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THE ABILITY OF POMEGRANATE TO AMELIORATE
SYMPTOMS ASSOCIATED WITH ALZHEIMER'S
DISEASE IN AGED TRANSGENIC MICE

BY
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
INTERDISCIPLINARY NEUROSCIENCE

UNIVERSITY OF RHODE ISLAND

2013

MASTER OF SCIENCE THESIS
OF
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2013

ABSTRACT

Accumulating research has demonstrated that polyphenolic compounds from natural products, such as pomegranate, may have antioxidant and neuroprotective properties in animal models of Alzheimer's disease (AD). However, the present study explores whether the administration of a pomegranate peel extract could have a rescuing effect on AD pathology in aged transgenic animal models of AD. These mice already have abundant AD pathology since amyloid beta ($A\beta$) deposition continues with time. Two doses of the extract or a control solution were fed daily to groups of transgenic mice (R1.40), ranging in age from 24-30 months. Treatment and behavioral assessment lasted thirty-seven days total. Mice were tested in the Morris water maze and the Y-maze for improvements in spatial, long-term and working memory functions. This was followed by the measurements of cortical amyloid precursor protein (APP) and $A\beta$ levels along with other relevant biomarkers for AD. The resulting data demonstrated a lack of change in cognitive performance in the mazes, as well as the precursor protein and other enzymes associated with the amyloidogenic pathway. However, biochemical analyses revealed an alteration in the levels and ratio of the $A\beta$ peptides that favored a diminution in AD pathogenesis. This was featured by the lowering of the more amyloidogenic $A\beta_{1-42}$ peptide and an increase in the $A\beta_{1-40}$ peptide. Further experiments revealed that this reversal could be the product of the modification of the gamma-secretase enzyme responsible for generating the more amyloidogenic form. In conclusion, pomegranate peel extract appears to contain

ingredients that act as gamma-secretase modulators, which may be identified and developed as compounds for use in future drug therapy.

ACKNOWLEDGMENTS

I would like to acknowledge a number of individuals who have inspired me to investigate this topic and have guided me in completing this scholarship. First is my professor and adviser, Dr. Nasser H. Zawia, whom since my undergraduate years has witnessed my growth and achievements as a young scientist. He has been an excellent mentor, friend, and source of encouragement. I would also like to acknowledge my professor Dr. Navindra P. Seeram for his continued guidance and contributions to my work. I thank him for providing the pomegranate extract used in this study. My co-workers and friends, Dr. Gehad Subaiea and Dr. Syed Bihaqi have been indispensable. They always guided me in the right direction with my research and answered my many questions. Finally, I would like to thank my family and friends for their support during this journey.

This study was made possible through NIH Grant Support (ES13022 and AG027246) awarded to Dr. Nasser H. Zawia.

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CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder that slowly impairs daily functioning through loss of memory and cognition. It is the most common cause of dementia, affecting 13% of people over the age of 65 (Hebert 2003). As populations of the United States and other countries begin to age, AD becomes a more common cause of death (Xie 2008). However, the effects that the disease has on daily living is not the only major concern. According to the latest data released by the Alzheimer's Association, the total spending for Alzheimer's care and other dementias was over \$200 billion in 2012 and it is expected to increase five-fold by 2050 (Thies 2013). In addition to increasing awareness and action among the public health community, it is imperative that an effective disease modifying treatment, or perhaps even a cure, is developed in the near future to combat this debilitating disease.

Most cases of AD show up in people aged 60 or above. This form of the disease is called late-onset Alzheimer's disease (LOAD). Although the causes for LOAD are still poorly understood, several studies have loosely linked it to a version of the apolipoprotein E (APOE) gene called APOE ϵ 4. The presence of this gene can increase a person's risk for developing LOAD (Corder 1993). AD can also have earlier origins in people, and these cases are called early-onset Alzheimer's disease (EOAD). Albeit rare, it occurs in individuals between the ages of 30 and 40. A majority of these early-onset cases are called Familial Alzheimer's disease (FAD),

where a genetic predisposition is the contributing cause. The main three genes commonly associated with FAD are: amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2). They occur on chromosomes 21, 14, and 1, respectively. A mutation in any of these three genes, or inheriting the mutated form, causes FAD in people (Ertekin-Taner 2007). Mutation in the gene leads a change in the structural arrangement of the coded protein, thus changing the way it is processed, as in the case of APP, and how it affects downstream enzymatic activity, as in the case of PS-1 and PS-2. The result of having one or more of these mutations is the increased likelihood of developing the characteristic hallmarks found in Alzheimer's disease. These familial AD genes are guiding the way for animal-based models to effectively study the disease and test novel therapies.

Pharmacological therapies that are currently being studied, including those under clinical trials, are focusing on interrupting the underlying pathogenesis of AD in the brain (Herrmann 2011). A common but general target is beta amyloid ($A\beta$) plaques, one of the major hallmarks of the disease that is known to cause neuronal cell death (Hardy 1992). However, there is still some uncertainty about which specific byproducts of the disease should be targeted and what their roles are in the overall pathogenesis of the disease. Pharmaceutical drugs that target a specific enzyme involved in AD may lead to moderate to severe side effects (May et al 2011, Svedruzic 2013). This may be due to the fact that that same enzyme also has non-pathological functions within the cell. Although there are still no proven ways to treat or prevent disease progression, there recently has been accumulating evidence that diet can affect risk (Solfrizzi 2011).

