EFFECT OF AMYLOID BETA (25-35) PEPTIDE ON MITOCHONDRIAL RESPIRATORY FUNCTION IN NEURONAL CELLS OVER-EXPRESSING APE1

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BY

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CERTIFICATE

I declare that the dissertation entitled “EFFECT OF AMYLOID BETA (25-35) PEPTIDE ON MITOCHONDRIAL RESPIRATORY FUNCTION IN NEURONAL CELLS OVER-EXPRESSING APE1” has been prepared by me under the guidance of Dr. Anil K. Mantha, Assistant Professor, Centre for Biosciences, 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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ABSTRACT

Effect of Amyloid beta (25-35) on Mitochondrial Respiratory Function in Neuronal Cells Over-Expressing APE1

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Alzheimer's disease (AD) is an important public health problem which affects millions of people worldwide. The major pathological hallmarks associated with AD are the accumulation of amyloid beta (Aβ) in senile plaques and neurofibrillary tangles (NFTs) made up of hyperphosphorylated tau proteins. Accumulating evidences point towards the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of AD. Aging is considered as one of the greatest risk factor for AD. In order to maintain genome integrity, base excision repair (BER) pathway is the predominant pathway for repairing oxidized base lesions in neuronal cells. APE1 is the central enzyme of the BER-pathway, having both repair and redox activities and shown to enhance neuronal survival after oxidative stress. In my study, effect of Aβ(25-35) on mitochondrial ROS/RNS levels and activities of respiratory complexes (I, III, & IV) in neuronal cells was studied with and without ectopic APE1 expression and the neuro-modulatory role of Ginkgolide B (from leaves of *G. biloba*) was evaluated. It was seen that Aβ(25-35) increases the ROS/RNS levels in these cells which was decreased when pre-treated with Ginkgolide B (G.B) before treating with Aβ(25-35). APE1 levels were found to be decreased on treating with Aβ(25-35) and were increased on pre-treatment with G.B and subsequent treatment with Aβ(25-35). These results indicate that ectopic APE1 expression in the mitochondria of the neuronal cells might overcome the oxidative damage caused by Aβ(25-35). Also, phytochemical G.B has shown to modulate the mitochondrial complex activity upon Aβ(25-35)-induced oxidative stress and modulate the ROS/RNS levels in the presence of APE1. Further studies are needed to understand the mechanism of action of APE1 in relation to the above results, which will be carried out during my Ph.D. work.

(Navrattan Kaur) **(Dr. Anil K. Mantha)**

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(Navrattan Kaur)

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LIST OF ABBREVIATIONS

Full Form	Abbreviation Used
[3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]	MTT
Alzheimer's Disease	AD
Amyloid Beta	Aβ
Amyloid Precursor Protein	APP
Apurinic/Apyrimidinic Endonuclease-1	APE1
Base Excision Repair	BER
Bovine Serum Albumin	BSA
Concentration	conc
Degree Celsius	ºC
Dimethyl Sulfoxide	DMSO
Ethylenediaminetetraacetate	EDTA
Ginkgolide B	G.B
Hour	hr
Huntington's Disease	HD
Micro Gram	µg
Micro Litre	µl

Micro Molar	µM
Micro Unit	µU
Milli Gram	mg
Milli Litre	ml
Milli Molar	mM
Minute	min
Molar	M
Nanometer	nm
Nitric Oxide	NO
Nuclear Respiratory Factor-1	NRF-1
Optical Density	OD
Oxidative Phosphorylation	OXPHOS
Parkinson’s Disease	PD
Phosphate Buffered Saline	PBS
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Redox Effector Factor-1	Ref-1

CHAPTER - 1

INTRODUCTION

Neurodegenerative diseases represent a range of diseases affecting the central nervous system (CNS) characterized by selective neuronal vulnerability and degeneration in specific regions of the brain. This causes disabling and debilitating conditions involving either impairment of memory (dementia) or movement-related disabilities (ataxia), ultimately leading to death. The degeneration is caused due to abnormal accumulation and aggregation of proteins in specific parts of the brain intracellular or extracellular as insoluble or soluble forms (Takalo *et al.*, 2013). The exact reason as to why these proteins begin to accumulate is not known but research has revealed that this may happen due to a number of reasons, majorly due to disruption of ubiquitin-proteosome machinery, autophagy failure and oxidative stress (Keller *et al.*, 2000; Lin *et al.*, 2006; Salminen *et al.*, 2013a). Some of the common neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Amyotrophic lateral sclerosis (ALS).

AD is the most common neurodegenerative disease and leading cause of dementia in people above 65 years of age. It is a progressive brain disorder in which a person's memory, thinking ability and behaviour is affected with the deterioration worsening with time. AD is characterized by the presence of two major neuropathological hallmarks which are extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) composed of tau causing neuronal dysfunction (Irvine *et al.*, 2008). Around 27 million people are estimated to be affected by this disease (Wimo *et al.*, 2006). As per the Annual Report (2010) of Alzheimer's and Related Disorders Society of India (ARDSI), the prevalence of AD in India is said to be 1 in 20 for people above 60 years of age. The cost of treating AD is estimated to be Rs.14,700 crores, posing a great burden on the society. Also, 1 in 85 people globally are likely to be affected by the disease by 2050 (Brookmeyer *et al.*, 2007).

Although the molecular mechanism(s) underlying the AD are not understood completely, various factors have been implicated in the development of AD. Accumulating evidences point towards the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of AD and recognize them as an early event in AD

development. Aging is considered the greatest risk factor for AD and is linked to oxidative stress that causes the accumulation of somatic mutations in mtDNA over time and leads to genome instability and mitochondrial dysfunction. In order to counteract oxidative stress and maintain genome integrity, various DNA repair pathways exist, with base excision repair (BER) pathway being the predominant pathway for repairing oxidized base lesions in neuronal cells. Apurinic/apyrimidinic endonuclease 1 (APE1) is the central enzyme of the BER-pathway, having both repair and redox activities and shown to enhance neuronal survival after oxidative stress. Newer studies are revealing the role of APE1 in maintenance of mitochondrial genome repair and function. In this scenario, antioxidant-based therapy, which could reduce oxidative stress and modulate the activities of APE1, can serve as an effective treatment providing neuroprotection in AD.

In order to understand the underlying mechanisms of pathogenesis and progression of AD, a number of cell models have been developed. These include different neuronal cell lines that are commonly used for neuronal *in vitro* culture system, such as rat pheochromocytoma PC12, human neuroblastoma IMR-32 and SH-SY5Y cell lines. Further, cell transfections with wild-type amyloid-precursor proteins, tau or mutant forms of these molecules can be performed in these cell lines. By using differentiating factors like retinoic acid, these cells are directed into neuronal lineage and display synaptic structures, functional axonal vesicle transport, and express neurospecific proteins. (Nordin, 2013) (Neill *et al.*, 1994). This will help to elucidate the role of cells of a particular type, their signalling mechanisms and help to screen potent drugs having therapeutic value for effective treatment of AD.

Phytochemical *Ginkgo biloba* (*G. biloba*) has been shown to improve the oxidative phosphorylation (OXPHOS) performance in presence of Aβ-induced oxidative stress. It is shown that *G. biloba* reduces the ROS levels and increases the ATP production (Rhein *et al.*, 2010). Other phytochemicals like soy isoflavones, curcumin, resveratrol, and polyphenols (quercetin) have shown to modulate APE1/Ref-1's repair and redox activities making them effective molecules towards

prevention and treatment of cancer and degenerative diseases (Silva *et al.*, 2008; Singh *et al.*, 2010; Yang *et al.*, 2005a). But, how mitochondrial APE1 functions in the presence of *G. biloba* when oxidative stress is induced by Aβ is not known so far. It is required to elucidate the function of APE1 at the molecular level that will give insights about how APE1 helps in maintaining mitochondrial integrity and finding out new therapeutics for treating AD.

Objectives of the study:

1. To study the effect of Aβ(25-35) on mitochondrial ROS/RNS levels and activities of respiratory complexes (I, III, & IV) in SH-SY5Y and IMR-32 neuronal cells.

2. To evaluate the neuronal survival mechanism(s) through over-expression of APE1 upon Aβ(25-35)-induced oxidative stress in SH-SY5Y and IMR-32 neuronal cells.

3. To identify the neuro-modulatory role of Ginkgolide B (from leaves of *G. biloba*) on mitochondrial functions after Aβ(25-35)-induced oxidative stress in SH-SY5Y and IMR-32 neuronal cells with and without APE1 ectopic-expression.

Significance of the study:

AD is an important public health problem which affects millions of people Worldwide. The major pathological hallmarks associated with AD are the accumulation of Aβ in senile plaques and NFTs made up of hyperphosphorylated tau. Although the molecular mechanism(s) underlying the disease is not identified completely, various factors have been implicated in the development of AD. Accumulating evidences point towards the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of AD and recognize them as an early event in AD development. It is not known whether accumulation of Aβ is the cause or outcome of declining mitochondrial function in AD. This study is aimed to provide insight into

understanding the pathogenesis of AD linking Aβ-induced oxidative stress, mitochondrial dysfunction, role of APE1 and *G. biloba* towards AD-therapeutics. The study will allow us to understand how APE1 ectopic-expression affects the neuronal cells in overcoming the oxidative stress induced by Aβ(25-35) using the SH-SY5Y and IMR-32 human neuroblastoma cells. The present study will also provide insights into how the phytochemical Ginkgolide B (from leaves of *G. biloba*) may help in modulating/protecting APE1's repair activity by reducing mitochondrial oxidative stress, and further which will reduce Aβ-induced dysfunction of mitochondrial respiratory system of the neuronal cells in maintaining cellular energy demands.

Thus, from the knowledge gained with this study and combining these parameters of oxidative damage with those of redox state, DNA-repair activity and antioxidant defence may help in designing future studies and serve as a useful therapeutic tool towards the treatment of AD.

CHAPTER - 2

REVIEW OF LITERATURE

2. Alzheimer's Disease (AD)

AD is an important public health problem which affects millions of people Worldwide. It is characterized by a gradual memory loss and deterioration of cognitive functions and presence of neuritic plaques containing Aβ and NFTs containing phosphorylated-tau protein.

AD is characterized by 3 distinct major neuropathological hallmarks: intracellular NFTs, extracellular plaques and neuronal loss (Butterfield *et al.*, 2003). NFTs are aggregates of the hyperphosphorylated form of microtubule-associated protein, tau that accumulate intracellularly within the neuronal cells. The normal function of tau is to stabilize microtubules by phosphorylation and dephosphorylation of the protein in healthy neurons. But, the phosphorylated form of tau cannot bind to microtubules and instead polymerizes with other tau molecules, forming straight filaments causing a failure of neuronal transport that eventually leads to cell death. It has been shown that the number and distribution of cortical tangles correlate positively with cognitive deficits and thus, serves as a good marker of disease progression (Braak *et al.*, 1998).

The major neuropathological hallmark of AD i.e. Aβ plaque, made up of a peptide of 39-42 amino acids, accumulates extracellularly in the neurons. The Aβ peptide is produced by the sequential cleavage of amyloid precursor protein (APP), which exists in the brain predominantly in three different isoforms viz. APP695 (expressed by neurons), APP751 and APP770 (expressed in glial cells). At extracellular sites within the brain parenchyma, Aβ forms deposits due to aggregation of mostly Aβ(1–42) peptide, which are associated with dystrophic neurites, activated microglia and reactive astrocytes. It has been reported that there exists a correlation between elevated levels of total Aβ peptide in the brain and cognitive decline as seen in AD (Revett *et al.*, 2013).

The areas displaying high densities of plaques and tangles in AD patients have degenerating neurons and synapses which lead to cognitive decline in these patients.

The regions of the brain which are badly affected are cerebral cortex, temporal lobe, parietal lobe, frontal cortex, cingulate gyrus and brainstem nuclei (locus coeruleus). It is found that glutamatergic neurons (present in hippocampus) are severely affected. Likewise, there is a reduction in activity of the cholinergic neurons in AD. It has been observed that there is an increase in glutamatergic synapses and choline acetyltransferase activity in individuals with mild cognitive impairment and a subsequent reduction in the enzyme activity during AD progression. This is thought to be due an initial compensatory mechanism in AD patients that worsens as AD progresses (Francis, 2003; Revett *et al.*, 2013).

2.1. Global burden of AD

It is indicated that 26.6 million persons Worldwide are currently living with AD (11.4–59.4 million). About 43% of prevalent cases of AD need a high level of care like that of a nursing home. It is projected that by the year 2050, 1 in 85 persons would be living with AD Worldwide and the Worldwide prevalence will quadruple to 106.2 million. The increase is a result of the aging of the World's population. It is projected that by the year 2050, the World's population of persons at least or above 80 years of age will increase by a factor of about 3.7 according to United Nations (UN) population division. If the development and progression AD could be delayed by 1 year, there would be nearly 9.2 million fewer cases of the disease in 2050 (Brookmeyer *et al.*, 2007).

2.1.1. AD Statistics in relation to Indian context

It is predicted that developing countries like India and China will be worst hit by AD in the next decade. It is estimated that around 50 % of AD cases occur in developing countries and which will rise to about 70 % by 2025. According to ARDSI, about 3.7 million Indians were suffering from dementia in 2010. This number is expected to double by 2030. A very few number of studies have been conducted in relation to Indian context. Recently, a 10-year follow-up study (2001-2011) conducted in Indian population found that incidence rates per 1000 person-years for AD was 11.67 for those aged above 55 years and higher (15.54) for people above 65 years of

age. The World age standardized incidence rate was 21.61 per 100,000 in Indian population, and standardized against the age distribution for the year 2000 U.S. Census (9.19 per 1000 person-years) in people above 65 years of age (Mathuranath *et al.*, 2012). Thus, the above statistics suggest that AD incidence rates were much higher in rural north India, comparable with China, and marginally lower than that reported from the western World.

2.2. Genetic basis of AD

Genetic mutations are the known cause of early-onset familial AD with a prevalence of less than 1% (Thies *et al.*, 2011). Thus, three forms of early-onset AD that are inherited as autosomal dominant traits involving 3 genes viz. APP on chromosome 21; presenilin 1 (PS1) on chromosome 14; presenilin 2 (PS2) on chromosome 1 (Roses, 1996) are recognized. Persons with Down's syndrome (trisomy 21) are at a greater risk of developing AD (Wilcock *et al.*, 2013). Additionally, an individual's chance for developing AD increases if he inherits an *APOE* ϵ4 allele, one of the 3 common alleles (ϵ2, ϵ3, ϵ4) of *APOE* gene, from his parents and has an increased risk if two *APOE* ϵ4 alleles are inherited (Thies *et al.*, 2011). Mutations in *APP* gene, *PS1* and *PS2* genes are recognized that make a person susceptible to early-onset familial AD (Roses, 1996). Several Genome-Wide Association Studies (GWAS) have identified and implicated many genes in the etiology of AD (Chapuis *et al.*, 2013; Hollingworth *et al.*, 2011; Lambert *et al.*, 2009). Apart from the *APOE* gene, a number of new genes are implicated in the pathogenesis of late-onset AD (LOAD). Bridging integrator 1 (BIN1), also known as Amphiphysin 2, is now recognized as an important genetic risk factor after *APOE4* for LOAD (Tan *et al.*, 2013). BIN1 transcripts levels were observed to be elevated in AD brains showing BIN1 as a genetic susceptibility locus in AD (Chapuis *et al.*, 2013). Also, decreased expression of drosophila BIN1 ortholog *Amph* supressed tau-mediated neurotoxicity highlighting the role of BIN1 in mediating AD risk and its role in modulating AD pathogenesis at the level of the tau pathway (Chapuis *et al.*, 2013). Thus, BIN1 can be thought of as a target for treatment of AD. In addition to *APOE* and *BIN1*, other susceptibility gene loci identified include phosphatidylinositol-binding clathrin assembly protein

(*PICALM*), ATP-binding cassette transporter (*ABCA7*), CD2-associated protein (*CD2AP*), clusterin (*CLU*), complement receptor 1 (*CR1*), CD33 antigen (*CD33*), ephrin receptor Eph-A1 (*EPHA1*) and cluster of membrane-spanning 4A (*MS4A*) genes (Hollingworth *et al.*, 2011; Lambert *et al.*, 2009; Schellenberg *et al.*, 2012; Tan *et al.*, 2013).

2.3. Other factors affecting AD-pathology

Although the neuropathological hallmarks of AD are known, the etiological factors involved in the pathogenesis of the disease are unknown. A number of studies have indicated some risk factors for the onset of disease, advancing age being the most important risk factor. Vascular factors are one of the major risk factors, which are also modifiable and can lower the onset of dementia by practicing healthy lifestyle habits. These include alcohol consumption, smoking, obesity, high body mass index (BMI), high blood pressure. Drinking alcohol poses a threat of developing AD at a later life. Heavy drinkers who are *APOE4* ϵ4 allele carriers are at a greater risk of developing AD at a later life than moderate alcohol drinkers (Anttila *et al.*, 2004). Many studies have found association between AD and cigarette smoking. For example, *APOE4* non-carriers are at a greater risk of developing AD (Ott *et al.*, 1998). People with high blood pressure in middle age are more susceptible to develop AD at a later stage in their life (Launer *et al.*, 2000). Obesity is also a risk factor for AD (Whitmer *et al.*, 2008). Higher BMI or obesity in middle age can increase the risk of developing AD at a later life (Kivipelto *et al.*, 2005). Higher serum cholesterol levels are also associated with development of AD at a later stage (Solomon *et al.*, 2007; Solomon *et al.*, 2009). Use of statins i.e. cholesterol lowering drugs poses a lower risk of AD (Haag *et al.*, 2009). In line with this, it was seen that people having a diet rich in polyunsaturated fatty acids and fish have a lower risk of AD (Huang *et al.*, 2005) as compared to those consuming saturated fatty acids in their diet (Laitinen *et al.*, 2006; Povova *et al.*, 2012). An association between diabetes and AD has been observed. Diabetic people are more prone to develop AD, with an increased risk if diabetes occurs in middle age (Xu *et al.*, 2009).

Mild cognitive impairment (MCI) is said to be a transitional stage between aging and development of AD (Petersen *et al.*, 1999). It can be described as a condition in which a person has memory impairment though it doesn't affect a person's daily activity. It is shown that those who have MCI are at a greater risk of developing AD at a later life (Petersen *et al.*, 1999). Also, people with moderate head injuries are at an increased risk of developing dementia than those without any head injuries. Those with severe head injuries are at the greatest risk of developing dementia at a later life.

Chronic brain inflammation is associated with AD. Activated microglia and reactive astrocytes are seen in close proximity to neuritic Aβ plaques in AD brain (Meda *et al.*, 2001). Studies have shown that complement system is activated in AD brains (Strohmeyer *et al.*, 2000; Webster *et al.*, 1997). In order to clear Aβ, the activated microglia and astrocytes secrete ROS, nitric oxide (NO), proteolytic components which further increase APP production and proteolytic processing of APP thus causing neuronal dysfunction (Rubio-Perez *et al.*, 2012). β-site APP-cleaving enzyme 1 (BACE1), a membrane-bound aspartic proteinase, is primarily expressed by the neurons and is associated with the generation of Aβ peptides from APP owing to its β-secretase activity (Sinha *et al.*, 1999). BACE1 expression has been observed in reactive astrocytes in AD brains while resting astrocytes do not display BACE1 at detectable levels, thus showing that activation of astrocytes may lead to development of AD (Rossner *et al.*, 2005). Another study showed that the transcription factor (TF) NF-κB acts as a repressor in neuronal and non-activated astrocytes while NF-κB acts as activator of BACE1 transcription in activated astrocytes and Aβ-exposed neuronal cells. Also, the presence of increased level of activated astrocytes with aging is well demonstrated, which may lead to increased processing of BACE1 causing increased Aβ resulting into chronic inflammation and subsequently astrocyte activation ending up forming a feedback loop (Bourne *et al.*, 2007). Thus, inflammation plays a major role in AD-pathology.

Impairment of neuronal housekeeping and protein quality control systems are implicated in the development of AD. One of these is autophagy, which is a lysosomal degradative process involving removal of toxic proteins and preventing protein aggregation. Recent studies have indicated Beclin-1 to be involved in autophagy regulation and modulation of APP metabolism (Salminen *et al.*, 2013b). Beclin-1 expression was also seen to be reduced in AD brain (Pickford *et al.*, 2008). Rapamycin is emerging as a potential neuroprotective agent for AD and functions via enhancement of autophagy (Cai *et al.*, 2013). A recent study has shown that Aβ(1-42) induced Beclin-1 expression was upregulated by Rapamycin and the Beclin-1-dependent autophagy can prevent neuronal cell death before occurrence of AD in PC12 cells (Xue *et al.*, 2013). Inhibition of Beclin-1-dependent autophagy was shown to speed up neuronal cell death. Thus, Beclin-1 dysfunction is associated with AD. Therefore, autophagy failure is a potential factor leading to accumulation of toxic Aβ plaques and tau in AD.

2.4. Processing of APP: The Amyloidogenic and Non-Amyloidogenic Pathways

The conventional view of AD is that much of the AD pathology is due to increased load of Aβ in the brain of AD patients (the 'Amyloid Hypothesis'). The Aβ protein is central to the amyloid cascade hypothesis, the prevailing hypothesis for more than 20 years, which explains the pathogenesis of AD. Though it does not pin-point towards a specific Aβ species in the etiology of AD, but several studies have implicated soluble oligomers of Aβ, rather than monomers or insoluble amyloid fibrils, in causing neuronal dysfunction in AD.

The 37–43 amino acid Aβ is generated by proteolytic processing from its precursor, the β-amyloid precursor protein (APP) in a physiologically normal pathway. APP is a type I, single-pass transmembrane protein with large extracellular domains and belongs to a family of proteins which include amyloid precursor-like proteins (APLP1 and APLP2) in mammals and the amyloid precursor protein-like (APPL) in drosophila (O'Brien *et al.*, 2011). APP contains 40 or 42 amino acid sequence i.e. Aβ(1-40) or Aβ(1-42) and three sites for cleavage by various proteinases, which are

designated as α, β, and γ secretases (Nunan *et al.*, 2000). APP is expressed in all tissues ubiquitously and present on the plasma membrane, majority of which localizes to endoplasmic reticulum (ER), golgi apparatus (GA), mitochondria (Pagani *et al.*, 2011). Two pathways exist for processing of APP viz. amyloidogenic pathway and non-amyloidogenic pathway. In the amyloidogenic pathway, the activity of β-secretase (also known as BACE1) at the beginning of Aβ domain of APP results into a soluble N-terminal fragment (sAPPβ) and an amyloidogenic C-terminal fragment of 99 amino acids (CTF99). Further γ-secretase cleaves this C-terminal fragment to mainly Aβ(1-40) and Aβ(1-42), which may accumulate in the mitochondria and other cellular compartments affecting the cellular functions and leading to mitochondrial dysfunction and hyperphosphorylation of tau. Non-amyloidogenic pathway is the major APP processing pathway in which the activity of α-secretase generates a large soluble N-terminal fragment (sAPPβ) and a non-amyloidogenic C-terminal fragment of 83 residues (CTF83) owing to the cleavage within the Aβ domain. This C-terminal fragment is then cleaved by γ-secretase resulting into non-amyloidogenic peptide (P3) and APP intracellular domain (AICD) which are non-toxic and degraded rapidly and occurs in majority of individuals including non-demented and healthy individuals (Reddy *et al.*, 2008) as shown in **Figure 2.1**. In case of early-onset AD, mutations in *APP, PS1* and *PS2* are known to activate β- and γ-secretases leading to generation of Aβ but in case of sporadic AD, it is proposed that oxidative stress activates BACE1 which increases the production of Aβ (Reddy *et al.*, 2008). Although the mechanism of APP trafficking is not known, but APP is said to be axonally transported, endocytosed and sorted to different compartments of the cell, thus leading to Aβ generation and accumulation in different cellular compartments thus, impairing normal cellular function. A recent study pointed towards the role of Huntington associated protein 1 in regulating APP trafficking to the non-amyloidogenic pathway resulting into reduced Aβ levels (Yang *et al.*, 2012). As excessive BACE1 expression and APP processing can lead to uncontrolled production of Aβ, different regulatory mechanisms are present (Tonelli *et al.*, 2004; Zuchner *et al.*, 2005). Apart from the transcriptional control, a complex network of neurotransmitter systems and translational regulation is present. Among the different systems viz. glutamatergic,

adrenergic, serotonergic, cholinergic and dopaminergic systems; the cholinergic system is known to be affected in the early stages of AD. A study by Zuchner *et al.*, showed that downregulation of M2 acetylcholine receptor in the brains of AD patients affects a number of AD-relevant genes including BACE1 (Zuchner *et al.*, 2005).

In the amyloidogenic pathway, different lengths of Aβ are produced ranging from 37 to 43 amino acids. Aβ(1-40) is the most dominant species produced from the processing of APP. In comparison to Aβ(1-40), only a small amount of Aβ(1-42) is produced in human brain in the ratio of approximately 99 to 1. But, Aβ(1-42) is the main component of the amyloid deposits associated with AD and has a tendency to form aggregates spontaneously and thus, may form oligomers. Therefore, Aβ(1-42) is considered more neurotoxic than Aβ(1-40) (Lublin *et al.*, 2010). While both Aβ(1-40) and Aβ(1-42) are capable of forming amyloid fibrils but Aβ(1-42) is said to make the fibrils much faster than Aβ(1-40). Using ion-mobility spectrometry it was shown that when Aβ(1-40) and Aβ(1-42) are present together in a solution, the Aβ(1-40) and Aβ(1-42) monomers form dimers, trimers and tetramers. But, when present alone, Aβ(1-42) tends to form pentamers and hexamers (paranuclei) which on self-association form dodecamers, protofibrils and fibrils. Aβ(1-40) alone produces oligomer distribution upto tetramer level only. This pointed out that when present together, Aβ(1-40) inhibits the oligomerization of Aβ(1-42) and inhibits protofibril and fibril formation by Aβ(1-42). Thus, as Aβ(1-40) is the predominant species present in the human brain in ~ 10 times the level of Aβ(1-42) in a healthy human brain, the former inhibits the oligomerization of Aβ(1-42) and prevents the development of AD (Murray *et al.*, 2009). Studies have shown that mutations in *APP, PS1* and *PS2* may lead to enhanced accumulation of the more toxic Aβ(1-42) species (Gandy, 2005; Hutton, 2004; Lublin *et al.*, 2010; Vassar *et al.*, 1999).

Various studies have recognized four endogenously produced Aβ oligomeric assemblies viz. dimers, trimers, Aβ*56 and annular protofibrils (APFs) which may alter neuronal function in human and transgenic mice and have different consequences on neuronal survival (Larson *et al.*, 2012). In case of AD, soluble Aβ

monomers may form higher-order assemblies ranging from low-molecular weight oligomers (dimers and trimers) to Aβ*56, then to APFs and fibrils, which are the primary components of amyloid plaques, characteristic of AD. Taken together, recent studies provide the evidence that Aβ oligomers are the more toxic species than insoluble fibrillar deposits relevant to AD pathology.

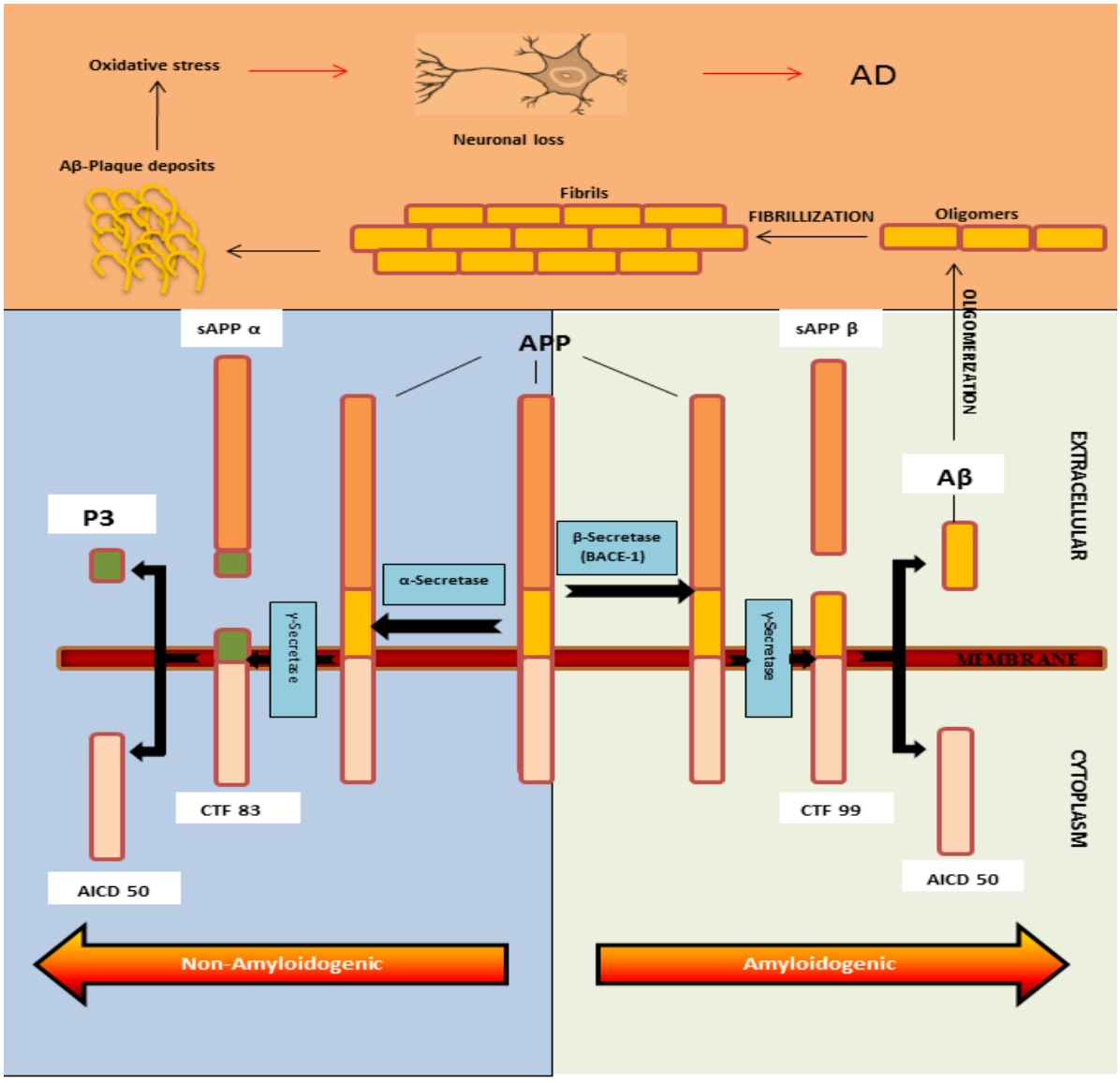


Figure 2.1. APP processing leads to the generation of cleavage products via the amyloidogenic and non-amyloidogenic pathways. Both oligomerization and aggregation of Aβ leads to the formation of senile plaques, a hallmark of AD phenotype (Kaur *et al.*, 2014).

2.6. Mitochondria and ROS

Mitochondria have an essential function in all cells. Apart from its role in ATP generation, it also controls cellular processes such as calcium (Ca^{2+}) homeostasis and apoptosis. During the process of respiration occurring in mitochondria, electrons of NADH and $FADH_2$, intermediates of the Kreb's cycle, are transferred from complex I to IV and finally to molecular oxygen. The proton gradient generated by pumping of protons from the mitochondrial matrix into the intermembrane space is utilized by complex V to generate ATP. However, upto 2% of electrons which are transferred through the respiratory chain lead to the formation of ROS [superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$)] at complex I and complex III (Starkov, 2008).

The superoxide anion might also react with other radicals such as nitric oxide (NO), producing reactive nitrogen species (RNS). NO and its derivatives (peroxynitrite, nitrogen dioxide or nitrosothiols) reversibly inhibit cytochrome oxidase in competition with oxygen (Brown, 2001). In healthy organisms, the endogenous antioxidant system such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) or glutathione peroxidase (GPx) detoxifies these harmful species. The aging process and age-associated neurodegenerative diseases, generation of high ROS/RNS levels, also described as "oxidative stress", lead to the accumulation of oxidized proteins, lipids and nucleic acids and impair cellular functions (Mattson *et al.*, 2006). Several of the proteins that are part of the respiratory chain (complex I-IV) and ATP synthase are encoded by mitochondrial DNA (mtDNA) rather than nuclear DNA. Thus, maintaining the integrity of mtDNA is essential for healthy cellular life (Trifunovic *et al.*, 2004).

When compared with other organs of the body, our brain is particularly vulnerable to oxidative stress due to three factors: **1**) high consumption of consumed oxygen; **2**) high levels of polyunsaturated fatty acids; and **3**) low extent of antioxidant defense compared to other organs (Clark *et al.*, 2010). Enhanced oxidative stress in the aging brain has been detected (Butterfield *et al.*, 1999). Elevated ROS levels in

the ageing brain has led to a theory that OXPHOS-generated ROS induce oxidative modifications of the respiratory chain and somatic mutations in the mtDNA which in turn lead to reduced OXPHOS function contributing to the pathogenesis of many neurodegenerative diseases like AD and PD (Marchi *et al.*, 2011). Linking aging with neurodegenerative diseases, the free radical theory of aging suggests that oxidative imbalance i.e. elevated levels of ROS have a role in the pathogenesis of many neurodegenerative diseases like AD (Clark *et al.*, 2010). There are a number of exogenous and endogenous sources of ROS, which increase the oxidative stress and lead to genome instability. Mitochondria are considered to be the major internal source of ROS. One study has showed that overexpression of antioxidant catalase, targeted to mitochondria reduces oxidative damage, thus highlighting the role of mitochondria as a source of these radicals (Schriner *et al.*, 2005). In line with this evidence, many studies have shown that mitochondria as a source as well as a target of ROS. The link between Aβ and free radical generation ultimately leading to increased ROS in neuronal cells has been well established (Behl *et al.*, 1994; Butterfield *et al.*, 2002; Hensley *et al.*, 1994; Manczak *et al.*, 2006).

Since post-mitotic cells like neurons of the central nervous system (CNS) are generally not replaced, thus DNA repair play an important role in maintaining brain homeostasis. ROS-induced oxidized DNA lesions represent a major type of neuronal DNA damage (Bosshard *et al.*, 2012). The O_2^- and ($OH^{\cdot}$) are produced during OXPHOS and present in high amounts in mitochondria. Apart from NO and its derivatives are also present which inhibit mitochondrial respiration. NO is mainly produced by the enzyme nitric oxide synthase (NOS), of which there are three known isoforms: nNOS [neuronal NOS], iNOS [inducible NOS], and eNOS [endothelial NOS] (Stuehr, 1999). NOS use substrates arginine, NADPH and oxygen, and produces citrulline, NADH and NO. It is shown that nM amount of NO specifically and reversibly inhibit cytochrome oxidase in competition with oxygen (Brown *et al.*, 1994). All these cause mutations in mtDNA and identification of these mutations in diseases characterized by neurological dysfunction suggests that neurons are particularly sensitive to mitochondrial dysfunction (Marchi *et al.*, 2011).

2.7. Role of mitochondria in AD-pathology

Mounting evidences are present which point to an association between aging and mitochondrial dysfunction. An earlier study pointed out that somatic mutation in mtDNA can lead to premature-onset of aging phenotypes (Trifunovic *et al.*, 2004). But it is not known whether the decline in mitochondrial function is a cause or outcome of aging. Of particular interest, a large number of evidences from studies involving experimental models and human samples suggest an association of APP and Aβ with mitochondria. In particular, a study involving a mouse model showed that Aβ, particularly Aβ(1-42), accumulates in mitochondria in the presence of a mutant APP gene and there is a decline in the activity of respiratory complexes III and IV. Also, Aβ was shown to accumulate in mitochondria very early before the extensive extracellular deposition takes place (Caspersen *et al.*, 2005). This points toward the involvement of mitochondria in pathogenesis of AD.

In addition, it is reported that age-related accumulation of somatic mutations in mtDNA can cause an increase in ROS production in the mitochondria (Greaves *et al.*, 2009; Larsson, 2010). It is known that oxidative stress induces an increased expression of BACE1 (Tamagno *et al.*, 2002; Tan *et al.*, 2013). Thus, it is speculated that with advanced age, an increase in ROS levels occur which increases Aβ levels owing to increased processing of APP by BACE1. Aβ induces production of free radicals (ROS & RNS) causing decline in mitochondrial function. Thus, a feedback loop exists in which age-related ROS levels cause increased production of Aβ and this Aβ further leads to increased production of ROS, resulting in memory impairment and cognitive decline associated with AD (Reddy *et al.*, 2012).

Several evidences point out that Aβ interacts with mitochondrial proteins and increase ROS levels finally leading to synaptic damage. Mounting evidences show that Aβ, in its soluble oligomeric form, disrupts the axonal mitochondrial transport and disturbs the mitochondrial fission-fusion balance (Pigino *et al.*, 2009; Wang *et al.*, 2010). Recently, it was shown that both monomers and oligomers of Aβ interact with mitochondrial fission protein, dynamin-related protein 1 (Drp1) and may be involved in

abnormal mitochondrial dynamics causing mitochondrial fragmentation leading to neuronal dysfunction in AD. In addition, expression levels of mitochondrial fission genes *Drp1* and *Fis1* (Fission 1) were increased and those of mitochondrial fusion genes *Mfn1* (Mitofusin 1), *Mfn2* (Mitofusin 2), *Opa1* (Optic atrophy 1) and *Tom40* were decreased in AD patients (Manczak *et al.*, 2011). This study also provided evidence that mitochondrial fragmentation may be initiated by interaction of Aβ with Drp1 causing abnormal mitochondrial dynamics, which increases as AD progresses. Thus, targeting these abnormal interactions can serve to minimize the neuronal damage caused by AD.

Poly (ADP-ribose) Polymerase-1 (PARP-1), a predominantly nuclear enzyme responsible for genome stability and transcriptional regulation, is thought to be involved in the pathogenesis of AD. Activation of PARP-1 by oxidative stress (induced by Aβ) is believed to be an early event in the pathogenesis of AD. Enhancement of PARP-1 activity and accumulation of PAR is observed in brains of AD patients (Strosznajder *et al.*, 2012). A recent study has established the role of PARP-1 in microglial activation by its interaction with NF-kB (Kauppinen *et al.*, 2011). Recently, the mitochondrial localization of PARP-1 and its interaction with mitochondrial protein, Mitofilin has been established (Rossi *et al.*, 2009). It was shown that in the absence of PARP-1, there is an accumulation of mtDNA damage, suggesting that mitochondrial PARP-1 has a role in mtDNA damage repair/signaling. Overexpression of PARP-1, Bax, p53 and altered mitochondrial function in presence of oxidative stress induced by Aβ has also been shown recently (Bayrakdar *et al.*, 2014). Additional studies are needed to ascertain the role of PARP-1 and mitochondrial dysfunction in AD pathogenesis.

2.8. Oxidative Stress and DNA Repair

Human DNA is constantly under stress by the presence of endogenous and exogenous agents that induce DNA lesions and cause DNA instability. This instability leads to blockage of DNA replication and transcription (Bohr, 2002). Different DNA repair pathways are present in the cells to maintain genomic integrity. Depending

upon the type of DNA lesion that has been generated, repair takes place by a specific pathway. Bulky lesions induced by UV light are removed by nucleotide excision repair (NER) pathway, whereas DNA lesions generated by ROS are repaired by the BER-pathway (Seeberg *et al.*, 1995). Mismatches in DNA are processed by the mismatch repair pathway (MMR) and lesions such as interstrand cross-links are removed by recombinational DNA repair (Bohr, 2002).

2.8.1. APE 1: an emerging neuroprotective enzyme

In mammals and higher organisms different organs consist of various cell types, some of them are dividing while others are non-dividing. In adults, cell types such as myocytes, adipocytes, skin cells and neurons are non-dividing cells, i.e. terminally differentiated (Crescenzi *et al.*, 1995; Iyama *et al.*, 2013). BER is the major pathway for oxidative DNA base damage caused by ROS/RNS as well as for abasic (AP) sites and single strand breaks (SSBs). APE1 is a primary BER-pathway enzyme, and responsible for repair and removal of AP sites and strand breaks (Hegde *et al.*, 2008; Hegde *et al.*, 2012; Mantha *et al.*, 2013).

