Neuroprotective Mechanisms of *Ginkgo Biloba* Extract (EGb761) in Alzheimer's Disease

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Abstract of thesis entitled:

Neuroprotective mechanisms of *Ginkgo biloba* extract (EGb761) in Alzheimer's Disease

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EGb761, a Ginkgo biloba extract, is a medicinal product for the treatment and prevention of cardiovascular and neuronal diseases, including Alzheimer's disease (AD). While considerable researches have documented its neuroprotective effects, its clinical effect is inconclusive and the precise neuroprotective mechanisms are not clearly known.

EGb761 consists of two major groups of substances, flavonoids and terpenoids. Using human neuroblastoma SH-SY5Y cells, the present study demonstrated that, EGb761 could block Aβ1-42 (a 42-amino acid cytoxic form of beta amyloid protein)-induced cell apoptosis, reactive oxygen species (ROS) accumulation, mitochondrial dysfunction and activation of c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt signaling pathways, possibly via its antioxidant and platelet activating factor (PAF) antagonizing activities. Two active constituents of EGb761, quercetin (a flavonoid) and ginkgolide B (a terpenoid) might contribute to the protective effects of EGb761. Quercetin but not ginkgolide B might be responsible for the antioxidant action of EGb761. Both compounds might be involved in the PAF antagonist activity of EGb761.

The effective dosage of EGb761 in the brain remains undetermined. Using SH-SY5Y cells, this study demonstrated that low doses of EGb761 (50-100 μg/ml) inhibited hydrogen peroxide (H_2O_2)-induced cell apoptosis via inactivation of Akt, JNK and caspase 3 while high doses of EGb761 (250-500 μg/ml) enhanced H_2O_2 toxicities via inactivation of Akt and enhancement of activation of JNK and caspase 3. Additional experiments suggested that the dosage effect of EGb761 on apoptotic signaling proteins might be correlated with regulation of the cell redox state.

The ability of EGb761 to cross the blood brain barrier (BBB) is unclear. In this study, the ability of EGb761 to cross the BBB was speculated through comparison of the effects of EGb761 on mitochondrial function between platelets and central nervous system in two animal models, the senescence accelerated prone 8 (SAMP8) mouse strain and ovariectomized rats. Mitochondrial function was evaluated as cytochrome c oxidase (COX) activity, mitochondrial ATP content and mitochondrial glutathione (GSH) content. SAMP8 mice have been widely used as a model of age-related cognitive decline with relevance to biochemical and genetic alterations in AD. Using two age groups (3-week-old and 40-week-old) of SAMP8 mice, this study found that, EGb761 protected against mitochondrial dysfunction in both platelets and hippocampi of old mice, but only showed protective effects on platelet mitochondria of young mice. Estrogen withdrawal was suggested to play a primary role in the onset of post-menopausal AD. Using ovariectomized middle-aged rats to mimic the post-menopausal pathophysiological changes, this study also demonstrated that, EGb761 protected against mitochondrial dysfunction in both platelets and hippocampi of ovariectomized rats. In contrast, in sham-operated rats, EGb761 increased mitochondrial GSH content in platelets but failed to show similar

effect on hippocampi. These results suggested that the effects of EGb761 on the brain might be interfered by the BBB permeability.

In conclusion, EGb761 may have beneficial effects in treatment and prevention of neurodegenerative diseases like AD. Its neuroprotective effects may be associated with constituent multiplicity, the dosage and BBB permeability.

中文摘要

EGb761 對 Alzheimer 氏病的神經保護機制

銀杏葉提取物 EGb761 被廣泛地用於心血管疾病及神經疾病如 Alzheimer 氏病（AD）的防治。儘管有研究顯示 EGb761 有神經保護作用，其臨床效應仍不確定。而且確切的作用機制也不清楚。

EGb761 主要包含黃酮類和萜類內酯。運用 β 澱粉樣蛋白（Aβ1-42）誘導人神經瘤細胞株 SH-SY5Y 損傷模型，本研究顯示 EGb761 可以阻斷 Aβ1-42 誘導的細胞凋亡、活性氧生成、線粒體機能紊亂及 JNK、ERK1/2 和 Akt 信號通路的啓動。這些效應可能與其抗氧化及拮抗血小板活性因子的活性有關。EGb761 的活性成分槲皮素（黃酮類）可能與抗氧化活性有關，而槲皮素和銀杏內酯 B（萜類內酯）都可能與拮抗血小板活性因子的活性有關。

EGb761 的中樞有效劑量仍不清楚。運用過氧化氫 (H_2O_2) 誘導 SH-SY5Y 細胞凋亡的模型，顯示低濃度的 EGb761（50-100 μg/mL）通過抑制 JNK、Akt 和半胱氨酸天冬酶的啓動，抑制了 H_2O_2 所誘導的凋亡；高濃度的 EGb761（250-500 μg/mL）通過增強 JNK 和半胱氨酸天冬酶的啓動，抑制 Akt 的啓動，增強了 H_2O_2 所誘導的凋亡。進一步的研究顯示，EGb761 的這種劑量效應可能與對細胞氧化還原狀態的調節有關。

EGb761 穿透血腦屏障的能力還不清楚。通過比較 EGb761 對 2 種動物模型（快速老化小鼠 SAMP8 和去勢大鼠）的血小板和海馬組織線粒體機能的作用（檢測指標包括細胞色素氧化酶活性、線粒體三磷酸腺苷及谷胱甘肽含量），本研究對 EGb761 穿透血腦屏障的能力進行推測。SAMP8 小鼠被廣泛地用於老化相關的認知功能減退的研究，並表現出類似 AD 的生化和基因改變。通過對 2 個年齡組的 SAMP8 鼠（3 周齡和 40 周齡）用藥前後的線粒體機能進行檢測，本研究顯示，EGb761 對老年鼠血小板和海馬線粒體機能有保護作用，但只對青年鼠血小板線粒體機能有保護作用。雌激素撤退被認爲與更年期後女性 AD 的發病有關。採用去卵巢中年大鼠，本研究顯示，EGb761 對去卵巢鼠血小板和海馬組織的線粒體機能有保護作用。相比之下，EGb761 增加了假手術鼠的血小板線粒體谷胱甘肽含量，而對海馬組織線粒體則無類似作用。提示 EGb761 的中樞效應可能受到血腦屏障通透性變化的影響。

總之，本研究結果提示 EGb761 對神經退行性疾病如 AD 有保護作用。其神經保護作用可能與成分多樣化、使用劑量以及血腦屏障通透性有關。

Abbreviations

Alzheimer's disease	AD
adenosine triphosphate	ATP
accelerated senescence-prone	SAMP
accelerated senescence-resistant	SAMR
all-trans-retinoic acid	RA
β-amyloid peptide	Aβ
Base Deactived Silica	BDS
blood-brain barrier	BBB
cyclic AMP response element-binding protein	CREB
c-jun N-terminal kinase	JNK
cytochrome c oxidase	COX
dimethyl sulfoxide	DMSO
2, 7-dichlorofluorescin dictate	DCF-DA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Eagle's minimum essential medium	DMEM
ethylenediaminetetraacetic acid	EDTA
extracellular signal-regulated kinase 1/2	ERK1/2
glutathione	GSH
Ginkgo Evaluation of Memory	GEM
hydrogen peroxide	H_2O_2
high performance liquid chromatography	HPLC
mitogen-activated protein kinase	MAPK
mitochondrial deoxyribonucleic acid	mtDNA
messenger RNA	mRNA
mild cognitive impairment	MCI
nuclear factor-kappaB	NF-kappaB
platelet activating factor	PAF
phosphate-buffered saline	PBS
reactive oxygen species	ROS
sodium dodecyl sulfate-polyacrylamide gel electrophoresis	S.D.S-PAGE
Statistical Package for the Social Sciences	SPSS
senescence-accelerated strain of mice	SAM
5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanineiodide	JC-1
tumour necrosis factor	TNF
Univariate analysis of variance	ANOVA

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Contents

1. Chapter I. General introduction

The leaves of the *Ginkgo* tree have a long history of being used for medicinal purposes. In the early 1970s, Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) successfully improved methodical procedures for the extraction and standardization of *Ginkgo biloba* preparation and produced highly concentrated and stable extracts from *Ginkgo biloba* leaves (Le Bars, 2003). The extract of *Ginkgo biloba* leaves (EGb761) since then has been standardized to contain 24% flavonoid glycosides (containing quercetin, kaempferol, isorhamnetin etc.), 6% terpenoids (in which 3.1% are ginkgolides A, B, C, and J and 2.9% is bilobalide), and 5–10% organic acids (Figure 1). The flavonoids and terenoids are suggested to be the pharmacologically active constituents of EGb761 (Smith et al., 1996). The organic acids in the extract contribute to its water solubility (Maclennan et al., 2002). So far, numerous preclinical studies evaluating EGb761 effects have been undertaken and suggest the neuroprotective effects of this herb (Luo, 2001; Ahlemeyer et al., 2003; Ponto et al., 2003; Christen, 2004; Smith and Luo, 2004; Luo, 2006; Saleem et al., 2008). Nowadays, EGb761 has been widely used for primary neurodegenerative dementias associated with aging, Alzheimer's Disease (AD), peripheral vascular diseases, and neurosensory problems (e.g., tinnitus) (Schneider, 1992; Luo, 2001; Holstein, 2001).

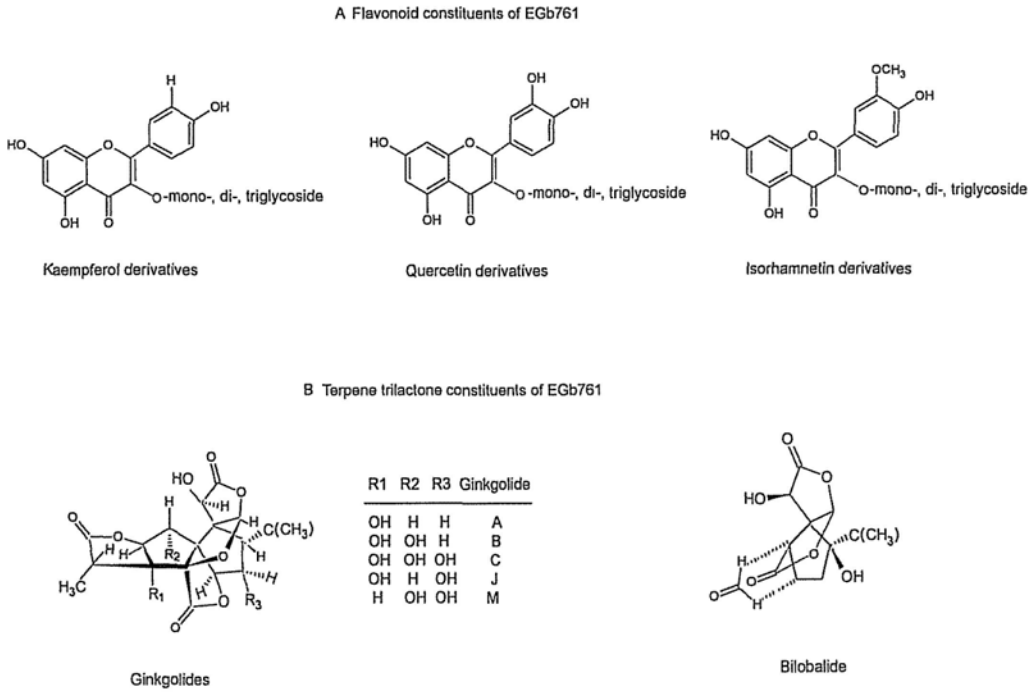

Figure 1. (A) Chemical structure of some representative flavonoid-*O*-glycosides and (B) terpene trilactone constituents of EGb761.

AD is one of the most common neurodegenerative disease associated with progressive cognitive and memory loss. The major molecular hallmarks of this disorder include extracellular deposition of the amyloid β peptide (Aβ) in senile plaques, intracellular neurofibrillary tangles, cholinergic deficit and neuronal loss in brain regions critical for memory and cognition (Castellani et al., 2006; Shi et al., 2008a; García-Ayllón et al., 2008; Dinamarca et al., 2008). A number of possible mechanisms for the pathological changes observed in AD brains include oxidative stress, inflammation, excitotoxicity and neuronal apoptosis (Bertoni-Freddari et al., 2003; Magaki et al., 2007; Culpan et al., 2007; Koutsilieri et al., 2007; Shi et al., 2008a; Gella and Durany, 2009; Cameron and Landreth, 2009). Several protective agents such as antioxidants, anti-inflammatory drugs, cholinergic agents, estrogens,

neurotrophic factors and calcium ion channel antagonists have been proposed for the prevention and treatment of AD, but none of them is proved to have a definite therapeutic effect (Zimmermann et al., 2002; Klegeris and McGeer, 2005; Castellani et al., 2006; Cole and Frautschy, 2007; Steele et al., 2007; Zhang et al., 2008; Akasofu et al., 2008; Kovács, 2009). In contrast, EGb761 has long been thought to be "multivalent" (Smith and Luo, 2004) and demonstrated to possess different profiles of action of various commonly-used drugs proposed for AD (Zimmermann et al., 2002). EGb761 thus has the potential to treat or prevent AD.

1.1 Mechanism of Neuroprotection by EGb761 and Its Constituents

1.1.1 Antioxidant Activity

Oxidative stress has long been thought to play a major role in the pathogenesis of AD (Gella and Durany, 2009). The proposal that the beneficial action of EGb761 is mainly due to its free-radical scavenging action is supported by numerous *in vitro* and *in vivo* studies (Smith and Luo, 2004). For example, pretreating cerebellar granule cells with EGb761 effectively attenuated oxidative damage triggered by $H_2O_2/FeSO_4$ (Wei et al., 2000). In another study using two AD models, Aβ-expressing neuroblastoma cell lineN2a and Aβ-expressing transgenic *Caenorhabditis elegans*, EGb761 was found to be able to attenuate the basal as well as the induced levels of H_2O_2-related reactive oxygen species (ROS) (Smith and Luo, 2004; Wu et al., 2006).

In addition to direct attenuation of ROS, EGb761 may also stabilize the cellular redox state by up-regulation of the protein level and activity of antioxidant enzymes (Ahlemeyer and Krieglstein, 2003a). For example, EGb761 was found to be able to

increase the protein level and activity of superoxide dismutase (SOD) and catalase in rat hippocampus and rat ileum (Ahlemeyer and Krieglstein, 2003a; Bridi et al., 2001; Colak et al., 1998). Moreover, activity of glutathione (GSH) reductase and gamma-glutamylcysteinyl synthetase, two enzymes critical for reduction and synthesis of GSH, were also enhanced by EGb761 (Rimbach et al., 2001; Sasaki et al., 2002; Ahlemeyer and Krieglstein, 2003a).

The flavonoid fraction is suggested to be mainly responsible for the antioxidant properties of EGb761. It is proposed that, the flavonoid fraction evokes antioxidant effects via direct scavenging ROS, chelating pro-oxidant transitional metal ions and up-regulate antioxidant proteins such as SOD and GSH (Maitra et al., 1995; Oken et al., 1998; Gohil and Packer, 2002; Smith and Luo, 2003; Smith and Luo, 2004). The polyphenol structure of flavonoids (Figure 1A) is thought to be responsible for their antioxidant actions (Saija et al., 1995; Smith and Luo, 2004). Quercetin (Sestili et al., 1998) and myricetin, two flavonoid constituents with such structure, especially effectively inhibit oxidation of *tert*-butylhydroperoxide (Ahlemeyer and Krieglstein, 2003a). In contrast, the antioxidant activity of terpene lactones is still in dispute (Ahlemeyer and Krieglstein, 2003b). There are conflicting data regarding the superoxide scavenging activity of bilobalide and the ginkgolides B, C and J (Scholtyssek et al., 1997; Pietri et al., 1997; Ahlemeyer and Krieglstein, 2003b). Ginkgolide A has been demonstrated to lack the ability to scavenge the superoxide (Ahlemeyer and Krieglstein, 2003b). The discrepancy on the antioxidant activity of the terpene lactones may be explained as being due to differences in the type of oxidative stress used as well as the experimental models (Ahlemeyer and Krieglstein, 2003b).

1.1.2 Protective Effects on Mitochondrial Function

Abnormalities in mitochondrial function are suggested to be associated with the pathological changes seen in AD (Castellani et al., 2002; Tendi et al., 2002). Recently, EGb761 has been proposed to have direct protective effects on mitochondria. This may also contribute to its antioxidant effects, as mitochondrial respiratory chain is both the major target and the major source of ROS. Using mitochondria isolated from PC12 cells, EGb761 and bilobalide were found to be able to up-regulate the gene expression of mitochondrial NADH dehydrogenase and decreased stage 4 respiration (Shi et al., 2009). In another study using PC12 cells and dissociated mice brain cells, as well as isolated mitochondria, mitochondrial abnormalities during aging were mimicked by using external insults (oxidative stress, nitrosative stress, serum deprivation and complexes inhibitors) and effects of EGb761 on mitochondrial function were evaluated (Abdel-Kader et al., 2007). It was found that, EGb761 protected mitochondrial function *in vitro* at concentrations as low as 0.01 mg/ml (Abdel-Kader et al., 2007). Despite these evidences, mechanisms underlying the protective effects of EGb761 and its constituents on mitochondrial function are still unclear and necessitate further studies.

1.1.3 Anti-apoptotic Effect

Apoptosis has been implicated in the pathogenesis of various neurodegenerative diseases like AD (Yuan and Yankner, 2000; Bertoni-Freddari et al., 2003; Smith and Luo, 2004). As summarized by Smith and Luo in a review, the anti-apoptotic actions of EGb761 are multifactorial and may act synergistically upon multiple intracellular signaling pathways involved in apoptosis (Maclennan et al., 2002; Smith and Luo,

2004). As for possible mechanisms underlying its anti-apoptotic action, EGb761 may maintain the integrity of the mitochondrial membrane; prevent cytochrome c release from the mitochondria, thereby blocking the formation of the apoptosome and the apoptotic caspase cascade; enhance the transcription of antiapoptotic Bcl-2-like protein; attenuate the transcription of pro-apoptotic caspase-12; inactivate pro-apoptotic *c-Jun* N-terminal kinase (JNK), thereby "turning off" downstream target *c-Jun*; inhibit the cleavage of the key effector protease caspase-3, thereby blocking the execution of apoptosis and prevent nuclear DNA fragmentation, the molecular hallmark of apoptosis (Smith and Luo, 2004).

The flavonoid fraction of EGb761 may be partly responsible for its anti-apoptotic properties. Despite the evidence that flavonoids prevent cell apoptosis induced by various oxidants (Saija et al., 1995; Maitra et al., 1995; Oken et al., 1998; Gohil and Packer, 2002; Smith and Luo, 2003), beneficial effects of EGb761 may go beyond its free-radical scavenging properties. Recently, evidences have accumulated to show that anti-apoptotic effects of flavonoids may be associated with modulation of specific proteins central to intracellular apoptotic signaling cascades such as the mitogen-activated protein kinase (MAPK) cascade. Quercetin, one of the major flavonoid constituents in EGb761 for example, inhibit JNK activity and apoptosis induced by hydrogen peroxide and 4-hydroxy-2-nonenal (Uchida et al., 1999; Wang et al., 2002; Spencer et al., 2003). It is proposed that, quercetin exerts its anti-apoptotic effects by inactivation of the peroxide-induced JNK-*c-Jun*/AP-1 pathway and extracellular signal-regulated kinase (ERK)-*c-Fos*/AP-1 pathway (Ishikawa and Kitamura, 2000; Spencer et al., 2003;). But low concentrations of quercetin could also promote cellular survival by activation of the MAPK pathway (ERK2, JNK1, and p38), leading to expression of downstream survival genes (*c-Fos*, *c-Jun*) and

defensive genes (phase II detoxifying enzymes; GSH *S*-transferase, quinone reductase) (Kong et al., 2000).

The terpene fraction of EGb761 may also contribute to its anti-apoptotic properties. Bilobalide, ginkgolide B, and ginkgolide J were demonstrated to be able to attenuate apoptosis in chick embryonic neurons caused by 24-h exposure to serum deprivation (Ahlemeyer et al., 1999; Defeudis, 2002). Bilobalide could also reverse apoptotic damage induced by 12-h staurosporine treatment of chick neurons (Defeudis, 2002). In mixed cultures of neurons and astrocytes from neonatal rat hippocampus, bilobalide rescued the neurons from serum deprivation-induced apoptosis, and both bilobalide and ginkgolide B attenuated staurosporine-triggered apoptotic damage (Defeudis, 2002). However, ginkgolide A failed to block apoptotic damage either in serum-deprived or in staurosporine-treated neurons (Ahlemeyer and Krieglstein, 2003). Analysis of DNA fragmentation and the activities of caspase-1- and caspase-3-like protease suggest that bilobalide can block neuronal apoptosis in the early stage by attenuating the elevations of c-myc, p53, and Bax and the activation of caspase-3 (Defeudis, 2002). Similarly, the anti-apoptotic effects of ginkgolides are also suggested to be associated with blockage of early signaling events in apoptosis. A recent study demonstrated that ginkgolide B inhibited ethanol-induced apoptotic cell death via suppression of activation of JNK and caspase 3 (Chan and Hsuuw, 2007). However, there are also contrasting data showing that the terpene constituents of EGb761 have no protective effect on cell apoptosis. For example, bilobalide failed to protect primary adult rat hippocampal neurons against apoptosis caused by a peroxyl radical-generator, 2,2′-azobis-2-amidinopropane (Rapin et al., 1998; Defeudis, 2002). In another study, the terpenes of EGb761 could not block hydroxyl radical-induced apoptosis in rat cerebellar granule cells (Xin et al.,

2000). This discrepancy may be due to the different types of cells and different methods for inducing apoptosis (Defeudis, 2002).

Despite the anti-apoptotic effects of EGb761 and its pharmacologically active components, the pro-apoptotic action of EGb761 and its constituents (e.g., quercetin and ginkgolide B) has also been demonstrated (Smith and Luo, 2003; Spencer et al., 2003; Chan and Hsuuw, 2007). Treatment dosage has been suggested to be one of the vital factors that determine the specific action of EGb761 constituents quercetin and ginkgolide B on apoptosis (Spencer et al., 2003; Chan and Hsuuw, 2007), but the precise mechanisms still remained unclear.

1.1.4 Anti-inflammatory Effect

Inflammation has been implicated in the pathology of AD (de Toledo, 2006). Cytokines, acute phase reactants, and other inflammatory mediators have been found to be up-regulated in pathologically vulnerable regions of AD brains (de Toledo, 2006). EGb761 has been demonstrated to have anti-inflammatory effects (Braquet, 1987; Braquet, 1991; Chan et al., 2007). These effects may be attributed to the combined actions of its ginkgolide and flavonoid constituents (Chan et al., 2007).

The anti-inflammatory action of ginkgolides may be associated with their platelet-activating factor (PAF) -antagonist activity. Substantial evidence suggests the role of PAF as a regulator of cytokines in inflammatory responses (Bonavida et al., 1994; Maclennan et al., 2002). Intracerebroventrical administration of PAF in rats could stimulate the synthesis of pro-inflammatory mediator leukotriene, particularly leukotriene C4 (Hynes et al., 1991; Maclennan et al., 2002). PAF can be synthesized in neurons following stimulation with neurotransmitters such as *N*-methyl-D-aspartic acid (NMDA) and glutamic acid and plays various roles in neuronal functions and

brain development (Aihara et al., 2000). However, increased concentrations of PAF in the brain are also implicated in neurodegenerative diseases such as AD (Farooqui and Horrocks, 2006; Bate et al., 2008). Ginkgolides display very specific and potent antagonist effects against PAF (Chan et al., 2007). Intracerebroventrical administration BN-52021 (ginkgolide B) in rats significantly attenuated PAF-induced rise in cerebrospinal fluid peptidoleukotriene levels (Hynes et al., 1991; Maclennan et al., 2002). BN-52021 could also reduce PAF-induced production of the eicosanoid and thromboxane B in a fetal rat brain (Kunievsky and Yavin, 1992; Maclennan et al., 2002). In addition to PAF-antagonizing activity, the inhibitory effects of ginkgolides A and B on pro-inflammatory cytokines tumour necrosis factor (TNF)-alpha and interleukin-1 production in lipopolysaccharide-stimulated rat microglial cultures were also observed (Du and Li, 1998; Maclennan et al., 2002).

On the other hand, the flavonoid fraction of EGb761 reportedly inhibits lipooxygenase that is concerned with the formation of leukotrienes (Chan et al., 2007).

1.1.5 Protective Effects against Amyloidogenesis and Aβ Aggregation

The accumulation of Aβ plaques has been proposed to be one of the most prominent mechanisms underlying the pathology of AD (Gerlai, 2001). Recently, the role of EGb761 in the protection against the Aβ-induced toxicity has received much attention. A number of recent reports indicate that EGb761 protects against Aβ-induced neurotoxicity by blockage of Aβ-induced events, such as ROS accumulation, glucose uptake, mitochondrial dysfunction, activation of AKT, JNK and ERK 1/2 pathways and apoptosis (Bastianetto et al., 2000; Smith and Luo, 2003). In addition to the protective effects against Aβ, EGb761 has also been shown to prevent

amyloidogenesis (Stackman et al., 2003; Colciaghi et al., 2004; Yao et al., 2004; Ramassamy, 2006). On hippocampal slices, Colciaghi *et al.* demonstrated that EGb761 could push amyloid precursor protein (APP) metabolism towards the α-secretase pathway, thereby increasing the release of the soluble form of APP (sAPPα) (Colciaghi et al., 2004; Ramassamy, 2006). Using the transgenic AD model Tg2576 mice, the consequence of the capability of EGb761 to influence positively the α-secretase pathway was also assessed *in vivo*. (Stackman et al., 2003; Ramassamy, 2006). It was found that, after EGb761 treatment, Tg-2576 mice exhibited an enhancement of spatial learning and memory comparable to wild type mice (Stackman et al., 2003; Ramassamy, 2006). It has been proposed that, EGb761 inhibits the production of brain Aβ by lowering the levels of circulating free cholesterol, as free circulating and intracellular cholesterol levels could affect APP processing and amyloidogenesis (Bodovitz and Klein, 1996; Howland et al., 1998; Simons et al., 1998; Yao et al., 2004; Ramassamy, 2006). Despite these evidences, further investigations are needed to identify the major constituents responsible for this anti-amyloidogenic effect.