Natural compounds found in certain fruits have been shown to possess neuroprotective and antioxidant abilities when studied with *in vivo* and *in vitro* models of AD (Choi 2012). In many transgenic mouse and rat studies, dietary intake of fruits such as blueberry, bilberry, and black currant at an early age slowed A β deposition in the brain and improved learning ability and overall cognition, as compared to controls (Andres-Lacueva 2005, Pannangrong 2011, Vepsalainen 2013). It is believed that such compounds, called polyphenols, can alleviate the oxidative stress that is caused by reactive oxygen species, which are normally induced by A β (Ortiz, 2004). Under a large nine-year clinical research study called the *Kame* project, dementia-free subjects that consumed vegetable and fruit juices three or more times a week demonstrated a decreased likelihood of being diagnosed with AD, compared to those who consumed less (Dai 2006). The diagnoses were based mainly on the criteria for the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association. Again, this study and others like it have provided supporting evidence that such natural products are effective in preventing the pathogenic events leading to AD. What is yet to be fully determined is the effectiveness of these natural compounds at reversing amyloidogenic processing and deposition in old age, and improving cognition in an already diseased brain. Such studies are necessary to evaluate the potential of natural products as forms of treatment, rather than just as preventative measures.

The hypothesis of this study is that a pomegranate peel extract, a fruit with high levels of antioxidant polyphenols, can be used as a short-term treatment to help alleviate the cognitive and biochemical features of AD in already aged transgenic

animal models. In a 2004 study, researchers looked at the effects of a blueberry supplemented diet on age-related deficits in rats. They found that polyphenolic compounds from the fruit were able to cross the blood brain barrier and localize to regions of the brain important for learning and memory (Andres-Lacueva 2005). Similarly, this study will not only reveal the effectiveness of naturally occurring foods as forms of treatment, but it will also help to better characterize the mechanism of the effects of pomegranate extract. The results of this study may help the research community realize the potential of alternative therapies towards alleviating the symptoms of late stages of AD, and dementia in general.

This hypothesis will be tested by first choosing an AD transgenic animal model that demonstrates the cognitive and behavioral decline that is seen in AD and exhibits the biochemical hallmarks of the disease. After mice are bred, aged, and genotyped, treatment and control groups will be determined. Administration of the extract at different doses, or the control solution, will follow for a short period. During treatment, animals will first be evaluated for their cognitive and learning abilities through various behavioral tasks. This will be followed by post-mortem tissue examination of regions of the brain that are known to be compromised during AD. The tissues will be used to measure levels of key enzymes shown to play a role in $A\beta$ development or degradation. Brain tissues will also be tested for levels of APP, a protein whose sequential proteolysis by two enzymes generates $A\beta$ peptides. This will reveal the efficacy of pomegranate at ameliorating the cognitive and biochemical features of the disease.

Pomegranate (*Punica granatum L.*) has been chosen as the fruit candidate for this study for several reasons. First, pomegranate has a long history of being used as a traditional remedy for a variety of illnesses. Only recently has this fruit been researched and characterized using scientific methodology. Secondly, it has been specifically shown to possess higher levels of antioxidant polyphenols, such as punicalagin, ellagic acid, and gallic acid, and antioxidant potency in comparison to other fruits and fruit juices (Kelawala 2004, Loren 2005, Seeram 2008). With regards to its bioavailability *in vivo*, a study on punicalagin-fed rats found that only a small fraction of this compound and its metabolites were found in feces and urine (Cerdeira 2003). This indicates its high absorption and bioavailability in animals. Studies have also identified pomegranate's antioxidant, anticarcinogenic, and anti-inflammatory activities through its application towards preventing cancer, diabetes, cardiovascular disease and dental conditions (Jurenka 2008). Extracts from the peel in particular have been shown to have significantly higher levels of antioxidant activity in comparison to the pulp and seed fractions (Li 2006). The peel also has high levels of antioxidant activity in the brains of adult rats (Abdel Moneim 2012). In an attempt to harness many of these properties of the pomegranate fruit, this study will use a crude extract obtained from the peel of the fruit. A crude extract may be beneficial because it is believed that the synergistic action of different compounds from a fruit is better than that of any single purified compound (Jurenka 2008, Seeram et al 2005).

CHAPTER 2

REVIEW OF LITERATURE

Thirty years ago, the scientific community knew very little about AD. Since then, researchers have made significant advances, resulting in a better understanding of its biochemical and clinical features. One of these advances is the development of transgenic animal models that carry copies of the mutated genes found in familial AD. With these genes, these models then develop the characteristic features of the disease, both behavioral and biochemical, and can be more easily studied. New treatments can also be tested on the animals to determine their global effects.

The two characteristic neuropathological hallmarks of AD are extracellular $A\beta$ plaques and intracellular tangles of the microtubule-stabilizing protein tau. It has been widely established that the hyperphosphorylation of tau sequesters normal tau into tangles of filaments, while the oligomerization of $A\beta$ peptides, a cleavage product of the transmembrane spanning APP, leads to the development of senile plaques (Alonso 2001). The plaque aggregates are mainly composed of $A\beta$ peptides ranging from 36-43 amino acids in length. These are generated after being sequentially cleaved from the protein APP by the beta-site APP-cleaving enzyme 1 (BACE1), also known as β -secretase, followed by γ -secretase, a protein complex with presenilin-1 as its catalytic site (Haass 2007). The non-amyloidogenic pathway in APP processing involves a different enzyme called α -secretase, which cleaves between the β - and γ - cleavage sites and thus precludes $A\beta$ formation (Haass 1993).

In AD, the $A\beta_{1-40}$ and $A\beta_{1-42}$ isoforms are the prime constituents of cerebral plaques. Even though the $A\beta_{1-40}$ species is ten times more prevalent than its counterpart, the $A\beta_{1-42}$ is more aggregation-prone and neurotoxic to cells (Walsh & Selkoe 2007). This extracellular aggregation of amyloid-beta into plaques has been shown to initiate the pathogenesis of AD, leading to extensive synaptic dysfunction, neuronal loss, and dementia. This cascade of events is collectively known as the amyloid hypothesis (Hardy 1992, Selkoe 1993). Recent studies also claim that the soluble clusters of $A\beta$ called oligomers, and the insoluble fractions, are the species responsible for cell toxicity and death. The soluble fraction has also been better correlated with disease severity in AD patients (Mc Donald 2010, McLean 1999).

