The Effects Of Oil Palm Phenolics On Inflammation And Oxidative Stress In Relation To Amyloid Beta Plaques In Fad-Mutant And Wild Type B103 Cells

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THE EFFECTS OF OIL PALM PHENOLICS ON INFLAMMATION AND OXIDATIVE
STRESS IN RELATION TO AMYLOID BETA PLAQUES IN FAD-MUTANT AND WILD
TYPE B103 CELLS

by

LINDSAY GODSEY

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2012

MAJOR: NUTRITION AND FOOD SCIENCE

Approved by:

____________________________________
Advisor                                                    Date
DEDICATION

Throughout my graduate studies, I worked as a rehabilitation assistant for adults with brain and spinal cord injuries. With the acquisition of a head trauma increasing an individual's risk for developing Alzheimer's disease, I was able to see first hand how this disease can affect someone's life. Taking care of an individual with Alzheimer's disease requires 24 hour care and at times becomes extremely exhausting. With no cure, I felt helpless watching my clients struggle with their daily activities. Although my thesis work is one small step in better understanding this disease, it was still very important to me. Despite my clients declining state, they maintained positive attitudes and showed perseverance to work through not only their disabilities from a head trauma but also from their dementia. My clients are the ones who provided me with the motivation and dedication to complete this thesis; therefore, I dedicate this thesis work to them.
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CHAPTER 1

INTRODUCTION

I. Dementia

Dementia affects approximately 35.6 million people in the world (33). This disease is a result of damaged brain cells and/or the damage of the connections between them. It results in a decline of memory and has been determined to be caused by other diseases or conditions ranging from severe head trauma to specific vitamin deficiencies. To be classified as dementia, the decrease in memory must be beyond the memory loss related to the normal ageing process and also severe enough to affect the individuals' daily activities. In addition, an individual with dementia experiences either a decline in the ability to speak or understand language, recognize or identify objects, complete motor activities, or problem solve and make accurate judgment calls. Due to dementia possibly being caused by another disease or condition, it is important for a physician to determine the cause of it early on. Early detection would allow the patients to receive treatment for their condition, sometimes leading to the reversal of their symptoms. Treatable conditions associated with dementia include delirium, depression, drug interactions, alcoholism, specific vitamin deficiencies, and thyroid complications. If an individual does not express any of these treatable conditions, the specific type of dementia needs to be determined in order for it to be treated properly. Some individuals may even express multiple types of dementia at one time making treatment more complex (29).
II. Alzheimer's disease

Alzheimer’s disease (AD) is a very progressive type of dementia. Within the United States, it has been determined to be the 6th leading cause of death within all age groups, and the 5th leading cause of death in those above the age of 65. AD is a disease that was discovered over 100 years ago; however, the pursuit for the discovery of a treatment did not become urgent until recently. With the baby boomer generation currently aging, approximately 10 million individuals will soon be added to the older, most at risk, population. This increase in the older population is evident when looking at the number of deaths associated with AD. There has been a 66% increase in AD associated deaths between the years 2000 and 2008 (29). Unfortunately, this number is only expected to continue to increase over time.

Every 69 seconds someone within the United States develops this disease and the outlook doesn’t look promising. By 2050 the rise in occurrence of AD is expected to increase to the point at which someone is diagnosed with this debilitating disease every 33 seconds, rather then every 69 seconds. In the year 2011, 5.4 million Americans were considered to have AD. Not only does the diagnosis of AD alter lives, but 61% of the individuals with AD who are 70 years of age are estimated to die before the age of 80. With 13% of the population over the age of 65 and 43% of those aged 85 years of age or older developing AD, the understanding and treatment of this disease is critical for the ever growing older population (29).

1. Signs and Symptoms of AD

Although some individuals are never diagnosed with AD due to their idea that their symptoms are contributable to the normal aging process, the symptoms of AD are
very prominent and debilitating. The first and most commonly recognized symptom occurs when an individual has difficulty remembering new information. This symptom is the result of the first stages of neuronal cell death occurring in the region of the brain involved in forming new memories. As AD progresses and more brain cells begin to die, the damage spreads into other regions of the brain and the individual’s symptoms worsen. Other recognizable symptoms of AD consists of confusion with orientation of time and place, trouble speaking and writing, difficulty solving problems, memory loss, misplacing of objects, alterations in mood and personality such as becoming withdrawn, and altered judgment. As symptoms worsen and become more frequent, their daily life becomes disrupted. Activities that require cognitive and functional abilities, such as making a cup of coffee, may become too difficult for the individual to complete alone (29).

The progression of this disease varies from individual to individual and can rate from moderate to severe. When AD reaches the severe state, the individual may become bed bound, unable to communicate, and may no longer be able to recognize their family and friends. Additional complications such as decreased ability to move around and malnutrition attributed to swallowing disorders, makes AD patients highly susceptible to acquiring pneumonia. For these reasons, pneumonia is the most common cause of death in AD individuals. With this disease being extremely progressive and life changing, a caregiver is a crucial key for the survival and health of these patients (29).

2. Care Giving

For most individuals with AD, approximately 70% are taken care of by their family members during the early stages of their disease. In 2010, approximately 14.9 million
unpaid family members and friends provided 17 billion hours of care to their loved ones with AD. If these individuals were to be paid, it would have totaled over $202 billion dollars. Despite not being paid for the care they provide, they also experience emotional, physical, financial, and work related stress. This stress increases when the AD patient has additional health issues. This situation is very common and became evident in 2004 when 26% of AD patients over the age of 65 who received Medicare beneficiaries were also diagnosed with coronary heart disease, 23% with diabetes, 16% with congestive heart failure, 13% with cancer, and 8% with Parkinson’s disease (29).

When the patient’s status becomes severe, around the clock care is usually needed. The most common solution is to place the individual in a nursing home. By the end of the year 2011, the healthcare, housing, and hospice services cost the United States approximately $183 billion. This total was calculated without including the cost of family members and caregivers who are unpaid. With the incidence of AD increasing, this number is expected to rise to $1.1 trillion in the year 2050 (29). These statistics show not only the emotional impact on society, but also the financial impact.

3. Types of AD

Individuals can fall into one of two categories; familial AD (FAD) or non-familial AD. FAD occurs in less than 1% of the population and has been discovered to develop due to rare genetic mutations. When an individual inherits a mutation on chromosome 21, the gene for the amyloid precursor protein, chromosome 14, the gene for the presenilin 1 protein, or on chromosome 1, which is the gene for the presenilin 2 proteins, it guarantees that they will develop AD. Some individuals with FAD can begin developing their symptoms as early as 30 years of age. Individuals without these genetic mutations
are said to have non-familial AD. This category can be further divided into two subcategories; late-onset AD and early-onset AD. When non-familial AD occurs in people under the age of 65, it is classified as early-onset. When an individual develops AD at or above the age of 65 it is considered to be late-onset. Of the two non-familial types of AD, late-onset is the most prevalent form (29). These types of AD are becoming better understood; however, the etiology remains unknown making this disease difficult to diagnose and ultimately treat (24).

4. Diagnosis

Currently, primary care physicians are not recommended to perform any assessment tests for AD on their patients unless the individual is presently displaying signs and symptoms. The pathway to diagnosis is not well established and is usually based on the person’s history in addition to cognitive and behavioral examinations performed by the physician (29). An individual may also undergo magnetic resonance imaging (MRI) to possibly identify some changes in the brain's physical appearance; however, it also can provide an inaccurate diagnosis. A diagnosis can only be confirmed after an individual has died and an autopsy of the brain can be conducted (37). Some studies suggest that approximately 80% of individuals with AD have not been diagnosed. The reasoning for this statistic is thought to be due to the lack of health care, or individuals assuming symptoms are a part of their normal aging process. If a person is diagnosed with FAD, the cause is known to be contributed to genetics; however, the cause of non-familial AD, which is more prevalent, is not well understood. Despite not having an accurate diagnostic tool, some risks factors for developing AD have been established and are often considered by the physician upon diagnosis (29).
5. Risk Factors

When an individual is being assessed by their physician, specific risk factors which consist of modifiable and non-modifiable factors are considered. Non-modifiable risk factors include increasing age, family history, the additional diagnosis of mild cognitive impairment (MCI) and genetics. Increasing age is the greatest risk factor. AD is not a part of the normal ageing process, but reaching the age of 65 greatly increases the chances of an individual developing this disease. MCI has also been discovered to be a risk factor for AD. It was found that 15% of those who have the condition MCI have progressed to dementia later on. MCI is considered to possibly aid in the progression of normal aging neuronal loss to the substantial neuronal deficits found in AD individuals (29).

Genetics has been found to play a major role in the development of FAD; however, it has also been discovered to act as a risk factor for non-FAD individuals. Apolipoprotein E is a protein that carries cholesterol within the bloodstream. There are 3 forms, ε2, ε3, and ε4. Each person inherits one form of the APOE gene from each parent. It has been found that those who inherit the APOE ε4 form have an increased risk for developing AD when compared to those who inherit the ε2 or ε3 forms. If an individual inherits APOE ε4 from both parents, their risk increases even more. Having this form of APOE gene does not guarantee the occurrence of AD but it has been shown to act as a risk factor for the development of it (29). All of these risk factors are non-modifiable making it impossible for an individual to control the acquisition of them; however, there are some that people can modify to help decrease their risk for developing AD.
Modifiable risks that can alter an individual's chances for developing AD include acquiring head traumas or traumatic brain injuries. When someone experiences a moderate head trauma, their chances for developing AD doubles. A moderate head trauma is characterized as a head injury that causes the loss of consciousness or amnesia that lasts for more than 30 minutes. A severe head trauma is when the loss of consciousness lasts for more than 24 hours. If this situation occurs, the individual's chances of acquiring AD increases by 4.5 times (29).

Additional modifiable risks include high cholesterol, type 2 diabetes, high blood pressure, a sedentary lifestyle, smoking and obesity; also referred to as cardiovascular risk factors. These risks are associated with the development of AD risks due to the idea that the heart ensures the brain receives enough oxygen and nutrient enriched blood in order for it to function normally (29). Without a healthy heart, the brain will not receive what it needs and may result in the decline of the brain's functionality. If a person avoids head traumas and takes care of their heart, their chances for developing AD have been shown to decrease.

Other factors have also been researched to determine if they play a role in the development of AD. These factors include race, gender, and socioeconomic status. In 2011, two thirds of the 5.2 million individuals diagnosed with non-familial AD were women. Despite this statistic, it can not be determined that women are more likely then men to develop AD. It can not be stated due to women living longer then men and this possibly affecting this statistic; however, it is a startling statistic that should be looked at further. Another factor considered is the socioeconomic status of an individual. It is thought that an individual with a lower socioeconomic status has a higher risk of
developing AD. This may be due to their lack of achieving a higher education and their inability to obtain adequate health care. Education may play a direct role because research has shown that learning allows your brain to grow which in return increases the number of neuronal synapses. Since AD decreases the number of neuronal synapses, the more you have to begin with may possibly delay the acquisition of the debilitating symptoms. Race was also looked at as another risk factor and was in fact determined to play a role in the risk for developing AD. It was discovered that African Americans are twice as likely then Caucasians to develop AD. Hispanics also have a higher risk then Caucasians and are approximately 1.5 times more likely to develop it. These differences are most likely due to the physiological variations among races (29). With the risks for the development of AD coming in from so many different angles, an individual should modify the risks they can and also be aware of those they can not.

6. Treatments

With the large number of risks and the increasing prevalence of AD, a treatment solution is necessary. Currently, two drugs, cholinesterase inhibitors and Namenda, are being prescribed to AD patients. These treatments do not prevent or cure this disease; however, they have been shown to delay the progression or onset of it. Cholinesterase inhibitors prevent the breakdown of acetylcholine which has been found to be important for memory and learning. The exact mechanism of acetylcholine and the effects it has on AD is not well known but it has been found to be effective for the prevention of symptoms from worsening. Namenda, N-methyl-D-aspartate antagonist, regulates the amount of glutamate within the brain. When glutamate levels are above the normal range, neuronal cell death has been shown to occur. Preventing the accumulation of
glutamate has been shown to be important for allowing patients to carry out their daily activities for an extended period of time (31).

Despite the benefits from these drugs, there are also negative side effects. In order to try and prevent them, individuals begin taking these drugs at low dosages and then slowly increase them over a period of time. The side effects can include nausea, vomiting, diarrhea, weight loss, loss of appetite, and muscle weakness while taking cholinesterase inhibitors. Side effects for Namenda include dizziness, headaches, constipation, and confusion. Due to these drugs effecting different chemicals and pathways in the body, it is possible for the patient to receive both of them at the same time (31). Although it is a step in the right direction, only half of the individuals receiving these drugs actually end up benefiting from them. Unfortunately, if an individual does benefit from taking them, it usually only delays their symptoms for approximately 6 to 12 months (29). To develop a more effective treatment, the causes and hallmarks of AD need to be better understood.

