Toxicity Study in Alzheimer's Disease Cell Model

by

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ABSTRACT

Alzheimer's disease (AD) is the most common type of dementia, affecting one in nine people age 65 and older. One of the most important neuropathological characteristics of Alzheimer's disease is the aggregation and deposition of the protein beta-amyloid. Beta-amyloid is produced by proteolytic processing of the Amyloid Precursor Protein (APP). Production of beta-amyloid from APP is increased when cells are subject to stress since both APP and beta-secretase are upregulated by stress. An increased beta-amyloid level promotes aggregation of beta-amyloid into toxic species which cause an increase in reactive oxygen species (ROS) and a decrease in cell viability. Therefore reducing beta-amyloid generation is a promising method to control cell damage following stress.

The goal of this thesis was to test the effect of inhibiting beta-amyloid production inside stressed AD cell model. Hydrogen peroxide was used as stressing agent. Two treatments were used to inhibit beta-amyloid production, including iBSec1, an scFv designed to block beta-secretase site of APP, and DIA10D, a bispecific tandem scFv engineered to cleave alpha-secretase site of APP and block beta-secretase site of APP. iBSec1 treatment was added extracellularly while DIA10D was stably expressed inside cell using PSECTAG vector. Increase in reactive oxygen species and decrease in cell viability were observed after addition of hydrogen peroxide to AD cell model. The increase in stress induced toxicity caused by addition of hydrogen peroxide was dramatically decreased by simultaneously treating the cells with iBSec1 or DIA10D to block the increase in beta-amyloid levels resulting from the upregulation of APP and beta-secretase.

DEDICATION

I want to dedicate this thesis first of all for my parents, Tony and Jubie, and my brother, Sean. Thank you so much mom and dad for all your love, for raising me to be the person that I am today. Without you two, I would not get to this point. Also, for Sean, thank you for being an amazing brother and especially for patiently listening to all my complaints. I know we fought so much when we were little, but we also had so many great memories.

This thesis goes to Huidan next. I would not finish this thesis on time if it were not for you. Thank you so much for pushing me to start on my thesis and for reminding me always to work on it. Thank you also for all the fun lab dates to help keep me from stressing out too much, I enjoy every second spent with you. Even if you don't realize it, you have been so much help. I know research has been very stressful for you too, but I promise I will help you the best I can (including learning how to make baozi and getting good at it).

Last but not least, thank you so much to all my friends, especially Yifei, Ricky, and Ploy. I am so blessed to have so many wonderful friends around me. Without you all, I would not be able to finish my master's. Thank you for being much needed distractions through all the hard times and thank you for pushing me when I needed some motivation. What I miss the most from school will be all the fun times spent with all of you.

ACKNOWLEDGMENTS

First of all, I would like to thank my advisor, Dr. Michael Sierks, for giving me the chance to work in his lab and for all his guidance and support throughout my time in the lab. The past five years have been a very rewarding learning experience. I would also like to express my gratitude for all the opportunities he has given me, including the projects he trust me with and conferences poster presentation.

Next, I would like to thank my committee members, Dr. David Nielsen and Dr. Lenore Dai for agreeing to be on my committee on a short notice. They are both wonderful teachers, I enjoyed both their classes so much and I learned a lot from them.

I would also like to thank Dr. Shanta Boddapati, my mentor, who was not only extremely intelligent, but also very patient. I want to thank her especially for teaching me not only how to do experiments, but also the reason behind all the experiments.

Finally, thank you so much to all the lab members, both current and past. I want to especially thank Phil for training me when I started in the lab, for all his help with ordering, and for his suggestions. I would also like to thank Stephanie for her help with ELISA and Sharareh & Wei for their help with cell culture. Last, a huge thank you for all the lab members, both current and past: Shanta, Phil, Stephanie, Wei, Lalitha, Huilai, Ping, Patrick, Sharareh, Srinath, Ricky, Now Bahar, Jessica, John, Tim, Taylor, Ankush, and Michael; you all have made the past five years working in this lab a wonderful and fun experience. I will miss you all.

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CHAPTER 1

INTRODUCTION

1.1 Alzheimer's Disease Background

Alzheimer's disease (AD) is the most common form of dementia and the sixth leading cause of death in the United States (Gaugler et al., 2013). It is a neurodegenerative disease that deteriorates the brain, affecting memory, speech, and mobility. Prevalence of this disease can be seen through the painting of an artist William Utermohlen. Figure 1.1 below shows a collection of his self-portraits. Utermohlen was diagnosed with AD in 1995 and as the disease progresses, his face in his paintings becomes harder and harder to discern.

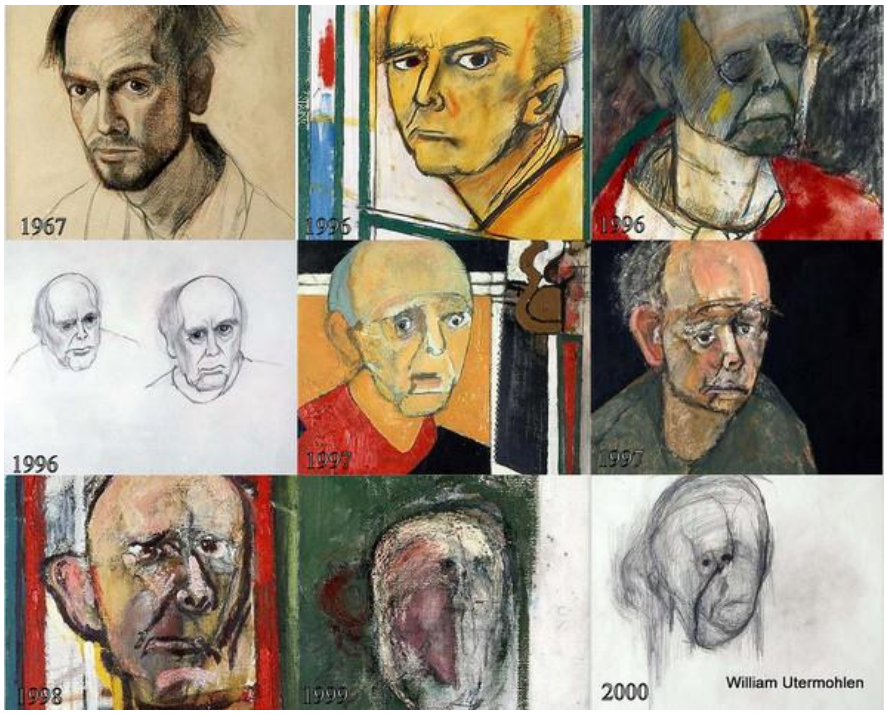

Figure 1.1. Self-portraits of artist William Utermohlen, diagnosed with AD on 1995 (http://www.williamutermohlen.org)

No cure is currently available for Alzheimer's disease. Treatments are given to slow down disease progression. Cholinesterase inhibitors like Donepezil and Galantamine are prescribed to slow down breakdown of acetylcholine receptors (Selkoe, 2001). Another type of medication prescribed is Memantine, used to regulate activity of glutamate which is a messenger chemical involved in memory and learning (Bond et al., 2012). Although these medications can be used to slow down AD progression, they are ineffective as long term treatment for the disease.

About 5.2 million people in the United States have Alzheimer's disease, costing the nation $203 billion in treatment and care; number of people with the disease is estimated to double by 2020 and triple by 2050 (Hebert et al., 2013). Moreover, an estimated 17.7 billion hours of unpaid care were given to Alzheimer's patients by family and friends, valued at more than $220 billion (Hurd et al., 2013). These facts highlight the importance of a more effective treatment for Alzheimer's disease.

1.2 Alzheimer's Disease Neuropathology

Compared to normal brain, Alzheimer's disease brain shows shrinkage; these shrinkage are especially more visible in the cerebral cortex and hippocampus region. Studies have also shown significant neuronal loss in several area of the brain, including hippocampus (West et al., 1994). Figure 1.2 below shows the difference between brain cell of normal and AD patient. Two very important neuropathological characteristics of AD are the deposition of beta-amyloid (Aβ) plaques and neurofibrillary tangles of hyperphosphorolated tau protein (Tiraboschi et al., 2004).

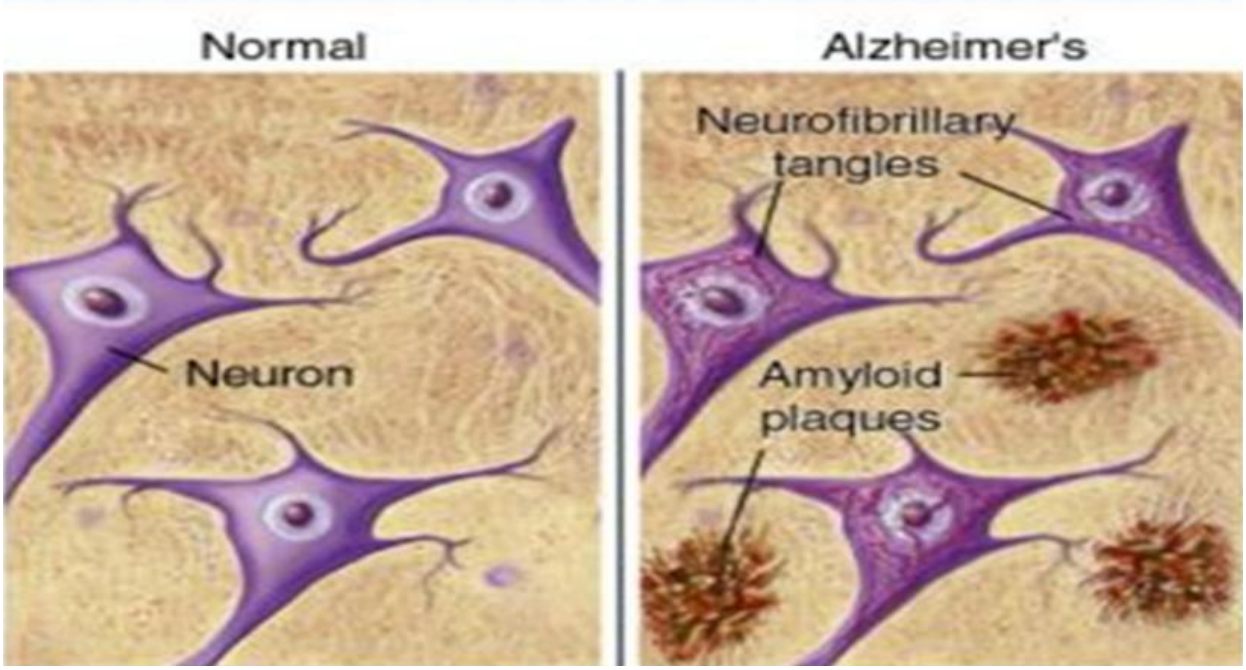

Figure 1.2. Brain cells of normal and AD patient. Neurofibrillary tangles of hyperphosphorolated tau protein and amyloid plaques of Aβ protein can be seen in brain of AD patient (Weiner et al., 2006).

1.2.1. Beta-amyloid plaques

Plaques in AD patients form as deposits between neurons from the aggregation of Aβ protein (Selkoe, 2001). They are spherical in shape and are usually found in limbic brain regions like hippocampus and amygdala (LaFerla et al., 2005). Beta-amyloid, typically a 40/42 amino acid protein, is formed from processing of amyloid precursor protein (APP) with the 42 amino acid long Aβ more prone to aggregation. These beta-amyloid when aggregates form beta sheet structures. Amyloid plaques also form in normal brain; however, plaques formation in AD brain is significantly higher (Tomlinson et al.,

1997). What cause plaques formation is still unclear, whether it is due to irregular processing of APP or improper accumulation of Aβ.

1.2.2. Neurofibrillary Tangles

Another hallmark of Alzheimer's disease is the intracellular formation of neurofibrillary tangles. These neurofibrillary tangles are composed of hyperphosphorolated tau protein (Iy et al., 1998). Tau are proteins whose primary function is to maintain structural stability of microtubules. AD patients show hyperphosphorolation of tau proteins, resulting in dissociation of microtubules and self-assembly of hyperphosphorolated tau. Braak staging is sometimes used to group the disease progression into six different stages based on the amount of neurofibrillary tangles in the brain (Braak et al., 1991).

