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Recent evidence indicates that oxidative stress contributes to neuronal death in Alzheimer's disease (AD). In addition, it has been suggested that AD is a systemic illness in which the development of the disease is only visible in the brain. The aim of this research is to develop experimental procedures using a simple cell model, the fibroblast, to determine if proteins derived from AD skin fibroblasts are more sensitive to oxidation by reactive oxygen species than non-AD cells, and to assess the ability of antioxidants to prevent this oxidative damage in AD fibroblasts. Preliminary findings suggest that changes in sensitivity are already detectable in fibroblasts from AD patients, probably as a consequence of a genetic component as well as other risk factors. Therefore, this biochemical marker might have the potential for identifying individuals at risk for AD.

ALZHEIMER'S FIBROBLASTS ARE MORE

SUSCEPTIBLE TO OXIDATIVE STRESS.

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ALZHEIMER'S FIBROBLASTS ARE MORE SUSCEPTIBLE TO OXIDATIVE STRESS.

THESIS

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ABBREVIATIONS

Alpha-2-Macroglobulin (**A2M**)
Alzheimer's Disease (**AD**)
Amyloid Beta Peptide (**Aβ**)
Amyloid Precursor Protein (**APP**)
Analysis of Variance (**ANOVA**)
Apolipoprotein E (**ApoE**)
Bicinchoninic acid (**BCA**)
Central Nervous System (**CNS**)
Cerebrospinal Fluid (**CSF**)
Computerized Cooled Digital camera(**CCD**)
Cyclin-Dependent-Kinase 5 (**cdk5**)
Deoxyribonucleic Acid (**DNA**)
Dilute Sodium Dodecyl Sulfate (**dSDS**)
2,4-dinitrophenol (**DNP**)
2,4-dinitrophenylhydrazine (**DNPH**)
Dulbecco's Modified Eagle Medium (**DMEM**)
Familial Alzheimer's Disease (**FAD**)
Fetal Bovine Serum (**FBS**)
Glutathione (**GSH**)
Heat Shock (**HS**)
Horseradish Peroxidase (**HRP**)
Human Leukocyte Antigen (**HLA**)
Hydrogen Peroxide (H_2O_2)
4-Hydroxy-2-Nonenal (**HNE**)
Hyperbaric Oxygen (**HBO**)
Immobilized pH Gradient (**IPG**)
Mini-Mental State Exam (**MMSE**)
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (**MTS**)
N-acetyl-L-cysteine (**LNAC**)
Neurofibrillary Tangle (**NFT**)
Oligomeric Proanthocyanidin Complex (**OPC**)
One-dimensional Polyacrylamide Gel Electrophoresis (**1-D PAGE**)
Paired Helical Fragments (**PHF**)
Phenazine methosulfate (**PMS**)
Phosphate Buffered Saline (**PBS**)
Polyvinylidene difluoride (**PVDF**)
Presenilin-1 (**PS-1**)
Presenilin-2 (**PS-2**)
Programmed Cell Death (**PCD**)
Reactive Oxygen Species (**ROS**)
Scutellaria baicalensis Georgi Extract (**SbE**)
3-morpholinosydnonimine hydrochloride (**SIN-1**)
Sodium Dodecyl Sulfate (**SDS**)
Sporadic Alzheimer's Disease (**SAD**)
Superoxide Dismutase (**SOD**)
Trichloroacetic Acid (**TCA**)
Two-dimensional Polyacrylamide Gel Electrophoresis (**2-D PAGE**)
Xanthine/Xanthine Oxidase (**X/XO**)

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INTRODUCTION

I. Background

Alzheimer's Disease (AD) is a neurodegenerative process that is characterized by a progressive loss of memory function, language skills, and perception. The onset of AD is typically gradual and eventually all AD patients become completely incapacitated. AD advances in stages from early, mild forgetfulness to severe dementia. On average, AD patients live from four to eight years after the initial diagnosis but usually die from other disorders, most commonly pneumonia [79].

While AD was originally thought to be a rare condition affecting only young people and was referred to as "presenile dementia", today, there are an estimated five million Americans with AD and the disease is the fourth leading cause of death of adults in the US [79]. The prevalence of AD escalates with increasing age so that one in two Americans at 85 years of age will be affected [29]. In this century, given the increasing mean age in the U.S., the number of people affected will increase to nearly 22 million by 2025 [44]. Currently, approximately 50% of the people in US nursing homes have AD; this number is also on the rise [79]. Because of this increasing trend, it is critical that more is known of the molecular basis of AD so that new treatments can be developed. These studies were designed to elucidate such mechanisms.

The first reported case of Alzheimer's disease was published in 1907 by a German psychiatrist, Alois Alzheimer. In his report, Alzheimer described his autopsy findings in a woman who died at the age of 51 with a severe dementia. Alzheimer used

the then new silver stains to reveal the presence of abnormal nerve cells in the cortex [3]. These methods are still used today to determine the presence of neurofibrillary tangles (NFT) and beta amyloid plaques. AD is only positively diagnosed upon postmortem examination of brain tissue for these characteristic plaques and tangles. As a result, doctors often rely on other techniques to diagnose "probable" AD. These exams include a thorough medical history, Mini-Mental State Exam (MMSE), and a complete physical to help to rule out other diseases. Currently, there is still no accurate diagnostic test for AD. Although AD is considered a disorder localized to the brain, several lines of evidence support the idea that AD could be a systemic deficiency manifested in all tissues. If this proves true, it may be possible to diagnose AD in peripheral tissues first, long before symptoms occur.

When the diagnosis is Alzheimer's, there is still no effective prevention of the disease. Because of this, AD represents not only an emotional crisis, but also a financial one. The financing for approximately five million AD patients in the U.S., including costs of diagnosis, treatment, nursing home care and in-home care, is estimated to be more than $100 billion each year [29]. The federal government covers $4.4 billion and the states another $4.1 billion. The patients and their families cover the remaining costs [81]. It is estimated that the average cost of health care is between forty and sixty thousand dollars per patient, per year – with nearly 360,000 new patients every year [79].

II. Genetics of AD

While the mechanism that leads to the death of neurons and clinical symptoms in the Alzheimer patient is currently unknown, it is thought that AD results from not only genetic risk factors but also environmental factors. Individuals suffering from AD can be divided into two distinct classes: Familial "Early-Onset" AD (FAD) and Sporadic "Late-Onset" AD (SAD). Familial AD represents less than 5% of all AD cases. Individuals with this form of the disease develop AD early, at an age between 30 and 65 years. FAD has been associated with mutations in the Amyloid Precursor Protein (APP), Presenilin-1 (PS-1), and Presenilin-2 (PS-2) genes.

The exact function of APP is not well understood but it has been shown to be neuroprotective and to function as a protease inhibitor. Alterations in the APP gene have been hypothesized to lead to AD in two ways: 1) overexpression and 2) increase in the amyloidogenic enzymatic cleavages. All the missense mutations discovered in the APP gene thus far have been clustered around the β-secretase cleavage site. Cultured fibroblasts that express a mutated FAD-linked APP produce threefold more Amyloid beta peptide (Aβ) than normal APP-producing cells [22,89].

PS-1 and PS-2 are homologous polytopic proteins that have been localized to the ER and Golgi in mammals. Presenilins are expressed at low abundance in most cell types, including neurons. Finding a mutation in the PS1 or APP gene has an absolute predictive value for the eventual development of AD [22].

Sporadic AD is the more common form and typically effects persons over age 65. No genes have been linked to this form of AD [80, 93,94]. However, several genetic risk

factors have been identified. A genetic risk factor increases the likelihood of getting the disease, without actually causing it. For example, persons carrying the specific form of Apolipoprotein E gene (ApoE), ApoE4, are more likely to develop sporadic AD. In this way, ApoE is considered to be a "risk factor" for SAD. Evidence has demonstrated that carriers of one or two ApoE4 alleles have a five to ten time higher risk. There is no causal connection or a strict inheritance pattern. AD is a multi-factorial genetic disease, and since the ApoE4 effect is an association and not a direct causal relation, the diagnostic value of ApoE genotyping is practically zero.

In America, 40-60% of AD cases are thought to be related to ApoE4. ApoE normally plays a role in the distribution of cholesterol for repairing nerve cells during development and after injury. There are three known forms: ApoE2, ApoE3, and ApoE4. Nearly half of all late-onset AD patients have the ApoE4 form. While ApoE2 and ApoE3 seem to have protective qualities that help to maintain nerve-cell structure, ApoE4 does not. E4 binds to beta amyloid and helps to destroy nearby brain cells [116]. However, a mutation in ApoE is not as damaging to a person as a PS1 or PS2 mutation is in early-onset AD cases. In fact, many people who inherit the mutation will never develop the disease, even if they live to 90.

The inheritance of a mutation in the gene encoding α2-macroglobulin (A2M) has also been associated with an increased risk for Sporadic AD. A2M is an abundant proteinase inhibitor present in serum and the brain that accumulates in neuritic plaques. It binds Aβ; this is similar to ApoE. Both A2M and ApoE bind the same receptor, Low Density Lipoprotein Receptor-Related Protein (LRP) [95]. However, unlike ApoE, A2M

inhibits Aβ aggregation. In fact, A2M may be involved in the clearance of Aβ from synaptic clefts in the brain. ApoE4 and A2M work in harmony with one another, balancing the tendency of Aβ to solubilize vs. aggregate [95]. Yet, ApoE4 and A2M mutations are neither necessary nor sufficient to cause AD on their own.

Other genes are suspect as well. Mutations in tau show an increase in other related forms of dementia such as Parkinson's disease, and α-synuclein, a protein with a tendency to aggregate and present in AD neuritic plaques, appears to cause some forms of Parkinson's disease [54]. New candidate genes linked by recent research to SAD include the serotonin transporter, human leukocyte antigen (HLA) gene, and butyrlcholinesterase K [12]. Table 1 summarizes the genes involved in AD.

III. Histological Features of AD

There is a pressing need for AD therapy and many scientists have focused on how Alzheimer's affects the brain. In fact, much of what is known today has come from studies on autopsied brain tissue. The AD brain is characterized by senile plaques located outside neurons and by the presence of neurofibrillary tangles (NFTs) within neurons [29,130]. Not all brain regions show these characteristic tangle and plaque formations – the areas most prominently affected are those related to memory. Moreover, these two "classical" lesions of AD can occur independently of the other.

NFTs are located in regions of the AD brain associated with not only memory but other higher learning centers such as the amygdala and hippocampus. Paired helical filaments (PHF) form the tangles; the components of PHF are normal gene products,

	Gene	Chromosome	Prevalence	Onset	Effect	Reference
Causal	APP	21	1%	45-65	Aβ Deposition	[89]
	PS-1	14	8%	28-50	Apoptosis	
					Ca++ deregulation	[22]
	PS-2	1	1%	40-55	Apoptosis	[22]
Risk	ApoE	19		60-80	Aβ Deposition	[95]
	Mito DNA	--		Late	Oxidative Stress	[12]
	A2M	12		Late	Aβ buildup/toxicity	[95]
	Tau	17			Neurodegeneration	
					Fibrillary Tangles	[54]
	α-synuclein	4			Fibrillary tangles,	
					Oxidative damage	
					Excitotoxicity	[54]

Table 1. Genes Involved in Alzheimer's disease. Mutations in APP, PS1 or PS2 are linked to early onset AD. Other genes have been identified which may increase the risk of developing AD late in life.

specifically tau and ubiquitin. Tau is a protein that is rich in serine and threonine residues and can be phosphorylated by many different kinases. In healthy brains, tau provides neurons with structural support, but in AD, this structual support collapses and becomes twisted and tangled. No mutations in tau have been linked to AD. It is suspected that the process of tangling begins when amyloid plaques build up to the extent that they begin to press up against other neurons. This pressure sets off a cascade of chemical changes within the neuron, resulting in a triggering of cyclin dependent kinase 5 (cdk5) [69]. Cdk-5 is deregulated in the brains of people with AD [108]. This enzyme escapes its normal restraint, in turn, triggering the abnormal phosphorylation of tau inside the neuron. Phosphorylation of tau weakens its affinity for the microtubules and tau can no longer stabilize these microtubules within the axon of the nerve cell. The microtubules aggregate and form fibrils and cause a collapse in the framework of the cell. It has been suggested that NFTs form secondary to plaque formation in the AD brain [108,107]. Figure 1 shows the current theory of how plaque and tangle formation are linked.

The hallmark of the AD brain is amyloid plaques. The major component of both categories of plaques is a 4-kDa peptide Amyloid beta (Aβ) [129,106]. Two basic categories of plaques were first described in 1988: diffuse and neuritic or senile plaques. Diffuse plaques are believed to be benign deposits of non-fibrillar amyloid peptide since they do not elicit an immune response. Diffuse plaques are present in non-demented aged individuals as well as AD patients. Neuritic plaques, on the other hand, are collections of dystrophied neurons surrounding an amyloid core. They also contain non-fibrillar amyloid. In addition, a number of activated microglia as well as reactive

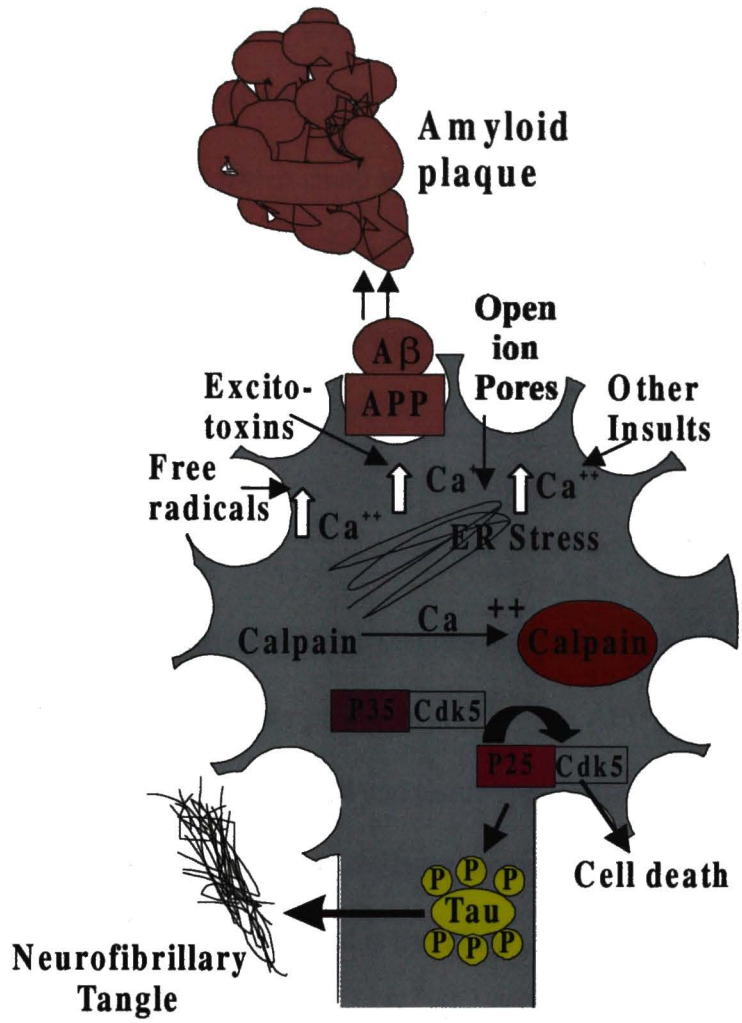


Figure 1. The current model of plaque and tangle formation. Aβ plaque formation is believed to begin the cascade of events leading to AD. Aβ generates free radicals and this oxidative stress triggers a protein protease, Calpain, to cleave the p35 control protein. p25 is released and redirects cdk5 to tau hyperphophorylation. When tau becomes hyperphosphorylated, it no longer holds microtubules within the dendrite together and tau tangles form.

astrocytes are present at the site of the plaque. The core of neuritic plaques is typically made up of Aβ42 while the bulk of the plaque is Aβ40. This is because Aβ40 aggregates much more rapidly when spiked with a small amount of Aβ42 [73]. Neuritic plaques also contain smaller amounts of several other proteins, which include ApoE, anti-chymotrypsin, complement C1, α-2-Macroglobulin, and α-synuclein [103,1]. These plaques are abundant in the AD brain and are not common in the normal aged brain.

