Neuroprotective effects of Resveratrol against oxidative stress and memory impairment *in vivo* and *in vitro*

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 6, 2016

Keywords: Resveratrol, Alzheimer's disease, amyloid beta, oxidative stress, diabetes, memory

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Abstract

Resveratrol is a polyphenolic phytoalexin known to exert anti-diabetic, anti-inflammatory and neuroprotective effects. Various studies have reported a link between obese diabetic state and Alzheimer's disease. The present study evaluates the neuroprotective action of resveratrol against oxidative stress and memory associated proteins in obese (*ob/ob*) mice, and Amyloid beta (Aβ) treated H19-7 rat hippocampal cell line. Resveratrol was administered orally at the dose of 25 mg kg-1 body weight daily for 3 weeks to lean, obese (*ob/ob*) mice. Cultured rat hippocampal H19-7 neuronal cell line was pretreated with 75μM of resveratrol for 2 hrs followed by 25μM of Aβ (1-40) for 24 hrs. Resveratrol treatment did not alter body weight or blood glucose levels in *ob/ob* mice. The lipid peroxide levels were significantly increased in the brains of obese mice, and Aβ-treated H19-7 cells. The enzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and non-enzymic antioxidants like tocopherol, ascorbic acid and glutathione were decreased in obese mice brains and Aβ-treated H19-7 cells. Formic acid fractions in the brains of *ob/ob* mice, and Aβ-treated H19-7 cells were found to have increased expression of Tau, phosphorylated forms of tau (CP13, S202/205; PHF1, S396/404) and glial fibrillary acidic protein whereas decreased expression levels of Insulin Degrading Enzyme (IDE), phospho GSK 3β, synaptophysin, PSD-95, and ARC were observed as compared to the control group. Resveratrol treatment attenuated lipid peroxide levels, up-regulated the antioxidant activities, and increased the expression of proteins such as IDE, synaptophysin, PSD-95, ARC, and phospho GSK3β in both obese mice

brains and Aβ- treated H19-7 cells. These findings suggest the neuroprotective effect of resveratrol in attenuating the oxidative damage and memory impairment *in vivo* and *in vitro.*

Acknowledgements

I would like to express my heartfelt gratitude and appreciation to my advisor, Dr. Ramesh B. Jeganathan for his expert guidance, encouragement and scholarly advice throughout my doctoral research. I would also like to thank Dr. Geetha Thangiah for her timely advice, instructive assistance and tremendous help during my research.

I would like to extend my sincere appreciation to my committee members Dr. Suresh Mathews, Dr. Kevin Huggins and Dr. Floyd Woods for their incredible support and guidance throughout my research pursuit. I am thankful to our collaborator Dr. Tom Broderick for his valuable contributions in support of my graduate research.

I especially owe a deep sense of gratitude and gratefulness to my parents for their understanding, unconditional love and endless support throughout my life. I would also like to thank my brother for being a constant source of inspiration and motivation. Special thanks to my fellow lab mates Mr. Chen Zheng, Ms. Sruthi Kumar and Ms. Andrea Carter for their contributions and, especially for being so co-operative and supportive throughout these years. It was always a great pleasure working together.

Last, but not the least, to all faculty members in the Department of Nutrition, Dietetics and Hospitality Management - Thank you for giving me the opportunity of a lifetime…it's been wonderful!

Reference Style

This document is referenced using the citation style of the journal Frontiers in Aging Neuroscience.

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List of Abbreviations

Aβ,	Amyloid beta
AD	Alzheimer's disease
APP	Amyloid precursor protein
AMPK	AMP-activated protein kinase
BBB	Blood Brain Barrier
BDNF	Brain derived-neurotrophic factor
PUFA	Poly unsaturated fatty acids
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Chapter 1: Introduction

Alzheimer's disease (AD) is the most common type of neurodegenerative disorder clinically characterized by a progressive loss of memory, and behavioral abnormalities such as anxiety, depression, aggression, and mood swings (Kolarova et al., 2012; Selkoe, 2001). AD affects rapidly aging population and, has become a serious public health problem, imposing an enormous strain on the social and economic development (Rodríguez-Gómez et al., 2014). Currently, the prevalence of AD in the United States is anticipated to rise from 5.3 million in 2015 to approximately 16 million in 2050 making AD the sixth leading cause of death in the United States and the fifth leading cause of death in Americans aged 65 years or older (Alzheimer's Association, 2015). Amyloid β protein (Aβ) is a peptide (36–43 amino acids), which originates from the proteolytic cleavage of the transmembrane amyloid precursor protein (APP) (Selkoe, 2002). Accumulation of extracellular neuritic plaques containing insoluble deposits of Aβ peptide and intracellular neurofibrillary tangles composed of hyperphosphorylated tau, together contributes towards the pathogenesis of AD (Kolarova et al., 2012; Selkoe, 2002). Several compelling evidences support the idea that Aβ induced neuronal cell death is mediated through the production of reactive oxygen species (ROS) (Butterfield et al., 2007). Enhanced oxidative stress is associated with the early development and progression of AD. Oxidative stress associated with an increase in levels of reactive oxygen species (ROS) is associated with the early development of AD, and further causes vascular and neuronal injury eventually resulting in memory impairment (Jomova et al., 2010; Praticò, 2008). Due to the vast

impact of AD on the quality of life effective therapeutic strategies should be undertaken to prevent the progression of this fatal disease. Although various pharmaceutical drugs such as acetyl cholinesterase inhibitors (tacrine, rivastigmine, galantamine and donepezil) and memantine, an NMDA receptor antagonist have been developed and targeted at slowing or stopping the neuronal dysfunction, none of these medications have been shown to delay or arrest the progression of the disease. Hence, the current treatment strategy lacks efficacy and is still unavailable (Hansen et al., 2008). Therefore, identifying and developing an acceptable, inexpensive, safe and effective non-pharmacological approach to delay or attenuate the progression of AD is currently a challenging issue because of the underlying pathogenesis of this disease. Non-pharmacologic therapies are often used with the goal of maintaining cognitive function, improving quality of life or reducing behavioral symptoms such as depression, mood swings, anxiety and aggression (Zec and Burkett, 2008). Phytochemical compounds with anti-oxidant and neuroprotective properties play a promising role in the treatment of AD by improving endogenous antioxidant defense system as well as cognitive function (Kim et al., 2010). Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene), a naturally occurring polyphenolic phytoalexin found in the skin and seeds of grapes and other plant species, exerts neuroprotective action by readily crossing the Blood Brain Barrier (BBB) (Soleas et al., 2001). Thus, the proposed study will evaluate the neuroprotective effect of resveratrol against oxidative stress and various memory associated proteins in obese *(ob/ob)* mice brains as well as in cell culture model of AD. Moreover, it would offer a natural therapeutic approach in the prevention and/or treatment of this debilitating neurodegenerative disease.

Chapter 2: Review of Literature

2.1 Alzheimer's disease (AD)

2.1.1 Epidemiology

Alzheimer disease (AD) is a chronic neurodegenerative disorder characterized by a gradual decline in cognitive function, which typically begins with memory impairment (Kolarova et al., 2012). Currently, around 33.9 million people have been diagnosed with AD globally and the prevalence of AD in the United States is anticipated to rise from 5.3 million to approximately 16 million in 2050 (Barnes and Yaffe, 2011). AD is considered to be the sixth leading cause of death in the United States and the fifth leading cause of death in Americans aged 65 years or older (Barnes and Yaffe, 2011). Based on its age of onset, AD is classified into early onset AD (EOAD, onset <65 years) accounting for 1–5% of all cases, and late-onset AD (LOAD, onset ≥65 years) accounting for >95% of affected. Approximately 5.1 million people age 65 and older have late onset AD and around 200,000 individuals under age 65 have younger-onset Alzheimer's (Alzheimer's Association, 2015). One in nine people age 65 and older (11 percent) has Alzheimer's disease. About one-third of people age 85 and older (32 percent) have Alzheimer's disease. Eighty-one percent of people who have Alzheimer's disease are age 75 or older (Alzheimer's Association, 2015).

2.1.2 The history and definition of Alzheimer's disease

Alzheimer's disease (AD) was first described in 1906 by Alois Alzheimer, a German neurologist and psychiatrist, and was renamed many years later by Emil Kraepelin (Möller and Graeber, 1998). The disease was initially observed in a 51-year-old woman named Auguste D. After noticing changes in her personality and behavior, she was brought to Dr. Alzheimer by her family in 1901. She had a striking cluster of symptoms that included difficulty speaking, impaired comprehension, and problems with memory (Möller and Graeber, 1998). Dr. Alzheimer later described Auguste as having an aggressive form of dementia, manifesting itself in memory, language and behavioral deficits (Hippius and Neundörfer, 2003). Dr. Alzheimer noted many abnormal symptoms, including difficulty in speech, agitation, disorientation, paranoia and confusion (Hippius and Neundörfer, 2003). Dr. Alzheimer continued to follow her case for five years, until her death in 1906 (Möller and Graeber, 1998). Following post-mortem autopsy of the patient's brain, Alzheimer found a significant reduction in brain mass, especially the hippocampus, as well as he discovered neurofibrillary senile plaques and tangles, which are now have become suggestive features of AD, by histopathological analyses (Möller and Graeber, 1998) (Fig. 1). The condition was first discussed in medical literature in 1907 and named after Alzheimer in 1910 (Hippius and Neundörfer, 2003).

A B

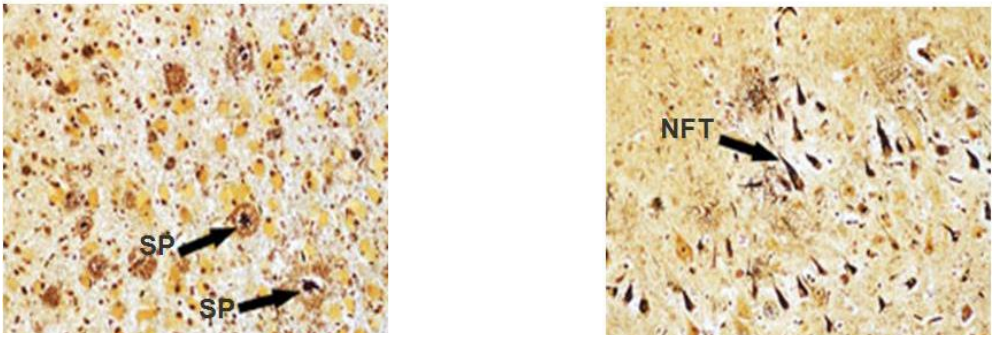


*Figure 1. Alzheimer disease (AD) brain histopathology: Silver stains of A) Senile Plaques (SPs) and B) Neurofibrillary Tangles (NFTs) (*Möller and Graeber, 1998)

Alzheimer's disease is an irreversible, chronic neurodegenerative disorder characterized by neuritic plaques (Vingtdeux et al., 2008), neurofibrillary tangles and synaptic loss together, which leads to a progressive decline in cognitive and behavioral abilities (Kolarova et al., 2012). It is the most common type of dementia that describes a group of symptoms affecting memory, and other cognitive abilities seriously enough to interfere with day-to-day activities (Alzheimer's Association, 2015). It accounts for around 60 to 80 percent of dementia cases (Alzheimer's Association, 2015). The hallmark pathologies of AD are the accumulation of the protein fragment beta-amyloid (plaques) outside neurons in the brain and twisted strands of the hyperphosphorylated protein tau (tangles) inside neurons (Kolarova et al., 2012; Selkoe, 2001) These changes further lead to the damage and death of neurons. Beta-amyloid is a 39–43 amino acid peptide fragment derived from the sequential proteolytic cleavage of APP by the enzymes beta (β) and gamma (γ) secretase (Huang et al., 2011). In 2000, around 25 million people were diagnosed with AD worldwide, and this number is expected to rise to 114 million by 2050 (Wimo et al., 2003).

2.1.3 Classification of AD:

There are three known types of Alzheimer's disease. They include:

Early-onset Alzheimer's: Early age onset AD is a rare form of AD diagnosed in the people younger than 65 years (Zhu et al., 2015). This type of AD is seen in less than 10% of all Alzheimer's disease patients (Zhu et al., 2015). More of the brain abnormalities in younger people who develop Alzheimer's disease are related to it. Early-onset AD is linked with a genetic abnormality on chromosome 14 (Cacace et al., 2016) Early onset Alzheimer's disease (EOAD) accounts for only 1–2 % of all AD cases, and it usually follows an autosomal dominant inheritance pattern where mutations in a single gene can cause the disease (Cacace et al., 2016; Zhu et al., 2015).

Late-onset Alzheimer's. This is the most common form of Alzheimer's disease occurring after the age of 65. Around 90% of cases accounts for this type (Wainaina et al., 2014). Late-onset Alzheimer's also known as sporadic AD affects nearly half of all people above the age of 85 and, occurs due to a complex combination of our genes, our environment, and our lifestyle. Aging is considered to be the greatest risk factor for developing sporadic Alzheimer's disease (Wainaina et al., 2014).

Familial Alzheimer's disease (FAD). This is an extremely rare inherited form of Alzheimer's disease (Borchelt et al., 1996). It accounts for less than 1% of all cases of Alzheimer's disease. In familial AD patients, mutations are seen in the APP, presenilin1 (PSEN1), and presenilin2 (PSEN2) genes (Selkoe et al., 2004). If a person has familial Alzheimer's disease, each of his/her children has a 50% chance of inheriting the disease-causing gene and developing Alzheimer's disease (Borchelt et al., 1996). The key factors contributing to the pathogenesis of both familial and sporadic forms of AD are Aβ peptides (Selkoe, 2001; Selkoe et al., 2004).

2.1.4 AD pathogenesis

Over the past several years, there has been considerable improvement in the understanding of AD pathogenesis, but the exact mechanism of AD is still ambiguous. Various hypothesis related to AD pathogenesis such as genetic mutations and polymorphisms, altered immune or inflammatory responses, oxidative stress, traumatic brain injury, Mild cognitive impairment (MCI), drug interactions have been proposed to address the pathological abnormalities of AD (Dong et al., 2012). Out of which the most important hypothesis are amyloid beta cascade and tau hyperphosphorylation. AD is characterized by two major hallmarks: extracellular accumulation of beta amyloid (Aβ) protein and intracellular accumulation of hyperphosphorylated tau protein (Dong et al., 2012; Kolarova et al., 2012). Abnormal deposition of Hyper-phosphorylated tau protein results in the formation of neurofibrillary tangles whereas insoluble deposits of β-amyloid (Aβ) peptide causes Aβ senile plaques (Golde et al., 1992).

2.1.5 Beta amyloid (Aβ)

Beta-amyloid is a 39–43 amino acid peptide fragment derived from the sequential proteolytic cleavage of Amyloid Precursor Protein (APP), a transmembrane protein, by the enzymes beta (β) and gamma (γ) – secretase (Huang et al., 2011). In most cell types, the parent protein, 695-770 amino acid APP, undergoes the non-amyloidogenic pathway. The non-amyloidogenic pathway further leads to the generation of the P3 peptide fragment, which contains 16 amino acids, and involves β -secretase cleavage followed by a β -secretase cut within the Aβ domain of the APP protein (Selkoe, 2001). The amyloidogenic pathway promotes the release 40-43 amino acid amyloid beta (Aβ) peptide by sequential cleavage of β secretase followed by the ϒ-secretase (Butterfield et al., 2007). Studies have reported that Aβ peptides cause hippocampal long-term potentiation (LTP) impairment, and also could suppress long-term

synaptic plasticity in the hippocampus (Chen et al., 2000). Moreover, it has also shown to induce neurotoxicity in rat hippocampal H19-7 cells, a cellular model of AD (Rege et al., 2015).

2.1.6 Tau

AD is also considered a taupathy due to abnormal aggregation of the tau protein. Each neuron is composed of a cytoskeleton partly made up of structures called microtubules (Medeiros et al., 2011). These microtubules act as neuronal transport system-guiding nutrients to the axons and back. Microtubules play an important role in various cellular functions, including mitosis, intracellular transport, and maintenance of cell shape (Hernández and Avila, 2007). Tau protein also known as a microtubule-associated protein stabilizes the microtubules when phosphorylated. In AD, tau becomes hyper phosphorylated upon certain chemical changes that take place and begins to aggregate as pairs of twisted filaments around one another (paired helical filaments) forming neurofibrillary tangles. This further disintegrates the neuron's transport system (Hernández and Avila, 2007).

As the disease progresses, symptoms such as language problems, difficulty completing familiar tasks at home, at work or at leisure, disorientation, confusion with time or place, difficulty in speaking or writing, mood swings and other behavioral abnormalities are observed (Alzheimer’s Association, 2015).

2.2 Oxidative stress

Oxidative stress occurs due to an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defenses to counteract the resulting damage (Jomova et al., 2010). Reactive oxygen species (ROS) or oxygen free radicals and reactive nitrogen species (RNS) are chemically reactive molecules that are produced in our body by various endogenous systems. ROS/RNS have both beneficial as well as deleterious effects on the biological systems. In

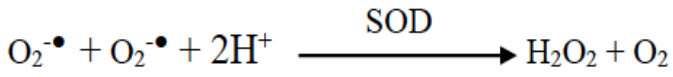
general, the majority of ROS found in the cell results from electron leakage from the ETC within the mitochondria, which leads to production of the superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) by partial reduction of molecular O_2 (Adam-Vizi, 2005). Furthermore, H_2O_2 gets converted to even more reactive hydroxyl radical ($OH^{\bullet}$), in the presence of transition metal ions (Thannickal and Fanburg, 2000). In the brain, levels of oxidative stress have been shown to increase with age, inducing protein and nucleic acid oxidation, lipid peroxidation, and apoptosis, leading to loss of synapses and neurons, and declining cognition (Praticò, 2008). Brain tissue is more prone to oxidative stress due to its higher oxygen rate consumption, increased peroxidizable fatty acids content, less regenerative capability, and minimal amounts of antioxidants. Thus, free radicals play a key role in the process of brain aging (Floyd, 1999; Honda et al., 2004). Elevated levels of ROS/RNS lead to the process of aging and various chronic diseases including neurodegenerative diseases (Jomova et al., 2010), cardiovascular diseases (Tsai et al., 2011), diabetes (Karunakaran and Park, 2013) and cancer (McCarty et al., 2010).

2.3 Antioxidants and Alzheimer's disease

The human body consists of enzymatic antioxidant defense system that includes superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) (Góth, 1991, 1991999). Enzymatic antioxidants catalyze reactions to block the formation of free radicals. The non-enzymatic anti-oxidant defense system involves ascorbic acid (vitamin C), α-tocopherol (α – TOH, vitamin E), glutathione (GSH), β-carotene and vitamin A, which react with activated oxygen species and are mainly involved in scavenging free radicals to reduce cellular damage (Naidu, 2003)

2.3.1 Enzymatic antioxidants

1) Superoxide Dismutases (SOD): SOD catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), and is an important antioxidant defense in nearly all living cells exposed to oxygen (Matés et al., 1999)



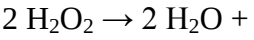
$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

H_2O_2 is also generated by direct reduction of O_2 in the peroxisomes, which is then converted to $OH^\bullet$ via the Fenton and Haber-Weiss reactions (Matés et al., 1999).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^-$$

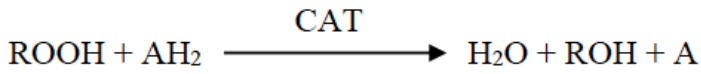
$$O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + OH^\bullet + OH^-$$

2) Catalase: It catalyzes the decomposition of hydrogen peroxide to water and oxygen



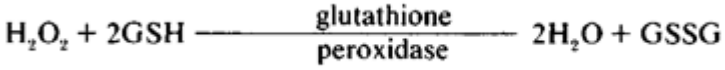
$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase is also capable of peroxidative activity, where low molecular weight alcohols serve as electron donors (Góth, 1991)



$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

3) Glutathione peroxidase (GPx): GPx-1 is a selenoprotein belonging to a family of peroxidases that scavenges and inactivates peroxides and hydroperoxides, thereby protecting the body against oxidative damage. GPx is a major intracellular antioxidant enzyme that is found in the cytoplasm and mitochondria of all cell types (Sunde and Hoekstra, 1980)



$$H_2O_2 + 2GSH \xrightarrow[\text{peroxidase}]{\text{glutathione}} 2H_2O + GSSG$$

4) Glutathione Reductase (GR): Glutathione reductase is an antioxidant enzyme found in the human cells and belongs to the larger family of flavoezymes, and converts oxidized glutathione (GSSG) to two molecules of reduced glutathione (GSH) (Masella et al., 2005) GSH is a direct scavenger of hydroxyl radicals and singlet oxygens, and acts as a cofactor in several detoxifying enzymes (Moron et al., 1979) It is readily involved in amino acid transport through the plasma membrane. It is capable of regenerating important antioxidants such as Vitamins E and C to their reactive forms. The redox state of GSSG/2GSH inside the cell determines the antioxidant capacity of Glutathione (GSH) (Masella et al., 2005)

$$GSSG + NADPH + H^{+} \xrightarrow[\text{reductase}]{\text{glutathione}} 2GSH + NADP$$

5) Glucose 6-phosphate dehydrogenase (G6PD): Glucose-6-phosphate dehydrogenase is a major antioxidant enzyme present in all types of cells, involved in the normal processing of carbohydrates. It is known for its role in ribose 5-phosphate production and the generation of NADPH, in the hexose monophosphate pathway. G6PD protects cells from reactive oxygen species and all the NADPH reactions produced prevent ROS build up to in the cells (Efferth et al., 2006). It plays a key role in red blood cells by protecting them from oxidative damage and premature destruction. Deficiency of functional glucose-6-phosphate dehydrogenase leads to increased susceptibility of red blood cells to toxic effects of ROS and contributes to rupture and premature break down of RBC''s (undergo hemolysis). This loss of red blood cells eventually causes the signs and symptoms of hemolytic anemia, a characteristic feature of glucose-6-phosphate dehydrogenase deficiency (Mehta et al., 2000).

$$G\text{-}6\text{-}P + NADP \xrightarrow[\text{dehydrogenase}]{\text{G-6-P}} 6\text{-}PG + NADPH + H^{+}.$$

2.3.2 Non- Enzymatic Antioxidants

The non-enzymatic anti-oxidant defense system involves ascorbic acid (vitamin C), α-tocopherol (α –TOH, vitamin E), glutathione (GSH), β-carotene and vitamin A (Matés et al., 1999), that react with activated oxygen species and are mainly involved in scavenging free radicals to reduce cellular damage.

1) Vitamin C: Vitamin C or L ascorbate is a water-soluble vitamin widely found in plants and is required for normal growth and development. Vitamin C is essential for maintaining healthy skin, tendons, ligaments, and blood vessels. It helps in the repair and maintenance of cartilage, bones, and teeth, promotes wound healing, forms scar tissue and facilitates iron absorption (Bsoul and Terezhalmy, 2004).

Vitamin C occurs in two forms L-ascorbic acid (AA) and dehydroascorbic acid (DHAA). AA is the most active form and DHAA is the oxidized form. AA is readily oxidized to DHAA, and thereby protects other compounds from oxidation. DHAA can also be easily converted (reduced) back into AA (Naidu, 2003). Ascorbate when reacts with a hydroxyl radical, results in the formation of semi dehydroascorbate radical and a water molecule. Two semidehydroascorbate radicals further combine to generate a molecule of ascorbate and dehydroascorbate. Dehydroascorbate breaks down further into the following products:

Ascorbate + $OH\cdot$ → Semidehydroascorbate radical + H_2O

2 Semidehydroascorbate → Ascorbate + Dehydroascorbate

Dehydroascorbate → Oxalate, threonate, etc.