7. Hallmarks of AD

Although some of the risks and symptoms of AD have been discovered, the real mechanisms that are known to be the hallmarks of this disease need to be better understood. There are two definitive characteristics of AD; senile plaques and intracellular neurofibrillary tangles (24). A normal adult brain contains approximately 100 billion neurons. Each neuron contains branching extensions referred to as dendrites which allow them to form over 100 trillion connections with other neurons known as synapses. These connections are vital for the brain to function and allow the neurons to pass information such as memories, thoughts, sensations, emotions, skills, and
movements amongst one another. In the brain of an individual with AD, the number of neuronal cells and synapses begin to cease due to the accumulation of senile plaques and the formation of neurofibrillary tangles (29).

### a. Tau Proteins

One known hallmark of AD is the intracellular accumulation of neurofibrillary tangles. Microtubule-associated proteins, also known as tau proteins, help to stabilize and promote the assembly of microtubules for neuronal cells within the normal brain (24). In an AD brain, tau proteins become hyperphosphorylated which in turn causes them to no longer function and begin to accumulate. As the number of hyperphosphorylated tau proteins build up, they eventually create neurofibrillary tangles. Due to these tangles forming amongst the neuronal cells, they begin to cause irregularities in axonal transport ultimately resulting in the prevention of the transportation of vital nutrients and chemicals (5, 29). In the end, the inability to receive these important chemicals leads to cell death and results in the progression of AD (24).

### b. Senile plaques

Senile plaques, also known as amyloid beta (Aβ) plaques, build up in the damaged axons of the neurofibrillary tangles and are another hallmark for AD (37). The amyloid precursor protein (APP) is approximately 120kDa in size and is expressed in mammalian cells (7). It is a vital protein that mediates neurite outgrowth and when cleaved, it produces chemicals that are important for the maintenance and formation of neuronal synapses (37). APP is processed post-transitionally and is first cleaved on the cells surface by α-secretase. This produces a molecule that is approximately 100kDa in size that is usually referred to as sAPPα. It has been discovered to aid in the health and
development of synapses. The remaining APP is then cleaved by β-secretase which produces the N-terminal fragment referred to as sAPPβ. This fragment is internalized, and for normal individuals, is vital for axonal pruning. For individuals with AD, the internalization of sAPPβ causes additional neuronal cell death which aids in the progression of their disease. The last cleavage of APP is carried out by γ-secretase. This protease has the ability to yield two different products; Aβ1-42 and the APP intracellular domain (AICD). The presenilin-1 (PS1) and presenilin-2 (PS2) genes play a role in whether or not the Aβ1-42 fragment is formed. This is evident when looking at those individuals diagnosed with FAD which have been found to have mutations on the PS1 and PS2. The Aβ1-42 fragments have aggregate properties; therefore, they accumulate and cause oligomerization of the fragments resulting in plaque formation. When the plaques continue to build up, vital mechanisms such as ion channels, calcium homeostasis, energy metabolism, and glucose metabolism becomes impaired (4).

Aβ1-42 fragments have been found in the cerebral spinal fluid of normal individuals and can be related to the normal aging process; however, normal brains are able to degrade some of these fragments preventing excessive accumulation (7). In 1992, J. Hardy developed the amyloid cascade hypothesis. His idea suggested that the amount of Aβ1-42 produced in the brains of AD patients is not equal to the amount cleared, ultimately causing the build up and accumulation of senile plaques (5). In FAD patients, it has been noted that the genetic mutations are the cause for the overproduction and accumulation of Aβ1-42 fragments, but unfortunately the mechanism for non-familial AD patients still remains unclear (17). The understanding for the overproduction of these plaques is necessary due to them playing a major role in
neuronal cell death via lipid peroxidation, imbalance of ion homeostasis, and programmed cell death; all of which results in the progression of AD (37).

8. Underlying Causes of AD Neurodegeneration

Based on the knowledge for the hallmarks for AD it may seem clear as to which issues need to be addressed in order to slow the progression of or even cure the disease; however, it is unfortunately not that simple. The formations of neurofibrillary tangles and Aβ plaques have been found to lead to the development of additional underlying causes of neurodegeneration (4). Two well known underlying causes are oxidative stress and inflammation. These characteristics can aid in the progression of AD eventually becoming detrimental to the individual. Reactive oxygen species formation and microglial activation have been found to play key roles in the development of these underlying causes of AD neurodegeneration (26).

a. Reactive Oxygen Species

Reactive oxygen species (ROS) carry an extra oxygen molecule making them highly reactive chemicals (3). They are formed as a byproduct of aerobic respiration carried out by mitochondria within the cell. In a normal cell, the high reactivity of ROS is balanced out by antioxidants and enzymes. For an individual with chronic diseases such as AD, ROS is produced in excessive amounts. As a result, the body can not balance out the disproportionate amounts causing highly reactive ROS chemicals to remain present (6). The formation of these chemicals not only aids in the development of the hallmarks of AD but is also linked to oxidative stress and inflammation.
ROS formation has been found to play a key role in the formation of tau proteins. When there is an increase in ROS formation it in turn activates kinases such as protein kinase C (PKC), protein kinase A (PKA), and extracellular signal-regulated protein kinase 2 (Erk2). In the normal brain, these kinases are not activated; therefore, their activation in the AD brain is considered to cause the irregularity or hyperphosphorylation of the tau proteins. Tau then becomes disassociated from the microtubule causing oligomerization of the Tau and destabilization of the microtubule. As a result, neurofibrillary tangles form intracellularly and neuronal cell apoptosis occurs (4).

The other hallmark of AD, Aβ plaque formation, has also been linked to ROS formation. ROS is thought to be produced as a result of the accumulation of Aβ1-42 fragments. As Aβ plaques form, ROS formation becomes over expressed. This excess formation of ROS leads to oxidative stress, membrane damage, and ultimately further neuronal apoptosis. Also, the ROS formed by Aβ plaques can in turn be used for additional hyperphosphorylation of tau proteins (4).

Oxidative stress and inflammation are additional consequences from increased ROS formation (4). It has been found that mitochondrial ROS can act as signaling molecules and activate pro-inflammatory agents (6). The shift in the balance of ROS formation and degradation leads to oxidative stress and lipid peroxidation which has been found to occur in the brains of AD individuals. The lipid peroxidation then causes membrane damage and oxidative stress results in the production and activation of p53, Bad and Bax. These three chemicals take part in cell death via apoptosis and excitotoxicity (3). As oxidative stress increases, there is an increase in the production of
poly (ADP-ribose) polymerase (PARP). This protein can alter many transcription factors including one that regulates the activation of microglial (28).

b. Microglial Activation

Microglial are macrophages whose key role is to act as an immune response cell within the central nervous system. The exact role they play in the brain is not well known; however, their activation associated with the aging of the hippocampus and neuropathic pain leads to neurotoxicity and over expression of inflammation (38). Inflammation begins when bioactive lipids that are produced by arachidonic acid known as eicosanoids are released. The most common eicosanoid released is prostaglandin E₂ (PGE₂) and due to its prevalence within the body during inflammation, it serves as a mediator for cytokine-target gene expression (16). When microglial become activated, many morphological changes occur as well as an increase in pro-inflammatory agents such as cytokines (27).

The body begins the process of inflammation when it detects injuries from certain stimuli such as damaged cells, pathogens, and irritants. When this process is working normally, it is vital for the body in order for it to return it to its homeostasis. It accomplishes this by eliminating the harmful stimuli. The problem occurs when the body’s response causes uncontrolled inflammation. This can actually induce more harm then good, making the regulation of inflammation important for the health of an individual (16). In someone with AD, Aβ plaques have been shown to cause microglia activation which then leads to cytokine production. This recruitment of the pro-inflammatory cytokines causes inflammation to be expressed beyond normal levels causing harm to the individual (4).
Microglial can cause the progression of AD through another pathway was well, the regulation of metalloproteinases (MMPs). When microglial become activated, MMPs are released. These molecules have the ability to open the blood brain barrier. This characteristic allows for direct neurotoxicity of the brain ultimately resulting in additional neuronal cell death (28).

Microglial activation and ROS formation plays vital roles in neurodegeneration. They seem to have their own direct mechanisms, but more information is needed. For example, when examined under experimental conditions, Aβ plaques caused activation of microglial; however, despite microglial releasing neurotoxic chemicals, they can also release neurotrophic agents that can aid in the reduction of Aβ plaques via phagocytosis (11). As each mechanism for the neurodegeneration of AD is discovered, many are beginning to be linked to together taking one step closer to the discovery of a treatment.
**Figure 1:** Amyloid plaque and neurofibrillary tangle formation in Alzheimer’s disease.

III. Biomarkers

Due to inflammation and oxidative stress being considered as underlying causes of AD neurodegeneration; two biomarkers will be looked at throughout this research: cyclooxygenase-2 (COX-2) and poly (ADP-ribose) polymerase (PARP). COX-2 will be examined due to it being a well known marker for inflammation in non-AD and AD individuals (37). PARP alters the transcription factor that regulates the activation of microglial which, as stated earlier, are known to regulate pro-inflammatory agents. These characteristics allow it to be used as an inflammation biomarker (28). Additionally, research shows PARP becomes activated and over expressed in the presence of oxidative stress also making it an oxidative stress marker (15). Due to the characteristics of these biomarkers, both have been used widely in AD research.

1. Cyclooxygenase

With researchers knowing inflammation is some how linked to Aβ plaque formation, they have recently discovered cyclooxygenase (COX) as a possible biomarker for it in individuals with AD. COX is considered to be a biomarker due to it being a rate-limiting enzyme that plays an important role in the production of bioactive prostaglandins (PGs) (37). PGs, as explained previously, are synthesized from arachidonic acid and are thought to influence the onset, magnitude, duration, and course of the inflammation process (16). This is accomplished by two isoforms of COX, COX-1 and COX-2. These isoforms are encoded from different genes and is noticed when looking at their amino acid sequences due to approximately 40% of their amino acid sequences varying. Attributable to their amino acid diversity, their pathophysiological roles differ as well. COX-1 is predominately found regulating the physiological functions
of the kidney and a variety of tissues such as platelets. Since, COX-2 is derived from prevalent inflammatory chemicals, PGs; it is expressed by a variety of stimuli such as cytokines and growth factors. Due to the characteristics of the COX-2 isoform, recent research has discovered that COX-2 may play a role in diseases such as cancer, neuronal disorders, and those that include the characteristic of inflammation such as AD (37).

There have been many studies that have been able to link inflammation to AD by looking at COX-2 as an inflammatory marker. One piece of evidence that suggests a relationship between AD and COX-2 discovered that individuals who received non-steroidal anti-inflammatory drugs (NSAIDs) compared to those who did not, delayed the onset and/or progression of AD (18). Most NSAIDs work by inhibiting COX causing the link between COX and AD to be looked at more closely (2). Additionally, it was found that the expression levels of COX-2 were higher in certain brain regions of individuals with AD when compared to non-AD patients. COX-2 was elevated in the frontal cortex, hippocampus, and thalamus regions of the AD brain. It was also determined that COX-1 levels were not significantly different within the hippocampus neurons between AD and non-AD patients; therefore, specifying that the COX-2 isoform is the one most commonly expressed (22). Further research discovered that there is a correlation between COX-2 immunoreactivity and the number of Aβ plaques present within the neurofibrillary tangles containing damaged axons. Based on this past research Yoshimoto et al. in 2001 found that the mRNA expression of APP was stimulated when COX-2 was overexpressed. As a result of this increase in APP production, the amount of sAPPβ and ultimately Aβ1-42 fragment production was also stimulated (37).
Along with clinical and cell models, animal models have also been used and displayed similar evidence. Cakata et. Al in 2007 injected Aβ intracerebroventricularly into twelve month old C57Bl6 mice. For the group injected with Aβ, the levels of COX-2 expressed at the protein level increased significantly. It was determined that the COX-2 immunoreactivity was elevated due to systemic inflammation. These characteristics coincided with behavioral changes as well. The mice injected with Aβ demonstrated an increase in memory disturbances via a lack in object recognition when compared to the control mice. Another group of mice also received the Aβ injection along with an additional injection of COX-2 inhibitor. This group of mice demonstrated no memory deficits or changes in motor skills (2). Wilcock et al. took the research one step further in 2011 by using transgenic AD mouse models. They altered specific inflammatory genes and showed that by decreasing the expression of them, it leads to significant reductions of Aβ fragments (34). With many researches carrying out experiments such as these, it poses the possibility of a link between inflammation, COX-2, and the accumulation of Aβ plaques. The observation of Aβ plaques and neurofibrillary tangles being surrounded by active inflammatory cells has also been noted, but the mechanistically effects inflammation has on AD remains controversial and not well understood (34).

2. Poly (ADP-ribose) polymerase (PARP)

A nuclear protein that activates the release of pro-inflammatory agents such as cytokines, ROS, and proteases, is known as poly (ADP-ribose) polymerase (PARP). There are many forms of this protein; however, PARP-1 accounts for over 90% of the total PARP activity within a cell (28). It is a protein that is well known for its ability to
repair DNA. Due to this characteristic, it also allows it to bind to certain transcription factors causing them to become altered (15).