EGb761 could also inhibit the formation of Aβ fibrils (Luo et al., 2002; Ramassamy, 2006). It is known that, the β-sheet structure of Aβ fibrils is mainly responsible for the neurotoxicity of Aβ and may also help Aβ escape from clearance via proteolytic degradation (Pike et al., 1991; Simmons et al., 1994; Soto et al., 1998; Ramassamy, 2006). Thus, inhibiting of formation of β-sheet structure of Aβ fibrils may also help to prevent Aβ toxicity. The interaction of Aβ with transition metal ions, notably iron, zinc and copper, could influence the aggregation state of Aβ. EGb761, through its ion chelating property, may inhibit Aβ fibrils formation (Luo et al., 2002; Ramassamy, 2006). EGb761 may also influence the formation of Aβ fibrils by

increasing gene expression of transthyretin (Watanabe et al., 2001), as transthyretin has been shown to prevent Aβ aggregation *in vitro* by sequestering Aβ monomers (Tsuzuki et al., 2000). The inhibitory effect of EGb761 on Aβ aggregation was also observed with bilobalide, ginkgolide J and flavonoid compounds (Luo et al., 2002; Ramassamy, 2006).

1.1.6 Other Mechanisms

Other mechanisms which may be involved in neuroprotective effects of EGb761 on AD are ion homeostasis, modulation of phosphorylation of tau protein, and induction of growth factor synthesis.

Ca^{2+} dyshomeostasis may be of pivotal importance in mediating neutotoxicity in AD (Shirwany et al., 2007; Berrocal et al., 2009). Our recent data suggested that, EGb761 could protect against Aβ (1–42)-triggered Ca^{2+} influx via *N*-methyl-D-aspartic acid receptors and possibly acted through its antioxidant and PAF-atagonizing activities. This effect was also observed with its constituents quercetin and ginkgolide B.

In AD brain, hyperphosphorylated microtubule-associated protein tau is aggregated as neuro-fibrillary tangles of paired helical filaments, which is a key event in the pathogenesis of AD (Liu et al., 2002; Ahlemeyer and Krieglstein, 2003). Using mRNA microarrays, Watanabe *et al.* found that EGb761 was able to up-regulate gene expression of microtubuli-associated tau protein as well as of neural protein phosphatase type 1, a serine/threonine protein phosphatase known to dephosphorylate hyperphosphorylated tau protein, in the hippocampus and cortex of normal mice (Watanabe et al., 2001; Ahlemeyer and Krieglstein, 2003).

Altered levels of nerve growth factor (NGF) have been detected in AD brains (Scott et al., 1995; Pierre et al., 1999; Peng et al., 2009). EGb761 was reported to be able to up-regulate mRNA expression of NGF such as growth hormone and prolactin in mouse cortex (Pierre et al., 1999; Ahlemeyer and Krieglstein, 2003). Furthermore, the mRNA and protein expression of glial-derived neurotrophic factor and vascular endothelial growth factor in cultured rat cortical astrocytes could also be up-regulated by a terpenoid constituent of EGb761, bilobalide (Zheng et al., 2000; Ahlemeyer and Krieglstein, 2003).

1.2 Current Status of Clinical Use of EGb761: Still a Long Way from Preclinical Promise

Despite the substantial body of preclinical evidence suggesting that EGb761 may be effective in treatment and prevention of AD, its clinical effect remains elusive. A recent meta-analysis of clinical studies (925 AD patients in 9 trials) on the use of EGb761 suggested that beneficial effects of EGb761 in AD are inconsistent (Birks and Grimley Evans, 2009). In addition, the *Ginkgo* Evaluation of Memory (GEM) study, the largest and longest randomized controlled trial of *Ginkgo biloba* extract concluded that EGb761 supplement was not effective in reducing either the overall incidence rate of dementia or AD incidence in elderly individuals with normal cognition or those with mild cognitive impairment (DeKosky et al., 2008). These reports suggest to us that, despite the preclinical promise that EGb761 may be effective in treatment and prevention of AD, this promise has not completely translated to clinical research benefits.

Certain confounding factors, as previously suggested (Le Bars, 2003), may interfere with EGb761's effect, and may be the source of the variations observed among EGb761 studies. First, the therapeutic effect of EGb761 may depend on the sensitivity of the study population to this drug. Thus the homogeneity of the study population may affect EGb761's efficacy (Le Bars, 2003). In two multicenter trials, EGb761 treatment showed a greater effect on the AD subgroup than the total mixed dementia population (AD and Multi-Infarct Dementia) (Le Bars, 2003). Moreover, it is still unclear whether age and sex influence population sensitivity to EGb761 treatment. Second, the therapeutic effect of EGb761 in AD may be associated with the severity of the impairment (Le Bars, 2003). In the group of patients with very mild to mild cognitive impairment, EGb761 effect could be considered in the term of improvement while in more severe dementia, EGb761 effect should be considered more in terms of stabilization or slowing down of dementia progression (Bodovitz et al., 1996; Le Bars, 2003). Third, the sensitivity of different cognitive outcome measurements such as the Syndrom-Kurztest and the Alzheimer's Disease Assessment Scale-Cognitive Subscale may affect the assessment of EGb761's effect, but this factor may be directly dependent on the severity of the cognitive impairment of the tested patients (Le Bars, 2003). Last but not least, there may be a dose-dependent effect of EGb761. Nowadays, a daily dose of 240 mg has been extensively used to slow down the disease progression in AD patients (Hofferberth, 1994; Oken et al., 1998; Yao et al., 2004; Kanowski et al., 1996). But whether doses higher than 240 mg/day further enhances the effect of EGb761 remains unclear (Le Bars, 2003). To

improve the clinical efficacy of EGb761, these factors should be considered in future studies.

1.3 Research objectives

A substantial body of preclinical evidence has suggested the neuroprotective effects of EGb761. Possible mechanisms suggested include anti-oxidation, anti-apoptosis and anti-inflammation etc. Despite these evidences, the effectiveness of EGb761 in the treatment and prevention of neurodegenerative diseases like AD is still inconclusive. Moreover, the precise neuroprotective mechanisms are not clearly known.

EGb761 consists of two major substances, the flavone glycosides (flavonoid fraction, 24%) and the terpene lactones (terpenoid fraction, 6%). Understanding of actions of individual EGb761 components may provide insights into direct mechanisms of neuroprotective effects of EGb761. In this study, protective effects of EGb761 and two of its constituents quercetin (a flavonoid) and ginkgolide B (a terpenoid) against Aβ 1–42-induced toxicity in human neuroblastoma SH-SY5Y cells were investigated. Aβ 1–42 is known as the main constituent of amyloid plaques in AD brains. The mechanism underlying Aβ 1–42-induced neurotoxicity is complex, involving several pathways as signaling events associated with apoptosis, oxidative stress and mitochondrial dysfunction etc (Longpré et al., 2006). Investigation of the effects of individual EGb761 components against Aβ toxicity may better our understanding of protective mechanism of EGb761 against neurodegenerative diseases such as AD.

The effective concentration of EGb761 in the brain is unclear. In this study, dosage effects of EGb761 on hydrogen peroxide (H_2O_2)-induced cell death in SH-SY5Y cells were investigated. H_2O_2 is a major reactive oxygen species (ROS) and once produced in excessive amounts, may induce oxidative stress (Halliwell, 1992; Crossthwaite et al., 2002). Oxidative stress has been widely implicated in apoptotic neuronal cell death that is associated with a variety of chronic neurodegenerative diseases such as AD (Behl, 1999; Onyango et al., 2005). The dosage effect of EGb761 on oxidative stress-induced cell death may contribute to better understanding of its effectiveness and complexity in the treatment and prevention of neurodegenerative diseases such as AD.

The ability of EGb761 to cross the BBB is unclear. Since EGb761 is a complex mixture of hundreds of chemical constituents, its ability to cross BBB is hard to monitor. In *in vitro* studies using neurons, peripheral non-neuronal cells and iolated mitochondria, EGb761 has been shown to protect mitochondrial function in terms of gene expression and activitity of electron transport chain enzymes as well as ATP synthesis (Tendi et al., 2002; Ahlemeyer and Krieglstein, 2003; Abdel-Kader et al., 2007). We thus proposed in this study that comparison of the *in vivo* effects of EGb761 on mitochondrial function between peripheral tissue cells and central nervous system may help to speculate the ability of EGb761 to cross the BBB. Recently, platelets, blood cells derived from fragmentation of precursor megakaryocytes, have been widely proposed as a peripheral biomarker in studies of mitochondrial function and aging-related diseases (Lenaz et al., 1998; Schapira, 1998; D'Aurelio et al., 2001; Merlo Pich et al., 2004; Xu et al., 2007b; 2007c; Shi et al., 2008b). The rationale of employing platelet rests on the assumption that alterations occurring in senescence and age-related diseases may be present in all cells, and that

changes occurring in platelet mitochondria may signal generalized bioenergetic deficiencies (D'Aurelio et al., 2001). Following these points, this study investigated effects of EGb761 on mitochondrial function in platelets and hippocampi in two animal models SAMP8 mice and ovariectomized rats. SAMP8 mice have been widely used as a model of age-related cognitive decline with relevance to biochemical and genetic alterations in AD. Our previous studies have demonstrated that mitochondrial dysfunction may be one of the mechanisms leading to age-associated degeneration in SAMP8 mice (Xu et al., 2007b). By contrast, ovariectomized middle-aged rats have been widely used to mimic the post-menopausal pathophysiological changes. Previous studies have demonstrated that estrogen withdrawal plays a primary role in the onset of post-menopausal AD, and mitochondrial dysfunction may be one of the mechanisms underlying neurodegeneration after estrogen withdrawal (Xu et al., 2008d; Shi et al., 2008a; Shi and Xu, 2008). Through investigation of the effect of EGb761 on mitochondrial function in platelets and hippocampi in the two animal models, we may better understand the *in vivo* pharmacological effects of EGb761 and its effectiveness and complexity in the treatment and prevention of neurodegenerative diseases such as AD.

2. Chapter II. EGb761 and its constituents quercetin and ginkgolide B protected against β-amyloid peptide-induced toxicity

2.1 Introduction

Accumulation of β-amyloid (Aβ) peptide in the brain is one of the pathological hallmarks of Alzheimer's disease (AD). Numerous *in vitro* and *in vivo* studies indicate that Aβ can directly induce neuronal death. The mechanism whereby Aβ induces neurotoxicity is complex, involving several pathways as apoptotic signaling events (Longpré et al., 2006). For example, nuclear factor-kappaB (NF-kappaB), ERK/p38 MAPK pathways (Mattson et al., 1997; Kaltschmidt et al., 1999; Lee et al., 2005) and JNK pathways (Yao et al., 2005) are shown to be involved in Aβ-mediated neuronal apoptosis.

Aβ-induced neurotoxicity also implicates oxidative stress that is manifested as an increase of lipid peroxidation, protein oxidation and DNA damage as well as alteration of antioxidants systems, as observed in the AD brains (Markesbery, 1997; Sayre et al., 1997; Sayre et al., 1997; Ramassamy et al., 1999; Ramassamy et al., 2000; Christen, 2000; Montine et al., 2005; Longpré et al., 2006). Aβ has been substantiated to generate oxygen free radicals spontaneously (Hensley et al., 1994; Monji et al., 2001a; 2001b; Longpré et al., 2006;) and the residue methionine 35 in the Aβ 1-42 peptide may be responsible for its pro-oxidant properties (Butterfield et al., 2005; Longpré et al., 2006). In addition, mitochondrial dysfunction is also

suggested to be involved in Aβ-induced oxidative cell death. Effects of the Aβ peptides (25-35 and 1-42) on NT2 cells with mitochondria and without mitochondria were investigated by Cardoso et al. (2001). It was found that, after treatment with Aβ peptides, cell viability was decreased in NT2 cells with mitochondria but not in NT2 cells without mitochondria, suggesting that Aβ peptides require functional mitochondria to induce cell toxicity (Cardoso et al., 2001). Therefore, in addition to the oxidative damage directly induced by Aβ, mitochondrial dysfunction may also contribute to Aβ-induced oxidative cell death. A possible synthesis of these results is that reactive oxygen species (ROS) derived directly from Aβ may induce mitochondrial dysfunction that then amplifies the ROS signal.

EGb761 is a well-known antioxidant (Abramov and Duchen, 2005). It has also been shown to protect against Aβ-induced neurotoxicity recently. For instance, EGb761 was able to protect primary hippocampal neurons and PC12 cells against the Aβ toxicity by blockage of Aβ-induced toxic events, such as ROS accumulation, glucose uptake and apoptosis in a concentration-dependent manner (Bastianetto et al., 2000). Another *in vitro* reconstitution study demonstrated that EGb761 dose-dependently inhibited the formation of Aβ-derived diffusible neurotoxic soluble ligands (Yao et al., 2001). Moreover, EGb761 restored the impaired phosphorylation of cyclic AMP response element-binding protein (CREB) (Xu et al., 2007a) and prevented the activation of NF-kappaB, ERK1/2, and JNK pathways in an Aβ-expressing neuroblastoma cell line N2a (Longpré et al., 2006). In addition, EGb761 was also found to be able to attenuate the basal as well as the induced levels of hydrogen peroxide (H_2O_2)-related ROS in both Aβ-expressing neuroblastoma cell line N2a and Aβ-expressing transgenic *Caenorhabditis elegans* (Smith and Luo,

2003).

Two major groups of constituents of EGb761, flavonoids (24%) and terpenoids (6%), have been suggested to be involved in the protective effects of EGb761 against Aβ toxicity (Smith and Luo, 2003). The ginkgolides, known as potent antagonists of platelet-activating factor (PAF) (Smith et al., 1996) are unique to the *Ginkgo biloba* tree (Nakanishi, 1967; Ivic et al., 2003; Jaracz et al., 2004). The flavonoids, such as quercetin, are involved in the antioxidant properties of EGb761. Despite the protective effects against Aβ toxicity, the underlying mechanisms of the actions of EGb761 and its single constituents are not clearly known.

Following the above-mentioned points, in this study, we used human neuroblastoma SH-SY5Y cells to test the effects of EGb761 and two of its constituents, quercetin and ginkgolide B, on Aβ-induced cell apoptosis, ROS accumulation, mitochondrial dysfunction, and activation of JNK, ERK1/2 and Akt signaling pathways. The results contribute to the better understanding of the neuroprotective mechanisms of EGb761.

2.2 Methods

2.2.1 Reagents

Eagle's minimum essential medium (DMEM) was purchased from American Type Culture Collection. Fetal bovine serum, penicillin and streptomycin, Donkey Anti-Goat IgG-HRP, Donkey Anti-Mouse IgG-HRP and Goat Anti-Rabbit IgG-HRP were

from Invitrogen (Grand Island, NY). EGb761, a *Gingkgo biloba* extract standardized to contain 24% flavonol glycosides, 6% terpene trilactones (of these, 2.9% ginkgolides A, B, C and J and 3.1% bilobalide), and <5ppm ginkgolic acidesis, was from Dr. Willmar Schwabe (Karlsruhe, Germany). Aβ 1-42 was from Biosource (USA). phospho-Akt (Thr308) antibody, total Akt antibody phospho-ERK1/2 (Thr202/Tyr204) antibody, phospho-JNK (Thr183/Tyr185) antibody and JNK1 antibody were from Santa Cruz Biotechnology (CA, USA). Cleaved caspase 3 antibody and total ERK1/2 antibody were from Cell Signaling Technology (USA). α-Tubulin antibody was from Thermo Scientific (USA). Unless stated otherwise, all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2.2 Cell culture

Human neuroblastoma SH-SY5Y cell line was grown in 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Monolayer cultures at a density of 10^4 cells/cm^2 were incubated in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in a 95% air, 5% CO_2 humidified atmosphere at 37℃. The culture medium was changed every 48 h.

Before the commencement of all experiments, cells were fully differentiated with all-trans-retinoic acid (RA) as previously described (Wu et al., 2009). Ten micromole RA was added to the medium one day after cell plating. After 5 days in the presence of RA, cells were washed three times with serum-free medium and transferred to a different experimental setup, as indicated. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cells were

exclusion assay, the cells were plated at a density of 5×10^4 cells in 35-mm-diameter plates. For flow cytometric analysis and measurement of cytochrome c oxidase (COX) activity as well as the rate of ATP synthesis, the cells were plated at a density of 1×10^6 per well in 6-well plates. For TUNEL apoptosis assay, the cells were plated at a density of 1×10^4 cells/well in 96-well plates. For western blot analysis, the cells were grown on a 10-cm dish at a density of 5×10^6 cells.

2.2.3 Aβ 1-42 toxicity and pharmacological treatments

Stock solutions of Aβ (1–42) peptide, 6 mg/ml in high performance liquid chromatography (HPLC)-graded water, were prepared according to the manufacturer's instructions and stored at -20℃. Before use, these stock solutions were thawed and incubated at 37℃ for 24 h to induce peptide aggregation and then diluted to the final concentration in medium. Stock solutions (2000×) of EGb761, quercetin and ginkgolide B were prepared in dimethyl sulfoxide (DMSO) and added to the culture medium at the indicated concentrations. PAF was dissolved in absolute ethanol to make a 1000× stock solution and added to the culture medium at the indicated concentration. DMSO or ethanol, at the final concentrations used (less than 0.1%), was found to have no effects on any of the parameters tested.

Cells were pre-incubated with serum-free culture medium containing indicated concentrations of EGb761 or quercetin, or ginkgolide B for 2 h. After that, 100 μg/ml Aβ 1-42, or 750 μM H_2O_2 or 50 μM PAF was added to the medium. Cells ($5\text{-}6 \times 10^6$) were then exposed to the medium containing Aβ 1-42 and EGb761 or quercetin, or ginkgolide B for another 24 h prior to next experiments. Cells in different treatments received equivalent volumes of vehicle.

2.2.4 Measurement of cell viability

Cell viability was determined by a colourimetric MTT assay according to Kobayashi et al. (2000). Briefly, cells were plated into 96-well plates. After cells were treated as described above, 10 μl 5 mg/ml MTT solution was added to each well of the 96-well plate, which was then incubated for a further 4 h at 37□. The medium containing MTT was then aspirated off and 200 μl of DMSO was added to each well. With DMSO as the blank, the absorbance was read with the Victor-2 Multilabel counter (EG&G Wallac, Gaithers- burg, Maryland, USA) at a wavelength of 490 nm.

2.2.5 Measurement of cell death

Oligonucleosomal DNA fragmentation in apoptotic cells was determined using the Cell Death Detection ELISA^{plus} Kit (TUNEL Apoptosis Assay Kit, product number: 11774425001) according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany) (Chan and Hsuuw, 2007). Cells were treated as described previously prior to ELISA detection, and spectrophotometric data were obtained using the same Victor-2 Multilabel counter at 405 nm against a reference wavelength of 490 nm.

In addition, the percentage of cell death in control cells was also determined with trypan blue exclusion assay. In brief, cells were collected and rinsed with phosphate-buffered saline (PBS). Cells were then immediately stained with 0.4% trypan blue solution, and the number of viable cells was counted using a hemocytometer under a light microscope.

2.2.6 ROS assay

Intracellular ROS levels were determined using a 2, 7-dichlorofluorescin dictate (DCF-DA) dye as previously descrived (Chan and Hsuuw, 2007). After treated as previous described, cells were incubated in 50 μl PBS containing 20 μM DCF-DA for 1 h at 37□. DCF-DA was added from a stock solution in DMSO. The quantity of DMSO never exceeded 0.1% and was also added to the blank. Intracellular ROS levels were measured using the same Victor-2 Multilabel counter (excitation 485 nm, emission 530 nm). An aliquot of the cell suspension was lysed in lysis buffer (50 nM Tris–HCl, pH 7.4, 150 nM sodium chloride, 5 nM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, 1% TritonX-100, 0.1 mg/ml phenylmethylsulfonyl, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin), and the protein concentration was determined with a DC protein assay kit (product number: 500-0121, Bio-Rad, Hercules, CA, USA).

2.2.7 Flow cytometric analysis of mitochondrial membrane potential using JC-1

Mitochondrial membrane potential was analyzed using an aggregate-forming lipohilic dye 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanineiodide (JC-1). JC-1 was stocked as 1 mg/ml DMSO solution and freshly diluted with culture medium. Cells in different treatments were loaded with JC-1 (at the final concentration of 5 μg/ml) at 37°C for 20 min. After that, cells were collected by centrifugation at 400×g for 3 min and the pellets were re-suspended in 0.5 ml PBS. Flow cytometry analysis was then performed within 10 min by using a flow cytometer (Coulter Epics AltraTM, Beckman Coulter, USA). Green and red fluorescence were analyzed on the FL1 (525 nm BP) and FL2 (575 nm BP) channels, respectively. The percentages of red and green fluorescence were quantified by

Expo32TM software (Beckman Coulter™) and mitochondrial membrane potential was expressed as red/green fluorescence ratio.

2.2.8 Measurement of COX activity

After cells were treated as described above, mitochondria were isolated using commercially available mitochondria isolation kit (product number: MITOISO2, Sigma, USA). Activity of COX was then measured using cytochrome c oxidase assay kit (product number: CYTOCOX1, Sigma, USA). Protein concentration of mitochondrial pellets was determined as previously described.

2.2.9 Measurement of the rate of mitochondrial adenosine triphosphate (ATP) synthesis by a HPLC-based method

The rate of mitochondrial ATP synthesis was measured as previously described (Shi et al., 2008a). Briefly, isolated mitochondrial pellets (1.0 mg/ml) were suspended in a stock solution consisting of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mM EDTA, 1.6 mM magnesium chloride, 5 mM succinate, 1 mg/ml bovine serum albumin (fatty acid-free), 0.5 μg/ml rotenone and 2.5 mM potassium phosphate buffer (pH 7.4). Ten seconds after the addition of adenosine diphoshate (at a final concentration of 100 μM), the reaction was terminated by adding ice-cold 0.3 M perchloric acid. The mixture was incubated on ice for 30 min and then centrifuged at 15,000 rpm for 5 min. The resulting supernatant was neutralized by adding an equal volume of saturated potassium hydroxide solution and centrifuged again at 15,000 rpm for 5 min.

ATP content in supernatants was then measured by a high performance liquid chromatograph (Waters, Milford, MA, USA). The gradient elution was performed on a 4.6-mm-i.d.× 30-cm, 5-μm-particle-size hypersil Base Deactived Silica (BDS) C-18 HPLC column with washing buffer (150 mM potassium dihydrogen phosphate) at a rate of 0.8 ml/min. Washing buffer with pH adjusted to 6.45 by potassium hydroxide was filtered through a Rainin 0.2-μm Nylon-66 filter and degassed in a flask linked with a vacuum pipe. Twenty microliters of standard mixture was first subjected to chromatography to obtain the retention time (about 15 min, as shown in Fig. 2.1) and UV monitored at 254 nm for about 15 min. Afterward, 20 μl of sample was injected. Peaks were identified by both the retention times and cochromatography with standards.

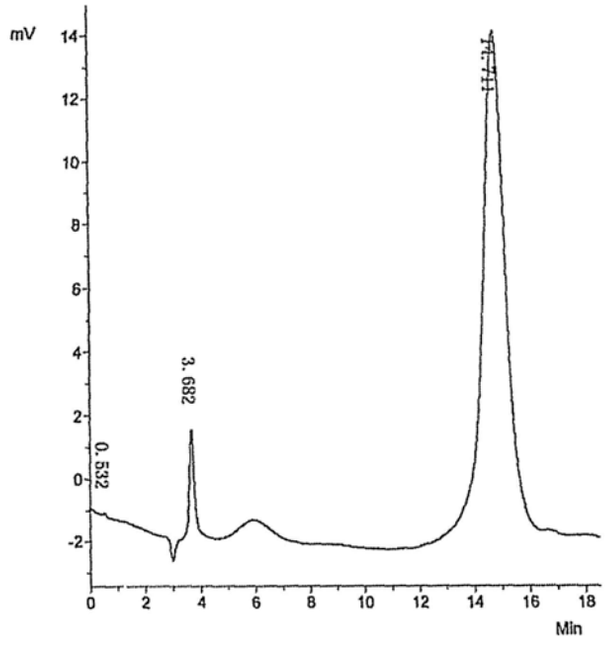

Fig. 2.1 Representative chromatogram of ATP standard (20 μM) showing the separation of ATP. Running time was 18 min.

2.2.10 Western blot analysis of signalling proteins involved in Aβ toxicity

The expression levels of cleaved caspase 3, phospho-JNK, JNK1, phospho-ERK1/2, total ERK1/2, phospho-Akt and total Akt in SH-SY5Y cells were examined by Western blot analysis. After treated as previous described, cells were lysed and the total protein concentration was measured as previously described. After boiled in 2-folds of loading buffer (100 nM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 200 nM dithiothreitol, 0.2% bromophenol blue, and 20% glycerol) for 5 min, aliquots of 20 μg proteins were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (S.D.S-PAGE) for electrophoresis at 100 V for 3 h, and then transferred onto nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). Transfer of proteins was done for 1 h at 10 V. Blots obtained were rinsed in PBST (100 nM phosphate buffer, pH 7.5, containing 150 nM sodium chloride and 0.05% Tween-20) and blocked with 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) in PBST for 1 h. They were then incubated with primary antibodies of cleaved caspase 3 (1:1000), phospho-JNK (Thr183/Tyr185) (1:1000), JNK1 (1:1000), phospho-ERK1/2 (Thr202/Tyr204) (1:1000), total ERK1/2 (1:1000), phospho-Akt (Thr308) (1:1000), total Akt (1:1000), or α-tubulin (1:1000) on a platform shaker overnight at 4°C. After rinsing with PBST, blots were incubated with 1:5000 secondary antibodies (Donkey Anti-Goat IgG-HRP for phospho-JNK and JNK1, Donkey Anti-Mouse IgG-HRP for α-tubulin, Goat Anti-Rabbit IgG-HRP for all others) for 2 h and then rinsed with PBST again. The protein band was visualized by using X-ray film and determined with BioRad GS800 densitometer equipped with "Quantity One" software package. Cleaved caspase 3 protein levels were determined by densitometry and plotted as ratios relative to the α-tubulin levels; phosphorylated

JNK protein levels were determined by densitometry and plotted as ratios relative to the levels of total JNK1; phosphorylated ERK1/2 protein levels were determined by densitometry and plotted as ratios relative to the levels of total ERK1/2; phosphorylated Akt protein levels were determined by densitometry and plotted as ratios relative to the levels of total Akt.

2.2.11 Statistics

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). All data were submitted to the Kolmogovor-Smirnov test for normal distribution and Levene's test of equal variances for variance homogeneity. In some cases, square root transformation was employed for correction for heterogeneity of variance. For one-factor design, one-way Univariate analysis of variance (ANOVA) followed by pairwise *t*-tests was performed. For two-factor design, two-way ANOVA followed by pairwise *t*-tests was used.

2.3 Results

2.3.1 Protective effect of EGb761 and its constituents quercetin and ginkgolide B against Aβ1-42-induced cell death in SH-SY5Y cells

As estimated by the MTT assay, cell viability dropped to about 50% after exposure to 100 μg/ml Aβ 1-42 for 24 h (Fig. 2.2 A-C). By contrast, incubation with different concentrations of EGb761 (50-200 μg/ml), or quercetin (1.5-6 μg/ml), or ginkgolide B (5-20 μg/ml) alone for 24 h did not affect cell viability (Fig. 2.2 A-C).

