NEURO-PROTECTIVE ROLE OF GINKGOLIDE B IN Aβ-INDUCED NEURODEGENERATION AND AChE ENZYME ACTIVITY IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

Research Project Submitted to Central University of Punjab

For the Award of

Master of Science

In

Life Sciences (Sp. Animal Sciences)

By

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May 2018

DECLARATION

I declare that the project entitled "**NEURO-PROTECTIVE ROLE OF GINKGOLIDE B IN Aβ-INDUCED NEURODEGENERATION AND AChE ENZYME ACTIVITY IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS**" has been prepared by me under the guidance of Dr. Anil K. Mantha, Associate Professor, Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Ankita Mukherjee has prepared her project entitled “**NEURO-PROTECTIVE ROLE OF GINKGOLIDE B IN Aβ-INDUCED NEURODEGENERATION AND AChE ENZYME ACTIVITY IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS**“ for the award of M. Sc. Degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ABSTRACT

TITLE: NEURO-PROTECTIVE ROLE OF GINKGOLIDE B IN Aβ-INDUCED NEURODEGENERATION AND AChE ENZYME ACTIVITY IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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Keywords: Alzheimer's disease (AD), Oxidative stress, Acetylcholinesterase (AChE), Amyloid beta (Aβ), Ginkgolide B (GB).

Ginkgolide B (GB) is being used as medicine in China for treating neurodegenerative diseases for a long time. Its neuroprotective role is getting well established. Alzheimer's disease (AD) is a neurodegenerative disease that has multiple factors associated with its onsetand is one of the most common causes of dementia in the world. GB is known to reduce the oxidative stress caused due to accumulation of amyloid beta (Aβ), a major hallmark of AD associated strongly with the production of oxidative stress via production of ROS. The increase in the expression of AChE has been reported and it has been associated with increased toxicity of Aβ. This study tried to decipher the relationship between Aβ, GB and AChE activity. In this study, it was found that $A\beta_{(25\text{-}35)}$-induced oxidative stress leads to increased production of ROS and decreased AChE activity. On the other hand, GB decreased ROS production and expression of AChE, thus pointing toward its protective effect. GB increased the activity of AChE, suggesting that due to its antioxidant potentialit possibly caused a decrease in protein oxidation, and thus increased the activity of the AChE enzyme. Therefore, the results of the present study show the modulatory role of GB an AChE enzyme activity under oxidative stress conditions as seen in AD, suggesting the potential of GB in AD therapeutics.

(Ankita Mukherjee) **(Dr. Anil K Mantha)**

ACKNOWLEDGEMENT

I would like to take this opportunity to thank those important people in my life whose presence has helped this dissertation to get completed in this smooth manner.

First and foremost, I would like to thank my advisor, Dr. Anil Kumar Mantha, Associate Professor, and Head,Department of Animal Science, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, who has guided me and supported me throughout my dissertation work.His continuous patience, support, knowledge in scientific research and encouraging suggestions has helped me to understand and enjoy my work. I consider myself lucky as I got a chance to work under his guidance.

Next I would like to express my sincere thanks to Dr. Monisha Dhiman for listening to my queries and giving valuable suggestions.Her continuous encouragement and support has always kept the work in flow.

I would also like to thank all my seniors Mr. Shishir Upadhyay, Ms. Navrattan Kaur, Mr. Kunj Bihari Gupta, and Ms. Sharanjot Kaur for providing me a friendly and cheerful environment in lab and giving critical comments and help during my work.

I am very thankful to my seniors Dr. Bibekananda Sarkar and Mrs. Iqball Gill for their complete support, encouragement and proper guidance during my dissertation work. Without them the completion of this dissertation work was not possible.

I am also very thankful to my classmates and friends in Central University of Punjab for being there for me and playing the role of my family during my stay away from home.

My special gratitude goes to my parents who if not for their support and prayers, I would not have made it this far. They are my role models for hard work and have never questioned my decision in choosing what I really wanted in life. I would also like to thank my little brother for being there for me through thick and thin and giving me advices on my decisions and supporting me in life.

Date: (Ankita Mukherjee)

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Alzheimer’s disease	AD
2	Amyloid beta	Aβ
3	Neurofibrillary tangles	NFT
4	Reactive oxygen species	ROS
5	Reactive nitrogen species	RNS
6	Oxidative stress	OS
7	Neurodegenerative disease	ND
8	Parkinson’s disease	PD
9	Huntington’s disease	HD
10	Oxygen	O_2
11	Acetylcholinesterase	AChE
12	Acetylcholine	ACh
13	Ginkgolide B	GB

14	Mild Cognitive Impairment	MCI
15	Senile plaques	SP
16	Amyloid Precursor Protein	APP
17	Tyrosine kinase	Trk
18	Mitogen-activated protein	MAP
19	Brain-derived neurotrophic factor	BDNF
20	Lactate dehydrogenase	LDH
21	Centimetre	Cm
22	Dulbecco’s Modified Eagle’s Medium	DMEM
23	Fetal Bovine Serum	FBS
24	Penicillin-Streptomycin	PS
25	Carbon dioxide	CO_2
26	Degree Celsius	˚C
27	Phosphate Buffer Saline	PBS
28	Ethylene diaminetetra acetate	EDTA
29	Millimetre	Mm

30	Milli Molar	mM
31	Dimethyl sulfoxide	DMSO
32	Micro Molar	µM
33	Hour	Hr
34	Nitroblue tetrazolium	NBT
35	Milligram	Mg
36	Millilitre	Ml
37	Molar	M
38	Potassium hydroxide	KOH
39	Optical density	OD
40	Ribo Nucleic Acid	RNA
41	Micro Litre	µL
42	Minute	Min
43	Deoxyribo Nucleic Acid	DNA
44	Complementary DNA	cDNA
45	Polymerase Chain Reaction	PCR

46	Messenger RNA	mRNA
47	Voltage	V
48	Kilo base	Kb
49	Sodium Dodecyl Sulfate	SDS
50	Protease Inhibitor	PI
51	Revolution per minute	RPM
52	Ellman’s reagent (5,5’-dithiobis-2-nitrobenzoic acid)	DTNB
53	Deoxyribonucleotide triphosphate	dNTP
54	Reverse Transcriptase	RT

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD), a chronic neurodegenerative disease is the most common form of dementia that causes problems with memory, thinking and behaviour, ultimately, leading to death. More than 37 million people are affected by it globally, with the highest prevalence seen in people ageing ≥65 years. It has been estimated that with increasing life expectancy and lifestyle changes, one in 85 people would be living with AD by the year 2050(Gaur *et al*., 2014). The major hallmark of AD is the accumulation of amyloid beta (Aβ) plaques between the nerve cells (neurons) in the brain and the neurofibrillary tangles (NFT) which are insoluble twisted Tau fibres found inside the brain cells (Hardy and Selkoe 2002; Brion, 1998). The various factors leading to the onset of this neurodegenerative disease may include oxidative stress, depression, hypertension, a history of head injury and others (Burns *et al.,* 2009).

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen or nitrogen species (ROS/RNS) and antioxidant defence, which may lead to tissue injury. It is suspected to play an important role in various neurodegenerative diseases (ND)including AD, Parkinson’s disease (PD), Huntington’s disease (HD), depression, and multiple sclerosis (Patel *et al.,* 2011). Since the brain utilizes 20% of total oxygen [O_2] consumption of the whole body, it makes it highly vulnerable to the damages caused by oxidative stress (Barja *et al*., 2004).

Aβ an exogenous oxidant has been found to play an important role in the development and progression of the AD. A peptide of 36-43 amino acids long, it is crucially involved as the main component of the amyloid plaques found in the brain cells of AD patients (Hamley, 2012). Deposition of Aβ plaques is considered as the causative agent of AD pathology and the NFT, cell loss, vascular damage, and dementia to follow as a direct result of this deposition (Hardy and Higgins 1992). Studies suggested that Acetylcholinesterase (AChE) interacts with Aβ to promote deposition of amyloid plaques in the brain of patients with AD (Rees *et al*., 2003).

Acetylcholinesterase (AChE) a cholinesterase in the body catalyzes the breakdown of acetylcholine and some of the other choline esters that functions as neurotransmitter (e.g. dopamine, serotonin, histamine, epinephrine, and norepinephrine). It is mainly found at the muscular junctions and in the chemical synapse of the cholinergic type, where its activity serves to terminate synaptic transmission. Since it is located on the post-synaptic membrane, it terminates the signal transmission by hydrolyzing Acetylcholine (ACh), Thus in a way resulting in improper functioning of the neurons, hence, causing various neuronal diseases (Whittaker *et al.,* 1990).

Ginkgolide B (GB) one of the subtypes of Ginkgolide (extracts of the leaves of *Gingko biloba* tree) is a phytochemical used as medicine for decades (Stromgaard and Nakanishi, 2004).It functions as an antioxidant, anti-depressant, as neuroprotector, and prevents neuroinflammation. It has specifically shown to minimize the inhibitory effect of amyloid-β peptides on cholinergic transmission (Lee *et al.,* 2004) and has displayed significant role in neuroprotective effect in various AD model studies (Kaur *et al.,* 2015; Gill *et al*., 2017).

Hypothesis

Treatment of GB, an antioxidant may play a protective role against $A\beta_{(25-35)}$ induced oxidative stress by modulating the expression of AChE enzyme in human neuroblastoma SH-SY5Y cells. It was hypothesised that GB might decrease the activity of AChE enzyme and might protect the cells from the effect of oxidative stress induced by $A\beta_{(25-35).}$

Objective of the study

- To study the neuro-protective effect of GB on the Aβ-induced oxidative stress responses and AChE activity in human neuroblastoma (SH-SY5Y) cells.

Significance of the study

This study will provide insight towards the potential antioxidant nature of GB against oxidative stress caused by $A\beta_{(25-35)}$ in human neuroblastoma (SH-SY5Y) cells. It may also reveal about the modulatory effect of GB on AChE enzyme activity in oxidative stress conditions and the relation between Aβ and AChE enzyme. This study may also advocate for future studies for the role of GB in medication to delay or prevent the onset of AD.

