CHAPTER II

LITERATURE REVIEW

Alzheimer's Disease and Neuropathology

Alzheimer's Disease (AD), one of the main causes of dementia, is a chronic neurodegenerative disorder affecting the elderly. AD is characterized clinically by progressive memory impairment, disordered cognitive function, altered behavior and progressive decline in language function. The histopathological hallmarks of AD include the formation of two main protein aggregates: senile plaques and neurofibrillary tangles in the brain which are involved in the extensive synaptic and neuronal loss (Beyreuther et al., 1991; Haass and Selkoe, 1993; Selkoe, 1996, 2001).

Senile plaques are composed of extracellular deposits of β-amyloid (Aβ). Aβ is a 39-43 amino acid long peptide which is derived proteolytically from amyloid precursor protein (APP) and exhibits toxicity to neurons either in vitro (Yarker, 1996) or in vivo (Ceula et al., 1998). Neurofibrillary tangles are typically found in neurons in close proximity to plaques and are identified as intracellular bundles of self-associated hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Kurt et al., 1997). These pathological lesions do not occur diffusely throughout the brain but are restricted to selective regions, particularly the hippocampus and neocortex. In addition, several structural and biochemical abnormalities are observed in AD brain including neuronal loss, reduced synaptic density and depletion of neurotransmitter (Selkoe, 1991; Sun and Alkon, 2001; Tran et al., 2002).

The pathology of AD is complex and involves many molecular, cellular, and physiological pathologies. The exact mechanism of AD pathogenesis is still obscure and current therapeutic approaches utilize various hypotheses for the treatment of this disease. Among those is the acetylcholine-deficiency-hypothesis which is based on the fact that AD is associated with a massive decrease of neurotransmitter acetylcholine (ACh) (Levin and Simon, 1998; Sun and alkon, 2001; Tran et al., 2002). Another hypothesis is the arthritis-of-the-brain-hypothesis of AD which focuses on the role of inflammation during AD-pathogenesis (Roger et al., 1992; McGeer and McGeer, 1998). In addition, the energy-depletion-hypothesis and the oxidative stress hypothesis of AD have been proposed (Hoyer, 1998; Behl, 1999).

There is an abundance of evidence supporting the idea that oxidative stress is associated with AD pathology. These include (I) increased levels of highly oxidized proteins, DNA and lipids, (II) higher amounts of free metal ions that can drive free radical generation, (III) the occurrence of advanced glycation end-products, and (IV) the observation that AD-associated Aβ can induce an oxidative environment of neurons (Retz et al., 1998; Behl, 1999). Furthermore, the amyloid cascade-hypothesis is supported by many genetic findings. Mutation in the genes for the APP, presenilin1 and presenilin 2 and apolipoprotein E which are linked to familial forms of the disease results in an abnormal APP processing and an increase Aβ production. These mutations may contribute to the pathogenesis of AD (Selkoe, 1999; Shastry, 2001a).

Aβ-induced neurotoxicity

In vitro, the neurotoxicity of Aβ was described for the first time in primary embryonic neurons (Yankner et al., 1989). Since then, the toxic effect of Aβ has been reproduced and extensively studies by many investigators using primary cultured neurons and clonal neuronal cell lines (Koh et al., 1990; Roher et al., 1991; Behl et al., 1992, 1994b; Mattson et al., 1992, 1993a; Yoshikawa et al., 1992; Shearman et al., 1994; Iversen et al., 1995; Yankner, 1996; Mattson, 1997; Behl, 1997). $A\beta_{25-35}$ is the shortest peptide sequence that contains biological activity comparable with that of full length (Pike et al., 1995). An important feature of Aβ toxicity is that it requires the peptide to be present in the form of an amyloid aggregate.

In vivo toxicity, using Aβ as a potential neurotoxin, is not consistent. While in some studies, a direct neurotoxicity after injection of Aβ into the rat brain could be observed (Frautschy et al., 1991; Kowall et al., 1991), cerebral infusion or implants of Aβ did not induce neurodegeneration in other approaches (Podlisny et al., 1993; Winkler et al., 1994). These conflicting results could partially be explained by different sources and the degree of fibrillization of Aβ used. Some studies have shown that Aβ injected into the rat brain can be toxic after the formation of fibril deposits (Snow et al., 1994; Giovenelli et al., 1995). The aggregation state appears to be crucial for Aβ's toxicity *in vitro*. This might also be relevant for the *in vivo* toxicity.

Molecular mechanisms of Aβ-induced neurotoxicity

Aβ-induced neurotoxicity is mediated by various mechanisms ranging from direct neurotoxic interactions with the neuronal membrane to Aβ's effects on membrane receptors and intracellular signaling pathways. Molecular mechanisms by which Aβ and its aggregates may cause neurotoxicity have been proposed as follows.

I. The disturbance of calcium homeostasis

Several reports, both *in vitro* and *in vivo*, provide evidence that the sustained elevation of intracellular free calcium concentration $[Ca^{2+}]_i$ and the appearance of $[Ca^{2+}]_i$-triggered neurotoxic cascades might be of pivotal importance in mediating the cellular neurotoxic action of Aβ (Mogensen et al, 1998; O' Mahony et al., 1998; Stix and Rreiser, 1998). In addition, Aβ alters Ca^{2+} homeostasis that can directly or indirectly lead to cell death via necrotic or apoptotic process (Mattson, 2000). Further, increased intracellular Ca^{2+} following neuronal exposure to Aβ can stimulate phospholipase A_2, leading to free fatty acid release. The latter is reported to cause tau protein to polymerize (Gamblin et al., 2000).

Previous studies proposed the selective interaction of Aβ with cell surface molecules. **Firstly**, cation (Ca^{2+})-selective channel-forming properties of Aβ fragments were proposed (Arispe et al., 1993, 1996) and this characteristic feature of $Aβ_{25-35}$ and $Aβ_{1-40}$ has been confirmed by others (Mirzabekov et al., 1994; Kawahara et al., 1997). After, Aβ peptides can insert into the neuronal plasma membranes of relevant target cells, change their structure and accumulated Aβ peptides become aggregated on the membrane. Finally, aggregated Aβ protein polymers form ion permeable channel, transport physiologically relevant ions such as Ca^{2+} and mediate cytotoxicity (Kawahara and kuroda, 2000; Kagan et al., 2002). This disruption of Ca^{2+} homeostasis by channel formation may be the molecular mechanism of Aβ-induce neurotoxicity. **Secondly**, the extensive pharmacological data from *in vivo* experiments indicate the involvement of common excitotoxic pathways in Aβ-induce brain injury (Morimoto et al., 1998a; O' Mahony et al., 1998) and pathways similar to that of N-methyl-D-aspartate (NMDA) neurotoxicity (Harkany et al., 1997, 1998a). It seems likely that Aβ binds to selective cell surface recognition sites mediating intracellular Ca^{2+}entry. **Thirdly**, preliminary studies suggest that Aβ binds to the receptor for advanced glycation end-products (RAGE) on endothelial cells in the vasculature (Yan et al., 1996). RAGE can act as a signal transducer molecule for an