The alteration of transcription factors that PARP plays a role in regulates the release of pro-inflammatory agents. PARP can regulate the expression of many inflammatory mediators; however, all of them are regulated by one factor; the nuclear factor-kappa β (NF-κβ) (36). NF-κβ is known to be regulated by the binding and altering of DNA via PARP (15). NF-κβ regulates macrophage and microglial activation (2). As discussed earlier, microglial activation can release pro-inflammatory agents and increase inflammation. Experimental data has shown that by knocking out the PARP gene, in mice and cells, reduction of both the activation and transcription of NF-κβ can be reduced, ultimately leading to the reduction of pro-inflammatory agents (36).

With PARP linked to inflammation, it has become looked at more carefully in AD research. Experimental data has discovered that within the brains of AD individuals, PARP is over expressed and highly active (11). The reasoning for this is thought to be due to PARP becoming activated by single strand breaks within the DNA possibly due to oxidative damage (36). Widespread over activation causes cells to become depleted of intracellular NAD+ and ATP pools ultimately leading to cell death (15, 36). This occurs due to PARP initiating the cycle of transferring ADP ribose units from NAD+ to a nuclear protein resulting in the consumption of the cells energy (36). This is problematic in AD individuals due to the accumulation of Aβ plaques causing an increase in oxidative stress leading to additional DNA damage and the over expression of PARP (15).

With the characteristics of PARP and ROS formation causing an increase in oxidative stress and inflammation, AD individuals face a constant battle. Based on
previous research, the Aβ plaques formed in AD individuals causes an increase in ROS formation. This can then lead to inflammation, oxidative stress, and an increase in tau protein hyperphosphorylation. Oxidative stress causes an over activation of PARP which in turn causes activation of microglial. Due to the microglial activation, additional pro-inflammatory agents are released and additional Aβ plaques are formed. The constant cycle between over expression of inflammation and oxidative stress makes the treatment of AD very difficult.

Due to ROS formation playing a key role in the neurodegeneration of AD individuals, antioxidants can potentially aid in the delay of AD progression. The high reactivity of ROS and imbalance in degradation and formation, leads to the underlying causes of AD neurodegeneration. With more antioxidants present it may be able to balance out the reactivity of ROS and reduce the amount of reactive ROS within the cells. By examining biomarkers such as PARP and COX-2, their expression may be able to be decreased by the use of antioxidants, such as oil palm phenolics.

IV. Oil Palm

Oil palm belongs to the species *Elaeis guineensis* and originates from the tropical forests in West Africa. Due to an increase in industrial cultivation of this species, other countries such as Malaysia and Indonesia are becoming large producers (30). Palm oil has a fatty acid composition of a 1:1 ratio of saturated to unsaturated fatty acids; this makes the oil semi-solid at room temperature. Due to this characteristic, margarine and shortening can be produced without hydrogenation, making it the second largest consumed oil in the world (27). Oil palm contains two separate components; fat-soluble compounds as well as large amounts of water-soluble phenolic compounds.
1. Fat Soluble Components

As oil palms popularity increases among consumers and researchers, components of it are beginning to be looked at more closely. Research has shown that individuals who are consuming palm oil as a part of a low-fat diet can better maintain their plasma cholesterol and lipoprotein cholesterol at more desirable levels (27). The fat-soluble components include vitamin E (30% tocophorels, and 70% tocotrienols) and carotenoids (23, 27). During the processing of palm oil, the carotenoids are usually removed and the levels of tocotrienols are highly reduced. This is due to the use of high temperature steam deodorizations. It has been shown that compounds within the fat-soluble components have anti-cancer properties so it is important to try and preserve these nutrients (27). With the fat-soluble components retaining health benefits, the water-soluble compounds are now being used in current research as well.

2. Water Soluble Components

Oil palm phenolics (OPP) consist of the water-soluble compounds retained after extraction of palm oil from the oil palm fruit (27). These constituents are extracted from oil palm by vigorous centrifugation and membrane filtrations (23). Current technology allows the extraction of OPP to maintain its vital compounds by using separation techniques that isolate each compound based on its molecular weight (27). OPP consists of three major phenolic acids, caffeoylshikimic acid isomers, protocatechuic acid and p-hydroxybenzoic acid. It has shown to not cause toxicity within various animal models; therefore, it has become a component in many upcoming research experiments including this one (23).
Phenolics have high antioxidant capacities due to their aromatic ring structure containing a high reduction-oxidation potential. This characteristic allows them to act as hydrogen donors, reducing agents, singlet oxygen quenchers and metal chelators. Antioxidants prevent the formation of prooxidants by scavenging for them or by inducing their decomposition. This is helpful due to prooxidants being linked to the triggering of chronic diseases and ageing via oxidization of macromolecules. This is carried out by hydroxyl radicals causing lipid peroxidation which in turn modifies DNA bases or damages proteins resulting in tissue damage. Prooxidants are also linked to inflammation due to their capability to regulate gene expression specifically the nuclear factor kappa-B and activator protein-1 (23).

OPP works as an anti-oxidant via alteration of genes by either silencing them or turning them on. It has been determined that OPP effects gene expression in the heart, liver and spleen (23). These changes in gene expressions were not drastic enough to cause harm or alterations to the organs or hematology; however, it did alter them enough to up-regulate lipid catabolism and down-regulate cholesterol biosynthesis in the liver (23). OPP also promoted aggregation of platelets in the spleen and slowed the ageing process in hearts. Due to prooxidants playing an important role in the link between oxidative stress and inflammation for the onset of chronic diseases, phenolics such as OPP have been shown to contain many possible therapeutic elements (23).

V. Hypothesis

The high number of reports linking inflammation and oxidative stress to Aβ plaques formation has opened the door to possibly using naturally occurring antioxidants such as OPP to decrease inflammation and ultimately retard the accumulation of Aβ
plaques. This information is a small step that will hopefully lead to the better understanding of AD and eventually the prevention and/or treatment of it. Thus, it was hypothesized that the addition of oil palm phenolics will decrease inflammation and oxidative stress in familial Alzheimer’s disease cells in vitro. To test this hypothesis, the specific aims stated below were carried out:

**Specific Aim I:** To determine the anti-amyloidogenic activities of OPP, if any, by measuring the inhibition of formation and destabilization of amyloid beta proteins using a thioflavin T (ThT) binding assay.

**Specific Aim II:**

**II (a):** To determine the effect of OPP on cell growth and viability of the FAD-mutant cell constructs with increase in passage using MTS assay.

**II (b):** To investigate the effects of OPP on the protein expression of APP, COX-2 and PARP, known biomarkers for AD and inflammation in FAD mutant and WT cells.

**Specific Aim III:** To investigate the effect of OPP on the mRNA expression of COX-2 and amyloid precursor protein (APP) in FAD mutant and WT cells using real time RT-PCR.
CHAPTER 2

METHODOLOGY

Specific Aim I: To determine the anti-amyloidogenic activities of OPP, if any, by measuring the inhibition of formation and destabilization of amyloid beta proteins using a thioflavin T (ThT) binding assay.

1. Preparation of Amyloid Beta Fibrils

For both the destabilization and deformation assays, the Aβ 1-42 proteins (Peptides Internationals, Louisville, KY) needed to first be dissolved and diluted to the concentrations described by Katayama, S., Ogawa, H., and S. Nakamura in 2011 (10). This was accomplished by dissolving 0.5 mg of Aβ 1-42 proteins in 400 ul of a 0.1% ammonia solution for a final concentration of 250 uM. It was then divided equally into 4 vials, sealed, and stored at –80 degrees Celsius until use. Using a 50 mM phosphate buffer containing 100 mM of NaCl and with a final pH of 7.5, the 250 uM Aβ 1-42 protein solution was diluted to a final concentration of 25 uM. For the destabilization experiment, the 25 uM Aβ 1-42 protein solution was incubated at 37 degrees Celsius for 24 hours in order to allow the amyloid beta fibrils to form. The inhibition of formation assay was carried out by adding the OPP concentration of 300 ul/10 ml directly to the 25 uM Aβ 1-42 protein solution and incubating it for 24 hours at 37 degrees Celsius.

The OPP concentration used in this experiment was 300 ul/10 ml. This concentration was used based on the results of the MTS assay showing that it was statistically significant when compared to the sample control. When the 25 uM Aβ 1-42 protein solution was created, each vial contained a total volume of 100 ul without OPP.
In order to achieve this desired concentration, 3 µl of OPP was added to the 100 µl of the 25 µM Aβ 1-42 protein solution.

Once the 24 hour incubation period was over, the OPP concentration of 300 µl/10 ml was added to the destabilization vials. Each vial, for both the destabilization and formation inhibition assay, was then vortexed and placed in a dark area. Thioflavin T (ThT) is a light sensitive fluorescence marker; therefore, the mixing and loading of this solution was done in a dark space. 1.2 ml of ThT solution containing 50 mM glycine, NaOH buffer with a final pH of 8.5 and a final concentration of 5 µM was added to 6 µl of each sample for both assays. Each tube was then vortexed and loaded into a black 96 well microtiter plate, in triplicates, with 250 µl of volume in each well. Multiple controls were also prepared and added to the microtiter plate. A ThT control was made by adding 6 µl of ethanol to 1.2 ml of ThT solution. A sample control was used to detect amyloid fibril formation without OPP and was created by adding 6 µl of 25 µM Aβ 1-42 protein solution to 1.2 ml of the ThT solution. Lastly, an OPP buffer control was prepared by adding 6 µl of OPP to 1.2 ml of the ThT solution. This was done in order to ensure the fluorescence readings were due to the ThT binding to the hydrophobic grooves of the β-sheet surface of the amyloid beta fibrils rather then the OPP itself creating fluorescence. Once all samples and controls were loaded into the microtiter plate, it was covered with aluminum foil in order to help prevent light from altering the samples.

2. Spectrofluorometer

After the samples were prepared and loaded onto a 96 well microtiter plate, it was then read using a Tecan GENios Microplate Reader (MTX Lab Systems Inc., Vienna, VA). The wavelengths were set at 450 nm for excitation and 492 nm for
emission. Shaking was turned on and the assay was conducted at room temperature. Each sample was read every 30 minutes for a total time period of 24 hours.

3. Statistical Analysis

Once all the readings were recorded, the OPP 300 ul/10 ml sample fluorescence was determined by averaging the three readings and subtracting the fluorescence averages of the OPP buffer fluorescence and ThT control. The sample control fluorescence readings were calculated by averaging the three readings and subtracting the average ThT control fluorescence. The OPP buffer control was not subtracted from the sample control due to no OPP being present. The sample control and OPP 300 ul/10 ml sample readings were then divided by the starting fluorescence value for the OPP 300 ul/10 ml sample. This was done in order to make all the values relative to 1. The change in fluorescence over a 12 hour time period was then graphed to illustrate the overall decrease in ThT fluorescence which is relative to the decrease in the number of Aβ plaques.

Specific Aim II (a): To determine the effect of OPP on cell growth and viability of the FAD-mutant cell constructs with increase in passage using MTS assay.

1. Cell Lines

The cell lines were kindly provided by Dr. Yilong Shu from the University of Alabama with permission of release from Dr. Lennart Mucke from the Gladstone Institute of Neurological Disease located at the University of California, where they were constructed. B103, rat neuroblastoma cells, were transfected with cDNA constructs. The FAD cells were constructed by the insertion of a mutant cDNA construct,
hAPP695mutant, in order to over express APP levels. The wild type cells also received a
cDNA construct, hAPP695wt, and were inserted to express APP at levels found in non-
AD individuals in order to be used as a control. These constructs were confirmed to be
stably transfected within the cell lines via Western blot analysis (19).

2. Passage Experiment

Since a rat neuroblastoma cell line was used, it was important to determine at
what passage the cells would begin to lose their construct. This was vital information in
order to know at which point the cells could no longer be used in experiments due to the
loss of their AD-like characteristics. Cells are considered to undergo one passage each
time trypsin/EDTA is added to detach the cells from the flasks wall. When the cells were
received from Dr. Yilong Shu’s lab they were in the first passage. Once the cells were
thawed and enough were grown in order to conduct an experiment, they were in the third
passage.

a. Re-Seeding of Cells

This experiment was initiated by thawing one vial of WT cells and one vial of FAD
cells received from Dr. Yilong Shu, and seeding them for growth. The cells received
were frozen within dimethyl sulfoxide (DMSO); therefore, they were thawed in a water
bath maintained at 37 degrees Celsius and then titrated. The contents of the FAD vial
were transferred into 13 ml of selection media in a T75 flask. The WT vial contents were
placed into a separate T75 flask also containing 13 ml of selection media. The selection
media was composed of 410 ml of 1X DMEM (Dulbecco’s Modified Eagle Medium)
(Cellgro, Manassas, VA), 50 ml of fetal bovine serum (FBS) (Gibco, Carlsbad, CA), 25
ml off horse serum (heat inactivated) (Gibco, Carlsbad, CA), 5 ml of
penicillin/streptomycin (Gibco, Carlsbad, CA), 5 ml of L-glutamine 100X (Gibco, Carlsbad, CA), and 5 ml of G418 (geneticin) (Gibco, Carlsbad, CA). This composition was optimized by Dr. Lennart Mucke’s lab. The T75 flasks containing the cells were then placed into an incubator maintained at 37 degrees Celsius, with 5% carbon dioxide and 95% humidity (19). The selection media was removed from the flask and replaced with 13 ml of fresh selection media the following day in order to remove any DMSO.