1.3. Alzheimer's Disease Pathogenesis

While the cause of Alzheimer's disease is still unknown, there are three main hypothesis to explain the disease using its hallmark: the cholinergic hypothesis, the amyloid hypothesis, and the tau hypothesis (Tanzi et al., 2005).

1.3.1. The cholinergic hypothesis

Acetylcholine is a neurotransmitter in both peripheral and central nervous system; it plays an important role in conscious awareness, attention, working memory, and additional mnemonic processes. Acetylcholine is synthesized from choline and acetyl-CoA by the enzyme choline acetyltransferase (Feldberb, 1945). The cholinergic hypothesis

started in 1970s when study of postmortem AD brains show reduced activity of choline acetyltransferase in hyppocampus and cerebral cortex (Davies et al., 1976; Perry et al., 1977); it states that cognitive decline in Alzheimer's patient is a result of loss of cholinergic function.

Many AD drugs have been developed based on this hypothesis. Acetylcholinesterase inhibitors are used to inhibit acetylcholinesterase enzyme from breaking down acetylcholine, thus increasing the level of acetylcholine receptors in the brain. Currently, four acetylcholinesterase inhibitors are FDA approved and prescribed for AD patients; they are tacrine (Cognex), donepezil (Aricept), rivastigmine (Exelon), and galantamine (Razadyne). While these medication can be used to slow down disease progression and provide symptomatic benefit, they cannot stop or reverse the disease progression.

1.3.2. The amyloid hypothesis

Two main neuropathology hallmark of Alzheimer's disease are the extracellular beta-amyloid plaques and intracellular neurofibrillary tangles of hyperphosphorolated tau protein. The amyloid hypothesis states that neurodegeneration in Alzheimer's disease is caused by accumulation of Aβ inside the brain. These accumulation are due to irregular processing of APP and improper clearance of beta-amyloid from the brain. Previously, the amyloid hypothesis states that the fibrillar Aβ are the toxic species causing cell death and hyperphosphorolation of tau protein (Hardy, 1992; Selkoe, 2001). However, recent studies have shown that oligomeric Aβ are the toxic species triggering cell apoptosis in AD brain (Walsh et al., 2002; Sierks et al., 2011).

Aβ is formed by cleavage of Amyloid Precursor Protein (APP) by beta and gamma secretases (figure 1.3). β-site APP cleaving enzyme-1 (BACE-1) has been reported to be the predominant beta secretase enzyme responsible during APP processing through amyloidogenic pathway (Vassar et al., 1999; Yan et al., 1999). After monomeric Aβ is formed through APP processing, it aggregates, forming oligomeric species (Glass et al., 2010). Further aggregation of these oligomeric species result in the formation of amyloid plaques, one of the hallmarks of AD.

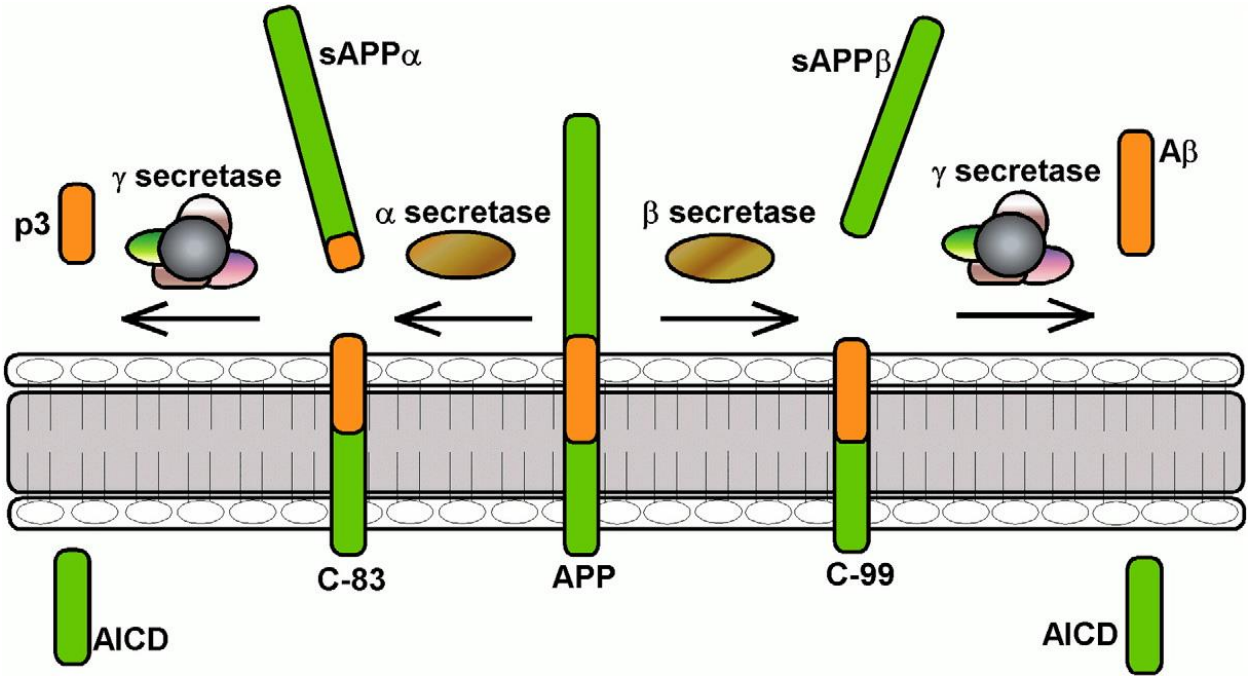

Figure 1.3. Proteolytic processing of APP. Non-amyloidogenic pathway of APP processing involves cleaving by α-secretase followed by γ-secretase, resulting in formation of membrane-bound protein p3. Amyloidogenic pathway involves cleaving by β-secretase instead of α-secretase, followed again by γ-secretase, yielding insoluble beta-amyloid protein (Boddapati et al., 2011).

Many drugs are currently developed based on the amyloid hypothesis; while many are in clinical trials, none of these drugs are FDA approved and prescribed for AD patients. One way to reduce amyloid in the brain is by clearing excess Aβ; Crenezumab is a monoclonal antibody currently in phase 2 clinical trial licensed to Genentech that was engineered to bind aggregated form of Aβ and clear them from the brain (Panza et al., 2014). Another way is to manipulate APP processing and reduce Aβ formation; this can be done by promoting cleavage of APP at alpha site, reducing APP cleavage at beta site, and reducing APP cleavage at gamma site. ASec1A is an example of proteolitic antibody designed to cleave APP at alpha site and iBSec1 is an example of monoclonal antibody against beta-amyloid cleaving enzyme 1 site of APP (Kasturirangan et al., 2009; Boddapati et al., 2011). Many research are also currently done in order to break and prevent aggregation of Aβ protein. Preventing accumulation of Aβ aggregates is a promising therapeutics for treatment against Alzheimer's disease.

1.3.3. The tau hypothesis

Tau hypothesis states that neurodegeneration in AD is caused by abnormalities in tau protein. Hyperphosphorolation of tau protein in AD cause dissociation of microtubules and formation of helical filaments and eventually neurofibrillary tangles (Iqbal et al., 1986; Alonso et al., 1994). Dissociation of microtubules leads to impaired axonal transport and synaptic transmission, contributing to neurodegeneration in Alzheimer's disease (Grundke-Iqbal et al., 1986). While neurofibrillary tangles are hallmark of AD, they also occur in several other neurological disorder, including subacute sclerosing panencephalitis and progressive supranuclear palsy (De la Torre et al., 2011; Muresan et al., 2009).

1.4. Oxidative stress and Alzheimer's disease

Increase in oxidative stress can be seen in brain of Alzheimer's disease patients compared to normal brain (Pratico, 2008). Buildup of oxidative stress lead to activation of c-Jun N-terminal kinase and p38, eventually leading to caspase-3 activity causing programmed cell death to occur (Marques et al., 2003). Oxidative stress is linked with beta-amyloid induced neurotoxicity, tau pathology, mitochondria dysfunction, and metal dyshomeostasis.

Many studies have been done on beta-amyloid and its relation with oxidative stress (Mattson, 1997). In vitro experiment in mammalian cell line showed that beta-amyloid addition increases reactive oxygen species (Behl et al., 1994). Consistently, experiments in AD transgenic mouse models upregulating beta-amyloid processing show increase in oxidative damage to the brain (Matsuoka et al., 2001; Smith et al., 1998; Apelt et al., 2004). Moreover, numerous studies have also shown that oxidative stress promotes beta-amyloid production. In vivo studies in transgenic mice model overexpressing APP show increase in beta-amyloid production as result of elevated oxidative stress and glucocorticoid stress hormone (Nishida et al., 2006; Green et al., 2006). Beta amyloid generation leads to increase in oxidative stress, thus leading to further beta-amyloid production; this creates a cycle that promotes the initiation and progression of Alzheimer's disease.

Oxidative stress is also linked with mitochondria dysfunction, metal dyshomeostasis, and tau pathology in AD. Biopsies from AD brain shows damage and reduction of mitochondria along with oxidative damage marked by 8-hydroxyguanosine and nitrotyrosine (Hirai et al., 2001). Abnormal level of copper, zinc, and iron in the brain

have also been observed in AD patient (Deibel et al., 1996); moreover, these metals are found inside amyloid deposits (Lovell et al., 1998). Reports have shown that reduction of these transition metals by beta-amyloid generate hydrogen peroxide and increase oxidative stress (Opazo et al., 2002; Rottkamp et al., 2001). Several studies have also linked oxidative stress with hyperphosphorolation of tau protein. P38 mitogen-activated protein kinase has been shown to phosphorolate tau in-vitro (Goedert et al., 1997). In hippocampal and cortical region of AD brain, activated p38 is found coimmunoprecipitated with hyperphosphorolated tau, suggesting that oxidative stress might be involved in tau pathology (Zhu et al., 2000).

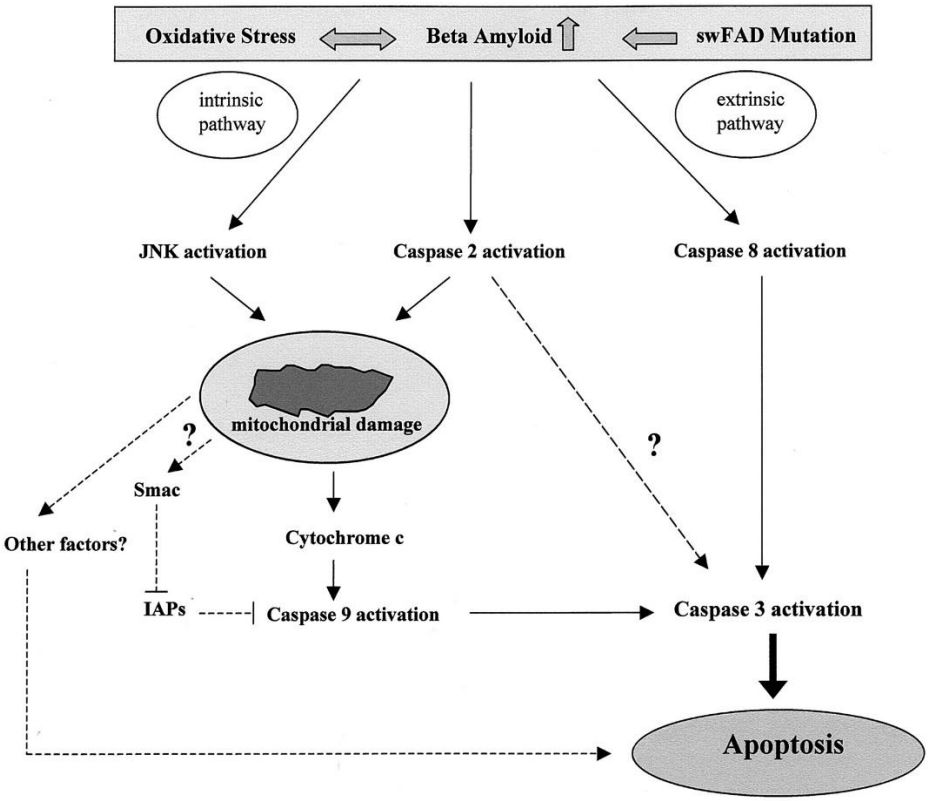

Figure 1.4. Sequence of event leading to apoptosis due to beta-amyloid generation (Marques et al., 2003).

