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Resveratrol + Ethanol Preconditioning and Neuroprotection

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Loyola University Chicago

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ACKNOWLEDGEMENTS

Sometimes you step out of your comfort zone. You step out because you want to move closer to your dreams. I still remember the moment I said goodbye to my established practice as a physician in my home town of Tabriz, Iran. I said goodbye to all my friends and family to accompany my husband in reaching his goal without having a specific vision for myself in the new country which is famous as the land of opportunity. I found myself in the middle of a metropolitan city among with millions of people running about to achieve their dreams. When I decided to apply to the Neuroscience program in Loyola Graduate School, I had no idea where it would lead me. I just knew, I wanted to be myself as a person who could help other people to live healthily. I envisioned a program based on my dreams. I chose Loyola because it was one the prestigious schools in Chicago. I remember the very first day I came to meet my adviser, Dr. Michael Collins, and the moment he told me that the next day they were going to interview students who they felt qualified for the program. I remember the very last minute that I decided to call Dr. Edward. J. Neafsey as the dean of neuroscience program to see if I was on the list of students who would be interviewed that day.

I would like to thank all of the people who made my dissertation possible in light of my background as a Medical Doctor. I would like to acknowledge Dr. Michael Collins who proved to be an excellent sounding board for me from the beginning of my time here even when an applicant of the program. He is an outstanding professor who from the very first meeting found me to be a fighter for my dreams. Also, I would like to extend my
sincere thanks to my co-adviser and director of the neuroscience program at Loyola graduate school, Dr. Edward J. Neafsey who guided and helped me like a father to a daughter. He shared his vision with me in my research. He always led and encouraged me during this arduous process. Additionally, I am grateful for my other committee member, Dr. Tony Pak, for his time, interest and comments throughout my research. I would like to express my sincere appreciation to all those who have offered me invaluable help during the two years of my study here at Loyola University Chicago.

Hereby, I would like to express my loving thanks and appreciation to my parents, Khalil Khodaie and Nasrin Maali, who guided me on my way toward completion and always encourage me for higher levels of education.

Finally and most importantly, I thank the love of my life, my best friend, and my better half, Kaveh Aflaki. I want to acknowledge Kaveh, my husband but also my real hero. He is a great man with a big heart and one who really believes in me unconditionally. He had and continues to be an invaluable source of support, inspiration, motivation and encouragement especially when I become frustrated in what I am doing. He was and is like a rock in bad times and good. I feel so fortunate in my new home, the U.S., only and only because of his supports.
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</tr>
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ABSTRACT

Our lab established that moderate ethanol preconditioning (MEP) for 6 days blocked the Aβ<sub>25-35</sub> toxicity in cerebellar mixed cell cultures and it has been shown that MEP cause the up regulation of the pre-survival enzymes such as proxiredoxin2 (PRX2), which suggests MEP can activate the cell survival pathways against upcoming toxins.

This study was performed to verify the possible combinatory effect of ethanol and resveratrol against Aβ<sub>25-35</sub> toxicity. Both ethanol and resveratrol sub-protective concentrations were detected against Aβ<sub>25-35</sub> in cerebellar mixed cell cultures. The combination of sub-protective levels of ethanol and resveratrol showed the significantly reduction in number of Aβ<sub>25-35</sub> toxicity compare to preconditioning with ethanol or resveratrol alone. The western blot analysis revealed that proxiredoxin2 levels were increased significantly in preconditioned cell cultures with combined sub-protective level of ethanol and resveratrol.

Combination of sub-protective levels of ethanol and resveratrol showed synergistic protection against Aβ<sub>25-35</sub> toxicity.
CHAPTER ONE
INTRODUCTION

i. Alcohol Toxicity and Preconditioning

There are strong correlation between heavy alcohol consumption and increased risk of developing cardiovascular disease, chronic pancreatitis, alcoholic liver disease, and cancer. Risk of damage to the central nervous system and peripheral nervous system is also increased by chronic alcohol abuse. However alcohol consumption and mortality show a J-shaped relationship, which means that in moving from alcohol abstinence to heavy drinking the rate of mortality is lower in light to moderate consumers than abstainers while heavy drinkers have higher rates of mortality from a number of causes (1).

Thus, while excessive alcohol consumption has been linked to a number of serious health and social problems, moderate alcohol consumption has been associated with some health benefits. Because the consumption of alcohol can be viewed as a “double-edged sword,”
Individual decisions regarding alcohol use should take into consideration scientific evidence regarding potential health benefits and risks as well as personal and family histories of health problems and addictions (2).

Definitions

*Standard alcoholic drink*

A standard alcoholic drink contains approximately 14 grams of alcohol, which is equivalent to 12 fluid ounces of beer, 5 fluid ounces of wine, 3.5 fluid ounces of fortified wine (e.g., sherry or port), or 1.5 fluid ounces of liquor (distilled spirits).

