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 NEUROPROTECTIVE EFFECT OF STABLE OVEREXPRESSION OF DNA TOPOISOMERASE IIß IN NEURALLY DIFFERENTIATED SH-SY5Y CELLS AS AN *IN VITRO* MODEL OF ALZHEIMER'S DISEASE INDUCED BY AMYLOID B1-42 PEPTIDES

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ÖZET

Nöral Farklılaştırılmış ve Amiloid β1-42 peptitleriyle in vitro Alzheimer Hastalığı Modeli Oluşturulmuş SHSY5Y hücrelerinde Gen İfadesi Kararlı Olarak Arttırılmış DNA Topoisomerase IIb'nın Nöroprotektif etkisinin araştırılması

Alzheimer hastalığı (AH) demansın en yaygın formudur ve yaşı ilerlemiş insanlar arasında en yaygın görülen nörodejeneratif hastalıktır. Davranışsal yetenekler, düşünme, hatırlama ve akıl yürütmenin yanı sıra nöronal kayıp sonucu bilişsel işlevlerde de azalmaya sebebiyet vermektedir. AH, beyin dokularında hücre dışı, nörotoksik amiloid-β (Aβ) plaklarının patolojik birikimi ve hiperfosforile mikrotübül ile ilişkili tau proteini içeren hücre içi nörofibriler yumaklar ile karakterize edilen ilerleyici bir nörodejeneratif hastalıktır. Bu değişikliklere yol açan mekanizmalar henüz tam olarak bilinmemektedir.

Topoizomeraz II (topo II), her iki DNA zincirinde kesik oluşturarak DNA süper katlanmasını çözen, replikasyon, transkripsiyon gibi hücresel olaylarda rol alan başlıca enzimdir. Bu enzimin iki izomerinden birisi, topo IIα, sadece bölünen hücrelerde bulunur ve bölünmeyle ilgili topolojik problemleri giderir. Topo IIβ ise bütün hücrelerde, özellikle gelişmekte olan beyinde post-mitotik nöron hücrelerinde bulunur. Topo IIβ aktivitesinin nöral farklılaşmanın önemli aşamalarında, nörit büyümesi ve akson uzamasında, görevli genlerin ifadesinin düzenlenmesinde rol oynadığı bilinmekte, topo IIβ geni baskılandığında daha kısa aksonlar gelişmektedir. Topo IIβ eksikliğinde aksonal gelişimin tamamlanamaması çeşitli çalışmalar tarafından gösterilmiştir. Bizim çalışmalarımızda da topo IIβ eksikliğinin, nörodejeneratif hastalıklar ile alakalı olabileceği *in vitro* hem Parkinson Hastalığı (PH) hem de Alzheimer Hastalığı (AH) modelleriyle ilişkilendirilmiştir. *In vitro* modellerde, topo IIβ eksikliğinin her iki hastalığın patalojisinde önemli rolü olabileceği gösterilmiştir. Önceki çalışmamızda, topo IIβ, nöral olarak farklılaşmış insan mezenkimal kök hücrelerinde (iMKH) susturulduğunda nöral farklılaşma veriminde ve nörit uzunluğunda kısalma belirlenmiştir. Olası nöroprotektif etkiyi göstermek için hücreler topo IIβ geni ile transfekte edilmiş, iMKH'de topo IIβ aşırı ifadesi daha uzun nöritler ve tüm hücrelerin nöral hücerelere farkılaşması ile sonuçlanmıştır.

Bu çalışmalardan yola çıkarak, bu tez çalışmasında nöronal farklılaştırılmış SH-SY5Y nöroblastoma hücre hattında kalıcı olarak aşırı ifade edilmiş DNA topo IIβ'nın,

Aβ42 fibrilleriyle oluşturulmuş *in vitro* AH modelinde nöroprotektif etkisini araştırılması amaçlanmıştır. Bu amaçla, TOP2B-GFP (Green Fluorescent Protein) genini içeren plasmid sentezlenip, topo IIß-GFP lentivirus (LV) üretilmiştir. Üretilen topo IIß-GFP lentivirüsleri, SHSY5Y hücrelerine transfekte edilerek kalıcı transgenik TOP2B-SHSY5Y transgenik hücre hattı elde edilmiştir. TOP2B gen ifadesi akış sitometrisi ile belirlenmiştir. Bu hücrelerde retinoik asit (RA) ve BDNF (insan beyin kaynaklı nörotrofik faktörü) muamelesi ile nöronal farklılaştırma yapılmış, sürecin 8. gününde hücreler $A\beta_{1-42}$ oligopeptidlerinden elde edilen fibriller ile muamele edilerek *in vitro* AH modeli oluşturulmuştur. AH modeli, Aß fibrillerinin tayini (Thioflavin S boyaması) ve AH'na özel belirteçler (tau, p-tau ve Aß1-42) ile doğrulanmıştır (immünfloresan boyama). Son olarak, topoIIß'nın nöroprotektif etkisi, hücre canlılık (MTT) analizi ve protein seviyesinde (western blot) ile belirlenmiştir.

Keywords: DNA topoizomeraz IIβ, SH-SY5Y Nöroblastoma Hücreleri, $Aß_{1-42}$ Peptidi, Alzheimer Hastalığı, Nörodejenerasyon.

ABSTRACT

Investigation of the Neuroprotective Effect of Stable Overexpression of DNA Topoisomerase IIß in Neurally Differentiated SH-SY5Y cells as an *in vitro* Model of Alzheimer's Disease Induced by Amyloid β1-42 peptides

Alzheimer's disease (AD) is the most common form of dementia and the most common neurodegenerative disease among older people. In addition to behavioral abilities, thinking, remembering and reasoning, it also causes a decrease in cognitive functions as a result of neuronal loss. AD is a progressive neurodegenerative disease characterized by the pathological accumulation of extracellular, neurotoxic amyloid-β (Aβ) plaques in brain tissues and intracellular neurofibrillary tangles containing hyperphosphorylated microtubule-associated tau protein. The mechanisms that lead to these changes are not yet fully understood.

Topoisomerase II (topo II) is the main enzyme that dissolves DNA superfolding by creating cuts in both DNA chains and plays a role in cellular events such as replication and transcription. One of the two isomers of this enzyme, topo IIα, is found only in dividing cells and resolves the topological problems associated with division. Topo IIβ is found in all cells, especially post-mitotic neuron cells in the developing brain. It is known that topo IIβ activity plays a role in regulating the expression of involved genes in important stages of neural differentiation, neurite growth and axon elongation, and when the topo IIβ gene is suppressed, shorter axons develop. Failure to complete axonal development in Topo IIβ deficiency has been shown by various studies. In our studies, topo IIβ deficiency has been associated with both Parkinson's Disease (PD) and Alzheimer's Disease (AD) models in vitro, which may be related to neurodegenerative diseases. In vitro models have shown that topo IIβ deficiency may have an important role in the pathology of both diseases. In our previous study, a shortening of neural differentiation efficiency and neurite length was determined when topo IIβ was silenced in neurally differentiated human mesenchymal stem cells (iMSCs). To demonstrate the possible neuroprotective effect, cells were transfected with the topo IIβ gene, and topo IIβ overexpression in iMSC resulted in longer neurites and differentiation of all cells into neural cells.

Based on these studies, this thesis study aimed to investigate the neuroprotective effect of DNA topo IIβ, permanently overexpressed in the neuronal differentiated SH-SY5Y neuroblastoma cell line, in the in vitro AD model formed with Aβ42 fibrils. For this purpose, the plasmid containing the TOP2B-GFP gene was synthesized and topo IIß-GFP lentivirus (LV) was produced. The produced topo IIß-GFP lentiviruses were transfected into SHSY5Y cells and the permanent transgenic TOP2B-SHSY5Y transgenic cell line was obtained. TOP2B gene expression was determined by flow cytometry. Neuronal differentiation was made in these cells by retinoic acid (RA) and BDNF (human brain-derived neurotrophic factor) treatment, and on the 8th day of the process, the cells were treated with fibrils obtained from Aβ1-42 oligopeptides to create an in vitro AD model. The AD model was confirmed (immunofluorescence staining) by detection of Aß fibrils (Thioflavin S staining) and AD-specific markers (tau, p-tau and Aß1-42). Finally, the neuroprotective effect of topoIIß was determined by cell viability (MTT) analysis and at the protein level (western blot).

Keywords: DNA Topoisomerases IIß, SH-SY5Y Nöroblastoma Cells, $Aß_{1-42}$ Peptide, Alzheimer's Disease, Neurodegeneration.

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FORM OF DECLARATION

Herewith I declare, that I obtained all the information and documents in this study within the framework of academic rules, presented all visual, auditory, and written information and results in accordance with scientific ethics, did not falsify the data I used, referred to the sources I used in accordance with scientific norms, that my thesis was original except in the cases cited, produced by me and written in accordance with the Thesis Writing Guide of Uskudar University Institute of Health Sciences.

28.06.2024

Tahire YURDAKUL

CONTENTS

INDEX OF TABLES

Page

INDEX OF FIGURES

Page

INDEX OF IMAGERY AND ABBREVIATIONS

AD: Alzheimer's Disease

Aβ: Amyloid beta.

APP: Amyloid precursor protein

BDNF: Brain derived neurotrophic factor

CNS: Central nervous system

DAPI: 4′,6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulfoxide

EDTA: Ethylenediaminetetraacetic acid

F12: Basal medium

FACS: Fluorescent activated cell sorter

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

GFP: Green fluorescent protein

HEK-293: Human embryonic kidney 293

LV: Lentivirus

mRNA: messenger RNA

NF-L: Neurofilament

NK: Natural Killer

PD: Parkinson Disease

PBS: Phosphate Buffered Saline

RA: Retinoic Acid

RTCA: Real time cell analysis

SH-SY5Y: Neuroblastoma cell type

TOP2A: Topoisomerase 2 alpha (Gene)

TOP2B: Topoisomerase 2 beta (Gene)

Topo I: Topoizomeraz I

Topo II: Topoizomeraz II

Topo IIß: Topoizomeraz II beta

Topo IIα: Topoizomeraz II alpha

β-ME: β-mercaptoethanol

µM: Micromolar

1. INTRODUCTION

Alzheimer's disease (AD) is the prevalent form of dementia. AD is a degenerative neurological disorder that gradually worsens over time. It is characterized by the abnormal buildup of amyloid-β (Aβ) plaques and tangled tau proteins within brain cells. The condition is characterized by profound memory loss and cognitive deterioration, and presently has just a few treatment choices that are successful. While individuals with AD have comparable neuropathological characteristics, recent research suggests that the illness has a multifaceted polygenic cause, with over 25 genetic loci associated with a higher risk of AD and dementia. Nevertheless, our capacity to comprehend the cellular and molecular pathways that contribute to the hereditary susceptibility to Alzheimer's disease, as well as its advancement and intensity, is nevertheless restricted.

Aß is generated by the proteolytic cleavage of the transmembrane amyloid precursor protein (APP). Within the central nervous system (CNS), neurons express a large amount of APP. This protein can be cleaved by endoproteases located in the cell membrane, resulting in the production of amyloidogenic fragments. These fragments are then discharged into the area outside the cells. The buildup of Aβ within neurons, namely in the form of soluble and oligomeric Aβ types, which are believed to be more harmful than insoluble forms, has been suggested as a significant factor in the development of Alzheimer's disease.

The TOP2B gene plays a crucial role in the development of the brain and the differentiation of neural cells. Topo IIβ has been demonstrated to have a function that might potentially contribute to the development of neurodegenerative illnesses including AD and Parkinson's disease (PD). Therefore, it indicates that mutations in TOP2B or alterations in topo IIβ expression levels may result in neurodevelopmental or neurodegenerative diseases.

The purpose of this study; to investigate the neuroprotective effect of stable overexpression of topo IIß in AD in the transgenic SH-SY5Y cell line.

2. GENERAL INFORMATION

2.1. DNA TOPOISOMERASES

DNA topoisomerases are essential for cells to continue their life cycle by regulating DNA topology and they are evolutionary well-conserved enzymes (Friedberg.,2003). Topoisomerase enzymes are named as I, II, III, IV, V, VI according to their historical numerology; Type I, II, IA, IB according to mechanical differences; Topo IA, IB, IC, IIA, IIB by evolutionary classification (Pommier., 2012). However, they are generally divided into two main classes, type I and type II. Type I topoisomerases create a temporary break in one of the two DNA strands, pass the unbroken strand through it and reconnect the broken ends while type II topoisomerases break both DNA strands (Figure 1).

Topoisomerases are essential for regulating the configuration of DNA. These may be categorized into two main families: Topoisomerase I (TOP1, which includes TOP1 and TOP1MT) and Topoisomerase II (TOP2, which includes TOP2A and TOP2B) (Davies et al., 1993).

Type I enzymes bind to the cut DNA strand at the 3' or 5' end by a temporary covalent phosphotyrosine bond (Baker et al., 2009). After the uncut chain is passed through the cut one, the fracture is reconnected. It has been reported that DNA relaxation occurs by DNA spins around itself (with controlled rotation). In bacteria this process is controlled by type I A enzymes via enzyme bridge by forming 5' covalent intermediates, and in eukaryotes and viruses it is controlled by type I B and I C through forming 3' covalent intermediates.

DNA Topoisomerase	Type	Structure	M.W. (kD)	DNA cleavage	Gene localization (Human chromosome)	Function
I	IB	Monomer	100	ssb	20	Replication, transcription, recombination
IIIα	IA	2 isoforms (alternative splicing)	110	ssb	17	Recombination, rDNA metabolism
IIIβ	IA	3 isoforms (alternative splicing)	96	ssb	22	Recombination
IIα	II	Homodimer	170	dsb	17	Chromosome condensation and segregation. Replication
IIβ	II	Homodimer	180	dsb	3	Not well defined

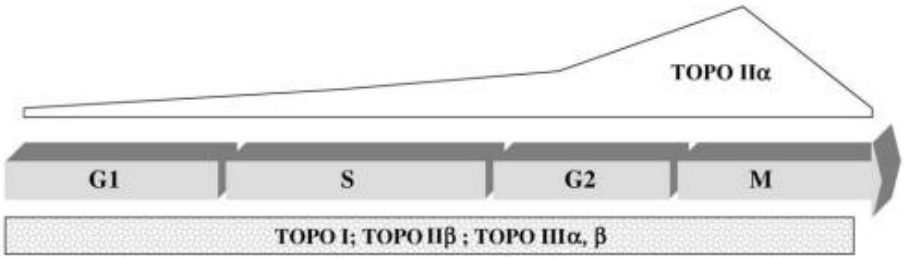

Figure 1: Human DNA Topoisomerases and Their Functions. Representation of human DNA topoisomerases through cell cycle. Type IA human topoisomerases are homologous to bacterial type topoisomerases. For the action of DNA cleavage, type I topoisomerases make a ss-break to relax the supercoiling of DNA molecule whereas type II topoisomerases form ds-break for relaxation. Type II topoisomerases have two isoforms which are Topoisomerase II α and Topoisomerase II ß. *(F. Cort´es et al.,2003).*

2.1.1. Type II DNA Topoisomerases

Type II DNA topoisomerases catalyze topological change of DNA by mediating transportation of one DNA double helix through another (Yang et al.,2000; Heng & Lee, 2010). DNA topoisomerase II takes important roles in several genetic activities such as DNA replication, transcription, recombination and mitotic processes like condensation, segregation of sister chromosomes (Tsutsui et al.,2001; Heng & Lee,2010). While low organization eukaryotes such as yeast or Drosophila encode only one single type II topoisomerase, vertebrates encode two different isoforms of this enzyme which are topoisomerase IIα (Topo IIα) and topoisomerase IIβ (Topo IIß) (Nitiss., 2009). These two enzymes share a very high amino acid sequence similarity (~70%), but they have different molecular weights (α form 170 kDa; β form 180 kDa). Also, they are encoded by different genes (McClendon and Osheroff, 2007). It has demonstrated that topo IIß has crucial role in the late stage of neural development (Tsutsui et al.,2001; Lyu et al.,2006; Sano et al.,2008). In addition to this, another isoform of DNA topoisomerase II, which is DNA topoisomerase IIα (topo IIα), mainly involves in chromosome segregation (Drake et al.,1987; Davies et al.,1993).

