ROLE OF CURCUMIN ON MONOAMINE OXIDASE (MAO) ENZYME EXPRESSION AND ACTIVITY AGAINST AMYLOID BETA (Aβ)-INDUCED OXIDATIVE STRESS IN HUMAN GLIOBLASTOMA U-87 MG CELLS

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In

Life Sciences (Sp. Animal Sciences)

By

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May, 2018

DECLARATION

I declare that the project entitled **"ROLE OF CURCUMIN ON MONOAMINE OXIDASE (MAO) ENZYME EXPRESSION AND ACTIVITY AGAINST AMYLOID BETA (Aβ)-INDUCED OXIDATIVE STRESS IN HUMAN GLIOBLASTOMA U-87 MG CELLS"** has been prepared by me under the guidance of Dr. Anil K. Mantha, Associate Professor, Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Nishibala N Behera has prepared her project entitled **"ROLE OF CURCUMIN ON MONOAMINE OXIDASE (MAO) ENZYME EXPRESSION AND ACTIVITY AGAINST AMYLOID BETA (Aβ)-INDUCED OXIDATIVE STRESS IN HUMAN GLIOBLASTOMA U-87 MG CELLS"** for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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Supervisor

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ABSTRACT

Role of Curcumin on Monoamine Oxidase (MAO) Enzyme Expression and Activity against Amyloid Beta (Aβ)-Induced Oxidative Stress in Human Glioblastoma U-87 MG Cells

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Keywords: Glioblastoma multiforme (GBM), Monoamine oxidase (MAO), oxidative stress, Curcumin, Amyloid Beta (25-35)

Glioblastoma (GBM) is the most common brain tumor in humans. The major factor responsible for its progression is oxidative stress. Oxidative stress leads to disruption of signaling pathways and damage to cells and tissues. Monoamine oxidase (MAO) is involved in oxidative deamination of endogenous biogenic amine neurotransmitters such as dopamine, serotonin, norepinephrine, and epinephrine. Therefore, MAO plays a key role in initiation and progression of GBM through oxidative stress. In the present study, Aβ(25-35) peptide treatment was used to induce oxidative stress in human glioblastoma (U-87 MG) cells. Aβ(25-35) is known to induce oxidative stress through altering the expression and activity of various antioxidant and mitochondrial enzymes. In this study, the expression and activity of MAO was evaluated through induction of oxidative stress by Aβ(25-35) and antioxidant treatment of Curcumin. It was found that Curcumin decreases the mRNA expression of MAO but its protein expression increases, whereas Aβ(25-35) showed little decrease in the mRNA expression of MAO and increase in its protein expression, thus pointing towards differential regulation of translation and transcription. The activity of MAO was found to be increased in Aβ(25-35), Cur and Cur+Aβ(25-35) . Therefore, Curcumin has little or no antioxidant effect in altering the expression and activity of MAO and Aβ showed its oxidative potential by increasing the expression and activity of MAO, although not very significant, possibly because it uses other pathways for inducing oxidative stress.

(Nishibala N Behera) (Dr. Anil K. Mantha)

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"While it is important to begin on your own, it is more important to get the support and warmth from people around you to finish well". Today I stand in a position where it would have never been possible to reach alone. I thank all whom in one way or another contributed in the completion of this research project.

At first, I thank the Almighty God for protection and ability to do work.

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Nishibala N Behera

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Alzheimer's Disease	AD
2	Amyloid Beta	Aβ
3	Amyloid Precursor Protein	APP
4	Beta Actin	β-actin
5	Bovine Serum Albumin	BSA
6	Complementary DNA	cDNA
7	Cyclin dependent kinase inhibitor 2A	INK2A
8	Degree Celsius	°C
9	Deoxynucleotide	dNTP
10	Diethyl pyrocarbonate	DEPC
11	Dimethyl sulfoxide	DMSO
12	Dithiothreitol	DTT
13	Dopamine	DA
14	Dulbecco's Modified Eagle's Medium	DMEM
15	Disodium hydrogen phosphate	Na_2HPO_4
16	Epidermal Growth Fator Receptor	EGFR
17	Ethylenediamine tetra acetic acid	EDTA
18	Extracellular matrix	ECM
19	Extracellular-regulated kinase 1/2	ERK 1/2
20	Fetal Bovine Serum	FBS
21	Glioblastoma Multiforme	GBM
22	Horse radish paradox	HRP
23	Hypoxia-inducible factor 1-alpha	HIF-1- α

24	Hour	hr
25	Inhibitor of differentiation	ID
26	Micro Litre	µL
27	Micro Molar	µM
28	Monoamine Oxidase	MAO
29	Monosodium dihydrogen orthophosphate	NaH_2PO_4
30	New England Biolabs	NEB
31	Norepinephrine	NE
32	Nuclear factor kappa-light-chain-enhancer of activated B cells	NF- κB
33	Parkinson's Disease	PD
34	Phenylethylamine	PEA
35	Phenylmethylsulfonyl fluoride	PMSF
36	Phosphatase and tensin	PTEN
34	Phosphatidylinositol-3 kinase	PI3K
38	Potassium chloride	KCl
39	Reactive nitrogen species	RNS
40	Reactice oxygen species	ROS
41	Receptor tyrosine kinases	RTK
42	Reverse transcriptase- Polymerase Chain Reaction	RT-PCR
43	Signal transducer and activator of transcription 3	STAT-3
44	Sodium chloride	NaCl
45	Sodium dodecyl sulphate	SDS
46	Temozolomide	TMZ
47	Tris Buffered Saline with Tween	TBST
48	Ultra violet rays	UV

49	Vascular endothelial growth factor	VEGF
50	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
51	5-hydroxytryptamine	5-HT

CHAPTER 1
INTRODUCTION

Oxidative stress is the major metabolic change that is found in various metabolic disorders including cancer. The metabolic reactions inside human body cells result in generation of reactive oxygen and nitrogen species (ROS/RNS). These redox reactions initiate biological pathways that alter normal metabolism and generate further oxidative stress and lead to various diseases. Oxidative stress is a key player in the aggressiveness of glioblastoma (GBM).

GBM is the most common, aggressive, malignant primary brain tumor in humans with an average life expectancy of nearly 15 months. It is originated from glial cells such as astrocytes and oligodendrocytes and is characterized by the presence of small areas of necrotizing tissue that are surrounded by anaplastic cells (Schwartzbaum *et al*., 2006). Amplification of oncogenes, such as EGFR; loss of tumor suppressors like PTEN, p53, or p16/INK4A; upregulation of Id1 and Id4 genes, genetically regulate and induce metastasis, apoptosis, promote angiogenesis, and drive brain tumor genesis through pathways such as Notch signaling (Sikder *et al*., 2003).

Monoamine oxidases are FAD-containing enzymes, involved in oxidative deamination of neurotransmitters such as dopamine, serotonin, norepinephrine, epinephrine and aniline. The reaction leads to the production of respective aldehyde and hydrogen peroxide,H_2O_2 (Shih *et al*., 1999). MAO-A has been reported to oxidize monoamine neurotransmitters resulting in ROS production (Kushal *et al*., 2016). Similarly, MAO-B activity is also reported to be increased in human glioma tumors, indicating key role of MAO in its progression (Callado *et al*., 2011; Sharpe *et al*., 2015).

Endogenous oxidants such H_2O_2 and Aβ(25-35) peptide exposure alter the expression of MAO-A and MAO-B. It has been reported that treatment with Aβ(25-35) has led to a substantial increase in MAO-B mRNA level in the astrocytes (Song *et al.,* 2000). Overtime, various research groups have shown evidence of treatment of age-related diseases such as Parkinson Disease (PD), Alzheimer Disease (AD), aging and cardiomyopathy by MAO inhibitors (Kumar *et al*., 2016). Recently, inhibitors of MAO-A have shown to decrease glioma progression (Kushal *et al.,* 2016).

OBJECTIVES OF THE STUDY

1. **To study the effect of Aβ-induced oxidative stress on MAO enzyme's expression and activity in human glioblastoma U-87 MG cells.**

2. **To study the effect of Curcumin on the expression and activity of MAO against Aβ-induced oxidative stress in human glioblastoma U-87 MG cells.**

HYPOTHESIS

Oxidative stress using non-cytotoxic doses of Aβ(25-35) peptide induces the expression of MAO-A and MAO-B. Curcumin may show the protective effect against induced oxidative stress in U-87 MG cells by altering the expression and activity of MAO.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Oxidative Stress

Reactive oxygen species (ROS) are chemically reactive species such as hydroxyl radical (OH), superoxide anion (O_2^-), transition metals such as iron and copper, nitric oxide (NO), and peroxynitrite ($ONOO^-$) etc.. ROS can be produced exogenously (pollutants, tobacco, smoke, drugs or radiation) or endogenously (plasma membrane, cytosol, peroxisomes, mitochondria or endoplasmic reticulum) in various physiological processes (Meo *et al.,* 2016).

Production of free radicals in the cell is a strictly regulated process which maintains cellular homeostasis, and act as messengers in the complex cellular processes such as mitogenic signal transduction, gene expression, cellular proliferation regulation, senescence/aging and apoptosis.

However, the disbalance between the production of oxidants and antioxidant defense leads to altered biological pathways and damaged biomolecules (DNA, RNA, proteins) which ultimately leads to oxidative stress (Halliwell*.,* 1996). Oxidative stress causes a wide variety of human diseases, such as neurodegenerative disease [e.g*.,* Alzheimer's disease (AD), Parkinson's Disease (PD), and Amyotrophic Lateral Sclerosis (ALS), inflammatory disease (e.g*.,* rheumatoid arthritis), cardiovascular disease (e.g*.,* muscular dystrophy), allergies, immune system dysfunctions, diabetes, ageing and cancer (Mariani *et al.,* 2005).

2.2 Oxidative Stress and Cancer

Cancer is an uncontrolled growth of abnormal cells inside the body tissues which can spread to other parts of the body. It is a major cause of mortality worldwide. Factors affecting tumorigenesis may be activation of oncogenes, inactivation of tumor suppressor genes, sedentary lifestyle involving diet, physical inactivity, obesity, alcohol and tobacco use, carcinogenic chemicals and radiation exposure (UV, X- ray, γ-ray) and heredity mutations.

