells and neuronal survival in the transwell co-culture system, with the possible modulatory effect in terms of neuroinflammation provided by the topo IIβ, in a LPS-induced *in vitro* microglia-mediated chronic neuroinflammation model.

The goal of the study, is to obtain positive results in neuronal survival (RTCA and MTT) and apoptosis (Annexin V) analyses in experimental groups as a result of topo IIβ overexpression and the modulation of neuroinflammation.

It is hoped that this study would be an important step towards reducing the impact of microglia cells in the chronic inflammation scenario and in various brain injuries, especially neurodegenerative diseases. With a co-culture design, it is planned to consider the bidirectional release of factors without allowing physical contact of cells and to examine the role of topoisomerase IIβ in microglia-mediated neuroinflammation.

2. METHODS

2.1. Preparing HMC3 Cells for Co-culture Experiments

2.1.1. Freezing and Thawing HMC3 Cells

Cells in culture were resuspended in FBS for long-term preservation. 950 L of cell suspension was added to each cryovial tube, which was then placed in an ice. 50 L of DMSO (Dimethyl Sulfoxide, GENAXXON, M6323.0100) was then added to them and held overnight at -80 C after being kept at -20 C for a couple of hours. Cells were moved to liquid nitrogen the following day and kept there for future use.

10 mL of pre-warmed MEM was added to the falcon tube before it was thawed. From the nitrogen tank, cryovial tubes were taken out, and they were agitated in a water bath until they dissolved. After that, it was put into a falcon tube with medium and centrifuged for five minutes at 2000 rpm. DMSO and other cellular wastes were eliminated in this technique. The pellet was resuspended after the supernatant was removed, and the cells were then seeded into flasks with 20% FBS (Fetal Bovine Serum; Thermo Fisher; 102701006) and antibiotics, which were then placed in an incubator.

2.1.2. Subculture of HMC3 Cells

The HMC3 microglia cell line was purchased as part of another project (ATCC, CRL-3304) and is stored in a liquid nitrogen tank for long-term use. When the cells reached 70-80% density, the old media were removed and the flask was washed with Phosphate Buffered Saline (PBS) (Phosphate Buffered Saline, Capricorn, PBS.1A). Then, the cells were removed from the flask base with trypsin/EDTA solution (Thermo Fisher, 25200056) in proportion to the flask size and kept in the incubator for 3 minutes. After observing the removal of cells under the microscope, a medium containing bovine serum

(FBS) up to 3 times the amount of trypsin was added, and the deactivation of trypsin was ensured. Then, the liquid was transferred to a 15 mL falcon tube and centrifuged at 2000 rpm for 5 min. The supernatant was thrown out and the pellet was dissolved in a medium containing 1 mL of FBS, antibiotic (Penicillin-Streptomycin or Primocin) (Invivogen, ant-pm-2), L-Glutamine (Capricorn, GLN.B), Sodium Pyruvate (Capricorn, NPY.B), and cell count was made by means of Thoma Slide (IsoLab).

20 µL of cell suspension was mixed with the same amount of trypan blue and loaded on both sides of Thoma. Counting under an Inverted Fluorescent Microscope (Zeiss), the required amount of cells were seeded with fresh media in a new flask, the remaining cells were frozen with the freezing protocol and stored in a liquid nitrogen tank.

2.1.3. Topoisomerase IIBeta Plasmid Isolation

The pEGFP-N1 plasmid containing the TOP2B gene (see Appendix 1) was obtained from Okayama University School of Medicine (Japan) by Prof. Ken Tsutsui.

2.1.3.1. Preparing LB Broth and LB Agar

LB broth was dissolved with distilled water and autoclaved on the liquid program (121C, 20 minutes). After autoclaving, 50 µL of kanamycin antibiotic was added in accordance with the plasmid construction and stored at 4 ^{0}C.

LB agar was likewise dissolved with distilled water and autoclaved on the liquid program (121C, 20 min). 50 µL of kanamycin antibiotic was added and poured into petri dishes and stored at +4 degrees.

Streak was thrown into agar using glycerol stock. It was kept in the incubator until colony formation was observed, after about 36 h, colonies were observed to form. Then, single droplets were selected from the formed colonies and grown in broth containing 1% kanamycin in a 37 ^{0}C shaking incubator for 24 h. After 24 h, the EGFP-N1 plasmid was isolated with the kit following the manufacturer's instructions. Concentration of isolated plasmid DNA was measured with LVis plate using Multimod Microplate reader (BMG LABTECH, FlouOstar Omega).

2.1.4. Overexpression of TOP2B

Prior to transfection, HMC3 cells were confirmed to reach 70-90% density in the Inverted Microscope (Zeiss). The Lipofectamine 3000 reagent (Invitrogen, L3000-008) has been optimized using the product's protocol. Briefly, LF3000 reagent and optimem were combined in one tube, then 50 μL of Opti-MEM, DNA and P3000 reagent were combined in a separate tube to dilute the DNA and these 2 tubes were mixed. It was incubated at room temperature for 15 min, then added dropwise onto the cells. It was incubated at 37 ^{0}C and the process was followed with a fluorescent microscope.

2.1.4.1. Separation of TOP2B Overexpressing Cells

Approximately 36 h after Lipofectamine 3000 transfection, transfected HMC3 cells were removed from the flask surface with 0.25% Trypsin/EDTA solution. It was centrifuged for 5 minutes at 1800 rpm at room temperature by adding FBS and transferring it to a 15 mL centrifuge tube. The cell pellet was resuspended in PBS containing 2% FBS.
Cells positive and negative for pEGFP-topo IIβ plasmid were separated from each other by FACS devices at Medipol University and Yeditepe University.
The scattering parameters for HMC3 cells were adjusted, excluding cells that might be duplicates, and aiming to have as homogeneous a positive cell population as possible.

2.1.5. Induction of the HMC3 Microglia Line by LPS and IFNγ

In order to activate microglia cells, LPS (Sigma-Aldrich, L4391-1MG) and IFNy (Gibco, PHC4031) agents were used in accordance with the literature. In this way, it is aimed to initiate neuroinflammation. 1 μg/mL LPS was applied for 24 h, after which M1 type microglia (pro-inflammatory) markers were confirmed by flow cytometry analysis. Activation analyses were also performed for interferon gamma concentration (CD16, CD86).

2.1.5.1. Flow Cytometry Analyses

Flow cytometry device was used to detect the expressions of neuroinflammatory markers of M1 phenotype (Beckman Coulter, B5-R3-V0 Cytoflex Flow Cytometer). First, cells were harvested after PBS washing with trypsin. Cells were fixed with fixation solution, followed by permeation solution. Then, by adding blocking solution, non-specific regions were blocked. Cell pellet was kept in antibody dilution solution, primary and secondary antibody applications were made. After adjusting the forward scatter (FSC) and side scatter (SSC) values, the device was started and data was collected.

2.2. Preparing SH-SY5Y Cells for Co-culture Experiments

2.2.1. Freezing and Thawing SH-SY5Y cells

Cells in culture were resuspended in FBS for long-term preservation. 950 L of cell suspension was added to each cryovial tube, which was then placed in an ice. 50 L of DMSO was then added to them, and they were held overnight at -80 C after first being kept at -20 C for 1-2 C. Cells were moved to liquid nitrogen the following day and kept there for future use.

10 mL of pre-warmed DMEM (Capricorn, DMEM.HXA) was transferred to a falcon tube before it was thawed. From the nitrogen tank, cryovial tubes were taken out, and they were agitated in a water bath until they dissolved. Then it was transferred to a falcon tube containing medium and centrifuged at 2000 rpm for 5 minutes. In this way, DMSO and other cellular wastes were removed. After removing the supernatant, the pellet was thawed and the cells were seeded into flasks containing 20% FBS and antibiotics and left in the incubator.