Human *APE gene* (~3 kb in size) is localized on chromosome 14q11.2-12 and consists of four introns and five exons (Fritz, 2000). The human APE *cDNA* is about 1.4 kb in length and encompasses a coding region of 954 nucleotides and encodes a protein comprising of 318 amino acids. APE1 is abundant (~10^5 copies per cell) in eukaryotic cells and has a relatively long half-life [~8 hr] (Tell *et al.*, 2000). APE1 is a dual function protein. Its C-terminus displays repair activity and its N-terminal contains a bi-partite nuclear localization signal, NLS (Barnes *et al.*, 2009; Bhakat *et al.*, 2009; Zaky *et al.*, 2008) and displays redox activity responsible for transcriptional regulation through redox based mechanisms (Bhakat *et al.*, 2009; Jiang *et al.*, 2009; Tell *et al.*, 2009).

2.8.2. Role of APE1 in oxidative DNA damage repair

Two isoforms of APE, APE1 and APE2, have been illustrated in mammalian tissues (Hanna *et al.*, 2004). APE1 is the main AP endonuclease in mammalian cells

taking part in the BER processes both in nucleus and mitochondria (Hanna *et al.*, 2004). BER, an evolutionarily conserved process, is responsible for repairing most endogenous lesions like oxidized bases, AP sites and SSBs in both nuclear DNA and mitochondrial DNA. The basic BER-pathway involves enzymes viz. DNA glycosylase, APE1, DNA polymerase and DNA ligase. APE1 has both repair and redox activity that is involved in signal transduction. APE1 participates in several important cellular mechanisms such as apoptosis, proliferation, differentiation functioning as a transcriptional co-activator (Bhakat *et al.*, 2009b). Attempts to generate APE1-null mice were not successful and lead to an early-embryonic death (Izumi *et al.*, 2005; Xanthoudakis *et al.*, 1996). Further attempts to generate cell lines from APE1-null embryos failed, showing the essentiality of APE1 in maintaining cell viability. A study pointing towards the role of APE1 in neuronal cell survival showed that overexpression of APE1 in hippocampal and sensory cells exposed to H_2O_2 lead to an increase in cell viability (Vasko *et al.*, 2005). Up-regulation of APE1 in cerebral cortical region of AD patients was also seen (Davydov *et al.*, 2003). An immunohistochemical study pointing towards the role of APE1 in regulating cellular response towards oxidative stress showed that increased nuclear expression of APE1 is present in cerebral cortical regions of AD patients (Marcon *et al.*, 2009). Another study showed the colocalization of APE1 with Aβ in the senile plaques in AD hippocampus (Tan *et al.*, 2009). This study also showed that varying concentrations of Aβ(1-42) regulates APE1/Ref-1 expression, thus pointing towards the neuroprotective role of APE1 in response to oxidative stress. A number of evidences point towards the role of cyclin-dependent kinase 5 (Cdk5) in mediating neuronal loss. In line with this, it was shown that Cdk5 complexes with p35 and phosphorylates APE1 at Thr232, causing reduction in APE1's endonuclease activity and leading to accumulation of DNA damage and neuronal loss (Huang *et al.*, 2010).

2.8.3. APE1- a neuronal hub protein

APE1's key role in the BER-pathway and in trans-activation of various TFs is well documented but less is known about the signaling pathways through which APE1 executes its neuronal functions. A recent study of Mantha *et al.,* has identified several

key neuronal proteins those are involved in various cellular functions are interacting with APE1 in response to Aβ(25-35)-induced stress in PC12 and SH-SY5Y cells (Mantha *et al.*, 2012). APE1 interacting proteins that were differentially expressed after treatment of PC12 neuronal cells with Aβ(25–35) peptide included cytoskeleton elements such as tropomodulin 3 (Tmod3) and the tropomyosin alpha-3 chain; energy metabolism proteins such as pyruvate kinase M2 (PKM2); *N*-acetyltransferase; sulfotransferase1c; and stress-responsive proteins such as leucine-rich and death domains, anti-NGF 30 and heterogenous nuclear ribonucleoprotein-H1 (hnRNP-H1) (Mantha *et al.*, 2012). Most of these APE1-associated proteins are involved in intracellular signaling regulating cell growth thus, it suggests that this could be due to an adaptive cellular response to the stress generated by exposure to Aβ(25–35) peptide. The increased association of various cellular proteins with APE1 suggests that it may play a role in neuronal protection against endogenous/exogenous oxidative stress via its DNA repair function and also by its transcription factor-activation function.Thus, it can be interpreted that APE1 has a role in providing protection against oxidative stress and helps to maintain cellular redox balance. It can be interpreted that APE1 has a major role in overcoming the oxidative stress and maintaining neuronal cell viability and integrity.

2.8.4. Mitochondrial APE1

Role of mitochondrial APE is unclear till now. It is still debated whether the mitochondrial APE is an N-terminal truncated product of APE1 (Mitra *et al.*, 2007). The deletion of the N-terminal residues containing the nuclear localization signal (NLS) has been described to induce a 3-fold increase in the specific activity of APE1 (Chattopadhyay *et al.*, 2006) and APE1 activity is higher in mitochondrial fractions than in nuclear fractions. As mitochondrial genome is small, most of the mitochondrial proteins are encoded by the nuclear genome and transported to mitochondria. The transcriptional activity of nuclear respiration factor 1 (NRF1), was elevated after H_2O_2-induced oxidative stress and its downstream mitochondrial target genes were upregulated (Li *et al.*, 2012). The activation of NRF1 DNA-binding activity by APE1 was demonstrated in a study showing that nuclear APE1 modulates nuclear-encoded

mitochondrial-related genes by regulating NRF1 activity in a redox-dependent manner (Li *et al.*, 2012). Thus, it is indicated that in response to oxidative stress, APE1 modulates the expression of some nuclear-encoded mitochondrial genes and consequently modulates mitochondrial functions.

2.8.5. Mitochondrial BER

BER in the mitochondria helps to cope up with the oxidized DNA lesions generated due to the presence of free radicals and thus maintains mtDNA stability. The basic mechanism by which mitochondrial BER (mtBER) acts remain the same as that of nuclear BER, but some specific BER enzymes are present in the mitochondria and these are coded by nuclear genes (Bohr, 2002). For a very long time, it was considered that only short patch BER (SN-BER) occurs in the mitochondria which include removal of a DNA lesion and incorporation of a single nucleotide. But, now it is believed that owing to the rate at which oxidized base lesions are generated in mtDNA, long patch BER (LP-BER) may also take place in mitochondria (Akbari *et al.*, 2008; Hegde *et al.*, 2011; Mantha *et al.*, 2013; Szczesny *et al.*, 2008).

The first step of mtBER pathway involving recognition of a damaged base is performed by a DNA glycosylase. Two monofunctional DNA glycosylases (M-DG) i.e. uracil-DNA glycosylase 1 (UNG1) and MutY homolog (MYH); four bifunctional DNA glycosylases (B-DG) viz. 8-oxoguanine DNA glycosylase (OGG1), endonuclease III-like 1 (NTH1), *Nei*-like-1 (NEIL1), and *Nei*-like-2 (NEIL2) are present in the mitochondria (Alexeyev *et al.*, 2013; Banerjee *et al.*, 2011). Next step of the mtBER-pathway involves processing of the AP sites by APE1, which is the main AP endonuclease of the mammalian cell and this mtAPE1 is believed to be an N-terminal truncated product of APE1. It is also shown that deletion of the 33 N-terminal residues increases the specific activity of mtAPE1 by 3-folds (Chattopadhyay *et al.*, 2006). The next step involves insertion of correct nucleotides by DNA pol γ which is followed by ligation of single-strand nick by DNA ligase III. This mitochondrial ligase III is derived from *LIG3* gene and is known to be independent of X-ray repair cross-complementing protein 1 **(**XRCC1) while the nuclear variant of DNA ligase III interacts with XRCC1

(Gredilla *et al.*, 2010; Lakshmipathy *et al.*, 2000). XRCC1 acts a SSB sensor protein and acts as a scaffold for BER proteins for SSB repair (Hegde *et al.*, 2008). XRCC1 also interacts and stimulates APE1 (Vidal *et al.*, 2001).

A recent study has shown that EXOG, a 5'-exo/endonuclease, is an essential component of BER/SSBR-pathway unique to the mitochondria and forms complex with APE1, DNA pol γ, DNA ligase III and involved in repairing endogenous SSBs in the mtDNA. Also, it was shown that depletion of EXOG increases ROS levels and induces apoptosis in normal cells (Tann *et al.*, 2011). 5' flap-endonuclease-1 (FEN1), part of DNA replication machinery, is shown to displace and cleave this 5' blocking group along with 4-6 nucleotides as a single stranded DNA flap. FEN1 is involved in repairing oxidative DNA damage via LP-BER in the mitochondria (Liu *et al.*, 2008). Cellular death and embryonic lethality in presence of gamma radiation-induced DNA damage was observed in *FEN1* gene knockout mice (Larsen *et al.*, 2003). The hDNA2, possessing nuclease, helicase and ATPase activities, is also involved in DNA replication and repair in the mitochondria. The hDNA2 forms a complex with pol γ and stimulates the polymerase activity. It is also involved in RNA primer removal during mtDNA replication. The hDNA2, owing to its nuclease property, can also process flap LP-BER intermediates. This points towards the synergistic roles of FEN1 and hDNA to process the 5'flap intermediates during DNA replication and repair in the mitochondria (Zheng *et al.*, 2008). Thus, all through these years we have gained knowledge about some of the repair pathways occurring in the mitochondria and identified different repair enzymes present in the mitochondria but much more needs to be understood towards establishing patho-physiologies which overtakes these repair processes.

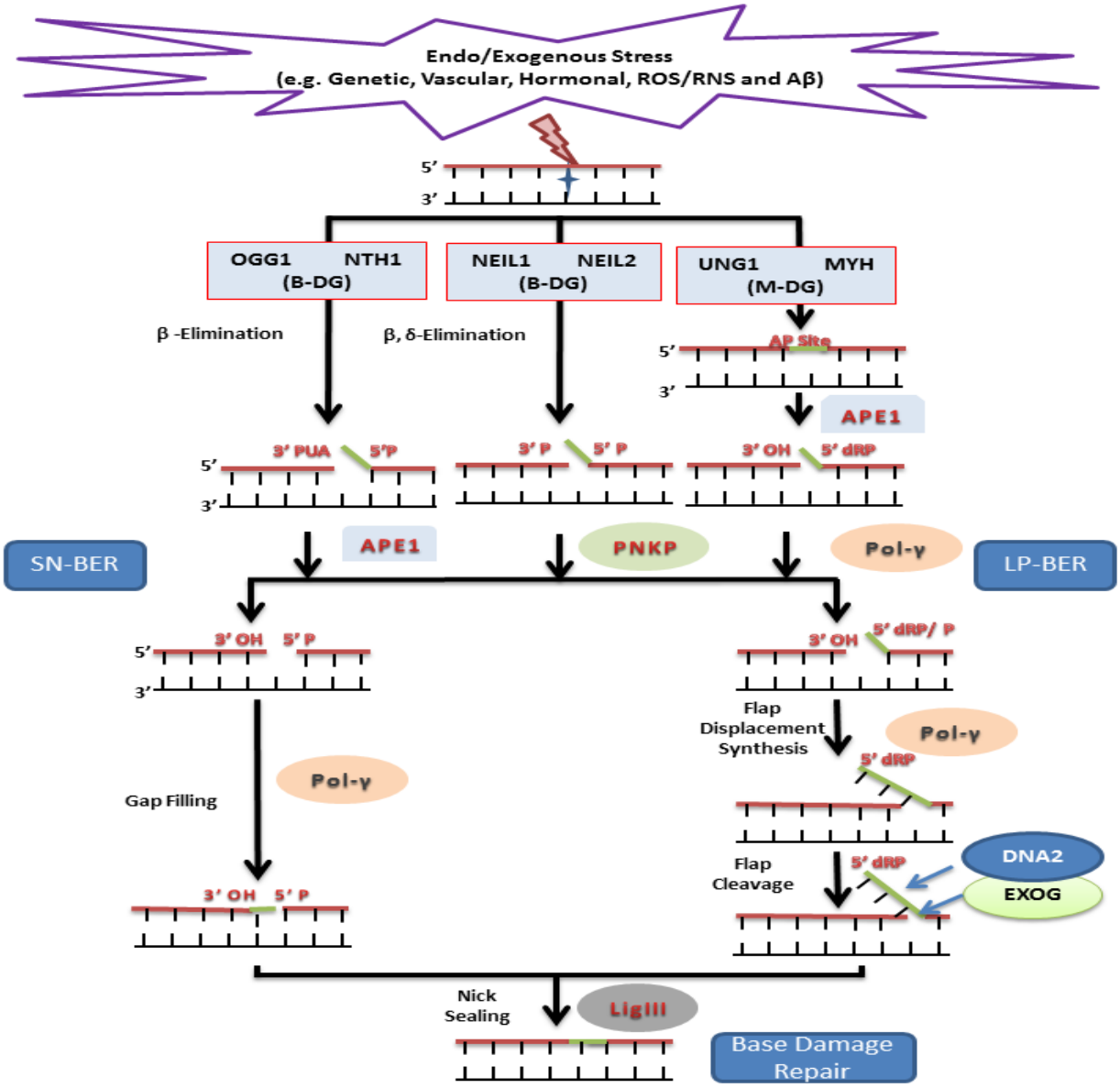


Figure 2.2. The basic mechanism of mitochondrial-BER (mtBER) remains the same as nuclear-BER pathway, but some specific enzymes are present in the mtBER pathway (Kaur *et al.*, 2014).

2.8.6. Phytochemical modulation

Human beings have used plant extracts for centuries for treating various types of ailments. As oxidative stress and mitochondria-derived oxidative stress is thought to play a role in pathogenesis of AD, a number of studies have shown that antioxidants may prevent or cure development and progression of AD. For the last few years scientists are trying to figure out the active ingredients present in the plant extracts responsible for the specific action and identify the molecular mechanism(s) by which these phytochemicals exert their action. Phytochemicals like resveratrol, isoflavones, curcumin, decursin, EGCG, L-carnitine and *Ginkgo biloba* extract have shown potent properties against neuronal disorders. It has been shown that resveratrol, a phytoalexin and a polyphenolic compound found in the seeds and wine made from grape cultivars, exerts protective effects against Aβ-induced neurotoxicity in rat hippocampal cells with the involvement of PKC (Han *et al.*, 2004). In addition, resveratrol oligomers from *V. amurensis* were shown to rescue Aβ-mediated oxidative stress in PC12 cells by inhibiting ROS production (Jang *et al.*, 2007). Resveratrol is also shown to regulate the redox activity of APE1/Ref-1 and is identified as a potent APE1 inhibitor (Yang *et al.*, 2005), upregulating AP-1 and NF-κB DNA binding activities, highlighting its anti-melanoma potential (Yang *et al.*, 2005b).

Curcumin is the main active flavonoid derived from the rhizome of *Curcuma longa* (Zingiberaceae). Curcumin has potent anti-inflammatory property due to its antioxidant activity resulting in the scavenging of the ROS generated inside the body under stress conditions (Kunchandy *et al.*, 1990). Curcumin owing to its antioxidant, anti-inflammatory action suppresses the oxidative damage and decreases amyloid deposition (Zhang *et al.*, 2010). Curcumin has a strong ability to cross BBB, and shown to reduce aggregation of Aβ(1-40) and cause disaggregation of Aβ(1-40). In addition, curcumin prevented Aβ(1-42) oligomer formation and toxicity, making it an effective molecule for prevention and treatment of AD (Yang *et al.*, 2005). Thus, this curry spice has a great potential in alleviating oxidative stress and improving cognitive decline in AD.

Soy isoflavones which include genistein, daidzein and glycitein, are the major flavonoids found in soybean, which have been a traditional food in Asia for a very long time and has potent neuroprotective abilities. A study showed that isoflavones, specifically genistein and glycitein have an anti-fibrillization, anti-oligomerization and fibril destabilizing potential on Aβ(1-40) and Aβ(1-42) *in vitro* and glycitein, in particular, binds directly to Aβ monomers, oligomers and fibrils and exhibit highest affinity for Aβ(25-35) (Hirohata *et al.*, 2012). An earlier study showed that genistein could attenuate the oxidative stress induced by Aβ(25-35) and reduce the ROS levels, and inhibit cell apoptosis possibly through Nrf/HO-1 signal pathway in PC12 cells (Bagheri *et al.*, 2011). Thus, isoflavones can be employed towards effective therapy to directly target amyloid assemblies for the treatment of AD.

The consumption of green tea and incidence of dementia, AD and PD are inversely correlated (Hu *et al.*, 2007; Mandel *et al.*, 2008). Numerous animal model studies have suggested that EGCG exerts neuroprotective effects against age-related cognitive decline and neurodegenerative diseases. EGCG has emerged as a mitochondrial restorative compound which was demonstrated to restore MMP, ROS levels and ATP levels in a double transgenic mouse model of AD. Thus, EGCG was shown to lessen the Aβ-induced mitochondrial dysfunction, which is implicated during the onset and progression of AD (Dragicevic *et al.*, 2011). These studies point out that EGCG, owing to its anti-amyloidogenic and mitochondrial restorative property, has a tremendous potential in AD therapy.

G. biloba is an herbal compound being used in traditional Chinese medicine for thousands of years to treat a variety of ailments. It has been shown to reduce memory loss, enhance brain activity and to slow down the degenerative effects of AD (DeKosky *et al.*, 2008). The clinical studies have demonstrated that extracts of *G. biloba* provide therapeutic benefits to AD similar to prescription drugs such as Donepezil or Tactrin. The ginkgolides present in *G. biloba* possess activities relevant to the disease mechanisms in AD such as antioxidant, neuroprotective and cholinergic activities according to the studies conducted by Medical Research Council

of New Castle General Hospital, USA (Perry *et al.*, 1999). An earlier study showed that *G. biloba* extract (EGb 761) protects and rescues hippocampal cells against nitric oxide-induced toxicity, thus EGb 761 acts as NO scavenger with neuroprotective abilities (Bastianetto *et al.*, 2000). Various clinical studies have indicated that 3-to 6-month treatment with 120-240 mg of *G. biloba* has produced significant effect in AD patients except for few reports of bleeding complications, nausea, vomiting (Mahadevan *et al.*, 2008). Another study showed that EGb 761 and its constituents Ginkgolide B have protective effects against Aβ(1-42)-induced toxicity in SH-SY5Y cells (Shi *et al.*, 2009). A study provided evidence that *G. biloba* improved metabolic energy pathways and improved OXPHOS performance and restore Aβ induced mitochondrial failure (Rhein *et al.*, 2010). Very few antioxidants have been shown to increase OXPHOS performance other than *G. biloba.* Thus, *G. biloba* exhibits positive effects on the mitochondrial function, provides stabilization of mitochondria and has free radical scavenging properties, in addition to its neuroprotective abilities.

It is a prerequisite for the neuronal cell to counter the oxidative stress responses elicited by different agents and mechanisms. APE1-mediated intervention along with phytochemicals, thus, emerges a new field of study to tackle the AD. The present study is aimed to provide insights into a new research molecule(s) to help in designing/screening of potential molecules as therapeutics for AD and other degenerative diseases. This will help to elucidate the function of APE1 at the molecular level that will give insights about how APE1 helps in maintaining mitochondrial integrity and finding out new therapeutics for treating AD.

CHAPTER - 3

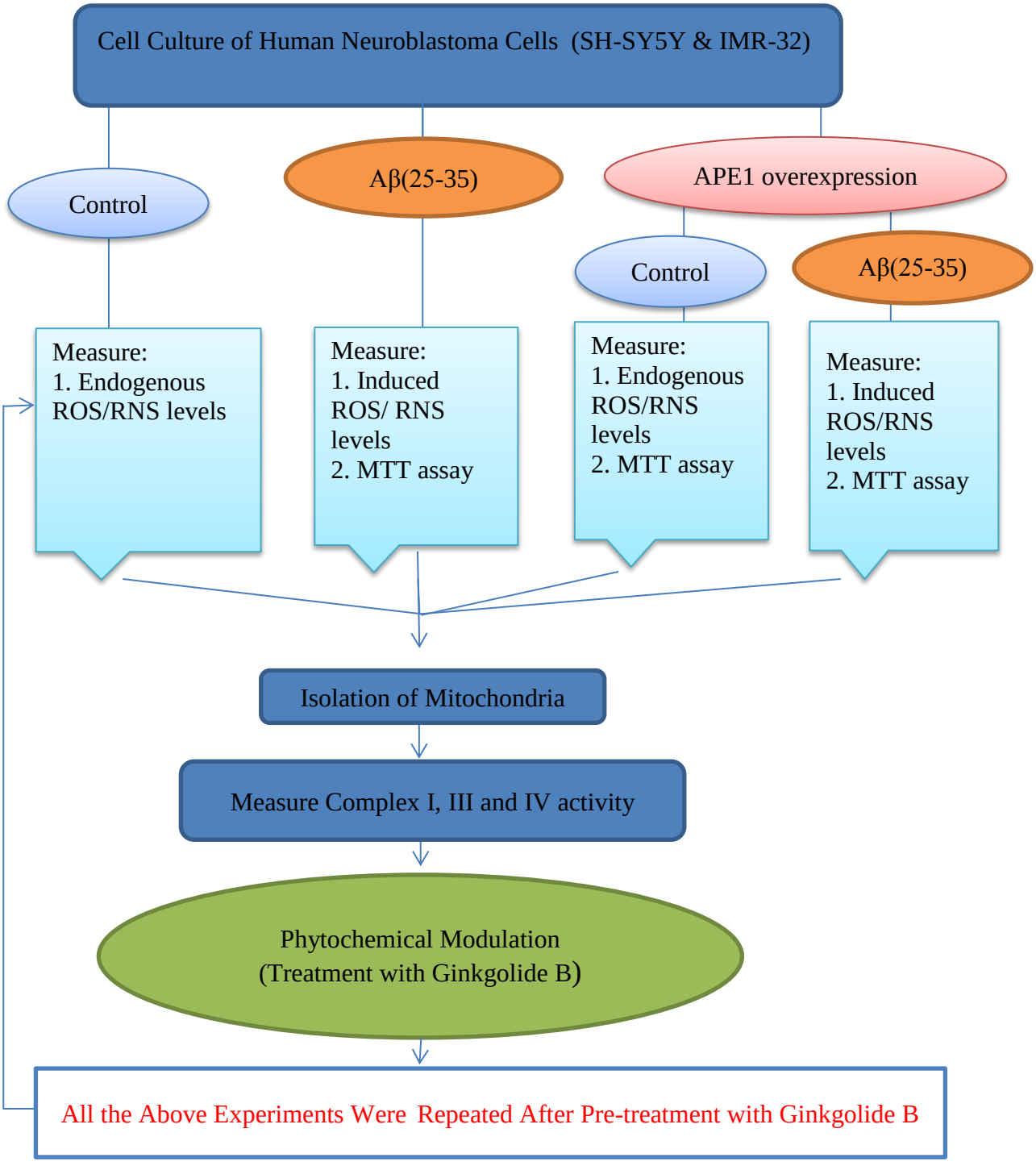
MATERIALS AND METHODS

3.1. Culturing of Human Neuroblastoma (SH-SY5Y) Cells. Human neuroblastoma SH-SY5Y cells generously gifted by Prof. Pankaj Seth, National Brain Research Centre (NBRC), Manesar were grown at 37 °C in complete medium: (1:1) DMEM/Ham's F12 supplemented with 10% fetal bovine serum (FBS)/10% horse serum (HS) and 1% penicillin-streptomycin (PS) and were maintained under 5% CO_2 atmospheric pressure as described earlier (Mantha *et al.*, 2012). When the cells became 70-80 % confluent, culture medium was removed and discarded. Then the cells were rinsed with phosphate buffered saline (PBS) to remove all traces of serum that contains trypsin inhibitor. After that, trypsin-EDTA 0.25 % (w/v) solution was added to the flask. It was then allowed to incubate until cells detached from the surface. Complete medium was added afterward for further culturing of the cells. Appropriate aliquots of the cell suspension were poured onto new culture flasks and maintained at 37 ^{0}C in a 5 % CO_2 humidified incubator, with few modifications (Mantha *et al.*, 2012).

3.2. Culturing of Human Neuroblastoma (IMR-32) Cells. The IMR-32 neuroblastoma cells procured from National Centre for Cell Science (NCCS), Pune were grown in DMEM medium containing 10 % FBS and 1% PS and were maintained at 37 ^{0}C in a 5 % CO_2 humidified incubator. When the cells became 70-80 % confluent, culture medium was removed and discarded. Then the cells were rinsed with PBS to remove all traces of serum that contains trypsin inhibitor. After that, trypsin-EDTA 0.25 % (w/v) solution was added to the flask. It was then allowed to incubate until cells detached from the surface. DMEM containing 10 % FBS and 1 % PS was added afterward for further culturing of the cells. Appropriate aliquots of the cell suspension were poured onto new culture flasks and maintained at 37^0 C in a 5 % CO_2 humidified incubator, with few modifications (Mantha *et al.*, 2012).

3.3 Preparation of Aβ(25-35). Aβ(25-35) peptide (GenScript) stock solutions were prepared freshly each time before treatment at 1000 µM in double-distilled deionized water and considered as soluble form (Mantha *et al.*, 2012).

EXPERIMENTAL APPROACH



3.4. Induction of Oxidative Stress. The SH-SY5Y and IMR-32 cells were seeded on 96-well plate at density of $1x10^5$ cells per well in complete media as described earlier with few minor modifications (Mantha *et al.*, 2012). The plates were incubated at 37º C in a humidified and sterile atmosphere containing 5 % CO_2 for 12 hr. After incubation, the medium containing serum was discarded, and new serum-free medium was added to each of the wells and incubated at 37º C in a sterile atmosphere containing 5 % CO_2 for 24 hr. Afterwards, treatment with Aβ(25-35) peptide at a range of 0-80 µM in the respective serum-free medium was done in both SH-SY5Y and IMR-32 cells. Then, the plates were incubated at 37º C in a humidified and sterile atmosphere containing 5 % CO_2 for 24 hr. Based on the findings, 20 µM was selected for further experimentation.

3.5. Treatment with Ginkgolide B. The SH-SY5Y and IMR-32 cells were seeded on 96-well plate at density of $1x10^5$ cells per well in complete media as described earlier with few minor modifications (Mantha *et al.*, 2012). The plates were incubated at 37º C in a humidified and sterile atmosphere containing 5 % CO_2 for 12 hr. After incubation, the medium containing serum was discarded, and new serum-free medium was added to each of the wells and incubated at 37º C in a sterile atmosphere containing 5 % CO_2 for 24 hr. Afterward, treatment with Ginkgolide B (Sigma Aldrich) in the range of 0-100 µM in the respective serum-free medium was done in both SH-SY5Y and IMR-32 cells (Ahlemeyer *et al.*, 2003). The plates were then incubated at 37º C in a humidified and sterile atmosphere containing 5 % CO_2 for different time points 12, 24, 48 and 72 hr. Based on the findings, 20 µM was found to be the optimum concentration for further experimentation.

3.6. Cell Viability Assay

Principle: The MTT assay was first described by Mosmann in 1983. MTT [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on cleavage of tetrazolium rings of pale yellow MTT by mitochondrial dehydrogenase enzyme from viable cells resulting in formation of dark blue formazan crystals. The formazan crystals get accumulated in healthy cells as they are impermeable to the cell

membrane. So, these are then solubilised using 0.6% acidified DMSO. The number of surviving cells is directly proportional to the level of formazan product formed measured at 570 nm.

Assay: SH-SY5Y and IMR-32 cells which were seeded on 96-well plate at density of $1x10^5$ cells per well and given treatments with Aβ and Ginkgolide B as indicated were assessed for cell viability using MTT to a final concentration of 0.5 mg/ml. On completion of respective incubation time of treatment, 10 µl of MTT (5 mg MTT dissolved in 1 ml PBS) was added to each well containing 100 ul of medium and incubated for 3 hr in dark at 37 ºC. After 3 hr, the formazan crystals formed were solubilised by adding 100 µl of 0.6% acidified DMSO to each of the wells (0.6 ml acetic acid per 100 ml of DMSO). The optical density of each well was measured at a wavelength of 570 nm using BioTek microplate reader according to the protocol described earlier (Dhiman *et al.*, 2012).

3.6. Measurement of ROS levels

Principle: The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (also known as dichlorofluorescin diacetate) is used as an indicator for ROS in cells. H_2DCFDA is a nonfluorescent chemically reduced form of fluorescein which is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) due to the cleavage of the acetate groups by intracellular esterases and oxidation. The H_2DCFDA fluorescence can be detected by a microplate reader at excitation wavelength of 478 nm and emission wavelength of 518 nm.

Assay: The treated and control SH-SY5Y and IMR-32 cells (1×10^5), cultured in 96-well plates were equilibrated in PBS and incubated in the dark for 30 min with 100µM of H_2DCF-DA (Invitrogen) [Ex_{478nm}/Em_{518nm}]. After washing twice with PBS, fluorescence was then read at the excitation wavelength of 478 nm and emission wavelength of 518 nm using BioTek Microplate reader as per the protocol described (Dhiman *et al.*, 2011).

3.7. Measurement of RNS levels

Principle: The reagent 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) is used to measure the intracellular RNS levels in the cells. DAF-FM, which is essentially nonfluorescent and cell-permeant, is used to detect and quantify low concentrations of NO in cells. When it encounters NO inside the cells, it reacts with it to form a fluorescent benzotriazole. The DAF-FM fluorescence can be detected by a microplate reader at excitation wavelength of 478 nm and emission wavelength of 515 nm.

Assay: The treated and control SH-SY5Y and IMR-32 cells (1×10^5), cultured in 96-well plates were equilibrated in PBS and incubated in the dark for 30 min with 20µM of DAF-FM (Invitrogen) [Ex_{478nm}/Em_{515nm}]. After washing twice with PBS, fluorescence was then read at the excitation wavelength of 478 nm and emission wavelength of 515 nm using BioTek microplate reader as per the protocol described (Keil *et al.*, 2004).

3.8. Cell Transfection. SH-SY5Y and IMR-32 cells were grown overnight in 96-well plates and 100 mm^2 dishes. Then the cells plated in 96-well plates were transiently transfected with 0.2 µg of plasmid DNA per well containing C-terminally FLAG-tagged full-length APE1 and 0.2 µg of empty vector using Lipofectamine 2000 (Invitrogen). The cells in 100 mm^2 dishes were transiently transfected with 2 µg of plasmid DNA per plate containing C-terminally FLAG-tagged full length APE1 and 2 µg of empty vector per plate using Lipofectamine 2000 as described earlier (Mantha *et al.*, 2012). At 6 hr after transfection, the cells were transferred to complete medium for 18 hr. Afterward, the cells were given treatments as indicated for 24 hr, followed by determination of cell viability, ROS/RNS levels and isolation of mitochondria.

3.9. Western Blotting. APE1 ectopic-expression in SH-SY5Y and IMR-32 cells after transfection was confirmed by Western blotting. Firstly, whole-cell extracts (WCE) were prepared from SH-SY5Y and IMR-32 cells using NP40 buffer (0.1% Nonidet P40, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, and protease inhibitors). 100 µg of WCE

prepared from control cells and cells ectopically expressing APE1 were separated by SDS electrophoresis. After transferring the proteins onto a nitrocellulose (NC) membrane overnight at 4 °C, 40 V, the membranes were blocked for 1 hr with 5% non-fat dry milk (NFDM) in 50 mM Tris-HCl (pH 7.4), containing 150 mM NaCl and 0.05% Tween 20 (TBST). The membrane was incubated with the mouse anti-APE1 mAb (1:2000) in 5% milk for overnight, and then washed 3 times for 15 min in TBST. The membrane was further incubated in secondary horseradish peroxidase-conjugated mouse Ab (1:3000) in the blocking buffer for 1 hr, then washed three times in TBST for 15 min. ECL reagent was used as chemiluminescent substrate followed by imaging and data analysis (Mantha *et al.*, 2012).

3.10. Composition of Lysis Buffer for Isolation of Mitochondria

The recipe of the lysis buffer contained the following at a final concentration dissolved in autoclaved water.

- 75 mM NaCl
- 1 mM NaH_2PO_4
- 8 mM Na_2HPO_4
- 250 mM Sucrose
- 0.05 % Digitonine
- Protease Inhibitor Cocktail (Sigma)

3.11. Isolation of Mitochondria. The control and treated SH-SY5Y and IMR-32 cells seeded in 100 mm^2 dishes were trypsinised and centrifuged at 2000 rpm for 10 min. The resulting cell pellet was resuspended in ice-cold lysis buffer as described above for 30 min on ice with vortexing after every 10 min. The cell suspension was centrifuged at 800 g for 10 min at 4 °C. The pellet was discarded, and the supernatant was again centrifuged at 20,000 g for 15 min at 4 °C. The resulting supernatant contained the cytosolic fraction while pellet contained the mitochondria. The mitochondrial pellet was resuspended in PBS and stored at 4 °C until use, followed by determination of protein content (Rhein *et al.*, 2010).

3.12. Determination of Protein Content. The amount of protein in the mitochondrial fraction of different treatment groups was determined using Bradford method. At first, a calibration curve was determined using Bovine Serum Albumin (BSA) as a standard. By adding required volume of the sample and bringing the volume up to 800 µl with PBS, 200 µl of 5X Bradford reagent was added and incubated at room temperature for 10 min. The absorbance of the samples was measured at 595 nm using BioTek microplate reader.

3.13. Measurement of APE1 Levels by ELISA (Enzyme-Linked Immunosorbent Assay). A 96-well microtiter plate was coated with 10 µg of mitochondrial protein for 16 hr at 4 °C. Wells were washed and incubated with blocking buffer (phosphate-buffered saline, 2% BSA, 0.1% Tween 20) for 1 hr at 37 °C. Following washing, APE1 antibody (anti-mouse, 1:500) was added and incubated for 2 hr at 37 °C. The wells were washed, and secondary antibody (anti-mouse IgG-horseradish peroxidase, 1:1000) was added and incubated for another 1 hr at 37 °C. Blue coloration was observed on addition of TMB (3,3',5,5'-Tetramethylbenzidine) as a chromogenic substrate. The reaction was terminated after 10 min with 2 M H_2SO_4, and the absorbance was read at 450nm using BioTek microplate reader (Dhiman *et al.*, 2009).

3.14. Determination of Complex I Activity. A total of 20 µg of mitochondrial protein was used to estimate the NADH-Ubiquinone Oxidoreductase activity. Firstly, the mitochondrial protein was incubated with 800 µl of water and incubated for 2 min at 37 °C. Then, 200 µl of 50 mM Tris (pH-8.0) medium supplemented with 5 mg/ml BSA, 800 µM NADH as donor, 1.2 mM KCN and 2 µM antimycin was added. The basal activity was measured at 340 nm for 3 min. The reaction was started by the addition of 50 µM of the acceptor decyl ubiquonone. The activity was measured at 30 °C by following the decrease in absorbance at 340 nm resulting from the oxidation of NADH for 3 min using BioTek microplate reader. The extinction co-efficient of NADH is 6.1 $mM^{-1}.cm^{-1}$ (Barrientos *et al.*, 2009; Rhein *et al.*, 2010).

3.15. Determination of Complex III Activity. A total of 10 µg of mitochondrial protein was used to estimate the Ubiquinol Cytochrome c Reductase activity. Firstly, decyl ubiquinol was freshly prepared by adding a few crystals of sodium borohydride to 10 mM of decyl ubiquinone in ethanol and mixed by pipetting till the solution becomes transparent. Few microliters of HCl was added to eliminate excess of sodium borohydride. Final pH of the solution was 2. The assay was carried out at 30 °C in a medium containing 80 uM decyl ubiquinol as the donor, 240 µM KCN, 4 µM rotenone, 200 µM ATP and 0.6 mM dodecyl β-D-maltoside. This was followed by incubation for 10 min at 30 °C. The reaction was started by addition of 40 µM oxidized cytochrome c. The activity was measured at 550 nm for 3 min using BioTek microplate reader. The extinction co-efficient of cytochrome c is 18.5 $mM^{-1}.cm^{-1}$ (Barrientos *et al.*, 2009; Rhein *et al.*, 2010).

3.16. Determination of Complex IV Activity. A total of 10 µg of mitochondrial protein was used to measure the Cytochrome C Oxidase activity. Briefly, cytochrome c (0.22 mM) was reduced using 5 µl of 0.1 M DTT. After 15 min of incubation, the colour changed from dark orange to pale purple which was measured by measuring absorbance at 550 nm and 565 nm. The ratio of 550/565 was between 10 to 20 indicating reduction of cytochrome c. The assay was carried out using 10 mM phosphate buffer (pH-6.5), 0.25 M sucrose, 1 mg/ml BSA and 2.5 mM dodecyl β-D-maltoside to permeabilize the outer membrane to cytochrome c. The reaction was started using 10 µg of mitochondrial protein. The absorbance was measured at 550 nm for 3 min using BioTek microplate reader. The extinction co-efficient of cytochrome c is 18.5 $mM^{-1}.cm^{-1}$ (Barrientos *et al.*, 2009; Rhein *et al.*, 2010).

The following formula was used to calculate the mitochondrial complex (I-IV) activity in using the mitochondrial protein obtained from control and treated SH-SY5Y and IMR-32 cells.

Activity (U/ml) = (ΔA/min x dil x 1) **/** (vol of enzyme) x $\Delta_{\epsilon mM}$

Where,

ΔA/min = A/min $_{(sample)}$ – A/min $_{(blank)}$

dil = dilution factor of enzyme or sample

vol of enzyme = volume of enzyme or sample in ml

$\Delta_{\epsilon mM}$ = extinction co-efficient

Unit definition: One unit will oxidize 1.0 μmole of substrate per minute at pH 7.0 at 25 °C.

3.17. Statistical Analyses. Data were presented as Mean ± Standard deviation. Results were analyzed using paired Student's t-test and Analysis of Variance for statistical evaluation of mean values for experimental and control samples. P values <0.05 were considered statistically significant.

CHAPTER - 4

RESULTS

4.1: Aβ(25-35)-induced oxidative stress and cell viability in SH-SY5Y cells: The SH-SY5Y cells were treated with various concentrations of Aβ(25-35) in a range of 0-80 μM for 24 hr. It was observed that upto 10 μM, there was no cytotoxic effect of Aβ(25-35) in these cells as compared with the control cells. Thereafter, a decrease in cell proliferation was seen with increasing concentration of Aβ(25-35) as compared to untreated cells, with a significant decrease of 18% and 28% at 40 μM and 80 μM respectively (**Figure 4.1** and **Table 4.1**). Based on the literature and available data, 20 μM was chosen to be used throughout the study for further experimentation.

4.2: Aβ(25-35)-induced oxidative stress and cell viability in IMR-32 cells: The IMR-32 cells were treated with various concentrations of Aβ(25-35) in a range of 0-80 μM for 24 hr. It was observed that there was a significant decrease in cell proliferation with increasing concentration of Aβ(25-35) from 10-80 μM as compared to the untreated cells. A decrease in cell proliferation by 12% was seen on treatment with 20 μM of Aβ(25-35) as compared to untreated cells, followed by a significant decrease of 16% and 32% at 40 μM and 80 μM respectively (**Figure 4.2** and **Table 4.2**). Based on the literature and available data, 20 μM was chosen to be used throughout the study for further experimentation.