To investigate the effect of EGb761 and two constituents quercetin and ginkgolide B on Aβ 1-42-induced cell death, we pre-incubated SH-SY5Y cells with various doses of EGb761, or quercetin, or ginkgolide B for 2 h, followed by simultaneous challenge with 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h and subsequent determination of cell viability. We found that treatment of cells with EGb761 concentration-dependently prevented Aβ 1-42-induced decrease of cell viability (Fig. 2.2 A). The protective effect of EGb761 was significant at the lowest concentration tested here (50 μg/ml) with a maximal and complete effect obtained at 100 and 200 μg/ml (Fig. 2.2 A). Quercetin and ginkgolide B also showed dose-dependent effects on Aβ 1-42-induced decrease of cell viability (Fig. 2.2 B, C). Quercetin attenuated Aβ 1-42-induced cell loss at the lowest concentration tested here (1.5 μg/ml) (Fig. 2.2 B). Higher doses of quercetin (3 or 6 μg/ml) showed no such protection (Fig. 2.2 B). By contrast, the best protective effect of ginkgolide B was seen at 10 μg/ml (Fig. 2.2 C). Ginkgolide B at 5 or 20 μg/ml could not display such protection (Fig. 2.2 C).

These findings were confirmed by TUNEL apoptosis assay, which showed that 24 h Aβ 1-42 treatment induced a 4.6-5-fold increase in apoptosis compared with control cells (Fig. 2.3). By contrast, incubation with EGb761 (100 μg/ml), or quercetin (1.5 μg/ml), or ginkgolide B (10 μg/ml) alone did not affect cell apoptosis (Fig. 2.3). Aβ 1-42-induced cell apoptosis could be completely prevented by EGb761 at 100 μg/ml and to a lesser extent, by quercetin (1.5 μg/ml) and ginkgolide B (10 μg/ml) (Fig. 2.3).

2.3.2 Inhibitory effect of EGb761 and its constituents quercetin and ginkgolide B on Aβ1-42-induced intracellular ROS accumulation in SH-SY5Y cells

Aβ-induced toxicity implicates oxidative stress (Behl et al., 1994; Sayre et al., 1997; Markesbery, 1997; Sayre et al., 1997; Ramassamy et al., 1999; Ramassamy et al., 2000; Christen, 2000; Montine et al., 2005) and consequently we studied the effects of EGb761 and its constituents quercetin and ginkgolide B on Aβ-induced intracellular peroxide accumulation. It was found that, a 24 h exposure of SH-SY5Y cells to Aβ 1-42 resulted in significant increase in intracellular ROS generation (Fig. 2.4). By contrast, incubation with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B alone for 24 h did not affect intracellular ROS level (Fig. 2.4).

A co-treatment with EGb761 (100 μg/ml) completely prevented Aβ 1-42-induced increase in intracellular ROS level (Fig. 2.4). A co-treatment with quercetin (1.5 μg/ml) or ginkgolide B (10 μg/ml) showed similar inhibitory effects, but to a lesser extent (Fig. 2.4).

2.3.3 Protective effect of EGb761 and its constituents quercetin and ginkgolide B against Aβ1-42-induced mitochondrial dysfunction in SH-SY5Y cells

As mitochondrial dysfunction was suggested to be involved in Aβ 1-42-induced cell apoptosis (Abramov and Duchen, 2005), effects of EGb761 and its constituents quercetin and ginkgolide B on Aβ 1-42-induced mitochondrial dysfunction were also investigated. In this study, mitochondrial function was evaluated by mitochondrial membrane potential, COX activity and the rate of ATP synthesis.

Mitochondrial membrane potential reflects mitochondrial integrity and bioenergetic function, and can also be considered as an early indicator of apoptosis. In the present study, 24 h incubation with Aβ 1-42 resulted in decease of mitochondrial membrane potential in SH-SY5Y cells (Fig. 2.5). By contrast, incubation with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B alone for 24 h did not affect mitochondrial membrane potential (Fig. 2.5). A co-treatment with EGb761 (100 μg/ml) completely prevented Aβ 1-42-induced decease of mitochondrial membrane potential (Fig. 2.4). A co-treatment with quercetin (1.5 μg/ml) or ginkgolide B (10 μg/ml) showed similar protective effects, but to a lesser extent (Fig. 2.5).

Respiratory chain enzyme activity and the efficiency of ATP synthesis are also direct indicators of mitochondrial functions. In the present study, COX activity and the rate of ATP synthesis decreased significantly after 24 h Aβ 1-42 treatment (Fig. 2.6-2.7). By contrast, incubation with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B alone for 24 h did not affect COX activity and the rate of ATP synthesis (Fig. 2.6-2.7). A co-treatment with EGb761 (100 μg/ml) completely blocked Aβ 1-42-induced decrease of COX activity and the rate of ATP synthesis (Fig. 2.6-2.7). Similarly, a co-treatment with ginkgolide B (10 μg/ml) showed less

potent protective effects. Further, a co-treatment with quercetin (1.5 μg/ml) had no such protective effects (Fig. 2.6-2.7).

2.3.4 Inhibitory effects of EGb761 and its constituents quercetin and ginkgolide B on Aβ1-42-induced activation of caspase 3, JNK, ERK1/2 and Akt in SH-SY5Y cells

Activation of caspase 3, JNK, ERK1/2, and Akt may be implicated in the early signaling events involved in Aβ toxicity (Mattson et al., 1997; Kaltschmidt et al., 1999; Yao et al., 2005; Lee et al., 2005), and consequently we investigated the ability of EGb761 and its constituents quercetin and ginkgolide B to modulate activation of these signaling proteins. It was found that, stimulation of SH-SY5Y cells with Aβ 1-42 produced a significant increase in levels of cleaved caspase 3, phosphorylated JNK, phosphorylated ERK1/2 and phosphorylated Akt (Fig. 2.8 A-D). By contrast, incubation with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B alone for indicated periods of time did not affect levels of cleaved caspase 3, phosphorylated JNK, phosphorylated ERK1/2 and phosphorylated Akt (Fig. 2.8 A-D).

When cells were co-treated with Aβ 1-42 and EGb761 (100 μg/ml), increase of cleaved caspase 3, phosphorylated JNK, phosphorylated ERK1/2 and phosphorylated Akt was completely suppressed (Fig. 2.8 A-D). When cells were co-treated with Aβ 1-42 and quercetin (1.5 μg/ml) or ginkgolide B (10 μg/ml), increase of cleaved caspase 3 and phosphorylated JNK could be attenuated (Fig. 2.8 A and B), and increase of phosphorylated ERK1/2 and phosphorylated Akt could be completely prevented (Fig. 2.8 C and D).

In addition, no measurable alteration in total levels of JNK1, ERK1/2 and Akt in response to Aβ 1-42 and/or EGb761, or quercetin, or ginkgolide B was detected (Fig. 2.8).

2.3.5 Protective effects of EGb761 and its constituents quercetin and ginkgolide B against H_2O_2- and PAF-induced cell death

Both H_2O_2 and PAF have been suggested to mediate Aβ toxicity (Behl et al., 1994; Bate et al., 2004a; 2004b; Bate et al., 2006; Bate et al., 2008). In this study, we also investigated effects of EGb761 and its constituents quercetin and ginkgolide B on H_2O_2- and PAF-induced cell death. SH-SY5Y cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h, followed by 24 h exposure to 750 μM H_2O_2 or 50 μM PAF in the presence of EGb761, or quercetin, or ginkgolide B. Cell death was then evaluated by the MTT and TUNEL apoptosis assay. It was found that, H_2O_2 at 750 μM or PAF at 50 μM could reduce the cell viability to about 50% and induced a 4.5-5.2-fold increase in apoptotic cells (Fig. 2.9-2.10), suggesting a damage extent similar to that of 100 μg/ml Aβ 1-42. H_2O_2-induced cell apoptosis could be completely prevented by EGb761 (100 μg/ml) and to a lesser extent, by quercetin (1.5 μg/ml), but not by ginkgolide B (10 μg/ml) (Fig. 2.9 A and B). By contrast, PAF-induced cell apoptosis could be completely blocked by EGb761 (100 μg/ml) and ginkgolide B (10 μg/ml), and to a lesser extent, by quercetin (1.5 μg/ml) (Fig. 2.10 A and B).

2.4 Discussion

Aβ peptide is known as the major protein component of senile plaque, the defining feature of AD neuropathology. Aβ 1-42 is one predominant form of Aβ peptide found in human brains (Selkoe, 2001; Butterfield and Boyd-Kimball, 2004). Therefore, blockage of Aβ 1-42-induced neurotoxicity may help to prevent the occurrence or progression of AD. In this study, protective effects of EGb761 and its constituents

quercetin and ginkgolide B on the cytotoxic action of Aβ 1-42 were tested. Aβ-induced apoptosis in SH-SY5Y cells was evaluated by DNA fragmentation, mitochondrial membrane potential and levels of cleaved caspase 3 in this study. It was found that, 24 h Aβ 1-42 treatment caused significant cell apoptosis in SH-SY5Y cells, which could be completely prevented by EGb761, and to a lesser extent, by its constituents quercetin and ginkgolide B. Protection of EGb761 against Aβ-induced apoptosis may be associated with blockage of Aβ-induced cytotoxic events, e.g., ROS accumulation, mitochondrial dysfunction, and activation of JNK, ERK1/2 and Akt pathways. Thus, effects of EGb761 and two constituents quercetin and ginkgolide B on Aβ-induced ROS accumulation, mitochondrial dysfunction, and activation of JNK, ERK1/2 and Akt pathways were also investigated in this study.

It has been demonstrated that, Aβ-induced neurotoxicity is mediated by free radicals, which may be initiated either directly by Aβ itself (Hensley et al., 1994; Zhang et al., 2001; Monji et al., 2001a; 2001b), or indirectly through an increase in intracellular generation of ROS (Behl et al., 1994; Bastianetto et al., 2000; Zhang et al., 2001). In this study, EGb761 and to a lesser extent quercetin and ginkgolide B, completely prevented Aβ-induced increase in intracellular production of ROS, suggesting that the protective effect of EGb761 against Aβ toxicity may be associated with blockage of Aβ-induced ROS accumulation, and both quercetin and ginkgolide B may be involved in this effect.

Recently, a large number of studies have shown that mitochondrial dysfunction is also involved in Aβ-induced toxicity, and Aβ-induced mitochondrial dysfunction may contribute to oxidative stress, the impairment of energy metabolism and neuronal apoptosis, as seen in AD (Takuma et al., 2005; Reddy, 2006). For

example, Aβ could inhibit activities of electron transport chain complexes, such as alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase and COX, and promoted calcium ion-mediated assembly of the permeability transition pore in isolated mitochondria (Takuma et al., 2005). In another study, Aβ treatment induced respiratory complex I inhibition and a decrease in cellular ATP content in both astrocytes and neurons (Casley et al., 2002; Keil et al., 2004). In support of these findings, our study also showed that, Aβ significantly decreased mitochondrial membrane potential, COX activity and ATP synthesis in SH-SY5Y cells.

EGb761 was found to be able to protect mitochondria from the attack of Aβ (Eckert et al., 2003). Consistent with this finding, our study also showed that, Aβ-induced mitochondrial dysfunction could be completely prevented by EGb761 and to a lesser extent, by its component ginkgolide B. By contrast, quercetin attenuated Aβ-induced mitochondrial membrane potential depolarization but could not improve the COX activity and the rate of ATP synthesis. Actually, similar effects of quercetin have been reported by a study with isolated mitochondria, which demonstrated that, quercetin could increase ROS production via inhibition of mitochondrial respiratory chain but also scavenged this specie as it was generated, the latter effect overcoming the former (Dorta et al., 2008). It seems that the inhibitory effect of quercetin on respiratory chain restricts its protective effect against Aβ-induced mitochondrial dysfunction in this study. Nevertheless, the protective effect of EGb761 against Aβ toxicity appears to be associated with stabilization of mitochondrial function and its component ginkgolide B may be involved in this effect.

In addition, a rapid phosphorylation of Akt, ERK1/2 and JNK in SH-SY5Y cells was also detected after Aβ treatment. It is known that, the serine/threonine

kinase Akt is one of main downstream effectors of phosphatidylinositol 3-kinase (PI3K) (Crossthwaite et al., 2002). Activation of Akt promotes cell survival by activation of downstream anti-apoptotic signaling pathways (Skaper et al., 1998; Kaplan and Miller, 2000). ERK1/2 and JNK are members of MAPK family. Signaling through ERK1/2 is known to be detrimental to neurons and that inhibition of ERK1/2 promotes cell survival (Murray et al., 1998; Satoh et al., 2000; Stanciu et al., 2000; Crossthwaite et al., 2002). However, activation of ERK1/2 is also found to be pro-survival, partly via activation of CREB (Bonni et al., 1999; Crossthwaite et al., 2002). By contrast, the importance of JNK and its downstream transcription factor *c-jun* has been clearly recognized, with substantial evidence linking activation of this pathway to neuronal apoptosis (Maroney et al., 1998;; Davis, 2000; Mielke and Herdegen, 2000; Whitfield et al., 2001; Crossthwaite et al., 2002). To determine which of the three pathways is dominant in Aβ-induced cell death, a previous study analyzed the early signaling mechanisms underlying Aβ toxicity using SH-SY5Y cells (Wei et al., 2002). It was found that, Aβ caused cell death and induced a 2-3-fold activation of JNK, which could be inhibited by overexpression of a dominant-negative SEK1 construct (Wei et al., 2002). Aβ also weakly activated ERK and Akt but inhibitors of ERK and PI3K pathways did not affect Aβ-induced cell death (Wei et al., 2002). Therefore, among these pathways, activation of the JNK pathway may be the initial signaling cascade in Aβ-induced cell death (Wei et al., 2002).

EGb761 was found to be able to completely block activation of JNK, ERK1/2 and Akt pathways induced by Aβ. Similarly, its constituents quercetin and ginkgolide B attenuated Aβ-induced activation of JNK and completely abrogated Aβ-induced activation of ERK1/2 and Akt. In support of these findings, recent studies have also

demonstrated that EGb761 and its constituents can block the early signaling cascades leading to Aβ toxicity. For example, EGb761, the flavonoid fraction (CP 205) and ginkgolide B could prevent activation of NF-kappaB, ERK1/2, and JNK pathways in an Aβ-expressing neuroblastoma cell line N2a (Longpré et al., 2006). In another study, EGb761 and one of its constituents ginkgolide C restored impaired phosphorylation of CREB in Aβ-expressing neuroblastoma cells through activation of ERK pathway (Xu et al., 2007a). Overall, these data suggest that protective effects of EGb761 may be associated with blockage of the early signaling pathways leading to Aβ toxicity and both quercetin and ginkgolide B may be involved in this action.

Considerable evidence suggests that, H_2O_2, a hydroxyl radical donor, mediates Aβ-induced toxicity. For example, Aβ induced intracellular accumulation of H_2O_2 and lipid peroxides (Behl et al., 1994; Bastianetto et al., 2000). The H_2O_2-degrading enzyme catalase and a number of antioxidants protected cells against Aβ toxicity (Behl et al., 1994; Butterfield et al., 1999; Castellani et al., 2006). Moreover, clonal cell lines selected for their resistance to Aβ toxicity were also found to be H_2O_2-resistant (Behl et al., 1994). Recently, PAF, a potent mediator of the inflammatory and immune response, has also been suggested to mediate Aβ-induced toxicity. Neurons pre-treated with PAF antagonists (Hexa-PAF, CV-6209 or ginkgolide B) were resistant to Aβ 1-42 (Bate et al., 2004a; 2004b; Bate et al., 2006; Bate et al., 2008). Moreover, high concentrations of PAF were shown to be able to reproduce many of the effects of Aβ 1-42 on neurons (Bate et al., 2006; Bate et al., 2008). Following these points, in the present study, effects of EGb761 and two constituents quercetin and ginkgolide B on H_2O_2- and PAF-induced cell apoptosis were also investigated. It was found that, EGb761 prevented both H_2O_2- and PAF-

induced cell apoptosis. Its antioxidant constituent quercetin showed similar effects, but to a lesser extent. By contrast, ginkgolide B, known as a potent PAF antagonist, blocked PAF-induced cell apoptosis but could not protect cells from H_2O_2-induced cell damage. These data suggest that the antioxidant and PAF antagonist activities of EGb761 may contribute to its protective effects against Aβ toxicity. Quercetin but not ginkgolide B is one of the components responsible for the antioxidant action of EGb761. Both quercetin and ginkgolide B may be involved in the PAF antagonist activity of EGb761.

Taken together, the present study demonstrated that EGb761 protected against Aβ-induced cell apoptosis and abrogated Aβ-induced cytotoxic events, e.g. ROS accumulation, mitochondrial dysfunction and activation of JNK, ERK1/2 and Akt signaling pathways. Both quercetin and ginkgolide B may be involved in the inhibitory effects of EGb761 on JNK, ERK1/2 and Akt signaling pathways. Ginkgolide B also helped to improve mitochondrial function but quercetin failed to show this effect. Additional experiments also reveal that, the antioxidant and PAF antagonist activities of EGb761 may contribute to its protective effects against Aβ toxicity. Quercetin but not ginkgolide B is one of the components responsible for the antioxidant action of EGb761. Both quercetin and ginkgolide B may be involved in the PAF antagonist activity of EGb761. Therefore, actions of individual EGb761 components may provide further insights into direct mechanisms underlying the neuroprotective effects of EGb761.

3. Chapter III. Dosage effects of EGb761 on hydrogen peroxide-induced cell death

3.1 Introduction

H_2O_2, one of the major ROS, is produced by superoxide dismutase and monoamine oxidase as a by-product of normal cellular metabolism (Crossthwaite et al., 2002). Under normal physiological conditions, H_2O_2 can be broken down by catalase and glutathione peroxidase in the brain (Crossthwaite et al., 2002). However, under certain pathological conditions where H_2O_2 is produced in excessive amounts, antioxidant defences can be overwhelmed and oxidative stress may be induced (Halliwell, 1992; Crossthwaite et al., 2002). Oxidative stress has been widely implicated in apoptotic neuronal cell death that is associated with a variety of chronic neurodegenerative diseases such as AD (Behl, 1999; Onyango et al., 2005). But the molecular mechanisms involved in oxidative stress-induced apoptotic neuronal cell death are not fully understood.

Recent studies suggest that an array of intracellular signalling cascades that are closely associated with cell death and cell survival may be implicated in oxidative stress-induced neuronal damage (Ruffels et al., 2004). Among them are members of the mitogen-activated protein kinase (MAPK) family, such as JNK and the serine/theonine kinase Akt (Crossthwaite et al., 2002). It is generally accepted that, activation of Akt promotes cell survival through activation of downstream anti-apoptotic signaling pathways (Skaper et al., 1998; Kaplan and Miller, 2000), whereas activation of JNK is associated with neuronal cell death (Xia et al., 1995; Brunet et

al., 2001; Harper and LoGrasso, 2001). For example, growth factor-induced activation of the PI3K/Akt pathway rescued primary neurons treated with high glutamate (Skaper et al., 1998; Crossthwaite et al., 2002) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Shimoke and Chiba, 2001; Crossthwaite et al., 2002). Insulin protected retinal neurons against oxidative stress-induced apoptosis via activation of the PI3K/Akt pathway (Yu et al., 2006). In addition, diminished Akt phosphorylation in neurons lacking glutathione peroxidase-1 led to increased susceptibility to oxidative stress-induced neuronal death (Taylor et al., 2005). On the other hand, the lipid peroxidation products 4-hydroxynonenal and oxidized low-density lipoprotein were reported to activate JNK (Camandola et al., 2000; Soh et al., 2000; Schroeter et al., 2001; Crossthwaite et al., 2002) and inhibition of JNK prevented necrotic cell death in an *in vitro* model of excitotoxic neuronal death (Arthur et al., 2007).

EGb761 has been widely used in clinical trials for its beneficial effects on brain functions (Maclennan et al., 2002; Luo et al., 2002). The assumption that the beneficial effects of EGb761 are mainly due to its free-radical scavenging action has been demonstrated in numerous *in vitro* and *in vivo* studies (Maitra et al., 1995; Pietri et al., 1997; Pierre et al., 1999; Smith and Luo, 2003; Smith and Luo, 2004; Sarikçioğlu et al., 2004). EGb761 was found to be more effective than water-soluble antioxidants (e.g., ascorbic acid, glutathione and uric acid), and as effective as lipid-soluble antioxidants (e.g., alpha-tocopherol and retinol acetate) in attenuating H_2O_2-induced oxidative stress in human erythrocytes (Köse and Doğan, 1995). The flavonoid constituents of EGb761, such as quercetin, have been reported to contribute to its anti-oxidative properties. Despite that the *in vivo* concentrations of flavonoids are always lower than those recorded for small molecule antioxidant

nutrients such as ascorbic acid and alpha-tocopherol (Halliwell and Zhao, 2000; Abd El Mohsen et al., 2002; Williams et al., 2004;), flavonoids are capable of protecting neurons from oxidative damage more effectively than ascorbic acid, even when the latter was used at 10-fold higher concentrations (Schroeter et al., 2000; Williams et al., 2004). These data suggest that, beneficial effects of EGb761 may go beyond its free-radical scavenging properties. Recently, there are increasing evidences showing that cellular effects of EGb761 may be associated with modulation of specific proteins central to intracellular signaling cascades. For instance, EGb761 enhanced phosphorylation of Akt in a human endothelial cell line and endothelial progenitor-cells (Koltermann et al., 2007; Dong et al., 2007). EGb761 also down-regulated JNK-activator protein-1 signaling pathway in human peripheral blood T cells (Cheng et al., 2003). In addition, a pre-treatment with EGb761 significantly inhibited ethanol-induced increase of JNK activity in gastric mucosa (Chen et al., 2005). However, whether EGb761 regulates divergent signaling through Akt/PKB and JNK in response to oxidative stress in neurons is presently unknown. Moreover, to determine whether the treatment dosage affects the interaction of EGb761 with these signaling proteins in neurons is also essential since the levels of EGb761 accumulated in the brain are unclear.

Therefore, in this study, we examined the dosage effects of EGb761 on H_2O_2-induced apoptosis of neuroblastoma SH-SY5Y cells, a widely used *in vitro* neuron cell model. The results presented suggest that, low doses of EGb761 (50-100 μg/ml) inhibit H_2O_2-induced cell apoptosis in SH-SY5Y cells via inactivation of Akt, JNK and caspase-3 whereas high doses of EGb761 (250-500 μg/ml) enhanced H_2O_2 toxicity via inactivation of Akt and enhancement of activation of JNK and caspase-3.

This dose-dependent effect on neuronal signaling pathways is important for our understanding of the specific actions of this drug.

3.2 Methods

3.2.1 Reagents

IRDye 680 Donkey Anti-Goat IgG, IRDye 800CW Donkey Anti-Mouse IgG and IRDye 800CW Donkey Anti-Rabbit IgG were purchased from LI-COR Biosciences (NE, USA).

3.2.2 Cell culture and drug treatments

Culture and differentiation of SH-SY5Y cells were performed as previously described. Cells were treated with or without various concentrations of EGb761 for 24 h. Then, the media containing EGb761 was aspirated off and cells were washed twice with PBS. After that, cells were directly subjected to next experiments, or, incubated with indicated concentrations of H_2O_2 for another 24 h. Normal control cells received medium-vehicle only.

3.2.3 MTT assay

Cell viability was determined by MTT assay as previously described.

3.2.4 TUNEL apoptosis assay

Oligonucleosomal DNA fragmentation in apoptotic cells was measured using the same Cell Death Detection ELISA^{plus} Kit (TUNEL Apoptosis Assay Kit) as previously described.

3.2.5 Flow cytometric analysis of mitochondrial membrane potential using JC-1

Mitochondrial membrane potential was analyzed using an aggregate-forming lipohilic dye JC-1 as previously described.

3.2.6 Western blot assay of signaling proteins

The expression levels of phospho-Akt, total Akt, phospho-JNK, JNK1 and cleaved caspase-3 in SH-SY5Y cells were examined by Western blot analysis. After treated as described previously, cells were trypsinized and collected by centrifugation at 400 × g for 3 min. The pellets were lysed in lysis buffer (50 nM Tris-HCl, pH 7.4, 150 nM sodium chloride, 5 nM ethylene dinitrilotetra-acetic acid, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 0.1 mg/ml Phenylmethylsulfonyl, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin), and the protein concentration was determined with the same DC protein assay kit (Bio-Rad, CA).

After boiled in twofold of loading buffer (100 nM Tris-HCl, pH 6.8, 4% S.D.S, 200 nM DTT, 0.2% bromophenol blue, and 20% glycerol) for 5 min, proteins were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (S.D.S-PAGE) at 100 V for 3 h, and then transferred onto nitrocellulose membrane

using a semi-dry transfer apparatus (Bio-Rad). Transfer of proteins was done for 1 h at 10 V. Blots thus obtained were rinsed in PBST (100 nM phosphate buffer, pH 7.5, containing 150 nM NaCl and 0.05% Tween-20) and blocked with 5% non-fat dry milk (Bio-Rad) in PBST for 1 h. They were then incubated with primary antibodies of phospho-Akt (Thr308) (1:1000), total Akt (1:1000), phospho-JNK (Thr183/Tyr185) (1:1000), JNK1 (1:1000), cleaved caspase-3 (1:1000) or α-tubulin (1:1000) on a platform shaker overnight at 4°C. After rinsing with PBST, blots were incubated with 1:5000 fluorescent secondary antibodies (IRDye 680 Donkey Anti-Goat IgG for phospho-JNK and JNK1, IRDye 800CW Donkey Anti-Mouse IgG for α-tubulin and IRDye 800CW Donkey Anti-Rabbit for all others) in the dark for 2 h and then rinsed with PBST again. Images were acquired with the Odyssey infrared imaging system and analyzed by the software program as specified in the Odyssey software manual. Phosphorylated Akt protein levels were determined by densitometry and plotted as ratios relative to the levels of total Akt; phosphorylated JNK protein levels were determined by densitometry and plotted as ratios relative to the levels of total JNK1; cleaved caspase-3 protein levels were determined by densitometry and plotted as ratios relative to the α-tubulin levels.

3.2.7 GSH determination

Intracellular glutathione (GSH) was measured by using commercially available Glutathione Assay Kit (product number: CS1020, Sigma, USA). Measurements were carried out in cell extract preparations from cells in different treatments with the same Victor-2 Multilabel counter at an excitation wavelength of 390 nm and

emission wavelength of 478 nm. Protein concentrations of cell extracts were determined as described previously.