oxidative stress pathway. It is hypothesized that RAGE mediates neurotoxic signaling of Aβ and is responsible for the movement of Aβ across the blood-brain-barrier, both of which are important in pathology of AD. All of these pathways converge to a pathological enhancement of intracellular Ca^{2+}entry via Ca^{2+}permeable channels and subsequent elevation of $[Ca^{2+}]_i$. It has been shown that sustained elevation of cytoplasmic Ca^{2+}levels can promote mitochondrial oxyradical (superoxide) production, membrane depolarization and ATP depletion (Guo et al., 1998b, 1999). These results suggest a contribution of altered Ca^{2+} homeostasis to mitochondrial dysfunction in AD. Recent studies have suggested that Aβ may be directly toxic to mitochondria (Casley et al., 2002) and it also causes loss of cytochrome C oxidase activity in cultured neurons (Kim et al., 2002).

II. Oxidative metabolic dysfunction

Extensive studies were launched investigating the link between the toxic Aβ-aggregates and oxidations (Harris et al., 1995; Zhou et al., 1996; Behl, 1997; Markesbery, 1997). Taken various results together, it can be summarized that aggregated Aβ can cause the intracellular accumulation of H_2O_2 and related peroxides. This may lead to formation of hydroxyl radicals via the Fenton reaction and ultimately to peroxidation of membrane lipids and cell death (Behl et al., 1994b; Schubert et al., 1995).

Recently, it has been reported that Aβ can induce lipid peroxidation, protein oxidation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation (Butterfield and Stadman, 1997; Varadarajan et al., 2000; Butterfield and Lauderback, 2002). This oxidative damage or membrane modification, resulting from reaction of the lipid peroxidation products 4-hydroxy-2-nonenal or arolein with enzymatic, transport, structural proteins, alters synaptic membranes that can lead to oxidative damage of synaptic terminals and eventually, lead to the death of the neurons (Butterfield and Stadtman, 1997; Butterfield and Lauderback, 2002). Aβ can induce nitric oxide synthetase and the resulting nitric oxide (NO) can react rapidly with superoxide radical leaked from mitochondria, producing highly reactive peroxynitrite. This latter agent can lead to oxidative damage of synaptic terminals. The enhanced NO formation may enhance ROS-mediated Aβ toxicity.

Aβ can also stimulate free radical production via receptor-mediated pathways. Aβ in fibrillar form can bind to RAGE or type 2 scavenger receptors to induce

oxidative stress (Yan et al., 1996) and can initiate an oxidative inflammatory response from activated microglia (Luwin and Bazan, 2000). Activation of microglia causes secretion of pro-inflammatory cytokines including tumor necrosis factor-alpha in a RAGE-dependent manner (Deane et al., 2003). Pro-inflammatory cytokines within the brain are known to cause neuronal death through production of ROS, NO and eventual cell death via caspase activation (Wang et al., 2003). In addition to the induction of oxidative stress after the reaction of aggregated Aβ with cellular membranes, Aβ itself can be transformed into a radical state and acts as a free radical (Hensley et al., 1994).

III. Increased vulnerability to excitotoxicity

The accumulation of excitatory amino acid such as glutamate can induce cell death by overactivation of specific glutamate receptor or by the induction of oxidative events. Neurons exposed to Aβ show increased vulnerability to excitotoxicity. Ion channel-linked glutamate receptors are up-regulated following exposure to Aβ and their overactivation may lead to further oxidative events that account for cumulative neural damage and death. Increased vulnerability to excitotoxicity is a mechanism of neuronal death that involves overactivation of glutamate receptor. Therefore, Aβ and excessive glutamatergic tone may act synergistically in a reinforcing manner to induce oxidative stress (Coyle and Puttfarcken, 1993).

It is likely that sustained elevation of $[Ca^{2+}]_I$ and excess generation of free radicals are closely related processes and directly regulate each other. An intracellular Ca^{2+} entry-coupled radical-generating cascade has been depicted that exposure of cultured neurons to Aβ triggers a rapid disintegration of plasma membranous structures indicated by fragmented microtubules and damage to inner mitochondrial membranes (Behl et al., 1994a). An extensive enhancement of lipid peroxidation supports membrane damaging properties of Aβ (Keller and Mattson, 1998). Furthermore, ligand-binding data indicate that Aβ selectively interacts with the NMDA receptor channel (Cowburn et al., 1997). The Aβ-induced activation of NMDA receptors and subsequent Ca^{2+} entry result in an increase of Ca^{2+}/calmodulin-activated NOS activity (O' Mahony et al., 1998). This study provides a possible coupling of $Ca^{2+}$and ROS-mediated neurotoxic pathways.

An increase in extracellular glutamate levels secondary to increased presynaptic release or altered astroglia clearance may initiate oxidative stress by a

different mechanism. **Firstly**, the accumulation of excitatory amino acids induces increased excitability of neurons and overstimulation of glutamate receptors. This situation promotes the formation of ROS and NOS. **Secondly**, glutamate indirectly depletes glutathione levels in astrocytes, oligodendrocytes and neurons by competition for the cysteine-antiportor system, thus indirectly promoting the accumulation of reactive species. The consequence is the peroxidation of membrane lipids as oxidative chain reaction, mitochondrial damage and cell lysis (Wolozin and Benl, 2000; Maccioni et al., 2001).

Neuroprotective effects of estrogen

The endogenous gonadal steroid 17β-estradiol (estrogen) plays an important role in the development, maturation and function of a wide variety of reproductive and non-reproductive tissues including those of the nervous system (Belcher and Zsarnovszky, 2001). In the brain, estrogen is well known as a fundamental regulator of physiology and behaviors required for reproduction. Estrogen also plays a significant role during normal development and in genderization of the mammalian central nervous system and it has important neurotrophic and neuroprotective functions in the brain (Beyer, 1999; Toran-Allerand et al., 1999; Wise et al., 2001).

Epidemiological studies have previously shown that estrogen and/or hormone replacement therapy (HRT) could reduce the risk of AD (Paganini-Hill and Henderson, 1996; Tang et al., 1996; Baldereschi et al., 1998; Brinton, 2001; Polo-Kantola and Erkkola, 2001). These studies reported that HRT delays the onset of AD and improves cognitive functions, including verbal fluency and verbal memory in postmenopausal woman.