2.2.2. Subculture of SH-SY5Y cells

The SH-SY5Y neuroblastoma cell line (ATCC, CRL-2266), which was obtained for use in another project, is kept in a liquid nitrogen tank for long-term storage. The old medium were removed after the cells achieved 70–80% density, and the flask was then rinsed with phosphate buffered saline (PBS). The cells were then separated from the flask base using trypsin/EDTA and left in the incubator for three minutes, depending on the size of the flask. Upon seeing cells separated under a microscope, trypsin deactivation was secured by adding FBS to a medium with three times the amount of trypsin. Then, the liquid was transferred to a 15 mL falcon tube and centrifuged at 2000 rpm for 5 min. The supernatant was thrown out and the pellet was dissolved in a medium containing 1 mL of FBS, antibiotic (Penicillin-Streptomycin / Primocin), L-Glutamine, Sodium Pyruvate and cell count was made by means of Thoma Slide (IsoLab).

2.2.3. Neuronal Differentiation of SH-SY5Y Cell Line

Cells were seeded into culture dishes at 1.25 x $10^4/cm^2$ for 96-well and 6.5 x $10^4/cm^2$ cells for 24-well. After 24 h, DMEM medium containing 15% FBS was withdrawn from the medium, combined with F12 differentiation medium containing Retinoic Acid (RA), antibiotics and FBS, to a concentration of 10 µM. The medium was changed every 48 h, after the 4th day, RA and FBS were withdrawn from the medium and the cells were treated with BDNF twice, one day apart. After the cells were treated in this way for 8 days, the neuronal differentiation process was finished **(Table 2).** The presence of neuronal markers (MAP2, NeuN) was confirmed by Immunofluorescent staining.

Table 2. Neuronal differentiation experiment schedule for SH-SY5Y cell line

Day	Treatment
-1	Cell seeding 1,25x10^4 for 96 well 6,5x10^4 for 24 well
0	F12 + Antibiotics + RA (10 uM)
1	-
2	F12 + Antibiotics + RA (10 uM)
3	-
4	F12 + BDNF
5	-
6	F12 + BDNF

2.2.4. Confirmation of Neuronal Markers by Immunofluorescent Staining in SH-SY5Y Cells

The cultured cells were fixed with paraformaldehyde for 15 min at room temperature after washing with PBS. Afterwards, permeability was created with the help of Triton X-

100 in PBS, and non-specific binding of proteins was prevented by incubating with 10% goat serum for 30 min. The primary antibody (MAP2) was diluted according to the characteristics of the product and incubated with the primary antibody at 4 ^{0}C overnight. Then, secondary antibody (GAM-Aleksa 594 and GAR Aleksa 488) was applied and incubated for 1 hour at room temperature and under dark conditions. It was washed three times with PBS for five minutes, incubated with 4',6-diamidino-2-phenylindole (DAPI), a dye used for imaging under fluorescent microscopy, and made ready for imaging. It was kept out of light and examined with a fluorescent microscope.

2.3. SH-SY5Y and HMC3 Co-culture Experiments

To examine the microglia-neuron relationship in the presence of chronic inflammation, it was planned to design a co-culture environment to allow cell lines to secrete factors bidirectionally. In a culture system called Transwell culture, the continuity of the cells was ensured and after the interaction between the cells, molecular analyses were made on the populations (Skaper and Facci, 2018; Lee et al., 2015). Using the transwell co-culture apparatus of the RTCA device (xCELLigence), which is planned to be used in the study, HMC3 (microglia) cell lines were seeded in the upper part and SH-SY5Y (neuroblastoma) cell lines in the lower part. The experimental groups planned in the study were formed as described below, and neuronal survival analyses were performed by making comparisons between the groups **(Table 3) (Table 4).**

Table 3. Experimental groups in RTCA system

Group(s)	Cell Line(s)	Treatment(s)
1	SH-SY5Y	None
2	SH-SY5Y	Differentiation
3	SH-SY5Y	Differentation + LPS
4	HMC3 + SH-SY5Y	Differentiation
5	HMC3 + SH-SY5Y	Differentiation + LPS
6	HMC3 + SH-SY5Y	Differentiation + TOP2B overexpression
7	HMC3 + SH-SY5Y	Differentiation + TOP2B overexpression + LPS

Table 4. Experiment schedule for co-culture system

Time	*HMC3*	*SH-SY5Y*
Day 1	Refresh	Seeding into RTCA/96/24 wells
Day 2	Refresh	Retinoic Acid
Day 3	Passage	-
Day 4	Transfection – 25cm2 Flask (1x106)	Retinoic Acid
Day 5	-	-
Day 6	FACS: Positive/Negative Seeding	BDNF
Day 7	-	-
Day 8	LPS & Plate Assembly	BDNF
Day 9	-	-
Day 10	-	Annexin V/MTT Assay End of RTCA

2.3.1. SH-SY5Y Neuronal Cell Index/Viability Analyses

2.3.1.1. Real Time Cell Analysis (RTCA, xCELLigence)

Real-time cell analysis device (Real Time Cell Analyzer: RTCA; Xcelligence, Roche) is a system used for real-time monitoring of the viability functions and behaviors of cells attached to the surface. Viability/cell index values and growth curves of SH-SY5Y cells were determined accurately and reliably by means of E-plates. The device was placed in the CO_2 incubator, then the cells were instantly followed through the software on the computer connected to this device and related results were revealed. SH-SY5Y cells will be plated in 96-well plates at $1,25x10^4$ cells per well in the transwell system. In order to monitor the positive/negative effects of pro-inflammatory factors released by microglia on SH-SY5Y cells, real-time growth curves of cells were obtained by obtaining cell index values from the RTCA system. Real-time growth curves were compared between groups. The RTCA device enables continuous and real-time analysis of cell proliferation and viability in a quantitative manner. After the data obtained from RTCA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analyses were performed.

2.3.1.2. MTT Assay

MTT (BOSTER, MTT Cell Proliferation and Cytotoxicity Assay Kit Cat No:AR1156) is a colorimetric test used to evaluate the metabolic activity of living cells. It identifies living cells by converting tetrazolium salt (yellow) into formazan crystals (blue). It is used to determine viability and cytotoxicity. SH-SY5Y cells were seeded at $1{,}25 \times 10^4$ in 96-well plates. Cells were incubated with MTT solution, then solubilizing solution was added to dissolve the formazan crystals and incubated overnight. The absorbance value was measured at 570 nm using a microplate reader (BMG LABTECH, FlouOstar Omega), including a reference point at 650 nm.

2.3.1.3. Apoptosis Assay

Detection of early apoptotic cells was made using Annexin V-FITC conjugate. Annexin V, a Ca2+ dependent phospholipid-binding protein, has high affinity for phosphatidylserine (PS), which is translocated from the inside of the apoptotic cell membrane to the outside. The staining was made according to manufacturer’s protocol. Briefly, after preparing the binding buffer, cells were incubated with 2.5% FITC mix, followed with the PI incubation. After the washing steps cells were fixed with 4% PFA and stained with 1:5000 DAPI. Anti-fade mountant has been added and the wells are covered with coverslips.

2.4. Statistical Analyses

The values obtained from the RTCA and MTT results were analyzed with the ANOVA test for statistical significance. After the ANOVA test, Tukey's test was used as multiple comparison. Results were expressed as the mean ± standard deviation (SD). GraphPad Prism software, version 9.5.0 was being used for these analyses. **(Table 5)** (GraphPad Software, Inc., La Jolla, CA, USA).

Table 5. P value format used for statistical analyses of the experiments (Graphpad, 2009). Analyses were completed according to this standardized chart.

Symbol	Meaning
ns	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

3. RESULTS

3.1. Optimization of Culture Conditions of the HMC3 Cell Line

HMC3 (ATCC, CRL-3304) microglia cells were regularly monitored for optimum growth curve and morphological health following their cultivation. As a result of the optimization studies, it was observed that the cells had the best growth conditions with MEM medium containing 10% FBS, 1% Sodium Pyruvate, 1% L-Glutamine, Primocin. Each time the cells were passaged when they reached 70-80% density, the medium was refreshed by 40-50% every other day, except for the passage, and were followed daily. In accordance with the amount of cells needed on the passage days, 20,000/cm^2 flasks and/or plates were seeded **(Figure 5)**.