1.5. Diagnosis and treatment

Currently, postmortem confirmational diagnosis of Alzheimer's disease is done through brain biopsy (Mckhann et al., 1984). Prior to death, presumed diagnosis can be done by measuring the amount of beta amyloid and tau protein in cerebrospinal fluid combined with PET scan, MRI, and other imaging methods (Prvulovic et al., 2011; Hampel et al., 2004; Risacher et al., 2009). However, AD pathogenesis occurs long before symptom starts to occur. Given the cost for treatment of AD and the predicted increase in AD cases within the next several years, more effective diagnosis methods and treatment must be developed.

Current AD treatment has been aimed to slow the disease progression. Primary drugs prescribed for AD are acetylcholinesterase inhibitors used to slow down the breakdown of acetylcholine receptors. However, these medications are ineffective in reversing or halting the disease progression, giving the need for a more effective treatment. Due to the current popularity of the amyloid hypothesis, many drugs are currently developed to reduce Aβ production and accumulation in the brain. Reducing Aβ production can be done by cleaving alpha-site of APP, blocking beta-site of APP, and blocking gamma-site of APP.

The use of single chain fragment variable (scFv) in therapeutics holds considerable promise. scFvs consist of the heavy and light chain of an antibody's antigen binding region linked together (figure 1.5). Just like antibodies, scFvs can also be engineered to be monoclonal and specific to the antigen they recognize. However, they are much smaller in size, allowing for better tissue penetration (Yokota et al., 1992); moreover, scFvs are less

immunogenic compared to antibodies because they do not have the fragment crystallizable region that cause inflammatory reactions (Miller et al., 2005).

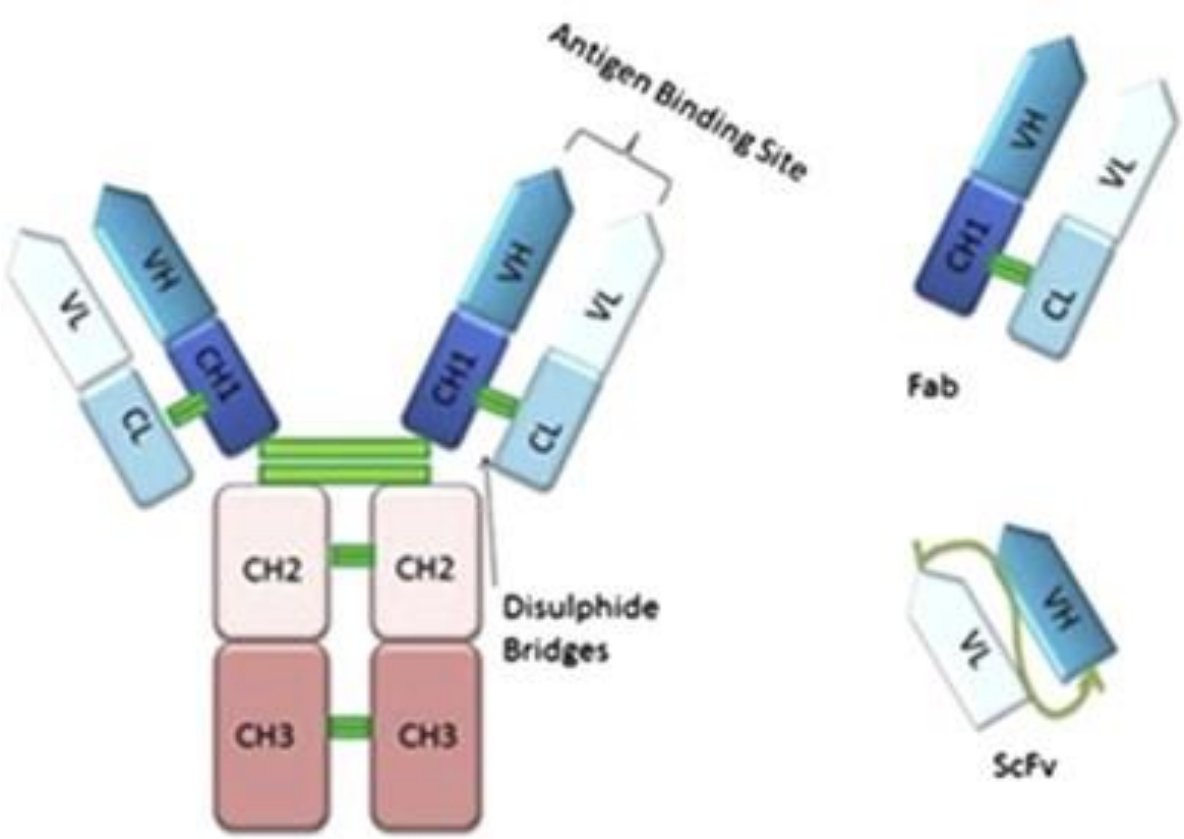

Figure 1.5. Illustration of immunoglobin and its antibody derivatives, fragment antigen binding (Fab) and single chain fragment variable (scFv) (Hairul Bahara et al., 2013).

1.6. Research objectives

Oxidative stress has been shown to accelerate AD progression both in-vitro and in-vivo. For this thesis, hydrogen peroxide is used to induce oxidative stress in AD cell model. iBSec1, a single chain fragment variable, and DIA10D, a bispecific tandem scFv, have been previously characterized and shown to reduce Aβ production (Boddapati et al., 2011;

Boddapati et al., 2012). The main objective of this thesis is to analyze the effect of inhibiting Aβ production using iBSec1 and DIA10D in stressed AD cell model.

This thesis consists of four chapters. The first chapter provides background information on Alzheimer's disease and the motivation behind this project. The second chapter deals with testing iBSec1 against stressed AD cell model. Chapter three presents the effect of DIA10D expression in stressed cell model of AD compared to non-AD cell model. Finally, chapter four provides summary and recommendation for future works.

Chapter 2

Effect of blocking beta-site of APP on stress induced toxicity in cell model of Alzheimer's disease

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2.1. Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in the United States (Gaugler et al., 2013). It is a neurodegenerative disease that deteriorates the brain, affecting memory, speech, and mobility. According to the 2013 AD Facts and Figures, there are about 5.2 million people diagnosed with AD in the United States at a cost of over $200 billion (Gaugler et al., 2013).

Two very important neuropathological characteristics of AD are the deposition of beta-amyloid (Aβ) plaques and neurofibrillary tangles of hyperphosphorolated tau protein. Aβ is formed by cleavage of Amyloid Precursor Protein (APP) by beta and gamma secretases. β-site APP cleaving enzyme-1 (BACE-1) has been reported to be the predominant beta secretase enzyme responsible during APP processing through amyloidogenic pathway (Vassar et al., 1999; Yan et al., 1999). After monomeric Aβ is formed through APP processing, it aggregates, forming oligomeric species (Glass et al., 2010). Further aggregation of these oligomeric species result in the formation of amyloid plaques, one of the hallmarks of AD (Hardy et al., 2002). Oligomeric forms of Aβ have been reported to be neurotoxic and are responsible for loss of neurons in AD patients (Gong et al., 2003; Glabe, 2008; Lesne et al., 2006). Depositions of Aβ can be reduced by promoting cleavage of APP at alpha site, reducing APP cleavage at beta site, and inhibiting Aβ aggregation.

Many studies have investigated the effect of stress on AD progression. Green et al (2006) showed that after injection of glucocorticoids, hormone upregulated by stress, Aβ and hyperphosphorolated tau inside AD mouse model increased. In other studies, stress induced by hydrogen peroxide has been shown to increase Aβ level in cell lines expressing

APP (Misonou et al., 2000; Shen et al., 2008). Increase in Aβ due to stress has been shown to correlate with decrease in cell viability as well as increase in both caspase-3 activity and reactive oxygen species (ROS) (Misonou et al, 2000; Casley et al., 2002; Reddy, 2006). Thus, ROS, caspase-3 activity, and cell viability are good measurements of stress-induced toxicity in cell models.

iBSec1 is a recombinant antibody fragment that selectively blocks BACE-1 processing of APP by binding the APP substrate as opposed to blocking the enzyme active site (Boddapati et al., 2011). Previous study on Chinese Hamster Ovaries (CHO) cell lines stably transfected with cDNA encoding mutant human APP751 (7PA2) showed that treatment of cells with 1.3 uM iBSec1 reduces both intracellular and extracellular Aβ by more than 40% (Boddapati et al., 2011). Here, we show that iBSec1 effectively protects cells from stress induced toxicity.

2.2. Materials and methods

2.2.1. Purification of iBSec1 scFv

IBSec1 was previously subcloned into PPNL9 expression vector and inserted into YVH10 strain of yeast. Expression and purification of iBSec1 were done as reported by Boddapati et al. (2011).

2.2.2. Cell culture

7PA2 cell line was a gift from Dr. Dennis Selkoe (Harvard Medical School, Boston, MA). Cells were grown in Dulbecco Modified Eagle Medium (Invitrogen, Grand Island,

CA) containing 10% fetal bovine serum (Invitrogen, Grand Island, NY), 2mM L-Glutamine (Invitrogen, Grand Island, NY), and 1% penicillin streptomycin (Invitrogen, Grand Island, NY). For selection purpose, 150 μg/mL of G-418 was added to media (Sigma-Aldrich, St. Louis, MO).

2.2.3. Reactive oxygen species (ROS) assay

2,7-dichlorodihydrofluoroscein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO) was used to measure the amount of reactive oxygen species inside cells. When DCFH-DA diffuses into cells, it is deacetylated to form 2,7-dichlorodihydrofluoroscein (DCFH). In presence of reactive oxygen species, DCFH is oxidized to form highly fluorescent 2,7-dichlorodihydrofluoroscein (DCF).

7PA2 cells were plated at density of 10^5 cells per mL in a 96-well plate. After 48 hours, the media was changed and treatments using hydrogen peroxide and antibodies were done. After 24 hour treatments, media was aspirated and new media containing 100 μM DCFH-DA was added. The media was aspirated after 45 minutes of exposure to DCFH-DA and amount of reactive oxygen species was measured using spectrophotometer by reading fluorescence at 480nm/535 nm.

2.2.4. Cell viability assay

Cell viability was measured using the XTT cell proliferation assay kit (ATCC, Manassas, VA). After entering the cell membrane, XTT is reduced into formazan by breaking apart the quaternary tetrazole ring. Reduction into formazan can be visibly seen

by a color change from slightly yellow to bright orange or quantified by reading the absorbance at 475nm.

7PA2 cells were plated at density of 10^5 cells per mL in a 96-well plate. After 48 hours, the media was changed and treatment using hydrogen peroxide and antibodies were done. After 24-hour treatments, the media was aspirated and new media containing XTT solution (2:1 ratio) was added. Absorbance was measured at 490nm (specific absorbance) and 650nm (non-specific absorbance/background) after 3 hours incubation at 37 ^{0}C.

2.3. Results and discussion

2.3.1. Purification of iBSec1 scFv

After expression, iBSec1 was purified using immobilized metal affinity chromatography as described in the materials and methods section. Western blot showed 35 kD band, size of iBSec1 scFv, inside 50mM, 100 mM and 500 mM elutions (figure 2.1). 100 mM and 500 mM imidazole elutions were combined and dialyzed using 10kD Macrosep column. Protein concentration was then quantified using bicinchoninic acid assay.