CHAPTER 2

REVIEW OF LITERATURE

Neurodegenerative diseases (ND):

Neurodegenerative disease (ND) is a broad term for a range of conditions primarily dealing with the affected neurons in the human brain. In these diseases, there is a progressive loss of structure or function of neurons, including death of the neurons (Gao and Hong, 2008).Neurons being the building blocks of the nervous system are found in the brain and spinal cord. Since they are unable to reproduce or replace themselves, when they become damaged or die, they cannot be replaced by the body and that loss causes major problems with the movement (ataxia), or the mental functioning (dementia). The various ND that have been discovered affecting millions of people worldwide include Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). These diseases are incurable and progresses with age and with the aging of the general population, the prevalence of these diseases is expected to rise dramatically in next few decades, with a resultant increase in the social and financial burden of these diseases. While aging play the role of the greatest risk factor, the various other risk factors of these diseases are mitochondrial DNA mutation, oxidative stress, etc. Dementia also responsible for the major part of ND, with Alzheimer's causing approximately 60-70% of dementia cases.

Alzheimer's disease (AD):

AD a progressive age-associated ND is the most common cause of dementia in the elderly, with a prevalence of 5% after 65 years of age, increasing to about 30% in people aged ≥85 years. This disease is characterised by progressive cognitive impairment, including impaired judgement, decision making, orientation in some cases accompanied by psychological disturbance or language impairment (Galimberti *et al.,* 2013; Ruszkiewicz *et al.,* 2015). This happens because the brain cells in the hippocampus, a part of the brain associated with learning are often the first to be damaged. Thus, causing memory loss, especially difficulty remembering recently learned information. According to the studies of 2017 by Alzheimer's Association, it was found that AD is the sixth leading cause of death in the United States and is the fifth leading cause of death in Americans aged ≥65 years. The deaths due to AD have been increasing greatly, between 2000 to 2013 deaths due to AD have increased by 71%.

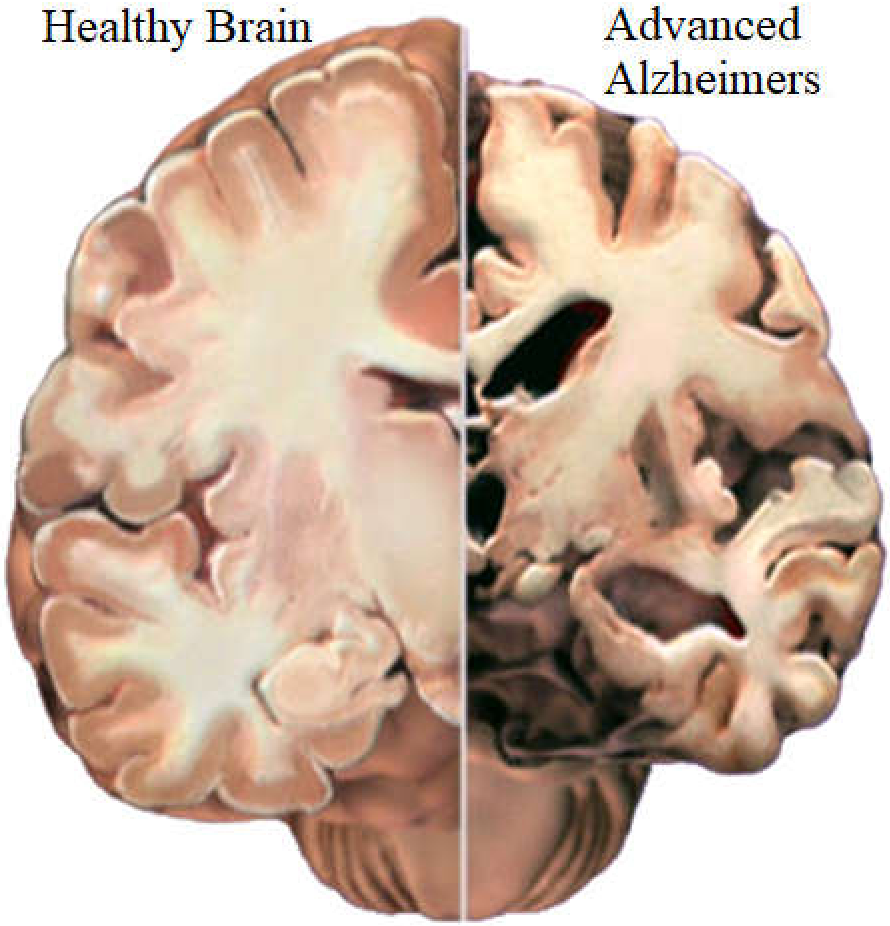


Figure 2.1: Comparison of healthy aged brain (left) and brain of AD patient (right) (http://www.alz.org/braintour/healthy_vs_alzheimers.asp).

By 2050, one new case of Alzheimer’s is expected to develop every 33 seconds, resulting in nearly 1 million new cases every year (Alzheimer’s Association 2016). Being age-associated progressive disease it begins slowly and is separated into three different categories: mild AD, moderate AD and severe AD. In the mild AD, a person may function independently but have problems with memory such as forgetting familiar words or the location of everyday objects. Moderate AD (middle stage) being typically the longest stage can last for many years with the progress of the disease towards severity. In this case the person gets confused with words, frustrated or angry, or may act in unexpected ways, this occurs due to the damage to the nerve cells in the brain making it difficult to express thoughts and perform routine tasks. With severe AD being the final stage or late stage of the disease, individuals lose the ability to respond to their environment, carry out conversation and eventually to control movement. As memory and cognitive skills continue to worsen, significant change in personality may take place (Alzheimer’s Association 2016).

The cause for most Alzheimer's cases is still largely unknown except for 1% to 5% cases where genetic differences have been identified. Age and genetics are the strongest risk factors for the occurrence and development of AD in a person along with family members suffering from Alzheimer’s, Mild Cognitive Impairment (MCI), cardiovascular diseases, traumatic brain injury playing some part in the occurrence and development of the same (Alzheimer’s Association 2016).

Accumulated evidences point towards the role of Aβ, the oxidative stress and mitochondrial dysfunction as the major cause in the pathogenesis of AD. The accumulation of Aβ plaques between the nerve cells (neurons) in the brain is the leading cause of oxidative stress in the neurons, leading to increased ROS, mitochondrial dysfunction, and eventually leading to death of the neurons (Mattson, 2004). The neuronal loss occurring due to the formation of amyloid plaques in the neuronal cells might contribute to 20-30% of brain-weight loss as reported in AD patients (Vina *et al*., 2007).

Amyloid beta (Aβ):

The aggregation of the Aβ-peptide into oligomers or fibrils is implicated as a key process associated with progression of AD (Hamley, 2012). Aβ forms highly insoluble and proteolysis resistant fibrils known as senile plaques (SP). Plaques are extracellular deposits of fibrils and amorphous aggregates of Aβ-peptide. The production of Aβ is the result of cleavage of the Amyloid Precursor Protein (APP), which is over-expressed in AD (Galimberti *et al.,* 2013). It is generated due to abnormal processing of APP in the neurons. The processing of APP occurs via two pathways: non-amyloidogenic and Amyloidogenic **(Figure 2.2)**. In the non-amyloidogenic pathway APP cleavage occurs by the α-secretase at residue 15-17 of the AβP sequence, this cleavage prevents the formation of full Aβ thus resulting in no amyloid deposition. In the amyloidogenic pathway, APP undergoes successive proteolysis by β- and γ-secretase located only six residues away from the cleavage site within the Aβ protein sequence, resulting into formation of Aβ and causing amyloid deposition. The cascade hypothesis suggests that other causes of AD act by initially triggering Aβ peptide deposition (Hardy and Higgins 1992) like recently it has been found that AChE promotes aggregation of Aβ peptide and formation of amyloid plaques (García-Ayllón *et al.,* 2011).

In the degenerated neurons of AD, there are abnormalities like increased oxidative damage and impaired energy metabolism which has been found to be instigated by Aβ (Mattson, 2004). Several lines of evidence indicate that Aβ induces oxidative stress. Oxidative stress that occurs within the lipid bilayer, hypothesized in the Aβ-induced oxidative stress hypothesis in which $A\beta_{(1\text{-}42)}$ inserts as oligomers into the bilayer and serves as a source of ROS, has been shown to initiate lipid per-oxidation **(Figure 2.3)** (Butterfield *et al.,* 2013).

Oxidative Stress:

ROS or RNS are generated both exogenously and endogenously within the cell causing oxidative stress. This increased ROS/RNS production leads to the damage of DNA, RNA, proteins and lipids, and has been associated with several neurological diseases including AD (Chen *et al.,* 2012). Oxidative stress occurs early in the course of AD, which would support its role in AD pathogenesis in relation with the presence of Aβ (Wang *et al.,* 2014). Also elevated levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ have been reported to be associated with increased levels of oxidation products from proteins, lipids and nucleic acids in AD hippocampus and cortex (Cheignon *et al.,* 2017).