Both FAD and WT flasks were viewed under a microscope and left to grow until they were approximately 90% confluent. Once confluent, the selection media was removed from the flask, cells were washed with 10 ml of 10X phosphate buffer saline (PBS) (Cellgro, Manassas, VA), and 1 ml of 1x trypsin/EDTA (TE) (Cellgro, Manassas, VA) was added. Any cells that were floating within the selection media were considered to have lost the cDNA construct and were removed when the cells were washed with PBS. After approximately 5 minutes of letting the flasks sit with TE, 9 ml of selection media was added to wash the cells from the flasks wall and to inactivate the TE. All contents were removed from the T75 flask and placed into a 15 ml centrifuge tube. The cells were centrifuged for 3 minutes at approximately 1,500 RPM. A pellet formed and the supernatant was removed. 1 ml of selection media was added and titrated to completely dissolve the pellet. 0.5 ml was placed into a T75 flask containing 13 ml of selection media, and the other 0.5 ml was placed into another T75 flask also containing 13 ml of selection media. This was completed with both the FAD and WT cells separately. The T75 flasks were placed in the incubator with the same parameters stated earlier and monitored until they were again approximately 90% confluent.
b. Counting and Freezing Cells

For each passage of WT and FAD cells, one T75 flask was frozen and the other was counted and then re-seeded as shown in figure 2. Once the cells became confluent, the T75 flasks were prepared as though they were going to be re-seeded following the same procedure stated earlier in section (a). Once the pellets were formed after centrifuging, one was frozen while the other was counted and then used to re-seed.

To count the cells, the pellet was re-dissolved in 1 ml of selection media followed by the addition of 9 ml of selection media. The 15 ml centrifuge tube was inverted to mix, and then 15 ul was aliquoted into a 1 ml centrifuge tube. 15 ul of trypan blue (Sigma Aldrich, Saint Louis, MO) was added and was thoroughly mixed by titrating approximately 100 times. The 30 ul mixture was placed under a clean cover slip situated on top of a hemacytometer. The hemacytometer is used for cell counting which allows an approximation of the number of cells present within the T75 flask to be calculated. This was accomplished by counting the number of cells within the squares located in the 4 corners and the square located directly in the middle of the hemacytometer. The cells located on the left and upper borders of each square designated for counting were considered to be a part of that square and were also counted. If over 90% of the cells were in contact with other cells, the sample would be re-done due to the calculations not being a correct representation. The number of cells within each of the 5 squares were added together then divided by 5, multiplied by 2, and then multiplied by 10^4. The final number represented the number of cells per ml. These numbers were recorded and used to observe the rates at which the cells were growing. The remaining selection media within the 15 ml centrifuge tube was used for re-seeding the cells. 4.9 ml was
placed into a new T75 flask and the remaining 4.9 ml was placed into another T75 flask, both containing 8 ml of selection media.

To freeze the cells, the pellets for FAD and WT were re-dissolved in 1 ml of freezing media. 10 ml of freezing media consisted of 1 ml of DMSO and 9 ml of FBS. The contents were then transferred into cyro tubes and placed at -20 degrees Celsius for approximately 1 hour. After, they were moved to -80 degrees Celsius to be stored overnight. For long term storage the cyro tubes were placed in liquid nitrogen.

When the cells were received they were currently in their third passage; therefore, the cells were observed, counted, frozen, and re-seeded, several more times in order to acquire cells at passages 4, 5, 6, and 7. This process can be viewed by looking at figure 2. Due to FAD cells growing at a faster rate then WT cells and them becoming confluent at different times, the cells were sometimes split into 3 or 4 flasks, allowing multiple vials of each passage for FAD and WT cells to be frozen and stored.
Figure 2: Passage experiment flow chart. Explanation of which T75 flasks were frozen and which ones were re-seeded in order to acquire cells at different passages.
3. MTS Assay

Once cells were obtained from varying passages, the IC$_{50}$ concentrations were determined after the addition of OPP by carrying out the CellTiter 96 AQ$_{uo}$ous One Solution Cell Proliferation Assay (Promega, Madison, WI). In order to prepare for this assay, a vial of FAD and WT cells from each passage were thawed and placed in separate T75 flasks containing 13 ml of media. They were allowed to grow until they became 90% confluent within an incubator with the same specifications as stated earlier in part 2. Each cell type, from each passage, was then counted using a hemacytometer following the same guidelines discussed in section 2(b). Once the number of cells/ml was determined, dilution factors were calculated in order to acquire approximately 5000 cells/100 ul. This was done in order to place roughly the same number of cells in each well of the 96 well microtiter plate. Selection media was used to achieve these desired dilutions. 100 ul of cells from each passage were then placed into all wells within the 96 well plates except for those located around the perimeter. A 96 well plate was used for each type of cell and for each passage; therefore, there were plates for FAD cells in passages 4, 5, 6 and 7, and for WT cells in passages 4, 5, 6, and 7.

The plates were placed into the incubator and left for approximately 24 hours to allow the cells to attach. After the 24 hours, the media was removed from the wells using a multi-channel pipette. 100 ul of fresh selection media was added to each well located around the perimeter of the plate to be used as blanks and was also added to the first and last columns of wells which contained cells in order to be used as controls. 100 ul of differing OPP, from Malaysia, concentrations explained in figure 3 were added to differing columns.
Once the plates were incubated for 48 or 72 hours with varying OPP concentrations, 10 ul of the CellTiter 96 AQueous One Solution Reagent was added to each well. It was also added to a few wells located around the perimeter of the plate in order to acquire blanks. The plates were placed on a shaker approximately 30 seconds and then incubated with the CellTiter 96 AQueous One Solution Reagent for 3 hours. Each plate was read at an absorbance of 490 nm using the Bio-Tek microplate reader (Bio-Tek, Winooski, VT). Later, the MTS assay was completed using cells in passage 4 or passage 7. This modification was made based on the results of the initial MTS assay and was done in order to obtain more precise and accurate conclusions.
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**Figure 3:** Volumes of OPP and selection media used to obtain specific OPP concentrations used for MTS assay.
4. **Statistical Analysis**

Based on the data obtained from the Bio-Tek microplate reader, the three values closest together were taken for each OPP concentration, control, and blank. These values were then averaged and Microsoft Excel was used to calculate the standard deviations. Data from different passages and OPP concentrations were compared using the application of SPSS software. Values considered to be statistically significant had a p value that was less than or equal to 0.5.

**Specific Aim II (b):** To investigate the effects of OPP on the protein expression of APP, COX-2 and PARP, known biomarkers for AD and inflammation in FAD mutant and WT cells.

1. **Addition of OPP**

In order to complete Western blot analysis, the cells had to be thawed from liquid nitrogen and re-seeded. Despite the cells growing efficiently within the selection media even at passage 7, it was determined that passage 4 of both FAD and WT cells would be used. This decision was based on the results of the MTS assay and in order to be certain the cells retained their AD characteristics. One cyro tube for each cell type, FAD and WT, were thawed and seeded into 13 ml of selection media within T75 flasks. The cells were incubated with the same specifications as stated earlier and left to grow until they became 90% confluent. The cells were prepared by following the same procedures for re-seeding stated in section 2 (a); however, instead of adding the dissolved pellet back into a T75 flask, five Petri dishes containing 10 ml of selection media each received 200 ul of the cells. This was completed for passage 4 of FAD and WT cells for a total of 10 Petri dishes. The Petri dishes were then rotated in order to evenly distribute
the cells and then placed into the incubator overnight. After 24 hours, the plates were viewed under the microscope to confirm attachment of the cells to the plate wall. If cells were floating within the selection media they were considered to have lost their cDNA construct; therefore, they needed to be re-seeded due to possible contamination.

After 24 hours, and the cells were confirmed to be attached to the Petri dishes, the media was removed and 10 ml of the differing OPP concentrations were added to different dishes. Based on the MTS assay results it was determined to use the OPP concentrations of 150, 300, and 600 ul/10 ml. One Petri dish was used as a control and 10 ml of fresh selection media was added to it. The fifth Petri dish received an additional OPP concentration of 600 ul/10 ml. This was done due to such a high concentration of OPP possibly causing the cells growth to be so low that one plate would not produce enough cells to carry out the experiment. The Petri dishes were then incubated for the optimal time period of 72 hours.

2. **Protein Extraction**

   Once the cells were incubated with the differing OPP concentrations, protein extraction was completed. This experiment was conducted on ice in order to prevent protein denaturation. The media was removed from each Petri dish and placed into separate 15 ml centrifuge tubes. Each Petri dish was then scrapped with the addition of 1-3 ml of PBS and transferred into the corresponding 15 ml tube. The tubes were centrifuged at 1,500 RPM for 3 minutes. The supernatant was removed and based on the size of the pellet; the appropriate amount of lysis buffer (10X lysis buffer was diluted to 1X) (Cell Signaling, Danvers, MA) containing protease inhibitor cocktail for mammalian cells (Sigma Aldrich, Saint Louis, MO) was added. 200 ul of protease
inhibitor was added to 1 ml of lysis buffer. Only 1 ml was mixed at a time due to the protease inhibitor losing its properties after 30 minutes. Each of the 8 pellets was dissolved into twice their volume of lysis buffer. Lastly, the contents were transferred into 1 ml centrifuge tubes and stored at -80 degrees Celsius overnight.

3. Protein Assay

After 24 hours, the protein samples were thawed and centrifuged. The cells were spun at approximately 1,500 RPM while being maintained at -4 degrees Celsius. The supernatant was then transferred into new 1 ml centrifuge tubes and the pellets were discarded. The protein assay was carried out by using the Pierce BCA Protein Assay Kit (Thermo Scientific, Logan, UT). By following the specifications made by this kit, the BSA standards and the BCA working reagent were prepared. In a 96 well microtiter plate, 25 ul of each standard and each sample was added into 3 consecutive wells. The remaining protein samples were placed back into -80 degrees Celsius until the Western blot analysis could be completed. 200 ul of the BCA working reagent was then added into each of the wells by using a multi-channel pipette. The plate was placed on a shaker for approximately 30 seconds followed by 30 minutes of incubation at 37 degrees Celsius. The plate was allowed to cool to room temperature and then read at an absorbance at 570 nm using a Bio-Tek microplate reader (Bio-Tek, Winooski, VT). If the absorbance of the samples exceeded the maximum standard value, the samples were diluted. This was carried out by adding 10 ul of each sample to 90 ul of deionized water before loading them into the plate.
4. Western Blot Analysis

Western blot analysis was carried out to see the effects OPP had on the protein level of expression of COX-2 and PARP for FAD and WT cells. The first step completed in order to carry out this experiment was to assemble the gel apparatus. The glass plates were checked for leaks after being assembled and clamped. Once it was ensured that there were no leaks, the separating and stacking gels were prepared.

The first gel prepared was a 20% polyacrylamide gel mix. The separating gel was mixed and consisted of 5 ml of 30% acrylamide, 3.75 ml of 1.5 M Tris pH 8.8 (Bio-Rad, Hercules, CA), 6.125 ml of deionized water, 75 ul of 20% sodium dodecyl sulfate (SDS), 150 ul of 10% ammonium sulfate, and 15 ul of tetramethyleneethylenediamine (TEMED) (Acros Organics, Geel, Belgium). These solutions were mixed within a 15 ml centrifuge tube and immediately added in between the 2 gel plates previously assembled. Using a 1 ml pipette the separating gel was added until it reached approximately 2.5 cm from the top. If air bubbles were present within the gel, it was re-done. Deionized water was added on top while the gel hardened. After approximately 20 minutes, the gel was hardened and the water was removed by inverting the plate apparatus. To aid in the removal of all the water, filter paper was carefully used to soak up any water that remained.

After the separating gel was hardened, the stacking gel was prepared. For the 20% polyacrylamide gel, 2 ml of 30% acrylamide, 3.75 ml of 0.5 M Tris pH 6.8 (Bio-Rad, Hercules, CA), 9.125 ml of deionized water, 75 ul of 20% SDS, 150 ul of 10% ammonium persulfate, and 15 ul of TEMED were mixed via inverting the 15 ml centrifuge tube. The stacking gel was added on top of the separating gel and a 10 well comb was
placed into the gel. The comb remained in the gel until it completely hardened, approximately 20 minutes.