Treatments for Alzheimer's are at the moment limited in number and their overall efficacy. Currently, there are four cholinesterase inhibitors and one N-methyl-D-aspartate receptor antagonist that have been FDA approved for the treatment of AD. These medications are not disease modifying and do not halt the progression of AD (Ozudogru 2012). Efforts to develop effective treatment strategies have generally focused on targeting $A\beta$ production and aggregation, since it has been closely linked to disease pathogenesis. This has been approached either by down-regulating APP generation or its amyloidogenic processing, by directly inhibiting $A\beta$ oligomerization, or by promoting $A\beta$ clearance enzymes such as neprilysin and insulin degrading enzyme (IDE) to decrease plaque deposition (Querfurth 2010).

Secondary to this is the regulation of inflammation and oxidative stress, both which are mediated by plaque deposition and lead to neuronal loss (Hardy 2002). Although these treatment mechanisms have been observed with pharmaceutical drugs

such as NSAIDs and statins, accumulating evidence has demonstrated that natural products may exhibit similar activities in the brain (Shukla 2008, Weggen 2003, Wilkinson 2012). One advantage, as already stated, is that these natural products have exceptional safety profiles. Many of them have long been used as traditional remedies for a number of ailments. A plethora of studies have already looked at the effects of various natural remedies in different model systems of AD. Some of these products have been shown to improve cognitive and behavioral symptoms when studied *in vivo*. In addition, reductions in $A\beta$ peptide formation and plaque deposition have been observed. As potential treatments for AD, a notable feature that is shared among many of these products is their abundance in bioactive polyphenolic antioxidants (Essa 2012). In addition to antioxidant activities, polyphenols have also displayed potent anti-amyloidogenic activity (Ono 2004, Riviere 2008). However, not all diets have therapeutic effects on AD.

There is accumulating evidence suggesting that certain diets can actually increase the risk of developing AD. A diet that is high in fat and cholesterol has been shown to accelerate Alzheimer's pathology in APP transgenic mice (Refolo 2000). Cholesterol-fed animals have even been used as models of AD (Sparks 2008). Trace amounts of inorganic copper that is found in drinking water and supplements has also been suggested to induce beta-amyloid plaque deposition (Sparks 2006). In addition, a diet high in the amino acid methionine, such as red meat and eggs, has been linked to elevated $A\beta$ levels and behavioral impairments in an APP transgenic mouse model (Zhuo 2010). However, even more important is the evidence in support of certain diets to help reduce the risk of AD.

The curry spice curcumin has been shown to suppress proinflammatory cytokines, oxidative proteins, and plaque pathology in AD mouse models (Cole 2007, Lim et al 2001). Similar effects have been found with pepper and cinnamon in other AD models (Chonpathompikunlert 2010, Frydman-Marom 2011). In addition, walnuts have been found to reduce age-related cognitive and motor deficits *in vivo* and ameliorate $A\beta$ -peptide induced cell death in PC12 cells (Muthaiyah 2011, Willis 2009). Even beverages such as green tea and coffee have displayed antioxidant effects and increased cognitive performance *in vivo* (Essa 2012). In addition to these products, a number of studies have revealed the beneficial effects of particular fruits and berries on AD and age-related deficits.

From biochemistry to bioavailability, the pomegranate fruit itself has already been extensively studied. There have already been a number of findings substantiating its numerous health benefits for AD. In one experimental study, long-term administration of pomegranate juice reduced hippocampal $A\beta_{1-42}$ and extracellular amyloid plaques in young APP transgenic mice. Cognitive performance and behavior was also improved (Hartman 2006). In another study, punicalagin and ellagic acid, the major polyphenols of pomegranate, were found to be BACE1 inhibitors found in the peel of the fruit (Kwak 2005). Furthermore, ellagic acid itself was found to reduce $A\beta_{1-42}$ cytotoxicity toward SH-SY5Y neuroblastoma cells by preventing its oligomerization (Feng 2009).

In addition to pomegranate, other fruits have also shown promising results. A study on aged blueberry-fed rats found that anthocyanins from the fruit were able to cross the blood brain barrier and localize in regions important for learning and

memory (Andres-Lacueva 2005). In a separate study, aged rats that were given an 18 gm/kg extract of blueberry had enhanced performance in the Morris water maze spacial navigation task (Joseph 1999). Additionally, researchers have found that a blueberry extract exhibits equal neuroprotection against $A\beta$ toxicity in both middle and old-aged rats as well as APP/PS1 mice through a specific mechanism involving protection against reactive oxygen species (ROS) (Brewer 2010).

Aside from blueberry, another study looked at the short term treatment of 20% red grape juice on AD modeled rats. Memory performance was improved in the passive avoidance task. In addition, free radicals were neutralized and oxidative stress was reduced, both of which are cardinal features of AD (Siahmard 2012). An eight-week diet supplemented with 2% blackberry also improved cognitive and motor performance to aged Fischer 344 rats. This was assessed by tasks that require balance and coordination as well as short-term memory function. These effects were mainly attributed to increased antioxidant and anti-inflammatory levels in the brain due to the action of polyphenols (Shukitt-Hale 2009). These studies demonstrate that fruits and other diets seem to be effective therapeutic options to decrease the risk of developing AD and other age-related deficits. However, what is yet to be fully evaluated is the effectiveness of such products in reversing AD pathogenesis in aged transgenic AD models.

The aim of this study was to evaluate the potential of a short-term treatment of pomegranate to reverse, not prevent, the cognitive deficits and aggregation of $A\beta$ in transgenic animal models as compared to vehicle control. This was accomplished by utilizing reliable cognitive tasks and analyzing performance. This study was also used

to help illustrate the specific mechanisms of pomegranate in the aging brain. By looking at the changes in important biomarkers for AD, such as APP and $A\beta$, it was possible to evaluate the activity of pomegranate.