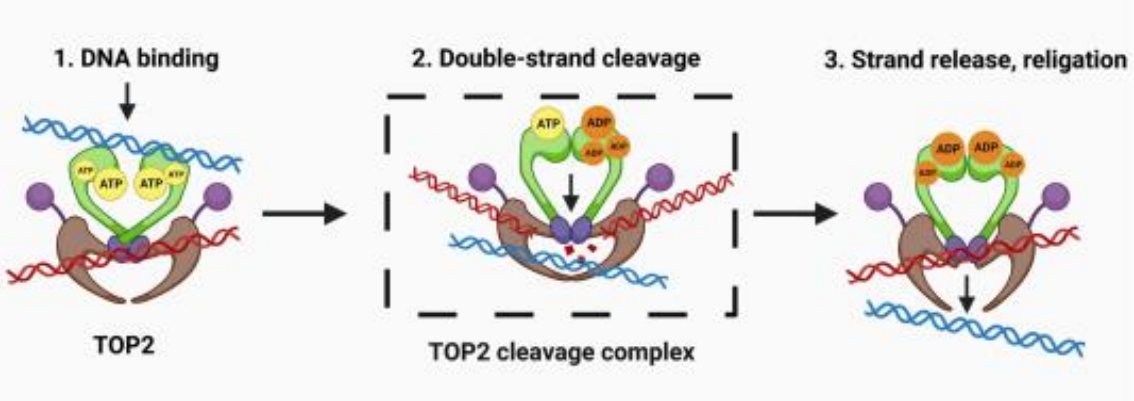

Figure 2: Working Principle of DNA Topoisomerase IIß for modifying DNA topology (Zhu et al.,2024).

2.1.2 DNA Topoisomerase IIß

DNA topoisomerase IIß (topo IIß) was discovered by purifying from murine P388 cells, and it is an enzyme that is one of the isoforms of type II DNA topoisomerase subfamily in mammals (Drake et al.,1987). The primary role of topo IIß is to regulate the configuration of DNA by cleaving, spinning, and rejoining its strands (Davies et al., 1993). Recent research has demonstrated that TOP2B is essential for preserving the shape of chromosomes, mending DNA, duplicating genes, and controlling transcriptional activity (Mcclendon & Osheroff, 2007). It has a crucial function in organisms, which includes not only controlling the regular cell cycle and development, but also influencing the development and treatment of cancer and other disorders associated to aging, as well as regulating the natural aging process (Lyu et al., 2006; Sano et al., 2008).

DNA topoisomerase IIß, that one of the isoforms of DNA topoisomerase II, is found at high amount in post-mitotic neuronal cells in developing brain. During neuronal cell differentiation, suppressing or knock downing the function of topoisomerase IIß may leads to depression in the induction of some neuronal genes. Topo IIß is involved enzymatically in an early stage of increased expression of neuronal genes. It is thought that this enzymatic activity occurs via decondensation of the chromatin structure of the gene containing region (Tsutsui et al., 2006; Heng & Lee, 2010). Even though topo IIß is functional on naked DNA, topo IIß is found in nucleoli as an enzyme and is unachievable to chromation DNA. Therefore, it is thought that DNA topo IIß could move forward into nucleoplasm as a response to appropriate signals to control structure of chromatin DNA (Tsutsui et al., 2006).

While TOP2B is present in several regions of the central nervous system, its expression in the cerebellum of adult rats is lower compared to young rats.

The presence of TOP2B has been identified in brain cells of elderly sheep. Animal models lacking TOP2B display several signs of accelerated aging, including retinopathies, Hoffman syndrome characterized by autism, defective NK cells and hearing loss (Hu et al., 2019).

Moreover, it has showed that topo IIß may have a role that contribute to pathogenesis of neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's Disease (PD) (Terzioglu-Usak et al., 2017).

2.1.3 DNA Topoisomerase IIß and Neurodegenerative Diseases

There have been many studies that showing the importance of DNA topoisomerase IIß (topo IIß) for neural differentiation and brain development. It involves in transcriptional regulation of some genes in neuronal cells. These genes affect important neuronal events such as migration, neurite outgrowth and axon guidance (Tsutsui et al., 2006; Nur-E-Kamal et al., 2007; Heng & Lee, 2010). In one study, it has demonstrated that inhibition of topo IIß blocks considerably the neurite outgrowth and growth cone formation (Figure 3) (Nur-E-Kamal et al., 2007). Another study showed that the motor axon is not able to make the connection with skeletal muscles and the sensory axon is not capable of entering the spinal cord in the cerebral cortex of topo IIß-null mice (Heng & Lee, 2010). Due to the shortening of the axons, neuronal networks are not provided efficiently. These properties are also seen in neurodegenerative diseases in common. Also, it has demonstrated that topo IIß may play a role in the pathogenesis of neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's Disease (PD) by regulating signal transduction pathways in neural differentiation (Terzioglu-Usak et al., 2017). In recent years, it has also shown that inhibition of topo IIß activity may play a role in the pathogenesis of neurodegenerative diseases by changing the balance in Rho-GTPase activities (Zaim & Isik, 2018). Also, in the latest study, it has reported that silencing of topo IIß activity may cause PD pathology through down regulation of the expression of tyrosine hydroxylase (TH) which is considered as a PD marker (Yeman & Isik, 2021).

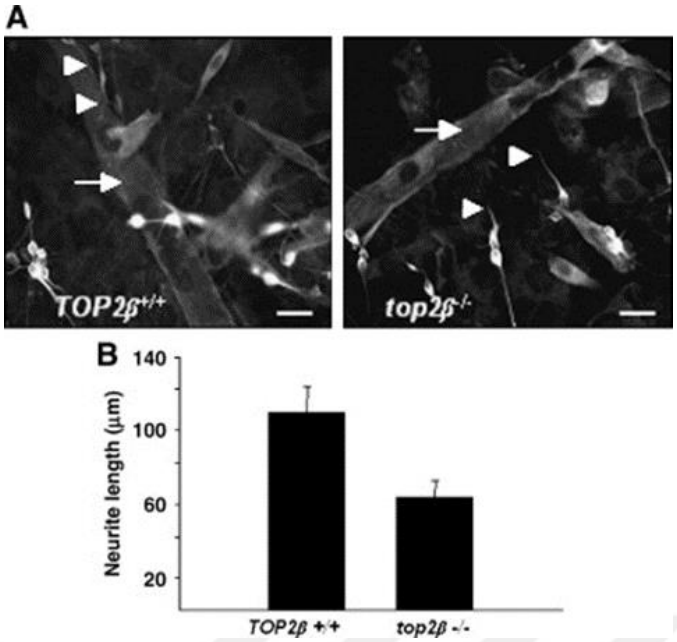

Figure 3: Topo IIß null neurons form shorter neurites. Cortical neurons that were isolated from wild type (*TOP2*$\beta^{+/+}$) and null (*top2*$\beta^{-/-}$) embryos. (B). Measuring of length of neurites. *(Nur-E-Kamal et al., 2007).*

Significant decreases in the protein levels and messenger RNA (mRNA) of TOP2B were seen in cerebellar granule neurons in a rat model of Alzheimer's disease. The reduction significantly impacts the suppression of nuclear receptor-related factor 1 (Nurr1), eventually exacerbating the progression of Alzheimer's disease.

2.2. Alzheimer's Disease

Alzheimer's Disease (AD) is a degenerative condition of the nervous system that is characterized by a gradual decline in cognitive function and is the primary cause of dementia in elderly individuals. Discovered by Dr. Alois Alzheimer in 1906, the disease is distinguished by a deterioration in cognitive function, loss of memory, and alterations in behavior. AD gradually impairs cognitive functions such as thinking, memory, reasoning, and behavior. These symptoms contribute to the high prevalence of this kind of dementia among the elderly population. The severity of AD is influenced by both hereditary factors and the patient's everyday lifestyle (Jia et al., 2024).

Although the exact onset mechanism remains unknown, the initial stages of Alzheimer's disease are characterized by memory lapses and difficulty in verbal expression. The main cause of these alterations in AD brains is the abnormal accumulation of amyloid fibrils and tau tangles, which are toxic. As the disease progresses, neurons gradually lose their functionality and capacity to form connections

with each other, ultimately resulting in their death. Over time, the damage progressively extends across the hippocampus, resulting in impairments to memory. As the number of neurons decreases, the damaged brain begins to undergo a reduction in size. During the ultimate phase of severity, there is a significant collapse of brain tissue (Rachmian et al., 2024) **(Figure 4).**

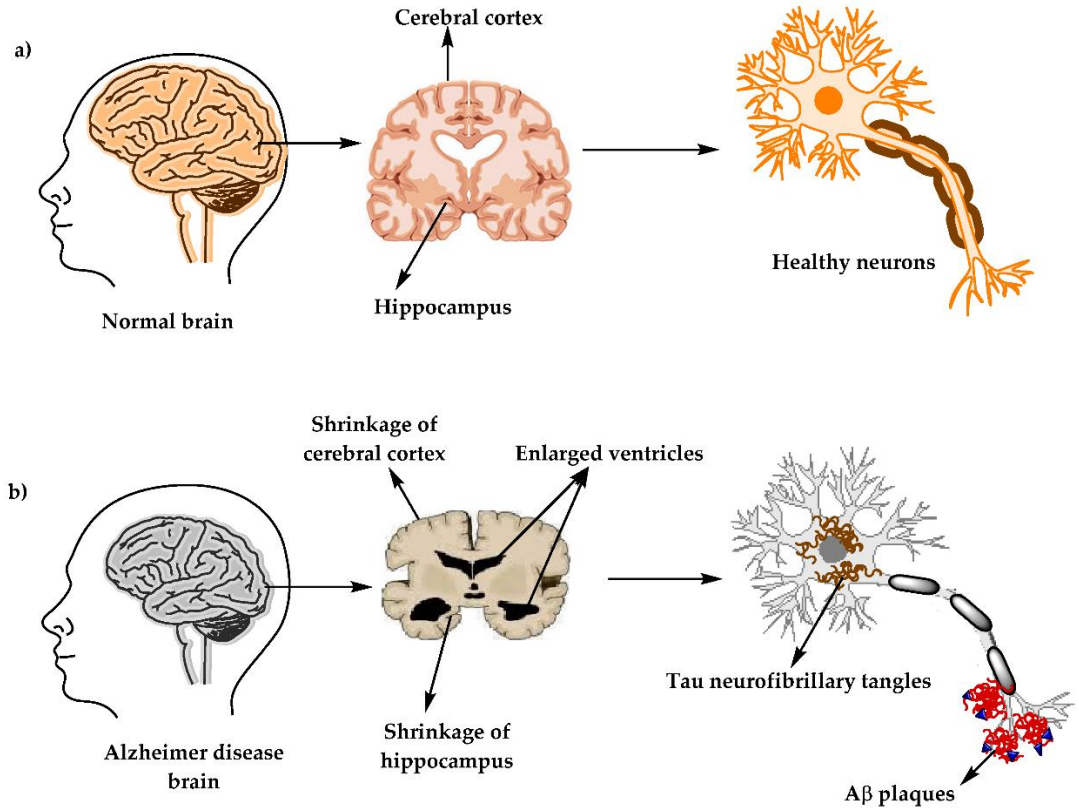

Figure 4: The anatomical structure of the brain and neurons in (a) normal brain and (b) brain affected by Alzheimer's disease (Breijyeh and Karaman, 2020).

Pathology

The characteristic features of Alzheimer's disease (AD) are the presence of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) made up of tau protein that has undergone hyperphosphorylation. Amyloid-beta plaques accumulate outside of cells, whereas neurofibrillary tangles grow inside neurons, causing disruption to cellular function (Figure 5). These irregularities are responsible for synaptic malfunction, inflammation in the nervous system, and the death of neurons, especially in brain areas crucial for memory and cognitive functions including the hippocampus and cortex.

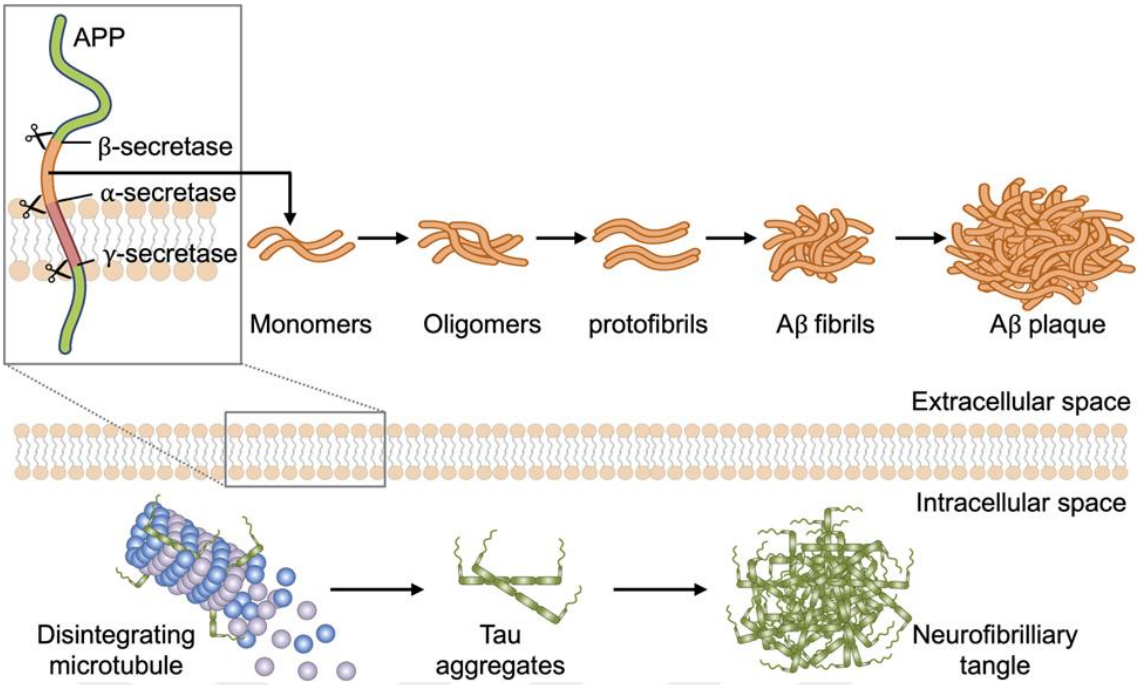

Figure 5: Aß plaques accumulates extracellularly while neurofbrillary tangle forms in intracellular space (SangJoon et.al, 2021).

AD is histologically defined by the presence of extracellular senile plaques consisting of Aβ peptides (Walker and Jucker, 2015). These peptides are formed by a series of enzymatic cleavages of amyloid precursor protein by secretases (O'Brien and Wong, 2011). The predominant Aβ peptides are composed of 40–42 amino acid residues. Among them, the peptide with 42 amino acid residues (Aβ1–42) has a higher neurotoxicity because it is more prone to aggregation (Kumar and Singh, 2015; Acosta et al., 2018). The presence of Aβ peptides initiates a pathogenic sequence that results in the development of a second histological characteristic of Alzheimer's disease (AD)-neurofibrillary tangles (Hardy and Selkoe, 2002). These tangles are composed of tau protein that has undergone hyperphosphorylation. Both Aβ and tau protein have important functions in neurons. Aβ regulates synaptic activity (Brothers et al., 2018), whereas tau protein stabilizes microtubules (Hervy and Bicout, 2019). Nevertheless, the abnormal accumulation of these substances gradually impairs the structure and function of neurons (Figure 6). Neuronal deterioration in Alzheimer's disease (AD) is mostly detected in the amygdala, neocortex, and hippocampus (Hussain et al., 2018; Niikura et al., 2006). Multiple ideas have been suggested on the etiology of AD. The amyloid cascade theory (Ricciarell, and Fedele, 2017) is the most commonly recognized hypothesis, followed by the cholinergic hypothesis (Francis et al., 1999), the tau hypothesis [36], and the more recent neuroinflammation hypothesis (Heneka et al., 2015). Each of these explanations

partially overlap and complement each other in describing the intricate causes of AD (Kumar and Singh, 2015).

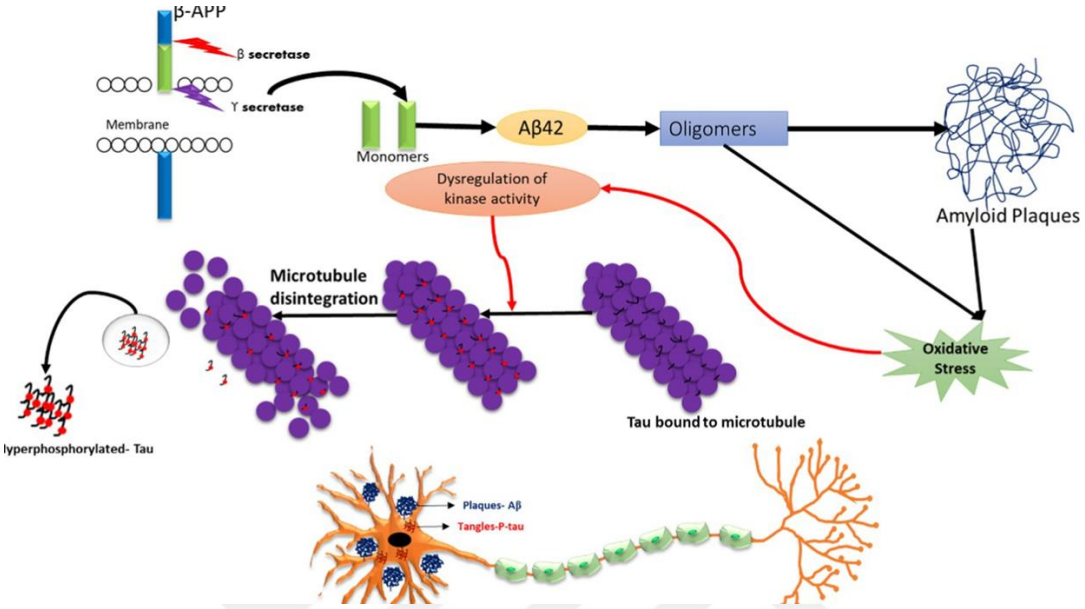

Figure 6: Intracellular (tau phosphorylation and extracellular (Aß fibril formation) mechanisms involved in AD ptahology (Prajapat et al., 2023)

Risk Factors

There are so many factors play roles in AD development. Aging, genetic factors, enviromental factors and gender are some of the rish factors in AD development. Notably, age remains the most significant risk factor, with the incidence doubling every five years above age 65 (Armstrong, 2019).