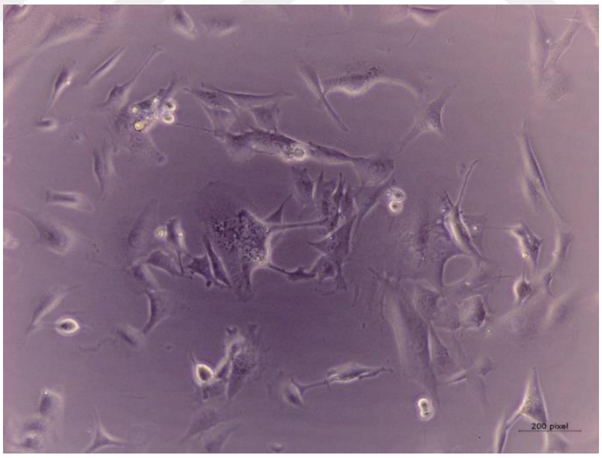


Figure 5. Morphological image of HMC3 cells seeded in 96-well plate. Image was taken with 10X, Inverted Fluorescent Microscope (ZEISS).

3.2. Overexpression of TOP2B Gene in HMC3 Cell Line

The Lipofectamine 3000 reagent has been optimized using the product's protocol. It was incubated at 37 degrees and the process was followed by fluorescent microscopy. Following TOP2B transfection with the reagent in accordance with the protocol, cells were regularly photographed with an inverted fluorescent microscope. Transfection efficiency has been calculated as approximately 15-20% from the acquired images **(Figure 6)** and ImageJ analysis.

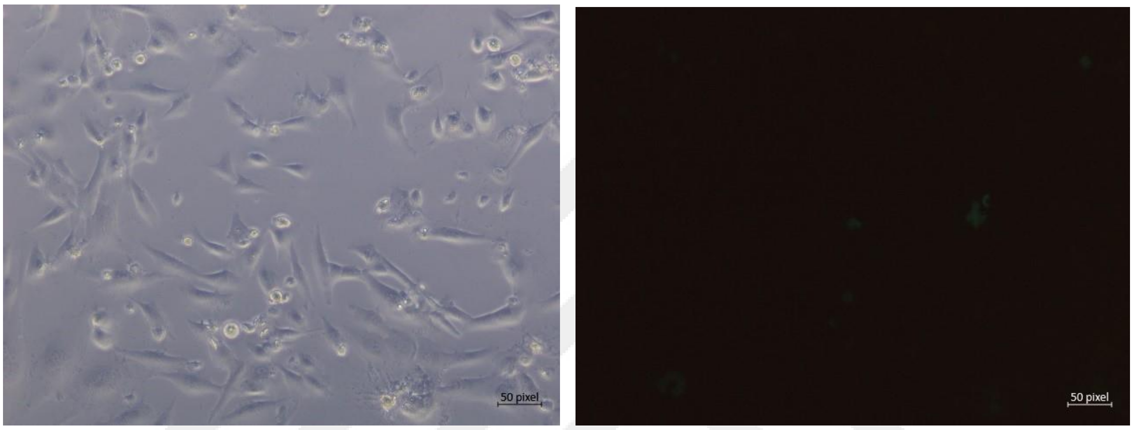


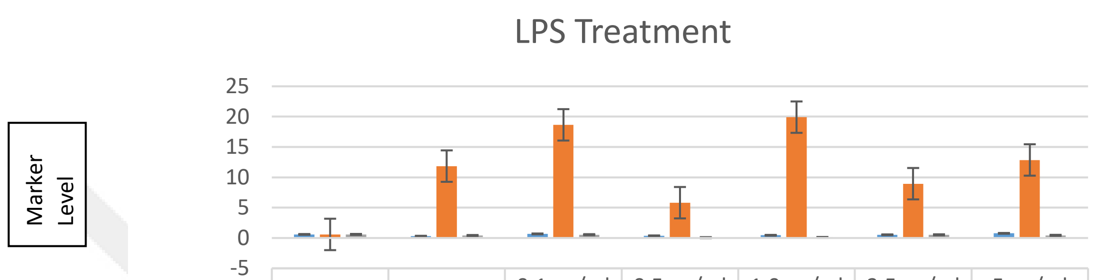
Figure 6. Phase and Fluorescent Images of HMC3 Lipofectamine Transfection, Respectively. Image was taken with 10X, Inverted Fluorescent Microscope (ZEISS).

3.3. LPS Treatment in HMC3 Cell Line

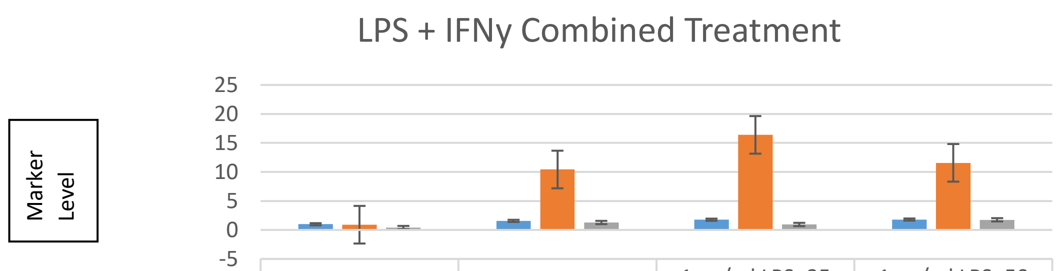
In order to activate microglia cells, LPS and IFNy agents were used in accordance with the literature. In this way, it is aimed to initiate neuroinflammation. 1 µg/mL LPS was applied for 24 h, after which M1 type microglia (pro-inflammatory) markers were confirmed by flow cytometry analysis. (CD16, CD86)

CD206, CD16, CD86 and CD45 antibodies were used to show the induced morphology of HMC3 cells after 24 and 48 h of treatments with different concentrations of LPS. As a result of the analysis of the staining, the most effective concentrations of LPS were found to be 0.1 ug/mL and 1 ug/mL. As a result of the analysis, it was seen that CD86, which is the M1 marker, increased significantly.

In addition to 1 ug/mL LPS decided within the scope of LPS optimization studies, 25 ng/mL and 50 ng/mL IFNy were used. At the end of the 24-h application, it was observed that the high concentration of IFNy caused a lower increase in neuroinflammation, contrary to expectations. This optimization was performed in our lab for regular experiments **(Figure 7)** (Akbayır, 2022).



	Unstained	Control	0.1 ug/ml LPS	0.5 ug/ml LPS	1.0 ug/ml LPS	2.5 ug/ml LPS	5 ug/ml LPS
CD206	0,59	0,3	0,68	0,36	0,45	0,53	0,77
CD86	0,59	11,85	18,65	5,82	19,92	8,95	12,86
CD16	0,59	0,41	0,55	0,06	0,09	0,53	0,44



	unstained	control	1 ug/ml LPS+25 ng/ml IFN-g	1 ug/ml LPS+50 ng/ml IFN-g
CD206	0,98	1,56	1,76	1,79
CD86	0,9	10,42	16,39	11,57
CD 16	0,41	1,28	0,94	1,75

Figure 7. Flow Cytometry Optimization for various LPS and LPS + IFNy doses (Akbayır, 2022). First graph stands for the different LPS doses and various levels of receptors related to microglia phenotype, while the second one is for LPS + IFNy doses with same receptors.