*Moderate Alcohol Consumption (as defined by the U.S. Department of Agriculture (3))*

- Men: No more than two standard alcoholic drinks/day
- Women: No more than one standard alcoholic drink/day*
- Adults over 65: No more than one standard alcoholic drink/day#

*Heavy Alcohol Consumption*

- Men: 15 or more standard alcoholic drinks/week or 5 or more alcoholic drinks on any given occasion
- Women: 8 or more standard alcoholic drinks/week or 4 or more alcoholic drinks on any given occasion*
- Adults over 65: 8 or more standard alcoholic drinks/week or 4 or more alcoholic drinks on any given occasion#

On average, women absorb and metabolize alcohol differently than men. In general, women have less body water than men of similar body weight, so after drinking equivalent amounts of alcohol women achieve higher blood alcohol concentrations (4). Women appear to be more sensitive to side health effects of heavy drinking than men. So, definitions of “moderate” or “heavy” drinking apparently are lower for women than men.

Age-associated decreases in lean body mass may result in decreased total body water and higher blood alcohol concentrations in older adults compared to younger adults after drinking equivalent amounts of alcohol (5).

*Moderate Alcohol Consumption Benefit for Cardiovascular Disease and Coronary Heart Disease*

More than 60 epidemiological studies have shown a significant reduction in the risk of coronary heart disease (CHD) with moderate alcohol consumption (6). Recent data from two large prospective cohort studies in the U.S. proposed that the amount of CHD risk reduction related with moderate alcohol consumption might be closer to 30%. In a 12-year study of more than 38,000 male health professionals, those who consumed alcohol at least 3-4 times weekly had a risk of myocardial infarction (heart attack) that was 32% lower than men who drank alcohol less than once weekly (7). When the results of 28 prospective cohort studies were combined in a meta-analysis, adults who consumed
an average of 25 grams/day of alcohol (the amount in two standard alcoholic drinks) had a risk of CHD that was 20% lower than adults who did not consume alcohol (8). Similarly, in a 20-year study of more than 120,000 men and women, those who reported consuming 1-2 alcoholic drinks daily had a risk of death from CHD that was 30% lower than those who did not drink alcohol (9). Coronary heart disease is a major cause of heart failure. A prospective study in a cohort of 126,236 men and women and another in a cohort of 21,601 men found that moderate alcohol intake might be inversely associated with increase of heart failure rate, especially heart disease related to CHD (10-11).

The formation of cholesterol plaque in the arteries (atherosclerosis), vascular inflammation, and platelet aggregation are characteristic for development of CHD (12). One of the roles of HDL is to carry cholesterol from tissues, such as arterial walls, back to the liver for elimination or recycling. Basically, higher levels of HDL-cholesterol lead to reductions in CHD risk (13). Most of randomized trials have consistently shown that moderate alcohol consumption significantly increases HDL-cholesterol levels that inhibit coagulation or enhance the dissolution of clots (14). Moderate alcohol intakes has shown an anti-inflammatory effect by reducing the serum levels of C-reactive protein (CRP), a systemic inflammation biomarker and sensitive predictor of CHD risk, compare to abstainers (15-18). Also, moderate alcohol consumption improves insulin sensitivity, lowers serum triglycerides, and reduces abdominal obesity (19).
Moderate consumption of wine, beer, and liquor has all been shown significant reductions in CHD risk. However wine is rich in phenolic compounds like resveratrol that have been found to have antioxidant, anti-inflammatory, and potentially anti-atherogenic properties in the *in vitro* experiments and in some in vivo models of atherosclerosis (20). The “French Paradox”—the observation that mortality from CHD is relatively low in France despite relatively high levels of dietary saturated fat and cigarette smoking—led to the idea that regular consumption of red wine might provide additional protection from CHD (21,22). Although moderate alcohol consumption consistently has been shown 20-30% reductions in CHD risk, nonalcoholic compounds abundant in red wine have not shown any additional CHD risk reduction. Other prospective studies found that wine drinkers were at lower risk of CHD than beer or liquor drinkers (9,27,28), others found no difference (8,29,30). Socioeconomic and lifestyle differences between people who prefer wine and those who prefer beer or liquor may explain part of the additional benefit observed in some studies. Several studies have demonstrated that wine drinkers have higher incomes, have more formal education, smoke less, and eat more fruits and vegetables and less saturated fat than people who prefer other alcoholic beverages (31-33).
Ischemic Stroke

A stroke, also known as a cerebrovascular accident (CVA), is the rapid loss of brain functions due to disturbance in the blood supply to the brain. This can be due to ischemia (Lack of blood flow) caused by blockage or a hemorrhage (leakage of blood). 83% of strokes are classified as ischemic strokes (34). Moderate alcohol consumption has been associated with a reduced risk of ischemic stroke but not hemorrhagic stroke in a number of observational studies (31-34). Combined meta-analysis results of 19 prospective cohort studies and 16 case-control studies of moderate alcohol consumption and the risk of stroke has shown a significant reduction in the risk of ischemic stroke (35). Overall, those who consumed one or two drinks daily had a 28% lower risk of ischemic stroke than those who did not consume alcohol.

Moderate alcohol and Dementia and Cognitive Decline

Although previous studies of alcohol consumption and the risk of dementia have shown conflicting results (49,50), two prospective studies that observed older adults without dementia for six years found that those who reported moderate alcohol intakes were significantly less likely to develop dementia than whose abstaining from alcohol (51,52). A recent prospective study of 11,102 women reported that moderate alcohol consumption was associated with improved cognitive function and less cognitive decline over a two-year period, compared to nondrinkers (53). Two epidemiological studies have
used MRI to examine relationships between alcohol intake and subclinical abnormalities in the brains of older adults. Although one study found that infarctions were less frequent in the brains of those reporting moderate alcohol intake compared to those who abstained from alcohol (54), another study found no relationship between alcohol intake and the presence of infarction (54). In contrast, measures of brain atrophy, a characteristic of Alzheimer’s disease and alcoholic dementia, were lowest in those who abstained from alcohol and increased with alcohol consumption in both studies (54-55).