CHAPTER 3

METHODOLOGY

Pomegranate extract preparation

The pomegranate peel extract that was used in this study (POMELLA®; <http://www.pomextract.com/index.php>) is a proprietary commercially available and patented botanical extract obtained from Verdure Sciences (Noblesville, IN, USA). It was standardized to polyphenol (61.5% gallic acid equivalents), punicalagins (29.5 %) and ellagic acid (2.3%) content by the Bioactive Botanical Research Laboratory at the University of Rhode Island (Principal Investigator: Dr. Navindra Seeram) according to previously reported methods (Jean-Gilles 2013, Seeram 2005). The powdered extract was dissolved in saline at two concentrations, 100 mg/kg and 200 mg/kg, by vortexing. Pure saline was prepared as the control. The extract solutions were stored at –20°C for the entire duration of the study.

Study population

The study was conducted on the B6.129-Tg(APP^{Sw})40Btla/Mmjax transgenic animal model, also known as R1.40. These transgenics were developed by Bruce T. Lamb by inserting the entire 400-kb human APP gene containing the Swiss familial AD (FAD) mutation using a yeast artificial chromosome (YAC) and a 250-kb flanking sequence. The R1.40 model develops amyloid beta deposits in the hippocampus and frontal cortex at 24 to 26 months of age (Lamb 1999). In addition, the total levels of

the $A\beta_{1-42}$ peptide are also 7 to 8-fold higher in the hemizygous groups, compared to hemizygotes for the wild-type human gene (Lamb 1997). Hemizygous colonies were ordered from Jackson Laboratory under an *Institutional Animal Care and Use Committee (IACUC)* approved protocol to be used for this study.

Preliminary studies from the University of Rhode Island (URI) have already established the learning and memory deficits associated with the R1.40 model (Subaiea 2013). Spatial memory functions were assessed in the Morris water maze and the Y-maze, both of which rely on the integrity of the cortex and hippocampal regions. Aged female APP transgenic mice (n=19) and control wild-type mice (n=18) were given daily trials for 8 days in the MWM, followed by probe trials. In the Y-maze, the spontaneous alternation ratio was measured for each group. Baseline results showed a significant impairment in long-term memory retention and working memory function compared to wild type controls (Subaiea 2013). Therefore, these animals can be deemed as useful models for the research of AD. During experimental preparations, animals were housed in standard mouse cages at the URI animal quarter rooms with a 12:12 hour light-dark cycle. Temperature was maintained at 22°C. Food and water was made available ad libitum. The protocols for this experiment were approved by the URI IACUC. Animals were kept under supervision by a veterinarian throughout the course of the entire study.

Transgenic animal experiments

Hemizygous YAC APP transgenic mice aged 24-30 months were given the pomegranate extract or a control solution daily to determine its overall effectiveness in

reversing AD. Mice were assigned to one of three groups in a fashion so as to equalize the age variation within and between the groups. The three groups were assigned as follows: 200 mg/kg/day extract in saline (n=8), 100 mg/kg/day extract in saline (n=8), and vehicle control (n=7). Doses of even higher concentration have been deemed safe to administer *in vivo* (Patel 2008). The animal numbers for each group were calculated as the minimum needed based on standard power analysis, justified for scientific and statistical purposes. The daily administration of the extract was done carefully through oral gavage to ensure the precise delivery of the extract or control solution. Treatment continued for three weeks. Previous studies on natural products and animal models of AD have demonstrated significant effects after only a single month of administration (Pannangrong 2011, Sehgal 2012). On day 15 of treatment, mice began the Morris water maze task, including probe trials. On day 36, the mice performed the Y-maze task for a single day. On day 37, mice were euthanized and brain tissue was extracted and stored at -80°C.

Morris water maze

Spatial memory functions were assessed in the hidden version of the Morris water maze (MWM) task. Through trial and error, mice had to spatially associate distal extramaze cues with a submerged escape platform in order to learn of its location (Gulinello 2009, Vorhees 2006). The apparatus consisted of a white pool with a 24" radius and 30" height. Water was added to a depth of 14", just above the height of a 10cm² Plexiglas platform. The water was mixed with a washable, non-toxic white paint to keep it opaque. The temperature was maintained at around 25°C during

experimentation. At the start of the third week of pomegranate administration, mice were given a habituation trial in which they swam freely for 60 seconds. On the following day, and for a total of 10 days, each animal was given three timed trials to find the escape platform. For each trial, the mouse was placed in a different quadrant from which to start its task. The position of the platform remained fixed throughout the daily training sessions. A successful trial was defined as any attempt by which the location of the platform was determined within a maximum of 60 seconds. Upon completion of a successful trial, the mouse was allowed to sit for a maximum of 10 seconds on the platform before being removed. If a trial was unsuccessful, the mouse was gently guided to the platform and allowed to sit for a maximum of 30 seconds. Probe trials on day 1 and day 11 after the last day of the 10 acquisition sessions were conducted to assess long-term memory retention. On these days, each mouse was given a single trial lasting 60 seconds to show preference for the quadrant in the maze that previously held the hidden platform. A digital video-tracking system recorded the swim path and latency of each animal while locating the platform, as well as the time spent in each quadrant, to allow for accurate data collection (ObjectScan; Clever Sys., Inc., Reston, VA, USA).