4.3: Assesment of cytotoxic response in SH-SY5Y and IMR-32 cells after treatment with Ginkgolide B (G.B): Different concentrations of G.B were used to treat SH-SY5Y and IMR-32 cells in a concentration range of 0-80 μM for different time points i.e. 12, 24, 48 and 72 hr. A decrease in cell proliferation by 25 % (significant) and 8% was seen at higher concentrations (80 μM) treated for 24 hr in SH-SY5Y and IMR-32 cell lines (**Figure 4.3** and **Table 4.3**) and (**Figure 4.4** and **Table 4.4**) respectively. When these cell lines were treated with G.B for 72 hr, there was a significant decrease in cell proliferation with increasing concentration of G.B in case of SH-SY5Y cells. Similar trend was seen in the case of IMR-32 cells treated for 72 hr, with a significant decrease at 80 μM. Although the lower concentration studied

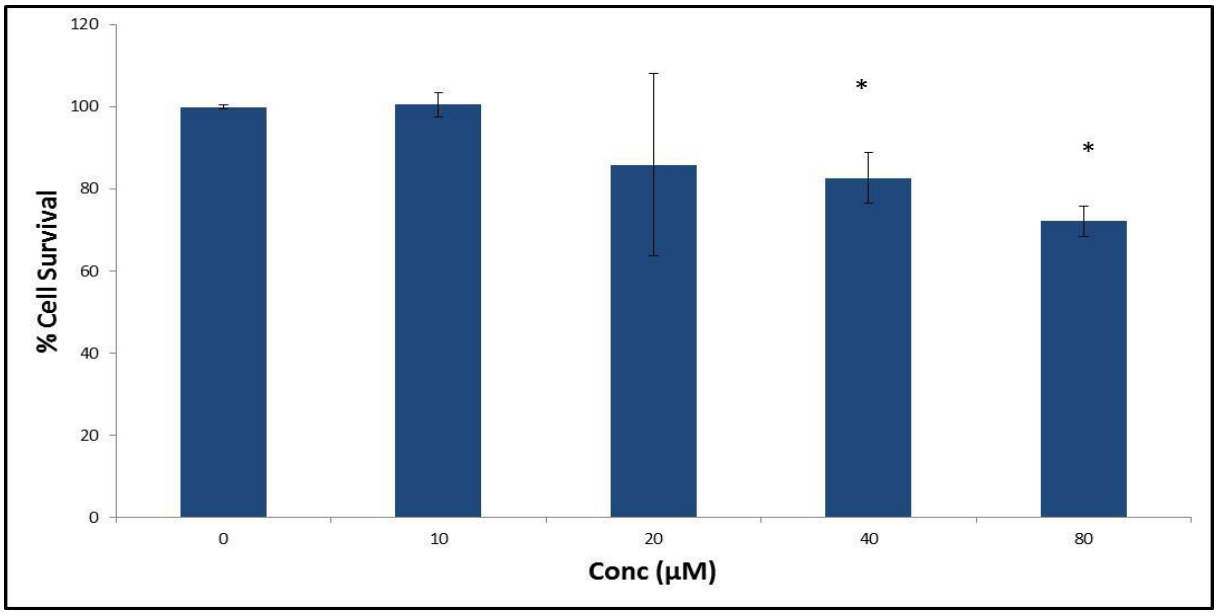


Figure 4.1: The percent cell viability in SH-SY5Y cells treated with various concentrations of oxidant Aβ(25-35) for 24 hr.

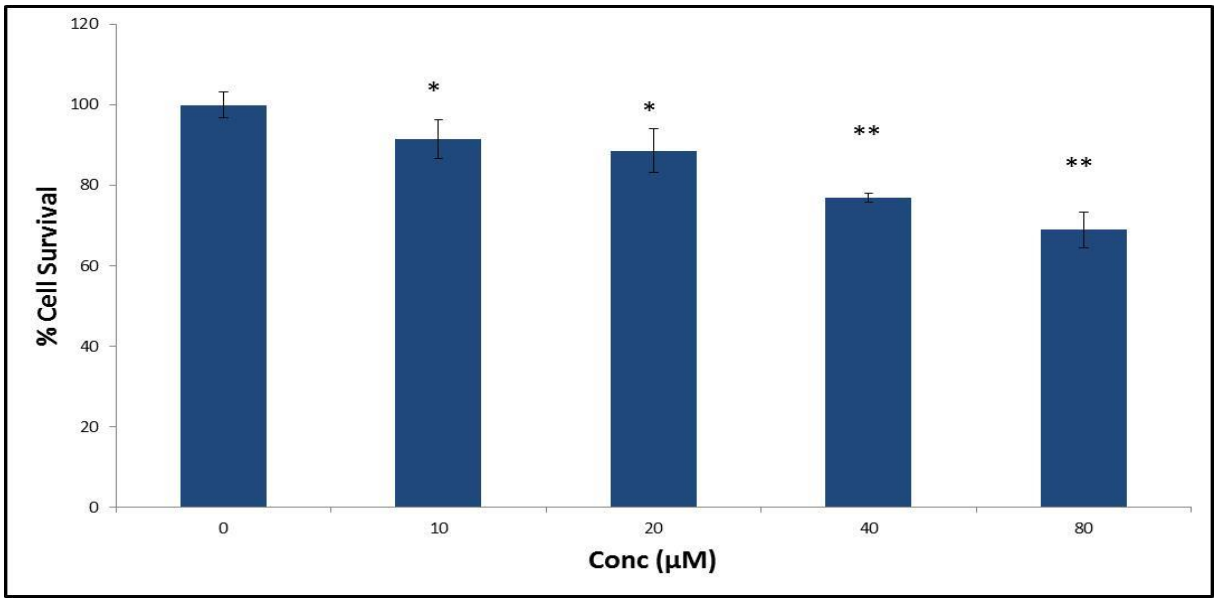


Figure 4.2: The percent cell viability in IMR-32 cells treated with various concentrations of oxidant Aβ(25-35) for 24 hr.

Table 4.1: The percent cell viability in SH-SY5Y cells treated with various concentrations of oxidant Aβ(25-35) for 24 hr.

Concentration of Aβ(25-35) (μM)	**% Cell Viability**
Control (0)	100±0.4
10	100±3
20	85±22
40	82±6*
80	72±3*

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells. The results were presented as mean ± standard deviation (n=3).

Table 4.2: The percent cell viability in IMR-32 cells treated with various concentrations of oxidant Aβ(25-35) for 24 hr.

Concentration of Aβ(25-35) (μM)	**% Cell Viability**
Control (0)	100±3
10	91±5*
20	88±5*
40	76±1**
80	68±4**

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells. The results were presented as mean ± standard deviation (n=3).

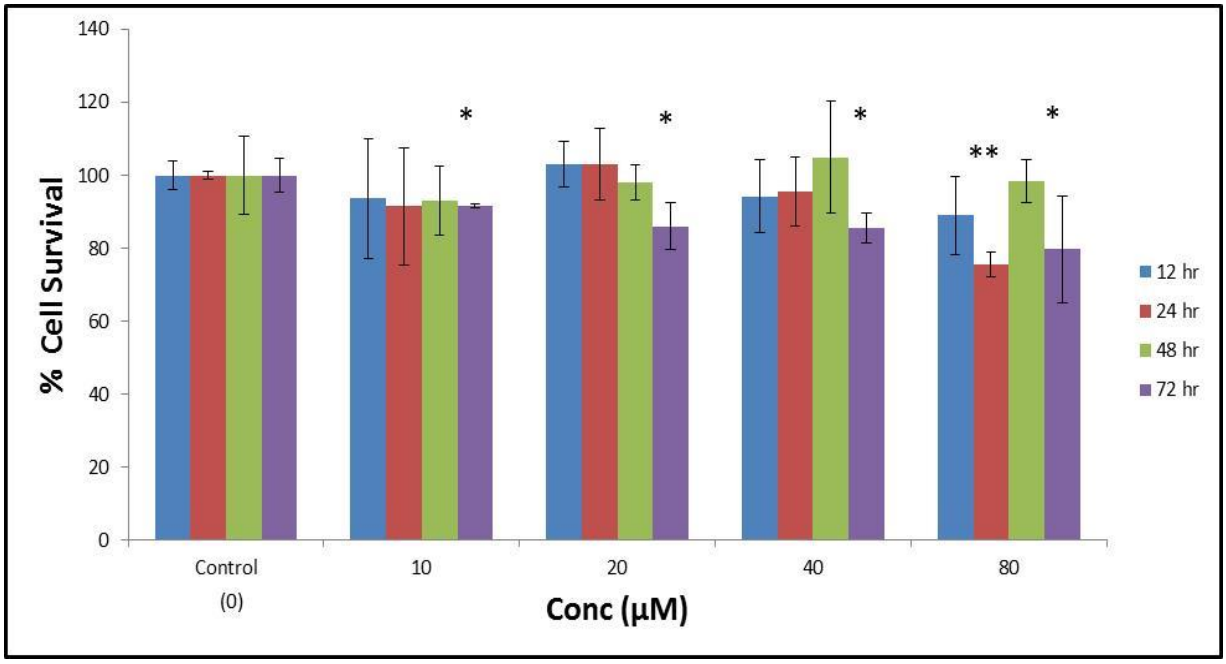


Figure 4.3: The percent cell viability of human neuroblastoma SH-SY5Y cells when treated with increased concentrations of Ginkgolide B for 12, 24, 48 and 72 hr.

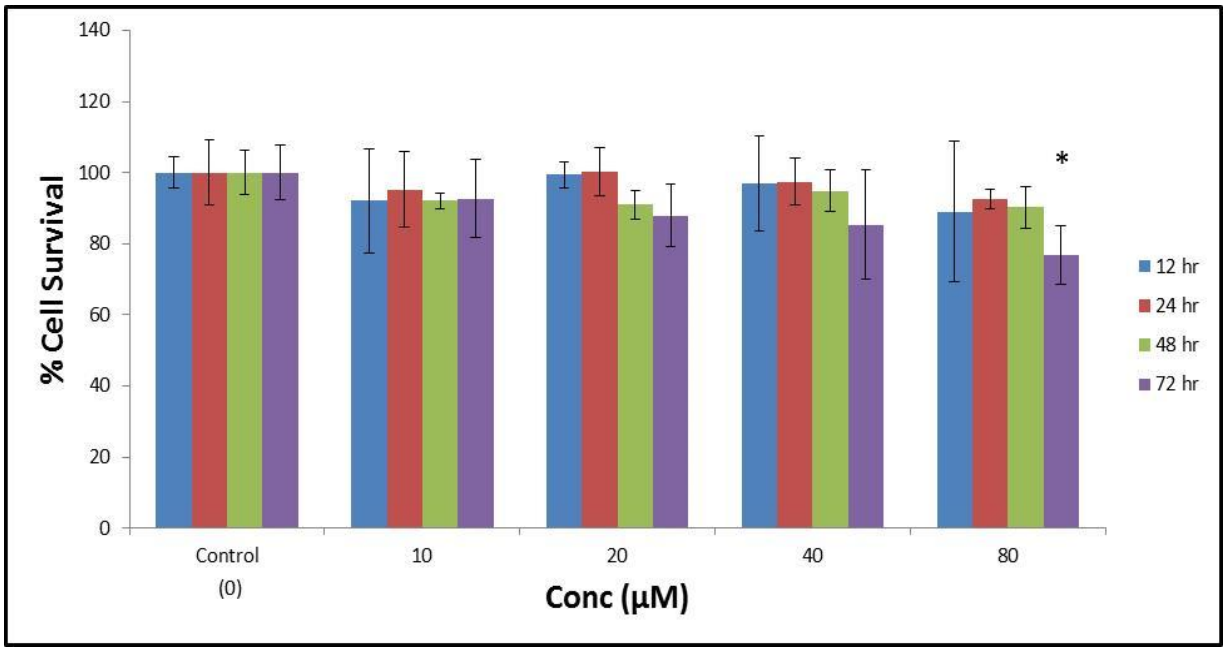


Figure 4.4: The percent cell viability of human neuroblastoma IMR-32 cells when treated with increased concentrations of Ginkgolide B for 12, 24, 48 and 72 hr.

Table 4.3: The percent cell viability of SH-SY5Y cells when treated with various concentrations of Ginkgolide B for different time periods.

Concentration of Ginkgolide B (μM)	**% Cell Viability**			
Time of Exposure	**12 hr**	**24 hr**	**48 hr**	**72 hr**
Control (0)	100±4	100±1	100±11	100±7
10	94±16	91±16	93±9	93±11*
20	103±6	103±10	98±5	88±9*
40	94±10	95±9	105±15	85±15*
80	89±10	75±3**	98±6	77±8*

The student (t) test was performed to evaluate the significance of the results. The data were considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

Table 4.4: The percent cell viability of IMR-32 cells when treated with various concentrations of Ginkgolide B for different time periods.

Concentration of Ginkgolide B (μM)	**% Cell Viability**			
Time of Exposure	**12 hr**	**24 hr**	**48 hr**	**72 hr**
Control (0)	100±4	100±9	100±6	100±7
10	92±14	95±10	92±2	92±11
20	99±3	100±7	90±4	87±9
40	96±13	97±6	94±6	85±15
80	89±20	92±2	90±6	76±8*

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells. The results were presented as mean ± standard deviation (n=3).

did not show any cytotoxicity. Based on these results, two concentrations i.e. 20 and 40 μM were chosen for further assays.

4.4: Assesment of cell viability in SH-SY5Y and IMR-32 cells with pre-treatment with Ginkgolide B (G.B) and after the treatment of Aβ(25-35): The cell viability assay was performed further using the chosen concentrations of G.B and Aβ(25-35) in the SH-SY5Y and IMR-32 cell lines for 24 hr. The results showed that at 20 μM of G.B, there was a comparable cell proliferation as the control in SH-SY5Y cells and an increase in cell proliferation by 9% in IMR-32 cells. An increase in cell proliferation by 12% (significant) and 4% respectively was seen on treatment with G.B at 40 μM for 24 hr in SH-SY5Y and IMR-32 cells. On treatment with Aβ(25-35), there was a decrease in cell viability by 19% and 6% in SH-SY5Y and IMR-32 cells respectively. The cell viability in SH-SY5Y and IMR-32 cells was increased by 16% and 4% respectively on pre-treating the cells with G.B (20 μM) for 3 hr prior to the treatment with Aβ(25-35) for 24 hr; on comparison with Aβ(25-35)-treated cells (**Figure 4.5** and **Table 4.5**) and (**Figure 4.6** and **Table 4.6**). When compared with the respective G.B-treated SH-SY5Y and IMR-32 cells, the cell proliferation was seen to be decreased by 7% and 11% respectively in G.B(20 μM)+ Aβ-treated cells and by 24% and 22% significantly in G.B(40 μM)+ Aβ-treated cells.

4.5: Measurement of Intracellular ROS levels in SH-SY5Y and IMR-32 cells with pre-treatment with Ginkgolide B (G.B) and after the treatment of Aβ(25-35): This assay was performed to measure the intracellular ROS levels in SH-SY5Y cells in presence of oxidative stress induced by 20 μM of Aβ(25-35) and modulation by phytochemical G.B at two concentrations i.e. 20 and 40 μM. ROS levels were found to be significantly increased by 76% and 7% at 40 μM of G.B as compared to the respective control SH-SY5Y and IMR-32 cells. Treatment with Aβ(25-35) increased the ROS levels significantly by 80% and 41% respectively as compared to the

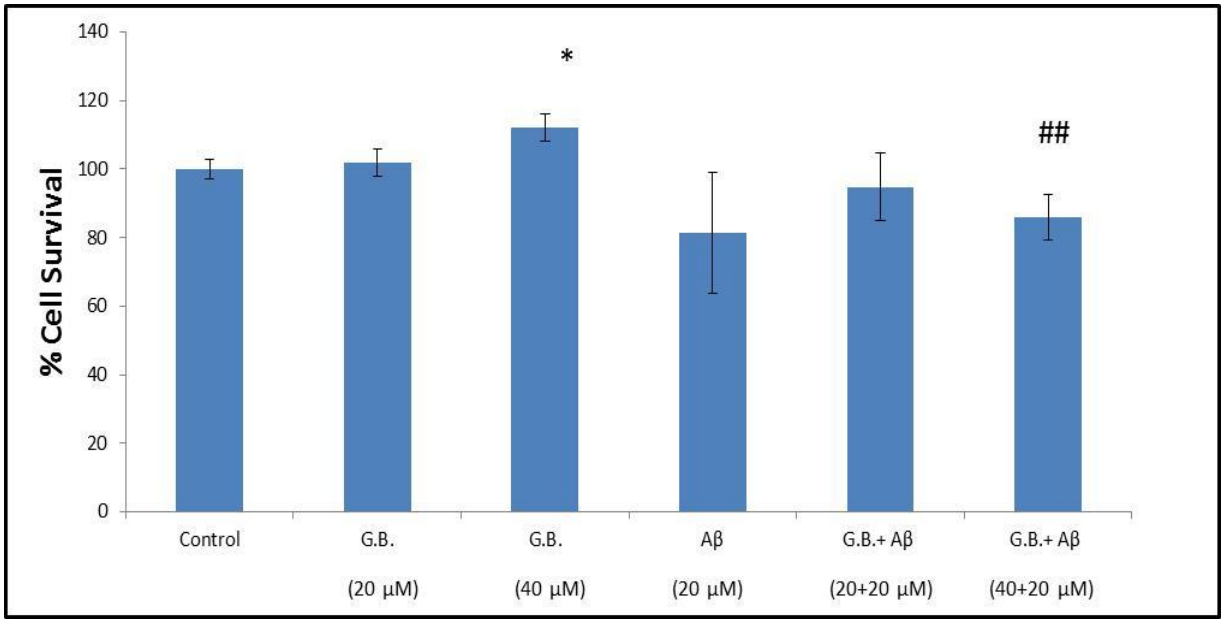


Figure 4.5: The percent cell viability in SH-SY5Y cells and cells those are treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) after 48 hr of experimental procedure.

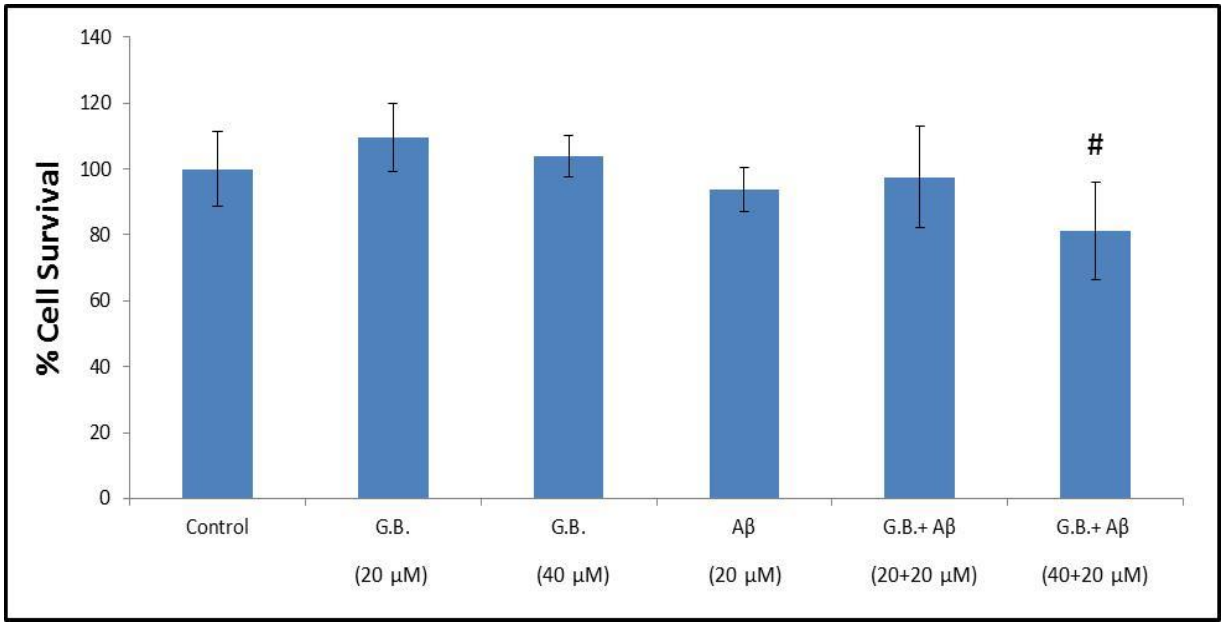


Figure 4.6: The percent viability in IMR-32 cells and cells those are treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) after 48 hr of experimental procedure.

Table 4.5: The percent cell viability in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) after 48 hr of experimental procedure.

Sample	**Concentration (μM)**	**% Cell Viability**
Control	-	100±3
G.B	20	102±4
G.B	40	112±4*
Aβ	20	81±17
G.B + Aβ	20+20	95±10
G.B + Aβ	40+20	86±7$^{\#\#}$

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with G.B. The results are presented as mean ± standard deviation (n=3).

Table 4.6: The percent cell viability in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) after 48 hr of experimental procedure.

Sample	Concentration (μM)	% Cell Viability
Control	-	100±11
G.B	20	109±10
G.B	40	104±6
Aβ	20	94±7
G.B + Aβ	20+20	97±15
G.B + Aβ	40+20	81±14$^{\#}$

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.005$ when G.B+Aβ treated cells were compared with G.B. The results are presented as mean ± standard deviation (n=3).

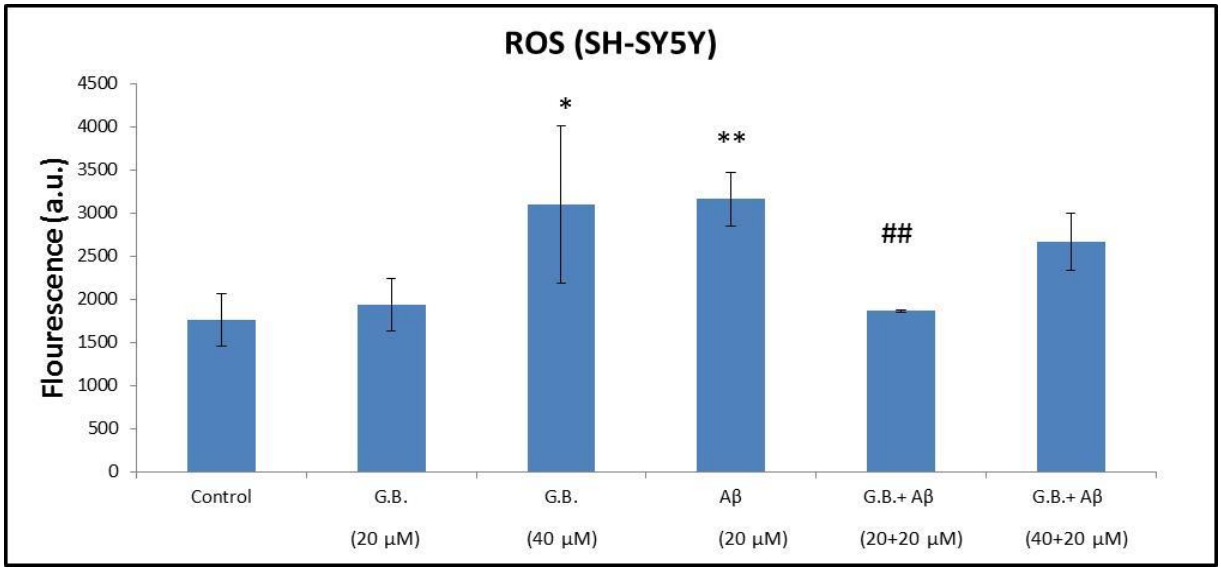


Figure 4.7(a): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

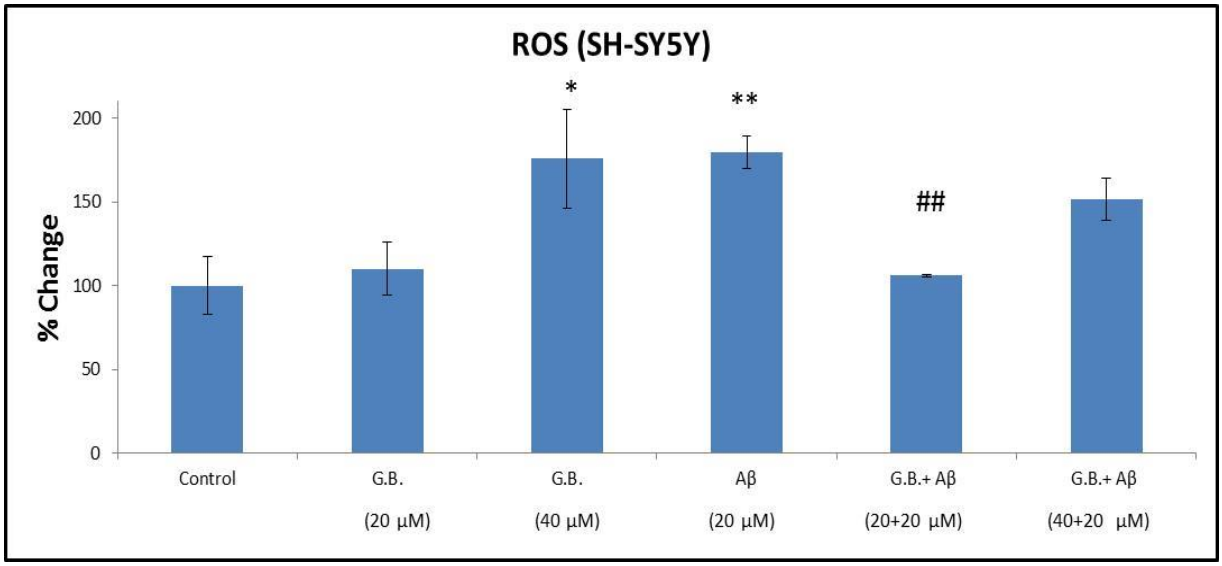


Figure 4.7(b): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.7: Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular ROS levels (a.u)	% Change
Control	-	1759±300	100±17
G.B	20	1937±301	110±15
G.B	40	3093±911*	176±29
Aβ	20	3161±313**	180±10
G.B + Aβ	20+20	1863±12$^{\#\#}$	106±0.6
G.B + Aβ	40+20	2665±330	151±12

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B and Aβ-treated cells and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with Aβ-treated cells. The results are presented as mean ± standard deviation (n=3).

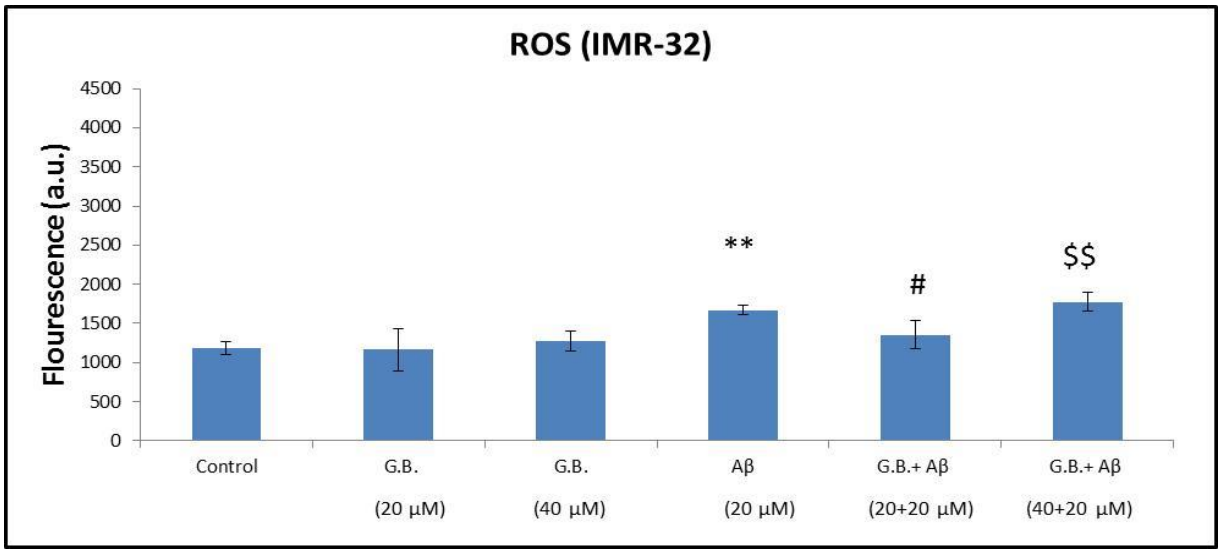


Figure 4.8(a): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

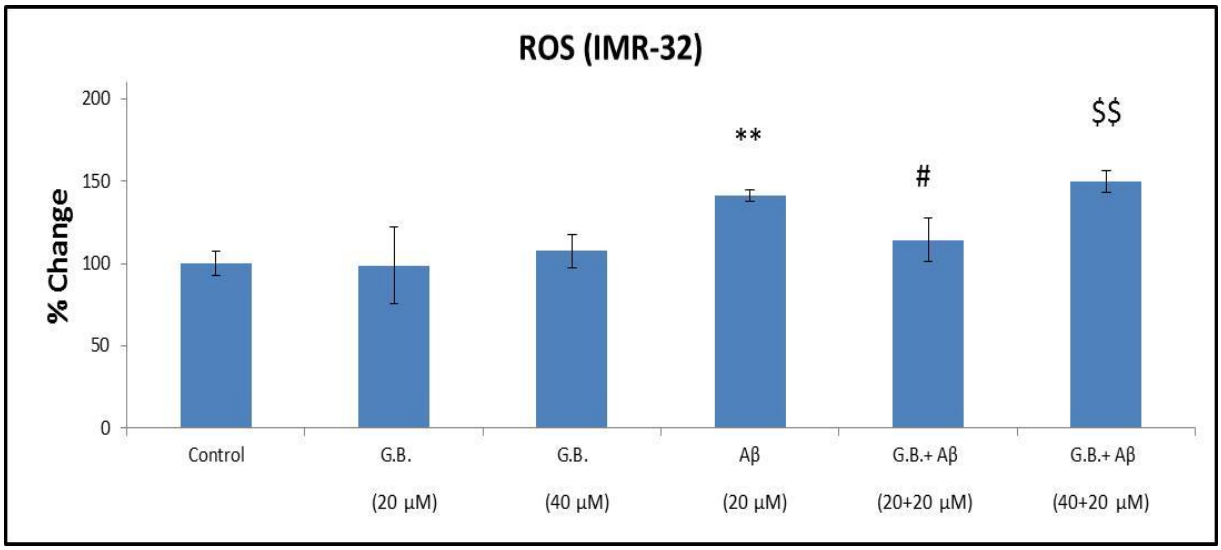


Figure 4.8(b): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.8: Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B).

Sample	**Concentration (μM)**	**Intracellular ROS levels (a.u)**	**% Change**
Control	-	1182±87	100±7
G.B	20	1168±271	99±23
G.B	40	1272±127	107±10
Aβ	20	1672±57**	141±3
G.B + Aβ	20+20	1352±180$^{\#}$	114±13
G.B + Aβ	40+20	1771±121$^{\$\$}$	150±7

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with Aβ-treated cells and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with Aβ-treated cells and $^{\$}$p≤0.05 and $^{\$\$}$p≤0.005 when G.B+Aβ are compared with G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

untreated SH-SY5Y and IMR-32 cells (**Figure 4.7** and **Table 4.7**) and (**Figure 4.8** and **Table 4.8**). On comparison with Aβ(25-35)-treated SH-SY5Y and IMR-32 cells, the G.B(20 μM)+Aβ treated cells showed significantly decreased ROS levels by 41% and 19% respectively. The G.B(40 μM)+Aβ treated cells showed decreased ROS levels by 26% and an increase of 6% as compared to their respective Aβ(25-35)-treated SH-SY5Y and IMR-32 cells. It was observed that there was a decrease in ROS levels by 4% and 14% in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated SH-SY5Y cells respectively. While, an increase in ROS levels by 15% and 39% (significant) in G.B(20 μM)+Aβ treated cells and G.B(40 μM)+Aβ treated IMR-32 cells was seen, on comparing with the respective G.B treated cells.

4.6: Measurement of Intracellular RNS levels in SH-SY5Y and IMR-32 cells with pre-treatment with Ginkgolide B (G.B) and after the treatment of Aβ(25-35): The fluorescent DAF-FM assay was used to measure the RNS levels in SH-SY5Y cells after treatment with G.B and Aβ(25-35) for 24 hr. Treatment with Aβ(25-35) showed an increase in RNS levels by 169% and 197% (significant) as compared to their respective SH-SY5Y and IMR-32 control cells. When the SH-SY5Y and IMR-32 cells were treated with G.B at a concentration of 20 μM, there was an increase in RNS levels by 30% and a decrease by 12% was observed respectively as compared with the untreated control cells. An increase of 178% (significant) was seen on treating the SH-SY5Y cells with 40 μM of G.B as compared with the untreated control cells while not much increase in RNS levels was seen in IMR-32 cells. On comparison with Aβ(25-35)-treated SH-SY5Y cells, there was a decrease by 45% and 9% respectively in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated cells while a decrease of 14% and 15% in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated IMR-32 cells was seen (**Figure 4.9** and **Table 4.9**) and (**Figure 4.10** and **Table 4.10**). An increase of 14% and a decrease by 12% was observed in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated SH-SY5Y cells respectively while an significant increase of 159% and 133% was observed in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated IMR-32 cells respectively when compared with their respective G.B-treated cells.

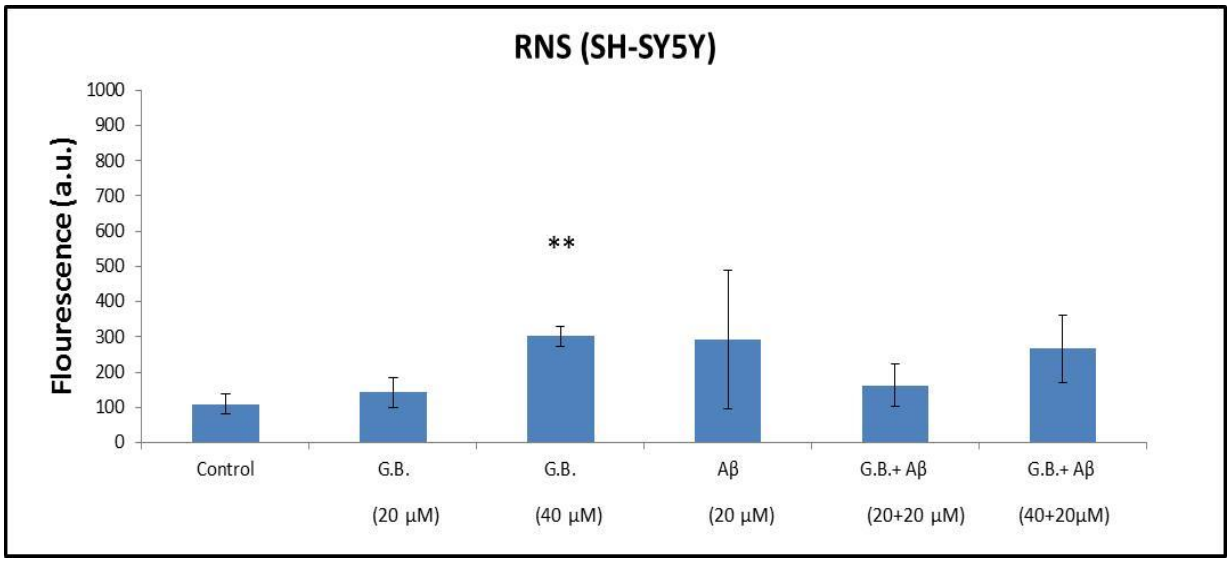


Figure 4.9(a): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

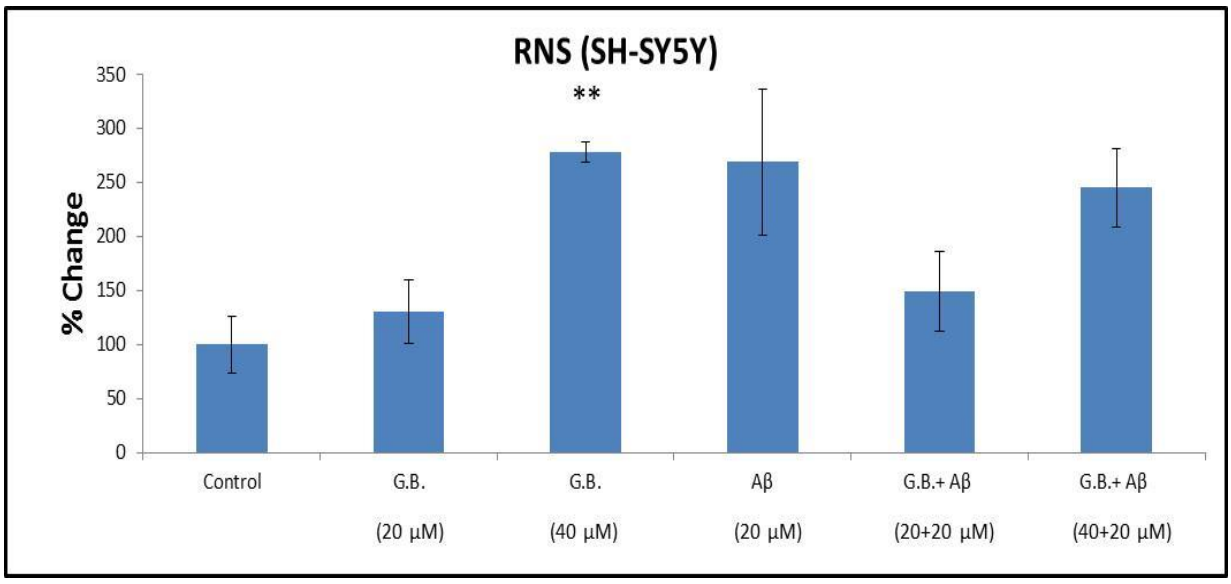


Figure 4.9(b): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.9: Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B).