**ii. Resveratrol**

Resveratrol (3,4', 5-trihydroxystilbene) is a member of a class of polyphenolic compounds called stilbenes (56). Resveratrol and other stilbenes are produced in some types of plants in response to stress, injury, fungal or bacterial infection, or ultraviolet (UV) radiation (57). Scientists became interested in exploring potential health benefits of resveratrol in 1992 when its presence was first reported in red wine (59) leading to the hypothesis that, resveratrol might help explain the “French Paradox”. The “French Paradox”—the observation that mortality from coronary heart disease is relatively low in France despite relatively high levels of dietary saturated fat and cigarette smoking—led to the idea that regular consumption of red wine might provide additional protection from cardiovascular disease (73, 74).
Metabolism and Bioavailability

Although \textit{trans}-resveratrol seems to be well absorbed by humans when taken orally, its bioavailability is low due to its rapid metabolism and elimination (61, 62). Results of another study suggested that the bioavailability of resveratrol from grape juice, which contains mostly glucosides of resveratrol, may be even lower than \textit{trans}-resveratrol (63).

Resveratrol metabolites are primarily detected upon oral exposure to \textit{trans}-resveratrol. In one experiment, six healthy men and women took an oral dose of 25 mg of \textit{trans}-resveratrol; only traces of the un-metabolized resveratrol were detected in plasma (blood). Plasma concentrations of resveratrol and metabolites peaked around 60 minutes later at concentrations around 2 micromoles/liter (491 micrograms/liter) (61).

Biological Activities

Direct Antioxidant Activity

In vitro, resveratrol effectively scavenges (neutralizes) free radicals and other oxidants (64) and inhibits low-density lipoprotein (LDL) oxidation (65, 66).
**Induction of Apoptosis in Cancer Cells**

Unlike normal cells, cancer cells proliferate rapidly and are resistant to cell death signals that initiate apoptosis. Resveratrol has been found to inhibit the proliferation of a variety of human cancer cell lines, including those from breast, prostate, stomach, colon, pancreatic, and thyroid cancers. Resveratrol has been found to inhibit proliferation and induce apoptosis in a number of cancer cell lines [reviewed in (57,67)]. In animal models, oral administration of resveratrol inhibited the development of esophageal (20), intestinal (76), and mammary (breast) cancer (67,77). Few studies have shown that oral resveratrol protects against colon cancer development in rats induced by the carcinogen, (78, 79, 80).

**Inhibition of Vascular Cell Adhesion Molecule Expression**

Inflammation is one of the earliest events in the development of atherosclerosis and begins with adhesion of white blood cells from the blood to the arterial wall by vascular cell adhesion molecules (68). Resveratrol has been found to inhibit the expression of adhesion molecules in cultured endothelial cells (69, 70).
Inhibition of Platelet Aggregation

Platelet aggregation leads to formation of blood clot can result in myocardial infarction or stroke, respectively. Resveratrol has been found to inhibit platelet aggregation in vitro (71, 72).

Activation of autophagy in parkinsonic cells

Excessive misfolded proteins and/or dysfunctional mitochondria, which may cause energy deficiency, have been implicated in the etiopathogenesis of Parkinson’s disease (PD). Enhanced clearance of misfolded proteins or injured mitochondria via autophagy has been reported to in resveratrol treated cell cultures. Resveratrol has neuroprotective role in PD models (93).

The neuroprotective effects of resveratrol against Aβ toxicity has been shown in rat hippocampal cells cultures via protein kinase C pathway (92).

Inhibition of atherosclerosis

Resveratrol and other polyphenolic compounds in the red wine, which have shown antioxidant, anti-inflammatory, and anti-atherogenic effects in vitro and in some animal models of atherosclerosis (95).
Alcoholic Encephalopathy

Alcoholic encephalopathy is one of the hallmarks of neurinflammation results from chronic and excessive consumption of alcohol due to increased oxidative nitrosative stress and activation of inflammatory cascade, which leads to neural apoptosis in cortex and hypothalamus.

Neuroprotection

Brief resveratrol pretreatment conferred neuroprotection against cerebral ischemia via SIRT activation. This neuroprotective effect produced by resveratrol was similar to ischemic preconditioning–induced neuroprotection, which protects against lethal ischemic insults in brain and other organ system (94).

Longevity

In yeast, caloric restriction stimulates the activity of an enzyme known as Sir2 (81), and also increased the activity of the homologous human enzyme (Sirt1) (60).

Treating the yeast with resveratrol increased Sir2 activity in the absence of caloric restriction and the replicative lifespan of yeast by 70% (60). A recent study reported that resveratrol extended lifespan of mice on a high-calorie diet such that their lifespan was similar to that of mice fed a standard diet (82). A recent aging study in mice
found that a low dose of dietary resveratrol changed gene expression in heart, brain, and skeletal muscle similar to that induced by caloric restriction. Like caloric restriction, resveratrol also blunted the age-related decline in heart function in this study. Clinical trials will be needed to determine if these findings are relevant to humans (83).