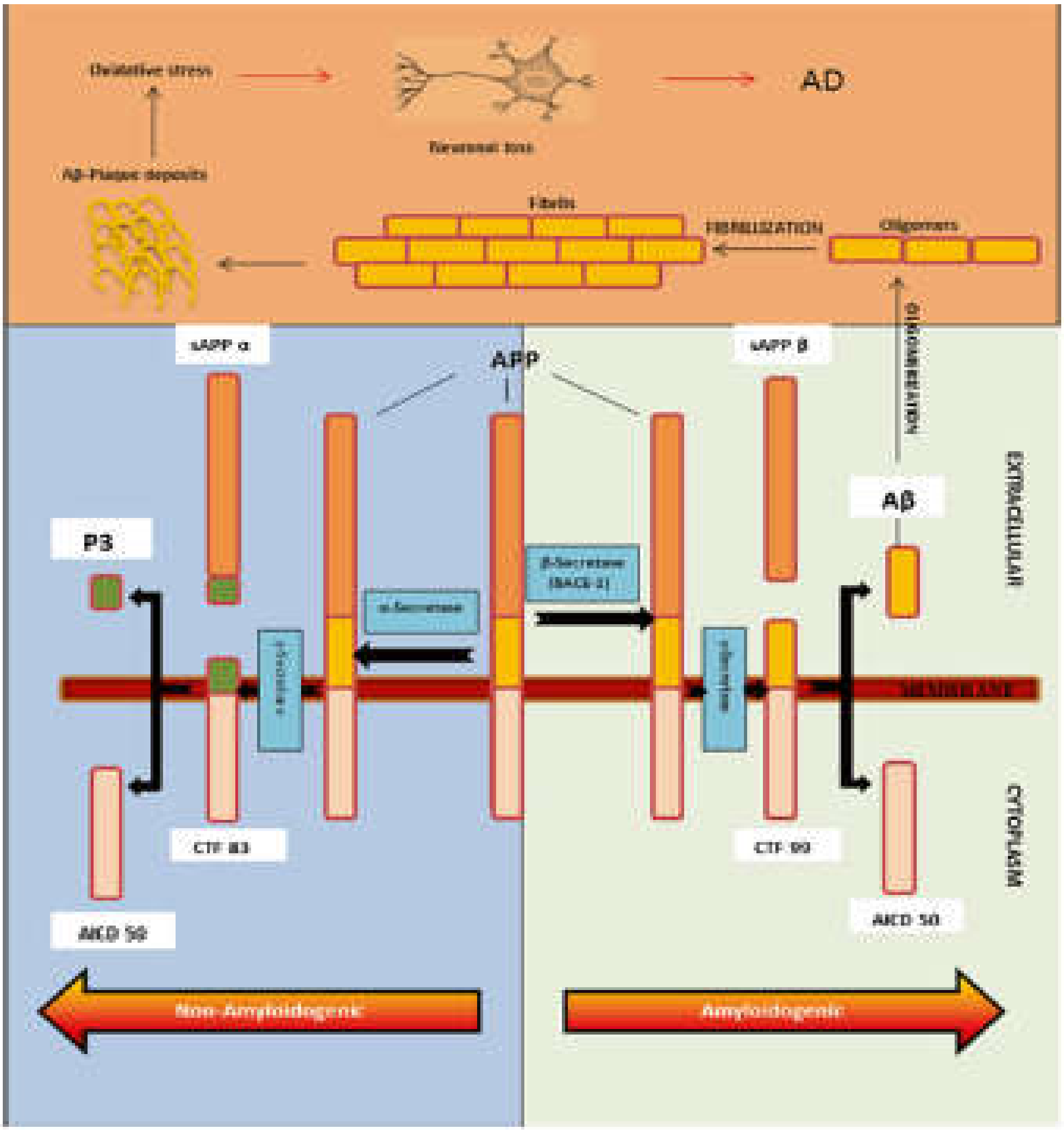


Figure 2.2: Non-Amyloidogenic and Amyloidogenic pathways for APP processing (Kaur *et al.*, 2015).

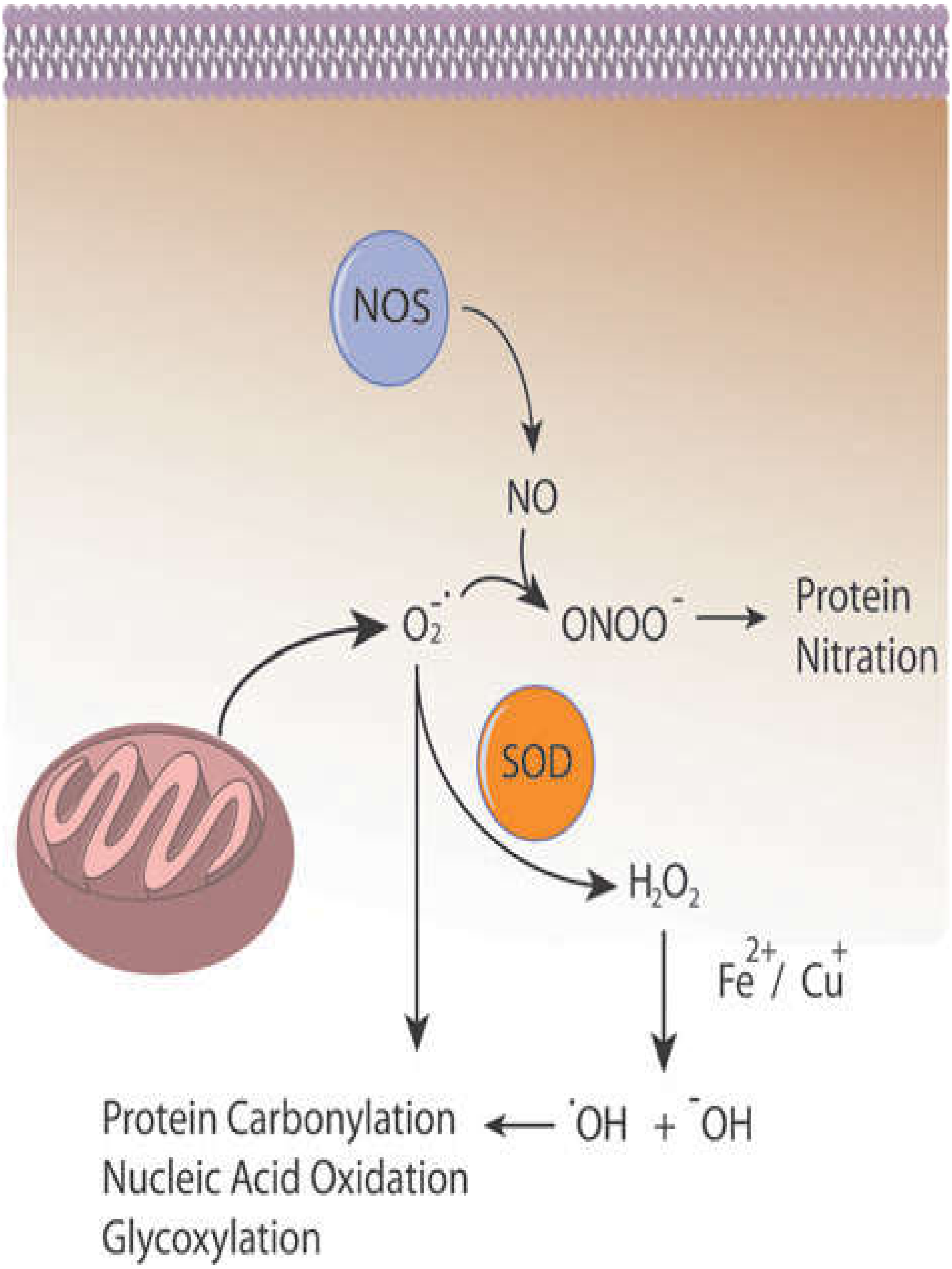


Figure 2.3: Consequences of elevated ROS and RNS (Butterfield *et al.,* 2013).

Acetylcholinesterase (AChE):

AChE (EC 3.1.1.7) functions in the central and peripheral nervous system, along with the acetylcholine (ACh) receptor, in the transmission of action potentials across nerve-nerve and neuromuscular synapses. The enzyme springs into action when ACh is released from the pre-synaptic nerve process in response to an action potential. AChE rapidly terminates the ACh receptor-mediated ion gating by hydrolyzing ACh **(Figure 2.4)**. A hallmark of the AD is developing cholinergic deficit as the disease progresses (Quinn, 1987).The only treatment for AD has been the use of inhibitors of AChE which is one of the several proteins associated with amyloid plaque deposits (Inestrosa *et al*., 2005). AChE consistently co-localizes with the amyloid deposits and is found to enhance the aggregation of the $A\beta_{(12-28)}$ and $A\beta_{(25-35)}$ peptides but not of the $A\beta_{(1-16)}$ fragment (Alvarez *et al.,* 1997; Inestrosa *et al*., 2008).

Phytochemicals:

Phytochemicals are constitutive metabolites that enable plants to overcome temporary or continuous threats integral to their environment while also controlling essential functions of growth and reproduction (Molyneux *et al*., 2007). There are many plant based compounds with antioxidant, anti-allergic, anti-inflammatory, antiviral, antiproliferative and anti-carcinogenic property like curcumin, ferulic acid, Ginkgolides (Hatcher *et al*., 2008; Balasubashini *et al*., 2004; Ahlemeyer and Krieglstein 2003). These phytochemicals show protective role against AD, PD, and HD along with many other phytochemicals (Gupta and Sharma 2017).

Ginkgolide B (GB):

The extract of *Gingko biloba* leaves (EGb761) has been standardised to contain 24% flavinoid glycosides (containing quercetin, kaempferol, isorhamnetin, etc.), 6% terpenoids (3.1% Ginkgolides A, B, C and J and 2.9% bilobalide), and 5-0% organic acids (Shi *et al.,* 2009). Numerous preclinical studies have shown the

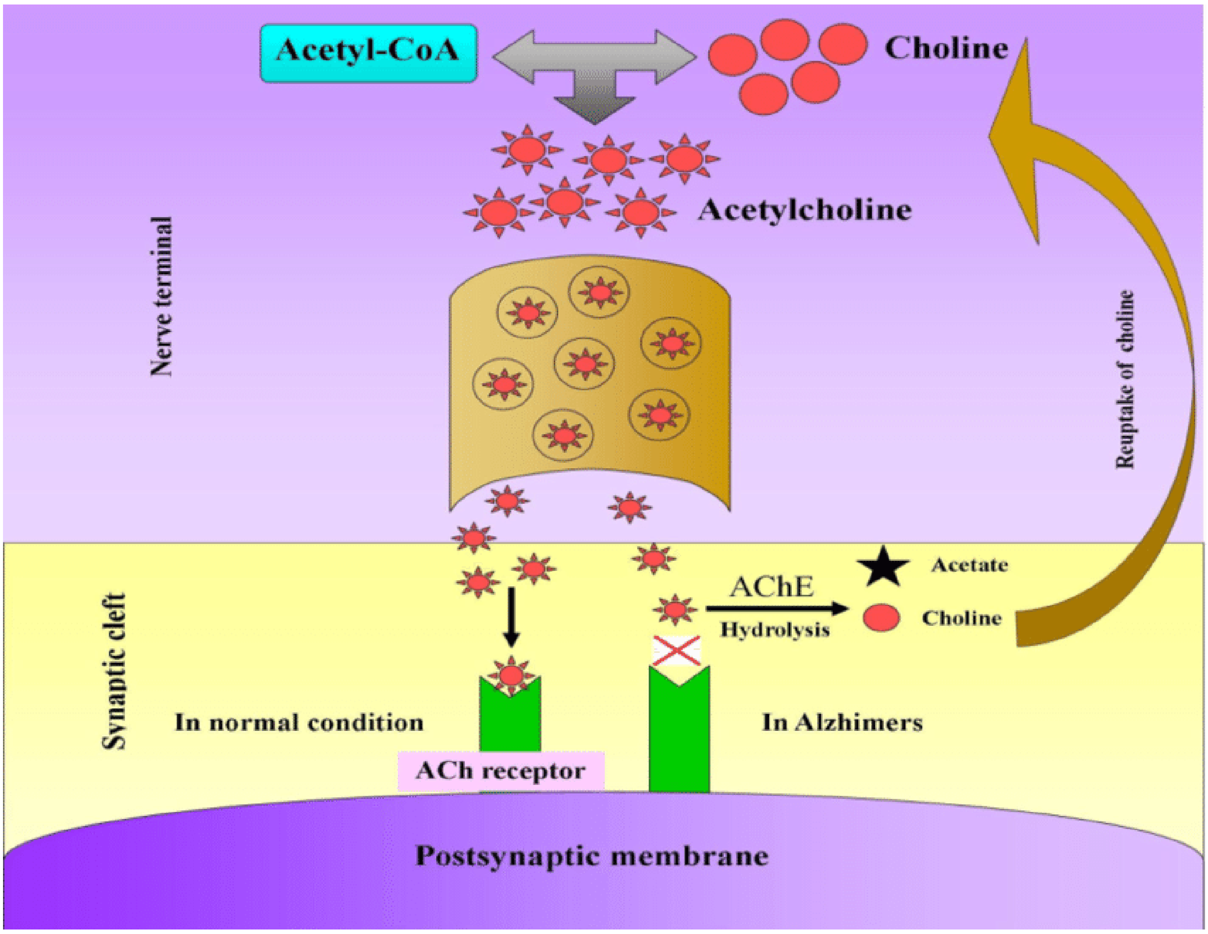


Figure 2.4: AChE mechanism of action (Sayeed, *et* al., 2016).