2) Vitamin E: Vitamin E is termed as a family of eight fat-soluble molecules with antioxidant activities: four tocopherol isoforms (α-, β-, γ-, and δ-tocopherol) and four tocotrienol isoforms (α-, β-, γ-, and δ-tocotrienol. α-tocopherol, is the only form readily used by

human body. In humans, α-tocopherol functions as a fat-soluble antioxidant (Shaikh et al., 2015). α-Tocopherol blocks the formation of peroxyl radicals and thus prevents a chain reaction of lipid oxidation. A molecule of α-tocopherol neutralizes a free radical, gets oxidized and loses its antioxidant capacity. Other antioxidants, such as vitamin C, facilitate the antioxidant capacity of α-tocopherol. α-tocopherol protects the fats in low-density lipoproteins (LDLs) from oxidation and maintains the integrity of cell membranes in humans (Shaikh et al., 2015).

3) Glutathione: GSH is an endogenous nonenzymatic antioxidant against reactive oxygen species in the cellular defense system. GSH is oxidized to glutathione disulfide (GSSG) by reactive oxygen species, thereby reducing the level of GSH. Glutathione reductase (GR) converts GSSG back to GSH by NADPH, which in turn is released by glucose-6-phosphate dehydrogenase (G6PDH) (A Meister and Anderson, 1983).

2.4 Lipid Peroxidation

Membrane lipids are prone to damage by free radicals, initiating a self-propagating chain reaction called lipid peroxidation (LP) (Mylonas and Kouretas, 1999). Free radical attack initiates a peroxidative sequence by removal of a hydrogen atom from a methylene group (CH2), leaving behind an unpaired electron on the carbon atom (•CH). The free radical then undergoes oxidation to form peroxyl radical, which further reacts with other PUFAs producing lipid hydroperoxide and another lipid free radical (Grotto et al., 2009; Mylonas and Kouretas, 1999) (Figure 2).

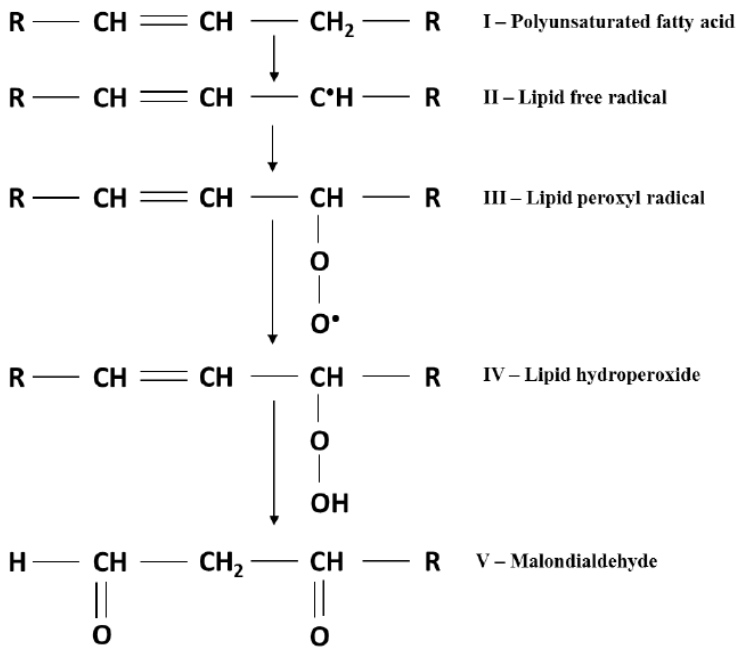


Figure 2. Schematic steps of MDA formation from polyunsaturated fatty acids (Grotto 2009)

Lipid hydroperoxide formed is unstable and is converted to malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are toxic to the cells and can potentially cause DNA damage (Grotto et al., 2009).

Antioxidant such as α-tocopherol is capable of terminating lipid peroxidation chain reactions by generating a more stable tocopherol phenoxyl radical that is not involved in further chain reactions. Other antioxidants such as vitamin C and GSH further recycle tocopherol phenoxyl radical (Devasagayam et al., 2004).

2.5 Current treatments for AD

Currently, there is no cure for Alzheimer's disease but researchers are looking for new ways to alter the course of Alzheimer's and improve the quality of life. Current drug treatments help to improve the symptoms of Alzheimers, but do not completely stop the disease progression or treat the underlying disease (Rodríguez-Gómez et al., 2014). Several drugs are in the final stages of developing and testing, and may be available for clinical trials soon. But only a few drugs are available currently as it costs upto $300,000,000 and takes around 12-25 years to develop and test before the drug reaches the market (Hansen et al., 2008). Currently, five drugs approved by the U.S. Food and Drug Administration (FDA) that temporarily ameliorates symptoms of Alzheimer's disease by elevating the levels of chemicals called neurotransmitters in the brain are cholinergic drugs such as tacrine (brand name Cognex), donepezil (brand name Aricept), rivastigmine (brand name Exelon) and galantamine (brand name Reminyl) and memantine, an NMDA receptor antagonist. But the drug effectiveness varies from individual to individual (Hansen et al., 2008). However, none of the treatments available so far slows or stops the disease progression and therefore makes the disease fatal.

Several factors such as the high cost of drug development, duration of drug development and testing, side effects of the drugs cause hindrance in the development of effective treatment strategy for Alzheimer's (Alzheimer's Association, 2015). Therefore, identifying and developing an acceptable, safe, inexpensive and effective non-pharmacological approach to delay or attenuate the progression of AD is somewhat a challenging task due to the underlying pathogenesis of this disease.

Non-pharmacologic therapies are often aimed at maintaining cognitive function, improving quality of life or reducing behavioral symptoms such as depression, mood swings, anxiety and aggression (Zec and Burkett, 2008). Phytochemical compounds with anti-oxidant and neuroprotective properties play a promising role in the treatment of AD by improving endogenous antioxidant defense system as well as cognitive function (Kim et al., 2010).

Phytochemicals are a large group of plant-derived bioactive compounds found in fruits, vegetables, grains, and other plant foods, classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Arts and Hollman, 2005).

2.6 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a naturally occurring polyphenolic compound, which belongs to the phytoalexin superfamily. This compound was first isolated from the roots of white hellebore (*Veratrum grandiflorum O. LOES*) and was named by Dr. Michio Takaoka in his thesis in 1940. The discovery of resveratrol by Dr. Takaoka was the prime step leading to establishing the scientific efficacy of the Chinese "material medica," a collection of traditional Asian medicines (Takaoka, 1940). In 1963, resveratrol was isolated from the roots of *Polygonum cuspidatum*, a traditional Chinese and Japanese medicine Ko-jo-kon (NONOMURA et al., 1963). Resveratrol is present in skin and seeds of more than 70 different plant species, including grapes, berries, grains, tea, and peanuts (Chen et al., 2000; Soleas et al., 1997). In the presence of an enzyme resveratrol synthase, the phytochemical resveratrol is synthesized in response to environmental stress such as heavy metal ions, injury, fungal infection, or UV irradiation from coumaroyl CoA and malonyl CoA (Singh et al., 2013). It is synthesized in the pericarp of grape berries, epidermis of grape berry leaf, and in the stalks and kernels of the berries (Creasy and Coffee,, 1988). It constitutes one of the primary components in red wine and is claimed to be an

essential factor in the French Paradox, a term frequently used to summarize the apparently paradoxical epidemiological observation that French people have a relatively low incidence of CHD despite having a diet relatively rich in saturated fats (Liu et al., 2007; Sun et al., 2008). The level of resveratrol in plants reaches its peak approximately 24 h after stress exposure and subsides after 42–72 h due to the activation of stilbene oxidase (Jeandet et al., 2002; Soleas et al., 2001). Resveratrol belongs to a group of compounds called the stilbene family, which contain two aromatic rings joined by a methylene bridge. Stilbene synthase (STS), which belongs to a multigene family of the type 3 group of the polyketide synthase superfamily, is the enzyme that controls the production of resveratrol in plant tissues (Bais et al., 2000). Resveratrol exists in two geometric isomers with trans and cis configuration (Figure 3). Trans-resveratrol is considered to be a non-toxic potential stereoisomer and is widely known to possess the reported beneficial health effects (Orallo, 2006).

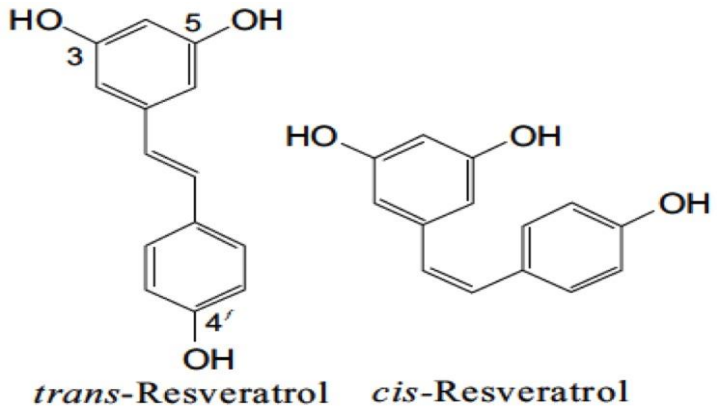


Figure 3: Isomers of Resveratrol: Chemical structures of trans and cis-resveratrol.

Indeed, resveratrol is also protective against oxidative stress, inflammation (Das and Das, 2007), and the development of cardiovascular diseases (Chen and Zhong, 2013), diabetes (Venturini et al., 2010), neurodegenerative diseases (Vingtdeux et al., 2008), and cancer (Kris-Etherton et al., 2002). Resveratrol plays a prominent role in the prevention of neurodegenerative diseases such

as AD, Parkinson's disease, cerebral ischemia as well as Huntington's disease because resveratrol enters the blood stream after the formation of glucuronide conjugates and can readily pass through the BBB; (Baur and Sinclair, 2006). Resveratrol (10–100 μM) is reported to exert neuroprotective effects in several studies (Richard et al., 2011). In this review, we discuss the several beneficial effects of resveratrol and newly designed resveratrol analogs in AD and its potential to promote human health.

2.6.1 Metabolism and bioavailability of resveratrol

Resveratrol is rapidly absorbed and metabolized on oral administration to form glucuronide and sulfate conjugates, which are excreted in urine (Vingtdeux et al., 2008). In humans, the primary metabolite of resveratrol is trans-resveratrol-3-O-glucuronide, whereas in mice and rats, trans-resveratrol-3-O glucuronide and trans-resveratrol-3-sulfate are the primary metabolites, respectively (Yu et al., 2002). Several studies conducted on the bioavailability of resveratrol indicate that poor absorption and rapid metabolism of resveratrol and its metabolites like glucuronides and sulfates results in low oral bioavailability of resveratrol (Wenzel and Somoza, 2005). Studies by (Walle, 2011) have shown the oral absorption rate of resveratrol to be 70–75% with respect to the urinary excretion of the total metabolites after radiolabelled doses. Plasma concentrations of resveratrol ranged from 1 to 5 ng/ml on administration of 25 mg resveratrol (Almeida et al., 2009), a concentration commonly used in experimental studies and associated with various beneficial effects on cardiovascular, endothelial, and neurologic function (Francis et al., 2009; Rege et al., 2013). Administration of higher doses up to 5 g led to a proportional increase in the plasma resveratrol to about 500 ng/ml (Boocock et al., 2007). Despite its poor bioavailability and rapid disposal, resveratrol does indeed accumulate in tissues, including brain, even after acute or short-term treatment. Acute administration of resveratrol by

oral gavage using a low dose of 80 μg/kg results in significant accumulation of resveratrol in brain within 4 h (Bertelli et al., 1999). Short term treatment using a concentration of 40 μg/kg by the same route of administration for a period of 15 days also increases resveratrol content in the brain (Bertelli et al., 1999). Resveratrol is known to have several beneficial effects in brain but its poor bioavailability or the matrix within which it is contained in the dietary media are issues of major concern for resveratrol delivery (Goldberg et al., 2003; Mohar and Malik, 2012).

2.6.2 Bioavailability of resveratrol and its analogs

The BBB is a highly selective permeable endothelial cell layer connected by tight junctions, sequestering the CNS tissue from vasculature. This barrier is permeable to the flow of water, some gases, nutrients, and lipid soluble molecules through passive diffusion. In addition, the BBB prevents entry of many neurotoxins by P-glycoprotein-mediated active transport. Polyphenols are accessible and enter the brain only if they cross the BBB (Vauzour, 2012). Based on in vitro studies, the permeability of polyphenols through the BBB depends on several factors such as the lipophilic state of the compound and increased capability of brain uptake by less polar polyphenols or metabolites (such as O-methylated derivatives; (Youdim et al., 2003). Resveratrol being a lipophilic compound can readily cross the BBB via transmembrane diffusion (Lin et al., 2010). Further, to effectively penetrate the BBB, molecules must be below 500 Da in molecular weight (Banks, 2009). Resveratrol, with its molecular weight of 228 Da (Amri et al., 2012) and lipid soluble properties, should easily cross the BBB. (Faria et al., 2010) demonstrated that similar sized flavonoids found in red wine such as quercetin and catechin can easily penetrate membranes in RBE-4 cells, an immortalized cell line of rat cerebral capillary endothelial cells.

However, resveratrol's low bioavailability originating from its poor water solubility and resulting from its short biological half-life, labile properties, rapid metabolism and clearance limits the efficacious concentrations of resveratrol to accumulate in plasma and target tissues (Cho et al., 2014; Walle, 2011). Therefore, several drug delivery systems are designed to improve these inherent biologic limitations of resveratrol, such as increasing its solubility and preventing resveratrol from rapid degradation while preserving its biological activity. Approaches aimed at controlling its release from the gastrointestinal tract to enhance its bioavailability are also considered (Augustin et al., 2013; Sessa et al., 2011). At present, several drug delivery systems for enhancing the bioavailability and solubility of resveratrol have been developed such as encapsulation in liposomal formulations, the design of resveratrol–protein complexes to favor resveratrol binding to protein, use of cyclodextrin complexes and solid lipid nanoparticles for enhanced matrix-based delivery, pectinate delivery systems, and chitosan microspheres (Augustin et al., 2013). In agreement with these novel delivery systems, recent evidence has shown that administration of 5 mg/kg of resveratrol in loaded-lipid core nanocapsules every 12 h intraperitoneally for 14 days is effective against the neurotoxicity induced by intracerebroventricular injection of Aβ1–42 in rats (Frozza et al., 2013). Also, the use of more potent analogs of resveratrol such as SRT501 (Howells et al., 2011) and resveratrol in combination therapy with piperine, a natural product obtained from black pepper, have proved to be efficient methods of enhancing its bioavailability (Johnson et al., 2011).

Recently, Csiszár et al. reported that encapsulation of resveratrol into novel fusogenic liposomes is more efficient than conventional liposomes. This approach enhances the delivery of polyphenol resveratrol into aged cells leading to the activation of cellular Nrf2-mediated antioxidant defense systems (Csiszár et al., 2014). Furthermore, the use of polyethylene glycol

(PEG) derivatives presents several advantageous features for delivery. PEG as an oral vehicle material is a non-toxic polymer, has high water solubility, is both non-teratogenic and non-immunogenic, and exhibits antigenic properties. Two types of amino acid PEGylated resveratrol conjugates developed to date demonstrate increased solubility of resveratrol up to 900 mg mL-1, clearly highlighting the potential of PEGylated compounds as an effective system for improving the solubility and bioavailability of resveratrol (Zhang et al., 2014).

Resveratrol exhibits good absorption rates but low bioavailability. An oral dose of 25 mg results in less than 5 μg/mL in the serum following absorption through the gastrointestinal tract, corresponding to approximately a 1000-fold decrease in bioavailability. A 125-fold lower dose of 0.2 mg, yet in the milligram amount, injected intravenously results in plasma levels in the low nanogram range (16.4–30.7 ng/mL) (Walle et al., 2004). This rapid clearance is due to the reduction in the amount of free resveratrol from conjugation by sulfation and glucuronidation via P 450 enzymes. Albumin and lipoproteins serve as the major carriers for resveratrol in plasma (Delmas et al., 2011). After ingestion of resveratrol, conjugation produces resveratrol-O-glucuronide and resveratrol-C-glucuronide (Cottart et al., 2010). More than 90% of free resveratrol is bound to human plasma lipoproteins, and 50% of the plasma trans-resveratrol-3-sulfate, trans-resveratrol-disulfates and the novel trans-resveratrol-C/O-diglucuronides are non-covalently bound to proteins as reported by (Burkon and Somoza, 2008). Evidence shows that resveratrol undergoes enterohepatic metabolism in both rats and humans (Timmers et al., 2012). After uptake by enterocytes, resveratrol is metabolized to sulfate and glucuronide conjugates, which may be deconjugated by gut microflora, reabsorbed, or excreted in the feces. Thus, the enterohepatic circulation decreases the amount of free compound reaching the target tissues. Hence, a small fraction of the ingested resveratrol reaches the tissues (Timmers et al., 2012). In a

brain ischemic model, results suggest that resveratrol enters the blood stream after its formation to glucuronide conjugates and could thereby cross the BBB (Wang et al., 2002). To overcome the potential limitations of low bioavailability and metabolism, a therapeutic approach in developing congeners and analogs of resveratrol should be undertaken. Modification of resveratrol's chemical structure by altering the number and position of the hydroxyl groups, intramolecular hydrogen bonding, double bonds, and stereoisomerism is crucial for improving the efficacy and enhancing the bioavailability. Stilbene monomers include methyl and methoxy group subsitutions, and variations in cis and trans configurations (Cottart et al., 2010). Systemic exposure to pterostilbene, a dimethyl derivative of resveratrol, resulted in significantly higher plasma levels when compared with resveratrol following administration at equimolar doses in male rats over 14 consecutive days. Treatment with pterostilbene also produced a sevenfold rise in its oral bioavailability than the parent resveratrol (Kapetanovic et al., 2011). A recent study by (Greer et al., 2014) aimed at improving the bioavailability of trans resveratrol (tRes) by modifying its structure to reduce glucuronidation revealed three new stilbene derivatives. These derivatives of tRes with hydroxyl, aromatic nitro and carboxyl substituents to create NI-ST-05, DNR-1, and NI-12a, respectively, were less prone to glucuronidation, suggesting that tRes analogs improve bioavailability and could be potentially developed as alternate therapeutics (Greer et al., 2014). Several other resveratrol analogs such as hexahydroxystilbene (M8), galic acid, digalloyl resveratrol exert free radical scavenging properties and anti-carcinogenic effects (Szekeres et al., 2010). Another naturally occurring resveratrol analog, 3,5,4′-trimethoxy-trans stilbene, had greater plasma exposure, a longer half-life and lower clearance rates in rats (Lin and Ho, 2009). Several studies have indicated resveratrol to be a potent activator of SIRT 1. Sirtuins are NAD+-dependent class III histone/protein deacetylase (HDAC) enzymes. SIRT 1

deacetylates nucleosomal histones at specific residues by translocating from cytoplasm to nucleus and contributes to transcriptional silencing of telomeres and life span expansion (Pallàs et al., 2013). Recent studies demonstrate that both natural and synthetic sirtuin activating compounds (STACs) promote allosteric SIRT 1 activation by binding of STACs to a conserved N-terminal domain in SIRT1. Recently sirtuins have gained considerable importance due to its key role in the calorie restriction (CR) response and as possible therapeutic drug targets. Amongst all the naturally occurring activators of SIRT 1, resveratrol is considered to be the most effective SIRT 1 activator. However, synthetic STACs have been documented to possess more potency, solubility and bioavailability as compared to natural STACs. The first synthetic STACs such as SRT1460, SRT1720, and SRT2183 were derivatives of an imidazothiazole scaffold and chemically different from the polyphenol resveratrol. Like resveratrol, SRT1720 compound was shown to activate SIRT1 by lowering the Km for the substrate peptide. The third generation STACs derived from benzimidazole and urea-based scaffolds were more potent than resveratrol itself (Hubbard and Sinclair, 2014).

2.6.3 Resveratrol analogs in the treatment of AD

Currently, several studies have reported various polyphenols exhibiting neuroprotective effects both in vivo and in vitro. Resveratrol and its derivatives have gained a prime importance amongst all these polyphenols due to their neuroprotective properties.

Piceatannol, a monohydroxylated derivative of resveratrol that differs by an additional hydroxyl group in 3′ of benzene ring, has shown to exhibit neuroprotective effects against beta-amyloid induced neural cell death by blocking Aβ-induced accumulation of ROS (Kim et al., 2007b). Pterostilbene has shown to be a potent modulator of cognition and cellular oxidative stress associated with AD (Chang et al., 2012). In addition to monomers, several dimers and oligomers

have been developed. Two new stilbene dimers, scirpusin A with an additional hydroxyl group, and ε-viniferin glucoside, with a glucose moiety, demonstrated a robust inhibition of fibril accumulation, thereby could be used as efficient fibril inhibitors in the treatment of AD (Rivière et al., 2010).

Lu and colleagues designed a novel series of resveratrol derivatives serving as multi-target agents in the treatment of AD. Amongst the synthesized compounds, 5d (E)-2-((4-(3,5-Dimethoxystyryl) phenylamino) methyl)-4-(dimethylamino) phenol and 10d (E)-5-(4-(5-(Dimethylamino)-2-hydroxybenzylamino) styryl)-benzene-1, 3-diol exerted significant inhibition of Aβ aggregation, metal-chelating ability, disintegration of highly structured Aβ fibrils and Cu (II)-induced Aβ aggregation, antioxidant activity and low neurotoxicity. Moreover, compound 5d could also cross the BBB in vitro and doses up to 2000 mg/kg were not associated with any signs of toxicity in mice (Lu et al., 2013). Lu and colleagues previously reported a series of stilbene derivatives based on the structure of resveratrol in which compound 7l (E)-5-(4-(isopropylamino)styryl)benzene-1-3-diol exerted potent β-amyloid aggregation inhibition activity (Lu et al., 2012). Novel synthetic compounds such as STACs confer remarkable health benefits in various animal models. SRT3025 is one such STAC, which penetrates the BBB; mimics the effects of CR on the brain and further reduces neurodegeneration (Hubbard and Sinclair, 2014). In conclusion, various resveratrol analogs developed with improved bioavailability possess neuroprotective properties and could be further used as novel multifunctional drugs in the treatment of AD.

2.7 Resveratrol content in wine and plants

Resveratrol occurs as free resveratrol and as 3 β-glucoside, a derivative of resveratrol both in grapes and wine (Vrhovšek et al., 1995). Concentrations of resveratrol in grape species range from 50 to 400 μg/g fresh weight in the leaves and fresh grape skin contains around 50–100 μg of resveratrol per gram (Jeandet et al., 1991). In grape juices, the concentration of free resveratrol is low as compared to cis- and trans-piceid derivatives of resveratrol (Romero-Pérez et al., 1999). The levels of resveratrol vary from 3 to 15 μg/L and 690 to 14,500 μg/L in grape juices (Romero-Pérez et al., 1999).

The concentration of resveratrol in wine varies considerably and is also largely dependent on the grape cultivar, geographic conditions and exposure to fungal infections. Typically, the total concentration of resveratrol in red wine is between 0.2 and 5.8 mg/L while white wine contains approximately 0.68 mg/L. Red wines have six times higher concentrations of trans-resveratrol than white wines while white wines contain high levels of cis-resveratrol. Red wine is extracted without removing the grape skin, whereas white wine is fermented only after removal of the skin (Prasad, 2012). Other sources of common foods containing resveratrol include dark chocolate, various berries, soy, and raw or boiled peanuts.