Y-maze

The Y-maze was performed on day 36 of the experiment, immediately following the second probe trial for the MWM task. This test is based on a rodent's innate curiosity to explore novel areas over areas that have already been visited. The apparatus consisted of a white maze with three paths or 'arms' that conjoined in the

middle. Each arm was 12" long, 3" wide, and with 8" high walls. For this task, the spontaneous alternation ratio was calculated for each animal to assess its spatial working memory functions. This ratio is defined as the number of new arm entries (an arm that is different from the previous two visited) to total arm entries minus two. Mice were placed into a single arm and allowed to roam between the arms for five minutes. Following each trial, the maze was cleaned with 70% ethanol. Trials were tracked and recorded using a computerized video-tracking system (ObjectScan; Clever Sys., Inc.), and the resulting data were analyzed.

Protein extraction and Western blotting

Cortex and hippocampal brain tissue was first homogenized using mechanical grinding in radioimmunoprecipitation assay (RIPA) lysis buffer made up of 10mM Tris-HCL (pH 7.4), 150mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1mM ethylenediaminetetraacetic acid, and 0.1% protease inhibitor cocktail. Homogenates were centrifuged at 10,000 x g for 20 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the BCA kit (Pierce Biotechnology, Inc, Rockford, IL, USA). Forty micrograms of total protein was first separated on precast 4-15% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad Laboratories, Inc, Hercules, California, USA). Protein was then transferred onto polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked for 20 minutes using Tris-Buffered saline with 0.1% Tween-20 (TBST, pH 7.4). The membranes were then incubated overnight at 4°C with a specific antibody diluted in TBST [1:1000 dilution of Anti-APP A4 antibody for

APP, 1:1000 dilution of Anti-Neprilysin antibody for neprilysin, and 1:000 dilution of PC730 Anti-IDE for insulin degrading enzyme (EMD Millipore Corporation, Billerica, MA, USA)]. Membranes were washed three times with TBST and then incubated for 1 hour with a 1:5000 dilution of anti-mouse or anti-rabbit IRDye 680 (Li-Cor Bioscience, Lincoln, NE, USA) at room temperature. Membranes were again washed three times with TBST. Images were captured using the Li-Cor Odyssey infrared imaging system (Li-Cor Bioscience). Membranes were also reprobbed with an antibody for the protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at a 1:5000 dilution (Sigma-Aldrich Corp., St. Louis, MO, USA) as a control to obtain the specific target-protein/GAPDH ratio. The intensities of the Western blot bands were measured using the Odyssey V1.2 software (Li-Cor Bioscience).

A β ELISA

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure levels of soluble A β_{1-40} and A β_{1-42} (Immuno-Biological Laboratories, Gunma, Japan). These kits are solid-phase ELISA with highly specific antibodies that are 100% reactive with mouse A β_{1-40} with a sensitivity of 5.00pg/mL, and 70% reactive with mouse A β_{1-42} with a sensitivity of 4.03pg/mL. Both fragments of A β were measured in protein samples from the cortex and hippocampus of each animal. The protocol for the assay was followed in accordance with a method that has already been described (Morishima-Kawashima 2000, Wu 2008). The A β levels in the samples were calculated based on the standard curve generated from each plate.

γ -secretase (GACE) ELISA

An ELISA kit highly specific for mouse γ -secretase was used to measure its concentration in cortical tissue homogenates. The kit specifically measured the concentration by using an immunogen that binds to the presenilin enhancer 2 (PEN-2) subunit of γ -secretase (Cusabio Life science, Wuhan, P.R. China). PEN-2 serves as an essential cofactor of the γ -secretase enzyme (Luo 2003). The kit is a sandwich ELISA with a minimum detection limit of 1.95pg/mL. The protocol used for the kit was strictly consistent with that of the company's. The γ -secretase levels in the samples were calculated based on the standard curve generated from the plate.

Data analysis

The results for each treatment group for all behavioral and biochemical tests were expressed as the mean $\pm$ the standard error of the mean (SEM). Significant differences between the various treatment groups for all data were determined by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison a posteriori analysis. For the MWM daily training sessions, performance within each group was measured by a two-tailed Student's t-test. All data were analyzed using the GraphPad InStat 3 software (GraphPad Software, La Jolla, CA, USA) where a p-value <0.05 was considered statistically significant.

CHAPTER 4

FINDINGS

Pomegranate treatment does not reverse the cognitive deficits in aged hemizygous R1.40 mice

The R1.40 model has been previously shown to possess learning and memory deficits during late age, as determined by the MWM and Y-maze tasks (Subaiea 2013). It was hypothesized that daily treatment with pomegranate extract would attenuate and reverse these deficits at this late age. Hemizygous R1.40 mice ranging from 24 to 30 months were treated with 100 and 200 mg/kg/d pomegranate extract for three weeks. In the MWM task, daily training produced a significant improvement in escape latency between the first and last day within each group (Fig. 1A). However, there were no significant differences between any of the groups in daily learning acquisition. The probe trials, which test for retention of long-term memory, also showed a lack of significance between the groups as determined by ANOVA when compared on day 11 (Fig. 1B). The Tukey-Kramer HSD post hoc test showed that both the 100 mg/kg/d (HSD=1.36, $p>0.05$) and the 200 mg/kg/d (HSD=1.16, $p>0.05$) failed to reach significance.

The ANOVA results from the spontaneous alternation ratio in the Y-maze showed a lack of significant improvement in working memory ($F(2,14)=2.02$, $p=0.169$) (Fig. 2). The Tukey-Kramer HSD post hoc test revealed a lack of improvement with both the 100 mg/kg/d and 200 mg/kg/d treatment groups.

Pomegranate treatment does not lower cortical and hippocampal APP protein levels

Levels of cortical and hippocampal APP were analyzed by Western blot for all the groups and normalized against the housekeeping protein GAPDH (Fig. 3). Levels of APP are expressed as the APP/GAPDH ratio. It was revealed by ANOVA that there were no significant differences in protein levels for both the cortex ($F(2,6)=0.252$, $p=0.785$) and hippocampus ($F(2,15)=3.13$, $p=0.073$). The Tukey-Kramer post hoc test confirmed that both the 100 mg/kg/d and 200 mg/kg/d treatments were not significantly different from the vehicle for both brain regions.