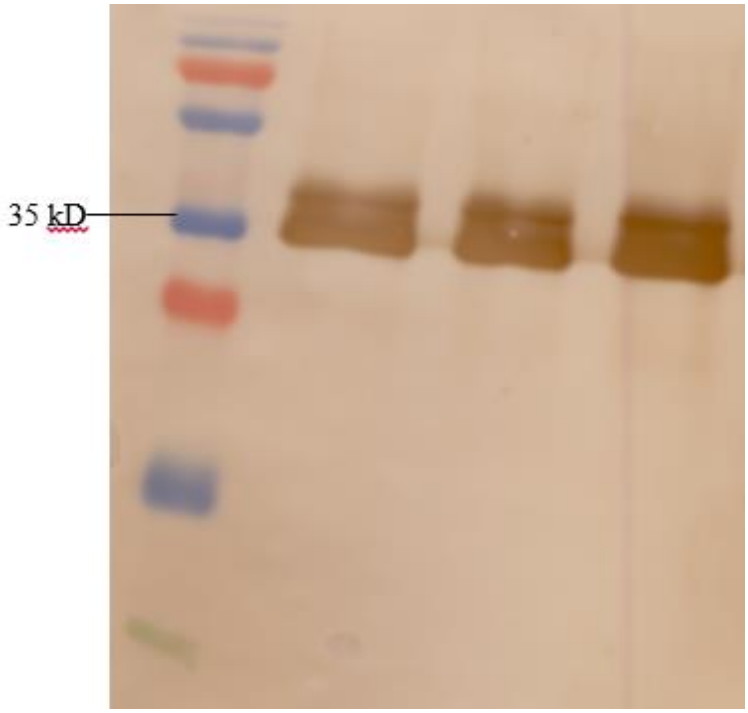

Figure 2.1. Western blot image of iBSec1 purification. Membrane shows in order from left to right: molecular weight marker, 50 mM imidazole elution, 100 mM imidazole elution, and 500 mM imidazole elution.

2.3.2. Reactive oxygen species assay

Hydrogen peroxide treatment has been reported to increase reactive oxygen species inside cell lines expressing APP. 7PA2 cells were stressed using hydrogen peroxide and iBSec1 was added to inhibit Aβ production. According to Boddapati et al., 1.3 μM of iBSec1 showed highest protection and Aβ inhibition in 7PA2 cells (2011). To show concentration dependent protection, 130 nM iBSec1 was also used for the experiment.

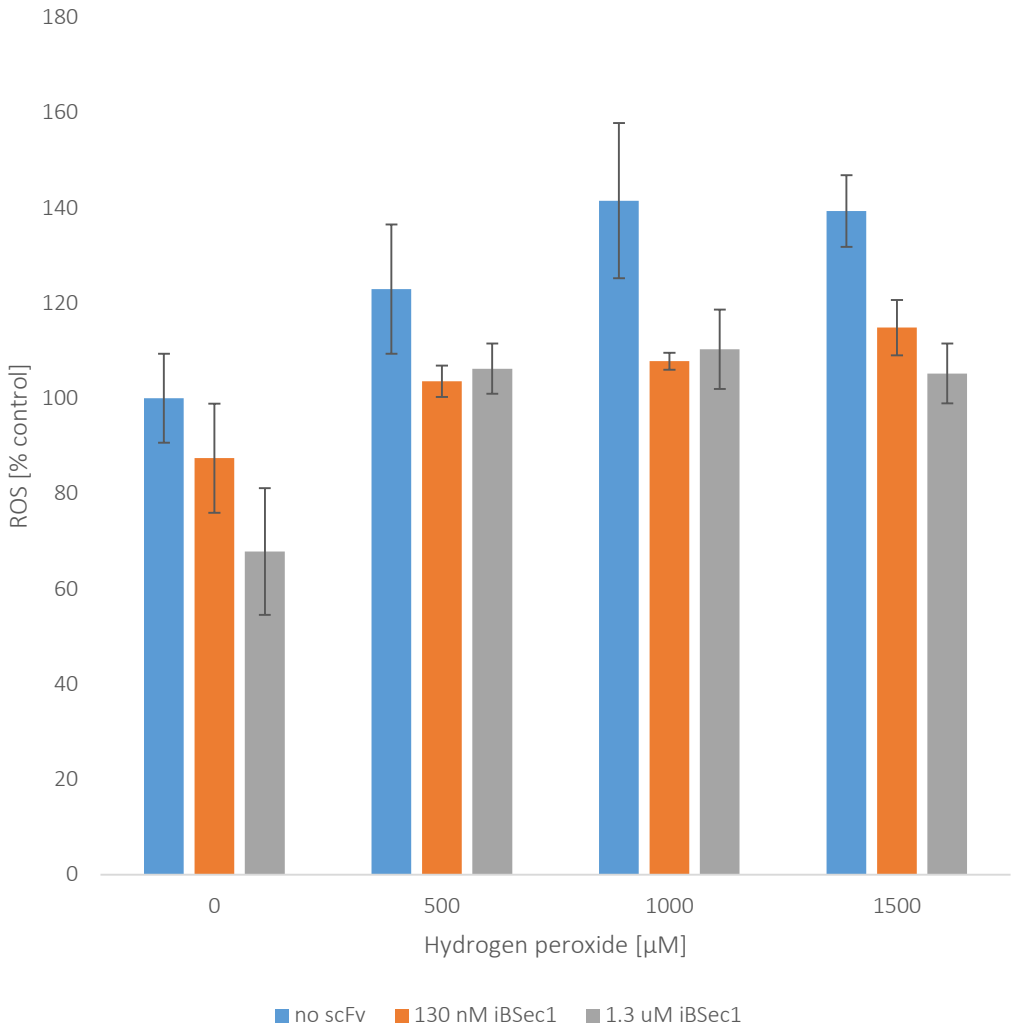

Figure 2.2. ROS levels for 7PA2 cells treated with different concentration of hydrogen peroxide and iBSec1. n=3. Error bars: +/-1 SE.

From figure 2.2, it can be seen that addition of hydrogen peroxide as oxidative stress to the cell increases ROS generation. Furthermore, co-incubation of cells with iBSec1 showed a concentration decrease in ROS. At concentration of 1,500 µM, decrease in ROS can be seen due to cell death (see figure 2.4 for cell viability result). Because DCFH-DA dye used for ROS measurement diffuses into cell and reacts with ROS generated intracellularly, ROS signals needed to be normalized to cell viability.

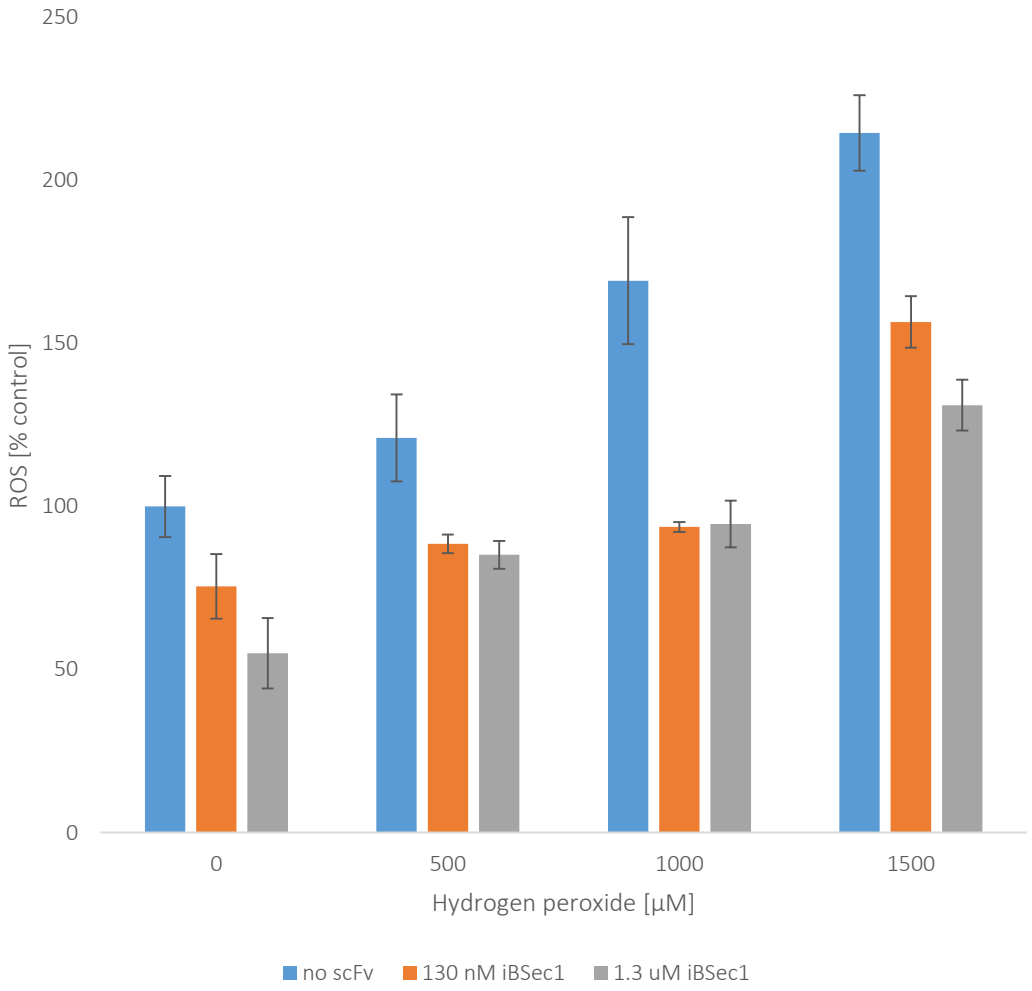

Figure 2.3. ROS levels for 7PA2 cells treated with different concentration of hydrogen peroxide and iBSec1 normalized to cell viability. n=3. Error bars: +/-1 SE.

Figure 2.3 showed ROS generation normalized to cell viability. P values were also computed using one-way ANOVA and recorded in table 2.1. Again, increase in ROS generation was seen with increasing concentration of hydrogen peroxide used. While addition of 500 µM hydrogen peroxide did not show a significant increase in ROS, addition of 1 and 1.5 mM showed statistically significant increase in ROS generation ($P<0.05$). Moreover, concentration dependent decrease in ROS generation was observed with addition of iBSec1. With the exception of 1.3µM treatment of iBSec1 on unstressed 7PA2

cells, addition of iBSec1 to unstressed and cells stressed with 500 μM hydrogen peroxide did not show statistically significant reduction in ROS signals ($P<0.05$). However, addition of iBSec1 to cells incubated with 1 mM and 1.5 mM hydrogen peroxide showed significant decrease in ROS generation. Addition of hydrogen peroxide in cell lines expressing APP has been reported to increase ROS generation and Aβ production (Misonou et al., 2000; Shen et al., 2008). Here, we showed that ROS generation can be downregulated by inhibiting Aβ production.

Group 1		Group 2		significance
H2O2 [μM]	scFv [nM]	H2O2 [μM]	scFv [nM]	(p<0.05)
		500	0	0.26691171
		1000	0	0.03283433
0	0	1500	0	0.00152889
		0	130	0.1462256
0	0	0	1300	0.03439308
		500	130	0.07610944
500	0	500	1300	0.06271206
		1000	130	0.01812285
1000	0	1000	1300	0.02290752
		1500	130	0.01441448
1500	0	1500	1300	0.00394039

Table 2.1. One way ANOVA analysis of ROS signal.

2.3.3. Cell viability assay

Accumulation of reactive oxygen species results in caspase-3 activation leading to programmed cell death, thus reduction in cell viability. Cell viability of 7PA2 cells treated with hydrogen peroxide and iBSec1 was measured using XTT kit.