3.4. Optimization of Culture Conditions of the SH-SY5Y Cell Line

SH-SY5Y (ATCC, CRL-2266) neuroblastoma cells were regularly monitored for optimum growth curve and morphological health following their seeding. As a result of the optimization studies, it was observed that the cells had the best growth conditions with DMEM medium containing 15% FBS, 1% Sodium Pyruvate, 1% L-Glutamine, Primocin. Each time the cells were passaged at 70-80% density, the medium was refreshed by 40-50% every other day and followed daily. In accordance with the amount of cells needed on the days of passage, 40,000/cm^2 flasks and/or plates were seeded **(Figure 8).**

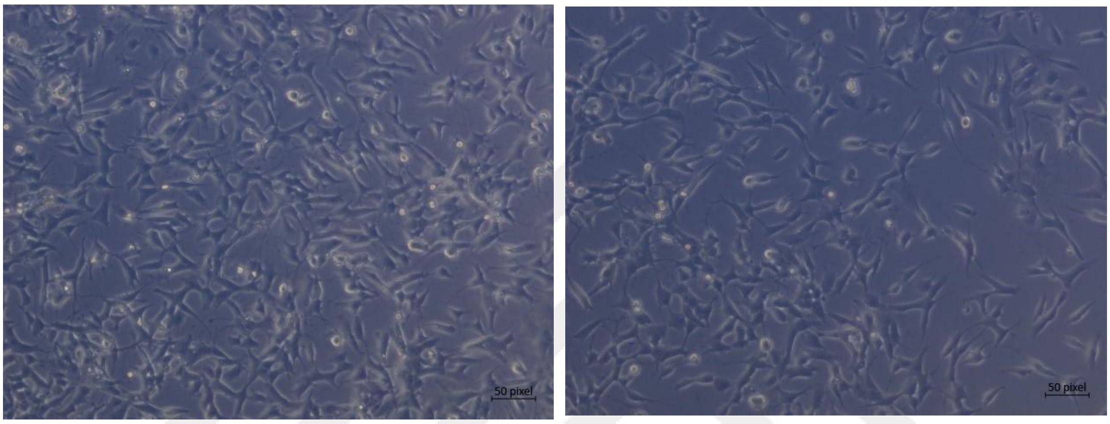


Figure 8. Morphological Images of SH-SY5Y Cells with different cell densities. Images were taken with 10X, Inverted Fluorescent Microscope (ZEISS).

3.5. SH-SY5Y Neuronal Differentiation Process

Cells were seeded at approximately 40,000/cm^2 in culture dishes where differentiation was desired and the differentiation protocol optimized by our lab was applied. After the day of seeding, RA application was made twice in 4 days, then 4 days for BDNF. In total, cells obtained 2 RA and 2 BDNF treatment **(Figure 9)**.

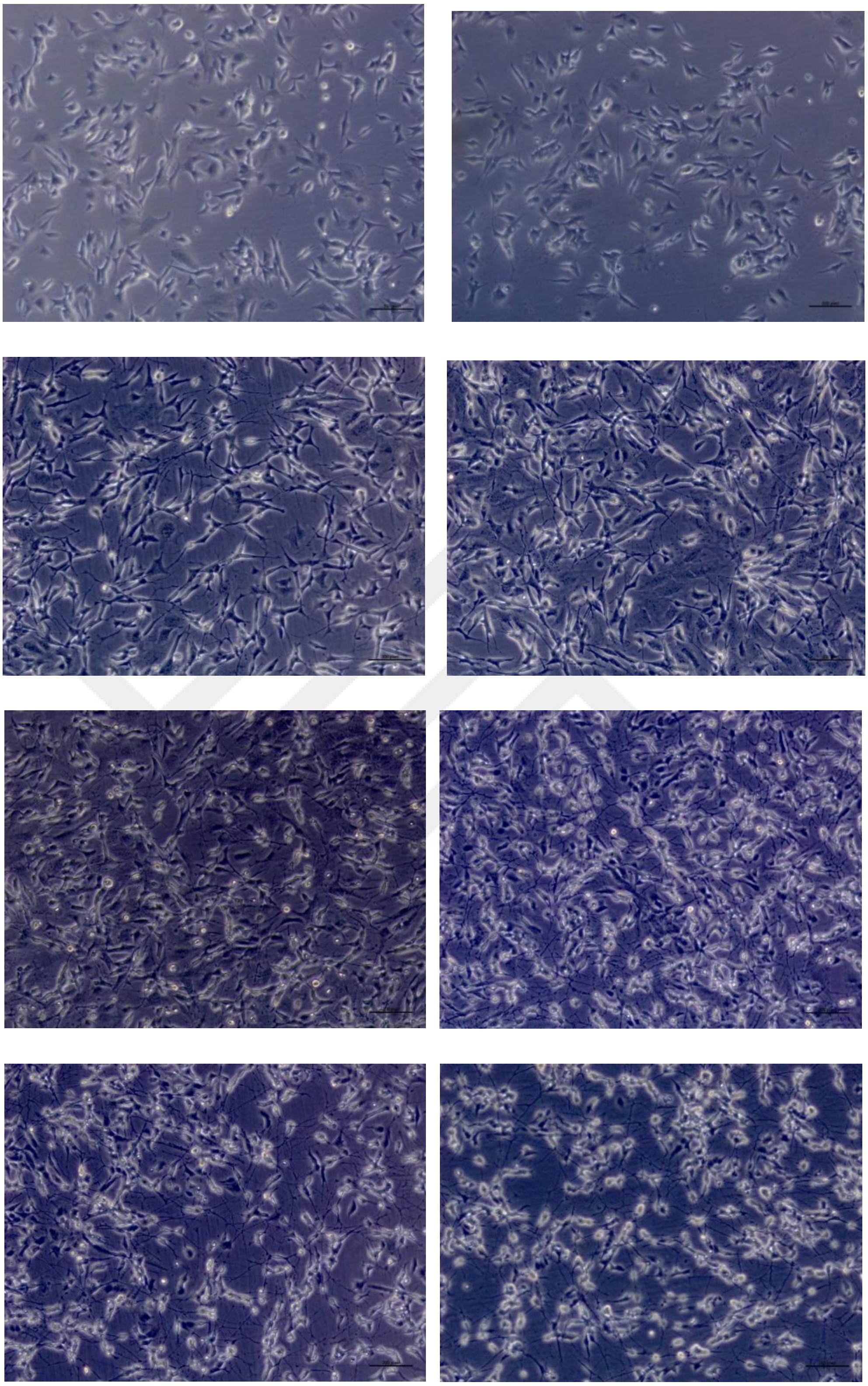


Figure 9. SH-SY5Y Neuronal Differentiation Images Day by Day, Respectively. Images were taken with 10X, Inverted Fluorescent Microscope (ZEISS).

3.5.1. Confirmation of Neuronal Markers by IF Staining

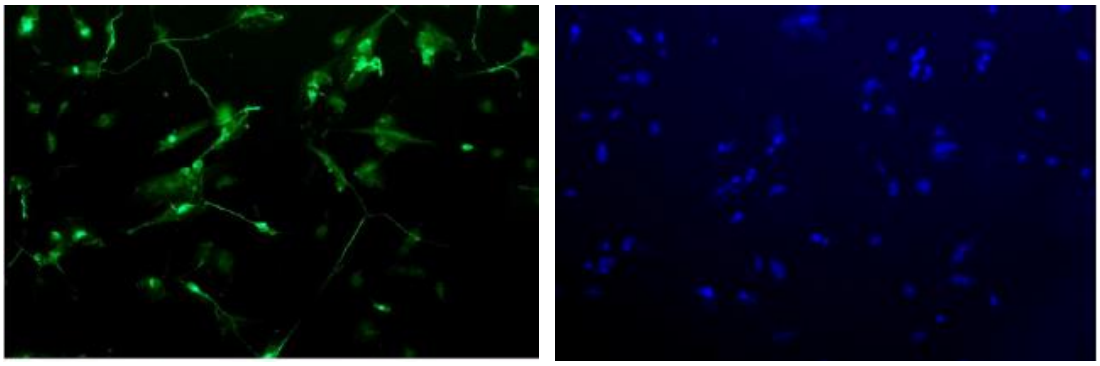


Figure 10. MAP2 and DAPI Stainings of Neural Differentiated Cells, respectively. Images were taken with 10X, Inverted Fluorescent Microscope (ZEISS).

This optimization was performed by our lab for regular experiments and the same protocol was being applied for years **(Figure 10)**. It is possible to say that the expression of the neuronal markers significantly has been increased and therefore the neurons are ready for the coculture experiments and the cell index/viability assays.

3.6. SH-SY5Y and HMC3 Co-culture Experiments

After the preparatory phases, co-culture experiments were started in order to perform neuronal survival analyses.