The ability of tumor cells to evade immune response against them makes them withstand adverse microenvironment inside the cell. They achieve this by the following:

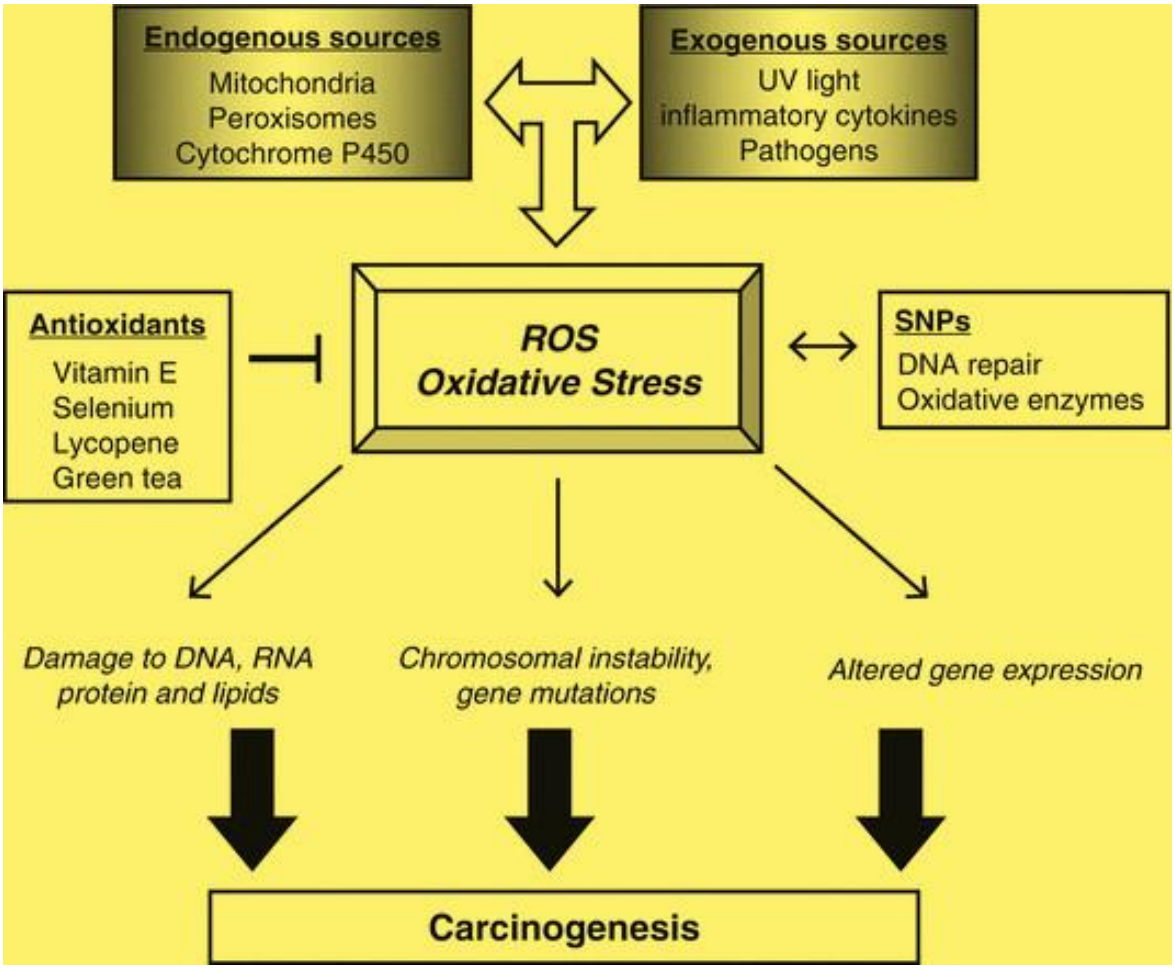


Figure 2.1: Role of reactive oxygen species and their role in the development of cancer (Pande *et al.* (2015)

- altering metabolic pathways
- upregulating antioxidant system to protect themselves from elevated ROS
- developing unusual mechanisms to maintain the genomic instability under adverse conditions (Lieu and Storz, 2010).

ROS can promote many aspects of tumor development and progression (Wiseman and Halliwell., 1996), such as:

(a) Cellular proliferation [e.g*.,* extracellular-regulated kinase 1/2 (ERK1/2) activation and ligand-independent RTK activation] (Liou *et al*., 2010)

(b) Evasion of apoptosis or anoikis [e.g*.,* Src, NF-κB and phosphatidylinositol-3 kinase (PI3K)/Akt activation] (Liou *et al*., 2010)

(c) Tissue invasion and metastasis (e.g*.,* metalloproteinase (MMP) secretion into the extracellular matrix (ECM), mesenchymal epithelial transition factor (MET) overexpression, and Rho-Rac interaction) (John *et al.*, 2001)

(d) Angiogenesis [e.g*.,* the release of vascular endothelial growth factor (VEGF) and angiopoietin] (Xia *et al*., 2007)

Oxidative stress is the key player in the aggressiveness of GBM, as discussed in the next section.

2.5 Amyloid Beta and Oxidative Stress

Amyloid beta (Aβ) peptide is a 4 kDa proteolytic fragment derived from the beta-amyloid precursor protein (APP), cleaved by β-secretase and γ-secretase. It is one of the hallmarks of Alzheimer's disease (AD). It is the characteristic feature of the amyloid plaque deposits in the brain of AD patients. The secretion of Aβ peptide also occurs by the glial cells.

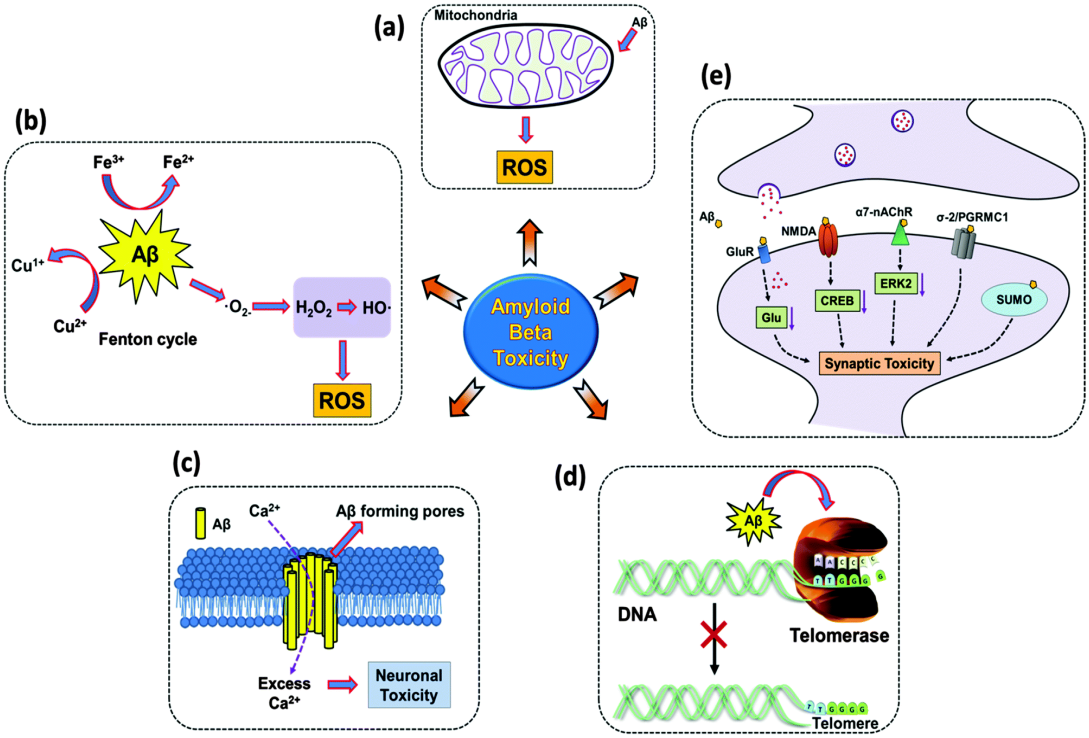


Figure 2.2 Aβ toxicity (a) Aβ causes mitochondrial dysfunction causing increases in ROS generation (b) Aβ oligomers cause an increase in oxidative stress (c) Cell membrane disruption caused by Aβ aggregates (d) Telomerase inhibition (e) Aβ interfering with signalling pathways causing synaptic dysfunction and toxicity (Rajasekhar et al., 2015).

Aβ peptides ranging from 36–43 are derived from the APP via two pathways viz. amyloidogenic and non-amyloidogenic. In the former, APP is cleaved by β-secretase producing APPsβ and β-CTF (b C-terminal fragment). Subsequent cleavage by γ-secretase on the transmembrane domain produces Aβ and AICD (amyloid precursor protein intracellular domain). In the non-amyloidogenic pathway, α-secretase cleaves APP leading to the formation of APPsα fragment and α-CTF. Successive action by γ-secretase cleaves the α-CTF releasing the P3 peptide and AICD (Rajasekhar *et al*., 2015) and as reviewed extensively (Kaur *et al*.,2016). Accumulation of Aβ is known to be associated with oxidative stress and toxicity n the cells by various mechanisms as shown in **Figure 2.2.**

2.3 Glioblastoma Multiforme (GBM)

GBM is the most common and aggressive malignant primary brain tumor in humans with an average life expectancy of nearly 14 months. WHO classifies it as “Grade IV astrocytoma”. It originates from various types of brain cells (astrocytes, oligodendrocyte progenitor cells, and neural stem cells). GBM is characterized by the presence of small areas of necrotizing tissue that are surrounded by anaplastic cells (Mao *et al*., 2012).

2.3.1 Affected Populations

GBM affects males more often than females, whereas adults are affected more than children. People in the age group of 40-60 years are mostly affected by this cancer. As per the available statistics, 2-3 people per every 1,00,000 are affected by GBM in the United States and many European countries (National Organization for Rare Diseases). When compared with the other primary childhood brain tumors, GBM incidence rate is around 3-4%. In case of India, Indian Council of Medical Research (ICMR) conducted a survey and reported that among males, Trivandrum had the highest glioma incidence rate of 28.2%, followed by Bangalore (6.7%) Mumbai (5.8%), Dibrugarh (5.6%) and Chennai (3.5%). On the other hand, Trivandrum again had the highest glioma incidence rate at 21.8%, followed by Chennai (7.5%), Mumbai (6.3%), Bangalore (5.6%) and Dibrugarh with 0% incidence rate among females (Shankarkumar *et al.*, 2011).

2.3.2. Causes

The exact cause underlying GBM is largely unknown. It is speculated that environmental factors including exposure to UV rays, ionizing radiation, genetic and immunologic abnormalities are the major factors that may contribute to the development of certain cancers. But, till now, no conclusive evidence is present which establishes the direct relationship between the development of gliomas and these factors. Amplification of oncogenes such as EGFR, and loss of tumor suppressors like PTEN, p53, or p16/INK4A are some of the most common genetic alterations seen in GBM (Lacob *et al.,* 2009). A recent discovery of somatic mutations in the metabolic enzyme viz. isocitrate dehydrogenase (IDH) has been identified in glioma (Rohle *et al.*, 2013). The inhibitor of differentiation genes (Ids) are involved in cell cycle control, tumorigenesis and angiogenesis through the negative regulation of helix-loop-helix transcription factors (Norton *et al.,* 1998). Ids regulate cell cycle and cell differentiation and have an important role in the control of stem cell self-renewal. Very recently, Id1 has been shown to be expressed in B1 type adult neural stem cells, having an important role in the regulation of the self-renewal capacity of these cells. In cancer, Id1 is found upregulated in several tumors and has been described to be involved in metastasis. Id4 has been reported to participate in neural stem cell differentiation, induce apoptosis in astrocytic cultures, promote angiogenesis, and drive brain tumor genesis through notch signaling (Sikder *et al*., 2003). Other than Id-1, transcription factors such as STAT-3 (Brantley & Benveniste, 2008), HIF-1-α (Sharpe, 2015) have been reported to control the development of GBM.

2.3.3 Treatment

The standard treatment therapy involves surgery followed by chemotherapy and radiation for GBM patients. Following surgery, treatment using anticancer drugs (chemotherapy) is commonly used procedure to individuals with GBM. Alkylating agent-based therapy such as Temozolomide (TMZ) shows effectiveness in the GBM patient, however, the survival of GBM patients is less than 15 months (Davis *et al*., 2016).

2.4 Monoamine Oxidase

Monoamine oxidases (MAO) are mitochondrial-bound enzymes involved in oxidative deamination of endogenous biogenic amine neurotransmitters such as dopamine, serotonin, norepinephrine, and epinephrine and also oxidizing xenobiotics such as aniline. The reaction leads to the production of respective aldehyde and hydrogen peroxide (Shih *et al*., 1999).