Sample	**Concentration (μM)**	**Intracellular RNS levels (a.u)**	**% Change**
Control	-	108±29	100±26
G.B	20	141±42	130±29
G.B	40	302±29**	278±9
Aβ	20	292±198	269±67
G.B + Aβ	20+20	162±60	149±37
G.B + Aβ	40+20	266±95	245±36

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

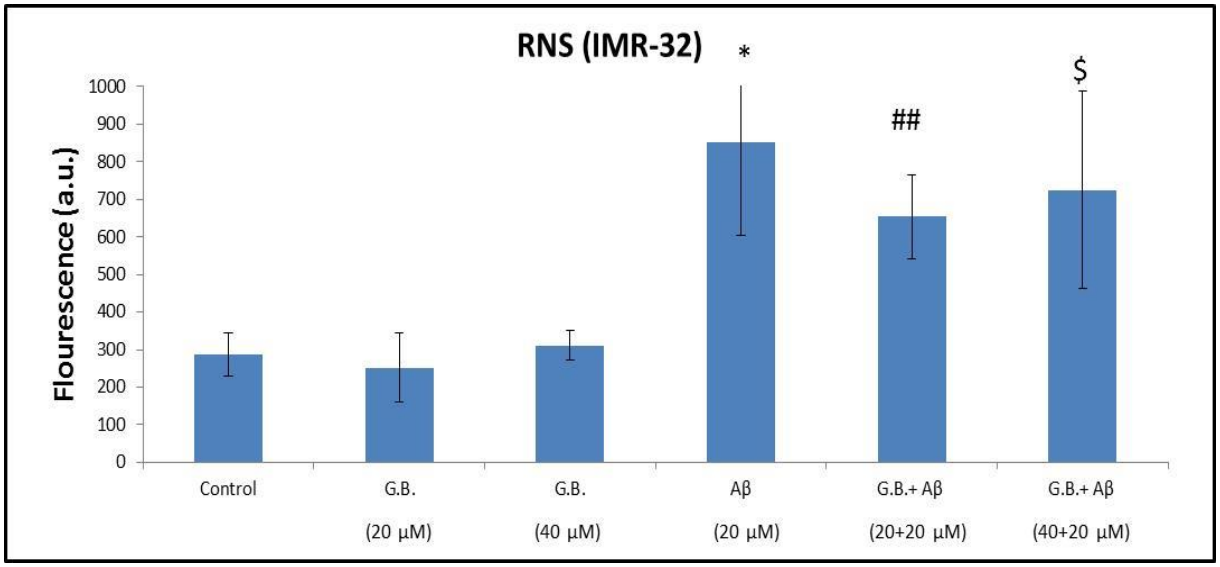


Figure 4.10(a): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

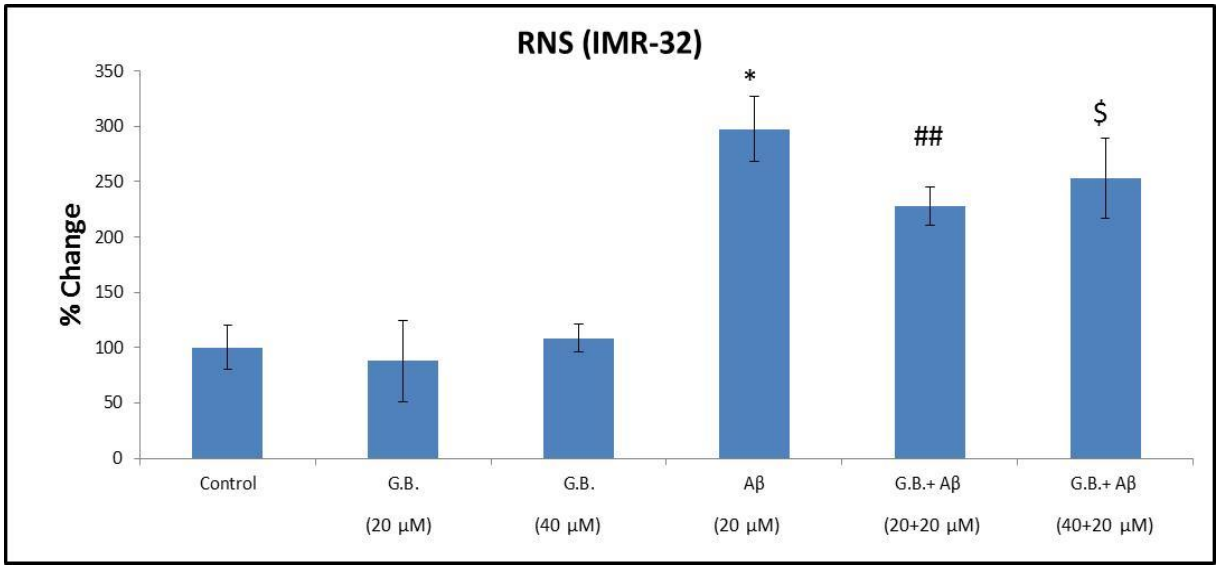


Figure 4.10(b): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.10: Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells by DAF-FM and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular RNS levels (a.u)	% Change
Control	-	286±57	100±20
G.B	20	251±92	88±36
G.B	40	311±40	108±13
Aβ	20	852±249*	297±29
G.B + Aβ	20+20	653±110$^{\#\#}$	228±17
G.B + Aβ	40+20	724±263$^{\$}$	253±36

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with Aβ-treated cells and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with the respective G.B-treated cells and $^{\$}$p≤0.05 and $^{\$\$}$p≤0.005 when G.B+Aβ are compared with the respective G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

4.7: Measurement of APE1 levels on induction of oxidative stress by Aβ(25-35) and modulation by Ginkgolide B (G.B): ELISA was done to measure APE1 levels under different treatment conditions in SH-SY5Y cells. It was found that Aβ(25-35)-treated cells caused a significant decrease of 39% in APE1 levels as compared to control cells. On treating with G.B, there was a decrease of 20% as compared with untreated control cells. Pre-treatment with G.B along with exposure to Aβ(25-35) caused an increase in 95% of APE1 levels in G.B(20 μM)+ Aβ treated cells when compared to control cells (**Figure 4.11** and **Table 4.11**). Thus, it points toward the modulation of APE1 by G.B in presence of Aβ(25-35)-induced oxidative stress. Subsequently, APE1 ectopic-expression studies were carried out to find out the neuro-modulatory role of G.B on mitochondrial functions after Aβ(25-35)-induced oxidative stress responses in SH-SY5Y and IMR-32 cells with ectopic APE1 expression.

4.8: Assessment of cell viability in SH-SY5Y and IMR-32 cells with ectopic APE1 expression with pre-treatment with Ginkgolide B (G.B) and after treating with Aβ(25-35): The cell viability assay was performed further after APE1 ectopic-expression in SH-SY5Y cells upon cell transfection (refer to the **Section 4.24**) using the chosen concentrations of G.B and Aβ(25-35) in the SH-SY5Y cells for 24 hr. The results showed that at 20 μM of G.B, there was an increase of 21% and 22% in cell proliferation as compared with the respective APE1 control SH-SY5Y and IMR-32 cells and an increase of 28% (significant) and 32% in SH-SY5Y and IMR-32 cells respectively on treatment with 40 μM of G.B. On treatment with Aβ(25-35), there was a decrease of 10% and 3% in SH-SY5Y and IMR-32 cells respectively. The cell viability was increased by 14% and 19% on pre-treating the respective SH-SY5Y and IMR-32 cells with G.B (20 μM) while an increase of 13% and 12% G.B on pre-treating the respective SH-SY5Y and IMR-32 cells cells with G.B (40 μM) for 3 hr prior totreating with Aβ(25-35) for 24 hr (**Figure 4.12** and **Table 4.12**) and (**Figure 4.13** and **Table 4.13**). The cell proliferation was seen to be decreased by 14% and 6% respectively in G.B(20 μM)+Aβ treated SH-SY5Y and IMR-32 APE1 expressing cells

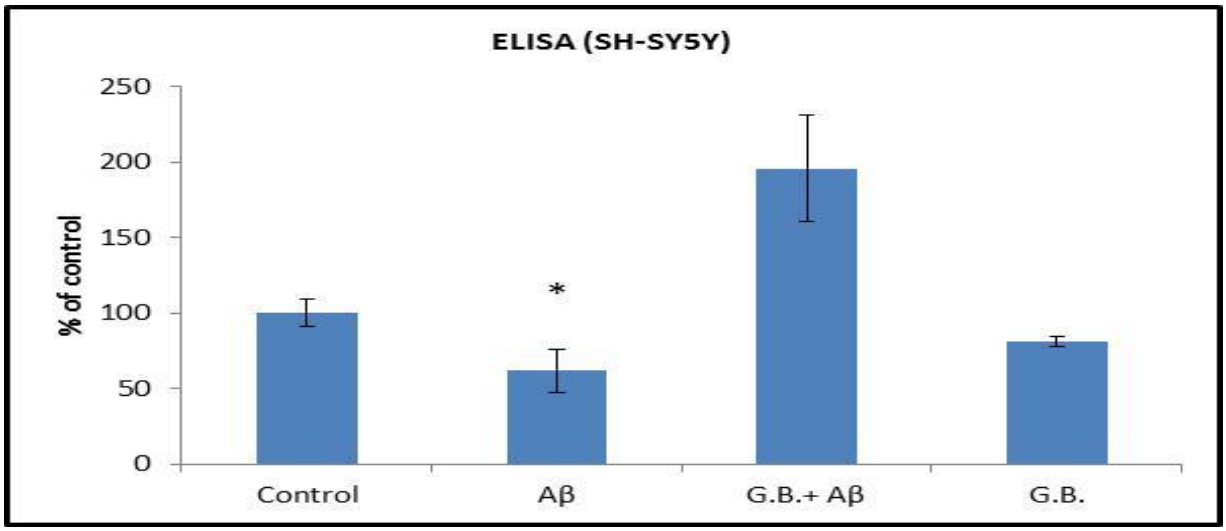


Figure 4.11: Measurement of APE1 levels performed by ELISA in order to check the APE1 levels in the presence of oxidant Aβ(25-35) and document the change in APE1 levels with the pre-treatment of Ginkgolide B (G.B) against Aβ-induced toxicity in SH-SY5Y cells.

Table 4.11: The APE1 levels as measured by ELISA after pre-treatment with Ginkgolide B (G.B) and treatment with oxidant Aβ(25-35) in SH-SY5Y cells.

Sample	**Concentration (μM)**	**APE1 levels (%)**
Control	-	100±9
Aβ	20	61±14*
G.B + Aβ	20+20	195±35
G.B	20	80±3

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with Aβ-treated cells. The results are presented as mean ± standard deviation (n=3).

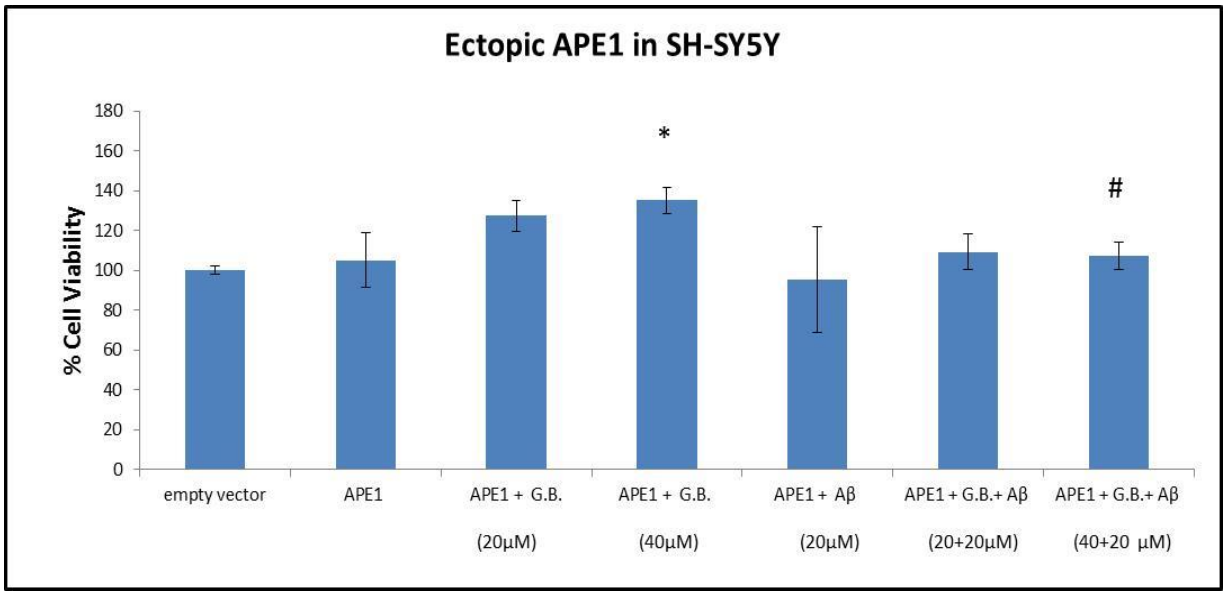


Figure 4.12: The percent cell viability in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with ectopic APE1 expression.

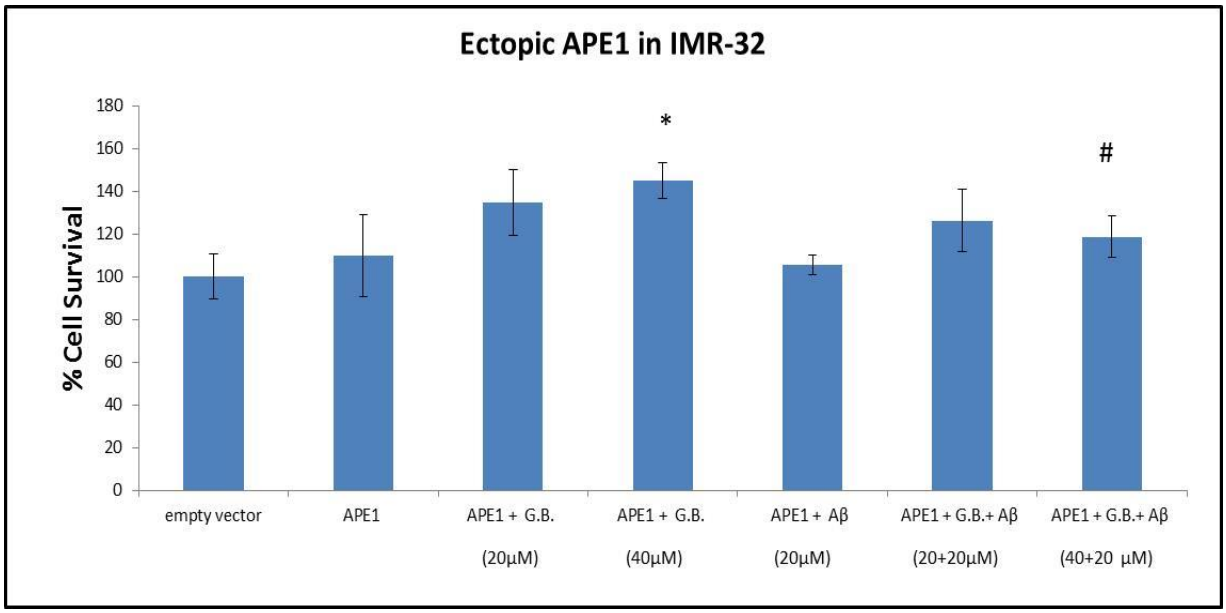


Figure 4.13: The percent cell viability in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with ectopic APE1 expression.

Table 4.12: The percent cell viability in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with ectopic APE1 expression.

Sample	**Concentration (μM)**	**% Cell Viability**
Empty Vector	-	100±2
APE1	-	105±13
APE1 + G.B	20	127±8
APE1 + G.B	40	135±6*
APE1 + Aβ	20	95±26
APE1 + G.B + Aβ	20+20	109±9
APE1 + G.B + Aβ	40+20	107±7$^{\#}$

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated APE1 cells were compared with APE1+G.B treated cells; and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when APE1+G.B+Aβ treated cells were compared with the respective APE1+G.B treated cells. The results are presented as mean ± standard deviation (n=3).

Table 4.13. The percent cell viability in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with ectopic APE1 expression.

Sample	Concentration (μM)	% Cell Viability
Empty Vector	-	100±10
APE1	-	110±19
APE1 + G.B	20	134±15
APE1 + G.B	40	145±8*
APE1 + Aβ	20	105±4
APE1 + G.B + Aβ	20+20	126±15
APE1 + G.B + Aβ	40+20	118±10#

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated APE1 cells were compared with APE1+G.B treated cells; and #p≤0.05 and ##p≤0.005 when APE1+G.B+Aβ treated cells were compared with the respective APE1+G.B treated cells. The results are presented as mean ± standard deviation (n=3).

and significant decrease by 21% and 18% respectively in G.B(40 μM)+Aβ treated SH-SY5Y and IMR-32 cells ectopically expressing APE1, when compared with respective G.B-treated cells.

4.9: Measurement of Intracellular ROS levels in SH-SY5Y and IMR-32 cells with ectopic APE1 expression with pre-treatment with Ginkgolide B (G.B) and after treating with Aβ(25-35): The fluorescent H_2DCF-DA assay was performed to measure the intracellular ROS levels in SH-SY5Y cells after ectopic APE1 expression in the presence of oxidative stress induced by 20 μM of Aβ(25-35) and modulation by phytochemical G.B at two concentrations i.e. 20 and 40 μM. ROS levels in SH-SY5Y and IMR-32 cells were found to be decreased significantly by 25% and 27% after treating with 20 μM and by significantly by 51% and 29% after treating with 40 μM of G.B as compared to the respective APE1 control cells. Treatment of SH-SY5Y and IMR-32 cells with Aβ(25-35)-increased the ROS levels by 5% and decreased the ROS by merely 2%. On comparison with Aβ(25-35)-treated cells with APE1 ectopic-expression, the G.B(20 μM)+Aβ cells showed decreased ROS levels by 8% and 14% in SH-SY5Y and IMR-32 cells respectively. Similarly, the G.B(40 μM)+Aβ-treated SH-SY5Y and IMR-32 cells showed a decrease in ROS levels by 21% and 5% respectively (**Figure 4.14** and **Table 4.14**) and (**Figure 4.15** and **Table 4.15**). It was observed that there was an increase in ROS levels by 29% and 16% (significant) respectively in G.B(20 μM)+Aβ-treated SH-SY5Y and IMR-32 cells with ectopic APE1 expression and a significant increase of 67% and 32% respectively in G.B(40 μM)+Aβ treated APE1 overexpressing SH-SY5Y and IMR-32 cells, on comparing with the respective G.B treated cells ectopically expressing APE1 only.

4.10: Measurement of Intracellular RNS levels in SH-SY5Y and IMR-32 cells with ectopic APE1 expression with pre-treatment with Ginkgolide B (G.B) and after treating with Aβ(25-35): The fluorescent DAF-FM assay was used to measure the RNS levels in APE1 expressing SH-SY5Y cells after treatment with Aβ(25-35) and G.B for 24 hr. Interestingly, a significant decrease of 23% was observed when ectopic APE1 expressing SH-SY5Y cells were compared with SH-SY5Y cells expressing

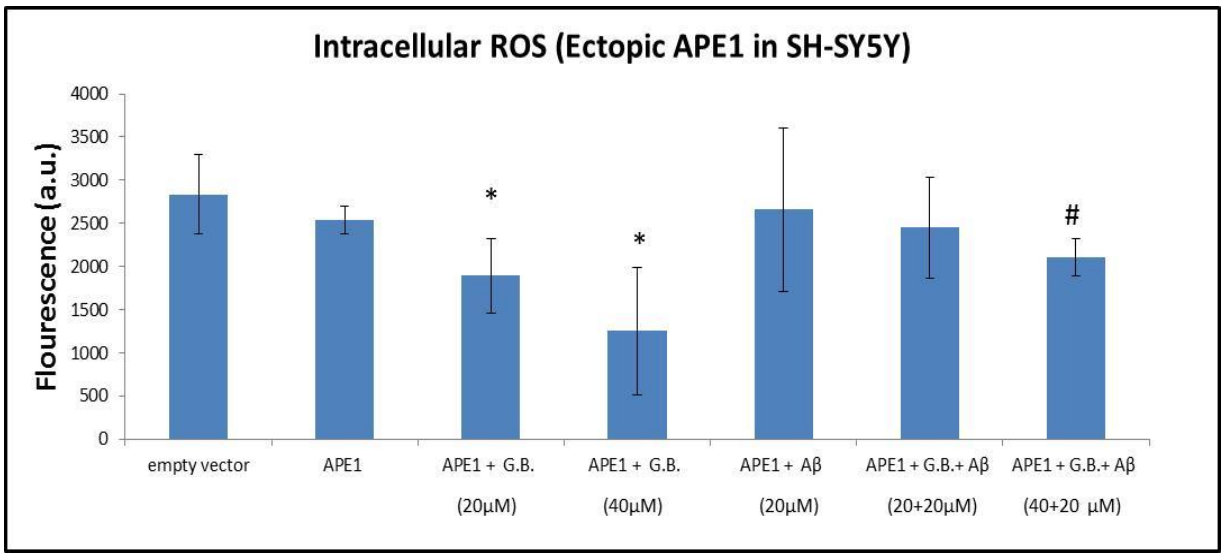


Figure 4.14(a): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

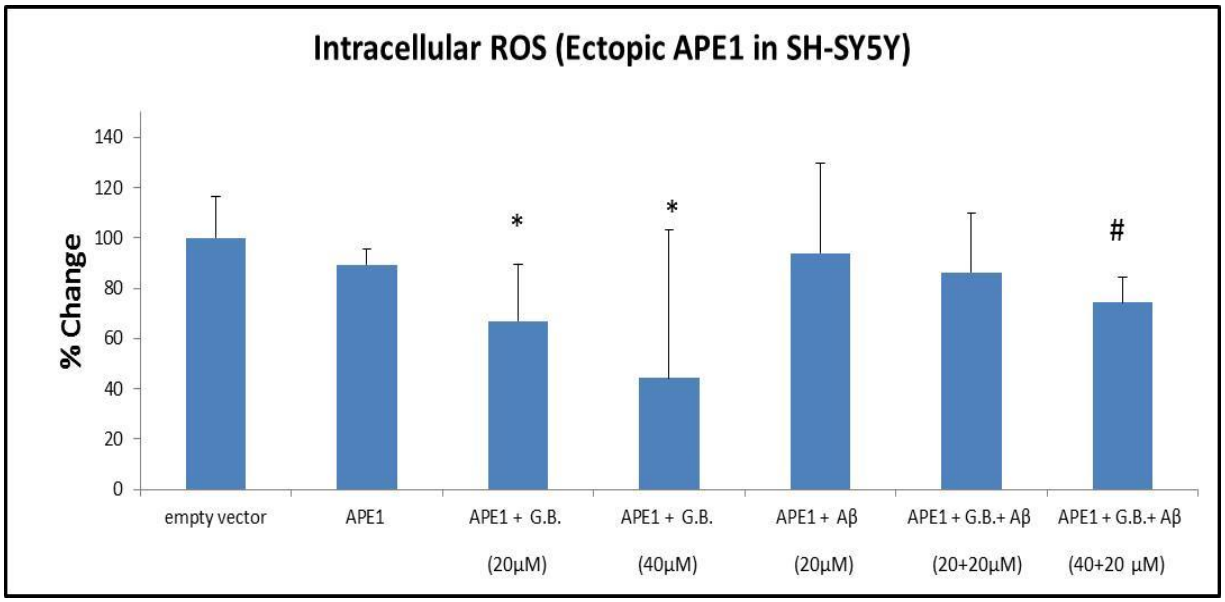


Figure 4.14(b): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.14: Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular ROS levels (a.u)	% Change
Empty Vector	-	2834±460	100±16
APE1	-	2536±157	89±6
APE1 + G.B	20	1897±430*	67±22
APE1 + G.B	40	1256±737*	44±58
APE1 + Aβ	20	2659±949	94±35
APE1 + G.B + Aβ	20+20	2449±579	86±23
APE1 + G.B + Aβ	40+20	2104±217#	74±10

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated APE1 cells were compared with APE1+G.B treated cells; and #p≤0.05 and ##p≤0.005 when APE1+G.B+Aβ treated cells were compared with the respective APE1+G.B treated cells. The results are presented as mean ± standard deviation (n=3).

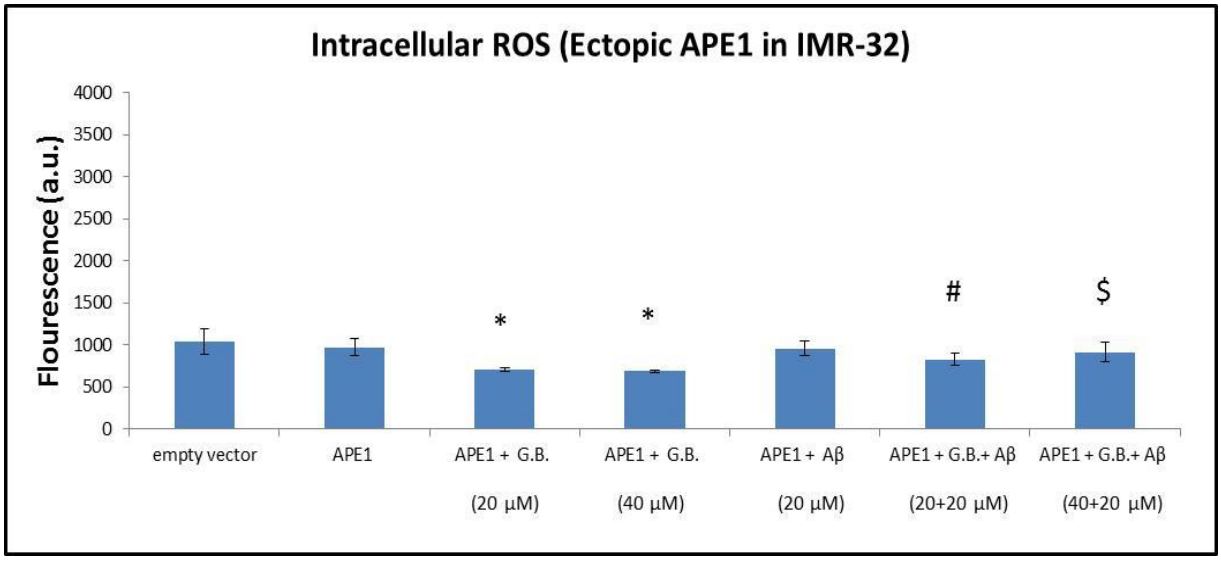


Figure 4.15(a): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

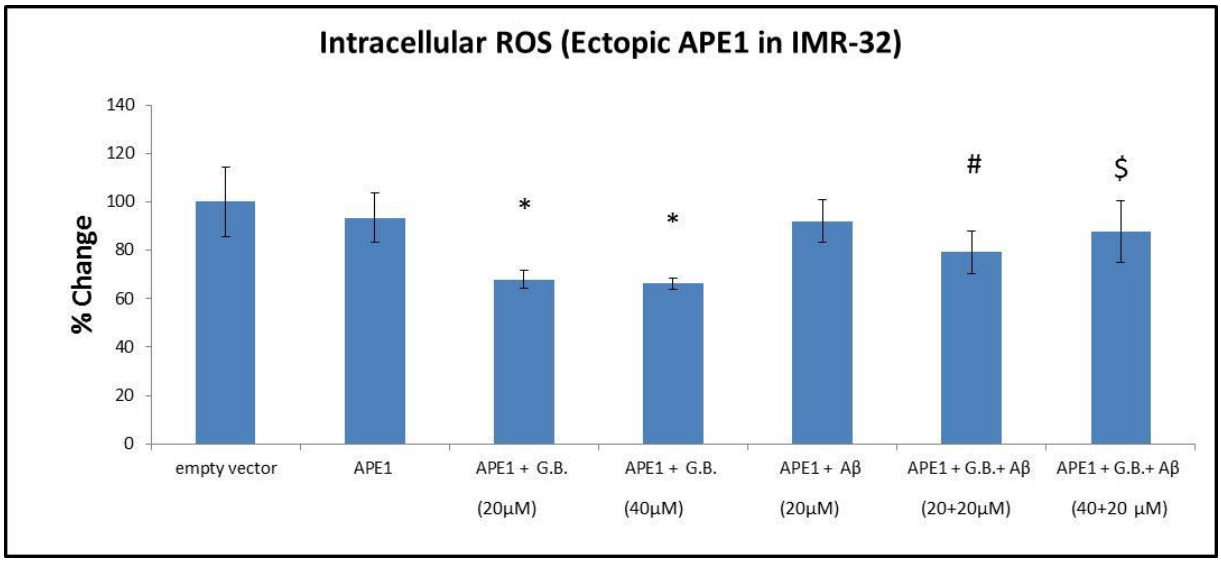


Figure 4.15(b): Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.15: Measurement of intracellular ROS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by H_2DCFDA and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular ROS levels (a.u)	% Change
Empty Vector	-	1042±150	100±14
APE1	-	972±99	93±10
APE1 + G.B	20	707±26*	68±4
APE1 + G.B	40	689±17*	66±2
APE1 + Aβ	20	959±86	92±9
APE1 + G.B + Aβ	20+20	824±72$^{\#}$	79±9
APE1 + G.B + Aβ	40+20	912±116$^{\$}$	87±13

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated APE1 cells were compared with APE1+G.B treated cells; $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with the respective G.B-treated cells; and $^{\$}$p≤0.05 and $^{\$\$}$p≤0.005 when G.B+Aβ are compared with the respective G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

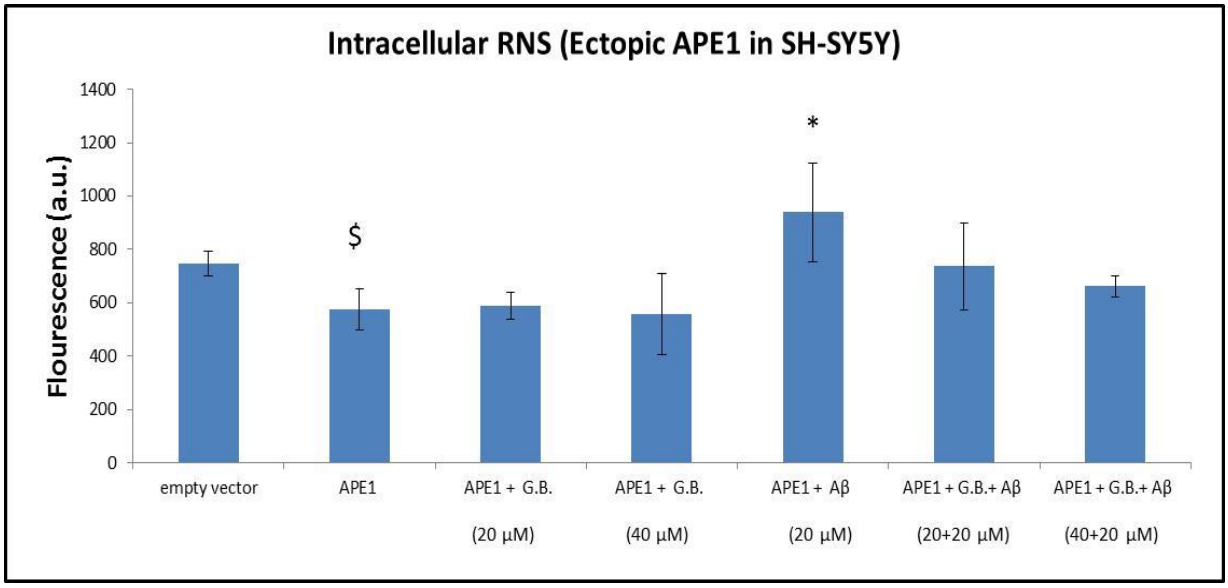


Figure 4.16(a): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

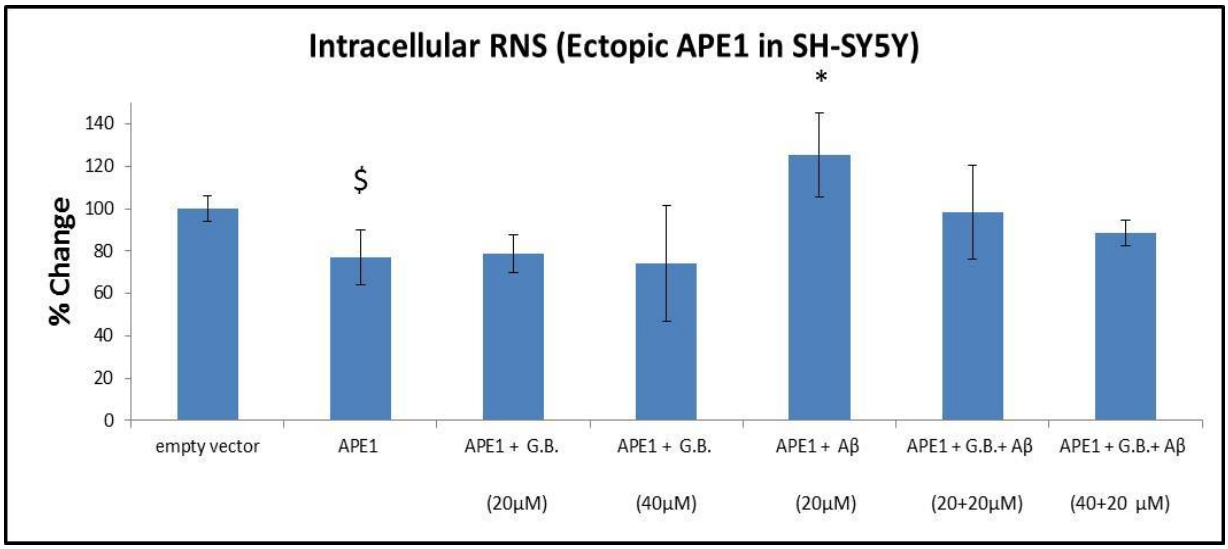


Figure 4.16(b): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.16: Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in SH-SY5Y cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular RNS levels (a.u)	% Change
Empty Vector	-	748±46	100±6
APE1	-	576±76$^{\$}$	77±13
APE1 + G.B	20	588±51	78±9
APE1 + G.B	40	556±151	74±27
APE1 + Aβ	20	939±185*	125±20
APE1 + G.B + Aβ	20+20	736±162	98±22
APE1 + G.B + Aβ	40+20	662±39	88±6

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\$}p\leq0.05$ and $^{\$\$}p\leq0.005$ when untreated cells with ectopic APE1 are compared with untreated cells expressing empty vector and $^{*}p\leq0.05$ and $^{**}p\leq0.005$ when untreated APE1 cells were compared with APE1+Aβ treated cells. The results are presented as mean ± standard deviation (n=3).

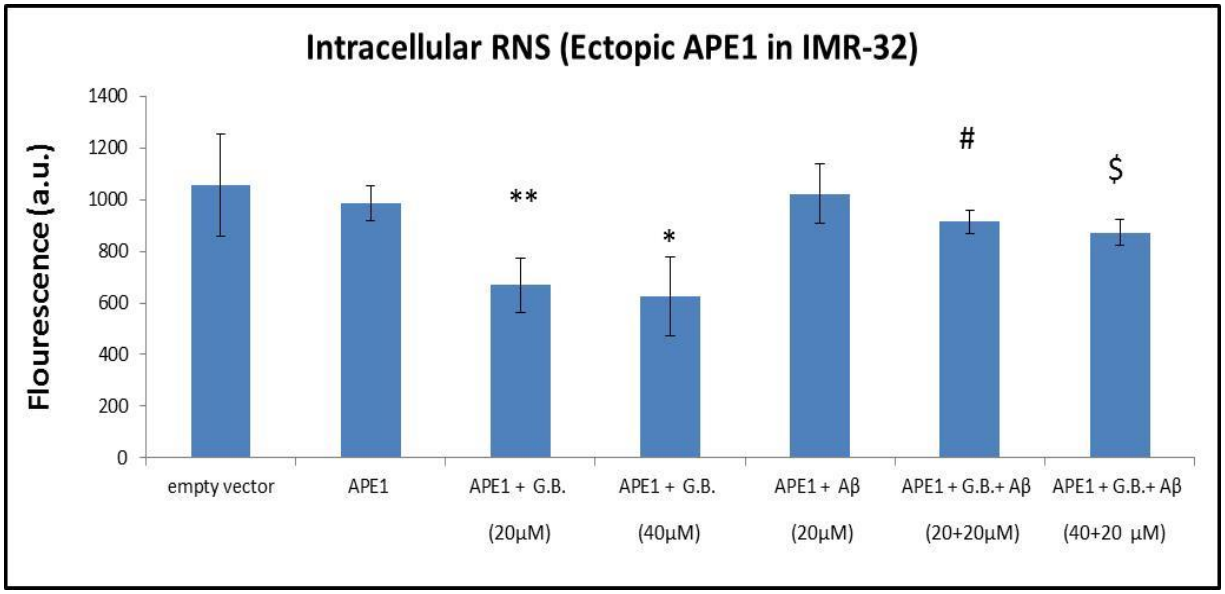


Figure 4.17(a): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed in fluorescent intensity (a.u.).

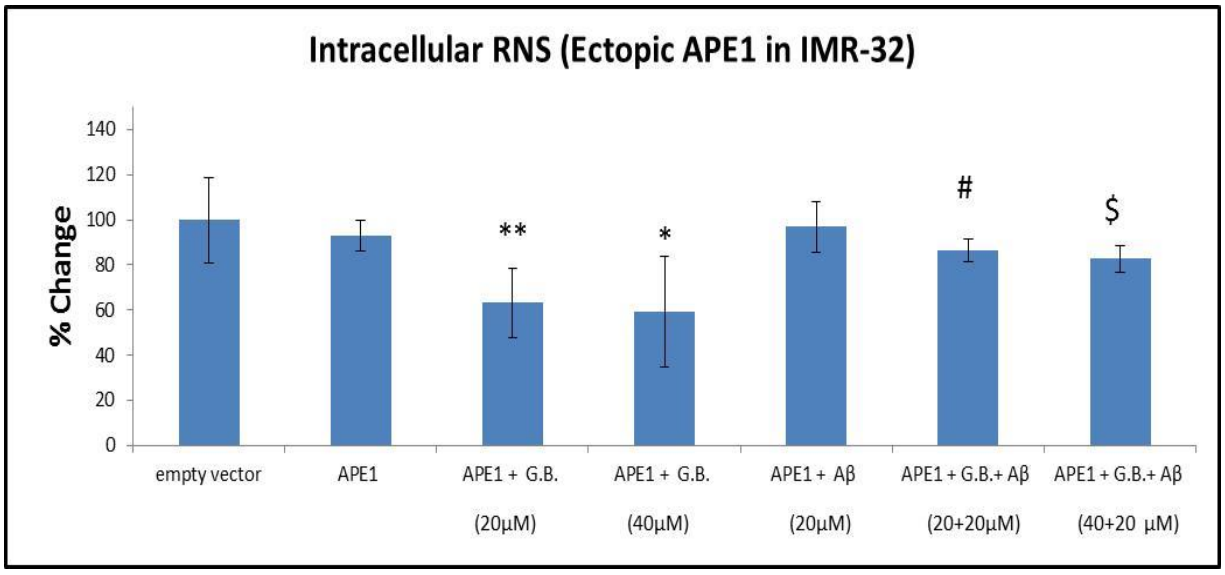


Figure 4.17(b): Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B) expressed as % change.

Table 4.17: Measurement of intracellular RNS produced after Aβ(25-35)-induced oxidative stress in IMR-32 cells with ectopic APE1 expression by DAF-FM and phytochemical modulation by Ginkgolide B (G.B).

Sample	Concentration (μM)	Intracellular RNS levels (a.u)	% Change
Empty Vector	-	1056±199	100±19
APE1	-	983±67	93±7
APE1 + G.B	20	668±103**	63±15
APE1 + G.B	40	625±154*	59±24
APE1 + Aβ	20	1023±113	97±11
APE1 + G.B + Aβ	20+20	913±45$^{\#}$	86±5
APE1 + G.B + Aβ	40+20	873±51$^{\$}$	82±6

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated APE1 cells were compared with APE1+G.B treated cells; $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when G.B+Aβ treated cells were compared with the respective G.B-treated cells; and $^{\$}$p≤0.05 and $^{\$\$}$p≤0.005 when G.B+Aβ are compared with the respective G.B-treated cells. The results are presented as mean ± standard deviation (n=3).

empty vector or control. Treatment with Aβ(25-35) in SH-SY5Y cells with APE1 ectopic-expression showed a significant increase in RNS levels by 63% as compared to the APE1 control SH-SY5Y cells (**Figure 4.16** and **Table 4.16**) and (**Figure 4.17** and **Table 4.17**). Not much increase was found in IMR-32 cells with APE1 ectopic-expression after treatment with Aβ(25-35). When the APE1 ectopically expressing in SH-SY5Y and IMR-32 cells were treated with G.B at a concentration of 20 μM, there was an increase in RNS levels by 2% and decrease by 32% (significant) respectively and a decrease by 4% and 37% (significant) respectively was seen when the APE1 ectopically-expressing SH-SY5Y and IMR-32 cells were treated with 40 μM of G.B as compared with the respective untreated APE1 control cells. On comparison with Aβ(25-35)-treated SH-SY5Y and IMR-32 cells with APE1 ectopic-expression, there was an decrease of 22% and 11% respectively in G.B(20 μM)+Aβ treated APE1 expressing cells while decreased RNS levels by 30% and 15% respectively were seen in G.B(40 μM)+Aβ treated APE1 expressing SH-SY5Y and IMR-32 cells (**Figure 4.16** and **Table 4.16**) and (**Figure 4.17** and **Table 4.17**). An increase of 25% and 36% (significant) and an increase of 19% and 39% (significant) was observed in G.B(20 μM)+Aβ and G.B(40 μM)+Aβ treated SH-SY5Y and IMR-32 cells respectively when compared with their respective G.B treated ectopically APE1 expressing cells.

