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Sulforaphane as a multifunctional neuroprotective molecule to prevent and slow down the progression of Alzheimer's disease

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1 INTRODUCTION

During normal aging the brain undergoes many changes resulting in a gradual but detectable cognitive decline that is associated with limited neuronal loss, glial proliferation in the cortex and gross weight decrease of 2-3% per decade. On the molecular level, the mechanism driving aging of the brain are not yet understood, but likely include mitochondrial DNA damage and chronic oxidative stress. This slow decline in cognitive ability does not interfere with normal function through at 100 years of life. In contrast, Alzheimer's disease (AD) is a debilitating neurodegenerative disorder associated with a rapid cognitive decline with an average of survival of 5-10 years after diagnosis. Furthermore, AD clearly differs from the normal aging in that it causes dramatic loss of synapsis, neurons and brain activity in specific anatomical regions, and results in massive atrophy and gliosis.

The factors that cause some individuals to depart from the relatively benign process of normal aging brain and instead undergo the pathological cascade that leads to AD are unknown (1).

Although memory loss is usually the initial and most prominent problem, deficits in cognitive domains other than memory can occur in the early stages of disease in some patients. Final deterioration leads to a bedridden, mute, incontinent, and unresponsive state, which mimics the persistent vegetative state. Families frequently say that they can live with the memory and other cognitive deficits of their loved ones, but that the behavioral (ex, psychosis, aggression, and agitation) and mood-related symptoms (ex, depression) are much more difficult to handle. Psychotic symptoms (ex, hallucinations, delusions), wandering, aggressive behavior, psychomotor agitation and depressive symptoms (ex. sadness, anxiety, depressed mood) are common in AD. Not all patients with AD, however, have these abnormal behaviors. It has been suggested that these behaviors are associated with specific phenotypes with a different natural history, and they may have a genetic component (2).

Because AD cannot be cured and is degenerative, management of patients is essential. The role of the main caregiver is often taken by the spouse or a close relative. Alzheimer's disease is known for placing a great burden on caregivers; the pressures can be wide-ranging, involving social, psychological, physical, and economic elements of the caregiver's life. In developed countries, AD is one of the most economically costly disease to society.

1.1 Epidemiology and risk factors

AD is the most common form of dementia, accounting for 50-60% of all cases. The prevalence of dementia is below 1% in individuals aged 60-64 years, but shows an almost exponential increase with age, so that in people aged 85 years or older the prevalence is between 24 and 33% in the Western World. Thus, with the vast majority of baby boomers now entering the dreaded 65 years, numerous occidental countries are facing a possible dantesque scenario for their respective health plans. Instead, in 2001, more than 24 million people had dementia, a number that is expected to double every 20 years up to 81 million in 2040 because of the anticipated increase in life expectancy, constituting a major public-health problem.

Besides ageing, which is the most obvious risk factor for the disease, epidemiological studies have suggest several potential association. Some can be linked to a decrease reserve capacity of the brain, including reduced brain size, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life. Moreover, several epidemiological studies have shown that head injury could be a risk factor. Whether brain trauma initiates the pathogenic cascade leading to plaque and tangle formation or whether it simply reduces the brain reserve capacity is still unclear.

Other risk factors are associated with vascular disease, including hypercholesterolemia, hypertension, atherosclerosis, coronary hearth disease, smoking, obesity and diabetes. Whether these are causal risk factors for AD, driving the pathogenic processes resulting in plaque and tangles formation, or whether they induce cerebrovascular pathology, which adds to clinically silent disease pathology thus exceeding the threshold for dementia, needs to be elucidate.

Furthermore, environmental factors might increase the risk of Alzheimer's disease, this form of the disease has been shown to have a significant genetic background. A large population-based twin study showed that the extent of heritability for the sporadic disease is almost 80% (3).

1.2 Genetics

The amount of risk of Alzheimer's disease that is attributable to genetics is estimated to be around 70%. Established genetic causes of Alzheimer's disease include dominant mutations of the genes encoding amyloid precursor protein (APP), and presenilin 1 (PSEN1) and PSEN2. These genes have been essential in our understanding of Alzheimer's disease mechanisms, although they are the cause of Alzheimer's disease in only 5% of patients, who usually develop onset of clinical symptoms in midlife.

SORL1 has also been identified as an important genetic cause of late-onset Alzheimer's disease, at least one further familial Alzheimer's disease gene is thought to exist, possibly located on chromosome 10. Several potential risk genes for Alzheimer's disease have also been identified. The most consistently associated risk gene is ApoE. Individuals with two ApoE ε4 alleles have a more than seven times increased risk of developing Alzheimer's disease compared with those with ApoE ε4 alleles. Many candidates risk genes have been also identified but not confirmed by initial studies.

PSEN1 and PSEN2 mutations affect concentrations of Aβ (1-42) because the presenilin proteins form part of γ secretase, which cleaves APP to produce Aβ. SORL1, one of the VPS10 domain-containing receptors families genes, reduces the interaction between APP and β secretase. ApoE also seems to affect the rate of Aβ clearance. Several other genes that affect Alzheimer's disease risk possibly have roles in the clearance or uptake of Aβ

Tau mutations result in tauopathies, such as corticobasal degeneration and frontotemporal dementias, but not Alzheimer's disease. However, tau is an important pathological substrate of Alzheimer's disease and is a potential treatment target because tau tangles are more closely associated with the severity of dementia than are Aβ plaques.

Polymorphisms of phosphokinases, such as DYRK1A, might be associated with an increased risk of Alzheimer's disease and might have a role in explaining the link between Aβ and tau pathology because DYRK1A is up regulated by Aβ. The TOMM40 gene is located in a region of chromosome 19 that is in linkage disequilibrium with APOE, and a repeat polymorphism in this gene affects the age of onset of late-onset Alzheimer's disease in patients with an APOE genotype. Furthermore, recent genome-wide association studies in patients with Alzheimer's disease have identified mutations in genes such as CLU and PICALM, but the associated risks were small and large cohorts and replication cohorts are needed (>10 000 people). These genes do not markedly assist in predicting Alzheimer's disease risk, but they might have important roles in identifying the pathways involved in the disorder and potential drug targets (4).

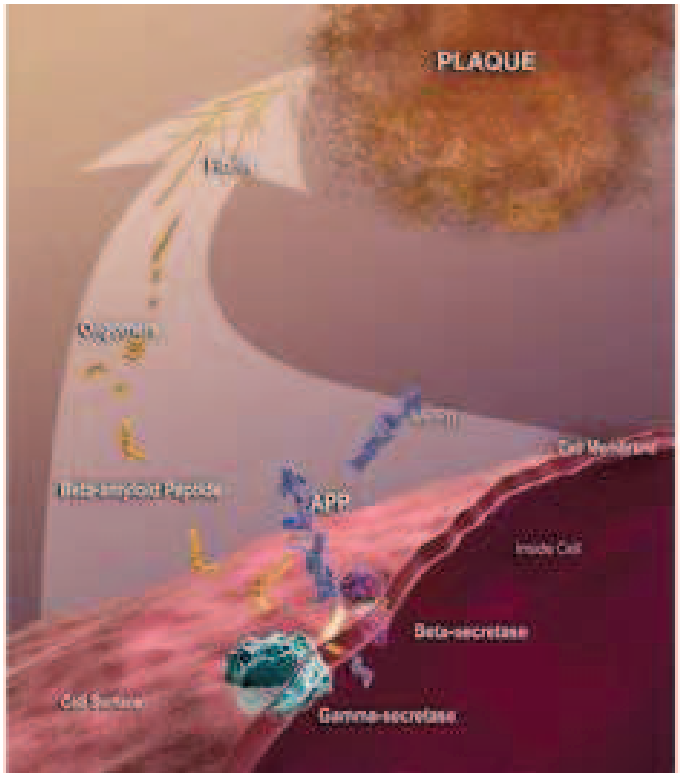
1.3 Pathogenesis

Most forms of AD are sporadic with the onset symptoms generally beginning after 65-70 years of age. A small proportion of cases, however exhibit a Mendelian pattern of inheritance and are referred to as familial Alzheimer's disease (FAD). Neuropathologically, both FAD and sporadic AD are remarkably similar and are characterized by two hallmark proteinaceous aggregates: amyloid plaques that are compact, spherical extracellular lesions, usually found in limbic brain regions, such as the hippocampus and amygdale, and also in specific cortical and subcortical areas, and neurofibrillary tangles that are intracellular aggregates, composed of hyperphosphorylated forms of tau protein. In addition to these proteinaceous aggregates the AD brain is also marked by additional neuropathological alterations, including the loss synapses, atrophy, the selective depletion of neurotransmitter system and Lewy bodies in a minority of cases (5).

- **Role of amyloid β peptide**

The finding of a correlation between plaque counts and dementia severity put great focus on the involvement of plaques in the pathogenesis of the disease. Because of their insolubility, attempts to identify then protein composition of plaques were fruitless until the mid-1980s when researchers succeeded in purifying plaque cores and identifying the amino acid sequence of Aβ the main plaque component. Initially, Aβ found in senile plaque was thought to be an abnormal protein. Therefore, an important finding was that Aβ is produced constitutively during normal cell metabolism. Under normal conditions, brain Aβ is degraded by the peptidases insulin-degrading enzyme, neprilysin and by endothelin-converting enzyme. Aβ is also cleared from the brain in a process balanced by the efflux, mediated by low-density lipoprotein receptor-related protein, and the influx, mediated by the receptor for advanced glycation end product, of Aβ across the blood-brain barrier. There is no evidence for any disturbances in these proteolytic enzymes or transport mechanisms in Alzheimer's

disease. The central hypothesis for the cause of Alzheimer's disease is the amyloid cascade hypothesis, which states that an imbalance between the production and clearance of Aβ in the brain is initiating event, ultimately leading to neuronal degeneration and dementia. Support for this hypothesis includes finding that the mutations implicated in the familial disease are present in the genes for both the substrates (APP) and the key enzyme (presenilin) for Aβ generation. Most APP mutation also cluster around the secretase sites, and both the APP and presenilin mutations increase Aβ (1-42) production (3). Aβ is generated by the sequential action of β and γ secretase enzymes on the transmembrane APP. β secretase cleaves APP at the Aβ N-terminus, resulting in a soluble form of APP and a 99-residue C-terminal fragment. This C-terminal fragment undergoes processing by γ-secretase within the transmembrane region of APP, resulting in the formation of Aβ peptides of various lengths (39-43 amino acid residues). The two most common primary isoforms generated are Aβ (1-40) and Aβ (1-42): the shorter form is typically produced by cleavage in the trans-Golgi network and is the more predominant isoform produced by neuron and other cells and accounts for over 70% of the total Aβ produced, whereas Aβ (1-42) which accounts for only 10-20% of total Aβ, is the major protein component of the neuritic plaques. Aβ (1-42) is more hydrophobic and has a greater propensity to form aggregates into fibrils and also greater neuronal toxicity in tissue culture models than Aβ (1-40), implying that Aβ (1-42) is more important moiety in AD plaque formation and pathogenesis. Aβ monomers are known to aggregates into higher molecular weight oligomers and fibrils. Aβ oligomers and fibrils can interact with neuronal membrane protein, which may induce change or loss of protein function (6).

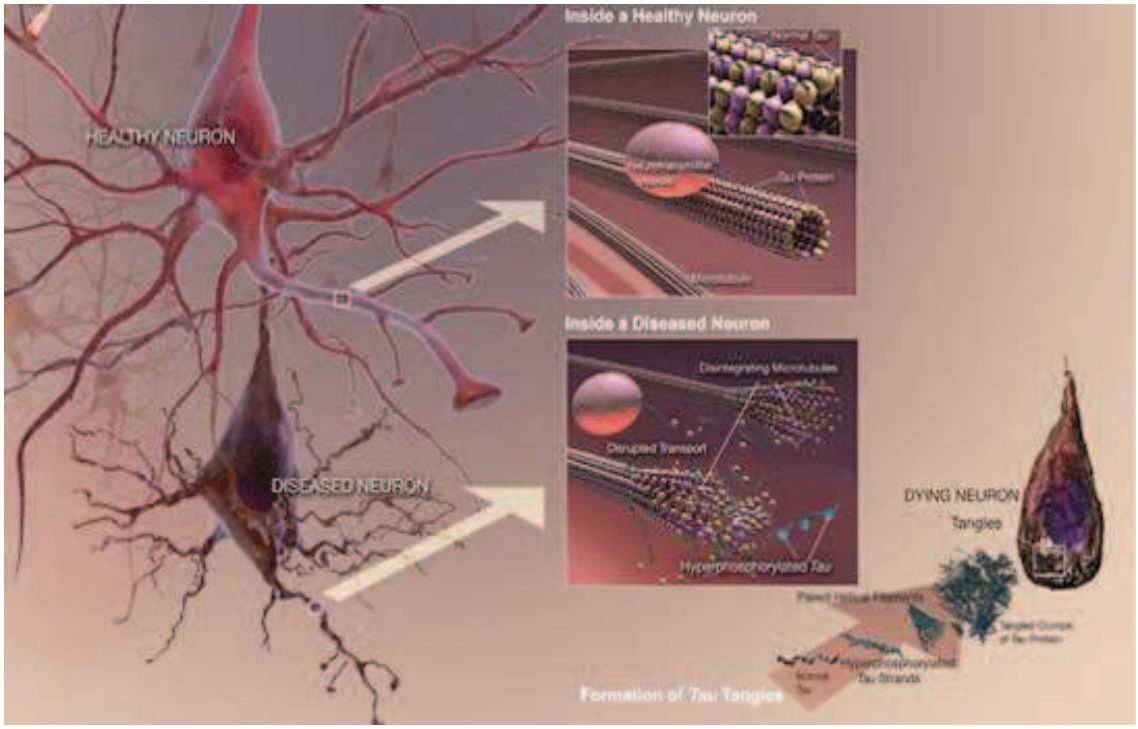


Beta amyloid cascade.The beta-amyloid cascade begins with cutting of APP by sequential action of β and γ secretase enzymes Aβ monomers are known to aggregates into higher molecular weight oligomers and fibrils, leading to the plaque formation.

- **Role of tau**

Almost in parallel with the identification of Aβ plaques, tangles were shown to be composed of abnormally hyperphopsorylated tau protein. Tau is a normal axon protein that binds to microtubules through its microtubule-binding domains, thereby promoting microtubule assembly and stability. Tau phosphorylation is regulated by the balance between multiple kinases (e.g., GSK3 and CDK5) and phosphates. Tau hyperphosphorylation in Alzheimer's disease starts intracellular and leads to sequestration of normal tau and other microtubule-associated proteins, which causes disassembly of microtubules and thus impaired axonal transport, compromising neuronal and synaptic function. Tau also becomes prone to aggregation into insoluble fibrils in tangles further compromising neuronal function (3). Neurofibrillary tangles also occur in other neurodegenerative

disorder including frontotemporal dementia with Parkinsonism linked to chromosome 17, Pick's disease, progressive sopranuclear palsy (PSP) and corticobasal degeneration (CBD) (5). In AD, tau pathology starts early in the transentorhinal region, spreads to the hippocampus and amygdale, and later to the neocortical association areas. Whether tau hyperphosphorylation and tangles formation are cause or consequence of Alzheimer's disease is unknown. This should not imply that tau pathology is irrelevant or innocuous in the pathogenesis of AD, because neurodegeneration induced by tau dysfunction might have a pivotal role in AD. This evidence further indicates that tau pathology can be triggered by different mechanisms, both dependent on and independent on Aβ (3). Further supporting the view that Aβ and tau interact, Oddo *et al.* (7) have shown that the removal of Aβ by immunotherapy leads to removal of early tau pathology. The removal of both lesions is hierarchical, because after a single intrahippocampal injection of an anti- Aβ antibody, the clearance of Aβ precedes the clearance of the early tau pathology. Moreover, after the antibody itself is cleared or degraded, the Aβ pathology reemerges prior to the tau pathology. Oddo *et al.* also showed that hyperhosphorylated and silver-positive tau lesions are resistant to clearance by Aβ immunotherapy. These findings are consistent with the existence of two different stages of tau pathology: an early stages, in which tau accumulates in the soma dendritic compartment, which is not stainable with the Gallya's silver-impregnation method and tau can be cleared by anti- Aβ intervention; and late stages, in which tau is stained by Gallya's but cannot be cleared by anti-Aβ interventions. Further studies also suggest that Aβ might be trigger or facilitate the development of tau pathology, indicating that Aβ can modulate the tangles accumulation.



Neurofibrillary tangles (NFT) formation. NFTs develop when threads of the protein, tau, become knotted inside brain and spinal cord neurons. Usually, tau helps to stabilize microtubules that form a scaffold-like support structure of these nerve cells. But in people with AD, the support threads of tau become tangled and the neuron support structure collapses. This collapse results in decreased chemical signaling between neurons (communication that is necessary for neuron health) and decreased nutrient transport within the affected neurons, eventually resulting in the death of affected neurons

- **Role of cerebrovascular dysfunction**

Some lines of evidence suggest that there may be converging pathogenic mechanism between cerebrovascular and Aβ plaque pathology. Several studies show comorbidity of cerebrovascular disease and AD, and also abnormalities in the brain microvascular system. The neurovascular hypothesis suggests that dysfunctional blood vessels could contribute to cognitive dysfunction by impairing delivery of nutrients to neurons and reducing Aβ clearance from the brain. Such cerebrovascular alterations could be initiated by down-regulation of the vascular differentiation gene MEOX2, with resulting loss of cerebral microvessels and reduced cerebral blood flow and Aβ efflux from the brain. Additionally, a polymorphism in the vascular endothelial growth factor (VEGF) gene might be

associated with the sporadic disease. Both human and experimental studies show that cerebrovascular pathology with ischemia results in upregulation of APP expression followed by Aβ deposition (3). Emerging evidence suggest that neurovascular dysfunction is a feature of cerebrovascular diseases and neurodegenerative condition such as AD. Hypertension is the strongest risk factor for AD and vascular dementia when these conditions are considered together. The penetrating arteries in the circle of Willis are particularly sensitive to the effects of hypertension and suffer early and selective damage during chronic hypertension. Hypertension is closely associated with atherosclerosis and vascular function, and in the brain this results in hypoperfusion and ischemic condition. Furthermore the blood brain barrier (BBB) that is essential for brain performance, id considered substantially compromised in a subpopulation of AD patients. Normal functioning of BBB is critical for proper neuronal function including synaptic transmission, remodeling, angiogenesis and neurogenesis. The BBB is characterized by tight junctions between adjacent brain endothelial cells providing a unique boundary that is highly specialized with different transport system. Integrity of the BBB is dependent on the health of the vasculature, and in AD; the total length of capillaries is reduced and microvascular endothethial is affected by degeneration processes. Altered brain endothelial cells in AD reflected dysfunctional angiogenesis, reduced lipoprotein receptor-related protein 1, and resultant impairment of Aβ efflux from the brain, providing evidence that impaired BBB contributes to the disease. Dysfunction of the BBB also has substantial effects on Aβ influx. Receptor for advanced glycation endoproducts (RAGE) is the major transporter of Aβ affecting influx of the peptide across the BBB and mediating pathophysiology response. Accumulation of RAGE ligands, such as advanced glycation endproducts and Aβ cause an increased cerebrovascular expression of RAGE resulting in the transcytosis of Aβ into the brain parenchyma where it binds the neurons (8).