3.2.8 Statistics

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). All data were submitted to the Kolmogovor-Smirnov test for normal distribution and Levene's test of Equal Variances for variance homogeneity. In some cases, square root transformation was employed for correction for heterogeneity of variance. For one-factor design, one-way Univariate analysis of variance (ANOVA) followed by pairwise *t*-tests was performed. For two-factor design, two-way ANOVA followed by pairwise *t*-tests was used.

3.3 Results

3.3.1 Dosage effects of EGb761 on H_2O_2-induced cell apoptosis

MTT assay of SH-SY5Y cells incubated with various doses of H_2O_2 revealed that the cell viability dose-dependently decreased in response to 250-1000 μM H_2O_2 (Fig. 3.1 A). This finding could be confirmed by TUNEL apoptosis assay, which showed that 250-1000 μM H_2O_2 dose-dependently increased the proportion of apoptotic cells (Fig. 3.2 A). By contrast, MTT and TUNEL apoptosis assay of SH-SY5Y cells incubated with various doses of EGb761 alone revealed that EGb761 at 50-500 μg/ml did not affect cell viability (Fig. 3.1 B and Fig. 3.2 B). To investigate the effects of various doses of EGb761 on H_2O_2-induced cell death, we pre-incubated SH-SY5Y cells with

various concentrations of EGb761 for 24 h, followed by challenge with 500 μM H_2O_2 for another 24 h. We found that low concentrations of EGb761 (50-100 μg/ml) prevented H_2O_2-induced cell apoptosis while high concentrations of EGb761 (250-500 μg/ml) exacerbated this effect (Fig. 3.1 C; Fig. 3.2 C). To test if a protective dose of EGb761 protects against cell death induced by a wide range of H_2O_2 doses, we also test the effect of 100 μg/ml EGb761 on various doses (250-1000 μM) of H_2O_2-induced cell apoptosis. It was found that, EGb761 at 100 μg/ml could protect against cell apoptosis induced by 250-1000 μM H_2O_2 (Fig. 3.1A and Fig. 3.2A).

3.3.2 Dosage effects of EGb761 on H_2O_2-induced depolarization of mitochondrial membrane potential

It has been proposed that opening the mitochondrial permeability transition pore and/or mitochondrial membrane permeabilization signals cell death by releasing apoptogenic factors, e.g., cytochrome c, from the mitochondria. To further investigate the effects of EGb761 on H_2O_2-induced cell death, changes in mitochondrial membrane potential were determined. It was found that, 500 μM H_2O_2 significantly decreased mitochondrial membrane potential in SH-SY5Y cells (Fig. 3.3A and Fig. 3.4). By contrast, incubation with different concentrations of EGb761 alone did not affect mitochondrial membrane potential in cells (Fig. 3.3B).

A pre-treatment of cells with low concentrations of EGb761 (50-100 μg/ml) prevented H_2O_2-induced decrease of mitochondrial membrane potential (Fig. 3.3 A; Fig. 3.4) whereas higher concentrations (250-500 μg/ml) exacerbated this effect (Fig. 3.3 A; Fig. 3.4).

3.3.3 Dosage effects of EGb761 on H_2O_2-induced activation of Akt

As activation of Akt has been strongly implicated in the regulation of neuronal survival and/or protection, the ability of EGb761 to modulate Akt activation in SH-SY5Y cells was tested. It was found that, stimulation of SH-SY5Y cells with 500 μM H_2O_2 significantly increased phosphorylated Akt levels (Fig. 3.5 A, B). By contrast, incubation with different concentrations of EGb761 alone showed no effects on phosphorylated Akt levels (Fig. 3.5 C). H_2O_2 or EGb761 alone did not affect the total level of Akt (Fig. 3.5 A).

When cells were pre-treated with low doses of EGb761 (50-100 μg/ml) prior to challenge with 500 μM H_2O_2, phosphorylated Akt levels declined to baseline levels (Fig. 3.5 A, B). In contrast, when cells were pre-incubated with high doses of EGb761 (250-500 μg/ml) prior to challenge with 500 μM H_2O_2, phosphorylated Akt levels declined to below baseline levels (Fig. 3.5 A, B). In addition, a pre-treatment with EGb761 at 500 μg/ml also decreased the total levels of Akt in H_2O_2-treated cells but lower concentrations (50-250 μg/ml) showed no such effects (Fig. 3.5 A).

3.3.4 Dosage effects of EGb761 on H_2O_2-induced activation of JNK and caspase-3

JNK and caspase-3 have been implicated in the pro-apoptotic signaling in neurons and consequently we investigated the ability of EGb761 to modulate the activation of these two signaling proteins. It was found that, treatment of SH-SY5Y cells with 500 μM H_2O_2 resulted in marked increase in levels of JNK phosphorylation (Fig. 3.6 A, B) and cleaved caspase-3 (Fig. 3.7 A, B). By contrast, incubation with different concentrations of EGb761 alone showed no effects on levels of JNK phosphorylation (Fig. 3.6 C) and cleaved caspase-3 (Fig. 3.7 C).

A pre-treatment of cells with low concentrations of EGb761 (50-100 μg/ml) suppressed 500 μM H_2O_2-induced activation of JNK and caspase-3 whereas higher doses (250-500 μg/ml) enhanced the activation of these two signaling proteins (Fig. 3.6 A, B and Fig. 3.7 A, B). But no measurable alteration in the total level of JNK1 in response to H_2O_2 and/or EGb761 was detected (Fig. 3.6 A).

3.3.5 Dosage effects of EGb761 on H2O2-induced decrease of intracellular GSH content

Since GSH can facilitate the metabolism of drugs to less active compounds and detoxify the free radicals (Malik et al., 1997; Arrick and Nathan, 1984; Meister, 1983), intracellular GSH content in SH-SY5Y cells was tested. It was found that, H_2O_2 treatment significantly decreased intracellular GSH content in SH-SY5Y cells (Fig. 3.8 A). In contrast, incubation with high doses of EGb761 (250-500 μg/ml) alone slightly decreased intracellular GSH content (to about 85%) while lower doses (50-100 μg/ml) alone showed no such effect (Fig. 3.8 B).

A pre-treatment of cells with low concentrations of EGb761 (50-100 μg/ml) attenuated or completely blocked 500 μM H_2O_2-induced decrease of intracellular GSH content whereas higher concentrations (250-500 μg/ml) exacerbated this effect (Fig. 3.8 A).

3.4 Discussion

H_2O_2-induced apoptotic neuronal cell death was demonstrated to be through extrinsic (death receptor-mediated) and intrinsic (mitochondria-dependent) pathways (Yamakawa et al., 2000; Chandra et al., 2000; Facchinetti et al., 2002; Ruffels et al.,

2004). Both pathways involve the activation of caspases (Strasser et al., 2000; Reed, 2001; Ruffels et al., 2004). The extrinsic pathway is initiated by actication of members of the TNF family such as TNFR1, Fas and TRAIL receptors (Ruffels et al., 2004). Stimulation of these receptors activates initiator caspases, particularly caspase-8, which then cleaves and activates downstream effector caspases such as caspase-3 (Ruffels et al., 2004). In contrast, the intrinsic pathway is triggered by dissipation of the mitochondrial membrane potential and release of cytochrome c from the mitochondria (Ruffels et al., 2004). Cytochrome c combines with Apaf1 in the cytosol resulting in the activation of the initiator caspase-9, which also cleaves and activates the effector caspase-3 (Ruffels et al., 2004). In support of these findings, the present study demonstrated that DNA fragmentation, mitochondrial membrane potential depolarization, and activation of caspase-3 in SH-SY5Y cells were dose-dependently enhanced by H_2O_2. Moreover, a rapid phosphorylation of Akt and JNK in SH-SY5Y cells was also detected in this study. As mentioned above, the PI3K/Akt signaling pathway has been recognized as one of the most critical pathways in regulating neuronal survival (Williams and Doherty, 1999; Kaplan and Miller, 2000) whereas activation of JNK and the downstream phosphorylation target *c-jun* has been implicated in neuronal death signaling (Maroney et al., 1998; Whitfield et al., 2001; crossthwaite et al., 2002). In this study, signaling through Akt appeared to have a pro-survival role but apparently could not protect neurons from H_2O_2-induced apoptosis. Pro-apoptotic signals through JNK should be dominant under this condition. It has been demonstrated that a balance of signals through Akt and JNK following H_2O_2 exposure determined the cell viability of primary cortical neurons (Crossthwaite et al., 2002) and it is plausible that a similar mechanism occurs in SH-SY5Y cells when exposed to oxidative stress.

EGb761 showed dosage effects on H_2O_2-induced cell toxicity in this study. Low doses of EGb761 (50-100 μg/ml) effectively prevented H_2O_2-induced cell apoptosis via inactivation of Akt, JNK and caspase-3, while high doses of EGb761 (250-500 μg/ml) enhanced activation of JNK and caspase-3, induced a deactivation of Akt accompanied by reductions in total Akt protein levels, and consequently enhanced H_2O_2-induced cell apoptosis. But these concentrations of EGb761 alone did not affect cell viability and active state of these signaling proteins.

Interestingly, previous studies have demonstrated that the pharmacological active components of EGb761 display similar dosage effects on signaling pathways involving Akt/PKB or JNK. Taking quercetin for example, treatment with high concentrations (30 μM) of quercetin led to sustained dephosphorylation of Akt and subsequent Akt cleavage by caspase-3 in mouse cortical neurons, whereas at lower concentrations (<10 μM) the inhibition of Akt phosphorylation was transient and reversible (Spencer et al., 2003). It has been suggested that, quercetin inhibits phosphorylation of Akt possibly through inhibition of the upstream partner PI3K at its ATP-binding site (Spencer et al., 2003). In another study, low concentrations of ginkgolide B (5–25 μM) inhibited ethanol-induced apoptotic cell death in human Hep G2 cells via inhibition of activation of JNK and caspase-3, but higher concentrations (50–100 μM) enhanced ethanol-induced cell apoptosis through enhancement of activation of JNK and caspase-3 (Chan and Hsuuw, 2007). These data suggest that, both flavonoid and terpenoid constituents may be involved in the dosage effects of EGb761 on H_2O_2-induced activation of these signaling proteins.

In this study, EGb761 also showed dose-dependent effects on intracellular GSH content. H_2O_2-induced decrease of intracellular GSH level was reversed by low concentrations of EGb761 while enhanced by higher concentrations of EGb761

treatment. Moreover, high doses of EGb761 alone slightly decreased intracellular GSH level whereas lower doses showed no such effect. Decrease of intracellular GSH content suggests that the intracellular redox equilibrium favors the oxidized state of thiol containing proteins (Clément et al., 1998). Thus, EGb761 showed both anti-oxidative and pro-oxidative effects in this study. Actually, polyphenolic flavonoids can be both pro-oxidative and anti-oxidative (Galati et al.,1999; Awad et al., 2000; Galati et al., 2001; Galati et al., 2002). It has been shown that, catalytic concentrations of flavonoids with phenol rings, upon oxidation by peroxidase/H_2O_2, form phenoxyl radicals which, in some cases are sufficiently reactive to cooxidize NADH, GSH or ascorbate accompanied by extensive oxygen uptake and ROS generation (Galati et al., 1999; Galati et al., 2002). In contrast, catalytic concentrations of polyphenolics with catechol rings can also cooxidize ascorbate, likely mediated by semiquinone radicals (Awad et al., 2000; Galati et al., 2002) and deplete GSH through formation of GSH conjugates, which is likely mediated by the o-quinone metabolites (Awad et al., 2000; Galati et al., 2002). EGb761 contains both phenol ring-containing polyphenolics (e.g. apigenin) and catechol B ring-containing polyphenolics (e.g. quercetin). It has been demonstrated that, GSH can be oxidized by apigenin phenoxyl radicals, whereas the formation of GSH conjugate involves the quinoid (quinone methide) product of quercetin (Galati et al., 2001). On the other hand, a recent study demonstrated that, low concentrations of ginkgolide B (5–25 μM) inhibited ethanol-induced ROS generation in human Hep G2 cells while high concentrations of ginkgolide B (50–100 μM) enhanced this effect (Chan and Hsuuw, 2007). These findings again suggest that, both flavonoid and terpenoid constituents may be involved in the dose-dependent regulatory effects of EGb761 on cell redox state. Cell redox state is an important regulator of cell fate decisions. In this study,

the dosage effects of EGb761 on H_2O_2-induced cell apoptosis are probably correlated with regulation of cell redox state. High doses of EGb761 increase susceptibility of cells to oxidative stress-induced cell death likely through attenuation of cellular antioxidant defense.

In addition, another issue needing consideration is the relationship between the concentrations of EGb761 used in this study versus those in *in vivo* studies, as well as the relevance of these concentrations to humans (Yao et al., 2004). EGb761 consists of hundreds of chemical constituents (Yao et al., 2004). As its terpene trilactone constituents ginkgolides represent approximately 2–3%, the *in vivo* concentrations of these compounds have recently been used to indicate the bioavailability of EGb761 (Biber, 2003; Yao et al., 2004). As suggested in this study, EGb761 at 100 μg/ml could protect against cell death induced by a wide range of H_2O_2 doses. Similar concentrations have been tested in *in vivo* studies. It has been demonstrated that, after oral administration of 100 mg/kg EGb761 in rats, the blood concentration of ginkgolides reached 2–3 μg/ml (Biber, 2003; Yao et al., 2004), a concentration indicating the presence of 100 μg/ml EGb761 in the blood. Similar concentrations of ginkgolides have also been detected in the blood of human subjects treated with EGb761 at 240 mg/day, a dose used in clinical trials to slow down the disease progression of AD patients (Hofferberth, 1994; Kanowski et al., 1996; Le Bars et al., 1997; Oken et al., 1998; Biber, 2003; Amri et al., 2003; Yao et al., 2004).

Although blood concentrations of this compound can be predicted and monitored after oral administration or direct intravenous injection, it is not easy to control the concentrations in the brain, since the ability of this compound to cross the blood-brain barrier (BBB) is still unclear. It is known that, under normal physiological conditions, the BBB controls tightly the entry of drugs into the central

nervous system. But disruption of BBB is now increasingly documented in both normal aging and neurodegenerative disorders such as AD (Popescu et al., 2009; Farrall and Wardlaw, 2009). Under pathological conditions such as AD, EGb761 was found to be able to cross the BBB effectively (Kanowski et al., 1996; Garcia-Alloza, 2009), suggesting that an effective concentration might be reached in the AD brain when treatment with appropriate doses.

Notably, oxidative stress has long been considered a major cause underlying neurodegenerative and neuroinflaummatory disorders and BBB dysfunction associated with them (Haorah et al., 2007). It has been demonstrated that, ROS augments BBB permeability and monocyte migration across BBB, possibly through activation of protein tyrosine kinase and matrix metalloproteinases (Haorah et al., 2007). Our study suggested that EGb761 shows pro-oxidant effects at higher concentraions. This raises the possibility that the pro-oxidant action of high-dose EGb761 may facilitate its entry across BBB. Nowadays, EGb761 has been used in clinical trials for cognitive and circulatory diseases at daily doses of 120-720 mg. Considering the above-mentioned points, this study also indicate a cautious use of high doses of EGb761 in clinical practice.

In summary, in this study, we found that, low concentration of EGb761 (50-100 μg/ml) inhibited H_2O_2-induced cell apoptosis in SH-SY5Y cells via inactivation of Akt, JNK and caspase-3 whereas high concentration of EGb761 (250-500 μg/ml) enhanced H_2O_2 toxicity via inactivation of Akt and enhancement of activation of JNK and caspase-3. Moreover, dosage effects of EGb761 on intracellular GSH content were also observed, which further suggests that, dosage effects of EGb761 on apoptotic signaling proteins may be correlated with regulation of cell redox state. Therefore, treatment dosage may be one of the vital factors that determine the

specific action of EGb761 on oxidative stress-induced cell apoptosis. A clear understanding of the mechanisms of dosage effects of EGb761 may have important clinical implications.

4. Chapter IV. EGb761 protects against aging-associated mitochondrial dysfunction in SAMP8 mice

4.1 Introduction

Aging is a complex biological process during which physiological functions of various cells, tissues and organs of an individual deteriorates with age. Until now, the molecular mechanisms underlying the aging process have not been completely understood. The free radical theory of aging (FRTA), one theory of aging proposed by Harman et al. in the 1950s suggests that aging is a result of accumulated macromolecular damage through reactions involving reactive oxygen species (ROS) (Gruber et al., 2008). In the 1980s, the idea in FRTA was extended to implicate mitochondria as source as well as target of ROS (Bossy-Wetzel et al., 2003; Miquel et al., 1980; Gruber et al., 2008). So far, considerable evidence has demonstrated that, aging is associated with mitochondrial dysfunction. Mitochondrial dysfunction during aging is manifested as accumulated mtDNA (mitochondrial deoxyribonucleic acid) mutations, deficient oxidative phosphorylation, decreased ATP synthesis, increased ROS production and abnormal mitochondrial ultrastructure, all of which may predispose to aging-associated disorders (Pallotti et al., 1996; Alemany et al., 1988; Cortopassi and Wong, 1999; Krause, 2007; Hiona and Leeuwenburgh, 2008; Shi et al., 2008b).

Many models have been developed for studying aging and aging-associated disorders (Kuro-o, 2001; Johnson, 2008; Cedar and Minger, 2008; Fernández-Gómez et al., 2008; Luo et al., 2008). Among them is the senescence-accelerated strain of

mice (SAM), one of the murine models of accelerated aging established by Takeda et al. (1997). The SAM strain of mice is comprised of nine bred strains of SAMP (accelerated senescence-prone) and three SAMR (accelerated senescence-resistant) strains (Takeda et al. 1997). Compared with the SAMR strains, the SAMP strains display a more accelerated senescence process that is manifested by a shorter lifespan and an earlier onset and more rapid progress of aging-associated pathological changes similar to human geriatric disorders (Takeda et al. 1997; Hosokawa, 2002; Chiba et al., 2009). In our previous study, the mitochondrial function of platelets and hippocampi was tested in SAMP8 (one of the nine substrains of SAMP) and SAMR1 (one of the three substrains of SAMR) at 2, 6 and 9 months of age (Xu et al., 2007b). The rationale of using platelets as a peripheral biomarker for mitochondrial damages rests on the assumption that alterations occurring in senescence may be present in all cells, and that changes occurring in platelet mitochondria may represent generalized bioenergetic deficiencies (Lenaz et al., 1998; Schapira, 1998; D'Aurelio et al., 2001; Merlo Pich et al., 2004; Xu et al., 2007b; 2007c; Shi et al., 2008b). It was found that, mitochondrial function of platelets and hippocampi in SAMP8, evaluated as mitochondrial membrane potential and ATP content, decreased at a relatively early age compared with SAMR1 (Xu et al., 2007b). These results suggest that mitochondrial dysfunction may be one of the mechanisms leading to age-associated degeneration in SAMP8 mice at an early age and platelets may serve as peripheral biomarkers of mitochondrial function and aging-associated disorders (Xu et al., 2007b).

EGb761 has been demonstrated to possess polyvalent properties, such as anti-oxidation, anti-apoptosis, and anti-inflammation (Smith and Luo, 2004). These properties may play a role in its purported "anti-aging" action (Sastre et al., 2000; Wu

et al., 2002; Christen, 2004). More recently, EGb761 has also been proposed to have direct protective effects on mitochondria. Using PC12 cells, dissociated mice brain cells and isolated mitochondria, Abdel-Kader et al. mimicked aging-associated mitochondrial abnormalities by using external factors (oxidative stress, nitrosative stress, serum deprivation and complexes inhibitors) and reported that EGb761 protected mitochondrial function *in vitro* at concentrations as low as 0.01 mg/ml (Abdel-Kader et al., 2007). Moreover, EGb761 could also reverse anoxia/reoxygenation-induced decrease of ADP phosphorylation versus oxygen consumption in isolated mitochondria from rat brain and rat liver and prevented the decrease of GSH/GSSG ratio in rat mitochondria during aging (Ahlemeyer and Krieglstein, 2003). In addition, EGb761 and its constituent bilobalide have also been shown to up-regulate gene levels of mitochondrial NADH dehydrogenase, thereby decreasing stage 4 respiration whose increase is indicative of mitochondrial oxidative damage (Tendi et al., 2002). These data suggest that protective effects of EGb761 on mitochondrial function may also contribute to its "anti-aging" property. Nowadays, EGb761 has become one of the most popular herbal supplements, taken for its purported "anti-aging" property (Smith and Luo, 2004). However, its "anti-aging" effect, particularly its effect on central nervous system, is still inconclusive.

Following these points, in this work, EGb761 was administrated orally to SAMP8 mice at 3 and 24 weeks of age for 12 weeks to test the preventive and rescue effects of this herb on age-associated mitochondrial dysfunction. As mentioned above, comparison of *in vivo* effects of EGb761 on mitochondrial function between platelets and hippocampi may help to speculate the ability of EGb761 to cross the BBB. In this study, effects of EGb761 on mitochondrial function in both platelets and hippocampi were tested.

4.2 Methods

4.2.1 Experimental animals and herb administration

Two groups of SAMP8 mice aged 3 weeks and 40 weeks, respectively, were provided by the Animal facility of Tianjin University of Traditional Chinese Medicine. Before experiments, they were kept under defined environmental conditions (25℃, 50% relative humidity, 12-h light-dark cycle) and were given standard diet and water *ad libitum* as previously described (Lorke et al., 2003). Mice in each age group were subdivided into two subgroups, i.e., control (n=15) and EGb761-treated subgroups (n=15). For the EGb761-treated subgroup, the SAMP8 mice were fed with EGb761 in 4% surcose with a daily dose of 100 mg/kg body weight. For the control subgroup, the SAMP8 mice were given 4% sucrose instead.

4.2.2 Isolation of mitochondria from platelets and hippocampi

Mice were anesthetized with 10 g/L sodium pentobarbital after 12 weeks of drug treatment. Collection of blood and isolation of platelets were performed as prevously described (Karpouza and Vakirtzi-Lemonias, 1997). In brief, after the thoracic cavity was opened, blood (0.8–1.5 ml) was immediately collected by cardiac puncture through a 0.6 mm-gauge needle into a plastic syringe containing 0.2 M ethylenediaminetetraacetic acid (EDTA) in a ratio of 1 part EDTA to 40 parts blood. Blood from five mice was pooled and centrifuged at 250×g for 15 min at room

temperature to prepare the platelet-rich plasma (PRP). PRP was centrifuged at 1,400 ×g for 15 min at 4□ to precipitate platelets. Platelet mitochondria were then isolated as previously described (Mancuso et al., 2003). Briefly, the isolated platelet pellets were suspended in a hypotonic medium (10 mM Tris–Cl, pH 7.6) for 4 min and then centrifuged at 1,500×g for 10 min at 4 ℃. The resulting supernatant was centrifuged at 10,000×g for 20 min at 4℃ to precipitate mitochondria.

After exsanguination, mice were immediately decapitated. The brain was removed and hippocampi were quickly dissected on a cold plate at −20℃. Hippocampi from three mice were pooled. Hippocampal mitochondria were then isolated using commercially available mitochondria isolation kit (product number: MITOISO1, Sigma, USA).

Half of the mitochondrial pellets isolated from blood or hippocampi of mice of each experimental subgroup were lysed in lysis buffer (50 nM Tris–HCl, 150 nM sodium chloride, 5 nM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl, 5 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin, pH 7.4) and used for determination of protein concentration and mitochondrial GSH content. The other half were resuspended in the storage buffer (10 mM HEPES, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, and 1 mM dithiothreitol, pH 7.4) for measurement of COX activity and mitochondrial ATP content.

The protein concentration was determined with the same DC protein assay kit (Bio-Rad, Hercules, CA, USA). Spectrophotometric data were obtained using the same Victor-2 Multilabel counter (EG&G Wallac, Gaithers- burg, Maryland, USA) at

a wavelength of 650 nm.

4.2.3 Determination of COX activity

COX activity was measured using the same cytochrome c oxidase assay kit (Sigma, USA) as previously described.

4.2.4 Measurement of mitochondrial ATP content by a HPLC-based method

Purine extraction from mitochondrial suspension was performed as previously described (Shi et al., 2008a). Briefly, ice-cold perchloric acid was added to 1 mg/ml mitochondrial suspension at the final concentration of 0.3 M. The mixture was then centrifuged at 15, 000 rpm for 5 min. The resulting supernatant was neutralized by adding an equal volume of saturated potassium hydroxide solution and then centrifuged at 15, 000 rpm for 5 min. ATP content was then measured by a high performance liquid chromatograph (Waters, Milford, MA, USA) as previously described.

4.2.5 Measurement of mitochondrial GSH content

Mitochondrial GSH was measured by using the same Glutathione Assay Kit (Sigma, USA) as previously described.

4.2.6 Statistical analysis

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). After the Kolmogovor-Smirnov test for normal distribution and Levene's test of Equal Variances for variance homogeneity, all data were submitted to one-way Univariate analysis of variance (ANOVA) followed by Least Significant Difference t test (LS.D.).

4.3 Results

4.3.1 Preventive and rescue effects of EGb761 on COX activity in platelets and hippocampi of SAMP8 mice

COX, or complex IV, is one of enzymes in the respiratory electron transport chain of mitochondria. In this work, COX activity was tested as an indicator of mitochondrial function. As shown in Fig. 4.1, with age, COX activity gradually decreased in both platelets and hippocampi of SAMP8 mice.

To investigate the preventive and rescue effects of EGb761 against decreased COX activity in SAMP8 mice, two groups of SAMP8 mice aged 3 weeks and 40 weeks, respectively, were given EGb761 at a daily dose of 100 mg/kg. After 12 weeks of EGb761 treatment, COX activity in platelets and hippocampi was tested. It was found that, 12 weeks of EGb761 treatment completely prevented the decrease of COX activity in platelet mitochondria in the young mice and rescued the decrease of COX activity in platelet mitochondria in the old mice (Fig. 4.1 A). In contrast, 12 weeks of EGb761 treatment rescued the decrease of COX activity in hippocampal mitochondria in the old mice, but failed to prevent the decrease of COX activity in hippocampal mitochondria in the young mice (Fig. 4.1 B).

4.3.2 Preventive and rescue effects of EGb761 on mitochondrial ATP content in platelets and hippocampi of SAMP8 mice

Mitochondrial ATP level is a direct indicator of mitochondrial function. In this work, mitochondrial ATP content was also tested. With age, mitochondrial ATP content also decreased gradually in both platelets and hippocampi of SAMP8 mice (Fig. 4.2). But compared with the decrease of COX activity, the decrease of mitochondrial ATP content was obviously delayed (Fig. 3.1 and 4.2). As shown in Fig. 4.2, no significant decrease of mitochondrial ATP content was detected until 40 weeks of age.

We simultaneously tested the protective effects of EGb761 against the decrease of mitochondrial ATP content in SAMP8 mice. We found that, 12 weeks of EGb761 treatment protected against the decrease of mitochondrial ATP content in both platelets and hippocampi of the old mice, but no obvious effect on mitochondrial ATP content was observed in both platelets and hippocampi of the young mice (Fig. 4.2).