Pomegranate treatment does not increase cortical IDE protein levels

A function of insulin degrading enzyme (IDE) is the cerebral degradation of $A\beta$ peptides (Kurochkin 1994). Western blotting was used to measure its levels in the

cortex and determine if they were affected by pomegranate treatment (Fig. 4). The band intensities were normalized against GAPDH to obtain the ratio. It was shown by ANOVA that there were no significant differences in IDE between the groups ($F(2,14)=1.36, p=0.290$).

Pomegranate treatment increases levels of $A\beta_{1-40}$, decreases levels of $A\beta_{1-42}$, and decreases the $A\beta_{42}/A\beta_{40}$ ratio, in both the cortex and hippocampus

ELISA was used to measure concentrations of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in both the cortex and hippocampus. Additionally, the $A\beta_{42}/A\beta_{40}$ ratio was obtained for each sample by dividing the levels of each peptide, which was obtained from ELISA. Studies have suggested that the ratio serves as a better predictor of AD plaque load in the brain (Yin 2007). ANOVA revealed that for the cortex, there was a significant increase in $A\beta_{1-40}$ ($F(2,15)=4.11$, $p=0.038$) (Fig. 5A). The Tukey-Kramer HSD post hoc test indicated that levels for the 200 mg/kg/d group were significantly higher (HSD=3.87, $p < 0.05$), whereas the 100 mg/kg/d group failed to reach significance (HSD=0.91, $p > 0.05$). For $A\beta_{1-42}$, ANOVA showed a significant decrease in their levels in the cortex ($F(2,16)=4.88$, $p=0.022$) (Fig. 5B). The HSD post hoc test showed that levels for both the 100 mg/kg/d (HSD=3.89, $p < 0.05$) and the 200 mg/kg/d

(HSD=3.66, $p<0.05$) treatment groups were lowered significantly. The $A\beta_{42}/A\beta_{40}$ ratio results were also analyzed and it was shown by ANOVA that it had decreased significantly ($F(2,14)=8.90$, $p=0.003$), but the HSD post hoc test showed that it had decreased significantly for the 200 mg/kg/d treatment group (HSD=5.94, $p<0.01$) and not the 100 mg/kg/d (HSD=3.27, $p>0.05$) (Fig. 5C).

For the hippocampus, ANOVA revealed that there were similar alterations in the $A\beta$ peptides, and thus, also the ratio. Levels of $A\beta_{1-40}$ increased significantly ($F(2,17)=6.62$, $p=0.007$), with the Tukey-Kramer HSD post hoc test showing a

significant increase for both the 100 mg/kg/d group (HSD=3.98, $p<0.05$) and the 200 mg/kg/d group (HSD=4.77, $p<0.01$), when comparing all columns (Fig. 6A). The levels of $A\beta_{1-42}$ changed even more significantly ($F(2,17)=62.4$, $p<0.0001$). The HSD post hoc test showed that the 200 mg/kg/d group was significantly lower than both the 100 mg/kg/d group (HSD=11.94, $p<0.001$) and the vehicle (HSD=15.16, $p<0.001$). The 100 mg/kg/d group (HSD=3.36, $p>0.05$) did not reach significance (Fig. 6B). As expected, the $A\beta_{42}/A\beta_{40}$ ratio was lowered significantly ($F(2,17)=38.28$, $p<0.0001$)

Fig. 6. Changes in hippocampal $A\beta_{1-40}$ and $A\beta_{1-42}$. Hippocampal concentrations of soluble $A\beta$ changed in hemizygous R1.40 mice after treatment with pomegranate. (A) Soluble $A\beta_{1-40}$. (B) Soluble $A\beta_{1-42}$. (C) $A\beta_{42}/A\beta_{40}$ ratio, obtained by dividing the ELISA result for each peptide. *Values are significantly different from the vehicle group. **Values are significantly different from the 100 mg/kg/d and vehicle group. Significance was determined by one-way analysis of variance with Tukey-Kramer post hoc test ($p<0.05$). Analysis of results was done using the GraphPad InStat 3 software.

(Fig. 6C). The HSD post hoc test reported a significant decrease in the 200 mg/kg/d group when compared to both the 100 mg/kg/d group (HSD=7.60, $p<0.001$) and the vehicle (HSD=12.32, $p<0.001$). The 100 mg/kg/d group (HSD=4.92, $p<0.01$) also reached significance.

Pomegranate increases levels of γ -secretase in the cortex

An ELISA kit was used to determine whether the levels of γ -secretase changed with pomegranate treatment. Any changes would help explain the shift in the preference for the $A\beta_{1-40}$ peptide, since γ -secretase provides the final cleavage of APP before generating $A\beta$. It's important to note that the results of this method would

reveal very little about the actual activity of this enzyme. After the results were obtained, ANOVA showed that there was significant increase in its levels ($F(2,17)=4.71$, $p=0.024$) (Fig. 7). The Tukey-Kramer HSD post hoc test revealed that

the 100 mg/kg/d group reached significance (HSD=3.98, $p<0.05$), but not the 200 mg/kg/d group (HSD=3.42, $p>0.05$) when comparing all columns.

CHAPTER 5

DISCUSSION AND CONCLUSION

In recent years, there has been an increase in evidence supporting the relation between the intake of certain natural products and the decreased risk of developing Alzheimer's disease (Essa 2012). Fruits in particular have received considerable attention because of their many disease-fighting compounds and antioxidant properties (Dai 2006, Hughes 2010). However, very little research has addressed these effects during the late stages of AD. In this study, a pomegranate peel extract, which has considerable levels of antioxidant polyphenols, was administered to hemizygous R1.40 mice aged 24-30 months for three weeks to determine its effects on both the cognitive and pathological features of the disease. In addition, the mechanism of action of pomegranate in the aging AD brain was analyzed. It was hypothesized that a short-term treatment of pomegranate peel extract would help to reverse cognitive decline and plaque deposition in the AD mouse model.