Herein, the most important risk factors are mentioned and explained their relationship with AD.

The main determinant of risk for AD is the process of aging. Alzheimer's disease is predominantly observed in adults aged 65 and above, making its occurrence in younger individuals uncommon (Guerreiro and Bras, 2015). The process of aging is a complex and permanent phenomena that impacts several organs and cellular systems. It entails a reduction in the size and weight of the brain, a decline in the connections between neurons, and an enlargement of fluid-filled spaces in certain areas, accompanied by the accumulation of senile plaques (SP) and neurofibrillary tangles (NFT). Furthermore,

aging can lead to several problems such as reduced glucose metabolism, imbalanced cholesterol levels, dysfunctional mitochondria, depression, and cognitive decline. These modifications are also evident in the natural aging process, making it difficult to distinguish between early instances of Alzheimer's disease. Alzheimer's disease (AD) can be categorized based on the age of onset (Hou et al., 2019). Early-onset AD (EOAD) is a less common variety, affecting around 1-6% of cases. EOAD is typically familial, meaning it occurs in families and is defined by the presence of AD in several generations. The age of onset for EOAD ranges from 30 to 60 or 65 years. The second category is known as late-onset Alzheimer's disease (LOAD), which is more prevalent in those who develop symptoms beyond the age of 65. Both forms may appear in individuals with a familial history to Alzheimer's disease and in families with a late-onset form of the disease (Bekris et al., 2010).

Genetic variables, have been shown to have a significant impact on the development of AD, have been uncovered by researchers over time. Genetic factors were responsible for 70% of the cases of AD. In particular, most cases of early-onset AD (EOAD) are inherited in a pattern where the dominant genes Amyloid precursor protein (APP), Presenilin-1 (PSEN-1), Presenilin-2 (PSEN-2) are mutated. The most significant genetic risk factor is apolipoprotein E (ApoE) and it is linked with late-onset AD (Khanahmadi et al., 2015).

The etiology of AD cannot be solely associated with aging and genetic risk factors. Various **environmental risk factors**, such as metals, diet, cardiovascular health, infections, air pollution and others (obesity, diabetes etc.) can trigger oxidative stress and inflammation, hence increasing the possibility of developing AD (Grant et al., 2002).

2.3. SH-SY5Y Cell Line

The SH-SY5Y cell line is derived from the SK-N-SH neuroblastoma cell line through three rounds of subcloning. This serves as a prototype for neurodegenerative illnesses since the cells may be converted into various types of fully functional neurons by introducing certain substances. Moreover, the SH-SY5Y cell line has been widely employed in experimental neurological studies, particularly for examining neuronal growth, metabolism, and function in connection to neurodegenerative processes, neurotoxicity, and neuroprotection (Riegerova et al., 2021).

This cell line was derived from the SH-SY subclone of the original SK-N-SH human neuroblastoma cell line. The SK-N-SH cell line originated from metastatic cells found in the bone marrow aspirate of a four-year-old female of unknown ethnicity in 1970 (Ross et al., 1983).

Differentiation of SH-SY5Y:

Cellular differentiation is a complicated process in which dividing cells alter their function or phenotype (Iwanami and Iwami, 2018). The neurons in the central nervous system (CNS) of humans are classified into many distinct subtypes, such as serotonergic, adrenergic, dopaminergic, cholinergic and GABAergic. Each subtype primarily expresses a distinct kind of neurotransmitter and the related proteins involved in its transport, production storage, and release (Nicoll, Malenka and Kauer, 1990). Cell culture media may be modified by introducing different chemicals to promote differentiation. An instance of elevation of dopaminergic markers (such as tyrosine hydroxylase and dopamine transporter) occurs in SH-SY5Y cells when treated with retinoic acid alone or combined with phorbol esters, namely phorbol-12-myristate-13-acetate (Avola et al., 2018). Combining retinoic acid with neurotrophins, such as brain derived neurotrophic factor, results in the development of a cholinergic phenotype. On the other hand, using dibutyryl cyclic AMP leads to the formation of an adrenergic phenotype (Xie, Hu and Li, 2010; Kume et al., 2008). An additional advantage of differentiating SH-SY5Y cells, in addition to reflecting the primary neuronal populations impacted in neurodegenerative disorders, is the enhanced translational significance of these models. SH-SY5Y cells that have undergone differentiation have elongated neurites, show mature neuronal markers, and have a notably reduced rate of proliferation, rendering them morphologically comparable to human primary cortical neurons (Kovalevich and Langford, 2013).

3. MATERIAL AND METHOD

3.1. SH-SY5Y Cell Culture

3.1.1. Freezing and Thawing of Cells

SH-SY5Y cell line (CRL-2266™) was purchased from ATCC (American Type Culture Collection). Cells were stored in liquid nitrogen at -196˚C for long-term storage.

To freeze the cells for long-term storage, after centrifugation step in subculture process defined as above, cell pellet was resuspended in FBS. 900 µl cell suspension including the density of $1.5x10^6$cells/ml was transferred to cryovial tubes which were placed on ice previously. 100 µl DMSO (Dimethyl Sulfoxide, GENAXXON) was added into each tube by mixing and drop by drop to avoid crystallization. Cells were frozen gradually by keeping them at -20˚C for 1 h, -80˚C for overnight and -196˚C for long term, respectively.

In order to culture frozen cells, they have to be thawn. Before thawing the cells, thawing medium (DMEM-HG, 1% penicillin/streptomycin) and complete DMEM, including increased FBS to 20%, 1% L-glutamine, 1% sodium pyruvate, 1% penisillin/streptomycin, were placed in water bath for warming. 9 ml prewarmed thawing medium was transferred to 15 ml centrifuge tube. A cryovial tube was taken out from liquid nitrogen tank then immediately placed and shaked in 37˚C water bath for maximum 2 minutes. Melted cells in cryovial tube was transferred to 15 ml centrifuge tube and centrifuged at 1800 rpm, for 5 minutes, at room temperature. Supernatant was removed and cell pellet was resuspended in 1 ml thawing medium. To completely remove DMSO from cells, 5 ml thawing medium was added in tube and centrifuged again at the same conditions. After second washing, cells were seeded into $25cm^2$ flask in 5 ml complete DMEM including 20% FBS. Cells were incubated at 37˚C, 5% CO_2. The day after, medium was refreshed to remove cell debris and dead cells.

3.1.2. Subculture of SH-SY5Y Cells

Cells were subcultured when the cell confluency reaches around %70-80. Before subculture, PBS (Phosphate Buffered Saline, Capricorn), 0.25% Trypsin/EDTA (Gibco; cat. no: 25200056) solution, washing medium (DMEM-HG, 10 %FBS, 1% penisillin/streptomycin) and complete medium including Dulbecco's Modified Eagle's Medium (DMEM-HG, Capricorn), 15% FBS (Fetal Bovine Serum, Capricorn), 1% L-glutamine (Capricorn), 1% sodium pyruvate (Capricorn), 1% pen/strep (Gibco) were warmed in 37˚C water bath. Old medium was discarded from the 25 cm^2 flask and cells were washed with 3 ml prewarmed PBS without calcium and magnesium. Cells were trypsinized with prewarmed 1 ml 0.25% Trypsin/EDTA solution for 2 minutes at 37˚C and they were checked under the Inverted Fluorescent Microscope (Zeiss), when the cells detach from the flask surface, trypsinization was stopped with washing medium in an amount of 3 times trypsin. Cells were transferred to in a 15 ml centrifuge tube with washing medium and centrifuged at 1700 rpm, at room temperature for 5 minutes. Supernatant was removed and cell pellet was resuspended in 1 ml complete medium. Cells were diluted 1:1 dilution ratio with Trypan Blue (Sigma; cat. no: T8154) dye and cell viability was determined on thoma slide under microscope. Then cells were seeded at a density of $4x10^4$cells/cm^2 with prewarmed DMEM-HG including 15% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% pen/strep in a new culture flask. Cells were grown at 37˚C, 5% CO_2 in the incubator. Medium was refreshed every 2 days and cells were subcultured every 7 days.

3.2. Neural Differentiation of SH-SY5Y Cells

3.2.1. Coating of Cell Culture Plasticware with Fibronectin

Fibronectin is a cell attachment protein and helps cell adhesion. Commercially purchased natural human fibronectin powder (lyophilized) (Corning; cat no: 354008) was equilibrated to room temperature. 1 mg fibronectin powder was resuspended in 1 ml sterile distilled water and incubated for 30 minutes without spinning or agitating. Then it was aliquoted into tubes each contains 50 µl solution and stored at -20˚C for further use.

Before cell seeding, surface of the cell culture plasticware, such as 6 well plate, were coated with 5 µg/ml fibronectin diluted from stock with cold PBS to mediate neural differentiation. Each well was incubated with diluted solution for 1 hour at room

temperature under sterile hood. Fibronectin was removed and each well was washed with autoclaved distilled water for 1 time. The plate was leaved to air-dry for couple minutes under sterile hood.

3.2.2. Induction of Neural Differentiation with Retinoic Acid (RA) and Brain Derived Neurotrophic Factor (BDNF) in SH-SY5Y Cells

Reconstitution of Retinoic Acid (RA): Commercially available 50 mg RA (Sigma; cat no: R2625) was resuspended in 1.25 ml DMSO as a stock concentration 100 mM. The solution was aliquoted into 100 µl and stored at -80˚C. One tube from stock was taking out from -80˚C and the solution was diluted to 10 mM final concentration with DMSO. It was aliquoted into tubes for 50 µl. Tubes were stored at -20˚C sealed with aliminium foil.

Reconstitution of recombinant human BDNF protein: Commercially purchased 10 µg recombinant human BDNF protein (Abcam; cat no: ab206642) vial was spinned down. 1 ml PBS was added on protein powder and it was dissolved very well. (DO NOT VORTEX!) For complete dissolving, it was allowed for several minutes and the solution was transferred into 100 µl aliquots. They were stored at -20˚C.

Neural Differentiation: Cells were subcultered as described above and seeded at a density of $1.25x10^4$cells/cm^2 into coated wells in complete DMEM and incubated at 37˚C, 5% CO_2. The day after, on day 0, medium was refreshed with Ham's F-12 including 15% FBS, 1% penicillin/streptomycin and RA as working concentration 10 µM. Every 48 hours, medium was refreshed with the same medium. Due to RA is sensitive to light, I worked on dark for RA incubation period. On day 4, RA and FBS were removed from medium and Ham's F-12 medium containing 1% penicillin/streptomycin, 50ng/ml recombinant human BDNF protein was replaced by previous medium. Cells were incubated with this medium for another 6 days by refreshing medium every 2 days. On day 10, neural differentiation process was completed (Figure 7).

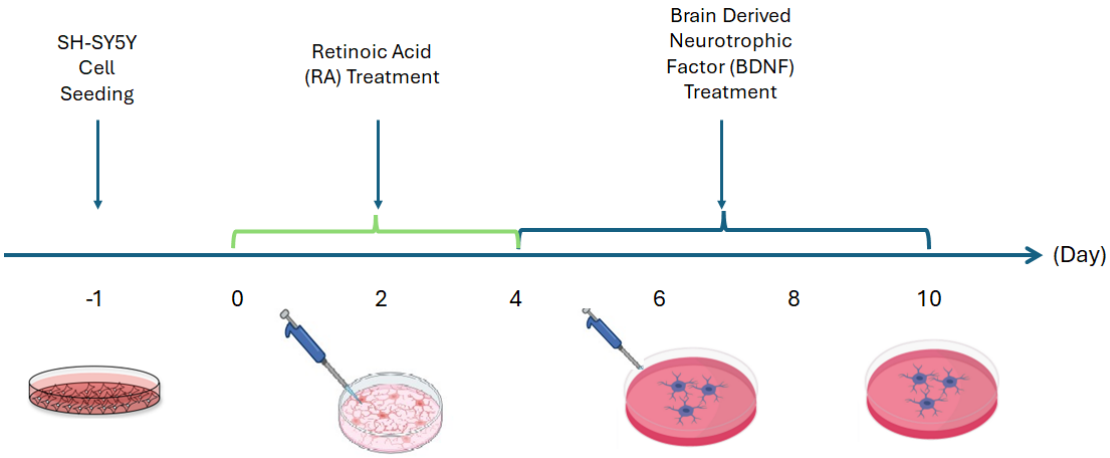

Figure 7: Neural Differentiation Timeplan.

3.3. Formation of Transgenic SH-SY5Y Cell Line Stable Overexpressed Topo IIß

3.3.1. Designing of Plasmid

The vector plasmid TOP2B_GFP_pHIV-EGFP was designed by my co-advisor Assistant Professor Cihan TAŞTAN and synthesized by GenScript and human topo IIß gene sequence was inserted into empty backbone plasmid which is DNA pHIV-EGFP **(**Addgene plasmid # 21373) (Figure 8). It is self-inactivating lentiviral plasmid for co-expression of gene of interest and EGFP. The cloning site was selected as XbaI/BamHI and the plasmid length is 4884bp.

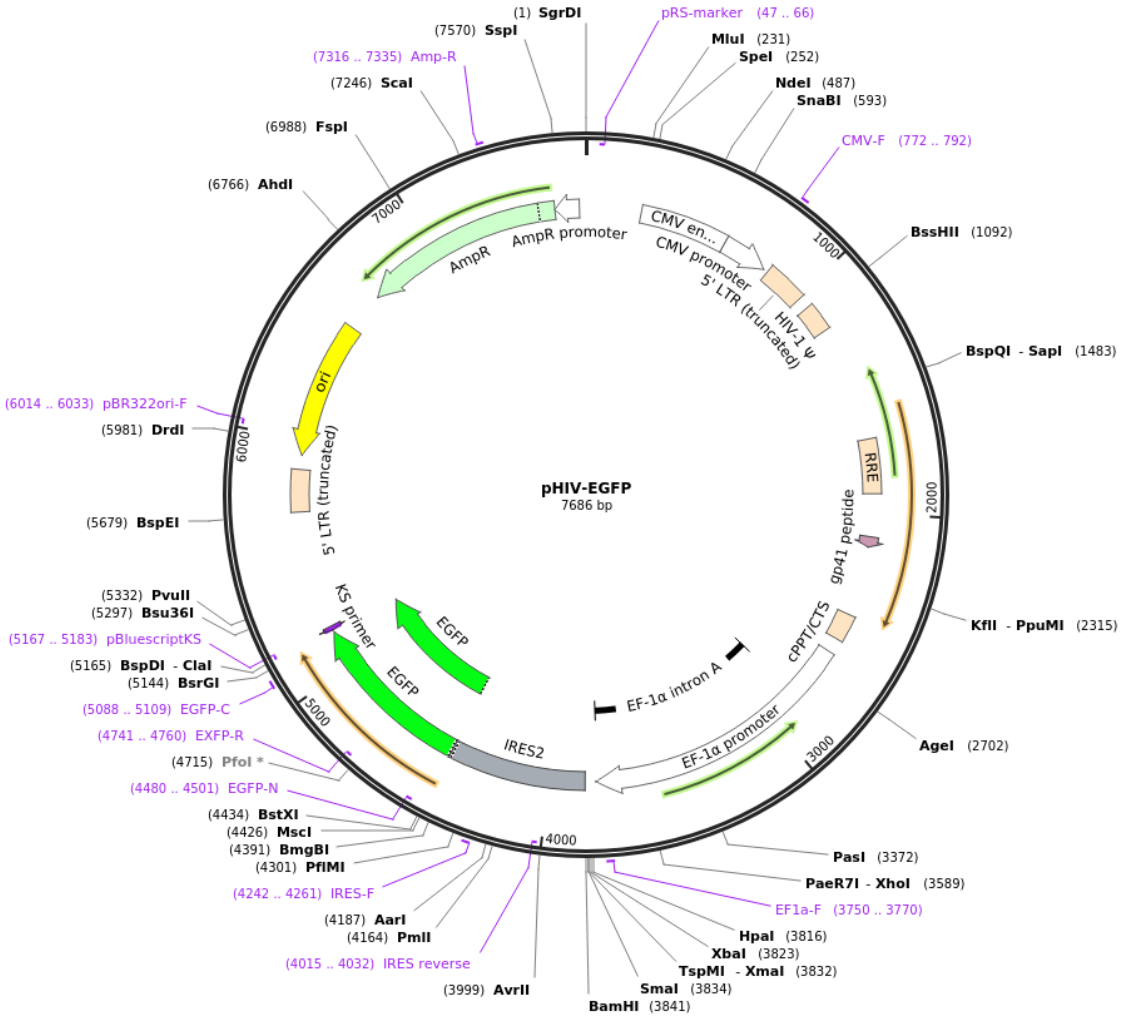

Figure 8: Empty backbone plasmid (Addgene plasmid # 21373).