- **Role of neuroinflammation**

Several other hypotheses have been proposed to explain the pathogenesis of AD, including abnormalities in proteins regulating cell cycle, inflammatory mechanism, oxidative stress and mitochondrial dysfunction with disruption in energy metabolism.

Inflammation is a complex cellular and molecular response to insults (stress, injury or infection), an attempt to defense against these insults. Inflammation is also a process that has been closely related to the onset of various neurodegenerative diseases, including AD. This inflammatory response in neurons includes activation of microglia, astrocytes, macrophages and lymphocytes, resulting in the release of inflammatory mediators such as cytokines, chemokines, neurotransmitters and ROS. The release of mediators leads to recruitment of monocytes and lymphocytes through the BBB as well as activation of additionally microglia, promoting their proliferation and resulting in further release of more inflammatory factors.

Microglia reside in the CNS, comprise approximately 12% of the brain (depending on brain region, health or pathology), and serve as the brain's immune defense. Microglia is unique from neurons, oligodendrocytes, and astrocytes in that they are not derived from the neuroectoderm. Instead, it is generally accepted that the original microglial population in the CNS differentiates from cells of the myeloid lineage that originate in the bone marrow, which occurs early in embryonic development. Once fetal macrophages come to reside in the developing CNS, they begin the differentiation process that will result in the formation of fully-matured microglia. Although the course of this differentiation is not fully understood, one of the early steps is the formation of rounded 'ameboid' microglia that cluster within distinct anatomical regions in the developing brain and may act as a source of microglial progenitors. Later in embryonic and early fetal development, these progenitors will follow a path of migration and differentiation leading to the mature microglia, a process that extends into neonatal development. Differentiation first involves the formation of partially ramified microglia followed by the development of fully ramified, or branched, microglia that express cell surface molecules characteristic of resting microglia. Analogous to the role of macrophages and lymphocytes in the periphery, one role of microglia is to act as

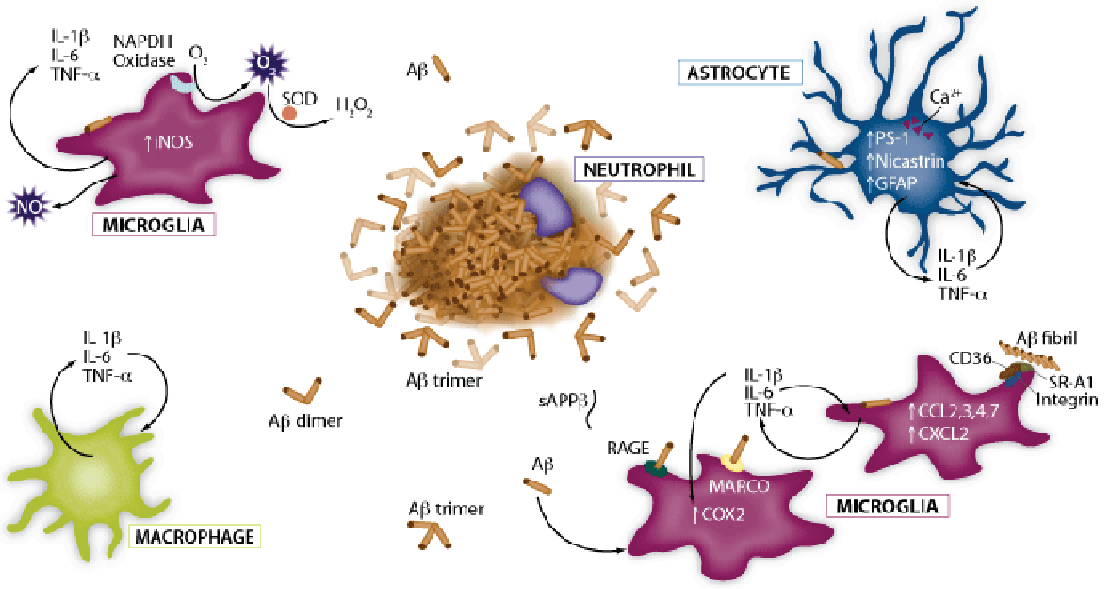
the brain's immune defense against disease and injury. In addition to these duties, however, microglia is involved in number of processes in the normal, healthy CNS. In a normal brain, microglia are said to be resting, and can be distinguished by both their morphology and pattern of gene expression. In this state, microglia takes on a ramified appearance, where long thin processes extend from the cell body and into the surrounding. When reacting to extracellular signals, such as the presence of pathogens, foreign material and dead or dying cells, microglia may undergo a morphological change into an ameboid shape with short or non-existent processes. This morphological change is also accompanied by changes in signaling and gene expression that can result in changes in surface receptor expression, the release of pro- or anti-inflammatory factors, recruitment molecules, and ROS, among others. The cumulative effect of these changes in morphology and phenotype is a shift from resting to activated microglia, (9).

As such the functions of microglia may form the first line of defense in CNS, through activation, proliferation, release of bioactive factors, removal of cellular debris and neutralization of pathogens. Although these functions are generally of benefit to the CNS, excessive activity of microglia may contribute to a variety of pathological conditions.

Microglia with a morphologically activated phenotype are present in a large numbers of CNS tissue from patients with chronic neurodegenerative disease including AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and prion disease. One of the hallmarks of Alzheimer's disease pathology is the existence of β-amyloid plaques that is normally cleared by microglia. Activated microglia and their toxic effects have been associated with Alzheimer's disease for decades. This has lead to research showing that not only is β-amyloid directly toxic to neurons, but it also causes microglia to cluster around plaques and become activated, which may perpetuate neuronal damage and death. In contrast to the beneficial housekeeping duties of resting and moderately activated microglia, over-activation of microglia, resulting in excess production of inflammatory mediators is in fact neurotoxic and microglial activation has been strongly linked to pathology in AD. The term over-activation describes the state in which microglia continually produce inflammatory mediators, such as pro-inflammatory cytokines

and ROS, which accumulate to levels that are harmful to neurons, and often in combination, lead to neurodegeneration. The degeneration of neurons and their processes might also activate microglia. The relationship between microglial activation and neurodegeneration is one of paradox. While the surveillance properties of microglia are essential for the maintenance of CNS integrity, excessive or uncontrolled microglial activation can have severe deleterious consequences. Many signaling molecules are released such as cytokines, reactive oxygen species, glutamate and nitric oxide, adversely affecting neuronal growth and survival (10).

Astrocytes are characteristic star-shaped glial cells in the brain and spinal cord and are most abundant cells in human brain. Astrocytes have various functional capacities including biochemical support of endothelial cells of BBB, supplying nutrients to nervous tissue, maintenance of extracellular ion balance, and healing the brain and spinal cord following traumatic injury. Similar to microglia, they also secrete various proinflammatory molecules as cytokines, prostaglandines, complement factors, and protease inhibitors. Senile plaque is known to induce development of reactive astrocytes and astrocystes cluster around Aβ deposits. This process in AD suggests that these lesions produce chemotactic molecules that induce astrocyte recruitment. Astrocytes activated by Aβ produce chemokines, cytokines and ROS that may result in neuronal damage and further microglia activation, with additionally release of proinflammatory products, thus increasing neuronal degeneration in the pathogenesis of AD (11).



Accumulation of A-beta as mature plaques and associated inflammation. During Alzheimer's disease (AD), A-beta molecules accumulate as mature A-beta plaques. In response to the deposition of A-beta and the release of chemoattractants from damaged neurons, activated astrocytes, microglia, and macrophages release pro-inflammatory cytokines such as IL-1beta, IL-6, and TNF-alpha. Although this initial response is an attempt to protect the brain, the prolonged state of chronic inflammation is believed to be a major detrimental factor during AD..

- **Role of Oxidative Stress**

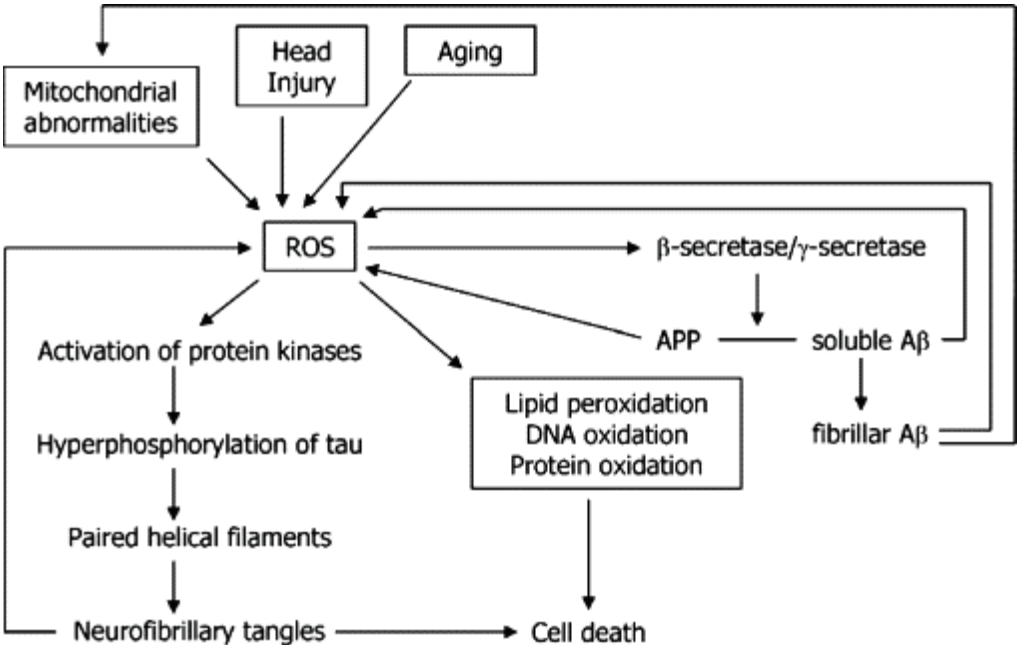
Oxidative stress has been implicated in the pathogenesis of a number of diseases including cancer, ischemia and neurodegenerative disorders. Free radicals and similar molecules are regularly classified collectively as reactive oxygen species (ROS), indicating their capacity to cause oxidative modifications within the cells. ROS plays an important role in cell signaling, a process termed redox signaling and they are a normal byproduct of the metabolic pathway of the oxidative phopshorylation during cellular respiration. Thus, to maintain proper cellular homeostasis, a balance must be struck between reactive oxygen production and consumption. Excessive ROS or free radicals need to be either quenched by converting them into metabolically nondestructive molecules or be scavenged/neutralized right after their formation. This protective mechanism is called the antioxidant defense system preventing free radical mediated damage of

cells leading to various diseases and aging. Oxidative stress is caused by the imbalance in the production of ROS and the biological system's inability to detoxify those species and repair the resulting damage. One of the most metabolically active organs of the body is the brain, including the spinal cord comprising central nervous system (CNS), which utilizes an estimated 20% of the total oxygen uptake. Consumption of oxygen leads to production of free radicals and the brain requirement of higher amount of oxygen leads to even high number of reactive oxygen/nitrogen species. Unexpectedly, it is relatively deficient in the enzymes that metabolize a number of oxygen-based redactants to innocuous species (12). It has been proposed that oxidative stress contributes to the pathogenesis of AD and there are many evidences suggesting that Aβ (1-42) oligomers might be critically important in the oxidative stress observed in AD brain (13). Several researchers have investigated associations between oxidative stress and Aβ; Aβ induces oxidative stress *in vitro* and *in vivo* and oxidative stress increases Aβ production. In addition, it was reported that AD patients have reduced ability to counteract oxidative stress because of declines in plasma of antioxidant levels and activity of antioxidant enzymes. These findings suggest that oxidative stress is one of the key pathological events in AD because it contributes to membrane damage, cytoskeletal alterations and neurons death. Oxidants and oxidative products increase APP expression and Aβ levels in brain cells. Moreover, it has been demonstrated that oxidative stress elevates the expression and activity of BACE 1, which plays a major role in the production of toxic Aβ. In addition, the accumulation of ROS results in damage to major cell components including the nucleus, mitochondrial DNA, membranes and cytoplasmic proteins. Accumulated evidence indicates that the increased levels of ROS may act as important mediators of synaptic loss and eventually promote formation of neurofibrillary tangles and senile plaques. Therefore a vicious circle between ROS and Aβ accumulation may accelerate progression of AD. Moreover, extensive oxidative damage observed in Mild Cognitive Impairment (MCI) brain regions suggest that oxidative stress may be an early event in the progression from normal brain to AD pathology. Based on these notions, it seems likely that increased production of ROS may act as

important mediator of synaptic loss and cell death and eventually promotes NTFs and senile plaque deposition (11).

The main ROS involved in neurodegenration are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical (HO•). Furthermore, reactive nitrogen species (RNS) such nitric oxide (NO) can also damage neurons. The mitochondrion has is a major site of cellular O_2^- production mainly derived from Complex I and Complex III. Superoxide dismutase (SOD) in the mitochondria (Mn-SOD) and cytoplasm (Cu/Zn-SOD) convert O_2^- to oxygen (O_2) and H_2O_2. In turn catalase and glutathione peroxidase convert H_2O_2 to water. These enzymes are critical for preventing oxidative damage to cells. Thioredoxin and glutathione thiol-based reducing system are important reductants in many oxidative stressors such as peroxides (14). In mild cognitive impairment (MCI) hippocampus, a brain region highly affected in AD, SOD and glutathione-S-transferase (GST) activity is decreased (15). Therefore, a biological approach to investigate whether new drugs have molecular mechanism of action targeted on antioxidant enzymes seems to be an interesting strategy in drug discovery. In addition, other antioxidant molecules, such as α-tocopherol improved the neurological performance and brain mitochondrial function in mice by presumably decreasing mitochondrial protein and lipid oxidation products. Mitochondria are not the only source of ROS involved in neurodegeneration. Transition metal ions are capable of stimulating free radical formation, and markers of oxidative stress precede pathological lesions in AD, including senile plaque and neurofibrillary tangles. The mail features of enhanced oxidative stress in the AD brain involve increased content of reduced copper and iron capable of stimulating free radical generation, increased protein and DNA peroxidation and enhanced lipid peroxidation. The pathologic interaction of cerebral Aβ with transition metals, such as zinc, copper or iron is possibly a neurochemical factor that initiates Aβ deposition. A deregulation of brain iron and copper homeostasis is a key factor to early neuropathological events in AD, including oxidative stress, inflammatory processes, Aβ deposition, tau phosphorylation and neuronal cell cycle regulatory fail, leading to apoptosis. However, the causes and consequences of oxidative

stress in AD are not fully understood, but considerably evidences indicate that oxidative stress, protein aggregation and redox active metal ions can all be considered potential targets for the treatment of neurodegeneration (14).



ROS production. Possible involvement of aging, mitochondria, head injury, APP, Aβ, and NFT in oxidative stress-induced cell death in AD. Head trauma and aging can induce production of ROS. On the other hand, ROS may activate the β– and γ–secretases to increase Aβ production from APP. Aβ and APP may also directly induce the production of ROS. Aβ can also induce the mitochondrial abnormalities which further increase the production of ROS. These ROS react with lipids, proteins, and nucleic acids and lead to cell death in AD. ROS may also activate protein kinases, leading to tau phosphorylation and NFT pathology, which in turn produce more ROS and result in cellular death.

- **Apoptosis in AD**

Apoptosis or programmed cell death is highly organized and orchestrated form of cell death that is important in tissue homeostasis and is common in a variety of biological processes including normal organ involution, immune response and embryogenesis. In CNS, neuronal apoptosis is physiological process that is an integral part of neurogenesis, and aberrant apoptosis has been implicated in the

pathogenesis of neurodegeneration. The recognition that loss of apoptotic control can underpin disease pathogenesis has spurred much research in the areas of apoptosis regulation and/or induction. Wide literature evidence links apoptosis to neurodegenerative process. Much progress in the understanding of apoptotic involvement and signaling in neurodegenerative disease was underpinned by the availability of cell systems as well as chemical- and genetic-based animal models. In addition, studies at the cellular, animal and human levels on neuroprotective strategies that target attenuation of oxidative stress and apoptosis, as well as preservation of neuronal mitochondrial integrity, offer promise for future development of treatment modalities against neurodegenerative processes.

Cellular apoptosis is a complex process that is triggered by extrinsic and intrinsic signal. The extrinsic (external) pathway involves the activation of death receptor upon binding of its ligand, recruitment of specific proteins at the “death domain”, and downstream signaling through a cascade of protein-protein interactions. The intrinsic pathway involves the mitochondria (mitochondrial pathway) at the release of pro-apoptotic factors into the cytosol with subsequent activation of executioner caspases.