3.6.1. SH-SY5Y Neuronal Survival Analyses

Neuronal survival analyses were demonstrated by 3 different experiments. Half an hour after SH-SY5Y cells were seeded in a 96-well plate, the RTCA device was started and the growth curve of the cells was followed instantly. On day 3, HMC3 cells were transfected with TOP2B, on day 5 positive cells were differentiated by FACS and seeded into inserts with control cells. Except for the negative control group, no application was made to all groups, except for the differentiation protocol, until the 7th day. On the 7th day, the co-culture system was started, LPS was given to the determined groups and neuronal differentiation was continued with BDNF application. On the 9th day (approximately 48 h in the co-culture system), RTCA system was terminated, MTT solution was given, Annexin V experiment was started. After 4 hof incubation of the MTT solution, solubilizing buffer was given, and measurements were taken the next day. Annexin V results were photographed on the same day.

3.6.1.1. RTCA Analysis Results

Results from the RTCA device were followed for approximately 225 h after cell seeding **(Figure 11)**. The groups are indicated with different colors and the experimental scheme is given below.

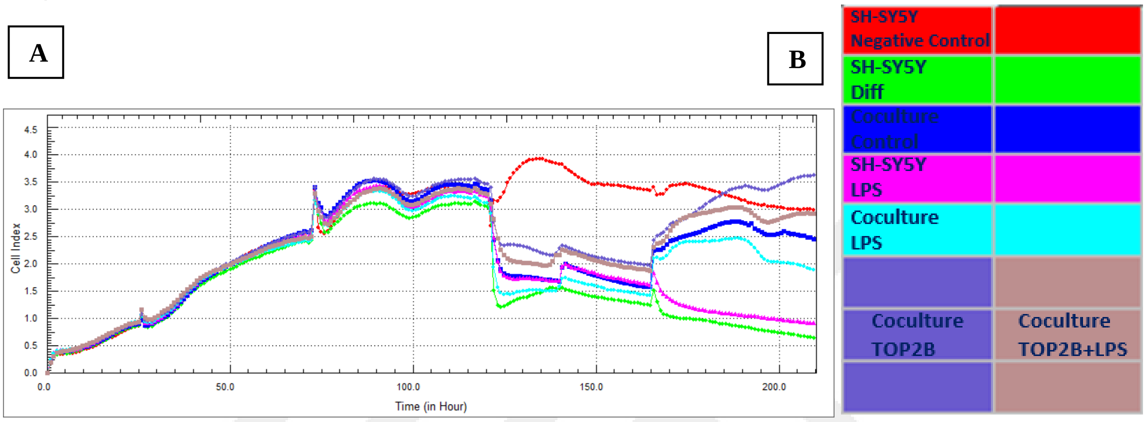
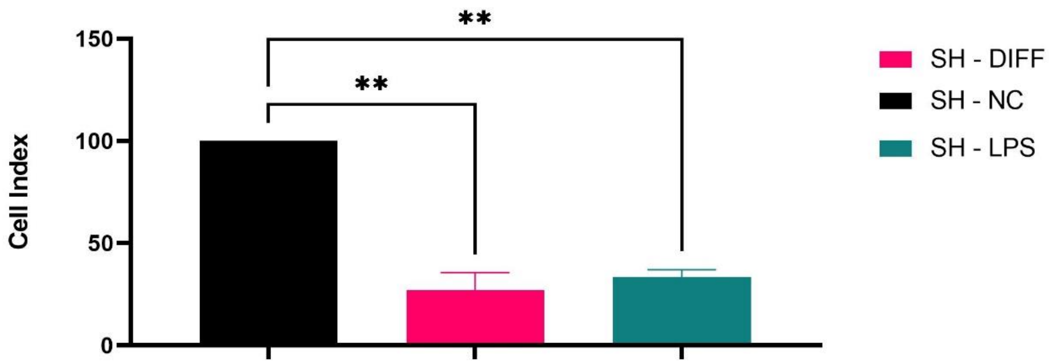


Figure 11. (A) Cell index values of the experimental groups followed instantly from the RTCA device **(B)** Experimental group layout. The graph shows the cell index for different groups and (B) shows the layout of the experiment for different groups. Red group is the control of the differentiation process. Every group underwent differentiation except the red one to obtain the neurons. Red, green, pink groups were containing SH-SY5Y cells only, while the dark blue, turqoise, purple and brown ones show the coculture groups. The graph and layout were obtained by the RTCA software (Agilent).

SH-SY5Y cells were seeded into the wells of the RTCA plate as 1.25 x 104/well and the follow-up was started. Approximately 24 h after seeding, retinoic acid was added to all wells except for the red group to initiate differentiation. After 48 h of incubation, a second RA supplement was also made. For approximately 120 h, no difference was observed between the groups in cell index values until BDNF administration. After 120 h, all groups except the red group received BDNF supplementation and there was a remarkable decrease in the cell index values of these groups, as expected from the literature. At approximately 170th hour, the upper-plate and lower-plate were assembled together, LPS was applied to the purple, turquoise, and brown groups, and all groups except the red group received BDNF supplementation. After this point, clear divergences began to be seen between the experimental groups, and statistically significant differences were expected between the groups before MTT and Annexin experiments were started **(Figure 12)**.

A



B

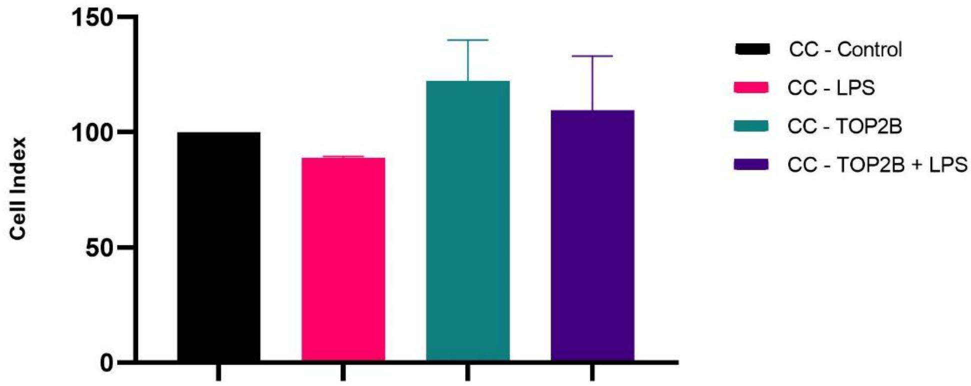


Figure 12. Calculation of cell index 24 h after co-culture assembly in RTCA, One-way ANOVA and Tukey's analyses. A) SH-SY5Y Only Groups B) Co-culture groups. (A) shows the cell index values of the SH-SY5Y cell groups. SH-NC: Negative Control; SH

As a result of the calculations, it was revealed that there was a significant decrease in cell index of the differentiation group (SH-SY5Y) compared to the control group. Similarly, the group receiving differentiation + LPS treatment experienced an almost identical decrease in cell index.

On the other hand, a decrease in cell index was observed in the group treated with LPS on HMC3 cells in the co-culture system compared to the control. While the peak cell index level was obtained in the TOP2B applied group, a higher index level was observed in the TOP2B+LPS group than the LPS group, but lower than the TOP2B only group.

After 24 h, after the results given above, there was no statistically significant change between the co-culture groups, and analyses were continued every 6 h. Significant values were reached among the desired groups after 48 h and the RTCA device was terminated **(Figure 13)**.