3.3.2. Transformation

Firstly, lyophilized 4µg TOP2B encoding plasmid was dissolved in 100µl sterile water by pipetting well and vortexing it. Then, 10µl plasmid was added to 50µl competent E. coli DH5α strain (NEB C2987H) and incubated on ice for 30 mins then it was heat shocked at 42˚C for 30 secs to allow integration. The competent cell/DNA mixture was added into 400 µl LB (without antibiotic) (Table 1) at room temperature in a 1.5 ml microcentrifuge tube and incubated at 37˚C, 150rpm for 1 hour in a shaking incubator. Then the mixture was added drop by drop on LB (+Amp) agar (Table 2) plate and spreaded. Plate was incubated at 37˚C for 16 h.

This transformation step was also applied lentivirus packacing plasmids (VSVG and psPAX2).

Table 1: Preperation LB Broth

	Amount	Final Concentration
LB Medium	20 g	20 g/L
dH_2O	Up to 1 L	
Total	1L	

Table 2: Preperation LB Agar

	Amount	Final Concentration
LB Agar Medium	35 g	35 g/L
dH_2O	Up to 1 L	
Total	1L	

3.3.3 Plasmid Isolation

Plasmid isolation was performed from selected bacterial colonies with the ZymoPure Plasmid Maxi Kit (#D4203, Zymo Research). DNA concentration was measured with microplate ELISA Reader (FLUOstar Omega) and the purity was evaluated whether it was between 1.8<A260/A280<2.0.

3.3.4 Lentivirus Production

For lentivirus production, isolated envelope, packaging and topo IIß plasmids were co-transfected to HEK-293 cells with the help of 100 mg transfection reagent (PolyScience 23966-100 Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI25K™)) in DMEM HG medium with L-Glutamine (10% FBS and 1% penicillin/streptomycin, Gibco). Packaged recombinant lentiviruses were harvested from the supernatant of HEK293T cell cultures at 72 h post-transfection. The produced topo IIß lentiviruses were concentrated with Lenti-X Concentrator (Takara Bio, 631232) to increase the virus concentration (20X-100X). Viruses were stored at -80°C.

3.3.5 Transduction of SH-SY5Y cells with lentiviruses expressing topo IIß

The Jurkat cell line kept in culture was prepared to 10,000 cells per well in 100 µl complete medium (RPMI containing 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine and 1% vitamin). Jurkat cells in 100 µl medium were seeded in a 96-well plates from A to H wells. The wells were adjusted to have 10

µl, 3 µl, 1 µl, 0.3 µl, 0.1 µl and 0.03 µl of 100 X concentrated topo IIß lentivirus solution each in 50 µl. 50 µl of virus solution of each concentration was transferred to a 10,000 Jurkat/100µl well, the total volume was 150 µl and the cells was incubated for 3-4 days. Analysis for the topo IIß expression marker EGFRt expression with anti-EGFR-A488 antibody (R&D Systems, FAB10951G) was performed using Cytoflex Flow Cytometry (Beckman Coulter, B5-R3-V0) and MOI (Multiplicity of Infection) value was calculated. Based on this value, same concentration of virus was transducted to SH-SY5Y cells for both topo IIß and GFP (control).

3.4. Aß$_{1-42}$ Fibrils Formation and Treatment of Cells with Aß$_{1-42}$

Commercially available lyophilized 1 mg ß-Amyloid 1-42 peptide (human) (Abcam; cat no: ab120301) was initially dissolved directly in 80 µl 1% NH_4OH. Due to impossibility of long-term storage of Aß$_{1-42}$ peptide in 1% NH_4OH, the peptide solution was immediately diluted to a concentration of 1 mg/ml with 1X PBS without Ca^{++} and Mg^{++}. It was vortexed gently (less than 1 minute) to mix. The solution (221,5 µM) was transferred into 100 µl aliquots and stored at -80°C.

In order to form Aß fibrils, pre-incubation is necessary. Soluble peptide suspensions corresponding to different concentrations were prepared in culture medium and incubated separately for 48 h at 37°C with gentle shaking. Neural differentiated SH-SY5Y and control cells were treated with 48 h pre-incubated Aß$_{1-42}$ fibrils for 48 h.

3.5 Establishment of *In Vitro* Alzheimer's Disease Model

3.5.1 MTT Assay

To create an Alzheimer's model, Aß1-42 solution was prepared at different concentrations and administered SH-SY5Y cells The effects of amyloid beta fibrils at different concentrations on the survival rates of neurons was observed during culture with MTT assay.

Cells were seeded at a density of 1.25x10^4 cells/well in a 96 well plate. The day after, neural differentiation was begin and in the 10th day of neural differentiation cells were exposed to different concentrations (5 µM, 7 µM,10 µM and 15 µM) of 48 h pre-

incubated Aß$_{1-42}$ peptide. Cells were incubated with Aß$_{1-42}$ peptide for 48h. Later, 110 µl MTT (ROCHE, cat no:11465007001) solution/well (Table 3) was given to cells. 4 h later, 100 µl solubilization buffer was added on MTT solution and incubated for overnight (16-18 h) at 37°C. The absorbance value was measured at 570 nm by microplate reader (BMG LABTECH, FlouOstar Omega).

Table 3: Preperation MTT Solution

	Amount	Final Concentration
MTT Reagent	100 µl	10 %
DMEM	1000 µl	
Total	1100 µl	

3.5.2 Thioflavin S Staining

Cells were seeded at a density of 4.5x10^4 cells/ well in a 48-well plate. At Day 8 of neuronal differentiation, 48 h pre-incubated Aß$_{1-42}$ peptides were given to cells. After 48 h Aß incubation cells were treated with 4% PFA/PBS solution (200 µl/well) to fix the cells for 10 mins on rocking shaker and wash the cells with 500 µl PBS for 5 mins. Then, 200 µl 0.005% ThS (Sigma, T1892) solution, diluted from 0.025% (filtrated with 0,45 µm filter) (Table 4), was added and cells were incubated for 10 mins. ThS is sensitive to light so worked in dark by then. When ThS incubation was over, wash the cells with 70% EtOH for 5 mins for 3 times and incubate the cells with 1:5000 Dapi for 5 mins for nuclei staining. Sabsequently wash the cells with 1X PBS for 1 times and dH_2O for 1 time, respectively. Immediately observe under the microscope.

Table 4: Preperation of ThS Solution

	Amount	Final Concentration
Thioflavin S	2,5 mg	0.025 %
EtOH/dH_2O (1:1)	10 ml	
Total	10 ml	

3.6 Analysis of Association between Topo IIß and AD

3.6.1 Western Blotting

3.6.1.1 Protein Isolation:

To isolate the protein, it was worked on ice during the experiment. Firstly, the old medium was removed from 6 well plate and each well were washed with 1.5 ml cold PBS. Subsequently, 80µL of RIPA buffer (Nacalai, 16488-34) consist of 1% SDS (Applichem, A1112,0500) (Table 5) and 1% protease inhibitor cocktail (PI, Applichem, A7779,0001) (Table 6) was added to lyse cells. Immediately, the scraper was used to thoroughly scrape the wells, and the collected cells were then put into a 1.5 ml centrifuge tube. The samples were homogenized using the MagNA Lyser (Roche) at a speed of 6000 rpm for 15 secs, repeated twice. They were then boiled at a temperature of 95ºC for 2 minutes and the protein samples were kept at -20 ºC to be used in the future.

Table 5: Preperation of 10% SDS Solution

	Amount	Final Concentration
SDS	1 mg	10 %
dH_2O	10 ml	
Total	10 ml	

Table 6: Preperation of RIPA Buffer Solution

	Amount	Final Concentration
10 % SDS	10 µl	1 %
PI	1 µl	1 %
RIPA	89 µl	
Total	10 ml	

3.6.1.2 Detection of Protein Concentration with BCA Assay:

The quantities of each proteins were determined using the Rapid Gold BCA Protein Assay Kit (Thermo Fisher, A53225). The BSA standards were diluted using a 1:5 ratio of RIPA-dH2O diluent solution, as specified in Table 7. This ratio was determined based on optimisation. Due to neural differentiation, protein concentration was very low so 1:5 ratio was enough to determine the protein concentrations. In order to achieve a dilution ratio of 1:5, the protein samples were diluted by adding 2 µL of protein to 8µL of dH2O. 10µL of each standard and sample were put to a 96-well plate. Subsequently working reagent was prepared. It had two components which were A and B. The quantity of the A component was determined using the formula: (#standards + #sample) x (# replication) x (0.2 ml). The amount of component B was estimated based on the ratio with reagent A, which is 1:50. Therefore, the volume of component A divided by 50 gives the volume of component B. Component A was light sensitive so working reagent was prepared in dark. Both the A and B components were combined in a 15 ml centrifuge tube and mixed vigorously for a short period of time. Subsequently, 200µL of the prepared working reagent was added onto each standard and sample well. Following a 5 minute incubation period at room temperature, the concentration was determined by measuring and comparing the absorbance of both standards and the sample using a microplate reader set to a wavelength of 480nm. The resulting sample concentration was multiplied by a factor of 5 due to the sample being diluted in a ratio of 1:5.

Table 7: Preperation of BSA Standards

Standard	Diluent volume (µL)	BSA volume (µL) and its source	Final BSA concentration (µg/mL)
A	0	300 (stock)	2000
B	62.5	187.5 (stock)	1500
C	100	100 (stock)	1000
D	100	100 (from B)	750
E	100	100 (from C)	500
F	100	100 (from E)	250
G	100	100 (from F)	125
H	160	40 (from G)	25
I	200	0	0

3.6.1.3 SDS PAGE

Gel Preparation:

Two different acrylamide gel percentage (6% and 20%) was prepared (Table 8, Table 9) to load the proteins. 6% gel was used to detect high molecular weight proteins and 20% was for low molecular weight proteins especially $A\beta_{1-42}$ peptide (4 kDa). All of the components of seperating gel were added in 15 ml tube except TEMED and APS. They were added lastly and seperating gels was poured immediately in Mini-PROTEAN Tetra Cell Casting Module (Biorad) then, it was filled with propanol to prevent formation of any air bubble and make the gels flat. The gels were left at room temperature to solidify for 20-30 mins. When solidification was completed, proponal was removed and gels were washed with dH_2O until the smells of propanol was gone. Stacking gels were prepared and poured onto separating gels and 10-lanes comb were placed into the stacking gels immediately. They were left for solidification as well as separating gels. Then gels may be used when they are ready, or they may be stored at 4ºC for further use covered with wet tissue in a wet box.

Table 8: Components of 6% Acrylamide Gel

	Seperating Gel	**Stacking Gel**
dH_2O	3.25 ml	2.212 ml
1M Tris HCl	3.75 ml (pH 8.8)	0.38 ml (pH 6.8)
40% acrylamide	1.5 ml	0.225 ml
2% APS	0.5 ml	0.150 ml
1% SDS	1 ml	30 µl
TEMED	10 µl	4 µl
Total Volume	10 ml	3 ml

Table 9: Contents of 20% Acrylamide Gel

	Seperating Gel	**Stacking Gel**
dH_2O	-	2.212 ml
1M Tris HCl	3.75 ml (pH 8.8)	0.38 ml (pH 6.8)
40% acrylamide	5 ml	0.225 ml
2% APS	0.5 ml	0.150 ml
1% SDS	1 ml	30 µl
TEMED	10 µl	4 µl
Total Volume	10 ml	3 ml

Sample Preparation:

Subsequently quantification of protein concentration, samples were prepared according to their concentrations. Same concentration of protein (11 µg) was added in a 0.2 ml centrifuge tube then, 6 µl Laemmli buffer (Biorad, 1610747) (Table 10) was added onto each sample and the volume was completed with dH_2O up to 24 µl which was maximum volume of one lane. When the samples were ready, they were loaded in gels and protein ladder was loaded lastly.

Table 10: Preperation of Laemmli Buffer for 1 lane

	Amount	Final Concentration
10 % SDS	5,4 µl	
PI	0,6 µl	10 %
Total	6 µl	

Table 11: Preperation of 10 X SDS PAGE Running Buffer

	Amount
Glycine	36 g
SDS	2.5 g
Tris	7.575 g
dH_2O	Up to 250 ml
Total	250 ml

After that samples were run in 1X running buffer (Table 11) in SDS PAGE gel running system (Figure 9) at constant voltage, 50 V, for 30 min, then the voltage increased to 150 V until running was completed.

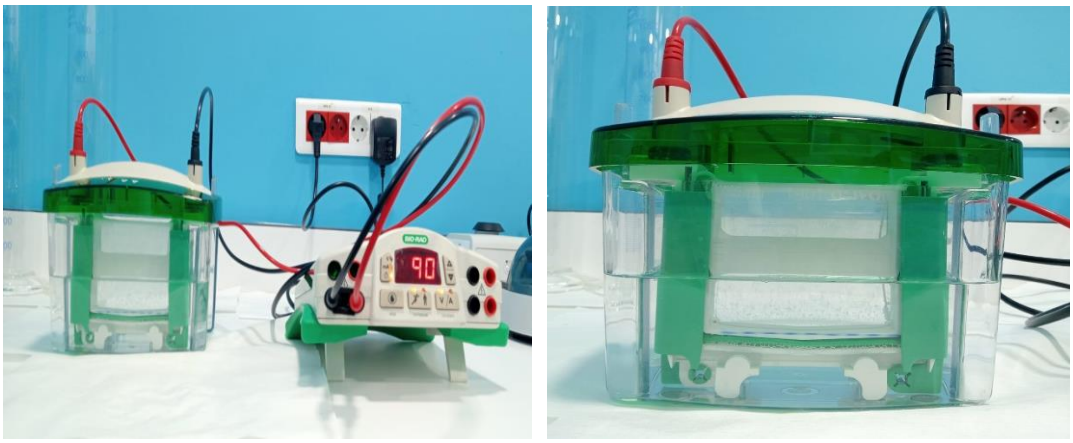

Figure 9: SDS Page Gel Running System

Blotting:

The Trans-Blot Turbo RTA Mini PVDF kit (Biorad, 1704272) was employed to facilitate the transfer of proteins that were in the the gel to the PVDF membrane. Following the kit instruction, two stacks of ion reservoirs were dampened with transfer buffer (Table 12). The PVDF membrane was rendered transparent by being immersed in absolute ethanol for a duration of 30-45 seconds. Subsequently, it was transferred to the transfer buffer. The sandwich was prepared in a Trans-Blot Turbo Transfer System cassette (Biorad) . The sandwich was prepared in the middle of the cassette. Firstly one stack was placed and the membrane was positioned on the stack. Bubbles were removed with the help of roller. On the membrane the gel was placed and another stack was put on the gel. The blotting process was conducted using a continuous current of 1A, while the voltage was restricted to a maximum of 25V. The duration of the blotting was 70 minutes.

Table 12: Preperation of 1X Transfer Buffer

	Amount	Final Concentration
5X transfer buffer	50 ml	1 X
EtOH	50 ml	
dH_2O	150 ml	
Total	250 ml	

After completing the blotting process, the membrane was moved to a blocking solution (Table 14) and placed on a rotary shaker (20 rpm) for 1 hour at room temperature. In order to assess the effectiveness of the blotting process, the gel was immersed in a Coomassie Blue solution (as specified in Table 15) for a duration of 45 minutes. Subsequently, the gel was transferred to a destaining buffer (as specified in Table 16) and left to incubate overnight on an orbital shaker. After completing the blocking process, the membrane was precisely sliced based on the specific position of the protein of interest. The membranes were immersed in primary antibody solutions for topo IIβ (BD, 611492), p-tau (cell signalling) and GAPDH and placed on a rotary shaker at a speed of 12 rpm at a temperature of 4ºC for the duration of one night. The antibody solution was prepared as specified in Table 17.The next day, the incubation was finished for a further 2 hours, at the room temperature. Subsequently, the membranes underwent a triple wash with TTBS (Table 19), lasting 5 minutes each. Subsequently, the membranes were placed in a solution containing secondary antibodies and incubated in darkness on a rotary shaker (12 rpm) for 1 hour. Later, the membranes underwent a triple wash with TTBS, lasting 5 minutes each. The membranes were rinsed to remove excess TTBS and then immersed in Western Bright ECL-HRP substrate (Advansta, ADV-K-12045-C20) for a duration of 1-2 minutes. Ultimately, the ECL was removed, and the membranes were seen using the C-Digit Blot Scanner (LI-COR) apparatus.