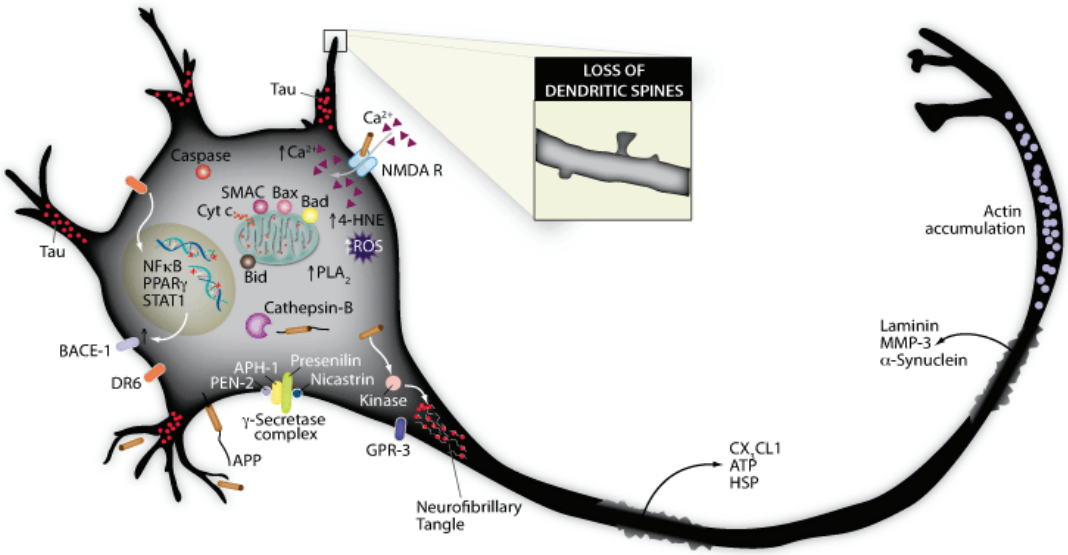
Death receptors belong to the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily and are transmembrane proteins. The best-characterized death receptors which function in apoptosis are Fas (CD95 or APO-1), TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and TRAIL-R2. The ligands that bind to the death receptors are TNF□□□Fas ligand (FasL) and TRAIL. Upon ligand binds the receptor, Fas- and TRAIL-associated death domain (FADD/TRADD) proteins further bind pro-caspase 8 and/or 10 and form DISC complex where the initiator caspases are activated, and apoptosis is mediated through a cascade of protein-protein interaction that converge in the activation of executioner caspase 3.

In the intrinsic pathway is well accepted that mitochondria are key players in the early induction and regulation of apoptotic cell death. Apoptotic stimuli such as DNA damage, ROS or Fas signaling, mediate mitochondrial cell death by a process that results in the release of small pro-apoptotic proteins that are normally located in the mitochondrial intermembrane space. Once in the cytosol, pro-apoptotic

proteins like cytochrome c, second mitochondria-derived activator of caspases/direct IAP binding protein of low pI (smac/Diablo), trigger caspase-dependent or-independent apoptotic death pathway. In the caspase-depend mechanism, cytochrome c binds the apoptotic protease-activating factor (Apaf-1), to form the apoptosome where pro-caspase 9 is activated in the presence of ATP. Caspase 9 further activates the effector caspase 3 and/or 7, leading to DNA fragmentation. Several mechanisms have been proposed to explain the permeabilization of the mitochondrial membrane that leads to the release of small pro-apoptotic molecules. One of the main mechanisms proposed involves member of the Bcl-2 pro- and anti-apoptotic proteins. The major anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x, are localized in to the mitochondrial outer membrane and to endoplasmic reticulum. Bax and Bak are pro-apoptotic members widely expressed in the nervous system. The involvement of apoptosis in AD brains was suggested by findings of DNA fragmentation as detected by TUNEL staining with concomitant upregulation of pro-apoptotic Bax protein and effector caspase 3. In addition, the intracellular Aβ (1-42) has been shown to induce human neuronal apoptosis through Bax activation that resulted in cytochrome c release and activation of caspase 6. Evidence of oxidative stress has been reported alongside neuronal apoptosis. For instance, enhanced formation of lipid peroxidation products, like 4-hydroxynonenal (HNE), were found in the brain and cerebrospinal fluid of AD patients, and elevated oxidized DNA bases in certain regions of AD brains. This increased oxidative stress was associated with decrease in glutathione-S-transferase (GST) activity. That the Aβ production is associated with generation of neuronal oxidative stress is well documented. For instance, Aβ induced the formation of hydroxyl and lipid radicals in PC12 cells, presumably due to the interaction of Aβ with transition metal. The specific Aβ (25-35) and Aβ (1-40) peptides have been shown to induce ROS production, lipid peroxidation and decreased cell GSH that collectively impair mitochondrial respiratory function, deplete intracellular ATP and induce mitochondrion oxidative DNA damage. It has been shown in various neuronal cell lines that Aβ-induced apoptosis involves the activation of JNK and p38 kinase pathway. In particular, JNK activation mediates

the translocation of pro-apoptotic Bcl-2 proteins to the mitochondria, a process that triggers mitochondrial apoptotic signaling, suggesting that oxidative stress and activation of JNK are associated with Aβ-induced apoptosis. Furthermore, microglia activation widely reported in AD, results in the secretion, among other toxic factors, of TNF α that can activate the death receptor (extrinsic) pathway.

Thus, pathologically neuronal cell death might be a direct consequence of toxic insult such Aβ or an indirect consequence of a complex interaction between neurons, microglia and toxic factor.



A-beta generation challenges neuronal viability and promotes apoptosis. Increased amyloidogenic processing of APP by BACE-1 and the gamma-secretase complex results in augmented levels of A-beta. In vulnerable neurons, A-beta peptides promote apoptosis, compromise mitochondrial integrity, and induce free radical generation, leading to the release of injury markers. A-beta is also thought to promote kinase activity, tau phosphorylation, the formation of neurofibrillary tangles, and the loss of dendritic spines.

- ## Role of Glycogen synthase 3 (GSK3)

GSK3 is a constitutively active, proline-directed serine/threonine kinase that plays a part in a number of physiological processes including glycogen metabolism, gene transcription and microtubule stability.

The evidence that GSK3 plays a central role in AD and that its deregulation accounts for many of the pathological hallmarks of the disease in both sporadic and familial AD cases. GSK3 activity is modulated by insulin and Wnt signaling, both pathways act in a negative regulatory manner. Many, but not all GSK3 substrates require pre-phosphorylation (priming) before phosphorylation by GSK3 can occur, so in both health and disease the activity of the priming kinase might limit GSK3 activity. Insulin signalling leads to the activation of PI3-kinase and subsequently the activation of Akt, which in turn phosphorylates free cytoplasmic GSK3 β and GSK3 α at serine (Ser) residues 9 and 21, respectively. Regulatory serine phosphorylation results in the generation of an intra-molecular pseudo-substrate, which blocks part of the active site preventing the enzymatic activity of GSK3. Thus, regulation of GSK3β activity is critically dependent on the phosphorylation state of its Ser 9. GSK3 is intimately involved in the hyperphosphorylation of tau, memory impairment, the increased production of Aβ and in inflammatory responses. GSK3 also reduces acetylcholine synthesis, which is in accordance with the cholinergic deficit present in AD. Moreover, GSK3 is a key mediator of apoptosis and thereby might directly contribute to neuronal loss in AD (16). Furthermore, GSK3 contributes to Aβ-induced neurotoxicity. Aβ is well-known to cause neuronal death when applied to cultured cells or to brain in vivo, and inhibition of GSK3 by a variety of methods significantly attenuates the neurotoxicity caused by Aβ, indicating a direct link between Aβ-induced GSK3 activation and neurotoxicity (17). Moreover, the GSK3 pathway has been emphasized in oxidative stress-induced neuronal cell death, by enhancing the expression of pro-apoptotic proteins and inhibiting the activity of anti-apoptotic proteins. It is well known that GSK3 directly affects CCAAT/enhancer binding protein, nuclear factor of activated T cells, Myc, HSTF-1, Tau, cyclic adenosine mono phosphate (AMP) response element binding protein, β catenin, NFkB, and p53, affects release of cytochrome c and caspase-3, and induces neuronal cell death. In addition, an increase in GSK3 activity could inhibit the antioxidant cell response through the phosphorylation of the Nrf2 (NF-E2-related factor 2) transcription factor, that is implicated in the regulation of the redox homeostasis.

Nrf2 is a basic leucine-zipper transcription factor that binds antioxidant response elements (AREs) located in the promoters of antioxidant and phase II drug-metabolizing genes (18). Under oxidant conditions, Nrf2 dissociates from Keap1 and escare Keap1-dependent degradation (Katsuoka et al. 2005). Released Nrf2 translocates to the nucleus, and up-regulates transcription of phase II genes, such as heme oxygenase-1, nicotinamide-adenine-dinucleotide-phosphate [NAD(P)H]: quinine oxidoreductase 1, glutathione S-transferases, glutamate-cysteine ligase, glutathione peroxidases, etc (19). Therefore, these evidences suggest that Nrf2 may be a critical element in taking survival and death decisions when neurons are exposed to an oxidant environment. *Rojo et al* have shown that GSK 3 down-regulates the transcription factor Nrf2 after oxidant damage, blocking its prosurvival function (20).

1.4 Therapeutic strategies for AD treatment

More than a decade after the first approval of the use of acetylcholine esterase inhibitor on patients with Alzheimer's disease, we still not have a single treatment or combination therapy that can effectively stop or reverse the progression of such neurodegenerative disease. There probably is not one single cause, but several factors are important to describe the etiology of the disease. Therefore, combination of compounds, which act at more than one target site, could be useful for AD treatment.

There are only 5 medications approved by Food Drug Administration (FDA) to treat AD. They include 4 acetylcholine esterase inhibitor (AchEIs) and one N-methyl-daspartate (NMDA) antagonist.

Cholinesterase Inhibitors

The cholinergic hypothesis of AD concludes that cholinergic system in the basal forebrain are affected early in the disease process including loss of acetylcholine neurons, loss of enzymatic function for acetylcholine synthesis and degradation, resulting in memory loss as well as deterioration of other cognitive and noncognitive functions such as neuropsychiatric symptoms. A strategy to enhance

the cholinergic transmission by using AChEIs to delay the degradation of acetylcholine between the synaptic cleft was then proposed. In 1993 the first FDA approved AChEI, tacrine boomed out but it was no longer used because of its high prevalence of hepatotoxicity. FDA approved another three AChEIs: donepezil (1996), rivastigmine (2000), and galantamine (2001) in the following years. These drugs have been regarded as the standard and first-line treatment for AD. Systemic reviews including many double-blinded, randomized, placebo-controlled trials (RCT) of these three AChEIs all showed benefit on cognitive functions, activities of daily living (ADL), and global function for patients with mild to moderate AD; there was no significant difference of efficacy between individual AChEI. Donepezil is also beneficial for severe AD. Systemic reviews showed that the incidence of gastrointestinal adverse effect, such as nausea, vomiting, diarrhea and abdominal cramp, was lower with donepezil than with rivastigmine and galantamine. The incidence of adverse effect was associated with higher therapeutic dose. The long-term efficacy of AChEIs remains controversial, but continuing treatment was beneficial and was suggested if well-tolerated. However, the efficacy of AChEI in protecting subjects with mild cognitive impairment from converting into AD remains inconclusive.

NMDA antagonist

In addition to the conventional cholinergic hypothesis of AD, there is great evidence that NMDA regulated excitotoxicity, closely related to neuronal plasticity and memory function, plays an important role in neurodegeneration. Aβ disturbs function of the postsynaptic NMDA receptor leading to excessive calcium influx into neurons and activation of NMDA-dependent downstream pathways. The cytosol and mitochondrial calcium overload results in a cascade of oxidative cytotoxicity and apoptosis. Memantine, a voltagegated and uncompetitive NMDA antagonist with moderate affinity, can protect neurons from excitotoxicity. It was approved by FDA in 2003 for treatment of the patients with moderate to severe AD. A systemic review of double-blinded, parallel-group, RCT studies of memantine showed improvement in cognitive function, ADL and behaviors in people with moderate to severe AD after 6 months. The memantine was usually

well tolerated, except small group of patients might develop agitation. Another systemic review included 6 RCT studies indicated that memantine may reduce behavioral and psychological symptoms of dementia.

Drug candidates with potential disease-modifying effect

Numerous studies focused on disease modifying therapeutics targeting anti-Aβ, anti-oxidative injury, anti-inflammatory or anti-tau-phosphorylation strategies against the pathogenesis of AD.

Drugs targeting amyloid

γ-Secretase inhibitors

Aβ peptides may result in synaptic dysfunction, LTP impairment, tau hyperphosphorylation and consequent oxidative neuronal death. All these contribute to the cognitive defect of AD. Aβ peptide comes from amyloid precursor protein (APP), an intra-membranous protein, catalyzed by β-secretase (BACE-1) and then γ-secretase. Thus blocking Aβ formation may show potential benefit for AD treatment.

γ-Secretase inhibitors can reduce Aβ synthesis and thereby prevent Aβ aggregation and reverse APP-induced cognitive deficits in preclinical models. Unfortunately, γ-Secretase cleaves transmembranes proteins in addition to APP. While physiologic role of γ-Secretase cleavage for most of these substrates is unknown, γ-Secretase cleavage of the Notch family of transmembrane receptors is required for Notch signaling. As a result of the inhibition of the Notch signaling, chronic dosing of γ-Secretase inhibitors causes changes in the gastrointestinal tract, spleen, and thymus that limit the extent of Aβ inhibition attainable in vivo. Despite this limitation γ-Secretase inhibitors can improve cognitive deficits at tolerable doses in preclinical models (22). Based on these findings, γ-Secretase inhibitor LY-450139, Semagacestat, has been tested in humans. Clinical trials have shown dose dependent decrease of the plasma but not the CSF Aβ. Semagacestat had no

cognitive or functional benefit for patients with mild to moderate AD in a multi-center, RCT phase II studies. It is disappointing that preliminary results from two phase-III clinical trials (IDENTIY and IDENTITY-2) showed that Semagacestat did not slow down disease progression and was associated with worsening of clinical measures of cognition and the ability to perform ADL. INDENTITY and INDENTITY-2 are two RCT studies including more than 2,600 patients with mild-to-moderate AD from 31 countries with a treatment period of about 21 months. In addition Semagacestat is associated with an increased risk of skin cancer. Clinical trial of LY450139 was thus halted (21).

β-Secretase Inhibitors

The identification of BACE 1 as the enzyme required for brain Aβ formation was met great enthusiasm, in particularly when the analysis of BACE1 knockout mice did not reveal any undesired phenotypes and shown an almost complete Aβ reduction in brain. This initial enthusiasm has, however, been dampened by two issues. First, several investigators have identified others phenotypes in BACE 1 knockouts; second, it has been difficult to design potent brain penetrant BACE 1 inhibitors.

Active immunization

Active vaccination with AN1792 which contains Aβ (1-42) aggregates, was first tested in humans based on impressive preclinical studies. Unfortunately, meningo-encephalitis in 6% of the vaccinated patients caused the termination of this study. The encephalitis is hypothesized to result from T-cell activation based on examination of post mortem tissue and studies in mice (21).

Second generation active immunization vaccines have been under investigation since then. These vaccines used various fragments of Aβ combined with promiscuous non-self T-cell epitope such as adenovirus vectors with Aβ cDNA, or combined DNA epitope. These second generation vaccines decrease insoluble Aβ and amyloid plaque in the brain but not the soluble Aβ and amyloid oligomers and have no effect on tauopathy (22).

Passive immunization

The positive findings with Aβ (1-42) immunization in mice led to similar studies using passive immunization with anti-Aβ antibodies. These studies in transgenic mice showed that peripherally administrated antibodies could reduce plaque burden and reverse cognitive deficits. While passive immunization doesn't likely avoid the encephalitis observed with active immunization, microhemorrhaging and increased vascular amyloid has been observed in transgenic mice.

Bapineuzumab is a monoclonal antibody which binds to the N-terminal of Aβ to enhance clearance of Aβ from the brain. Bapineuzumab was injected intravenously into human bodies to act in a passive immunotherapy manner. A phase II RCT study which included 234 patients with mild to moderate AD, showed no significant advantage on cognitive and ADL functions after 78 weeks. In this trial, patients who did not carry Apo lipoprotein E4 (APOE4) allels, performed better and had lower adverse effect (vascular edema) than the others. This demonstrates the complicacy of AD mechanism that both genetic and environmental factors play a role. Further investigation on the relationship between APOE4, Aβ, and bapineuzumab should be done. Currently long-term phase II/III trials are now under investigation. Solanezumab is a humanized anti-Aβ immunoglobulin G-1 monoclonal antibody. It has passed a phase II trial including 52 patients with mild to moderate AD which demonstrated increased Aβ in plasma and CSF. However, there was no significant change in cognitive function or quantity of brain Aβ. But there was no adverse effect such as brain inflammation revealed by MRI. It will undergo two phase III RCT studies focusing on efficacy on cognition and ADL in patients with mild to moderate AD (21).

Aβ aggregation Inhibitors

Tramiprosate (3-amino-1-propanesulfonic acid) is a glycosaminoglycan compound that binds to Aβ monomers preventing formation of cytotoxic Aβ oligomers thus

enhancing Aβ clearance from the brain. A phase II RCT study on patients with mild to moderate AD showed that Aβ (1-42) level in cerebral spinal fluid (CSF) was decreased in a dose-dependent manner. However, the North American phase III RCT study on 1052 patient with mild to moderate AD failed to demonstrate a beneficial effect on the primary outcome, neither change in cognition or dementia staging after 18-month intervention. The negative result was considered to be from unexplained high inter-site variation. In spite tramiprosate was well tolerated.

Scyllo-inositol can directly bind to Aβ oligomers and restore LPT of neurons and synaptic plasticity in hippocampus. In preclinical trials, scyllo-inositol can penetrate BBB, and can reduce insoluble Aβ (1-40), Aβ (1-42), and amyloid plaque in the brain, and can improve performance of learning. Now oral ELND005 is under a phase II clinical trial evaluating safety and efficacy for mild to moderate AD patients.

Receptors for advanced glycation end products (RAGE) is a molecule of immunoglobulin superfamily localized in neurons, microglia, astrocytes and BBB. RAGE enhanced in AD can help transport Aβ from vascular circulation to the brain. Aβ binding to RAGE interferes with synaptic LPT resulting in neuronal stress, cytotoxicity, inflammation and consequent memory and learning deficits. RAGE enhances generation and accumulation of the Aβ in CNS by modulating BACE 1. Soluble RAGE can bind to systemic Aβ decreasing accumulation of the brain Aβ and improving learning and memory of transgenic mice. The effort on RAGE proteolysis as a therapeutic target for AD has been carried on. *PF04494700*, an RAGE antagonist, is currently under evaluation for AD treatment in a phase II trial.