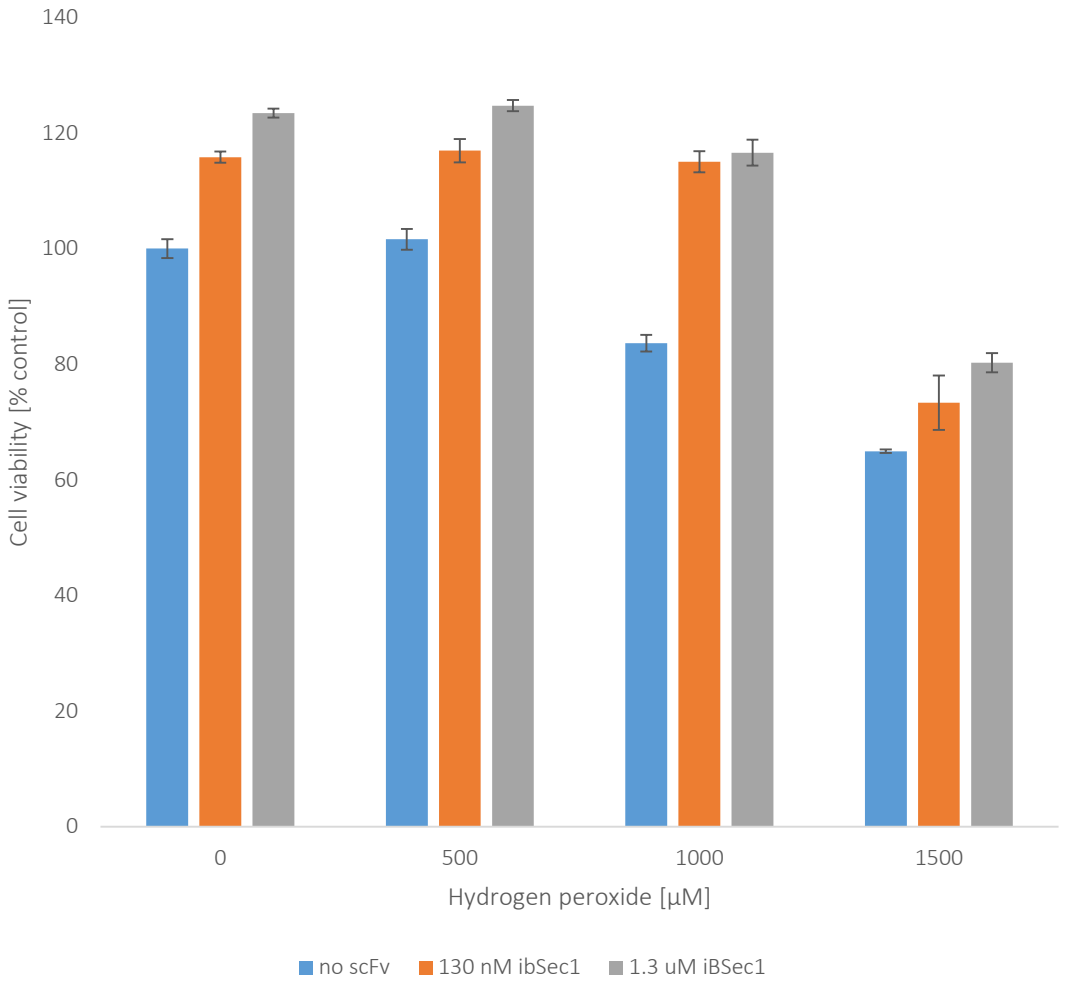

Figure 2.4. Cell viability for 7PA2 cells treated with different concentration of hydrogen peroxide and iBSec1. n=3. Error bars: +/-1 SE.

Figure 2.4 shows that cell viability of 7PA2 cells started to decrease with addition of 1 mM hydrogen peroxide. Addition of 1.5 mM hydrogen peroxide decreased cell viability further to 65%. One way ANOVA showed statistical significant decrease in cell viability with addition of 1 and 1.5 mM hydrogen peroxide. Addition of iBSec1 showed significant concentration dependent increase in cell viability ($P<0.05$) in stressed and unstressed cells with the exception of 130 nM addition of iBSec1 to cells incubated with 1.5 mM hydrogen

peroxide. Results showed that iBSec1 effectively reduce stress induced toxicity in 7PA2 cell line.

Group 1		Group 2		significance
H2O2 [uM]	scFv [nM]	H2O2 [uM]	scFv [nM]	(p<0.05)
		500	0	0.544276
		1000	0	0.001674
0	0	1500	0	0.000030
		0	130	0.001134
0	0	0	1300	0.000202
		500	130	0.004836
500	0	500	1300	0.000356
		1000	130	0.000169
1000	0	1000	1300	0.000252
		1500	130	0.148857
1500	0	1500	1300	0.000825

Table 2.2. One way ANOVA analysis of cell viability.

Chapter 3

Effect of promoting alpha-site cleavage and blocking beta-site of APP In stressed cell model of Alzheimer's disease

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3.1. Introduction

Alzheimer's disease is one of the most prominent neurodegenerative disease associated with aging. It is the sixth leading cause of death in United States, affecting one in nine people age 65 and older (Gaugler et al., 2013). First reported case of AD came from a Bavarian psychiatrist, Dr. Alois Alzheimer in 1906; after the death of one of his patient, postmortem brain biopsy showed microscopic lesions of amyloid plaques and neurofibrillary tangles (Selkoe, 2011). Since then, many advancement have been made on AD; however, no effective treatment has been found for the disease.

Two neuropahological hallmarks of the disease are intracellular neurofibrillary tangles and extracellular amyloid plaques (Selkoe, 2001). Intracellular neurofibrillary tangles are composed primarily from hyperphosphorolated tau protein (Iqbal et al., 1986). Tau is a microtubule-binding protein used to stabilize axonal microtubule assembly. In AD, hyperphosphorolation of tau destabilizes microtubules, causing impairments in axonal transport and neuronal dysfunction (Grundke-Iqbal et al., 1986). Extracellular amyloid plaques are composed of beta-amyloid protein (Aβ). Aβ is formed from cleavage of amyloid precursor protein (APP) at beta and gamma site (figure 3.1). Aβ then aggregates, forming oligomeric species; oligomeric Aβ has been widely reported to be the primary toxic species causing neuronal death in AD (Walsh et al., 2002; Sierks et al., 2011). Further aggregation of oligomeric Aβ form fibrillar Aβ and amyloid plaques (Glass et al., 2010).

Studies have been done on relation between Aβ and oxidative stress. Oxidative damage to the brain was reported in AD mouse models with upregulated beta-amyloid processing (Matsuoka et al., 2001; Smith et al., 1998; Apelt et al., 2004). In vitro, Aβ

addition to mammalian cell line B12 was also reported to increase reactive oxygen species (Behl et al., 1994). Many studies have also reported increase in Aβ as a result of oxidative stress. In vitro study in mammalian cell culture reported increase in Aβ production with addition of hydrogen peroxide as oxidative stress in mammalian cell lines expressing APP (Misonou et al., 2000). In vivo studies in transgenic mice model overexpressing APP show increase in beta-amyloid production as result of elevated oxidative stress and glucocorticoid stress hormone (Nishida et al., 2006; Green et al., 2006). Beta amyloid generation leads to increase in oxidative stress, thus leading to further beta-amyloid production; this creates a cycle that promotes the initiation and progression of Alzheimer's disease.

DIA10D is a bispecific tandem scFv engineered to cleave APP at alpha-site and block beta-site of APP. Previous study shows stable transfection of DIA10D on human neuroglioma cell lines overexpressing APP reduces intracellular Aβ by 15% and extracellular Aβ by more than 50% while at the same time increasing production of neuroprotective sAPPα by 20% (Boddapati et al., 2012). When stably expressed in Chinese Hamster Ovaries (CHO) cell lines transfected with cDNA encoding mutant human APP751 (7PA2), DIA10D effectively protects against stress induced toxicity.

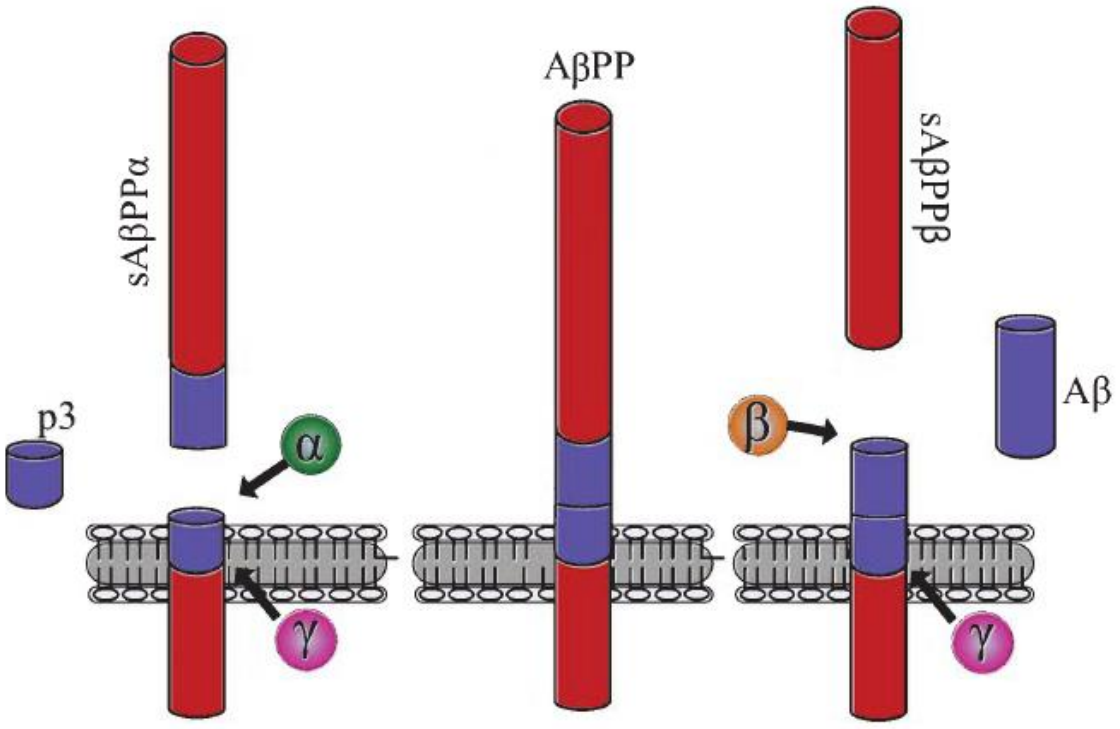

Figure 3.1. Schematic showing APP processing. In non-amyloidogenic pathway, APP is cleaved by alpha and gamma-secretase enzyme, producing membrane-bound protein p3. In amyloidogenic pathway, APP is cleaved by beta and gamma-secretase enzyme, producing toxic Aβ (Boddapati et al., 2012).

3.2. Materials and methods

3.2.1. Plasmid purification

DIA10D was previously constructed, inserted into PSECTAG vector, and cloned into DH5α cell by Boddapati et al. (2012). Purification of DNA was done using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. DNA concentration was obtained using NanoDrop 2000 (Thermo Scientific, Vernon Hills, IL).

3.2.2. Cell culture

Two cell lines were used, Chinese Hamster Ovaries (CHO) (ATCC, Manassas, VA) and CHO cell line stably transfected with cDNA encoding mutant human APP751 (7PA2). 7PA2 cell line were a gift from Dr. Dennis Selkoe (Harvard Medical School, Boston, MA). Cells were grown in Dulbecco Modified Eagle Medium (Invitrogen, Grand Island, CA) containing 10% fetal bovine serum (Invitrogen, Grand Island, NY), 2mM L-Glutamine (Invitrogen, Grand Island, NY), and 1% penicillin streptomycin (Invitrogen, Grand Island, NY). For selection purpose, 150 μg/mL of G-418 was added to 7PA2 cell media (Sigma-Aldrich, St. Louis, MO). Cells were incubated in a 5% CO_2 humidified incubator at 37^0C.

3.2.2. Transfection and generation of stable cell lines

Transfection was performed using Fugene HD Transfection Reagent (Roche, Pleasanton, CA). CHO and 7PA2 cells were plated at density of 10^5 cells per mL in a 6-well plate with 3 mL media. After reaching 50-70% confluency, transfection was performed. 3μg DNA and 6 μg reagent were incubated for 15 minutes in Dulbecco Modified Eagle Medium containing 2mM L-Glutamine and 1% penicillin streptomycin without serum. Media was aspirated from the wells, followed by addition of new media containing DNA and transfection reagent. Cells were then incubated at 37^0C for 6 hours, and media was replaced by complete media containing 10% serum. After 48 hours in complete media, zeocin was added at concentration of 400 μg/ml.