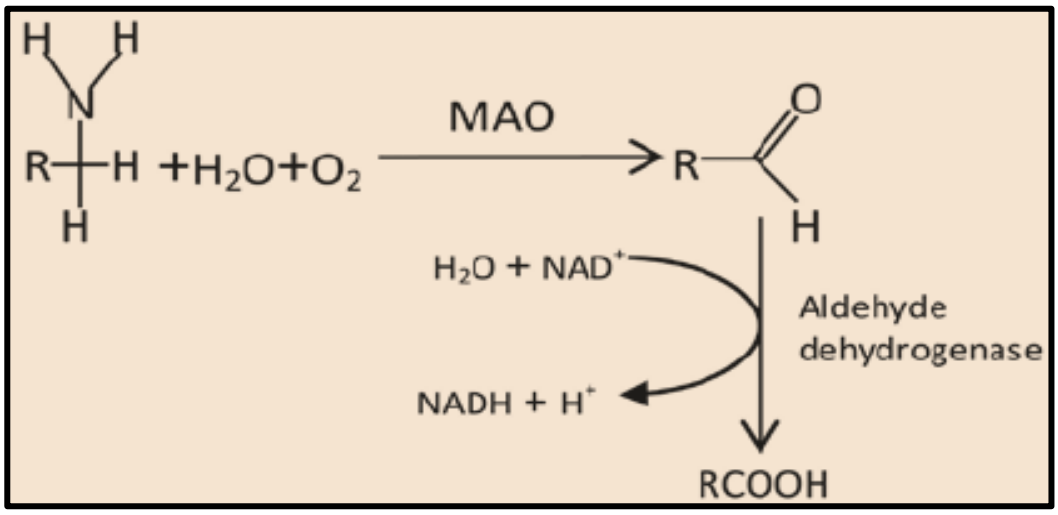


Figure 2.3 A typical reaction catalyzed by MAO. The reaction of a base (R, arbitrary group) with one ammonia and two hydrogen components plus water and oxygen, catalyzed by MAO, generates the aldehyde, ammonia and H_2O_2. (Cai *et al*., 2014)

2.4.1 Types of MAO

Johnston (1968) defined two subtypes of MAO enzyme, MAO-A and MAO-B (Westlund *et al*., 1988). MAO-A has a higher affinity for the substrates serotonin (5-HT), norepinephrine (NE), dopamine (DA), and the inhibitor clorgyline, whereas MAO-B has a higher affinity for phenylethylamine (PEA), benzylamine, and the inhibitor deprenyl (Kushal *et al.*, 2016; Kumar *et al.,* 2016).

Biophysical techniques, such as immunohistochemistry, or *in-situ* hybridization have shown the localization of MAO-A predominantly in catecholaminergic neurons and MAO-B in serotonergic and histaminergic neurons and glial cells. Both MAO-A and B forms are located throughout the brain in the

outer membrane of mitochondria. The distribution of MAO in the periphery also varies within the same organism. Some tissues, such as human platelets and bovine liver and kidney mainly contain MAO-B while others, like human placenta and bovine thyroid, predominantly contain MAO-A (Westlund *et al*., 1988). In humans and rodents, MAO-A level is almost same as at birth, whereas MAO-B activity increases several-fold with aging.

MAO-A and MAO-B are encoded by MAO-A and MAO-B genes respectively, adjacent to each other at Xp11.3. These genes have been shown to have 70% amino acid sequence identity. Their deletion may lead to disorders like Norrie disease (Shih *et al*., 1999). MAO-A expression is increased in human glioma tissues and cell lines. MAO-A oxidizes monoamine neurotransmitters resulting in reactive oxygen species (Kushal *et al*., 2016). MAO-B activity is also reported to be increased in human glioma tumors (Callado *et al*., 2011; Sharpe *et al*., 2015). A study found that endogenous oxidants such H_2O_2 and Aβ(25-35) peptide exposure alters the expression of MAO-A and MAO-B. Aβ(25-35) peptide was shown to induces MAO-B activity. Treatment with Aβ(25-35) also led to a substantial increase in MAO-B mRNA level in the astrocytes (Song *et al.,* 2000). Also, Aβ(25-35) was able to selectively induce MAO-B expression in rat astrocytes (Song et al., 2000).

Over time, various research groups have shown evidence of treatment of age-related diseases such as Parkinson's Disease (PD), Alzheimer's Disease (AD), aging and cardiomyopathy by MAO inhibitors (Kumar *et al*., 2016). Recently MAO-A inhibitors have shown to decrease the glioma progression (Kushal *et al.,* 2016).

2.5 Curcumin

Curcumin appears as crystalline compound with a bright orange-yellow color. Curcumin is commonly used as coloring agent, food additive, herbal supplement and cosmetics ingredient. It occurs naturally in rhizome of Turmeric (*Curcuma longa)*, an indispensable ingredient of Indian cuisine. It is a diarylheptanoid and together with demethoxycurcumin and bis-demethoxycurcumin form curcuminoid, suitable for drug formation (Kawamori *et al*., 1999). The yellow colour of Curcumin also results due to these diaryl heptanoids.

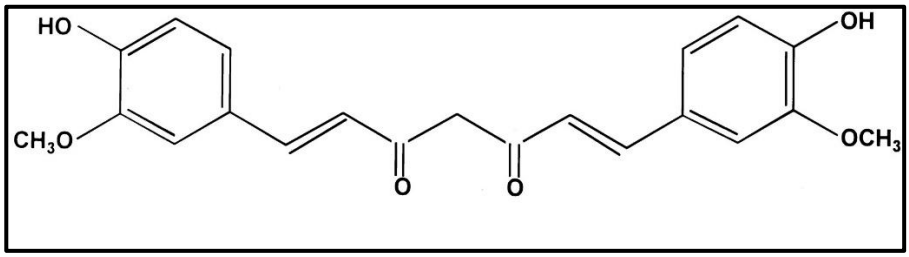


Figure 2.4: Structure of Curcumin (Kawamori *et al*., 1999)

2.5.1 Potential of Curcumin in GBM treatment

The putative benefits of curcumin as a treatment for GBM have been studied by numerous groups. Curcumin has been shown to induce autophagy by suppression of the protein kinase B (AKT)/mammalian target of rapamycin (mTOR)/p70S6K and activation of the extracellular-signal-regulated kinase (ERK1/2) pathways in U-87 MG and U-373 MG human malignant glioma cells harboring a phosphatase and tensin homolog (PTEN) mutation (Akoi *et al*., 2007). It is reported that curcumin activates p21 in U-87 MG human GBM cells via ERK and c-JUN N-terminal protein kinase signaling (Choi *et al*., 2010). Senft *et al.* (2010) studied cell lines from human primary and recurrent GBM and showed that curcumin reduced cell growth, inhibited migration and decreased invasiveness due to its inhibition of the JAK/STAT3 pathway. Similarly, Dhandapani *et al.* (2012) showed that curcumin enhanced cell death by reducing the activity of activator protein 1 (AP-1) and nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB) binding in human and rat glioma cell lines. Curcumin caused a reduction in brain tumor volume in C6 implant rat glioma model (Zanotto-Filho *et al*., 2012). Curcumin has also been shown to suppress the growth of human glioma U-87 MG cells xenografted into athymic mice (Perry *et al*., 2010). Fong *et al.* (2010) showed that curcumin may have the potential to target cancer stem cells in rat C6 glioma cells. Curcumin is also found to induce differentiation of glioma-initiating cells and inhibit their growth via autophagy (Zhuang *et al*., 2012; Sordilo *et al.,* 2015).

Recently, curcumin nanoparticles have been proposed to slow-down GBM growth through the inhibition of cell proliferation and a reduction in the stem-like tumor cell. curcumin coupled to a monoclonal antibody caused a 120-fold increase

in the death of human GBM cells in culture compared to curcumin alone. In addition, mice implanted with GBM cells had an extended survival time and a reduction in the size of the brain tumor mass with this treatment (Lim *et al*., 2010).

Thus, curcumin is found to possess anticancer activities in different types of carcinomas including GBM, thus, pointing towards the putative role of curcumin as an adjunct to traditional chemotherapy in the treatment of gliomas.

CHAPTER 3

Materials and Methods

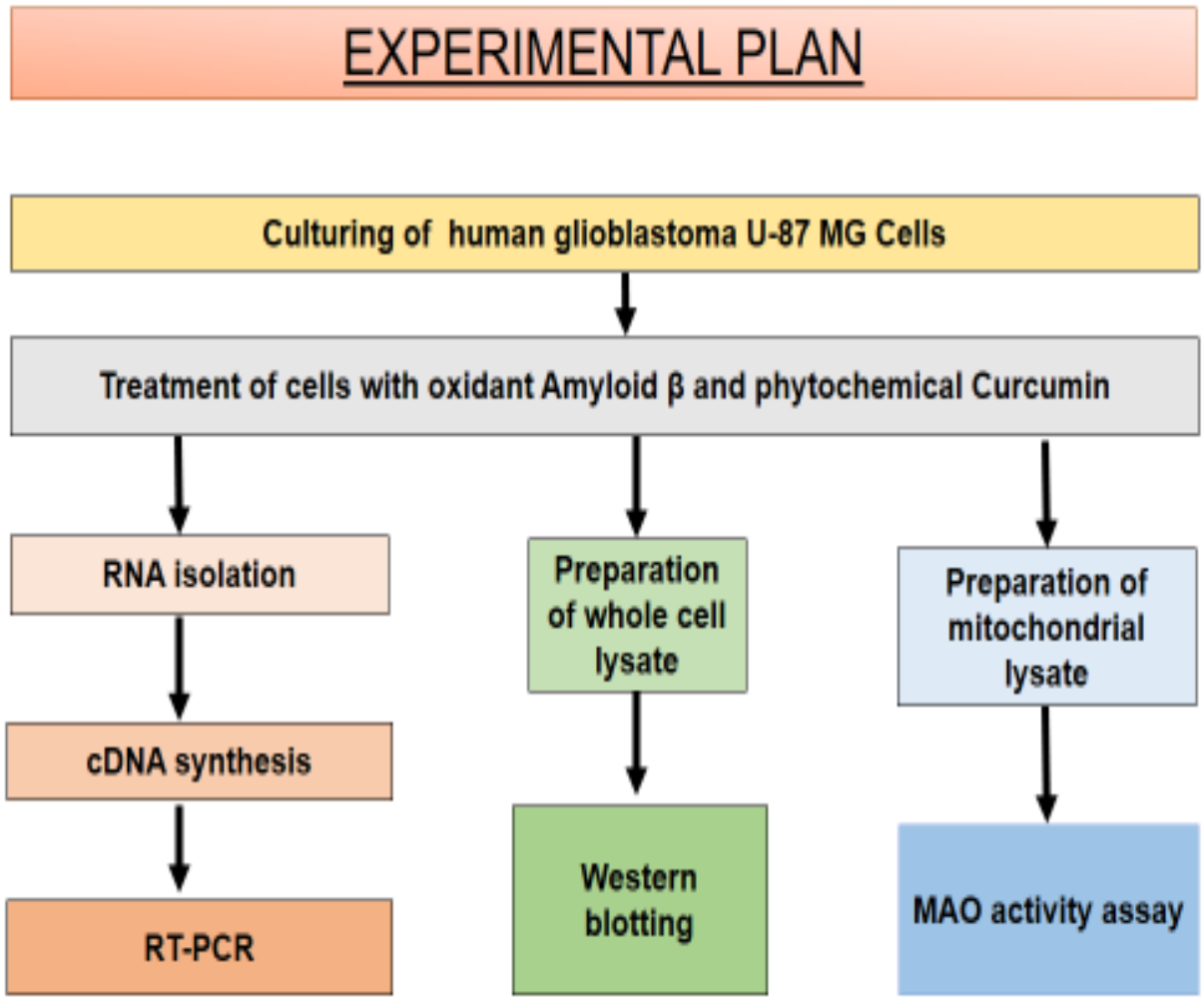


Figure 3.1: Summary of the experiments performed during the study

3.1 Cell Culture

U-87 MG (ATCC®HTB-14™) is an adherent epithelial cell line derived from brain tissue (glial cells) of Homo sapiens. Prior to cell culture, all the apparatus such as tip boxes, reagent bottles, filter assembly were thoroughly cleaned and autoclaved. Then, DMEM media was prepared by filter-sterilization. Following media preparation, U-87 MG cells were thawed and cultured in DMEM medium supplemented with 10% FBS and 1% 1X Penicillin/Streptomycin under 5% CO_2 humified atmosphere at 37°C. After 24 hr, the confluency was checked. At 80% confluency, cells were split further (Cholia *et al*., 2017).