2.8 Antioxidant properties of resveratrol in AD

Resveratrol exhibits strong antioxidant properties as shown by in vitro and in vivo studies (Sönmez et al., 2007; Venturini et al., 2010). Oxidative stress occurs due to an imbalance between pro-oxidant and antioxidant activities in the body leading to the excessive production of ROS, free radicals and peroxides (Barnham et al., 2004). Brain tissue is more susceptible to oxidative stress due to its greater rate of oxygen consumption, high content of peroxidizable fatty acids, less regenerative capability, and low amounts of antioxidants. Thus, free radicals seem to

play a crucial role in the process of brain aging (Floyd, 1999; Honda et al., 2004). AD is an age-related disorder, most often diagnosed in individuals over 65 years of age and hence aging is strongly implicated in the pathogenesis of this disease (Jayasena et al., 2013).

Oxidative stress caused by an excessive production of ROS in the brain has been considered as the underlying cause for the pathogenesis of a number of neurodegenerative disorders. An increase in levels of ROS, reactive nitrogen species, or some malfunction of the cellular antioxidant systems can damage protein and membrane poly unsaturated fatty acids, causing lipid peroxidation and further leads to loss of membrane integrity and increased permeability to Ca2+ in the plasma membrane (Floyd, 1999; Rege et al., 2013; Sun et al., 2008). Moreover, it causes injury to neural membranes and ultimately memory impairment (Sun et al., 2010). Several in vivo and in vitro studies have reported that ROS increases Aβ production and Aβ induces oxidative stress, which may together accelerate the progression of AD (Murakami et al., 2005; Tabner et al., 2005). However, plant derived dietary antioxidants can be regarded as potential useful targets for the prevention of neuronal damage in neurodegenerative disorders.

Resveratrol suppresses oxygen free radical formation by inhibiting pro-oxidative genes such as nicotinamide adenine dinucleotide phosphate oxidase and myeloperoxidase, and inducing various antioxidant enzymes like SOD, catalase, thioredoxin and glutathione peroxide (GSH-Px); (Carrizzo et al., 2013; Wang et al., 2012), while lowering the activity of enzymes involved in the development of oxidative stress (Carrizzo et al., 2013; Figure 4). Thus resveratrol is a direct scavenger of free radicals production in tissues. It is interesting that resveratrol has proven to be effective in suppressing iNOS production, which is involved in the Aβ-induced lipid peroxidation and heme oxygenase-1 (HO-1) downregulation, thereby protecting the rats from Aβ-induced neurotoxicity (Table 1A; Huang et al., 2011; Figure 4). One of the major

pathological features in AD is cerebral metal ion imbalance. Ions of copper, iron, zinc, and aluminum act as key cofactors in various neuronal functions, including cellular respiration, cellular redox homeostasis, nerve transmission, oxygen transport and functioning of the channels. Dysregulation in the metal ion balance plays a key role in driving neurodegeneration, which is likely to impact cellular function and ultimately neuronal survival. Decreased levels of copper lead to ROS generation and neuronal inflammation in association with Aβ deposition. Resveratrol administration with a high affinity copper chelator may attenuate copper imbalance and ROS production. Similarly, resveratrol can prevent the accumulation of free iron and iron mediated ROS generation and can also counteract the iron-induced mitochondrial dysfunction by suppressing GSK3β activity. Excessive accumulation of zinc and aluminum also promotes ROS production, increases neuroinflammation eventually leading to AD. However, resveratrol has not shown to have direct effects on the levels of zinc but can prevent further development of zinc-related ill effects. Moreover, resveratrol seems to cause an ameliorative change in aluminum induced neurotoxicity (Granzotto and Zatta, 2011). Findings of Granzatto et al. suggest that resveratrol acts as a neuroprotectant against Aβ as well as against Aβ-metal complexes. In addition, resveratrol exerts ROS scavenging properties and reduces toxicity against Aβ-Fe, Aβ-Cu, and Aβ-Zn, but fails to completely block Aβ-Al and Aβ-Cu toxicity (Table 1B; Granzotto and Zatta, 2011). A substantial amount of research has attributed this polyphenol for its anti-antioxidant and cytoprotective actions in oxidative stress-induced brain pathologies. Consequently, resveratrol appears to improve glial, oxidative and inflammatory responses by enhancing the expression of HO-1 and extracellular GSH content in H2O2-induced C6 cells (Quincozes-Santos et al., 2013). Moreover, resveratrol also protected PC12 cells against amyloid-induced cytotoxicity, cell death, and intracellular ROS accumulation and also

suppressed beta-amyloid-induced activation of NF-KB in PC12 cells (Jang and Surh, 2003). Another key enzyme known as Poly (ADP-ribose) polymerase-1 (PARP-1) plays a key role in the regulation of Aβ precursor protein metabolism processing. Studies have reported that over-activation of PARP-1 due to oxidative stress leads to an accumulation of the novel signaling molecule poly-ADP-ribose (PAR), which induces neuronal cell death associated with AD pathogenesis (Strosznajder et al., 2012) (Figure 4). Findings by Lee et al. indicated resveratrol reduced PARP-1 cleavage and protected SH-SY5Y neuroblastoma cells from apoptosis (Lee et al., 2007). Resveratrol being a robust activator of SIRT1 has shown to possess anti-amyloidogenic activity through the activation of SIRT 1 in the brains of Tg2576 mice and protects the cells against oxidative damage (Kelsey et al., 2010) (Figure 4). Furthermore, resveratrol prolongs the synthesis of Aβ in neuronal cultures expressing APP and reduces Aβ production by stimulating SIRT 1 activity (Tang and Chua, 2008). Also, resveratrol protects neocortical neurons cultured from the senescence-accelerated mouse strain SAMP8 against increased susceptibility to oxidative damage via SIRT 1 activation (Cristòfol et al., 2012) (Table 1C). Thus, SIRT 1 appears to be a promising new avenue for therapeutic intervention in age related AD.

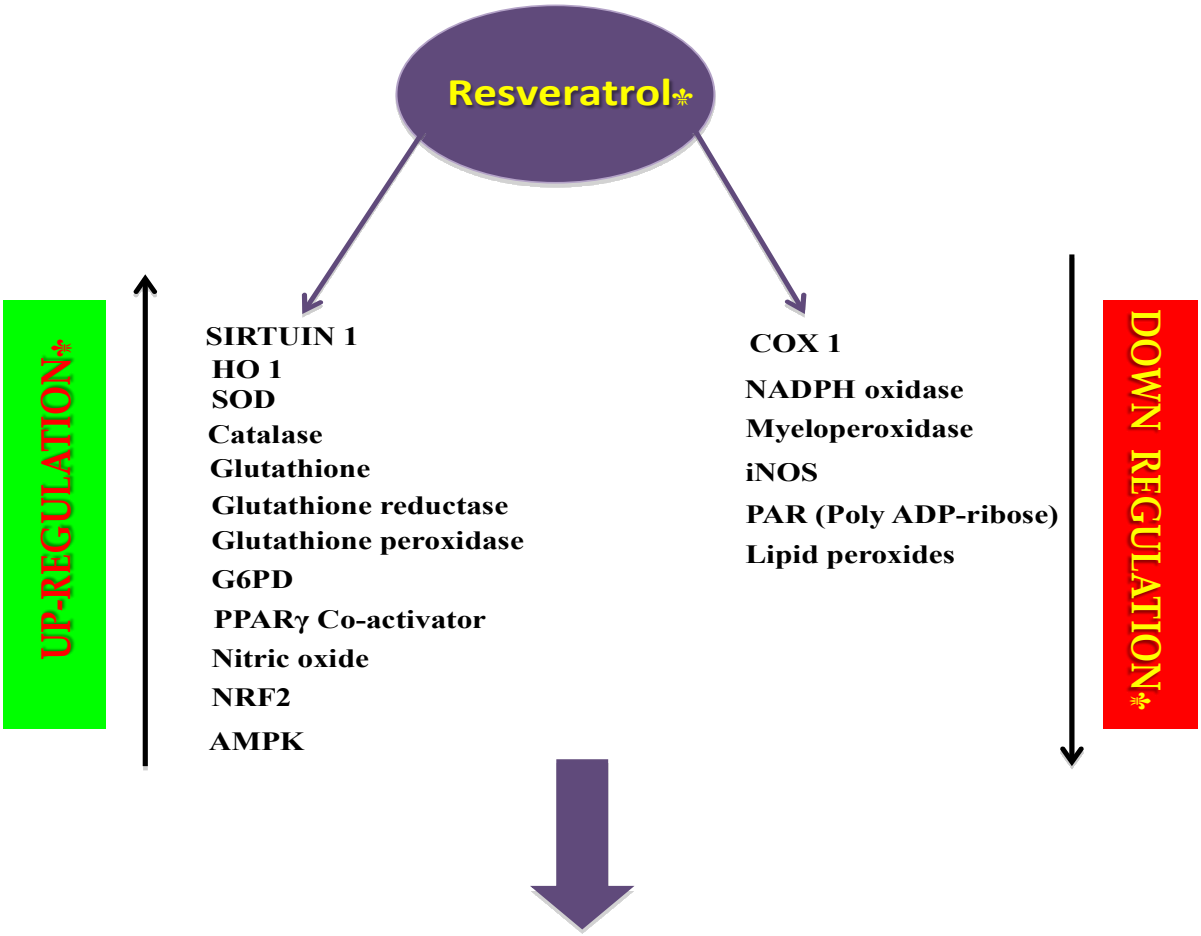


Figure 4. Schematic overview of biological activity of resveratrol. Resveratrol, a natural antioxidant, upregulates the activity of SIRT1, enzymatic antioxidants, PPARγ Co-activator, NO, NRF2, AMPK and downregulates the activity of COX1, NADPH oxidase, Myeloperoxidase, iNOS, PAR, Lipid peroxides thereby prevents apoptosis and inflammation and reduces neurodegeneration.

2.9 Beneficial effects of resveratrol on neuronal inflammation in AD

Neuronal inflammation promotes the pathogenesis of several chronic neurodegenerative diseases, including AD. Various reports show that the inflammatory responses occurring in central nervous system such as activation of microglia, astrocytes, lymphocytes and

macrophages triggers numerous pro- and anti-inflammatory mediators such as ROS, NOS, cytokines, and various neurotransmitters (Moore and O'Banion, 2002). Activation of microglia releases highly ROS such as hydroxyl radicals, superoxide and per oxy radicals, hydroxyl peroxide, and thereby causes oxidation of proteins, lipid peroxidation, and DNA fragmentation. These processes eventually lead to neuronal inflammation and cell death (Liu and Hong, 2003). Amyloid β peptides, the major component of amyloid plaques interact with various Toll-like receptors (TLRs) such as TLR4 and can trigger microglial activation. Anti-inflammatory action of resveratrol has shown to prevent lipopolysaccharide (LPS, a TLR4 ligand)-induced activation of murine RAW 264.7 macrophages and microglial BV-2 cells. It also prevented proinflammatory effect of Aβ on macrophages by inhibiting activation of STAT 1 and STAT3 and NFκB activation by interfering with IKK and IκB phosphorylation (Capiralla et al., 2012). In addition, oral administration of resveratrol in a mouse model of cerebral amyloid deposition significantly reduced microglial activation related to amyloid deposition (Table 1D; Capiralla et al., 2012). Since NF-κB signaling is involved in Aβ-induced neuronal cell death, another link between AD and neuroprotective action of resveratrol is its potential to decrease the expression of iNOS, prostaglandin E2 (PGE2), cathepsin and NO modulated by NF-κβ (Kim et al., 2006). Lu and colleagues reported that resveratrol attenuates LPS-stimulated NF-κB activation in murine primary microglia and astrocytes and LPS-induced inflammatory responses could be modulated by different potencies of resveratrol (Lu et al., 2010). Studies have shown that astrocytes in brain have both positive and negative effects on the central nervous system. They serve as a source of nutrients to neurons and aid in the maintenance of extracellular ion balance as well as in the clearance and degradation of Aβ (Lee et al., 2010; Wyss-Coray et al., 2003). Astrocytes also secrete prostaglandins, interleukins, leukotrienes, thromboxanes, and form

bunches around Aβ deposits(Sidoryk-Wegrzynowicz et al., 2011) (Sidoryk-Wegrzynowicz et al., 2011). A study by Simao et al. showed resveratrol pretreatment (30 mg/kg) significantly reduced NF-κB and JNK activation, and decreased the global cerebral ischemia-induced astroglial and microglial activation and iNOS and COX-2 regulation (Simão et al., 2012). Resveratrol reduces the concentration of 8-iso-prostaglandin F2α, an indicator of free radical production in LPS-activated rat microglial cells, and is considered to be involved in the downregulation of neuroinflammatory responses (Candelario-Jalil et al., 2007). Resveratrol treatment decreased lipid peroxidation, thereby causing an upregulation in the antioxidant status in the senescence-accelerated mouse model. It also prevented cerebral mitochondrial deletion and decreased the impairment in learning and memory (Liu et al., 2012) (Table 1E).

Table 1. Neuroprotective effects of resveratrol in animal models and cultured cell systems

Experimental animals	Model	Effects	Dosage	Treatment/ Time of incubation	References
a) Sprague Dawley Rats	AD	• Decreased hippocampal Aβ accumulation • Improved Aβ induced spatial memory • Reversed Aβ induced iNOS expression • Enhanced HO 1 expression and reduced lipid peroxidation	100μM/5μl	7 days for 30 min	(Huang et al., 2011)
b) SH-SY5Y Human Neuroblastoma	-	• Protected against Aβ as well as Aβ metal complexes. • Acts as a ROS scavenger. • Reduced Aβ and Aβ metal complex toxicity.	15- 200 μM for 24 hrs	Aβ & Aβ complexes	(Granzotto and Zatta, 2011)
c) Neocortical neurons-SAMP8 mice	-	• Increased expression of SIRT 1 • Decreased susceptibility to oxidative damage.	50 μM for 2hr and 24 hrs	BSO (DL-Buthionine-sulfoximine)	(Cristòfol et al., 2012)
d) APP/PSI transgenic mice	AD	• Decreased Aβ associated microglial activation. • Lowered cerebral Amyloid deposition	350mg/kg BW	15 days	(Capiralla et al., 2012)

e) Senescence accelerated mice models	AD	• Increased antioxidant status and decreased lipid peroxidation. • Prevents cerebral mitochondrial deletion. • Increased learning and memory impairment.	25, 50, 100 $kg^{-1}d^{-1}$	8 weeks	(Liu et al., 2012)
f) p 25 transgenic mouse	AD	• Decreased hippocampal neurodegeneration • Increased cognitive performance • Decreased acetylation of SIRT 1 substrates PGC-1 alpha and p53	5 µg/µl injected bilaterally	2-3 times/week	(Kim et al., 2007)
g) PC 12	-	• Inhibited β-amyloid induced cell apoptosis. • Up regulated SIRT 1 levels. • Down regulated ROCK 1 expression.	12.5-100 µM for 24-48 hrs	Amyloid -β	(Feng et al., 2013)
h) APP-HEK_{293} & APP- N_{2a}	-	• Lowered Aβ levels by activating AMPK pathway. • Induced autophagy & lysosomal degradation of Aβ.	40 µM for 24 hrs	$Aβ_{1-40}$ & $Aβ_{1-42}$	(Vingtdeux et al., 2010)
i) Tg19959 transgenic mice	AD	• Reduced plaque counts and burden in medial cortex, striatum and hypothalamus • Increased brain cysteine levels to 54% and decrease in brain glutathione levels to 21%.	300 mg/Kg BW	45 days	(Karuppagounder et al., 2009)
j) SAMP8 mice	AD	• Increased life expectancy. • Decreased cognitive impairment. • Increased both SIRT 1 and AMPK levels. • Decreased P53 acetylation. • Reduced amyloid deposition and favored non- amyloidogenic pathway in hippocampus.	1g/kg BW	7 months	(Porquet et al., 2013)

k) Wistar rats (Colchicine-induced)	AD	• Decreased cognitive impairment • Decreased lipid peroxidation and nitrite levels • Increased acetylcholinesterase activity • Restoration of GSH levels	10-20mg/kg	25 days beginning 4 days prior to colchicine injection	(Kumar et al., 2007)
l) Primary cortical neurons from mice	-	• HO1 production-acts as neuroprotection	5-100μM for 6 hrs	Resveratrol alone	(Zhuang et al., 2003)

3.0 Anti-amyloidogenic effects of resveratrol

Resveratrol exhibits its neuroprotective effects in the inhibition of β-amyloid production and aggregation and in the destabilization of the Aβ fibrils (Ono et al., 2006). Resveratrol also decreases the accumulation of Aβ in cell cultures and lowers Aβ secretion from different cell lines. Since it has no effect on the Aβ producing enzymes, β and γ secretases, it does not suppress Aβ production but promotes proteolytic clearance of Aβ through a mechanism that implicates a proteasome and not NEP (neprilysin) ECE-1 and ECE-2 (endothelin converting enzyme 1 and 2) or IDE (insulin degrading enzyme) (Marambaud et al., 2005). Chronic administration of resveratrol proved to be effective in protecting animal models of AD from Aβ-induced neuronal loss, cell death, accumulations of lipid peroxide products, inhibition of hippocampal iNOS production, and the elevation of HO-1 expression. In accordance with this, resveratrol showed recovery from Aβ-induced spatial memory impairment in the animal models of AD (Huang et al., 2011). Further, consumption of red wine significantly reduces the impairment of spatial memory function and Aβ neuropathology in Tg2576 mice (Wang et al.,

2006). Another study by Lu et al. suggested that administration of resveratrol lowered MPTP-induced deterioration of motor coordination and neuronal loss caused by excessive production of free radicals (Lu et al., 2008). A marked reduction in neurodegeneration in the hippocampus was observed on administration of intra cerebroventricular injection of resveratrol, which was caused by a decrease in the acetylation of SIRT1 substances such as peroxisome proliferator-activated receptor gamma co-activator and p53 (Kim et al., 2007a). This eventually prevented learning deficit in the p25 transgenic mouse model of AD (Table1F) Kim et al., 2007a). Moreover, an in vitro model of PC12 cells using Aβ25-35 provided new compelling evidence on the protective effect of resveratrol against Aβ induced neurotoxicity. Resveratrol protected PC12 cells and inhibited Aβ-induced cell apoptosis through the upregulation of SIRT 1 expression and downregulation of Rho-associated kinase 1 (ROCK 1). Thus, anti-apoptopic actions of resveratrol were partially mediated through the SIRT1-ROCK 1 pathway (Table 1G; (Feng et al., 2013). Resveratrol is also found to exert its neuroprotective actions via the activation of key metabolic sensor proteins, such as the AMP-activated protein kinase (AMPK; Figure 2). Resveratrol induced AMPK activation results in the inhibition of AMPK target mTOR (mammalian target of rapamycin), initiation of autophagy and promotion of lysosomal clearance of Aβ (Vingtdeux et al., 2010). Studies indicate that resveratrol lowers Aβ accumulation in the cortex due to activation of AMPK signaling by enhancing cytosolic Ca2+ levels and CaMKKβ-dependent phosphorylation of AMPK in primary neuronal cultures (Table 1H; (Vingtdeux et al., 2010). It has also been shown to decrease the formation of plaques in specific regions of brain thereby slowing down the process of neurodegeneration (Table 1I; (Karuppagounder et al., 2009). A recent study by Porquet and colleagues reported that dietary resveratrol supplementation at the dose of 1 g/kg body weight to SAMP8 mice, an age-related model of AD,

activates AMPK pathways, prosurvival routes such as SIRT1 and reduces amyloid accumulation, tau hyperphosphorylation and cognitive impairment (Table 1J; (Porquet et al., 2013). Resveratrol at dosages of 10 and 20 mg/kg manifests a neuroprotective action against colchicine-induced cognitive impairment and oxidative damage in Wistar rats (Table 1K; (Kumar et al., 2007). Furthermore, resveratrol treatment has also shown to suppress the levels of NOS and the expression of COX-2 in beta-amyloid treated C6 glioma cells (Kim et al., 2006). Another key player in the regulation of cellular antioxidant mechanism is nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 serves as a chief regulator of cellular resistance to oxidants and genes encoding antioxidant proteins such as HO-1, NAD (P) H-quinone oxidoreductase, GST and glutathione synthetase (GSS; (Scapagnini et al., 2011). Under normal unstressed conditions, Nrf2 is anchored by Keap 1 (Kelch-like ECH-associating protein 1) in the cytoplasm, which causes polyubiquitination, and proteasome mediated degradation. It has also been shown to induce HO1 via Nrf2 and PI3K/AKT pathways and thereby reduce ROS induced oxidative damage in PC 12 cells (Chen et al., 2005). Resveratrol is known to promote HO-1 expression through the activation of Nrf2 in primary neuronal cultures (Table 1L; (Zhuang et al., 2003); Figure 4). Thus, Nrf2 serves as a promising target for resveratrol in the prevention/treatment of certain neurodegenerative diseases.

Resveratrol has been recognized as a potential therapeutic agent for treating wide array of health conditions/diseases such as inflammation, pain, tissue injury, diabetes, and cancer. However, emerging evidence focuses strongly on its potential beneficial effects against several neurodegenerative diseases. In this review, we discussed the antioxidant properties as well as neuroprotective effects of resveratrol in the pathogenesis of AD. For example, in AD, resveratrol promotes clearance of Aβ peptides, anti-amyloidogenic cleavage of APP, its ability to reduce

oxidative stress and neuronal cell death. Consequently, it is plausible to recommend resveratrol as one of the promising tools in the development of drug therapy for AD. Moreover, it is non-toxic, cost effective, and widely available. However, the efficacy and utility of resveratrol also depends upon its solubility and bioavailability. Therefore, future research on the design and synthesis of novel analogs needs to be conducted to address these issues.

4.0 Study Objectives

Resveratrol (3, 5, 4'-tihydroxy-trans-stilbene), a polyphenolic phytoalexin found in the skin and seeds of grapes, is known to exert antioxidant, anti-inflammatory, anti-diabetic, and neuroprotective effects. Various studies have reported a link between obese diabetic state and Alzheimer's disease.

The overall goal of this proposal was to provide a natural therapeutic approach in the treatment of Alzheimer Disease by demonstrating a potential neuroprotective role of resveratrol in improving the oxidative stress and memory loss associated with AD.

We proposed to test the hypothesis that the natural plant based phytochemical resveratrol can reduce oxidative stress and protect memory-associated proteins due to its antioxidant and neuroprotective potential. The following were the study objectives:

Objective 1:

a) Evaluate the neuroprotective effect of resveratrol against oxidative damage in the brains of obese (*ob/ob*) mice.

Objective 2:

a) Evaluate the neuroprotective effect of resveratrol on the expression levels of memory-associated proteins in obese (*ob/ob*) mice.

Objective 3:

a) Determine the neuroprotective action of resveratrol on β- amyloid induced oxidative stress in Amyloid β treated rat hippocampal (H19-7) neuronal cells.

b) Evaluate the neuroprotective effect of resveratrol on the expression levels of memory-associated proteins in Amyloid β treated rat hippocampal (H19-7) neuronal cells.

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Resveratrol Protects the Brain of Obese Mice from Oxidative Damage

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3.1 Abstract

Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin that exerts cardioprotective, neuroprotective, and antioxidant effects. Recently it has been shown that obesity is associated with an increase in cerebral oxidative stress levels, which may enhance neurodegeneration. The present study evaluates the neuroprotective action of resveratrol in brain of obese (ob/ob) mice. Resveratrol was administered orally at the dose of 25mg kg−1 body weight daily for three weeks to lean and obese mice. Resveratrol had no effect on body weight or blood glucose levels in obese mice. Lipid peroxides were significantly increased in brain of obese mice. The enzymatic antioxidants superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and nonenzymatic antioxidants tocopherol, ascorbic acid, and glutathione were decreased in obese mice brain. Administration of resveratrol decreased lipid peroxide levels and upregulated the antioxidant activities in obese mice brain. Our findings indicate a neuroprotective effect of resveratrol by preventing oxidative damage in brain tissue of obese mice.