**Food Sources**

Resveratrol is found in grapes, wine, grape juice, peanuts, and berries such as blueberries, bilberries, and cranberries (84, 85, 86). In grapes, resveratrol is found only in the skins (87). The amount of resveratrol in grape skins varies with the grape cultivar, its geographic origin, and exposure to fungal infection (88). The amount of fermentation time a wine spends in contact with grape skins is an important determinant of its resveratrol content. Consequently, white and rosé wines generally contain less resveratrol than red wines (59). Red or purple grape juices may also be good sources of resveratrol (58). The predominant form of resveratrol in grapes and grape juice is *trans*-resveratrol glucoside (*trans*-piceid), but wines also contain significant amounts of resveratrol aglycones, thought to be the result of sugar cleavage during fermentation (84).

The total resveratrol content of some beverages and foods are listed in the tables below. These values should be considered approximate since the resveratrol content of foods and beverages can vary considerably.
<table>
<thead>
<tr>
<th>Beverage</th>
<th>Total resveratrol (mg/liter)</th>
<th>Total resveratrol in a 5-oz glass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White wines (Spanish)</td>
<td>0.05-1.80</td>
<td>0.01-0.27</td>
</tr>
<tr>
<td>Rosé wines (Spanish)</td>
<td>0.43-3.52</td>
<td>0.06-0.53</td>
</tr>
<tr>
<td>Red wines (Spanish)</td>
<td>1.92-12.59</td>
<td>0.29-1.89</td>
</tr>
<tr>
<td>Red wines (global)</td>
<td>1.98-7.13</td>
<td>0.30-1.07</td>
</tr>
<tr>
<td>Red grape juice</td>
<td>1.14-8.69</td>
<td>0.17-1.30</td>
</tr>
</tbody>
</table>

**Table 1**: Total Resveratrol Content of Wines, Grape Juice
<table>
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<tr>
<th>Food</th>
<th>Serving</th>
<th>Total resveratrol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanuts (raw)</td>
<td>1 cup (146 g)</td>
<td>0.01-0.26</td>
</tr>
<tr>
<td>Peanuts (boiled)</td>
<td>1 cup (180 g)</td>
<td>0.32-1.28</td>
</tr>
<tr>
<td>Peanut butter</td>
<td>1 cup (258 g)</td>
<td>0.04-0.13</td>
</tr>
<tr>
<td>Red grapes</td>
<td>1 cup (160 g)</td>
<td>0.24-1.25</td>
</tr>
</tbody>
</table>

*Table 2: Total Resveratrol Content of Selected Foods*
iii. Alzheimer Disease

The burden of Alzheimer Disease

According to the Alzheimer’s Association’s Alzheimer’s Disease Facts &Figures 2010, there are as many as 5.3 million Americans living with Alzheimer disease (AD) and every 70 seconds someone in America develops AD. In 2010, there were half a million new cases of AD. The Alzheimer’s Association estimates that total U.S. payments for health and long term care service for people with AD will amount to $172 billion in 2010. Nearly 11 million U.S. family members and other unpaid caregivers provided 12.5 billion hours of care for people with Alzheimer’s and other dementias, valued at $144 billion. The total estimated worldwide costs of dementia are $640 billion in 2010. In the World Alzheimer Report 2009, estimated that there are 35.6 million people living with dementia worldwide, and this will increase to 65.7 million by 2030.

Alzheimer Disease Pathology

Alzheimer's disease is characterized by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. Amyloid plaques and neurofibrillary tangles are clearly visible by microscopy in brains of those afflicted by AD (90). Plaques are dense deposits of amyloid beta peptide and cellular material outside and around neurons. Neurofibrillary tangles are aggregates of the microtubule-associated protein tau, which have become
hyperphosphorylated and which accumulate inside the cells themselves (91). Sporadic AD, constituting the most of AD cases with a late age of onset (65 years and older)(92); In contrast, familial AD constitutes only a small portion of all AD patients (younger than 65 years)(93). Both familial and sporadic AD, Aβ peptide, a cleavage product of Aβ precursor protein (APP) that is due to β and γ secretases, is a key factor in pathology of AD (92, 94).

**iv. Moderate Ethanol Preconditioning**

Our lab has shown that moderate ethanol preconditioning for 6 days has a neuroprotective effect against Aβ on *in vitro* model. Preconditioning refers to the phenomenon that a sub-lethal, brief stress leads to protection against subsequent, normally lethal injury. Moderate ethanol preconditioning (MEP; 20-30 mM) for 6 days protects rat cerebellar and hippocampal cultures from neurotoxins such as Aβ. The neuroprotective mechanism of ethanol is unclear, but it conceivably involves sensors→ transducers→ effectors, analogous to other preconditioning modalities.
Peroxiredoxins (Prxs) are family of cytoplasmic anti apoptotic and anti oxidative enzyme that catalyze the reduction of peroxides (92, 93). Family members possess a redox active peroxidative cysteine sulfenic acid (-SOH). This oxidized form of PRX must then reduced to the –SH Form by reduced Thioredoxin (Trx) (92, 93). Prx2 is expressed exclusively in neurons of cerebral cortex, cerebellum, basal ganglia, substantia nigra and spinal cord (94). Overexpression of Prx2 protects primary neurons against Aβ toxicity (95). In other brain preconditioning models, Peroxiredoxin 2 overexpression protects cortical neuronal cultures from ischemic and oxidative injury (96). The PRX2 level was detected in cell lysates by Western blot using an anti-PRX2 antibody.
CHAPTER TWO