Drugs targeting tau

Tau phosphorylation is regulated by kinase and phosphatase. It is believed that Aβ induces hyperphosphorylated kinase cascade and will lead to tau phosphorylation. Thus to inhibit kinase or to enhance phosphatase may disturb the formation of abnormal tau. The major target kinases are GSK3 and cdk5/p25, whereas GSK3 plays a key role in AD neurodegeneration which also enhances Aβ production. Lithium not only can inhibit GSK3 but also can modulate neuron apoptosis. It can

decrease abnormal tau in vitro and in vivo; however the benefit of lithium in neuroprotection and cognitive function remains controversial. In patients with amnesic MCI, the effect of attenuating cognitive deficits and modification of biological markers are apparent in a phase II trial. There are other potent selective inhibitors of GSK-3 that showed promising results in preclinical models. Another promising compound is the methylthioninium chloride (MTC), with antioxidative ability through mitochondrial modulation that can reduce Aβ oligomerization and can bind to the domain responding for tau aggregation. In a preclinical study, it reverses the learning impairment in a cognitive deficit model. A phase II RCT study of MTC monotherapy on patients with mild to moderate AD showed a significant improvement of cognitive function with long term benefit. It also showed that MTC can restore brain activity in brain tauopathy region. These exciting results should be validated in a coming large-scale phase III clinical trial. The main phosphatase of phosphorylated tau is PP2A and PP2B. PP2A is the major phosphatase downregulated in Alzheimer's disease. Pin1, the peptidyl prolyl cis/trans isomerase can facilitate dephosphorylation of phosphatase. It can be activated by Aβ- oligomers induced tau-phosphorylation, and can modulate APP. The possibility of regulating this tau-related molecule may develop into new therapeutics. Vaccines consist of phosphor-tau epitope can reduce aggregated tau in the brain and can prevent or slow down progression of the tangle-related behavioral and cognitive impairment in animal models.

Drug candidates based on epidemiology

Epidemiological studies have served as the theoretical basis for several treatment approaches.

Anti-inflammatory drugs

A role for neuroinflammation in AD is suggested by the observation of activated inflammatory markers on microglia, astrocytes and neuron near plaques, the ability of Aβ to bind and activate microglia and the reduced incidence of AD with non steroidal anti-inflammatory drugs (NSAIDs) usage. Neurotoxicity may result

from this chronic inflammation response via complement, cytokines, reactive oxygen species, eicosanoides, excitatory amino acids, proteases, nitric oxide and acute phase proteins. These data led to critical trials with tradition anti-inflammatory therapeutics, including prednisone, naproxen, celecoxib and rofecoxib in AD, but all of the trials are negative. Because inflammation in AD brain is relatively mild and involves different cell types than classical peripheral inflammation, failure of these trials may not reflect the possible benefits of neuroinflammatory inhibitors. New clinical trials may better address this hypothesis. One example is the cytokine modulator, VP-025 which is being prepared for phase II trials for AD. PPAR agonist (rosiglitazone) may also have an anti-inflammatory effect in addition to normalizing insulin regulation and possibly also reducing Aβ levels (22).

Statins

APOE is a cholesterol carrier in CNS and APOE4 may aggravate Aβ deposition and tau hyperphospohrylation. Carrying APOE4 allel is a significant risk factor for sporadic AD. Epidemiological study showed that people with a higher cholesterol level had a higher risk of developing AD. In animal studies, lowering cholesterol level may slow down the expression of Alzheimer's pathology, reduce NFT and amyloid burden, and reverse learning and memory. A cohort study suggested that elective statin use can significantly reduce risk of AD in individuals taking anti-inflammatory agents. But review of RCT studies of statin revealed decreasing cholesterol level without reducing risk of AD or dementia. An RCT study (LEADe) of atorvastatin 80 mg/day for 72 weeks on 640 patients with mild to moderate AD showed no effect on cognitive or global function (21).

DHA (docosahexaenoic acid*)*

DHA is an omega-3 polyunsaturated fatty acyl chain concentrated in phospholipids of brain and retina. It can attenuate Aβ secretion and enhance synthesis of the neuroprotectin (NDP1), which can repress inflammation, oxidative stress, and cell apoptosis induced by Aβ (1-42) and promote neuronal survival. DHA and NDP1

were reduced in AD patients. In animal models, DHA presented a beneficial effect on decreasing AD pathology, cognitive impairment, synaptic dysfunction, and tau hyperphospohrylation. Animals on DHA depletion diet exhibited learning and memory impairment; increased inflammatory and oxidative damage to neurons and synapses in the brain. Epidemiological studies showed conflicting results between fish intake, DHA blood level and the incidence of AD. A systemic review showed no evidence that dietary or supplemental omega-3 polyunsaturated fatty acid could reduce risk of cognitive impairment or dementia in healthy elderly.

Estrogens

Epidemiological studies have reported an association between reduced risk of dementia and postmenopausal estrogen supplementation. Animal studies also suggest that estrogens could have several beneficial effects on neuronal function. However, large randomized controlled clinical trials of estrogens have not shown a reduced risk of disease development.

Antioxidants

Oxidative stress is thought to play an important role in the pathogenesis of AD, a disease that likely begins years, if not decades, prior to clinical onset of dementia. In line with this hypothesis, experimental data support the notion that antioxidants protect against neurodegeneration (23).

One well known therapeutic approach related to oxidative stress is the administration of direct antioxidant drugs. Supplementation with vitamin E was used in transgenic AD mice for its ability to reduce ROS. Authors found that vitamin E reduced lipid peroxidation in both young and aged mice, but reduced plaque burden only when the drug was administered at early ages. In combination with vitamin C, vitamin E reduced memory deficits but did not affect amyloid deposition in transgenic mice with both AβPP and PS1 mutations. Interestingly, genetic depletion in vitamin E in transgenic AD mice resulted in increased lipid peroxidation, and amyloid deposition and oligomerization by affecting Aβ

clearance. Melatonin is another potent antioxidant drug used in transgenic AD animal models. Melatonin receptors affect mechanisms of learning and memory in mice, especially electrophysiological processes such as long-term potentiation. In transgenic AD mice, administration of melatonin reduced oxidative stress and pro-apoptotic markers. It also elevated levels of SOD and glutathione, two mitochondrial enzymes involved in free radical scavenging. Long term administration of melatonin improved cognitive performance and reduced amyloid deposition in transgenic AD mice. Another study showed that melatonin treatment in mice injected with Aβ protofibrils reduced ROS and intracellular calcium levels. Spices are other sources of antioxidants. Curcumin in particular has been widely used to reduce oxidative stress. In transgenic AD mice, low and high doses of curcumin decreased oxidized proteins, soluble and insoluble Aβ, as well as amyloid plaques. *In vitro* and *in vivo*, curcumin also reduced Aβ aggregation. As a possible mechanism for plaque clearance, curcumin can bind Aβ and increase Aβ uptake from macrophages. Interestingly, short term administration of curcumin partially restored distorted neurites in transgenic AD mice. Several other natural antioxidants have been studied in the context of AD therapy, such as blueberry and red grape, particularly for their content of resveratrol. Fruit extracts and resveratrol itself have shown beneficial effects including lowering plaque burden and improving behavioral deficits in transgenic AD mice possibly via AMP-activated protein kinase activation pathway (19).

Unfortunately, clinical trials have shown no benefit of antioxidant supplements for AD, the wider variety of antioxidants in food sources is not well studied in relation to dementia risk; a small number of studies have yielded inconsistent results with varying lengths of follow-up (23).

Maybe if we consider the highly complex system for fine regulation of cellular redox balance in the human body, it is no wonder that extrinsic in vitro antioxidants may show only limited effects on reduction of oxidative damage in biologic system. Therefore, we should survey ways to activate our intrinsic system to reduce oxidative damage, which may be effective in the retardation of disease progression. Efficacy may be greater in prevention of AD and reducing risk of cognitive decline with aging. Future trials of antioxidants are likely to be more

successful if focused on prevention or on very early stages of disease, and if a broad increase in the entire network of antioxidant defense systems is targeted.

2 AIM OF THE STUDY

AD is a devastating neurodegenerative disorder that leads to severe cognitive impairment. Extracellular amyloid plaques and intracellular neurofibrillary tangles are the pathological hallmarks of AD. Aβ is a major component of the plaques, and it plays important roles in pathogenesis of AD. Aβ exists in several forms, including oligomeric forms. Aβ is usually composed of 40 or 42 amino acid residues, Aβ (1–40) and Aβ (1–42), respectively, of which Aβ1-42 is more prone to aggregate, more resistant to degradation, and more toxic. There is accumulating evidence that the toxicity of Aβ is associated to a large extent with the formation of soluble Aβ oligomers and that Aβ accumulation at early stages of AD can promote neuronal death, preceding senile plaque formation. One possible mechanism of Aβ neurotoxicity is an increase in neuronal sensitivity to oxidative stress, which has been extensively implicated in AD. There is considerable evidence indicating increased levels of oxidative stress in AD brains, particularly in the brain regions involved in the regulation of cognition. Several researchers have investigated associations between oxidative stress and Aβ; Aβ induces oxidative stress *in vitro* and *in vivo* and oxidative stress increases Aβ production. In addition, it was reported that AD patients have reduced ability to counteract oxidative stress because of declines in plasma of antioxidant levels and activity of antioxidant enzymes. Recent evidence suggests that inflammatory responses also may significantly contribute to the progression and chronicity of AD. Inflammatory changes, including the presence of activated microglia, are observed throughout the AD brain, but particularly at amyloid deposits. Aβ also activates microglia directly by binding to receptors, including the receptor for advanced glycation end products, the lipopolysaccharide (LPS) receptor CD14, and other scavenger receptors. Prolonged simulation of microglia induces the release of large amounts of pro-inflammatory mediators, including complement components, cytokines such as TNFα, IL-1β and IL-6, various free radicals and nitric oxide (NO), all of which potentially contribute to further neuronal dysfunction and eventual death (24).

For these reasons, growing attention has focused on oxidative mechanism of Aβ toxicity as well as the search for novel neuroprotective agents. Indeed, such information may be useful for developing therapeutic strategies with the hope that early treatment will slow or prevent the progression of AD. A strategy to prevent the oxidative stress in neurons may be the use of chemopreventive agents as inducers of antioxidant and phase 2 enzymes. Sulforaphane [1-

isothiocyanate-(4R)-(methylsulfinyl) butane] is a natural compound widely studied since the 1980s decade given that it has chemotherapeutic properties including anti- proliferative and anti-angiogenic properties. However, beyond its anticancer properties, current research has also strongly focused on the effects of sulforaphane against important pathologies including hyperglycemia and the damage to brain, kidney, liver, heart, and muscle among others. Sulforaphane (SF) is a dietary isothiocyanate which is synthesized from a precursor found in cruciferous vegetables of the genus Brassica such as cauliflower, broccoli, kale, colecrops, cabbage, collards and brussels sprouts, mustard and cress as well as in other genera as radish (Raphanus sp.). The 4-methylsulfinylbutylglucosinolate (glucoraphanin) is the SF precursor, which is generally found in high concentration in broccoli (0.8–21.7 mol/g of dry weight). Glucosinolates are physically segregated from the myrosinase, which comes into contact with its substrate when the plant is injured in processes such as pathogen attack, chewing, chopping or preparing for human consumption and as a result, the enzymatic hydrolyzes formation of SF as the major reaction product (25).

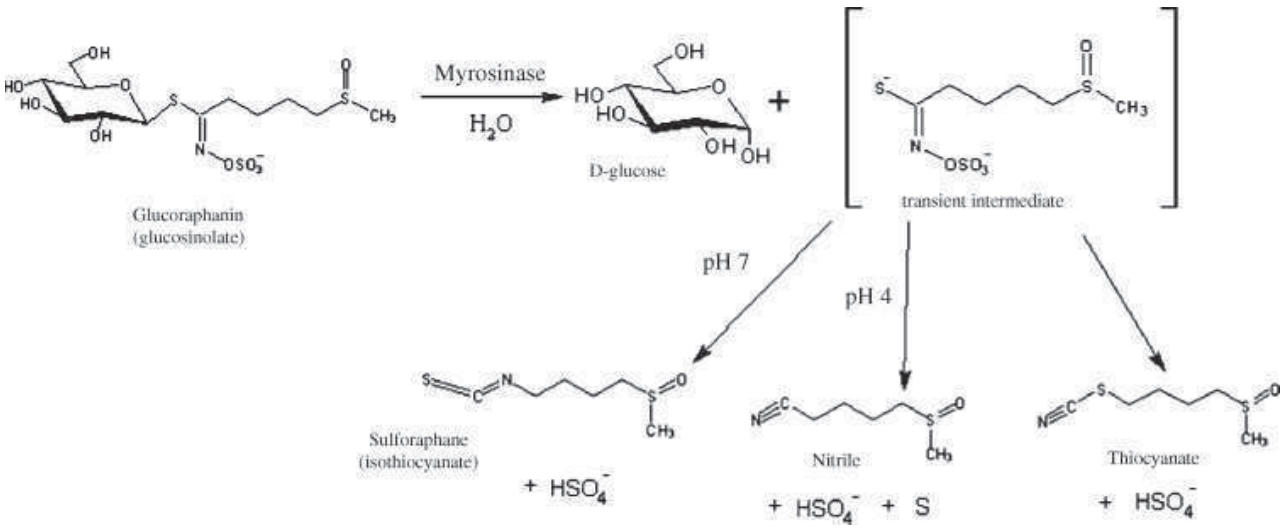


Fig. 1 Hydrolysis of glucoraphanin. Glucoraphanin is the major glucosinolate in broccoli and it is hydrolyzed by the myrosinase enzyme to form both d-glucose and sulforaphane, however, thiocyanates and nitriles could also be formed under acid conditions.

The SF anticancer activity is thought to be related to the induction of a phase II detoxification response promoting a disruption of nuclear factor E2-factor related factor (Nrf-2)-Kelch-like ECH-associated protein (Keap1) interactions and mitogen-activated protein kinase activation. As a result, Nfr2 modulates gene expression via antioxidant response element (ARE). ARE-

driven targets include NAD (P) H: quinone oxidereductase (NQO1), heme oxygenase-1 (HO-1) and γ-glutamylcysteine ligase (γGCL) and the induction of these enzymes has been observed both *in vitro* and *in vivo* experiments after SF treatment. For example, dried broccoli sprouts (200mg/day) were able to attenuate oxidative stress, hypertension and inflammation in stroke-prone spontaneously hypertensive rats. In addition, the antihypertensive effect of broccoli sprouts was accompanied by an enhancement in the glutathione (GSH) concentration and in the activities of glutathione reductase (GR) and glutathione peroxidase (GPx) in heart, kidney, aorta and carotid. A cardioprotective effect was found after feeding broccoli for 30 days to rats in isolated heart preparations submitted to ischemia and reperfusion. Rats fed with broccoli had an increased post-ischemic ventricular function and reduced myocardial infarct size along with reduced cardiomyocyte apoptosis. These protective effects were associated with prevention in the decrease of thioredoxin, glutaredoxin and peroxiredoxin, HO- 1, superoxide dismutase (SOD) and SOD2 and Nrf2 as well as enhanced induction of the survival signaling proteins including Bcl2, Akt, extracellular signal-regulated kinase 1/2 and down- regulation of the proteins (e.g., Bax, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase) of the death signaling pathway induced by ischemia and reperfusion. Zhao et al. (26) investigated the effect of SF on regulation of Nrf2/ARE pathway in liver injury induced by intestinal ischemia/reperfusion. They showed that the pretreatment with SF ameliorates the ischemia–reperfusion- induced intestinal and liver injury. This protection was associated to the increase in liver expression of Nrf2 and HO-1.

SF has neuroprotective effects in several experimental paradigms. Zhao et al. (26) showed that a single ip SF administration was able to reduce the infarct size in rats induced by ischemia and reperfusion by increasing HO-1 expression in brain. It is important to notice that HO-1 is an inducible enzyme that catabolizes free heme into carbonmonoxide, iron and biliverdin, which is converted to bilirubin by biliverdin reductase. The same protective effect was observed in a neonatal hypoxia-ischemia brain injury model (27) in which SF decreases malondialdehydeand 8-hydroxy-2-deoxyguanosine levels. In addition, a model of oxygen and glucose deprivation in immature neurons and in astrocytes (28; 29), SF protects the cells activating the Nrf2/ARE pathway, increases the gene transcription, protein levels and activity of anti oxidant enzymes including NQO1, HO-1, and GCL modifier subunit. Interestingly, SF also protects blood brain barrier after brain injury which was accompanied by the enhanced expression of Nrf2-driven genes (30). SF injection protected against cerebral damage induced by intrastriatal injection of autologous blood (30) and also activated Nrf2 in intracerebral

hemorrhage (ICH)-affected brain tissue and reduced neutrophile count, oxidative damage, and behavioral deficits caused by ICH.Nrf2-deficient mice showed more severe neurologic deficits after ICH and did not benefit from the protective effect of SF. The above described protection was not observed in mice lacking the nrf2 gene and suggesting that this protection was dependent on Nrf2 (30). In another brain experimental model, lipopolysaccharide-induced inflammation was attenuated by SF pretreatment with Nrf2 induction and HO-1 expression in the hippocampus of these brain animals (31). The role of Nrf2 in this protective effect was confirmed by using Nrf2 deficient mice. To add strength, in vitro studies have been done using SF and results also corroborate the protective SF effect and add more information about its mechanism of protection. For example, in BV2 microglial cells, the protective effect of SF against the oxidative effect of lipopolysaccharide was associated with HO-1induction (31). In another cell culture, the dopaminergic cell death, induced by a compound that produces dopamine quinone: 6- hydroxydopamine and tetrahydrobiopterin, was also attenuated by SF preincubation (32).

Consistent with this evidence this study is aimed to identify the SF ability to prevent and counteract the oxidative damage inducted by oligomers of Aβ (1-42) in terms of impairment in the intracellular redox state and cellular death in differentiated human neuroblastoma. Chronic treatment with retinoic acid has previously been reported to inhibit SH-SY5Y proliferation and induce a significant proportion of cells to adopt a neuronal phenotype (33). Thus, differentiated SH-SY5Y could serve as a suitable *in vitro* cell model of AD.