4.11: Determination of Complex-I Activity in SH-SY5Y and IMR-32 cells with APE1 ectopic-expression after treatment with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B): The activity of complex-I in the mitochondria was found to be decreased by 33% and 22% in SH-SY5Y and IMR-32 cells respectively upon treatment with Aβ(25-35) as compared to control SH-SY5Y and IMR-32 cells. Treatment with G.B showed that the activity was increased by 19% and decreased by 11% as compared to the respective untreated control SH-SY5Y and IMR-32 cells respectively. A significant increase by 79% was observed when the SH-SY5Y cells were pre-treated with G.B for 3 hr prior to treating with Aβ(25-35) as compared to Aβ(25-35)-treated SH-SY5Y cells while in case of IMR-32 cells, there was a mere 2% increase in the activity. Further, there was an increase of 27% when ectopically APE1 expressing SH-SY5Y cells were compared with untreated control

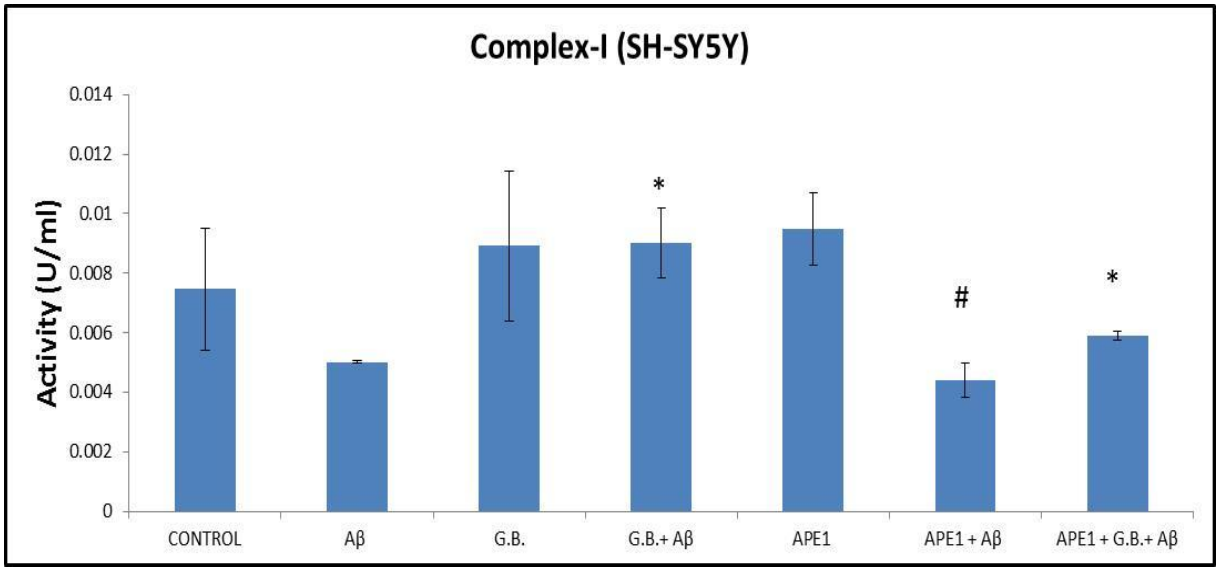


Figure 4.18(a): The mitochondrial complex-I activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

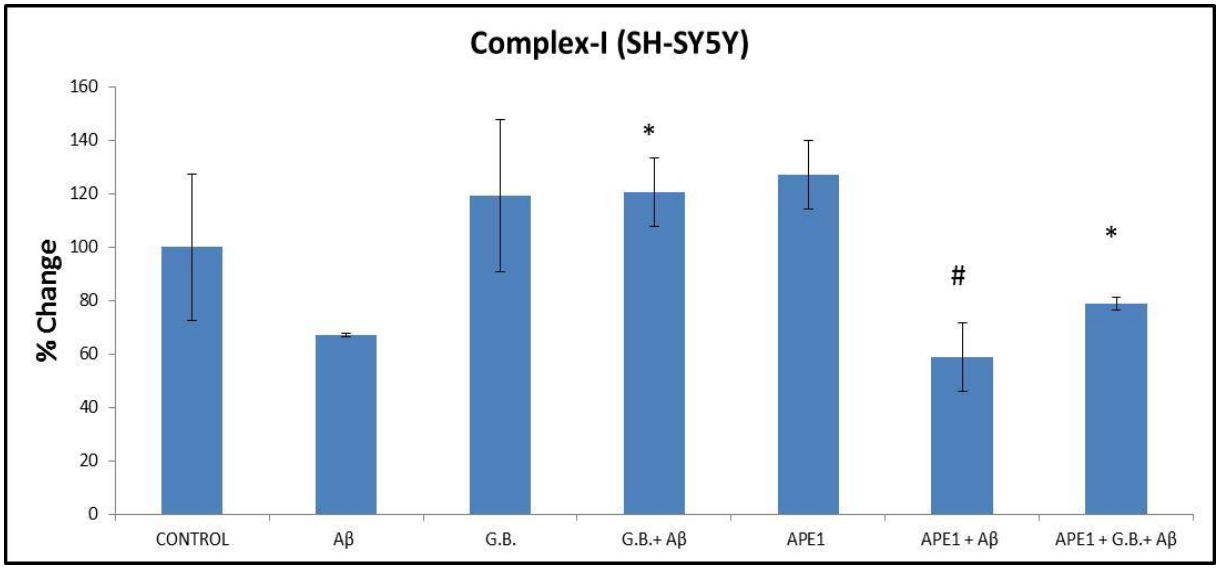


Figure 4.18(b): The mitochondrial complex-I activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.18: The mitochondrial complex-I activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.007±0.002	100±27
Aβ	20	0.005±0.000	67±0.6
G.B	20	0.008±0.002	119±28
G.B + Aβ	20+20	0.009±0.001*	120±13
APE1	-	0.009±0.001	127±13
APE1 + Aβ	20	0.004±0.0005$^{\#}$	58±13
APE1 + G.B + Aβ	20+20	0.005±0.0001*	79±2.3

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when the respective Aβ-treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=2).

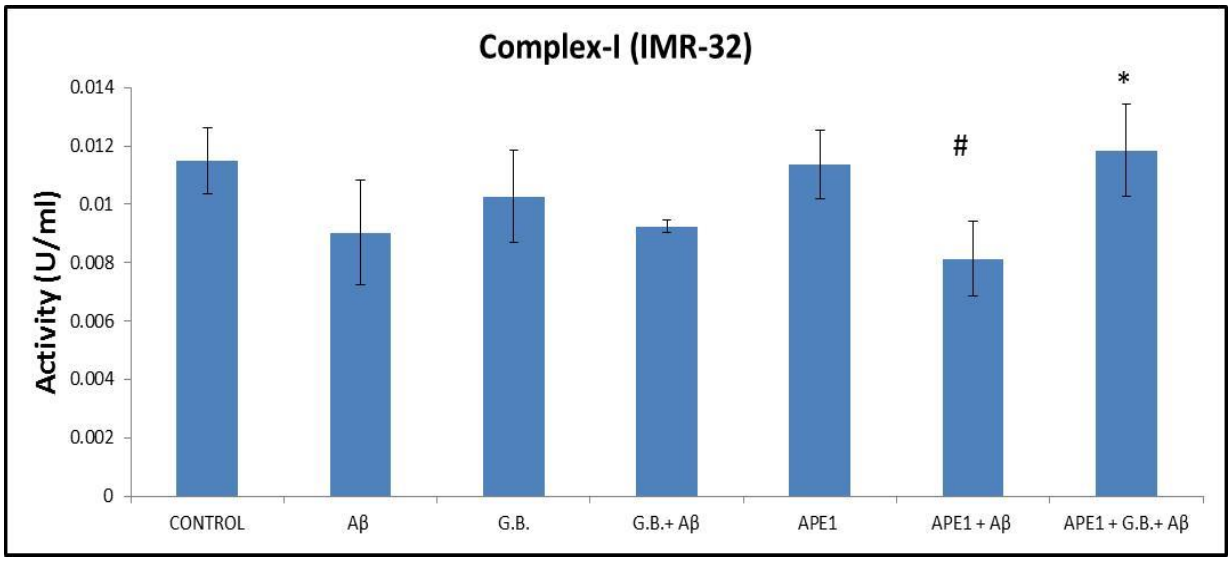


Figure 4.19(a): The mitochondrial complex-I activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

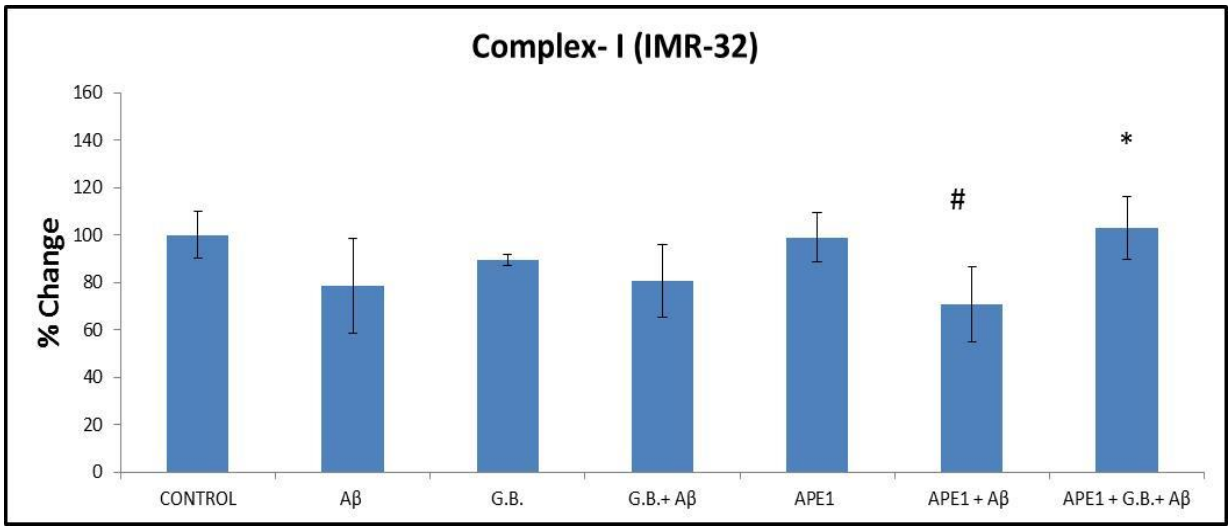


Figure 4.19(b): The mitochondrial complex-I activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.19: The mitochondrial complex-I activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.011±0.001	100±10
Aβ	20	0.009±0.001	78±20
G.B	20	0.01±0.001	89±15
G.B + Aβ	20+20	0.009±0.0002	80±15
APE1	-	0.01±0.001	99±10
APE1 + Aβ	20	0.008±0.001$^{\#}$	71±16
APE1 + G.B + Aβ	20+20	0.011±0.001*	103±13

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when the respective Aβ-treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=3).

cells while the IMR-32 cells with ectopic APE1 expression showed comparable activity to that of the untreated controls (**Figure 4.18** and **Table 4.18**) and (**Figure 4.19** and **Table 4.19**). It was also observed that Aβ(25-35)-treatment to SH-SY5Y and IMR-32 cells with ectopic APE1 expression caused a significant decrease of 54% and 29% respectively as compared to the respective APE1 control cells. Furthermore, a significant increase of 34% and 46% respectively was observed when Aβ(25-35)-treated ectopically APE1 expressing SH-SY5Y and IMR-32 cells were pre-treated with G.B on comparison with only Aβ(25-35)-treated cells with ectopic APE1 expression.

4.12: Determination of Complex-III Activity in SH-SY5Y and IMR-32 cells with ectopic APE1 expression after treatment with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B): The activity of complex-III in the mitochondria was found to be increased by 52% and 33% in SH-SY5Y and IMR-32 cells respectively on treating with Aβ(25-35) as compared to control SH-SY5Y and IMR-32 cells, may be due to some compensatory mechanism operating in the cell. Treatment with G.B showed that the activity was increased significantly by 120% in SH-SY5Y cells as compared to the untreated control cells. Not much increase in activity was seen in case of IMR-32 cells. A significant decrease of 43% and 29% was observed when the SH-SY5Y and IMR-32 cells respectively were pre-treated with G.B for 3 hr prior to treatment with Aβ(25-35) as compared to Aβ(25-35)-treated cells. There was a significant increase of 79% and 203% respectively when ectopic APE1 expressing SH-SY5Y and IMR-32 cells were compared with the respective untreated control cells. It was observed that Aβ(25-35)-treatment to both SH-SY5Y and IMR-32 cells with ectopic APE1 expression caused an increase of 44% (significant) and 7% (**Figure 4.20** and **Table 4.20**) and (**Figure 4.21** and **Table 4.21**) respectively. Furthermore, a significant decrease of 37% and 11% respectively was observed when Aβ(25-35)-treated ectopic APE1 expressing SH-SY5Y and IMR-32 cells were pre-treated with G.B on comparison with only Aβ(25-35)-treated cells with ectopic APE1 expression, which is much higher to the activity as seen in untreated control cells.

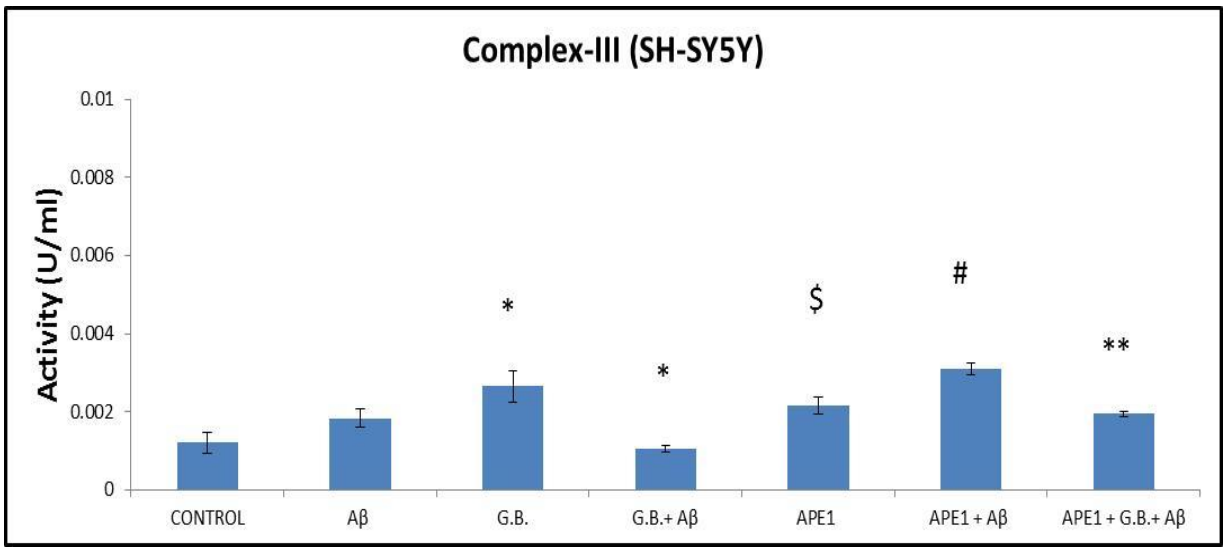


Figure 4.20(a): The mitochondrial complex-III activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

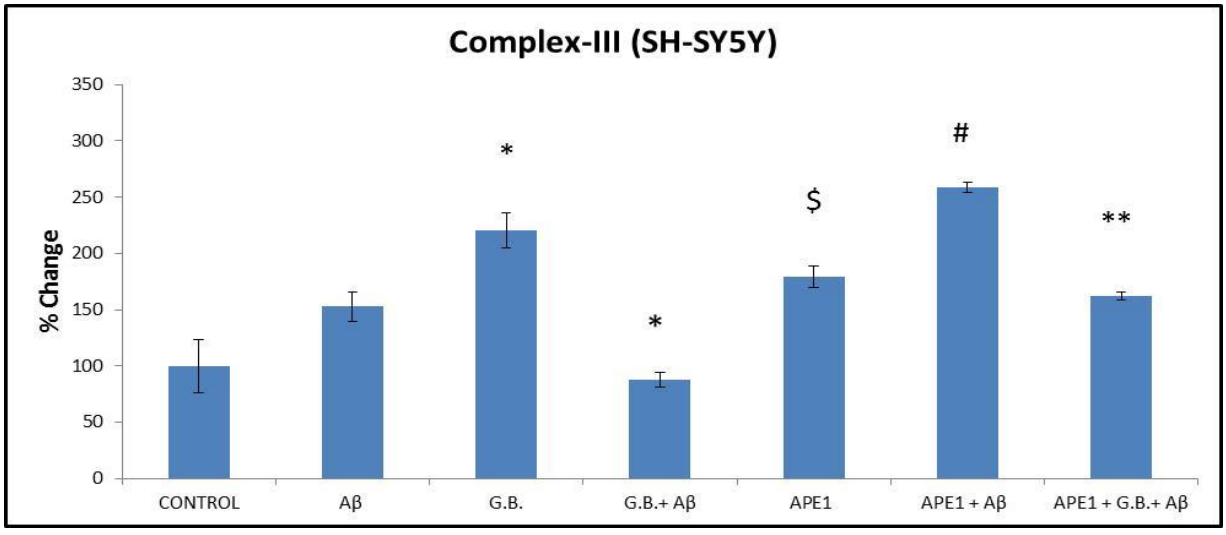


Figure 4.20(b): The mitochondrial complex-III activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.20: The mitochondrial complex-III activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.0012±0.0002	100±23
Aβ	20	0.0018±0.001	152±13
G.B	20	0.0026±0.0004*	220±16
G.B + Aβ	20+20	$0.001\pm0.0000^{*}$	87±7
APE1	-	$0.0021\pm0.00021^{\$}$	179±10
APE1 + Aβ	20	$0.0031\pm0.0001^{\#}$	258±4
APE1 + G.B + Aβ	20+20	$0.0019\pm0.0000^{**}$	162±3

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\$}p\leq0.05$ and $^{\$\$}p\leq0.005$ when untreated cells were compared with untreated cells with ectopic APE1 expression; $^{*}p\leq0.05$ and $^{**}p\leq0.005$ when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}p\leq0.05$ and $^{\#\#}p\leq0.005$ when the respective Aβ-treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=2).

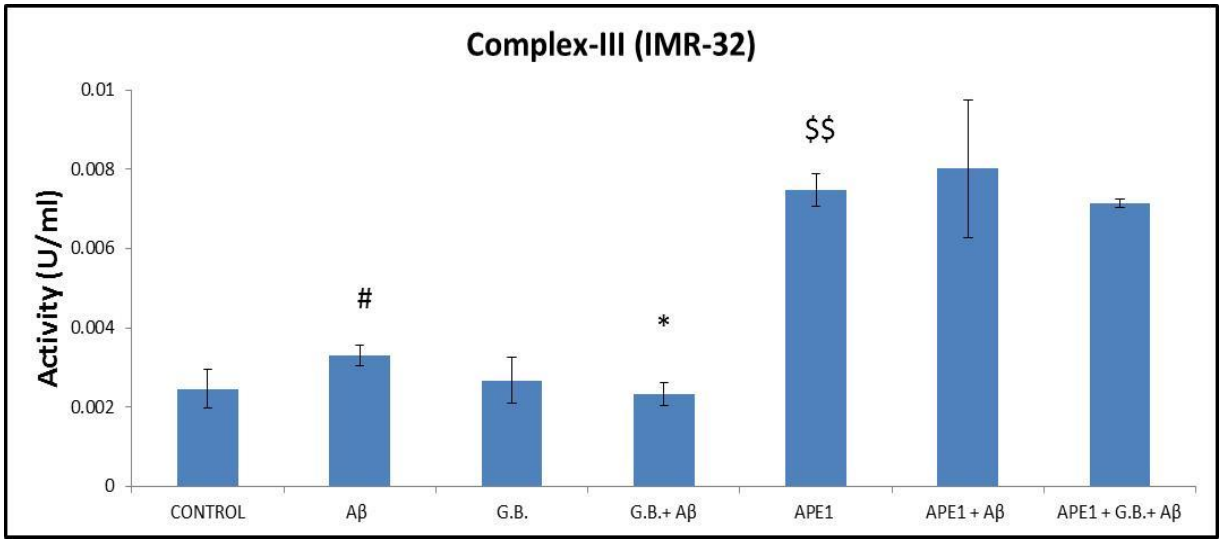


Figure 4.21(a): The mitochondrial complex-III activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

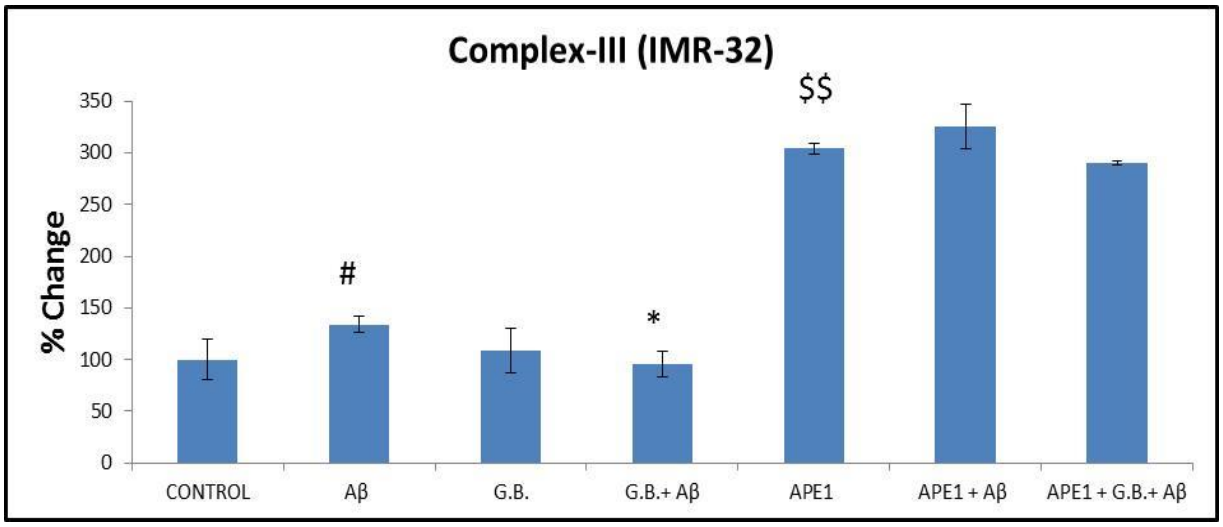


Figure 4.21(b): The mitochondrial complex-III activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.21: The mitochondrial complex-III activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.0024±0.0004	100±19
Aβ	20	0.0032±0.0002$^{\#}$	133±8
G.B	20	0.0026±0.0005	108±21
G.B + Aβ	20+20	0.0023±0.0002*	95±12
APE1	-	0.007±0.0004$^{\$\$}$	303±5
APE1 + Aβ	20	0.008±0.001	326±21
APE1 + G.B + Aβ	20+20	0.0071±0.0001	290±1

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\$}p\leq0.05$ and $^{\$\$}p\leq0.005$ when untreated cells were compared with untreated cells with ectopic APE1 expression; *p≤0.05 and **p≤0.005 when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}p\leq0.05$ and $^{\#\#}p\leq0.005$ when the respective Aβ treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=3).

4.13: Determination of Complex-IV Activity in SH-SY5Y and IMR-32 cells with ectopic APE1 expression after treatment with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B): The mitochondrial complex-IV activity calculated was seen to be significantly decreased by 59% (significant) and 57% respectively in Aβ(25-35)-treated SH-SY5Y and IMR-32 cells as compared with the respective untreated control cells. A significant increase by 111% and 155% was seen when the SH-SY5Y and IMR-32 cells were pre-treated with G.B prior to treating with Aβ(25-35) as compared to the respective Aβ(25-35)-treated cells. Further, SH-SY5Y cells treated alone with G.B had comparable activity to that of control SH-SY5Y untreated cells. While, an increase of 31% was seen in case of IMR-32 cells. When cells with ectopic APE1 expression were compared with untreated control, there was a decrease of 33% complex-IV activity. IMR-32 cells with ectopic APE1 expression had comparable activity to that of untreated control. The complex-IV activity was observed to be increased by 19% and decreased by 7% respectively in Aβ(25-35)-treated APE1 expressing SH-SY5Y and IMR-32 cells as compared to the respective APE1 control cells (**Figure 4.22** and **Table 4.22**) and (**Figure 4.23** and **Table 4.23**). Further, a significant increase in activity by 27% was seen in ectopically APE1 expressing SH-SY5Y cells treated with G.B+Aβ(25-35), which is comparable with that of untreated control SH-SY5Y cells and a 92% significant increase was seen in ectopically APE1 expressing IMR-32 cells treated with G.B+Aβ(25-35), which is much higher as compared to untreated IMR-32 control cells.

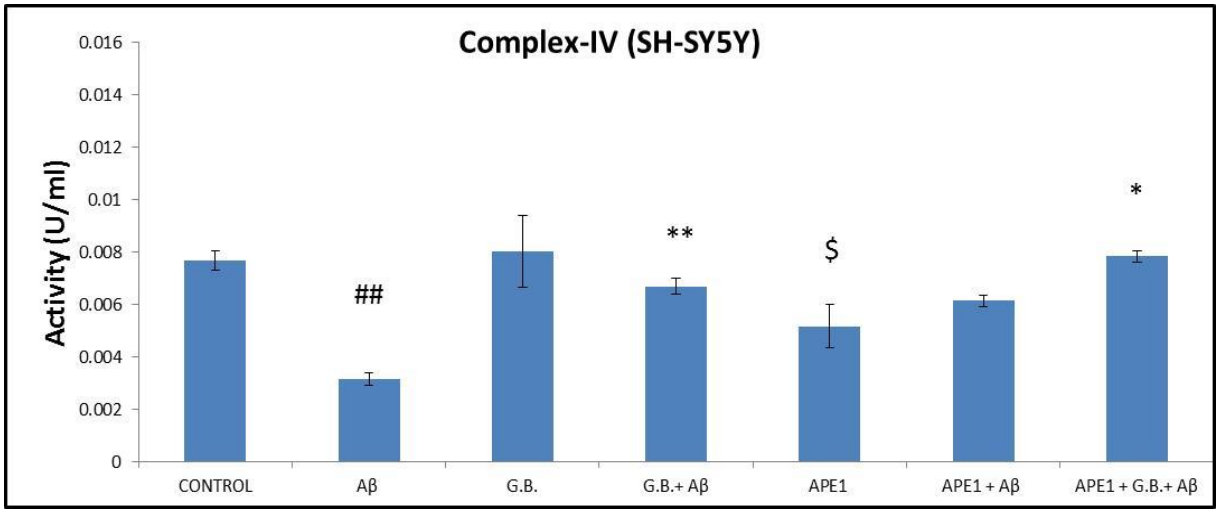


Figure 4.22(a): The mitochondrial complex-IV activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

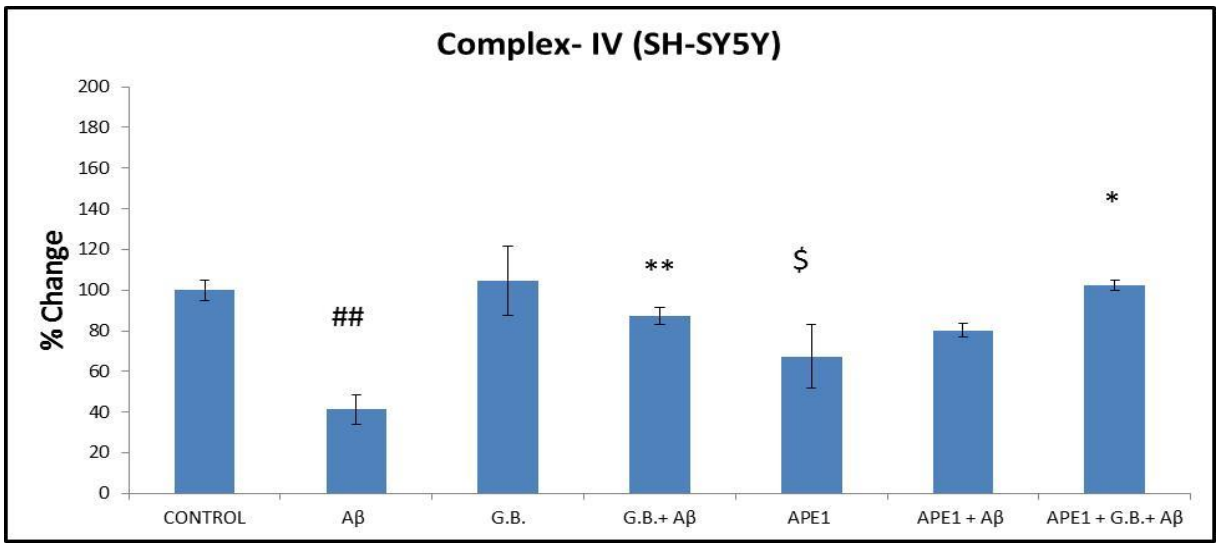


Figure 4.22(b): The mitochondrial complex-IV activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.22: The mitochondrial complex-IV activity in SH-SY5Y cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.0076±0.0003	100±5
Aβ	20	0.0031±0.0002$^{\#\#}$	41±7
G.B	20	0.008±0013	104±17
G.B + Aβ	20+20	0.0066±0.0002**	87±4
APE1	-	0.0051±0.0008$^{\$}$	67±16
APE1 + Aβ	20	0.0061±0.0002	80±3
APE1 + G.B + Aβ	20+20	0.0078±0.0002*	102±2

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\$}p\leq0.05$ and $^{\$\$}p\leq0.005$ when untreated cells were compared with untreated cells with ectopic APE1 expression; $^{*}p\leq0.05$ and $^{**}p\leq0.005$ when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}p\leq0.05$ and $^{\#\#}p\leq0.005$ when the respective Aβ-treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=2).

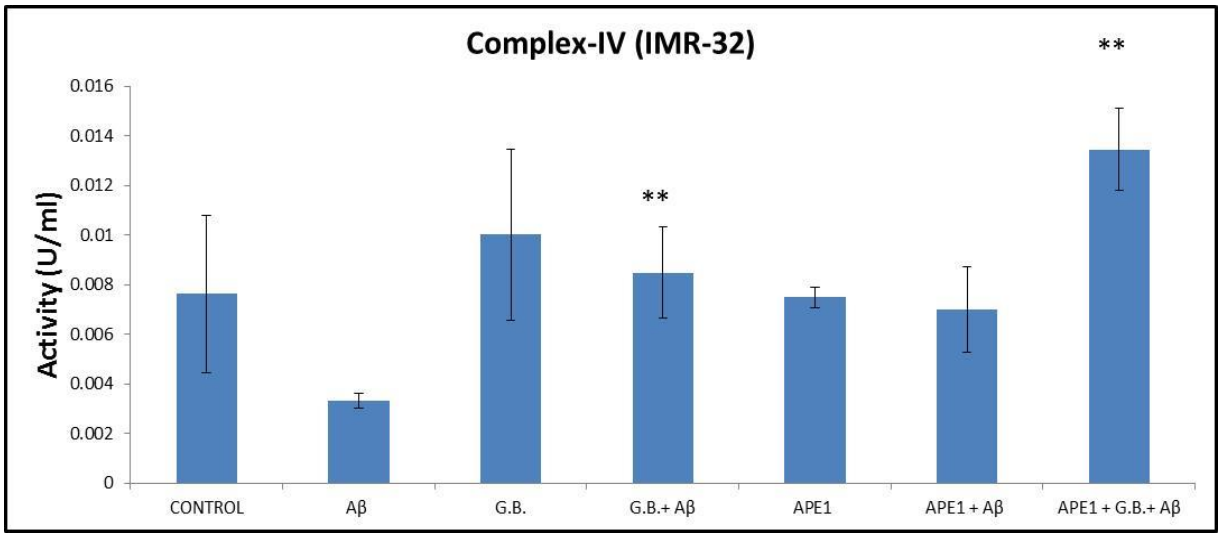


Figure 4.23(a): The mitochondrial complex-IV activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as activity in U/ml.

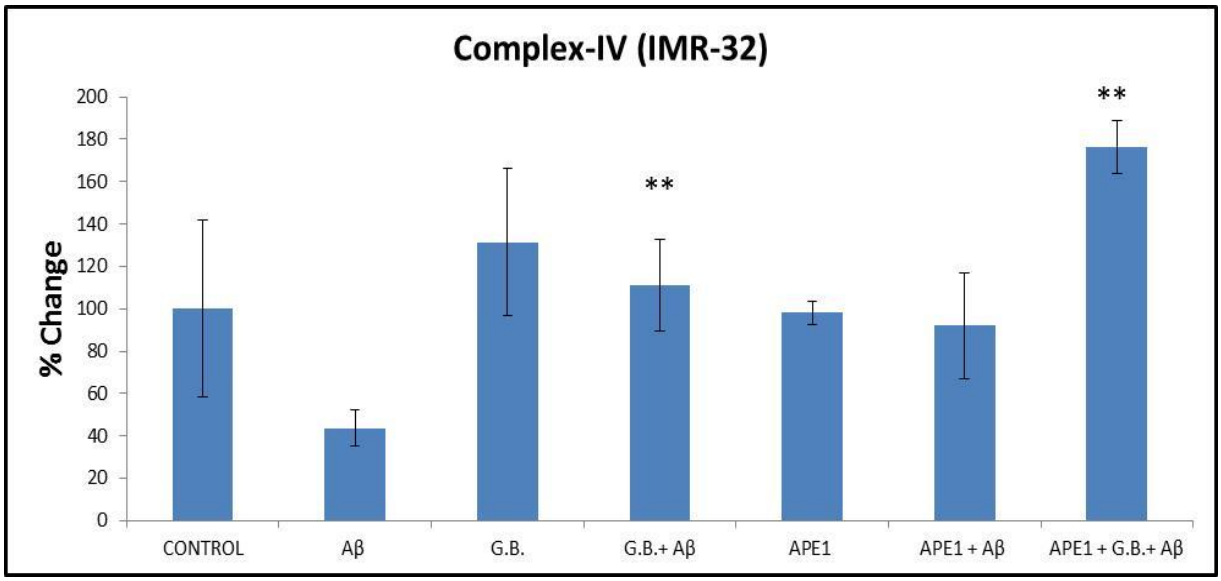


Figure 4.23(b): The mitochondrial complex-IV activity in IMR-32 treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression expressed as % change.

Table 4.23: The mitochondrial complex-IV activity in IMR-32 cells treated with the oxidant Aβ(25-35) and phytochemical Ginkgolide B (G.B) with and without ectopic APE1 expression.

Sample	Concentration (μM)	Activity (U/ml)	% Change
Control	-	0.0076±0.003	100±41
Aβ	20	0.0033±0.0002	43±8
G.B	20	0.01±0.003	131±34
G.B + Aβ	20+20	0.0084±0.0018**	111±21
APE1	-	0.0074±0.004	98±5
APE1 + Aβ	20	0.007±0.0017	91±25
APE1 + G.B + Aβ	20+20	0.013±0.0016**	176±12

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\$}p\leq0.05$ and $^{\$\$}p\leq0.005$ when untreated cells were compared with untreated cells with ectopic APE1 expression; $^{*}p\leq0.05$ and $^{**}p\leq0.005$ when the respective G.B+Aβ treated cells were compared with the respective Aβ-treated cells; and $^{\#}p\leq0.05$ and $^{\#\#}p\leq0.005$ when the respective Aβ-treated cells were compared with the respective untreated control cells. The results are presented as mean ± standard deviation (n=3).

4.24: Western Blotting. APE1 ectopic-expression in SH-SY5Y and IMR-32 cells after cell transfection was confirmed by Western blotting. It was observed that as compared to the empty vector (**lane no. 1, Figure 4.24**), there was an increase in APE1 expression in IMR-32 and SH-SY5Y cells (**lane no. 2 & 3, Figure 4.24**) which were ectopically expressing APE1 upon cell transfection. APE1 expression was seen at 37kDa, the molecular weight of APE1, by using a marker.

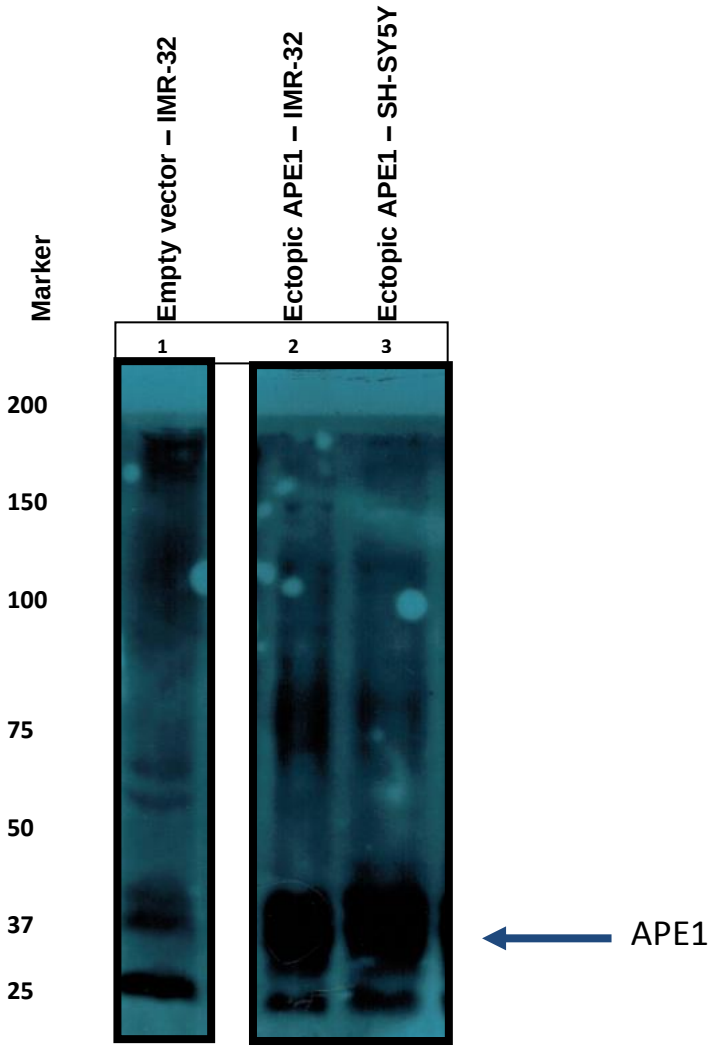


Figure 4.24: Confirmation of ectopic APE1 expression in SH-SY5Y and IMR-32 cells upon cell transfection by Western blotting.

CHAPTER - 5

DISCUSSION

The present study was aimed to provide insight into the understanding the pathogenesis of AD linking Aβ-induced oxidative stress, mitochondrial dysfunction, and the role of APE1. In this study, the aim was to see how APE1 ectopic-expression affects the neuronal cells in overcoming the oxidative stress induced by Aβ(25-35) using SH-SY5Y and IMR-32 human neuroblastoma cells. In addition, modulation/protection of APE1's activities by the phytochemical Ginkgolide B (from leaves of *G. biloba*) was also studied. It was hypothesized that the presence of Ginkgolide B (G.B) may help in reducing mitochondrial oxidative stress, and further this will reduce Aβ-induced dysfunction of mitochondrial respiratory system of the neuronal cells in maintaining cellular energy demands.

Mitochondria play an important role in the pathogenesis of neurodegenerative diseases and aging. Linking aging with neurodegenerative diseases, the free radical theory of aging suggests that oxidative imbalance i.e. elevated levels of ROS have a role in the pathogenesis of many neurodegenerative diseases like AD (Clark *et al.*, 2010). Various studies have attempted to find a link between the oxidative stress-related neurodegeneration and decreased mtDNA repair capacity in neuronal cells. Altered levels of free radical scavengers and oxidative stress with aging may lead to mitochondrial dysfunction, which may reduce the capacity of neuronal cells to repair mtDNA damage and protect against mutations in the mitochondrial genome.

Extensive studies in the recent years have linked many neurodegenerative studies to altered DNA repair mechanisms i.e. BER, double strand break (DSB) and single strand break (SSB) repair, mitochondrial defects and oxidative stress. While the presence of nucleotide excision repair (NER) pathway in mitochondria has not yet been clarified, emerging evidence reveals the existence of mismatch repair (MMR) and BER-pathway (Hegde *et al.*, 2012). Oxidative DNA damage repair is the major pathway observed in neurodegenerative diseases and the best-known DNA repair system studied in mitochondria. BER-pathway is the known to be the predominant pathway in mitochondria for removal of the oxidized base lesions generated in presence of oxidative stress. APE1 is the central enzyme of the BER-

pathway, acting on AP-sites and exerting its reduction–oxidation (redox) modification activity on some TFs including AP-1, NF-kB and p53, thus regulating their DNA-binding activity (Bhakat *et al.*, 2009; Hegde *et al.*, 2012; Izumi *et al.*, 2003).

In order to induce oxidative stress in SH-SY5Y and IMR-32 cells, Aβ(25-35) peptide was used. On the basis of the cell viability assay, 20 μM of Aβ(25-35) was used throughout the study for both SH-SY5Y and IMR-32 cells. On exposure to Aβ(25-35), the levels of ROS/RNS produced intracellularly were determined. It was found that in the presence of Aβ(25-35), there was a significant increase in the intracellular ROS levels in SH-SY5Y cells as compared to the control untreated SH-SY5Y cells. Similar trend was seen in the intracellular ROS levels in IMR-32 cells on exposure to Aβ(25-35). These results are consistent with various studies showing Aβ(25-35) as a ROS generator and as neurotoxic agent (Barger *et al.*, 1995; Jang *et al.*, 2005; Kang *et al.*, 2001).