While the gels were hardening, the supernatant of the protein samples acquired earlier were prepared for loading. This was accomplished by first determining the protein concentrations and the amount of volume needed for each sample. This was calculated by using the data from the Pierce BCA Protein Assay Kit performed previously. The 3 values of protein concentrations provided by the Bio-Tek microplate reader in ug/ml were averaged and then converted to ug/ul for each sample. The ug/ul value was divided into 40 producing the value for the amount of protein needed of that sample. Once the calculations were computed, the samples were re-thawed by centrifuging them at -4 degrees Celsius at 2,000 RPM for approximately 1 minute. 5 ul of 6X loading buffer was added to the appropriate amount of protein followed by the addition of enough 1X loading buffer to bring the total volume of each sample up to 30 ul. These calculations were preformed in order to have an equal volume of protein for each sample. Once the protein samples were prepared, they had to be denatured. This was accomplished by wrapping them with parafilm and placing them in boiling water for 5 minutes.

After the gel and protein samples were prepared, the samples were loaded into the gel. The 10 well comb was removed and 30 ul of each sample was loaded into separate wells. In order to be able to compare the samples to a standard, one well received 30 ul of the Full Range Rainbow Marker (Fisher Scientific, Pittsburgh, PA) and another well received 30 ul of the Magic Mark XP Western Standard (Invitrogen, Carlsbad, CA). The gels were then ran at 50 mA in a Bio-Rad electrophoresis (Bio-Rad, Hercules, CA) with 1X electrode tris-glycine-SDS (TGS) (Bio-Rad, Hercules, CA) running
buffer. Once the bands passed through the stacking gel and entered the separating gel, the voltage was reduced to 35 mA. When the samples reached the bottom of the gel the machine was turned off.

After, the samples were run through the gel; it then had to be transferred onto a nitrocellulose membrane (Whatman, Piscataway, NJ). The first step in this process was to carefully remove the gels from the glass plates and remove the stacking portion. The gel was then placed on an equally sized piece of the nitrocellulose membrane which was placed on top of 3 equally sized pieces of filter paper. Once the gel was carefully placed and all air bubbles were removed, 3 more pieces of filter paper were placed on top and all components were placed on a Semi-Dry Transfer Blot Apparatus (AA Hoefer, Holliston, MA). Transfer buffer was used to soak the membrane and filter papers and was also added to the transfer apparatus. This buffer consisted of, 700 ml of deionized water, 100 ml of TGS buffer (Bio-Rad, Hercules, CA) and 200 ml of methanol. The transfer apparatus was ran for 90 minutes at a voltage of 80 mA.

After the gel was transferred to the membrane, it was incubated with the primary antibody. First, the membrane was washed with 1X phosphate buffered saline (PBS) for 5 minutes. 10X PBS was prepared by dissolving 80 grams of sodium chloride (NaCl), 2 grams of potassium chloride (KCl), 14.4 grams of disodium hydrogen phosphate (Na2HPO4), and 2.4 grams of potassium dihydrogen phosphate (KH2PO4) in 800 ml of deionized water. The pH was adjusted to 7.4 by adding either HCl or NaOH and then enough deionized water was added to bring the total volume to 1 liter. The 10X PBS was then diluted to 1X PBS with the use of deionized water. Secondly, the membrane was blocked with 25 ml of 5 grams of bovine serum albumin (BSA) (Sigma Aldrich, Saint
Louis, MO) dissolved in 100 ml of 1X PBS (5% BSA) for 2 hours. Once it was blocked, it was washed for 10 minutes with 1X PBS three times. The primary antibodies used were COX-2 (Novus Biologicals, Littleton, CO), 22C11 Anti-Alzheimer Precursor Protein A4 (Millipore, Temecula, CA), beta tubulin (Novus Biologicals, Littleton, CO), PARP 46D11 (Cell Signaling, Danvers, MA) and beta-actin (Cell Signaling, Danvers, MA). Each primary antibody was diluted to a 1 to 1000 ratio; therefore, 10 ul of the antibody was added to 10 ml of 5% BSA. The membrane was placed into a plastic bag, the diluted antibody was added, and all sides of the bag were sealed. The membrane was placed at 4 degrees Celsius on a shaker with high agitation overnight.

After the membrane was incubated with the primary antibody, it was then incubated with the secondary antibody. This was accomplished by first washing the membrane with PBS-Tween (PBS-T) (0.1% of tween added in 1 liter of 1X PBS) (Fisher Scientific, Pittsburgh, PA) 3 times with each wash lasting for 10 minutes. It was then incubated with the secondary antibody for 2 hours. Depending on the primary antibody used, either the goat anti-rabbit HRP conjugate (Bio-Rad, Hercules, CA) or the anti-mouse monoclonal antibody (Cell Signaling, Danvers, MA) was used. The goat anti-rabbit antibody was used for the primary antibodies, beta-actin, COX-2, PARP and beta tubulin, while the anti-mouse antibody was used for 22C11. These antibodies required different dilutions, however, it was discovered that they both worked the best with a 1:4000 dilution. This meant that 6.25 ul of the secondary antibody was added to 25 ml of 5% BSA. After the 2 hour incubation period, the membrane was washed with PBS-T four times, each one lasting for 5 minutes. Once the washes were completed, the membrane was read using the Bio-Rad ChemiDoc XRS molecular imager (Bio-Rad, Hercules, CA). The membrane was sprayed evenly 2 to 3 times with a Calbiochem RapidStep ECL
reagent (Biocompare Billerica, MA). After spraying, the Bio-Rad molecular imager was used to calculate the band densities for COX-2, APP, PARP, beta tubulin, and beta-actin. Using beta-tubulin or beta-actin primary antibodies, the results for COX-2, APP, and PARP were normalized against it.

Due to a membrane only being able to be incubated with one primary antibody at a time, the membrane had to be stripped in order to achieve results for an additional biomarker. This was accomplished by stripping the membrane with 10 ml of Restore Western Blot Stripping Buffer (Thermo Scientific, Logan, UT) and placing it on high agitation for 10 minutes. The membrane was then washed with 1X PBS for 5 minutes followed by repeating the procedures for blocking with 5% BSA, primary incubation, and secondary incubation. Once the band densities for the desired biomarkers were obtained by using Quantity One software along with the Bio-Rad Molecular imager, calculations were made and imported into Microsoft Excel.

**Specific Aim III:** To investigate the effect of OPP on the mRNA expression of COX-2 and amyloid precursor protein (APP) in FAD mutant and WT cells using real time RT-PCR.

1. **Preparation of Cells**

   The cells were prepared for real-time PCR by following the same guidelines discussed in specific aim II, step 1. Cells for FAD and WT that were maintained in liquid nitrogen at passage 4 were incubated in Petri dishes with OPP concentrations of 150, 300, and 600 ul/10 ml.
2. RNA Extraction

RNA extractions were completed for all samples using a miRNeasy Mini Kit and QIAzol Lysis reagent (QIAGEN Inc., Valencia, CA). All steps were carried out while on ice in order to try and prevent RNA denaturation. The cells were collected from the Petri dishes by following the procedures discussed in specific aim II, step 2, but stopping before lysis was added. Once the pellets were formed, they were loosened by flicking the tubes. 700 ul of QIAzol Lysis reagent was then added. Each tube was vortexed for 1 minute or until cells were homogenized completely and placed at room temperature for 5 minutes. 140 ul of chloroform was added to each tube and mixed thoroughly by shaking vigorously. The samples were then left to stand at room temperature for 2 to 3 minutes. With the centrifuge being maintained at -4 degrees Celsius the tubes were spun at 12,000 x g for 15 minutes. This caused the samples to separate in 3 different phases. The phase containing the RNA was the upper, colorless, aqueous phase; therefore, this phase was removed and placed into a new collection tube provided by the miRNeasy mini kit.

After acquiring the RNA phase, 525 ul of 100% ethanol was added to the collection tubes and mixed thoroughly via titration. An Rneasy mini column was placed into a 2 ml collection tube and 700 ul of each sample was added. The samples were subsequently centrifuged at 10,000 RPM for 15 seconds while at room temperature. The content collected within the 2 ml collection tubes was discarded. The remainder of the sample was added in 700 ul increments until the entire sample was spun through the Rneasy column. After each spin, the content in the collection tube was discarded. 700 ul of Buffer RWT was added into the Rneasy column and then centrifuged at 10,000 RPM.
for 15 seconds. The flow through was discarded. 500 ul of Buffer RPE was then added into the Rneasy column and also centrifuged at 10,000 RPM for 2 minutes. The addition of Buffer RPE and centrifugation was repeated in order to dry the Rneasy column membrane. The Rneasy columns were placed into new 2 ml collection tubes and centrifuged at full speed for 1 minute. To collect the RNA, the Rneasy columns were then transferred into 1.5 ml collection tubes and 50 ul of Rnase-free water was added onto the column. The tubes were centrifuged for 1 minute at 10,000 RPM. The contents in the 1.5 ml collection tubes were placed back through the Rneasy column and re-centrifuged. This step was completed in order to increase the yield of RNA collected. The contents within the 1.5 ml collection tubes contained the desired RNA.

It should be noted that these steps were completed on ice unless otherwise stated by the miRNeasy mini kit and all materials used were Rnase free. Most tubes were provided by the kit; however, for any additional steps completed such as ensuring the Rneasy column was dry, the tubes were only used if they were new and Rnase free. This was done to prevent denaturation and to ensure the collection of purified RNA. A nanodrop spectrophotometer machine (Thermo Scientific, Logan, UT) was used to quantify the RNA.

3. Complementary DNA (cDNA)

Once the RNA was extracted and purified, it was immediately converted to cDNA. This was carried out by using a high capacity RNA-to-cDNA master mix (Applied Biosystems, Carlsbad, CA). Calculations were completed to determine the amount of each sample needed in order to achieve approximately 1ug/ul in a 20 ul volume. Once the samples were diluted to the same concentrations using Rnase free water, 12 ul of
the diluted RNA sample was mixed with 12 ul of Rnase free water, and 6 ul of Complete Master Mix. Each sample was titrated and then placed in an Eppendorf Realplex thermocycler (Eppendorf, Hauppauge, NY). The parameters used to produce the cDNA were as follows; 5 minutes at 25 degrees Celsius, 30 minutes at 42 degrees Celsius, 5 minutes at 85 degrees Celsius, and held at 4 degrees Celsius. The samples were then stored at -80 degrees Celsius until amplification of specific genes could be carried out.

4. Real-Time Polymerase Chain Reaction (PCR)

Once cDNA was made from RNA, the genes of interest were amplified. The first step in this process was preparing the forward and reverse primers. The control gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), (Invitrogen, Carlsbad, CA) was used to normalize genes expressing COX-2 and APP. The forward primer for APP was 5’-GATGTCTTTGGCCAA CATGAT-3’ and the reverse primer was 5’-CATCTGCTCAAAGAACCTTGTA-3’ (Invitrogen, Carlsbad, CA). The forward primer used for COX-2 was 5’-GACAGCAGCC CTCTCATTTC-3’ and the reverse primer was 5’-CTGCTCCAAGACTGGCTCTC-3’ (Invitrogen, Carlsbad, CA). Each primer was reconstituted and then diluted to 20% using nuclease water. For each gene, GAPDH, COX-2, and APP, 12.5 ul of SYBR green (Applied Biosystems, Carlsbad, CA), 2 ul of the reverse primer, 2 ul of the forward primer, and 6.5 ul of nuclease free water were combined together. For a total of 8 samples, triplicates for each sample, and with a control, a total of 25 reactions were needed for each gene. Once the genes were prepared, 23 ul of each one was placed into 25 wells of a 96 well plate. This was completed for GAPDH, COX-2 and APP. The cDNA samples were removed from -80 degrees Celsius and centrifuged. 2 ul of each sample was added and titrated into 3 wells
for each gene. After all 8 samples were added, 2 ul of nuclease free water was added to one well for each gene in order to be used as a negative control. The plate was capped, spun down at -4 degrees Celsius, and amplified using an Eppendorf Realplex thermocycler. The samples were amplified with the parameters set at 95 degrees Celsius for 10 minutes, 40 cycles of 95 degrees Celsius for 15 seconds followed by 60 degrees Celsius for 1 minute, 95 degrees Celsius for 1 minute, 60 degrees Celsius for 30 seconds, temperature increase to 95 degrees Celsius over a 20 minute time period and the final step of 95 degrees Celsius for 30 seconds. Once the amplification process was completed, calculations were done to determine the fold increase with GAPDH being used to normalize the samples. A change less then a two fold difference was considered to be insignificant.
CHAPTER 3

RESULTS

Specific Aim I: To determine the anti-amyloidogenic activities of OPP, if any, by measuring the inhibition of formation and destabilization of amyloid beta proteins using a thioflavin T (ThT) binding assay.

This experiment was conducted to determine if OPP has any anti-amyloidogenic activities. This was accomplished by carrying out a ThT binding assay to see if OPP can prevent the formation of and/or aid in the destabilization of Aβ1-42 proteins. Although for both assays the data was collected every 30 minutes over a 24 hour period, the number of data points plotted was reduced in order to better show the trend.