3.2 Cell Treatment

U-87 MG cells were treated with Aβ(25-35) at a concentration of 10 µM in order to generate oxidative stress. Phytochemical Curcumin treatment was given to the cells at 10 µM. For this, a stock solution of Curcumin (100 mM) was prepared in DMSO. Further, a 3 hr pretreatment of Curcumin (10 µM) followed by Aβ (10 µM) treatment was given to the cells for 24 hr.

3.3. Total RNA Isolation

Before proceeding with RNA isolation, all the apparatus were treated with DPEC water and dried in oven. For isolating RNA, U-87 MG cells were seeded in a 6-well plate and given treatments as described above.

After 24 hr of treatment, Trizol (300 µl) was added to the treatment groups. Cells were firstly homogenized by pipetting up and down and incubated at room temperature. Then, 200 µL of chloroform was added. After incubation for 2-3 min, the mixture was centrifuged at 12000 g for 15 min at 4°C. This led to the formation of 3 layers/phases. The upper aqueous phase containing RNA was transferred to a new tube. After this, 200 µL of isopropanol was added to the aqueous phase contained in the tube. The samples were again centrifuged for 10 min at 12000 g at 4°C. Finally, RNA could be seen precipitated as white gel like pellet at the bottom of the tube. The supernatant was discarded and the pellet was resuspended in 1 ml of 75% ethanol. The samples were vortexed briefly and then centrifuged for 5 min at 7500 g at 4°C. The supernatant was discarded with the help of pipette and the pellet

was air dried for 5-10 min. This pellet was then resuspended in 40µL of RNase-free water and incubated in water bath at 55°C for 10-15 min. Lastly, RNA quantification was done by Nanodrop Spectrophotometer. The pertocol for RNA isolation was followed as described earlier (Gupta *et al.,* 2018).

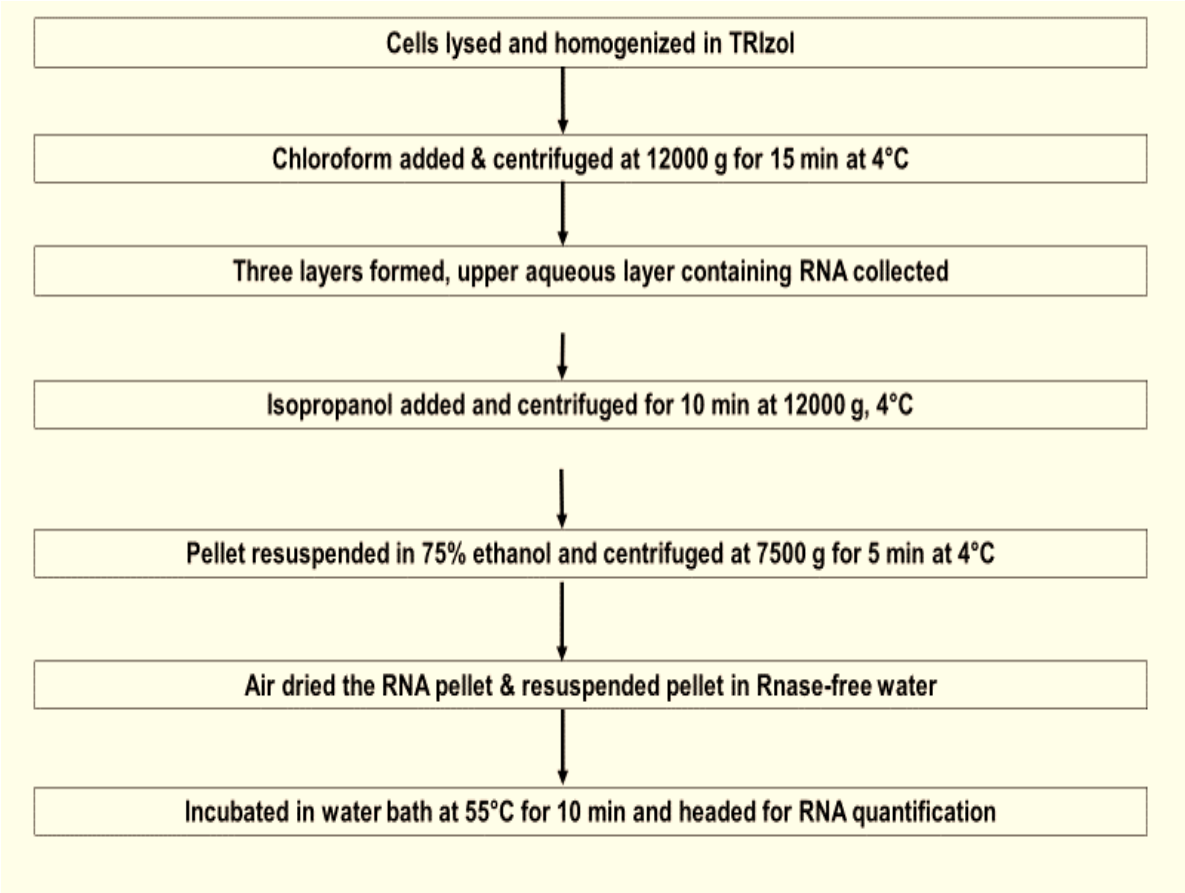


Figure 3.2: Steps followed for isolation of total RNA

3.4 Synthesis of cDNA

Upon RNA isolation and quantification, cDNA synthesis was done by using Thermo Scientific Verso cDNA synthesis kit. The reaction mix was prepared according to the manufacturer's protocol as described in **Table 3.1**. This reaction mixture was then incubated for 30 min at 42°C and for 2 min at 95°C in Thermal Cycler (SimpliAmp™).

Table 3.1: Various components and their volumes used during cDNA synthesis

Components	Volume (µL)
5x cDNA Synthesis buffer	4 µL
dNTP Mix	2 µL
Random Primer	1 µL
RT Enhancer	1 µL
Verso Enzyme Mix	1 µL
Template RNA	2-7 µL
Nuclease Free Water	Upto 20 µL
Total Volume	20 µL

3.5 PCR for expression of MAO-A

PCR for amplification of MAO-A was performed using Quick load 2X master mix (NEB). Each reaction consisted of 7µL of master mix, 1µL of 10µM forward MAO-A (5'-TTCAGCGTCTTCCAATGGGAGCT -3') primer, 1µL of 10µM reverse MAO-A (5'- TGCTCCTCACACCAGTTCTTCTC -3') primer to make final volume of 10µL. This was followed by incubation in a thermal cycler (Thermo Fisher) using 61°C annealing temperature (Wang *et al*., 2011).

3.6 PCR for expression of β-actin

PCR for amplification of β-actin was performed using Quick load 2X master mix (NEB). Each reaction consisted of 7µL of master mix, 1µL of 10µM forward β-actin (5'-CTAAGTCATAGTCCGCCTAGAAGCA-3') primer, 1µL of 10µM reverse β-actin (5'-TGGCACCCAGCACAATGAA-3') primer to make final volume of 10µL.

This was followed by incubation in a thermal cycler (Thermo Fisher) using 59°C annealing temperature (Gupta *et al.,* 2018).

3.7 Analysis of mRNA Expression

After the PCR was completed, the samples were electrophoresed on 1.2% agarose gel at 75 V for 30 min. The gel images were taken with BioRad GelDoc system and bands were analyzed by Image Lab Software. Further, densitometric analysis was done for all the samples for quantitative measurement of band intensities on an agarose gel (Cholia *et al*., 2017).

Table 3.2: Various components and their volumes used in RT-PCR

Components	Volume
Master mix	7µL
Forward primer	1µL
Reverse primer	1µL
cDNA	1µL
Total	10µL

Table 3.3: Summary of primers used, annealing and amplification cycles

Gene	Base pairs	Primer sequence	Annealing temp	Amp cycle
MAO-A	23 bp	F: 5’ -TTCAGCGTCTTCCAATGGGAGCT-3’	61°C	34X
		R: 5’- TGCTCCTCACACCAGTTCTTCTC -3’		
β-actin	186 bp	F: 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’	59°C	34X
		R: 5’-TGGCACCCAGCACAATGAA-3’		

3.8 Preparation of Whole Cell Lysates

Cells were cultured in DMEM media supplemented with 10% FBS and 1% PS. At maximum confluency, they were treated with Aβ (10µM), Curcumin (10 µM) and pretreated Curcumin+Aβ (10 µM+ 10 µM). After 24 hr of treatment, cells were

lysed with Triton X-100 lysis buffer (containing 150 mM NaCl, 0.5 M EDTA, 1 M Tris, 0.5 % Triton X-100, 20 % SDS, 1 mM PMSF, 5 % Glycerol and Protease Inhibitor Cocktail). The cells were mixed homogeneously and centrifuged at 20,000 g for 20 min. Lastly, the supernatant was collected and stored at -20°C, followed by estimation of protein concentration by Bradford assay (Mantha *et al*.,2012).

3.10 Bradford Assay

The Bradford protein assay determines the concentration of protein in solution by the binding of Coomassie Brilliant Blue G-250 dye to proteins.

For the assay, a stock solution of 0.1 mg/ml BSA was used for making a standard curve at a concentration range of 0-70 µg/mL by plotting graph of absorbance vs. concentration. All experiments were carried out in dark and plate was kept on shaker for 15-20 min, to develop a blue color. Absorbance was read at 595 nm and the concentration of unknown samples was calculated using the standard curve prepared using BSA (Cholia *et al*., 2017).

3.11 Western Blotting

3.11.1 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for separating proteins by electrophoresis (the separation of macromolecules in an electric field). It uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins.

To perform SDS-PAGE, a 10% resolving gel (containing 1.5 M Tris-Cl (pH 8.8), 30% Bis/Acrylamide (29:1), 20 % SDS, 10% APS, TEMED and water) and a 4% stacking gel (containing 0.5 M Tris base (pH 6.8), 30 % Bis/Acrylamide (29:1) 30%, 20% SDS, 10% APS, TEMED and water) was prepared by using BIORAD gel casting assembly. After heating at 95°C, the protein samples were loaded onto the gel. The gel was run at 70V (Mantha *et al.,* 2012).