IV. APP and Aβ

Aβ is the main constituent of neuritic plaques and much of the AD research has focused on the structure/function of its precursor, APP. The APP gene was isolated in 1987 and is expressed in virtually every cell and tissue type in the body [35]. However, the only cell type that constitutively expresses large amounts of APP is the nerve cell [100]. APP can be alternatively spliced and is therefore produced in three forms. APP's function may be to help repair injured brain cells. It may help keep brain tissue healthy and boost brain repair activities. In addition, it has also been shown to function as a protease inhibitor that regulates blood clotting. It has been suggested that in some cases, AD may be caused by the failure of APP to protect against p53, a protein which when activated triggers a cell death pathway. In effect, APP exerts control over cell death because activation of p53 is not blocked when APP is mutated [128].

APP can be processed in two different ways. Through protease action, the protein is cleaved at specific sites to either produce an amyloidogenic or non-amyloidogenic sequence [42]. As shown in Figure 2, the non-amyloidogenic pathway works through the

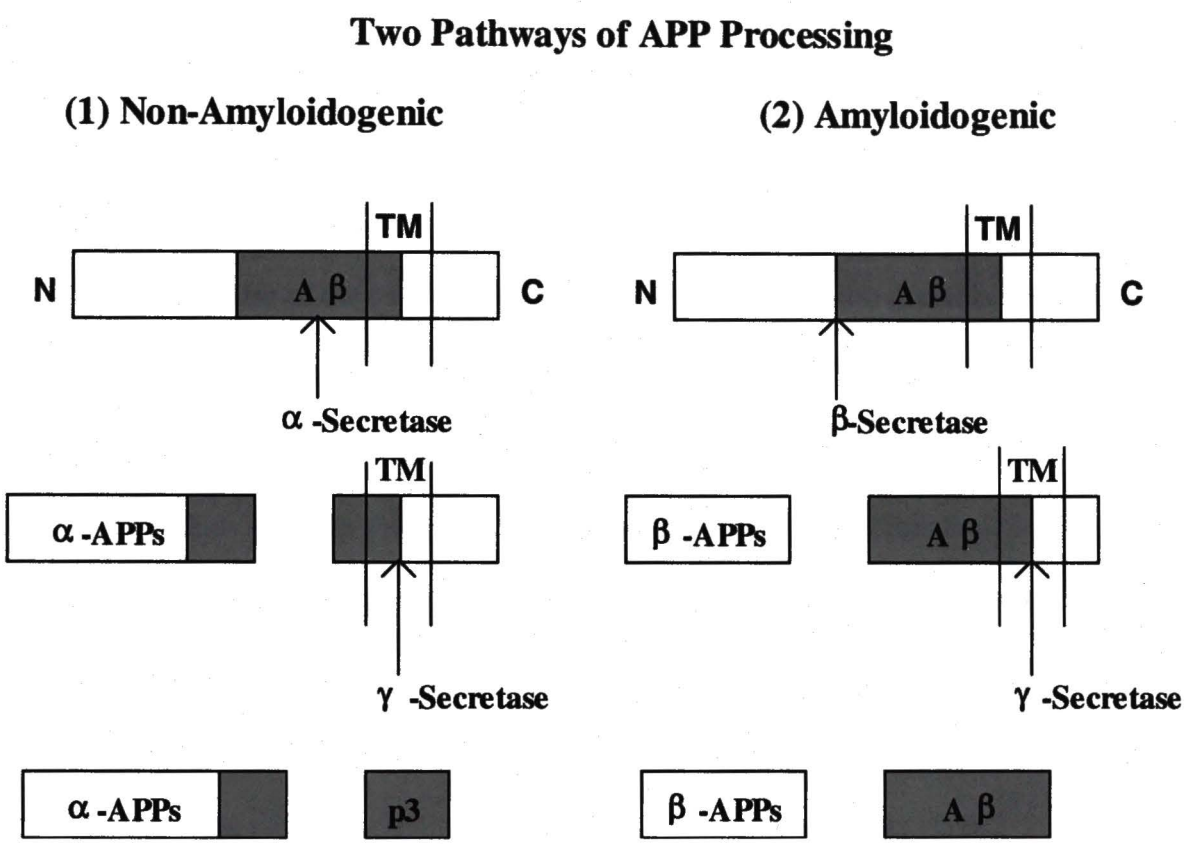
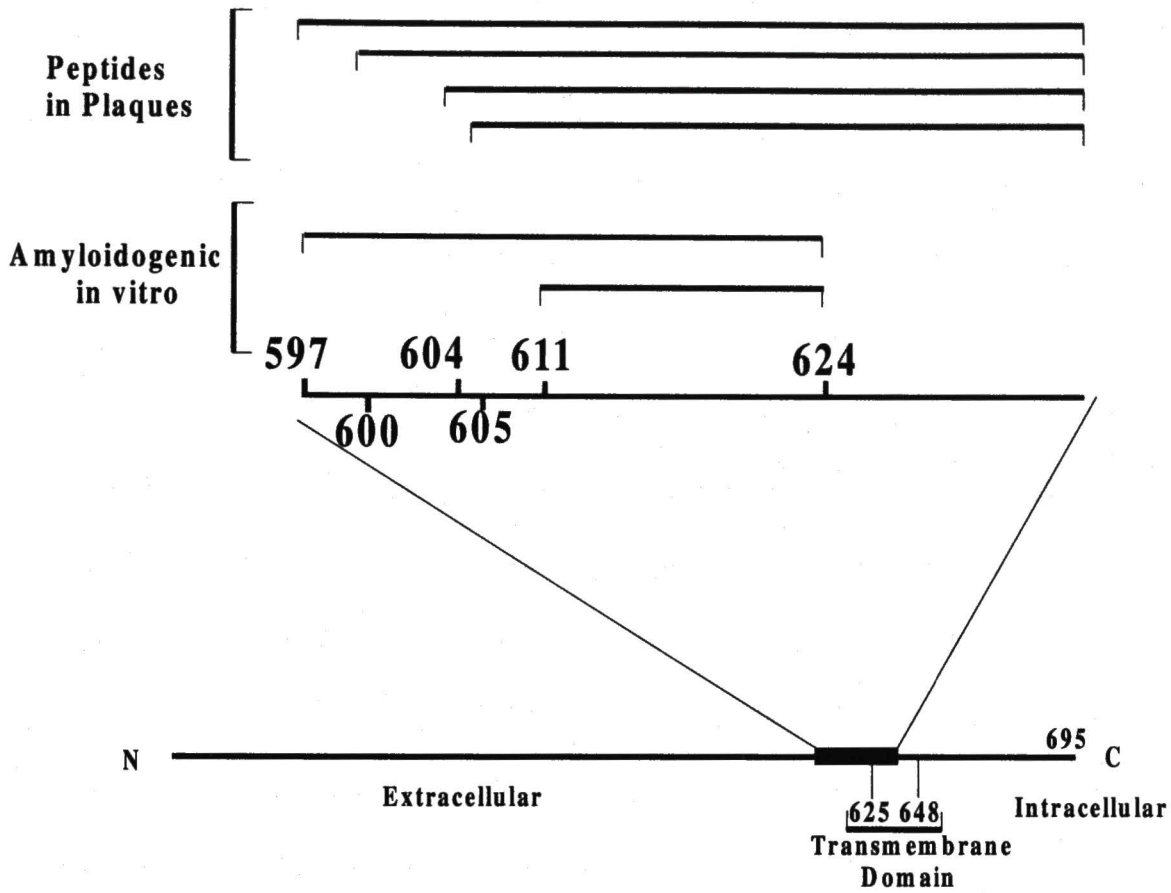


Figure 2. The two pathways of APP processing. (1) shows the non-amyloidogenic pathway which is characterized by the action of α-secretase. This enzyme cleaves within the beta amyloid sequence and thus prevents the formation of an intact Aβ. (2) is the competing amyloidogenic pathway, characterized by the action of β-secretase. This enzyme cleaves the intact Aβ at the N terminus. After cleavage by these two enzymes, a third enzyme, γ-secretase, cleaves at the C terminus of the Aβ region. A p3 segment and an intact Aβ fragment are formed, depending on the pathway [42]. Abbreviations: amyloid precursor protein (APP); Transmembrane (TM); amino terminal (N); carboxy terminus (C); amyloid beta protein (Aβ).

action of α-secretase, which cleaves APP within the Aβ sequence so that an intact Aβ sequence is not produced. This pathway product is soluble and thus does not give rise to the pathological lesions of AD. In contrast, the amyloidogenic pathway works through the action of two enzymes, β and γ-secretase. These two proteases form an intact Aβ product.

Because of the two ways APP is cleaved, there are two possible forms of Aβ. These two major types differ in their amino acid length: Aβ1-40 and Aβ1-42 (see Figure 3). Roughly 90% of the total secreted amyloid is the shorter form. The first 28 amino acid residues make up the extracellular domain while 29-40/42 functions in anchoring the peptide in the lipid membrane [31]. The hydrophobic region of the peptide is extremely important as is illustrated when comparing the two forms of Aβ; the shorter form does not aggregate as easily. The increase in aggregating ability in the longer form leads to its neurotoxicity [49].

The neurotoxic action of Aβ was localized to the 25-35-residue region (GSNKGAIIGLM), which has been shown to produce cell death [69]. This short sequence of the peptide has been shown to increase cytosolic calcium levels, an action that is directly cytotoxic to neurons [74,88]. Aβ 1-40 and 1-42 are found in normal cells but their function is not understood. However, it is obvious that Aβ accumulates in the fluid surrounding neurons to form the plaques. Overproduction does not seem to be the problem: a typical AD sufferer makes Aβ at the same rate as a healthy person. The problem lies in the clearance of Aβ from extracellular space.



SEQUENCE of Aβ1-42: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile- Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

Figure 3. The 695 amino acid Amyloid Precursor Protein. The Aβ protein sequence is indicated with a bar and shown in more detail above the precursor. The four Aβ sequences that have been identified in neuritic plaques are shown, as well as sequences that form amyloid fibrils *in vitro*, including the minimum amyloidogenic sequence of amino acids 611 to 624 [69].

Aβ is normally present at very low levels. While the function of Aβ is less understood, it appears to play a role in blood clotting because it stimulates platelet aggregation and it may also play a role in regulating cholesterol uptake by cells, as it is bound by ApoE and A2M. Aβ spontaneously forms beta-pleated sheets. Several *in vitro* studies have shown that β-pleated sheet Aβ fibrils are neurotoxic in AD [64]. There are several types of amyloidosis involving different APPs. The deposition of amyloid occurs in many different diseases (atherosclerosis, renal failure, different types of cancer, Creutzfeldt-Jakob disease, prion diseases, and sclerosis) in which aggregated proteins are deposited mainly in the extracellular space of tissues, producing cell damage and organ dysfunction. In addition to Aβ, several other amyloidogenic proteins are toxic to cells in culture suggesting a common mechanism of pathology induced by amyloid [64].

Recently, researchers addressed the relationship between Aβ and oxidative stress *in vivo*, using a transgenic mouse model of AD, and found a correlation. These mice overexpress APP and, as in AD, develop characteristic Aβ deposits. These animals also show the same type of oxidative damage that is found in AD and this damage directly correlates with the presence of Aβ [110,102]. Thus, it has been suggested that the process of Aβ aggregation can induce a reactive oxygen species (ROS) formation [131].

V. Oxidative Stress in AD

A. Reactive Oxygen Species

A number of studies have indicated that Aβ plays a central role in the development of AD [19,106]. In fact, a great majority of this research has focused on Aβ

peptides as initiators of oxidative stress-induced cell damage and death through the generation of ROS [17,18]. Exposure to Aβ has also been shown to increase levels of various oxygen radicals and increases free radical mediated damage to membrane lipids, proteins and deoxyribonucleic acid (DNA). This is strong support for the oxidative stress role in AD. Exposure of neurons to Aβ greatly increases their vulnerability to subtle stress [125,45,63]. In addition, cultured neurons exposed to Aβ become insensitive to acetylcholine, impairing their ability to communicate with one another. It is widely thought that if Aβ peptide fibril formation could be prevented, neuritic plaque formation could be diminished [17,18,47]. Figure 4 shows the pathological cascade of oxidative damage that eventually leads to AD development, neuronal cell death, and dementia.

ROS include free radicals, which are molecular species that contain an unpaired electron. Consequently, they are extremely reactive species. Because of the need to pair its single electron, a free radical attacks another molecule, causing the formation of another free radical and promoting a chain reaction of radical propagation. Free radicals can arise from the diet, smoking, alcohol, etc. The body produces free radicals to help cells to fight infection. This makes up the vast majority of free radicals. Under normal conditions, such as metabolism, free radical intermediates are produced. The major sources of free radicals are modest leakages from the electron transport chain. However, increased free radical production can become harmful. Free radicals are highly reactive; they readily react with vital molecules nearby such as the lipid membrane of the cell, its DNA, or protein.

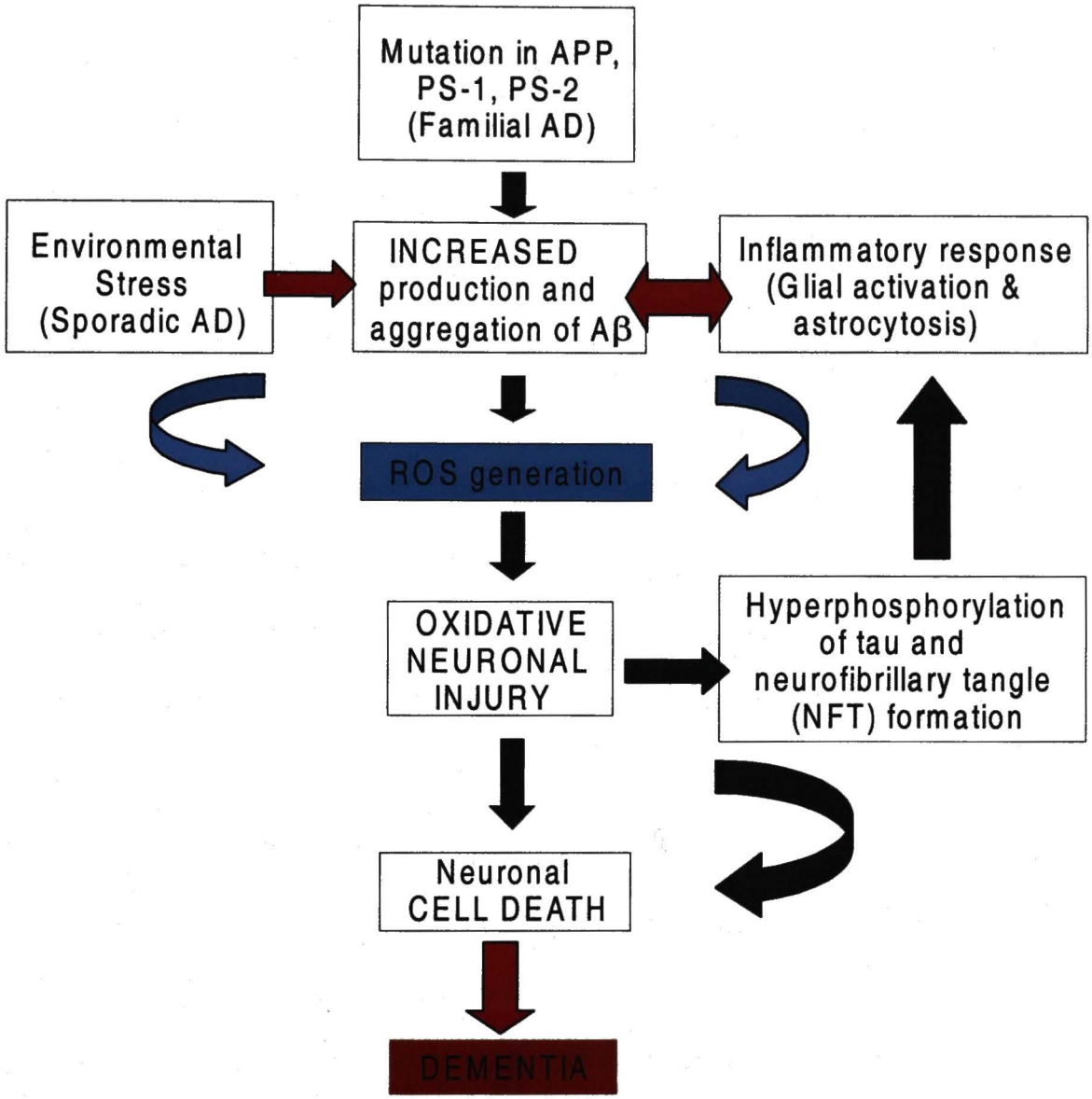


Figure 4. Pathological cascade that gives rise to Alzheimer's disease. Genetic predisposition and environmental stress can lead to increased Aβ deposition and the production of reactive oxygen species (ROS), which leads to neuronal cell death and dementia [107].