Figure 4 shows the data obtained from the destabilization assay. The ThT fluorescence is relative to the amount of Aβ plaques formed. The fluorescence for the sample control, no OPP, remained low and nearly constant, ±0.2 units. Thus, this represents no effect on Aβ plaque destabilization. The sample with an OPP concentration of 300 ul/10 ml, displayed a decrease in fluorescence, indicating a decrease in the number of Aβ plaques. This is evident when looking at the starting value which was calculated to be approximately 1 while the end value was 0.4. Figure 4 shows the decrease in fluorescence during a 12 hour period for both the OPP control sample and the OPP 300 ul/10 ml sample. It illustrates that the OPP 300 ul/10 ml sample had a greater decrease in the number of Aβ plaques then the OPP control sample. Although the values are relative to 1, the data shows that OPP is indeed aiding in the destabilization of Aβ plaques in comparison to the sample control.
While the destabilization assay showed the effect OPP had on Aβ plaques; unfortunately, the inhibition for formation assay did not. The effect of OPP on Aβ formation needs to be investigated further. Experimental error may have played a part in this assay; therefore, this information cannot be reported without confirmation.
**Figure 4**: Destabilization ThT binding assay. The destabilization of Aβ 1-42 proteins with the addition of an OPP concentration of 300 ul/10 ml in comparison to Aβ 1-42 proteins without OPP. Differences in values shown between the first 12 hours. ThT fluorescence values were determined by averaging three values and subtracting appropriate controls. Values are relative to 1.
**Specific Aim II (a):** To determine the effect of OPP on cell growth and viability of the FAD-mutant cell constructs with increase in passage using MTS assay.

This assay was conducted in order to determine the number of passages the cells could go through without losing their cDNA construct. This was accomplished by counting the number of cells in each flask as well as growing them in a selection media. If either the FAD or WT cells lost their cDNA construct, they would not be able to grow in the flask. When completing the passage assay, the cells continued to grow in the selection media up to and through passage 7. The numbers of cells were also counted each time they were removed to be re-seeded and frozen. With the cells still viable to grow in the selection media, the number of cells increased with each passage. This assay showed that the B103 rat neuroblastoma cells would maintain their cDNA construct through passage 4, 5, 6, and 7, meaning that the FAD mutant cells would maintain their AD-like characteristics.

The MTS assay was conducted in order to determine the IC$^{50}$ concentrations of the FAD and WT cells in passage 4 and 7 when incubated for 48 or 72 hours with varying OPP concentrations. The purpose of this experiment was to find the optimal incubation period, cell passage, and OPP concentration. These parameters were vital to find at the beginning in order to use them throughout the rest of the experiments and maintain consistency. Since the cell lines used were rat neuroblastoma cells, the optimal concentration of OPP needed to show a significant effect on the cells without causing excessive cell death.

The first MTS assay completed tested the effects of OPP concentrations between 100 and 450 ul/ 10 ml on cells in passage 4 for a 48 hour incubation period.
Figure 5 shows the effects these parameters had on FAD cells. Using SPSS software, it was calculated that all OPP concentrations were statistically significant from the control (no OPP). The procedures for these calculations was described in the materials and methods section 2 (4). The IC$_{50}$ concentration was calculated to be approximately 250 ul/10 ml. Figure 6 shows FAD cells in the same passage with identical OPP concentrations; however, the incubation period was extended from 48 hours to 72 hours. This was completed to compare varying time periods of incubation and determine the optimal period to be used for following experiments. When the FAD cells were incubated for 72 hours, data analysis determined that all OPP concentrations were statistically significant when compared to the control. There was a difference in the IC$_{50}$ concentration when comparing 48 hours vs. 72 hours; the IC$_{50}$ concentration at 72 hours was approximately 225 ul/10 ml.

Once the incubation time periods were compared for FAD cells, the passage at which they were frozen and then used was tested. Using B103 rat neuroblastoma cells transfected with a cDNA mutation means the more passages you carry out with them, the higher the possibility that they will begin to lose their mutation characteristics. Passage 4 was the state at which the cells were received and then allowed to grow; therefore, that passage was compared to passage 7 to see if there were any variances in the data. Figure 7 shows the effects of varying OPP concentrations on FAD cells in passage 7 after 48 hours of incubation. All OPP concentrations were found to be statistically significant from the control and the IC$_{50}$ concentration was determined to be approximately 300 ul/10 ml. FAD cells in passage 7 were also incubated for 72 hours and can be seen in figure 8. Unlike the previous data, all varying OPP concentrations except for 100 ul/10 ml were determined to be statistically significant from the control.
The calculated IC$_{50}$ concentration was approximately 325 ul/10 ml. After the effects of differing parameters on FAD cells were established, WT IC$_{50}$ concentrations were determined for passages 4 and 7 during incubation time periods of 48 and 72 hours with varying OPP concentrations.
**Figure 5:** MTS assay results for FAD cells in passage 4 and with a 48 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.

**Figure 6:** MTS assay results for FAD cells in passage 4 and with a 72 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.
**Figure 7:** MTS assay results for FAD cells in passage 7 and with a 48 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.

**Figure 8:** MTS assay results for FAD cells in passage 7 and with a 72 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.
Once the effects of varying parameters on FAD cells were determined, WT cells were then tested. As with the FAD cells, WT cells in passage 4 were first incubated for 48 hours with OPP concentrations varying between 100 and 450 ul/10 ml. As shown in figure 9, all OPP concentrations were found to be statistically significant from the control. The IC\text{50} concentration was calculated to be approximately 275 ul/10 ml. Increasing the incubation time period to 72 hours was then completed and that data is shown in figure 10. Again, it was found that all of the OPP concentrations were statistically significant when compared to the control. The IC\text{50} concentration was calculated to be approximately 250 ul/10 ml.

After the incubation time periods were compared while the WT cells were in passage 4, they were then tested in passage 7. Figure 11 shows the effects of varying OPP concentrations on WT cells incubated for 48 hours while in passage 7. The data shows that all OPP concentrations except for 100 ul/10 ml were statistically significant from the control. The IC\text{50} concentration was also calculated and determined to be approximately 250 ul/10 ml. WT cells in passage 7 were then incubated for 72 hours. This data can be seen in figure 12. Unlike the other data, this graph shows a greater variation between the differing OPP concentrations. The only OPP concentrations shown to be statistically significant when compared to the control were between 250 ul/10 ml and 450 ul/10 ml. Despite the larger variations, the IC\text{50} concentration was calculated to be the same as the 48 hour incubation period with passage 7; an OPP concentration of 250 ul/10 ml.
**Figure 9:** MTS assay results for WT cells in passage 4 and with a 48 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.

**Figure 10:** MTS assay results for WT cells in passage 4 and with a 72 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.
**Figure 11:** MTS assay results for WT cells in passage 7 and with a 48 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.

**Figure 12:** MTS assay results for WT cells in passage 7 and with a 72 hour incubation period with various OPP concentrations. *P < 0.05 when compared to the untreated control.
The optimal parameters were determined after all the data was collected and calculated. This assay was completed three different times all producing similar results. When the data between the cells in passage 4 and passage 7 are compared, it is evident that the passages are behaving differently. The cells in passage 4 illustrated a more uniform graph showing a slow, clean, dose dependent curve at both time points. This is evident when comparing figure 6 showing FAD cells in passage 4 for a 72 hour incubation period to figure 8 showing FAD cells in passage 7 for a 72 hour incubation period. Using the same data from figures 6 and 8, figure 13 further illustrates this point. The absorbances for both passage 4 and passage 7 were graphed on a scatter plot and a trendline with an order of 4 was added. Based on these results, it is logical to select passage 4 for the following experiments.

The other important parameter determined with the MTS assay was the optimal incubation time period. When comparing 48 hours vs. 72 hours, it was decided that for the remaining experiments the cells would be incubated with the OPP concentrations for 72 hours. This decision was based upon the data showing greater variances in the absorbances of samples after 72 hours of incubation. The data for 48 hours showed a steady decrease in the samples absorbances as the OPP concentrations increased; however, the larger variances in the sample absorbances after 72 hours illustrated that the OPP had a greater effect on the cells. This can be confirmed by comparing the results of the first assay, which can be seen by looking at figure 5 and figure 6. For FAD cells in passage 4 with a 48 hour incubation period, the control sample absorbance was calculated to be 1.202 and the OPP 450 ul/10 ml sample absorbance was determined to be 0.295. For FAD cells incubated for 72 hours, the control sample absorbance was 1.424 while the OPP 450 ul/10 ml sample absorbance was calculated to be 0.187. The
greater decrease in samples absorbance when compared to the controls was evident during the 72 hour incubation period. This variance also occurred for WT cells in passage 4. For this reason, both the FAD and WT cells would be incubated with the OPP for a total of 72 hours for the remaining experiments.

Lastly, the optimal OPP concentration was determined to be 300 ul/10 ml. This conclusion was based on the calculated IC_{50} concentrations. The IC_{50} concentrations ranged between 225 and 325 ul/10 ml for FAD cells and between 250 and 275 ul/10 ml for WT cells. Due to these calculations, it was decided that the optimal OPP concentration to be used for the remaining experiments would be 300 ul/10 ml.

Although comparing the incubation time periods, OPP concentrations, and passages were important in order to find the optimal parameters to be used in following experiments, comparing the WT to FAD cells also provided essential information. To compare FAD vs. WT cells the absorbance of the controls were compared against the values of the absorbance for the samples containing an OPP concentration of 450 ul/10 ml. This was done in order to see if OPP had a greater effect on the FAD cells when compared to the WT cells. Since the optimal parameters were determined to be cells in passage 4 with a 72 hour incubation period, figure 6 and figure 10 were compared. The FAD control absorbance was calculated to be 1.424 while the OPP 450 ul/10 ml sample absorbance was 0.187. This made the difference between the control and OPP sample to be 1.237. The WT control absorbance was determined to be 0.671 while the OPP 450 ul/10 ml sample absorbance was 0.154. The difference calculated between these two values was 0.517. These calculations show that the addition of OPP had a greater effect on FAD cells when compared to WT cells, do to the larger decrease in absorbance.
between the control and the sample containing an OPP concentration of 450 ul/10 ml. This comparison is further illustrated in figure 14. It should also be noted that the OPP 450 ul/10 ml samples for both the FAD and WT cells had an absorbance of approximately 0.19.
**Figure 13:** Graph showing the absorbance for FAD cells incubated for 72 hours in passage 4 vs. FAD cells incubated for 72 hours in passage 7. A trendline with an order 4 was added in order to illustrate that the cells in passage 4 produced a more uniform, clean, dose dependent curve when compared to the cells in passage 7.
Figure 14: Graph showing the absorbance for FAD cells incubated for 72 hours in passage 4 vs. FAD cells incubated for 72 hours in passage 7. A trendline with an order 4 was added in order to illustrate that the cells in passage 4 produced a more uniform, clean, dose dependent curve when compared to the cells in passage 7.
Figure 15: The change in absorbance between the sample control and OPP 450 ul/10 ml sample for FAD and WT cells in passage 4 for 72 hour incubation. Shows a greater effect of OPP on FAD cells than when compared to WT cells.
Specific Aim II (b): To investigate the effects of OPP on the protein expression of APP, COX-2 and PARP, known biomarkers for AD and inflammation in FAD mutant and WT cells.

The protein expression levels of APP, COX-2 and PARP were measured using western blot analysis. The optimal OPP concentration was determined to be approximately 300 μl/10 ml; therefore, the cells were incubated with OPP concentrations of 150, 300, and 500 μl/10 ml. APP was tested in order to determine if OPP could directly affect the amount of APP produced at the protein level within the cells. The picture of the membrane incubated with the antibody 22C11 (Millipore, Temecula, CA) was taken using the BioRad molecular imager and is shown in figure 15a. The 22C11 antibody recognizes three specific isoforms of APP. Using the molecular weight marker it was determined that the bands measured were at the molecular weights specified by the 22C11 antibody; ~110 kda, ~120 kda, and ~130 kda. The isoform at ~110 kda represents immature APP, while the isoform at ~120 kda corresponds to sAPP and ~130 kda signifies the mature APP isoform.

With the bands from the membrane confirmed to be the isoforms of APP, they were then measured as a whole and normalized against the band densities of beta actin obtained on the same membrane. This data is represented in figure 15b. These results show that the highest amounts of total APP expressed were found in the FAD control sample. This result was expected due to the FAD cells having a mutation to produce excess amounts of APP such as in the brain of an individual with AD. Additionally, as the concentration of OPP increased, the amount of total APP expressed in the FAD cells decreased. Not only did the OPP decrease the total amount of APP expressed, but it
was shown to lower it to a level expressed in the WT control sample. Further, OPP shows a slight reduction in APP levels even in the WT cells. These results show that an OPP concentration of 300 ul/10 ml may reduce the amount of APP expressed at the protein level to that of cells without the AD mutation. When extrapolated to humans, it reflects reduction of APP levels to those of an individual without AD.