Studies have shown that there is an over-production of NO owing to up-regulation of iNOS expression in neurons and glia. This is due to various inflammatory responses found in the brain under a variety of neurodegenerative diseases like AD and PD (Togo *et al.*, 2004). In my study, it was examined whether Aβ(25-35) induces NO within cells, by using a fluorescence probe, DAF-FM, which can directly detect NO. It was found that there was an increased NO production in presence of Aβ(25-35) in SH-SY5Y cells as compared to the control. Likewise, a similar significant increase in intracellular NO was found in IMR-32 cells on exposure to Aβ(25-35).

Many studies have indicated that polyphenols and flavonoids acting as antioxidants and their ability to scavenge ROS (Chen *et al.*, 2006; Lagoa *et al.*, 2011; Lin *et al.*, 2012). Some plants have been shown to exhibit health benefits, particularly for medicinal properties in the brain. *Ginkgo biloba* extracts have been reported to protect neurons against oxidative stress, but the underlying mechanisms are not fully understood (Ahlemeyer *et al.*, 2003; Maclennan *et al.*, 2002). In the present study, it was studied that the effect of Ginkgolide B, a terpene lactone component of

standardized extract EGb 761, on the intracellular ROS/RNS levels induced by Aβ(25-35). Two concentrations of G.B i.e. 20 µM and 40 µM were chosen based on the cell viability assay. It was found that G.B+Aβ treated cells showed attenuation of ROS production when compared with Aβ(25-35)-treated cells in both SH-SY5Y and IMR-32 cells. More attenuation of ROS was seen when cells were pre-treated with 20 µM of G.B in both the cell lines. This result is in line with a finding that showed the neuroprotective effect of G.B against Aβ(25-35)-induced neurotoxicity leading to inhibition of ROS accumulation in SH-SY5Y cells (Shi *et al.*, 2009). Also, cells treated alone with 20 µM of G.B showed comparable ROS levels as compared to the control in both SH-SY5Y and IMR-32 cells.

On studying the intracellular NO levels, it was found that there is a significant increase in NO levels when the SH-SY5Y cells were treated with 40 µM of G.B alone as compared to the untreated cells whereas with 20 µM of G.B there was much less increase in NO production as compared to the control SH-SY5Y cells. In support of this finding, there are reports which show that *Ginkgo biloba* increases NO production in human endothelial cells (Persson *et al.*, 2008). Next, Aβ(25-35) caused increased NO levels in both SH-SY5Y and IMR-32 cells as compared to their respective untreated control cells, which was brought down to a greater extent on pre-treating the cells with 20 µM G.B than with 40 µM G.B In case of IMR-32 cells, a significant increase in NO levels was seen in G.B+Aβ treated cells as compared to cells treated with G.B alone, for both 20 µM and 40 µM of G.B An earlier study showed that EGb 761 significantly reduces gentamycin-induced NO production in human cochlear cells (Yang *et al.*, 2011). Thus, pointing that Aβ increased the intracellular NO levels significantly in IMR-32 cells, which was brought down by pre-treating with G.B

APE1 plays a central role in the cellular response to oxidative stress. Attempts have been made to link increased levels of APE1 with protection in neuronal cells. One study has showed that there is an increased nuclear expression of APE1 in cerebral cortical regions of AD patients (Marcon *et al.*, 2009). Another study showed the colocalization of APE1 with Aβ in the senile plaques in AD hippocampus (Tan *et*

al., 2009). Also, APE1 immunostaining was found to be relatively low in control AD brain sections but was seen to be increased in regions of neuronal injury (Tan et al., 1998). These results suggest that over-expression of APE1 in injured neurons may be part of a response to oxidative stress and an attempt to repair damaged DNA in AD.

APE1 is found to be present preferentially in nucleus with conditional distribution in the mitochondria. Considering the importance of the mitochondria in the cellular response to oxidative stress, studies have been conducted to find out the role of APE1 in the mitochondria. Recent studies have confirmed the existence of APE1 in the mitochondria (Chattopadhyay *et al.*, 2006; Li *et al.*, 2010). Under normal cellular condition, the level of APE1 is minimal, which increases in presence of oxidative stress in the cell. An earlier study showed that APE1 rapidly re-localizes into mitochondria following H_2O_2 activation and might exert a protective function (Frossi *et al.*, 2002).

In the present study, modulation of APE1 expression during Aβ(25-35)-induced oxidative stress was studied. Indirect ELISA was done to measure APE1 levels in the mitochondria under different treatment conditions in SH-SY5Y cells. An earlier study conducted by Tan *et al.,* showed that high concentrations of Aβ(1-42) caused reduction of APE1 expression leading to neuronal death while lower concentrations of Aβ(1-42) transiently induced APE1 expression and was associated with prolonged neuronal survival (Tan *et al.*, 2009). In my study, it was found that 20 µM of Aβ(25-35) caused a significant decrease in mitochondrial APE1 levels as compared to control SH-SY5Y cells. Pre-treatment with G.B along with exposure to Aβ(25-35) caused an increase in APE1 levels in the mitochondria when compared to the control cells. In support of this finding, a recent study showed a decrease in APE1 levels against $AlCl_3$-induced neurotoxicity, which were up-regulated on treatment with the phytochemical resveratrol providing protection against neuroinflammation induced by $AlCl_3$ (Zaky *et al.*, 2013). Thus, it points towards the modulation of APE1 by G.B in the presence of Aβ(25-35)-induced oxidative stress. Subsequently, APE1 ectopic-expression studies were carried to find out the neuromodulatory role of G.B on

mitochondrial functions after Aβ(25-35)-induced oxidative stress in SH-SY5Y and IMR-32 neuronal cells with ectopic APE1 expression.

Although, studies have demonstrated that there is a reduction in the expression of APE1 in neural tissues after neuronal insult, the role of APE1 in regulating neurotoxicity remains to be identified. An earlier study showed that over-expression of wild-type APE1 in hippocampal and sensory cells resulted in a significant increase in cell viability after exposure to various concentrations of H_2O_2 (Vasko *et al.*, 2005). In the present study, cell viability was checked in SH-SY5Y and IMR-32 cells with ectopic APE1 expression in presence of the oxidant, Aβ(25-35). The results showed that Aβ(25-35) caused a decrease in cell survival when compared to the respective SH-SY5Y and IMR-32 cells ectopically expressing APE1. But, cell survival was increased in Aβ(25-35)-treated APE1 ectopically-expressing cells as compared to the respective Aβ(25-35)-treated SH-SY5Y and IMR-32 cells without APE1 expression. An increase in cell viability in untreated cells with APE1 expression as compared to untreated cells expressing empty vector was seen in both SH-SY5Y and IMR-32 cells. When SH-SY5Y cells with APE1 over-expression were treated with the phytochemical G.B, there was a rise in cell survival as compared to APE1 control cells. Similar trend was seen in IMR-32 cells when treated with the phytochemical G.B. On comparison with cells without APE1 ectopic-expression, it was found that treatment with G.B caused much increased cell viability in the respective SH-SY5Y and IMR-32 cells with ectopic APE1 expression.

Furthermore, cell viability assay was done to see modulation of APE1 by G.B in presence of Aβ(25-35) showed that pre-treatment with G.B at both the concentrations of 20 µM and 40 µM prior to treating with Aβ(25-35) increased the cell survival in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression much more than in the respective cells without APE1 expression given the same treatment. This shows that pre-treatment with G.B might modulate the activity of APE1 providing neuro-protection to cells from the damage caused by Aβ(25-35)-induced oxidative stress, leading to increased neuronal survival.

To see how APE1 modulates oxidative stress in the cell, intracellular ROS levels were measured. The results showed that ectopic APE1 expression in both SH-SY5Y and IMR-32 cells decreased the ROS levels as compared to cells expressing the empty vector. In both SH-SY5Y and IMR-32 cells, there was attenuation of ROS levels when cells with ectopic APE1 expression were treated with G.B (20 and 40 μM) as compared to APE1 control cells. There were comparable ROS levels as compared to APE1 control cells in the presence of oxidative stress induced by Aβ(25-35) in both the cell lines which are expressing APE1. When compared with cells without ectopic APE1 expression, it was seen that APE1 expression lead to attenuation of ROS levels on treatment with Aβ(25-35) in both SH-SY5Y and IMR-32 cells. Also, similar trend was seen on treatment with G.B. Pre-treatment with G.B at both the concentrations of 20 μM and 40 μM prior to treating with Aβ(25-35) caused a decrease in ROS levels in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression as compared to the respective cells without ectopic APE1 expression. These results advocates for neuroprotective role of APE1 in regulating neurotoxicity induced by Aβ(25-35) in the presence of the phytochemical G.B.

Subsequently, intracellular RNS levels were also measured in SH-SY5Y and IMR-32 cells after ectopic APE1 expression. A decrease in RNS levels in untreated cells with APE1 over-expression as compared to untreated cells expressing empty vector was seen in both SH-SY5Y and IMR-32 cells. It was observed that Aβ(25-35) caused a significant increase in NO production in SH-SY5Y cells when compared to the SH-SY5Y untreated cells ectopically expressing APE1. But, comparable NO levels were seen when IMR-32 cells with ectopic APE1 expression treated with Aβ(25-35) were compared to the IMR-32 cells ectopically expressing APE1. NO production was seen to be decreased in Aβ(25-35)-treated APE1 expressing cells as compared to the respective Aβ(25-35)-treated SH-SY5Y and IMR-32 cells without APE1 expression. When SH-SY5Y cells with ectopic APE1 expression were treated with the phytochemical G.B, there was a decrease in RNS levels as compared to APE1 control cells. Similar trend was seen in IMR-32 cells when treated with G.B. On comparison with cells without ectopic APE1 expression, it was found that treatment

with G.B caused much decreased RNS levels in respective SH-SY5Y and IMR-32 cells with ectopic APE1 expression. Pre-treatment with G.B at both the concentrations 20 µM and 40 µM prior to treating with Aβ(25-35) caused a decrease in NO levels in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression as compared to the respective cells without ectopic APE1 expression. This points out that G.B treatment prevents the oxidative damage in both SH-SY5Y and IMR-32 cells by modulating APE1 function in response to Aβ(25-35)-induced oxidative stress responses.

A general decline in CNS function is associated with normal aging. In relation to this, certain mutations in mitochondrial genes have been implicated in the etiology of various age-related neurodegenerative diseases like AD. The pathogenesis resulting from mtDNA mutations is believed to be involved in impaired OXPHOS with a concomitant increase in ROS production. In order to study the effect of Aβ(25-35) at the mitochondrial level at OXPHOS performance, it was planned to see the modulation of ETC activities of complex-I, III & IV by APE1 in the presence of G.B.

Numerous studies have shown that G.B protects against aging-associated mitochondrial dysfunction and reduces oxidative stress in the mitochondria (Abdel-Kader *et al.*, 2007; Shi *et al.*, 2010). The results obtained showed that complex-I activity in the mitochondria was reduced in both SH-SY5Y and IMR-32 cells in the presence of Aβ(25-35)-induced stress. This was seen to be increased after treating the cells with G.B. When treated with G.B alone, the SH-SY5Y cells had more activity while the IMR-32 cells showed comparable activity as compared to their respective control cells. In support of this finding, a recent study performed by Rhein et al., showed increased activity of complex-I on treatment with G.B+Aβ(25-35) and comparable activity on treatment with G.B alone in SH-SY5Y cells (Rhein *et al.*, 2010).

Further, ectopic APE1 expression studies were conducted under the same treatment conditions as above for determining complex-I activity. A previous study has provided evidence that APE1 regulates the mitochondrial function against

oxidative stress due to its role in redox regulation (Li *et al.*, 2012). The activity of complex-I was more in untreated SH-SY5Y cells with ectopic APE1 expression as compared to untreated control SH-SY5Y cells without APE1 expression, showing the neuroprotective effect of APE1 in these cells. Comparable activity of complex-I was found in IMR-32 cells with ectopic APE1 expression as compared to IMR-32 cells without APE1 expression. A significant decrease in the activity of complex-I was seen in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression in the presence of Aβ(25-35)-induced stress as compared to APE1 control cells. On pre-treating with G.B, the decrease in activity of complex-I in the presence of Aβ(25-35)-induced stress was subsequently found to be increased significantly in both SH-SY5Y and IMR-32 cells. This activity as measured in the presence of G.B+Aβ(25-35) was comparable to the untreated IMR-32 control cells. Thus, it shows that APE1 has a neuromodulatory effect on complex-I activity in bringing the activity of complex-I to the basal level in the presence of G.B and protecting the cellular energy levels through OXPHOS.

Interesting results were found when complex-III activity was determined. It was found that there was an increased complex-III activity when cells (SH-SY5Y and IMR-32) were given Aβ(25-35) treatment as compared to the respective controls. This result is in line with a previous finding done on SH-SY5Y cells (Rhein *et al.*, 2010). This could be due to some compensatory mechanism to rescue Aβ(25-35)-induced mitochondrial defects. Treatment with G.B caused an increase in the activity in SH-SY5Y cells, but comparable activity in IMR-32 cells as compared to the respective control cells. Pre-treatment with G.B prior to treating with Aβ(25-35) led to comparable activity as compared with the control cells in both the cell lines (SH-SY5Y and IMR-32), bringing the complex-III activity down to the basal level. Further, same treatments in ectopic APE1 expression showed that there was a significant increase in the complex-III activity in APE1 expressing SH-SY5Y and IMR-32 cells as compared to the respective cells without APE1 over-expression. Again, an increase in complex-III activity was seen in the presence of Aβ(25-35) in both the cells (SH-SY5Y and IMR-32) with ectopic APE1 expression. Pre-treatment with G.B, prior to treating with Aβ(25-35) showed a rise in complex-III activity in both IMR-32 and SH-SY5Y

cells, which was much more than the basal level activity. This points towards the protection of complex-III activity by APE1 in presence of increased ROS (due to electron leakage occurring at complex-III) in presence of G.B.

Lastly, complex-IV activity was determined in the SH-SY5Y and IMR-32 cells with and without ectopic APE1 expression. The activity of complex-IV was found to be reduced significantly in the presence of Aβ(25-35)-induced stress responses in SH-SY5Y cells as compared to the untreated control SH-SY5Y cells. Similar trend was seen in the IMR-32 cells. There was a comparable activity in G.B treated SH-SY5Y cells but much increase in complex-IV activity in G.B treated IMR-32 cells when compared to their respective controls. There was a significant up-regulation of complex-IV activity in G.B+Aβ(25-35)-treated cells as compared to only Aβ(25-35)-treated respective cells. Comparable activity of complex-IV was found in IMR-32 cells with ectopic APE1 expression as compared to cells without APE1 expression. But, a decrease in complex-IV activity was found in SH-SY5Y cells with ectopic APE1 expression as compared to cells without APE1 expression. The activity was further increased upon treatment with Aβ(25-35) in SH-SY5Y cells as compared to APE1 control SH-SY5Y cells. In case of IMR-32 cells, Aβ(25-35) treatment led to comparable activity as APE1 control IMR-32 cells. Pre-treatment with G.B, prior to treating with Aβ(25-35) showed a rise in complex-IV activity in IMR-32 cells and comparable activity to that of untreated control in SH-SY5Y cells.

Oxidative stress, especially the mitochondria-derived oxidative stress is thought to have an important role in the pathogenesis of AD. Our experimental data suggests that ectopic APE1 expression helps the neuronal cells in overcoming the oxidative stress induced by Aβ(25-35) in SH-SY5Y and IMR-32 cells. Also, the phytochemical G.B has shown to modulate APE1 activities by reducing mitochondrial oxidative stress, and further reduces Aβ-induced dysfunction of mitochondrial ETC-associated with OXPHOS of the neuronal cells in maintaining cellular energy demands. Thus, from the knowledge gained with this study and combining these parameters of oxidative damage with those of redox state of the neuronal cells, DNA-

repair activity, antioxidant defence, and neuroprotective role played by APE1 may help in designing future studies and also serve as a useful therapeutic tool towards the treatment of AD in combination with phytochemical modulation.

SUMMARY AND CONCLUSION

Mitochondria have a central role in aging-related neurodegenerative diseases like AD. Many lines of evidence have suggested that oxidative damage in association with mitochondrial dysfunction play a major role in AD progression and development. In case of late-onset, sporadic AD, an increase in ROS levels with increasing age has been thought to play a role in AD progression and development, while mitochondrial damage through oxidative stress play a significant role in the progression of familial AD. Various studies have attempted to find a link between the oxidative stress-related neurodegeneration and decreased mtDNA repair capacity in neuronal cells. But, the mechanistic link between oxidative damage, altered DNA repair mechanisms and mitochondrial dysfunction in AD development and progression is still unclear. To address this issue, the present study was designed to understand how ectopic-expression of APE1, the major BER-pathway enzyme, supports the neuronal cells in overcoming the oxidative stress induced by Aβ(25-35) *in vitro* using the neuroblastoma (SH-SY5Y and IMR-32) cell lines. In addition, it was determined whether the phytochemical Ginkgolide B (from leaves of *G. biloba*) helps in modulating/protecting APE1's functions in reducing oxidative stress and Aβ-induced dysfunction of mitochondrial ETC of the neuronal cells in maintaining cellular energy demands.

The results of the present study demonstrated that in the presence of Aβ(25-35), there was a significant increase in the intracellular ROS levels in both SH-SY5Y and IMR-32 cells, which is consistent with various studies showing Aβ(25-35) as a ROS generator and as neurotoxic (Barger *et al.*, 1995; Jang *et al.*, 2005; Kang *et al.*, 2001). Likewise, a similar increase in intracellular NO was found in SH-SY5Y and IMR-32 cells on exposure to Aβ(25-35). The neuroprotective effects of Ginkgolide B, the terpene lactone component of standardized extract EGb 761, were evaluated on the intracellular ROS/RNS levels induced by Aβ(25-35) in both the cell lines. Attenuation of ROS production was seen in G.B+Aβ treated SH-SY5Y and IMR-32 cells. The increase in intracellular NO in response to Aβ(25-35) in both SH-SY5Y and IMR-32 cells was brought down to a greater extent by pre-treating the cells with G.B.

Further, modulation of APE1 expression during Aβ(25-35)-induced oxidative stress was studied using indirect ELISA. The results demonstrated that there is a significant decrease in mitochondrial APE1 levels in presence of Aβ(25-35). Pre-treatment with G.B along with exposure to Aβ(25-35) caused an increase in APE1 levels in the mitochondria. This points towards the modulation of APE1 by G.B in presence of Aβ(25-35)-induced oxidative stress. Subsequently, ectopic APE1 expression studies were carried to find out the neuro-modulatory role of G.B on mitochondrial functions after Aβ(25-35)-induced oxidative stress in SH-SY5Y and IMR-32 cell lines with ectopic APE1 expression. The results showed that cell survival was increased in Aβ(25-35)-treated APE1 expressing cells as compared to the respective Aβ(25-35)-treated SH-SY5Y and IMR-32 cells without ectopic APE1 expression. When SH-SY5Y and IMR-32 cells with APE1 expression were treated with the phytochemical G.B, there was a rise in cell survival rate. A recent study has showed that pre-treatment with G.B caused attenuation of ROS levels and increased cell viability in SH-SY5Y cells in response to stress induced by bupivacaine (Li *et al.*, 2013). Subsequently, on comparison with cells without ectopic APE1 expression, it was found that treatment with G.B caused much decreased ROS/RNS levels in the ectopic APE1 expressing SH-SY5Y and IMR-32 cells, pointing towards the modulatory role of APE1 in regulating neurotoxicity induced by Aβ(25-35) in the presence of the phytochemical G.B. In support of this finding, an earlier study has shown that the *G. biloba* extract (EGb 761) protects against the Aβ(1-42)-induced oxidative stress in SH-SY5Y cells by blocking the Aβ(1-42)-induced apoptosis and ROS accumulation (Shi *et al.*, 2009). Another study has shown EGb 761 restores the Aβ(1-42)-induced mitochondrial failure in SH-SY5Y cells.

Further, effect of Aβ(25-35)-induced stress and modulation by G.B on mitochondrial function i.e. complex (I, III & IV) activity was studied with and without APE1 expression. Earlier studies have shown that EGb 761, which contains G.B, protects against aging-associated mitochondrial dysfunction and reduces oxidative stress in the mitochondria (Abdel-Kader *et al.*, 2007; Rhein *et al.*, 2010; Shi *et al.*, 2010). The results of my study showed that complex-I activity was reduced in both

SH-SY5Y and IMR-32 cells in presence of Aβ(25-35)-induced stress, which was increased after treating the cells with G.B. When treated with G.B alone, the SH-SY5Y cells had more activity while the IMR-32 cells showed comparable activity as compared to their respective control cells. After ectopic -expression of APE1, the activity of complex-I was found to be more as compared to untreated control SH-SY5Y cells without APE1 expression, showing the protective effect of APE1 in these cells. On pre-treating with G.B, the decrease in activity of complex-I in presence of Aβ(25-35) was subsequently found to be increased significantly in both SH-SY5Y and IMR-32 cells with ectopic- APE1 expression, pointing towards the modulatory effect of APE1 on complex-I activity in bringing the activity of complex-I to the basal level in the presence of G.B.

In relation to complex-III, an increase in the activity was found when cells (SH-SY5Y and IMR-32) were given Aβ(25-35) treatment as compared to the respective control. This could be due to some compensatory mechanism to rescue Aβ(25-35)-induced mitochondrial defects. Treatment with G.B caused an increase in the activity in SH-SY5Y cells, but comparable activity in IMR-32 cells as compared to the respective control cells. In support of this finding, a recent study showed that pre-treatment with G.B preserved the mitochondrial complex-III activity in SH-SY5Y cells (Li *et al.*, 2013). Further, same treatments with ectopic APE1 expression showed that there is an increase in complex-III activity was seen in presence of Aβ(25-35) in both cells lines studied (SH-SY5Y and IMR-32) with ectopic APE1 expression. Pre-treatment with G.B, prior to treatment with Aβ(25-35) showed a rise in complex-III activity in both IMR-32 and SH-SY5Y cells, which was much more than the basal level activity. This points toward the protection of complex-III activity by APE1 in presence of increased ROS (due to electron leakage occurring at complex-III) in the presence of G.B.

On evaluation of complex-IV activity, it was observed that there was decreased activity in Aβ(25-35)-treated SH-SY5Y and IMR-32 cells. An increase in complex-IV activity was seen when the SH-SY5Y and IMR-32 cells were pre-treated with G.B

prior to treating with Aβ(25-35) as compared to the respective Aβ(25-35)-treated cells. This is in accordance with an earlier study showing the beneficial effect of standardized *G. biloba* extract (LI 1370) on SH-SY5Y cells in response to Aβ(1-40)-induced stress in APP cells (Rhein *et al.*, 2010). Further, an increase in the activity was seen when IMR-32 cells were treated with G.B alone while the activity was close to the basal level in case of SH-SY5Y cells. Further, an increase in the complex-IV activity was observed in Aβ(25-35)-treated APE1 over-expressing SH-SY5Y cells while decreased activity was seen in IMR-32 cells. Further, an increase in activity was seen in APE1 ectopically-expressing SH-SY5Y and IMR-32 cells treated with G.B+Aβ(25-35), which was close to the basal level in case of SH-SY5Y cells and much higher than the basal level in IMR-32 cells. This suggests that G.B together with APE1 has shown to ameliorate the complex-IV activity in response to oxidative stress induced by Aβ(25-35).

The experimental data presented here suggests that APE1 ectopic-expression helps the neuronal cells in overcoming the oxidative stress induced by Aβ(25-35) in human neuroblastoma (SH-SY5Y and IMR-32) cells. Phytochemical G.B has shown to modulate the ROS/RNS levels in presence of APE1. The study demonstrates that Aβ(25-35) interferes with mitochondrial function as seen from the decline in complex-I & IV activity in presence of Aβ(25-35). Phytochemical G.B together with APE1 has shown to modulate the Aβ(25-35)-induced oxidative stress and restores OXPHOS. Thus, G.B may act as an antioxidant, reducing ROS/RNS generated by electron leakage at complexes I & III. The precise mechanism of mode of action of G.B on OXPHOS performance needs further evaluation. Further studies are needed to establish the mechanism of action of APE1 in relation to the above results. The results of the present study suggest that mitochondrial targeted therapies may be effective in AD therapeutics. Thus, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation hold great promise.

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Abstract

Alzheimer's disease (AD) is an important public health problem which affects millions of people worldwide. The major pathological hallmarks associated with AD are the accumulation of amyloid beta (Aβ) in senile plaques and neurofibrillary tangles (NFT) made up of hyperphosphorylated tau proteins. New findings suggest that oligomeric Aβ is a more toxic species than fibrillar Aβ relevant to AD pathology. Although the molecular mechanism(s) underlying the disease is not identified completely, various factors have been implicated in the development of AD. Accumulating evidences point towards the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of AD and recognise them as an early event in AD development. Ageing is considered the greatest risk factor for AD and is linked to

oxidative stress which causes accumulation of somatic mutations in mitochondrial DNA (mtDNA) over time and leads to genome instability and mitochondrial dysfunction. Recent studies on AD patients and transgenic mouse models suggest that amyloid precursor protein (APP) and Aβ localise to mitochondria, interact with mitochondrial proteins, disrupt electron transport chain (ETC), increases reactive oxygen species (ROS) level, impair axonal mitochondrial trafficking, thus leading to synaptic damage and cognitive decline associated with AD. It is not known whether accumulation of Aβ is the cause or outcome of declining mitochondrial function in AD. In order to counteract oxidative stress and maintain genome integrity, various DNA repair pathways exist, with base excision repair (BER) pathway being the predominant pathway for repairing oxidised base lesions in neuronal cells. APE1 is the central enzyme of the BER pathway, having both repair and redox activities and shown to enhance neuronal survival after oxidative stress. Newer studies are revealing the role of APE1 in maintenance of mitochondrial genome repair and function. In this scenario, antioxidant-based therapy, which could reduce oxidative stress and modulate the activities of APE1, can serve as effective treatment providing neuroprotection in AD. This review summarises some recent developments in understanding the pathogenesis of AD linking Aβ-induced oxidative stress, mitochondrial dysfunction, role of APE1 and phytochemicals towards AD therapeutics.

Keywords (separated by "-")	Alzheimer's disease - Oxidative stress - Amyloid beta - Mitochondria - APE/Ref-1 - Phytochemicals

Oxidative Stress Events and Neuronal Dysfunction in Alzheimer's Disease: Focus on APE1-/Ref-1-Mediated Survival Strategies

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Abstract

Alzheimer's disease (AD) is an important public health problem which affects millions of people worldwide. The major pathological hallmarks associated with AD are the accumulation of amyloid beta (Aβ) in senile plaques and neurofibrillary tangles (NFT) made up of hyperphosphorylated tau proteins. New findings suggest that oligomeric Aβ is a more toxic species than fibrillar Aβ relevant to AD pathology. Although the molecular mechanism(s) underlying the disease is not identified completely, various factors have been implicated in the development of AD. Accumulating evidences point towards the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of AD and recognise them as an early event in AD development. Ageing is considered the greatest risk factor for AD and is linked to oxidative stress which causes accumulation of somatic mutations in mitochondrial DNA (mtDNA) over time and leads to genome

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instability and mitochondrial dysfunction. Recent studies on AD patients and transgenic mouse models suggest that amyloid precursor protein (APP) and Aβ localise to mitochondria, interact with mitochondrial proteins, disrupt electron transport chain (ETC), increases reactive oxygen species (ROS) level, impair axonal mitochondrial trafficking, thus leading to synaptic damage and cognitive decline associated with AD. It is not known whether accumulation of Aβ is the cause or outcome of declining mitochondrial function in AD. In order to counteract oxidative stress and maintain genome integrity, various DNA repair pathways exist, with base excision repair (BER) pathway being the predominant pathway for repairing oxidised base lesions in neuronal cells. APE1 is the central enzyme of the BER pathway, having both repair and redox activities and shown to enhance neuronal survival after oxidative stress. Newer studies are revealing the role of APE1 in maintenance of mitochondrial genome repair and function. In this scenario, antioxidant-based therapy, which could reduce oxidative stress and modulate the activities of APE1, can serve as effective treatment providing neuroprotection in AD. This review summarises some recent developments in understanding the pathogenesis of AD linking Aβ-induced oxidative stress, mitochondrial dysfunction, role of APE1 and phytochemicals towards AD therapeutics.

Keywords
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1 Introduction

In 1907, Alois Alzheimer, a German psychiatrist and neuropathologist, described the hallmark lesions associated with 'presenile dementia', which later came to be known as Alzheimer's disease AD [1], a progressive and always fatal disorder characterised clinically by memory loss and behavioural abnormalities. The hallmarks described were extracellular plaques composed of amyloid beta (Aβ) and intracellular neurofibrillary tangles (NFTs) made up of a protein called tau (τ). Since then, although it has been more than 100 years that the neuropathological hallmarks of the disease have been described, the underlying molecular mechanism(s) of the pathogenesis of AD are yet to be elucidated. Since 1992, the 'Aβ cascade hypothesis' has been the main model describing the pathogenesis of AD. According to it, accumulation of Aβ, owing to increased processing of amyloid precursor protein (APP), induces biochemical, histological and clinical changes associated with the disease [2]. Various modifications of the model have taken place over the time and currently, oligomers, the soluble form of Aβ, rather than insoluble fibrillar Aβ, are considered to be the more toxic species and relevant to AD pathogenesis [3]. Many studies have proposed that oxidative stress plays an important role in the pathogenesis of AD and consider it to be one of the early changes associated with AD [4]. Also, mitochondrial dysfunction is seen as an early event in the pathogenesis of AD [5]. Thus, these studies have led to the formulation of a new hypothesis, viz. 'mitochondrial cascade hypothesis', indicating the role of the mitochondria and its dysfunction initiating late-onset AD pathologies particularly in relation to sporadic AD [6]. [AU1]

Ageing is considered to be the greatest risk factor for AD. Linking ageing with neurodegenerative diseases, the free radical theory of ageing

suggests that oxidative imbalance, i.e. elevated levels of reactive oxygen species (ROS), has a role in the pathogenesis of many neurodegenerative diseases like AD [7]. The brain is particularly vulnerable to oxidative stress due to a low level of antioxidant system and high consumption of oxygen [7]. There are a number of exogenous and endogenous sources of ROS, which increase the oxidative stress and lead to genome instability. Mitochondria are considered to be the major internal source of ROS. One study has showed that overexpression of antioxidant catalase, targeted to the mitochondria reduces oxidative damage, thus highlighting the role of the mitochondria as a source of these radicals [8]. In line with this evidence, many studies have shown the mitochondria as a source as well as a target of ROS. The link between Aβ and free radical generation ultimately leading to increased ROS in neuronal cells has been well established [9–12]. There is an emerging body of evidence revealing that Aβ enters the mitochondria and induces generation of free radicals causing oxidative damage, thus providing a link between Aβ and mitochondrial dysfunction in the pathogenesis of AD [13].

Postmitotic cells like neurons are terminally differentiated cells which cannot be replaced. Neurons are particularly sensitive to oxidative damage. Accumulation of DNA base modifications especially 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) are the major factors leading to genome instability [14, 15]. Base excision repair (BER) is the predominant pathway in the nucleus as well as in the mitochondria that removes these oxidised base lesions [16, 17]. Amongst the BER enzymes, apurinic/apyrimidinic endonuclease (APE1) is known to be a multifunctional enzyme involved in DNA repair and redox regulation of various transcription factors (TFs), thus is also known as redox effector factor 1, Ref-1 [17–19]. Elevated nuclear expression of APE1/Ref-1 in cerebral cortical regions of AD patients highlights the role of APE1 in neurons in response to oxidative stress [20]. But mitochondrial APE1's function is still not clear. Recently, it was shown that nuclear APE1 regulates nuclear-encoded mitochondrial-related genes by modulating the DNA-binding activity of nuclear respiratory factor-1 (NRF1) in a redox-dependent manner [21]. This indicates that APE1 regulates the expression of some nuclear-encoded mitochondrial constitutive genes and consequently modulates mitochondrial functions in response to oxidative stress.

There are a number of phytochemicals which have shown their neuroprotective abilities in rescuing the Aβ-induced oxidative stress in vitro and in vivo. Some of them have also shown modulation of APE1/Ref-1's repair and redox activities, making them effective molecules for prevention and treatment of cancers and degenerative diseases [22–24]. Thus, phytochemicals may serve as effective treatments providing neuroprotection in AD. This review summarises the available literature in the field and suggests a link between oxidative stress, mitochondrial dysfunction, APE1 and phytochemical-based interventions towards AD therapeutics.

2 Neurodegenerative Diseases

Neurodegenerative diseases represent a range of diseases affecting the central nervous system (CNS) characterised by selective neuronal vulnerability and degeneration in specific regions of the brain. This causes disabling and debilitating conditions involving either impairment of memory (dementia) or movement-related disabilities (ataxia), ultimately leading to death. The degeneration is caused due to abnormal accumulation and aggregation of proteins in specific parts of the brain intracellular or extracellular as insoluble or soluble forms. These diseases are also known as protein conformational diseases as the aggregated proteins bear a β-sheet conformation that aid in protein aggregation and fibril formation [25, 26] which are the pathological hallmarks associated with these diseases. The exact reason as to why these proteins begin to accumulate is not known but research has revealed that this may happen due to a number of reasons, majorly due to disruption of ubiquitin-proteosome machinery, autophagy failure and oxidative stress. Some of the common neurodegenerative

diseases include AD, Parkinson's disease (PD), Huntington's disease (HD) and amyotropic lateral sclerosis (ALS). Although the mechanism of the pathogenesis of these diseases is not understood to date, some genetic and environmental factors have been implicated which confer susceptibility to these diseases.

2.1 Alzheimer's Disease

AD is the most common neurodegenerative disease and leading cause of dementia in people above 65 years of age. It is a progressive brain disorder in which a person's memory, thinking ability and behaviour is affected with the deterioration worsening with time. Around 27 million people are estimated to be affected by this disease [27]. AD is characterised by the presence of two major neuropathological hallmarks which are extracellular Aβ plaques and intracellular NFTs composed of tau causing neuronal dysfunction [28]. Ageing is the greatest risk factor for AD with majority of patients above 65 years of age [29]. But cases of early-onset of AD are also present in those 35–60 years of age [30].

Genetic mutations are the known cause of early-onset familial AD with a prevalence of less than 1 % [29]. Thus, three forms of early-onset AD which are inherited as autosomal dominant traits involving three genes, viz. APP on chromosome 21, presenilin 1 (PS1) on chromosome 14 and presenilin 2 (PS2) on chromosome 1 [30], are recognised. Mutations in any of these three genes can make an individual susceptible to AD. Apolipoprotein E, type ϵ4 (APO ϵ4), is a risk factor for late-onset familial and sporadic AD, which accounts for majority (>99 %) of the AD cases [31]. Several genome-wide association studies (GWAS) have identified and implicated many genes in the aetiology of AD [32–34].

Accumulating evidences have shown that oxidative stress and mitochondrial dysfunction are important events occurring during the development of AD. In relation to this, decline in mitochondrial function with advancing age owing to accumulation of somatic mutations in mtDNA is reported [35]. Aβ is also known to induce free radicals [10, 11, 36] and cause decline in mitochondrial function. Oligomeric Aβ is considered to be more toxic than the insoluble fibrillar Aβ [3], which localises to the mitochondria and interacts with a number of mitochondrial proteins, causing synaptic damage and leading to memory impairment and cognitive decline, associated with AD [37, 38]. But the underlying mechanism(s) of the pathogenesis of AD is not known as yet. At present, there is no cure for AD but symptomatic treatments are available to relieve the symptoms and slowdown the impairment of memory associated with AD.

2.2 Factors Governing/ Responsible for AD Pathology

AD, a disabling and fatal disease, has become an important public health problem and poses an enormous economic and social burden for affected individuals, their caregivers and society. Though the neuropathological hallmarks of AD are known, the etiological factors involved in the pathogenesis of the disease are unknown. A number of studies have indicated some risk factors for onset of disease, advancing age being the most important risk factor. Other potential risk factors for AD are described in the following sections.

2.2.1 Genetic Factors

First, an individual having a family history of AD, is susceptible to develop it, especially if he is a first-degree relative of an affected person [29]. In comparison to males, females are more prone to develop AD. Persons with Down's syndrome (trisomy 21) are at a greater risk of developing AD [39]. Additionally, an individual's chance for developing AD increases if he inherits an *APOE* ϵ4 allele, one of the three common alleles (ϵ2, ϵ3, ϵ4) of *APOE* gene, from his parents and has an increased risk if two *APOE* ϵ4 alleles are inherited [29]. Mutations in *APP* gene and *PS1* and *PS2* genes are recognised to make a person susceptible to early-onset familial AD [30]. Apart from the *APOE* gene, a number of new genes are implicated in the pathogenesis of late-onset AD

(LOAD). Bridging integrator 1 (BIN1), also known as amphiphysin 2, is now recognised as an important genetic risk factor after *APOE4* for LOAD [40]. BIN1 transcripts levels were observed to be elevated in AD brains, showing BIN1 as a genetic susceptibility locus in AD [32]. Also, decreased expression of drosophila BIN1 ortholog *Amph* suppressed tau-mediated neurotoxicity, highlighting the role of BIN1 in mediating AD risk and its role in modulating AD pathogenesis at the level of the tau pathway [32]. Thus, BIN1 can be thought of as a target for treatment of AD. In addition to *APOE* and *BIN1*, other susceptibility gene loci identified include phosphatidylinositol-binding clathrin assembly protein (*PICALM*), ATP-binding cassette transporter (*ABCA7*), CD2-associated protein (*CD2AP*), clusterin (*CLU*), complement receptor 1 (*CR1*), CD33 antigen (*CD33*), ephrin receptor Eph-A1 (*EPHA1*) and a cluster of membrane-spanning 4A (*MS4A*) genes [33, 34, 40, 41].

2.2.2 Vascular Factors

Drinking alcohol poses a threat of developing AD at a later life. Heavy drinkers who are *APOE4* ϵ4 allele carriers are at a greater risk of developing AD at a later life than moderate alcohol drinkers [42]. Many studies have found association between AD and cigarette smoking. For example, *APOE4* noncarriers are at a greater risk of developing AD [43]. People with high blood pressure in middle age are more susceptible to develop AD at a later stage in their life [44]. Obesity is also a risk factor for AD [45]. Higher body mass index (BMI) or obesity in middle age can increase the risk of developing AD at a later life [46]. Higher serum cholesterol levels are also associated with development of AD at a later stage [47, 48]. Use of statins, i.e. cholesterol lowering drugs, poses a lower risk of AD [49]. In line with this, it was seen that people having a diet rich in polyunsaturated fatty acids and fish have a lower risk of AD [50] as compared to those consuming saturated fatty acids in their diet [51, 52]. An association between diabetes and AD has been observed. Diabetic people are more prone to develop AD, with an increased risk if diabetes occurs in middle age [53]. All these factors are modifiable risk factors that can be modulated by adopting healthy eating habits and an active life thus, lowering the risk of dementia and AD.

2.2.3 Head Trauma and Head Injury

Increased risk of AD development is associated with head injury and head trauma [29]. People with moderate head injuries are at an increased risk of developing dementia than those without any head injuries. Those with severe head injuries are at the greatest risk of developing dementia at a later life. Susceptibility of developing AD increases if a person carries an APOE ϵ4 allele and has suffered any head injury [54]. Thus, boxers and football players are at greater risk of AD at a later life [29].

2.2.4 Mild Cognitive Impairment

Mild cognitive impairment (MCI) is said to be a transitional stage between ageing and development of AD [55]. It is a condition in which a person has memory impairment though it doesn't affect a person's daily activity. It is shown that those who have MCI are at a greater risk of developing AD at a later life [55].