Oxidative stress is defined as when the production of damaging reactive oxygen species (ROS) overwhelms the body's natural defense mechanisms. The oxidative stress cascade has been greatly supported as a causative mechanism for AD [4]. There is evidence that hydrogen peroxide (H_2O_2), a ROS, mediates Aβ toxicity, since Aβ causes the intracellular accumulation of H_2O_2 [37]. In addition, Dyrks et al. reported that radical treatment transforms non-aggregated Aβ into stable, and toxic, aggregates [9]. This is thought to be the result of amino acid oxidation and protein cross-linking [25]. Even with this evidence, the exact relationship between Aβ's secondary structure, radical formation, aggregation, and toxicity are still unclear.

Aβ peptides can generate free radicals directly and indirectly. As Aβ aggregates, the insoluble fibrils release free radicals, resulting in a local inflammatory response. Free radicals produced by Aβ may be capable of damaging proteins and lipids embedded in the cell membrane, and inducing lipoperoxidation. It has been suggested that Aβ interacts with and causes membrane lipid peroxidation damage in a specific and selective manner [43]. Damage to the lipid membrane proteins can result in loss of membrane potential and homeostasis. This in turn may lead to an increase in intracellular calcium concentrations [6]. Increases in free intracellular calcium can result in a further increase in oxidative stress through the generation of oxygen radicals, and an excess of intracellular calcium has a devastating effect on the mitochondria: electron transport becomes leaky and malfunctioning [75]. This Ca^{2+} influx into the nerve cells results in not only further dysfunction of the neuron but also an increase in production of

neurofibrillary tangles (NFTs) and apoptosis [97]. Aβ is also suspect in DNA damage. A threefold increase in oxidative DNA damage has been discovered in the AD brain [10].

B. Protein Oxidation

Aβ has been implicated in protein oxidation. Proteins form the cytoskeleton, giving cells their shape and they play crucial roles in nearly all biological processes. Protein oxidation enhances protein catabolism and clearance [113,114]. This is deleterious to the normal function of the organism, especially if oxidation occurs at an important area of protein structure, such as the active site of the enzyme molecule. Many amino acids undergo specific irreversible modifications when a protein is oxidized. All amino acid residues of proteins are susceptible to attack by ROS, but some are more sensitive. Oxidative damage to proteins may produce an increase in the carbonyl content of the protein due to oxidation of sensitive amino acids such as histidine, proline, arginine, and lysine [112]. The carbonyl content of proteins can be used as one measure of protein damage [59,60].

The amino acid sequence of a protein, the ligands it associates with, and a protein's interactions with its neighbors are all important in determining a protein's oxidative susceptibility [105]. For example, while Aβ contains 40-42 amino acids, only the methionine in position 35 is readily oxidized. Peptides that contain the same amino acids but in a scrambled order are not vulnerable to oxidation of the methionine residue [124]. Furthermore, while Aβ can generate free radicals, its ability to do so is eliminated when Met35 is substituted with cysteine [131].

Oxidative damage is not unique to the AD brain. However, the brain is particularly vulnerable to free radical damage. This is true for several reasons. One, the brain has high levels of iron that catalyze the formation of ROS. Secondly, the brain exhibits high oxidative metabolism. It also has an increased amount of polyunsaturated fatty acids, which are subject to peroxidation. Moreover, the brain is incapable of neuronal regeneration. Perhaps the most important factor is the brain's relatively low levels of antioxidants (e.g. glutathione) and protective enzymes such as catalase and superoxide dismutase [29]. Taken together, the brain is extremely susceptible to oxidative attack. Once cells in the brain are damaged, they have long been considered dysfunctional for life. This widespread neuron degeneration leaves gaps in the brain's messaging network that interferes with communication between cells.

Oxidation products accumulate not only in the brains and cells of AD patients but normal subjects as well [50,83]. This is due to a progressive decrease with age in the normal defense mechanisms that protect against oxidation. Much evidence has pointed to the extensive oxidative damage to cellular proteins in AD patients [93]. While increased oxidation of proteins in the brains of AD patients has been documented [68], it is still not known whether these oxidized proteins are unique to AD brains, or are found in other cells or tissues.

C. Antioxidants

There is no known cure for AD, and treatment focuses on lessening symptoms and an attempt to slow the progress of the disease. The Food and Drug Administration

has approved drugs that increase or improve the function of acetylcholine, the neuro-transmitter affecting memory. Current research concentrating on the prevention of AD has focused on antioxidants as a possible therapeutic approach. One antioxidant, Vitamin E, has recently been shown to delay a significant functional decline in patients with a moderate AD dementia [98]. Pycnogenol, a member of the flavonoid family, has also been shown to be a potent scavenger of free radicals [122]. In addition, sex hormones have been shown to protect neurons from oxidative stress-induced damage. Estrogen and progesterone have been shown to be significant contributors to the protection of hippocampal neurons when treated with toxic Aβ [37]. These beneficial effects observed in AD patients indicate that oxidative damage is central to the pathogenesis of AD.

VI. The Fibroblast Model

Even with all of the recently gained knowledge into AD, there are still critical gaps in our understanding of this disease. These include the following questions: What types of oxidation are most critical?, What proteins are easiest oxidized in normal and/or AD subjects?, Are proteins more oxidized in AD than non-AD and Why?, and How can oxidation be prevented? In the following thesis, I have attempted to answer some of these questions.

Measurements of autopsied brain are an important strategy for studying AD, but due to postmortem delays and because the stage of AD is hard to diagnose, this approach has serious limitations. Secondly, although a large number of AD brains are now available for research, the number of control brains is markedly lower. Because of these

limitations, it is important to pursue alternative models for elucidating the mechanisms of AD. The oxidative damage of AD may not be restricted to proteins in the brain [50]. Because antioxidant defenses are lower in the AD brain [43,40] and ROS produced can cross the blood brain barrier, it is a reasonable conclusion that specific oxidized proteins might be found in blood, cerebrospinal fluid, or other cell types in the body.

Only a few research studies have focused on cerebrospinal fluid or peripheral cells, including cultured fibroblasts. While the research on autopsied brain has concentrated on the molecular characterization of neuritic plaques and neurofibrillary tangles, the use of peripheral cells is based on the hypothesis that AD is a systemic disease, affecting not only the brain but also other tissues in the body.

The fibroblast model poses some of the same problems as other models but the advantages far outweigh the disadvantages in this system. Growth properties and biological age in culture can have profound effects on properties of cultured skin cells. This point is critical. While some authors argue that results do not differ, reproducible and interpretable results with the AD fibroblasts model require attention to replicability of culture conditions, such as state of confluency, aging of cultures *in vitro*, and different growth conditions. Stage of the disease is also an important factor to consider. When culture conditions are carefully standardized, especially by passage number, these problems may be minimized.

In spite of some limitations, cultured cells offer many advantages for the study of AD. Any drugs or diets that the patient may be on are diluted millions of times, since fibroblasts are passed numerous times in culture. These cells are relatively easy to obtain

and maintain under rigid conditions. Finally, cells from Alzheimer's patients and age-matched controls show similar growth so that their differences can be assessed. Because such experiments can and are being done in fibroblasts, data obtained can help to identify primary mechanisms leading to AD and can be used to test hypotheses. The fibroblast model can provide a test for potential therapeutic approaches, and provide a system in which to assess potential cell biological markers by comparing diseased and normal fibroblasts. In fact, the study of fibroblasts enhances and complements the brain approach to studying AD.

Cultured fibroblasts have been used extensively to study basic properties of aging and in the study of other neurological disorders. For example, the enzyme deficiency, which describes Tay Sachs disease, was characterized in cultured fibroblasts [39]. Skin fibroblasts were also used in the study of Lesch-Nyhan disease to determine if the disease was due to an enzyme deficiency [39].

While AD is normally considered a central nervous system (CNS) disorder, numerous changes in tissues outside the CNS have been reported to show associations with AD. These peripheral abnormalities have been found in platelets, blood cells, and peripheral vessels [39]. In fact, the first studies of a role for oxidative stress were done on fibroblasts from AD patients and controls [39]. In these reports, several key enzyme abnormalities were found in the cells from AD patients. Two oxidative abnormalities were frequently found, deficiencies in thiamine requiring enzymes [34] and mitochondrial uncoupling [14]. Alterations in the phosphoinositol second messenger

system have also been well documented [39]. These first studies established that at the biochemical level, metabolic abnormalities are not limited to the brain.

Subsequently, many other abnormalities have been observed in fibroblasts from AD patients such as an altered signal transduction including calcium homeostasis and oxidative metabolism, oxidative deficits, and altered amyloid protein precursor (APP) metabolism [39]. APP exists and is processed in fibroblasts just as in other cells. In addition, fibroblasts from AD patients exhibit a unique vulnerability to oxidative stress [39]. The source of this increased sensitivity could be due to decreased free radical defenses, increased ROS production, or both. Such susceptibility might have significance for better understanding of the pathophysiology of the disease. Thus, a history exists for using cultured fibroblasts from patients, and supports the idea that at least some of the manifestations of the disease are systemic.

Using the hypothesis that AD increases cellular oxidation in non-neuronal cells, the aim of the present work was to develop experimental procedures for the use of a simple cell model, the fibroblast, to determine if proteins derived from AD skin fibroblasts are more sensitive to oxidation by reactive oxygen species than non-AD cells and to assess the ability of antioxidants, such as glutathione and N-acetyl-L-cysteine, to prevent this oxidative damage in AD fibroblasts.

MATERIALS & METHODS

Cell culture media (Dulbecco's Modified Eagle Media [DMEM]), fetal bovine serum (FBS), and other cell culture reagents were supplied by Fisher Scientific, Gibco, and Summit Biotech. Cell culture plates, flasks, and other plastics were supplied by NalgNunc. Aβ peptide was supplied by Bachem. Hydrogen peroxide, Xanthine /Xanthine Oxidase, and 3-morpholinosydnonimine hydrochloride (SIN-1) were supplied by Fisher Scientific. Antioxidants GSH and LNAC were supplied by Fisher. *Scutellaria baicalensis Georgi* extract was a gift from Chun-Su Yuan from the University of Chicago. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) Assay was supplied by Promega, Inc. Primary rabbit anti-2-4-dinitrophenol antibody was supplied by Molecular Probes; goat -anti-rabbit (HRP-conjugated) secondary antibody was supplied by Sigma.

Cell Culture

Human fibroblasts were obtained from the National Institute of Aging Cell Repository. They included 4 AD and 4 age-matched controls (Table 2). Cell lines were at the same passage for all measurements. Cells were maintained in complete culture medium until just before the experiments. Human fibroblasts were maintained as described previously [118]. Cells were incubated in T-75 flasks at 37°C in 6% CO_2 until

Cell Line ID #	Age/Gender	Condition	Passage#
AG 11011	61/M	Normal	8
AG11017	87/M	Normal	8
AG07143	80/M	Normal	8
AG11357	62/F	Normal	8
AG08537	61/M	AD	8
AG10788	87/M	AD	8
AG08257A	80/M	AD	8
AG14149	62/F	AD	8

Table 2. NIA Cell Repository AD and non-AD Cell Lines. Eight age and sex-matched fibroblast lines, four confirmed AD and four normal, and all at the same passage, were used.

confluent and ready to use. Protein concentration for each sample was determined using a bicinchoninic acid (BCA) protein dye (Pierce).

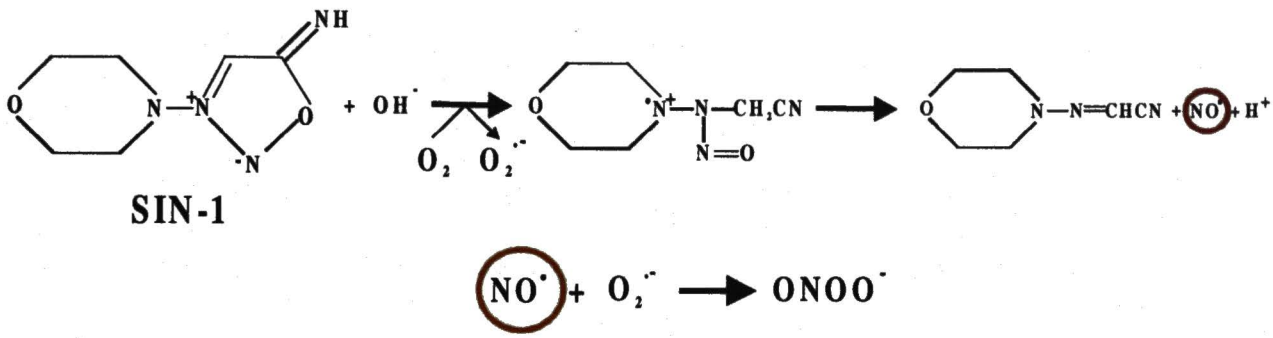
Oxidative Stress Treatment

Beta-amyloid

$A\beta_{25-35}$ peptide was pre-diluted to a concentration of 1mg/ml in sterile water just before experimentation. Cells were plated in 30mm plates at a density of 125,000 cells per plate. The peptide solution was then diluted to 50, 150, or 250μM in culture medium before addition to the cells. Cell plates were incubated for 24 hours for cell viability assays. For 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE), fibroblasts were plated in 100mm culture dishes at a density of 2 million cells per plate and treated with 150 μM Aβ for 24 hours.

SIN-1

3-morpholinosydnonimine hydrochloride (SIN-1) was prepared in sterile water to a concentration of 1mg/ml. Fibroblasts were plated in 96 well plates at a density of 4000 cells per well. SIN-1 was diluted in DMEM for final concentrations of 50, 100, and 200 μM. In every experiment, untreated control non-AD and AD fibroblasts were incubated. For cell viability experiments, 96 well plates were used for various time points of 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours.