There are many data supporting a neuroprotective role of estrogen in both culture and animal models. Estrogen-mediated neuroprotection has been widely reported in several neuronal cultures *in vitro* model of neurodegeneration including serum deprivation (Bishop and Simpkins, 1994; Green et al., 1997a, 1997b; Gollalpudi and Oblinger, 1999), Aβ-induced toxicity (Behl et al., 1995,1997; Goodman et al., 1996; Green et al., 1996; Gredley et al., 1997, 1998; Mattson, 1997; Bonnefont et al., 1998; Pike, 1999; Roth et al., 1999), oxidative stress (Behl et al., 1995; Goodman et al., 1996; Bonnefont et al., 1998; Sawada et al., 1998; Singer et al., 1998; Mosman and Behl, 1999) and excitotoxicity (Goodman et al., 1996; Regan and Guo, 1997; Weaver et al., 1997; Sawada et al., 1998; Singer et al., 1998, 1999;

Zaulynov et al., 1999) The effective concentrations for estrogen-mediated neuroprotection range from 0.1 nM to 50 μM (Brinton et al., 1997; Green et al., 1997a; Sawada et al., 1998; Bae et al., 2000). The possible mechanisms of neuroprotection include activation of the nuclear ER, alteration of Bcl-2 expression, activation of cAMP and the MAPK signal transduction pathway, modulation of intracellular homeostasis and enhancement of antioxidant activity (Singer et al., 1996, 1998, 1999; Weaver et al., 1997; Watters and Dorsa, 1998; Pike, 1999; Singh et al., 1999)

In addition, estrogen-mediated neuroprotection has also been described in different animal models *in vivo*. Several animal studies have shown that estrogen improves memory performance in ovariectomized rats (Simpkins et al., 1997a; Bimonte and Denenberg, 1999; Fader et al., 1999). Estrogen treatment has been shown to improve memory performance in male rats as well (Lannert et al., 1998). These findings suggest that estrogen may have a general enhancing effect on neural activity and may be involved in the normal maintenance of neural functions. The protective effects of estrogen have been documented in rats (Simpkins et al., 1997b; Dubal et al., 1998; Pelligrino et al., 1998; Zhang et al., 1998; Rusa et al., 1999; Wang et al., 1999), mice (Culmsee et al., 1999) and gerbils (Sudo et al., 1997; Chen et al., 1998). Estrogen has neuroprotective properties against 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine (MPTP)-induced neurotoxicity in dopaminergic system of ovariectomized mice (Dluzen et al., 1996a, 1996b; Dluzen, 1997, 2000; Callier et al., 2000). Estrogen also has neuroprotective effects against MPTP-induced neurotoxicity in castrated male animals (Dluzen et al., 1996a, 1996b). However, the interpretation of neuroprotective effects of estrogen in male animals is complicated by the possible influence of androgens. These studies show some discrepancies concerning the optimal dose of estradiol needed to exert a neuroprotective effect *in vivo*. It may be that different brain areas require different optimal hormone concentrations. The severity of the lesion and the route of administration may also be an important determinant in hormone concentrations required to counterbalance the damage. In spite of the discrepancies, most studies show a neuroprotective effect at low estradiol doses.

Mechanisms of neuroprotection by estrogen

Increasing evidence supports the role of estrogen as a neuroprotective agent. Estrogen may exert neuroprotective effects through several mechanisms. It may serve a protective role through ER-dependent and ER-independent, genomic and non-genomic means to attenuate neuronal injury.

ER-dependent mechanism

For long-term genomic effect, estradiol may act on conventional nuclear receptors and regulate transcription of growth factor receptors, cytoskeletal proteins and antiapoptotic molecules (Garcia-Segura et al., 2001). Estrogen activates ERs (ERα, β) which translocate to the nucleus and bind to estrogen responsive elements on the genomic DNA. There, ERs might induce the transcription of modulators of neuroprotections such as activation of growth factor receptor and induction of non-amyloidogenic APP processing, leading to the generation of soluble APPs (Xu et al., 1998a; Behl and Holsboer, 1999).

In addition to ERs that act through transcription factors within nuclei, there is interesting evidence that ER may mediate the effect of estrogen via interaction with intracellular signaling cascades (Mermelstein et al., 1996; Toran-Allerand, 1996; Lagrange et al., 1997; Kelly and Wagner, 1999; Singer et al., 1999; Singh et al., 1999). These effects are rapid and depend on the presence of ER or putative membrane receptor but not on protein synthesis (rapid non-genomic effect).

Recently, there is much evidence concerning rapid non-genomic actions of estrogen in the CNS. It was suggested that these rapid effects are of major important for normal development and function of the brain (Kelly and Wagner, 1999; Toran-Allerand et al., 1999; Woolley, 1999). Extensive studies have demonstrated that estrogen rapidly activated G-protein-coupled receptors resulting in altered neuronal excitability (Kelly and Wagner, 1999).

Estrogen has also been shown to play an important role in neuronal differentiation. In developing murine midbrain dopaminergic neurons, estrogen rapidly stimulated Ca^{2+} release from intracellular stores (Beyer and Raab, 1998) and could activate a protein kinase A (PKA) signaling cascade that results in activation of the cAMP-responsive element binding protein (CREB) (Beyer and Karolczak, 2000). This estrogen-induced mechanism is involved in estrogen-mediated differentiation of midbrain dopaminergic neurons.

Regarding other rapid actions, estrogen has also been shown to influence additional signaling pathways and results in increased neuronal survival. In cultured cortical neurons, estrogen can rapidly activated PI3-K (Honda et al., 2000) and MAPK signaling pathway (Singer et al., 1999; Singh et al., 1999). Both estrogen-activated mechanisms have been shown to increase cell survival and to protect cortical neurons from excitotoxic cell death. Additionally, estrogen rapidly protects hippocampal neurons from excitotoxicity through mechanism that requires MAPK activation (Bi et al., 2000) and can rapidly activate MAPK signaling in developing cerebellar neurons (Wong and Belcher, 2000). Furthermore, Singh (2001) recently reported that estradiol activates Akt/protein kinase B which can mediate the anti-apoptotic signaling pathway through increased expression of anti-apoptotic protein Bcl-2 (Singh, 2001). Taken together, it was suggested that rapid actions of estrogen are pleiotrophic, are not specific to reproductive tissues and are not specific to regions of the brain associated with reproductive or neuroendocrine functions.