According to the results obtained from the Morris water maze daily trials, all of the groups showed a significant decrease in escape latency throughout the course of the daily acquisition trials. This performance is expected with relative learning behaviors. However, the treated groups did not show significant improvement in spatial learning compared to the control. In addition, there was no significant improvement in long-term memory retention, as shown by the delayed probe trials on day 1 and 11 following the MWM. There was also a lack of improvement in working memory, as tested by the Y-maze. It has been suggested that recent spatial memory is

hippocampal dependent, while remote spatial memory is dependent on the medial prefrontal cortex (Frankland 2005, Teixeira 2006).

Working memory is also cortical and hippocampal dependent (Lalonde 2002). In the R1.40 mouse model, both the frontal cortex and hippocampus have diffuse $A\beta$ deposits at 24-26 months of age (Lamb 1999). Deposition of $A\beta$ peptides, especially $A\beta_{1-42}$, leads to increased oxidative stress and neuroinflammation, resulting in the neuronal loss and AD pathogenesis (Butterfield 2002). It was hypothesized that the antioxidant polyphenols from pomegranate could inhibit this cascade of events at this late stage in the treated groups, perhaps at multiple points in the pathway, and result in a gain of neuronal function. It can be concluded that there was already extensive neuronal loss in this model at the start of the study and treatment with pomegranate does not reverse these losses. These findings, however, support the reliability of this transgenic animal as a model for researching AD. Improvements in spatial learning and memory have already been seen in a previous study using this model. Treatment with a pharmacological drug at an early age produced significant cognitive improvements compared to vehicle controls (Subaiea 2013).

The effects of pomegranate extract on the biochemical aspects of AD were determined by focusing on changes in important AD biomarkers in the brain. The levels of the amyloid precursor protein (APP), which is the precursor to $A\beta$ peptides, did not change between the groups. This was found with both brain regions. These results are consistent with previous findings with pomegranate (Hartman 2006). Since APP remained unaffected, its proteolytic processing into $A\beta$ peptides was the next target for consideration.

An interesting effect was seen with the soluble levels of $A\beta_{1-40}$ and $A\beta_{1-42}$. It appears that treatment significantly inhibited the production of the longer $A\beta_{1-42}$ subtype in favor of the shorter $A\beta_{1-40}$ one. These trends were consistent in both the cortex and hippocampus. This shift in preference resulted in significant decreases in the $A\beta_{42}/A\beta_{40}$ ratios, especially with the 200 mg/kg/d treatment. It has been previously reported that although the $A\beta_{1-40}$ fragment appears at levels ten times more than its longer counterpart in AD, it exhibits neuroprotective properties by inhibiting the aggregation and deposition of $A\beta_{1-42}$ in the brain (Kim 2007, Wang 2006, Yan 2007). It has also been found that a decreased production of the $A\beta_{1-40}$ subtype due to altered function in γ -secretase leads to exacerbated plaque pathology in mice (Deng 2006). Studies also suggest that lowering the $A\beta_{42}/A\beta_{40}$ ratio has more implications for AD therapy than simply lowering the absolute amount of $A\beta$ (Kuperstein 2010). The $A\beta$ results from this study point to a specific mechanism of action for pomegranate in combating AD pathogenesis. In order to explain the changes in these $A\beta$ peptides, a number of hypotheses had to be proposed. Alterations in $A\beta$ could be due to changes in its clearance rates. It also could have been due to changes in its generation. To test the first, levels of insulin degrading enzyme (IDE) were measured.

IDE regulates the levels of both insulin and $A\beta$ in the body through their degradation (Kurochkin 1994). In this study, it was found that the levels of IDE protein remained unaffected by pomegranate extract treatment. It can be strongly asserted that pomegranate does not alter the $A\beta$ profile by inducing its clearance from the brain. These results pointed to a more specific target for pomegranate; a target that is more determinative about the quality of $A\beta$ that is generated, as shown by the

changes in the $A\beta_{42}/A\beta_{40}$ ratios, instead of quantity of $A\beta$. This target would most likely be γ -secretase.

BACE1 is the initial enzyme that cleaves APP extracellularly and creates two fragments; the soluble extracellular fragment termed sAPP β and a cell membrane-bound fragment known as C99. The latter fragment serves as the substrate for cleavage by γ -secretase and thus the generation of $A\beta$. γ -secretase is responsible for the heterogeneous cleavage of this fragment into a variety of $A\beta$ subtypes, and thus represents the target for consideration in this study. ELISA was used to measure γ -secretase and determine its changes in the brain after treatment with pomegranate extract. This test was limited in that it told only about the changes in the concentration of γ -secretase, but not its catalytic activity. However, changes in its concentration can perhaps be directly associated with its levels of catalytic activity in the brain. The results showed that the relative amount of γ -secretase increased with treatment, but significantly only with the 100 mg/kg/d group. The 200 mg/kg/d group failed to reach significance by a narrow margin. This disquieting lack of significance can perhaps be explained by noting that gamma-secretase was measured only in the cortex, and not in the hippocampus. When analyzing the changes in the $A\beta$ subtypes from the cortex, it appears that the 200 mg/kg/d group is showing levels that are within range of the levels of the 100 mg/kg/d group. However, the treatment's specific effects are more apparent when analyzing the hippocampus region. It appears that the $A\beta$ trends were statistically stronger in the hippocampus tissue than in the cortex. The ANOVA tests revealed that the p -values were considerably smaller for the both $A\beta$ subtypes and ratio in the hippocampus, in comparison to the cortex. Perhaps the treatment has more

favorable outcomes in one brain region compared to another. Conducting the γ -secretase ELISA on the hippocampus would help confirm these results.