neuroprotective effect of EGb761 and support the notion that it may be effective in the treatment and prevention of neurodegenerative disorders such as AD (Ahlemeyer and Krieglstein 2003; Shi *et al.,* 2010). GB a phytochemical derived from the root bark and leaves of *Gingko biloba* tree are present in less than 0.1 to 0.25% in the tree and has a structure of diterpenoidtrilactone with six five membered rings **(Figure 2.5)** (Dewick 2002; Andersen *et al.,* 2010). The mechanisms associated with the neuroprotective role of GB are, activation of Trk/Ras/MAP, induces secretion of brain derived neurotrophic factor (BDNF) and reduces the ROS, LDH, caspase3, and pro-apoptotic factors (Zhang *et al.,* 2011). GB when pre-treated has shown protection against Aβ-toxicity in human neuroblastoma SH-SY5Y cells (Kaur *et* al., 2015; Gill *et al*., 2017).

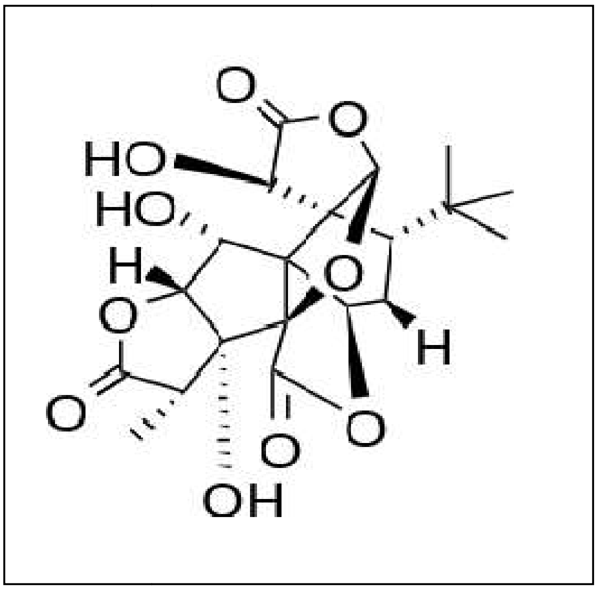


Figure 2.5: Structure of Ginkgolide B (Andersen *et al.,* 2010).

Thus, owing to its antioxidant nature, ability to counter Aβ-induced neurotoxicity and neuroprotective effect, GB has a strong potential for its use in AD treatment and management, the mechanisms and signalling pathways of which need further insights and investigation.

CHAPTER 3

MATERIALS AND METHODS

EXPERIMENTAL PLAN

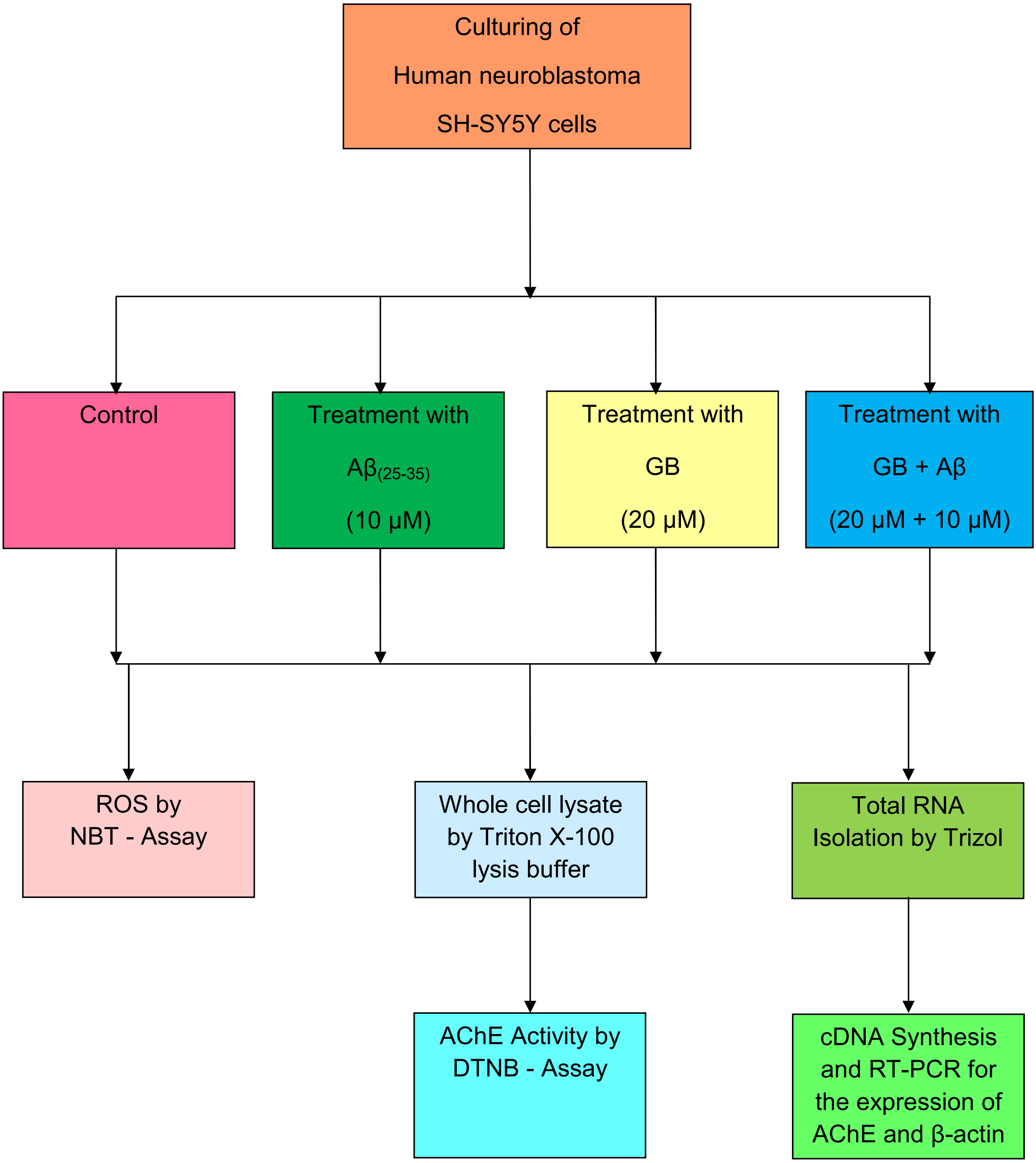


Figure 3.1: Experimental design of the study

3.1 Culturing of SH-SY5Y cells

SH-SY5Y cells were cultured in 25 cm^2 canted neck cell culture flasks, at a density of 1 × 10^6 cells/cm^2, in4 ml of complete media containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution Penicillin-Streptomycin (PS). The cells were incubated in an incubator maintaining 5% CO_2 and 37°C under atmospheric pressure (Mantha *et al.*, 2012). The cells were grown upto a confluence of 70-80% while changing the media at regular intervals. After reaching proper confluence, then the cells were washed with 1 × PBS, trypsinized with 0.25% Trypsin-EDTA (1×) and split at proper intervals into (1:2) 100 mm^2 culture dishes for further experimentation and subculturing.

3.2 Cell Treatments

The cells were first seeded in 100 mm^2 dishes in complete media at a density of 1 × 10^6 cells/cm^2 for treatment and grown upto a confluence of 70-80% maintaining 5% CO_2 and 37°C under atmospheric pressure in the incubator. The treatments were given with $A\beta_{(25-35)}$ for the production of oxidative stress and phytochemical GB was used as an anti-oxidant.

3.2.1 Preparation and Treatment of GB and $A\beta_{(25-35)}$

Fresh stock solution of GB (10 mM) was prepared in 100% DMSO and cells were pre-treated for 3 hr with 20 µM concentration of GB in treatment media containing DMEM and 1% PS (Kaur *et al.*, 2015). Afterwards oxidative stress was induced with 10 µM $A\beta_{(25-35)}$treatment. Freshly prepared stock solution of $A\beta_{(25-35)}$(1mM) in double-distilled deionised water, considered as the soluble form (Mantha *et al.*, 2012). This treatment method was followed for all the treatments in further experiments.

3.3 Determination of ROS Produced by NBT assay

SH-SY5Y cells were seeded in 96 well plates at a density of 1 × 10^4 cells/well and treatment was given after growing the cells for 24 hr. Fresh solution of Nitroblue tetrazolium (NBT) a yellow coloured substance was prepared in 1× PBS for the concentration of 1 mg/ml. The cells after treatment of 24 hr were incubated for 2 hr in 200 µL of NBT solution (Choi *et al.,* 2006). After the incubation period, the NBT solution was removed and the cells were washed with 1 × PBS, and then fixed with chilled Methanol, then air-dried. The NBT crystals deposited inside the cells were then dissolved, first by adding 120 µL of 2 M KOH for solubilising the cell membranes, then followed by 140 µL of DMSO to dissolve the blue fomazan by shaking for 10-15 min at room temperature. The absorbance was read on BioTek®Synergy H1 (Sarkar *et al*., 2017).

$$\textbf{Percentage of ROS produced} = \frac{\textbf{O.D. Of treated cells}}{\textbf{O.D. Of untreated cells}} \times 100$$

3.4 Total RNA Isolation

For total RNA isolation, SH-SY5Y cells were seeded in 60 mm dishes in complete media (DMEM, 10% FBS, 1% PS) at a density of 1 × 10^6 cells/cm^2. After 24 hr of treatment, 200 µL of Trizol (Invitrogen) was added to the cells and incubated for 5 min. The cells were then thoroughly homogenized by pipetting and were transferred to properly labelled eppendorf tubes. 200 µL of chloroform was added to each tube, and after incubation of 2-3 min, the mixture was centrifuged at 4°C for 15 min at 12,000×g. After centrifugation, the mixture got separated into 3 layers, upper aqueous layer (containing RNA), middle white layer (containing DNA) and bottom pink layer (containing proteins). The upper aqueous layer containing RNA was carefully transferred to a fresh tube by pipetting, to this 200 µL of Isopropanol was added. After incubation for 10 min, the samples were again centrifuged at 4°C for 10 min at 12,000×g. The RNA precipitated as white gel like pellet at the bottom of the tube. The supernatant was discarded carefully and the pellet was re-suspended in 1 ml 75% Ethanol after washing once with 75% Ethanol. Then all samples were vortexed briefly and then centrifuged at 4°C for 5 min at 7500×g. The supernatant was discarded carefully with the help of pipette and the RNA pellets obtained in each sample were air-dried briefly. The pellets were then re-suspended in 1 ml of Nuclease free water, followed by incubation in water bath at 55°C for 5-10 min. Quantification of the RNA obtained was performed by Nanodrop Spectrophotometer and Agarose Gel Electrophoresis.