Formation of Peroxynitrite

Serum Deprivation

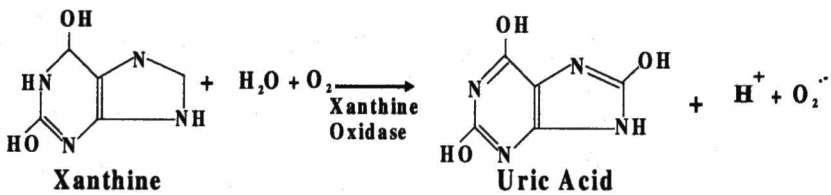
Fibroblasts were plated in 96 well plates at a density of 4000 cells per well in full media for cell viability experiments. After incubation for 4-6 hours, total media was aspirated and replaced with sera-free media. In every experiment, untreated control non-AD and AD fibroblasts were incubated. Cells were serum deprived for periods ranging from 0-72 hours.

Hydrogen Peroxide

Fibroblasts were plated in 96 well plates at a density of 4000 cells per well. Hydrogen peroxide (H_2O_2) was diluted in DMEM for final concentration of 350μM. The cytotoxic effect of H_2O_2 was measured by incubating the reaction mixture with cells at a concentration of 350 μM for 30 min., 1 hour, 2 hours, and 3 hours.

Xanthine/Xanthine Oxidase

Fibroblasts were plated in 96 well plates at a density of 4000 cells per well. X/XO was diluted in culture media for final concentrations of 1mM xanthine/12.5, 25, and 50mU/ml xanthine oxidase, where one unit equals the amount of enzyme which causes the oxidation of 1μM of xanthine per minute. In every experiment, untreated control non-AD and AD fibroblasts were incubated. For cell viability experiments, 96 well plates were used for various time point experiments of 30 min., 1 hour and 3 hours.



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

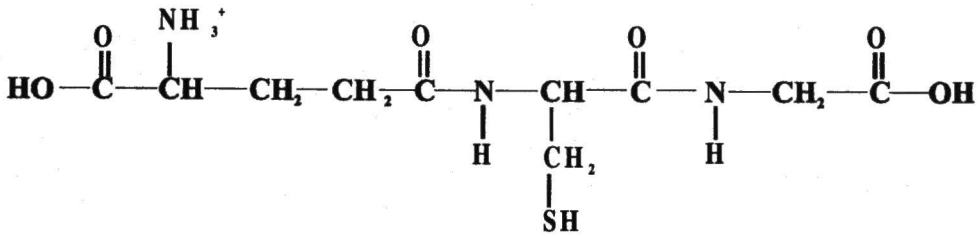
Formation of Superoxide Radicals and Hydrogen Peroxide

Antioxidant Treatment

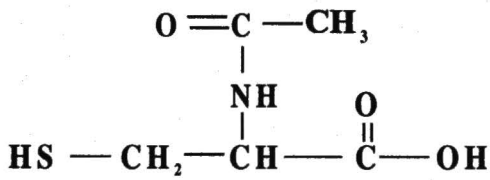
Glutathione (GSH) and N-Acetyl-L-Cysteine (LNAC)

To examine the effect of antioxidants on cell survival/protection, fibroblasts were incubated with both an oxidative stress and an antioxidant. For cell viability experiments, fibroblasts were plated in 96 well plates at a density of 4000 cells per well. Concurrently with an oxidative stress, fibroblasts were exposed to either GSH or LNAC at final concentrations of 0.5mM, 1mM, or 5mM. For 2-D PAGE analysis, fibroblasts

were plated in 100mm plates at a density of 2 million cells per plate. Cells were treated with 150μM Aβ and at the same time were treated with 5mM GSH for 24 hours.



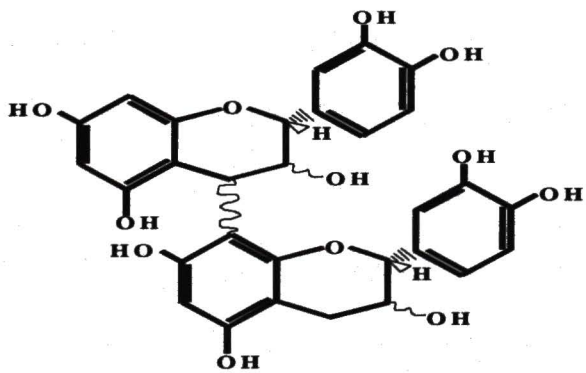
Structure of Glutathione



Structure of N-acetyl-L-cysteine

***Scutellaria baicalensis* Grape Seed extract (SbE)**

For cell viability experiments, fibroblasts were plated in 96 well plates at a density of 4000 cells per well. Cells were serum deprived or treated with 50mU X/XO or 150μM Aβ and concurrently exposed to SbE at final concentrations of 1.25 mg/ml, 6.2 mg/ml, and 12.5 mg/ml.



Structure of Oligomeric Proanthocyanidin Complex (OPC) from Grape Seed extract

Cell Viability -- MTS Assay

Cell survival was evaluated using the MTS assay. This assay is a colorimetric method for determining the number of viable cells in proliferation. The assay is composed of an MTS tetrazolium compound that is bioreduced by cells into a formazan that is soluble in tissue culture medium. It is also composed of an electron-coupling reagent phenazine methosulfate (PMS). The absorbance of the formazan at 490nm can be measured directly from 96 well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenases in metabolically active mitochondria inside the cell. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture [23].

Cell Extraction/Preparation

Fibroblasts were scraped in media into 15ml centrifuge tubes and centrifuged for 10 min. at 2000xg. The media was aspirated without disturbing the pellet. The pellet was washed twice with 1X phosphate buffered saline (PBS). 250 µl of hot dSDS solution (.3% SDS. 1% beta-mercaptoethanol (BME), 0.5M Tris HCl [pH8.0]) was added to the pellet and the pellet was dissolved by pipet action. The samples were then boiled for 1-3 min. Samples were cooled in an ice bath and 10µl protease inhibitor, 12µl DNase with 25µl of DNase buffer (10X), and 12µl of RNase were added. After the additions, samples were incubated at 37°C for 1 hour. Samples were gently vortexed for several

minutes to avoid foaming and spun for 15 min. Proteins were then precipitated using trichloroacetic acid (TCA)/acetone [61].

2-D PAGE

Identification of specific proteins that were oxidized and the levels of oxidation were achieved using high resolution 2-D PAGE. The samples were rehydrated, isoelectically focused, and separated by molecular weight according to Amersham Pharmacia Biotech as shown in Figure 5 [118]. Approximately 250μg of fibroblast proteins were mixed with Laemmli sample buffers and absorbed onto an 18cm immobilized pH gradient (IPG) strip (pH 3-10), in a re-swelling tray. The 18cm IPG strips (pH 3-10) containing the samples were then electrofocused on an ISO-DALT® (Amersham Pharmacia) for 25,000 Volt/Hours. The IPG strips were then derivitized using a 10mM 2,4 dinitrophenol (DNP) solution and equilibrated in preparation for the second dimension. The strips were loaded into polyacrylamide gels, overlayed with agarose, and run overnight.

Silver Staining

Duplicate samples of the derivatized proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (e.g. 10% acrylamide and 0.1% SDS). Proteins in one gel were silver stained [118]. The silver staining procedure was a modification of a method that was described previously [82,91,92]. Proteins were fixed in the gel using ethanol:acetic acid (40:1) for two hours or overnight. Two separate sensitizers were used (e.g. glutaraldehyde (1% w/v)/sodium acetate (0.5M) and 2,7-napthalene disulfonic acid

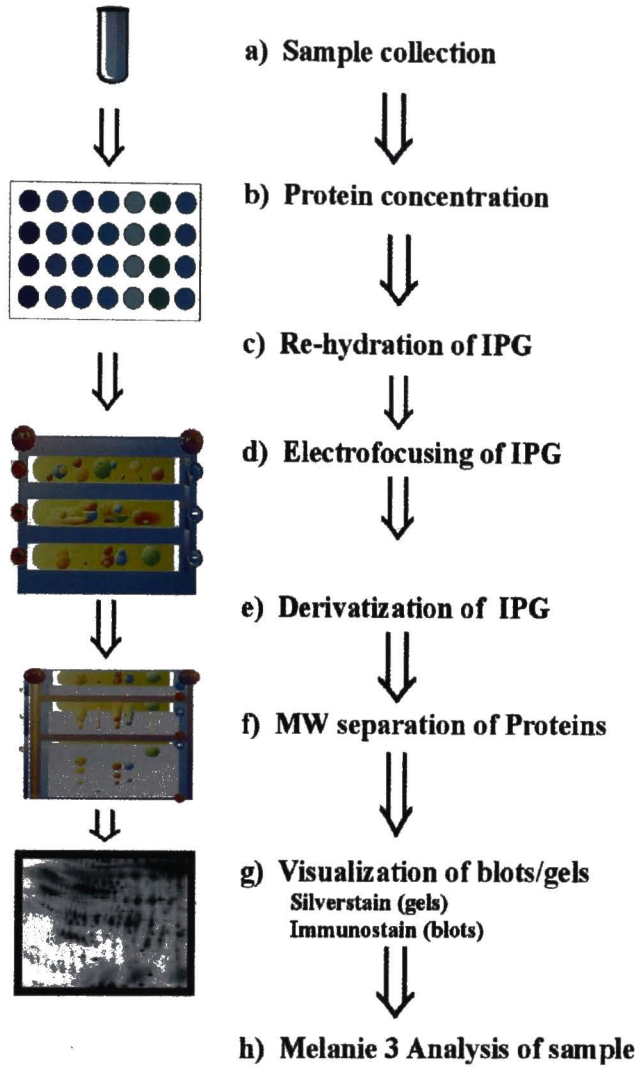


Figure 5. 2-D Polyacrylamide Gel Electrophoresis Protocol for Fibroblast Proteins.

(0.05% w/v) to increase sensitivity. Silver diamine staining solution (500 mM AgNO3, 500 mM NH_4OH, 20 mM NaOH) and the developer (citric acid (0.01% w/v)/formaldehyde (0.01%v/v)) were used to visualize the proteins followed by stop solution (Tris base (5% w/v)/acetic acid (2% v/v)). Silver stained gels were digitized using a Scan Maker 4 optical scanner (Microtec) at a resolution of 300 x 300 dpi.

Electroblotting

Replicate slab gels that were not visualized using standard silver staining procedures were electroblotted to polyvinylidene difluoride (PVDF) and immunostained for protein carbonyls. These slab gels were equilibrated in electrotransfer buffer (25mM-Tris; 192mM-glycine; 15%-methanol). The PVDF membranes were prepared for electroblotting according to manufacturer's directions. The Hoeffer electroblotting cassette was set up with blotting paper, PVDF membrane, and the slab gel as indicated by the manufacturer's instructions. The electroblotting cassette was closed and electroblotted at 80V for 90 min. at 4°C.

Antibody (2,4-DNPH)

Oxidation often results in the formation of new carbonyls in proteins, and carbonyl content is often used to quantitate the oxidation of mixtures of proteins [112,59,60,78]. To visualize the extent of oxidative damage, carbonyl groups were reacted with 2,4-dinitrophenylhydrazine (DNPH), which forms a protein-bound hydrazone that can be detected from its absorbance at 360-370 nm. Similarly,

immunospecific detection of oxidized proteins is possible using antibodies directed against this hydrazone derivative [96]. Duplicate SDS-PAGE of the derivatized proteins was carried out in 10% polyacrylamide gels containing 0.1% SDS. Proteins in one gel were silver stained [118], and proteins from the other gel were electroblotted to PVDF.

Immunostaining

PVDF membranes were immunostained using a modification of the protocol described by Mansfield [70]. The membranes were removed from the electroblotting apparatus and directly incubated with 5% milk (w/v) in phosphate buffered saline and Tween (3%) for 1 hour. Membranes were incubated overnight at 4°C with the primary antibody solution consisting of a 1:16,000 dilution of the rabbit anti-2,4-dinitrophenol (2,4-DNP) antibody in PBS-Tween solution containing 5% milk. The membranes were then washed three times with PBS-Tween solution for 15 min. each, and incubated with a 1:6,000 (v/v) dilution of the goat anti-rabbit (HRP-conjugated) secondary antibody in PBS-Tween containing 5% milk (w/v) for 1 hour at 4°C. The membranes were then washed 3 times for 15 min. each in PBS-Tween solution before visualization.

2-D Image Analysis

A computerized cooled digital (CCD) camera-based imaging system (Alpha Innotech) was used to visualize and record the stained proteins. Silver stained gels were illuminated from below using white fluorescent light source. A chemiluminescence kit (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce) was used to visualize

the immunostained blots. Following exposure to the chemiluminescent chemicals, the membranes were placed in a light-tight cabinet and a cooled CCD camera captured the light produced by the chemical reaction with the enzyme that is linked to the secondary antibody. The CCD camera shutter remained open for several minutes to capture the light produced. Protein bands and spots were quantified using Melanie 3. Melanie 3 was used for the analyzing, annotating and querying of the 2D gel samples. In addition, Melanie 3 offers sophisticated state-of-the-art analysis for the identification, quantification and matching of gels.

Statistical Analysis

Statistical analysis was done using the Minitab® statistics computer program. An extension of a single-classification ANOVA, the Nested Analysis of Variance, was used. The nested ANOVA is designed to represent the complexity of the given experiments and to extract all the relevant information from them. In these experiments, the one-way ANOVA would have been inadequate.

For example, two different cell types were used, and for each cell type, there were four different cell lines. In addition, many different forms of oxidative treatments were employed and for some of those treatments, different amounts of a drug (i.e. Aβ) or different time points were used. Statistical analysis was important not only in detecting differences among cell lines and then cell types, but also in detecting differences among various treatments/stresses and the time points of those treatments.

The analysis of such experiments called for a nested ANOVA because subordinate classifications (i.e. oxidative stress) were nested within the higher classification of cell type. Tables of the statistical differences are shown below the relevant charts. Fibroblasts that exhibited significant differences ($p<0.05$ level) in percent of cell survival were indicated by different letter superscripts. For example, if the percentage of cell survival of AD fibroblasts treated with an oxidative stress is 69^{c}, while that of the non-AD fibroblasts under the same conditions is 90^{b}, b and c are different letter subscripts, and hence, the difference is significant.

RESULTS

Fibroblasts from AD subjects are More Susceptible to Oxidation

Eight separate age-matched AD and non-AD fibroblast cell lines were plated at a density of 125,000 cells in a 30mm cell culture plates and allowed to attach to the plate for 4 hours. Cells were then subjected to different types of stress conditions (e.g. Aβ, heat shock, and hyperbaric oxygen) and cell survival was determined 24 hours after each oxidative insult. In general, each stress caused a decrease in survival; however, the survival in fibroblasts derived from AD patients was consistently, and significantly, lower than fibroblasts derived from non-AD controls (Figure 6). At 50μM Aβ, the non-AD and AD cells had a differential response. For example, non-AD fibroblasts showed a 10% increase in survival over the control non-AD fibroblasts not subjected to the Aβ insult. In contrast, the AD-fibroblasts showed a 15% decrease in survival compared to non-AD fibroblasts not subjected to the Aβ insult. This represents a 25% lower survival rate for AD compared to non-AD fibroblasts. Both results (increased proliferation [104,67], and decreased survival [27,13]) are consistent with previous reports from different cell lines, but have not previously been reported in AD and non-AD fibroblasts.