Cross-talk of estrogen with intracellular signal transduction pathways

The interaction between estrogenic activity and growth factor signaling is well established in the brain. It was indicated that neurotrophic growth factors and estrogen may act in concert on the same neuron to regulate the expression of specific genes that may influence neuronal survival and regulate the differentiation of their target neurons (Toran-Allerand, 1996). These data suggest that there is a direct interaction between components of the neurotrophin-activated and estrogen-mediated MAPK signaling pathways at the level of B-raf (Toran-Allerand et al., 1999).

ER-independent mechanism

There is a strong evidence supporting mechanisms independent of ER activation. It was indicated that estradiol has antioxidant properties and suppresses the oxidative stress in neurons and neuronal cell lines induced by hydrogen peroxide, superoxide anions and other pro-oxdidants (Behl et al., 1995, 1997; Bonnefont et al., 1998; Sawada et al., 1998; Moosmann and Behl, 1999; Behl and Manthey, 2000). The antioxidant property of estradiol is related to the C3 position on the phenolic A ring of the steroid structure (Behl et al., 1997; Green et al., 1997b). A major concern in several previous studies was the high micromolar concentrations of estrogens used to exert an antioxidant effect (Goodman et al., 1996). The phenolic A ring estrogens are inhibitors of lipid peroxidation with an efficacy equivalent to α-tocopherol (Green et al., 2000). Based on these data, it was demonstrated that pharmacological levels of

estradiol can act as potent antioxidant and inhibit lipid peroxidation. The mechanisms underlying pharmacological actions (high micromolar concentration) of estrogen are rapid and do not appear to involve transcription of new genes.

In addition to antioxidant effect, estradiol may interact with estrogen binding sites in the plasma membrane and activate membrane associated signaling cascades that result in neuroprotection (Ramirez and Zheng, 1996). It may have many different rapid effects on neuronal excitability (Moss and Gu, 1999) and neuronal transmission (Bicknell, 1998). Estradiol may be in part related to the modulation of neurotransmitter receptors such as NMDA receptor. Estradiol may protect the nigrostriatal system by regulation of dopamine release and via inhibition of dopamine uptake by decreasing the affinity of the transportor of dopamine (Disshon and Dluzen, 1997; Disshon et al., 1998; Dluzen, 2000).

Phytoestrogens

Phytoestrogens are defined as the family of plant compounds that found ubiquitously in many food plants such as fruits, vegetables, legumes, whole-grains and especially soy products (Kurzer and Xu, 1997; Setchell, 1998). They are structurally similar to the natural and synthetic estrogens and can mimic the action of steroid estrogen. They have ability to selectively bind to ERs and regulate gene expression through estrogen responsive element. They exhibit not only some estrogen agonist-like properties (Mäkelä et al., 1995; Stahl et al., 1998) but also partial ER antagonists (Bowers et al., 2000). The estrogenic or antiestrogenic activity of phytoestrogens depends on the ability of the compound to interact with the ER. The antiestrogenic activity of phytoestrogens may be partially explained by their competition with endogenous estrogen for ERs. They have been categorized as endrocrine disruptors that cause environmental problems and deleterious effects on reproductive systems (Kanno et al., 2002, Wuttke et al., 2003). Some phytoestrogens are well known enzyme inhibitors (Agarwal, 2000).

Phytoestrogens possess many interesting effects including estrogenic (Mäkelä et al., 1994; Wang et al., 1996; Kuiper et al., 1998) and antiestrogenic effect (Adlercreutz et al., 1995; Wang et al., 1996), induction of cancer cell differentiation (Sartorelli, 1985; Constantinou and Huberman, 1995), inhibition of tyrosine kinase (Chang and Geahlen, 1992; Boutin, 1994) and DNA topoisomerase activities

(Constantinou et al., 1990; Kiguchi et al., 1990), suppression of angiogenesis (Fotsis et al., 1993) and antioxidant effect (Hendrich et al., 1994; Wei et al., 1995).

Accumulating evidence from molecular and cellular biology experiments, animal studies, and to a limited extent, human clinical trials, suggests that phytoestrogens may potentially confer health benefits related to cardiovascular diseases, cancer, osteoporosis and menopausal symptoms (Kurer and Xu, 1997; Murkies et al., 1998; Tham et al., 1998). This potential health benefits are consistent with the epidemiological evidence that rates of heart disease, various cancers, osteoporotic fractures and menopausal symptoms are more favorable among populations that consume plant-based diets, particularly among cultures with diets that traditionally high in soy products.

Chemistry and classification

The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. Flavonoids are the most abundant polyphenols in human diet and represent a subclass of polyphenol with a C6-C3-C6 backbone structure. The multifunctional effects of flavonoids are very intimately connected with the structure and its functional groups. Flavonoid aglycone consists of a benzene ring (A) fused with a pyrone ring (C) that in position 2 or 3 carries a phenyl ring (B) as a substituent (Figure 2). Besides, the important role in the activity play C2-C3 double, hydroxyl group at C3 and the number of hydroxyl groups at B-ring which act as electron donors and are responsible for radical scavenging activity of flavonoids (Rice-Evans et al., 1996). Flavonoids are mainly present in plants as glycosides. Aglycone (the forms lacking sugar moieties) occur less frequently. The large number of flavonoids is a result of the many different combinations of flavonoid of agycones and sugars. The most common sugar moieties include D-glucose and L-rhamnose. The glycosides are usually O-glycosides with the sugar moiety bound to the hydroxyl group at the C-3 or C-7 position.

Phytoestrogens are divided into three classes: isoflavones, coumestans and lignans (Albertazzi and Purdie, 2002; Branca, 2003). The common characteriastic of these three classes is that they are diphenolic compounds with structural similarities to natural and synthetic oestrogens and anti-oestrogens (an aromatic A ring with one hydroxyl group and a second hydroxyl group on the same plane of the a ring, Figure 3). The main phytoestrogens currently recognized are soyabean isoflavones, namely

genistein, daidzein and glycitein and their glycosides (genistin, daidzin, glycitin). Besides, isoflavones are present chick peas and lentis. Lignans (lariciresinol, isolariciresinol, secoisolariciresinol, matairesinol) are mainly present in wholemeal cereals, linseed and wild berries (Marzur et al., 2000). Coumestans (coumestrol) are mainly present in young sprouting legumes, clover (*Trifolium* spp.) and Lucerne (*Medicago sativa*) sprouts.