3.2 Introduction

Obesity is a major risk factor for the development of type 2 diabetes. Roughly 30 percent of obese people are diabetic, and 85 percent of diabetics are obese. Other obesity-related conditions include heart disease, stroke, and certain types of cancer. According to the National Institutes of Health around 97 million Americans are affected by these conditions, which is the second leading cause of death. Recently obesity has been shown to increase the level of cerebro cortical reactive oxygen species and impair brain function (Freeman et al., 2013), suggesting that obesity may increase the risk for neurodegenerative conditions such as Alzheimer's disease (Luchsinger et al., 2007; Naderali et al., 2009).

Oxidative stress is associated with an increase in oxidizing species that destructs the vascular and neuronal cells in central nervous system. Oxidative stress is due to the imbalance between the oxygen free radicals generated and the antioxidant defense system to detoxify the reactive intermediates (Balaban et al., 2005). Oxidative stress changes the signaling pathways that may induce cellular responses such as inflammation, cell proliferation, and cell survival and death (Kregel and Zhang, 2007). Reactive oxygen species (ROS) are chemically reactive molecules that consist of oxygen ions and peroxides that include hydrogen peroxide, singlet oxygen, nitric oxide, peroxynitrite, and superoxide free radicals. The release of peroxides and free radicals is toxic to the cell, which may lead to cell death. The antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and peroxidases, and non-enzymatic free radical scavengers (ascorbic acid, α-tocopherol, and GSH) convert the reactive oxygen species to water and oxygen, the stable molecules. These antioxidants are known to protect the cells and tissues against oxidative injury caused by reactive oxygen species (Uttara et al., 2009). Obesity has found to increase the levels of total reactive oxygen species in brain, thereby increasing susceptibility to oxidative

stress and neurodegeneration (Freeman et al., 2013). Resveratrol (3,5,4□-trihydroxystilbene) a naturally occurring polyphenol belonging to the phytoalexin family is found in peanuts (Chen et al., 2002), skin, and seeds of grapes (Soleas et al., 1997). Evidence indicates that resveratrol exerts neuroprotective effects against diabetes-induced oxidative damage (Ates et al., 2007; Venturini et al., 2010). Resveratrol is also cardioprotective (Li et al., 2012), anti-inflammatory (Bertelli et al., 1999) prevents certain cancers (Aggarwal et al., 2004) and improves insulin sensitivity in diet-induced obese mice (Lagouge et al., 2006). The present study was designed to evaluate the neuroprotective action of resveratrol on obese (*ob/ob*) mice induced oxidative stress.

3.3 Materials and Methods

3.3.1. Reagents. Resveratrol (trans) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.3.2. Animals. This study was approved by the Midwestern University Research and Animal Care Committee. Male 8- week-old B6.V-Lep/J *ob/ob* mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The *ob/ob* mouse was selected because it exhibits metabolic abnormalities including hyperglycemia and hyperinsulinemia that phenotypically resembles human type 2 diabetes and severe obesity. Age matched C57BL/J6 mice were used as lean controls. Mice were housed 2 per cage and were provided with food and water provided ad libitum, maintained in a room with alternating twelve hour light/dark cycle, and kept at 22∘ C. Mice were maintained on a standard pellet diet (Lab Diet 5001, PMI Nutrition International, Inc., Brentwood, MO, USA) for 21 days, including the 24-hour period of when food intake was measured. The composition of the diet (based on chemical composition) according to the manufacturer was as follows: 23.9% protein, 10.7% fat, 5.1 fiber, 48.7% carbohydrate, 7% mineral mixture, and 4.6% vitamin mixture.

3.3.3. Experimental Design. After one week of acclimatization, obese and lean control mice were divided into four groups consisting of six animals each: lean control, lean control resveratrol treated, obese, and obese-resveratrol treated. Trans-resveratrol was mixed with a 1% solution of methylcellulose (Sigma-Aldrich, MO, USA), with viscosity 25 cP to form a colloid, which was administered by oral gavage in volume of 0.5 to 0.75mL at a concentration of 25mg/kg body weight once daily for a period of 21 days. Lean mice received the vehicle only. This methylcellulose formulation was well tolerated by mice, and there was no evidence of

gastrointestinal distress, changes in behavior and ambulatory activity, or dramatic weight loss in mice. This concentration of resveratrol and duration of treatment were selected based on previous studies highlighting its insulin-mimetic properties, beneficial effects on cardiac and endothelial function, and anti-inflammatory and neuroprotective effects (Clark et al., 2012; Sharma et al., 2011; Zhang et al., 2009, 2010).

Recent evidence also indicates that significant levels of resveratrol are detected in rat brain following 3 daily doses of 25mg/kg of trans-resveratrol (Clark et al., 2012), despite the short half-life and bioavailability of this conjugated form in plasma (Walle et al., 2004).

3.3.4. Food Intake. Mice from each group were placed individually in metabolic cage systems (Mini Mitter, Bend, OR) between 8:00 and 9:00 am during the final week of study for a 24-hour period for measurement of food intake.

3.3.5. Blood and Tissue Sampling. After the 3-week treatment period, mice were sacrificed in the morning between 10am and 1 pm. Following CO2 asphyxiation, a sternotomy was performed to expose the heart, and then blood was obtained by cardiac puncture from the right ventricle. Blood was centrifuged (3,500 rpm at 4°C, for 5 min), and plasma was separated from the erythrocytes for the assay of glucose using a commercially available kit (Wako Chemical, VA). Brain tissues were excised immediately, and a 10% homogenate was prepared in 100mM Tris HCl (pH 7.4) using a Potter- Elvehjem homogeniser and used for the estimation of biochemical parameters. Adipose tissue was dissected and then weighed.

3.3.6. Estimation of Protein. The amount of protein in the tissue homogenate was measured by the method of Pierce using BSA as standard. 150 μL of protein assay reagent was added to 10 μLof the brain tissue homogenate, and the color developed was read after 5min at 630nm. The levels of protein are expressed as mg/mL.

3.3.7. Lipid Peroxidation. Lipid peroxidation was estimated in brain tissue homogenate by themethod of Högberg et al., 1974 using thiobarbituric acid. The release of malondialdehyde as an end product of peroxidation of lipids served as the index of the intensity of oxidative stress. Given the limitation of this method, direct measurement of lipid hydroperoxide was also carried out using a commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA).

3.3.8. Enzymic Antioxidants. Antioxidant enzymes were estimated in brain tissue homogenate of experimental groups. Superoxide dismutase (SOD) isoforms SOD1 and SOD2 were assayed using the kit (CaymanChemical Co., AnnArbor, MI, USA) according to the manufacturers' standard procedures. Catalase activity was assayed by the method of Sinha, 1972. Glutathione peroxidase (GPX) was assayed by the method of Rotruck et al., 1973. The utilization of glutathione was used to express the activity. Glutathione reductase (GR), that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was measured by the method of Staal et al., 1969. The activity of glucose-6-phosphate dehydrogenase (G6PDH) was assayed by the method of Ells and Kirkman, 1961.

3.3.9. Nonenzymatic Antioxidants. Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. Dehydroascorbic acid reacts with 2,3-dinitrophenyl hydrazine to form the derivative of 2,4-dinitrophenyl hydrazine. This compound in strong sulphuric acid undergoes a rearrangement to form a product with absorption maxima at 520 nm. The reaction was run in the presence of thiouria to prevent the interference of non-ascorbic acid chromogens (Omaye et al., 1979). α-Tocopherol was estimated by the method of Quaife et al., 1949; the reduced glutathione levels (GSH) and oxidized glutathione (GSSG) were quantified according to kit manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA). The redox index was calculated as (GSH = 2GSSG)/(2GSSG × 100) reported by (Oztürk and

Gümüşlü, 2004).

3.3.10. Statistical Analysis. All values are expressed as mean ± standard deviation (S.D.) in each group. Statistical difference between groups was assessed by one-way ANOVA followed by Tukey-Kramer analyses with equal variance. Significance was set at $P < 0.05$.

3.4 Results

3.4.1 Resveratrol Had No Effect on Body Weight of Obese Mice. The effects of resveratrol treatment on physical characteristics of obese mice are illustrated in Table 1(a). Confirming the phenotype of the *ob/ob* mouse, body weight and fat pad weight were significantly greater ($P < 0.001$) in obese mice compared to lean control mice. However, body weight was not altered with resveratrol treatment of obese mice. There was no significant difference in the fat pad weight between obese and obese-resveratrol treated groups, although fat pad weight tended to be lower by ~15% in obese mice treated with resveratrol.

3.4.2 Resveratrol Did Not Reduce the Blood Glucose in Obese Mice. Blood glucose levels in lean and obese mice after resveratrol treatment are shown in Table 1(b). Also confirming the characteristics of the *ob/ob* mouse, blood glucose levels were significantly elevated ($P < 0.001$) in obese mice compared to lean control mice. Treatment with resveratrol had no effect on plasma glucose levels in both lean and obese mice. Intriguingly, in obese mice, blood glucose levels were increased by ~19% following resveratrol treatment. Food intake in lean and obese mice with and without resveratrol treatment is illustrated in Table 1(b). Resveratrol had no significant effect on food intake in both lean and obese mice. However, food intake was highest ($P < 0.05$) in the obese-resveratrol treated group compared to lean groups.

3.4.3 Resveratrol Ameliorated Lipid Peroxidation Induced in Obese Mice. Figure 1 shows the effect of resveratrol on malondialdehyde (Figure 1(a)) and lipid peroxides (Figure 1(b))

levels in brain of the lean and obese mice. The levels were substantially increased ($P < 0.001$) in obese mice brain compared to lean control mice. Administration of resveratrol for 3 weeks to obese mice significantly reduced ($P < 0.001$) the lipid peroxidation when compared to obese untreated mice.

3.4.4 Resveratrol Improves the Enzymic Antioxidants in Obese Mice. Table 2 presents the levels of antioxidant enzymes in lean and obese mice with or without resveratrol administration. We found that in obese control mice brain the activities of antioxidant enzymes: SOD, catalase, GPX, GR, and G6PD were significantly declined ($P < 0.001$) when compared to lean control mice. Resveratrol administration to the obese mice enhanced ($P < 0.001$) the enzymic antioxidant activities in brain to a significant extent compared to obese control mice. However resveratrol administration did not show any significant change in SOD2 activity. There were no significant changes in the levels of enzymic antioxidants of lean mice administered resveratrol in comparison to untreated lean mice.

3.4.5 Resveratrol Enhanced the Nonenzymatic Antioxidants in Obese Mice. The non enzymatic antioxidants were reduced in obese mice brains to the same extent as those observed for the enzymatic antioxidants (Table 3). The levels of ascorbic acid, α-tocopherol, GSH, and GSH/GSSG ratio were significantly decreased in obese control mice brain compared with lean control mice. Resveratrol administered to obese mice enhanced the levels of ascorbic acid ($P < 0.001$), α- tocopherol ($P < 0.05$), and GSH ($P < 0.01$) levels compared to obese-untreated mice. There were no marked changes observed in resveratrol treated lean mice.

3.5 Discussion

Oxidative stress leads to neurodegeneration due to insufficiency of the antioxidant defense mechanisms in the brain to counteract the increased reactive oxygen species formation (Bruce-Keller et al., 2010; Gemma et al., 2007; Head et al., 2008; Mariani et al., 2005). Dietary supplements containing antioxidants might be favorable in maintaining the brain function (Liu et al., 2003).

For instance, in PC12 cells, the antioxidant resveratrol has found to be neuroprotective against oxidative stress (Sun et al., 1997), by attenuating the generation of free radicals. Evidence also indicates that resveratrol suppresses oxidative stress-induced neuronal cell death (Virgili and Contestabile, 2000) and blocks lipid peroxidation (Tadolini et al., 2000).

Resveratrol has been shown to improve the memory loss and protect the rats from Aβ-induced neurotoxicity by reducing the inducible nitric oxide synthase and lipid peroxides (Huang et al., 2011). The present study was designed to examine the salubrious effects of resveratrol as a neuroprotective antioxidant on brain of *ob/ob* mice, a model of severe obesity with insulin resistance, resulting from defective leptin signaling. We chose the representative concentration of resveratrol of 25mg/kg based on the observations that this polyphenol improves the overall diabetic state and accumulates in tissues, including heart, liver, kidney, and brain, following acute and chronic treatments (Bertelli et al., 1996; Clark et al., 2012; Palsamy and Subramanian, 2009; Su et al., 2006; Zhang et al., 2009). We demonstrate that obesity has a negative impact on the oxidative stress in the central nervous system of *ob/ob* mice. Indeed, brain from obese mice exhibits increased lipid perioxidation, along with a decrease in the levels of key neuroprotective antioxidants. Resveratrol was clearly beneficial by reversing lipid peroxidation and improving

the antioxidant status.

In brain, polyunsaturated fatty acids exposed to reactive oxygen species result in the production of toxic lipid peroxidation intermediates (Keller et al., 2004). A significant increase in lipid peroxidation was observed in brain of *ob/ob* mice, confirming earlier reports (Freeman et al., 2013). Recent evidence indicates that a diet high in fat was found to increase the level of lipid peroxidation in rat brain (Charradi et al., 2012). Lipid peroxidation is also increased in brain of streptozotocin-induced diabetic rats, suggesting that lipid peroxidation is not solely related to defective leptin signaling and hyperglycemia (Ates et al., 2007). Administration of resveratrol reduced the lipid peroxidation level of the obese mice significantly. The beneficial effect of resveratrol on lipid peroxidation in brain was also reported in streptozotocin-induced diabetic rats (Ates et al., 2007).

The cellular antioxidant defense mechanism against reactive oxygen species includes enzymatic defense systems such as SOD, catalase, and GPX. SOD converts the superoxide radical to H2O2, which, in turn, is further eliminated by catalase and GPX. The activities of these enzymes are reduced in *ob/ob* mice compared to lean mice. Mice fed with high fat diet demonstrated a significant decrease in GPX activity in cortex but no difference in SOD in cortex and hippocampus (Freeman et al., 2013). Obesity increases the total reactive oxygen species and superoxide in brain (Freeman et al., 2013), which might explain the decreased activities of SOD, catalase, and GPX observed in obese mice.

In brain from obese mice, catalase activity might be reduced due to reduced NADPH levels, since the regeneration of catalase from its inactive form requires NADPH. GPX activity is also reduced in brain from obese mice due to reduction of GSH levels and its inactivation by the accumulation of superoxide radicals. In this study, we are the first to report that administration of

resveratrol significantly increased the enzymic antioxidant activities in obese mice.

In addition to enzymic antioxidants, non-enzymatic antioxidants also protect the brain from oxidative damage. GSH is an endogenous non-enzymatic antioxidant against reactive oxygen species in the cellular defense system. GSH is oxidized to glutathione disulfide (GSSG) by reactive oxygen species, thereby reducing the level of GSH. Glutathione reductase (GR) converts GSSG back to GSH by NADPH, which in turn is released by glucose-6-phosphate dehydrogenase (G6PDH). The level of these antioxidants is reduced in both obese mice and diabetes mellitus (Baydas et al., 2003; Dincer et al., 2002; Tachi et al., 2001). Administration of resveratrol increased the activities of these antioxidants in obese mice, as well as that of α-tocopherol and ascorbic acid, which are also reduced in obese mice brain. Ascorbic acid is a water-soluble antioxidant (Lykkesfeldt and Moos, 2005), which prevents the degradation of tocopherol to tocopheroxyl radical (Zhang and Omaye, 2001). α-Tocopherol is a lipophilic antioxidant and functions as a peroxyl radical scavenger. It blocks lipid peroxidation by reacting with free radicals, thereby forming α-tocopherol radical, which is then oxidized by ascorbic acid and converted back to its reduced state (Traber and Stevens, 2011). In the present study, elevation of ascorbate and α-tocopherol was observed in obese mice brain treated with resveratrol. The levels of ascorbate and α- tocopherol might be elevated due to increase in GSH activity on resveratrol administration.

Early investigations have addressed the kinetics of absorption and bioavailability of resveratrol and its conjugated forms in serum. It is known that conjugated resveratrol is less biologically active than resveratrol provided or ingested in its natural red wine matrix and that absorption of resveratrol may by altered by other dietary media such as juice homogenates (Goldberg et al., 2003; Mohar and Malik, 2012). In diabetic mice, it is possible that elevated brain glucose levels

(Jacob et al., 2002), may interfere with accumulation of resveratrol and limit its antioxidant properties.

Yet, the treatment regimen used in this study has been linked to several beneficial neuroprotective effects, indicating that trans-resveratrol does accumulate in tissues after short-term treatment, and overcoming the metabolic perturbations associated with hyperglycemia in brain of diabetic rodents (Jacob et al., 2002).

Further, earlier feeding studies in the rat have demonstrated that redwine administered acutely by oral gavage at the much lower dose of 80 μg/kg resulted in a significant accumulation of total resveratrol in heart, liver, and kidney within 30 to 240 minutes. Reducing the dosage by one-half with continued treatment over 15 days also produced dramatic increase in tissue resveratrol content (Bertelli et al., 1996). In this study, bioavailability of trans-resveratrol is clearly not rate limiting with evidence indicating that accumulation of resveratrol in brain is seen following short-term administration of resveratrol at the concentration of 25mg/kg (Clark et al., 2012). It was interesting to observe that food intake in *ob/ob* mice treated with resveratrol was elevated compared to non-diabetic mice. Food intake measurements were initiated after the observation that resveratrol decreased body weight in *ob/ob* mice, believing that effect was secondary to perhaps a reduction in food intake. Although administration of resveratrol is linked to a reduction in food intake in diabetic rodents (Su et al., 2006), food intake was increased in the ob/ob mice compared to non obese mice, suggesting improvements in overall oxidative metabolism occurring to resveratrol. To our knowledge, a direct central nervous system-mediated hyperphagic response of resveratrol has not been reported in this model of obesity and diabetes. However, there is evidence that resveratrol, by activating the sirtuin system in peripheral tissues, improves glucose metabolism, mitochondrial function, and biogenesis, and may be hormetic in

nature (Mohar and Malik, 2012; Palsamy and Subramanian, 2009).

3.6 Conclusions

This study suggests that resveratrol is effective in preventing against obesity-induced oxidative damage in brain. Indeed, in brain of *ob/ob* mice, the reduction in the antioxidative status is attenuated, indicating that resveratrol exerts both antioxidant and neuroprotective properties. Our findings provide the rationale for further studies directed in understanding of mechanism of resveratrol in preventing neurodeterioration.

Conflict of Interests

The authors of this study have no conflict of interests to disclose.

Acknowledgments

This work was supported by the New Faculty Start-up Fund from Auburn University to JRB and Midwestern University Office of Research and Sponsored Programs to TLB.

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Figure Legends

Fig 1: Effect of resveratrol on the levels of malondialdehyde (a) and hydroperoxide (b) in lean and obese mice brains. Values are expressed as mean ± S.D. for six mice in each group. Values are statistically significant at *** $P < 0.001$.

Table 1: Effect of resveratrol on the body weight and blood glucose level in lean and obese mice. The body and fat pad weight (A), blood glucose level and food intake (B) were estimated in lean and obese mice.

A.

Parameter	**Lean**	**Lean + Res**	***ob/ob***	***ob/ob* + Res**
Body weight (g)	25.1±1.58	23.9±1.26	$49.3\pm6.19^{a***}$	47.2 ± 4.50^{bNS}
Fat pad (g)	1.24±0.24	1.08±0.11	$13.12\pm3.71^{a***}$	11.23 ± 2.75^{bNS}

B.

Parameter	**Lean**	**Lean + Res**	***ob/ob***	***ob/ob* + Res**
Blood glucose (mg/dl)	124.2±19.40	111.8±21.24	$242.2\pm58.1^{a***}$	288.4 ± 44.4^{bNS}
Food intake (g/day)	4.44±0.11	4.96±1.61	6.58 ± 3.24^{aNS}	$9.56\pm3.28^{c*}$

Values are expressed as mean ± S.D. for twelve mice in each group. Values are statistically significant at *** $P < 0.001$ and * $P < 0.05$. a) *ob/ob* mice were compared with lean control mice; b) *ob/ob* mice were compared with *ob/ob*-resveratrol treated mice; c) lean mice were compared with *ob/ob* resveratrol treated mice. NS represents non significant.

Table 2. Effect of resveratrol on the levels of enzymic antioxidants in lean and obese mice brains.

Parameter	**Lean**	**Lean + Res**	***ob/ob***	***ob/ob* + Res**
SOD1	71.80±3.82	72.62±3.53	$46.96\pm3.56^{a***}$	$63.75\pm3.26^{b***}$
SOD2	80.11±4.93	80.61±5.14	$71.41\pm4.43^{a**}$	77.15 ± 4.74^{bNS}
Catalase	6.93±0.49	6.95±0.61	$4.07\pm0.37^{a***}$	$6.07\pm0.49^{b***}$
GPx	7.44±0.99	7.66±0.84	$4.37\pm0.52^{a***}$	$6.60\pm0.59^{b***}$
GR	0.42±0.04	0.43±0.03	$0.19\pm0.02^{a***}$	$0.38\pm0.03^{b***}$
G6PD	571.2±28.6	586.1±23.9	$292.9\pm20.4^{a***}$	$496.9\pm19.2^{b***}$

Values are expressed as mean ± S.D. for six mice in each group. SOD: amount of enzyme required to exhibit 50% dismutation of superoxide radical/mg protein; catalase: nmoles of H_2O_2 consumed/min/mg protein; GPX: mmoles of GSH oxidized/min/mg protein; GR: nmoles of NADPH consumed/min/mg protein; G6PD: nmoles of NADPH liberated/min/mg protein. Values are statistically significant at ** $P < 0.01$ and ***$P < 0.001$. a *ob/ob* mice were compared with lean control mice; b *ob/ob* mice were compared with *ob/ob*-resveratrol treated mice. NS represents non significant.

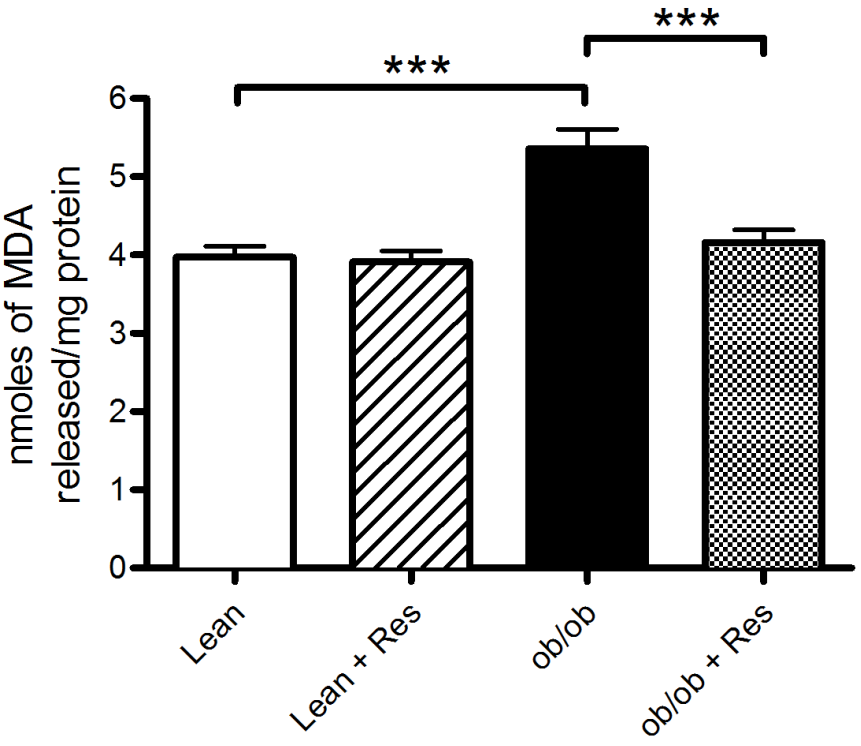
Table 3: Effect of resveratrol on the levels of nonenzymic antioxidants in lean and obese mice brains.