HYPOTHESIS AND SPECIFIC AIMS

I hypothesize that combination of non-protective level of resveratrol with non-protective level of ethanol have synergistic protective effect to protect rat brain culture against Aβ neurotoxicity. For reaching this goal, the overall project is broken down into five specific aims.

i. **Aim I** is to determine whether a shorter duration of MEP can protect cerebellar mixed cell cultures against Aβ. To accomplish this aim I will carry out time course study of MEP to find the duration for neuroprotection. The percent of dead cells will be counted in Hoechst 33342 stained cerebellar mixed cell cultures after 3 and 6 days of MEP and one day of exposure to Aβ.

ii. **Aim II** is to determine a non-protective level of ethanol in the 3 days of MEP. The cerebellar mixed cell cultures be treated with different level of ethanol 30, 20, 10 mM for 3 days and one day of exposure to Aβ, and the percent of dead cell will be counted in Hoechst 33342 stained treated cell cultures and compared with control cell culture not treated with MEP.

iii. **Aim III** is to determine a non-protective of resveratrol. Cerebellar mixed cell cultures will be treated with different concentrations of resveratrol 2, 5, 10 and 25 μM for 3 days and then one more day of exposure to Aβ. The effects of treatment with different concentrations of resveratrol were measured determining the percent of dead cells in Hoechst 33342 stained in treated cell cultures.
iv. **Aim IV** is to determine a synergistic effect of non-protective levels of both ethanol and resveratrol to protect against Aβ toxicity. Cerebellar mixed cell cultures were treated with both 5μM of resveratrol and 10mM ethanol for 3 days and then one day of exposure to Aβ. The dead cells were counted in Hoechst33342 stained cell cultures and compared to counts in control cell cultures.

v. **Aim V** is to determine if peroxiredoxin 2 is involved in the synergistic effect of non-protective levels of ethanol and resveratrol co-treatment pathway. Our lab has shown Peroxiredoxin 2 increased significantly after 6 days of MEP. Co-treated cell cultures with 5μM resveratrol and 10mM ethanol will be harvested and amount of peroxiredoxin2 protein measured by western blotting. To accomplish this aim, three groups of cultures on 10th day *in vitro* treated with 10 mM ethanol, 5μM resveratrol and or the combination of 10mM ethanol and 5μM resveratrol for 3 days. Another group of cultures were kept untreated as control group for 3 days.
CHAPTER THREE
MATERIALS AND METHODS

Chemical reagents and antibodies

Neurobasal–A media, B27, trypsin and fetal bovine serum (FBS) were from Gibcoco. (Corlsbad, CA). Hoechst 33342, deoxyribonuclease I (DNAse), tweener 20, protease and phosphatase inhibitor cocktails, and penicillin/streptomycin were provided by Sigma Co. (St. Louis MO). Aβ 25-35 was from AmericanPeptide Co. (Sunnyvale, CA). Polyacrylamide gels and Western blot enhanced chemiluminescence detection (ECL) solutions were from Thermo scientist (Rockford, IL). The peroxiredoxin antibodies (1:1000-10000) provided from Epitomics (Burlingame, CA), and the goat anti rabbit antibodies (1:20000) were from Santa Cruz Co (Santa Cruz, CA), and actin anti rabbit IgG (1:1000-20000) was from Cell Signaling (Danvers, MA).
Sprague-Dawley rat pups (7-10 days old) were anesthetized in a CO₂ chamber for 30 seconds and then rapidly decapitated. The brain was carefully removed and placed in ice-cold Gays balanced salt solution (GBSS) supplemented with 4.5 mg/ml glucose. The cerebellum was taken and placed into ice-cold MEM supplement with 6.5 mg/ml glucose. Meninges were removed, and the cerebellas were divided into approximately 6 pieces each. Pieces were transferred into calcium/magnesium-free Hanks balanced salt solution (CM-HBSS) containing 0.25% trypsin and 0.5 mg/ml DNAse at 37°C. Tissue were incubated for 10 min, and then transferred into MEM with 10% fetal bovine serum (FBS) and 5% horse serum (HS) to inactivate trypsin. Cells were dispersed by triturating with two fire polished pipettes that had decreasing size. Cells were then passed through a 70 µm cell strainer. Viable cell density was determined by trypan blue exclusion on a hemocytometer: cells were diluted to a concentration of 800,000 cells/ml in 10% fetal bovine serum in Neurobasal-A media supplemented with 2% B27 (Gibco), 500 µg L-glutamine and 1% Penicillin-streptomycin. Then cells were plated on poly-l lysine coated 12 well plates at 700 µl/well. Half of the media was changed after 24-36 hours, and then every 3 days half of the media was replaced with serum-free Neurobasal-A media supplemented with 2% B27 (Gibco), 500 µg L-glutamine and 1% Penicillin-streptomycin. At DIV10 100% of media was changed.
Preconditioning methods

At DIV 10, the cell cultures were placed in plastic Tupperware container with different concentrations of ethanol in the media (30mM=MEP30, 20mM=MEP20, 10mM=LEP10, 5mM=LEP5) or ethanol free media for 3 or 6 days (100% serum free media). A small open container containing 90, 60, 30, 15 mM ethanol and 50 ml of double distilled water were placed in the Tupperware container with the ethanol-treated cultures to prevent loss of ethanol due to evaporation from the cultures. Control plates were treated with the same method, but water was added to media and small container instead of ethanol. The container was kept 10 min open to equilibrate with incubator and then sealed for 3 or 6 days.