The protein level of expression of COX-2 was also measured using the western blot analysis method. The same OPP concentrations of 150, 300, and 500 ul/10 ml were used. The membrane was incubated with the primary antibody COX-2 which produced the raw data that can be seen in figure 16a. The molecular weight marker was compared to the bands produced on the membrane to confirm that they represented COX-2. Once confirmed, the band densities were measured and then normalized against the measured band densities for beta actin. The band densities were graphed and can be viewed by looking at figure 16b.

The normalized data shows that the FAD control expressed the highest amount of COX-2. Once OPP was added, the amount of COX-2 expressed decreased significantly. Again, it should be noted that the addition of OPP decreased the levels of COX-2 to approximately the same level as the WT control.

Lastly, the PARP protein expression levels were measured. A membrane containing samples with OPP concentrations of 150, 300 and 500 ul/10 ml was incubated with the PARP 46D11 primary antibody. The membrane was viewed using the BioRad molecular imager and the bands were confirmed to represent PARP. The raw membrane can be seen in figure 17a. The bands for PARP were then measured and normalized against the band densities for beta actin. The normalized data can be viewed
by looking at figure 17b. As with COX-2 and APP, the highest levels of PARP protein expression was observed in the FAD control. A dose dependent decrease in PARP expression is seen upon addition of OPP. It should be noted that due to a small sample of cells, the membranes were stripped in order to measure more then one antibody per assay. The band densities were measured immediately following the western blot; however, the pictures were taken at a different time. Due to these procedures, the pictures of the membranes may seem to differ from the data obtained and then plotted.
**Figure 16a:** The membrane from western blot analysis incubated with the 22C11 antibody. This was used in order to recognize the protein levels of expression of the three isoforms of APP (immature at ~110 kda, sAPP at ~120kda, and mature at ~130kda) for both the controls and varying OPP concentrations for FAD and WT B103 cells.

**Figure 16b:** The band densities (INT/mm²) for total amyloid precursor protein normalized against the band densities for beta actin (INT/mm²) for both FAD and WT B103 cells for controls and each varying OPP concentrations (ul/10 ml).
Figure 17a: The membrane from western blot analysis incubated with COX-2 antibody. It was used in order to recognize the protein expression levels of cyclooxygenase-2 for both the controls and varying OPP concentrations for FAD and WT B103 cells.

Figure 17b: The band densities for cyclooxygenase-2 (INT/mm2) normalized against the band densities for beta actin (INT/mm2) for both FAD and WT B103 cells including controls and each varying OPP concentrations (ul/10 ml).
**Figure 18a:** The membrane from western blot analysis incubated with the cleaved-PARP antibody. It was used in order to recognize the protein expression levels of poly-(ADP-ribose) polymerase for both the controls and varying OPP concentration incubations for FAD and WT B103 cells.

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**Figure 18b:** The band densities for poly-(ADP-ribose) polymerase (INT/mm2) normalized against the band densities for beta actin (INT/mm2) for both FAD and WT B103 cells for controls and each varying OPP concentrations (ul/10 ml).
Specific Aim III: To investigate the effect of OPP on the mRNA expression of COX-2 and amyloid precursor protein (APP) in FAD mutant and WT cells using real time RT-PCR.

Once the protein expression levels were calculated and obtained, the experimentation was taken one step further. This specific aim was conducted in order to see if OPP could effect the expression of COX-2 and APP at the mRNA level. This was accomplished by using real time PCR. The OPP concentrations used for these experiments were 150, 300 and 600 ul/10 ml. After the data was collected it was normalized against GAPDH. Analysis was then done to convert the data into a fold difference. Any fold change greater then 2 times is considered to be significant.

The first real time PCR completed tested the mRNA expression levels of APP for FAD cells. The fold change can be seen by looking at figure 18. Although slight increases upon the addition of OPP are seen, this is not statistically significant. This is due to there being less then a two fold difference between the values. The mRNA expression levels were then tested for WT cells. This data can be seen in figure 19. For this assay, the control sample and OPP 150 ul/10 ml sample were considered to be statistically insignificant. This was found to be the same for the OPP 300 ul/10 ml and OPP 500 ul/10 ml samples as well. However, when comparing the OPP control and OPP 150 ul/10 ml sample to the OPP 300 ul/10 ml and OPP 500 ul/10 ml samples, they were found to be statistically significant from one another. This shows that in WT cells, an OPP concentration of 300 or 500 ul/10 ml will significantly decrease the levels of APP expression.
Figure 19: The fold change of the mRNA expression of amyloid precursor protein normalized with GAPDH for FAD control and samples with the OPP concentrations of 150, 300, and 600 (ul/10ml). * = statistically significant due to a two fold difference or greater.
**Figure 20**: The fold change of the mRNA expression of amyloid precursor protein normalized with GAPDH for WT control and samples with the OPP concentrations of 150, 300, and 600 (ul/10ml). * = statistically significant due to a two fold difference or greater.
Lastly, the mRNA expression levels of COX-2 were measured in FAD and WT cells. Figure 20 shows the fold differences for FAD cells. The OPP 150 ul/10 ml sample was calculated to be statistically significant from the FAD control as well as the OPP 300 and OPP 600 ul/10 ml samples. The OPP 150 ul/10 ml sample was shown to have the highest level of COX-2 mRNA expression. COX-2 was then tested in WT cells. This data is shown in figure 21. For WT cells it was calculated that the OPP 300 ul/10 ml sample was statistically significant from the WT control and OPP 150 ul/10 ml sample. Also, the OPP 600 ul/10 ml was determined to be statistically significant from all other samples; WT control, OPP 150 and OPP 300 ul/10 ml.
Figure 21: The fold change of the mRNA expression of cyclooxygenase-2 normalized with GAPDH for FAD control and samples with the OPP concentrations of 150, 300, and 600 (ul/10ml). * = statistically significant due to a two fold difference or greater.
Figure 22: The fold change of the mRNA expression of cyclooxygenase-2 normalized with GAPDH for WT control and samples with the OPP concentrations of 150, 300, and 600 (ul/10ml). * = statistically significant due to a two fold difference or greater.
Alzheimer’s disease (AD) is a very debilitating type of dementia that is affecting approximately 35.6 million people in the world today (33). Of that population, 5.4 million reside in the United States. AD is a progressive disease and has been determined to be the 6th leading cause of death within the United States. Currently, an individual develops AD every 69 seconds (29). Due to the baby boomer population vastly approaching the at risk age of 65 years old, the importance for finding a treatment is becoming more crucial. By the year 2050, the prevalence of AD is expected to rise to the point at which an individual develops it every 33 seconds. With a 66% increase in AD associated deaths between the years 2000 and 2008 and with an increasing at risk population; a treatment discovery is vital (29).

The first sign of AD occurs when an individual is unable to retain new information. This is due to neuronal cell death occurring in the region of the brain responsible for forming new memories. Damage from neuronal cell death begins to spread to other regions of the brain causing the individuals symptoms to worsen. Besides certain recognizable symptoms such as confusion with orientation of time and place, the diagnosis of AD is difficult. Often times the individual attributes their loss of memory to the normal ageing process causing the diagnosis to go undetected (29). However, even when the symptoms are recognized as AD, a diagnosis can only be confirmed once the individual has passed away and an autopsy of the brain can be completed (37). Without having accurate assessment tests, physicians often rely on the patients’ history and an MRI of the brains physical appearance. Due to the difficulty of diagnosing AD, some studies suggest approximately 80% of the AD cases have gone
unnoticed (29). Without the etiology of AD being well known, it is difficult to diagnose and/or treat (24).

AD can be further defined by either non-familial AD or familial AD (FAD). Non-familial AD can be classified as early-onset, under the age of 65, or late-onset, over the age of 65. Non-familial AD is the most prevalent form while approximately only one percent of the population is diagnosed with FAD. The etiology of FAD and non-familial AD is not well known; however, there have been rare genetic mutations discovered to cause FAD (24, 29). These mutations affect the chromosomes responsible for the production of the amyloid precursor protein, presenilin 1 proteins, and for the presenilin 2 proteins (29). These proteins contribute to the development and progression of certain AD characteristics.

One of the most prevalent and well known hallmarks of AD is the production and formation of amyloid beta (Aβ) plaques. The amyloid precursor protein (APP) is a vital protein that mediates neurite outgrowth and produces chemicals that are important for the maintenance and formation of neuronal synapses (37). The problem for AD individuals occurs when γ-secretase yields the Aβ1-42 fragment at a higher rate then in non-AD individuals. Due to established genetic mutations for FAD, it is known that the presenilin 1 and presenilin 2 proteins play a role in this rate of production (4). In non-AD individuals, their body is able to clear out most of the Aβ1-42 fragments produced; however, AD individuals produce an excessive amount ultimately creating a buildup of the fragments (7). Once the Aβ1-42 fragments accumulate and create a plaque, additional pathways are triggered causing microglial activation, ROS formation, oxidative stress and an increase in inflammation (4).
With Aβ plaques somewhat understood, certain biomarkers have been established to look at the progression and/or development of AD. This study focused primarily on two biomarkers; cyclooxygenase-2 (COX-2) and poly-(ADP-ribose) polymerase (PARP). COX-2 plays a vital role in the inflammatory pathway. Looking at figure 22 it can be seen that COX-2 is a rate limiting enzyme that regulates the production of prostaglandins and cytokines (37). This is important due to prostaglandins influencing the onset, magnitude, duration, and course of the inflammatory process (16). As shown in figure 23, inflammation in AD individuals creates a continuous cycle of cell apoptosis. ROS formation causes an increase in pro-inflammatory agents which then leads to cell apoptosis, microglial activation, and additional pro-inflammatory agents (28). Due to inflammation playing such an important role in the progression of AD, COX-2 was examined in this study.

The other inflammatory biomarker looked at during this study was PARP. This biomarker is known to regulate the release of pro-inflammatory agents by binding to and altering the nuclear factor-kappa β (NF-κβ) transcription factor located on the DNA (36, 15). The NF-κβ is responsible for the regulation of macrophage and microglial activation (11). As shown in figure 23, single strand DNA breaks, as a result from an increase in oxidative stress, causes PARP to bind to the DNA and alter the NF-κβ transcription factor (36). This then depletes cells energy pools leading to additional cell death. In addition, this leads to an over production of microglial activation, which in turn increases pro-inflammatory agents (15, 36).
Figure 23: Flow chart showing the production and use of cyclooxygenase in the inflammatory process.
Figure 24: Flow chart showing the link between amyloid beta plaques, poly-(ADP-ribose) polymerase, call apoptosis, and the inflammatory process.
The inflammatory biomarkers, PARP and COX-2, were used in this study because of the strong link between inflammation and AD; however, they were also chosen due to oil palm phenolics having high antioxidant capabilities. Oil palm phenolics (OPP) are the water-soluble portions of the palm fruit. Currently, the fat-soluble portion is being used as palm oil due to its rare fatty acid composition (27). While the fat soluble portion is being used readily, the water-soluble portion is still being researched. It was discovered that within the water-soluble portion, three major phenolics are present. Due to the aromatic ring structure of phenolics, they have a high reduction-oxidation potential, meaning they act as antioxidants (23). For this reason, this study examined the effects OPP may have on the inflammatory biomarkers, PARP and COX-2, and its association with AD.

First, OPP was examined to determine if it possesses any anti-amyloidogenic capabilities. The ThT binding assay was conducted to test the effects of OPP on the destabilization and also the inhibition of formation of Aβ1-42 plaques. The inhibition of formation assay was inconclusive. Possibly due to experimental error, the effect OPP has on the formation of Aβ plaques needs to be investigated further before a report can be made. Unlike the inhibition of formation assay, the destabilization assay illustrated that OPP did in fact aid in the destabilization of Aβ plaques. This is evident when looking at the decrease in the ThT fluorescence over a 12 hour period for the sample containing an OPP concentration of 300 ul/10 ml when compared to the sample control. The decrease in the OPP 300 ul/10 ml sample fluorescence was approximately 0.6 while the sample control fluorescence only decreased by approximately 0.06. Based on these results it can be concluded that OPP may aid in the destabilization of Aβ plaques.
After the ThT binding assay, a rat neuroblastoma B103 cell line, transfected with a mutated cDNA construct to over express APP levels, were used. The first two assays were conducted to determine if the cells maintained their AD characteristics throughout different passages and also to discover the optimal parameters that would be used in the following experiments. The passage experiment concluded that the cells maintained their AD characteristics up to and through passage 7. The MTS assay determined that the optimal OPP concentration was approximately 300 ul/10 ml of media, the optimal incubation time period was 72 hours, and that using cells in passage 4 was ideal. Along with these findings, the MTS assay also showed that OPP had a greater impact on FAD cells then it did on WT cells. This could possibly show that due to FAD cells having an increase in inflammatory agents, the OPP was able to use its antioxidant capabilities more so then it could on the WT cells.