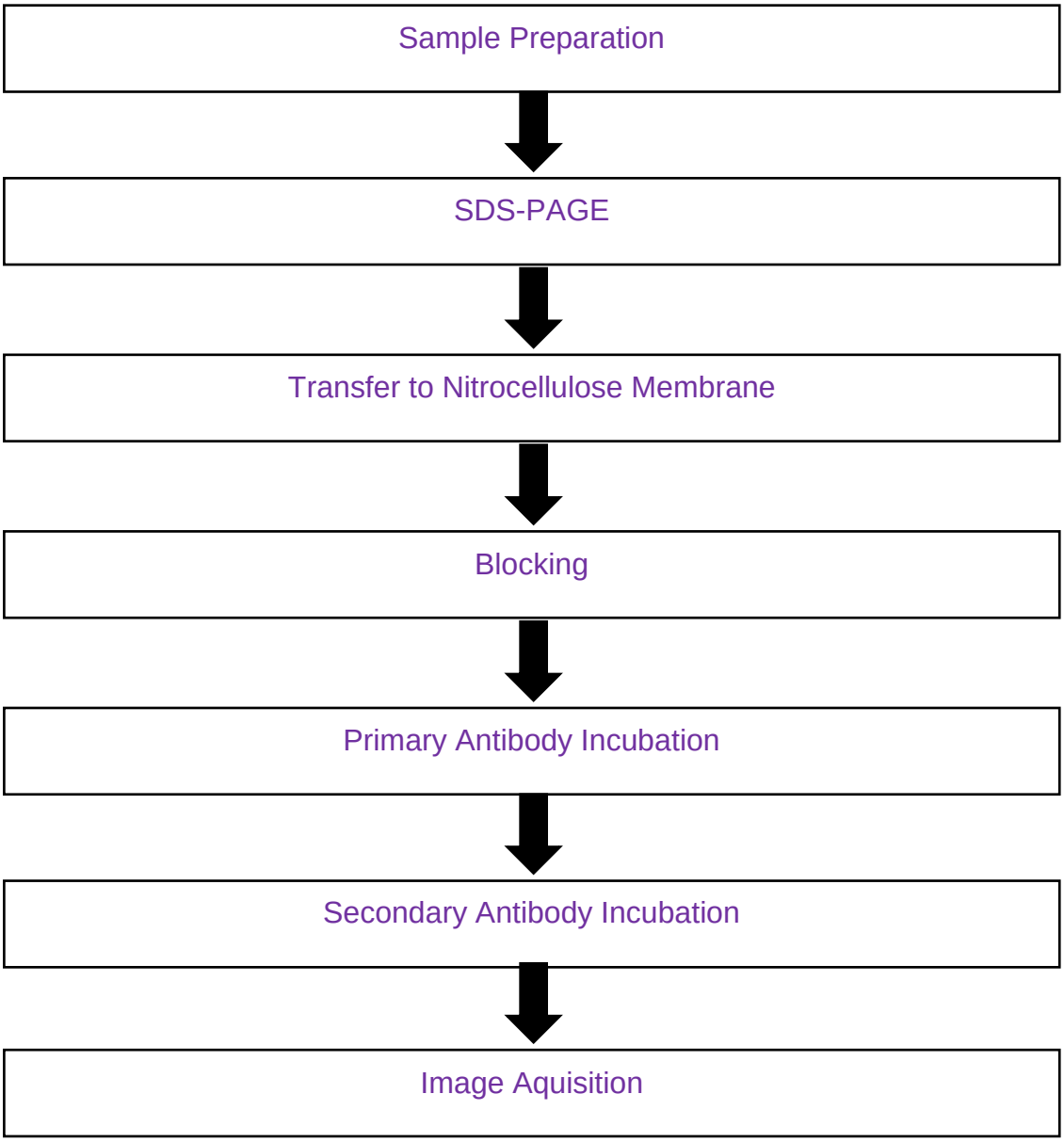


Figure 3.3: Overview of steps in Western blotting

3.11.2 Transfer to Nitrocellulose Membrane

After SDS-PAGE, the protein bands on the gel were made to transfer using transfer assembly (BIORAD) by wet transfer technique. The Gel-Membrane-Filter sandwich was placed in the transfer tank, suspending in 1X transfer buffer (containing 25mM Tris base, 192mM Glycine, 10% Methanol) vertically. The set was allowed to run at 30 V overnight at 4°C. Proteins were transferred from the gel to the membrane under electric field produced by electrode plate paralleled to the sandwich.

3.11.3 Blocking and Antibody Incubation

After overnight incubation, the membrane was stained with Ponceau stain to visualize the bands. The membrane was further rinsed in TBST (containing 1M Tris (pH 7.5), 136mM NaCl, 0.1 % Tween-20) and blocked in 5% non-fat dry milk, prepared in TBST, for 2 hr. Then, the membrane was washed with TBST with interval of 10 min, thrice. The membrane was further incubated in rabbit polyclonal MAO-A/B (H-50) antibody (santa cruz biotechnology) at a dilution of 1:1000 at 4°C overnight.

After overnight incubation, the membrane was washed in TBST thrice with an interval of 10 min to remove any unbound primary antibody. The membrane was then exposed to HRP conjugated anti-rabbit secondary antibody with a dilution of 1:5000 for 1 hr. The membrane was then washed with TBST for 15 min with time interval of 5 min, then headed for image development.

3.11.4 Image Development

Chemiluminescence is a common detection method for western blotting. After secondary antibody conjugation, the membrane is incubated with a solution containing the chemiluminescent substrate. In the presence of peroxide, the HRP enzymes catalyzes the oxidation of luminol, which then generates light. An enhancer is included in the substrate solution to increase the longevity and intensity of the emitted light. This light resulting from this reaction can be detected with either film or a digital imaging system.

Image of membrane was developed in Chemidoc using Imagelab software at DNA Lab facility. ECL (BioRad) chemiluminiscent detection system was used. Further densitometry was performed for image quantification (Cholia *et al.,* 2017).

3.12 Stripping of Membrane

Mild stripping was done by 1x stripping buffer (containing 0.1 g SDS, 1.5 g Glycine, 1 ml Tween 20) (2.2 pH) (5 min twice), then washed with PBS (10 min, twice), TBST (5 min, twice) and finally added in blocking solution (2 hr). The same procedure was repeated till image development as explained above.

The membrane was incubated in mouse monoclonal β-Actin (Sigma Aldrich) primary antibody with dilution 1:10000 and HRP conjugated anti-mouse secondary antibody with dilution 1:5000 (Cholia *et al*., 2017).

3.13 Preparation of Mitochondrial Lysate

The U-87 MG cells were cultured in 150 mm culture plate containing DMEM medium supplemented with 10% FBS and 1% 1x PS, maintained at 37°C and 5% CO_2. At maximum confluency, cells were treated with Aβ (10μM), Cur (10μM) and 3 hr pretreatment of Curcumin (10μM) followed by Aβ(25-35) (10μM). After 24 hr, the cells were trypsinised and centrifuged at 1500 rpm for 10 min at 4°C. The resulting cell pellets were resuspended in ice-cold lysis buffer (containing 75 mM NaCl, 250 mM sucrose, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 0.05 % Digitonin and PI cocktail) for 30 min with vortexing every 10 min. Then, the cell suspension was centrifuged at 800 g for 10 min at 4°C. The nuclear pellet obtained 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 and the pellet contained the mitochondria. The mito-pellet was further washed with wash buffer (containing 20 mM HEPES (pH 7.4), 250 mM Sucrose, and 1mM DTT) twice for 5 min. Lysis buffer (containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM DTT, 300 mM KCl, 5% glycerol, 0.5% Triton X-100, 1 mM PMSF) was added, vortexed properly and incubated on ice for 15 min, and centrifuged at 1000 g for 10 min at 4°C. The

supernatant was collected and protein estimation of the obtained sample was done by Bradford assay (Kaur *et al*., 2016).

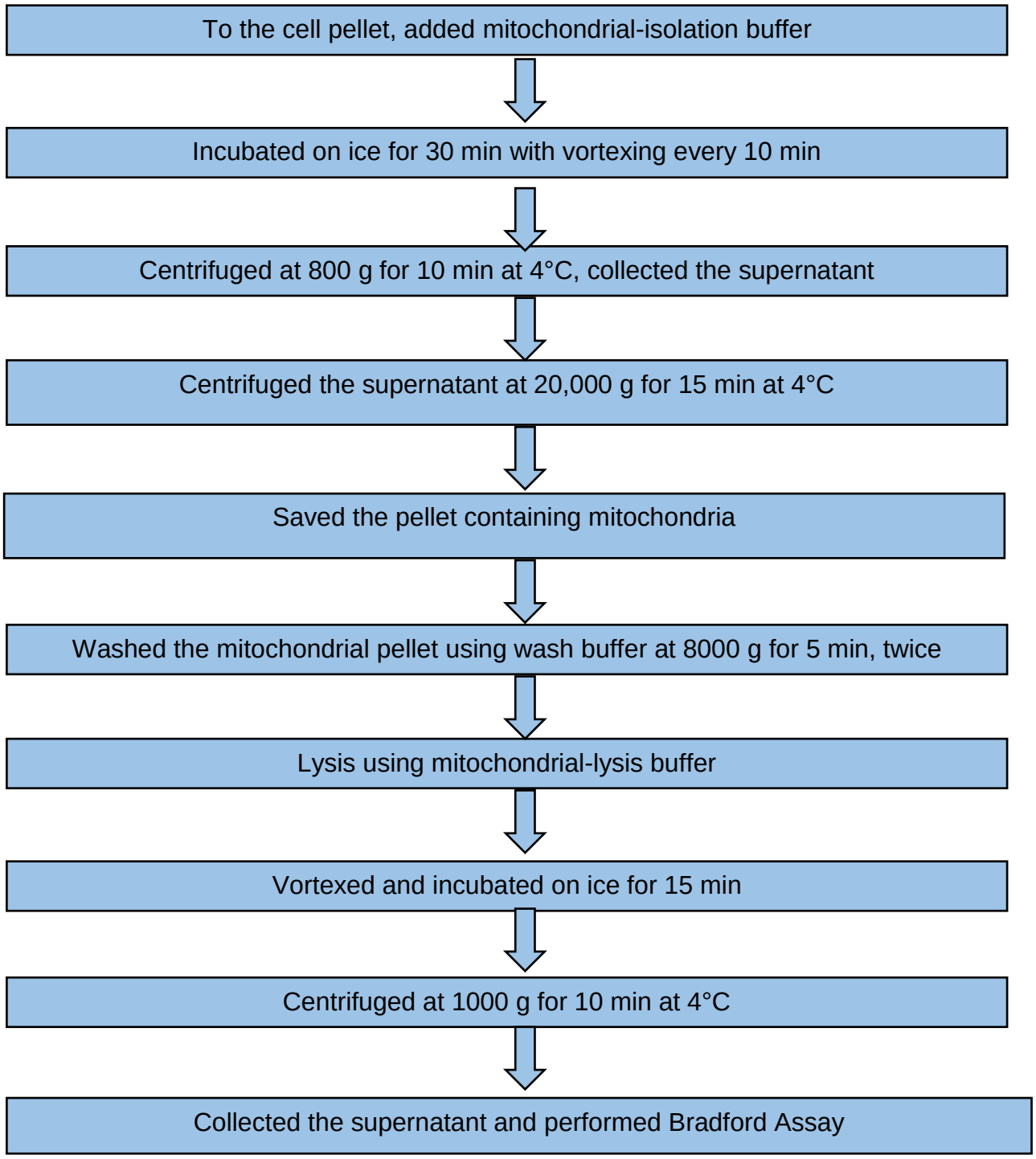


Figure 3.4: Steps of preparation of Mitochondrial Lysates (Kaur *et al.,* 2016)

3.14 MAO activity assay

The reaction was started by adding 0.05 mM Tris Cl (pH 7.4), 0.22 mM kynuramine dihydrobromide, 0.08 mM $MgCl_2$, mitochondrial protein as per the protocol described (Mantha et al., 2006) with few newer modifications. After incubation of 90 min, at 37°C, the reaction was stopped by adding 0.5 M NaOH and 0.1 M $ZnSO_4$. It was heated in dry bath at 100°C for 10 min and then centrifuged at 10,000 rpm for 10 min. The amount of reaction product, i.e, 4-hydroxyquinoline formed was determined spectrophotometrically in the supernatant at 330 nm using Gen5 software. Blank was prepared by replacing Kynuramine with H_2O. A standard curve of Kynurenic acid was prepared to determine the concentration of 4-hydroxyquinoline. For this, a stock solution of 50μg/ml of Kynurenic acid was prepared. Different concentrations of Kynurenic acid from 2μg/mL to 20μg/mL were prepared and heated at 100°C. After 10 min, they were centrifuged at 10,000 rpm for 10 min. The supernatant was then collected in a 96-well plate and reading was taken in spectrophotometer at 330 nm.