3.5 cDNA Synthesis form Isolated Total RNA

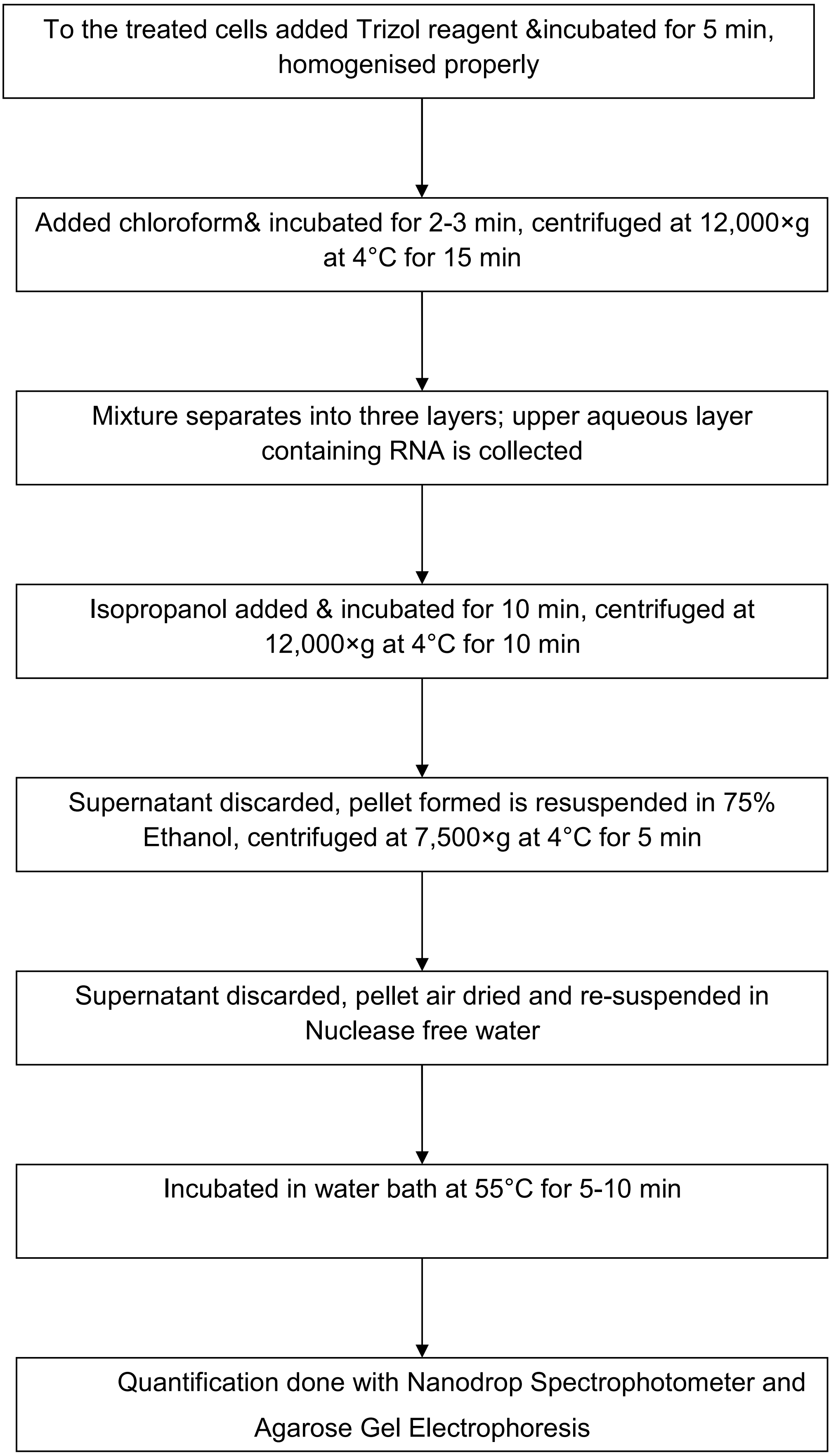
Thermo Scientific Verso cDNA synthesis kit was used for the synthesis of the cDNA. The reaction mixture for cDNA synthesis was prepared according to the instructions given by the company **(Table 3.1)**. The reaction mixture was incubated for 30 min at 42°C and for 2 min at 95°C in Thermal Cycler (SimpliAmp^{TM}). cDNA synthesis was carried for all the four treatment groups in well labelled PCR tubes (Gupta *et al*., 2018).

3.6 PCR for expression for AChE

Red Dye PCR Master Mix(GeNei^{TM}) was used for PCR of all the four treatment samples. Each microfuge PCR tube contained 5 µL of Master Mix, 1 µL of 10 µMforward AChE (5’-CTTCCTCCCCAAATTGCTC-3’) primer, 1 µL of 10 µMreverse AChE (5’-TCCTGCTTGCTGTAGTGGTC-3’) primer and nuclease free water to adjust final volume to 10 µL was added in each tube. The tubes were incubated in Thermal Cycler (SimpliAmp^{TM}) for PCR at 59°C as annealing temperature (Gupta *et al*., 2018).

3.7 PCR for expression for β-actin

Red Dye PCR Master Mix (GeNei^{TM}) was used for PCR of all the four treatment samples. Each microfuge PCR tube contained 5 µL of Master Mix, 1 µL of 10 µM forward β-actin (5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’) primer, 1 µL of 10 µM reverse β-actin (5’-TGGCACCCAGCACAATGAA-3’) primer and nuclease free water to adjust final volume to 10 µL was added in each tube. The tubes were incubated in Thermal Cycler (SimpliAmp^{TM}) for PCR at 60°C as annealing temperature (Gupta *et al*., 2018).



To the treated cells added Trizol reagent &incubated for 5 min, homogenised properly

Added chloroform& incubated for 2-3 min, centrifuged at 12,000×g at 4°C for 15 min

Mixture separates into three layers; upper aqueous layer containing RNA is collected

Isopropanol added & incubated for 10 min, centrifuged at 12,000×g at 4°C for 10 min

Supernatant discarded, pellet formed is resuspended in 75% Ethanol, centrifuged at 7,500×g at 4°C for 5 min

Supernatant discarded, pellet air dried and re-suspended in Nuclease free water

Incubated in water bath at 55°C for 5-10 min

Quantification done with Nanodrop Spectrophotometer and Agarose Gel Electrophoresis

Figure 3.2: Steps carried out for RNA Isolation

3.8 mRNA expression analysis by Agarose Gel Electrophoresis

After completing PCR, mRNA expression analysis was carried out by electrophoresis in 1.2% agarose gel at 75 V for 30 min, along with 1Kb plus DNA ladder (Invitrogen) for reference. Agarose gel images were captured with BioRad ChemiDoc system and bands on gel were analysed by Image Lab Software (Gupta *et al*., 2018).

3.9 Whole cell lysate preparation

For preparation of whole cell lysates of SH-SY5Y cells, the cells were seeded in 100 mm dishes in complete media (DMEM, 10% FBS, 1% PS) at a density of 1 × 10^6 cells/cm^2. After 24 hr of treatment the cells were at first gently rinsed twice with ice-cold 1× PBS and then lysed by adding 300-400 µL of Triton X-100 lysis buffer [0.5M NaCl, 0.5M EDTA, 1M TrisHCl (pH 7.4), 0.5% Triton-X 100, 20%SDS, 100mM PMSF, 5% Glyerol and protease inhibitor (PI) cocktail (10 µLof per 100 µL of lysis buffer)] per plate. The cells were then scraped thoroughly and homogenized by pipetting and were then transferred to properly labelledmicrofuge tubes. After the transfer, the sample containing tubes were vortexed for 30 min for 3 times at an interval of 5 min, all steps were carried out on ice (Mantha *et al*., 2012). After proper mixture, total contents were centrifuged at 4°C for 20 min at 13,000 rpm. The supernatant produced was transferred to fresh labelled microfuge tubes and stored at -20°C for further experiments and the pellet was discarded (Sarkar *et al*., 2017).Estimation of protein was carried by Bradford assay.

3.10 AChE Enzyme Assay by DTNB

$$\text{Acetylcholine} \xrightarrow{\text{AChE}} \text{Thiocholine + Acetate}$$

$$\text{Thiocholine + DTNB} \longrightarrow \text{5-Thio-2-nitro-benzoic acid (yellow)}$$

The enzyme AChE efficiently catalyzes the hydrolysis of Acetylcholine to Thiocholine and Acetate. Thiocholine, in the presence of highly reactive

Dithiobisnitro-benzoate (DTNB) ion produces yellow colour, which is visible and can be measured spectrophotometrically. The enzyme mixture contained in 200 µl, 180 µl Ellman's reagent, 10 µl substrate and 10 µl of enzyme (whole cell lysates of un-treated and treated cells) samples (Mantha *et al*., 2006;Benabent *et al*., 2014). The blank was set by replacing 10 µl of enzyme samples with 10 µl of 0.1 M of Sodium-Phosphate buffer (pH 7). The enzyme activity was measured spectrophotometrically at 405 nm at an interval of 30 sec for 10 min.

$$\textbf{AChE activity} = \frac{\textbf{Absorbance change/min} \times \textbf{Dilution of the extract (sample)}}{\textbf{Volume of extract} \times \textbf{13.6/ Total reaction volume}}$$

Where, 13.6 is a molar extinction coefficient of Nitrobenzoate ion. One unit of AChE activity is defined as the amount of enzyme that produces 1 µM of thiocholine/gm protein/min at 25 °C.