Parameter	**Lean**	**Lean + Res**	***ob/ob***	***ob/ob* + Res**
Ascorbic acid	1.57±0.20	1.51±0.21	$0.76\pm0.12^{a***}$	$1.32\pm0.18^{b***}$
α-Tocopherol	1.62±0.19	1.58±0.19	$1.06\pm0.20^{a***}$	$1.42\pm0.19^{b*}$
GSH	4.73±0.60	5.01±0.43	$3.77\pm0.31^{a**}$	$4.44\pm0.37^{b**}$
GSSG	1.71±0.20	1.88±0.24	1.65 ± 0.20^{aNS}	1.73 ± 0.19^{bNS}
GSH/GSSG	2.69±0.16	2.74±0.19	$2.30\pm0.12^{a***}$	$2.57\pm0.11^{b**}$
Redox index	0.023±0.001	0.024±0.001	$0.021\pm0.001^{a***}$	$0.023\pm0.001^{b**}$

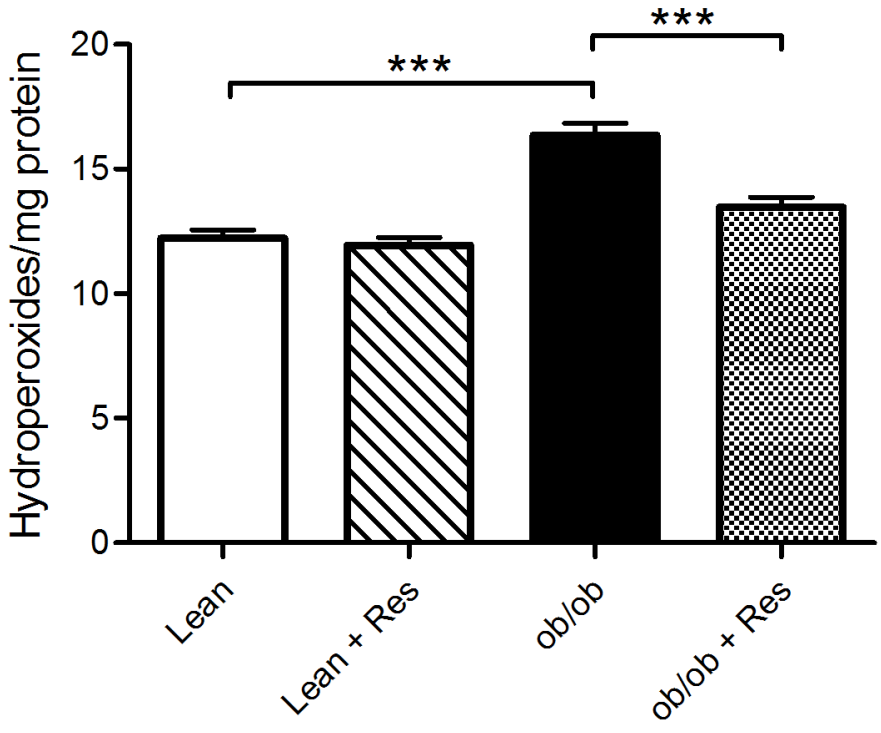
Values are expresses as mean ± S.D. for six mice in each group. Ascorbic acid, mg/mg protein; *α*-tocopherol:mg/mg protein; GSHand GSSG: nmol/mg protein. Values are statistically significant at ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. [a] *ob/ob* micewere compared with lean control mice; [b] *ob/ob* micewere compared with *ob/ob*-resveratrol treated mice. NS represents nonsignificant.

Fig 1.

A.


***
***
nmoles of MDA released/mg protein
0
1
2
3
4
5
6
Lean
Lean + Res
ob/ob
ob/ob + Res

B.


***
***
Hydroperoxides/mg protein
0
5
10
15
20
Lean
Lean + Res
ob/ob
ob/ob + Res

Resveratrol Protects Memory Associated Proteins in the Brains of Obese Mice and H19-7 Hippocampal Neuronal Cells.

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4.1 Abstract

Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is a polyphenolic phytoalexin known to exert anti-diabetic, anti-inflammatory and neuroprotective actions. Several studies report that obesity associated insulin resistance (IR) and T2DM represents a major risk of developing Alzheimer's disease (AD). The present study evaluates the neuroprotective action of resveratrol in the *ob/ob* mouse; a model of severe obesity and diabetes as well as in Aβ induced H19-7 rat hippocampal cells. Resveratrol was administered orally at the dose of 25 mg kg-1 body weight daily for 3 weeks to male lean and *ob/ob* mice. Cultured rat hippocampal H19-7 neuronal cell line was pretreated with 75 μM of resveratrol for 2 hrs followed by 25 μM of Aβ (1-40) for 24 hrs. Our results showed increased expression of tau, phosphorylated forms of tau (CP-13, S202/205; PHF1, S396/404), glial fibrillary acidic protein (GFAP) and pJNK in the brain of *ob/ob* mice whereas expression of synaptophysin, PSD95, Arc, insulin degrading enzyme (IDE), ADAM10, phospho glycogen synthase kinase 3 ($GSK3\beta^{p}$), pAMPK, CREB and BDNF was decreased compared to the brain of lean mice. Similar results were observed in Aβ treated H19-7 cells. Resveratrol mediated neuroprotective effects on amyloid burden and tau hyperphosphorylation, the two major hallmarks of AD, improved the expression of memory-associated proteins, activated APMK pathways and enhanced CREB levels to subsequently promote BDNF synthesis in *ob/ob* mice brain and Aβ treated H19-7 cells. These findings demonstrate a potential neuroprotective effect of resveratrol in the brain of *ob/ob* mice and Aβ treated H19-7 cells.

4.2 Introduction

Obesity is strongly correlated with insulin resistance, which in turn may contribute to an increased risk for Type 2 Diabetes Mellitus (Olefsky and Glass, 2010). It has truly become a global epidemic afflicting an estimated 500 million adults worldwide and nearly two third of the adult population in the United States (Finucane et al., 2011). Insulin is known to play a major role in brain function such as regulation of energy metabolism, growth, survival and differentiation. Insulin, by a saturable receptor mediated process crosses the blood brain barrier (BBB) and facilitates glucose uptake in various type of neurons (Moreira et al., 2013). Insulin receptors are widely expressed throughout the Central Nervous system (CNS), at particularly higher concentrations in the hippocampus and medial temporal cortex, suggesting that insulin may have region - specific functions in brain and does influence memory and learning (Craft, 2009). The brain is one of the most metabolically active organs in the body and requires glucose to fuel its activities; thus, the regulation of glucose homeostasis by insulin is essential for energy metabolism, growth, survival and differentiation of neurons. Therefore, optimum insulin levels in the brain improve memory and facilitate synaptic plasticity in the hippocampus and are considered to be neuroprotective and neurotrophic (van der Heide et al., 2005; Zhao et al., 2004). Obesity associated insulin resistance (IR) and T2DM are now recognized as the major risk factors in the development and progression of Alzheimer's disease (AD) (Businaro et al., 2012; Petanceska, 2007; Profenno et al., 2010; Schrijvers et al., 2010). AD is an age-related neurodegenerative disorder, characterized clinically by progressive cognitive decline and pathologically by extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) (Chen and Zhong, 2013; Matsuzaki et al., 2010; Umegaki, 2010). Insulin resistance is accompanied by reduced insulin sensitivity resulting in peripheral hyperinsulinemia, associated

with impaired insulin transport across the BBB and reduction of insulin levels in the brain. Therefore, impairment in insulin signaling influence the onset of AD and is believed to mediate AD through neuronal inflammation, cerebral oxidative stress, increased Aβ accumulation, tau protein phosphorylation and eventually neuronal cell death and memory impairment (Calvo-Ochoa and Arias, 2014). Thus, therapeutic strategies that target the link between insulin resistance and AD as well as the development of ideal diagnostic biomarkers might benefit in AD treatment.

Resveratrol (3, 5, 4' trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin found in skin and seeds of more than 70 different plant species including grapes, grains, tea and peanuts (Li et al., 2012; Zhang et al., 2013). Studies suggest resveratrol exerts anti-diabetic, anti-inflammatory, anti-carcinogenic and neuroprotective actions (Bertelli et al., 1999; Rege et al., 2013). Our recent study has shown that resveratrol exerts a neuroprotective effect on the cerebral oxidative stress in obese diabetic mice (Rege et al., 2013). Based on these encouraging findings, we sought to evaluate the neuroprotective effect of resveratrol on various proteins associated with memory impairment in obese (*ob/ob*) mice, a model of severe obesity and diabetes. Furthermore, we also extended these studies by examining the resveratrol-mediated neuroprotective action on several specific Alzheimer's markers in Aβ induced H19-7 rat hippocampal cells.

4.3 Materials and Methods

In Vivo studies

4.3.1 Reagents. Resveratrol (trans) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Antibodies against synaptophysin, activity-regulated cyto skeleton-associated protein (Arc (C-7)), glial fibrillary acidic protein (GFAP), Tau (C-7), CDK-5 and BDNF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and insulin degrading enzyme (IDE), postsynaptic density protein (PSD95), ADAM and BACE from Abcam (Cambridge, MA). Antibodies specific to CP13 and paired helical filaments (PHF1) were obtained from Dr. Peter Davis, Albert Einstein College of Medicine, Bronx, New York. Phospho- GSK3β (Ser 9) that recognizes phosphorylation at serine 9, GSK3β that detects endogenous levels of total GSK3β protein, pAMPK, AMPK, CREB, JNK were purchased from Cell Signaling Technology (Danvers, MA). pJNK was purchased from Biolabs and Beta actin was purchased from Sigma (St. Louis, MO, USA).

4.3.2 Animals. This study was approved by the Midwestern University Research and Animal Care Committee. Male 8- week-old B6.V-Lep/J *ob/ob* mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The *ob/ob* mouse was selected because it exhibits metabolic abnormalities including hyperglycemia and hyperinsulinemia that phenotypically resembles human type 2 diabetes and severe obesity. Age matched C57BL/J6 mice were used as lean controls. Mice were housed 2 per cage and were provided with food and water provided ad libitum, maintained in a room with alternating twelve hour light/dark cycle, and kept at 22∘C. Mice were maintained on a standard pellet diet (LabDiet 5001, PMI Nutrition International, Inc.,

Brentwood,MO, USA) for 21 days. The composition of the diet (based on chemical composition) according to the manufacturer was as follows: 23.9% protein, 10.7% fat, 5.1 fiber, 48.7% carbohydrate, 7% mineral mixture, and 4.6% vitamin mixture.

4.3.3 Experimental Design. After one week of acclimatization, obese and lean control mice were divided into four groups consisting of six animals each: lean control, lean control resveratrol treated, obese, and obese-resveratrol treated. Trans-resveratrol was mixed with a 1% solution of methylcellulose (Sigma-Aldrich, MO, USA), with viscosity 25 cP to form a colloid, which was administered by oral gavage in volume of 0.5 to 0.75mL at a concentration of 25mg/kg body weight once daily for a period of 21 days (Rege et al., 2013). Lean mice received the vehicle only. This methylcellulose formulation was well tolerated by mice, and there was no evidence of gastrointestinal distress, changes in behavior and ambulatory activity, or dramatic weight loss in mice.

4.3.4 Tissue Sampling. After the 3-week treatment period, mice were sacrificed in the morning between 10am and 1 pm and brain tissues were excised immediately, and a 10% homogenate was prepared in 100mM Tris HCl (pH 7.4) using a Potter- Elvehjem homogeniser and used for the estimation of biochemical parameters.

***In Vitro* studies**

4.3.5 Reagents. Resveratrol (trans) purchased from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in ethanol as a 200 mM stock solution and stored in aliquots at -20°C. Beta amyloid (Aβ1-40) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Aβ1-40, a neurotoxic peptide fragment derived from APP (Cleary et al., 1995) , was dissolved in deionized distilled water at a concentration of 1 mM and stored at - 20°C. The stock solutions were diluted to the desired concentrations immediately before use and added to the culture medium without

the aging procedure. All other chemicals and solvents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Antibodies against CDK-5 and BDNF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and ADAM and BACE from Abcam (Cambridge, MA). Antibodies specific to pAMPK, AMPK, CREB, JNK were purchased from Cell Signaling Technology (Danvers, MA). pJNK was purchased from Biolabs and Beta actin was purchased from Sigma (St. Louis, MO, USA).

4.3.6 Cell Culture

The H19-7 cell line was derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos and immortalized by retroviral transduction of temperature sensitive tsA58 SV40 large T antigen. All cells were cultured in poly-D-lysine-coated culture dishes and were maintained in 10% fetal bovine serum (FBS), 0.001mg/ml puromycin, and 0.2 mg/ml G418 at 34°C. Cells were serum starved overnight prior to treatment.

4.3.7 Cell Treatment

Cultured cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM Aβ1-40 for 24 h. After treatment, cells were lysed with HEPES lysis buffer (50 mM HEPES, pH 7.6), 150 mM NaCl, 20mM sodium pyrophosphate, 10 mM NaF, 20 mM β-glycerophosphate, 1% Triton, 1 mM Na3VO4, 1 mM phenylmethyl sulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin (Rege et al., 2015)

4.3.8 Estimation of Protein

The amount of protein in the cells was measured using Pierce 660nm Protein Assay and BSA as standard (Rege et al., 2013). 150 μL of protein assay reagent was added to 10 μL of the cell

lysate, and the optical density was measured after five min at 630 nm. Protein concentrations are expressed as mg/mL.

4.3.9 Western Blot Analysis

Lysates from the brain of *ob/ob* mice and amyloid β –induced H19-7 cells were prepared and samples were boiled in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 10% SDSPAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Temecula, CA), analyzed by western blotting with appropriate antibodies, and detected by enhanced chemiluminescence.

4.3.10 Statistical Analysis

All values are expressed as mean ± standard error of mean (SEM) in each group. Statistical difference between groups was assessed by one-way ANOVA followed by Tukey-Kramer analyses with equal variance. Significance was set at $*P < 0.05$. Quantification of all the western blots was done using Image J software.

4.4 Results

4.4.1 Resveratrol ameliorates markers of neurodegeneration. Synaptic plasticity is the ability of neurons to change the number and strength of their synapses and efficient synaptic transmission is crucial for neuronal signaling and memory formation. Synaptic loss is an early event in AD pathogenesis that correlates with memory impairment in AD subject (Shankar and Walsh, 2009). Therefore, to determine synaptic activity in the brain of obese mice, expression of synaptophysin, a constituent of synaptic vesicles and a marker for synaptic plasticity was examined using western blotting. As illustrated in Fig. (1A), we further analyzed the effect of resveratrol administration on the expression of PSD-95 in obese mice brain. PSD-95 is a membrane-associated guanylate kinase, known to play an important role in the regulation of

synaptic function and organization of neurotransmitter receptors in the synaptic cleft (Shao et al., 2011). We also measured the expression levels of ARC, a protein found in the dendrites of hippocampal neurons, essential for synaptic plasticity and formation of long term memory (Dorostkar and Herms, 2012). Moreover, we analyzed the effect of resveratrol administration on the expression of GFAP, a marker specific for astroglial cells. GFAP is intermediate filament protein highly expressed in astrocytes. Abnormal GFAP regulation as well as associated glial scarring is seen in various neurodegenerative diseases, including AD (Eng et al., 2000). Hence, brain lysates from *ob/ob* mice were western blotted with antibodies specific to synaptophysin, PSD-95, ARC and GFAP. Obese mice group exhibited decreased expression levels of synaptophysin, PSD-95, ARC and increased expression levels of GFAP as compared to the lean mice. Resveratrol administration significantly increased the expression of synaptophysin, PSD-95, ARC and decreased GFAP expression levels as compared to the obese mice group.

4.4.2 Resveratrol decreases Amyloid Beta Deposition in the brain of obese mice and Aβ-Induced H19-7 Cells. We further wanted to examine whether Aβ accumulation is associated with reduced IDE activity. IDE is a zinc metalloprotease responsible for insulin degradation is also known to play a crucial role in Aβ peptide degradation (Reitz and Mayeux, 2014). Cerebral insulin dysregulation disrupts insulin uptake across the blood brain barrier resulting in low insulin levels in the brain and reduced expression levels of IDE and Aβ degradation mediated by IDE. This further leads to an increased Aβ accumulation in neurons (Chen and Zhong, 2013). Hence, brain lysates from *ob/ob* mice were western blotted with antibody specific to IDE. Our results revealed decreased expression levels of IDE in obese mice brain when compared with the control group. Resveratrol administration enhanced the expression of IDE when compared with

the obese mice group (Fig 1A). In addition, we also analyzed the expression levels of two enzymes responsible for the pro amyloidogenic/non-amyloidogenic processing of APP, the β- (BACE) and α- (ADAM10) secretases. No alterations were observed in the expression of pro-amyloidogenic BACE enzyme whereas there was a significant decrease in the expression levels of non-amyloidogenic ADAM-10 enzyme in the obese mice brain. Resveratrol treatment had no significant effect on the expression levels of ADAM 10 enzyme in the obese mice group (Fig 1A). Furthermore, we also wanted to see whether resveratrol treatment favors non-amyloidogenic pathway in Aβ-Induced H19-7 Cells. Hence, lysates from Aβ-treated H19-7cells were prepared and western blotted with antibody specific to BACE and ADAM-10. Interestingly, we could see increased expression levels of non-amyloidogenic ADAM-10 enzyme in Aβ-Induced H19-7 Cells treated with resveratrol whereas no modifications were observed in the expression levels of pro-amyloidogenic BACE enzyme in Aβ-Induced H19-7 Cells treated with resveratrol (Fig 2A).

4.4.3 Resveratrol decreases Phosphorylated Tau in the brain of obese mice and Aβ-Induced H19-7 Cells. The levels of hyper phosphorylated tau forming aggregates such as PHFs or straight filaments serve as a promising marker of the severity of AD (Hu et al., 2002). Hyperphosphorylated tau promotes the formation of NFTs - one of the hallmarks of AD. Therefore, to determine the effect of resveratrol administration on the phosphorylation of tau, western blot analysis of phospho-specific tau species using the brain lysates from obese mice was performed. The monoclonal antibody CP13 that detects phosphorylated tau at serine 202 (S 202) in both early and more advanced stages of NFT accumulation, and phosphorylation-dependent antibody PHF1 that recognizes phosphorylated tau at serine 396 were used (Andorfer et al., 2003). In addition, phospho tau antibody, which recognizes hyper phosphorylated tau

protein, was used as a marker for NFTs. The expression of phosphorylated forms of tau and phospho tau were markedly increased in the brain of obese mice group as compared to the lean mice group. Resveratrol administration significantly reduced the expression of phosphorylated forms of tau (S202 and S396 residues) and phosphorylated tau when compared with the obese mice group (Fig 1B).

Further, we wanted to determine the effect of resveratrol administration on GSK-3β phosphorylation in obese mice brain. GSK-3β plays a vital role in tau pathology (LLorens-Martin et al., 2014). Abnormal brain insulin signaling results in tau hyper phosphorylation caused due to inhibition of PI3K/Akt and increased GSK-3β activation. The activation of GSK-3β causes reduced GSK-3β phosphorylation, thereby leading to phosphorylation of tau protein and formation of NFTs (Hohman et al., 2014). Hence, lysates from obese mice brain were used against antibodies specific to phospho GSK-3β. Western blot analysis showed reduced expression of phospho-GSK3β was reduced in obese mice brain as compared with the lean mice group. Resveratrol administration increased the expression of phospho-GSK3β when compared to obese mice brain group (Fig 1B). Interestingly, our recent findings have also demonstrated similar compelling results in the cell culture model of AD (Rege et al., 2015).

Moreover, tau hyperphosphorylation is mediated by activation of several other kinases in the brain (Porquet et al., 2013) Therefore we wanted to see the effect of resveratrol administration on the activation of various tau kinases such as CDK5 and JNK in the brain of obese mice. The expression levels of CDK5 and phosphorylated levels of JNK were analyzed using brain lysates from obese mice western blotted with antibodies specific to CDK5 and pJNK. No significant reduction in the expression of CDK5 protein levels was observed in the brain of obese mice group upon resveratrol administration. Increased phosphorylated levels of JNK were observed in

obese mice brain group as compared to the lean control mice whereas a drop in phosphorylated JNK levels were observed in resveratrol administered obese mice brain (Fig 1B). We further wanted to confirm if resveratrol treatment also exerts neuroprotective action on the activation of CDK5 and JNK tau kinases in Aβ-treated H19-7cells. Hence, lysates from Aβ-treated H19-7cells were prepared and western blotted with antibody specific to CDK5 and pJNK. Our findings revealed a significant increase in the expression levels of phosphorylated JNK in Aβ group but resveratrol treatment did not show any alterations in the expression of CDK5 as well as pJNK (Fig 2B).

4.4.4 Resveratrol enhances the expression of AMPK, CREB and BDNF in obese mice brain and Aβ-treated H19-7cells. AMP-activated protein kinase (AMPK), a cellular energy sensor plays a major role in glucose and lipid metabolism. Studies indicate that AMPK activation ameliorates disrupted neuronal energy metabolism observed in AD pathogenesis. Moreover, activation of AMPK signaling pathway can regulate amyloid protein (Aβ) generation (Vingtdeux et al., 2010; Won et al., 2010) and tau hyper phosphorylation (Thornton et al., 2011; Vingtdeux et al., 2011). Hence, we wanted to examine the effect of resveratrol on AMPK activation. Lysates from obese mice brain and Aβ-treated H19-7cells were western blotted using antibodies specific to phosphorylated AMPK (pAMPK) and total AMPK. Results showed that resveratrol significantly upregulated the expression levels of pAMPK in obese mice brain as well as Aβ-treated H19-7cells suggesting its role in AMPK activation (Fig 1C).

CREB plays a crucial role in synaptic plasticity and memory formation in the brain (Silva et al., 1998). Synaptic activity causes certain genes to turn on or off which further helps in the regulation of synaptic function that underlie learning and memory. CREB protein turns on the gene for BDNF (brain derived neurotrophic factor), a protective protein that promotes growth,

survival and differentiation of neurons and synapses. Reduced BDNF levels are seen in human AD brains and AD mouse models and are correlated with cognitive impairment. (Dickey et al., 2003; Phillips et al., 1991).

Therefore we examined if the expression levels of CREB and BDNF are regulated by resveratrol in obese mice brain as well as Aβ-treated H19-7cells. Lysates from obese mice brain and Aβ-treated H19-7cells were western blotted using antibodies specific to CREB and BDNF. Results showed that resveratrol significantly enhanced the expression of CREB in obese mice brain (Fig 1C) whereas no significant increase was observed in the expression of CREB in Aβ-treated H19-7cells (Fig 2C). Also, the level of BDNF, a CREB regulated gene was also increased in resveratrol treated obese mice brain (Fig 1C). On the contrary, no significant change was observed in the expression level of BDNF in Aβ-treated H19-7cells (Fig 2C). Significant results from the obese mice study suggest that up regulation of CREB protein might further facilitate BDNF synthesis (Zhao et al., 2013). These findings may suggest the role of resveratrol in improving cognitive function through a CREB- BDNF-mediated mechanism.