3.2.3. Fluorescence staining of cell

Cells were plated in at density of 10^5 cells per mL in a 6-well plate with 3 mL media. After 48 hours, media was aspirated and cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature. Blocking was done using 3% FBS + 0.1% tween-20 in PBS for 30 minutes followed by labeling using 1:200 dilution of monoclonal anti-c-Myc antibody (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour and 1:300 dilution of Alexa Fluor 488 goat anti-mouse FITC antibody (Life Technologies, Carlsbad, CA) in PBS for 30 minutes in room temperature. Cells were visualized using a Zeiss AxioObserverD1 fluorescence microscope.

3.2.4. Western blot assay

SDS-PAGE sample buffer was added to cell supernatant at 1:1 ratio and boiled for 5 minutes. Samples were then separated on a 15% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membrane was then blocked using 5% milk in PBS for 2 hours in room temperature, followed by overnight incubation in 1:1000 dilution of monoclonal anti-c-Myc antibody in PBS overnight at 4^0C and 1:1000 dilution of goat anti-mouse HRP antibody (Sigma-Aldrich, St. Louis, MO). DAB chromogenic substrate was used to develop the blot as per manufacturer's protocol.

3.2.5. Reactive oxygen species (ROS) assay

2,7-dichlorodihydrofluoroscein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO) was used to measure the amount of reactive oxygen species inside cells. Briefly, 100 µl of cells were plated at density of 10^5 cells per mL in a 96-well plate. After 48 hours, the

media was changed and treatments using hydrogen peroxide and antibodies were done. After 24 hour treatments, media was aspirated and new media containing 100 μM DCFH-DA was added. The media was aspirated after 45 minutes of exposure to DCFH-DA and amount of reactive oxygen species was measured using spectrophotometer by reading fluorescence at 480nm/535 nm.

3.2.6. Cell viability assay

Cell viability was measured using the XTT cell proliferation assay kit (ATCC, Manassas, VA). Briefly, 100 μl of cells were plated at density of 10^5 cells per mL in a 96-well plate. After 48 hours, the media was changed and treatment using hydrogen peroxide and antibodies were done. After 24-hour treatments, the media was aspirated and new media containing XTT solution (2:1 ratio) was added. Absorbance was measured at 490nm (specific absorbance) and 650nm (non-specific absorbance/background) after 3 hours incubation at 37 ^{0}C.

3.3. Results and discussion

3.3.1. Fluorescence staining of cell

Fluorescence staining was done following transfection and addition of zeocin as resistance. It can be seen from figure 3.2 that CHO and 7PA2 showed no green fluorescence. Transfected CHO and 7PA2 with DIA10D showed green fluorescence, showing expression of protein with c-myc tag inside cell.

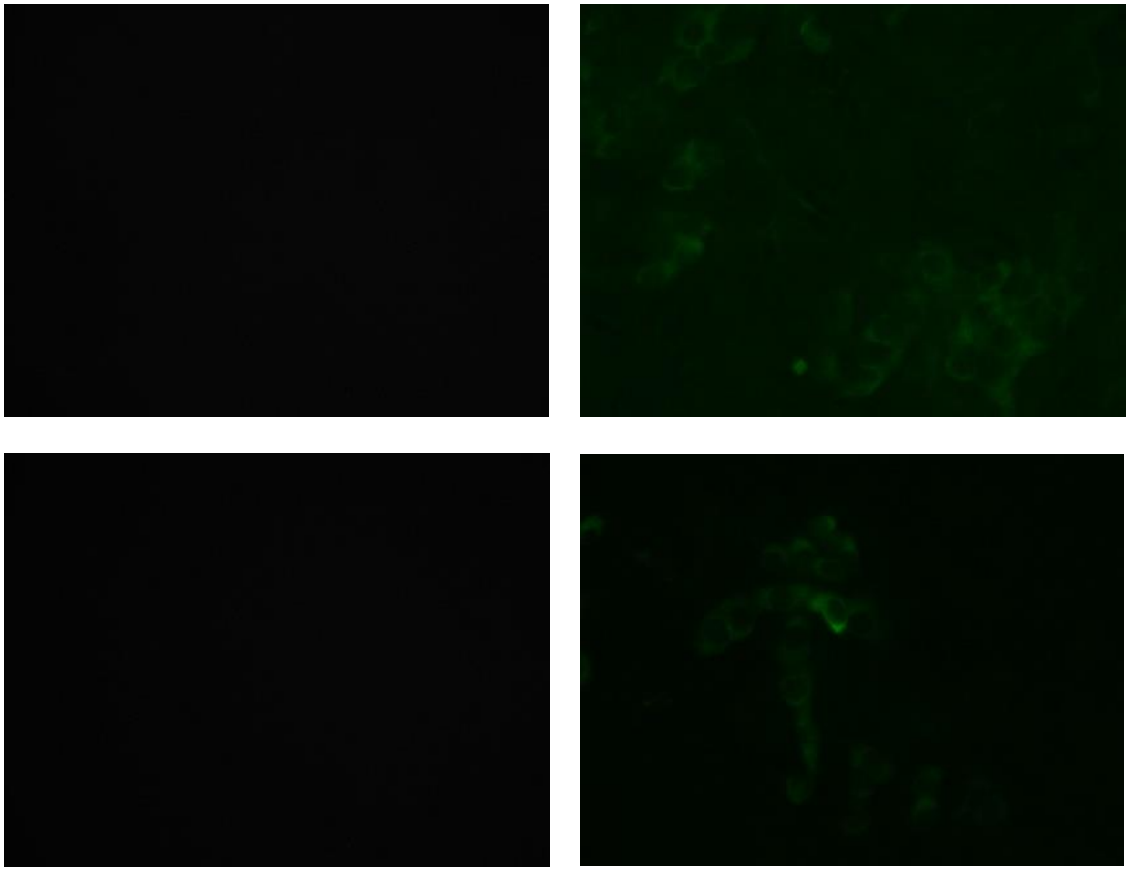

Figure 3.2. Fluorescence cell staining of CHO (top left), CHO+DIA10D (top right), 7PA2 (bottom left), and 7PA2+DIA10D (bottom right).

3.3.2. Western blot assay

Supernatant of cells were collected 48 hours after plating and ran on an SDS-PAGE gel. Samples were then transferred onto a nitrocellulose membrane and developed. Band corresponding 56 kDa protein can be seen in both transfected CHO and 7PA2 cells, showing full DIA10D expression.

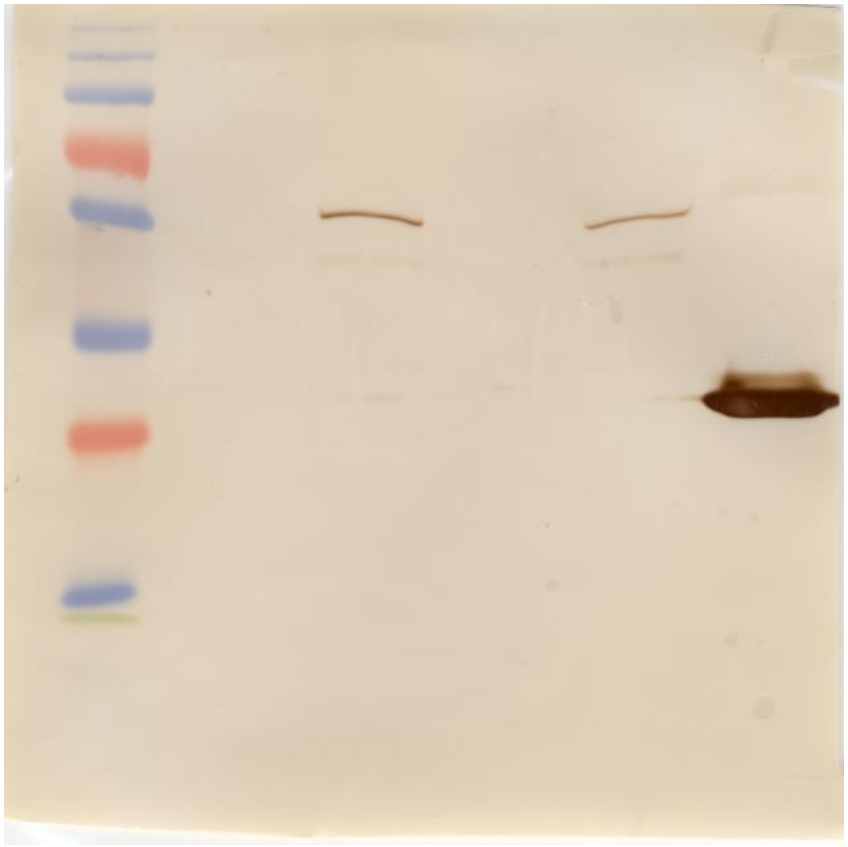

Figure 3.3. Western blot of supernatant after 48 hours. From left to right: marker, CHO, CHO+DIA10D, 7PA2, 7PA2+DIA10D, αPLB (+).

3.3.3. Reactive oxygen species assay

Transfected and non-transfected CHO and 7PA2 cells were plated and hydrogen peroxide was added. Intracellular reactive oxygen species was measured using DCFH-DA fluorescence dye after 24 hours treatment. ROS signals were normalized to cell viability.

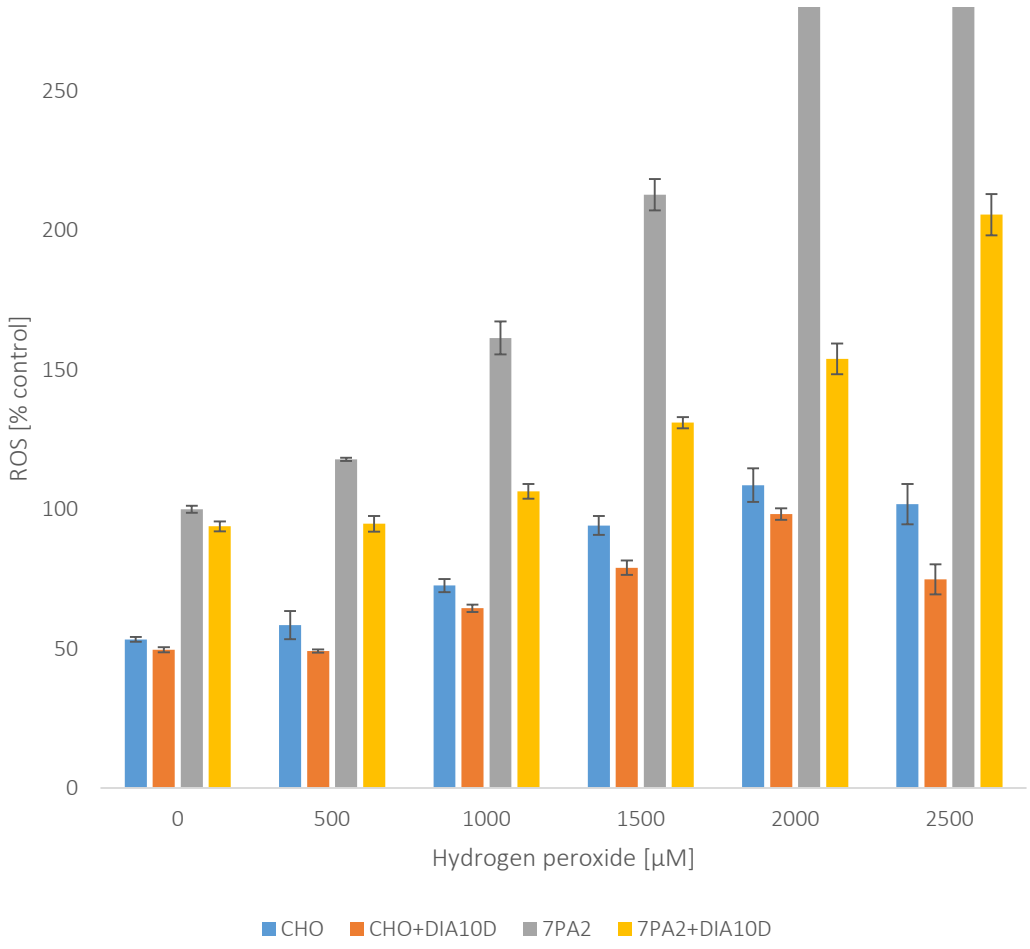

Figure 3.4. ROS for cells treated with different concentration of hydrogen peroxide normalized to cell viability. n=3. Error bars: +/-1 SE.