2.2.5 Autophagy Failure

Accumulation of Aβ plaques and tau protein in neurons shows that the neuronal housekeeping and protein quality control systems are impaired in AD. One of these is autophagy, which is a lysosomal degradative process involving removal of toxic proteins and preventing protein aggregation. Recent studies have indicated beclin-1 to be involved in autophagy regulation and modulation of APP metabolism [56]. Beclin-1 expression was also seen to be reduced in the AD brain [57]. Rapamycin is emerging as a potential neuroprotective agent for AD and functions via enhancement of autophagy [58]. A recent study has shown that Aβ(1-42)-induced beclin-1 expression was upregulated by rapamycin and that the beclin-1-dependent autophagy can prevent neuronal cell death before occurrence of AD in PC12 cells [59]. Inhibition of beclin-1-dependent autophagy was shown to speed up neuronal cell death. Thus, beclin-1 dysfunction is associated with

AD. Therefore, autophagy failure is a potential factor leading to accumulation of toxic Aβ plaques and tau in AD.

2.2.6 Brain Inflammation

Chronic brain inflammation is associated with AD. Activated microglia and reactive astrocytes are seen in close proximity to neuritic Aβ plaques in the AD brain [60]. Studies have shown that complement system is activated in the AD brains [61, 62]. The microglia, astrocytes along with complement system components, cytokines and chemokines are involved in inflammatory responses against Aβ. Microglias are cells of CNS involved in the protection of the brain as first-line defence against any invading pathogen and pathological conditions. On the other hand, astrocytes provide trophic support to neurons and are involved in Aβ clearance [63]. In order to clear Aβ, the activated microglia and astrocytes secrete ROS, nitric oxide (NO) and proteolytic components which further increase APP production and proteolytic processing of APP, thus causing neuronal dysfunction [63]. β-site APP-cleaving enzyme 1 (BACE1), a membrane-bound aspartic proteinase, is primarily expressed by the neurons and is associated with the generation of Aβ peptides from APP owing to its β-secretase activity [64]. BACE1 expression has been observed in reactive astrocytes in the AD brains while resting astrocytes do not display BACE1 at detectable levels, thus showing that activation of astrocytes may lead to the development of AD [65]. Another study showed that the TF NF-κB acts as a repressor in neuronal and nonactivated astrocytes, while NF-κB acts as activator of BACE1 transcription in activated astrocytes and Aβ-exposed neuronal cells. Also, the presence of increased level of activated astrocytes with ageing is well demonstrated, which may lead to increased processing of BACE1 causing increased Aβ resulting into chronic inflammation and subsequently astrocyte activation ending up forming a feedback loop [66]. Thus, inflammation plays a major role in AD pathology.

2.2.7 Hormones

Levels of reproductive hormones change with advancing age and are considered as risk factors for AD. Studies have shown that an elevated level of luteinising hormone (LH) increases the risk for developing AD [67]. Subsequently, using a transgenic mouse model of AD, it was shown that leuprolide {a gonadotropin-releasing hormone (GnRH) agonist} lowered serum LH levels, improved working memory and decreased Aβ deposition [68]. A study has also suggested that spatial memory impairment observed in post-menopausal women or female rats after ovariectomy is attributed to high LH levels [69]. Thus, reduction in LH levels may serve as potential therapeutics for AD. Also, in males there is a significant reduction in testosterone and elevation in LH levels with ageing. This is a potential risk factor for development of AD in males. A study showed that gonadectomised (GDX) mice had increased levels of Aβ, and the levels of Aβ were significantly lowered on treatment with testosterone [70]. This suggests potential of androgen therapy for treating AD in hypogonadal men.

2.2.8 Pathogens

Intracellular bacterial pathogen *Chlamydophila* (*Chlamydia*) *pneumoniae* is a risk factor for AD. Studies have shown presence of *Chlamydia* in the brains of LOAD patients [71]. Also, the presence of herpes simplex virus type 1 (HSV1) in the brain of *APOE4*-ϵ4 carriers is a risk factor for development of AD [72]. In a recent finding, association between periodontal disease and AD was established by the presence of elevated serum antibodies against periodontal infection, caused by bacteria, in individuals who later developed AD as compared to serum antibody levels in control individuals [73]. The mechanisms leading to the pathogenesis of AD due to the presence of these pathogens in the brain need to be understood to a greater extent.

2.2.9 Metal Exposure

Exposure to metals has been linked to AD from a very long time. A recent study has shown that elevated magnesium (Mg^{2+}) in the brain has a synapto-protective effect and improves cognition deficits by reducing Aβ plaques and stabilising BACE1 expression in a transgenic mouse model of AD [74]. An earlier study had shown that reduced Mg^{2+} levels are present in AD patients

[75]. This shows that restoring brain Mg^{2+} levels has a potential to treat AD. Elevated levels of aluminium (Al) are seen in the serum of AD patients [76]. In line with this study, recently it was shown that Al may mediate liver toxicity in AD patients and lead to free copper (Cu^{2+}) in serum, as seen in AD patients [77]. This study has put forward a likely mechanism showing that Al toxicity to liver leads to abnormal synthesis of ceruloplasmin and ATPase7B causing increase in Cu^{2+} levels in serum, and these free Cu^{2+} may cause accumulation of Aβ and neuronal dysfunction associated with AD [77]. Thus, reducing Al levels and reviving normal liver function can be thought of as a treatment for AD. Another significant study has showed that drinking silicon (Si)-rich mineral water reduced Al levels, leading to the removal of Al via urine and thus improved cognition in AD patients [78]. Lead (Pb) exposure has also been linked to the pathogenesis of AD. Pb is shown to increase APP expression and change methylation patterns of *APP* gene, making it hypomethylated in PC12 cells [79]. Another study showed that Pb exposure facilitates Aβ fibril formation and increases Aβ deposition in a transgenic mouse brain [80]. Another evidence supporting the role of Pb in the pathogenesis of AD showed increased fibrillisation of tau protein on exposure to Pb via interaction with His330 and His362, the His mutants of wild-type tau [81]. Inorganic Cu^{2+}, present in drinking water and Cu^{2+} supplements, has also been linked to AD and cognitive impairment in the elderly [82]. A recent study has demonstrated that exposure to iron (Fe^{2+}) leads to upregulation of a disintegrin and metalloproteinase 10 (ADAM10) and increased transcription levels of BACE1, and these were reported to be associated with increased expression of APP-α-CTF and APP-β-CTF, respectively, in PC12 cells, suggesting that Fe^{2+} induces enhanced expression of ADAM10/BACE1 leading to altered APP carboxyl-terminal processing [83]. Increased intracellular calcium (Ca^{2+}) levels brought about by oligomeric Aβ are associated with impaired synaptic plasticity. A study pointed towards the synaptic loss of phosphorylated (active) Ca^{2+}/calmodulin-dependent protein kinase II-α[p(Thr286)CaMKII], a critical enzyme mediating synaptic events, in the MCI and AD hippocampal regions. The loss of p(Thr286) CaMKII was also observed in mice hippocampal regions on treatment with oligomeric Aβ. This was shown to be prevented by inhibiting the phosphatase calcineurin (CaN). Thus, dysregulated Ca^{2+} signaling is associated with AD and MCI [84]. Altered Zinc (Zn^{2+}) homeostasis is implicated in AD. Higher concentrations of releasable Zn^{2+} are present in synaptic vesicles as compared to extracellular fractions in AD hippocampus [85]. In line with this, Bjorklund et al. also showed that in the case of non-demented with AD neuropathology (NDAN) individuals, Aβ oligomers are absent in hippocampal postsynapses along with lower total Zn^{2+} levels, thus leading to intact cognitive function in NDAN individuals [86]. Excess exposure to Zn^{2+} leads to enhanced APP processing, Aβ accumulation and memory impairment as seen in transgenic mice and SH-SY5Y human neuroblastoma cells overexpressing the Swedish mutant form of human *APP* (*APPsw*). Thus, Zn^{2+} overload has a toxic role in AD pathogenesis [87].

2.2.10 Air Pollution

There is a possible association between air pollution and development of AD. Studies have shown that air pollution causes accumulation of Aβ(1-42), increase in cyclooxigenase-2 (COX-2) expression and brain inflammation that cause neuronal dysfunction leading to pathological hallmarks that are associated with AD [88]. Damage to olfactory bulb, olfactory mucosa and frontal regions of the brain, as observed in AD brains, is associated with exposure to air pollution [89]. Ozone (O_3) is a powerful gaseous air pollutant and oxidising agent. It is shown that memory impairment, motor deficiency and lipid peroxidation are caused by exposure to different doses of O_3 in rats [90]. Dysregulation of inflammatory processes, progressive neurodegeneration, chronic loss of brain repair in the hippocampus and brain plasticity changes occurred in rats on exposure to low doses of O_3 [91]. Thus, air pollution is an important environmental hazard which may play a role in development of AD. Additional studies are needed at the

population level to clearly understand the role of air pollution in pathophysiology of AD.

2.3 Amyloid Proteins (Oligomers and Fibrils)

The Aβ protein is central to the amyloid cascade hypothesis, the prevailing hypothesis for more than 20 years, which explains the pathogenesis of AD. Though it does not pinpoint towards a specific Aβ species in the aetiology of AD, several studies have implicated soluble oligomers of Aβ, rather than monomers or insoluble amyloid fibrils, in causing neuronal dysfunction in AD [2, 92, 93].

Abnormal processing of APP in the neurons leads to generation and accumulation of Aβ. APP is a type I, single-pass transmembrane protein with large extracellular domains and belongs to a family of proteins which include amyloid precursor-like proteins (APLP1 and APLP2) in mammals and the amyloid precursor protein-like (APPL) in drosophila [94]. APP contains a 40- or 42-amino acid sequence, i.e. Aβ(1-40) or Aβ(1-42), and 3 sites for cleavage by various proteinases, which are designated as α, β and γ secretases [95]. APP is expressed in all tissues ubiquitously and present on the plasma membrane, majority of which localises to endoplasmic reticulum (ER), Golgi apparatus (GA) and mitochondria [96]. Two pathways exist for processing of APP, viz. amyloidogenic pathway and non-amyloidogenic pathway as illustrated in Fig. 1. In the amyloidogenic pathway, the activity of β-secretase (also known as BACE1) at the beginning of Aβ domain of APP results into a soluble N-terminal fragment (sAPPβ) and an amyloidogenic C-terminal fragment of 99 amino acids (CTF99). Further γ-secretase cleaves this C-terminal fragment to mainly Aβ(1-40) and Aβ(1-42), which may accumulate in the mitochondria and other cellular compartments affecting the cellular functions and leading to mitochondrial dysfunction and hyperphosphorylation of tau. Non-amyloidogenic pathway is the major APP processing pathway in which the activity of α-secretase generates a large soluble N-terminal fragment (sAPPβ) and a non-amyloidogenic C-terminal fragment of 83 residues (CTF83) owing to the cleavage within the Aβ domain. This C-terminal fragment is then cleaved by γ-secretase resulting into non-amyloidogenic peptide (P3) and APP intracellular domain (AICD) which are non-toxic and degraded rapidly and occurs in majority of individuals including non-demented and healthy individuals [97]. In case of early-onset AD, mutations in *APP, PS1* and *PS2* are known to activate β- and γ-secretases leading to generation of Aβ, but in case of sporadic AD, it is proposed that oxidative stress activates β-secretase which increases the production of Aβ [97]. Although the mechanism of APP trafficking is not known, APP is said to be axonally transported, endocytosed and sorted to different compartments of the cell, thus leading to Aβ generation and accumulation in different cellular compartments thus, impairing normal cellular function. A recent study pointed towards the role of huntingtin-associated protein 1 in regulating APP trafficking to the non-amyloidogenic pathway resulting into reduced Aβ levels [98]. As excessive BACE1 expression and APP processing can lead to uncontrolled production of Aβ, different regulatory mechanisms are present [99, 100]. Apart from the transcriptional control, a complex network of neurotransmitter systems and translational regulation is present. Amongst the different systems, viz. glutamatergic, adrenergic, serotonergic, cholinergic and dopaminergic systems, cholinergic system is known to be affected in the early stages of AD. A study by [99] showed that downregulation of M2 acetylcholine receptor in the brains of AD patients affects a number of AD-relevant genes including BACE1 [99].

In the amyloidogenic pathway, different lengths of Aβ are produced ranging from 37 to 43 amino acids. Aβ(1-40) is the most dominant species produced from the processing of APP. In comparison to Aβ(1-40), only a small amount of Aβ(1-42) is produced in the human brain in the ratio of approximately 99 to 1. But Aβ(1-42) is the main component of the amyloid deposits associated with AD and has a tendency to form aggregates spontaneously and, thus, may form oligomers. Therefore, Aβ(1-42) is considered more neurotoxic than Aβ(1-40) [101]. While both Aβ(1-40) and Aβ(1-42) are capable of forming

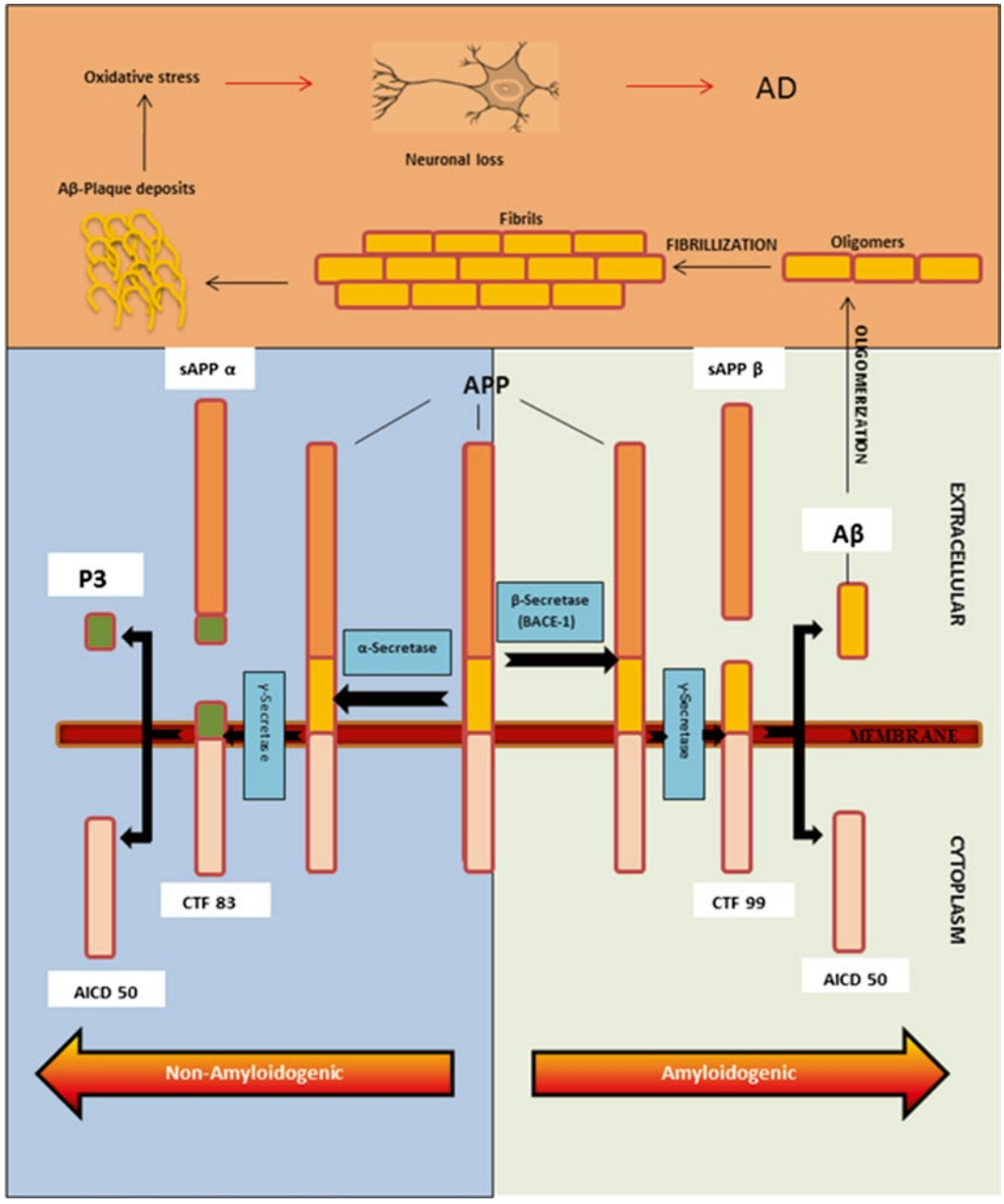

Fig. 1 APP processing leads to the generation of cleavage products via the amyloidogenic and non-amyloidogenic pathways. Both oligomerisation and aggregation of Aβ lead to the formation of senile plaques, a hallmark of AD phenotype. APP processing occurs via two pathways: amyloidogenic and non-amyloidogenic pathway. In the amyloidogenic pathway, β-secretase cleaves APP at the beginning of Aβ domain generating a soluble N-terminus fragment (*sAPPβ*) and amyloidogenic C-terminal fragment of 99 residues (*CTF 99*). Next, γ-secretase acts on CTF 99 and generates Aβ and APP intracellular domain (*AICD 50*). While in the α-secretase-based non-amyloidogenic pathway, α-secretase cleaves within the Aβ domain of APP and generates a soluble N-terminal fragment (*sAPPα*) and a non-amyloidogenic C-terminal fragment of 83 amino acid residues (*CTF 83*). This C-terminal fragment, i.e. CTF 83, is further cleaved by γ-secretase and generates a non-amyloidogenic peptide (*P3*) and APP intracellular domain (*AICD 50*). The amyloidogenic pathway, which generates the toxic Aβ, occurs in 10 % of individuals who have chances of developing AD in the later life. The generated Aβ(1-40) or Aβ(1-42) may further undergo different conformations including oligomerisation resulting into generation of oligomers which are considered to be the most toxic species of Aβ implicated in AD. Further fibrillisation may take place leading to fibril formation and deposition in the neurons, a characteristic feature of AD

amyloid fibrils, Aβ(1-42) is said to make the fibrils much faster than Aβ(1-40). Using ion-mobility spectrometry, it was shown that when Aβ(1-40) and Aβ(1-42) are present together in a solution, the Aβ(1-40) and Aβ(1-42) monomers form dimers, trimers and tetramers. But, when present alone, Aβ(1-42) tends to form pentamers and hexamers (paranuclei) which on self-association form dodecamers, protofibrils and fibrils. Aβ(1-40) alone produces oligomer distribution up to tetramer level only. This pointed out that when present together, Aβ(1-40) inhibits the oligomerisation of Aβ(1-42) and inhibits protofibril and fibril formation by Aβ(1-42). Thus, as Aβ(1-40) is the predominant species present in the human brain in ~10 times the level of Aβ(1-42) in a healthy human brain, the former inhibits the oligomerisation of Aβ(1-42) and prevents the development of AD [102]. Studies have shown that mutations in *APP*, *PS1*and *PS2* may lead to enhanced accumulation of the more toxic Aβ(1-42) species [101, 103–105].

It is reported that some NDAN individuals have significant amounts of amyloid plaques without displaying cognitive decline. A study pointed towards a resistance mechanism contributing towards the maintenance of cognitive function in these individuals. This study showed that oligomeric Aβ is absent in hippocampal postsynapses in NDAN brains. In addition, normal levels of phosphorylated (active) CREB, a transcription factor important for synaptic plasticity, are present in NDAN individuals advocating for the normal functionality of synapses in these in these individuals [86]. A number of studies have indicated that amyloid plaque formation does not correlate with AD pathogenesis [106]. Studies have also shown that deleterious changes associated with AD occur very early before the accumulation of amyloid plaques [107]. Accumulating evidences reveal that oligomers, the soluble form of Aβ, are the more toxic species and associated with cognitive decline in AD, rather than the insoluble fibrillar deposits [108]. Further, different approaches have been employed which have supported the above findings and implicated soluble Aβ in AD. Hippocampal long-term potentiation (LTP) is one such approach, which is correlated with learning and memory, and is shown to be inhibited by synthetic and naturally secreted human Aβ oligomers. Behavioural studies performed on living wild-type rats showed that rats developed learning and memory deficiency after human oligomers were infused in the hippocampus, which were readily observed in morris water maze models [101]. Recently, a possible role of cellular prion protein (PrPc) acting as a neuronal receptor for oligomeric Aβ was speculated. It was shown that there was no inhibition of LTP after treatment with oligomeric Aβ in mice lacking PrPc, thus pointing towards the role of PrPc in Aβ neurotoxicity [109].

Oligomeric Aβ(1-42) is also demonstrated to be involved in inducing toxicity in cholinergic neurons leading to cholinergic dysfunction and progressive basal forebrain cell loss, assumed to be an early event in the pathogenesis of AD. Heinitz et al. showed that on treating SN56.B5.G4 cells with oligomeric Aβ(1-42), many genes of the ER and GA involved in protein modification and degradation were affected. This indicated a possible role of ER-mediated stress in oligomeric Aβ(1-42) toxicity in cholinergic neurons and leading to cholinergic dysfunction in AD [110]. In line with this, another study by Joerchel et al. showed that SN56.B5.G4 cells when treated with oligomeric Aβ(1-42) affects the expression of a number of proteins, viz. calreticulin, MAPK kinase 6c, γ-actin, Rho-GDP dissociation inhibitor (Rho-GDI), ubiquitin carboxyl-terminal hydrolase-1 (UCHL-1) and α6-tubulin, which are known to be affected in the brains of AD patients, thus pointing towards the role of Aβ in affecting the integrity of the proteome in AD [111].

The amyloid cascade hypothesis has reached another level of complexity with recent studies revealing the appearance of multiple types of Aβ oligomers and their role in the pathogenesis of AD [2, 112, 113]. Various studies have recognised four endogenously produced Aβ oligomeric assemblies, viz. dimers, trimers, Aβ*56 and APFs (annular protofibrils), which may alter neuronal function in human and transgenic mice and have different consequences on neuronal survival [114]. In case of AD, soluble Aβ

monomers may form higher-order assemblies ranging from low-molecular weight oligomers (dimers and trimers) to Aβ*56, then to APFs and fibrils, which are the primary components of amyloid plaques, characteristic of AD.

Taken together, recent studies provide the evidence that Aβ oligomers are the more toxic species than insoluble fibrillar deposits relevant to AD pathology (Fig. 1). Also, soluble monomers can form higher-order assemblies finally leading to fibril formation which is the characteristic component of amyloid plaques. Thus, development of antibodies against specific oligomeric species can be an effective approach for treating AD.

2.4 Role of Mitochondria in AD Pathology

The 'Mitochondrial cascade hypothesis' was formulated in 2004, which provides an explanation of the aetiology of sporadic AD [115]. According to the 'mitochondrial cascade hypothesis', ageing and sporadic AD are two convergent events, and the etiological factors for autosomal dominant and sporadic AD are not the same. Also, mitochondrial dysfunction has been viewed as the common element between autosomal dominant and sporadic AD forms. In addition to sporadic AD, this hypothesis also predicts the aetiology of autosomal dominant AD forms. In case of autosomal dominant forms, it points out that excessive Aβ causes mitochondrial dysfunction and this Aβ-induced dysfunction further initiates histopathologies associated with AD. For sporadic AD cases, it is believed that age-related mitochondrial changes cause mitochondrial dysfunction and activate downstream cellular changes as observed in sporadic AD which include processing of APP to Aβ, tau phosphorylation, synaptic loss and finally neurodegeneration [6]. Thus, mitochondrial cascade believes Aβ accumulation as a downstream event in the cascade.

Mounting evidences are present which point to an association between ageing and mitochondrial dysfunction. An earlier study pointed out that somatic mutations in mtDNA can lead to premature onset of ageing phenotypes [116]. But it is not known whether the decline in mitochondrial function is a cause or outcome of ageing.

Of particular interest, a large number of evidences from studies involving experimental models and human samples suggest an association of APP and Aβ with the mitochondria. In particular, a study involving a mouse model showed that Aβ, particularly Aβ (1-42), accumulates in the mitochondria in the presence of a mutant *APP* gene and there is a decline in the activity of respiratory complexes III and IV. Also, Aβ was shown to accumulate in the mitochondria very early before the extensive extracellular deposition takes place [117]. These point towards the involvement of the mitochondria in the pathogenesis of AD (Fig. 2).

In addition, it is reported that age-related accumulation of somatic mutations in mtDNA causes an increase in ROS production in the mitochondria [118, 119]. It is known that oxidative stress induces an increased expression of BACE1 [120, 121]. Thus, it is speculated that with advanced age, an increase in ROS levels occurs which increases Aβ levels owing to increased processing of APP by BACE1. Also, this generated Aβ induces an increased production of free radicals (ROS and RNS), causing decline in mitochondrial function. Thus, a feedback loop exists in which age-related ROS levels cause increased production of Aβ, and this Aβ further leads to increased production of ROS, resulting in memory impairment and cognitive decline associated with AD [36].

Several evidences point out that Aβ interacts with mitochondrial proteins and increase ROS levels, finally leading to synaptic damage. Mounting evidences show that Aβ, in its soluble oligomeric form, disrupts the axonal mitochondrial transport and disturbs the mitochondrial fission-fusion balance [122, 123]. Mitochondrial trafficking/transport is a phenomenon in which synaptic mitochondria, synthesised in the cell body, are transported down the axon/dendrite to areas of high energy demand, thus serving cellular energy demands [97]. During the transport, the

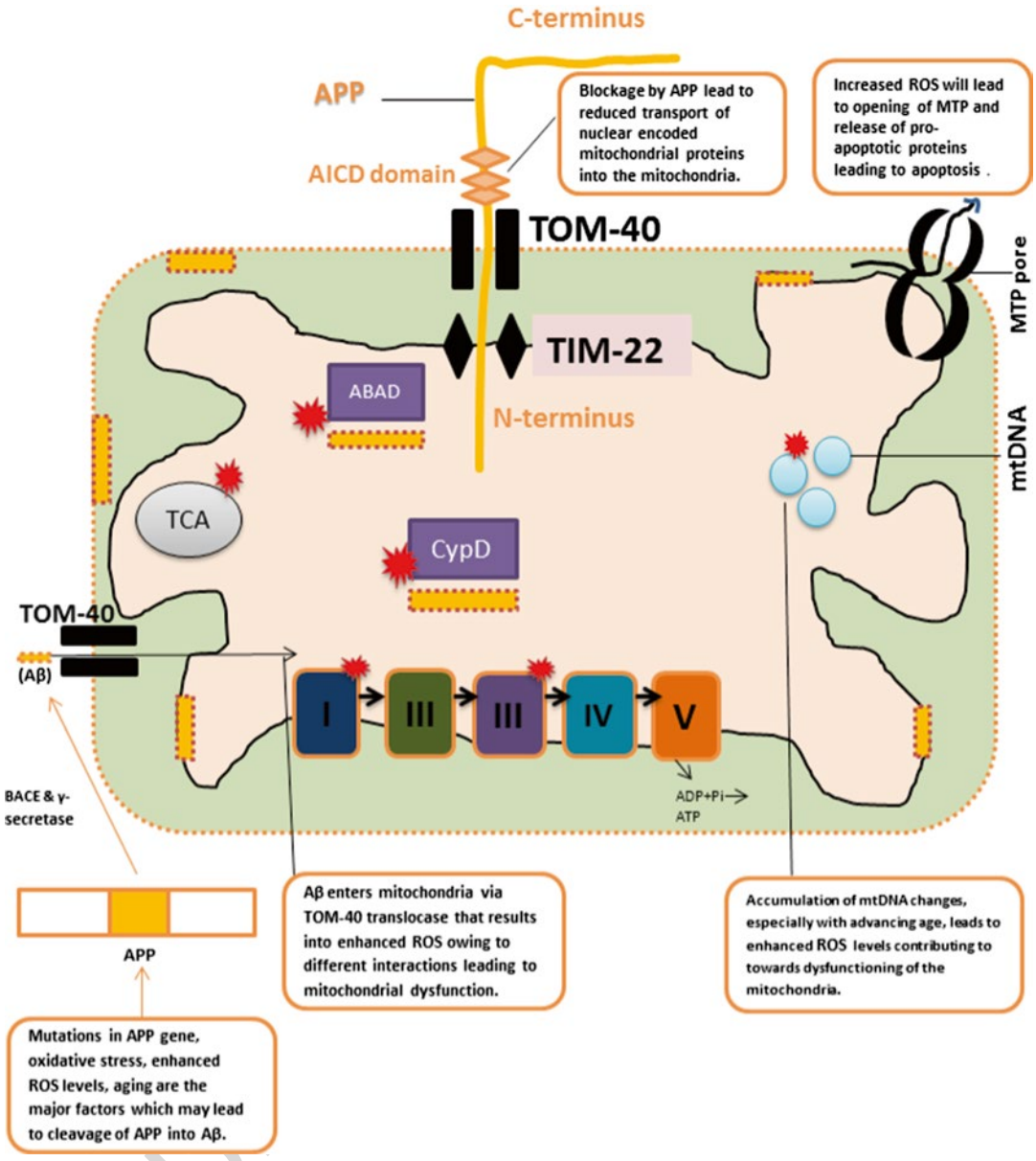

Fig. 2 Aβ induces mitochondrial dysfunction that results in neurodegeneration and AD. A mitochondrion is surrounded by two lipid membranes: outer membrane and inner membrane. The outer membrane is porous, while the inner membrane restricts ionic flow and harbours the electron transport chain (*ETC*). ETC is composed of complexes I–V and is responsible for the generation of mitochondrial ATP via oxidative phosphorylation. Electron leaks in complexes I and III are responsible for the generation of free radicals in the mitochondria. Components of tricarboxylic acid cycle (*TCA*), viz. α-ketoglutarate dehydrogenase and beta oxidation, are present in the mitochondrial matrix and generates superoxide radicals. These generated radicals thus cause lipid peroxidation and protein and DNA oxidation. In case of early-onset AD, genetic mutations in *APP*, *PS I* and *PS II* activate beta- and gamma-secretase and lead to increased processing of APP to Aβ. This Aβ is found to accumulate in the outer, inner mitochondrial membrane and the matrix. When associated with the outer membrane, it causes blockage of the entry of nuclear-encoded mitochondrial proteins. On being localised to inner membrane, it directly induces free radical production, decreases cytochrome oxidase activity, interferes with complex activities and impairs ATP production. Once inside, Aβ interacts with mitochondrial matrix proteins (ABAD and cyclophilin D) which increases the oxidative stress and worsens the mitochondrial damage. In case of late-onset AD, ageing is one of the major factors contributing to increased free radical production owing to mutations in mtDNA. APP is also said to be localised to mitochondrial outer membrane and forms complexes with the translocases of outer (*TOM*) and inner membrane (*TIM*). But the importation of C-terminus of APP is blocked due to the AICD domain of APP and thus blocks the mitochondrial pores. This prevents the entry of nuclear-encoded mitochondrial proteins causing impairment of mitochondrial enzyme activities, increased oxidative stress, neuronal damage and cognitive decline

mitochondria can encounter each other, leading to fusion (aided by fusion proteins) and subsequently resulting in exchange of mitochondrial content. This is necessary to maintain genome stability as it allows exchange of highly pathogenic mtDNA and helps to maintain mitochondrial function [124]. Mitochondrial fission plays an important role in apoptosis. Unbalanced fission and fusion results in disruption of mitochondrial dynamics, causing mitochondrial fragmentation and contributing to mitochondrial dysfunction [125], which has been speculated to be the underlying mechanism(s) causing synaptic degeneration in AD. Recently, it was shown that both monomers and oligomers of Aβ interact with mitochondrial fission protein, dynamin-related protein 1 (Drp1) and may be involved in abnormal mitochondrial dynamics causing mitochondrial fragmentation leading to neuronal dysfunction in AD. Also, expression levels of mitochondrial fission genes *Drp1* and *Fis1* (Fission 1) were increased and those of mitochondrial fusion genes *Mfn1* (mitofusin 1), *Mfn2* (mitofusin 2), *Opa1* (optic atrophy 1) and *Tom40* were decreased in AD patients [37]. This study also provided evidence that mitochondrial fragmentation may be initiated by interaction of Aβ with Drp1 causing abnormal mitochondrial dynamics, which increases as AD progresses. Thus, targeting these abnormal interactions can serve to minimise the neuronal damage caused by AD.

It has been shown that interaction of Aβ with Aβ-binding alcohol dehydrogenase (ABAD) in the mitochondria causes enhanced ROS production and apoptosis in AD patients and transgenic mice [126]. Some recent studies have implicated mitochondrial permeability transition pore (mPTP) formation in Aβ-mediated mitochondrial dysfunction [127]. The mPTP consists of cyclophilin D (CypD) in the mitochondrial matrix, voltage-dependent anion channel (VDAC) in outer mitochondrial membrane and adenine nucleotide translocase (ANT) in inner mitochondrial membrane. CypD has a role in opening of mPTP by binding with ANT and VDAC after its release from the matrix [38]. In relation to VDAC1, a recent study has shown that interaction of Aβ and phosphorylated tau with VDAC1 blocks mitochondrial pore leading to mitochondrial dysfunction in AD patients and transgenic APP mice [12]. Another mitochondrial protein CypD is shown to enhance mitochondrial and neuronal stress by interacting with Aβ. This interaction promotes ROS which leads to recruitment of CypD to the inner mitochondrial membrane and results in opening of mPTP causing cell death [128]. In addition, this study showed that CypD deficiency improved learning and memory in a mouse model of AD. A recent study has provided a new insight into the role of CypD in the disruption of axonal mitochondrial transport [38]. It was shown that depletion of CypD protects the Aβ-induced axonal mitochondrial transport damage and improves mitochondrial motility and dynamics. An increase in axonal mitochondrial density and bidirectional transport of the axonal mitochondria was observed on CypD depletion. CypD in the presence of Aβ promoted the opening of mPTP and, thus, disrupted Ca^{2+} homeostasis and increased accumulation of ROS, further activating P38 MAPK signal transduction pathway, causing synaptic injury. It was also demonstrated that CypD-mediated mPTP blockade improved synaptic function against Aβ toxicity using CypD-deficient mice. This study suggests a relation between CypD-mediated disruption of axonal mitochondrial trafficking and Aβ-induced mitochondrial dysfunction leading to synaptic injury, thus speculates a mechanism of mitochondrial dysfunction involved in the pathogenesis of AD (Fig. 2).

Another study has shown that age-related accumulation of Aβ occurs to a larger extent in the synaptic mitochondria as compared to the non-synaptic mitochondria and this accumulation in the synaptic mitochondria occurs very early as compared to its accumulation in the non-synaptic mitochondria. This led to altered mitochondrial transport in murine primary axons [129]. Thus, it can be ascertained that the synaptic mitochondria are more probable targets of Aβ-induced oxidative stress. Sirtuin 3 (SIRT3), a deacetylase has an essential role in maintaining mitochondrial function. Studies have highlighted its neuroprotective role and its role in energy homeostasis by maintaining basal ATP levels for survival of the cells [130, 131]. A recent study has shown upregulation of SIRT3 in relation to Aβ-accumulation in AD patients and transgenic AD mouse model. Also, it was shown that mitochondrial ROS levels regulated SIRT3 expression

[132]. This speculates that upregulation of SIRT3, seen in AD patients and mouse model, may be due to Aβ-induced mitochondrial oxidative stress. This suggests that in response to Aβ-induced oxidative stress in the mitochondria, upregulation of SIRT3 occurs to promote neuronal survival.

Recently, the RanBP9-cofilin pathway has been implicated in AD when it was shown that RanBP9, a scaffolding protein, generates Aβ and promotes Aβ-induced neurotoxity along with activating cofilin, having a key role in regulating actin dynamics and mitochondria-mediated apoptosis in a mouse model of AD pathology [133]. Also, suppression of Aβ and RanBP9-induced apoptosis by siRNA knockdown of cofilin confirmed the role of RanBP9-cofilin pathway in AD and can be the probable therapeutic target for lowering Aβ-induced neurotoxicity.

A study by Kopeikina et al. showed that soluble tau species are more toxic than tau aggregates and cause mitochondrial distribution deficiencies in a mouse model of tautopathy and in the human AD brains, possibly due to axonal transport deficiencies resulting in mitochondrial and neuronal dysfunction [134]. This suggests that soluble tau like the oligomeric Aβ are more toxic than aggregated form and involved in neuronal dysfunction associated with AD.

Poly (ADP-ribose) polymerase-1 (PARP-1), a predominantly nuclear enzyme responsible for genome stability and transcriptional regulation, is thought to be involved in the pathogenesis of AD. Activation of PARP-1 by oxidative stress (induced by Aβ) is believed to be an early event in the pathogenesis of AD. Enhancement of PARP-1 activity and accumulation of PAR is observed in the brains of AD patients [135]. A recent study has established the role of PARP-1 in microglial activation by its interaction with NF-kB [136]. Recently, the mitochondrial localisation of PARP-1 and its interaction with mitochondrial protein, mitofilin, has been established [137]. It was shown that in the absence of PARP-1, there is an accumulation of mtDNA damage, suggesting that mitochondrial PARP-1 has a role in mtDNA damage repair/signaling. Overexpression of PARP-1, Bax and p53 and altered mitochondrial function in the presence of oxidative stress induced by Aβ has also been shown recently [138]. Additional studies are needed to ascertain the role of PARP-1 and mitochondrial dysfunction in AD pathogenesis.

Taken together, these studies suggest that mitochondrial dysfunction is associated with ageing and AD. Along with Aβ, age-related accumulation of somatic mutations in mtDNA increases mitochondrial ROS levels leading to a decline in mitochondrial function. Recent studies have pointed out the role of oligomeric Aβ in the pathogenesis of AD. Oligomeric Aβ and APP are said to localise to mitochondria, mainly synaptic mitochondria, interact with mitochondrial proteins and disrupt axonal mitochondrial trafficking, causing synaptic injury and cognitive impairment. In relation to this, the use of mitochondria-targeted antioxidants can be seen as important approaches to treat AD.

3 Apurinic/Apyrimidinic Endonuclease (APE1): An Emerging Neuroprotective Enzyme

There are many external and internal agents, which bring human genome under stress and finally bring modification in the genomic stability. These threats are mainly produced internally from mitochondrial electron transport chain (ETC) or externally by different biological, chemical and physical agents like ultraviolet (UV) rays, ionising radiation (IR), chemotherapeutic agents, pollutants and heavy metals [139]. ROS attacks DNA readily and generates a variety of DNA base lesions [140]. DNA damage is a continuous process and ~10^4 DNA lesions are estimated to be produced in a mammalian genome each day as a result of spontaneous decay, errors in replication and cellular metabolism. To maintain genomic integrity, a cell has an internal regulatory mechanism which maintains DNA damage and repair in a balanced condition. There are two pathways by which cells maintain genome integrity: (i) antioxidants which quench the ROS/RNS, nonenzymatic antioxidants (e.g. α-tocopherol, β-carotene,

lycopene and ascorbic acid) and enzymatic antioxidants (e.g. superoxide dismutase (SOD), glutathione (GSH), peroxidases and catalase) and (ii) DNA repair by different processes. The DNA repair system comprises of base excision repair (BER), transcription-coupled repair (TCR), global genome repair (GGR), mismatch repair (MMR), homologous recombination (HR) and nonhomologous end joining [NHEJ] [141].

In mammals and higher organisms, different organs consist of various cell types; some of them are dividing while others are nondividing. In adults, cell types such as myocytes, adipocytes, skin cells and neurons are nondividing cells, i.e. terminally differentiated [142, 143]. BER is the major pathway for oxidative DNA base damage caused by ROS/RNS as well as for abasic (AP) sites and single-strand breaks (SSBs). Apurinic/apyrimidinic endonuclease (APE1) is a primary BER enzyme and responsible for repair and removal of AP sites and strand breaks [16, 17, 144].

Human *APE gene* (~3 kb in size) is localised on chromosome 14q11.2-12 and consists of four introns and five exons [145]. The human APE *cDNA* is about 1.4 kb in length and encompasses a coding region of 954 nucleotides and encodes a protein comprising of 318 amino acids. APE1 is abundant (~10^5 copies per cell) in eukaryotic cells and has a relatively long half-life [~8 h] [146]. APE1 is a dual function protein. Its C-terminus displays repair activity and its N-terminal contains a bipartite nuclear localisation signal, NLS [18, 147, 148] and displays redox activity responsible for transcriptional regulation through redox-based mechanisms [18, 149, 150].