MAO activity was calculated by the following formula :-

$$\textbf{MAO activity} = \frac{\textbf{Conc of 4 - hydroxyquinoline} \times \textbf{Dilution of extract} \times \textbf{Vol of assay}}{\textbf{Vol of extract}}$$

One unit of enzyme activity is equal to micromoles of 4-hydroxy quinolone produced per minute per gram at 25° C.

3.15 Statistical Analysis

The students (t) test was performed for the evaluation of the significance of the results. The data was considered statistically significant at $p \leq 0.05$ across all treatment group. Data was presented as mean ± standard error deviation (n=3).

CHAPTER 4

RESULTS

4.1 Assessment of relative mRNA expression of MAO-A

RT-PCR was performed to assess the mRNA expression of MAO-A in U-87 MG cells that were treated in different groups, namely, Control, 10μM Aβ(25-35), 10μM Cur and 10μM Cur+10μM Aβ(25-35). It was observed that Curcumin decreased the mRNA expression of MAO by 65% as compared to control, whereas Aβ(25-35) caused 22% decrease in its expression as compared to the untreated control. The pre-treatment of Cur followed by Aβ(25-35) treatment after 3 hr caused 58% decrease in mRNA expression of MAO as compared to Aβ(25-35) treatment (**Figure 4.1 & Table 4.1**).

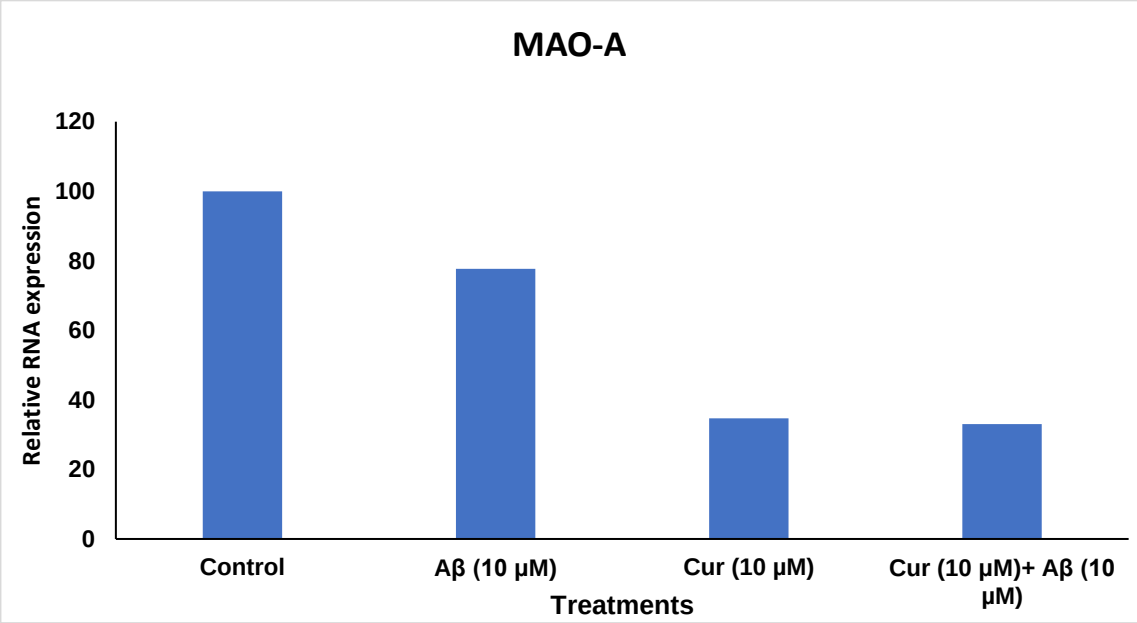
4.2 Measurement of protein concentration by Bradford assay

Bradford protein assay was performed to determine the protein concentration in the U-87 MG cells treated with 10μM Aβ(25-35), 10μM Cur and 10μM Cur+10μM Aβ(25-35). For determining the protein concentration, a standard curve using a known protein viz. BSA was prepared. A stock solution of 0.1 mg/ml BSA was used for making a standard curve at a concentration range of 0-70 μg/mL by plotting graph of absorbance vs. concentration. Upon blue color development, absorbance was read at 595 nm and the concentration of unknown samples was calculated using the standard curve prepared using BSA (**Figure 4.2**). Further, protein concentrations in the total cell lysates (**Table 4.2a**) and mitochondrial lysates (**Table 4.2b**) were determined using the respective standard curve prepared.

4.3 Assessment of relative protein expression of MAO-A/B

Western blot analysis was done to check the protein expression of MAO-A/B in whole cell lysates of various treatment groups, i.e., control, 10μM Aβ(25-35), 10μM Cur and 10μM Cur+10μM Aβ(25-35). It was found that Aβ(25-35) causes 2 fold increase in the protein expression of MAO, whereas Curcumin caused 5 folds increase in its expression. Pre-treatment of Curcumin along with Aβ(25-35) treatment after 3 hr caused 44% decrease in MAO expression as compared to Curcumin treatment (**Figure 4.3 & Table 4.3**).

(a)



(b)

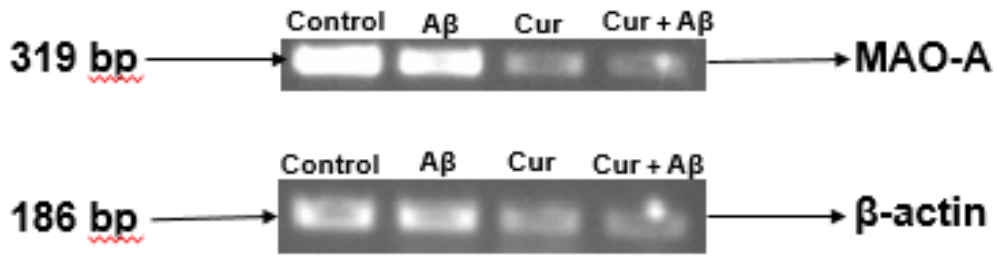


Fig 4.1: (a) Graphical representation of densitometric analysis of mRNA expression of MAO-A in different treatment groups i.e., Control, Aβ (10μM), Cur (10μM), and pretreatment with Cur (10μM) for 3 hr followed by trearment with Aβ (10μM) for 24 hr. (b) Agarose gel image representing the bands obtained in different treatment groups.

Table 4.1: Analysis of mRNA expression pattern of MAO-A in Control, Aβ (10μM), Cur (10μM), and Cur+ Aβ (10μM + 10μM) treated U-87 MG cells

Treatments	**Concentration (μM)**	**Relative mRNA expression (% of control)**
Control	-	100
Aβ(25-35)	10	78
Cur	10	35
Cur+Aβ(25-35)	10+10	33

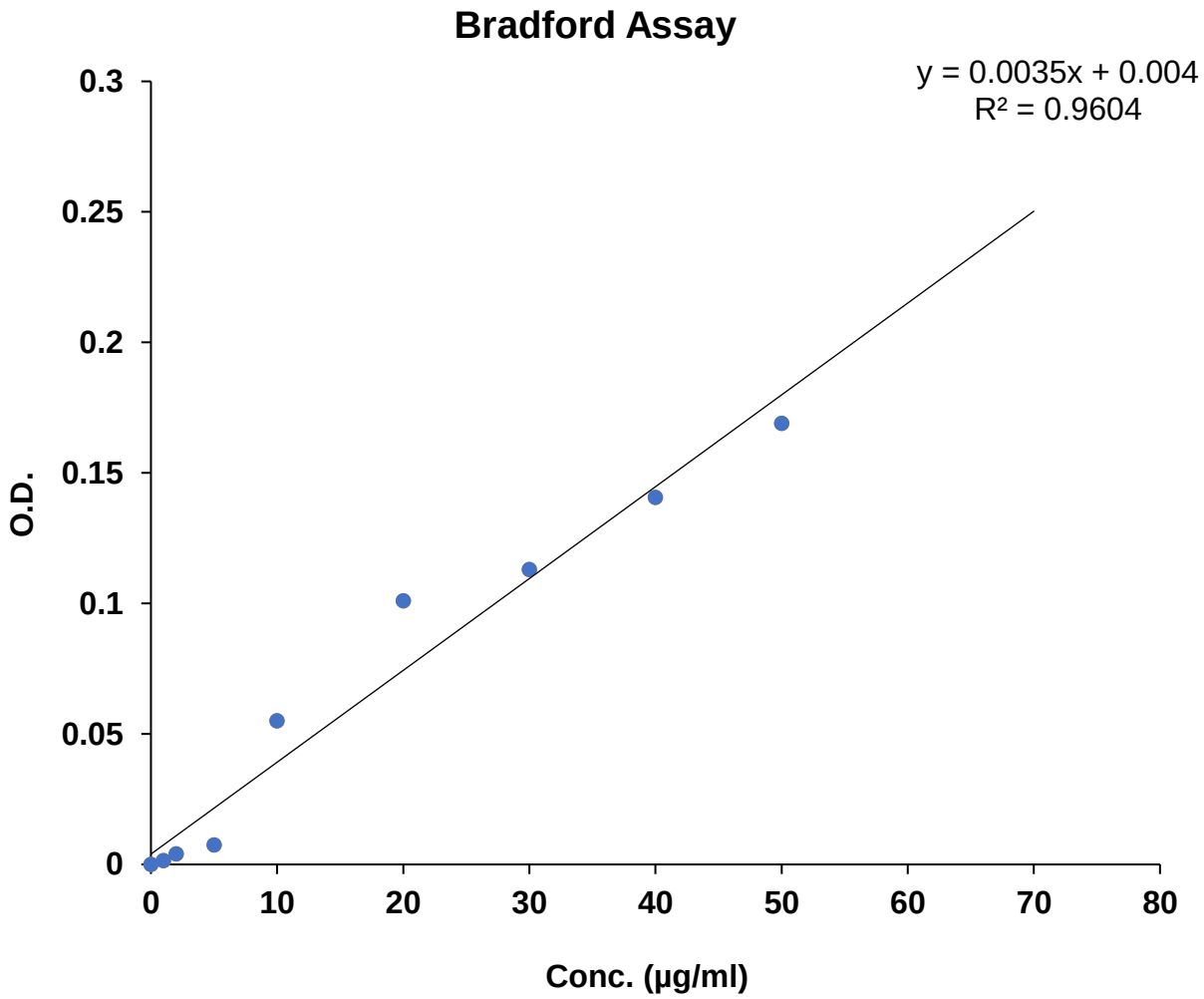


Figure 4.2: Standard curve representing known concentrations of BSA (µg/ml) corresponding to O.D. The concentration of protein in total cell lysates and mitochondrial lysates was determined using Bradford's reagent through standard curve obtained by known concentrations of BSA.