Table 13: Preperation of New TBS

	Amount
1M Tris-HCl (pH 7.5)	10 ml
5M NaCl	30 ml
dH_2O	960 ml
Total	1000 ml

Table 14: Preperation of Blocking Solution

	Amount	Final Concentration
Skim milk	1.250 g	5 %
TWEEN-20	25 µl	0.1 %
New TBS	25 ml	
Total	25 ml	

Table 15: Preperation of Coomassie Blue

	Amount	Final Concentration
Coomassie blue	0.5 g	1.1 %
MeOH	200 ml	40 %
AcOH	5 ml	1 %
dH_2O	295 ml	
Total	500 ml	

Table 16: Preperation Destaining Solution

	Amount	Final Concentration
MeOH	200 ml	40 %
AcOH	5 ml	1 %
dH_2O	295 ml	
Total	500 ml	

Table 17: Preperation of Antibody Solution

	Amount	Final Concentration
Skim milk	1.5 g	5 %
TWEEN-20	300 µl	1 %
New TBS	30 ml	
Total	30 ml	

Table 18: Preperation of 10 X TBS

	Amount	Final Concentration
1M Tris-HCl (pH 7.5)	200 ml	200 mM
NaCl	292.2 g	5 M
dH_2O	Up to 1000 ml	
Total	1000 ml	

Table 19: Preperation of TTBS

	Amount	Final Concentration
10 X TBS	100 ml	1 X
TWEEN-20	0.5 ml	% 0.05
dH_2O	900	
Total	1000 ml	

3.6.2 Immunoflorescence Staining

The old medium of SH-SY5Y cells was removed from the 24-well plate. Then, the cells were incubated with 4% PFA/PBS for 10 minutes at room temperature with shaking to fix the cells. After removing 4% PFA/PBS, it was washed three times with 1X PBS for 5 minutes each. Then, cells were incubated with TZN buffer to permeabilize cell membrane for nuclear proteins. Subsequently, cells were blocked with 5% NGS (Normal Goat Serum, Cell Signaling, 5425) and 5% NHS (Normal Horse Serum, Sigma-Aldrich, H0146-10ML) in 0.3% PBS/Triton X (PBS-Tx) for 30 min at room temperature. Blocked cells were incubated for overnight with diluted primary antibodies, specific to the targeted protein, in 0.3% PBS-Tx solution containing 3% normal horse serum at 4°C. The plate was covered with parafilm during incubation.

Next day, the wells were washed with 0.3% PBS-Tx 3 times of 5 minutes each with shaking. Cells were then treated with Alexa Fluor-labeled secondary antibodies with a host appropriate to the primary antibody for 1 hour at room temperature. The plates were sealed with parafilm and covered with aluminum foil because secondary antibodies are sensitive to light. After incubation with the secondary antibody, the cells were washed with 1X PBS three times for 5 min on rocking shaker. Then, the cells were incubated with 1X PBS DAPI (NEOFROXX, 1322MG005) at a ratio of 1:5000 for 10 minutes at room temperature. DAPI was diluted on ice and in the dark. Washing steps were performed again with 1X PBS and distilled water, respectively, with shaking three times for 5 min each. Cells were covered with mounting medium (Prolong Gold Antifade Kit, Invitrogen) and coverslips. Cells were observed under a fluorescence microscope (Zeiss, Axio Observer 3) and photographed. Topo IIß (1:100, BD), $Aß_{1-42}$ (1:500, Abcam), p-tau (1:100, Cell Signaling), NFL (1:100,Cell Signaling), (as the primary antibody.

Secondary antibody: Anti-rabbit IgG Alexa Fluor Conjugate 594 Conjugate (1:500, Cell Signaling, 4412).

Table 20: Experimental Design

	Differentiation	Topo IIß (+)	Aß (+)	Topo IIß (+) + Aß (+)
Day -2	Cell Seeding	Cell Seeding	Cell Seeding	Cell Seeding
Day -1		Topo IIß Overexpression	Topo IIß Overexpression	Topo IIß Overexpression
Day 0	RA Treatment	RA Treatment	RA Treatment	RA Treatment
Day 1				
Day 2	RA	RA	RA	RA
Day 3				
Day 4	BDNF Treatment	BDNF Treatment	BDNF Treatment	BDNF Treatment
Day 5				
Day 6	BDNF	BDNF	BDNF	BDNF
Day 7				
Day 8	BDNF	BDNF	BDNF+Aß	BDNF+Aß
Day 9				
Day 10	Protein Isolation, IF, ThS Staining, MTT	Protein Isolation, IF, ThS Staining, MTT	Protein Isolation, IF, ThS Staining, MTT	Protein Isolation, IF, ThS Staining, MTT

4. FINDINGS

4.1. SH-SY5Y Cell Culture

SH-SY5Y cells were followed and optimized in culture conditions. Two types of cell morphology were observed which were epithelial-like and neuroblastic cells with multiple, short neurites. They tended to making clusters and cultures reach high density by forming many layers consisting of numerous clusters of cells.

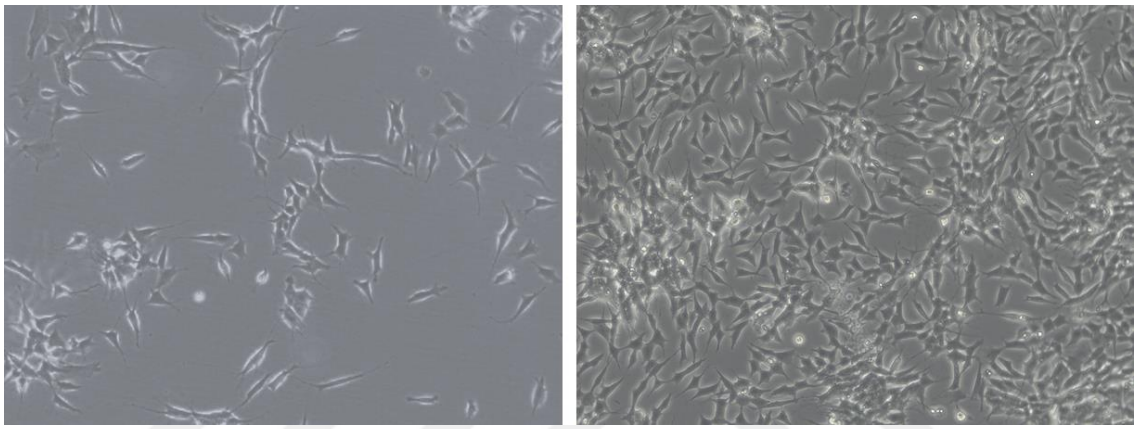

Figure 10: Morphological images of SH-SY5Y cells from low density (left), high density (right).

Cell morphology images in culture were shown in Figure 10 and they are parallel to the cell morphology results in ATCC. Optimum ulture conditions was optimized according to cell growth and it was decided that cells were subcultered once in a week and refreshed culture medium every 2 days. Cell density was decided as $4x10^5/cm^2$. It was decided DMEM medium consisting of 15% FBS, 1% L-glutamine, 1% sodium pyruvate and 1 % penicillin/streptomycin.

4.2. Neural Differentiation of SH-SY5Y Cells

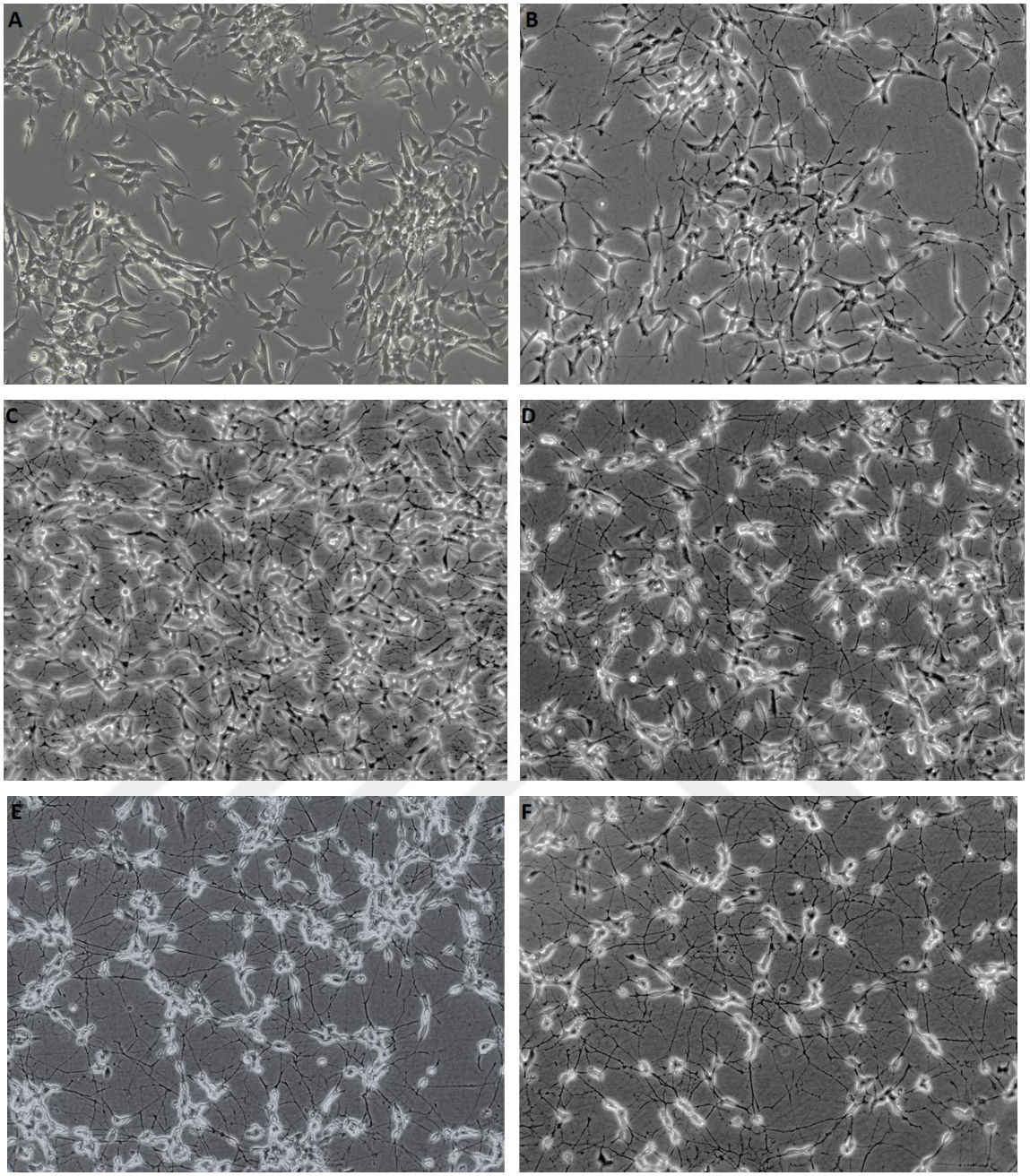

Figure 11: Neural Differentiation Images of SH-SY5Y Induced by RA and BDNF. A. 24h after from seeding. B. 2nd day of differentiation, before RA refreshment C. 4th day, before first BDNF treatment. D. 6th day. E. 8th day, before last BDNF refreshment. F. 10th day (the last day) of neural differentiation.

From the second day of differentiation, short neurites were started to be seen. SH-SY5Y cells were expanded during RA treatment and expansion stopped by serum free BDNF treatment. Cell nuclei was smaller and almost round as the neural differentiation progressed. At the end of the neural differentiation, neural network became clear, and cells were interacted with each other (Figure 11). There were no more undifferentiated cells at the last day of neural differentiation.

In real time cell analysis (RTCA) experiment, cell index was measured every 10 mins for 11 days and it was shown that neural differentiated cells expanded in size during RA treatment. Cell number and cell surface increased by effect of RA and cell index was higher compared to control SH-SY5Y cells. At 4th day, cell surface became smaller and cell index started decrease with BDNF treatment compared to undifferentiated controls (Figure 12).

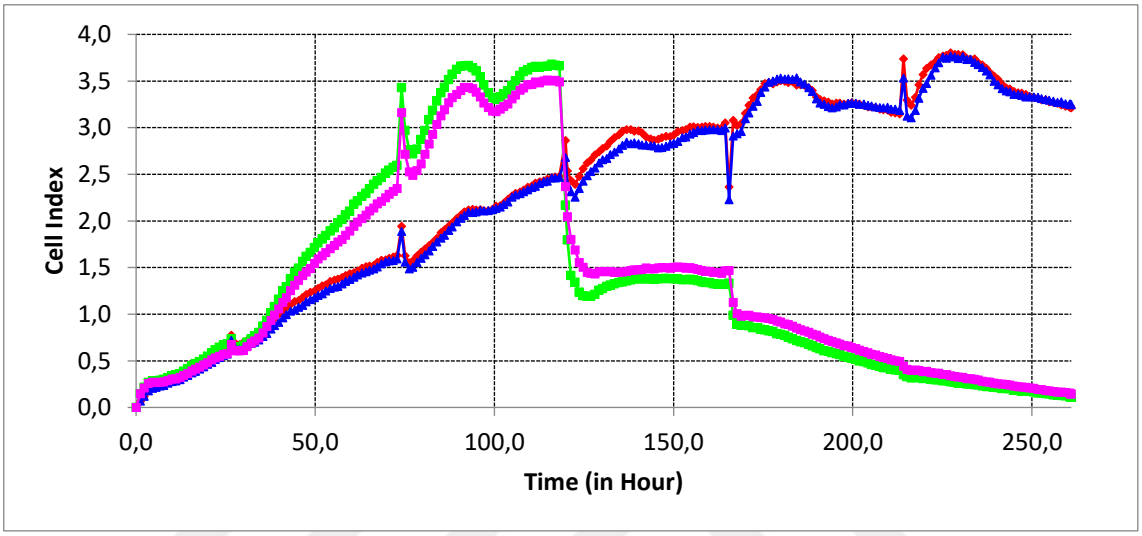

Figure 12: Growth curve of neural differentiated and control (undifferentiated) SH-SY5Y cels.

To examine the effect of topo IIß on neural differentiation, one of the experimental groups was exposed to topo IIß overexpression before starting neural differentiation and in the other group neural differentiation was started without topo IIß overexpression. It was observed that topo IIß has an effect on neural differentiation. In topo IIß overexpressed group, there were no undifferentiated cell while there were some undifferentiated cells in the control group shown by black arrow (Figure 13).

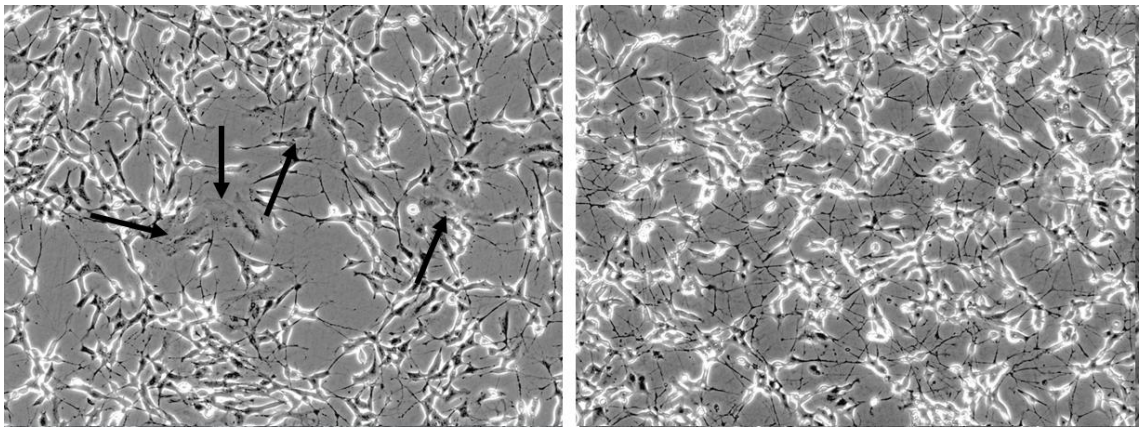

Figure 13: Neural differentiation images at day 10. Topo IIß was overexpressed before neural differentiation (right), control cells (left).

4.3. Formation of Transgenic SH-SY5Y Cell Line Overexpressed Topo IIß

4.3.1. Lentivirus Production

HEK-293 cells was co-transfected with topo IIß and Pspax 2, pVSV-G which are virus envelope and packaging proteins as well as GFP controls to check if transfection is succesful or not. Polyethylenimine (PEI) was used as the transfection reagent and transfection conditions was optimised as DMEM HG medium with L-Glutamine (10% FBS and 1% penicillin/streptomycin, Gibco) for 72 h. Both plasmid were transfected to HEK-293 cells succesfully. However, topo IIß expression efficiency was lower than GFP expression under microscope. Lentivirus production was verified since pHIV-EGFP fluoresence signal was detected by Inverted Fluorescent Microscope (Zeiss) (Figure 14).