4.3.3 Preventive and rescue effects of EGb761 on mitochondrial GSH content in platelets and hippocampi of SAMP8 mice

As mentioned above, mitochondrial dysfunction and mitochondrial oxidative stress may be cause and effect of each other (Miquel et al., 1980; Bossy-Wetzel et al., 2003). We consequently determined the mitochondrial GSH content in platelets and hippocampi of SAMP8 mice. With age, mitochondrial GSH content gradually decreased in both platelets and hippocampi of SAMP8 mice (Fig. 4.3). A 12-week

EGb761 treatment completely prevented the decrease of mitochondrial GSH content in platelets of the young mice and rescued the decrease of mitochondrial GSH content in platelets of the old mice (Fig. 4.3 A). In contrast, 12 weeks of EGb761 treatment rescued the decrease of mitochondrial GSH content in hippocampi of the old mice, but failed to prevent the decrease of mitochondrial GSH content in hippocampi of the young mice (Fig. 4.3 B).

4.4 Discussion

The SAMP strain of mice as a model for aging and aging-associated disorders has been widely researched. It has been demonstrated that, the early presence of high oxidative status and mitochondrial dysfunction is one of the conspicuous features of SAMP8 mice (Chiba et al., 2009). In one study, aging-associated changes in the oxidative status of the brain were investigated using SAMP8 and SAMR1 mice at 2, 4–8, 20–28, and 40–56 weeks of age (Sate et al., 1996; Takeda, 2009). It was found that, brain contents of lipid peroxide and protein carbonyl were transiently increased in 4 to 8-week-old SAMP8 mice (Sate et al., 1996; Takeda, 2009). Moreover, the net production of H_2O_2 in brain cells dissociated from SAMP8 mice at 4–8 weeks of age was also increased (Sate et al., 1996; Takeda, 2009). In addition, the activities of ROS-sensitive glutamine synthetase and catalase were decreased in the cerebral cortex of 4 to 8-week-old SAMP8 mice (Sate et al., 1996; Takeda, 2009). In contrast, acyl-CoA oxidase (microperoxysomal H_2O_2-producing enzyme) activity in the cerebral cortex of 4 to 8-week-old SAMP8 mice were found to be significantly increased, compared with age-matched SAMR1 mice (Sate et al., 1996; Takeda, 2009). In order to characterize the mitochondrial dysfunction in the early stage, Fujibayashi et al. investigated mitochondrial DNA deletions and respiratory chain

enzyme activities in the brain of SAMP8 mice at 4 and 8 weeks of age (Fujibayashi et al., 1998). Increased mitochondrial DNA deletion and decreased activities of mitochondrial complex I and III was detected in the brain of 4- and 8-week-old SAMP8 mice (Fujibayashi et al., 1998). In this work, age-related changes in mitochondrial function were further investigated using SAMP8 mice at 3, 15, 40 and 52 weeks of age. We found that, mitochondrial dysfunction and mitochondrial oxidative damage in platelets and hippocampi of SAMP8 mice tended to aggravate with age. These age-related changes in mitochondrial function observed in SAMP8 mice including the early decrease of COX activity and mitochondrial GSH content and the delayed reduction of mitochondrial ATP content support previous studies showing that decrease of the activity of a respiratory chain complex did not affect the rate of mitochondrial respiration or ATP synthesis until a critical value was reached (Letellier et al., 1994; Rossignol et al., 2003), and suggest a decreased functional reserve of mitochondria during aging. Therefore, a high oxidative status and mitochondrial dysfunction from a very early age, preceding the manifestation of aging, may be critical in inducing degenerative processes later in SAMP8 mice (Takeda, 2009) and an early protection against oxidative stress and mitochondrial dysfunction may help to delay aging and aging-associated degeneration.

EGb761 has long been considered a natural antioxidant (Smith and Luo, 2004). More recently, it has also been proposed to have direct protective effects on mitochondrial function (Tendi et al., 2002; Ahlemeyer and Krieglstein, 2003; Abdel-Kader et al., 2007). These properties of EGb761 make it a potential anti-aging agent. However, its "anti-aging" effect, particularly the effect on central nervous system, still remains elusive. In this work, 12 weeks of EGb761 treatment protected against the mitochondrial dysfunction in platelets of two age groups of SAMP8 mice (15-

week-old and 52-week-old), suggesting the peripheral effect of this herb in the prevention and treatment of age-associated degeneration. In contrast, in hippocampi, protective effects of EGb761 were observed only in the old mice, suggesting an age-associated central effect. Actually, similar central effect of EGb761 has been reported by previous studies. For example, in the study by Abdel-Kader et al. (2007), two age groups of NMRI mice (2–3-month-old and 15–16-month-old) were fed with EGb761 at 100 mg/kg/day for 14 days. EGb761 was found to be able to protect both the dissociated brain cells and isolated brain mitochondria from old mice against carbon monoxide-induced toxicity (Abdel-Kader et al., 2007). In contrast, in young mice, only a slight improvement in the mitochondrial membrane potential was observed after EGb761 treatment (Abdel-Kader et al., 2007). Moreover, EGb761 showed a significant protective effect on complexes I, IV and V in the brain of the old mice but no effect on the young mice was observed (Abdel-Kader et al., 2007).

In fact, many *in vivo* factors may affect the central effect of EGb761. A potential one is the permeability of BBB, as for the pharmacological active components of EGb761 to modulate central mechanisms, they have to cross the BBB and accumulate in the brain. BBB function in SAMP8 mice has been investigated by Ueno et al. using radiolabelled human serum albumin (125I-HSA) (Ueno et al., 1993; Takeda, 2009). The results showed that the brain transfer rates in 13-month-old SAMP8 mice and 22-month-old SAMR1 mice were significantly higher than in 3-month-old mice of the same strain, respectively (Ueno et al., 1993; Takeda, 2009). Another study done by Ueno et al. using a quantitative immunocytochemical procedure to detect endogenous albumin reported a larger percentage of capillaries and microvessels in the olfactory bulb in SAMP8 mice showing albumin leakage than in SAMR1 mice (Ueno et al., 1996; Takeda, 2009). Using horseradish

peroxidase, Ueno et al. also found an age-related increase in permeability of horseradish peroxidase in the hippocampus, thalamus and cerebral cortex of SAMP8 mice (Ueno et al., 1997; Takeda, 2009). Moreover, a recent study done by Pelegri et al. determined the extravasation of endogenous IgG from brain microvessels in SAMP8 mice aged 3, 7 and 12 months and in age-matched control SAMR1 mice and disclosed a higher level of IgG extravasation in the hippocampi of 12-month-old SAMP8 mice compared to SAMR1 mice (Pelegrı´ et al., 2007; Takeda, 2009). In addition, using Evans blue, Del valle et al. analyzed BBB permeability in three brain regions (cortex, hippocampus and hippocampal fissure) of SAMP8 mice at 3, 6, 9, 12 and 15 months of age and reported that BBB permeability in SAMP8 mice increased from 6 to 15 months in the three studied regions (Del Valle et al., 2009).

These results all indicate an increased permeability of BBB in aged SAMP8 mice. Similarly, BBB permeability increases during human aging (Farrall and Wardlaw, 2009). Furthermore, BBB permeability, as assessed by biochemical and imaging methods, significantly increases in neurodegenerative disorders such as AD as compared to normal aging subjects (Popescu et al., 2009). Under pathological conditions such as AD, EGb761 was suggested to be able to cross the BBB effectively and retain its neuroprotective properties (Kanowski et al., 1996; Garcia-Alloza et al., 2006), but there is a lack of evidence for the ability of EGb761 to cross the intact BBB under normal physiological conditions. In this study, EGb761 protected platelet mitochondria against mitochondrial dysfunction in both young and old mice but protective effects of EGb761 on hippocampus mitochondria were observed only in the old mice, suggesting that the central effect of EGb761 may be associated with age-associated increase of BBB permeability, i.e., EGb761's effects may mainly be limited to the outside of central nervous system in the presence of the

functionally intact BBB, but the permeability of BBB increase with age, which may facilitate EGb761 brain penetration, thus enhancing its central effects.

Taken together, using two age groups of SAMP8 mice (3-week-old and 40-week-old), this work investigated the preventive and rescue effects of EGb761 against aging-associated mitochondrial dysfunction in platelets and hippocampi. EGb761 protected against mitochondrial dysfunction in platelets of both young and old mice suggesting the peripheral effect of this herb in the prevention and treatment of age-associated degeneration. In contrast, in hippocampi, protective effects of EGb761 were observed only in the old mice, probably due to an age-associated increase in BBB permeability. Therefore, while EGb761 has potential anti-aging effect, its central effect can be affected by *in vivo* factors such as the BBB permeability.

5. Chapter V. EGb761 protects against mitochondrial dysfunction in ovariectomized rats

5.1 Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders of the central nervous system associated with progressive cognitive and memory loss. Molecular hallmarks of this disease are characterized by extracellular deposition of Aβ peptide in senile plaques, intracellular neurofibrillary tangles, basal forebrain cholinergic deficit, extensive neuronal loss and synaptic changes in brain regions critical for memory and cognition such as cortex and hippocampus (Nohl and Hegner, 1978; Chance et al., 1979; Henderson et al., 1994; Beal, 2000; Lemere et al., 2008; García-Ayllón et al., 2008; Dinamarca et al., 2008).

Epidemiological studies have shown that the incidence of female AD increases significantly after menopause by two to three times that of males, suggesting that estrogen withdrawal may be critical in inducing the onset of AD in post-menopausal women (Henderson et al., 1994). Estrogen has been suggested to be responsible for the lower oxidant generation and the higher antioxidant expression in mitochondria from females as compared with those from males of the same chronological age (Borrás et al., 2003; Viña et al., 2005; Borrás et al., 2007). This raises the possibility that estrogen withdrawal may trigger mitochondrial dysfunction. To test this possibility, we examined in the hippocampal CA1 region of 2-month-old Sparague-Dawley rats the mitochondrial ultrastructure, the ATP content and ATP synthesis at different phases after ovariectomy (Xu et al., 2008d; Shi et al., 2008a; Xu and Shi, 2008). Significantly, we observed a temporal alteration of mitochondrial

ultrastructure and a transient lowering of ATP content and ATP synthesis in mitochondria within 12 days after ovariectomy (Xu et al., 2008d; Shi et al., 2008a; Xu and Shi, 2008). These results suggest a mitochondrial dysfunction after estrogen withdrawal. To further determine whether aging could increase the vulnerability of the brain to estrogen withdrawal-induced mitochondrial dysfunction, we also measured the COX activity and mitochondrial ATP content in hippocampi of ovariectomized (OVX) Wistar rats of 2 and 9-months of age (Shi and Xu, 2008). Interestingly, 9-month-old OVX rats exhibited a prolonged lowering of COX activity and mitochondrial ATP content in hippocampi (>12 days) as compared with 2-month-old OVX rats (within 15 days) (Shi and Xu, 2008). These results suggest that, the functional reserve of mitochondria decreases with age, and estrogen withdrawal-triggered mitochondrial dysfunction may persistently present in the brain on the background of decreased functional reserve of mitochondria (Shi and Xu, 2008). Given that ovariectomy induces decrease in estrogen levels similar to that of menopause, these data further suggest that estrogen withdrawal and age-related decrease in the functional reserve of mitochondria may induce chronic mitochondrial dysfunction, which may be critical in inducing degenerative processes in the brain later in post-menopausal women.

EGb761 has now been widely used for geriatric memory disorders including neurodegenerative dementia. Several clinical trials have suggested the efficacy of EGb761 in the treatment of mild-to-moderate dementia of different etiology, but negative data do also exist (Kanowski et al., 1996; Le Bars et al., 1997; Le Bars et al., 2000; Solomon et al., 2002; Schneider et al., 2005; Napryeyenko et al., 2007; Abdel-Kader et al., 2007; DeKosky et al., 2008). Beneficial effects of EGb761 on these diseases were assumed to be due to its free radical scavenging properties (Pietri et al.,

1997; Pierre et al., 1999; Wei et al., 2000; Wu et al., 2006) but more recently it is also proposed that EGb761 has direct protective effects on the mitochondria. Using PC12 cells and dissociated mice brain cells, as well as isolated mitochondria, Abdel-Kader et al. (2007) demonstrated that, EGb761 could protect mitochondrial function against external insults (oxidative stress, nitrosative stress, serum deprivation and complexes inhibitors) at concentrations as low as 0.01 mg/ml. Moreover, EGb761 was found to be able to prevent anoxia/reoxygenation-induced decrease of ADP phosphorylation versus oxygen consumption in mitochondria isolated from rat brain and rat liver, and reverse the decrease of GSH/GSSG (oxidized form of GSH) ratio in rat mitochondria during aging (Du and Li, 1998; Ahlemeyer and Krieglstein, 2003). Recently, EGb761 and bilobalide have also been shown to up-regulate NADH dehydrogenase messenger RNA level, whose increase is indicative of the enhancement of mitochondrial antioxidant capacity (Tendi et al., 2002).

Following these points, in this study, ovariectomized middle-aged rats were used to mimic the post-menopausal pathophysiological changes in women, and effects of EGb761 on estrogen withdrawal-induced mitochondrial dysfunction were investigated. As mentioned above, comparison of *in vivo* effects of EGb761 on mitochondrial function between platelets and hippocampi may help to speculate the ability of EGb761 to cross the BBB. In this study, mitochondrial effects of EGb761 in both platelets and hippocampi of OVX rats were tested.

5.2 Methods

5.2.1 Experimental animals and drug administration

Forty female Wistar rats aged 9 months were provided by the animal facility of Sun-Yat Sen University. Before experiments, they were kept under defined environmental conditions (25□, 50% relative humidity, 12-h light-dark cycle) with free access to water and food. Rats were divided into four groups, i.e., sham-operated, OVX, EGb761-only and EGb761-OVX groups (n=10). For both OVX and EGb761-OVX groups, the bilateral ovariectomy was performed as previously described (Shi et al., 2008a) (Fig. 5.1). For both sham-operated and EGb761-only groups, the sham operation was performed as previously described (Xu et al., 2007e). Briefly, about 1 g of adipose tissue around the ovaries was removed from the rats. Rats in both EGb761-only and EGb761-OVX groups were given EGb761 in 4% surcose from the second day after operation at a daily dose of 100 mg/kg body weight. Rats in the sham-operated and OVX groups were fed with 4% sucrose instead.

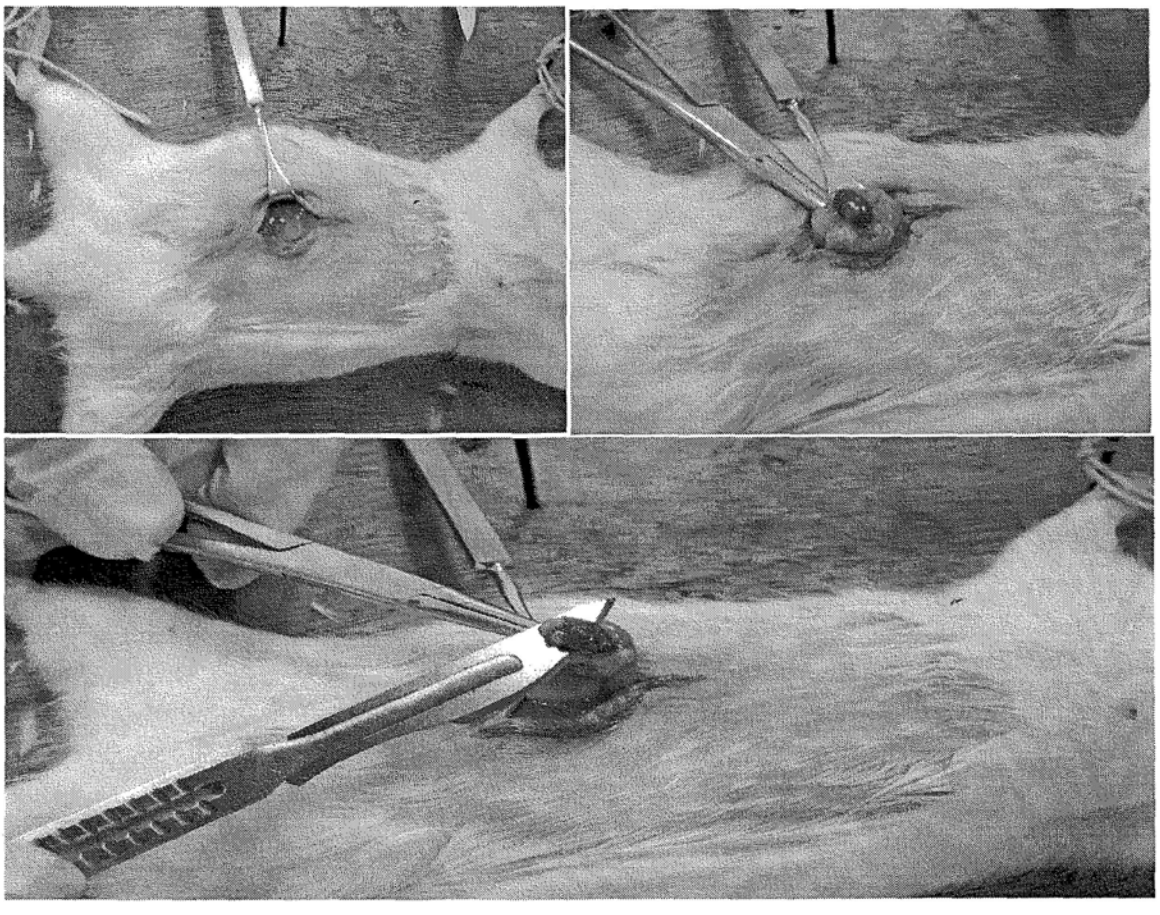

Figure 5.1 Ovariectomy was performed as previously described (Shi et al., 2008a; Xu et al., 2007e). Rats were anesthetized by intraperitoneal injection of 10 g /L sodium pentobarbital. For ovarietomy, the rats were fixed in a pronated position and an incision of 2–3 cm in the lumbar vertebral region along the midline of the back was made. Subsequently, psoas muscles under the ribs along the scapular line were opened bilaterally. The ovaries and cornua uteri were isolated and removed, followed by closure of the incisions of skin and muscles.

5.2.2 Isolation of mitochondria from platelets and hippocampi

Isolation of mitochondria from platelets and hippocampi and measurement of protein concentration were performed as previously described.

5.2.3 Determination of COX activity

COX activity was measured using the same cytochrome c oxidase assay kit (Sigma, USA) as previously decribed.

5.2.4 Measurement of mitochondrial ATP content by a HPLC-based method

Purine extraction from mitochondrial suspension and measurement of mitochondrial ATP content were performed as previously described.

5.2.5 Mitochondrial GSH content

Mitochondrial GSH was measured by using the same Glutathione Assay Kit (Sigma, USA) as previously descrived.

5.2.6 Statistical analysis

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). After the Kolmogovor-Smirnov test for normal distribution and Levene's test of Equal Variances for variance homogeneity, all data were submitted to one-way Univariate analysis of variance (ANOVA) followed by the Student-Newman-Keuls (S-N-K) test.

5.3 Results

5.3.1 Protective effects of EGb761 on COX activity in platelets and hippocampi of ovariectomized and sham-operated rats

COX, or complex IV, is the last enzyme in mitochondrial respiratory electron transport chain. In this work, COX activity was tested as an indicator of mitochondrial function. As shown in Fig. 5.2, after ovariectomy, COX activity in both platelets and hippocampi were significantly decreased.

To investigate the protective effect of EGb761 on COX activity in both ovariectomized and sham-operated rats, rats were given EGb761 at a daily dose of 100 mg/kg. After 18 days of EGb761 treatment, COX activity in both platelets and hippocampi were tested. An 18-day of EGb761 treatment completely reversed the decrease of COX activity in both platelets and hippocampi of OVX rats (Fig. 5.2). In contrast, in either hippocampi or platelets of sham-operated rats, no obvious effect of EGb761 on COX activity was observed (Fig. 5.2).

5.3.2 Protective effects of EGb761 on mitochondrial ATP content in platelets and hippocampi of ovariectomized and sham-operated rats

Mitochondrial ATP content directly reflects mitochondrial function. In this work, mitochondrial ATP content was also tested. After ovariectomy, mitochondrial ATP content in both platelets and hippocampi were decreased (Fig. 5.3).

We simultaneously tested the protective effects of EGb761 on mitochondrial ATP content in both ovariectomized and sham-operated rats. We found that, 18 days of EGb761 treatment could completely prevent the decrease of mitochondrial ATP content in both platelets and hippocampi of OVX rats (Fig. 5.3). By contrast, no obvious effect of EGb761 on mitochondrial ATP content was observed in either hippocampi or platelets of sham-operated rats (Fig. 5.3).

5.3.3 Protective effects of EGb761 on mitochondrial GSH content in platelets and hippocampi of ovariectomized and sham-operated rats

Mitochondrial dysfunction and mitochondrial oxidative damage are cause and effect of each other (Tendi et al., 2002). We consequently determined the mitochondrial GSH content in platelets and hippocampi of OVX rats. We found that, after ovariectomy, mitochondrial GSH content in both platelets and hippocampi were decreased (Fig. 5.4). A 12-week EGb761 treatment was able to reverse the decrease of mitochondrial GSH content in both platelets and hippocampi of OVX rats (Fig. 5.4). In contrast, treatment of sham-operated rats with EGb761 for 18 days had no clear effect on mitochondrial GSH content in hippocampi but increased mitochondrial GSH content in platelets (Fig. 5.4).

5.4 Discussion

It is known that, the effects of ovariectomy on female Sprague-Dawley rats are manifested by progressive learning and memory deficits, central cholinergic neural system degeneration as well as differentiation/apoptosis imbalance (Feng et al., 2004). Therefore, the ovariectomized rats have been widely used used to mimic the post-menopausal pathophysiological changes in women. Using the ovariectomized rats, we have previously demonstrated that, estrogen withdrawal through ovariectomy can trigger mitochondrial dysfunction, similar to aging, which may aggravate this mitochondrial dysfunction (Xu et al., 2008d; Shi et al., 2008a; 2008b; Shi and Xu, 2008). In support of this finding, we demonstrated in this study that, mitochondrial function, evaluated as COX activity, mitochondrial ATP content and mitochondrial GSH content, was decreased in both platelets and hippocampi of middle-aged (9-month-old) OVX rats. As mentioned above, estrogen is suggested to be responsible for the lower oxidant generation and the higher antioxidant expression in mitochondria from females as compared with those from males of the same chronological age (Borrás et al., 2003; Viña et al., 2005; Borrás et al., 2007). Mitochondrial dysfunction in middle-aged OVX rats may be a result of estrogen withdrawal-induced oxidative damage and aging-related decrease in the functional reserve of mitochondria.

The standardized *Ginkgo biloba* extract EGb761 has long been considered a natural antioxidant. The proposal that the beneficial effects of EGb761 are due to its free-radical scavenging action is supported by numerous *in vitro* and *in vivo* studies (Pierre et al., 1999; Pietri et al., 1997: Wei et al., 2000; Wu et al., 2006). Recently, EGb761 has also been demonstrated to have direct protective effects on the mitochondria (Du and Li, 1998; Tendi et al., 2002; Ahlemeyer and Krieglstein, 2003; Abdel-Kader et al.. 2007;). These effects of EGb761 make it a potential protective

agent against estrogen withdrawal-associated degeneration in post-menopausal women. In this work, EGb761 effectively reversed the decrease of COX activity, mitochondrial ATP content and mitochondrial GSH content in both platelets and hippocampi of ovariectomized middle-aged rats, suggesting its peripheral and central effects against estrogen withdrawal-associated degeneration. In contrast, in sham-operated middle-aged rats, EGb761 increased mitochondrial GSH content in platelets but failed to show similar effect on hippocampi, suggesting that EGb761 may help to enhance the functional reserve of mitochondria, but this effect was limited to the outside of the central nervous system.

As mentioned above, the permeability of BBB may be one of the factors affecting the central effect of EGb761. Despite evidences suggesting that EGb761 was able to cross the BBB effectively under pathological conditions such as AD (Kanowski et al., 1996; Garcia-Alloza et al., 2006), there is a lack of evidence for the ability of EGb761 to cross the intact BBB under normal physiological conditions. In this study, the protective effect of EGb761 was observed only in platelets of sham-operated rats, suggesting that EGb761's effects may mainly be limited to the outside of central nervous system under normal physiological conditions. If so, the application of EGb761 in the prevention of age-related neurodegenerative diseases such as AD may be limited.

However, estrogen withdrawal through ovariectomy may alter the selective permeability of the BBB. In the study by Wilson et al. (2008), BBB permeability in OVX rats was investigated by using Evan's blue dye. They found that, while the selective permeability of the BBB normally constricts the passage of molecules $\geq$ 100 Da, ovariectomy increased the permeability of the BBB, allowing Evan's blue dye with molecular weight $\geq$ 961 Da to cross the BBB, possibly via changes in junctional

proteins. In another study done by Cipolla et al. (2009) using Lucifer Yellow, a marker of transcellular transport, BBB permeability increased 5-fold in OVX animals. These findings suggest an increased BBB permeability following estrogen withdrawal, and also raise the possibility that estrogen withdrawal-induced increase in BBB permeability may enhance the central effect of EGb761. In support of this possibility, Takuma et al. (2007) reported that, EGb761 was able to attenuate hippocampal neuronal loss and cognitive dysfunction induced by chronic restraint stress in OVX rats. In this study, EGb761 displayed similar protective effects on platelets and hippocampi of OVX rats. Therefore, while the central effect of EGb761 may be affected by the selective permeability of the BBB under normal physiological conditions, EGb761 may have beneficial effects in the prevention and treatment of central neurodegeneration in post-menopausal women.

Taken together, in this work, the protective effects of EGb761 on mitochondrial function in platelets and hippocampi of sham-operated and OVX rats were investigated. EGb761 protected against mitochondrial dysfunction in platelets and hippocampi of OVX rats, suggesting its peripheral and central effects against estrogen withdrawal-induced degeneration. In contrast, in sham-operated rats, EGb761 increased mitochondrial GSH content in platelets but failed to show similar effect on hippocampi, suggesting that EGb761 may help to enhance the functional reserve of mitochondria, but this effect was limited to the outside of the central nervous system. EGb761 displayed similar effects on platelets and hippocampi of OVX rats but showed differential effects on platelets and hippocampi of sham-operated rats, possibly because estrogen withdrawal induced an increase in BBB permeability. Therefore, while the effect of EGb761 may be limited to the outside of the nervous system under normal physiological conditions, EGb761 may be a

potential protective agent against central neurodegeneration in post-menopausal women.