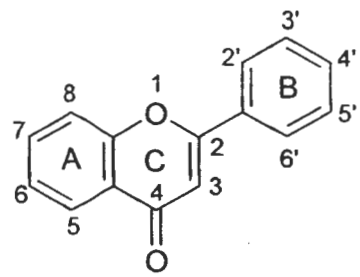


Figure 2 Basic flavonoid structure

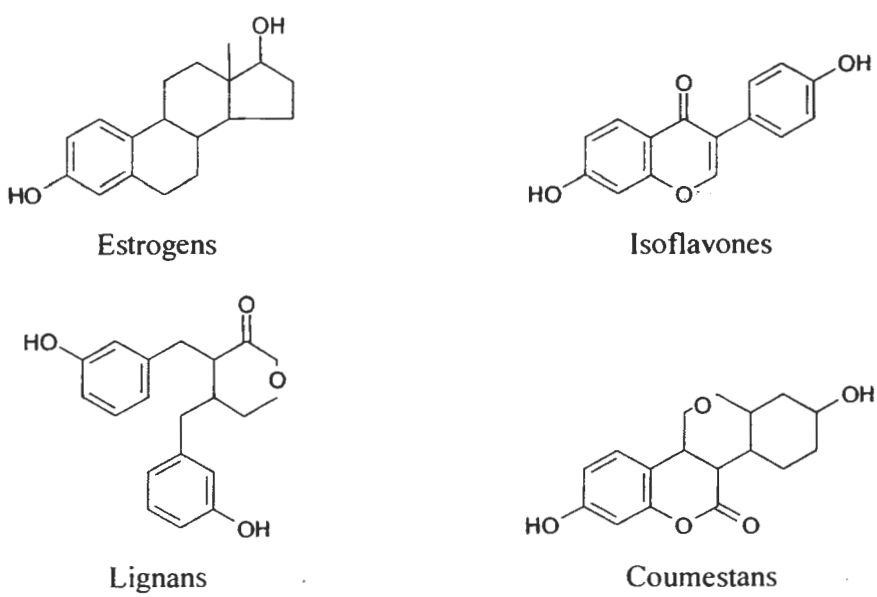


Figure 3 Chemical structures of the three major classes of phytoestrogens compared with those of the estrogens.

Neuroprotective effects of phytoestrogens

In regard to neuroprotective effect, it was reported that genistein retarded disease onset in male familial amyotrophic lateral sclerosis (FALS) mice but had no protective effect on female FALS mice (Trieu and Uckun, 1999). In addition, it also protected both male and female mice against oxygen singlet damage in a murine model of stroke. These data suggested that genistein has both estrogen-dependent and estrogen-independent neuroprotective activities.

The effects of α-and β-estradiol with plant-derived kaempferol on β-amyloid peptide-induced toxicity in PC12 neuroblastroma and T47D human breast cancer cells were compared (Roth et al., 1999). The results demonstrated that kaempferol exhibited a protective effect comparable to that observed with estradiol. In addition, the effects of the weak estrogen receptor agonists α-estradiol and kaempferol were found to be similar to the effects of the strong estrogen receptor agonists β-estradiol. These result indicated that a mode of action was independent from the nuclear estrogen receptor.

A study with primary cultured striatal neurons demonstrated that kaempferol (30 μM) had a potent intracellular inhibitor of oxidized low-density lipoprotein-induced neuronal cell death, possibly by counteracting JNK, c-Jun and caspase-3 activation (Schroeter et al., 2001).

It was reported that kaempferol and quercetin have been showed significant neuroprotection against cell death induced by glutamate in the mouse hippocampal cell line HT-22 (Ishige et al., 2001). Quercetin had the effective half maximal concentration of 2.2 μM. Kaempferol and quercetin at 10 μM were also effective protectant of primary cortical neurons from glutamate toxicity. Regarding the addition of H_2O_2, quercetin almost completely blocked cell death induced by H_2O_2 in HT-22 cells, whereas kaempferol showed marginal effects. In addition, quercetin at 10 μM increased basal level of GSH and accumulated low level of ROS as well as could also quench ROS, indicating the antioxidive property. Furthermore, HT-22 cells treated with quercetin at 10 μM showed low level of intracellular Ca^{2+}. These results demonstrated that the flavonol type showed three distinct mechanisms of protection. These included increasing intracellular GSH, directly lowering levels of ROS and preventing the influx of Ca^{2+}.

The neuroprotective effects of phytoestrogens on Aβ-induced toxicity had been shown in rat cortical neurons (Wang et al., 2001). It was found that 50 μM kaempferol and apigenin decreased Aβ-induced cell death. Apigenin blocked the release of cytochrome c and activation of caspase cascade induced by Aβ. Kaempferol only inhibited the activation of caspase cascade. In addition, kaempferol exhibited antioxidative activity and decreased the ROS accumulation induced by Aβ whereas apigenin lacked antioxidative activity. Furthermore, 50 μM quercetin and probucol potentiated the neuroprotective effect of apigenin against Aβ-mediated toxicity. These results indicated that the blockade of caspase activation conferred the major neuroprotective effects of phytoestrogens on Aβ-mediated neurotoxicity.

Linford et al. (2001) showed that genistein was toxic to rat primary cortical neurons (50 μM) whereas daidzein did not show toxicity at similar concentrations. This study suggested that genistein induced apoptosis through activation of p42/p44 MAPK and p38 MAPK as well as the release of intracellular Ca^{2+}.

Linford and Dorsa (2002) have shown that 50 nM genistein significantly reduced neuronal apoptosis in estrogen receptor-dependent manner and develop a model of apoptosis toxicity in primary cortical neurons by using the endoplasmic reticulum (ER) calcium-ATPase inhibitor, thapsigargin, to test potential anti-apoptotic effects of 17β-estradiol and genistein. Both 17-β-estradiol and genistein reduced the number if apoptotic neurons and reduced the number of neurons containing active caspase-3. This effect was blocked by co-addition of ICI 182,780. These results demonstrated that 17-β-estradiol and genistein have comparable anti-apoptotic properties in primary cortical neurons and that these properties are mediated through estrogen receptors.

Zhao et al. (2002) have reported that in cultured hippocampal neurons, six phytoestrogens, genistein, genistin, daidzein, daidzin, formononectin and equol induced a modest but significant reduction in LDH release following exposure to glutamate excitotoxicity and β-amyloid. They also showed that the neurotrophic potential of genistein (100 and 1,000 ng/ml) and daidzein (10, 100 and 1,000 ng/ml) that exerted a significant reduction in LDH release did not promote hippocampal neuron process outgrowth. These results indicated that phytoestrogens that include genistein could exert some neuroprotective effects similar to those of antioxidants but

these phytoestrogens are not functionally equivalent to endogenously active 17β-estradiol or to estrogen replacement formulations.