4.5 Discussion

Alzheimer's disease (AD) a progressive, irreversible neurodegenerative disorder is characterized by two primary neuropathological hallmarks: beta amyloid plaques and neurofibrillary tangles resulting in neuronal cell death and memory impairment (Kolarova et al., 2012; Selkoe, 2002). Currently, around 33.9 million people are diagnosed with AD worldwide and the rate of prevalence is expected to triple over the next 40 years (Barnes and Yaffe, 2011). The exact cause of AD is still unknown. Emerging research reports obesity, Insulin resistance and Type 2 diabetes as contributing risk factors in AD pathogenesis (Ho et al., 2004; Kohjima et al., 2010; Profenno et al., 2010; Schrijvers et al., 2010). Obesity is a global health issue affecting over one-

third of US adult population, with two-thirds overweight or obese (Li et al., 2005).

Obesity causes alterations in glucose and lipid metabolism and also has been shown to increase inflammation and oxidative stress (Qatanani and Lazar, 2007). Obese individuals develop insulin resistance which is characterized by peripheral hyperinsulinemia associated with reduced insulin transport across the blood brain barrier, resulting in impaired activation of insulin receptors in neurons and low insulin levels in the brain (Hribal et al., 2002; Saltiel and Kahn, 2001). Insulin plays a major role in memory and brain function; therefore, impaired insulin signaling promotes increased Aβ deposition and tau protein phosphorylation, both of which further contributes to nerve cell death and cognitive impairment (Calvo-Ochoa and Arias, 2014).

A growing body of literature has shown that resveratrol, a polyphenolic phytoalexin, exerts many beneficial and neuroprotective effects (Carrizzo et al., 2013). Therefore, the present study was designed to examine the neuroprotective effects of resveratrol on various proteins associated with memory impairment in the brain of *ob/ob* mice, a model of severe obesity with insulin resistance, resulting from defective leptin signaling as well as in Aβ-induced rat hippocampal cells, a cell culture model of AD.

Furthermore, to demonstrate a possible link between obesity associated insulin resistance and the development of AD, primarily the expression levels of markers involved in the process of neurodegeneration were examined. We observed reduced expression of synaptophysin, a synaptic marker in *ob/ob* mice brain. Also, GFAP, a marker specific for glia expressed in astrocytes provides structural stability to astrocytic processes by modulating the shape of cells and astrocyte movement. Astrocytes undergo molecular and morphological changes in response to CNS injuries or diseases, a condition termed as astrogliosis. It is characterized by rapid synthesis of GFAP and increase in GFAP protein content (Hol et al., 2003). Our results also

showed increased expression levels of GFAP in the brain of *ob/ob* mice. Resveratrol administration increased the expression of synaptophysin and decreased GFAP levels, indicating resveratrol could be a potential therapeutic strategy for preventing neurodegenerative processes in AD pathogenesis.

Further, we examined the effect of resveratrol on two hallmarks of AD: plaques composed of Aβ deposits and neurofibrillary tangles consisting of hyper phosphorylated tau. Another mechanism which links Insulin and IR to AD is mediated through Insulin degrading enzyme (IDE), a zinc metalloprotease responsible for clearance of Aβ; and both insulin and Aβ are substrates competing for IDE (Farris et al., 2003). Peripheral hyperinsulinemia causes a significant down regulation of insulin uptake across the BBB, resulting in low insulin levels in the brain and subsequent decrease in the expression of IDE and Aβ degradation mediated by IDE. This further increases accumulation of Aβ in the neuronal cells (Chen and Zhong, 2013; Farris et al., 2003). Our results support this notion, and a drop in the expression levels of IDE was observed in obese mice brain, which was improved on resveratrol administration. Moreover, we wanted to examine if resveratrol promotes non-amyloidogenic/ pro amyloidogenic processing of APP by modulating α- (ADAM10) and β- (BACE) secretases. ADAM 10 (A Disintegrin and Metalloproteinase 10), is one such α-secretase, which cleaves APP, inhibits the generation of neurotoxic Aβ and facilitates the production of secreted alpha cleaved APP fragment (sAPPα), which has been shown to exhibit neuroprotective effects (Prox et al., 2012). On the contrary, Aβ peptide is formed by sequential cleavage of APP by β secretase (BACE) and ϒ secretases (Vassar et al., 1999). Our findings reported increased expression levels of ADAM10 protein by resveratrol in both *ob/ob* mice brain and Aβ induced H19-7 cells. Resveratrol treatment showed no alterations

in BACE protein in *ob/ob* mice group whereas decreased expression levels of BACE were observed in Aβ induced H19-7 cells.

Moreover, several kinases are implicated in the regulation of tau hyperphosphorylation, another hallmark of AD. Our findings are consistent with the previous studies that show hyperphosphorylation of tau is mediated by the activation of various tau kinases such as CDK5, GSK3β, or JNK (Canudas et al., 2005; Porquet et al., 2013). Furthermore, glycogen synthase kinase-3β (GSK-3β) is one such kinase known to mediate tau hyper phosphorylation. Insulin signaling dysfunction leads to inhibition of PI3K/Akt and increased GSK-3β activation, hyper phosphorylation of Tau protein and accumulation of neurofibrillary tangles (NFTs) (Hohman et al., 2014). Our results showed that resveratrol treatment decreased the expression levels of phosphorylated tau, and phosphorylated forms of tau at S 202 and S 396, upregulated the phosphorylation of GSK-3β and downregulated the expression levels of both CDK5 and JNK in both ob/ob mice brain as well as Aβ induced H19-7 cells. Overall findings suggest the neuroprotective of resveratrol on IDE, ADAM 10, BACE and CDK5, GSK3β, JNK in the regulation of increased amyloid beta content and tau hyper phosphorylation, both of which are associated with memory impairment, a characteristic of AD.

AMPK is a chief regulator of cellular energy homeostasis. It also plays a major role in glucose and lipid metabolism (Cai et al., 2012). AMPK signaling regulates amyloid beta accumulation and tau hyperphosphorylation, the two major hallmarks of AD (Cai et al., 2012; Salminen et al., 2011). AMPK activation is caused by phosphorylation of Thr-172 by LKB1 complex due to elevation in the AMP/ATP ratio and by calmodulin-dependent protein kinase kinase-beta due to increased Ca (2+) levels, which facilitates regulation of Aβ generation (Cai et al., 2012; Porquet et al., 2013). AMPK is also known to upregulate the phosphorylation of tau at Ser-262. In

addition, AMPK activation promotes autophagy by lowering mTOR-signaling activity and causes lysosomal degradation of Aβ (Porquet et al., 2013). Our results showed that resveratrol treatment increased expression levels of phosphorylated AMPK in both *ob/ob* mice brain as well as Aβ induced H19-7 cells demonstrating that resveratrol activates and favors AMPK pathways. Furthermore, resveratrol treatment also upregulated the expression of CREB protein and level of BDNF, a CREB downstream target both in *ob/ob* mice brain as well as Aβ induced H19-7 cells. Our findings collectively suggest the importance of resveratrol in ameliorating cognitive function via CREB-BDNF mechanism and may demonstrate a potential therapeutic strategy in the treatment of AD and cognitive decline.

4.6 Conclusion

This study demonstrates a potential link between obesity associated insulin resistance, Type 2 diabetes and Alzheimer's disease. Indeed, resveratrol treatment not only favors AMPK pathways but also exerts neuroprotective effect on Tau hyper-phosphorylation and increased Aβ deposition, the two major AD hallmarks known to be associated with memory impairment. Further, resveratrol also protects the proteins associated with memory impairment and improves cognitive function via CREB-BDNF mechanism. Taken together, these findings demonstrate a potential neuroprotective effect of resveratrol in *ob/ob* mice brain and Aβ-induced rat hippocampal cells and additionally suggest a novel therapeutic strategy for AD.

Conflict of Interest: The authors of this study have no conflict of interest to disclose. This work was supported by the Alabama Agricultural Experimental Station, Hatch /Multistate Funding Program to JRB and Ida Belle Young Research award and New Faculty Grant in aid to TG.

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Figure legends:

Fig. 1 A. Effect of resveratrol on PSD95, ARC, synaptophysin, GFAP, IDE, ADAM and BACE in the brain of obese mice. Lysates from the obese brain tissue were analyzed by western blotting with PSD95, ARC, synaptophysin, GFAP, IDE, ADAM and BACE antibodies. B. Effect of resveratrol on CP 13, PHF1, Tau, CDK5, pJNK, JNK, GSK3βp and GSK3β in the brain of obese mice. Lysates from the obese mice brain tissue were analyzed by western blotting with CP 13, PHF1, Tau, CDK5, pJNK, JNK, GSK3βp and antibodies. C. Effect of resveratrol on pAMPK, AMPK, CREB, BDNF and Actin in the brain of obese mice. Lysates from the obese mice brain tissue were analyzed by western blotting with pAMPK, AMPK, CREB, BDNF and Actin antibodies. D. PSD95, ARC, synaptophysin, GFAP, IDE, ADAM, BACE, CP 13, PHF1, Tau, CDK5 protein levels were normalized to actin and pJNK, GSK3βp and pAMPK protein levels were normalized to total JNK, total GSK3β and total AMPK using Image J software. Values are expressed as Mean ± SEM, $*P < 0.05$, $**P < 0.01$ vs Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs *ob/ob* group.

Fig. 2 A. Effect of resveratrol on ADAM and BACE in Aβ-induced H19-7 cells. Cell lysates were analyzed by western blotting with ADAM and BACE antibodies. (B). Effect of resveratrol on CDK5, pJNK and JNK in Aβ-induced H19-7 cells. Cell lysates were analyzed by western blotting with CDK5, pJNK and JNK antibodies. (C) Effect of resveratrol on pAMPK, AMPK, CREB, BDNF and Actin in Aβ-induced H19-7 cells. Cell lysates were analyzed by western blotting with pAMPK, AMPK, CREB, BDNF and Actin antibodies. (D) ADAM, BACE, CDK5, CREB, BDNF protein levels were normalized to actin and pJNK, and pAMPK protein levels were normalized to total JNK and total AMPK using Image J software. Values are expressed as Mean ± SEM, $*P < 0.05$, vs Control, $^{\#}P < 0.05$ vs Aβ group

Fig 1.

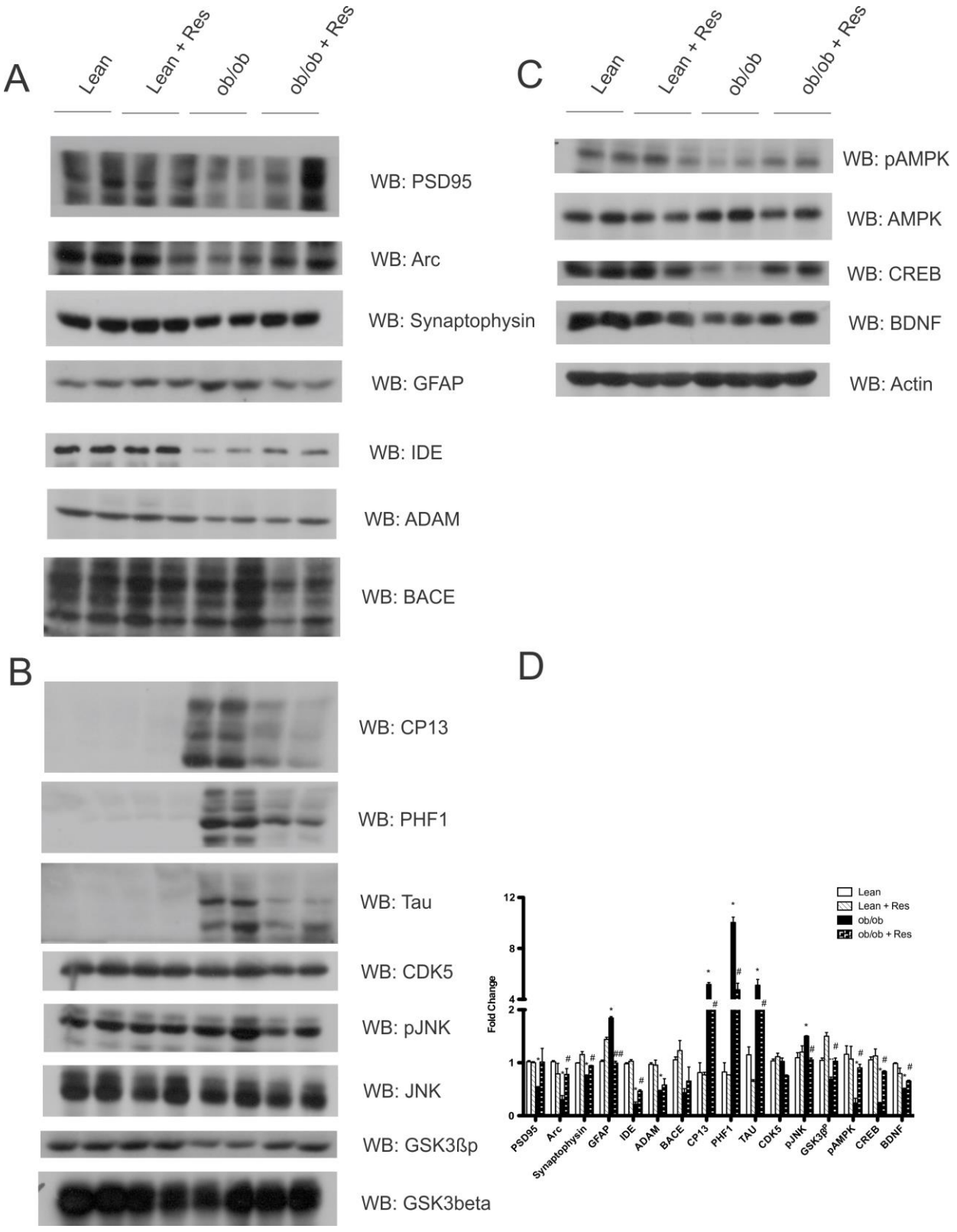
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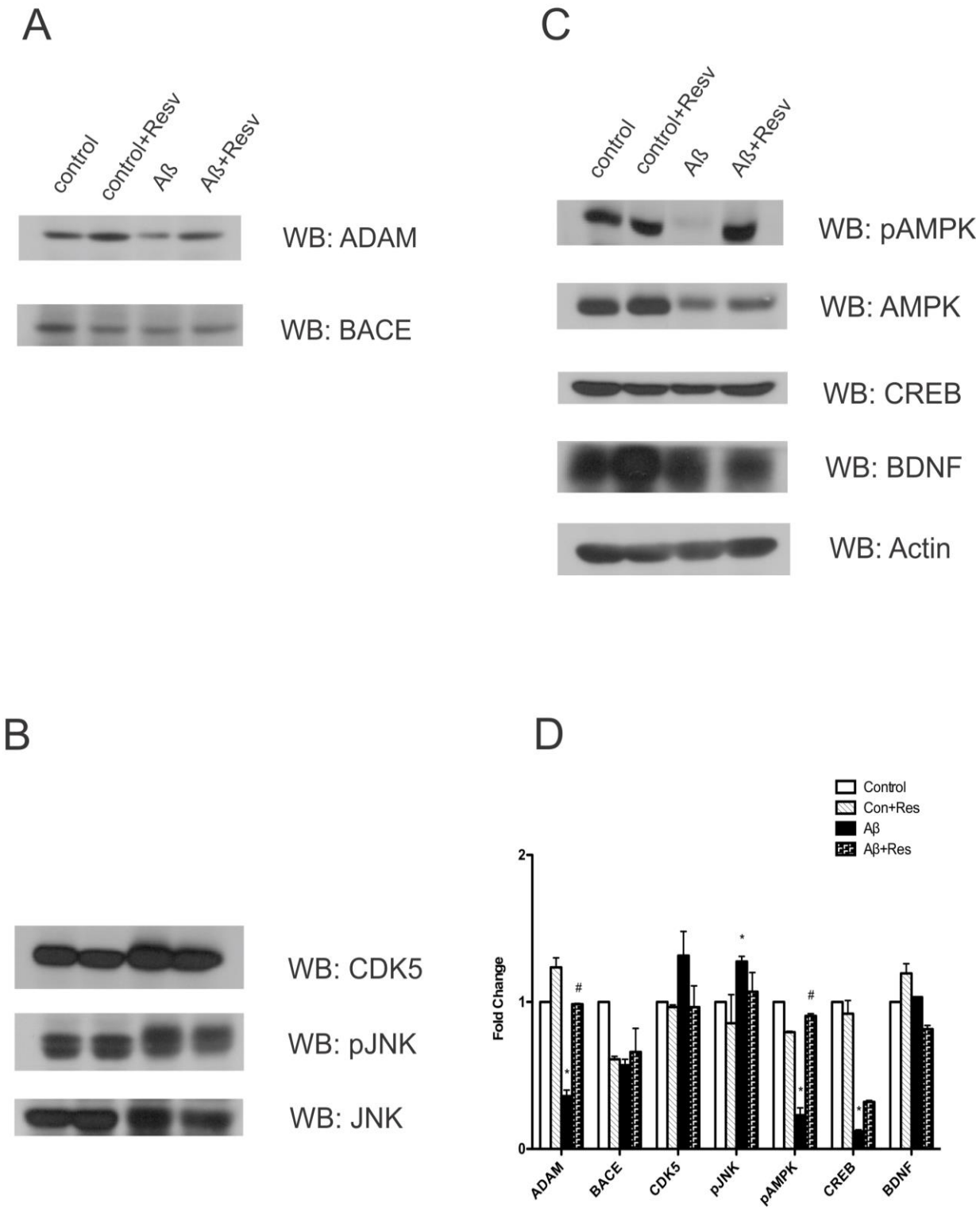
Fig 2.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Resveratrol Protects β amyloid-Induced Oxidative Damage and Memory Associated Proteins in H19-7 Hippocampal Neuronal Cells

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5.1 Abstract

Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is a polyphenolic phytoalexin known to exhibit antioxidant and neuroprotective effects in several experimental models. Amyloid β peptide (Aβ), a core component of extracellular senile plaques accumulates in the brains of patients with Alzheimer's disease and is related to the development of cognitive impairment and neuronal loss. The present study evaluates the neuroprotective action of resveratrol on Aβ-induced oxidative stress and memory loss. Cultured rat hippocampal H19-7 neuronal cell line were pretreated with 75 μM of resveratrol for 2 hrs followed by 25 μM of Aβ (1-40) for 24 hrs. H19-7 cells treated with Aβ exhibited increased lipid peroxide levels. Enzymatic antioxidants including superoxide dismutase, catalase, glutathione reductase, and non-enzymatic antioxidants such as tocopherol, ascorbic acid and glutathione were decreased in the Aβ treated group when compared to the control group. Aβ treatment also increased the expression of total tau as well as phosphorylated forms of tau (CP13, S202/205; PHF1, S396/404) and decreased the expression of insulin degrading enzyme (IDE), phospho glycogen synthase kinase 3 β involved in Aβ degradation and tau hyper phosphorylation. Expression of PSD-95 and Arc proteins, essential for synaptic maturity and plasticity, was decreased by Aβ treatment. Resveratrol treatment attenuated the accumulation of lipid peroxide levels, up-regulated the antioxidant activities and improved the expression of memory-associated proteins in Aβ treated H19-7 cells. These findings highlight the neuroprotective effect of resveratrol in preventing Aβ-induced oxidative damage and memory loss in vitro.

Keywords: Alzheimer's disease, β-amyloid, Insulin resistance, memory, oxidative stress, resveratrol, Type 2 diabetes.

5.2 Introduction

Alzheimer's Disease (AD) is the most common type of neurodegenerative disorder clinically characterized by a progressive loss of memory, cognitive function, and behavioral abilities (Kolarova et al., 2012). Currently, over 34 million people are diagnosed with AD worldwide and the prevalence is anticipated to triple over the next 40 years (Barnes and Yaffe, 2011). Despite this prevalence of AD, efficient disease modifying strategies and therapeutic options are still unavailable. Further, developing novel therapeutic approaches for AD is currently a challenging issue because of the multifactorial nature of the underlying pathogenesis of this disease.

Amyloid β protein (Aβ) is a peptide (36–43 amino acids) derived from the sequential cleavage of the transmembrane amyloid precursor protein (APP). It is a key component of amyloid plaques involved in the development and pathogenesis of AD (Huang et al., 2011; Selkoe, 2001). Extracellular senile plaques containing insoluble deposits of Aβ peptide and intracellular neurofibrillary tangles composed of hyper-phosphorylated tau, leading to premature neuronal death, are hallmarks of AD (Selkoe, 2002). Evidence indicates that Aβ-induced neuronal cell death is mediated through the excessive production of reactive oxygen species (ROS) (Butterfield et al., 2007). ROS are chemically reactive molecules that consist of oxygen ions and peroxides, including hydrogen peroxide singlet oxygen, and nitric oxide, peroxynitrite, and superoxide free radicals. The release of peroxides and free radicals induces cytotoxicity in neurons, which plays a key role in the etiology of neurodegeneration and neuronal death. Oxidative stress occurs due to the imbalance between the excessive generation of oxygen free radicals and antioxidant defense systems (Barnham et al., 2004; Rege et al., 2013). Oxidative stress alters multiple signaling pathways that may generate cellular responses such as

inflammation, cell proliferation, cell survival, and cell death. Oxidative stress associated with an increase in levels of ROS leads to the injury and destruction of vascular and neuronal cells and ultimately memory impairment (Barnham et al., 2004; Butterfield et al., 2007).

To effectively counteract these effects, the antioxidant enzymes such as superoxide dismutase (SOD), catalase, peroxidases, and non-enzymatic free radical scavengers (ascorbic acid, α-tocopherol, and GSH) convert the ROS to stable molecules such as water and oxygen. These antioxidants are either produced endogenously or obtained exogenously through the diet, and protect cells and tissues against oxidative stress induced by cellular toxicity (Irshad and Chaudhuri, 2002). One approach to prevent the cellular or tissue damage induced by ROS is to increase the production of natural endogenous antioxidant defense systems through dietary or pharmacological intake of antioxidants.

Resveratrol (3, 5, 4' trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin found in skin and seeds of commonly ingested plant species, including grapes, grains, tea, and peanuts (Li et al., 2012; Zhang et al., 2013). A growing body of evidence suggests resveratrol exerts anti-diabetic, anti-inflammatory, and anti-carcinogenic effects (Bertelli et al., 1999). We have recently demonstrated that resveratrol exerts a robust neuroprotective effect on the brain of obese diabetic mice (Rege et al., 2013). Indeed, treatment of obese diabetic mice with resveratrol prevented the accumulation of lipid peroxide levels and enhanced antioxidant function, including the activity of SOD and catalase. The present study was designed to evaluate the neuroprotective effects of resveratrol against Aβ-induced oxidative damage and other proteins associated with memory impairment in Aβ-induced cultured rat hippocampal (H19-7) cells.

5.3 Materials and methods

5.3.1 Reagents. Resveratrol (trans) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Resveratrol was dissolved in ethanol as a 200 mM stock solution and stored in aliquots at -20°C. Beta amyloid ($A\beta_{1-40}$) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). $A\beta_{1-40}$, a neurotoxic peptide fragment derived from APP (Cleary et al., 1995), was dissolved in deionized distilled water at a concentration of 1 mM and stored at -20°C. The stock solutions were diluted to the desired concentrations immediately before use and added to the culture medium without the aging procedure. All other chemicals and solvents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Antibodies against synaptophysin, activity-regulated cytoskeleton-associated protein (Arc (C-7)), glial fibrillary acidic protein (GFAP) and Tau (C-7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and insulin degrading enzyme (IDE), postsynaptic density protein (PSD95) (6G6-1C9) from Abcam (Cambridge, MA). Antibodies specific to CP13 and paired helical filaments (PHF1) were obtained from Dr. Peter Davis, Albert Einstein College of Medicine, Bronx, New York. Phospho- GSK3β (Ser 9) that recognizes phosphorylation at serine 9 and GSK3β that detects endogenous levels of total GSK3β protein were purchased from Cell Signaling Technology (Danvers, MA). Beta actin was purchased from Sigma (St. Louis, MO, USA).