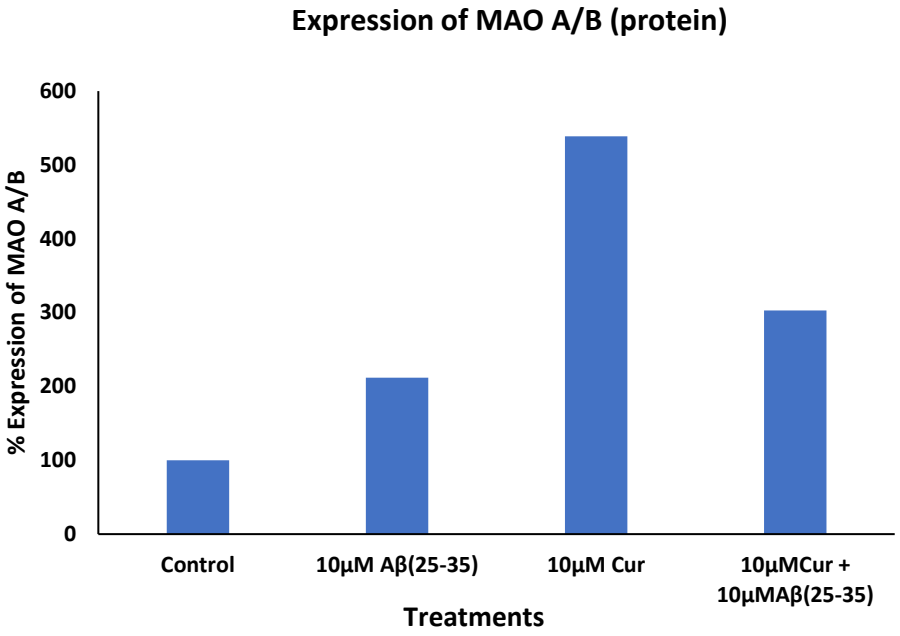
Table 4.2a: Measurement of protein concentration of total cell lysates in different treatments of U-87 MG cells by Bradford assay

Treatments	Conc.(µg/µL)	60 µg protein load (µL)
Control	1.18	50.5
Aβ(25-35)	1.3	45.5
Cur	1.65	36.5
Cur+ Aβ(25-35)	1.36	44

Table 4.2b: Measurement of protein concentration of mitochondrial lysates in different treatments of U-87 MG cells by Bradford assay

Treatments	Conc.(µg/µL)		5 µg protein load (µL)	
	Set 1	**Set 2**	**Set 1**	**Set 2**
Control	8.4	0.3	8.4	13.4
Aβ(25-35)	4.8	0.5	4.8	8.6
Cur	8.9	1.0	4.9	4.5
Cur+ Aβ (25-35)	5.4	0.4	5.4	12.0

(a)



(b)

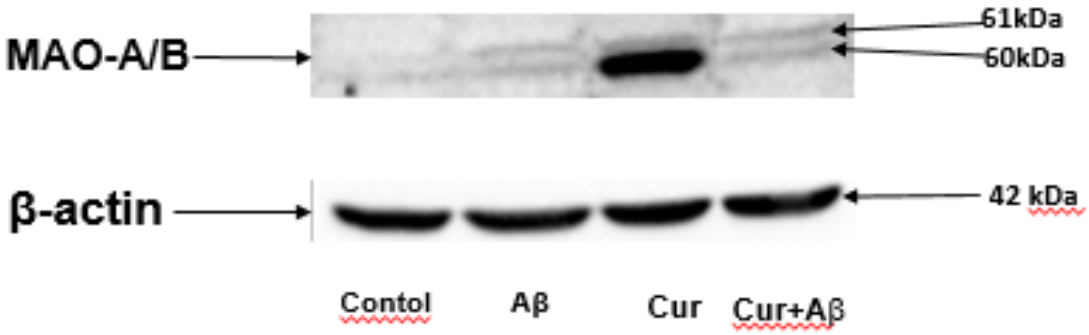


Figure 4.3: (a) Graphical representation of densitometric analysis of protein expression of MAO-A/B in different treatment groups i.e., Control, Aβ (10µM), Cur (10µM), and pretreatment with Cur (10µM) for 3 hr followed by treatment with Aβ (10µM) for 24 hr (b) Chemiluminiscence representing the bands obtained in different treatment groups (n=2).

Table 4.3: Analysis of protein expression pattern of MAO-A in Control, Aβ (10μM), Cur (10μM) and Cur+ Aβ (10μM + 10μM) treated U-87 MG cells

Treatments	Concentration (μM)	Relative protein expression (% of control)
Control	-	100
Aβ(25-35)	10	212
Cur	10	539
Cur+Aβ(25-35)	10+10	303

4.4 Assessment of MAO activity

MAO activity assay involves the formation of a yellow-colored product viz. 4-hydroxyquinoline upon the action of MAO on the substrate kynuramine dihydrobromide. To determine the concentration of 4-hyroxyquinoline, a standard curve was prepared using different concentrations in the range from 2µg/mL to 20µg/mL of Kynurenic acid (**Figure 4.4 & Table 4.4**).

Further, the enzyme activity of MAO was analyzed in mitochondrial lysates of different treatment groups and it was found that Aβ(25-35) treatment increased the activity of MAO by 11% as compared to the control, whereas Curcumin caused 3 fold increase in the activity of MAO as compared to the untreated control. Curcumin pre-treatment followed by Aβ(25-35) treatment after 3hr caused 1.5 folds increase in the enzyme activity of MAO as compared to Aβ(25-35) treated U-87 MG mitochondrial lysates (**Figure 4.5 & Table 4.5**).

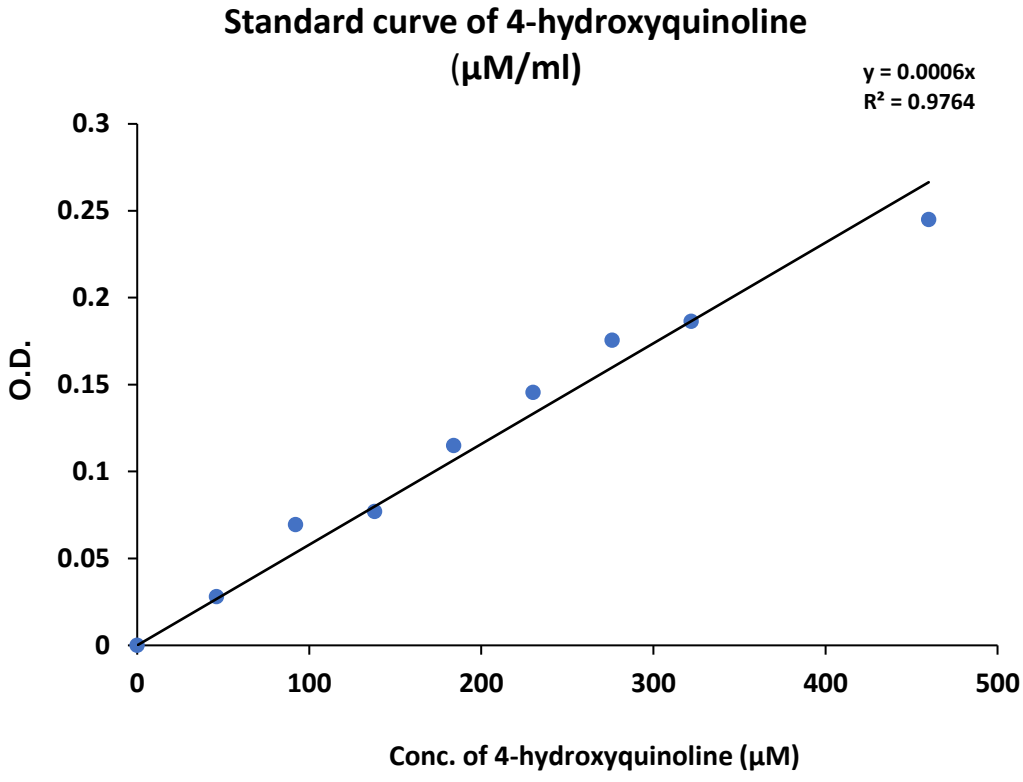
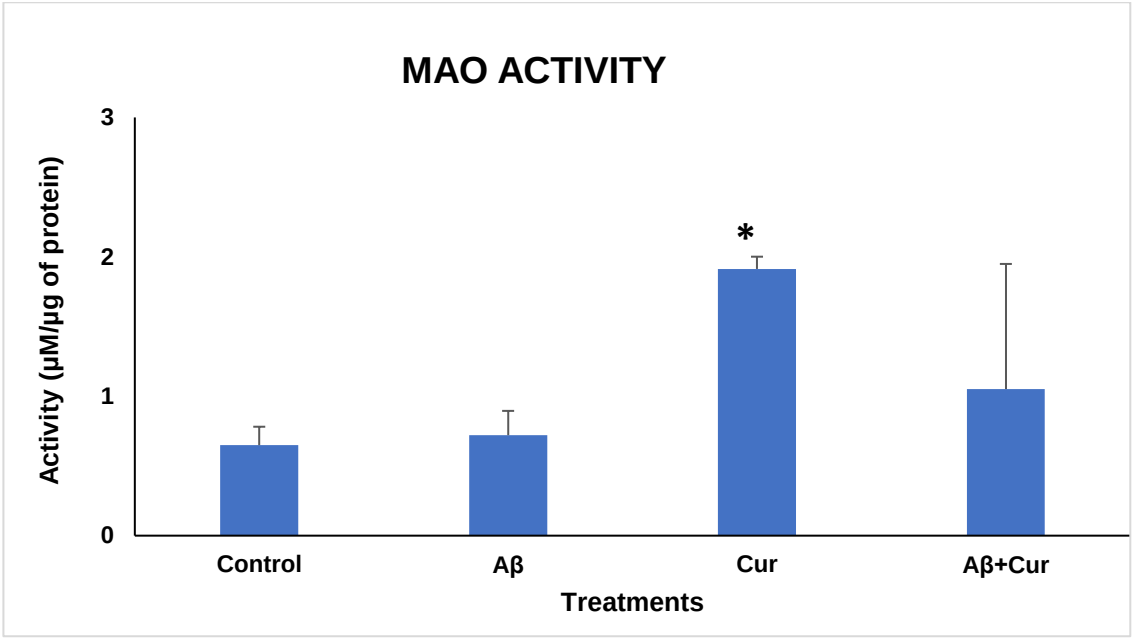


Figure 4.4: The standard curve of 4-hydroxyquinoline (kynurenic acid). The curve was used to calculate conc. of 4-hydroxyquinoline in treatment samples to determine the MAO enzyme activity.

Table 4.4: Working range for 4-hydroxyquinoline standard curve

Conc. of 4-hydroxyquinoline (µg/ml)	**Vol of NaOH (µL)**	**Vol of 4-hydroxyquinoline (µL)**
0	200	0
2	192	8
4	184	16
6	176	24
8	168	32
10	160	40
12	152	48
14	144	56
16	136	64
18	128	72
20	120	80

(a)



(b)

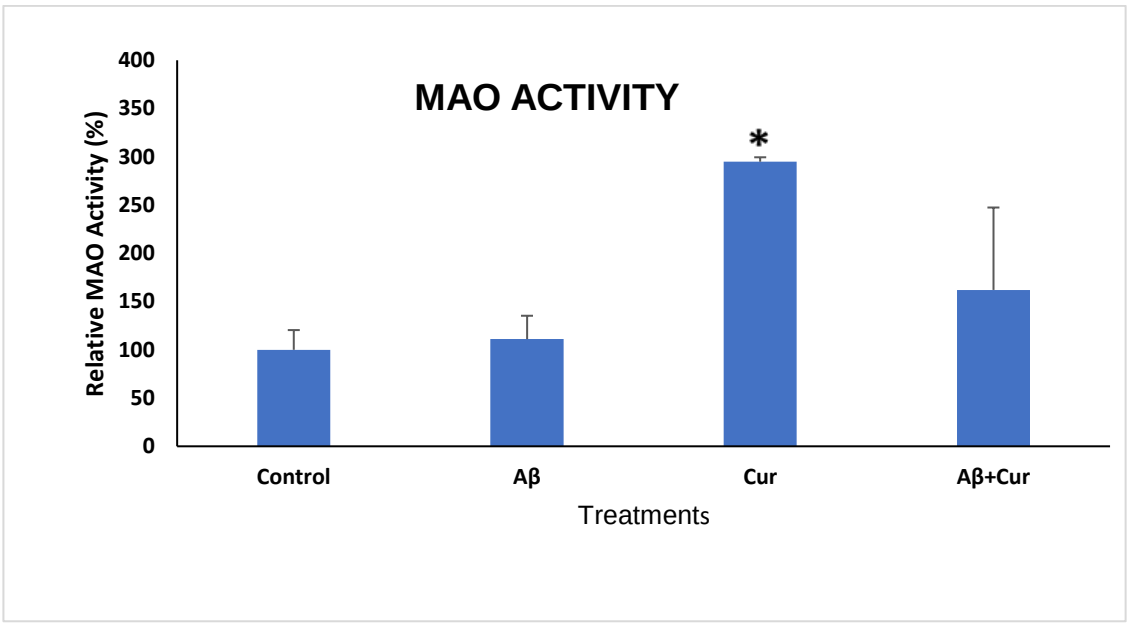


Figure 4.5: (a) Graphical representation of MAO activity in mitochondrial lysates of different treatment groups. (b) Graphical representation of MAO-A/B activity in mitochondrial lysates of different treatment groups expressed as % change as compared with that of control cells (n=3).