The possible role of quercetin in protecting against oxygen-glucose deprivation (OGD)-, excitotoxins- and free radical-induced neuronal injury was investigated in mouse cortical cell cultures (Ha et al., 2003). Pre-and co-treatment with quercetin (100 μM) inhibited 50 min OGD, 20 μM N-methyl-D-aspartate- and 50 μM kainate-induced neurotoxicity. Regarding free radical-induced neuronal injury, quercetin significantly ameliorated neuronal injury caused by buthionine sulfoximine (BSO), sodium nitroprusside (SNP), $ZnCl_2$ and $FeCl_2$. 30-100 μM concentrations of quercetin significantly reduced the neurotoxicity induced by BSO (1 mM) and $FeCl_2$ 40 μM. Quercetin at concentration ranging from 10 to 100 μM almost blocked 50 μM SNP-induced neuronal injury. Quercetin (1-100 μM) significantly inhibited the neurotoxicity induced by 30 μM $ZnCl_2$. These result suggested that quercetin may contribute a neuroprotective action against ischemic neuronal injury, partially via antioxidant actions.

Heo and Lee (2004) have investigated the protective effects of quercetin on H_2O_2-induced neurodegeneration in PC12 cells. Results showed that cell viability was clearly improved with quercetin and quercetin showed a higher protective effect than vitamin C. These results supported that quercetin contributed significantly to the protective effects of neuronal cells from oxidative stress-induced neurotoxicity.

Recently, Zeng et al. (2004) have reported that genistein (100 nM) protects neurons from Aβ-induced damage via the ER pathway and high concentration of genistein (40 μM), neuroprotective effect of genistein is mediated mainly by its antioxidative properties. These data suggested that genistein attenuated neuronal apoptosis induced by Aβ via various mechanisns. These include the blockade of Ca^{2+} influx, ROS accumulation and caspase-3 activation.

It has been reported that SH-SY5Y human neuroblastoma cells treated with genistein (1 nM) or 17β-estradiol (2 nM) in addition to Aβ exhibited less severe morphological changes compared to those treated with Aβ only (Bang et al., 2004). These result indicated that 1 nM genistein protects against Aβ-induced neuronal cell death that is similar to the protective action by a physiological dose (2 nM) of 17-β-estradiol. Additionally, both genistein (1 and 10 nM) and 17β-estradiol (2 and 20 nM) raised the number of trypan blue dye-excluding cells significantly more than cells

treated with Aβ only. These results indicated that genistein and 17β-estradiol showed comparable levels of protection against Aβ-induced deaths of cultured SH-SY5Y human neuroblastoma cells. Furthermore, it was found that these protective effects were blocked by an estrogen receptor antagonists, ICI 182,780. On the other hand, 17β-estradiol but not genistein induced proliferation of uterine endometrial cells. These results suggested that genistein might be used as a potential alternative to estrogen in the treatment of AD.

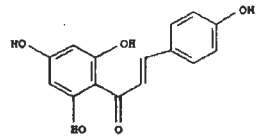
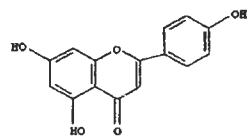
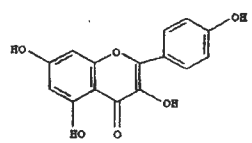
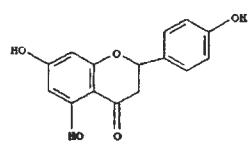
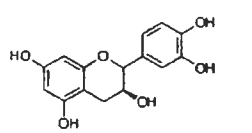
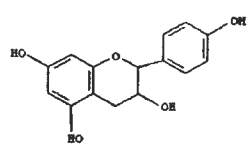
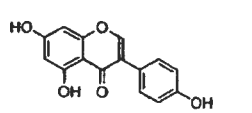
Kaempferol efficiently blocks the oxidative stress associated with the early stages of low K^+-induced cerebellar granule cell apoptosis and completely blocks the execution of this apoptotic process (Samhan-Arias et al., 2004).

Flavonoids and Human Health

Flavonoids comprise a large group of compounds occurring widely throughout the plant kingdom. Daily flavonoid intake (typically present in onion, apple, grape, wine, herbs and spices) in the human diet is highly variable, with estimations ranging from 23 mg/day (Hertog et al., 1993) to more than 500 mg/day (Manach etal., 1996). Flavonoids exert several biological activities, which are mainly related to their ability to inhibit enzymes and/or to their antioxidant properties, and are able to regulate the immune response (Hollman et al., 1995). These activities may explain the beneficial effects that flavonoid intake exerts in different human pathologies, including hypertension, inflammatory conditions and even cancer (Middleton et al., 2000).

In addition, some flavonoids such as apigenin, quercetin, kaempferol and naringenin, are also classed as phytoestrogens (Branca, 2003). Based on their structural features (variations in the heterocyclic C-ring), flavonoids are typically subdivided into seven types including chalcones, flavones, flavonols, flavanones, flavanols, anthocyanins and isoflavones (Table 1) (Moon et al., 2006). They are all polyphenol and many of them are structurally similar to the natural and synthetic estrogens.

Table 1 Major subclasses of flavonoids, chemical structures and major food sources (adapted from Moon et al., 2006).

	Structure	Example	Major food sources
Chalcone	 Chalcone	Hop chalcones (xanthohumol and dehydrocyclo-xanthohumol hydrate)	Hops, beer
Flavone	 Apigenin	Acacetin Apigenin Baicalein Chrysin Diosmetin Luteolin Tangeretin	Parsley, thyme, celery, sweet red peppers, honey, propolis
Flavonol	 Kaempferol	Galangin Kaempferol Morin Myricetin Quercetin	Onions, kale, broccoli, apples, cherries, berries, tea, red wine
Flavanone	 Naringenin	Eriodictyol Hesperetin Homoeriodictyol Naringenin	Citrus
Flavanol	 Epicatechin	Catechin Epicatechin Proanthocyanidins	Cocoa, green tea, chocolate, red wine, hawthorn, bilberry, motherwort, and other herbs
Anthocyanin	 Cyanidin	Cyanidins Pigmented compounds	Cherries, grapes, berries, red cabbage
Isoflavone	 Genistein	Biochanin A Genistein Diadzein Equol Formononetin	Red clover, alfalfa, peas, soy and other legumes

Biological and cellular properties of flavonoids

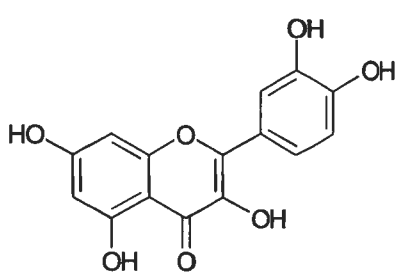
Flavonoids exhibit various effects on mammalian cells with interesting implications for cardiovascular diseases (Hertog et al., 1993; Hirvonen et al., 2001), cancer (Edenharder et al., 1993) and inflammation (Middleton and Kandaswami, 1992), involving the modulation of redox functions, calcium homeostasis (Yule et al., 1994), the activity of various enzymes, proliferation and differentiation (Middleton et al., 2000). There has been considerable interest in recent year in the cytoprotective

and neuroprotective effects of flavonoids, especially with respect to their mode of action as antioxidants. Efficient antioxidant activity of these molecules is generally assumed to be related to the presence of (I) a 3′,4′-dihydroxy (=catechol) moiety, (II) a C4=O keto group, (III) a 3-hydroxy substituent and (IV) a C2=C3 double bond (Rice-Evan et al., 1996).