6. Chapter VI. General discussion

A substantial body of preclinical evidence has suggested the neuroprotective effects of EGb761. Despite these evidences, its effectiveness in the treatment and prevention of neurodegenerative diseases such as AD is still inconclusive. Moreover, the precise neuroprotective mechanisms are not clearly known. We suggested in this study that the neuroprotective effects of EGb761 may be associated with constituent multiplicity, the dosage and BBB permeability.

EGb761 consists of two major groups of substances, the flavone glycosides and the terpene lactones. Understanding of the actions of individual EGb761 components may provide insights into direct mechanisms underlying the neuroprotective effects of EGb761. Using SH-SY5Y cells, the present study demonstrated that, EGb761 blocked Aβ-induced cell apoptosis and abrogated Aβ-induced toxic events e.g. ROS accumulation, mitochondrial dysfunction and activation of JNK, ERK1/2 and Akt signaling pathways, possibly through its antioxidant and PAF antagonist activities. Both quercetin and ginkgolide B were involved in the protective effects of EGb761. Quercetin but not ginkgolide B might be responsible for the antioxidant action of EGb761. Both compounds might be involved in the PAF antagonist activity of EGb761. These results suggested that the constituent multiplicity of EGb761 might contribute to its polyvalent properties. To further confirm this proposal, in future studies, we may test the effects of EGb761 and its consitutents on neurotoxicity induced by phosphorylated tau protein, another AD-associated pathological change in the brain.

The effective concentration of EGb761 in the brain remains undetermined. Using SH-SY5Y cells, this study demonstrated that, EGb761 had dosage effects on

H_2O_2-induced cell apoptosis, which might be correlated with regulation of cell redox state. Moreover, the protective effects of EGb761 were observed at lower concentrations (50-100 μg/ml). In support of these findings, previous studies have demonstrated similar dosage effects of quercetin and ginkgolide B (Spencer et al., 2003; Chan and Hsuuw, 2007). In addition, most of the protective effects of EGb761 observed *in vitro* were reported at lower concentrations (≤ 200 μg/mL) (Ahlemeyer et al., 1996; Wei et al., 2000; Xin et al., 2000; Bastianetto et al., 2000; Rimbach et al., 2001; Tendi et al., 2002; Luo et al., 2002; Yao et al., 2004; Shi et al., 2009). Therefore, only within a certain range of dosage, EGb761 showed protective effects.

For modulation of central mechanisms, the pharmacologically active components of EGb761 have to cross the BBB and accumulate in the brain to reach the effective concentration. In the present study, the ability of EGb761 to cross the BBB was speculated by comparing the effects of EGb761 on mitochondrial function between platelets and hippocampi in two animal models, SAMP8 mice and OVX rats. Interestingly, EGb761 protected against mitochondrial dysfunction in platelets and hippocampi of old SAMP8 mice but only showed protective effects on platelet mitochondria of young SAMP8 mice. Similarly, EGb761 displayed similar protective effects on mitochondrial function in platelets and hippocampi of OVX rats but showed differential effects on platelets and hippocampi of sham-operated rats. These data suggested that the effects of EGb761 on the brain might be interfered by the BBB permeability. To validate this possibility, in future studies, we may simutaneously test the BBB permeability and brain concentrations of the effective consitituents of EGb761 in animal models.

Overall, these results suggested that EGb761 might have beneficial effects in prevention and treatment of neurodegenerative diseases such as AD. Both flavonoid

and terpenoid constituents of EGb761 might contribute to its beneficial effects. Dosage and BBB permeability might be two important factors that interfere with the *in vivo* effect of EGb761. Following these points, identification of the effective constituents, improvement of the ability to cross the BBB and control of the brain concentrations may contribute to the neuroprotective effects of EGb761 in future medical practice.

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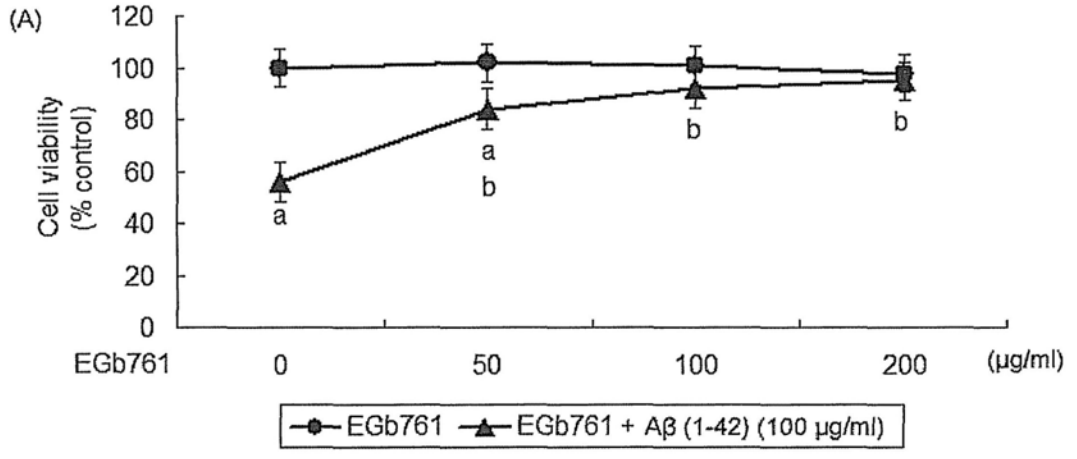
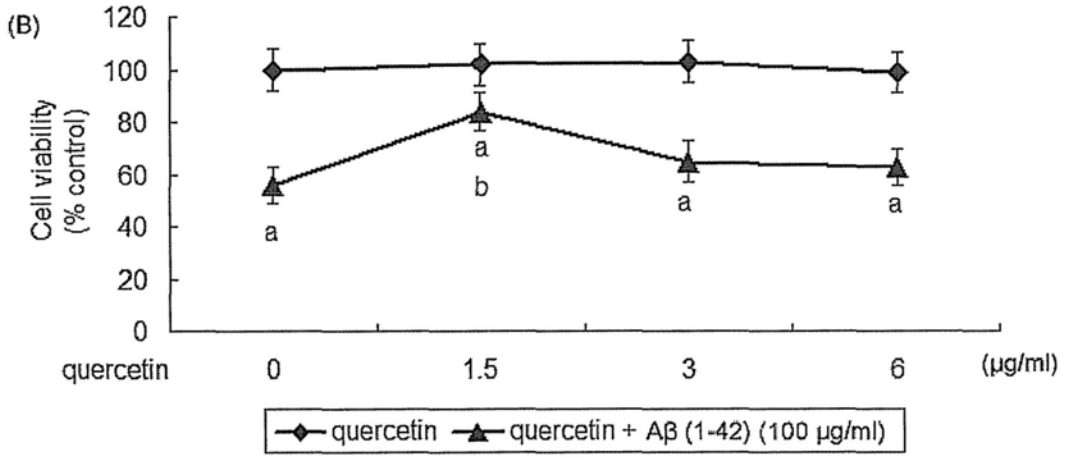
Figures


(A)
Cell viability (% control)
120
100
80
60
40
20
0
a
a
b
b
b
EGb761
0
50
100
200
(μg/ml)
EGb761
EGb761 + Aβ (1-42) (100 μg/ml)


(B)
Cell viability (% control)
120
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quercetin
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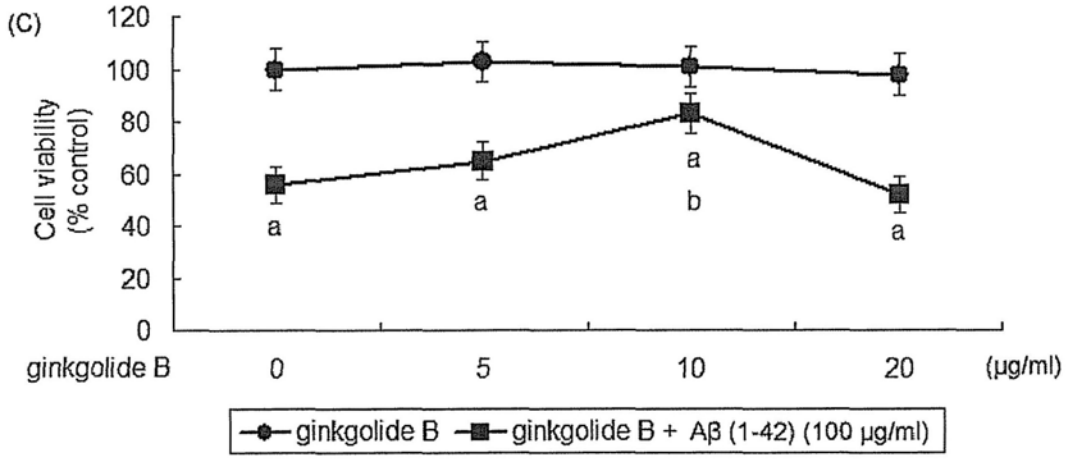
(μg/ml)
quercetin
quercetin + Aβ (1-42) (100 μg/ml)


(C)
Cell viability (% control)
120
100
80
60
40
20
0
a
a
a
b
a
ginkgolide B
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5
10
20
(μg/ml)
ginkgolide B
ginkgolide B + Aβ (1-42) (100 μg/ml)

Figure 2.2 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced decrease of cell viability in SH-SY5Y cells. Cells were pre-treated with various concentrations of EGb761, or quercetin, or ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. Cell viability was then determined by MTT assay. (A) Cells treated with Aβ 1-42 and/or various concentrations of EGb761. (B) Cells treated with Aβ 1-42 and/or various concentrations of quercetin. (C) Cells treated with Aβ 1-42 and/or various concentrations of ginkgolide B. Values are presented as mean ± S.D. of eight determinations. [a]: Compared with corresponding drug-treated cells (Aβ-free), $p<0.01$; [b]: Compared with Aβ 1-42-treated cells (drug-free), $p<0.01$.

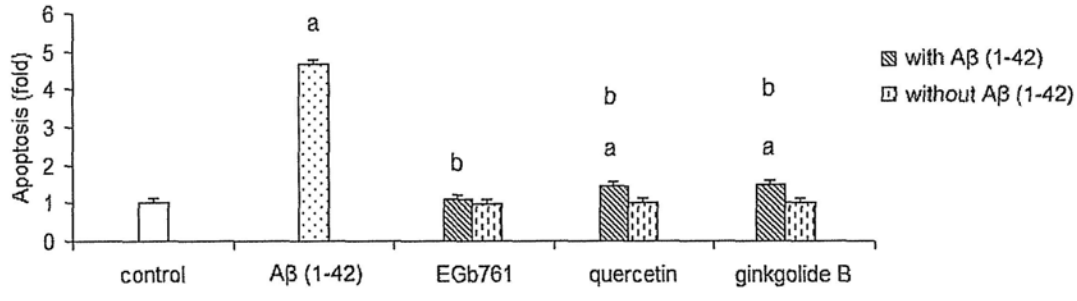


Figure 2.3 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced cell apoptosis in SH-SY5Y cells. Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. Cell apoptosis was then evaluated using the Cell Death Detection ELISA Kit. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$. (Note: Trypan blue exclusion assay suggested that the percentage of cell death in normal control cells was 9-12%).

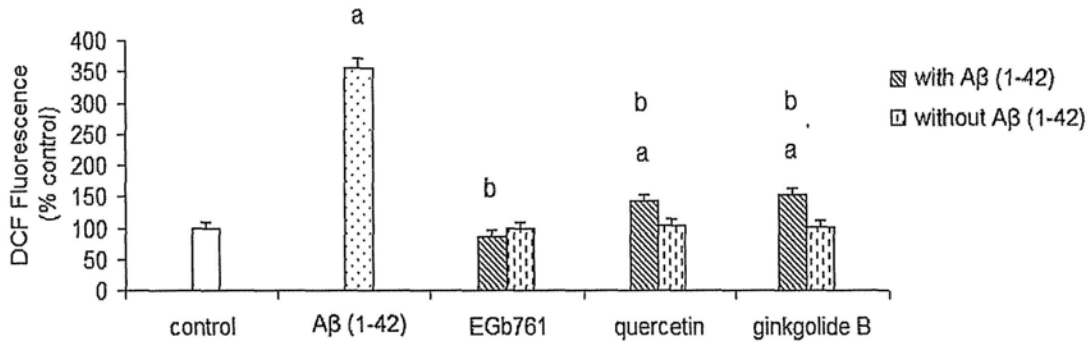


Figure 2.4 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced ROS generation in SH-SY5Y cells. Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. ROS generation was then assayed using DCF-DA. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.

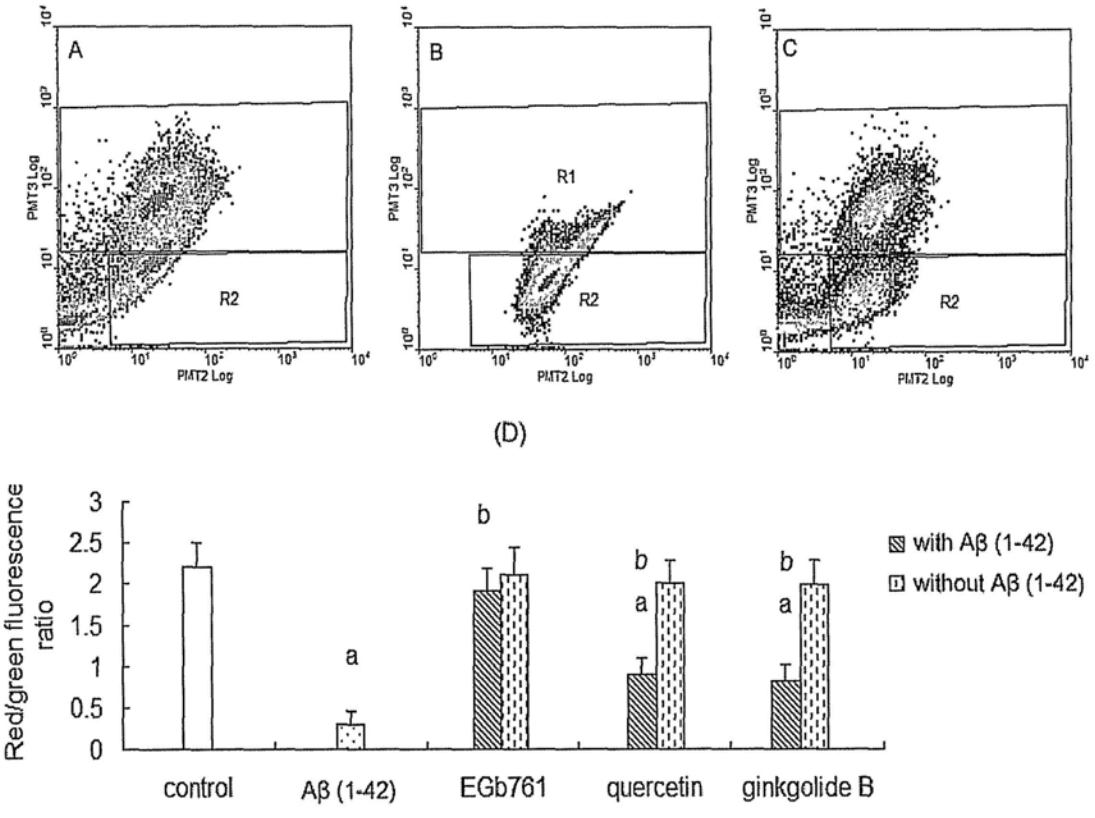


Figure 2.5 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced mitochondrial membrane potential depolarization in SH-SY5Y cells.Representative flow cytometry dot plots showing JC-1 staining of SH-SY5Y cells. Changes in the mitochondrial membrane potential were determined with the lipophilic dye JC-1.

Binding to mitochondria was detected by a shift in fluorescence from green, which is characteristic of its monomeric form, to red, which reflected florescent aggregation in mitochondria. Therefore, cells stained with JC-1 exhibited a heterogeneous distribution of polarized (red fluorescing JC-1 aggreregions, as shown in the R1 region) and depolarized (green fluorescing monomers, as shown in the R2 region) mitochondria. It could be seen that, compared with the pattern of untreated SH-SY5Y cells (A), after exposure of cells to Aβ (1–42) for 24h, fluorescence in R1 region (PMT3, red direction) decreased with a corresponding increase in R2 region (PMT2, green direction) fluorescent intensity (B), which indicated a compromise of mitochondria. This change could be reversed by 100 μg/ml EGb761 (C). (D) Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. Mitochondrial membrane potential was then determined with JC-1 staining. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.

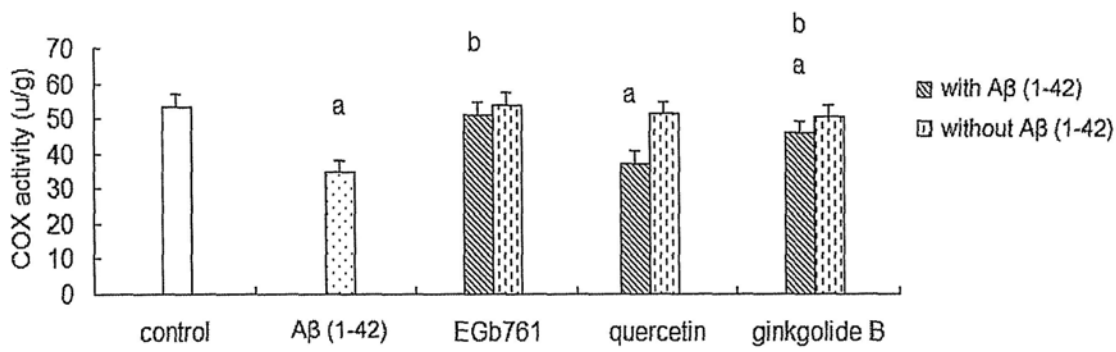


Figure 2.6 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced COX activity reduction in SH-SY5Y cells. Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. COX activity was then determined using cytochrome c oxidase assay kit. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.

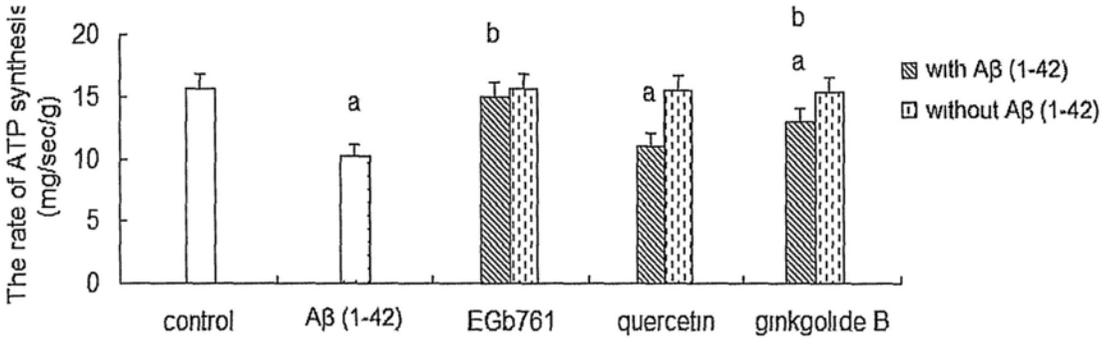


Figure 2.7 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced decrease of the rate of ATP synthesis in SH-SY5Y cells. Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. The rate of ATP synthesis was then determined using a HPLC-based method. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.

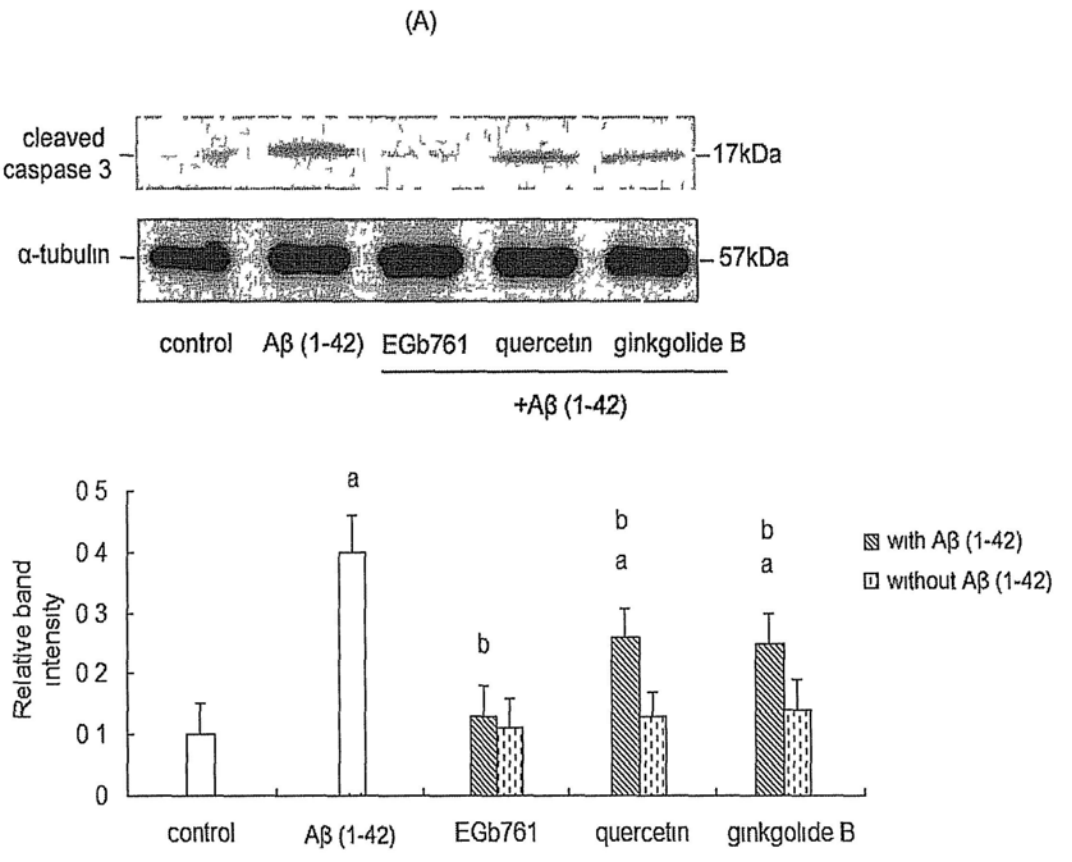
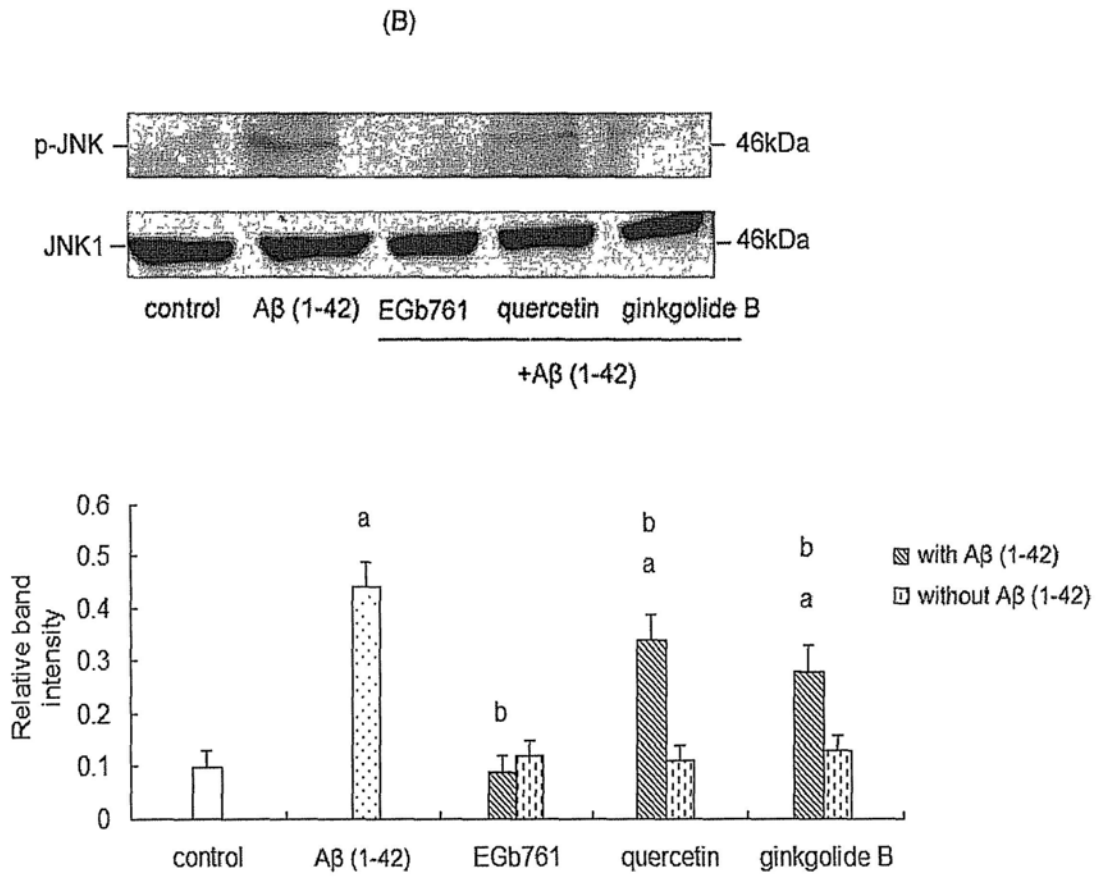
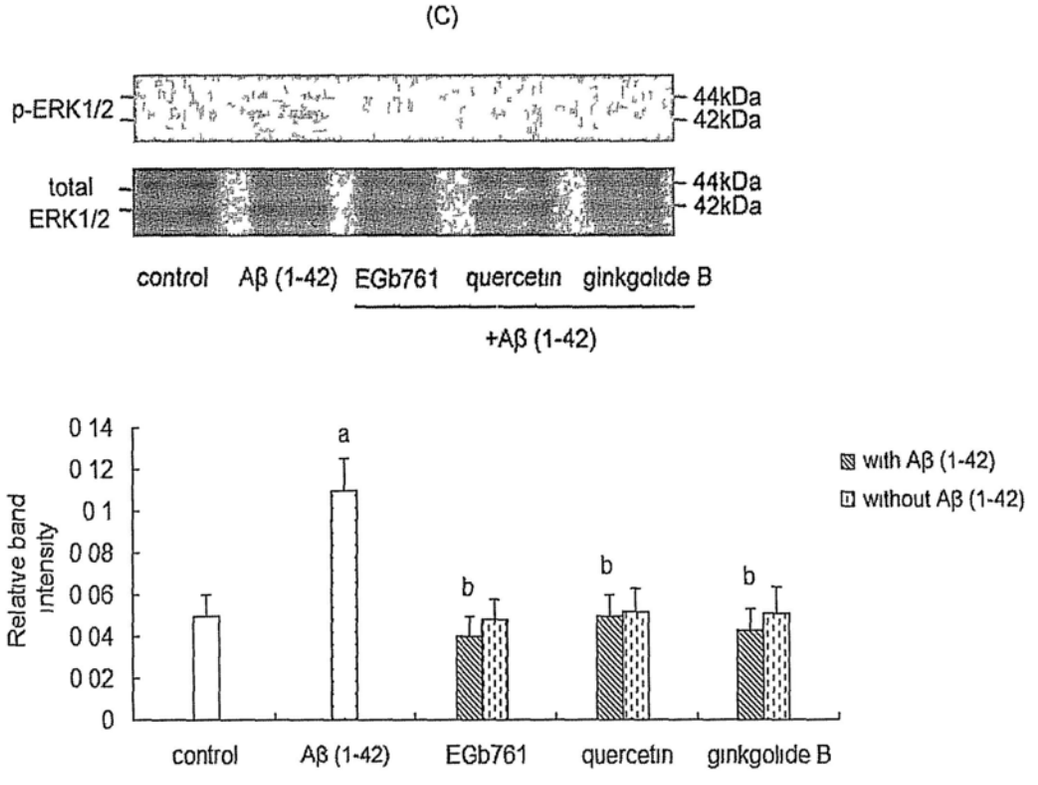