5.3.2 Cell culture. The H19-7 cell line was derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos and immortalized by retroviral transduction of temperature sensitive tsA58 SV40 large T antigen. All cells were cultured in poly-D-lysine-coated culture dishes and were maintained in 10% fetal bovine serum (FBS), 0.001mg/ml puromycin, and 0.2 mg/ml G418 at 34°C. Cells were serum starved overnight prior to treatment.

5.3.3 Cell treatment. Cultured cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM $A\beta_{1-40}$ for 24 h. After treatment, cells were lysed with HEPES lysis buffer (50 mM HEPES, pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM β-glycerophosphate, 1% Triton, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin.

5.3.4 Lactate dehydrogenase assay. This assay was done using a CytoTox 96 non- radioactive cytotoxicity assay kit from Promega (Madison, WI), according to the manufacturer's instructions. H19-7 cells were seeded in poly-D-lysine precoated 96-well plates (about $1x10^5$ cells $well^{-1}$) for 24 h for cytotoxicity analysis. Cells were cultured at 34°C in 95 % humidified atmosphere with 5% CO2 until use. After various treatments, medium from each well was collected to measure the amount of released LDH. Cells in separate sister wells were exposed to lysis buffer (9% Triton X100) and media was collected to measure the total amount of cellular LDH. Each experimental and control reaction was performed in triplicate. The amount of LDH from each sample was measured at the wavelength of 490 nm by a BioTek EL-808 microplate reader. The percentage of released LDH vs. total intracellular LDH was calculated and reflected the amount of cell death (Jiang et al., 2009; Lu et al., 2010)

5.3.5 Estimation of protein. The amount of protein in the cells was measured using Pierce 660nm Protein Assay and BSA as standard. 150 μL of protein assay reagent was added to 10 μL of the cell lysate, and the optical density was measured after five min at 630 nm. Protein concentrations are expressed as mg/mL.

5.3.6 Lipid peroxidation. Lipid peroxidation was estimated in the cell lysates by the method of Hogberg et al. (Högberg et al., 1974) using thiobarbituric acid, an end-product assay widely used

to assess oxidative damage. The release of malondialdehyde (MDA) as an end product of peroxidation of lipids served as an index of the intensity of oxidative stress.

5.3.7 Enzymatic antioxidants. Antioxidant enzymes were estimated in the cell lysates. SOD activity was determined by using the pyrogallol assay as described by Marklund and Marklund (Marklund and Marklund, 1974). Catalase activity was assayed by the method of Sinha (Sinha, 1972) and glutathione peroxidase (GPX) by the method of Rotruck et al. (Rotruck et al., 1973). The oxidation of glutathione was used to express the activity. Glutathione reductase (GR) which utilizes NADPH for the conversion of oxidized glutathione (GSSG) to the reduced form (GSH) was measured by the method of Staal et al. (Staal et al., 1969). The activity of glucose-6-phosphate dehydrogenase (G6PDH) was assayed by the method of Ells and Kirkman (Ells and Kirkman, 1961).

5.3.8 Non-enzymatic antioxidants. Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. Dehydroascorbic acid reacts with 2,3-dinitrophenyl hydrazine to form the derivative 2,4-dinitrophenyl hydrazine. In strong sulphuric acid, this compound undergoes a rearrangement to form a product with absorption maximum at 520 nm. The reaction was run in the presence of thiouria to prevent the interference of non-ascorbic acid chromogens. α-Tocopherol was estimated by the method of Quaife et al. (Quaife et al., 1949). Glutathione activity was assayed by the method of Moron et al. (Moron et al., 1979). All spectrophotometric analyses were performed with a Beckman Coulter DU ® 730 UV/Vis spectrophotometer.

5.3.9 Western blot analysis: Insoluble formic acid fractions from amyloid β-induced H19-7 cells were prepared and samples were boiled in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 10% SDS-PAGE, transferred onto

polyvinylidene fluoride (PVDF) membranes (Millipore, Temecula, CA), analyzed by western blotting with the appropriate antibodies, and detected by enhanced chemiluminescence.

5.3.10 Statistical Analysis. All values are expressed as mean ± standard error of mean (SEM) in each group. Statistical difference between groups was assessed by one-way ANOVA followed by Tukey-Kramer analysis with equal variance. Significance was set at $P < 0.05$. Quantification of all the western blots was done using Image J software.

5.4 Results

5.4.1 Effect of resveratrol on cytotoxicity in H19-7 cells To examine whether resveratrol induces cell death in H19-7 rat hippocampal cells, H19-7 cells were treated with different doses of resveratrol (0, 25, 50, 75, 100 and 125 μM) for 24 hours. The extent of cell death was measured after 24 hours by LDH release assay (Fig. 1), since the amount of LDH released from dying cells into the culture medium is proportional to the amount of cell death. Resveratrol at the concentrations of 25 μM (97.13 ± 0.16 %), 50 μM (98.24 ± 0.25 %), 75 μM (98.62 ± 0.14 %) of control did not show cytotoxicity in H19-7 rat hippocampal cells when compared to untreated control cells. On the Contrary, a significant increase in resveratrol-induced LDH release was observed at the concentrations of 100 μM (142.57 ± 0.09 %; $*P < 0.05$) and 125 μM (157.99 ± 0.09 %; $*P < 0.05$) when compared to untreated control cells. Highlights of the results are summarized in figure 1. These results suggest that no signs of resveratrol-mediated toxicity were observed at the dose of 75 μM and therefore was selected for the study. In accordance with the results obtained in our study, various previous studies have also reported cytoprotective and neuroprotective properties of resveratrol with 75 μM concentration in neuronal cell cultures (Hsu et al., 2011; Kairisalo et al., 2011; Nguyen et al., 2013; Rubiolo et al., 2008; Sakata et al., 2010).

5.4.2 Resveratrol attenuates lipid peroxidation in Aβ-induced H19-7 cells: To determine the anti-oxidative activity of resveratrol on Aβ-induced oxidative stress, the levels of lipid peroxidation were assayed by measuring the levels of MDA. Figure 2 shows the effect of resveratrol on MDA. The levels of MDA were substantially increased ($P < 0.01$) in Aβ-induced H19-7 cells (1.46 ± 0.02) when compared with the control group (1.35 ± 0.02). Resveratrol pre-treatment of cells for 2 h reduced ($P < 0.01$) the lipid peroxidation (1.34 ± 0.02) when compared with Aβtreated H19-7 cells (1.46 ± 0.02).

5.4.3 Resveratrol upregulates the enzymatic antioxidants in Aβ-induced H19-7 cells We also studied the effect of resveratrol on the antioxidant enzyme activity levels in Aβ-treated H19-7 cells. Figure 3 presents the levels of antioxidant enzymes in control and Aβ-induced H19-7 cells with or without resveratrol treatment. We found that in Aβ-induced H19-7 cells, the activities of antioxidant enzymes SOD, catalase, and GR were reduced [SOD (0.65 ± 0.02), catalase (1.23 ± 0.02): $P < 0.01$; GR (0.90 ± 0.14): $P < 0.05$] when compared with the control group [SOD (0.74 ± 0.01), catalase (1.33 ± 0.02), GR (1.35 ± 0.15)]. On the other hand, the activity of GPX was increased in the Aβ-induced H19-7 cells (0.42 ± 0.00, $P <0.05$) whereas there was no significant difference observed in the activity of G6PD in Aβ-induced H19-7 cells (1.50 ± 0.30) when compared with the control group (1.18 ± 0.02). Resveratrol pre-treatment for 2 h enhanced ($P < 0.05$) SOD (0.72 ± 0.01), catalase (1.30 ± 0.02), and GR activity (1.35 ± 0.15) compared with Aβ-treated H19-7 cells. However, resveratrol pre-treatment for 2 h did not cause any significant change in GPX (0.43 ± 0.00) and G6PD (1.38 ± 0.24) activity.

5.4.4 Resveratrol enhances the non-enzymatic antioxidants in Aβ-induced H19-7 cells: We further analyzed the effect of resveratrol on the non-enzymatic antioxidant activities in Aβ-treated H19-7 cells. Figure 4 represents the levels of non-enzymatic antioxidant in control and

Aβ-induced H19-7 cells with or without resveratrol pre-treatment. The non-enzymatic antioxidants were reduced in Aβ-induced H19-7 cells. The levels of ascorbic acid (1.23 ± 0.02; $P<0.001$), α–tocopherol (2.02 ± 0.12; $P<0.05$), and GSH (0.64 ± 0.02; $P < 0.001$) were significantly decreased in Aβ-induced H19-7 cells compared with the control group [ascorbic acid (1.38 ± 0.02), α–tocopherol (2.42 ± 0.12), GSH (0.78 ± 0.02]. Resveratrol pre-treatment for 2 h of Aβ-induced H19-7 cells enhanced the levels of ascorbic acid (1.36 ± 0.02; $P < 0.01$), α -tocopherol (2.38 ± 0.12; $P < 0.05$), and GSH (0.75 ± 0.02; $P < 0.01$) compared with Aβ treated H19-7 cells.

5.4.5 Resveratrol treatment improves markers of neurodegeneration: Neural function is dependent on efficient synaptic transmission, and synaptic loss is a pathological feature that correlates with cognitive impairment in subjects with AD. Synaptic activity is required for synaptic plasticity and formation of memory (Gouras et al., 2010). Therefore, to determine synaptic activity in Aβ-cultured hippocampal cells, expression of synaptophysin, a constituent of synaptic vesicles and a marker for neurodegeneration, was examined using the western blot procedure. As illustrated in Fig 5A, we further analyzed the effect of Aβ treatment on PSD-95, a membrane-associated guanylate kinase, which is involved in the regulation of synaptic function and organization of neurotransmitter receptors in the synaptic cleft (Shao et al., 2011). We also measured the expression of ARC, a protein enriched in the dendrites of hippocampal neurons, that regulates synaptic strength and mediates long-term memory formation (Dorostkar and Herms, 2012). In addition, we analyzed the effect of Aβ administration and resveratrol treatment on the expression of GFAP, a marker specific for astroglial cells. GFAP is expressed in astrocytes. Aberrant GFAP regulation and subsequent glial scarring is observed in various disease states, including AD (Eng et al., 2000).

H19-7 cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM Aβ1-40 for 24 h. Insoluble protein fractions obtained from Aβ-treated rat H19-7 cells were western blotted with antibodies specific to synaptophysin, PSD-95, ARC, and GFAP. Aβ administration decreased the expression levels of synaptophysin, PSD95, and ARC, and increased the levels of GFAP as compared the control group. Resveratrol pre-treatment for 2 hr increased the expression of synaptophysin, PSD-95, and ARC, and decreased GFAP levels compared to Aβ-treated cells (Fig. 5 A).

5.4.6 Resveratrol treatment decreases amyloid beta deposition in Aβ-induced H19-7 cells

We next wanted to test whether Aβ accumulation is associated with reduced IDE activity, a zinc metalloprotease responsible for insulin degradation that has been reported to play a role in Aβ peptide degradation (Reitz and Mayeux, 2014). Impaired insulin signaling promotes increased Aβ deposition and tau protein phosphorylation. In compensatory hyperinsulinemia, insulin uptake across the blood brain barrier is decreased, resulting in low insulin levels in the brain and a subsequent decrease in the expression of IDE and Aβ degradation mediated by IDE. This further leads to an increased deposition of Aβ in neurons (Chen and Zhong, 2013). Hence, lysates from insoluble formic acid fractions from Aβ-treated H19-7 cells were prepared and western blotted with antibody specific to IDE. Our results revealed that the expression of IDE was decreased in Aβ-treated H19-7 cells when compared with the control group. Resveratrol pre-treatment for 2 hr enhanced the expression of IDE when compared with the Aβ-treated cells (Fig. 5A).

5.4.7 Resveratrol treatment decreases phosphorylated tau in amyloid β-induced H19-7 cells

In AD, tau is hyperphosphorylated-forming aggregates such as PHFs or straight filaments and thereby promotes the formation of NFTs - one of the hallmarks of AD. To determine the effect of

Aβ administration on the phosphorylation of tau, western blot analysis of phospho-specific tau species in insoluble protein fractions obtained from Aβ-treated rat hippocampal (H19-7) cells was performed. The monoclonal antibody CP13, specific to phosphorylated tau at serine 202 (S 202), was used in the detection of tau pathology in both early (pre-tangle) and more advanced stages of NFT accumulation, and phosphorylation-dependent antibody PHF1 was used as a marker for tangles because it recognizes phosphorylated tau at serine 396 (Andorfer et al., 2003). In addition, the tau antibody, which recognizes total tau protein, was used as a marker for NFTs. The expression of phosphorylated forms of tau and total tau were substantially increased in Aβ-treated H19-7 cells compared with the control group. Resveratrol pre-treatment for 2 hr significantly reduced the expression of phosphorylated tau (S202 and S396 residues) and total tau when compared with Aβ-treated neuronal cultures (Fig 5B).

Further, we wanted to determine the effect of resveratrol treatment on GSK-3β phosphorylation in Aβ-treated neuronal cultures. GSK-3β is one of the kinases implicated in tau pathology (LLorens-Martin et al., 2014). Cerebral insulin dysfunction can result in hyper-phosphorylation of tau due to inhibition of PI3K/Akt and increased activation of GSK-3β. The activation of GSK-3β leads to decreased GSK-3β phosphorylation, thereby causing phosphorylation of tau protein and the formation of NFTs (Hohman et al., 2014). Lysates from insoluble formic acid fractions from Aβ-treated H19-7 cells were prepared and western blotted with antibody specific to phospho-GSK3β. Western blot analysis revealed that the expression of phospho-GSK3β was reduced in Aβ-treated H19-7 cells compared with the control group. Resveratrol pre-treatment for 2 hr increased the expression of phospho-GSK3β when compared to Aβ-treated cells (Fig. 5C).

5.5 Discussion

AD is a progressive age-related neurodegenerative disorder that poses increasing challenges to the global healthcare system and economic development. AD is characterized by extracellular neurotic plaques composed of Aβ deposits and intracellular neurofibrillary tangles composed of hyper-phosphorylated tau with progressive loss of synapses in the brain (Selkoe, 2002). Evidence demonstrates a potential link between oxidative stress (Jang and Surh, 2003) and AD development (Calvo-Ochoa and Arias, 2014). Indeed, elevations in the levels of ROS can cause destruction of proteins, nucleic acids, and membrane polyunsaturated fatty acids, causing lipid peroxidation. In addition, ROS leads to deficits in membrane integrity, reductions in mitochondrial membrane potential, and increased permeability of the plasma membrane to Ca^{2+} (Sun et al., 2008). Down-regulation in antioxidant defense mechanisms and elevated ROS generation leads to oxidative stress-mediated neurodegeneration (Gemma et al., 2007).

Compelling evidence has shown that resveratrol, a polyphenolic phytoalexin, exerts many beneficial and neuroprotective effects (Carrizzo et al., 2013). Recently, resveratrol has been shown to exert neuroprotective effects in rats from Aβ-induced neurotoxicity by reducing the inducible nitric oxide synthase and lipid peroxides, thereby reducing memory loss (Huang et al., 2011). The present study was designed to examine the effects of resveratrol against Aβ-induced oxidative stress and memory impairment in rat hippocampal cells.

Resveratrol has been shown to exert neuroprotective actions against amyloid β-induced oxidative stress in PC12 cells (Jang and Surh, 2003). It has also been found to inhibit oxidative stress-mediated neuronal cell death and subsequently suppress lipid peroxidation (Tadolini et al., 2000; Virgili and Contestabile, 2000). Exposure of polyunsaturated fatty acids to ROS leads to the generation of toxic lipid peroxidation products in the brain. Similarly, an increase in the levels of

lipid peroxidation was observed in Aβ-induced rat hippocampal cells, confirming previous reports (Keller et al., 2004). Enzymatic antioxidants such as SOD, catalase, and GPX act as the cellular antioxidant defense mechanism against free radicals. Since NADPH is required for the regeneration of catalase from its inactive form, catalase activity might be decreased in Aβ-induced rat hippocampal cells due to reduced NADPH levels. In this study, we have reported that resveratrol treatment significantly increased the enzymatic antioxidant activities in Aβ-induced rat hippocampal cells. In addition, non-enzymatic antioxidants also exhibited beneficial neuroprotective effects against oxidative stress. GSH is an endogenous non-enzymatic antioxidant that prevents damage to cellular components caused by ROS such as free radicals and peroxides. GSH is oxidized to glutathione disulfide (GSSG) by ROS, thereby causing a reduction in the level of GSH. GR reduces GSSG to GSH via NADPH, which in turn is released by glucose-6-phosphate dehydrogenase (Dincer et al., 2002). Resveratrol treatment upregulated the activity of these antioxidants in Aβ-treated rat hippocampal cells. Resveratrol treatment also increased the levels of α–tocopherol and ascorbic acid, which were reduced in Aβ-treated rat hippocampal cells.

Ascorbic acid is a water-soluble antioxidant that neutralizes the degradation reaction of tocopherol to form tocopheroxyl radical (Lykkesfeldt and Moos, 2005). α -tocopherol is a lipophilic antioxidant that acts as a peroxyl radical scavenger. It inhibits lipid peroxidation and leads to formation of the α–tocopherol radical, which is then oxidized by ascorbic acid and converted to its reduced state (Traber and Stevens, 2011). In the present study, a significant rise in the levels of ascorbate and α tocopherol was noted in rat hippocampal cells treated with resveratrol. This could be due to an increase in GSH upon resveratrol treatment.

In addition to oxidative stress, a strong association between insulin resistance and the development of AD has been demonstrated. Several studies have reported that insulin resistance (IR), an underlying characteristic of type 2 diabetes, is an important risk factor for AD and causes an increase in age-related memory impairment (Luchsinger et al., 2004; Schrijvers et al., 2010). Insulin regulates neuronal function after crossing the blood brain barrier by a saturable receptor-mediated process and by facilitating glucose uptake in neurons (Moreira et al., 2013). Insulin receptors are localized in hippocampus and medial temporal cortex, which suggests that insulin influences memory and learning (Craft, 2009). Optimal cerebral insulin levels augment memory and facilitate synaptic plasticity in the hippocampus and are considered to be neuroprotective (van der Heide et al., 2005; Zhao et al., 2004). Early stages of IR are marked by peripheral hyperinsulinemia associated with impaired insulin transport across the blood brain barrier, resulting in impaired activation of insulin receptors in neurons and reduction of insulin levels in the brain. Therefore, impaired insulin signaling promotes increased Aβ deposition and tau protein phosphorylation (Calvo-Ochoa and Arias, 2014). Another mechanism that links insulin and IR to AD is mediated through IDE, an enzyme responsible for clearance of Aβ; and both insulin and Aβ are substrates that compete for IDE (Farris et al., 2003). In patients with IR, compensatory hyperinsulinemia causes downregulation of insulin uptake across the blood brain barrier resulting in low insulin levels in the brain and a subsequent decrease in the expression of IDE and the Aβ degradation mediated by IDE. This leads to an increased deposition of Aβ in the neuronal cells (Chen and Zhong, 2013, 201; Farris et al., 2003). Furthermore, GSK-3β is one such kinase implicated in the regulation of tau hyper-phosphorylation. Insulin signaling dysfunction leads to inhibition of PI3K/Akt and increased GSK-3β activation resulting in hyper-phosphorylation of tau protein and accumulation of NFTs (Hohman et al., 2014). These findings

suggest a role for IDE and GSK-3β in the regulation of increased Aβ content and tau hyper-phosphorylation, both of which are associated with memory impairment. In addition to these proteins there are several other enzymes implicated in both amyloid and tau pathology.

Therefore, to demonstrate a possible link between type 2 diabetes and AD progression through these described mechanisms and to establish a relationship between Aβ deposits and AD-related neurodegeneration in Aβ-induced rat hippocampal cells, the levels of the synaptic marker, synaptophysin, which is involved in the process of neurodegeneration, were examined. Synapses are known to be the early sites of pathology and synaptic loss is considered to correlate with memory loss in AD patients (Tampellini et al., 2010). We observed decreased expression of synaptophysin in Aβ-induced rat hippocampal cells. Aβ treatment induced elevated expression of GFAP, a marker specific for glia expressed in astrocytes in the central nervous system. GFAP is thought to play a role in controlling the shape of cells and modulates astrocyte movement by providing structural stability to astrocytic processes. Injury caused by disease or trauma causes astrocytes to become highly reactive and respond rapidly in a characteristic way, a condition termed as *astrogliosis*. It is characterized by rapid synthesis of GFAP and is demonstrated by elevations in GFAP protein content (Hol et al., 2003). This supports the idea that synaptic dysfunction induced by Aβ is the fundamental marker of AD and precedes neuronal cell death. Synaptic activity is essential for synaptic plasticity and memory formation. Hence, we further wanted to investigate the effect of Aβ induction on PSD-95, a membrane-associated guanylate kinase and potent regulator of synaptic strength, which is essential for synaptic maturation and plasticity (Leuba et al., 2008), and ARC , a protein enriched in the dendrites of hippocampal neurons essential for synaptic plasticity and long-term memory formation (Kerrigan and Randall, 2013). Aβ administration decreased the levels of PSD95 and ARC. Our results showed

resveratrol treatment increased the expression of synaptophysin, PSD95, and ARC and decreased GFAP levels, indicating resveratrol could potentially be developed as a therapeutic strategy for preventing neurodegenerative processes involved in AD.

Further, we examined the effect of resveratrol on two hallmarks of AD: neurofibrillary tangles composed of phosphorylated tau and plaques consisting of Aβ deposits. In AD, tau becomes abnormally phosphorylated, aggregates into PHFs and forms NFTs. Insulin is known to be a principle cause of tau protein hyper-phosphorylation. In accordance with these effects, insulin was found to temporarily increase tau phosphorylation in primary cortical neurons, and hyperinsulinemia eventually resulted in tau hyper-phosphorylation in rat brains (Schubert et al., 2003). Peripheral hyperinsulinemia inhibits insulin transport across the blood brain barrier causing a drop in the brain insulin content. Impaired insulin signaling results in hyper-phosphorylation of tau due to inhibition of PI3K/Akt, and leads to GSK-3β dephosphorylation and activation (Neumann et al., 2008).

An increase in GSK-3β activation might also lead to an elevation in Aβ production and an increase in tau phosphorylation associated with NFT formation (Phiel et al., 2003). Our findings demonstrated increased expression of phosphorylated tau, and phosphorylated forms of tau at S 202 and S 396, and decreased amounts of phosphorylated GSK3β levels in our cell culture model of AD, all of which were efficiently reversed by resveratrol treatment. Another link between insulin resistance and amyloid pathogenesis is associated with IDE, a zinc metalloprotease responsible for the clearance of Aβ deposits. Peripheral hyperinsulinemia eventually may lead to a decrease in the activity of IDE and consequently the degradation of Aβ thereby increasing the accumulation of Aβ in neurons (Farris et al., 2003). Our findings support this notion, and a decrease in the expression of IDE was observed in Aβ-induced rat hippocampal cells, which was

ameliorated with resveratrol treatment. Thus, our study outlines the potential mechanisms linking type 2 diabetes and AD and reveals a natural therapeutic approach to the treatment of AD by demonstrating the potential neuroprotective role of resveratrol.