Heat stress (HS) has been shown to unfold proteins that can lead to increased oxidation and/or cell death [55,56]. The mild heat shock conditions (e.g. 42.5°C for 1 hour) that have been used in many different cell types to induce heat shock proteins

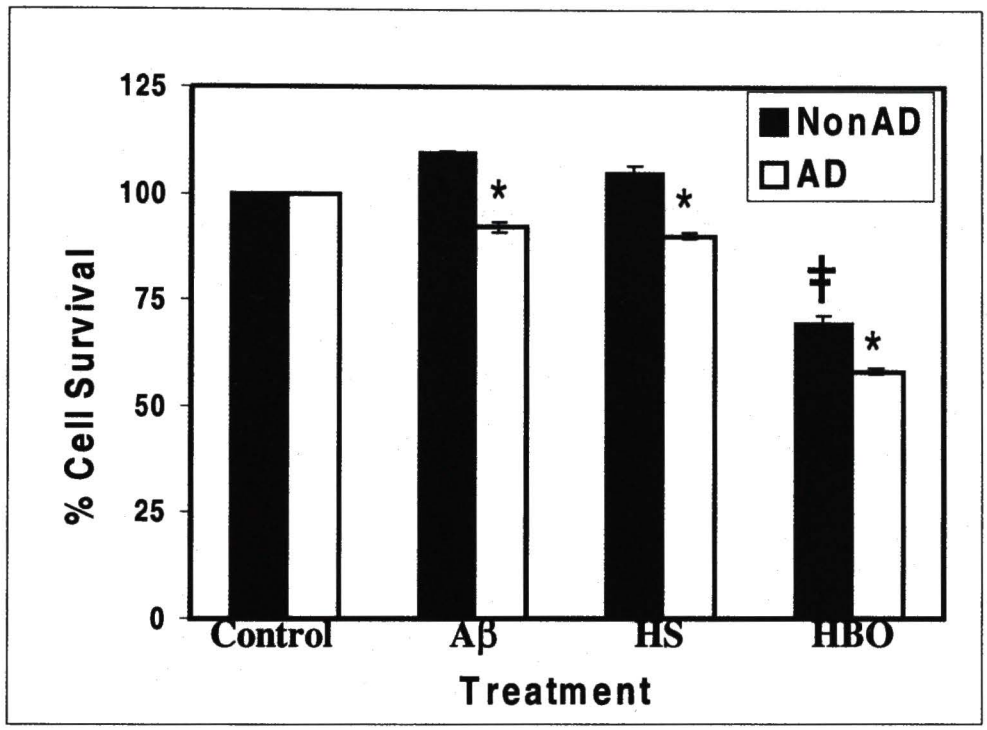


Figure 6. Survival of non-AD and AD fibroblasts following exposure to the $A\beta_{25-35}$ peptide, hyperbaric oxygen (HBO), and heat shock (HS). Non-AD(■) and AD (□) fibroblasts were grown to a density of 125,000 cells and then incubated for 24 hours as follows: Control (no treatment); 50μM Aβ; 4 ATM HBO; 42.5°C HS. Each bar represents the average and SEM of four separate 30 mm culture dishes. Asterisks (*) represent significant differences among cell groups as analyzed by ANOVA ($p<0.05$). ‡ represents differences among treatment groups as analyzed by ANOVA ($p<0.05$).

without decreasing survival were also used in these experiments (Figure 6-HS) [55,41]. Although there was no decreased survival in non-AD fibroblasts exposed to mild heat shock conditions, the survival in AD fibroblasts was significantly decreased compared to both non-AD fibroblasts (22%) and AD non-stressed control (25%).

Hyperbaric oxygen therapy (HBO) is an established therapeutic intervention in diving medicine and may play a role in wound healing [115]. However, brief exposures to high pressures of oxygen result in cell death and DNA damage occurs at relatively low pressures of oxygen [33,85]. To examine the effects of HBO on AD and non-AD cell death, four sets of AD and non-AD cells were plated in 30mm culture plates at a density of 125,000 cells per plate and exposed to 4 atm O_2 for 12 hours and then incubated at 37°C for an additional 12 hours. Although both groups of cells experienced increased cell death, the survival of the AD fibroblasts was significantly decreased: 28% non-AD compared to 71% AD cell death.

Cytotoxic Effect of Aβ

Cultured AD and non-AD skin fibroblasts were exposed to three different concentrations (50, 150 and 250μM) of $A\beta_{25-35}$. While Aβ proved toxic to both non-AD and AD fibroblasts, the AD lines were significantly more susceptible to Aβ toxicity (Figure 7). This toxicity was dose-dependent. Statistical analysis (ANOVA) showed the major difference between the two groups to be at 150μM Aβ. These results are consistent with previous reports of $A\beta_{25-35}$ toxicity [69,74,88]. However, $A\beta_{25-35}$

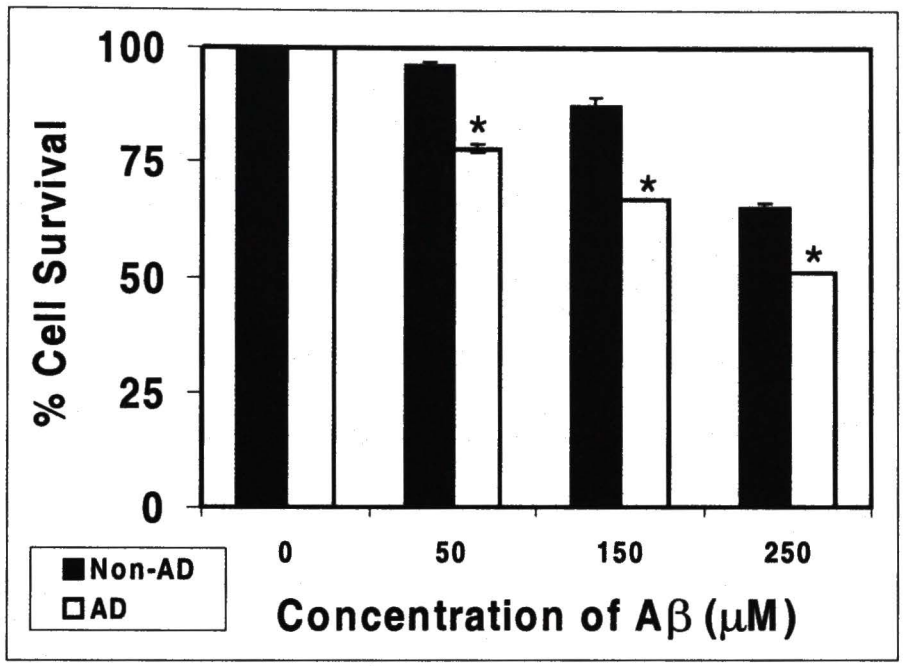


Figure 7. Survival of non-AD and AD fibroblasts following exposure to the $A\beta_{25-35}$ peptide. Non-AD (■) and AD (□) fibroblasts were grown to a density of 125,000 cells and then incubated as follows for a period of 24 hours. Control (no treatment); 50 μM Aβ; 150 μM Aβ; and 250 μM Aβ. Each bar represents the average and SEM of four separate 30 mm culture dishes. Astericks (*) represent significant differences between the two cell groups as analyzed by ANOVA.

induced cell death has not been shown in AD and non-AD cells and no comparisons have been made regarding susceptibility.

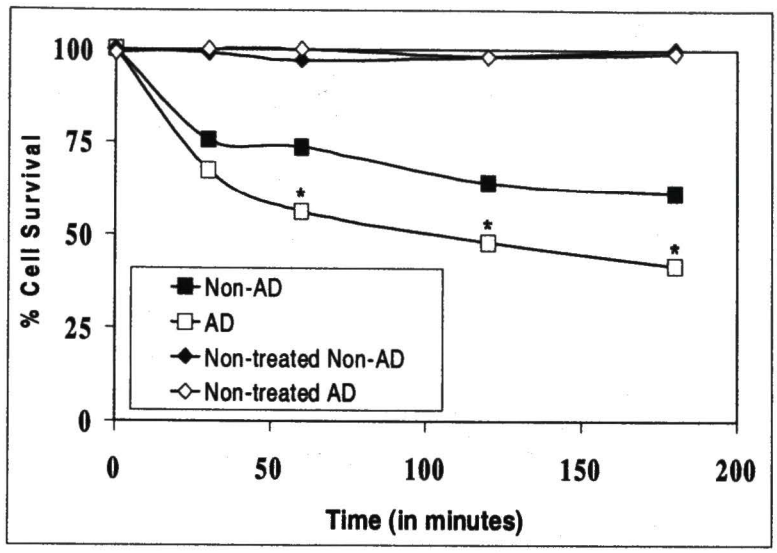
Effect of Hydrogen Peroxide on AD and Non-AD Fibroblasts' Survival

Four sets of AD and non-AD cells were plated at a density of 125,000 cells in 30mm culture plates and allowed to attach for 4-6 hours. The cells were then subjected to a bolus dose of hydrogen peroxide. Hydrogen peroxide (H_2O_2) was cytotoxic to both AD and non-AD fibroblasts and its toxicity was both dose dependent (data not shown) and time dependent. Figure 8A shows the effect of exposure to 350μM H_2O_2. The kinetics of the resulting killing was biphasic for both types of cells. The initial rate was much faster and significantly greater for the AD fibroblasts. The second, slower rate was essentially the same for the AD and normal cells. These data point out a problem associated with the direct addition of unstable ROS to cells in culture (see Discussion).

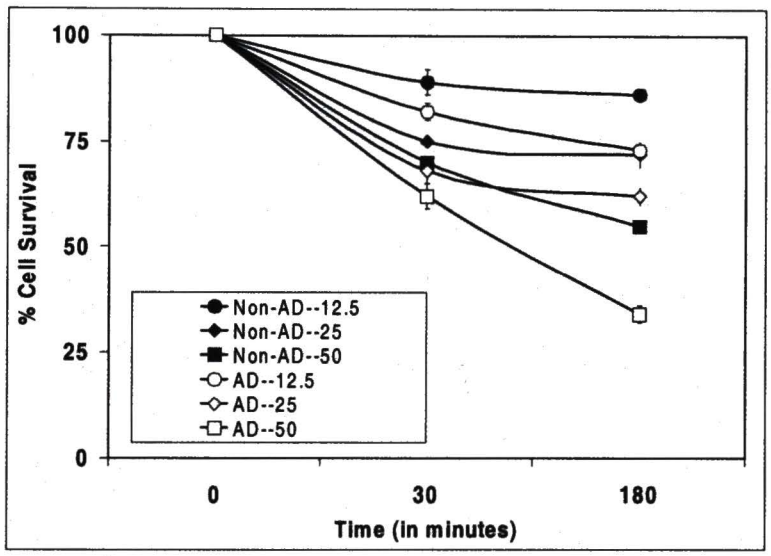
Xanthine-Xanthine Oxidase (X/XO) is an enzyme system that generates superoxide. The superoxide is then converted to H_2O_2 by superoxide dismutase (SOD). This approach has the distinct advantage of being a relatively stable system compared to a bolus dose of H_2O_2. Four sets of AD and non-AD fibroblasts were plated in 96-well plates at a density of 4,000 cells per well and incubated with various concentrations of xanthine and xanthine oxidase. The X/XO system was toxic to cultured skin fibroblasts. The survival of cells exposed to the X/XO system was found to be strongly dependent on time of exposure and concentration (Figure 8B). Statistical analysis (ANOVA) of cell survival percentages revealed highly significant differences between AD and non-AD

Figure 8. Panel A: Survival of non-AD and AD fibroblasts following exposure to a bolus of hydrogen peroxide. Non-AD(■) and AD (□) fibroblasts were plated in 96 well plates 4000 cells per well) and then incubated as follows: Control (no treatment); the rest were exposed to 350mM H_2O_2 for 0–3 hours. Each bar represents the average and SEM of four separate sets of AD and normal cells which were age-matched and at the same passage number.
Panel B: Survival of non-AD and AD fibroblasts following exposure to *in situ* generated ROS from xanthine/xanthine oxidase. Non-AD(■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and then incubated as follows: Control (no treatment); the rest were given various concentrations of Xanthine (1mM)/Xanthine Oxidase (12.5, 25, or 50 mUnits) for various timepoints (0-3 hours). Each bar represents the average and SEM of four separate wells.
Panel C is a table of the statistical analysis of the effect of *in situ* generated ROS on non-AD and AD Fibroblasts. Fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 12.5 mUnits X/XO for 3 hours is significantly different from non-AD fibroblasts treated with 12.5 mUnits X/XO for 30 minutes, but not different from AD fibroblasts treated with 25 mUnits X/XO for 30 minutes.

A



B



C

Xanthine Oxidase Treatment

Time	Cell Line	NonTreated	12.5 mUnits	25 mUnits	50 mUnits
30 min.	Non-AD	99 ± 1^{a}	89 ± 3^{b}	75 ± 3^{c}	70 ± 1^{c}
30 min.	AD	100 ± 2^{a}	82 ± 2^{b}	68 ± 3^{cd}	62 ± 1^{d}
3 hours	Non-AD	100 ± 2^{a}	86 ± 2^{b}	77 ± 1^{bc}	55 ± 3^{e}
3 hours	AD	97 ± 3^{a}	73 ± 1^{c}	62 ± 2^{de}	34 ± 1^{f}

fibroblasts. At higher concentrations and longer exposure to stress, the AD fibroblasts exhibited an enhanced susceptibility to the xanthine oxidase system.

Cytotoxic Effect of Peroxynitrite

Peroxynitrite is generated when nitric oxide and superoxide anion react. Cultures of non-AD and AD skin fibroblasts were exposed to three different concentrations of 3-morpholinosydnonimine hydrochloride (SIN-1), a chemical that mimics the effects of peroxynitrite. Table 3 shows that at 50μM SIN-1, as measured by the MTS assay, treated non-AD cells showed decreased viability (a 10-30% reduction) over a period of 1-24 hours. However, AD fibroblasts showed a significantly marked decrease in cell survival in a time-dependent manner, showing a 10% decline at 1 hour and exhibiting a 70% decrease at 24 hours. The differences between the two groups became more significant at higher concentrations of SIN-1, indicating a stronger susceptibility to this form of oxidative stress.

Cytotoxic Effect of Serum Deprivation

Serum deprivation causes significant oxidative events in cultured cells [87,11]. Serum starvation was cytotoxic to both AD and non-AD fibroblasts (Figure 9). However, cell survival is greatly decreased in the AD fibroblasts, reflecting a 50% decrease in cell death at 48 hours and continuing to decrease to 36% at 72 hours. These data support the observation that serum deprivation produces cell death [87]. However, increased

Time	Cell Line	NonTreated	50μM	100μM	200μM
1 hour	Non-AD	103 ± 1^{a}	91 ± 2^{b}	90 ± 2^{b}	88 ± 2^{b}
1 hour	AD	98 ± 2^{a}	92 ± 1^{b}	88 ± 1^{b}	85 ± 5^{b}
3 hours	Non-AD	100 ± 1^{a}	91 ± 2^{b}	90 ± 2^{b}	84 ± 2^{b}
3 hours	AD	97 ± 2^{a}	86 ± 2^{b}	83 ± 1^{b}	71 ± 2^{c}
6 hours	Non-AD	101 ± 2^{a}	73 ± 2^{c}	69 ± 2^{c}	68 ± 1^{c}
6 hours	AD	99 ± 3^{a}	43 ± 3^{d}	37 ± 1^{de}	32 ± 2^{e}
12 hours	Non-AD	98 ± 2^{a}	71 ± 3^{c}	72 ± 2^{c}	69 ± 3^{c}
12 hours	AD	99 ± 1^{a}	39 ± 2^{d}	32 ± 3^{e}	30 ± 2^{e}
24 hours	Non-AD	101 ± 2^{a}	68 ± 1^{c}	67 ± 2^{c}	63 ± 2^{c}
24 hours	AD	101 ± 1^{a}	37 ± 1^{d}	29 ± 2^{ef}	23 ± 2^{f}

Table 3. Effect of Peroxynitrite on survival of non-AD and AD Fibroblasts. AD and non-AD fibroblasts were treated with three different concentrations of SIN-1, a chemical that mimics peroxynitrite. Cell survival was measured by the MTS Method. Values represent the mean percent survival ± SEM for data obtained from four wells in a 96-well plate. The data were analyzed statistically by the ANOVA test, and those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 200μM SIN-1 for 6 hours is significantly different from non-AD fibroblasts treated with 200μM SIN-1 for 6 hours, but not different from AD fibroblasts treated with 200μM SIN-1 for 12 hours.