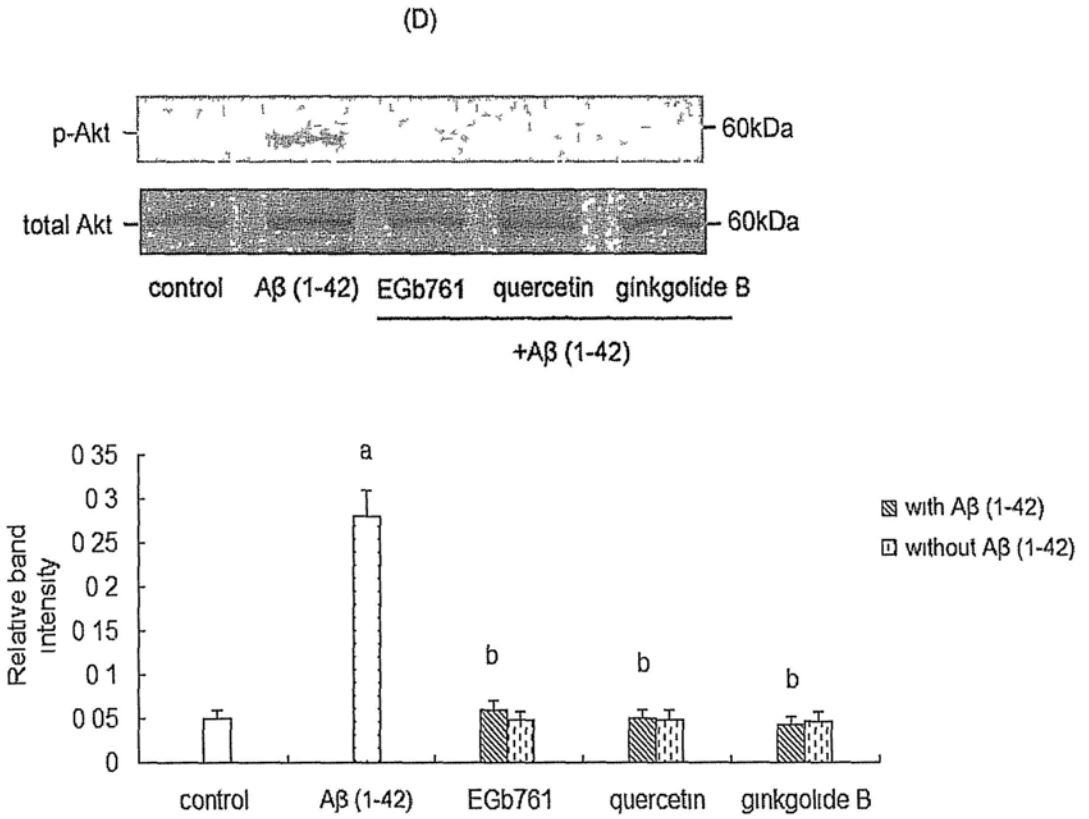
Figure 2.8 Effects of EGb761, quercetin and ginkgolide B on Aβ 1-42-induced activation of JNK, ERK1/2, Akt and caspase 3 pathways in SH-SY5Y cells. (A) Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 24 h. Cell lysates (20 μg) were analyzed for activation of caspase 3 by Western blotting using a cleaved caspase 3 antibody. The same crude lysates (20 μg) were also analyzed on a separate blot using an antibody that recognizes the α-tubulin. Protein bands representing cleaved caspase 3 were quantified using the Odyssey software. The relative band densities for cleaved caspase 3 were determined by normalizing against α-tubulin band intensity. Values for cells in different treatments were presented as mean ± S.D. of four determinations in a bar chart. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$



(B) Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 2 h. Cell lysates (20 μg) were analyzed for activation of JNK by Western blotting using a phospho-specific JNK (Thr183/Tyr185) antibody. The same crude lysates (20 μg) were also analyzed on a separate blot using an antibody that recognizes total JNK1. Protein bands representing phospho-JNK and total JNK1 were quantified using the Odyssey software. The relative ratios of phospho-JNK to total JNK1 band density for cells in different treatments were presented as mean ± S.D. of four determinations in a bar chart. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.



(C) Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 1 h. Cell lysates (20 μg) were analyzed for activation of ERK1/2 by Western blotting using a phospho-specific ERK1/2 (Thr202/Tyr204) antibody. The same crude lysates (20 μg) were also analyzed on a separate blot using an antibody that recognizes total ERK1/2. Protein bands representing phospho-ERK1/2 and total ERK1/2 were quantified using the Odyssey software. The relative ratios of phospho-ERK1/2 to total ERK1/2 band density for cells in different treatments were presented as mean ± S.D. of four determinations in a bar chart. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.



(D) Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 100 μg/ml Aβ 1-42 and EGb761, or quercetin, or ginkgolide B for another 1 h. Cell lysates (20 μg) were analyzed for activation of Akt by Western blotting using a phospho-specific Akt (Thr308) antibody. The same crude lysates (20 μg) were also analyzed on a separate blot using an antibody that recognizes total Akt. Protein bands representing phospho-Akt and total Akt were quantified using the Odyssey software. The relative ratios of phospho-Akt to total Akt band density for cells in different treatments were presented as mean ± S.D. of four determinations in a bar chart. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h Aβ 1-42-treated cells, $p<0.01$.

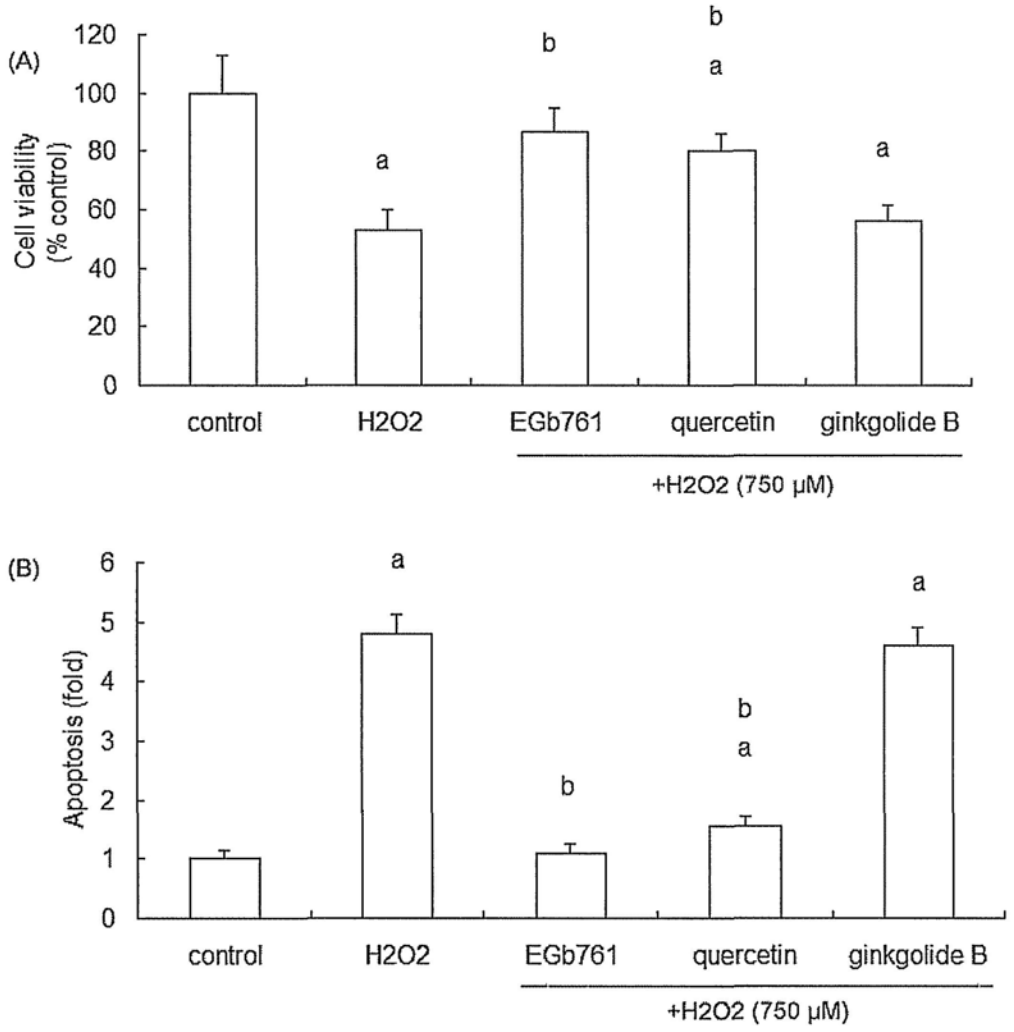


Figure 2.9 Effects of EGb761, quercetin and ginkgolide B on H_2O_2-induced cell apoptosis. Cells were pre-treated with various concentrations of EGb761, or quercetin, or ginkgolide B for 2 h and then exposed to the medium containing 750 μM H_2O_2 and EGb761, or quercetin, or ginkgolide B for another 24 h. Cell viability and cell apoptosis were then determined by MTT assay (A) and TUNEL apoptosis assay (B) respectively. Values are presented as mean±SD of five determinations. [a]: compared with control cells, $p<0.01$; [b]: compared with 24 h H_2O_2-treated cells, $p<0.01$.

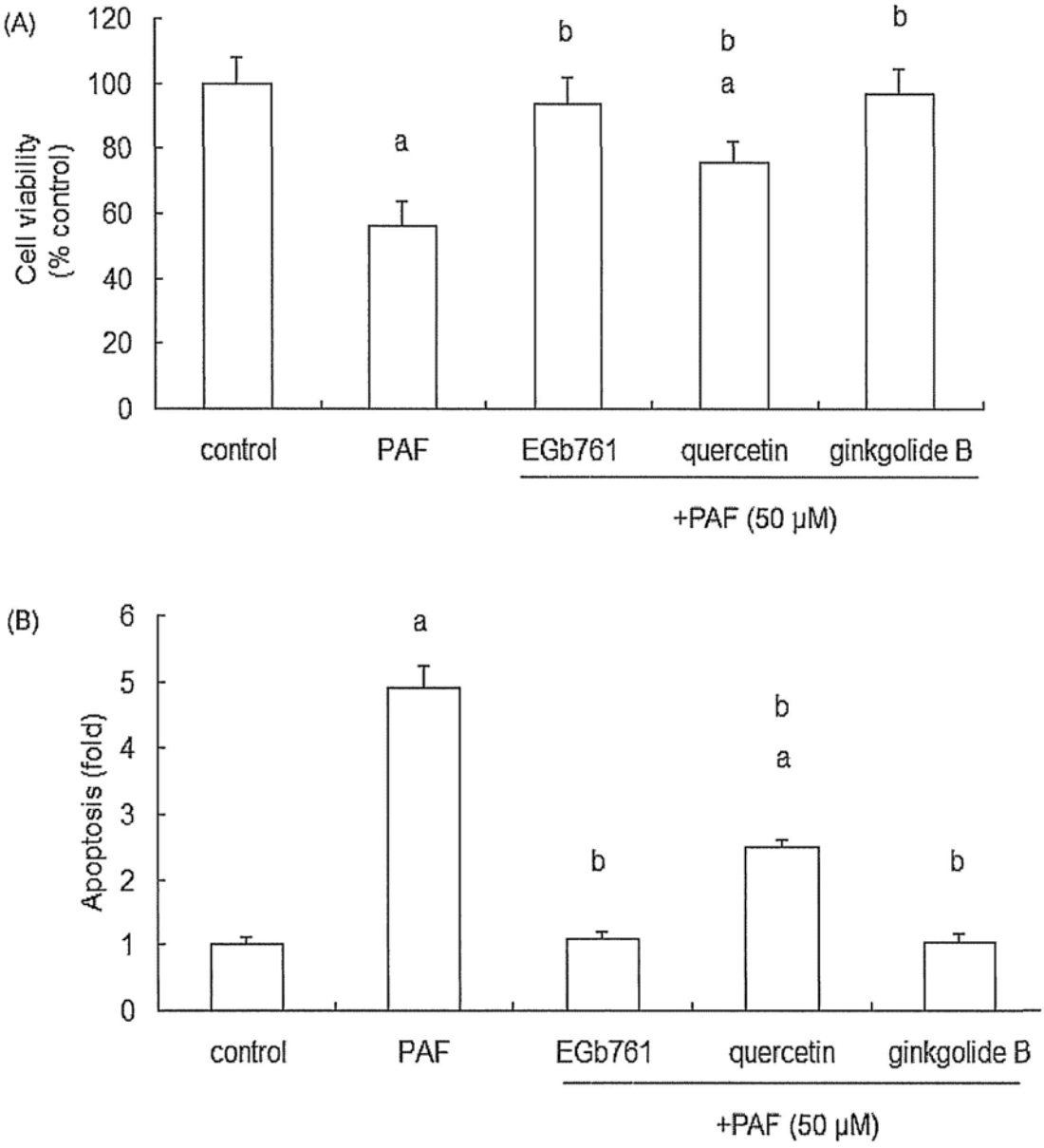


Figure 2.10 Effects of EGb761, quercetin and ginkgolide B on PAF-induced cell death. Cells were pre-treated with 100 μg/ml EGb761, or 1.5 μg/ml quercetin, or 10 μg/ml ginkgolide B for 2 h and then exposed to the medium containing 50 μM PAF and EGb761, or quercetin, or ginkgolide B for another 24 h. Cell viability and cell apoptosis were then determined by MTT assay (A) and TUNEL apoptosis assay (B), respectively. Values are presented as mean ± S.D. of five determinations. [a]: Compared with control cells, $p<0.01$; [b]: Compared with 24 h PAF-treated cells, $p<0.01$.

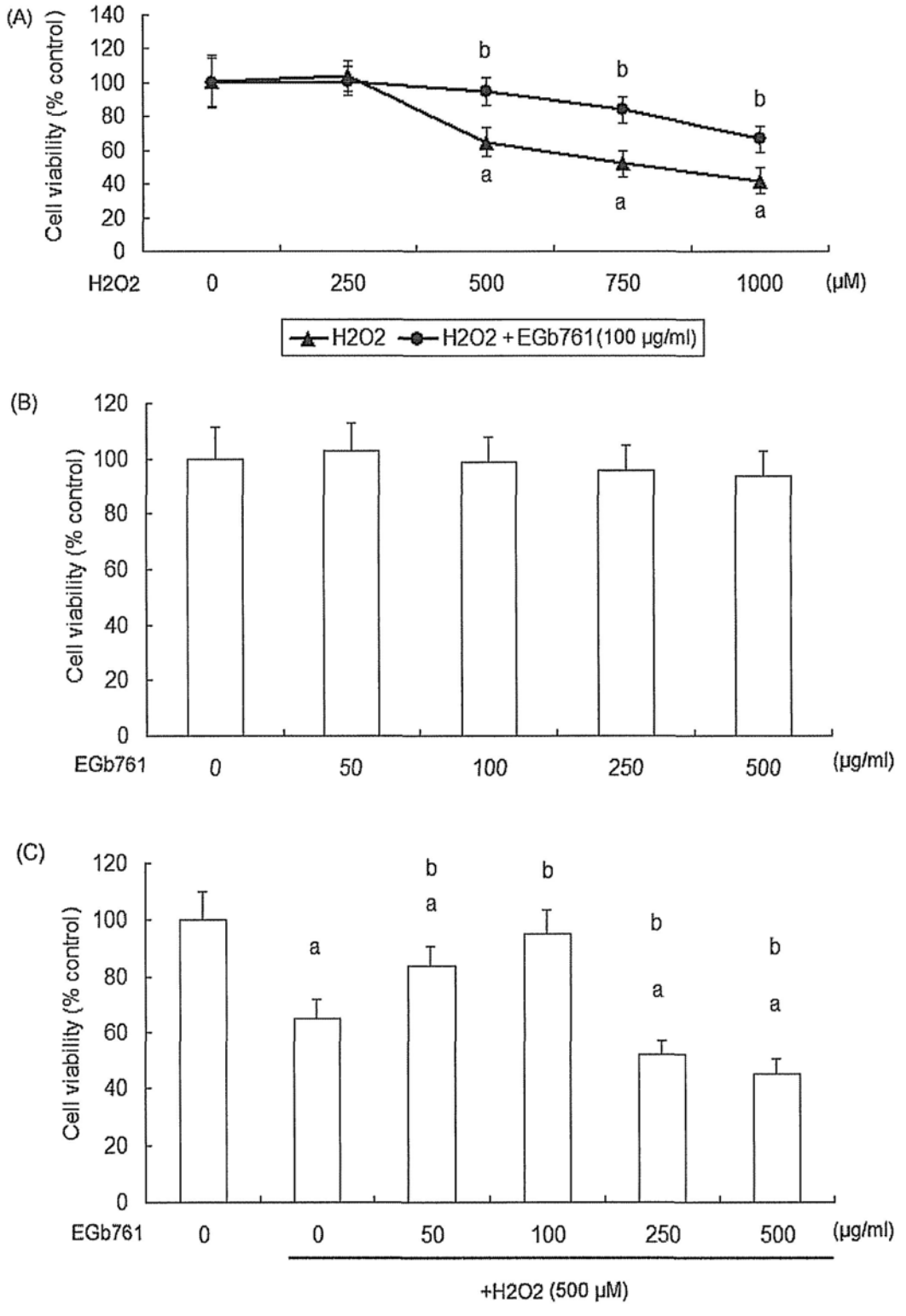


Figure 3.1 Dosage effects of EGb761 on cell viability of H_2O_2-treated SH-SY5Y cells. *(A)* Cells were incubated with or without 100 μg/ml EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged

with various concentrations of H_2O_2 for another 24 h. Cell viability was determined by MTT assay. Values are presented as mean ± S.D. of six determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus corresponding H_2O_2-treated group (EGb761-free). (B) Cells were incubated with various doses of EGb761 alone for 24 h. Cell viability was determined by MTT assay. Values are presented as mean ± S.D. of five determinations. (C) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 24 h. Cell viability was determined by MTT assay. Values are presented as mean ± S.D. of six determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus the H_2O_2-treated group (EGb761-free).

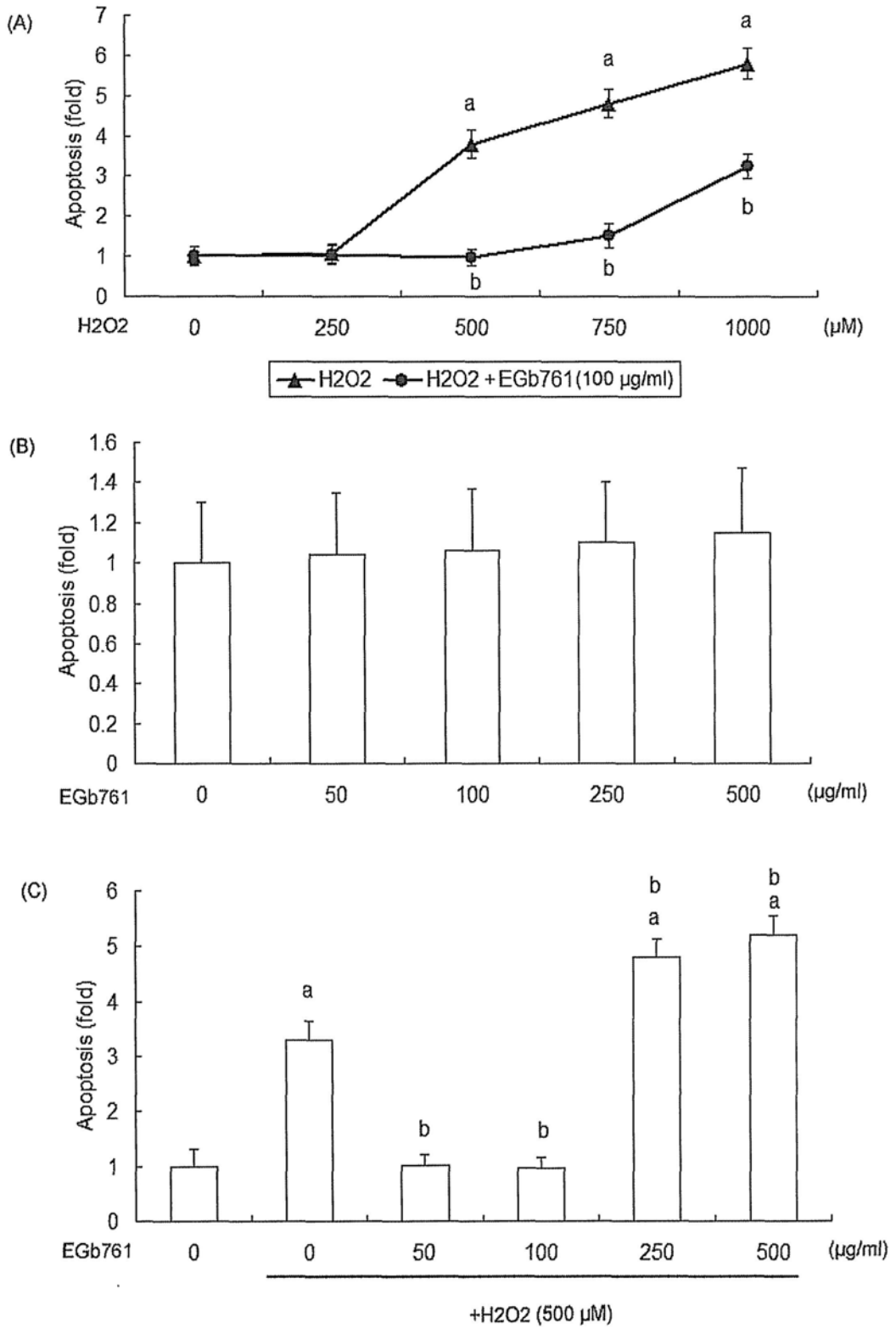


Figure 3.2 Dosage effects of EGb761 on H_2O_2-induced apoptosis in SH-SY5Y cells. *(A)* Cells were incubated with or without 100 μg/ml EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with various concentrations of H_2O_2 for another 24 h. Apoptosis was evaluated using the

Cell Death Detection ELISA Kit. Values are presented as mean ± S.D. of five determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus corresponding H_2O_2-treated group (EGb761-free). (B) Cells were incubated with various doses of EGb761 alone for 24 h. Apoptosis was evaluated using the Cell Death Detection ELISA Kit. Values are presented as mean ± S.D. of five determinations. (C) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 24 h. Apoptosis was evaluated using the Cell Death Detection ELISA Kit. Values are presented as mean ± S.D. of five determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus the H_2O_2-treated group (EGb761-free).

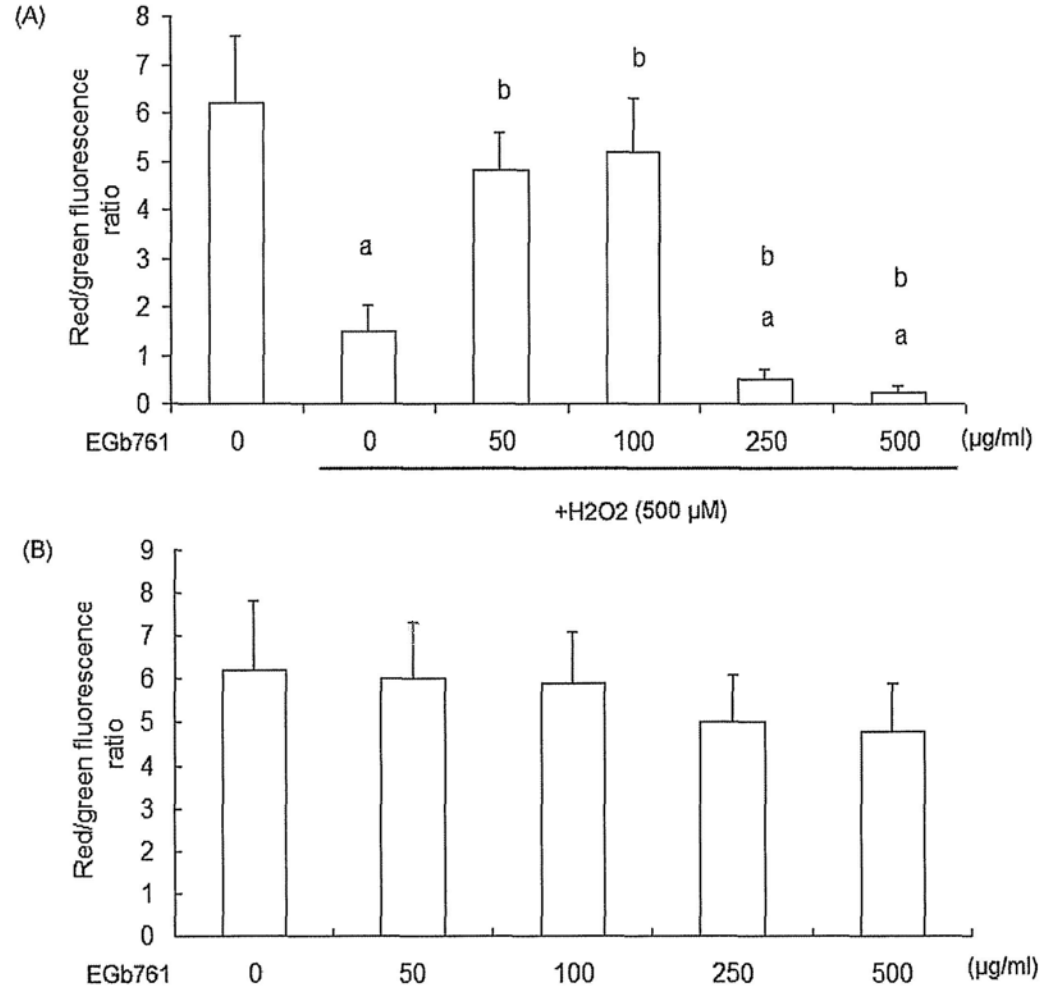


Figure 3.3 Dosage effects of EGb761 on H_2O_2-induced mitochondrial membrane potential deplolarization in SH-SY5Y cells. (A) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 24 h.