There are two general explanations as to why the γ -secretase levels are increasing with treatment. First, the mRNA expression of its subunits could be increasing, thus changing its amount of translation and assembly. Secondly, it's possible that with treatment, its eventual degradation from the cell is being downregulated. Regardless of these explanations, the question is how an increase in its levels relates to a change in the $A\beta_{42}/A\beta_{40}$ ratio. It can be hypothesized that the catalytic activity of γ -secretase is indeed being modulated, perhaps to favor the production of the shorter $A\beta$ peptide.

Even though there have been many studies elucidating the structure and function of γ -secretase, little is known about the mechanisms that regulate its cleavage specificity. This specificity is crucial for the pathogenesis of AD. It has already been suggested that mutations in the transmembrane portion of C99 can alter the interaction between C99 and γ -secretase, thus changing its cleavage specificity (Lichtenthaler 1999). More recently, it has been proposed that altering single amino acid residues at the cytosolic face of the C99 substrate alters the specificity of γ -secretase binding, thus modifying the levels of each $A\beta$ species that is produced (Ousson 2013). Nevertheless, to further investigate the implications of changing γ -secretase levels, it is important to look at its likely structure and function within this model.

The structure of γ -secretase has been found to be made up of four subunits, with PS-1 or presenilin-2 (PS-2) present at its catalytic core (Ogura 2006). Expression of all four proteins is necessary for its activity (Edbauer 2003). It has been widely

established that mutations in PS-1 cause significant elevations in extracellular $A\beta_{1-42}$, promoting its deposition into plaques (Scheuner 1996). However, since the model used for this study does not have the PS-1 mutation, the focus shifts to whether or not the wild-type PS-1 plays a role in γ -secretase activity and AD pathogenesis. Interestingly, it has been found that the presence of the wild-type PS-1 form in a PS-1 mutant mouse leads to lessened amyloid plaque pathology, compared to its absence. This establishes a protective role for wild-type PS-1 (Wang 2006). However, whether or not it exhibits any protection in the presence of the APPsw mutation used in this study remains to be elucidated. It would be interesting to measure the levels of wild-type PS-1 between the groups and determine any changes in its expression and activity. This also applies to the other subunits of the γ -secretase complex.

Another interesting target for consideration is BACE1. It has been previously found that the pomegranate peel contains compounds that work to inhibit BACE1 activity (Kwak 2005). Any inhibition of BACE1 by pomegranate would decrease APP processing and formation of $A\beta$ peptides. However, BACE1 is unlikely to be the target for pomegranate activity in this study because its inhibition would create less of the C99 fragment, and thus decrease both the $A\beta_{1-40}$ and $A\beta_{1-42}$ species that are generated from its cleavage by γ -secretase. The results of this study indicate a different effect.

Considering the changes in the $A\beta_{42}/A\beta_{40}$ ratio and the γ -secretase levels, it appears as though pomegranate is acting as a modulator of γ -secretase. Modulators of this enzyme have already been extensively studied as therapeutic agents for AD. Shifting the formation of $A\beta$ into the shorter, less toxic peptides has been a recent target for AD therapy (Peretto 2008). A large phase-3 clinical study on a drug called

tarenflurbil found that it shifts γ -secretase activity to form less of the $A\beta_{1-42}$ subtype in favor of shorter forms such as $A\beta_{1-38}$. However, this drug failed to improve cognition in patients with mild-AD, possibly due to low brain penetrability (Marder 2010).

γ -secretase modulators have been more promising for AD therapy compared to inhibitors of this enzyme. It is known that in addition to cleaving the C99 fragment of APP, γ -secretase also cleaves a transmembrane protein called Notch. Cleavage of Notch into an intracellular domain induces a signaling pathway that mediates cell-cell communication and is important for cell-fate decisions (Baron 2003). Inhibition of γ -secretase can prevent the necessary processing of Notch and thus lead to a variety of side effects and developmental issues (Geling 2002). In addition to being a modulator of γ -secretase instead of an inhibitor, pomegranate possesses no toxicity associated with its consumption and thus represents an ideal fruit for further investigation.

Some limitations of this study to note are as follows. First, the highest dose used for treatment was dependent on the solubility of the powdered extract in saline, which was the control treatment. Use of additional measures, such as heat, to dissolve more extract may have compromised its integrity. Secondly, this study employed aged transgenic mice of AD. The successful administration of the treatment and completion of the behavioral tasks was contingent on the animals' ability to withstand these stress-inducing measures. The animals' health was continuously monitored to ensure that they were capable of continuing the trials. Possible reductions in numbers were taken into consideration when determining the initial sample size of each group.

In conclusion, this study looked at the efficacy of a pomegranate peel extract in rescuing the cognitive deficits of aged R1.40 transgenic mice and modifying AD

plaque pathology. Even though a lack of effect was seen with learning and memory, a specific mechanism of action can be proposed for pomegranate in the AD brain. This mechanism involves the modulation of the enzyme γ -secretase. It is not exactly known which class of compounds from pomegranate may be promoting this modulatory effect. It is known, however, that pomegranate has a number of polyphenols with strong antioxidant activities in the brain. Whether it is acting through a separate mechanism by selectively targeting toxic $A\beta_{1-42}$ species remains to be known. In addition, further investigation on the workings and activity of γ -secretase on the APP/C99 fragment is needed to help determine on a molecular basis how modulatory compounds must be exerting their effects. In addition, a thorough evaluation of how antioxidants enter the brain to reduce $A\beta$ plaque pathology and interact with and protect neurons is necessary. A thorough understanding of how certain therapies alleviate the symptoms of AD is contingent on the understanding of the basic molecular implications of AD in the aging brain.

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