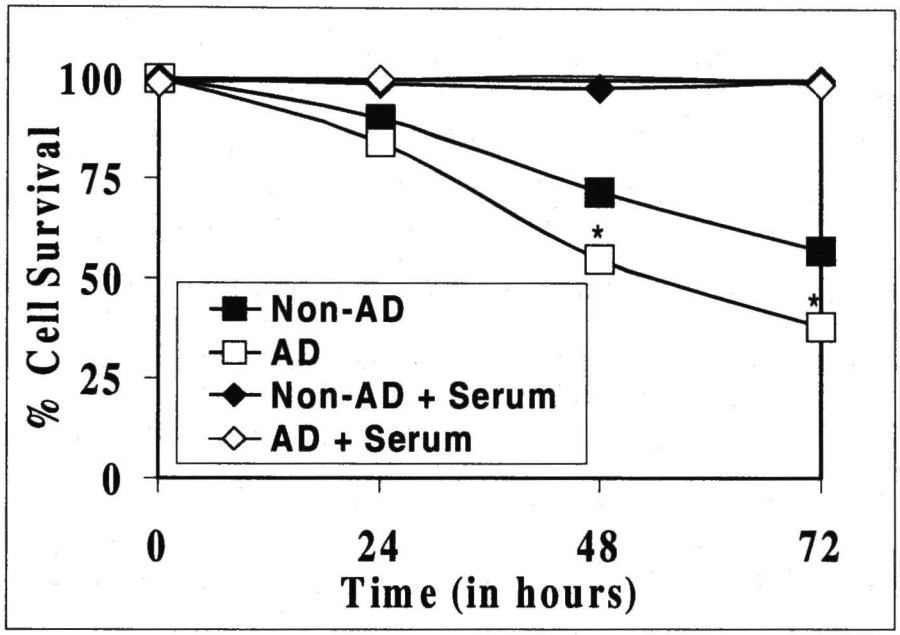


Figure 9. Survival of non-AD and AD fibroblasts following exposure to serum deprivation. Non-AD (■) and AD (□) fibroblasts were grown to a density of 2 million cells in 100 mm cell culture plates and then incubated as follows: Control (Serum Added); the rest were serum deprived for 24, 48, and 72 hours. At each time point, cell viability was measured using the MTS Assay. Asterisks (*) represent significant *p*-value differences of <0.05 between groups as analyzed by ANOVA.

vulnerability of AD fibroblasts to this form of oxidative stress has not previously been reported.

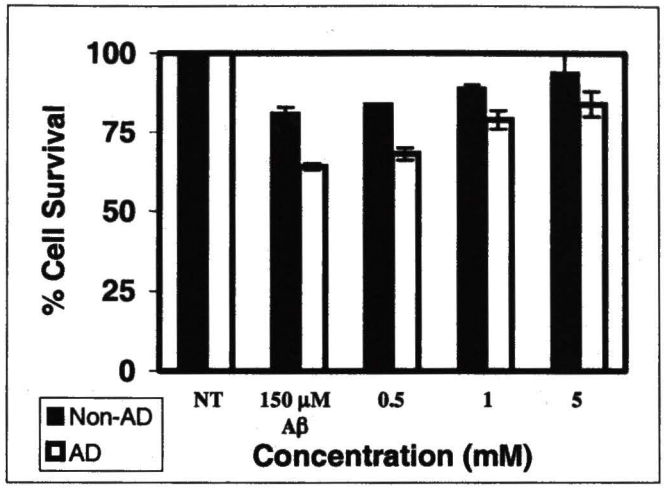
Effects of Antioxidants

The action of antioxidant added to the cells concurrent with different oxidants decreased the toxic effects of X/XO, serum deprivation, and Aβ. Fibroblasts were protected from oxidant treatment after treatment with glutathione (GSH), N-Acetyl-L-Cysteine (LNAC), and grape seed extract (SbE), as compared to untreated fibroblasts. Cell survival was increased in a dose-dependent manner and increased significantly above the level of stressed controls. Protection by SbE reached 100% in the AD fibroblasts, while that of GSH and LNAC was slightly lower.

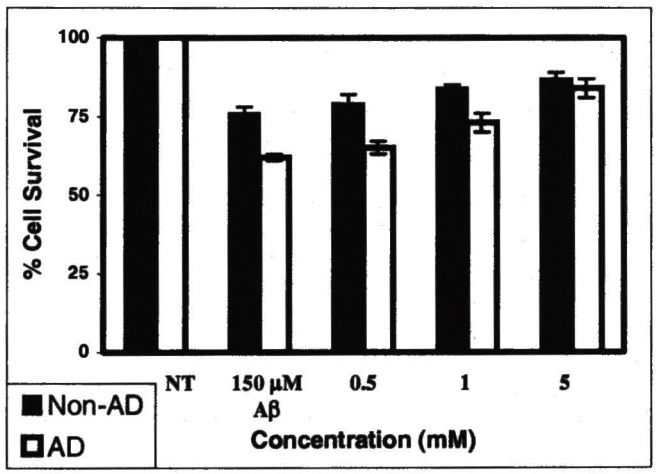
When cells were incubated with LNAC before Aβ exposure (Figure 10A), the toxicity of the peptide was eliminated at the highest concentration of LNAC (5mM). At lower concentrations, LNAC increased cell survival over stressed levels but had a more significant impact on the AD cells. GSH was not as effective against Aβ-induced cell death (Figure 10B). At the highest concentration of GSH, 5mM, both cell types still exhibited significant cell death (13% non-AD, 16% AD). However, AD cells showed a more significant improvement over non-AD cells under GSH exposure (11% non-AD vs. 22% AD increased survival). In the case of SbE, Aβ toxicity was virtually eliminated at its highest concentration of 12.5 mg/ml in both sets of cells (Figure 11). Survival was increased from 82 to 113% in non-AD cells while AD survival increased from 61% to 99%.

Figure 10. Panel A: Influence of N-acetyl-L-cysteine on the survival of non-AD and AD fibroblasts following exposure to beta-amyloid. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (No Treatment); 150μM Aβ; 150μM Aβ + 0.5mM NAC; 150mM Aβ + 1mM LNAC; and 150μM Aβ + 5mM LNAC for 24 hours. Each bar represents the average and SEM of four separate wells.
Panel B: Influence of Glutathione (GSH) on the survival of non-AD and AD fibroblasts following exposure to beta-amyloid. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); 150μM Aβ; 150μM Aβ + 0.5mM GSH; 150μM Aβ + 1mM GSH; and 150μM Aβ + 5mM GSH for 24 hours. Each bar represents the average and SEM of four separate wells.
Panel C represents the statistical analysis of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 1mM NAC is significantly different from AD fibroblasts treated with 0.5mM LNAC, but not different from non-AD fibroblasts treated with 1mM LNAC.

A



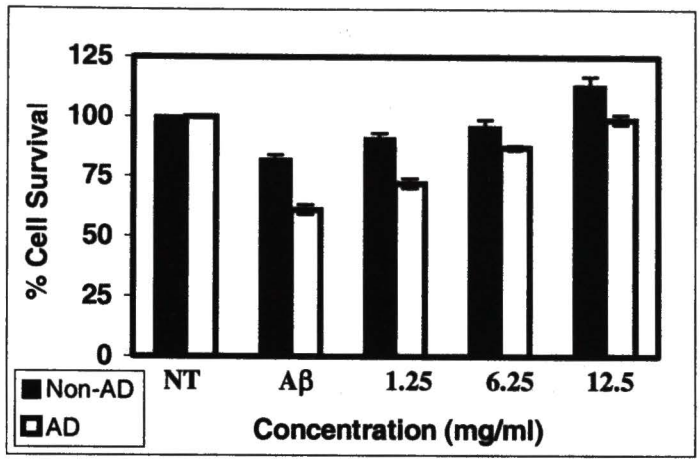
B



C

	Non-AD	AD
Non-Treated	101 ± 2[a]	98 ± 3[a]
Aβ (No Antioxidant)	76 ± 2[bc]	62 ± 1[d]
GSH (.5mM)	79 ± 3[bc]	65 ± 2[cd]
GSH (1mM)	84 ± 1[b]	73 ± 3[c]
GSH (5mM)	87 ± 2[b]	84 ± 3[b]
Non-Treated	97 ± 3[a]	99 ± 2[a]
Aβ (No Antioxidant)	83 ± 2[b]	65 ± 2[c]
LNAC (.5mM)	83 ± 0[b]	74 ± 2[c]
LNAC (1mM)	88 ± 1[b]	82 ± 4[b]
LNAC (5mM)	100 ± 6[a]	104 ± 4[a]

A



B

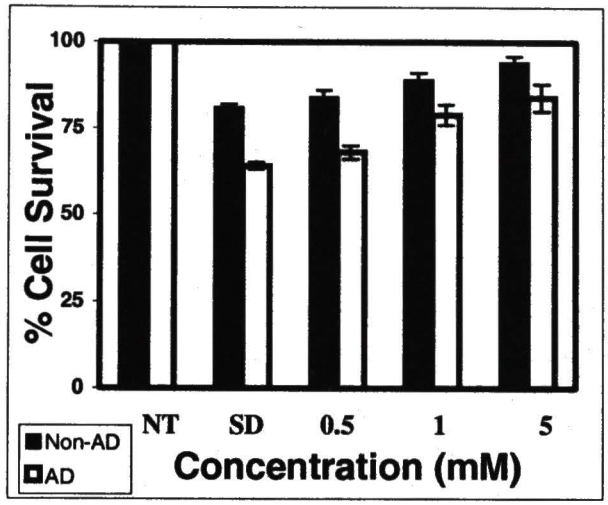
	Non-AD	AD
Non-Treated	100 ± 1[a]	98 ± 2[a]
Aβ (No Antioxidant)	82 ± 2[b]	61 ± 2[d]
SbE (1.25 mg/ml)	91 ± 2[ab]	72 ± 2[c]
SbE (6.25 mg/ml)	96 ± 3[a]	87 ±1[b]
SbE (12.5 mg/ml)	113 ± 4[a]	99 ± 2[a]

Figure 11. *S. baicalensis georgi* extract (SbE) increased the survival of non-AD and AD fibroblasts following exposure to beta-amyloid. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); 150 μM Aβ; 150 μM Aβ + 1.25 mg/ml SbE; 150 μM Aβ + 6.25 mg/ml SbE; and 150 μM Aβ + 12.5 mg/ml SbE for 12 hours. Each bar represents the average and SEM of four separate wells.

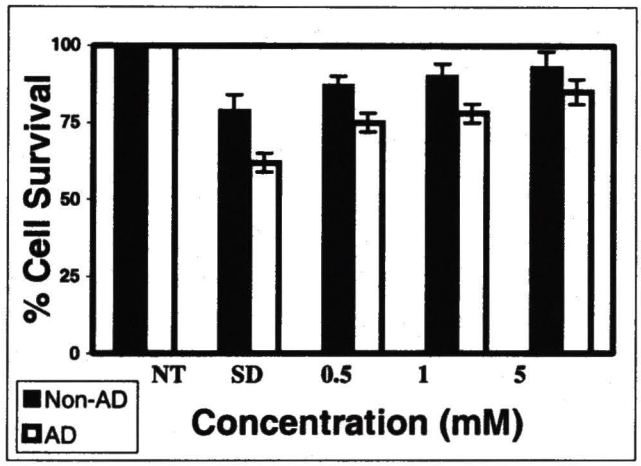
Panel B represents the statistical analysis (ANOVA) of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 12.5 mg/ml SbE is significantly different from AD fibroblasts treated with 6.25 mg/ml SbE, but not different from non-AD fibroblasts treated with 6.25 mg/ml SbE.

Figure 12. Panel A: Influence of N-acetyl-L-cysteine (LNAC) on the survival of non-AD and AD fibroblasts following exposure to serum deprivation. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (No Treatment); SD; SD + .5mM LNAC; SD + 1mM LNAC; and SD + 5mM LNAC for 48 hours. Each bar represents the average and SEM of four separate wells.
Panel B: Influence of Glutathione (GSH) on the survival of non-AD and AD fibroblasts following exposure to serum deprivation. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); SD; SD + .5mM GSH; SD + 1mM GSH; and SD + 5mM GSH for 48 hours. Each bar represents the average and SEM of four separate wells.
Panel C represents the statistical analysis (ANOVA) of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 1mM LNAC is significantly different from AD fibroblasts treated with 0.5mM LNAC, but not different from non-AD fibroblasts treated with 1mM LNAC.

A



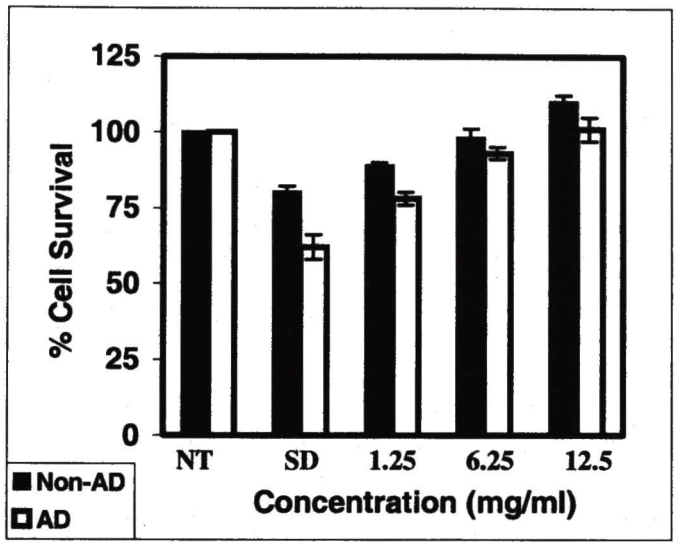
B



C

	Non-AD	AD
Non-Treated	98 ± 2[a]	99 ± 1[a]
SD (No Antioxidant)	79 ± 5[bc]	62 ± 3[d]
GSH (.5mM)	87 ± 3[b]	75 ± 3[c]
GSH (1mM)	90 ± 4[ab]	78 ± 3[bc]
GSH (5mM)	93 ± 5[ab]	85 ± 4
Non-Treated	101 ± 2[a]	99 ± 2[a]
SD (No Antioxidant)	81 ± 1[b]	64 ± 1[c]
LNAC (.5mM)	84 ± 2[b]	68 ± 2[c]
LNAC (1mM)	89 ± 2[ab]	79 ± 3[b]
LNAC (5mM)	94 ± 2[a]	84 ± 4[b]

A



B

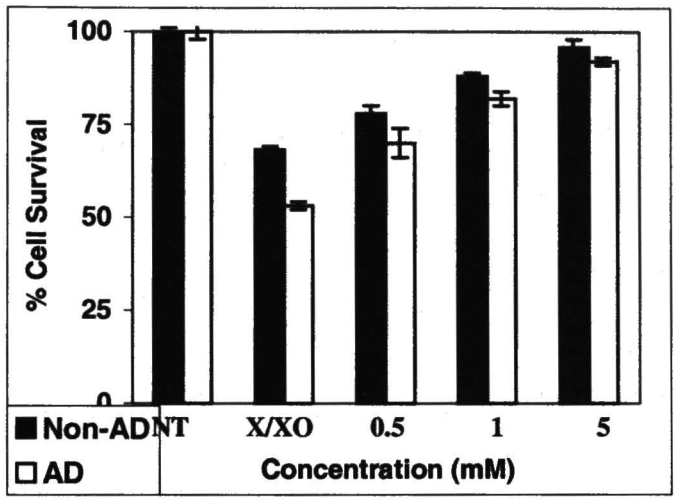
	Non-AD	AD
Non-Treated	97 ± 2 [a]	99 ± 1 [a]
SD (No Antioxidant)	80 ± 2 [bc]	62 ± 4 [d]
SbE (1.25 mg/ml)	89 ± 1 [ab]	78 ± 2 [c]
SbE (6.25 mg/ml)	98 ± 3 [a]	93 ± 2 [a]
SbE (12.5 mg/ml)	110 ± 2 [a]	101 ± 4 [a]

Figure 13. *S. baicalensis georgi* extract (SbE) increased the survival of non-AD and AD fibroblasts following exposure to serum deprivation. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); SD; SD + 1.25 mg/ml SbE; SD + 6.25 mg/ml SbE; and SD + 12.5 mg/ml SbE for 48 hours. Each bar represents the average and SEM of four separate wells.