3.11 Statistical Analysis

The students (t) test was performed for the evaluation of the significance of the results. The data was considered statistically significant at $*p \leq 0.05$ and $**p \leq 0.005$.All the experiments were carried out in duplicates or triplicates throughout the study.

Table 3.1:Various components and their volume used in cDNA synthesis

Components	**Volume (µl)**
5× cDNA synthesis buffer	4 µl
dNTP Mix	2 µl
Random Primer	1 µl
RT Enhancer	1 µl
Verso Enzyme Mix	1 µl
Template RNA	1-5 µl
Nuclease free water	Upto 20 µl
Total Volume	**20 µl**

Table3.2: Total reaction mixture prepared for PCR of each sample

Components	**Volume(μl)**
Red Taq Mix	5 μl
Forward Primer	1 μl
Reverse Primer	1 μl
cDNA	1 μl
Nuclease-free water	2 μl
Total volume	**10 μl**

Table 3.3: Summary of primers used, annealing temperature and number of amplification cycles in each PCR

Gene	Base pairs	Primer sequences	Annealing temperature	Amplification cycles
AChE	132bp	F: 5’-CTTCCTCCCCAAATTGCTC-3’	59°C	34×
		R: 5’-TCCTGCTTGCTGTAGTGGTC-3’		
β-actin	186bp	F: 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’	60°C	34×
		R: 5’-TGGCACCCAGCACAATGAA-3’		

CHAPTER 4

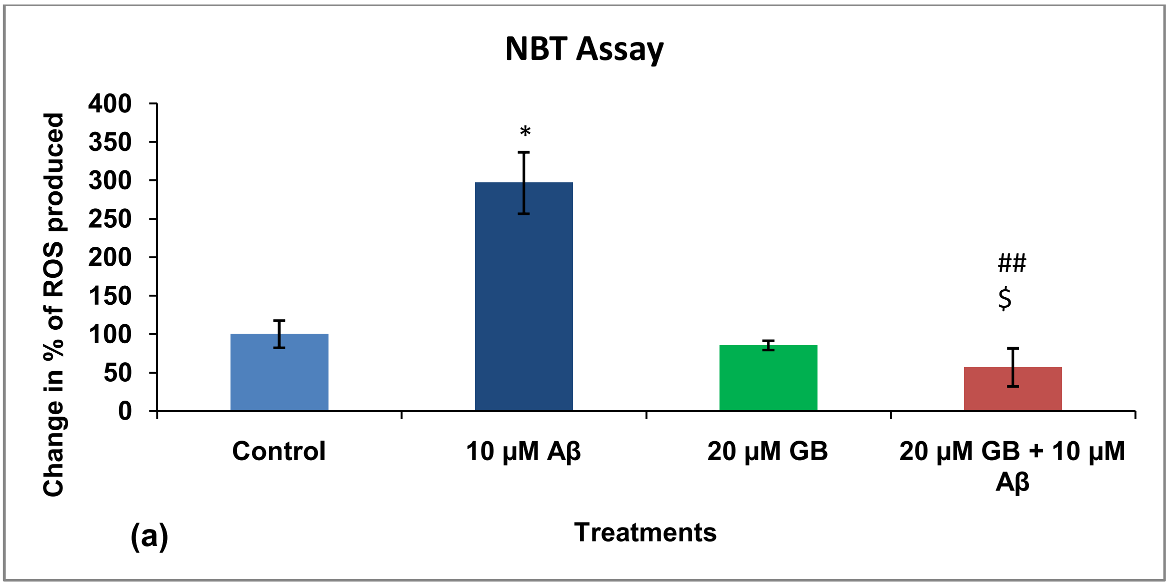
RESULTS

4.1. Measurement of level of ROS produced in SH-SY5Y cells after the treatment with GB, Aβ$_{(25\text{-}35)}$ and GB + Aβ$_{(25\text{-}35)}$ for 24 hr

The levels of ROS production were analysed in different treatment groups and it was found that Aβ$_{(25\text{-}35)}$(10 μM) cause significant increase by three folds in the levels of ROS production as compared to control SH-SY5Y cells. GB (20 μM) treatment caused 14% decrease in the levels of ROS production as compared to control. The pre-treatment of GB for 3 hr and then treatment of Aβ$_{(25\text{-}35)}$for 24 hr, caused decrease by 43% in ROS production as compared to the control untreated SH-SY5Y cells. Also, the pre-treatment of GB for 3 hr and then treatment of Aβ$_{(25\text{-}35)}$ (10 μM) for 24 hr time point caused significant decrease in ROS levels by two and half folds as compared to Aβ$_{(25\text{-}35)}$ (10 μM) treated SH-SY5Ycells (**Table 4.1 and Figure 4.1**).

4.2. Assessment of mRNA expression of *AChE*genein SH-SY5Y cells after the treatment with GB, Aβ$_{(25\text{-}35)}$ and GB + Aβ$_{(25\text{-}35)}$ for 24 hr

The expression change of *AChE* genes was analysed in different treatment groups and it was found that Aβ$_{(25\text{-}35)}$ (10 μM) treatment caused an increase by 8% in the expression of *AChE* gene as compared to the control untreated SH-SY5Y cells. GB (20 μM) treatment caused 9% decrease in the expression of *AChE* gene as compared to control untreated SH-SY5Y cells. The pre-treatment of GB for 3 hr and then treatment of Aβ$_{(25\text{-}35)}$ for 24 hr, caused 20% decrease in the expression of *AChE* gene as compared to the untreated control SH-SY5Y cells. Further analysis of pre-treatment of GB for 3 hr and then treatment of Aβ$_{(25\text{-}35)}$ (10 μM) for 24 hr time point caused decrease in *AChE* gene expression by 28% as compared to only Aβ$_{(25\text{-}35)}$ (10 μM) treated SH-SY5Ycells (**Table 4.2 and Figure 4.2**).



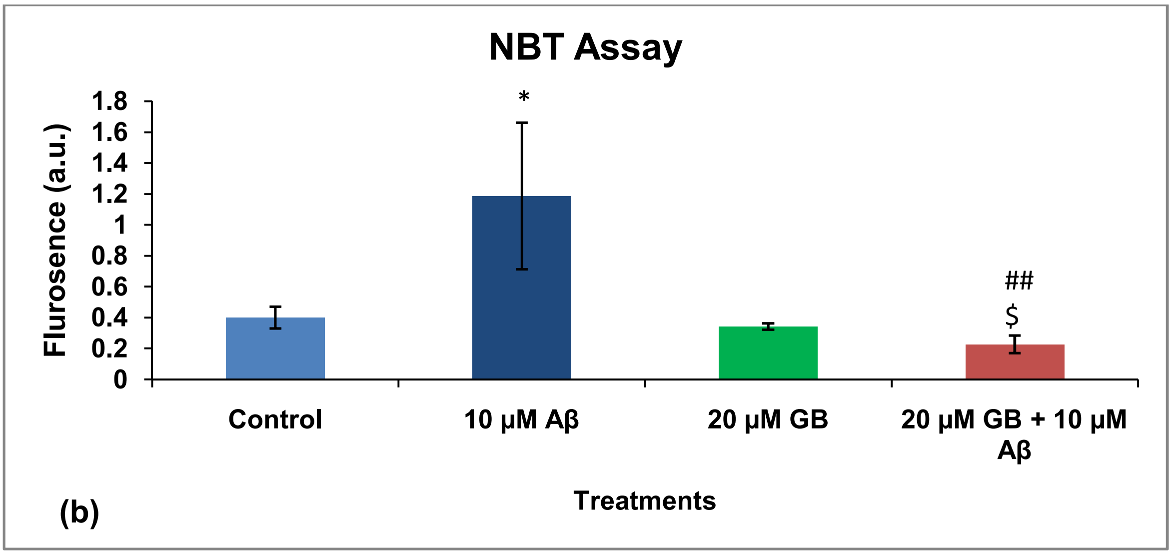


Figure 4.1:Analysis of ROS levels in SH-SY5Y cells, treated with $Aβ_{(25-35)}$ (10 µM), GB (20 µM), and GB (pre-treated for 3 hr @ 20 µM) followed by $Aβ_{(25-35)}$(10 µM) treatment for 24 hr. (a) Expressed as change in percentage, and (b) Expressed as fluorescence intensity (a.u).The data was considered statistically significant at *p≤0.05 when $Aβ_{(25-35)}$ (10 µM), GB (20 µM) and GB (pre-treated for 3 hr @ 20 µM) followed by $Aβ_{(25-35)}$(10 µM) treated cells were compared with control (untreated) cells; and $^{\#\#}$p≤0.005 when GB (pre-treated for 3 hr) (20 µM) followed by $Aβ_{(25-35)}$(10 µM) treated cells were compared with only $Aβ_{(25-35)}$ treated cells; and $^{\$}$p≤0.05 when GB (pre-treated for 3 hr @ 20 µM) followed by $Aβ_{(25-35)}$(10 µM) treated cells were compared with only GB treated cells. The results are presented as mean ± standard deviation (n = 4).

Table 4.1: Measurement of ROS production in SH-SY5Y cells

Sample	**Concentration (μM)**	**ROS level (a.u)**	**% Change**
Control	-	0.4 ± 0.07	100 ± 18
Aβ$_{(25\text{-}35)}$	10	1.18 ± 0.47*	297 ± 40*
GB	20	0.34 ± 0.02	86± 6
GB + Aβ$_{(25\text{-}35)}$	20 + 10	0.22 ± 0.05$^{\#\#\ \$}$	57± 25$^{\#\#\ \$}$

The students (t) test was performed for the evaluation of the significance of the results. The data was considered statistically significant at $^*p \leq 0.05$ when Aβ (10 μM), GB (20 μM) and GB (pre-treated for 3 hr @ 20 μM) followed by Aβ (10 μM) treated cells were compared with control (untreated) cells; and $^{\#\#}p \leq 0.005$ when and GB (pre-treated for 3 hr) (20 μM) followed by Aβ (10 μM) treated cells were compared with only Aβ$_{(25\text{-}35)}$ treated cells; and $^{\$}p \leq 0.05$ when GB (pre-treated for 3 hr @ 20 μM) followed by Aβ (10 μM) treated cells were compared with only GB treated cells. The results are presented as mean ± standard deviation (n = 4).