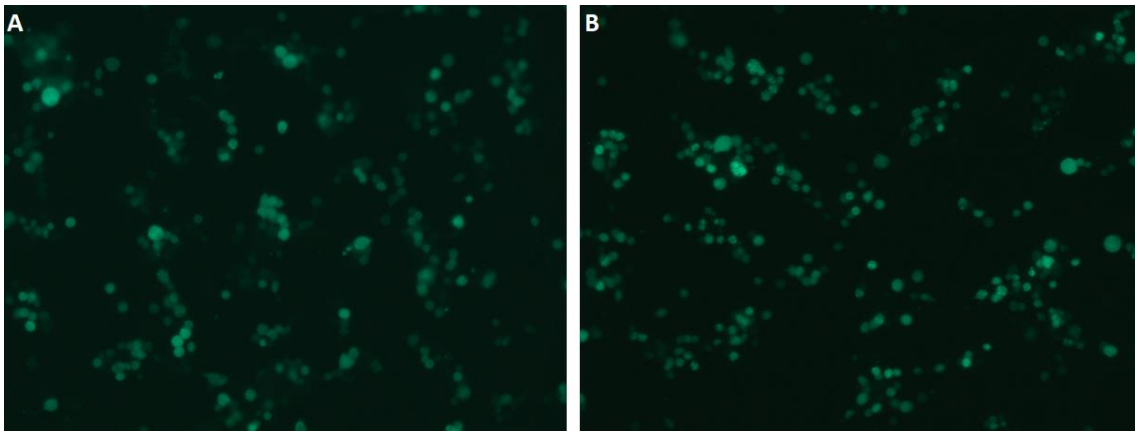

Figure 14: Transfection of **(A)** control GFP and **(B)** Topo IIß lentivirus in HEK 293 cells.

4.3.2.Transduction of SH-SY5Y cells with lentiviruses expressing topo IIß

SH-SY5Y cells (p#3) were transducted with concentrated topo IIß lentiviruses. Different concentrations of lentiviruses, 1 MOI (Multiplicity of infection) and 2 MOI, were applied. The cells, treated with 2 MOI viruses, were died mostly. GFP signals were seen (Figure 15) in the cells at day 7 in the 1 MOI group under microscope and signals increased day by day. However, all of the cells were not have GFP signal and topo IIß expressed cell efficiency was not enough.

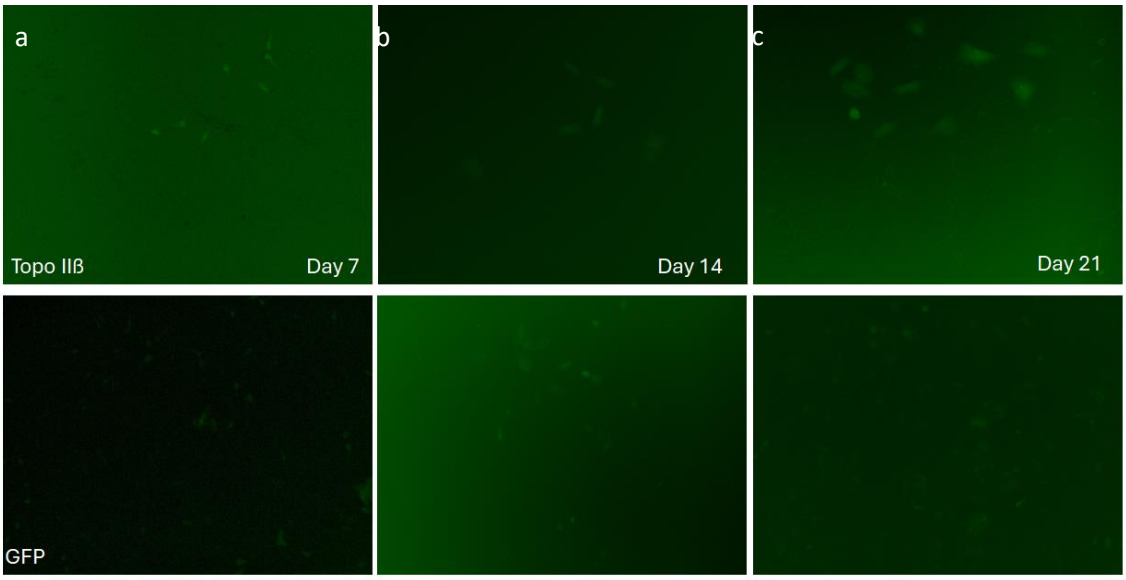

Figure 15: Fluorescent Images of Topo IIß and GFP Lentivirus Transduction at **(a)** 7th, **(b)** 14th and **(c)** 21st day in SH-SY5Y Cells.

Due to insufficient overexpression efficiency, we decided to sort negative and positive cells. Before sorting, there were 12% positive topo IIß overexpressed cells in culture according to flow cytometry results and cell viability was 76% (Figure 16). The percentage of GFP controls fluorescence was higher than topo IIß overexpressed cells, which was 48% and cell viability was %89. Flow cytometry results coincide with the microscope images.

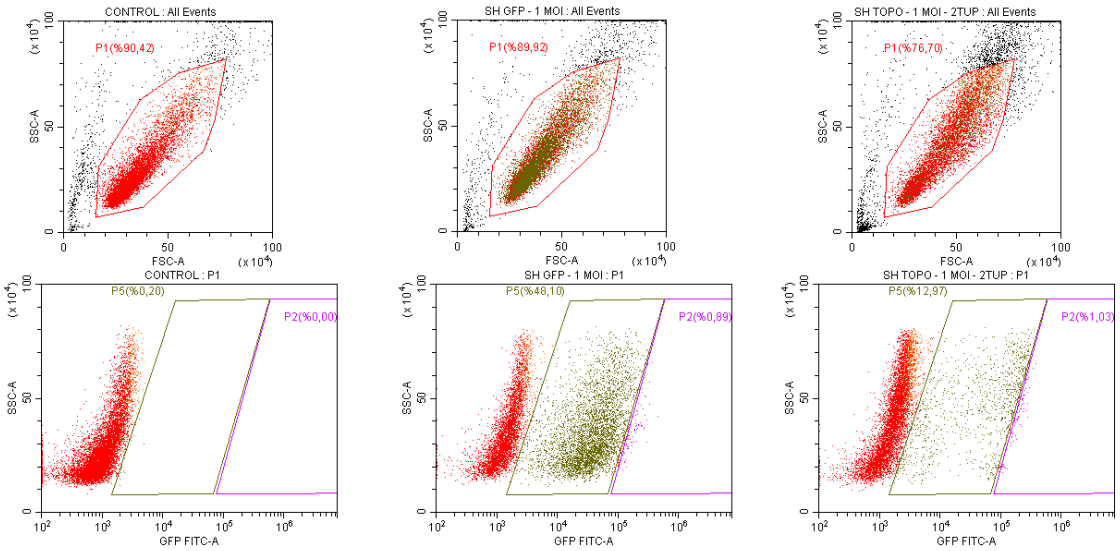

Figure 16: Topo IIß and GFP expression results with flow cytometry.

After sorting, we obtained only topo IIß overexpressed cells and started to grow the cells in culture. It was shown that GFP fluorescence remained until day 45 (Figure 17). With these results, we have obtained a transgenic SH-SY5Y cell line that permanently overexpresses the TOP2B gene. Grown cells were freezed to long term storage.

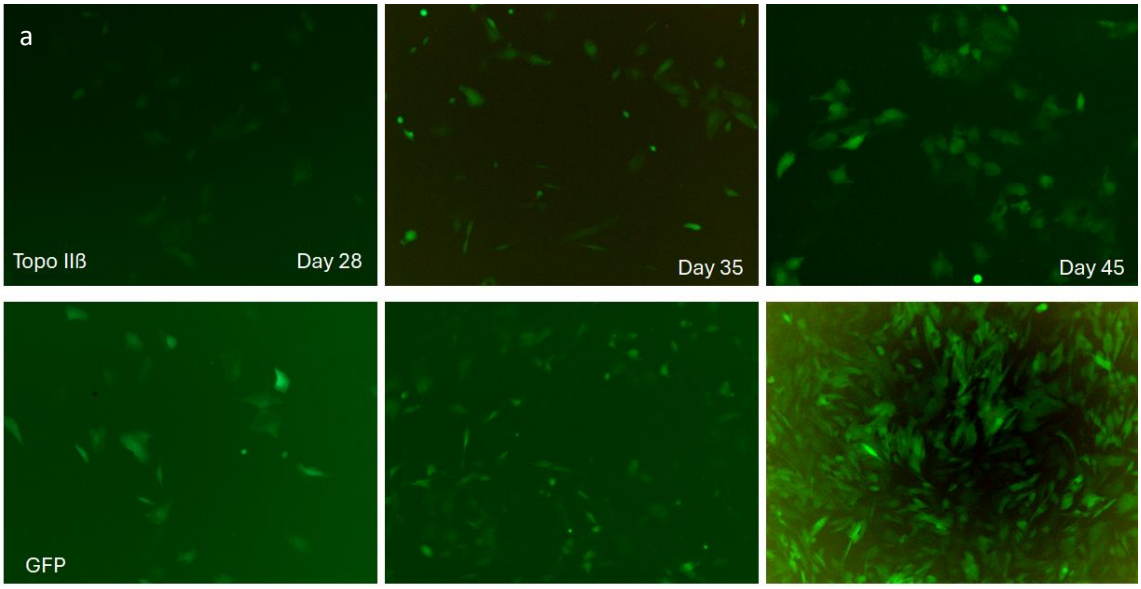

Figure 17: Fluorescent Images of transgenic SH-SY5Y Cells expressing Topo IIß and GFP stably after cell sorting at **(a)** 28th**, (b)** 35th and **(c)** 45th day.

4.4. Aß$_{1-42}$ Fibrils Formation

Aß$_{1-42}$ fibrils formation was checked by Thioflavin S staining. Cells were treated with 48 h pre-incubated Aß$_{1-42}$ peptides. After 48 h incubation with cells, ThS staining was performed. As a result it was shown that Aß$_{1-42}$ fibrils were formed succesfully. In the control group, cell nuclei were detected by DAPI staining and there was no fibrils observed with ThS staining while in the Aß$_{1-42}$ treated group there was ThS stain which stains formed Aß$_{1-42}$ fibrils (Figure 18). Aß$_{1-42}$ fibrils were like cloud and they sticked around cells.

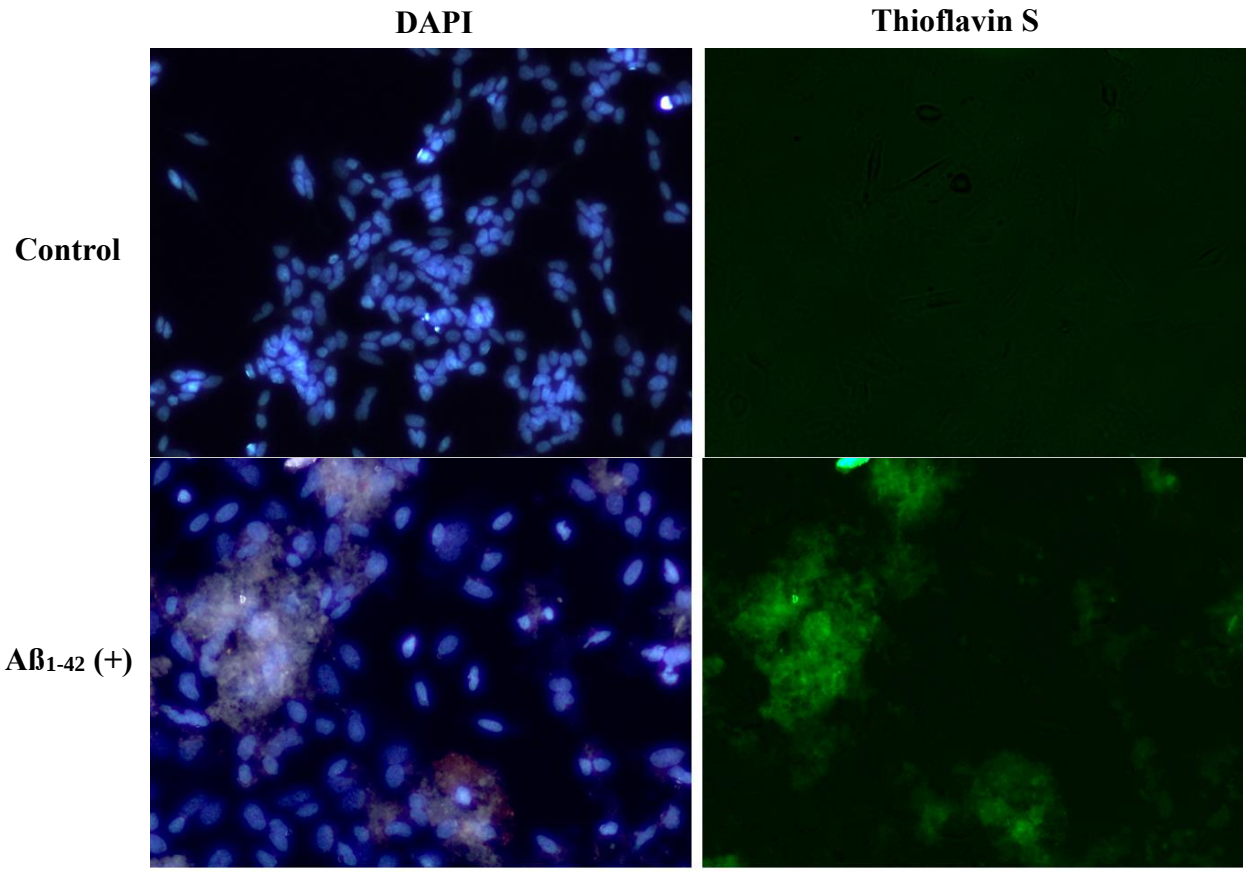

Figure 18: Thioflavin S and DAPI Staining of Aß$_{1-42}$ treated cells and their controls.

4.5. Establishment of *In Vitro* Alzheimer's Disease Model

To confirm the in vitro Alzheimer's Disease model, different concentrations (7 µM, 10 µM and 15 µM) of 48h pre-incubated $A\beta_{1-42}$ fibrils were applied to cells at the day 8 of neural differentiation and MTT analysis was performed to decide cell viability. Based on the MTT result (Figure 19) 15 µM $A\beta_{1-42}$ fibrils was very toxic to cells and it killed almost half of the cell in culture. On the other hand, 7 µM $A\beta_{1-42}$ concentration had almost no effect on cell viability and it was very close to viability of control (neurally differentiated) cells. We decided to continue with 10 µM $A\beta_{1-42}$ concentration because it had effect on cell viability and was not toxic as 15 µM $A\beta_{1-42}$ as.

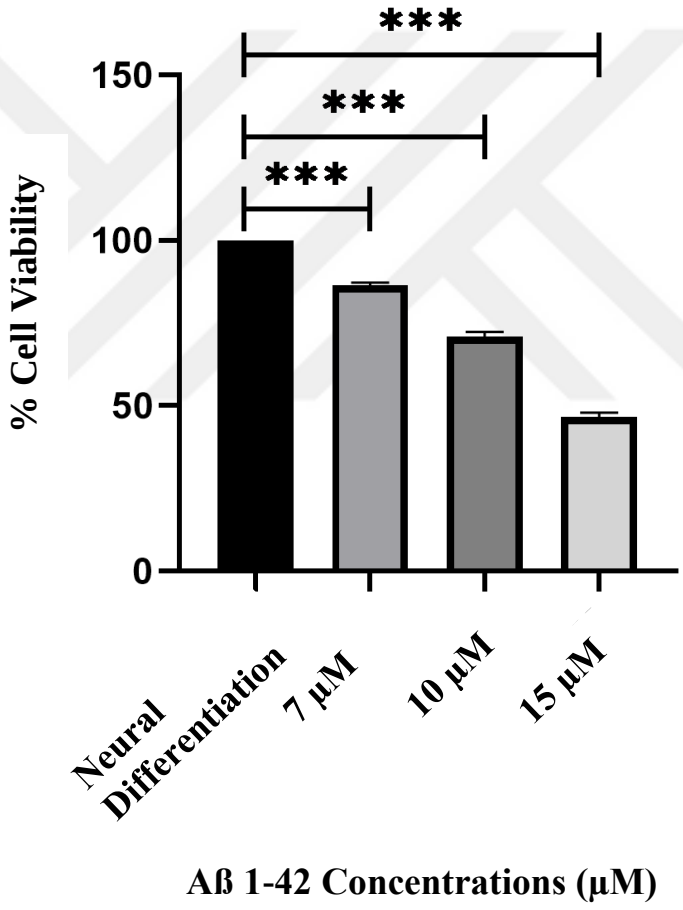

Figure 19: Cell viability after 48 h pre-incubated 7µM, 10µM and 15µM $A\beta_{1-42}$ incubation.