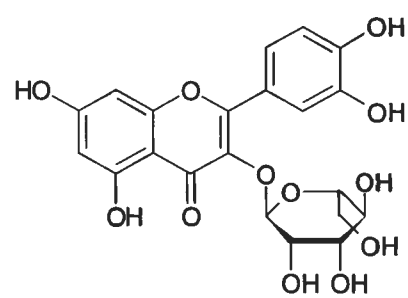
Flavonoids: Quercetin and its glycosides

Quercetin belongs to a group of polyphenolic substances known as flavonoids. Quercetin is a member of the subclass of flavonoids called flavonols. Quercetin is mainly present in plants as its glycosylated forms. For instance, quercetin glycone conjugates including glycone quercitrin and glycone rutin are glycosides and possess rhamnose (quercetin 3-O-rhamnoside) and rutinose (quercetin 3-O-rutinoside) at the C3 position of aglycone quercetin as 3-O-glycoside, respectively. The chemical structures of quercetin and its glycosides are shown in Figure 4.

Quercetin is widely distributed in higher plants in rinds and barks and found in vegetable and fruits. Especially rich sources of quercetin include onions, kale, broccoli, apples, cherries, fennel, sorrel, berries and tea (Ross and Kasum, 2002).



Quercetin



Quercitrin

Figure 4 Chemical structures of quercetin (3,3',4,5,7-pentahydroxyflavone) and glycoside quercitrin (quercetin 3-O-rhamnoside).

Biological properties of quercetin and quercitrin

Quercetin has been reported to exhibit antioxidative (Hayek et al., 1997; Chopra et al., 2000), anticarcinogenic (Deschner et al., 1991; Pereira et al., 1996), anti-inflammatory (Kim et al., 1998; Comalada et al., 2005; Nair et al., 2006), anti-aggregatory (Pignatelli et al., 2000), vasodilating (Perez-Vizcaino et al., 2002) and

gastroprotective (Alarcon de la Lastra et al., 1994) effects. It prevented secondary complications of diabetics (Varma and Kinoshita, 1976).

The mechanisms of these actions are largely unknown, however, it is possible that several different types of biochemical events precede them. For instance, antioxidation could be a result of metal chelation (Ferrali et al., 1997; Sestili et al., 1998), scavengimg of radicals (Huk et al., 1998; Aherne et al., 2000), enzyme inhibition (Da Silva et al., 1998; Nagao et al., 1999) and/or induction of the expression of protective enzymes (Myhrstad et al., 2002). Anticarcinogenesis, on the other hand, could result from enzyme inhibition (Agullo et al., 1997; Huang et al., 1997), antioxidation, or effect on gene expression (Piantelli et al., 2000; Xing et al., 2001). Altered gene expression could lie behind the anti-inflammatory effect as well (Kobuchi et al., 1999). Additionally, quercetin can inhibit aldose reductase which is the first enzyme of polyol pathway and this has been linked to diabetic complications such as cataracts, retinopathy, neuropathy, and neuropathy. Regarding anticarcinogenesis, quercetin by itself had been considered a carcinogen because it showed mutagenicity in the Ames test (Bjeldanes and Chang, 1977). However, a number of long term animal studies subsequently performed with different species have indicated that this is not the case. On the contrary, quercetin has been shown to inhibit carcinogenesis in laboratory animals (Stavric 1994).

Little is known so far about the biological activities of quercetin conjugates. The effect of glycosylation on their biological functions is still undefined. It has reported that quercetin, but not rutin and quercitrin, exhibited significant cytotoxic effect on human promyeloleukemic HL-60 cells. This evidence indicated that the addition of rhamnose or rutinose attenuated the apoptosis-inducing effect of quercetin and that the caspase 3 cascade but not free radical production was involved (Shen et al., 2003). In contrast, both quercitrin and rutinoside have shown to exert intestinal anti-inflammatory effects in experimental models of rat colitis (Cruz et al., 1998; Camuesco et al., 2004).

The sugar portion bound to the aglycone portion increases the solubility in polar solvents and consequently improves absorption, through the utilization of glucose transporters that are present in the intestinal mucosa (Gee et al., 1998). However, the majority of the studies have been carried out with the aglycone form and little is known about the biological properties of glycoside forms until the recent

years, due to the lack of commercial standards (Scalbert et al., 2002). At present, quercitrin had been shown to possess inhibitory effect on aldose reductase (Varma et al., 1975, 1977) and carbonyl reductase activities (Gebel and Maser, 1992; Maser and Netter, 1991), antileishmanial activity (Muzitano et al., 2006) , antioxidant activities (Omisore et al., 2005; Peng et al., 2003; Galvez et al., 1994), free radical scavenging activity (Hanamura et al., 2005), antinociceptive action (Gadotti et al., 2005), sedative activity (Kang et al., 2000), antidiarrheal activity (Galvez et al., 1995), anti-inflammatory effect (Comalada et al., 2005; Camuesco et al., 2004, 2006; Sanchez de Medina et al., 1996, 2002), cytoprotective effect (Yokozawa et al., 1999), and preventive effect on lipid peroxidation (Wagner et al., 2006).

Recent study analyzed the potency and efficacy of both quercetin and quercitrin as anti-inflammatory compounds (Comalada et al., 2005). It was reported that the *in vivo* effects of quercitrin in the experimental models of rat colitis induced by dextran sulfate sodium could be mediated by the release of quercetin generated after glycoside's cleavage by intestinal microbiota. In addition, querecetin, but not quercitrin, was able to down regulate the inflammatory response of bone marrow-derived macrophages *in vitro*. Moreover, it has demonstrated that querecetin inhibited cytokine and inducible nitric oxide synthase expression through of NF-κB pathway both *in vitro* and *in vivo*. These result suggested that quercitrin released quercetin in order to perform its anti-inflammatory effect which is mediated through the inhibition of the NF-κB pathway. It is important to note that when the glycosylated forms of quercetin are assayed, there is usually a loss of activity in these effects in comparison with those obtained with the aglycone, due to the presence of the sugar moiety in the flavonoid structure.