Table 4.5: Analysis of activity pattern of MAO in Control, Aβ (10μM), Cur (10μM), and Cur+ Aβ (10μM + 10μM) treated U-87 MG cells

Treatments	Concentration (μM)	Relative MAO Activity (μM/μg)	Relative MAO Activity (% of Control)
Control	-	0.6	100
Aβ(25-35)	10	0.7	111
Cur	10	1.9	295 *
Cur+Aβ(25-35)	10+10	1.0	162

The student t-test was performed to evaluate the significance of results & data was considered statistically significant with $*p \leq 0.05$, when Cur-treated cells were compared with control (n=3).

CHAPTER 5
DISCUSSION

Glioblastoma is the most common form of brain tumor in humans representing 15% of tumors. It develops in astrocytes localized in the frontal and temporal lobes of cerebrum. The average survival of persons diagnosed with GBM is 12-15 months (Kushal *et al.*, 2016). Initial symptoms of it are no specific but as the tumor grows it puts intracranial pressure on surrounding tissues which causes headache, drowsiness nausea and seizures. Various factors are thought to be associated with its progression, oxidative stress being one of them. Oxidative stress plays a major role in its progression through altering several signaling pathways in the cells (Sharma *et al*., 2007).

MAO are the mitochondrial bound enzymes involved in oxidative deamination of endogenous biogenic amine neurotransmitters such as dopamine, serotonin, norepinephrine, and epinephrine. It is a major enzyme that is known to induce ROS production through degradation of neurotransmitters. Oxidative stress is a major player in several metabolic disorders. The inhibition of MAO activity through inhibitors is a key target of research in treatment of various diseases, like cancer and neurodegenerative diseases (Kumar *et al.*, 2018). In the present study, the activity and expression of MAO at the transcriptional and translational level against Aβ-induced oxidative stress and in the presence of the phytochemical Curcumin was evaluated.

Starting with, in this study, Aβ(25-35) was used to induce oxidative stress in the U-87 MG cells. In this regard, several studies have earlier pointed towards the oxidative nature of Aβ (Kanski *et al*., 2002; Villareal *et al*., 2016). Aβ is also known to induce oxidative stress by reducing activities of antioxidant enzymes, causing production of excess ROS and decreasing cell viability (Kanski *et al*., 2002; Villareal *et al*., 2016; Yoon *et al*., 2009). In relation to AD, Aβ(25-35) has been found to act as a toxic peptide relevant to AD pathology (Ferreira *et al*., 2007). In a recent study, Aβ(25-35) was found to induce oxidative stress by increasing ROS production and by reducing activity of mitochondrial complexes in SH-SY5Y cells (Kaur *et al*., 2015). It was also found to decrease the activity of antioxidant enzymes in SH-SY5Y cells (Gill *et al.*, 2017). The intracellular ROS levels and oxidative DNA damage was also found to increase by the treatment of Aβ(25-35) in SH-SY5Y cells (Gill *et* al., 2017). Therefore, Aβ(25-35) was used as an oxidant in this study.

Curcumin was used as an antioxidant in this study. Many studies in the past have proved the antioxidant and anti-inflammatory nature of Curcumin (Ringman *et al*., 2005). It has been also found that it is a good scavenger of free radicals (Zhao *et al*., 1989). According to a study, Curcumin mediated a decrease in ROS levels against Aβ(25-35)-induced oxidative stress in SH-SY5Y cells, suggesting the antioxidant potential of curcumin. It also caused a decrease in the expression of iNOS against Aβ(25-35) mediated oxidative stress in SH-SY5Y cells. Thus, it was suggested that Curcumin alters several pathways through its antioxidant and protective nature (Sarkar *et al*., 2017). In the present study, the role of oxidative stress and antioxidant (Curcumin) in modulating the expression and activity of MAO enzyme was evaluated in human glioblastoma (U-87 MG) cells.

Firstly, the expression of MAO at the transcriptional levels was evaluated in the presence of oxidative stress and Curcumin. It was found that the mRNA expression of MAO was found to be decreased in the presence of Aβ-induced oxidative stress. Also, the mRNA expression in Curcumin treated U-87 MG cells was found to be decreased as compared to Aβ(25-35) treatment, pointing towards the antioxidant role of Curcumin. The pre-treatment of Curcumin for 3 hr, followed by oxidative stress induction by Aβ(25-35) for 24hr showed decrease in mRNA expression of MAO as compared to Aβ(25-35) treatment alone. These results indicate that Curcumin shows protective effect by decreasing the mRNA expression of MAO against oxidative stress conditions by its anti-oxidant nature. According to a study, Curcumin acts as an anti-depressant by inhibiting the enzyme MAO (Kulkarni *et al*., 2008).

Secondly, the protein expression of MAO was determined. It was observed to be increased in Aβ(25-35) treated U-87 MG cells, suggesting that Aβ(25-35) induces oxidative stress in these cells. The pre-treatment of Curcumin for 3 hr and then oxidant treatment of Aβ(25-35) showed a decrease in MAO expression as compared to Curcumin, pointing towards antioxidant and protective nature of curcumin against oxidative stress. Several studies indicate protective and antioxidant properties of curcumin against oxidative stress (Kulkarni *et al*., 2008; Yen *et al*., 2013).

Unlikely, Curcumin treatment led to a decrease in the mRNA expression of MAO, while its protein expression increased. This possibly suggests that MAO

mRNA is being translated at a much higher rate as compared to transcriptional rate and the protein is more stable than mRNA. Similarly, another study also pointed out that some proteins possess different mRNA and protein expression (Guo *et al*., 2008).

Lastly, the activity of MAO enzyme was evaluated. It was found to be increased in Aβ(25-35) treatment. This supported the oxidative nature of Aβ. The Curcumin+Aβ(25-35) treatment caused a further increase in its activity. The antioxidant potential of Curcumin reduces protein oxidation, thus possibly causes an increase in the activity of the enzyme. According to a study, the expression of antioxidant enzymes increase in response to oxidative stress but their activities decreased due to protein oxidation in the presence of oxidative stress conditions (Omar *et al*., 1999).

Surprisingly, a difference in the mRNA expression and protein activity of MAO enzyme upon Curcumin treatment has been observed in our study. The discrepancy between the activity and mRNA expression of MAO could be due to the molecular mechanisms occurring at the nucleic acid level, as was also observed in the study conducted by Ozarowski *et al*., (2013). In that study, it was observed that treatment with Rosmarinic acid (RA), a phenolic acid isolated form the leaves of *Rosmarinus officinalis*, led to a different mRNA expression profile of Acetylcholinesterase (AChE) enzyme and a different AChE activity pattern (Ozarowski *et al.*, 2013). It was also speculated that prevalence of different molecular forms of AChE may be a factor causing this variation. Thus, based on the above study, assessment of the MAO activity and separate mRNA expression analysis results of the different isoforms of MAO (MAO-A and MAO-B) may attribute towards better understanding and insight into the mechanisms leading to this variation.

Taken together, the results of the study show that Aβ(25-35) induces oxidative stress by increasing the expression and activity of MAO. On the other hand, Curcumin reverses the oxidative effects of Aβ(25-35), partly by decreasing the mRNA expression of MAO. Oxidative stress plays a major role in the progression of glioblastoma, so the protective effects of Curcumin may be beneficial by ameliorating the Aβ-induced oxidative stress, which needs further studies toprovide a better understanding of the mechanisms associated in GBM.

Chapter 6

Summary and Conclusion

Glioblastoma (GBM) is an aggressive primary brain tumor, with a survival time of 14 months from its time of diagnosis. Monoamine oxidase (MAO), a mitochondrial-bound enzyme, catalyzes the oxidative deamination of monoamine neurotransmitters such as serotonin, norepinephrine, dopamine. It promotes tumor initiation and progression by causing DNA damage in the cancer cells. Thus, inhibiting MAO may be a putative approach for the treatment of brain tumors. In this regard, the present study was aimed to study the role of oxidative stress and antioxidant in modulating the expression and activity of MAO enzyme in human glioblastoma (U-87 MG) cells. Aβ(25-35) was used to induce oxidative stress to cells while Curcumin was administered as a potent antioxidant. The expression of MAO at transcriptional and translational level; and its activity was evaluated in presence of the phytochemical Curcumin and oxidative stress induced by Aβ in U-87 MG cells in this study.

The results in the present study suggest that Aβ(25-35) induces oxidative stress in these cells and Curcumin shows protective effect against oxidative stress conditions by its anti-oxidant nature. The protein expression of MAO was found to be increased in Aβ(25-35) treated U-87 MG cells, suggesting that Aβ(25-35) induces oxidative stress in these cells. The combined treatment of Curcumin and Aβ(25-35) showed decrease in MAO expression as compared to Curcumin, pointing towards antioxidant and protective nature of curcumin against oxidative stress.

The mRNA expression of MAO was determined in this study to check the effect of the phytochemical treatment in presence of oxidative stress in glioblastoma cells. The results showed that the mRNA expression in Aβ(25-35)-treated U-87 MG cells was found to be decreased, whereas the mRNA expression in Curcumin treated U-87 MG cells was found to be decreased as compared to Aβ(25-35) treatment, pointing towards the antioxidant role of Curcumin. The pre-treatment of Curcumin for 3hr, followed by oxidative stress induction by Aβ(25-35) for 24hr showed decrease in mRNA expression of MAO as compared to Aβ(25-35) treatment alone. These results indicate that Curcumin shows protective effect by decreasing mRNA expression of MAO against oxidative stress conditions by its anti-oxidant nature.

Next, Western blotting was performed to check the protein expression of MAO in these oxidatively stressed U-87 MG cells. The protein expression was found to be increased in Aβ(25-35)-treated U-87 MG cells, suggesting that Aβ(25-35) induced oxidative stress in these cells. The pre-treatment of Curcumin for 3hr and then oxidant treatment of Aβ(25-35) showed decrease in MAO expression as compared to Curcumin, pointing towards antioxidant and protective nature of curcumin against oxidative stress. Several studies indicate protective and antioxidant properties of Curcumin against oxidative stress. The mRNA expression of MAO decreased in only Curcumin treatment, while protein expression increased. This possibly suggests that MAO mRNA is being translated at a much higher rate as compared to transcriptional rate and the protein is more stable than mRNA.

The activity of MAO enzyme was found to be increased in Aβ(25-35) treatment as compared to control. This supported the pro-oxidant nature of Aβ(25-35). The Cur+Aβ treatment caused a further increase in its activity. The antioxidant potential of Curcumin reduces protein oxidation, thus possibly causing an increase in the activity of the MAO enzyme. According to various studies, the expression of antioxidant enzymes increase in response to oxidative stress but their activities decrease due to protein oxidation in the presence of oxidative stress conditions.

In conclusion, the present study showed that Aβ(25-35) induces oxidative stress by increasing the expression and activity of MAO. Curcumin reverses the oxidative effects of Aβ(25-35), partly by decreasing the mRNA expression of MAO. Oxidative stress plays a major role in the progression of glioblastoma, so the protective effects of Curcumin may be beneficial atleast partly by ameliorating oxidative stress, which needs further studies to provide a better understanding of the mechanisms underlined in the tumor microenvironment.

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