The study will evaluate the ability of oligomers of Aβ (1-42) to induce ROS production in differentiated SH-SY5Y and in particular it will characterize which oxidant species are released during Aβ (1-42) –induced neurotoxicity. Subsequently, the research will investigate the ability of SF to prevent and counteract the oxidative damage and the mechanism underling the neuroprotective activity of SF. In particular we will investigate the SF ability to modulate the intracellular levels of glutathione (GSH), the most prominent antioxidant in the brain, and we will determinate if SF is able to decrease the GSK3 activity, because the GSK3 pathway has been emphasized in oxidative stress-induced neuronal cell death, by enhancing the expression of pro-apoptotic proteins and inhibiting the activity of anti-apoptotic proteins. It well known that exposure of neurones to Aβ increases GSK3 activity through the inhibition of PI3-kinase signalling and blockade of either GSK3 expression or activity prevents Aβ-

induced neurodegeneration (34). Considering these notions, the inhibition of GSK3 could be a promising strategy to modulate Aβ-induced neurotoxicity.

3 EXPERIMENTAL PROCEDURES

3.1 Materials

Amyloid β petptide

Aβ (1-42) peptides (Alexis) were first dissolved in hexafluoroisopropanol to 1mg/ml, sonicated, incubated at room temperature for 24 hours and lyophilized. The resulting unaggregated Aβ (1-42) film was dissolved with dimethysulfoxide and stored at -20°C until use. The Aβ (1-42) aggregation to oligomeric form was prepared by the incubation in D'PBS for 48 hours at 4°C. The morphology of the obtained oligameric Aβ (1-42) form was checked by transmission electron microscopy (TEM).

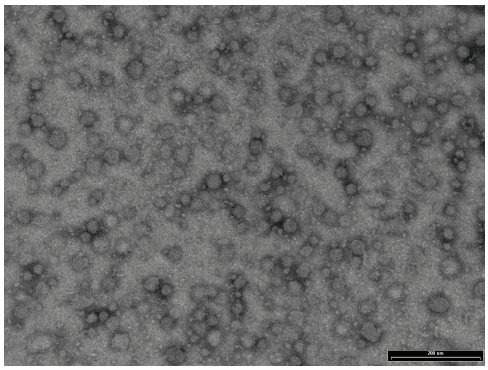
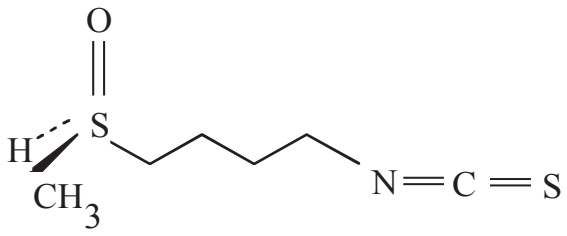


Fig.1 TEM micrographs of oligomeric Aβ (1-42) obtained after 48 hours of spontaneous aggregation. Total magnification: ×92,000; scale bar = 200 nm.

Sulforaphane

The Sulforaphane (SF) were purchased from LDK laboratories (St. Paul, MN, USA). SF was first dissolved in dimethylsulfoxide to obtain a stock concentration of [10μM], stored at -20°C.



Sulforaphane chemical structure

Cell cultures

Human neuronal-like SH-SY5Y were purchased from Interlab Cell Line Collection (ICLC Genova-Italy) and were routinely grown at 37°C in humified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2mM glutamine, 50 U/ml penicillin and 50 µg/ ml streptomycin (Lonza Group Ltd-Switzerland). To induce differentiation, the cells were seeded at $1 \cdot 10^5$ treated with 10 µM retinoic acid in DMEM with 1% heat-inactivated FBS for 6 days. The retinoic acid promotes the cells to establish a neurite network and increase the number of acetylcholine receptors (fig.1)

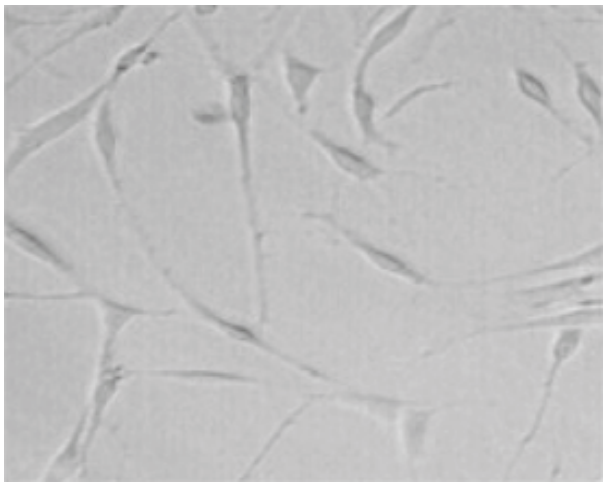


Fig.1 Differentiated SH-SY5Y cells.

Microglial cells were prepared from 3- to 7-day-old Wistar rat pups (Harlan, Udine, Italy) as described by Kingham and Pocock (35). After the isolation microglia cells are seeded at $1 \bullet 10^6$ cell/well in a 6-well plate, at 37°C in a humidified atmosphere of 5% CO2 in air and used after 1 day in vitro. Microglial cultures were > 99% pure as demonstrated by OX-42 reactivity (35) (fig.2). All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National (Ministry of Health) laws and policies. Care was taken to minimize the number of experimental animals and to take measures to minimize their suffering.

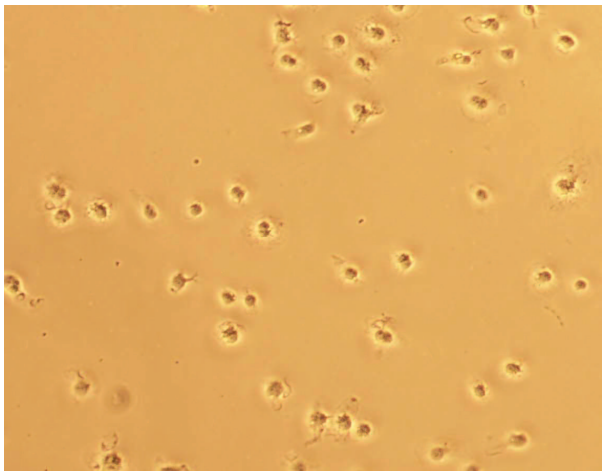


Fig.2 Microglia primary cultures
from rat.

3.2 Methods

Apoptosis assay

To determine the neuronal apoptosis, the Annexin-V-FLUOS Staining kit (Roche Diagnostic, Manheim, Germany) was used according to the manufacturer's instructions. Briefly, differentiated SH-SY5Y cells were treated with Sulforaphane

[0.6-2.5 µM] for 24 hours before or during the 24 hours treatment with oligomeric Aβ (1-42) [2 µM] . At the end of the treatment the cells are scraped, suspended at 2.5×10^5/mL, and washed with PBS. The cells were incubated with 100 µL of Annexin-V-FLUOS labeling solution at 24°C in the dark for 15 min. To determine the percentage of stained cells, four randomly selected areas with 50–100 cells in each were examined under a fluorescence microscope (Zeiss Axio Imager M1, Oberkochen, Germany). The values are expressed as percentage of apoptotic cells and calculated by the formula: (Annexin-V-positive cells/n total cells) × 100.

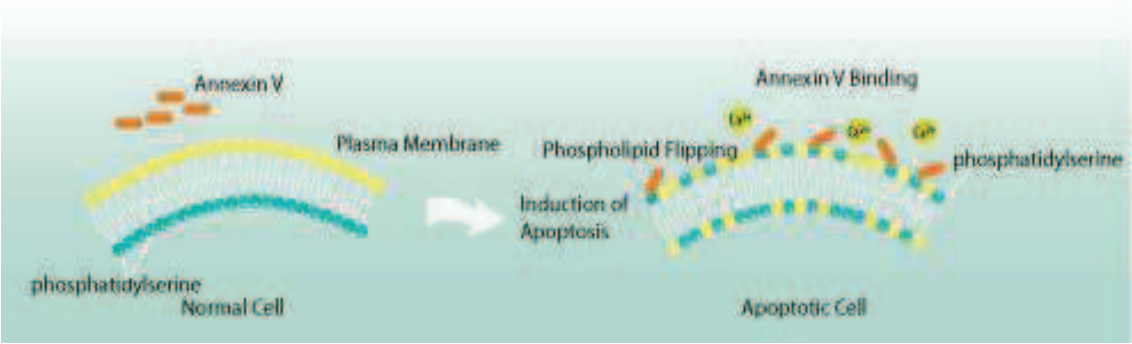


Fig.3 Schematic Representation of Apoptosis-Induced Membrane Changes Recognized by Annexin V. Reorganization of the plasma membrane occurs early in the apoptotic process disrupting phospholipid asymmetry and leading to the exposure of phophatidylserine on the outer leaflet of the cytoplasmic membrane. In the presence of calcium, Annexin V binds to phosphatidylserine allowing apoptotic cells to be easily identified by fuorescence microscopy.

Determination of ROS intracellular formation

ROS formation was determined using the fluorescent probe 2′,7′-dichlorodihydroflurescein diacetate, DCFH-DA (Sigma Aldrich) ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 535 nm). Briefly, differentiated SH-SY5Y cells were treated with Sulforaphane [1.25-5µM] for 24- 15-3 hours before the 3 hours-treatment with oligomeric Aβ (1-42) [2 µM] (pre-treatment) or they were treated at the same time with Sulforaphane [1.25-5µM] and oligomeric Aβ (1-42) [2 µM] for 3 hours

(co-treatment). Microglial primary culture were co-treated for 8 hours with Sulforaphane [5µM] and oligomeric Aβ (1-42) [2 µM]. At the end of the treatment, the cells were washed and incubated with DCFH-DA (5 µmol/L) for 15 min in the dark. After removal of the probe, cells were washed with PBS and incubated with DMEM serum-free for 1 h at 37°C. Intracellular ROS formation was measured under a fluorescence microscope (Zeiss Axio Imager M1). Fluorescence images were captured with an AxioVision image recording system computer. Four randomly selected areas with 20-30 cells in each were analyzed and the values obtained are expressed as densitometry/cell. The results were expressed as fold increase versus control.

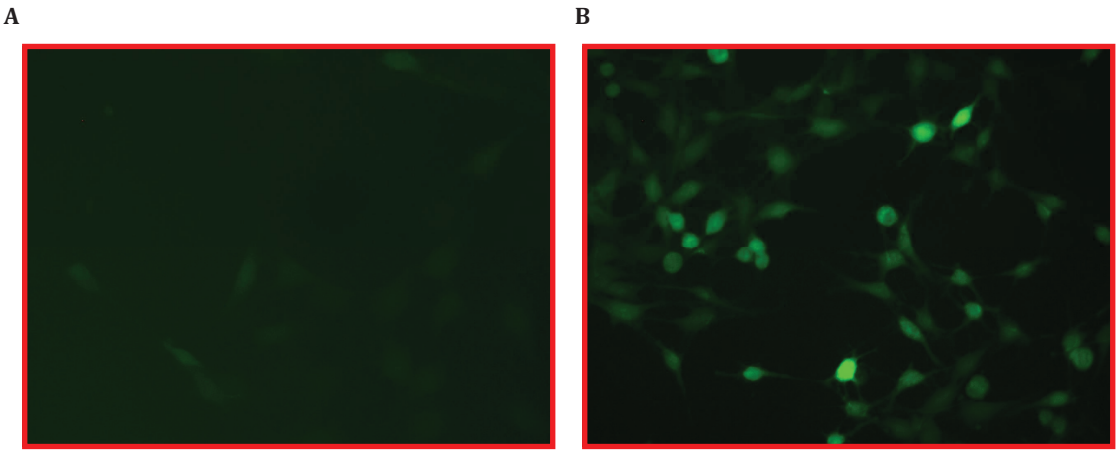


Fig.4 Representative images of ROS intracellular formation using DCFH-DA probe. Cellular esterases first hydrolyse DCFH-DA to DCFH, which is then oxidized by reactive species and originates 2,7 dichlorofluorescein (DCF), a fluorescent compound (**B**).

Determination of H_2O_2 release

To determine the hydrogen peroxide (H_2O_2), the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used according to the manufacturer's instructions. Differentiated SH-SY5Y cells were treated with Sulforaphane [5µM] for 24 hours before the 3 hours-treatment with oligomeric Aβ (1-42) [2 µM] (pre-treatment) or they were treated at the same time with

Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM] for 3 hours (co-treatment). Microglial primary culture were treated for 24 hours with Sulforaphane [5μM] before a treatment of 6 hours with oligomeric Aβ (1-42) [2 μM] and co-treated for 6 hours with Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM]. At the end of the treatment, 50μl of supernatant of each sample is collected and used to perform the assay. The Amplex® Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. The assay has been performed spectrophotometrically using 560nm with microplate spectrofluorometer (TECAN Genios, Mannendorf, Switzerland). The results were expressed as fold increase versus control.

Determination of intracellular superoxide levels

Dihydroethidium (DHE; Sigma Aldrich) was used to evaluate intracellular superoxide levels.

Briefly, differentiated SH-SY5Y cells were treated with Sulforaphane [1.25-5μM] for 24 hours before the 3 hours-treatment with oligomeric Aβ (1-42) [2 μM] (pre-treatment) or they were treated at the same time with Sulforaphane [1.25-5μM] and oligomeric Aβ (1-42) [2 μM] for 3 hours (co-treatment). Microglial primary culture were co-treated for 8 hours with Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM]. At the end of the treatment, the cells were washed and incubated with DHE [10 μM] for 15 min in the dark. After removal of the probe, cells were washed with PBS and incubated with DMEM serum-free for 1 h at 37°C. Intracellular superoxide formation was measured under a fluorescence microscope (Zeiss Axio Imager M1). Fluorescence images were captured with an AxioVision image recording system. The results were expressed as fold increase versus control.

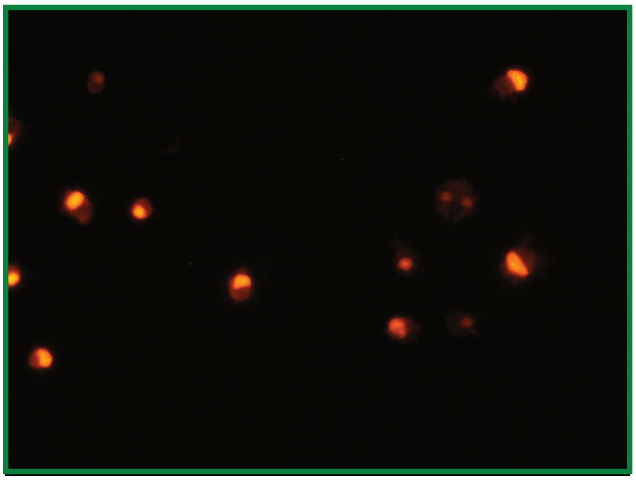


Fig.5 Representative images of Superoxide intracellular formation using DHE probe. In presence of superoxide anion, DHE oxidizes to ethidium and incorporates into the nuclear DNA emitting fluorescence

Determination of intracellular glutathione levels

To measure the intracellular glutathione (GSH) formation, monochlorobimane (MCB, Fluorescent Dyes, Los Angeles, CA, U.S.A) was used. Briefly, differentiated SH-SY5Y cells were treated with Sulforaphane [1.25-5μM] for 24-15-6-3 hours before the 3 hours-treatment with oligomeric Aβ (1-42) [2 μM] (pre-treatment) or they were treated at the same time with Sulforaphane [1.25-5μM] and oligomeric Aβ (1-42) [2 μM] for 3 hours (co-treatment). At the end of the treatment, the medium was eliminated from the wells and the cells were incubated with MCB [10 μM] for 30 minutes in the dark. The GSH levels were measured by microplate spectrofluorometer (TECAN Genios, Mannendorf, Switzerland). The results were expressed as fold increase versus control.

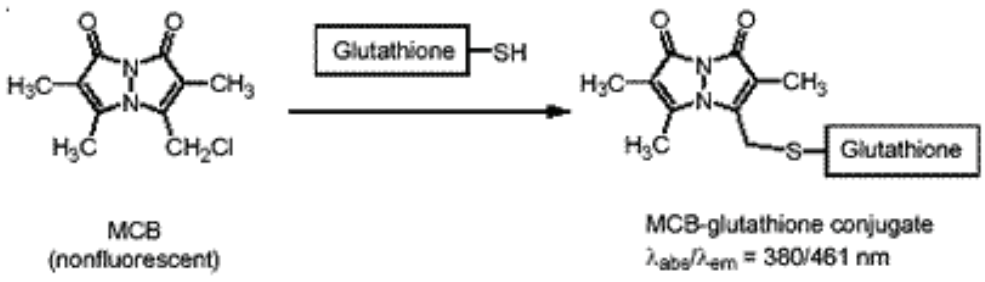


Fig. 6 Conjugation glutathione to MCB converts the nonfluorescent MCB to highly fluorescent MCB-glutathione conjugate.

Multiplex Luminex Assay

Bio-Plex Pro™ Rat Cytokine Kit (Bio-Rad) was used to determine the cytokine release from primary rat microglia. Microglial primary culture were co-treated with Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM] and co-treated with Sulforaphane [5μM] and LPS [1 μg/ml] for 15 hours. At the end of the treatment the supernatant of each sample was collected and used to perform the assay, according to the manufacturer's instructions.