4.6. Analysis of Association between Topo IIß and AD

Immunoflourescence staining was performed to establish association between topo IIß and Alzheimer's Disease. As an neural marker NFL (neurofilament) was selected to show neural differentiation. According to Figure 20, NFL expression in neural differentiation group (B) was higher than controls (A). It means that neural differentiation was completed succesfully. Also it was shown that NFL expression has increased in topo IIß overexpreesed cells (C) while it has decreased in Aß treated cells (D). Compare to Aß treated group NFL expression was increased in topo IIß overxpressed & Aß treated group (E).

In Figure 21, it was observed that topo IIß expression was effected from Aß and significantly decreased in Aß treated group (D) and it was increased in topo IIß overexpressed & Aß treated group (E). The enhanced topo IIß expression in neural differentiation group (B) was observed compare to control group (A). Also, its expression was highest in overexpression (C).

Almost no p-tau expression was seen in control group (A) while it was highest in Aß treated group (D) (Figure 22). Neural differentiation (B) exhibited little expression of p-tau.

In addition, $Aß_{1-42}$ expression was only seen in two groups: Aß treated (D) and topo IIß overexpressed & Aß treated group (E) (Figure 23). It was observed that there was no significant change in $Aß_{1-42}$ expression in topo IIß overexpressed & Aß treated group (E) compare to Aß tretaed (D).

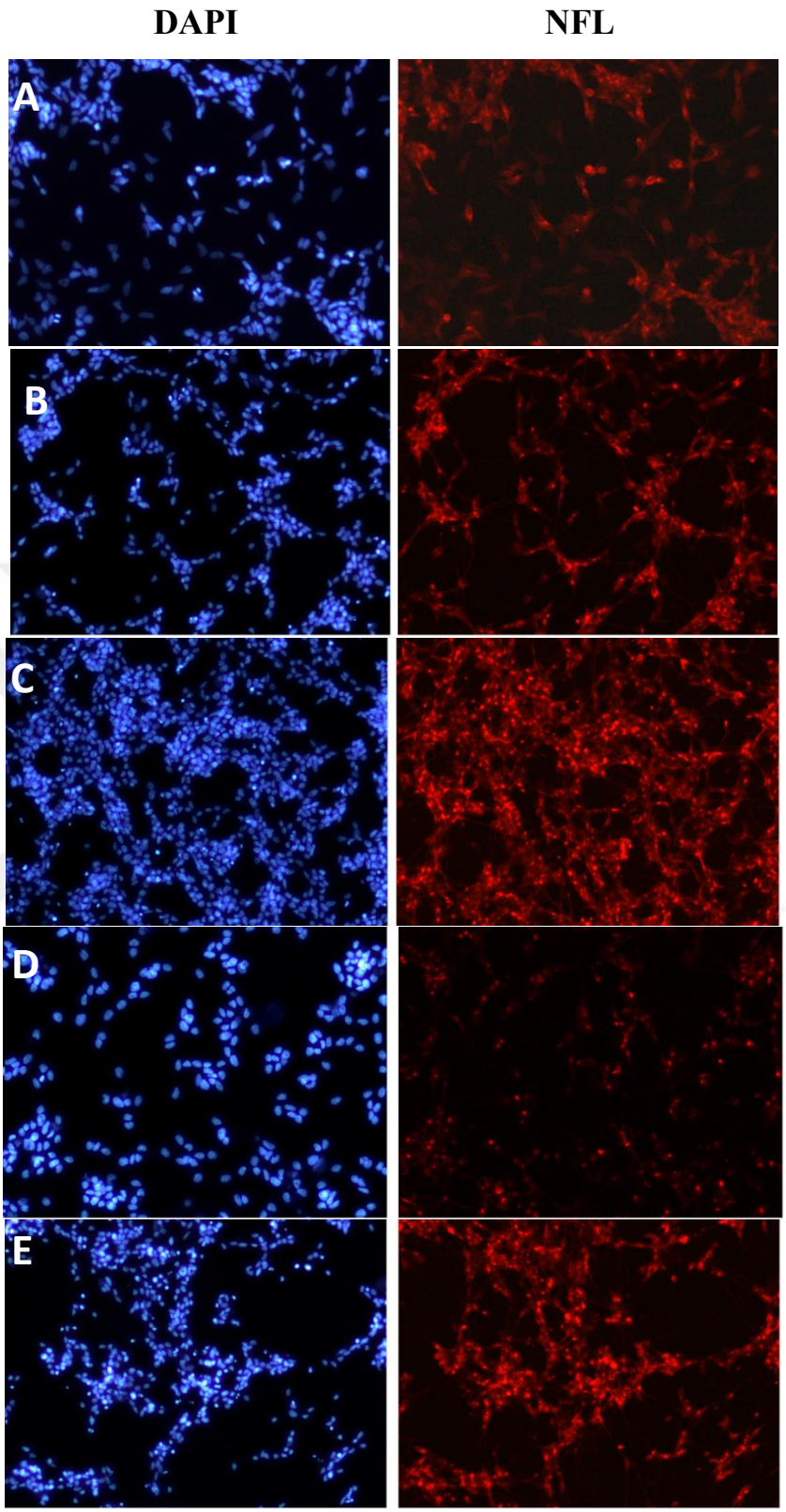

Figure 20: DAPI and NFL Immunoflorescence Images of **(A)** control, **(B)** Differentiation, **(C)** Topo IIß Overexpression, **(D)** Aß treated, **(E)** Topo IIß overexpressed & Aß treated. (All of the groups were neurally differentiated except A). Images were taken under 20X magnification.

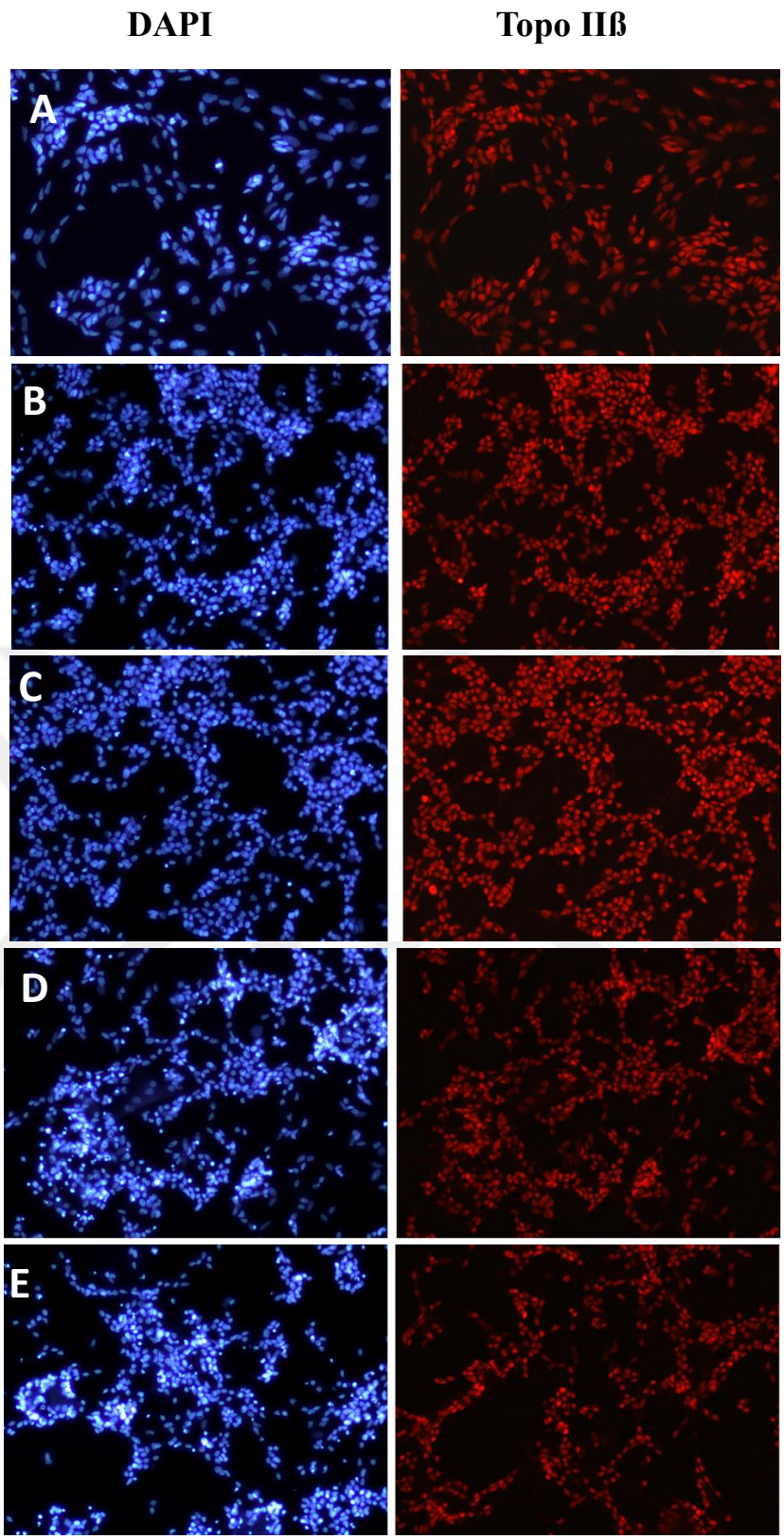

Figure 21: DAPI and Topo IIß Immunoflorescence Images of **(A)** control, **(B)** Differentiation, **(C)** Topo IIß Overexpression, **(D)** Aß treated, **(E)** Topo IIß overexpressed & Aß treated. (All of the groups were neurally differentiated except A). Images were taken under 20X magnification.

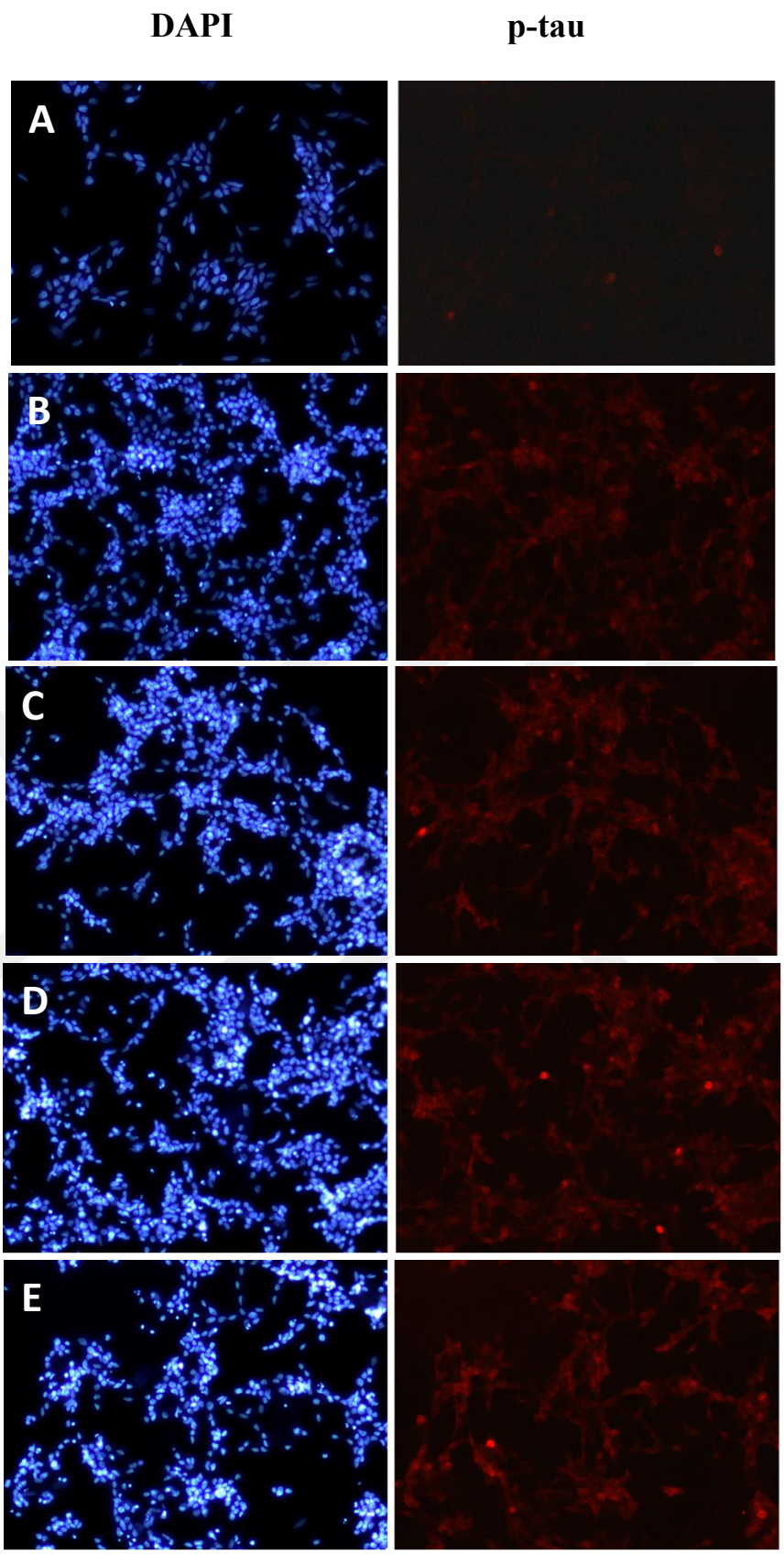

Figure 22: DAPI and p-tau Immunoflorescence Images of of **(A)** control, **(B)** Differentiation, **(C)** Topo IIß Overexpression, **(D)** Aß treated, **(E)** Topo IIß overexpressed & Aß treated. (All of the groups were neurally differentiated except A). Images were taken under 20X magnification.

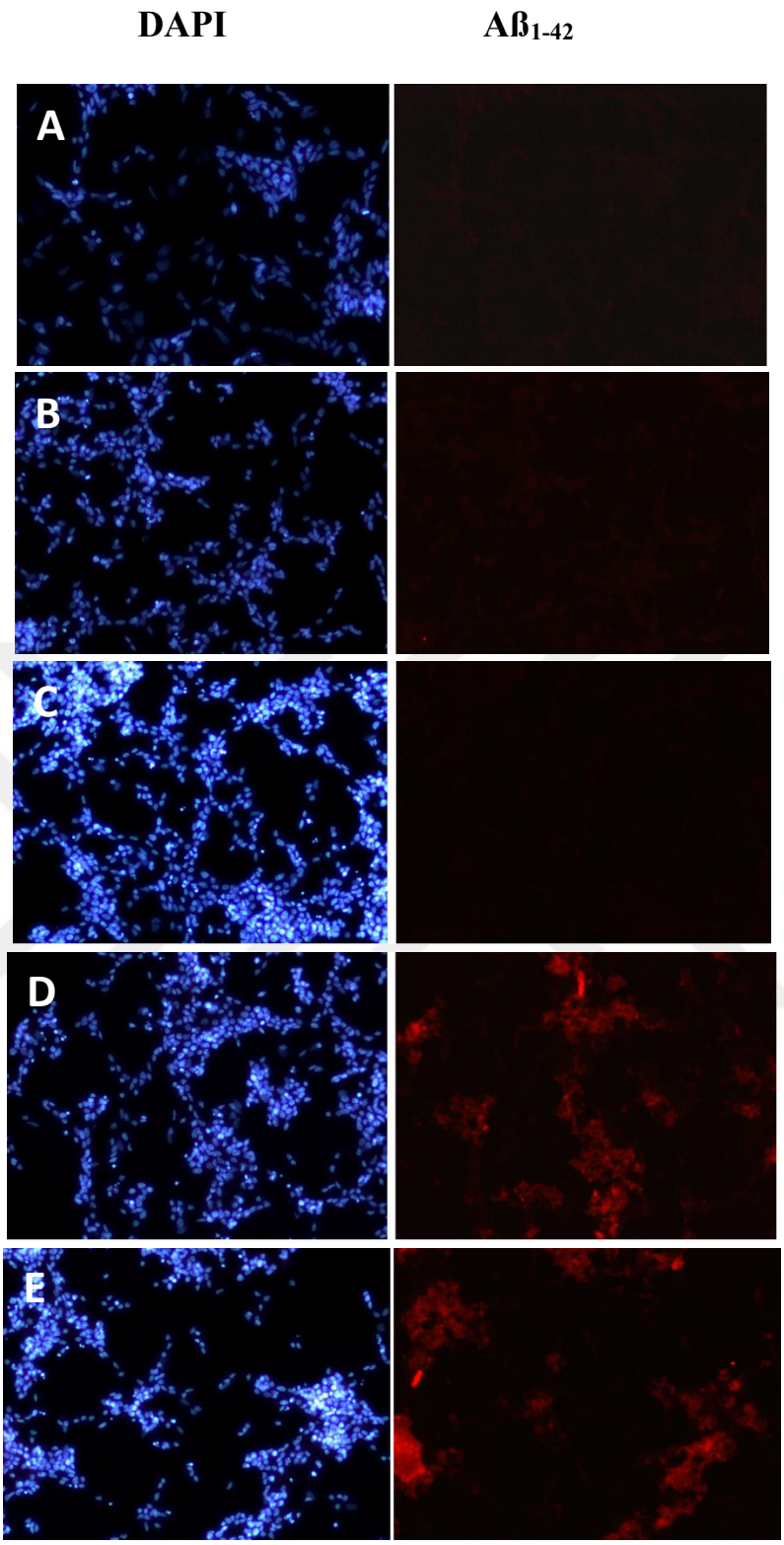

Figure 23: DAPI and Aß$_{1-42}$ Immunoflorescence Images of **(A)** control, **(B)** Differentiation, **(C)** Topo IIß Overexpression, **(D)** Aß treated, **(E)** Topo IIß overexpressed & Aß treated. (All of the groups were neurally differentiated except A). Images were taken under 20X magnification.