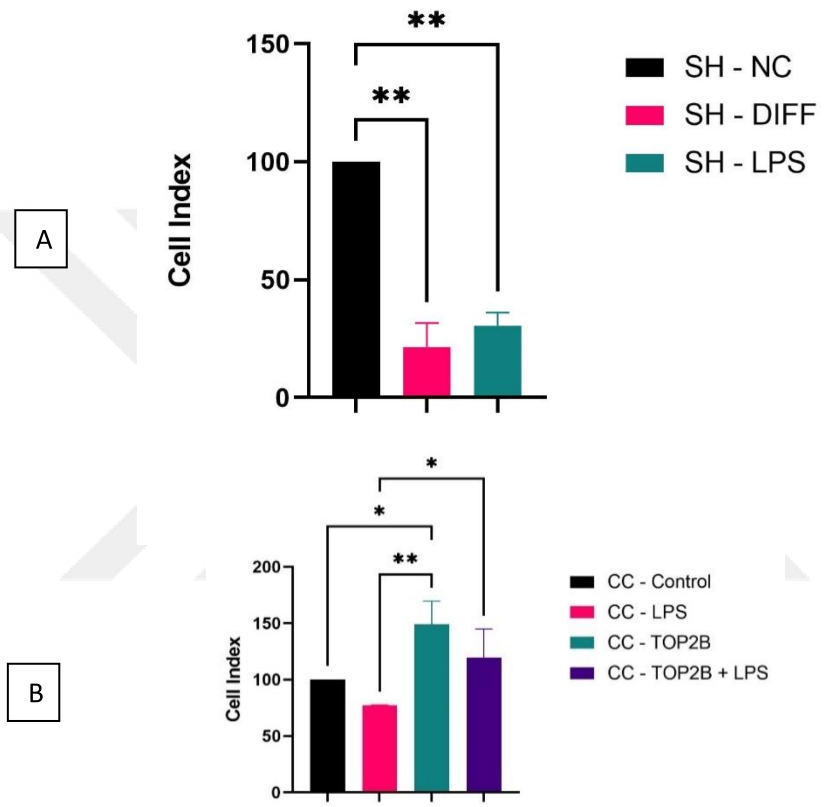


Figure 13. Calculation of cell index 48 h after co-culture assembly in RTCA, One-way ANOVA and Tukey's analyses. **A)** SH-SY5Y Only Groups **B)** Co-culture groups. (A) shows the cell index values of the SH-SY5Y cell groups. SH-NC: Negative Control; SH-DIFF: Differentiation; SH-LPS: Differentiation + LPS. (B) shows the cell index values of the coculture groups. CC – Control: HMC3 + SHSY5Y (Diff), CC – LPS: HMC3 + SHSY5Y (Diff+LPS), CC-TOP2B: HMC3 + SHSY5Y (Diff+TOP2B), CC – TOP2B + LPS (Diff+TOP2B+LPS). Graphs were created by GraphPad software. Error bars represent the means ± standard deviation; *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001

As a result of the calculations, the same relations obtained in 24 h were obtained in 48 h. It was revealed that the differentiation group (SH-SY5Y) experienced a significant decrease in the same cell index compared to the control group. Similarly, the group receiving differentiation + LPS treatment experienced an almost identical decrease in cell index.

Among cocultures, cell index went down to 73% in the LPS-treated group, while this value increased to approximately 150% in the TOP2B group. In the TOP2B + LPS applied group, a cell index increase was observed approximately 1.25 times that of the control group.

After 48 h, statistically significant changes were observed between the co-culture control, LPS, TOP2B and TOP2B + LPS groups, and the first part of neuronal survival analysis was completed.

3.6.1.2. MTT Assay

MTT solution was given to a plate that was duplicated with the plate placed in the RTCA device, as soon as the RTCA experiment was completed, solubilizing buffer was given after the incubation period of the solution for 4 h, and the values were measured with a reader the next day after it was left overnight **(Figure 14)**.

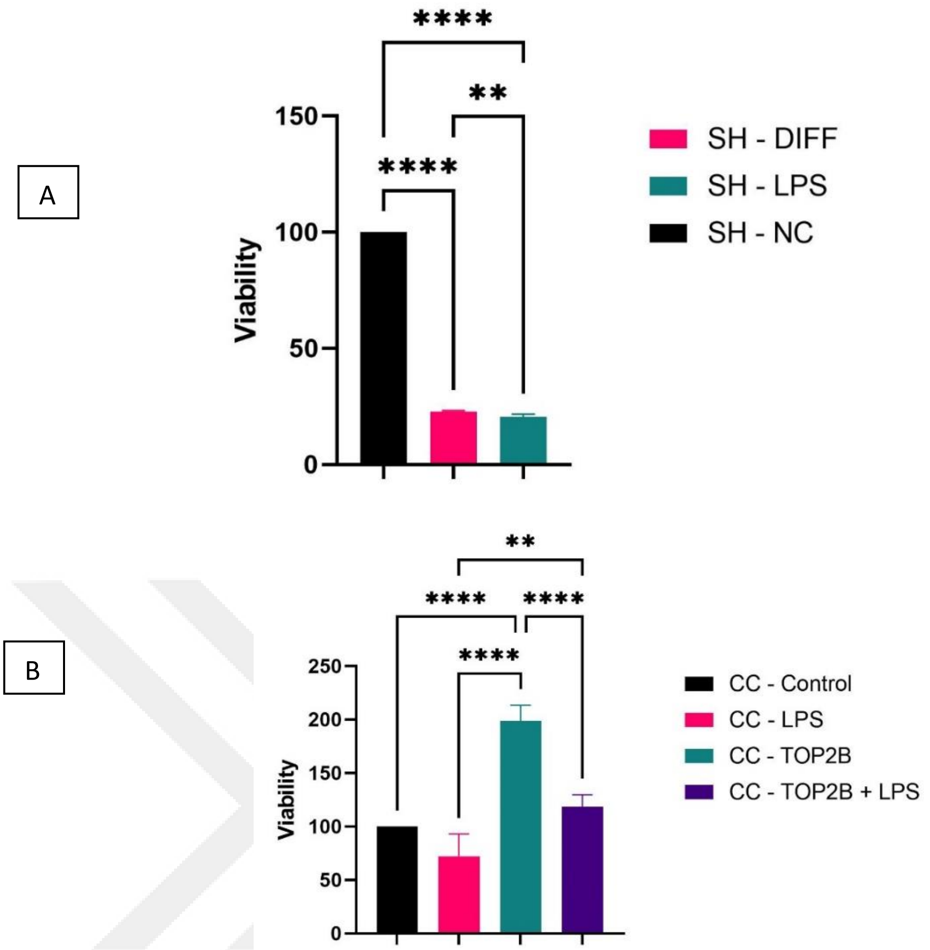


Figure 14. Calculation of cell viability 48 h after co-culture assembly in MTT, One-way ANOVA and Tukey's analyses. **A)** SH-SY5Y Only Groups B) Coculture groups. (A) shows the cell index values of the SH-SY5Y cell groups. SH-NC: Negative Control; SH-DIFF: Differentiation; SH-LPS: Differentiation + LPS. **(B)** shows the cell index values of the coculture groups. CC – Control: HMC3 + SHSY5Y (Diff), CC – LPS: HMC3 + SHSY5Y (Diff+LPS), CC-TOP2B: HMC3 + SHSY5Y (Diff+TOP2B), CC – TOP2B + LPS (Diff+TOP2B+LPS). Graphs were created by GraphPad software. Error bars represent the means ± standard deviation; *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001

As the results of MTT assay, the cell viability of the SHSY5Y experimental group decreased by approximately 75% in the Diff and Diff + LPS groups compared to the control group.

Among cocultures, a decrease in cell viability of approximately 25% was observed in the LPS applied group, while this value increased to approximately 2 times that of the control in the TOP2B group. In the TOP2B + LPS applied group, a cell viability increase was observed approximately 1.25 times that of the control group.

3.6.1.3. Apoptosis Test

Almost at the same time with MTT test, the Annexin V test was performed to perform the apoptosis test after the termination of the RTCA experiment **(Figure 15) (Figure 16)**.