Western blot analysis was also completed. This experiment provided the band densities for FAD and WT cells after being incubated with varying OPP concentrations. The first biomarker looked at was complete APP. Looking at this data, it can be concluded that OPP does in fact decrease the expression of APP at the protein level. By comparing the band density of the FAD control to the FAD 300 ul/10 ml and FAD 500 ul/10 ml band densities, it shows a dramatic decrease in APP levels. Also, the addition of 500 ul/10 ml of OPP decreased the protein level of APP expression to the level at which the WT control APP levels were measured at. This data shows that OPP may play a vital role in directly decreasing the protein expression levels of APP, so much so, that it may bring them back within normal levels.
Other studies have also shown that antioxidants can decrease APP levels. In 2005, researchers used APP transgenic mice to examine the capabilities of certain antioxidant enzymes. They discovered that the overexpression of APP caused a reduction in the levels of the antioxidant enzymes. Also, they antioxidants were found to be directly protective against APP toxicity (25).

Western blot analysis was also used to look at the differences in the COX-2 protein expression levels among the various OPP concentrations. After the data was collected and graphed, OPP was found to produce a decrease in COX-2 protein levels of expression. The FAD control band density was measured at approximately 20 INT/mm2 while all other samples of FAD that were incubated with OPP produced a band density measured well below 5 INT/mm2. As in the APP results, the FAD samples containing 300 ul/10 ml and 500 ul/10 ml of OPP, had COX-2 levels reduced to a level close to that of the WT control. In addition, the FAD control band density was approximately 20 times greater then the WT control. This showed that the FAD control contained a much higher level of COX-2.

Additional studies have found that the FAD model had higher levels of COX-2 and that the use of antioxidants decreased those levels. Hwang et al. found that according to their western blot data, the COX-2 protein levels were 1.5 to 2 folds higher in the brains of transgenic mice with presenilin 2 protein mutations when compared to the control mouse. They were also able to link the location of the COX-2 immunopositive neuronal cells to the same staining pattern as the Aβ-42 fragments (9). More similarly to this study, in Aβ neuronal cells it was found that there were higher levels of COX-2 when compared to the control. These cells also had a higher rate of apoptosis (22). Lastly, in
2008, it was discovered that the tocotrienol-rich fraction of palm oil increased anti-inflammatory activity by inhibiting the expression of COX-2 in monocyctic cells. It was discovered that the antioxidants suppressed the release of prostaglandin $E_2$ resulting in the downregulation of COX-2 (35).

The final western blot analysis conducted was completed in order to determine if OPP could decrease the PARP protein expression levels. As expected from the previous data, the addition of OPP dramatically decreased the levels of PARP in the FAD samples. The FAD control was measured to have a band density of approximately 4 INT/mm². With the addition of just 100 ul/10 ml of OPP, the PARP expression levels decreased to such that the FAD 150 ul/10 ml sample band density was measured to only be approximately 0.8 INT/mm². All samples, except for the FAD control, had a band density measurement of less than 1 INT/mm².

The decrease in PARP levels with the addition of an antioxidant such as OPP is supported by other previously conducted experiments. One study showed that a tocotrienol rich fraction of palm oil had an inhibitory effect on the activation of NF-κB in monocyctic cells. This was shown by using the western blot analysis as well (35). In 2009, a study conducted discovered that certain naturally occurring polyphenols such as curcumin, prevented DNA damage and PARP-1 activation. They also took the experiment one step further and showed that because of the inhibition of the PARP-1 activation, cells maintained their NAD+ pools ultimately preventing cell apoptosis (1).

APP, COX-2, and PARP protein expression levels decreased after OPP was added. This shows that OPP may in fact positively affect the inflammatory pathway and the APP levels. This is important due to APP being directly cleaved and producing Aβ
plaques, and also because the inflammatory pathway plays such a crucial role in oxidative stress and apoptosis in AD individuals. Although this data proves to be in the right direction of OPP possibly decreasing certain inflammatory biomarkers associated with AD, this study took one step further to try and determine if the cells were affected at the mRNA level. This was accomplished by carrying out real time polymerase chain reaction (PCR).

Real time PCR was the last experiment conducted in this study. For this experiment, COX-2 and APP were examined. Once the protein was extracted and the cDNA created, the data was collected and converted into a fold change graph. Looking at the mRNA expression levels for APP in FAD cells, it was determined that due to the fold increase being less than 1, there was no statistical significance between the control and samples containing OPP. This may be due to the OPP not affecting the cells at the mRNA level. This idea was further investigated by looking at the mRNA level of APP expression in WT cells. Here it was discovered that there was a statistically significant fold difference when comparing WT control and WT 150 to WT 300 and WT 600 ul/10 ml samples.

The real time PCR data for the expression of COX-2 at the mRNA level also showed a difference between the FAD and WT cells. FAD cells containing an OPP concentration of 150 ul/10 ml was determined to be statistically significant from the FAD control and other samples containing OPP. It had the highest level of COX-2 expression. This data does not coincide with the information obtained from the western blot analysis. Since it was the only sample to have been significantly different, there may have been contamination to that sample; therefore, this assay needs to be revisited. The fold
change graph for COX-2 expression in WT cells is similar to the graph for APP expression. The WT control and WT 150 ul/10 ml sample was calculated to be statistically significant from the WT 300 ul/10 ml sample. The control and 150 ul/10 ml samples were also determined to be statistically significant from the WT 600 ul/10 ml sample. In addition, the WT 300 ul/10 ml sample was statistically significant from the WT 600 ul/10 ml sample.

The variations in this data do not allow this study to accept or reject the idea that OPP affects the cells at the mRNA level. The WT samples for COX-2 and APP expression seem to show that the addition of OPP does in fact decrease the expression levels of these biomarkers. The inconsistency is present when looking at the data for the FAD cells. The expression levels of COX-2 and APP were expected to decrease once OPP was added. For COX-2 expression, the FAD 150 ul/10 ml sample had the highest, while for APP expression there was no differences between any of the FAD samples. Although this data does show that the OPP decreased COX-2 and APP in WT cells, the decrease should have been more prevalent and noticeable in the FAD cells. For this reason, this experiment was not able to determine if OPP affects the cells at the mRNA level.

Throughout the duration of this study there were many positive discoveries; however, certain improvements could have been made in order to make these findings more beneficial. First, a more appropriate cell line could have been chosen to use. The B103 rat neuroblastoma cells were ideal for this study based on their ability to maintain the cDNA mutant construct throughout multiple passages and also due to their extensive use in AD research. However, the problem is that in AD, you want to preserve the
neuronal cells while decreasing the inflammation and the number of Aβ plaques. A more appropriate cell line would allow for biomarkers to be evaluated without having some measurements recorded based on cell death, such as in the MTS assay.

In addition to possibly using a more appropriate cell line, the results from the ThT binding assay should have been confirmed using the Congo red test. According to recent experiments, polyphenols contain aromatic ring structures that are connected by conjugated systems. This characteristic creates strong electronic transitions ultimately making them intrinsically fluorescent. This may make the ThT binding assay unsuitable for evaluating the effects of phenolics compounds such as OPP (8). Even though a control was used in attempt to accommodate for that, the results for the inhibition of formation assay were inconclusive and the results for the destabilization assay were relative to 1; therefore, the results should have been confirmed using a different type of experiment such as the Congo red test.

Lastly, the real time PCR should have been further optimized. These results did not coincide with previous studies or with the data obtained in this study. According to previous research, OPP can in fact alter certain gene expressions. For example, it has been found to alter the expression of many genes on the spleen, liver, and heart (12). With data showing OPP altering gene expression in certain tissues, real time PCR needs to be further optimized and re-done in order to be sure of the data produced in this study.

Despite certain setbacks, this study has provided more knowledge about how the antioxidant, OPP, may aid in the decrease of inflammatory responses in AD individuals. However, this study is not alone at looking into the effects antioxidants may have on AD
as well as other similar diseases. For example, AD and Down syndrome are two diseases that have neuronal cell death that is possibly caused by oxidative stress. In recent Down syndrome cases, antioxidants have been found to delay premature apoptotic death in neuronal cells (20). Also, recent studies have shown that when patients with moderate AD are given antioxidants such as vitamin E and monoamine oxidase B, they were found to deteriorate at a slower rate when compared to the placebo group. In addition to this study, it was discovered that menopausal women undergoing estrogen replacement therapy have a reduced risk of AD. This is thought to be due to women receiving antioxidants that are estrogen derivatives. These results relate back to the idea that as an individual ages, their antioxidant defense system increases; however, their ROS formation also increases ultimately exceeding the antioxidant capabilities and increasing oxidative stress (20). Based on this study and previous ones, OPP may play an important role in decreasing APP, COX-2 and PARP expressions at the protein level; therefore, its capabilities need to further evaluated.

Since OPP did show some antioxidant capabilities against AD characteristics, the next step would be to take this study into the animal model. A recent study by Cole et al. showed that non-steroidal anti-inflammatory drugs (NSAIDs) decreased the risk for AD in the animal model. This was due to a significant suppression of inflammation. The problem with using NSAIDs is that some also inhibit the activation of COX-1. Not having COX-1 activated can lead to gastrointestinal, liver, and kidney toxicity (14). For these reasons, a naturally occurring antioxidant such as OPP would be an excellent alternative for AD patients.
There have been many promising studies with OPP and other antioxidants in the animal model representing AD. For example, curcumin has been discovered to suppress specific inflammatory cytokines, reduce oxidative stress, and decrease the formation of Aβ plaques. This was shown in the animal model and because of that it also provided information relating to memory deficit. Using the Morris water maze, researchers found that the animals who received curcumin in their diet showed lower Aβ induced memory deficit when compared to the control mice (14). OPP has also been shown to act an anti-inflammatory agent in the animal model. OPP promoted anti-inflammatory cytokine IL-13 and decreased the pro-inflammatory cytokine IL-12 (13). Theses studies show that antioxidants, including OPP, can be used in the animal model to provide more information on diseases such as AD.

Based on experiments such as the ones stated above and the one conducted within this thesis, the next step to be taken would be to continue experiments such as these in the animal model. There has been a lot of positive data and information obtained from previous AD animal model studies; however, additional information is needed. The etiology of non-familial AD is one important aspect that needs to better understood. FAD has well known causes making the models easy to create, but with only 1% of the population having FAD, non-familial AD needs to be examined and researched more closely. Also, OPP has been found to not be toxic in the animal model (12). This allows the optimal concentration to be further evaluated before pursuing additional clinical studies.

As the baby boomer population continues to increase, AD needs to become better understood. This study was completed as one small part in the ever expanding
exploration of AD. With additional research, it is the hope of this study to find a natural antioxidant such as OPP to help better the lives of AD individuals. Using OPP in the animal model will hopefully provide more information and lead to additional discoveries for the diagnosis, treatment, and hopefully some day the cure for AD.
REFERENCES


ABSTRACT

THE EFFECTS OF OIL PALM PHENOLICS ON INFLAMMATION AND OXIDATIVE STRESS IN RELATION TO AMYLOID BETA PLAQUES IN FAD MUTANT AND WILD TYPE B103 CELLS

by

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Alzheimer’s disease (AD) affects approximately 35.6 million people in the world today (35). It is expected that by year 2050, an individual will develop AD every 33 seconds (8). Unfortunately, the etiology is not well known. Familial Alzheimer’s disease (FAD) occurs due to specific genetic mutations, making it possible to replicate them in cells. Using B103 rat neuroblastoma cells transfected with a cDNA construct, wild type cells and FAD mutant cells were used in this research.

Natural ingredients such as oil palm phenolics (OPP) contain antioxidants. After finding the optimal experimental parameters, OPP was found to aid in the destabilization of Aβ plaques and also decrease the protein expression levels of amyloid precursor protein (APP), and the inflammatory biomarkers poly (ADP-ribose) polymerase (PARP) and cyclooxygenase-2 (COX-2). This research is one small step in better understanding AD and how using ingredients such as OPP may help in one day developing a treatment for this debilitating disease.
AUTOBIOGRAPHICAL STATEMENT

I began my undergraduate studies at Michigan State University located in East Lansing, MI as a pre-medical student. The first three years I spent dedicated to enhancing my resume for medical school applications. Due to this I took a research class my senior year. There I discovered that what I thought was a passion for medical school was in fact nothing compared to the excitement I felt while conducting research experiments. Knowing that I could potentially aid in the discovery of a cure for a disease or even the prevention of one, and possibly save thousands of lives, I made the decision to pursue my passion for research further.

After graduating from Michigan State University with a B.S. degree in nutritional sciences, I continued my education and attended Wayne State University located in Detroit, MI to obtain my M.S. degree in nutrition and food science. With experience from my undergraduate studies giving me some knowledge in animal studies I took the challenge of completing this thesis using the cell culture model. Conducting this thesis from beginning to end was a growing experience and one that confirmed my passion for research.

For the future I hope to continue to expand my knowledge in research especially in diseases and nutrition. Although pharmaceuticals have become necessary to save and better the lives of many, I feel if they can be solved or prevented with natural ingredients the individual will receive better results with fewer side effects. Eventually I would like to pursue my PhD and conduct research that will hopefully help thousands of individuals one day.