Significant difference can be seen from intracellular ROS between CHO and 7PA2 cells ($P<0.005$). Higher ROS in 7PA2 cells are believed due to overexpression of APP leading to higher Aβ production. Increase in Aβ has been reported to increase oxidative

damage both in vivo and in vitro (Matsuoka et al., 2001; Smith et al., 1998; Apelt et al., 2004; Behl et al., 1994). Moreover, increase in ROS can be seen with increasing treatment of hydrogen peroxide both in CHO and 7PA2 cells with 7PA2 showing a much higher increase in ROS generation. Higher ROS generation in 7PA2 cells is believed to be due to upregulated Aβ production; Aβ has been reported to increase oxidative stress while oxidative stress has been reported to upregulate Aβ production, creating a cycle that promotes Aβ production and neurotoxicity (Practico, 2008).

Significant decrease in ROS can be observed in 7PA2 cells stably expressing DIA10D compared to normal 7PA2 cells ($P<0.05$). We believe that decrease in ROS is due to both reduction in Aβ production and increase in sAPPα, which has been shown to be neuroprotective (Yang et al., 2007). Smaller effect of DIA10D on ROS generation was observed in CHO cells, with statistical significance ($P<0.05$) observed on untreated cells and stressed cells with 1, 2, and 2.5 mM hydrogen peroxide added. This is due to minimal amount of APP expression and processing by CHO cells, giving statistically insignificant effect of DIA10D.

Group 1		Group 2		significance
H2O2 [uM]	cell line	H2O2 [uM]	cell line	(p<0.05)
		500		0.37362169
		1000		0.001499527
		1500		0.000310913
		2000		0.000825381
0	CHO	2500	CHO	0.002643096
		500		0.000195012
		1000		0.00053847
		1500		4.07088E-05
		2000		0.000174443
0	7PA2	2500	7PA2	0.000121992
	CHO		CHO+DIA10D	0.042067024
	CHO		7PA2	6.55369E-06
0	7PA2	0	7PA2+DIA10D	0.047574079
	CHO		CHO+DIA10D	0.143811658
	CHO		7PA2	0.000311991
500	7PA2	500	7PA2+DIA10D	0.001364097
	CHO		CHO+DIA10D	0.038890532
	CHO		7PA2	0.000155769
1000	7PA2	1000	7PA2+DIA10D	0.001062011
	CHO		CHO+DIA10D	0.024165425
	CHO		7PA2	5.61215E-05
1500	7PA2	1500	7PA2+DIA10D	0.000166618
	CHO		CHO+DIA10D	0.17926686
	CHO		7PA2	0.000186341
2000	7PA2	2000	7PA2+DIA10D	0.000236544
	CHO		CHO+DIA10D	0.040172425
	CHO		7PA2	0.000123099
2500	7PA2	2500	7PA2+DIA10D	0.000151848

Table 3.1. One way ANOVA analysis of ROS for CHO and 7PA2 cells.

3.3.4. Cell viability assay

Transfected and non-transfected CHO and 7PA2 cells were plated and hydrogen peroxide was added. Cell viability was measured using XTT kit following 24 hours treatment.

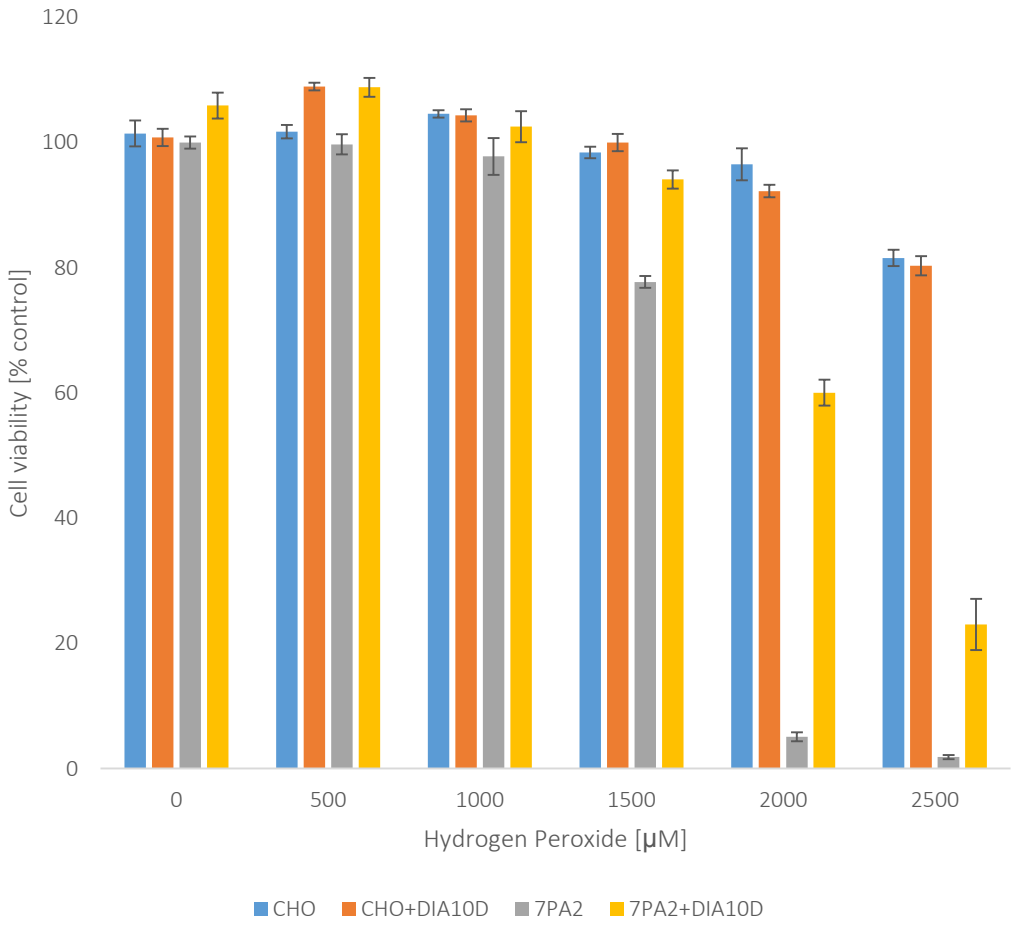

Figure 3.5. Viability for cells treated with different concentration of hydrogen peroxide. n=3. Error bars: +/-1 SE.

Cell viability of 7PA2 cells started to decrease with addition of 1.5 mM hydrogen peroxide while cell viability of CHO did not decrease until addition of 2.5 mM hydrogen peroxide. Moreover, no significant change of cell viability was observed with transfection of DIA10D into CHO cells with the exception of with treatment of 500 mM hydrogen peroxide. On the other hand, significant change in cell viability was observed in transfected 7PA2 cells compared to non-transfected 7PA2 cells with 1.5, 2, and 2.5 mM treatment of hydrogen peroxide. This result is believed to be due to overexpression of APP in 7PA2 cell

line. DIA10D was reported to effectively reduce toxicity, reduce intra and extracellular Aβ production, and increase sAPPα production in cell line overexpressing APP (Boddapati et al., 2012). Data shows that DIA10D effectively protect 7PA2 cells from apoptosis due to oxidative stress while it does not protect CHO cells from oxidative stress.

Group 1		Group 2		significance
H2O2 [uM]	cell line	H2O2 [uM]	cell line	(p<0.05)
		500		0.90730725
		1000		0.21988991
		1500		0.25506834
		2000		0.20776741
0	CHO	2500	CHO	0.00123499
	7PA2	500		0.88145587
		1000		0.51635167
		1500		7.9403E-05
		2000		1.5923E-07
0		2500	7PA2	7.5294E-08
	CHO		CHO+DIA10D	0.81296819
	CHO		7PA2	0.56394767
0	7PA2	0	7PA2+DIA10D	0.06160497
	CHO		CHO+DIA10D	0.00440084
	CHO		7PA2	0.35522036
500	7PA2	500	7PA2+DIA10D	0.01457165
	CHO		CHO+DIA10D	0.85498482
	CHO		7PA2	0.08691236
1000	7PA2	1000	7PA2+DIA10D	0.28526818
	CHO		CHO+DIA10D	0.39354347
	CHO		7PA2	9.5262E-05
1500	7PA2	1500	7PA2+DIA10D	0.00067009
	CHO		CHO+DIA10D	0.1940388
	CHO		7PA2	4.1813E-06
2000	7PA2	2000	7PA2+DIA10D	1.4641E-05
	CHO		CHO+DIA10D	0.57201129
	CHO		7PA2	4.6131E-07
2500	7PA2	2500	7PA2+DIA10D	0.00673071

Table 3.2. One way ANOVA analysis for cell viability of CHO and 7PA2 cells.

CHAPTER 4

CONCLUSION AND FUTURE WORK

4.1. Conclusion

About 5.2 million people in the United States have Alzheimer's disease, costing the nation $203 billion in treatment and care; number of people with the disease is estimated to double by 2020 and triple by 2050 (Hebert et al., 2013). Aβ upregulation leads to higher ROS generation, thus upregulating Aβ production further; this creates a cycle promoting initiation and progression of AD (Practico, 2008). Therefore, one promising method to reduce cell damage following stress is to reduce Aβ production. The goal of this thesis was to test stress induced toxicity following treatment with iBSec1 and DIA10D to reduce Aβ on AD cell lines.

Results showed a concentration dependent increase in ROS and decrease in cell viability following addition of hydrogen peroxide as oxidative stress. Addition of iBSec1 and DIA10D to cells showed statistically significant reduction of ROS in both unstressed and stressed 7PA2. Cell viability assay verified these results, showing that higher concentration of hydrogen peroxide is needed to reduce cell viability in 7PA2 cells treated with iBSec1 and DIA10D. However, expression of DIA10D in regular CHO cells showed inconclusive result for ROS and no difference in cell viability compared to non-transfected CHO cells. These results show that both iBSec1 and DIA10D effectively blocked stress-induced toxicity in AD cell models and are promising therapeutics for treatment of Alzheimer's disease.

4.2. Future work

Based on results, future work includes:

1. Quantification of Aβ in supernatant and cell lysate samples: Intracellular and extracellular Aβ can be measured using sandwich ELISA or western blot. Total Aβ can be probed using 6E10 monoclonal antibody while oligomeric Aβ can be probed using A4 and E1 scFv (Kasturirangan et al., 2012).
2. Transfection and toxicity study of SH-SY5Y cells with DIA10D: SH-SY5Y is a brain derived neuroblastoma cell line that can be differentiated into cholinergic cell. Addition of hydrogen peroxide to undifferentiated SH-SY5Y cell has been reported to increase Aβ production. In-vitro neuroprotection of DIA10D can be tested by toxicity test of both undifferentiated and cholinergic SH-SY5Y.
3. In-vivo study of iBSec1 and DIA10D: Effect of iBSec1 and DIA10D can also be tested in AD mouse model; this includes their ability to cross the blood brain barrier, delivery into brain, and neuroprotection in AD mouse model.

REFERENCES

Alonso A. D., Zaidi T., & Grundke-Iqbal I. (1994) Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. Proc Natl Acad Sci U S A 91, 5562-5566.

Apelt, J., Bigl, M., Wunderlich, P., & Schliebs, R. (2004). Aging-related increase in oxidative stress correlates with developmental pattern of beta-secretase activity and beta-amyloid plaque formation in transgenic Tg2576 mice with Alzheimer-like pathology. International Journal of DevelopmentalNeuroscience, vol. 22,no. 7, pp. 475–484.

Behl, C., Davis, J. B., Lesley, R., & Schubert, D. (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. Cell, vol. 77, no. 6, pp. 817–827.

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. Acta neuropathologica, 82(4), 239–259.

Brody, D. L. & Holtzman, D. M. (2008). Active and passive immunotherapy for neurodegenerative disorders. Annu Rev Neurosci 31, 175-93.