Chow et al. (2005) examined the protective mechanism of quercetin on oxidative stress-induced cytotoxic effect in RAW264.7 macrophages. Results showed that quercetin, but not rutin and quercitrin prevented H_2O_2-induced apoptosis via antioxidant activity and heme oxygenase-1 gene expression.

Recently, *In vitro* study, Wagner et al., (2006) investigated the antioxidant action of quercitrin, the glycoside form of quercetin, in rat brain lipid peroxidation induced by different agents. Additionally, the effect of quercitrin on the Fenton reaction was investigated. They found that both Fe^{2+} and Fe^{2+} plus EDTA caused a significant stimulation of brain TBAR formation, while quercitrin caused a reduction

quercitrin caused a reduction in the TBAR production induced by Fe^{2+} or Fe^{2+} plus EDTA. Quinolinic acid is a potent neurotoxin and a selective NMDA subtype of the glutamate receptor agonist. Sodium nitroprusside has been suggested to cause cytotoxicity via the release of cyanide and/or nitric oxide. Either quinolinic acid or sodium nitroprusside also caused a significant increase in brain TBAR production and effects of both neurotoxins were abolished by quercitrin. In addition, quercitrin was able to prevent the formation of TBARS induced by pro-oxidant agents tested. Furthermore, quercitrin caused a significant decrease in deoxyribose degradation (the Fenton reaction) that was not dependent on the concentration. These results indicated that quercitrin exhibits a scavenger and antioxidant role and these effects probable might mediated via different mechanisms involving the negative modulation of the Fenton reaction and NMDA receptor.

Experimental Models of Neurodegenerative Disorders

Embryonic neurons are well established experimental models for glutamate and Aβ toxicity that occur in neurodegenerative disorders (Mattson and Kater, 1989; Yankner et al., 1990). In these models, oxidative stress is an integral part of the cascade of events leading to cell injury and death (Chan, 2001; Coyle and Puttfarcken, 1993). It implies imbalance in the neutralization of reactive oxygen species (ROS) such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), which are normally scavenged by the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat). SOD converts $^{\bullet}O_2^-$ to H_2O_2, which is further detoxified by GPx or Cat. GPx is far more prevalent in the brain than is Cat, and GPx, not Cat, is assumed to be the critical downstream enzyme rescuing neuronal cells from H_2O_2 toxicity. Among other small molecular antioxidants, reduced glutathione (GSH) has been reported to be present in relatively high concentrations in brain and is considered vital for cell survival. With the activity of GPx and glutathione reductase, it serves to detoxify H_2O_2 to H_2O and molecular oxygen. It helps maintain the reduced state of the cysteinyl-thiol groups of proteins and rescues cells from apoptosis by buffering an endogenously induced oxidative stress (Filomeni et al., 2002; Schulz et al., 2000). It has been postulated that decreased levels of reduced GSH would be a marker for increased susceptibility to oxidant injury. Brain damage associated with an oxidative stress has been reported following GSH depletion (Gupta et al., 2000; Mytilineou et al., 2002; Papadopoulos et al., 1997; Vexler et al., 2003).

There is mounting evidence that different brain regions have different activities and responses of antioxidant enzymes to oxidative stress (Candelario-Jalil et al., 2001; Cardozo-Pelaez et al., 2000; de Haan et al., 1994).

In experimental models of neurodegenerative disorders, toxicity that leads to cell death occurs either by passive necrosis or a highly regulated active programmed cell death called apoptosis (Clarke, 1990). If ATP levels remain above a threshold, then an apoptotic program of gradual death can be orchestrated to prevent necrotic rupture with spillage of cytoplasmic enzymes (Nicotera and Leist, 1999). Studies with inhibitors support a sequence of events involving initiation at the plasma membrane or mitochondrion or endoplasmic reticulum (Vila and Przedborski, 2003), associated with or preceded by oxyradical generation and calcium overload (Atlante et al., 1998; Kruman et al., 1998; Lipton and Nicotera, 1998; Coppola and Ghibelli, 2000; Annunziato et al., 2003; Polster and Fiskum, 2004) leading to caspase activation (Riedl and Shi, 2004) and terminal steps of cytoskeletal and chromosomal fragmentation with nuclear condensation (Sahara et al., 1999).

In neonatal brain, the severity of brain hypoxia-ischemia injury varies between and within regions, with the hippocampus being the most affected region in CD1 mice and other inbred strains (Schmidt-Kastner and Freund, 1991; Sheldon et al., 1998, 2004). In an *in vitro* study, primary neurons cultured from hippocampus were five times more vulnerable than neurons from cortex after 50 μM H_2O_2 exposure (Koshy et al., 1998). It was shown that developing hippocampal neurons in primary culture are more sensitive to OGD than cortical neurons (Jiang et al., 2004). These results suggest that developing hippocampus may be more sensitive and vulnerable than other brain areas during oxidative stress. But whether this difference is due to the intrinsic properties of neurons, and the mechanisms underlying this difference are still unclear. An imbalance between excess prooxidant production (increased nNOS expression, and nitric oxide and reactive oxygen species production) and insufficient antioxidant defenses created by reduced glutathione peroxidase activity and GSH levels may explain the higher susceptibility to OGD of immature hippocampal neurons (Jiang et al., 2004). Recently, Shirai et al. (2006) showed that 7-DIV primary hippocampal neurons were susceptible to irradiation of 30 Gy but 21-DIV primary hippocampal neurons were resistant to this irradiation even though they are both postmitotic cells.

Thus, the susceptibility of postmitotic neurons may depend on their developmental stage.

In the present study, using Aβ exposure as an *in vitro* injury paradigm, we employed immature hippocampal neurons in primary culture as the *in vitro* model of neurodegeneration due to their higher sensitivity and vulnerability to oxidative damages. Neuronal cell injury and death were measured by using MTT reduction and LDH release assays. To investigate the contribution of prooxidant and antioxidant mechanisms in oxidative stress-induced neuronal cell injury and death after Aβ, we studied the production of ROS and lipid peroxidation levels, intracellular GSH levels, SOD and GPx activities. To investigate the contribution of apoptotic cell death mechanisms, we studied caspase-3 activity, Bcl-2 and Bax protein contents, and cytochrome *c* release. Quercitrin, a member of polyphenol flavonols that resembles phytoestrogens, was tested for its potential neuroprotective effects with this *in vitro* neurodegenerative model. Estrogen receptor antagonist, MEK and PI3K inhibitors, were also used to investigate the probable routes of quercitrin's neuroprotective action.