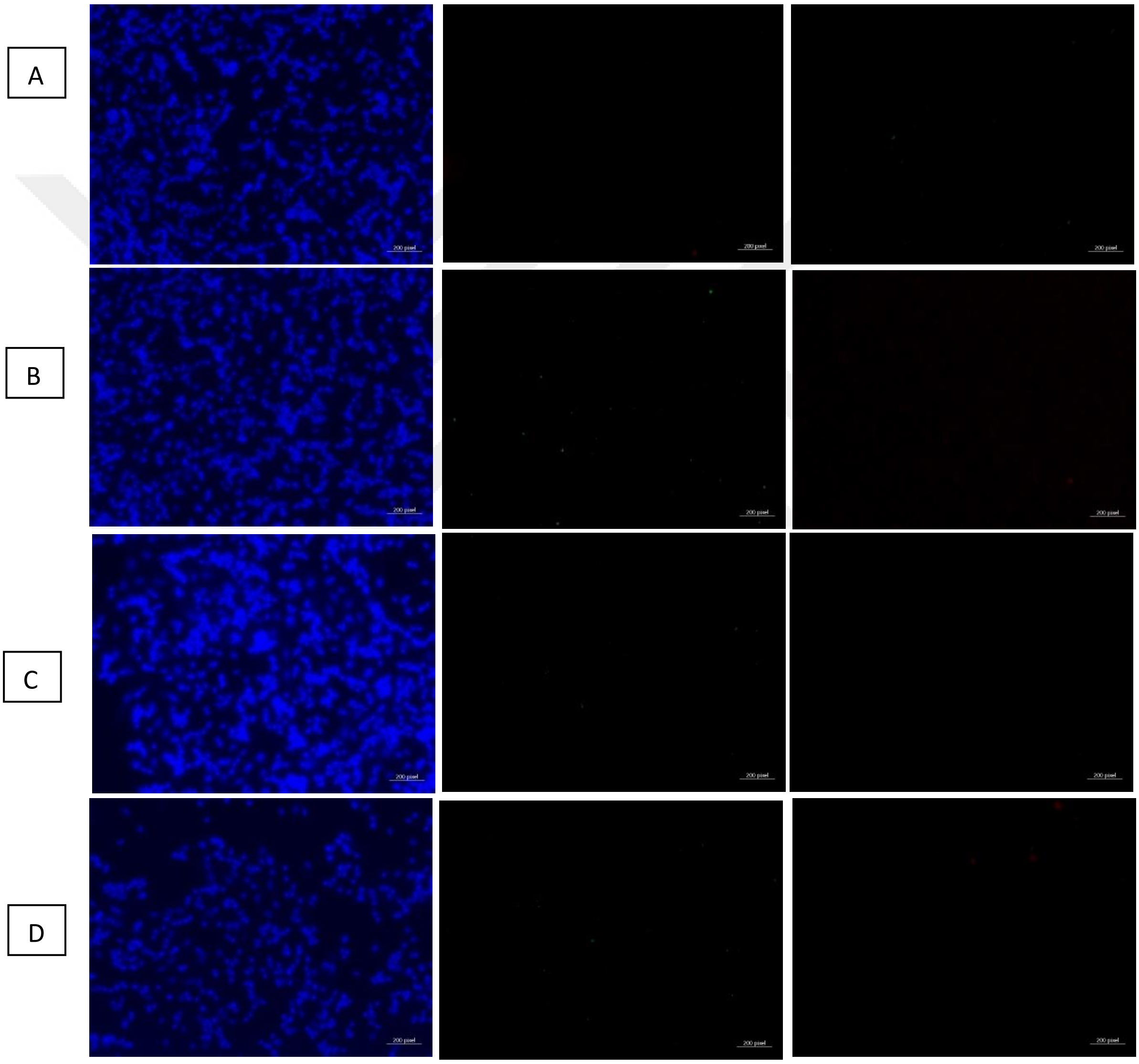


Figure 15. DAPI-Annexin-PI Stainings of Co-culture Groups, respectively. **A)** Control **B)** LPS-treated **C)** TOP2B only **D)** TOP2B + LPS treated. Images were taken by inverted fluorescent microscope, 10X (ZEISS)

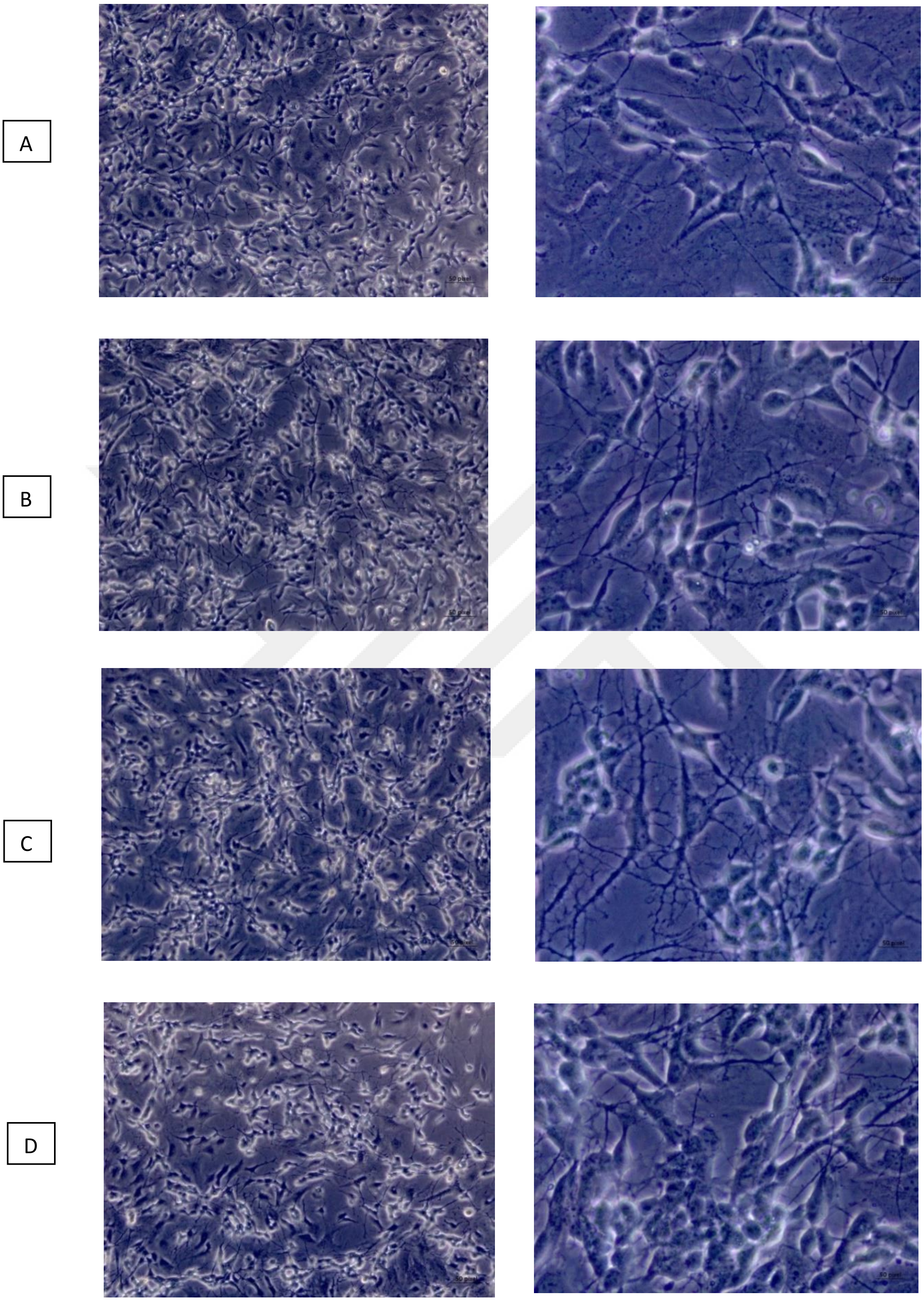


Figure 16. Morphological Images of Co-culture groups before the viability experiments, respectively. **A)** Control **B)** LPS-treated **C)** TOP2B only **D)** TOP2B + LPS treated. Images were taken by inverted fluorescent microscope, 10X and 40X respectively (ZEISS)

4. DISCUSSION

It is known that topo IIβ has a great importance in neuronal development and differentiation. Topo IIβ activity plays an important role in differentiation by activating the genes responsible for neural differentiation (Nur-E-Kamala et al., 2007, Tsutsui et al., 2001, Işık et al., 2015). In addition, silencing of topo IIβ may worsen the scenario of the disease by participating in the pathogenesis of neurodegenerative diseases because it suppresses neural differentiation. Topo IIβ has been shown to act on axons by regulating Rho GTPases; Silencing of topo IIβ causes neuronal axon degeneration in human bone marrow mesenchymal stem cells (MSCs), and its overexpression provides protection of axons and increases the neural differentiation potential of MSCs (Zaim and Işık, 2018).