Boddapati, S., Levites, Y. & Sierks, M. R. (2011). Inhibiting beta-secretase activity in Alzheimer's disease cell models with single-chain antibodies specifically targeting APP. J Mol Biol 405, 436-47.

Boddapati S., Levites Y., Suryadi V., Kasturirangan S., & Sierks M. R. (2012). Bispecific Tandem Single Chain Antibody Simultaneously Inhibits beta-Secretase and Promotes alpha-Secretase Processing of AbetaPP. J Alzheimers Dis 28: 961–969.

Bond, M., Rogers, G., Peters, J., Anderson, R., Hoyle, M., Miners, A., Moxham, T., et al. (2012). Memantine for the treatment of Alzheimer’s disease (review of Technology Appraisal No.111): a systematic review and economic model, 16(21).

Casley, C. S., Canevari, L., Land, J. M., Clark, J. B. & Sharpe, M. A. (2002). Beta-amyloid inhibits integrated mitochondrial respiration and key enzyme activities. J Neurochem 80, 91-100.

Davies, A. P., Moloney, A. J. F. (1976) Selective loss of cholinergic neurons in Alzheimer’s disease. Lancet ii, 1403.

De la Torre, J. C. (2011). "Thre postulates to help identify the cause of Alzheimer's disease." Journal of Alzheimers Disease 24: 657-668.

Deibel, M. A., Ehmann, W. D., & Markesbery, W. R. (1996). Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer’s disease: possible

relation to oxidative stress. Journal of the Neurological Sciences, vol. 143, no. 1-2, pp. 137–142.

Feldberg, W. (1945) Synthesis of acetylcholine by tissue of the central nervous system. J Physiol 103, 367-402.

Gaugler, J., James, B., Johnson, T., Scholz, K., & Weuve, J. (2013). 2013 Alzheimer's disease facts and figures. Alzheimers & Dementia volume 9 issue 2.

Glabe, C. G. (2008). Structural classification of toxic amyloid oligomers. J Biol Chem 283, 29639-43.

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C. & Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell, 140, 918–934.

Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A., & Cohen, P. (1997). Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases," FEBS Letters, vol. 409, no. 1, pp. 57–62.

Gong, Y., Chang, L., Viola, K. L., Lacor, P. N., Lambert, M. P., Finch, C. E., Krafft, G. A. & Klein, W. L. (2003). Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc Natl Acad Sci U S A 100, 10417-22.

Green, K. N., Billings, L. M., Roozendaal, B., McGaugh, J. L., LaFerla, F. M. (2006) Glucocorticoids increase amyloid-β and tau pathology in a mouse model of Alzheimer's disease. J Neurosci 26:9047–9056.

Grundke-Iqbal, I., Iqbal, K., & Quinlan, M. (1986) Microtubuleassociated protein tau: a component of Alzheimer paired helical filaments. J Biol Chem 261, 6084-6089.

Hairul Bahara, N. H., Tye, G. J., Choong, Y. S., Ong, E. B., Ismail, A., & Lim, T. S. (2013). Phage display antibodies for diagnostic applications. Biologicals Jul; 41(4): 209–216.

Hampel, H., Mitchell, A., Blennow, K., et al. (2004). Core biological marker candidates of Alzheimer's disease – perspectives for diagnosis, prediction of outcome and reflection of biological activity. J Neural Transm; 111:247-72.

Hardy, J. (1992). An anatomical cascade hypothesis for Alzheimer's disease. Trends Neurosci 15, 200-201.

Hardy, J. & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, 297, 353–356.

Hebert L. E., Weuve J., Scherr P. A., & Evans D. A. Alzheimer's disease in the United States (2010-2050) estimated using the 2010 Census. Neurology.

www.neurology.org/content/early/2013/02/06/WNL.0b013e31828726f5. Published online before print, Feb. 6, 2013.

Hirai, K., Aliev, G., Nunomura A. et al. (2001). Mitochondrial abnormalities in Alzheimer's disease. Journal of Neuroscience, vol. 21, no. 9, pp. 3017–3023.

Hurd, M. D., Martorell, P., Delavande, A., Mullen K. J., & Langa K. M. (2013). Monetary costs of dementia in the United States. N Engl J Med 2013;368:1326–34.

Iqbal, K., Grundke-Iqbal, I., & Zaidi, T. (1986) Defective brain microtubule assembly in Alzheimers disease. Lancet, 2, 421-426.

Iy, J. W., Wu, Q., Smith, A., Grundke-iqbal, I., & Y, K. I. (1998). Tau is phosphorylated by GSK-3 at several sites found in Alzheimer disease and its biological activity markedly inhibited only after it is prephosphorylated by A-kinase. FEBS Letters, 436, 28–34.

Kasturirangan, S., Brune, D. & Sierks, M. (2009). Promoting alpha-secretase cleavage of beta-amyloid with engineered proteolytic antibody fragments. Biotechnol Prog 25, 1054-63.

Kasturirangan, S., Li, L., Emadi, S., et al. Nanobody specific for oligomeric beta-amyloid stabilizes nontoxic form. Neurobiol Aging. 2012; 33(7): 1320-8.

Klafki, H. W., Staufenbiel, M., & Kornhuber, J. (2006). Therapeutic approaches to Alzheimer's disease. Brain; 129(Pt 11): 2840-55.

LaFerla, F. M. and Oddo, S. (2005). Alzheimer's disease: Aß, tau, and synaptic dysfunction. Trends in Molecular Medicine 11, 170-176.

Lesne, S., Koh, M. T., Kotilinek, L., Kayed, R., Glabe, C. G., Yang, A., Gallagher, M. & Ashe, K. H. (2006). A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440, 352-7.

Lovell, M. A., Robertson, J. D., Teesdale, W. J., Campbell, J. L., & Markesbery, W. R. (1998). Copper, iron and zinc in Alzheimer's disease senile plaques. Journal of the Neurological Sciences, vol. 158, no. 1, pp. 47–52.

Marques, C. A., Keil, U., Bonert, A., Steiner, B., Haass, C., Muller, W. E. & Eckert, A. (2003). Neurotoxic mechanisms caused by the Alzheimer's disease linked Swedish amyloid precursor protein mutation: oxidative stress, caspases, and the JNK pathway. J Biol Chem 278, 28294-302.

Matsuoka, Y.,Picciano, M., La Francois, J., & Duff, K. (2001). Fibrillar beta-amyloid evokes oxidative damage in a transgenic mouse model of Alzheimer's disease. Neuroscience, vol. 104, no. 3, pp. 609–613.

Mattson, M. P. (1997). Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. Physiological Reviews, vol. 77, no. 4, pp. 1081–1132.

McKhann, G., Drachman, D., Folstein, M., et al. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology*; 34(7): 939-44.

Mendez M.F. (2006). The accurate diagnosis of early-onset dementia. Int J Psychiatry Med.; 36(4): 401-12.

Miller, T. W. & Messer, A. (2005). Intrabody applications in neurological disorders: progress and future prospects. *Mol Ther* **12**, 394-401.

Misonou, H., Morishima-Kawashima, & Ihara, Y. Oxidative Stress Induces Intracellular Accumulation of Amyloid β-Protein (Aβ) in Human Neuroblastoma Cells. Biochemistry 2000 39 (23), 6951-6959

Muller, W. E., Eckert, A., Kurz, C., Eckert, G. P. & Leuner, K. (2010). Mitochondrial dysfunction: common final pathway in brain aging and Alzheimer's disease therapeutic aspects. Mol Neurobiol 41, 159-71.

Muresan V. & Muresan, Z. (2009) Is abnormal axonal transport a cause, a contributing factor or a consequence of the neuronal pathology in Alzheimer's disease? Future Neurol 4, 761-773.

Nishida, Y., Yokota, T., Takahashi, T., Uchihara, T., Jishage, K., & Mizusawa, H. (2006). Deletion of vitamin E enhances phenotype of Alzheimer disease model mouse. Biochemical and Biophysical Research Communications, vol. 350, no. 3, pp. 530–536.

Opazo, C., Huang, X. Cherny, R. A. et al. (2002). Metalloenzyme-like activity of Alzheimer's disease beta-amyloid: Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H2O2. Journal of Biological Chemistry, vol. 277, no. 43, pp. 40302–40308.

Panza, F., Solfrizzi, V., Imbimbo, B. P., Tortelli, R., Santamato, A., & Logroscino, G. (2014). Amyloid-based immunotherapy for Alzheimer's disease in the time of prevention trials: the way forward. Expert Rev Clin Immunol. Mar;10(3):405-19.

Perry, E. K., Gibson, P. H, Blessed, G., Perry, R. H., & Tomlinson, B. E. (1977). Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. J Neurol Sci 34, 247-265.

Pratico, D. (2008). Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. Trends in Pharmacological Sciences, vol. 29,no. 12, pp. 609–615.

Prvulovic, D., Hampel, H. (2011). Amyloid beta and phosphor-tau as biomarkers in Alzheimer's disease. Clin Chem Lab Med; 49:367-74.

Reddy, P. H. (2006). Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. J Neurochem 96, 1-13.

Reitz, C. & Mayeux, R. (2014). Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochemical Pharmacology, 88(4), 640-651.

Risacher, S. L., Saykin, A. J., West, J.D., et al. (2009). Baseline MRI predictors of conversion from MCI to probable AD in the ADNI cohort. Curr Alzheimers Res; 6:347–61.

Rottkamp, C. A., Raina, A. K., Zhu X. et al. (2001). Redox-active iron mediates amyloid-beta toxicity. Free Radical Biology and Medicine, vol. 30, no. 4, pp. 447–450.

Selkoe, D. J. (2001). Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81, 741-66.

Selkoe, D. J. (2001). Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. J Alzheimers Dis 3, 75-80.

Selkoe, D. J. (2011). Alzheimer's disease. Cold Spring Harb. Perspect. Biol. 3, a004457.

Shen, C., Chen, Y., Liu, H., Zhang, K., Zhang, T., Lin, A., & Jing, N. (2008). Hydrogen peroxide promotes Aβ production through JNK-dependent activation of gamma-secretase. J Biol Chem; 283: 17721-30.

Sierks, M. R., Chatterjee G., & McGraw C. (2011). CSF levels of oligomeric alpha-synuclein and beta-amyloid as biomarkers for neurodegenerative disease. Integr Biol. 3(12): 1188-96.

Smith, M. A., Hirai, K., Hsiao, K, et al. (1998). Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress. Journal of Neurochemistry, vol. 70, no. 5, pp. 2212–2215.

Tanzi, RE & Bertram, L. (2005). Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell; 120(4): 545-55.

Tiraboschi, P., Hansen, L. A., & Thal, L. J. (2004). The importance of neuritic plaques and tangles to the development and evolution of AD. Neurology. 62(11): 1984-9.

Tomlinson, B. E., Blessed, G., & Roth, M. (1997). Observations on the Brains of Demented Old People. Observations on the Brains of Non-Demented Old People. Journal of, 12, 785–790.

Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P. et al. (1999). β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science, 286, 735–741.

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., & Selkoe, D. J. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416, 535-539.

Weiner, H. L. & Frenkel, D. (2006). Immunology and immunotherapy of Alzheimer's disease. Nat Rev Immunol 6, 404-16.

West, M., Coleman, P., Flood, D., & Troncoso, J. (1994). Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. The Lancet, 344(8925), 769–772.

Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M. et al. (1999). Membraneanchored aspartyl protease with Alzheimer's disease β-secretase activity. Nature, 402, 533–737.

Yang, H. Q., Pan, J., Ba, M. W., Sun, Z. K., Ma, G. Z., Lu, G. Q., Xiao, Q. & Chen, S. D. (2007). New protein kinase C activator regulates amyloid precursor protein processing in vitro by increasing alpha-secretase activity. *Eur J Neurosci* **26**, 381-91.

Yokota, T., Milenic, D. E., Whitlow, M., & Schlom, J. (1992). Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Research*, 3402–3408.

Zhu, X., Rottkamp, C. A., Boux, H., Takeda, A., Perry, G., & Smith, M. A. (2000). Activation of p38 kinase links tau phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer's disease. Journal of Neuropathology and Experimental Neurology, vol. 59, no. 10, pp. 880-888