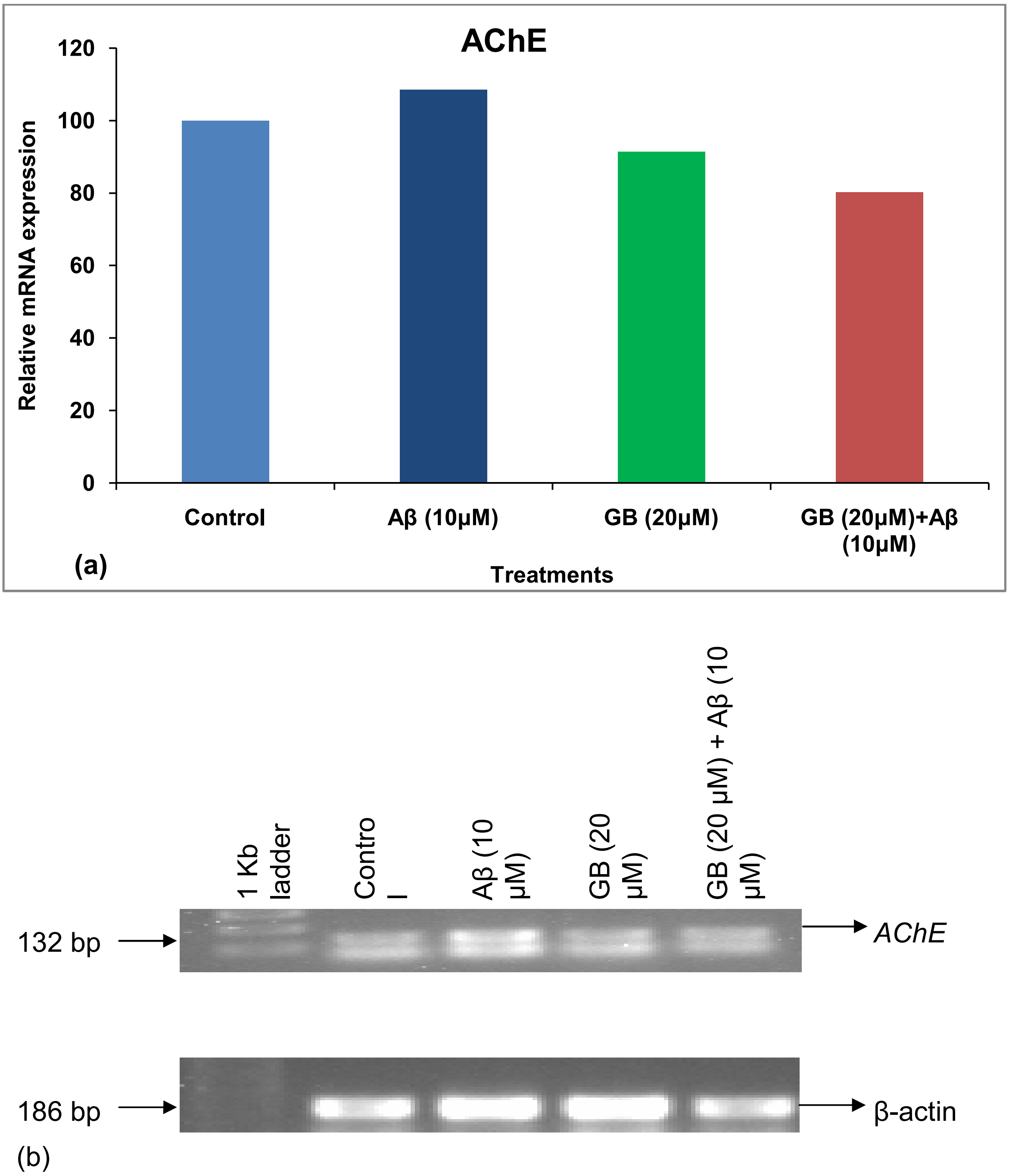


Figure 4.2:Analysis of mRNA expression of *AChE* gene in SH-SY5Y cells treated with Aβ$_{(25\text{-}35)}$ (10 μM), GB (20 μM) and GB (pre-treated for 3 hr @ 20 μM) followed by Aβ$_{(25\text{-}35)}$(10 μM) treatment for 24 hr. (a) Graphic representation as obtained by densitometric analysis of bands and (b) Agarose gel image showing bands of mRNA expression of *AChE* and β-actin sampled as house-keeping gene for normalization.

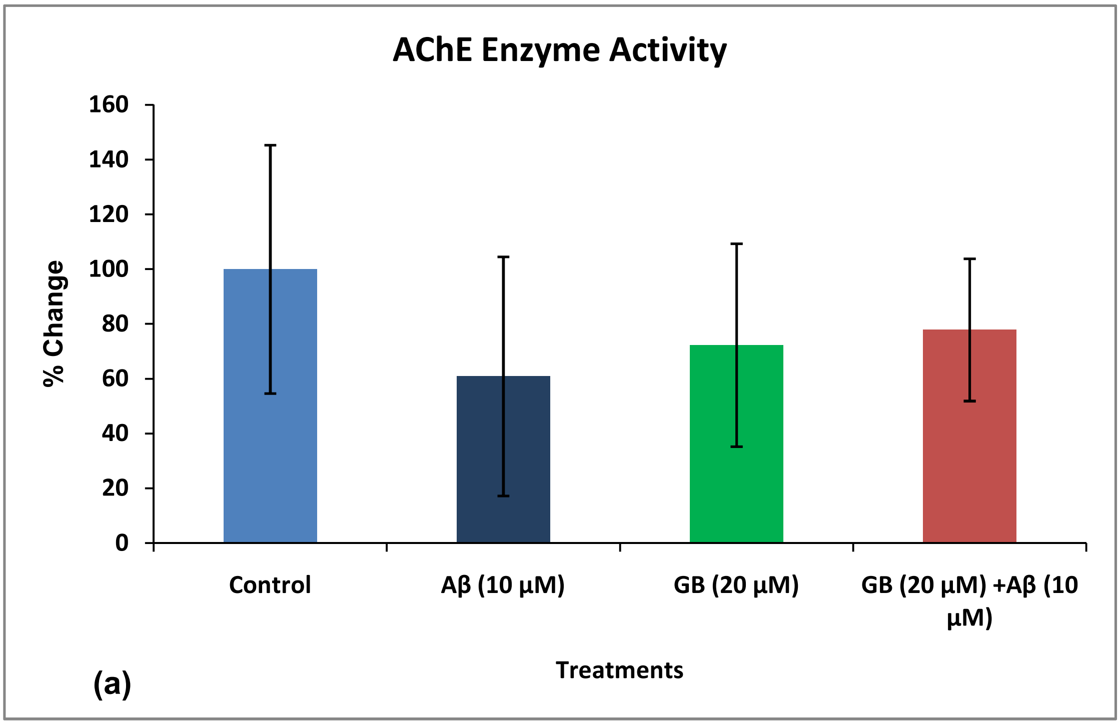
Table 4.2: Analysis of relative mRNA expression of *AChE* gene in SH-SY5Y cells

Treatments	**Concentration (μM)**	**Relative mRNA expression (%)**
Control	-	100
A$\beta_{(25\text{-}35)}$	10	108
GB	20	91
GB + A$\beta_{(25\text{-}35)}$	20 + 10	80

4.3 Acetylcholinesterase (AChE) Enzyme Activity in SH-SY5Y cells after the treatment with GB, Aβ$_{(25\text{-}35)}$ and GB + Aβ$_{(25\text{-}35)}$ for 24 hr

The change in AChE activity was analysed in different treatment groups and it was found that Aβ$_{(25\text{-}35)}$ (10 μM) treatment caused an increase by 39% in the AChE activity as compared to the control untreated SH-SY5Y whole cell lysates. GB (20 μM) treatment caused 28% decrease in the AChE activity as compared to the control whole cell lysates. The pre-treatment of GB for 3 hr and then the treatment of Aβ$_{(25\text{-}35)}$for 24 hr, caused decrease in the AChE activity by 22% as compared to the control cell lysates.

Further analysis of pre-treatment of GB for 3 hr and then treatment of Aβ$_{(25\text{-}35)}$ (10 μM) for 24 hr time point increased in AChE activity by 17% as compared to only Aβ$_{(25\text{-}35)}$ (10 μM) treated whole cell lysate of SH-SY5Y cells (**Table 4.3 and Figure 4.3**).



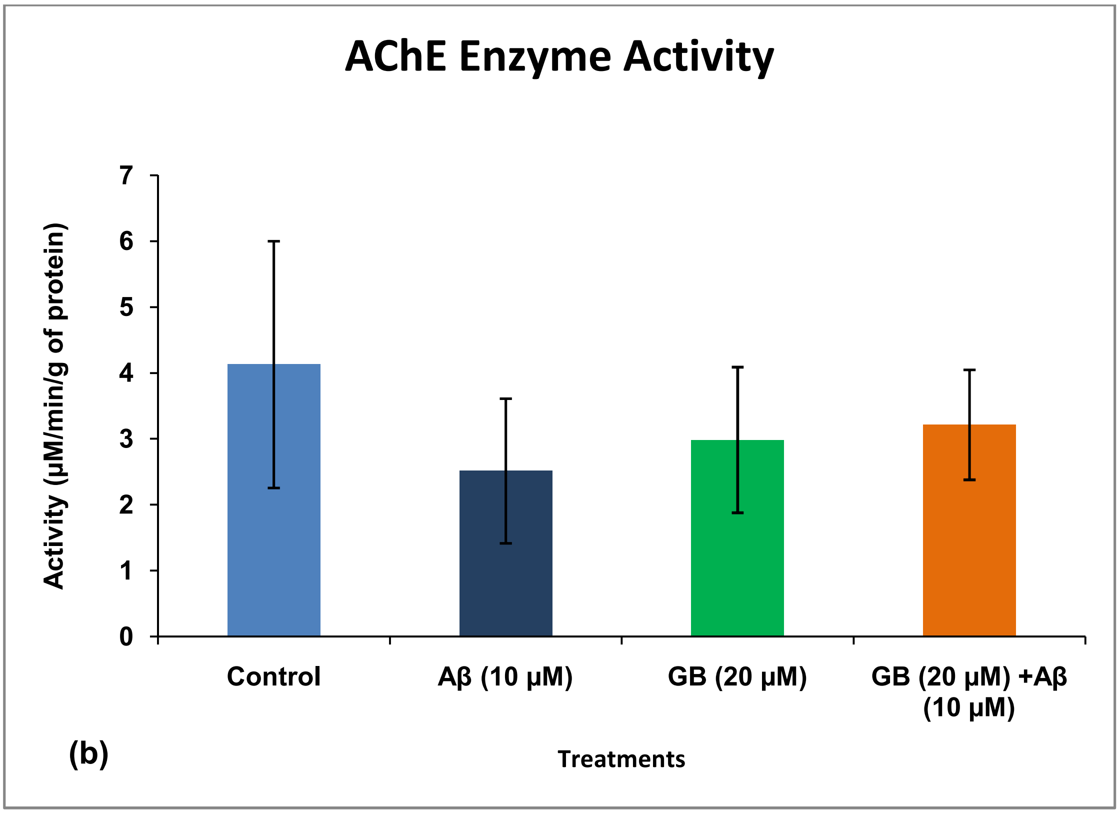


Figure 4.3: Analysis of AChE activity in whole cell lysates of SH-SY5Y cells treated with Aβ$_{(25\text{-}35)}$ (10 µM), GB (20 µM), and GB (pre-treated for 3 hr @ 20 µM) followed by Aβ$_{(25\text{-}35)}$(10 µM) treatment for 24 hr. (a) Expressed the AChE activitychange in AChE activity percentage and (b) Expressed as change in activity (µM/min/gm of protein).