Western Blotting

Western blotting analysis was used to detect phosphorylated GSK-3 (Cell Signaling)and iNOS (abcam) protein levels. Differentiated SH-SY5Y were co-treated for 1 hour with Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM] . Microglia primary cultures were co-treated with Sulforaphane [5μM] and oligomeric Aβ (1-42) [2 μM] and co-treated with Sulforaphane [5μM] and LPS [1 μg/ml] for 8 hours. At the end of the drug treatment times, cells were washed twice with ice-cold PBS and the harvested at 4°C in a lysis buffer (25 mM Tris buffer pH 7.4, containing 150 mM NaCl, 100 μM sodium orthovanadate, 1.5 mM $MgCl_2$, 1.0 mM leupeptin, 5 μg/ml aproptin). After 20 minutes on ice, cells were centrifuges at 14000 rpm for 10 minutes at 4°C. Supernatant aliquots were used for determination of protein concentration by Bradford method. Protein were

diluted in Laemli-SDS sample buffer and boiled for 5 minutes. Equal amount of protein were loaded into each lane of 12% SDS-PAGE gel (Biorad Mini protean pre-cast gels) and resolved at 200 V constant. Gels were transferred onto nitrocellulose membrane (Sigma Aldrich) at 100 V constant for 60 minutes. Membranes were then blocked in in blocking buffer (PBS, 0.1% Tween-20 with 5% w/v non fat dry milk) for 2 hours. Blots were incubated overnight at 4°C with specific primary antibodies. All primary antibodies were diluted 1:1000 in primary antibody dilution buffer (PBS, 0.1% Tween-20 with 2.5% w/v non fat dry milk). After washing three times for 15 minutes each with washing buffer (PBS, 0.1% Tween-20) membranes were exposed to a secondary antibody diluted 1:2500 for 1 hour at room temperature. Immunocomplexes were visualized using enhancing chemiluminescence ECL kit, (GE-Healthcare, Milano-Italy) detection system. Densitometry analysis were performed for the quantification of immunoblots using the Quantity One 1-D Analysis Software (Bio-Rad).

Statistical Analysis

Data were reported as mean ± SEM of at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA and the Dunnet or Bonferroni *post-hoc* test was used. Differences were considered significant at $p<0.05$. Analysis were performed using PRISM 4 software on a Windows platform.

4 RESULTS

4.1 SF ability to prevent Aβ–induced neurotoxicity in neurons

The study first determined the ability of SF 0.6-2.5 µmol/L to prevent the neuronal death in terms of apoptosis, induced by oligomers of Aβ (1-42) 2µmol/L. by Annexin V staining and fluorescence microscopy. The SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 2 m*M* L-glutamine. To induce differentiation, the cells were treated with 10 µ*M* RA in DMEM with 1% heat-inactivated fetal calf serum in the dark for 4–6 days. The retinoic acid promotes the cells to establish a neurite network and increase the number of acetylcholine receptors. Differentiated SH-SY5Y are exposed to various non-cytotoxic concentrations of SF (0.6-2.5 µmol/L) for 24 hours before or during the treatment with oligomers of Aβ (1-42) in concentration of 2µmol/L for 24 hours.

The pre-treatment with SF for 24 hours is able to prevent Aβ (1-42) induced apoptosis in differentiated SH-SY5Y, leading to a significant decrease of apoptosis at SF 2.5 µmol/L (Fig. 1).

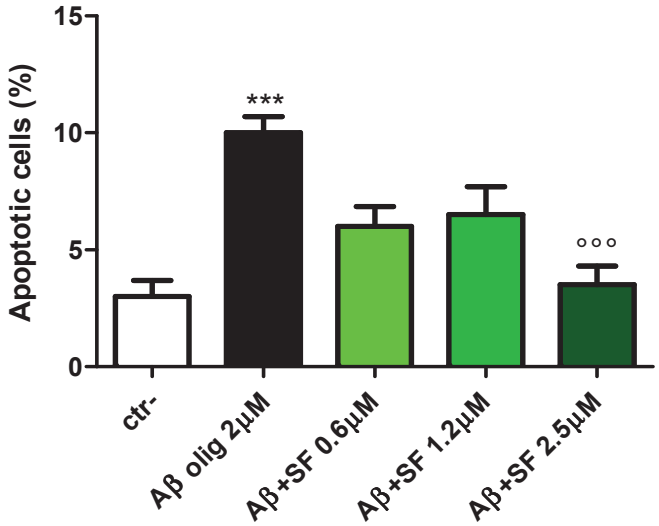


Fig. 1 SF (0.6-2.5 µmol/L) prevents oligomeric Aβ (1-42) induced apoptosis in differentiated SH-SY5Y. Differentiated SH-SY5Y were incubate for 24 hours with SF (0.6-2.µmol/L) and then treated with oligomeric

Aβ (1-42) 2 μmol/L for 24 hours. At the end of incubation the apoptosis was determined with Annexin V and fluorescence microscope. Four randomly selected areas with 50-100 cells in each were analyzed under a fluorescence microscope. The values are expressed as percentage of total number of cells and a shown as mean ± SEM of 3 independent experiments.***p<0.001 versus control, °°°p<0.001 versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

Several studies have indicated that Aβ induces apoptosis and neuronal cell death by producing ROS (36). Thus, to determine whether the recorded antiapoptotic effect of SF in the pre-treatment could be ascribed to its ability to counteract oxidative stress-mediated neuronal death, the study also investigated the Aβ-induced intracellular ROS formation in differentiated SH-SY5Y cells pre-treated with SF (1.25-5 μmol/L) for different times (3, 6, 15 and 24 hours), using DCFH-DA (5μmol/L), a fluorescent probe and a fluorescence microscope.

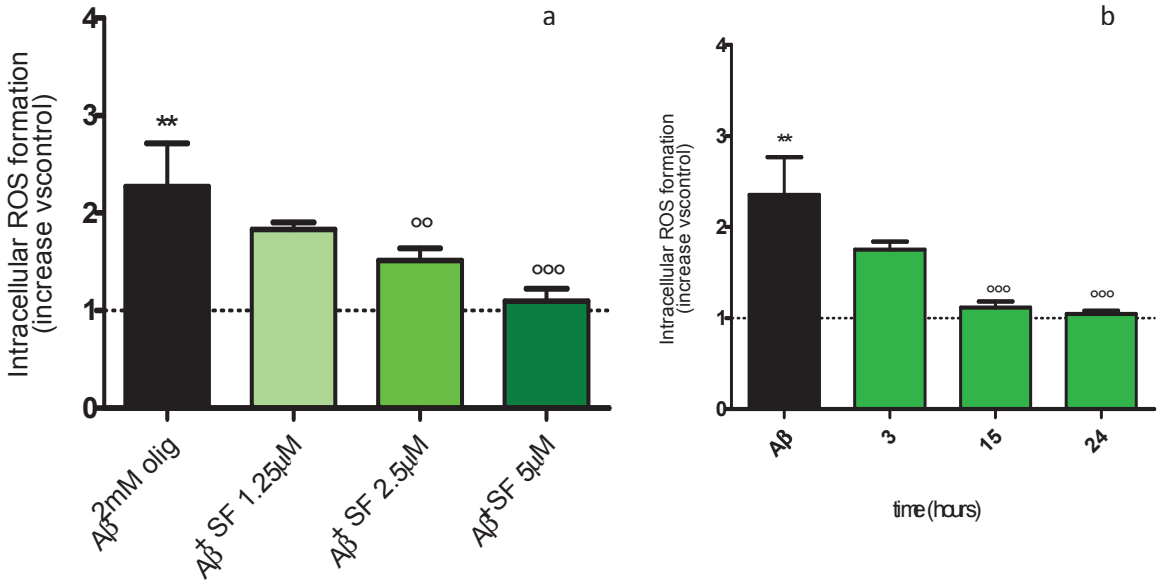


Fig.2 SF prevents Aβ-induced intracellular ROS formation in differentiated SHSY5Y cells. Differentiated SH-SY5Y cells were incubated with SF (1.25-5 μmol/L) for 24 hours, then were treated with oligomeric Aβ (1-42) 2 μmol/L for 3 hours (a). In (b) the differentiated SH-SY5Y cells were incubate with SF (5 μmol/L) for 3-6-15 and 24 hours and then they were treated with oligomeric Aβ (1-42) 2 μmol/L for 3 hours. At the end of incubation, ROS formation was determined using a fluorescence probe, DCFHDA-DA. Four randomly selected areas with 15-30 cells in each were analyzed under a fluorescence microscope. Values are expressed as a fluorescence increase versus control and shown as mean ± SEM of 3 independent experiments.***p<0.001

versus control, °°°p< 0.001 versus Aβ treated cells, °°p<0.01 versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

The pre-treatment with SF exhibits a significant dose-dependent inhibition in ROS formation (fig.2 a), moreover the ROS formation decrease shows a time-dependent relationship with the SF (5 µmol/L) pre-treatment (fig.2 b).

Subsequently we investigated the mechanism underlying the antiapoptotic effect and the inhibitory effect on intraneuronal ROS formation of SF. We determined if SF (1.25-5 µmol/L) could cause elevation of total GSH levels in differentiated SH-SY5Y cells. Treatment of SH-SY5Y with SF (1.25-5 µmol/L) for 24 hours resulted in a significant increase in cellular GSH content (fig.3 a). Moreover, the enhancement in cellular GSH levels induced by SF (5 µmol/L) also exhibits a treatment time-dependent relationship (fig.3 b).

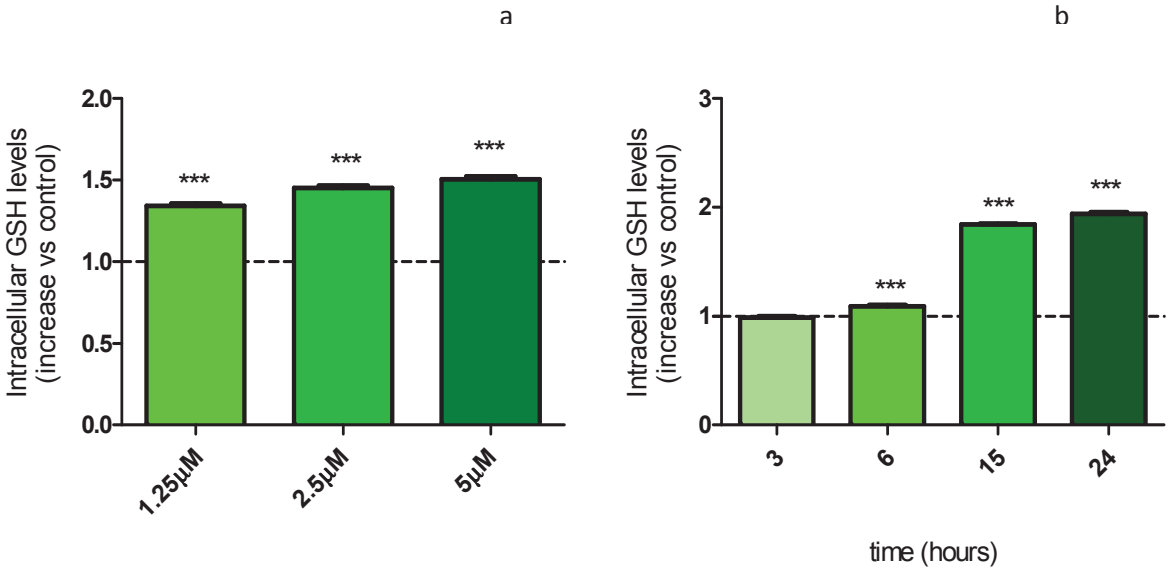


Fig. 3 SF enhances the total GSH levels of differentiated SH-SY5Y cells. The differentiated SH-SY5Y cells were incubated with various concentrations of SF (1.25-5 µmol/L) for 24 hours (a) and with SF (5 µmol/L) for several times (3-24 hours) (b). At the end of incubation, GSH levels were measured using Monochlorobimane (MBC, Molecular Probes). The values are shown as mean ± SEM of 3 independent experiments. ***p<0.001 versus untreated cells at ANOVA with Dunnet *post hoc* test.

Moreover, to verify the main role of GSH in the neuroprotective effects displayed by SF, the study used buthionine sulfoximine (BSO), which irreversibly inhibits gamma-glutamylcysteine synthetase, the first enzymes in the GSH biosynthesis pathway. The addition of BSO (400 µmol/L) to treatment of differentiated SH-Y5Y cells with SF (5 µmol/L) for 24 hours abolished the antiapoptotic effect observed with SF alone against toxicity induced by oligomeric Aβ(1-42) 2 µmol/L. for 24 hours (fig.4).

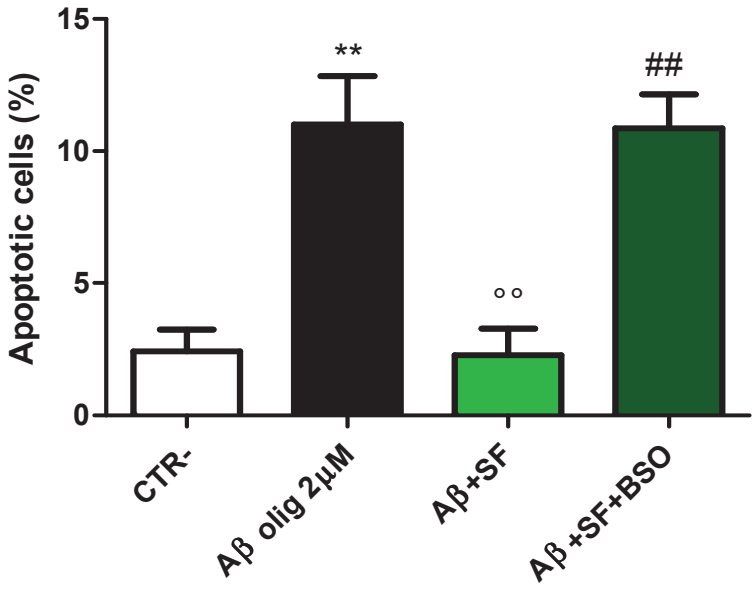


Fig.4 SF counteracts Aβ-induced apoptosis in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were incubate with SF (5 µmol/L) in the absence or presence of BSO 400 µmol/L and then treated with oligomeric Aβ (1-42) 2 µmol/L for 24 hours. At the end of incubation, the neuronal death in terms of apoptosis was determined by Annexin V and fluorescence microscope. The values are expressed as percentage of total number of cells and shown as mean ± SEM of 3 independent experiments.**$p<0.01$ versus control, °°$p< 0.01$ versus Aβ treated cells, ##$p<0.01$ versus SF pre-treated and Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

Subsequently, to characterize the intracellular ROS formation induced by oligomeric Aβ (1-42) it has been used Dihydroethidium (DHE), that is a widely used sensitive superoxide anions probe and the Amplex® Red Hydrogen Peroxide/peroxidase Assay Kit, to detect hydrogen peroxide (H_2O_2). Then, it has been determined the SF ability to prevent the production of these specific forms of ROS.

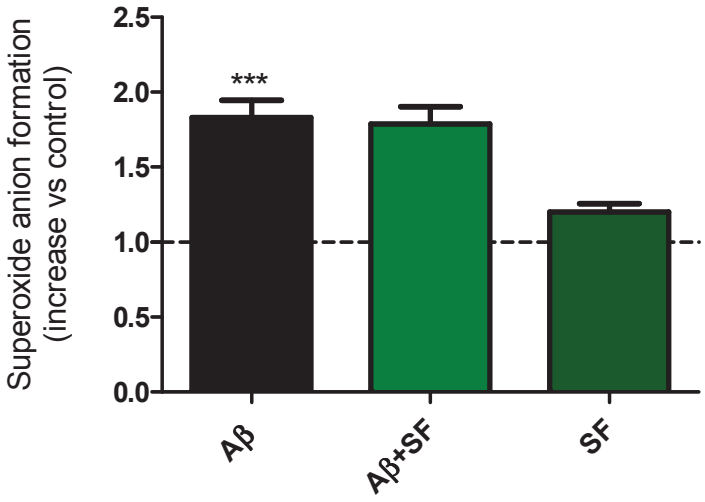


Fig.5 Differentiated SH-SY5Y were incubate with SF (5 μmol/L) for 24 hours and then they were treated with oligomeric Aβ (1-42) 2μ mol/L for 3 hours (a). At the end of the incubation intracellular superoxide anions production is determined using DHE (1 μmol/L) and fluorescence microscope. Values are expressed as a fold fluorescence increase versus control and shown as mean ± SEM of 6 replicates of one representative experiments. ***p<0.001 versus control, at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

The Amplex® Red reagent, in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2 released from cells.

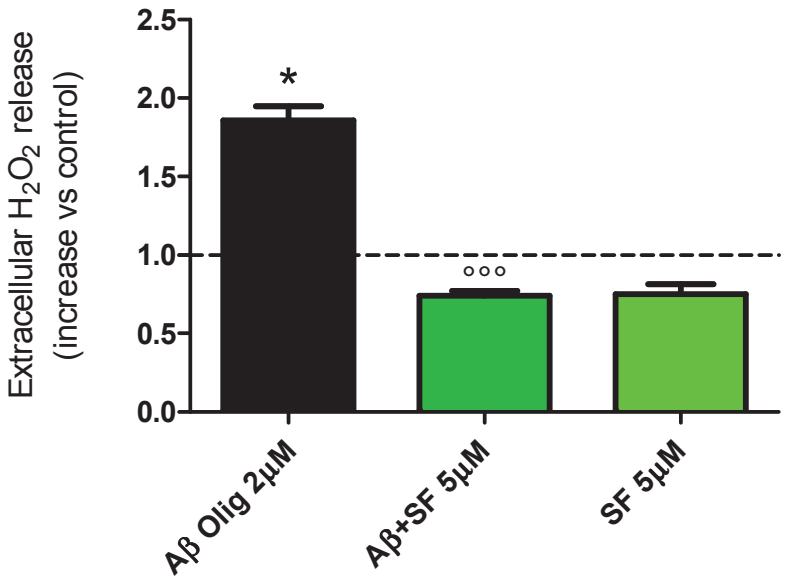


Fig.6 Differentiated SH-SY5Y were incubate with SF (5 μmol/L) for 24 hours and then they were treated with oligomeric Aβ (1-42) 2 μmol/L for 3 hours. At the end of the incubation H_2O_2 release is determined using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit following the protocol providing by the kit. Values are expressed as mean ± SEM of 3 replicates of 2 independent experiments. *$p<0.05$ versus control, °°°$p<0.001$ versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

4.2 SF ability to counteract Aβ–induced neurotoxicity in neurons

The study subsequently determined the ability of SF 0.6-2.5 μmol/L to counteract the neuronal death in terms of apoptosis, induced by oligomers of Aβ(1-42) 2μmol/L. by Annexin V staining and fluorescence microscopy in the differentiated SH-SY5Y.

The co-treatment with SF for 24 hours is able to counteract Aβ (1-42) induced apoptosis in differentiated SH-SY5Y, leading to a significant decrease of apoptosis for all SF concentrations, and the maximum inhibition at SF 2.5 μmol/L (Fig. 7).