Neuroinflammation, specifically, microglia-mediated neuroinflammation may contribute to the pathogenesis of neurodegeneration. However, the relationship between neuroinflammatory processes and neurodegenerative diseases is an indisputable issue. In our previous study, silencing of topo IIβ in neurally induced MSCs led to a significant increase in the expression of genes associated with inflammation (Işık et al., 2015). Topo IIβ enzyme may be one of the key regulatory proteins that control neuroinflammatory pathways and may prevent neurodegeneration in the future. This enzyme could be an important future step towards reducing the effect that microglia cells create the scenario of chronic inflammation and in various brain injuries. In line with these data, overexpression of topo IIβ enzyme in microglia is expected to positively affect neuronal survival in the environment by modulating chronic inflammation and reducing cell death.

In the study, microglia – neuron coculture was created to form an accurate brain microenvironment, but it should be kept in mind that microglia are not the only cell type in this context. In this direction, it is possible to create coculture models with other cells such as oligodendrocytes and astrocytes, then their effects on the pathways can be analyzed with a more comprehensive study with advanced models such as tri-coculture.

It is known that there is a scenario of chronic inflammation in neurodegenerative diseases, which is of great interest to our team. In addition, it is known that chronic inflammation scenario occurs in important issues such as stroke, brain damage and infection. Therefore, it will be extremely beneficial to carry the studies to an *in vivo* dimension and to examine the disease through animal models with different subtypes.

In line with a co-culture design, it was planned to consider the bidirectional release of factors without allowing physical contact of cells and to examine the role of topo IIβ in microglia-mediated neuroinflammation. The results we reached after the planned experiments confirm our hypotheses mentioned above. Recently, it was demonstrated that topo IIβ overexpression modulates the cytokines related to microglia phenotype in an inflammation model induced by LPS and IFNy (Akbayır, 2022). The results of the study groups can be discussed as follows:

RTCA - SHSY5Y only groups: After differentiation and differentiation + LPS treatments, cell index values were 21.61% and 30.56%, respectively. One of the reasons for the decrement is that after differentiation, cells gain a neuronal phenotype while losing their rounded one as the differentiation process goes on. In the Differentiation + LPS group, although the cell index was 30.56%, it was not statistically significant compared to differentiation only. Daily phenotype analysis under microscope also confirm the neuronal phenotype of cells. It is known from the literature that differentiation using RA + BDNF, as in our study, increases neurite outgrowths by forming amorphous cell bodies and then multiple neurites, forms an adult neuron phenotype in the end (Kou et al., 2008).
RTCA - Coculture groups: All groups were subjected to differentiation process. There was a 23% decrease in the LPS group compared to the control, a 49% increase in the TOP2B group, and an approximately 20% increase in the cell index in the TOP2B+LPS group. It is known that CD86+, an important M1 marker, and iNOS, which is an activated microglia marker, increase in microglia exposed to LPS-induced neuroinflammation (Akbayır et al., 2022). It is known that LPS administration in HMC3 cells removes the resting phenotypes and activates the cells, initiates the inflammatory state. It is seen that this inflammatory state causes a decrease in cell index values by disrupting the adhesion

of neuronal cells in the same environment. The 49% increase in cell index in the TOP2B overexpressed group without LPS showed that overexpression of this gene in non-activated microglia positively affected the spreading of cells in culture. In the TOP2B + LPS group, however, the 20% increase seen in the control group despite the presence of LPS indicates that this overexpression partially eliminates the damage of adhesion and spreading caused by LPS-induced neuroinflammation. From other studies of our team, it was observed that CD206, which is considered as the M2 phenotype marker, increased in HMC3 cells after TOP2B overexpression (Akbayır et al., 2022). In this case, it can be said that TOP2B overexpression brings microglia cells to the M2 phenotype, positively affects the status of the cells in culture and partially eliminates the bad scenario despite the presence of inflammation. After the RTCA results, the MTT assay was applied in the same plan. MTT exhibits the cell viability value.

Within the MTT – SHSY5Y only group, differentiation and differentiation + LPS treatments decreased cell viability values to 22.8% and 20.4%, respectively, compared to the control. This indicates that direct application of LPS to SHSY5Y cells produced an increase in cell index, but a decrease in cell viability. From other studies in the literature, it is known that LPS application causes a decrease in differentiated SHSY5Y cell viability after a certain period of time, and this situation is also observed by us (Pandur et al., 2019).

Within the MTT – Coculture groups, cell viability decreased to 72% in the LPS group, reached approximately 200% in the TOP2B group, and reached 118% in the TOP2B + LPS group. This shows that LPS successfully created the chronic inflammation scenario in HMC3 microglia cells and negatively affected the cell viability of neurons in the same environment after 48 h. It is known from other studies that IL-6 and TNF-α levels, which are inflammation markers, increase 48 h after LPS administration in microglia cells (Pandur et al., 2019). In this case, it is clear that the inflammation scenario described is damaging to neurons. Cell viability was almost doubled in the microglia TOP2B overexpressed group compared to the control group. In the TOP2B + LPS group, cell viability increased by 18% compared to the control. In this case, we show that despite the presence of chronic inflammation, topo IIβ modulates this scenario and makes a contribution to cell viability. Additionally, overexpression of TOP2B in resting microglia made an even better contribution to cell viability, approximately doubling. The relationship given was also verified with the Annexin V assay.

These results are for both adhesion and viability conditions, where we obtained a positive effect on cell viability by TOP2B. TOP2B overexpression is known to have an effect on neuronal induced mesenchymal stem cells via Rho GTPases (Yeman and Işık, 2021). This overexpression increases axon conservation by suppressing the RhoA/ROCK2 pathway. At the same time, it is known that suppression of RhoA in microglia can reduce M1 phenotype markers and convert microglia cells to M2 phenotype (Chen et al., 2017). In the later stages of the study, there is a need to conduct further analysis on this pathway and examine the pathway given above at the protein and mRNA espression level. This analysis will make a great contribution in terms of molecular pathways and will help to understand the reasons that increase viability. At this stage, the measurement of specific protein levels and gene expression level on suspicious pathways by performing analysis such as Western Blot, RT-qPCR is of great importance for the study.

5. CONCLUSION

In this study, it was aimed to investigate the neuroprotective effect of topo IIβ on neuronal cell viability against microglia mediated neuroinflammation *in vitro*. This was realized by overexpressing TOP2B gene in microglia cell line HMC3 in a co-culture system with a neuronal cell line SHSY5Y. Cell viability of neuronal cells increased in a condition of TOP2B overexpressed microglia cells in the absence or presence of LPS and INFγ. Increase in cell viability was confirmed by cell viability assays RTCA and MTT and apoptosis assay Annexin V assay.

Although this overexpression state has been shown to have a positive effect on the LPS-induced neuroinflammation model, the molecular pathways through which it does this still remain as a deficiency. In this sense, in order to better understand the molecular pathways affected by overexpressed topo IIβ in microglia and the effect of these pathways on neuronal viability, advanced analyses should be performed and the pathways should be analyzed at protein and mRNA levels. Since this enzyme is known to suppress the RhoA/ROCK2 pathway in neural induced mesenchymal stem cells, the result here will probably lead to a novel conclusion that will provide important data on the inhibitory effects of topo IIβ. Further in vivo studies are needed to clarify these pathways underlying the neuroprotective effect of topo IIb against microglia mediated neuroinflammation.

Considering how many people's lives worldwide are affected by neurodegenerative diseases, the enzyme which we have proven its positive viability effect is a starting point for many studies to be designed and promises great hope for the future as an end-product.

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APPENDIX

Appx. 1. Map of pEGFP-N1 (4.7 kb) vector