For resveratrol preconditioning, at DIV 10 different amounts of resveratrol (RES) 25, 10, 5 and 2 µM was added to 100% serum free cell media for 3 days.

For the combined preconditioning with resveratrol and ethanol, at DIV 10 Plates were placed in the Tupperware container and treated with a combination of non-protective level of ethanol and resveratrol (5 µM of resveratrol and 10 mM of ethanol)(LEP10+RES5) in 100% serum free media for 3 days. A small open container containing 30mM ethanol and 50 ml of double distilled water were placed in Tupperware containing ethanol-treated cultures to prevent loss of ethanol due to evaporation from the cultures. Control plates were treated with the same method, but water was added to the media and small container instead of ethanol. The container was kept open for 10 min to equilibrate with the incubator and then sealed for 3 days.
At the end of pretreatment the media were completely changed to media contained 25µM/ml of Aβ_{25-35}. This media was added in to the cultures except the control group cultures.

For measuring cytotoxicity, the plates were incubated with 4% paraformaldehyde for 20 min, washed with iced-cold PBS, incubated with 100% methanol for 20 min, and then with 2µl Hoechst 33342 stain stock solution in one ml of PBS for 20 min. The picture of fixed and stained plates were taken with a Nikon fluorescent microscope. Five pictures per well from predetermined areas (top right and left, central and bottom right and left were taken). Live cells with faintly stained nuclei and dead cells with intensely stained nuclei were easily recognized. For consistency in counting, cells that were cut by the edge or corners of pictures or clusters of cells were not counted. The mean value, standard deviation, and standard error of dead and live cells per each well are calculated by excel program after adding the numbers of each picture. The final numbers are obtained by taking the mean of mean value per picture, and standard deviation and standard error of dead and live cells per each well of each dish were calculated and analyzed by JBstat program for statistical analysis.

Cell fraction

The cell cultures were washed with iced-cold PBS, lysed in lysis buffer (20mM Tris-HCl, PH 7.5, 5mM EDTA, 10µl/ml phosphatase inhibitor cocktail), homogenized by sonication on a 550 Sonic Dismembator (Fisher Scientific; Pittsgurgh, PA) for 7 pulses,
and spun in a microcentrifuge for 5 minutes at 1000 rpm and 4°C. The supernatant was collected, and 25 µg of protein (measured by protein assay) was used for western blot analysis.

**Measurement of protein**

Protein was measured by using the bicinchoninic Acid (BCA) protein assay (Pierce Rockford IL). Aliquots (25µl) were collected and added to individual wells of a 96-well plate. Each sample was incubated with 200 µl of the BCA reagent for 30 minutes at 37°C. The absorbance of each sample was measured spectrophotometrically at 625 nm. The protein levels of samples were calculating by calibrating the absorbance of the individual samples with a standard curve generated with known concentrations of bovine serum albumin (BSA).

**Western blotting**

25µg/well of samples were electrophoresed on 10%SDS-PAGE gel with constant voltage at 120V for ~ 60 mins. The separated proteins were transferred to nitrocellulose membranes by electrical current and then blocked for 1 hour with 5% milk solution. The membrane was incubated overnight at 4°C with 1µl/ml of rabbit IgG anti-peroxiredoxin-2 for detection of peroxiredoxin-2 proteins and then washed 3 times with TBST followed by incubation for 1 hour with secondary antibody. The loaded samples were probed with
anti-GAPDH antibody for detection of GAPDH as a loading control. The proteins were detected by using the Pierce super signal West pico detection kit and detected with X-ray film. Western blots were quantified using NIH image software. The values of the band intensities were normalized with GAPDH standards. The levels of peroxiredoxin2 of treated cultures were compared with those of untreated control cultures.

Statistics

Each experiment was done 3-4 times. For experiments with Hoechst 33342 staining the number per group was 8-12 and for experiments with western blot the numbers per group was 3-4.

The data were analyzed by one-way ANOVA with Tukey-Kramer post-hoc tests for multiple comparisons between pairs. For experiments with two groups the means of data were analyzed by independent sample t tests. P<0.05 was considered significant the western blotting data for Peroxiredoxin-2 were analyzed by mixed effects ANOVA followed by post-hoc Tukey-Kramer tests.
CHAPTER IV

RESULTS

i. AIM I: MEP for 3 days blocks Aβ toxicity in cerebellar mixed cell cultures

Figure 1 illustrates the percentage of dead cells in the four different groups: Control for 6 days (CON6) and control for 6 days with 24 hours of Aβ toxicity (CON6+Aβ), MEP for 6 days (MEP6), and MEP for 6 days followed by 24 hours of Aβ exposure (MEP6+Aβ).