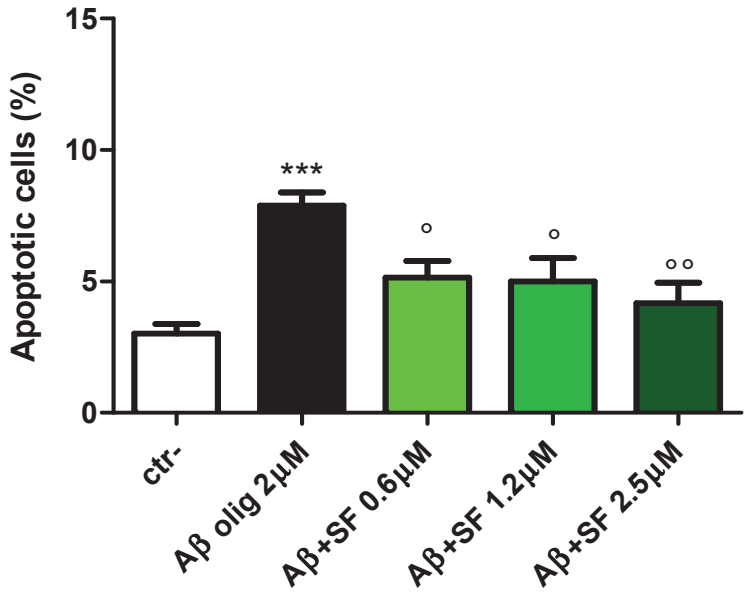


Fig. 7 SF (0.6-2.5 μmol/L) counteract prevents oligomeric Aβ (1-42) induced apoptosis in differentiated SH-SY5Y. Differentiated SH-SY5Y were incubate for 24 hours with SF (0.6-2.μmol/L) and with oligomeric Aβ (1-42) 2 μmol/L for 24 hours. At the end of incubation the apoptosis was determined with Annexin V and fuorescence microscope. Four randomly selected areas with 50-100 cells in each were analyzed under a fluorescence microscope. The values are expressed as percentage of total number of cells and a shown as mean ± SEM of 3 independent experiments.***$p<0.001$ versus control, °°$p<0.01$ versus Aβ treated cells and °$p<0.05$ versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

To determine whether the recorded antiapoptotic effect of SF in the co-treatment could be ascribed to its ability to counteract oxidative stress-mediated neuronal death, the study also investigated the Aβ-induced intracellular ROS formation in differentiated SH-SY5Y cells co-treated with SF (1.25-5 μmol/L) using DCFH-DA (5μmol/L), a fluorescent probe and a fluorescence microscope. . As shown in fig. 8, SF induces a significant decrease of intracellular ROS formation at 2.5 and 5 μmol/L.

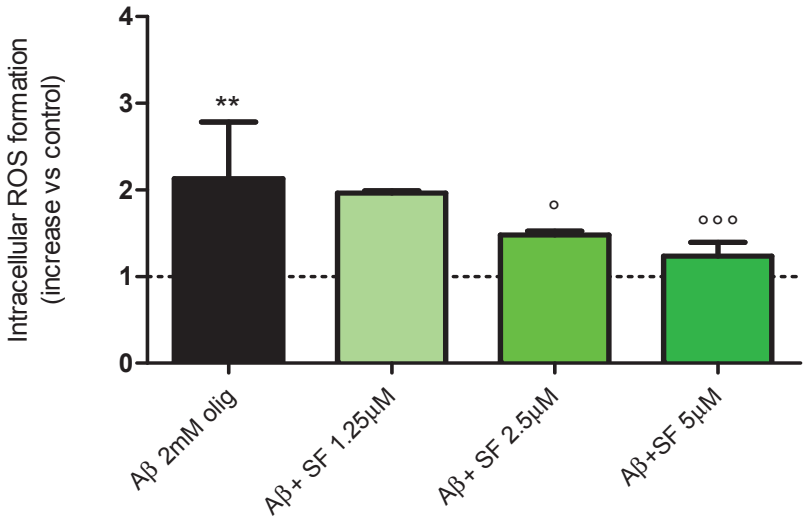


Fig.8 SF counteracts Aβ-induced intracellular ROS formation in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were incubated with SF (1.25-5 μmol/L) and with oligomeric Aβ (1-42) 2 μmol/L for 3 hours. At the end of the incubation, intracellular ROS formation was determined using a fluorescence probe, DCFHDA-DA. Four randomly selected areas with 15-30 cells in each were analyzed under a fluorescence microscope. Values are expressed as a fold increase versus control and shown as mean ± SEM of 3 independent experiments. **p<0.01 versus control, °°°p< 0.001 versus Aβ treated cells, °p<0.05 versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

Subsequently, to characterize the intracellular ROS formation induced by oligomeric Aβ (1-42) it has been used Dihydroethidium (DHE), that is a widely used sensitive superoxide anions probe and the Amplex® Red Hydrogen Peroxide/peroxidase Assay Kit, to detect hydrogen peroxide (H_2O_2). Then, it has been determined the SF ability to counteract the production of these specific forms of ROS (fig. 9)

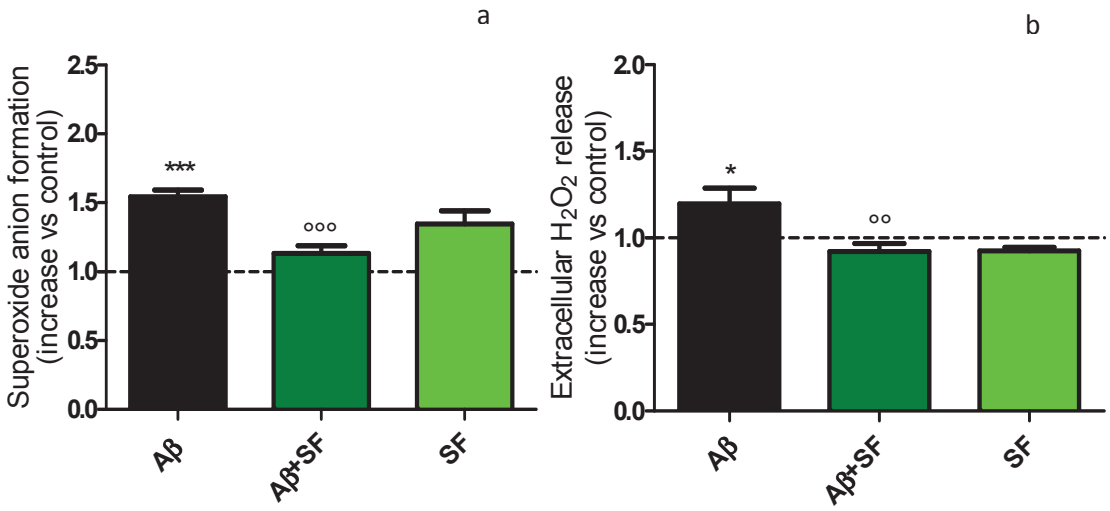


Fig. 9 Differentiated SH-SY5Y cells were incubate at the same time with SF (5 µmol/L) and oligomeric Aβ (1-42) 2µmol/L, for 3 hours, At the end of the incubation intracellular superoxide anions production is determined using DHE (1 µmol/L) and fluorescence microscope (a) and the hydrogen peroxide extracellular release is evaluated using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit following the protocol providing by the kit. . Values are expressed as a fold fluorescence increase versus control and shown as mean ± SEM of 6 replicates of one representative experiments. ***p<0.001 versus control, *p<0.05 versus control, °°p< 0.01 versus Aβ treated cells, °°°p<0.001 versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

To evaluate the mechanism underlying the antiapoptotic and the inhibitory effect of SF on the intracellular ROS formation in the co-treatment, the study investigated if SF (1.25-5 µmol/L) could cause elevation of total GSH levels in differentiated SH-SY5Y cells. Treatment of SH-SY5Y with SF at all concentrations doesn't exhibit the ability to enhance intracellular GSH levels (fig. 10).

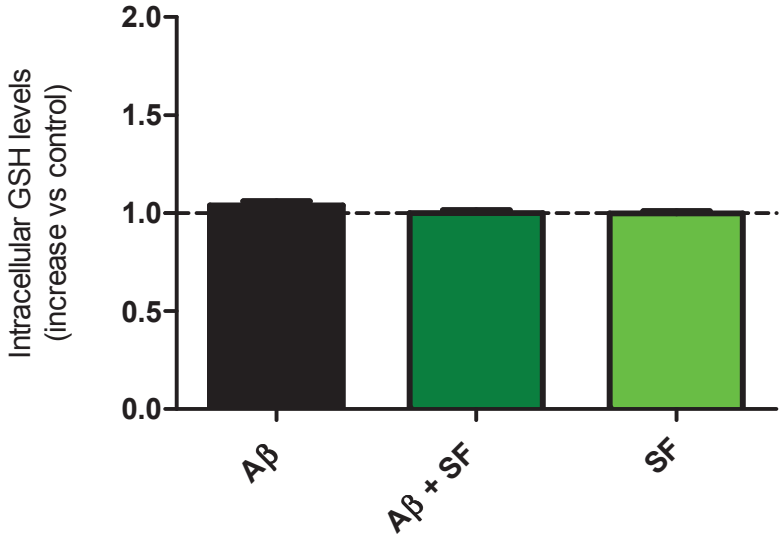


Fig. 10 SF doesn't enhance intracellular GSH levels in differentiated SH-SY5Y. The differentiated SH-SY5Y cells were incubated with SF /(5 μmol/L) for 3 hours (b). At the end of incubation, GSH levels were measured using Monochlorobimane (MBC, Molecular Probes). Values are expressed as fold increase versus control and shown as mean ± SEM of 3 independent experiments, Values are expressed as a fluorescence increase versus control and shown as mean ± SEM of 3 independent experiments.

To clarify the mechanism underlying the inhibitory effect of SF on the intracellular ROS formation in the co-treatment, the study also evaluates the potential direct antioxidant activity, investigating the SF ability to counteract the ROS formation induced by Menadione and H_2O_2 (fig.11) and measuring the free radical scavenging activity of our compound using the DPPH (1,1-diphenil-2 picrylhydrazyl) method.

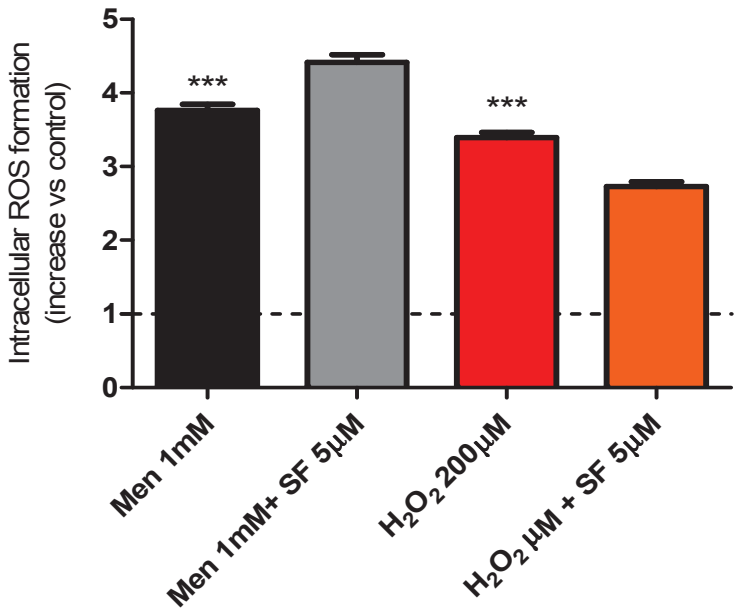


Fig. 11 Differentiated SH-SY5Y cells were incubate at the same time with SF (5 µmol/L) and Menadione 1mM and SF (5 µmol/L) with H_2O_2 (200µM) for 30 minutes. At the end of the incubation intracellular ROS production is determined using a fluorescence probe, DCFHDA-DA DHE (1 µmol/L) and fluorescence microscope. Values are expressed as a fold increase versus control and shown as mean ± SEM of 3 independent experiments.

The scavenging of the DPPH radical is a simple model reaction providing information about the relative ability of antioxidant compound to scavenge free radicals. N-acetyl-L-cysteine (NAC) were used as reference standard. The DPPH formation, observed at 517 nm, decreases stoichiometrically with respect to the number of electrons taken up. The decrease in the peak depends on the radical scavenging activity of the tested compound, the absorbance was recorded at various concentration of SF (0.6-5 µM). SF did not interact with DPPH radical at any concentration of SF used in the experiment (data not shown). This result confirm that SF does not exhibits any direct antioxidant properties.

Thus, to evaluate the mechanism underlying the antiapoptotic and the inhibitory effect of SF on the intracellular ROS formation in the co-treatment, in absence of any increase in the GSH intracellular levels and excluding the direct antioxidant activity of the SF, the study investigated if SF (5 µmol/L) could promote the inhibition of GSK3 (increasing the protein phosporylation) in differentiated SH-SY5Y cells.

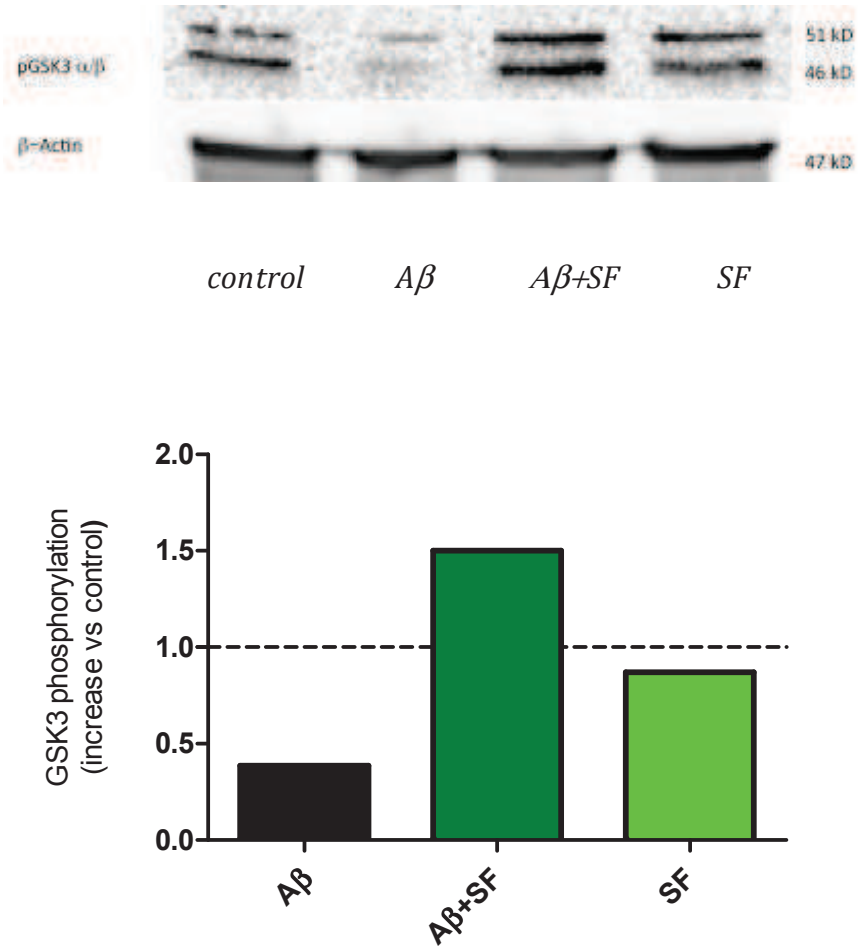


Fig.12 GSK3-phosphorylation after a co-treatment of 1 hours SF 5 µmol/L and oligomeric Aβ (1-42) 2µmol/L. Values normalized versus β Actin protein levels and express as fold increase versus control.

4.3 SF ability to counteract Aβ–induced neurotoxicity in microglia

Emerging evidence suggests that neuroinflammation contributes to the development of neurodegenerative disease, like AD. Microglia are found in close association with the neuritic plaques in AD brain and Aβ–induced inflammatory response mediated by microglia are thought to contribute to neuronal toxicity (37).

Based on the ability of SF to counteract the oligomeric Aβ (1-42)–induced ROS formation in differentiated SH-SY5Y, the study evaluated if this effect was recordable also in primary microglia cultures.

As shown in fig. 13, the co-treatment for 8 hours with SF (5 μmol/L) is able to induce a significant decrease of the ROS intracellular formation induced by oligomeric Aβ (1-42) 2μmol/L and LPS 1μg/ml.

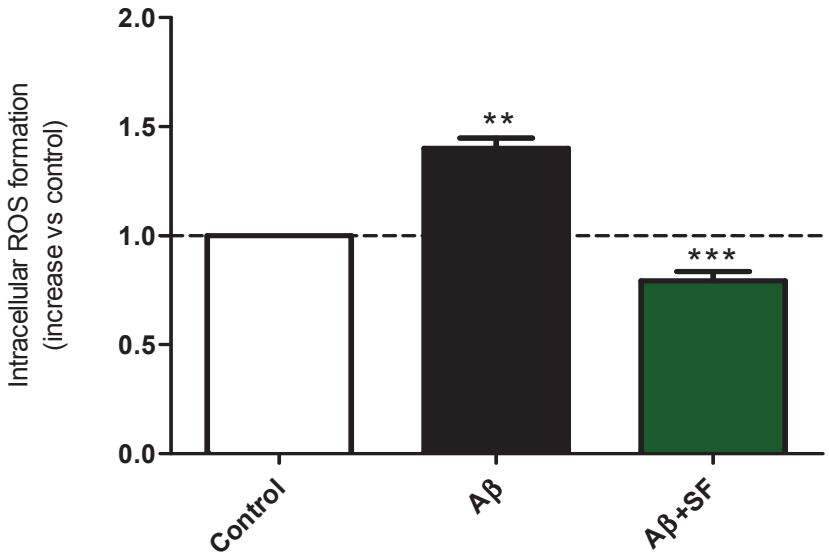


Fig.13 SF counteracts Aβ-induced intracellular ROS formation in microglia primary cultures. Microglia were incubated with SF (5 μmol/L) and with oligomeric Aβ (1-42) 2 μmol/L for 8 hours. At the end of the incubation, intracellular ROS formation was determined using a fluorescence probe, DCFHDA-DA. Four randomly selected areas with 15-30 cells in each were analyzed under a fluorescence microscope. Values are expressed as a fold increase versus control and shown as mean ± SEM of 3 independent experiments. **p<0.01 versus control, °°°p< 0.001 versus Aβ treated cells, at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

Subsequently, to characterize the intracellular ROS formation induced by oligomeric Aβ (1-42) it has been used Dihydroethidium (DHE), that is a widely used sensitive superoxide anions probe and the Amplex® Red Hydrogen Peroxide/peroxidase Assay Kit, to detect hydrogen peroxide (H_2O_2). Then, it has been determined the SF ability to counteract the production of these specific forms of ROS. As shown in fig.14, SF (5 µmol/L) is able to reduce the release of both, H_2O_2 and superoxide anion.