According to western blot results, the highest topo IIß protein expression was seen in topo IIß overexpressed (+) group although transfection was transient and performed with lipofectamine 3000 transfection reagent. An increased was observed with neural differentiation. Also, $Aß_{1-42}$ fibrils affect topo IIß protein expression adversely and it was observed a recovery with topo IIß overexpression.

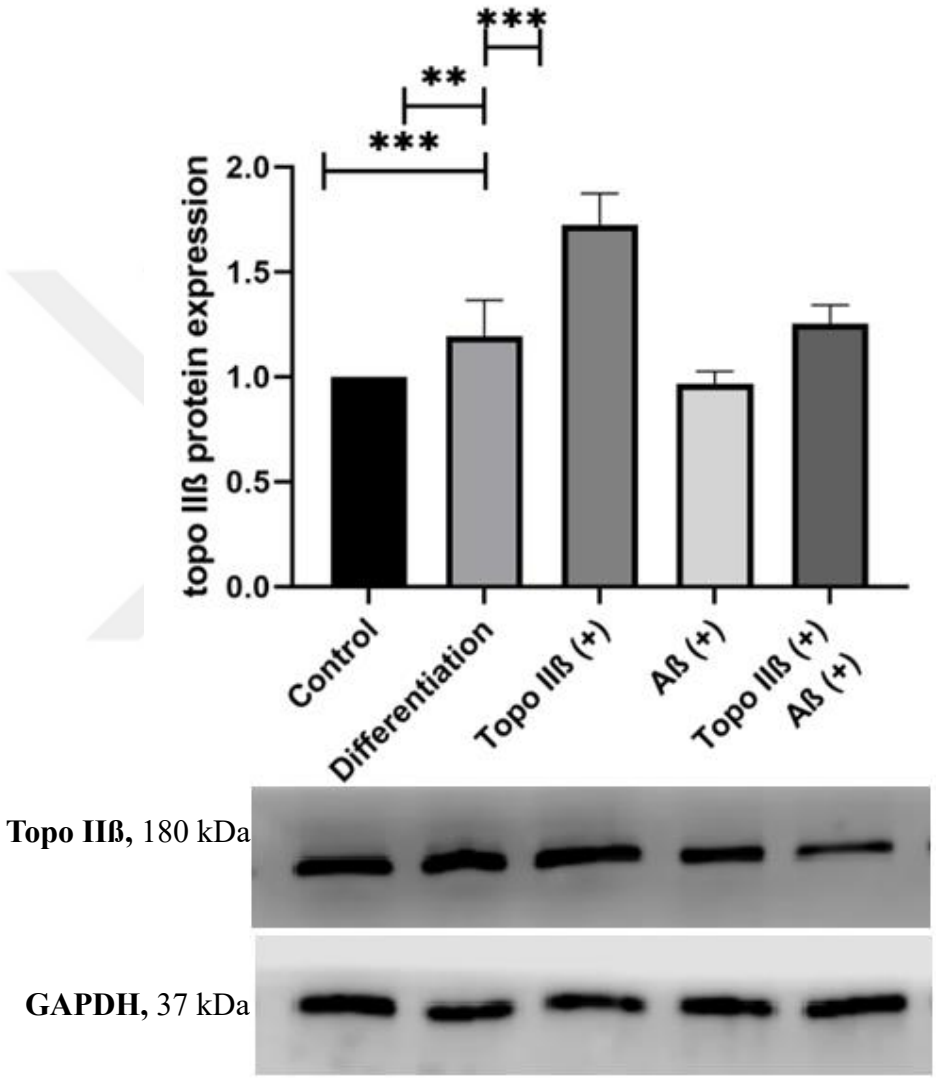

Figure 24: Topo IIß protein expression levels in experimental groups.

Phosphorylation of tau indicates the intracellular AD pathology mechanism and the highest p-tau protein expression level was observed in AD group. This western blot result confirmed our *in vitro* AD model. However, in both AD and topo IIß overexpressed group an improvement in p-tau expression level was found and its expression significantly decreased (Figure 25).

According to a previous study has performed by Heicklen-Klein et al, they showed that retinoic acid caused increase in tau expression with the progression of neural differentiation process. In our result, increased p-tau expression was observed in neural differentiation but in the presence of topo IIß overexpression p-tau expression level was decreased. Topo IIß has an effect on p-tau level which is the intracellular AD pathology mechanism.

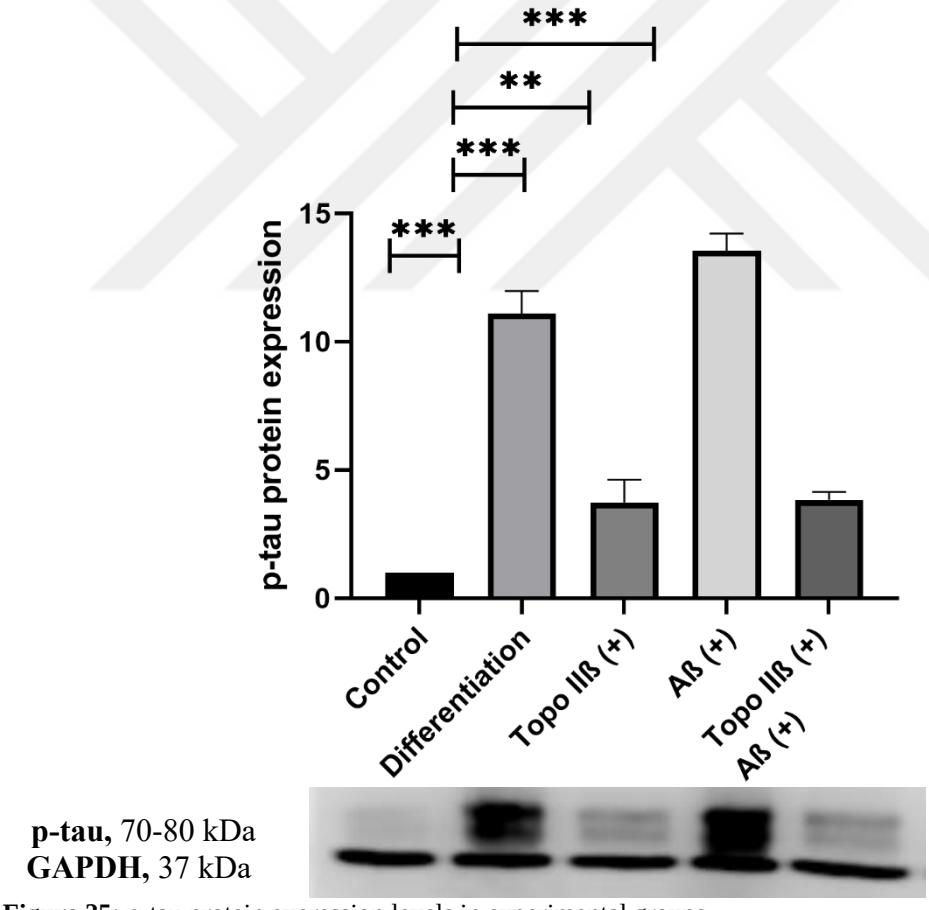

Figure 25: p-tau protein expression levels in experimental groups.

According to MTT result, in vitro AD model was formed succesfully and there were 70% cell viability in AD group. Due to transfection reagent, toxicity was observed in topo IIß overexpression (+) group. Since combination of transfection reagent and Aß toxicities, there were no neuronal survival was found.

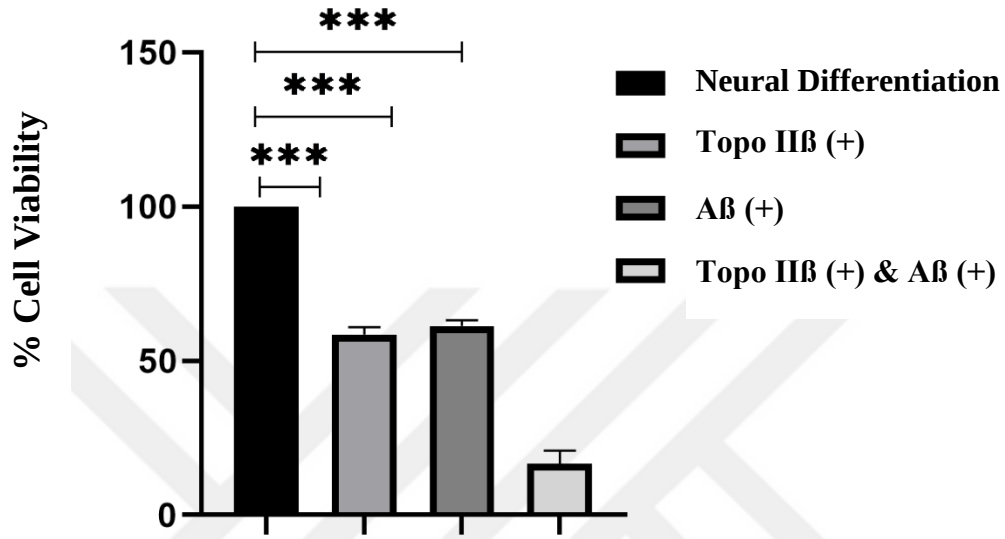

Figure 26: % Cell viability calculation.

5. DISCUSSION

Studies have shown that the TOP2B gene is a gene that has an important role in brain development and neural differentiation. It has been reported that increased expression of the TOP2B gene protects neurons from death (Zaim and Isik, 2018). Therefore, it is thought that mutations in TOP2B or changes in topo IIβ expression levels may lead to neurodevelopmental or neurodegenerative disorders.

Studies conducted on neural cells have reported that topo IIβ expression is suppressed in vitro or in vivo, resulting in both morphological and functional changes in the nervous system. In these studies, it was reported that due to the absence of topo IIβ, axon development was not completed and remained short, neuromuscular connections did not occur, important genes were not expressed during neural differentiation, and DNA damage increased (Nur-E-Kamal 2007).

In another study, it was observed that TOP2B gene expression decreased in the AD model created with Aβ1-42. It has also been reported that there is an increase in the expression levels of AD-related genes with the silencing of topo IIβ (Terzioglu et al., 2017). These results suggest that topo IIβ may have a protective and regulatory effect in AD.

Our previous studies have shown that topo IIβ regulates neural polarization in the regulation of cell signal transduction pathways in neural differentiation, and topo IIβ deficiency has a role that may contribute to the pathogenesis of neurodegenerative diseases such as AD and PD. In one of these studies, the first molecular evidence that the absence of topo IIβ is associated with AD-like pathology through the regulation of axonal growth was presented (Isik et al. 2015). In the subsequent study, it was shown that silencing topo IIβ causes AD-like pathology by increasing the expression of AD-related genes (Cofilin and Presenilin) and RhoA gene expression, which causes neuronal degeneration. On the other hand, topo IIβ is suppressed at the gene level in the in vitro AD model has been shown (Isik et al., 2014, Terzioğlu-Uşak et al. 2017). It is envisaged that by increasing the expression level of Topo IIβ by restoring it to neural cells externally, RhoA gene expression can be suppressed, axonal destruction in neuronal cells can be prevented, cells can be prevented from entering apoptosis, and therefore neuronal

deaths seen in AD can be prevented and possible new treatment methods can be developed.

In order to examine the possible neuroprotective role of topo IIß in AD, we performed an *in vitro* AD model in permanently topo IIß overexpressed SH-SY5Y neuroblastoma cell line.

Since the TOP2B gene is a large gene, there have been some difficulties in increasing gene expression above a certain level and maintaining that level in studies conducted so far. To date, no studies in which the TOP2B gene has been persistently overexpressed have been performed either from SH-SY5Y cells or other cell lines. With this thesis, the TOP2B gene was permanently overexpressed in the SH-SY5Y cell line and this problem was eliminated.

The most favorable results in increasing topo IIβ expression in SH-SY5Y cells were obtained by 1 MOI lentivirus concentration. The higher lentivirus concentration the higher cell death was observed. 12% transfection efficiency was obtained with 1 MOI TOP2B lentivirus transduction in SH-SY5Y cells. To increase the efficiency of topo IIß positive cells, FACS (fluorescence-activated cell sorting) was employed to sort the cells and sorted positive cells were used in experiments. TOP2B expression remained until day 56 after post infection in SH-SY5Y cells.

The effect of topo IIß on neural differentiation was known from previous studies (Zaim and Isik, 2018). In this study, it was shown that cells that overexpressed Topo IIß exhibited higher differentiation capability compared to the control cells. Even though it was the 4^{th} day of differentiation, all cells were differentiated and there was no undifferentiated SH-SY5Y cell. This result showed that, topo IIß has an important role in neural differentiation and brain development. Also, we checked the cell index difference between neurally differentiated SH-SY5Y cells and their undifferentiated controls with real time cell analysis system (Roche, x-Celligence). A decrease in the cell index was observed as the nuclei of the differentiated cells became smaller and the surface area decreased along with the formed axons.

In order to establish *in vitro* AD model, $Aß_{1-42}$ peptides were pre-incubated in 37˚C for 48 h with shaking. The formation of fibrils was demonstrated by Thioflavin S staininig. Formed fibrils were given to SH-SY5Y cells. The optimum concentration of

Aß peptide concentration was selected as 10 μM for differentiated cells and almos 70% cell viability was obtained. It was found that the higher concentrations leads to toxicity and lower concentrations has an no effect on cells. On the other hand, differentiated cells became more sensitive to Aß fibrils compare to undifferentated cells (un-published data).

In IF staining, a decrease was observed in the expression level of neuronal marker NFL and topo IIß in the Aß treated group, while an increase was observed in the level of p-tau, a disease pathology marker.

At the protein level, it was found that topo IIß protein expression increased with neural differentiation process. Aß has an adverse effect on topo IIß expression and caused to decrease in topo IIß expression. Neural differentiation may have a role in increasing p-tau expression due to retinoic acid which was used in first 4 days of neural differentiation process. According to literature, it was shown that retinoic acid leads to increase in p-tau expression so the increase in p-tau expression in neural differetiation was meaningful in Figure 24. However, when the topo IIß overexpressed, p-tau expression decreased which shows that topo IIß has an important role in AD pathology marker. The highest p-tau protein expression level was seen in *in vitro* AD model group. With the progression of the disease, tau proteins became phosphorylated and the level of phosphorylated tau increased. This result confirmed our *in vitro* AD model in addition to Aß toxicity. Also, in the overexpression of topo IIß, p-tau expression level significantly decreased in AD group as in neural differentiation.

Moreover, topo IIß had no significant effect on neuronal survival. However, the MTT experiment was performed by doing transient transfection and will be repeated with a permanently topo IIß overexpressing cell line. According to the results of other studies conducted by our group, the lipofectamine 3000 reagent used in transient transfection causes neuroinflammation. So, this result will be repeated by getting rid of the effect of lipofectamine 3000.

Also, due to stability of Aß fibrils, there was no role topo IIß in Aß clearence was observed.

6. CONCLUSION & RECOMMENDATIONS

In this study, it was aimed to investigate the neuroprotective role of topo IIβ on neuronal cell viability against in vitro Alzheimer's disease model. This was conducted by permenant overexpressing TOP2B gene in neurally differentiated SH-SY5Y neuroblastoma cell line. Topo IIß expression increased with the help of lentivirus and this increase was confirmed with flow cytometry analysis and western blot analysis at the protein level. Neural differentiation process was completed succesfully until day 10 by using retinoic acid and BDNF and topo IIβ has an important role in neuronal development.

Also, it was concluded that 10µM $Aß_{1-42}$ peptide concentration was selected to establish *in vitro* AD model and fibrils formation was confirmed by Thioflavin S staining. Additionally, Aß has an effect on topo IIß expression and Aß fibrils cause a decrease in topo IIß expression level.

Moreover, topo IIß has a regulatory role in AD pathology. Overexpression of topo IIß has a healing effect through p-tau, which is the intracellular AD mechanism.

According to the results of the previous studies, it is thought that permanently increasing the expression of topo IIβ in neural cells, as a factor preventing neuronal degeneration, may be a new strategy in the treatment of AD. However there are still unknown mechanisms that are linked with association between topo IIß and AD. Additional *in vivo* investigations are required to elucidate the mechanisms that underlie the neuroprotective impact of topo IIß against AD.

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