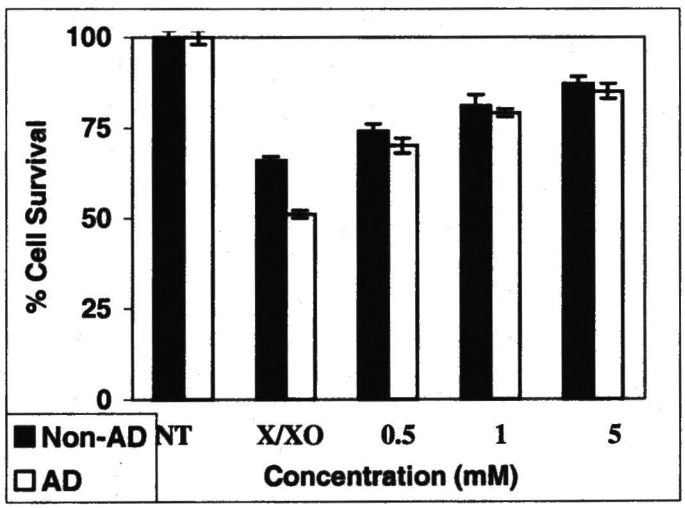
Panel B represents the statistical analysis (ANOVA) of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 6.25 mg/ml SbE is significantly different from AD fibroblasts treated with 1.25 mg/ml SbE, but not different from non-AD fibroblasts treated with 6.25 mg/ml SbE

Figure 14. Panel A: Influence of N-acetyl-L-cysteine (LNAC) on the survival of non-AD and AD fibroblasts following exposure to xanthine/xanthine oxidase. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (No Treatment); 1mM xanthine /50mU Xanthine Oxidase; X/XO + .5mM LNAC; X/XO + 1mM LNAC; and X/XO + 5mM LNAC for 2 hours. Each bar represents the average and SEM of four separate wells.
Panel B: Influence of Glutathione (GSH) on the survival of non-AD and AD fibroblasts following exposure to xanthine/xanthine oxidase. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); 1mM xanthine /50mU Xanthine Oxidase; X/XO + .5mM GSH; X/XO + 1mM GSH; and X/XO + 5mM GSH for 2 hours. Each bar represents the average and SEM of four separate wells.
Panel C represents the statistical analysis (ANOVA) of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 1mM LNAC is significantly different from AD fibroblasts treated with 0.5mM LNAC, but not different from non-AD fibroblasts treated with 1mM LNAC.

A



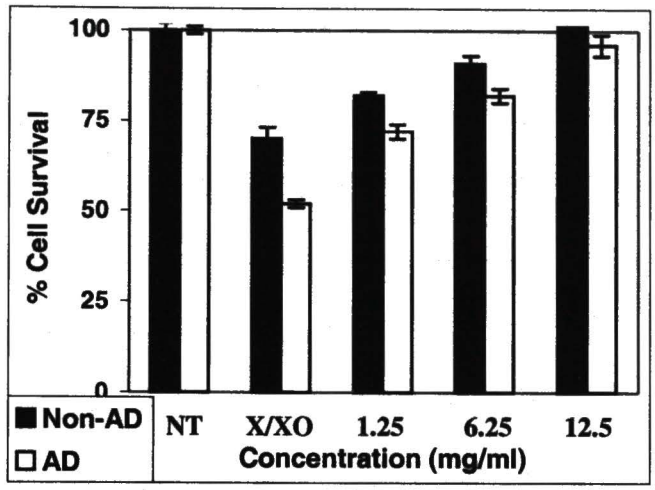
B



C

	Non-AD	AD
Non-Treated	100±2[a]	100±2[a]
X/XO (No Antioxidant)	66±1[d]	51±2[e]
GSH (.5mM)	74±2[c]	70±2[d]
GSH (1mM)	81±3[bc]	79±1[c]
GSH (5mM)	87±2[b]	85±2[b]
Non-Treated	100±1[a]	100±2[a]
X/XO (No Antioxidant)	68±1[c]	53±1[e]
NAC (.5mM)	78±2[c]	70±4[cd]
NAC (1mM)	88±1[ab]	82±2[b]
NAC (5mM)	96±2[a]	92±1[a]

A



B

	Non-AD	AD
Non-Treated	100±2[a]	100±1[a]
X/XO (No Antioxidant)	70±3[c]	52±1[d]
SbE (1.25 mg/ml)	82±1[b]	72±2[c]
SbE (6.25 mg/ml)	91±2[a]	82±2[b]
SbE (12.5 mg/ml)	102±2[a]	96±3[a]

Figure 15. *S. baicalensis georgi* extract (SbE) increased the survival of non-AD and AD fibroblasts following exposure to xanthine/xanthine oxidase. Non-AD (■) and AD (□) fibroblasts were plated in 96 well plates (4000 cells per well) and incubated as follows: Control (no treatment); 1mM xanthine/ 50mU Xanthine Oxidase; X/XO + 1.25 mg/ml SbE; X/XO + 6.25 mg/ml SbE; and X/XO + 12.5 mg/ml SbE for 2 hours. Each bar represents the average and SEM of four separate wells.
Panel B represents the statistical analysis (ANOVA) of the data. Those fibroblasts that show a significant difference ($p<0.05$ level) in percent of cell survival are indicated by different letter superscripts. For example, the percentage of cell survival of AD fibroblasts treated with 6.25 mg/ml SbE is significantly different from AD fibroblasts treated with 1.25 mg/ml SbE, but not different from non-AD fibroblasts treated with 1.25 mg/ml SbE

When the fibroblasts were exposed to the X/XO system, exposure to the three antioxidants revealed a similar pattern. LNAC was very effective, increasing survival to 96 and 92%, non-AD and AD respectively (Figure 12A). GSH was not as effective but significantly increased survival compared to stressed fibroblasts (Figure 12B). SbE was slightly more effective than LNAC against X/XO induced toxicity, bringing survival levels to 102% non-AD and 96% AD (Figure 13).

When both groups of cells were exposed to LNAC or GSH prior to serum withdrawal, the results were basically the same (Figures 14A and B). Both LNAC and GSH appeared to have the same effect on cell survival. At their highest concentration of 5mM, both antioxidants increased survival to 94% non-AD and 85% AD. SbE eliminated oxidative stress associated with serum deprivation (Figure 15). AD survival was 101% when treated with 12.5 mg/ml while that of non-AD was 110%.

Protein Oxidation

Cells were nontreated or exposed to 150μM Aβ or a combination of 150μM Aβ and 5mM GSH for 24 hours. Cellular proteins were extracted and separated using both 1-D and 2-D PAGE. Figure 16 is a 1-D PAGE of non-AD and AD fibroblasts. Lanes 1 and 2 are the untreated fibroblasts. There is very little difference in carbonyl content between the two groups. However, the Aβ treated AD fibroblasts, shown in Lane 4, show a two-fold increase in carbonyl content compared to the Aβ treated non-AD fibroblasts shown in Lane 3. This increase in carbonyl content was reduced when the AD fibroblasts were treated with the antioxidant glutathione (Lane 6).

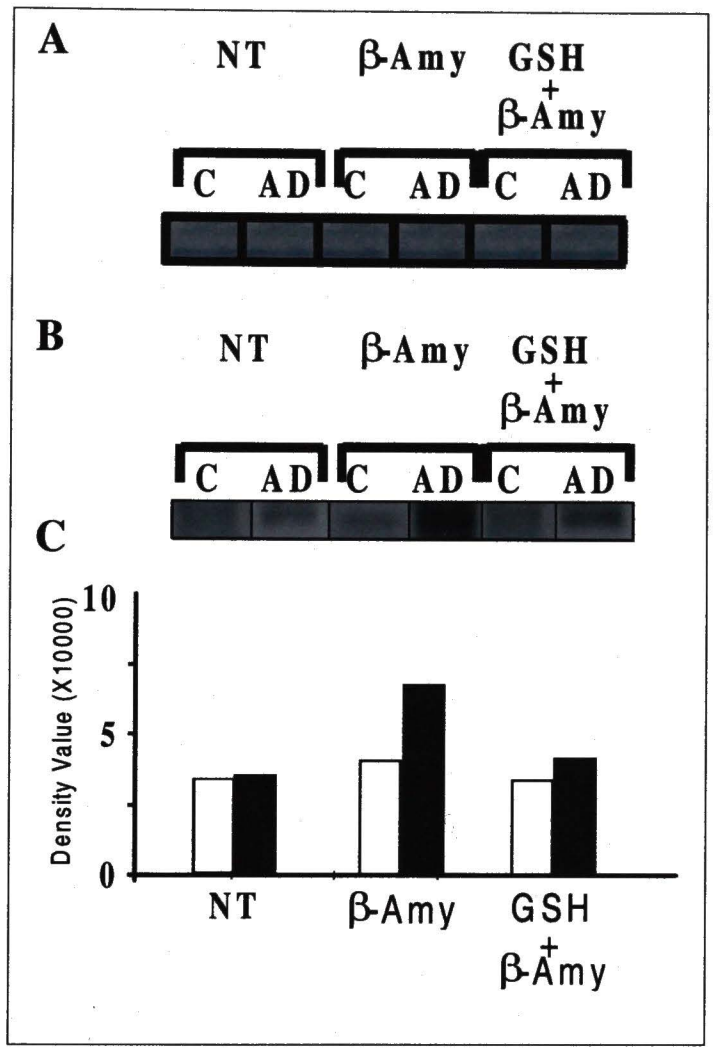
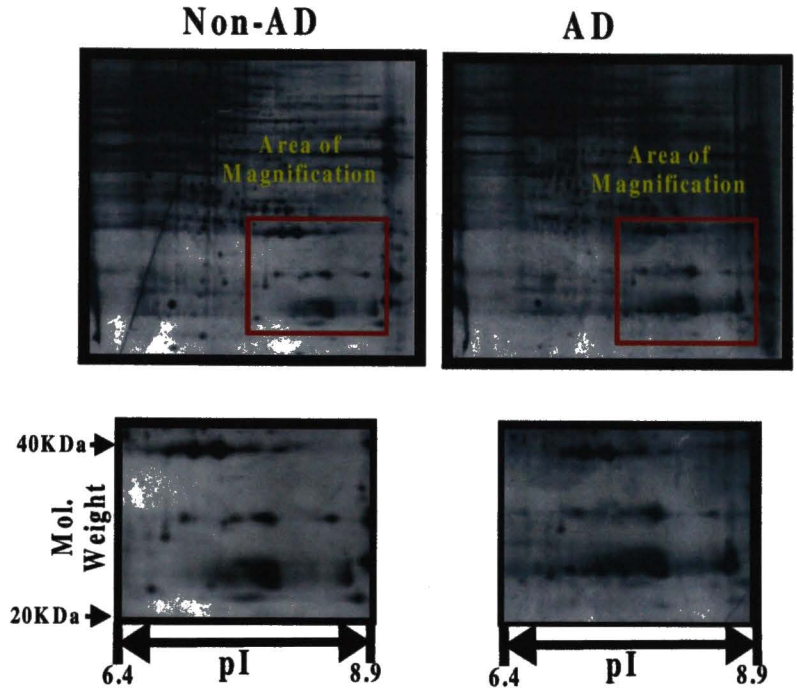


Figure 16. Effect of Aβ stress on specific fibroblast proteins. Following a 24 hour exposure of cells to Aβ and glutathione, protein carbonyl content was measured by derivatization with DNP as described in methods. Panel A represents the total protein stain of the 1-D gel. Panel B shows the western blot of carbonyl containing proteins from cells exposed to no oxidative stress, 150μM Aβ, or Aβ + GSH. Non-treated non-AD and AD fibroblasts are shown in Lanes 1 and 2, respectively. Lanes 3 and 4 are Aβ treated non-AD and AD fibroblasts. Non-AD (Lane 5) and AD (Lane 6) fibroblast samples were then treated with both Aβ and 5mM glutathione. Panel C shows a histogram of the results in the western blot.

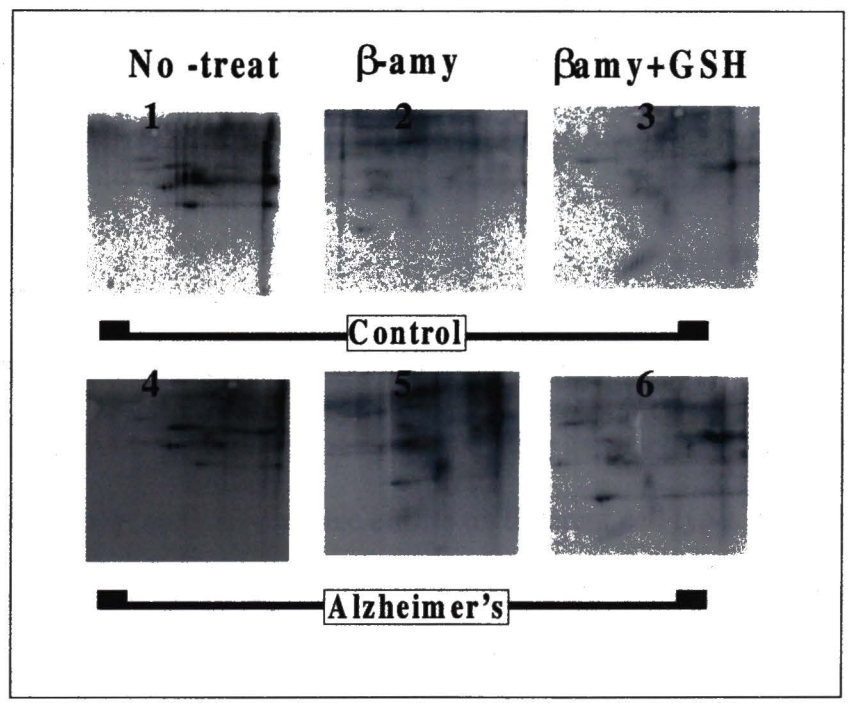
Figures 17A and B represent 2-D PAGEs of the non-AD and AD fibroblasts. Figure 17A shows the total protein stains. The total protein profiles indicate the presence of some protein spots in the AD fibroblasts not present in the non-AD cells. Figure 17B are the immunoblots of the same gels. As shown in Figure 17B, little difference was seen between the non-treated non-AD and AD fibroblasts. However, Aβ treated AD fibroblasts revealed more oxidized proteins than Aβ treated non-AD fibroblasts. In addition, these specifically oxidized proteins decreased upon co-treatment with the antioxidant glutathione.