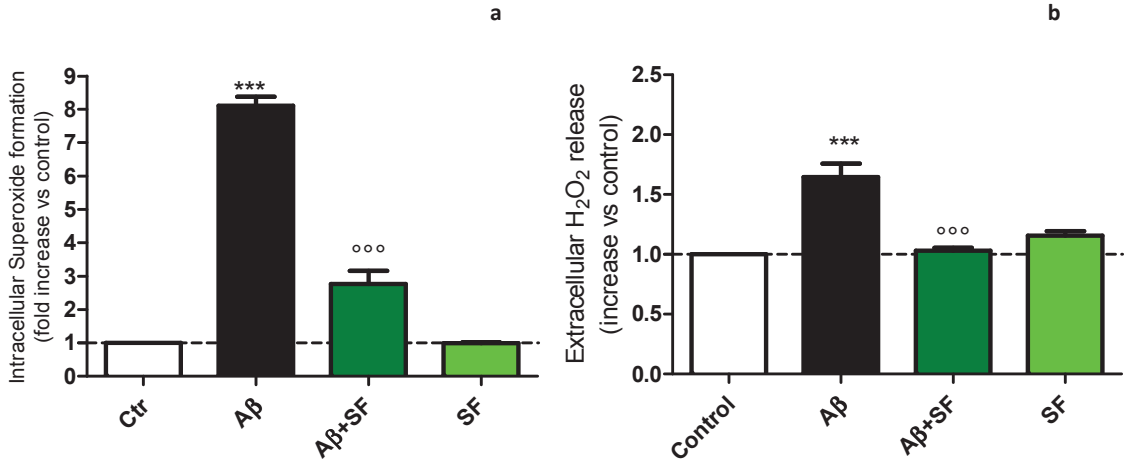


Fig.14 Microglia primary cultures were incubate at the same time with SF (5 µmol/L) and oligomeric Aβ (1-42) 2µmol/L, for 8 hours, At the end of the incubation intracellular superoxide anions production is determined using DHE (1 µmol/L) and fluorescence microscope (a) and the hydrogen peroxide release is evaluated using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit following the protocol providing by the kit. . Values are expressed as a fold increase versus control and shown as mean ± SEM of 5 replicates of one representative experiments. ***p<0.001 versus control, *p<0.05 versus control, °°p< 0.01 versus Aβ treated cells, °°°p<0.001 versus Aβ treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test.

Treatment of microglia cells with Aβ has previously been shown to increase the levels of inflammatory molecules (Shukla and Sharma 2011). Thus we examined whether SF has any effect on oligomeric Aβ (1-42)-induced release of cytokines (TNFα, IL1β and IL6) from microglia primary cultures, using multiplex luminex assay. LPS treatment was used as a activation microglia positive control .

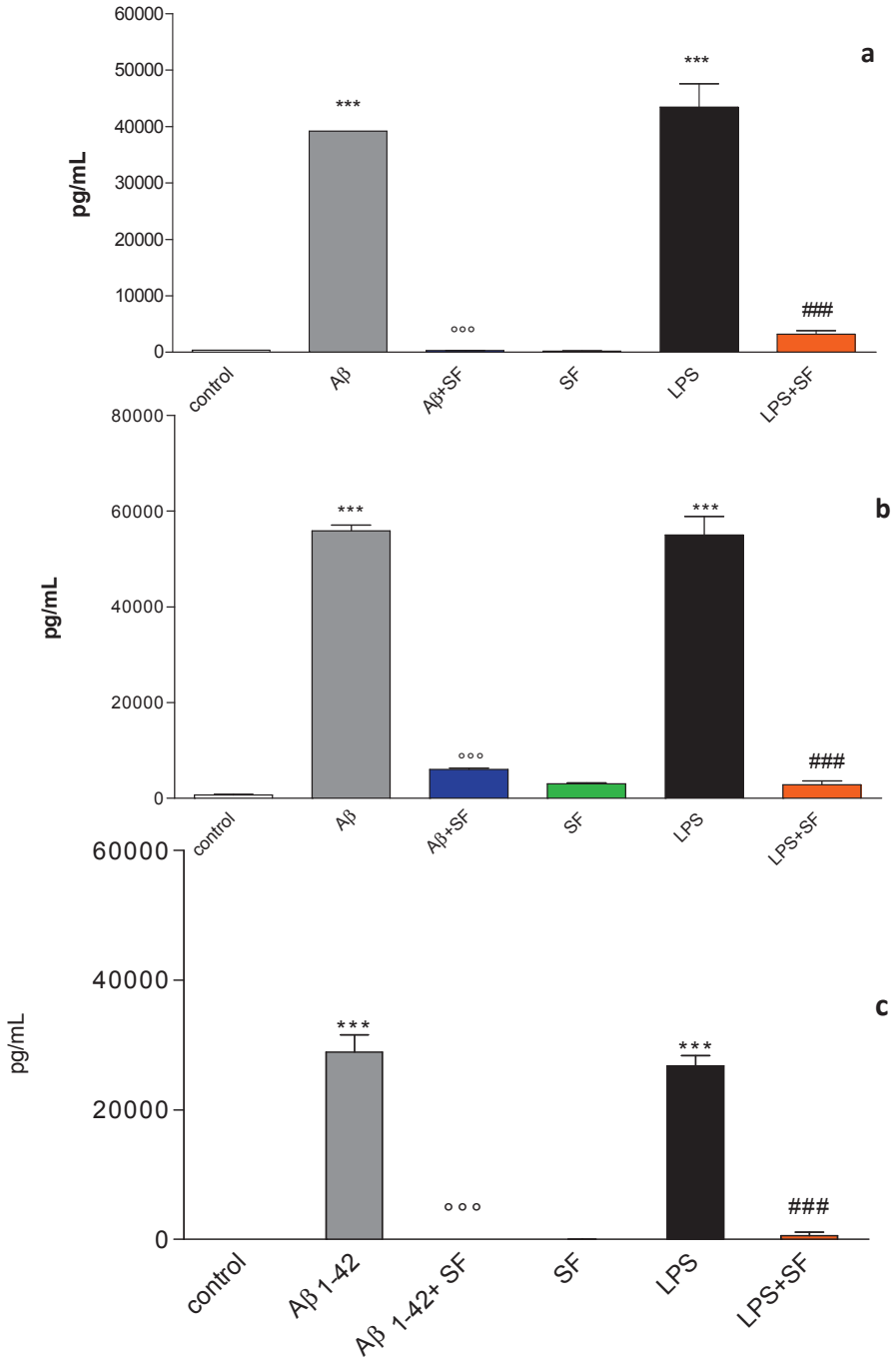


Fig. 15 Microglia primary cultures were exposed for 15 hours to SF (5 µmol/L), oligomeric Aβ (1-42) 2µmol/L and LPS (1µg/ml). At the end of treatment time, supernatant of each sample was collected and processed by the multiplex luminex assay, following the protocol providing by the kit. Values are expressed as pg/ml of cytokine and shown as mean ± SEM of 3 replicates of one representative experiments. ***p<0.001 versus control, °°°p< 0.01 versus Aβ treated cells, ###p<0.001 versus LPS treated cells at ANOVA with Bonferroni's Multiple Comparison *post hoc* test. (a) TNFα levels. (b) IL 1β levels. (c) IL 6 levels.

As shown in fig. 15, SF attenuates microglia activation, inducing a significant decrease in the production of pro-inflammatory cytokines TNFα, IL1β and IL6.

Subsequently, we evaluated the SF ability to modulate the Aβ- and LPS- induced expression of iNOS, enzyme known to be responsible for the production of NOS and ROS. As shown in fig.16, SF is able to attenuates the Aβ- and LPS-induced expression of iNOS.

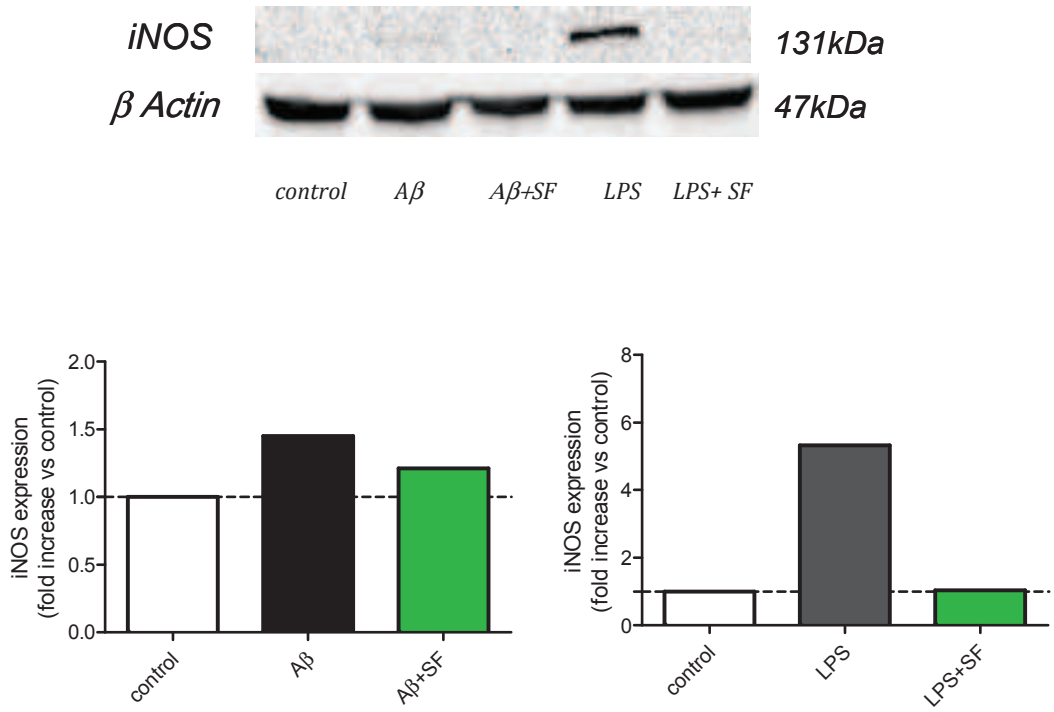


Fig.16 iNOS expression after a co-treatment of 8 hours with SF 5 µmol/L, oligomeric Aβ (1-42) 2µmol/L. and LPS 1µg/mL. Values normalized versus β Actin protein levels and expressed as fold increase versus control.

5 DISCUSSION

Oxidative stress has been implicated in the pathogenesis of a number of disease including neurodegenerative disorders, cancer, ischemia, etc. Under physiological conditions, there is a balance between the pro-oxidant and anti-oxidant levels; however certain environmental factors, stressor or disease may cause an imbalance leading to increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS may react with biomolecules including proteins, lipids, DNA and RNA, leading to oxidative damage of these biomolecules. Oxidative modification of biomolecules has been shown to lead to cellular dysfunction. A large number of hypotheses were proposed for AD mechanism, which include the amyloid cascade, excitotoxicity, oxidative stress and inflammation hypotheses, and all of these are based , to some extent, on the role of Aβ. In particular, a number of in vitro and in vivo studies showed that Aβ(1-42) is the mayor component of senile plaques and is more toxic of Aβ(1-40) and that small oligomers of Aβ were suggested to be the most toxic species (24).

Experimental data from in vitro and in vivo studies indicate that Aβ deteriorates a variety of neuronal and glial functions, thereby ultimately leading to apoptotic death in these cells (38). In particular, increasing evidence support that Aβ–caused neuronal cell death is mediated via oxidative stress (39; 40). The neurotoxic effect of Aβ are at least in part mediated by generation of ROS and RNS, leading to cell apoptosis (41). Aβ can directly cause neuronal toxicity but can also affect microglia cells to produce inflammatory and toxic factors that then can affect the viability of neurons. Considerable evidence indicates that Aβ induces ROS generation in microglia cells (42) and NO (43), that are implicated in the pathogenesis of AD. In addition Aβ activates microglia leading to the release of pro-inflammatory cytokines, like TNFα, IL 1β and IL 6, which may contribute to pathology, by promoting further inflammation and neuronal toxicity.

The study was aimed to evaluated the potential antioxidant activity of SF against ROS production and in particular against H_2O_2 and superoxide, induced by

oligomeric Aβ (1–42) in differentiated SH-SY5Y. Furthermore, the SF potential antioxidant activity was measured before and during Aβ-induced ROS formation. This experimental approach provided the opportunity to evaluated the potential neuroprotective and/or neurorescue effect of SF. In addition the study determined the SF ability to counteract the ROS formation and in particular against H_2O_2 and superoxide, and cytokines (TNFα, IL 1β and IL 6) release induced by oligomeric Aβ (1–42), in microglia primary cultures from rat.

Initially, we determined that pre-treatment with SF (2.5μmol/ml) is able to prevent the Aβ-induced apoptosis in differentiated SH-SY5Y. Several studies indicates that Aβ induces apoptosis by promoting ROS production (13; 36). Consistent with this evidence, we found that Aβ is able to promote ROS formation, and in particular H_2O_2 and superoxide production. Subsequently we investigated whether the recorded SF-antiapoptotic effect could be ascribed to its capacity to prevent ROS formation and we determined that pre-treatment for 15 and 24 hours with SF (2.5-5 μmol/ml) was able to promote a significant decrease of ROS, and in particular of H_2O_2.

An important way of preventing ROS in brain tissues, may be to replenish lost GSH levels by increasing the synthesis of GSH or slowing its degradation. Thus, we analysed the SF ability to enhance GSH levels, and we found that treatment with SF results in a significant increase in cellular GSH content, and the effect is dose- and time-dependent. This finding is particularly important since GSH can play an important role in the detoxification of H_2O_2, that is an important oxidant species, induced also by Aβ, and involved in promoting neuronal death. In particular, we also showed that antiapoptotic effect of SF could be ascribed to its ability to increase GSH levels, because of SF neuroprotective effect recorded in the pre-treatment, was abolished by inhibition of GSH synthesis, using BSO, supporting the predominant role of GSH in the indirect antioxidant effects displayed by SF.

Subsequently the study determined the antiapoptotic and antioxidant effects of SF during a co-treatment with Aβ. The results obtained after the co-treatment of 3 hours, showed that SF is able to counteract Aβ-induced apoptosis and Aβ-induced

ROS production, in particular is able to decrease the formation of both, H_2O_2 and superoxide. Contrary to the pre-treatment, the antioxidant ability of SF could not be ascribe to its capacity to increase cellular GSH content. Furthermore, we excluded SF ability to counteract ROS production with a direct antioxidant activity, measuring the free radical scavenging activity of SF with DPPH method and testing the SF capacity to modify the altered redox state of the cell induced by Menadione and H_2O_2.. Thus, to evaluate the mechanism underlying the antiapoptotic and inhibitory effect on ROS formation observed in the co-treatment, in absence of GSH induction and excluding a direct antioxidant activity, the study investigated if SF was able to promote the inhibition of GSK3 activity. Aberrant activation of GSK3 plays a critical roles in oxidative stress-induced neuronal death mechanisms (44; 45). Several studies show that GSK3 pathway promotes neuronal death by both enhancing the expression of pro-apoptotic proteins and inhibiting the activity of anti-apoptotic proteins (46). In particular, *Rojo et al.* showed that GSK3 is the main responsible of the inhibition in the transcriptional activity of Nrf2, blocking its antioxidant and cytoprotective functions (20). The induction of Nrf2 pathway by SF, it is the best known effect of this compound. Thus, the recorded SF ability to inhibit Aβ-induced GSK3 activity in our experiment could suggest that SF is able to induce Nfr2 expression genes through the GSK3 inhibition.

The absence of SF effect on counteracting the superoxide production induced by Aβ could be ascribed to its inability to induce SOD, the main enzyme involved in the detoxification of superoxide.

Among neuronal cells, microglia have been reported to play important roles in the neurodegenerative processes (47). In response to a variety of neurotoxic stimuli, microglia become active and secrete a number of pro-inflammatory cytokines and cytotoxic molecules such as ROS and RNS. The molecular mechanism of Aβ–mediated toxicity is not fully revealed, but there is potential evidence supporting the involvement of oxidative stress in this process (39).

In our study, oligomeric Aβ showed to induce ROS generation, pro-inflammatory cytokines (TNFα, IL 1β and IL 6) release and iNOS expression, in microglia primary

cultures. The co-treatment with SF has been able to significantly decrease the generation of ROS and in particular of H_2O_2 and superoxide, to promote a strong inhibition in the cytokines release and to reduce the iNOS expression induced by Aβ and LPS. However additional study will be necessary to investigate the mechanisms involved in the SF antioxidant activity exhibited in microglial cells.

In conclusion, the results showed the neuroprotective antioxidant activity of SF against Aβ -mediated oxidative stress in neuronal cells and in microglia cultures. Considerable evidence points to an important role for Aβ in hte pathogenesis of AD. With regards to neurotoxicity, Aβ can exert its toxic effect direct on neurons or can affect microglial cells to produce pro-inflammatory cytokines and toxic molecules that then affect the viability of neurons, promoting further neurodegeneration.

We propose that the dual effects of SF can produce additive neuroprotective effects and may be more beneficial in the prevention of neurodegenerative disease. In addition, SF has shown many advantage such as a good pharmacokinetics and safety after oral administration as well as the potential ability to penetrate the blood –brain barrier and deliver its neuroprotective effects in the central nervous system (48; 49). Based on these considerations, SF could be a promising compound with neuroprotective properties that may play an important role in the prevention and slowing down the neurodegeneration characteristic of AD.

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