Several behavioral studies have reported that hippocampus plays a major role in learning and memory depending on the structural and functional changes that occur in hippocampus (Squire, 1992) and especially it is the brain area that is widely affected during the development of AD (Mu and Gage, 2011). As, H19-7 cell line is derived from the embryonic rat hippocampus, it is known to possess the characteristics of hippocampal neurons (Bhargava et al., 2002). Also, this cell line has shown to express morphological and phenotypical markers of neuronal, glial or bipotential lineage and thereby widely used in the studies on development and plasticity in hippocampal neurons. (Eves et al., 1992; Morrione et al., 2000). Hippocampal neurons are involved in learning and functioning of memory and as these areas are commonly seen to be affected in AD; H19-7 cell line is therefore used for better understanding of AD pathogenesis (Mu and Gage, 2011; Shin et al., 2011). Hence, this cell-based system, which correlated with AD, was used in our study (Jung et al., 2010; Oh et al., 2008; Pugazhenthi et al., 2003; Shin et al., 2011).

5.6 Conclusion

This study demonstrated a potential link between oxidative stress, type 2 diabetes, and AD progression. Indeed, resveratrol treatment was found to ameliorate cellular antioxidant defense systems. Tau hyper-phosphorylation and increased amyloid beta deposition that are known to be associated with memory impairment were reversed with resveratrol treatment. Further, resveratrol also protected the proteins associated with memory loss. Taken together, these

findings demonstrate a potential neuroprotective effect of resveratrol in Aβ-induced rat hippocampal cells.

Abbreviations

AD, Alzheimer's disease; Aβ, Amyloid beta; ARC, Activity-regulated cytoskeleton-associated protein; IDE, Insulin-degrading enzyme; BBB, Blood brain barrier; GFAP, Glial fibrillary acidic protein; GSK 3 β, Glycogen synthase kinase 3 beta; GPx, Glutathione peroxidase; GR, Glutathione reductase, GSH, Glutathione, G6PD, Glucose-6-phosphate dehydrogenase, GSSG, Glutathione disulfide; NFT, Neurofibrillary tangles; PHFs, Paired helical filaments; PSD-95, Postsynaptic density protein-95; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

Conflict of Interest

The authors of this study have no conflict of interest to disclose. This work was supported by the Alabama Agricultural Experimental Station, Hatch /Multistate Funding Program to JRB and Ida Belle Young Research award and New Faculty Grant in aid to TG.

Acknowledgements

Conceived and designed the experiments: JRB TG TLB. Performed the experiments: SDR. Analyzed the data: SDR JRB TG TLB. Contributed reagents and materials: JRB. Wrote the paper: SDR JRB TG TLB.

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Figure legends

Fig. 1. Effect of resveratrol on cytotoxicity in H19-7 cells. Cells were treated with resveratrol at various concentrations (0, 25, 50, 75, 100 and 125 μM) for 24 h. Cytotoxicity was assayed with LDH release assay. Relative LDH release as percentage of control is shown in the bar graph. Values are expressed as mean ± SEM of each experimental and control experiment performed in triplicates. Values are statistically significant at *P <0.05.

Fig. 2. Effect of resveratrol on the levels of malondialdehyde in Aβ-induced H19-7 cells. Cultured cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM $A\beta_{1-40}$ for 24 h. Lipid peroxidation was estimated in the cell lysates using spectrophotometric analysis. Values are expressed as mean ± SEM for each group. Values are statistically significant at $^{**}P < 0.01$.

Fig. 3. Effect of resveratrol on the enzymatic antioxidants in Aβ-induced H19-7 cells. Cultured cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM $A\beta_{1-40}$ for 24 h and the enzymatic antioxidant levels of A) SOD, B) Catalase, C) GPx, D) Glutathione reductase and E) G6PD were estimated in the cell lysates using spectrophotometric analysis. Values are expressed as mean ± SEM for each group. Values are statistically significant at $^{**}P < 0.01$ and $^{*}P < 0.05$. NS represents non-significant.

Fig. 4. Effect of resveratrol on the levels of non-enzymatic antioxidants in Aβ-induced H19-7 cells. Cultured cells were pre-treated for 2 h with 75 μM resveratrol followed by exposure to 25 μM $A\beta_{1-40}$ for 24 h and the non-enzymatic antioxidant levels of A) Vitamin C, B) Vitamin E and C) GSH was estimated in the cell lysates using spectrophotometric analysis. Values are expressed as mean ± SEM for each group. Values are statistically significant at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

Fig. 5 A. Effect of resveratrol on IDE, synaptophysin, PSD95, ARC and GFAP in Aβ-induced H19-7 cells. Insoluble formic acid fractions were analyzed by western blotting with IDE, synaptophysin, PSD95, ARC, GFAP, and Actin antibodies. **B.** Effect of resveratrol on CP 13, PHF1 and Tau in Aβ-induced H19-7 cells. Insoluble formic acid fractions were analyzed by western blotting with CP 13, PHF1 and Tau antibodies. **C.** Effect of resveratrol on $GSK3\beta^{p}$ and total GSK3β in Aβ-induced H19-7 cells. Insoluble formic acid fractions were analyzed by western blotting with $GSK3\beta^{p}$ and GSK3β antibodies. **D.** IDE, synaptophysin, PSD95, ARC, GFAP, CP 13, PHF1 and Tau protein levels were normalized to actin and $GSK3\beta^{p}$ protein levels were normalized to total GSK3β using Image J software.

Fig 1.

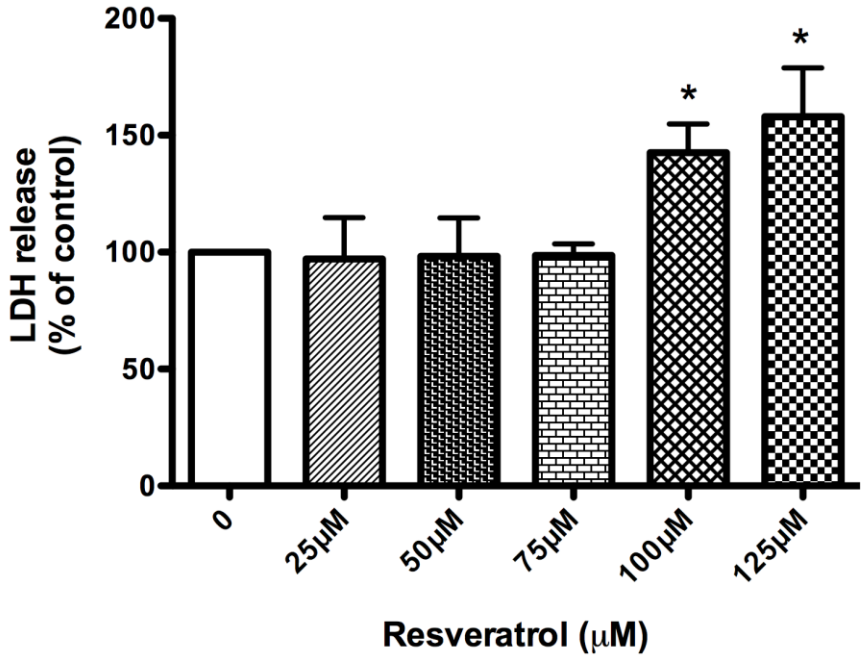

200
150
100
50
0
LDH release
(% of control)
*
*
0
25μM
50μM
75μM
100μM
125μM
Resveratrol (μM)

Fig 2.

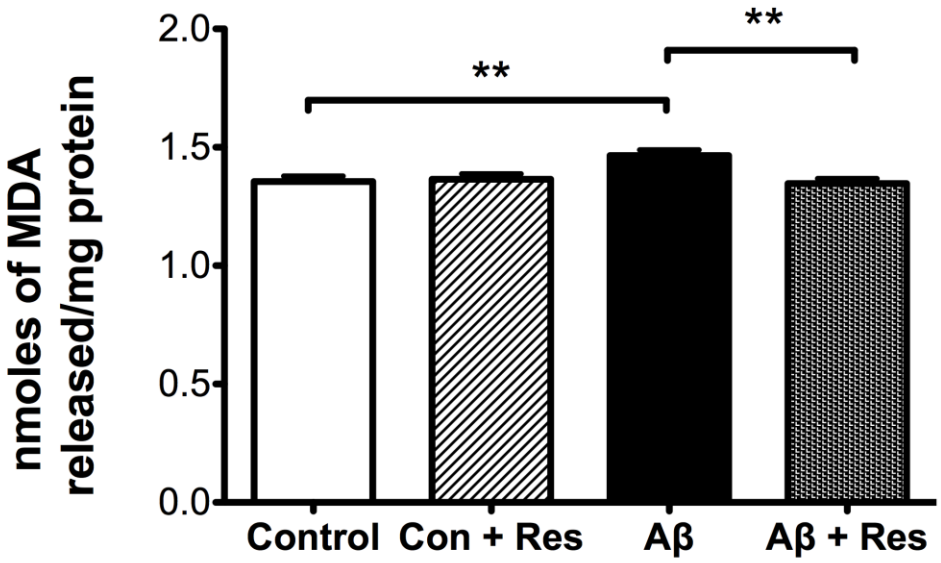

nmoles of MDA released/mg protein
2.0
1.5
1.0
0.5
0.0
**
**
Control
Con + Res
Aβ
Aβ + Res

Fig 3.

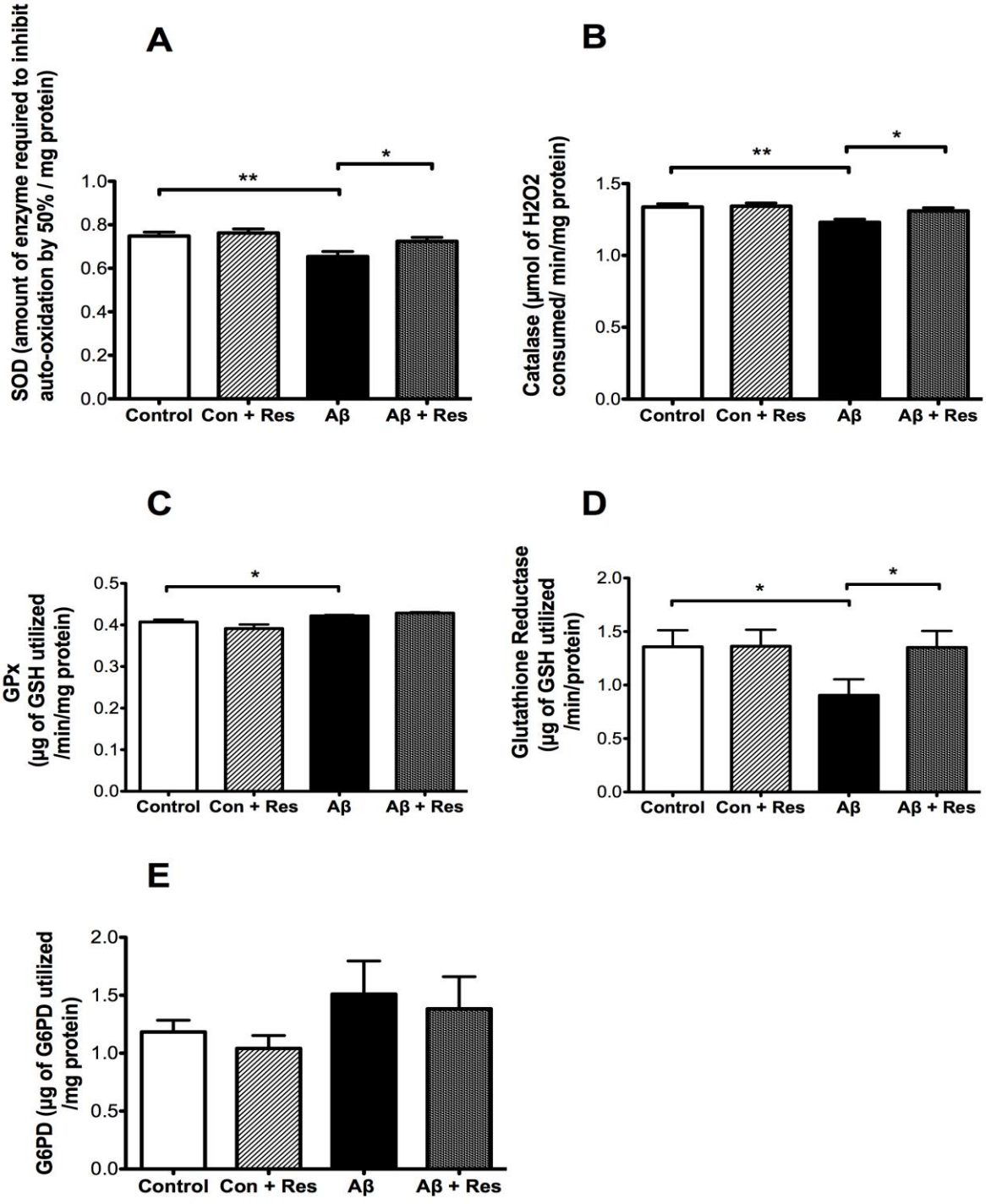

A
SOD (amount of enzyme required to inhibit auto-oxidation by 50% / mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Control
Con + Res
Aβ
Aβ + Res
B
Catalase (µmol of H2O2 consumed/ min/mg protein)
1.5
1.0
0.5
0.0
**
*
Control
Con + Res
Aβ
Aβ + Res
C
GPx (µg of GSH utilized /min/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Con + Res
Aβ
Aβ + Res
D
Glutathione Reductase (µg of GSH utilized /min/protein)
2.0
1.5
1.0
0.5
0.0
*
*
Control
Con + Res
Aβ
Aβ + Res
E
G6PD (µg of G6PD utilized /mg protein)
2.0
1.5
1.0
0.5
0.0
Control
Con + Res
Aβ
Aβ + Res

Fig 4

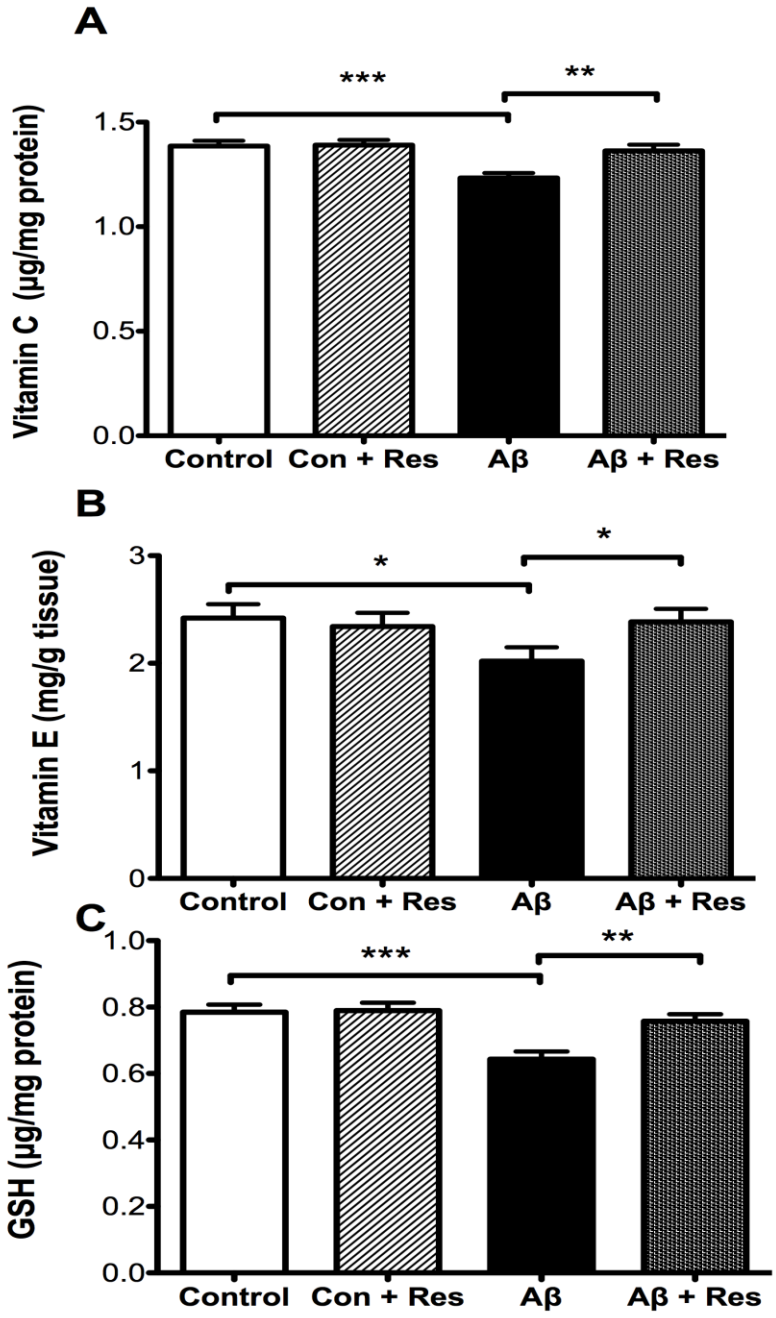
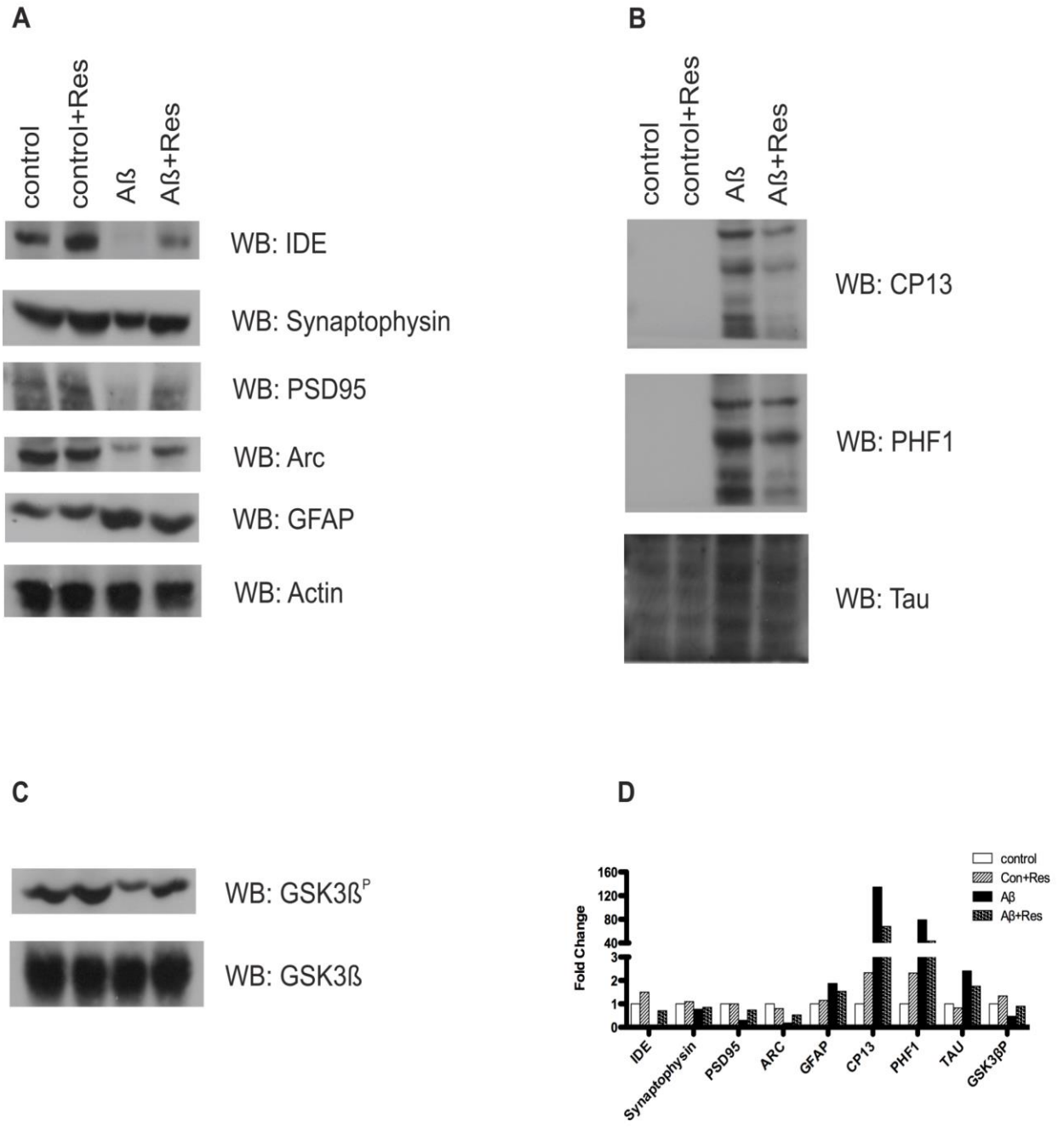

A
***
**
Vitamin C (µg/mg protein)
1.5
1.0
0.5
0.0
Control
Con + Res
Aβ
Aβ + Res
B
*
*
Vitamin E (mg/g tissue)
3
2
1
0
Control
Con + Res
Aβ
Aβ + Res
C
***
**
GSH (µg/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Con + Res
Aβ
Aβ + Res

Fig 5.


A
control
control+Res
Aß
Aß+Res
WB: IDE
WB: Synaptophysin
WB: PSD95
WB: Arc
WB: GFAP
WB: Actin
B
control
control+Res
Aß
Aß+Res
WB: CP13
WB: PHF1
WB: Tau
C
WB: GSK3ß^P
WB: GSK3ß
D
control
Con+Res
Aβ
Aβ+Res
Fold Change
160
120
80
40
3
2
1
0
IDE
Synaptophysin
PSD95
ARC
GFAP
CP13
PHF1
TAU
GSK3βP

Chapter 6: Summary and Conclusion

Alzheimer's disease (AD) is the most common type of neurodegenerative disorder clinically characterized by a progressive loss of memory, cognitive function, and behavioral abilities. It has truly become a major public health problem, posing burden on the global healthcare system and economic development. Many attempts have been made to prevent or cure the progression of AD such as developing pharmaceutical drugs like tacrine, rivastigmine, galantamine, donepezil and memantine, but administration of these drugs is known to often produce undesirable side effects. Therefore, it is essential to develop an acceptable, cost effective, safe and efficient non-pharmacological therapeutic approach to delay or attenuate the progression of AD.

Resveratrol, a polyphenolic phytochemical that exists in the seeds and the skin of grapes and berries is an excellent functional component, which exerts significant neuroprotective health benefits in recent studies. In this regard, resveratrol could be considered as a potential therapeutic option that has been suggested to have neuroprotective properties.

Our studies demonstrated that resveratrol is effective in preventing obesity-induced cerebral oxidative stress. Indeed, amelioration in cellular antioxidant defense systems was observed in the brains of *ob/ob* mice, indicating that resveratrol exerts both antioxidant and neuroprotective properties. Moreover, our studies reported a potential link between oxidative stress, type 2 diabetes, and AD progression. Increased amyloid beta accumulation and tau hyper phosphorylation that are known to be associated with memory impairment were reversed with resveratrol treatment. Furthermore, resveratrol also protected the proteins associated with memory impairment and showed considerable improvement in the cognitive function via CREB-

BDNF mechanism. Taken together, our findings would provide a better understanding of the mechanisms by which resveratrol exerts neuroprotection. Moreover, resveratrol being non-toxic cost effective, and widely available could potentially be developed as a therapeutic strategy for preventing neurodegenerative processes involved in AD, and could serve as one of the promising tools in the development of drug therapy for AD. This would further advance the field of AD and provide novel targets to combat the progression of this debilitating neurodegenerative disease.