Figure 17: Comparison of AD and non-AD fibroblasts treated with amyloid beta peptide (25-35) or a combination of Aβ and glutathione. Cell proteins were isoelectrically focused on IPG strips (pH 4-7), as described in Materials and Methods. The resultant proteins were then separated using 2-D PAGE, and either silver stained for total protein content or immunostained using an anti-DNP antibody. Panel A. Total protein stains of non-treated non-AD and non-treated AD fibroblast proteins.
Panel B. Immunoblot comparisons of AD and non-AD fibroblasts treated with Aβ or a combination of Aβ and glutathione. Non-treated non-AD (B-1), non-treated AD (B-2), Aβ treated non-AD (B-3), Aβ treated AD (B-4), Aβ and GSH treated non-AD (B-5) and Aβ and GSH treated AD fibroblasts (B-6) are shown.

A


Non-AD
AD
Area of Magnification
Area of Magnification
40KDa
Mol. Weight
20KDa
6.4
pI
8.9
6.4
pI
8.9

B


No -treat
β-amy
βamy+GSH
1
2
3
Control
4
5
6
Alzheimer's

DISCUSSION

The abnormalities that have been described by several groups in AD fibroblasts, suggest that AD fibroblasts reflect the disease. This makes the AD fibroblast model a valid tool for the study of AD. Increasing evidence suggests that oxidative damage to proteins is a prominent feature of the pathophysiology of AD [32,71]. In addition, several lines of evidence indicate that AD has systemic expression, manifested as alterations in biochemical processes outside the central nervous system [28]. The use of cultured fibroblasts complements other approaches in AD research. In order to establish which oxidants play a pathogenic role in AD, several types of oxidative stressors were employed in AD and control fibroblasts. These stresses included Aβ, hydrogen peroxide, peroxynitrite, and superoxide. An understanding of the effects of stress in fibroblasts may provide further insight into the pathophysiology of AD.

Cell Viability

Amyloid beta peptide (Aβ) has been hypothesized to play a major role in AD neurodegeneration based on its free radical generating capacity. Aβ has been shown to have both neurotoxic and neurotrophic effects on different cell types depending on the concentration of the amyloid peptide [13,27,67,104]. The level of toxicity induced by Aβ may depend on the presence or absence of other proteins or cofactors. For example,

Boggs [15] has shown that Aβ1-40 is toxic to fibroblasts. Treatment of cells with Aβ may induce a set of genes in the cells that can alter the phenotype in ways that increase susceptibility to oxidative damage or lead to apoptosis.

Aβ has been shown to enhance oxidative stress and stimulate increases in hydrogen peroxide (H_2O_2) [9]. It has also been proposed that Aβ induces death possibly through the generation of peroxynitrite from superoxide and nitric oxide [120]. Cell culture studies have shown that Aβ can disrupt calcium homeostasis and induce apoptosis by a mechanism involving oxidative stress. In fact, Aβ can induce caspase-dependent apoptosis in cultured neurons [120]. To evaluate Aβ toxicity to non-neuronal cells, fibroblasts from non-AD and AD patients were treated with Aβ.

AD and non-AD fibroblasts treated with low levels of Aβ, then placed under stress conditions, reacted differently (Fig 6 and 7). When treated with Aβ alone, AD fibroblasts exhibited a significant decrease in survival compared to non-AD cells. This difference between the two cell types is exacerbated when cells are placed under HBO conditions. These results support previous observations that the $A\beta_{25-35}$ peptide is cytotoxic to cultured fibroblasts [69] and also supports previous findings that Aβ, on its own, directly generates free radicals [18]. More importantly, these results also demonstrate an enhanced killing effect in the AD cell lines. These data suggest that sensitivity to oxidative stress could be systemic to AD patients and not localized to neurons.

A widespread occurrence of nitrotyrosine, an indicator of peroxynitrite involvement, has been detected in the neurons of AD patients [36,111]. This suggests that peroxynitrite, formed when superoxide and nitric oxide react, could be implicated in the oxidative damage in AD. Peroxynitrite is a strong oxidizing and nitrating agent that can react with all classes of biomolecules. The toxicity of peroxynitite results from the modification of these molecules, especially DNA, and the induction of an apoptotic pathway [65]. A recent report demonstrated that Aβ is able to induce an accumulation of mitochondrial peroxynitrite [76,119]. Once it is formed, peroxynitrite can inhibit components of the mitochondrial respiratory chain leading to a cellular energy deficiency state [8,46].

The cytotoxic action of peroxynitrite can be mimicked by 3-morpholino-sydnonimine hydrochloride (SIN-1), which liberates both nitric oxide and superoxide [5]. The results shown in Table 3 confirmed that oxidative stress generated *in vitro* is more damaging to AD fibroblasts than to non-AD control cells.

These results are supported by previous research which revealed that SIN-1 represents a strong stress which can lead to increases in heat shock protein expression, mitochondrial membrane depolarization, and cell death by apoptosis [2]. The data suggest that AD cells are more susceptible to oxygen radical damage than are normal controls. The significant difference between the two groups strengthens the theory that oxidative susceptibility in AD is not restricted to the brain.

In addition to oxidative stress by Aβ and peroxynitrite, the effects of hydrogen peroxide (H_2O_2) were also examined. H_2O_2 is an intermediate molecule produced during

enzymatic antioxidant defense in cells. It results from the dismutation of superoxide anions by superoxide dismutases. A number of forms of biological damage, such as lipid peroxidation, and DNA damage, have been linked to H_2O_2. It has been shown to induce apoptosis in different cultured cell lines. Hydrogen peroxide damages DNA in the presence of metals and causes cell death in fibroblasts and bacteria, mutagenicity in bacteria, and has been shown to cause tumors in Drosophila embryos. It also slowly but irreversibly inactivates the enzyme superoxide dismutase (SOD), increases p53 translocation to the nucleus, and can activate mitogen-activated protein kinase kinase kinase [121,53]. In addition, H_2O_2 has been demonstrated to be the mediator of the cytotoxic action of Aβ protein in AD.

H_2O_2 alone is cytotoxic to fibroblasts but the rate of killing does not differ between the two cell groups (Figure 8, Panel A). This indicates that a one-time bolus treatment with the unstable H_2O_2 is not an effective tool for studying oxidative stress.

The effects of the endothelium-associated enzyme xanthine/xanthine oxidase (X/XO) system were evaluated against a one-time bolus of hydrogen peroxide (H_2O_2). The X/XO system continuously generates $O_2^{\cdot -}$, which is converted to H_2O_2 through the action of SOD. Xanthine/xanthine oxidase has been shown to significantly alter cell function and increase intracellular concentrations of calcium and ROS [16]. Increased intracellular calcium is known to enhance the conversion of xanthine dehydrogenase (which metabolizes xanthine to uric acid plus NADH) to xanthine oxidase (converts xanthine to uric acid plus superoxide radical).

The actual mechanism by which cells become damaged while exposed to this system is still uncertain. There is some evidence that $O_2^{\cdot -}$ alone formed extracellularly does not cause death [43]. Instead, the SOD enzyme is responsible for the formation of H_2O_2, the major cytotoxic product formed in the X/XO system [48,62,127]. X/XO triggered cell death is associated with DNA fragmentation and increases in ROS occur before cell death is initiated [101].

When treated with the X/XO system, rates of killing were significantly different between the non-AD and AD fibroblasts (Figure 8, Panel B). This indicates that a one-time bolus treatment of H_2O_2 is not as effective as *in vitro* generation of H_2O_2. Consistent with the previous results, these data demonstrate that the survival of AD fibroblasts exposed to the X/XO oxidation system is significantly less than that of non-AD fibroblasts.

Serum deprivation has also been shown to induce apoptosis. It causes significant oxidative events in cultured cells [87,24]. These oxidative events include inhibition of cystine uptake, a concurrent depletion of the antioxidant glutathione (cystine is preferentially directed from protein synthesis to GSH production), and consequent oxidative stress leading to the death of the cell [11]. Apoptosis upon serum withdrawal results from the leakage of cytochrome c to cytosolic portions of the cell and activation of caspases. This release of cytochrome c is due to an absence of glycolytic production of ATP as well as limited redox potential of the mitochondria that results from a reduced supply of substrates for oxidative respiration [11]. This leads to a reduced mitochondrial membrane potential and subsequently, leakage of cytochrome c.

To study how serum deprivation and subsequent increases in intracellular oxidation effects cell viability, AD and non-AD fibroblasts were grown in serum free DMEM for various time periods up to 3 days. The control fibroblasts were maintained in complete growth medium. No significant survival differences were observed between the two groups at 24 hours (Figure 9). However, at both the 2 and 3 day time-periods, significant differences were observed between the AD and non-AD fibroblasts. Significantly increased levels of cell death were observed over control cultures ($p<0.05$). These data suggest that under serum deprivation, the subsequent increase in oxidative stress is more detrimental to AD fibroblasts than non-AD fibroblasts, inducing cells to apoptose. AD fibroblasts exhibit an enhanced vulnerability to conditions where there is an absence of growth and survival factors. Serum also contains antioxidants. This marked decrease of survival seen in the AD cells could be explained by their deficient antioxidant defense system.

Antioxidants

The oxidative stress hypothesis of AD suggests that prevention of Aβ induced oxidative damage to neurons, may slow or stop such damage. Recent reports suggest that the levels of antioxidant enzymes are altered in the brains of patients with dementia of the Alzheimer's type, supporting the idea that the AD brain is under increased oxidative stress [57,66,123].

Evidence that antioxidant therapies may slow the progression of AD has been reported. Many free radical scavengers such as vitamin E have produced promising

results in relation to AD [76]. In-vitro Aβ neurotoxicity has been prevented by antioxidants [9]. Clinical trials on elderly human populations show that antioxidants are associated with improved memory and learning performance [86]. Moreover, a preliminary study of patients with moderate cases of AD has found that supplementation with antioxidants could significantly delay AD related complications [99]. Antioxidants in general, have been shown to decrease free radical induced cell damage. Three antioxidants, glutathione (GSH), N-acetyl-L-cysteine (LNAC), and grape seed extract (SbE), were tested against Aβ, superoxide, and serum deprivation in order to examine their ability to prevent cell death against oxidative stress.

Oxidative stress-mediated cell death may be initiated by a decline in GSH [20]. Glutathione (GSH) is an important part of the body's antioxidant defense system. It is an important cerebral mitochondrial antioxidant maintaining both vitamin E and vitamin C in their reduced state and removing potentially damaging peroxides. Incubation with $A\beta_{25-35}$ has been shown to reduce the intraneuronal level of GSH [77]. GSH depletion can enhance oxidative stress, and it has been suggested that this deficiency may also increase the levels of excitotoxic molecules. Blood levels of glutathione decline with age and low glutathione levels are associated with a higher incidence of diseases in the elderly [90]. In fact, the susceptibility of different cell types to nitric oxide and peroxynitrite may be dependent on the intracellular glutathione concentration [46].

The AD brain is characterized by oxidative stress, manifested by protein oxidation, lipid oxidation, oxidized glutathione, and decreased activity of glutathione S-transferase [20]. To examine whether elevated levels of GSH would offer protection

against free radical-induced oxidative stress, fibroblasts were treated with both GSH and NAC, a known precursor of glutathione, prior to stress treatments.

As seen in Figures 10B, 12B, and 14B, GSH was significantly protective against Aβ, superoxide, and serum deprivation induced cell death. The protective effect of GSH is confirmed by past studies that showed that GSH protects cultured cells against various oxidative stressors including Aβ [4,10].

Interestingly, exposure to antioxidants fifteen minutes prior to Aβ incubation drastically reduces cell death caused by the peptide. In Figures 10A, 12A, and 14A, the protective effects of LNAC are demonstrated. LNAC elevates intracellular levels of GSH [81]. It has been shown to provide protection against free radicals as well as a broad range of toxic hazards [81]. The key to this protection is the sulphur and sulfhydryl groups that it contains [52]. In addition, LNAC is a good precursor of glutathione [81]. LNAC proved to be very effective against toxicity of Aβ. Both non-AD and AD fibroblasts showed significant increases in survival against all three oxidative stressors. These data support previous observations that LNAC prevents apoptotic death during serum deprivation [30], and protects against Aβ and superoxide induced cytotoxicity [51,126].

In addition to NAC and GSH, a third antioxidant, grape seed extract (SbE), was examined. Studies have reported that *S. baicalensis* prevents lipid peroxidation [32]. Actually, its antioxidant properties have been traced to several of its flavones. Grape seed extract is a potent extract, rich in flavonoids, which is used for fighting free radicals.

Flavonoids improve the bioavailability of vitamin C. One group of the flavonoids have antioxidant properties, the oligomeric proanthocyanidin complexes or OPCs.

OPCs have been reported to provide significantly greater protection against free radicals than vitamins C and E [7]. Gao et. al reported that GSE prevents human fibroblast cell damage by reducing hydroxyl and superoxide radicals [32]. Furthermore, Shao et. al demonstrated that SbE markedly suppressed oxidative stress in cardiomyocytes [109]. In Figures 11, 13, and 15, the effects of SbE protection against Aβ, superoxide, and serum deprivation were studied. The results of this experiment support previous observations with SbE. SbE significantly increased fibroblast survival of both non-AD and AD cell lines under all three oxidant stressors. In fact, SbE increased AD fibroblast survival to 100% in all three cases.

These results are consistent with previous experiments that antioxidant therapy in neurodegenerative diseases associated with oxidative stress, including AD, may be promising.

Protein Oxidation

Protein oxidation often results in the formation of new carbonyl groups. Utilizing this carbonyl addition, it is possible to visualize specific sites of oxidation. This is accomplished by forming a hydrazone derivative using 2,4-dinitrophenylhydrazine (DNPH). The ability to identify specific proteins that are most susceptible to oxidative modifications may be the key to the development of methods for early diagnosis,

assessment of new potential therapies and understanding the overall Alzheimer's disease mechanism.

Encouraged by the cell survival results, I utilized high resolution 2-D PAGE and western blotting of the fibroblast proteins to more specifically identify individual oxidized proteins from AD and non-AD controls. Figure 16 represents the 1-D PAGE. Although no change in carbonyl content occurs between the non-treated non-AD and AD cells, treatment with Aβ resulted in a two-fold increase in carbonyls in the AD fibroblasts (Lane 4). Moreover, treatment with GSH 15 min. prior to incubation with Aβ resulted in a decrease in carbonyl content almost to the original, non-treated level in the AD fibroblasts (Lane 6).

Oxidation 2-D PAGE profiles (Figure 17B) indicated a much stronger oxidation signal in the AD samples (panel 4), even though similar amounts of protein were loaded on the blots (Figure 17A). Some of these specific oxidized fibroblast proteins could be used as a biomarker(s) for the detection and monitoring of AD. In addition, the use of the antioxidant GSH reduced the carbonyl content and strength of the oxidation signal (Figure 16B, panel 6).

Conclusions

This study confirms that oxidative stress generated *in vitro* is damaging to cultured skin fibroblasts. Moreover, the results indicate that fibroblasts from AD patients are more susceptible to several different types of oxidants. These results are in addition to the findings of elevated apoptotic features in the brain [58,117] and in peripheral blood

cells of AD patients [26]. It is clear that genetic and other risk factors relevant for AD lead to a similar pattern of cell death not only in brain cells, but also in peripheral cells. The observation that antioxidants can prevent free radical mediated cell death suggests possible therapeutic agents against AD associated damage.

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