Figure 2 shows a representative picture of each group, to demonstrate the effect of 6 days of MEP on cerebellar mixed cell cultures.
Figure 1: Quantification Hoechst 33342 staining on dead cells of cerebellar mixed cell cultures. The experiment was done 3 times and result from representative experiment is shown. Data represented as mean ± S.E.M. N=8 and the ** shows the p<0.001 (CON6+Aβ VS CON6 or MEP6), which indicates Aβ toxicity and * depicts p<0.05. (CON6+Aβ VS MEP6+Aβ).
Figure 2: Demonstrates the effect of 6 days of MEP on cerebellar mixed cell cultures. The four pictures were taken by fluorescent microscope from each well of treated cell cultures.
Figure 3 illustrates the percentage of dead cells in the four different groups: Control for 3 days (CON3) and control for 3 days with 24 hours of Aβ toxicity (CON3+Aβ) and MEP for 3 days (MEP3) and MEP for 3 days followed by 24 hours of Aβ exposure (MEP3+Aβ).

Figure 4 shows a representative picture of each group; demonstrate the effect of 3 days of MEP on cerebellar mixed cell cultures.
Figure 3: Quantification Hoechst 33342 staining on dead cells of cerebellar mixed cell cultures. The experiment was done 3 times and result from representative experiment is shown. Data represented as mean ± S.E.M. N=8 and the ** shows the p<0.001 (CON3+Aβ VS CON3 or MEP3), which indicates Aβ toxicity and * depicts p<0.05 (CON3+Aβ VS MEP3+Aβ).
Figure 4: Demonstrates the effect of 3 days of MEP on cerebellar mixed cell cultures. The four pictures were taken by fluorescent microscope from each well of treated cell cultures.
ii. Aim II: determine the sub-protective level of ethanol

The purpose of this aim was to find the non-protective level of ethanol against Aβ toxicity in cerebellar mixed cell cultures. Figure 5 shows the effect of different concentrations of ethanol. The results of one-way ANOVA showed that there were significant differences between the numbers of dead cells in the control group and the groups of cells treated with Aβ and 10 or 5 mM ethanol, but there was no significant difference in the number of dead cells between the control group and the group of cells treated with Aβ and 20 mM ethanol (Fig 5). Aβ toxicity was verified by comparing the number of dead cells between control cultures and the cultures were incubated with Aβ for 24 hours. The one-way ANOVA results indicated the significant difference in number of dead cells between two compared groups (Fig 5). Figure 6 shows a representative picture of each group, which is shown in figure 5. Based on the LEP 10 group (10 mM ethanol) was chosen as the sub-protective level.
Figure 5: 20 mM ethanol confers neuroprotection against Aβ toxicity in cerebellar mixed cell cultures. The experiment was done 3 times and the result from representative experiment is shown. Data represented as mean+S.E.M. p<0.05 was considered the threshold for significance. ** P<0.001 (CON+ Aβ vs CON and MEP20+Aβ). N=8
Figure 6: Demonstrates the effect of different concentration of ethanol on cerebellar mixed cell cultures. 12 well dishes were used for cerebellar mixed cell cultures. The red arrows point to dead cells. The pictures represent here are one of the sixty pictures per each group of cell cultures.
iii. **Aim III:** Determine the protective and sub-protective levels of resveratrol in cerebellar mixed cell cultures against Aβ<sub>25-35</sub> toxicity.

The goal of this aim was to find the non-protective level of resveratrol against Aβ induced cell death in cerebellar mixed cell cultures. As shown in Figure 7, cultures treated with 25, 5 and 2 µM of resveratrol and incubated with Aβ<sub>25-35</sub> for 24 hours did not show a significant decrease in the percentage of dead cells compared to the control cultures treated with Aβ (CON+Aβ), while cultures treated with 10 µM resveratrol and incubated with Aβ<sub>25-35</sub> did show a significantly decreased in percentage of dead cells (Fig. 7). Aβ<sub>25-35</sub> toxicity was established in cerebellar mixed cell cultures by comparing the percentage of dead cells to total number of cells in the control (CON) to the control+ Aβ group (CON+Aβ). The percentage of dead cells was significantly increased in control+ Aβ group by one-way ANOVA (Fig. 7).

Figure 8 illustrates a picture of each group of figure 7, which presents an effect of different concentrations of resveratrol on cerebellar mixed cell cultures. Based on the RP5 method, 5µM resveratrol was chosen as the sub-protective level.
Figure 7: protective effects of resveratrol against Aβ_{25-35} toxicity in cerebellar mixed cell cultures. Cells (day 10 in vitro) were treated with resveratrol at various concentrations for 3 days before the addition of Aβ_{25-35} for 24 hours. The experiment was done three times and data from a representative experiment is shown. Data represented as mean±S.E.M. p< 0.05 was considered the threshold for significance. * P<0.05(CON+Aβ vs RP5+Aβ), ** P<0.001 (CON+Aβ vs RP10+Aβ). N=12
Figure 8: Demonstrates the effect of different concentration of resveratrol on cerebellar mixed cell cultures. 12 well dishes were used for cerebellar mixed cell cultures. The red arrows point to dead cells. The pictures represent here are one out of the sixty pictures per each group of cell cultures, which has been described in Fig 7.
**iv. Aim IV:** Determine if co-treatment with non-protective levels of ethanol and resveratrol blocks Aβ_{25-35} induced cell death.