3.1 Role of APE1 in Oxidative DNA Damage Repair

The ROS-induced damage to the DNA is implicated in a number of human diseases including neurodegenerative diseases like AD, PD, HD and cancers [16, 17, 151]. It is thus very important to repair the ROS-induced DNA damage in order to maintain the genomic integrity. BER, an evolutionarily conserved process, is responsible for repairing most endogenous lesions like oxidised bases, AP sites and SSBs in both nuclear DNA and mitochondrial DNA. The basic BER pathway involves enzymes, viz. DNA glycosylase, APE1, DNA polymerase and DNA ligase. APE1 is involved in the repair of oxidised base lesions generated in the DNA as a result of oxidative damage. Attempts to generate APE1-null mice were not successful and lead to an early embryonic death [152, 153]. Further attempts to generate cell lines from APE1-null embryos failed, showing the essentiality of APE1 in maintaining cell viability. A study pointing towards the role of APE1 in neuronal cell survival showed that overexpression of APE1 in hippocampal and sensory cells exposed to H_2O_2 lead to an increase in cell viability [154]. Upregulation of APE1 in cerebral cortical region of AD patients was also seen [155]. An immunohistochemical study pointing towards the role of APE1/Ref-1 in regulating cellular response towards oxidative stress showed that increased nuclear expression of APE1/Ref-1 is present in cerebral cortical regions of AD patients [20]. Another study showed the colocalisation of APE1/Ref-1 with Aβ in the senile plaques in AD hippocampus [156]. This study also showed that varying concentrations of Aβ(1-42) regulates APE1/Ref-1 expression, thus pointing towards the neuroprotective role of APE1/Ref-1 in response to oxidative stress. A number of evidences point towards the role of cyclin-dependent kinase 5 (Cdk5) in mediating neuronal loss. In line with this, it was shown that Cdk5 complexes with p35 and phosphorylates APE1 at Thr232, causing reduction in APE1's endonuclease activity and leading to accumulation of DNA damage and neuronal loss [157]. It can be interpreted that APE1 has a major role in overcoming the oxidative stress and maintaining neuronal cell viability and integrity.

3.1.1 Nuclear BER Pathway

A number of DNA repair pathways operate in the nucleus. Amongst them, BER pathway is the most

versatile repair pathway operating in the nucleus in response to oxidative damage for repairing alkylated and oxidised DNA lesions, AP sites and SSBs. Two models of BER are present: short-patch BER (SN-BER) and long-patch BER (LP-BER). SN-BER involves removal of a DNA lesion and incorporation of a single nucleotide, while a patch size of 2–8 nucleotides is associated with LP-BER [17, 144, 158]. The choice of the pathway depends on factors like type of lesions, AP sites and nature of 5′ terminus. The first step of the BER pathway is recognition and excision of a damaged base by DNA glycosylase. Two types of DNA glycosylases are present: monofunctional and bifunctional. Monofunctional DNA glycosylases (M-DG) include thymine DNA glycosylase (TDG), uracil-DNA glycosylase 1 (UDG1) and MutY homolog (MUTYH) and excise the substrate base, e.g. alkylated bases and uracil, generating an AP site which is later processed by APE1. Bifunctional DNA glycosylases (B-DG) which include 8-oxoguanine DNA glycosylase (OGG1) and *Nei*-like-1 (NEIL1), *Nei*-like-2 (NEIL2) and endonuclease III-like 1 (NTH1) have an additional lyase activity specific for oxidised bases and incise the DNA backbone 3′ to the AP site via β or β,γ elimination [144, 159–161]. The second step of BER involves processing of the generated AP site by APE1 that generates a nick containing a 3′OH residue and dRP at 5′ end, by cleaving the phosphodiester bond 5′ to the AP site. During the third step of the BER pathway, repair of the AP site is catalysed by polymerase (pol) β. If an unaltered group in deoxyribose is present, then pol β owing to its dRP lyase activity can carry SN-BER. The LP-BER occurs when AP sites are further oxidised by ROS and pol β cannot remove the 5′ blocking groups. 5′ flap-endonuclease-1 (FEN1), part of DNA replication machinery, is shown to displace and cleave this 5′ blocking group along with 4–6 nucleotides as a single-stranded DNA flap. PCNA also has a role to play by acting as a sliding clamp in LP-BER. The last step of BER involves nick sealing by DNA ligase which in case of LP-BER involves DNA pol ϵ/δ and DNA ligase I, and in case of SN-BER involves DNA ligase IIIα/XRCC1 complex [144, 160]. PARP-1 is known to modulate the capacity of BER and efficiently recognise and repair SSBs, thus acts as a DNA damage sensor and signal transducer [17, 162]. It was shown that Aβ is involved in the activation of PARP-1 through NO cascade in adult hippocampus [163]. Another protein XRCC1 also acts a SSB sensor protein and acts as a scaffold for BER proteins for SSB repair [144]. XRCC1 also interacts and stimulates APE1 [164]. Thus, it can be said that BER pathway is indeed a versatile pathway involved in maintaining genome integrity and involving a number of enzymes and interactions.

3.1.2 Mitochondrial BER Pathway

It is well known that the mitochondria have a role in the ageing process. Mitochondrial DNA (mtDNA) is more prone to oxidative DNA damage due to its close proximity to the site of ATP production in the inner mitochondrial membrane [165]. Thus, DNA damage repair in the mitochondria appears to be very important for maintaining proper functioning of the cell, especially during ageing. Various studies have shown that DNA repair actively takes place in the mitochondria, which was earlier thought to be present only in the nucleus. Recent studies have identified new DNA repair enzymes that participate in the DNA repair pathways operating in the mitochondria. Amongst the different repair pathways, BER is considered to be the major DNA repair pathway taking place in the mitochondria (Fig. 3). BER in the mitochondria helps to cope up with the oxidised DNA lesions generated due to the presence of free radicals and thus maintains mtDNA stability. The basic mechanism by which mitochondrial BER acts remains the same as the nuclear BER, but some specific BER enzymes are present in the mitochondria and these are coded by nuclear genes [166]. For a very long time, it was considered that only SN-BER occurs in the mitochondria which include removal of a DNA lesion and incorporation of a single nucleotide. But now it is believed that owing to the rate at which oxidised base lesions are generated in mtDNA, LP-BER may also take place in the mitochondria [17, 160, 167, 168]. The first step of the mtBER pathway involving recognition of a damaged base is

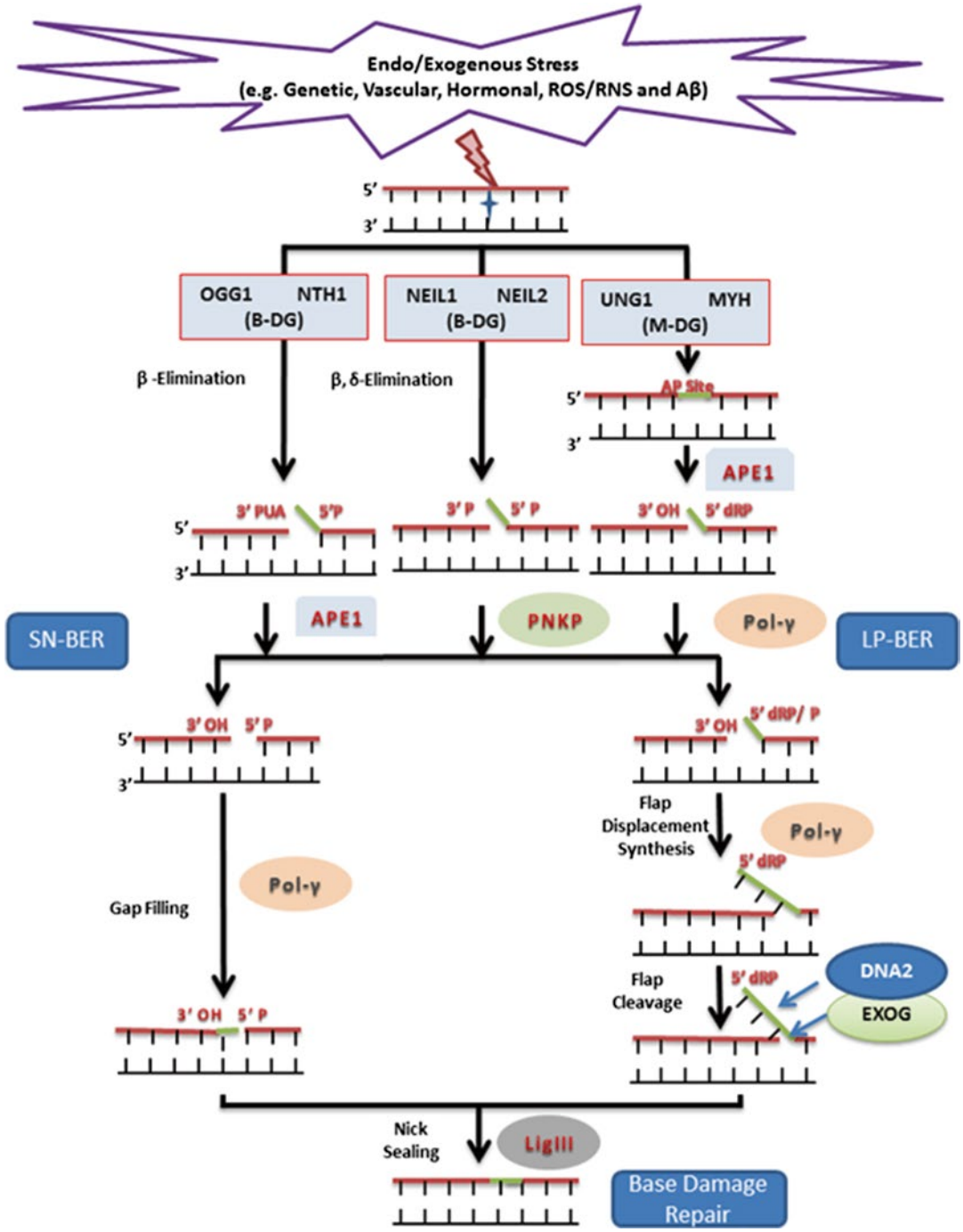

Fig. 3 Mitochondrial BER has more or less similar repair machinery as nuclear BER. DNA damage repair in the mitochondria is believed to be important in maintaining genomic integrity, especially during ageing. The basic mechanism of mitochondrial BER (*mtBER*) remains the same as nuclear-BER pathway, but some specific enzymes are present in the mtBER pathway. Both SN-BER and LP-BER are believed to take place in the mitochondria. Two M-DG, i.e. UDG1 and MYH, and four B-DG, viz. OGG1, NTH1, NEIL1 and NEIL2, are present in the mitochondria. APE1 in the mitochondria is found to be an N-terminal truncated product of APE1. In the mitochondria, DNA polγ is the only polymerase both in LP-BER and SN-BER pathways. Ligation of single-strand nick is performed by DNA ligase III. In addition, EXOG is an essential component of BER/SSBR pathway unique to the mitochondria and forms complex with APE1, DNA pol γ and DNA ligase III and is involved in repairing endogenous SSBs in the mtDNA

[AU6]

[AU7] performed by DNA glycosylase. Two M-DG, i.e. UDG1 and MYH, and four B-DG, viz. OGG1, NTH1, NEIL1 and NEIL2, are present in the mitochondria [169, 170]. Next step of the mtBER pathway involves processing of the AP site by APE1, which is the main AP endonuclease of the mammalian cell, and this mtAPE1 is believed to be an N-terminal truncated product of APE1. It is also shown that deletion of the 33 N-terminal residues increases the specific activity of mtAPE1 by threefold [171]. The next step involves insertion of correct nucleotides by DNA pol γ which is followed by ligation of single-strand nick by DNA ligase III (Fig. 3). This mitochondrial ligase III is derived from *LIG3* gene and is known to be independent of XRCC1 while the nuclear variant of DNA ligase III interacts with XRCC1 [165, 172]. A recent study has shown that EXOG is an essential component of BER/SSBR pathway unique to the mitochondria and forms complex with APE1, DNA pol γ and DNA ligase III and is involved in repairing endogenous SSBs in the mtDNA. Also, it was shown that depletion of EXOG increases ROS levels and induces apoptosis in normal cells [173]. FEN1 is involved in repairing oxidative DNA damage via LP-BER in the mitochondria [174]. Cellular death and embryonic lethality in presence of gamma radiation-induced DNA damage was observed in *FEN1* gene knockout mice [175]. The hDNA2, possessing nuclease, helicase and ATPase activities, is also involved in DNA replication and repair in the mitochondria. The hDNA2 forms a complex with pol γ and stimulates the polymerase activity. It is also involved in RNA primer removal during mtDNA replication. The hDNA2, owing to its nuclease property, can also process flap LP-BER intermediates. This points towards the synergistic roles of FEN1 and hDNA to process the 5′flap intermediates during DNA replication and repair in the mitochondria [176]. Thus, all through these years we have gained knowledge about some of the repair pathways occurring in the mitochondria and identified different repair enzymes present in the mitochondria but much more needs to be understood towards establishing pathophysiologies which overtakes these repair processes.

3.2 Role of APE1 in Redox and Transcriptional Regulation

Owing to APE1's N-terminal domain which contains the NLS and redox regulatory domain, APE1/Ref-1 is considered to be an important mammalian redox regulator of transcription. It is identified that Cys65 is the redox active site in APE1/Ref-1 and that Cys93 interacts with Cys65 via disulphide bond formation and thus these two Cys residues contribute to alter the redox state of a number of TFs [177]. A number of studies have shown that APE1/Ref-1 modifies the DNA-binding ability of several TFs, such as activator protein-1 (AP-1), Fos and Jun, NF-κB, p53, Myb, early growth response-1 (Egr-1), polyoma virus enhancer-binding protein-2 (PEBP-2), activating transcription factor/cAMP response element-binding protein (ATF-CREB), hypoxia-inducible factor (HIF-1α) and HIF-like factor [18, 149, 178]. The reduction of Cys in the DNA-binding domains of the TFs by APE1/Ref-1 enhances the DNA-binding ability of TFs. It was earlier shown that reduction of the conserved Cys residue in the DNA-binding domain of c-Jun by APE1/Ref-1 enhances the DNA-binding activity of AP-1 in vitro [179]. Also, the ability to reactivate Fos-Jun DNA-binding declines on oxidation of APE1, which can be restored on treatment with thioredoxin, TRX [177]. Thus, it can be said that alterations in the redox state could lead to alterations in gene expression of key cellular signaling and other regulatory proteins. While APE1 is considered as a redox activator of several TFs like AP-1, p53, HIF-1α, it also acts as a trans-acting factor which causes Ca^{2+}-dependent repression of parathyroid hormone (PTH) and renin genes [180, 181]. An increase in extracellular Ca^{2+} causes binding of APE1 to negative Ca^{2+} response element (nCaRE: nCaRE-A and nCaRE-B) in the respective gene promoters causing repression of PTH and renin gene expression. Acetylation of APE1 is also shown to modulate APE1's transcriptional regulatory function. In addition, APE1 interacts stably with other trans-acting factors like HIF 1-α, STAT 3

and CBP/P300 [18, 153, 182]. Thus, it points towards the redox-independent functions of APE1 in regulating transcription.

3.3 Other Functions

Apart from being the major DNA repair enzyme of the BER pathway and redox activator of several TFs, newer studies have shown that APE1 serves some other important functions as discussed below.

3.3.1 APE1 as Endoribonuclease

For a very long time, RNA decay in eukaryotes was considered to be an exoribonucleolytic process, while in prokaryotes it was considered to be an endoribonucleolytic process. But numerous evidences in the recent past have demonstrated that endoribonucleases also have a significant role in eukaryotic RNA metabolism and contribute to RNA turnover in eukaryotes [183]. Recently, APE1 was identified as an endoribonuclease that cleaves within the UA and CA dinucleotides of *c-myc* (a proto-oncogene) mRNA and regulates the *c-myc* mRNA levels [7, 147]. It was shown that APE1 knockdown in HeLa cells led to increased *c-myc* mRNA levels and its half-life [147]. In line with this, a study identified the active site of APE1 and found that common active site is shared for endoribonuclease and nuclease activities of APE1 but the mechanisms of cleavage of RNA and DNA are not identical [184]. Thus, the role of APE1 in controlling the levels and turnover of other mRNAs in the neuronal as well as other cell types need to be understood.

3.3.2 Maintenance of Cellular Homeostasis

Maintenance of proper cellular redox balance is an essential prerequisite for proper functioning of biological systems. An increase in the ROS/RNS levels beyond the normal physiologic limits could disturb the redox homeostasis leading to cell death and disease development. To cope up with the increased oxidative stress build-up in the cell, different enzymatic/nonenzymatic antioxidant systems are present. APE1/Ref-1 is known to act as an important redox regulator of the cell which helps in maintaining proper levels of ROS/RNS for cell survival and proliferation. An earlier study showed that APE1/Ref-1 helps in regulating oxidative stress by inhibiting ROS production and NF-κB activation by modulating the activation of rac1 GTPase and inhibits apoptosis [185, 186]. Overexpression of APE1/Ref-1 was shown to increase SH-SY5Y cell viability following exposure to H_2O_2 [150]. A recent study of Mantha et al. has identified several key neuronal proteins those are involved in various cellular functions are interacting with APE1 in response to Aβ(25-35)-induced stress in PC12 and SH-SY5Y cells [187]. Thus, it can be interpreted that APE1/Ref-1 has a role in providing protection against oxidative stress and helps to maintain cellular redox balance.

4 Importance of Phytochemicals in Modulating Functions of APE1/Ref-1 Towards AD Therapeutics

Human beings have used plant extracts for centuries for treating various types of ailments. For the last few years, scientists are trying to figure out the active ingredients present in the plant extracts responsible for the specific action and decipher the molecular mechanism(s) by which these phytochemicals exert their action (Fig. 4). Phytochemicals like resveratrol, isoflavones, curcumin, decursin, EGCG, L-carnitine and *Ganoderma lucidum* extract have shown potent properties against neuronal disorders. In addition, some of them have also shown their effect on modulation of APE1/Ref-1 repair as well as redox activity in relation to cancer. Limited studies warrant additional studies to see how these phytochemicals may affect APE1/Ref-1 functions in neurodegenerative diseases like AD. The following sections describe these phytochemicals as having potential therapeutic effect against AD.

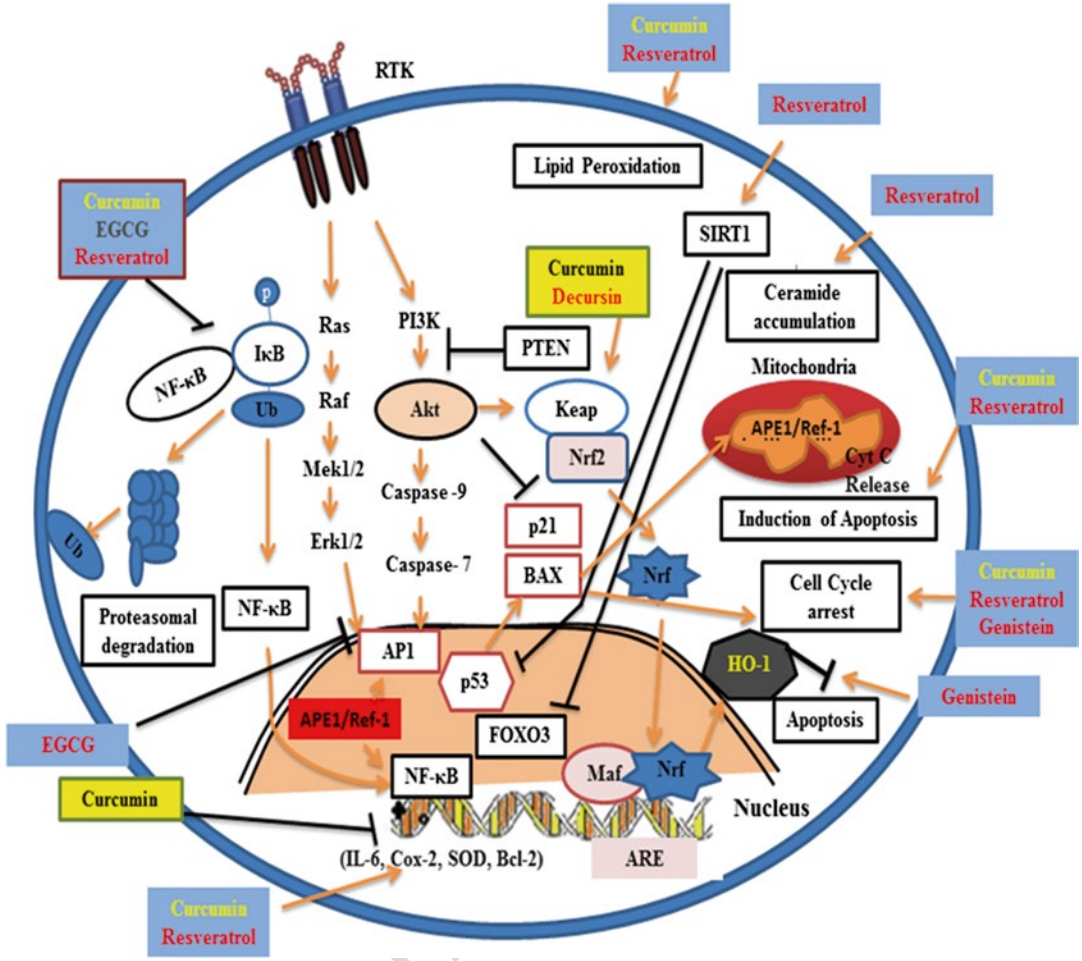

Fig. 4 Phytochemicals regulate different cellular interactions and modulates APE1/Ref-1 activity towards AD therapeutics. The phytochemical resveratrol triggers apoptosis through ceramide accumulation. Resveratrol activates SIRT1 and inhibits the transcription regulator p53 and FOXO3. Curcumin and decursin activate Nrf and rescue cell from oxidative stress. EGCG is an active polyphenol in green tea and inhibits multiple signal transduction pathways, including AP-1 and NF-κB, whereas curcumin and resveratrol activate different cellular factors like IL-6, Cox-2, SOD and Bcl-2. Curcumin, genistein and resveratrol also induce apoptosis and cell cycle arrest via functionally activated p53

4.1 Resveratrol

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a phytoalexin and a polyphenolic compound found in the seeds and wine made from grape cultivars which provides natural protection to the plant against environmental stresses such as UV radiation and fungal infections. Resveratrol is the possible explanation for the French Paradox. According to French Paradox, France has a low rate of coronary heart disease (CHD) in spite of high intake of saturated fats, thus presents a situation which is paradoxical when compared with other countries having comparable diet rich in saturated fats and subsequently high CHD [188]. Wine intake is highest in France and studies have pointed out that drinking red wine confers cardioprotection and this is attributable to resveratrol present in it along with other polyphenols [189, 190], thus explaining the paradox. Therefore, resveratrol has assumed a great importance over time. The protective abilities of resveratrol have

been attributable to its antioxidant properties [191]. Several lines of studies have shown that drinking red wine also confers neuroprotection and reduces the incidence of neurological diseases like AD [192, 193]. It has been shown that resveratrol exerts protective effects against Aβ-induced neurotoxicity in rat hippocampal cells with the involvement of PKC [194]. Resveratrol showed anti-apoptotic effect and interference in cell cycle progression in SH-SY5Y neuroblastoma cells [195]. The anti-Aβ potential of resveratrol in clearing Aβ via a mechanism involving activation of proteasome was also identified [196]. In addition, resveratrol oligomers from *V. amurensis* were shown to rescue Aβ-mediated oxidative stress in PC12 cells by inhibiting ROS production [197]. Resveratrol was found to stimulate NO production and reduce the oxidative stress after a focal cerebral ischaemia (FCI) injury in rats [198]. Downregulation of iNOS and enhancement of HO-1 expression by resveratrol rescues the Aβ-induced neurotoxicity [199]. Upregulation of iNOS is associated with Aβ levels [200], indicating a connection between iNOS and Aβ in the progression of AD. Resveratrol is also shown to be a SIRT-1 activator protecting the neuroblastoma cells from oxidative damage caused by Aβ [201].

SIRT-1 has an important role in maintaining genome integrity through regulation of BER pathway. An increase in association of APE1 with XRCC1 under genotoxic stress is reported, while the knockdown of SIRT-1 decreases this association. Resveratrol has been shown to promote binding of APE1 to XRCC1 by a mechanism involving activation of SIRT-1 [202]. Resveratrol is also shown to regulate the redox activity of APE1/Ref-1 and is identified as a potent APE1/Ref-1 inhibitor [24]. In this study, an increase in expression of Ref-1 was seen in human melanoma cells which may be partly due to mitochondrial dysfunction owing to high ROS levels and presence of oxidised melanin in these cells. Overexpressing Ref-1 led to increase in basal NF-κB transcription activities. In addition, in response to APE1/Ref-1 antibody, reduced AP-1 and NF-κB DNA-binding activities were observed. Thus, resveratrol seems to act as an APE1/Ref-1 inhibitor upregulating AP-1 and NF-κB DNA-binding activities, highlighting its anti-melanoma potential [24]. In a recent finding, it was shown that resveratrol mitigates the $AlCl_3$-induced direct neuroinflammation in rats [203]. Also, an increase in APE1 level and decrease in β-secretase and Aβ levels were observed. In addition, a decrease in expression of TNF-α, IL-6 and iNOS in the rat brain was seen on treatment with resveratrol, thus revealing the anti-inflammatory effects of resveratrol [203]. Taken together, these findings suggest resveratrol as a potent phytochemical for treating oxidative stress-induced mitochondrial dysfunction and inflammation in neurodegenerative diseases like AD and which might alter the APE1/Ref-1 function to mediate neuronal cell viability to counter AD.

4.2 Curcumin

Curcumin is the main active flavonoid derived from the rhizome of *Curcuma longa* (Zingiberaceae). Curcumin has potent anti-inflammatory property due to its antioxidant activity resulting in the scavenging of the ROS generated inside the body under stress conditions [204]. Curcumin owing to its antioxidant and anti-inflammatory action suppresses the oxidative damage and decreases amyloid deposition [205].

Curcumin acts as a strong metal chelator and has the ability to repress the inhibition of DG, NEIL1 caused by divalent metals like Cu and Fe in SH-SY5Y neuroblastoma cells [206, 207]. Curcumin acts as a potential therapeutic agent owing to its two effects – reduction of oxidative stress and acting as a metal chelator [160, 207]. Curcumin is shown to increase the heme oxygenase1 (HO-1) expression in cultured hippocampal neurons in response to glucose oxidase (GO)-mediated oxidative damage [208]. Curcumin has also shown to reduce the formation of Aβ and decrease plaque burden in transgenic AD mice [209]. Moreover, curcumin has a strong ability to cross blood-brain barrier (BBB) and shown to reduce aggregation of Aβ(1-40) and cause disaggregation of Aβ(1-40). In addition, curcumin prevented Aβ(1-42) oligomer formation and toxicity,

making it an effective molecule for prevention and treatment of AD [210]. Thus, this curry spice has a great potential in alleviating oxidative stress and improving cognitive decline in AD.

4.3 Decursin

Decursin (D) and decursinol angelate (DA) are the major coumarins present in the roots of *Angelica gigas* Nakai (Umbelliferae). The roots of *Angelica gigas* Nakai have been used in traditional Korean medicine for treating anaemia and as a sedative and an anodyne agent [211]. Many reports highlight the antitumour [211], antibacterial [211], anti-nematodal [212] and antioxidant [213] properties of *Angelica gigas* Nakai, mainly due to the presence of D and DA. A study demonstrated the anti-amnesic property of D which rescued the impairment induced by scopolamine through the inhibition of acetylcholinesterase (AChE) in the hippocampus of treated mice [211]. Decursin was shown to cross the BBB [212], thus showing a potential to intervene the CNS to treat disorders like AD. The neuroprotective role of D and DA in rescuing the glutamate-induced oxidative stress in primary cortical cells was highlighted in a study [214]. Another study showed the neuroprotective ability of D and DA and its role in nuclear factor erythroid 2-related factor (Nrf2) activation and elevation of antioxidant levels in rescuing Aβ-mediated oxidative stress in PC12 cells [213]. Both D and DA were shown to inhibit Aβ fibrillation. This study indicated that D and DA can be utilised as an important antioxidant to help reduce the oxidative stress induced by Aβ in AD. In a recent finding, it was shown that in response to Aβ(23-35)-induced oxidative stress, treatment with D leads to decreased ROS levels and activation of mitogen-activated protein kinases (MAPK) signal pathways, leading to Nrf2 activation and upregulation of HO-1 expression, thus protecting the PC12 cells from Aβ-mediated neurotoxicity [215]. Taken together, these findings suggest that D and DA can protect neurons from Aβ-mediated oxidative stress. Further studies are needed to show the potential of D and DA in modulating APE1/Ref-1's functions to limit neurodegeneration and increase cell survival in AD.

4.4 Soy Isoflavones

Soy isoflavones are the major flavonoids found in soybean, which have been a traditional food in Asia for a very long time. Apart from isoflavones, soy is also rich in phytic acid, trypsin inhibitors and saponins [216]. But soy isoflavones have dragged attention in the recent past due to its numerous health benefits particularly its neuroprotective effects. Soy isoflavones are also referred to as phytoestrogens due to their beneficial effects on estrogenic problems [217]. Soy isoflavones include genistein, daidzein and glycitein [218]. An earlier study showed that genistein could attenuate the oxidative stress induced by Aβ(25-35) and reduce the ROS levels and inhibit cell apoptosis possibly through Nrf/HO-1signal pathway in PC12 cells [219]. Another study showed that genistein improves the short-term spatial memory in rats by mitigating Aβ(1-40)-induced impairment via an estrogenic pathway [219]. Soy isoflavones suppressed the production of inflammatory cytokines and downregulated NF-κB activity, which was induced by Aβ(1-42) and improved the learning and memory impairment in rats [220]. Another study showed that isoflavones, specifically genistein and glycitein, have an anti-fibrillisation, anti-oligomerisation and fibril-destabilising potential on Aβ(1-40) and Aβ(1-42) in vitro and that glycitein, in particular, binds directly to Aβ monomers, oligomers and fibrils and exhibit highest affinity for Aβ(25-35) [221]. Thus, isoflavones can be employed towards effective therapy to directly target amyloid assemblies for the treatment of AD. In addition, genistein showed neuroprotection and increased cell viability and protein kinase C (PKC) activity in PC12 cells which were treated with Aβ(25-35) and this involved PKC signaling pathway, which is known to regulate neuronal survival in AD [222]. In addition, downregulation of *PS1*, involved in Aβ generation, by treatment with genistein was shown [223]. A recent finding showed that soy

isoflavones reduced the oxidative stress in the mitochondria induced by Aβ(1-42) in the rat brain and increased the mitochondrial membrane potential (MMP) and antioxidant function [217]. As a result, isoflavones help to maintain redox balance in the brain. Together, these findings show that isoflavones with potential can improve mitochondrial function and maintain redox balance for neuronal survival.

4.5 (−)-Epigallocatechin-3-Gallate (EGCG)

(−)–Epigallocatechin-3-gallate (EGCG) isolated from the leaves of green tea (*Camellia sinensis*) and a type of catechin [224]. It has a number of beneficial health effects owing to its neuroprotective, anticarcinogenic and anti-inflammatory property [225, 226]. The consumption of green tea and incidence of dementia, AD and PD are inversely correlated [227, 228]. Numerous animal model studies have suggested that EGCG exerts neuroprotective effects against age-related cognitive decline and neurodegenerative diseases. An earlier study using in vitro and in vivo models has shown that EGCG elevates the levels of soluble APP-α (an N-terminal cleavage product) and promotes the cleavage of α-C-terminal fragment of APP. This shows that EGCG promotes α-secretase activity leading to decreased Aβ levels and plaque formation [229, 230]. EGCG is shown to bind to the β-sheet-rich aggregates and bring about a conformational change remodelling mature α-synuclein and Aβ fibrils into smaller amorphous nontoxic protein aggregates and reduce cellular toxicity [231]. Another in vivo study involving passive avoidance and water maze tests showed that EGCG reduces the Aβ(1-42)-induced memory dysfunction dose-dependently and suppresses the activities of β- and γ-secretase. In addition, an inhibition in the activation of extracellular signal-regulated protein kinase (ERK) and NF-κB by EGCG was observed in the Aβ(1-42)-injected mouse brains [232]. EGCG has also emerged as a mitochondrial restorative compound which was demonstrated to restore MMP, ROS levels and ATP levels in a double-transgenic mouse model of AD. Thus, EGCG was shown to lessen the Aβ-induced mitochondrial dysfunction, which is implicated during the onset and progression of AD [230]. These studies point out that EGCG, owing to its anti-amyloidogenic and mitochondrial restorative property, has a tremendous potential in AD therapy.

4.6 L-Carnitine

L-carnitine is a derivative of the amino acid, lysine. Its name is derived from the fact that it was first isolated from meat (*carnus*). Acetyl L-carnitine (ALCAR), an L-carnitine ester of acetic acid, crosses the BBB and modifies acetylcholine production in the brain [233]. ALCAR is involved in regulation of mitochondrial energetics and oxidative stress associated with ageing [234]. An earlier study pointed towards induction of HO-1 and upregulation of Nrf-2, a redox-sensitive TFs, on treating astrocytes with ALCAR [235]. ALCAR was also shown to increase the synthesis of nerve growth factor receptors (NGFR) in PC12 cells and thus elicits neurite outgrowth by stimulating NGF uptake in these cells [236]. ALCAR is a physiological activator of the mitochondrial fatty acid metabolism and has been reported to improve cognitive deficits in aged animals and to slow deterioration in AD patients [237]. A study showed that ALCAR promotes α-secretase activity and physiological APP metabolism by facilitating the delivery of ADAM10 to the postsynaptic compartment regulating α-secretase activity towards APP, leading to release of a non-amyloidogenic product [238]. A study by Abdul et al. showed that ALCAR displayed neuroprotective effect towards Aβ(1-42)-induced oxidative stress in cortical neurons by upregulating the levels of glutathione (GSH) and heat shock proteins [HSPs] [239]. Thus, ALCAR displays neuroprotection and modulates mitochondrial function and oxidative stress, thus has a potential and can be employed in AD therapy upon further studies.

4.7 Ganoderma Lucidum

G. lucidum is a medicinal fungus used clinically in many Asian countries for health and longevity. A study showed the neuroprotective effect of *G. lucidum* in which the extract induced the neuronal differentiation of PC12 cells and prevented NGF-dependent PC12 neurons from apoptosis. This effect was thought to be mediated by the activation of ERK and CREB signaling pathways that maintained the survival of the NGF-dependent neurons [240]. An earlier study by Pillai et al. had demonstrated that an aqueous extract of *G. lucidum* protected against the radiation-induced nuclear DNA damage [241]. *G. lucidum* polysaccharides (GLP) was shown to reduce the expression of Caspase-3 and FasL leading to improved cognition and learning ability in Aβ(25-35)-injected mice [242]. Another study provided evidence that *G. lucidum* increased the non-amyloidogenic protein secretion, i.e. sAPPα secretion, in SH-SY5Y cells involving phosphatidylinositol 3 kinase (PI3K) and ERK signaling pathways [243]. A study pointing towards the antioxidant properties of *G. lucidum* has shown that the activities of heart TCA enzymes and mitochondrial complex (I-IV) improved on treating aged mice with an ethanolic extract of *G. lucidum* [244]. In line with this, another study showed that *G. lucidum* elevated the activities of mitochondrial dehydrogenases, i.e. succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KGDH) and pyruvate dehydrogenase (PDH), as well as complex I and II activities in the mitochondria of aged Wistar rat brains. Also, the level of lipid peroxidation was decreased in the *G. lucidum*-treated rats [245]. A recent in vivo study involving Sprague-Dawley rats showed that *G. lucidum* spore (GLS) improved mitochondrial functioning, alleviated oxidative stress and protected the hippocampal neurons from apoptosis, improving cognition in these rats [86]. Another recent study showed that *G. lucidum* promoted neurite outgrowth in differentiating N2a cells [246]. Thus, *G. lucidum* seems to have a great therapeutic importance in reviving brain and cognitive health in AD patients.

Although some of the phytochemicals described here are not studied directly with relation to APE1/Ref-1's functions, their beneficial effects as discussed further suggest testing them to understand their role in modulating repair, redox and other newly discovered roles of APE1/Ref-1 towards neuronal cell survival. It is a prerequisite for the neuronal cell to counter the oxidative stress responses elicited by different agents and mechanisms as discussed in this review and, further, APE1-/Ref-1-mediated intervention along with phytochemicals, thus, emerges a new field of study to tackle the AD.

5 Conclusions and Future Perspectives

AD is a disabling and debilitating disease affecting millions worldwide and is projected to affect many more. The pathological hallmarks of the disease are known from a very long time, but the molecular mechanism(s) underlying the AD is not known to date. Researchers have tried to understand the various factors responsible for the progression of this fatal disease. Some risk factors have been associated with the disease. These are mutations in the *APP*, *PS1* and *PS2* genes, which are responsible for the accumulation of Aβ, the main culprit, in the neurons leading to development of early-onset AD. A significant number of studies pointing out that Aβ oligomers are the more toxic species rather than the insoluble fibrillar deposits. Other risk factors for AD include the presence of *APOE* ϵ4 allele. Ageing is the greatest risk factor for AD. In the recent past, studies have implicated oxidative stress and mitochondrial dysfunction in the pathogenesis of AD. It is also observed that mitochondrial dysfunction occurs very early in AD pathogenesis. Studies have pointed out that accumulation of somatic mtDNA mutations over time causes genome instability and mitochondrial dysfunction. It is well established that Aβ causes oxidative stress. Recent studies have shown that Aβ and APP localise to the mitochondria, interact with mitochondrial proteins, increase ROS/RNS levels and cause mitochondrial dysfunction. Recent studies have provided evidence that Aβ accumulates more

in the synaptic terminals of neurons and interferes with the axonal mitochondrial transport, leading to synaptic damage and cognitive decline associated with AD. During the mitochondrial trafficking, fusion process occurs which is essential for exchange of pathogenic mtDNA. Unbalanced fusion/fission has been implicated in various neurodegenerative diseases like AD. BER is the predominant pathway responsible for repairing oxidised base lesions in the nucleus as well as in the mitochondria, with APE1 being the central repair enzyme of this pathway. Importance of APE1/Ref-1 for the cell can be discerned by the fact that attempts to generate APE1-null mice failed as it led to their early embryonic death. APE1/Ref-1 has been shown to play a major role in overcoming the oxidative stress and maintaining neuronal cell viability and integrity owing to various roles played by it, viz. as a redox and transcriptional regulator, as an endoribonuclease and as a regulator of cellular homeostasis. Phytochemicals like soy isoflavones, resveratrol and curcumin have been shown to modulate APE1/Ref-1 activity both in vitro and in vivo. In addition to these, decursin, L-carnitine, *Ganoderma lucidum* and EGCG have shown to lower the oxidative stress induced by Aβ in various studies. Thus, these phytochemicals have a potential to reduce the oxidative stress and modulate functions of APE1/Ref-1 and can be used as an effective approach to treat AD by protecting APE1/Ref-1's functions.

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Author Queries

Chapter No.: 13 0002180271

Queries	Details Required	Author's Response
AU1	Please check if edit to sentence starting "Thus, these studies…" is okay.	
AU2	Please check if edit to sentence starting "Mutations in APP…" is okay.	
AU3	Please check if edit to sentence starting "In order to…" is okay.	
AU4	Please check if edit to sentence starting "Overexpression of PARP-1…" is okay.	
AU5	Please check if edit to sentence starting "Monofunctional DNA glycosylases…" is okay.	
AU6	Please check if edit to sentence starting "Two M-DG, i.e…" is okay.	
AU7	Please check if edit to sentence starting "Two M-DG, i.e…" is okay.	
AU8	Please check if "(−)–Epigallocatechin" should be changed to "Epigallocatechin" throughout text.	
AU9	Please check if edit to sentence starting "In line with…" is okay.	
AU10	Please check if edit to sentence starting "Although some of…" is okay.	
AU11	References [24, 207], [147, 187], [150, 180], [144, 160] and [18, 149] were found to be similar in the manuscript. Hence, repeated reference have been deleted from the list and the rest renumbered. Please check if appropriate.	