Table 4.3: Acetylcholine Esterase (AChE) Activity in SH-SY5Y Cells

Sample	Concentration (μM)	AChE Activity (μmoles/min/gm)	% Change
Control	-	4.12 ± 1.87	100 ± 45
A$\beta_{(25\text{-}35)}$	10	2.51 ± 1.09	61 ± 44
GB	20	2.98 ± 1.105	72± 37
GB + A$\beta_{(25\text{-}35)}$	20 + 10	3.21 ± 0.83	78 ± 26

CHAPTER 5

DISCUSSION

Alzheimer’s disease (AD) is a neurodegenerative disorder associated with the loss of cognitive ability. The various factors associated with its occurrence are Aβ peptide accumulation, tau phosphorylation, oxidative stress, mitochondrial dysfunction and ageing. The initial accumulation of Aβ further exacerbates the stress conditions in the brain by altering multiple pathways. One includes elevation of AChE activity, which further contributes to the progression of the disease. The current study seeks to provide insight into the pathogenesis of AD, linking Aβ-induced oxidative stress, AChE activity and the role of GB and to determine how phytochemical GB affects the expression and activity of AChE in the neuronal cells to overcome the oxidative stress induced by Aβ.

Several studies have shown the oxidative or pro-oxidant nature of Aβ, as reviewed by Swomeley *et al*., (2014) and Kaur *et al*., (2015). It induces oxidative stress by various pathways, such as by reducing activity of antioxidant enzymes through protein oxidation and by reducing the cell viability by altering various pathways (Guo *et al.,* 2008; Villareal *et al.,* 2016). According to a study, the peptide fragment, Aβ$_{(25\text{-}35)}$, causes ROS production in cell free incubation assays (Hensley *et al*., 1994). In another study, Aβ$_{(25\text{-}35)}$ caused lipid peroxidation, thus pointing towards its potential in mediating oxidative stress (Butterfield *et al*., 1994). Aβ$_{(25\text{-}35)}$ has also been found to increase intracellular ROS level causing oxidative stress and decrease the activities of mitochondrial complexes thus causing mitochondrial dysfunction (Kaur *et* al., 2015). Therefore, in this study Aβ$_{(25\text{-}35)}$ was used to induce oxidative stress in human neuroblastoma(SH-SY5Y) cells.

GB is a phytochemical that has been shown to possessing anti-oxidant potential. It has been shown by several studies that it has protective effects against oxidative stress (Kaur *et al.,* 2015; Gill *et al.,* 2017). GB has been shown to decrease ROS production against Aβ$_{(25\text{-}35)}$ induced oxidative stress in SH-SY5Y cells. It has also been found to increase the activity of mitochondrial complexes and thus protected SH-SY5Y cells against Aβ$_{(25\text{-}35)}$ induced mitochondrial dysfunction (Kaur *et al*., 2015). In another study, GB reversed the effects of Aβ$_{(1\text{-}42)}$

in SH-SY5Y cells by decreasing intracellular ROS production and by decreasing oxidative DNA damage against oxidative stress induced by Aβ (Gill *et al*., 2017).

The amount of ROS produced was evaluated in untreated, Aβ$_{(25\text{-}35)}$ (10 μM), GB (20 μM), and GB (20 μM) + Aβ$_{(25\text{-}35)}$ (10 μM) treated SH-SY5Y cells in the present study. It was observed that as compared to the control, the amount of ROS produced has increased in Aβ$_{(25\text{-}35)}$ treated cells showing its oxidative nature and decreased upon pre-treatment and only GB treated cells. This points toward the oxidative potential of Aβ$_{(25\text{-}35)}$ and the protective and antioxidant nature of GB. The pre-treatment of GB for 3 hr, followed by oxidative stress induction by Aβ$_{(25\text{-}35)}$for 24 hr showed decrease in the ROS production as compared to Aβ$_{(25\text{-}35)}$ treatment alone(Bastianetto *et al*., 2000).Therefore, GB reverses the oxidative effect of Aβ$_{(25\text{-}35)}$ by inhibiting the excess ROS production. According to a study, neurons treated with GB were resistant to Aβ induced oxidative stress (Bate *et al*., 2004). GB has shown inhibitory effect in ROS production against Aβ induced oxidative stress (Kaur *et al*., 2015; Gill *et al*., 2017).

In this study, the expression of *AChE* gene at transcriptional level and its activity was assessed in untreated, Aβ$_{(25\text{-}35)}$ (10 μM), GB (20 μM), and GB (20 μM) + Aβ$_{(25\text{-}35)}$ (10 μM) treated SH-SY5Y cells. The mRNA expression of *AChE* gene in Aβ$_{(25\text{-}35)}$ treated SH-SY5Y cells was found to be increased. This is consistent with another study which showed an increase in *AChE* gene mRNA within dentate gyrus (DG) neurons of AD patients (Berson *et al*., 2007). On the other hand, the mRNA expression in GB treated SH-SY5Y cells was found to be decreased as compared to both control and Aβ$_{(25\text{-}35)}$ treated SH-SY5Y cells, suggesting the neuroprotective nature of GB. The pre-treatment of GB for 3 hr, followed by oxidative stress induction by Aβ$_{(25\text{-}35)}$ for 24 hr showed decrease in the mRNA expression of AChE as compared to Aβ$_{(25\text{-}35)}$ treatment alone. These results indicate that GB shows protective effect by decreasing the mRNA expression of *AChE* against oxidative stress conditions by its antioxidant nature. Increase in *AChE* expression has been associated with AD progression and cognitive ability, thus inhibiting *AChE* expression may play a protective role in AD pathology

(Garcia-Ayllon *et al*., 2011).In an another study, knockdown of *AChE* in SH-SY5Y cells by siRNA decreased presenilin-1 (PS1) levels against Aβ induced increase in PS1 (Silveyra *et al*., 2012). These studies suggest that inhibiting the expression of *AChE* may show neuro-protection by altering several pathways.

The present study also evaluated the relation between GB, Aβ$_{(25\text{-}35)}$ and AChE activity (a cholinesterase). It was observed that SH-SY5Y cells upon treated with GB displayed increase in the AChE activity as compared to Aβ$_{(25\text{-}35)}$ treated cell lysates, and there has also been an increase in the levels of AChE activity in GB (pre-treated) and Aβ$_{(25\text{-}35)}$ treated in combination as compared to that of Aβ$_{(25\text{-}35)}$ treatment alone. The difference in mRNA expression and activity of *AChE* may be due to its different molecular forms and its diverse location (Ozarowski *et al*., 2013).

Oxidative stress leads to oxidation of proteins, therefore the enzymes might get oxidized and their activities thus decrease under the oxidative stress conditions. In the presence of a natural antioxidant, ROS/RNS are scavenged and thus proteins are less liable to be oxidized. This may increase the activity of any enzyme in the presence of antioxidants. According to a study, Ferulic acid (an antioxidant) increases the activity of antioxidant enzymes against Aβ$_{(25\text{-}35)}$ induced oxidative stress in SH-SY5Y cells (Kaur, 2016). The activity of antioxidant enzymes decrease, but their expressions remain increased under the oxidative stress (Omar *et al*., 1999). Thus, it can be inferred that GB plays a critical role of an anti-oxidant by increasing the activity of AChE enzyme.

Oxidative stress is a major factor that contributes to the progression of AD. It leads to protein oxidation, lipid peroxidation, oxidative DNA damage and thus alters several pathways in the neurons. Cumulatively these alterations lead to neurodegeneration. In this study, Aβ$_{(25\text{-}35)}$ induced oxidative stress by increasing the ROS production, and the natural phytochemical GB showed protective effect by countering the challenges caused. Aβ, by an unknown mechanism, increases the expression of *AChE*. Simultaneously GB caused decrease in the mRNA expression of *AChE* against A$\beta_{(25\text{-}35)}$ mediated increase. The increase in the activity of AChE by GB points toward its antioxidant potential. Thus, GB shows neuroprotection by acting as an antioxidant and by inhibiting the expression of AChE against A$\beta_{(25\text{-}35)}$ induced oxidative stress. Further studies are required to elucidate the exact mechanisms which are involved directly or indirectly.

CHAPTER 6

CONCLUSIONS

Alzheimer’s disease (AD) is a progressive neurodegenerative disease which is characterised by the loss of neurons, synapses, synaptic functions and mitochondrial dysfunction, etc. The formation of neurotoxic Aβ oligomers along with the tau proteins work as the major causative factor for the pathology of this disease.

AChE a cholinergic marker is a widely investigated topic since the discovery of cholinergic deficit in the AD. Studies have shown that AChE forms a stable complex with the senile plaque components through the peripheral anionic site and increases the neurotoxic property of amyloid beta protein.

Ginkgolide B, a phytochemical, is a polyvalent agent with possible therapeutic use for the treatment of multifactorial neurodegenerative disease, like AD. The present study advocates for the neuroprotective role of GB. Finding show that the presence of GB protects the human neuronal SH-SY5Y cells against $A\beta_{(25-35)}$ induced oxidative stress by decreasing ROS production and *AChE* expression. The increase restoration in activity of AChE by GB against Aβ(25-35) induced oxidative stress, suggests the antioxidant potential of GB. Thus, based on these findings it is also possible that GB may alter other pathways as well, which are associated in the progression of AD. Further research studies may help in finding a new way for dealing with the prognosis and treatment of AD.

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