The objective of Aim IV was to examine the potential synergistic protective effect of non-protective levels of ethanol and resveratrol against Aβ_{25-35} toxicity. Based on the result of aim II and III, 10 mM ethanol and 5 µM resveratrol were chosen as the sub-protective levels. To investigate the synergistic effect, five groups of cerebellar mixed cell cultures were treated on the 10th day *in vitro*. There were two untreated cell cultures; one of them was exposed for 24 hours with Aβ_{25-35} to establish Aβ_{25-35} induced toxicity (CON and CON+Aβ). The remaining of three cell cultures was treated with 5 µM resveratrol alone (RP5), 10 mM ethanol alone (LEP10) and the combination of 10 mM ethanol and 5 µM resveratrol (LEP10+RP5). All pretreated cell cultures were exposed for 24 hours to Aβ_{25-35}. The cultures were then fixed and stained by Hoechst 33342.

Five random pictures were taken from each well of dishes by florescent microscopy, and the mean percentage of dead cells to total cells was calculated for each group. A one-way ANOVA analysis revealed that the number of dead cells increased significantly in Aβ_{25-35} added to the untreated cell cultures compared to the untreated group (Fig 9). It also showed a significantly increased number of dead cells in cell cultures with non-protective concentrations of ethanol alone or resveratrol alone in cell medium (Fig 9). Changes in cell morphology were assessed by florescent microscopic examination (Fig 10). Exposure of cells to Aβ_{25-35} at 10mM ethanol or 5µm resveratrol or without any thing in cell medium resulted in more cell death than untreated cell cultures.
(Fig 10). However, the combined treatment with 10mM ethanol and 5μM resveratrol provide significantly reduced Aβ toxicity.
Figure 9: synergistic effect of non-protective level of ethanol and resveratrol confers neuroprotection against Aβ toxicity in cerebellar mixed cell cultures. The experiment was done 3 times and the result from representative experiment is shown. Data represented as mean±S.E.M. p<0.05 was considered the threshold for significance. ** P<0.001 (CON+Aβ VS CON and LEP10+RP5+Aβ). N=12
Figure 10: Demonstrates the synergistic effect of non-protective level of ethanol and on cerebellar mixed cell cultures. 12 well dishes were used for cerebellar mixed cell cultures and the treated cells were fixed and stained with 33342 Hoechst stain. The red arrows point to dead cells. The pictures represent here are one of the sixty pictures per each group of cell cultures.
v. Aim V: Determine the synergistic effect of non-protective concentration of ethanol and resveratrol induces the expression of peroxiredoxin 2 (PRX2).

The purpose of this Aim was to find out if the synergistic protective effect of combined non-protective concentrations of ethanol and resveratrol alone induces the expression of peroxiredoxin 2 in cerebellar mixed cell cultures. The results revealed that cerebellar mixed cell cultures, treated by combined non-protective level of ethanol and resveratrol showed increased level of PRX2 (Fig 10). Figure 10 represent the non-protective levels of ethanol or resveratrol alone did not induce the expression of PRX2.
Figure 11: Level of PRX2 in cerebellar mixed cell cultures after 3 days treatment with non-protective level of either ethanol or resveratrol and combination of them. Results are the mean ± S.E.M of two independent experiments. ** p<0.001 vs. control, N=8
CHAPTER V

DISCUSSION

Our Lab has established the neuroprotective effect of moderate ethanol (30mM) preconditioning against Aβ_{25-35} induced cell toxicity in cerebellar mixed cell cultures (Collins MA et al 2010). However the legal blood alcohol level in US is 80milligrams in 100 milliliter of blood, which is equal to 17.3 mM of ethanol. Three to four glasses of wine (5 fluid ounces of wine is one unite are needed to increase the alcohol blood level up to 0.08. To reaching the protective level of alcohol based on our lab studies it would be necessary to drink six glasses of wine.

It has shown that after on oral dose of 25mg (110 µM) of resveratrol the plasma concentration reached about 2µM after 1 hour (walle et.al 2004). However the protective dose of resveratrol against Aβ_{25-35} in hippocampal neural cell cultures is 25 µM, more than 10 times the oral dose (Han et.al 2004). To reach a 25µM resveratrol in plasma based of low resveratrol bioavailability a 312mg oral dose of resveratrol should be taken, which is amount of resveratrol in 24 liters of wine (highest amount of resveratrol in wine is 13mg/liter)!

The present study has shown that the combinatory effect of ethanol and resveratrol reduced the protective levels of either ethanol or resveratrol alone. 10mM ethanol combined with 5µM resveratrol blocked the Aβ_{25-35} toxicity. About two glasses
of wine (10 fluid ounce) can provide 10mM ethanol and almost 5 liter of wine can provide 5µM resveratrol in plasma.


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VITA

Nastaran Khodaie was born in Tabriz, Iran. She attended Eram High school in Tabriz, Iran and graduated as top 10 students from that top ranked high school in 1998. After attending 7 years medical school of Tabriz University, She received the Medical Doctor degree September 2005. She started her career as a family doctor and drug and substance abuse counselor in different hospitals in Tabriz. She practiced for 3 years as a family doctor in Tabriz, Iran.

During her practice as a physician, based on her experience, she noticed there is huge gap between physicians and medical scientists in finding practical solution for different diseases. She got so interested in medical research especially in neuroscience. After moving to United States she got her admission for neuroscience graduate program in medical school of Loyola University Chicago.

In the winter 2010 she joined Dr. Collins’s Research Lab to start her research on alcohol effect on central neural system. This thesis is a result of 2 years dedicated research in that lab.