Mitochondrial membrane potential was determined with the lipophilic dye JC-1. Values are presented as mean ± S.D. of five determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus the H_2O_2-treated group (EGb761-free). (B) Cells were incubated with various doses of EGb761 alone for 24 h. Mitochondrial membrane potential was determined with the lipophilic dye JC-1. Values are presented as mean ± S.D. of five determinations.

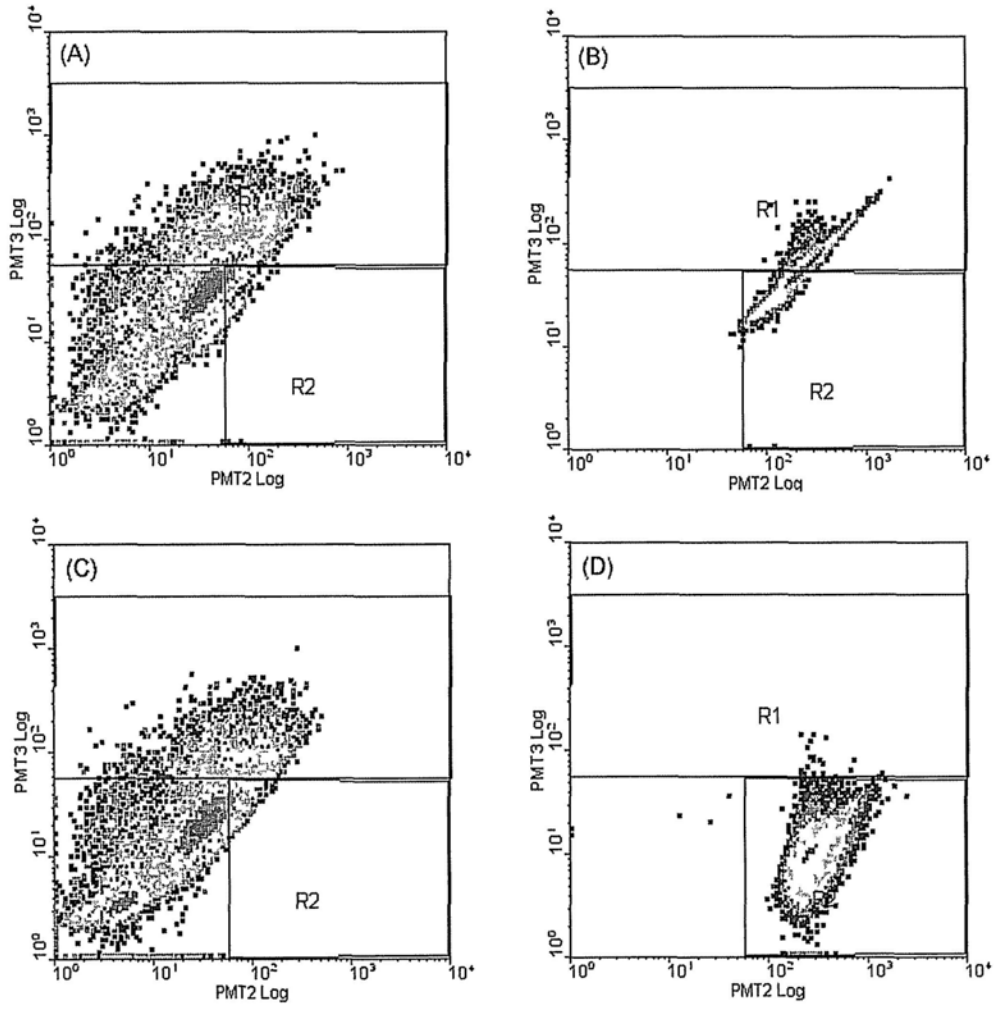


Figure 3.4 Representative flow cytometry dot plots showing JC-1 staining of SH-SY5Y cells. It could be seen that, compared with the pattern of untreated SH-SY5Y cells (A), after exposure of cells to 500 μM H_2O_2 for 24h, fluorescence in R1 region (PMT3, red direction) decreased with a corresponding increase in R2 region (PMT2, green direction) fluorescent intensity (B), which indicated a compromise of mitochondria. This change could be reversed by low dose of EGb761 (50 μg/ml) (C), whereas exacerbated by high dose of EGb761 (500 μg/ml) (D).

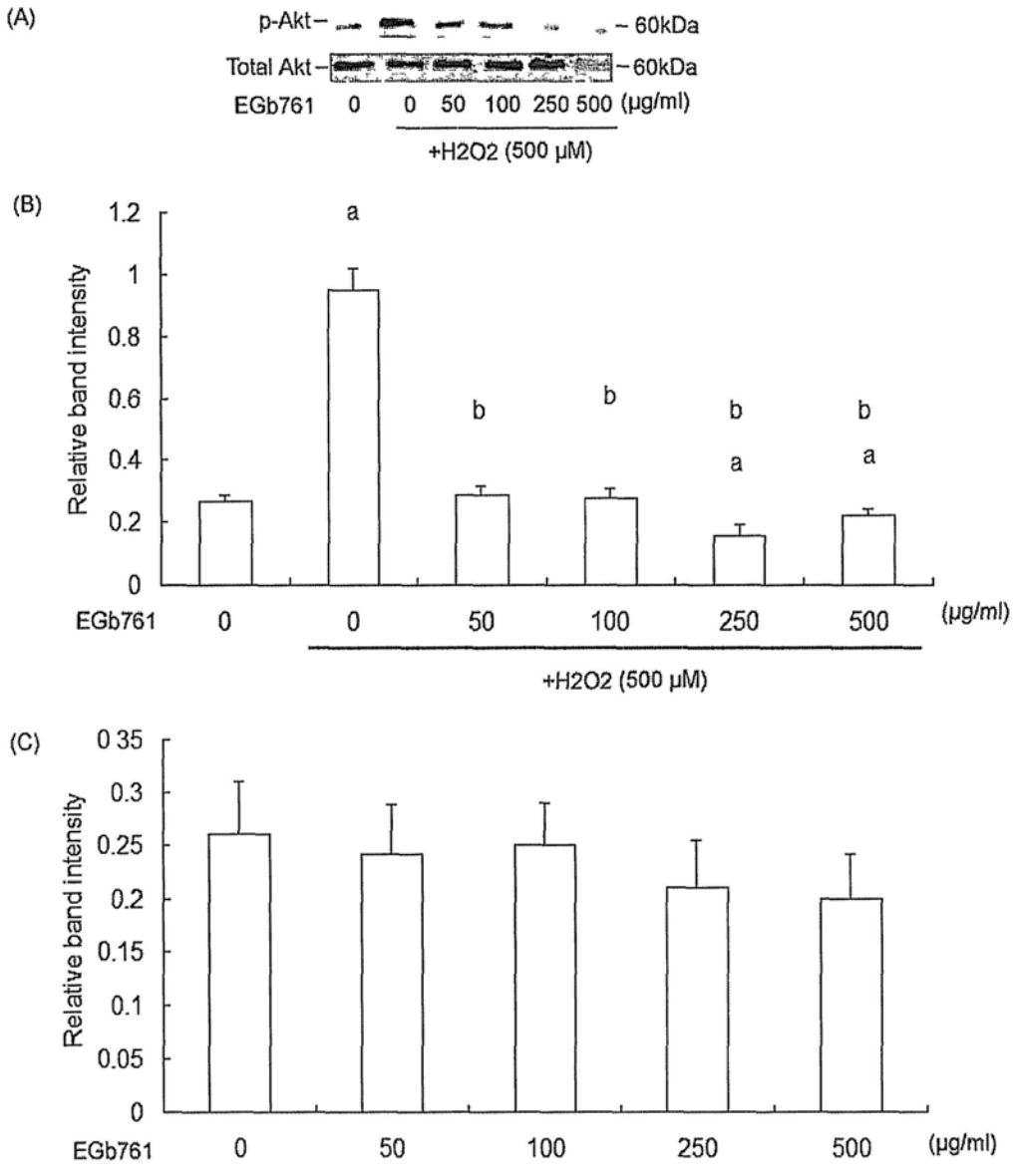


Figure 3.5 Dosage effects of EGb761 on H_2O_2-induced phosphorylation of Akt in SH-SY5Y cells. (A) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 1 h. Cell lysates (20 μg) prepared from cells in different treatments were analyzed for Akt activation by Western blot using a phospho-specific Akt (Thr308) antibody. The same crude lysates (20 μg) was also analyzed on a separate blot using an antibody that recognizes total level of Akt. (B) Protein bands representing phospho-Akt and total Akt were quantified using the Odyssey software. The relative ratios of phospho-Akt to total Akt band density were presented as mean ± S.D. of four determinations. [a]*P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b]*P< 0.01* versus the H_2O_2-treated group (EGb761-free). (C) Cells were incubated with various doses of EGb761 alone for 24 h. Akt

phosphorylation were also analyzed by Western blot as described previously. Values are presented as mean ± S.D. of four determinations.

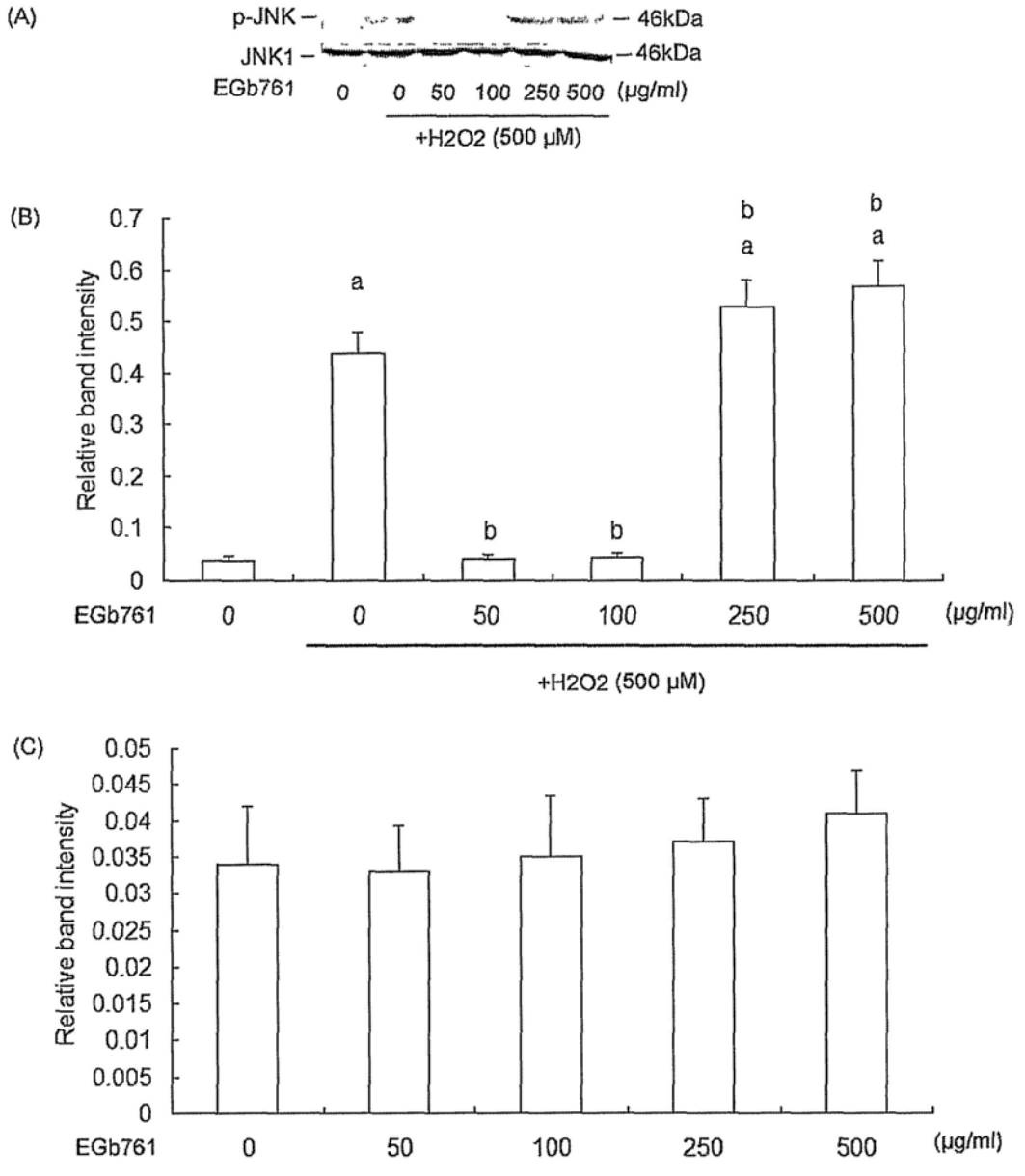


Figure 3.6 Dosage effects of EGb761 on H_2O_2-induced phosphorylation of JNK in SH-SY5Y cells. (A) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 2 h. Cell lysates (20 μg) prepared from cells in different treatments were analyzed for JNK activation by Western blot using a phospho-specific JNK (Thr183/Tyr185) antibody. The same crude lysates (20 μg) was also analyzed on a separate blot using an antibody that recognizes total JNK1. (B) Protein bands representing phospho-JNK and JNK1 were quantified using the

Odyssey software. The relative ratios of phospho-JNK to JNK1 band density were presented as mean ± S.D. of four determinations. [a] *P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b] *P< 0.01* versus the H_2O_2-treated group (EGb761-free). (C) Cells were incubated with various doses of EGb761 alone for 24 h. JNK phosphorylation was also analyzed by western blot as described previously. Values are presented as mean ± S.D. of four determinations.

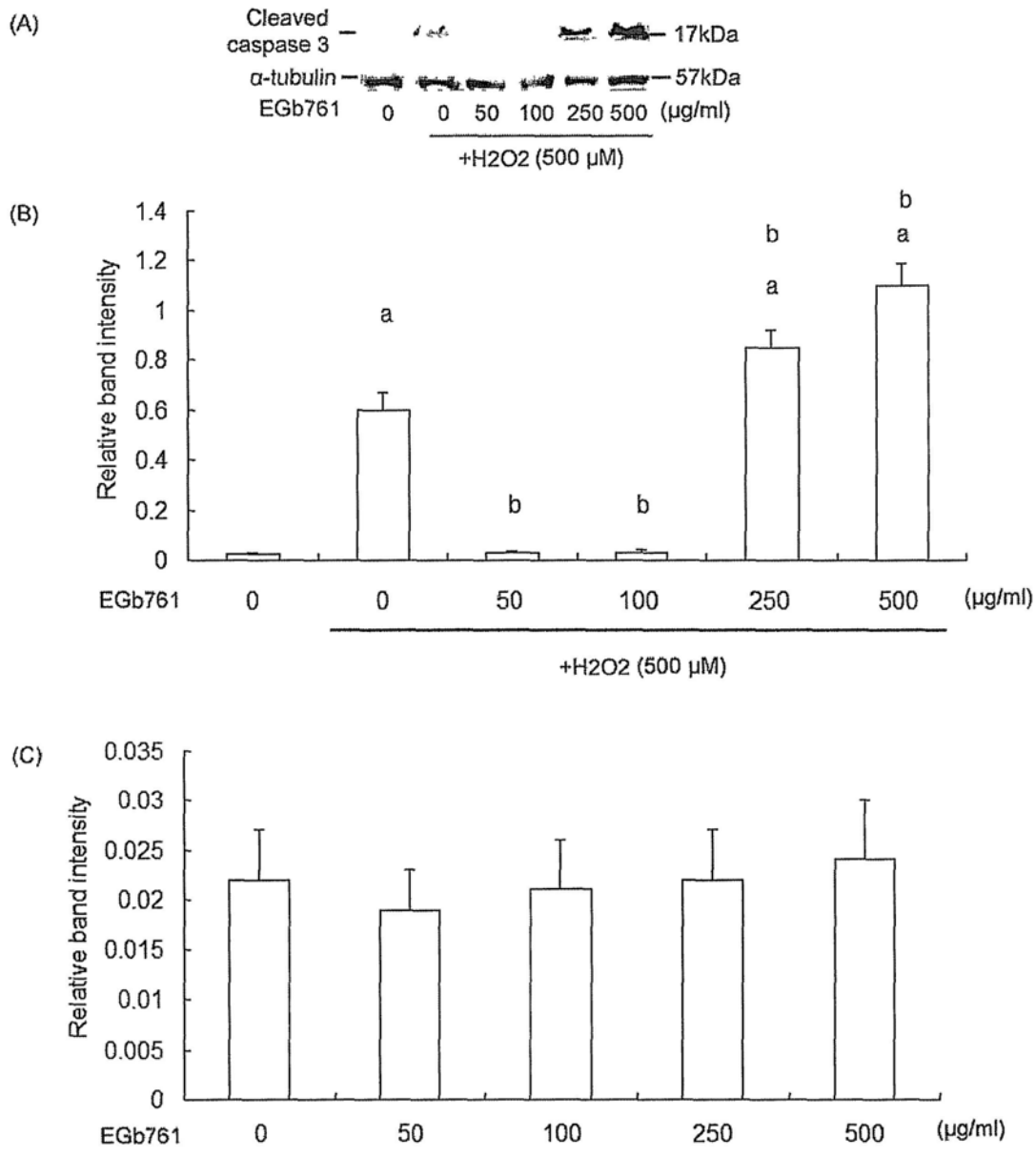


Figure 3.7 Dosage effects of EGb761 on H_2O_2-induced activation of caspase-3 in SH-SY5Y cells. (A) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 24 h. Cell lysates (20 μg) prepared from cells in different treatments were analyzed for cleaved caspase-3 by Western

blot using a cleaved caspase-3 antibody. The same crude lysates (20 μg) was also analyzed on a separate blot using an antibody that recognizes α-tubulin. (B) Protein bands representing cleaved caspase-3 and α-tubulin were analyzed using the Odyssey software. The relative band densities for cleaved caspase-3 were determined by normalizing against α-tubulin band intensity. Values were presented as mean ± S.D. of four determinations. [a] *P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b] *P< 0.01* versus the H_2O_2-treated group (EGb761-free). (C) Cells were incubated with various doses of EGb761 alone for 24 h. Cleaved caspase-3 was also analyzed by Western blot as described previously. Values are presented as mean ± S.D. of four determinations.

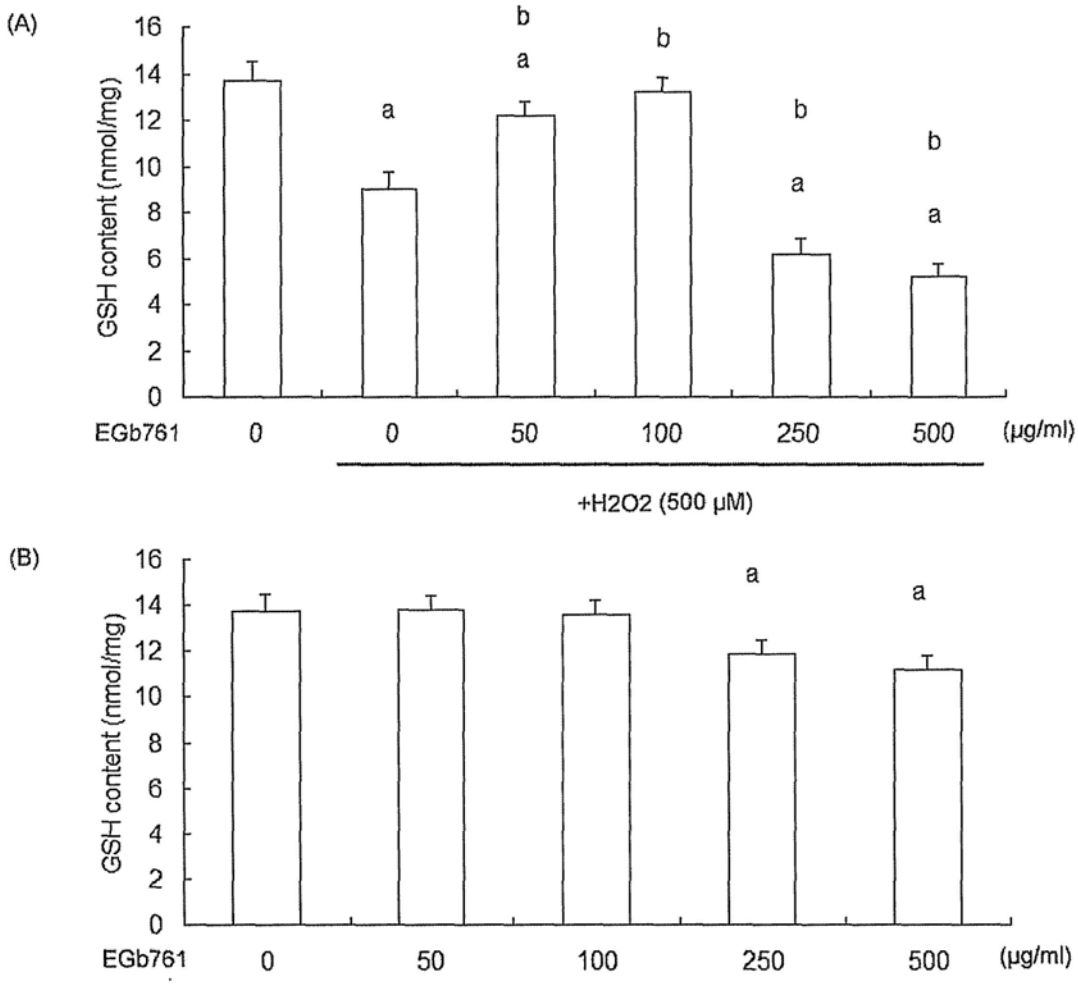


Figure 3.8 Dosage effects of EGb761 on H_2O_2-induced decrease of intracellular GSH content in SH-SY5Y cells. (A) Cells treated with or without various concentrations of EGb761 for 24 h. After that, the media containing EGb761 was aspirated off and cells were then challenged with 500 μM H_2O_2 for another 24 h. Intracellular GSH content was then determined using Glutathione Assay Kit. Values are presented as

mean ± S.D. of five independent experiments. [a] *P< 0.01* versus the control group (EGb761- and H_2O_2-free); [b] *P< 0.01* versus the H_2O_2-treated group (EGb761-free). (B) Cells were incubated with various doses of EGb761 alone for 24 h. Intracellular GSH content was then determined using Glutathione Assay Kit. Values are presented as mean ± S.D. of five independent experiments. [a] *P< 0.01* versus the control group (EGb761- and H_2O_2-free).

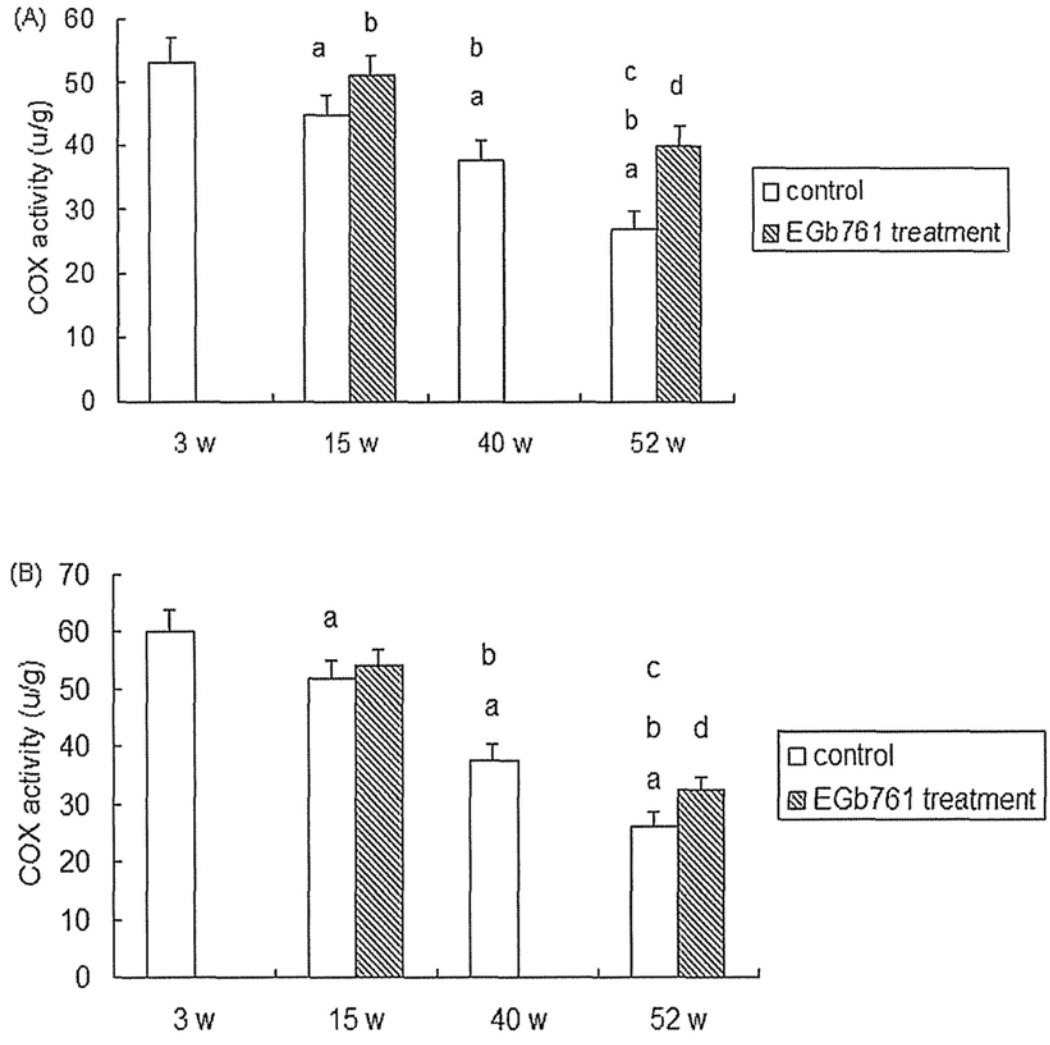


Figure 4.1 Preventive and rescue effects of EGb761 on COX activity in platelets and hippocampi of SAMP8 mice. Two groups of SAMP8 mice aged 3 weeks and 40 weeks, respectively, were given EGb761 at a daily dose of 100 mg/kg. After 12 weeks of EGb761 treatment, COX activity in platelets (A) and hippocampi (B) was tested by using cytochrome c oxidase assay kit. Values are presented as mean ± S.D. of five determinations. [a]: compared with 3-week-old controls, $p<0.01$; [b]: compared with 15-week-old controls, $p<0.01$; [c]: compared with 40-week-old controls, $p<0.01$; [d]: compared with 52-week-old controls, $p<0.01$.

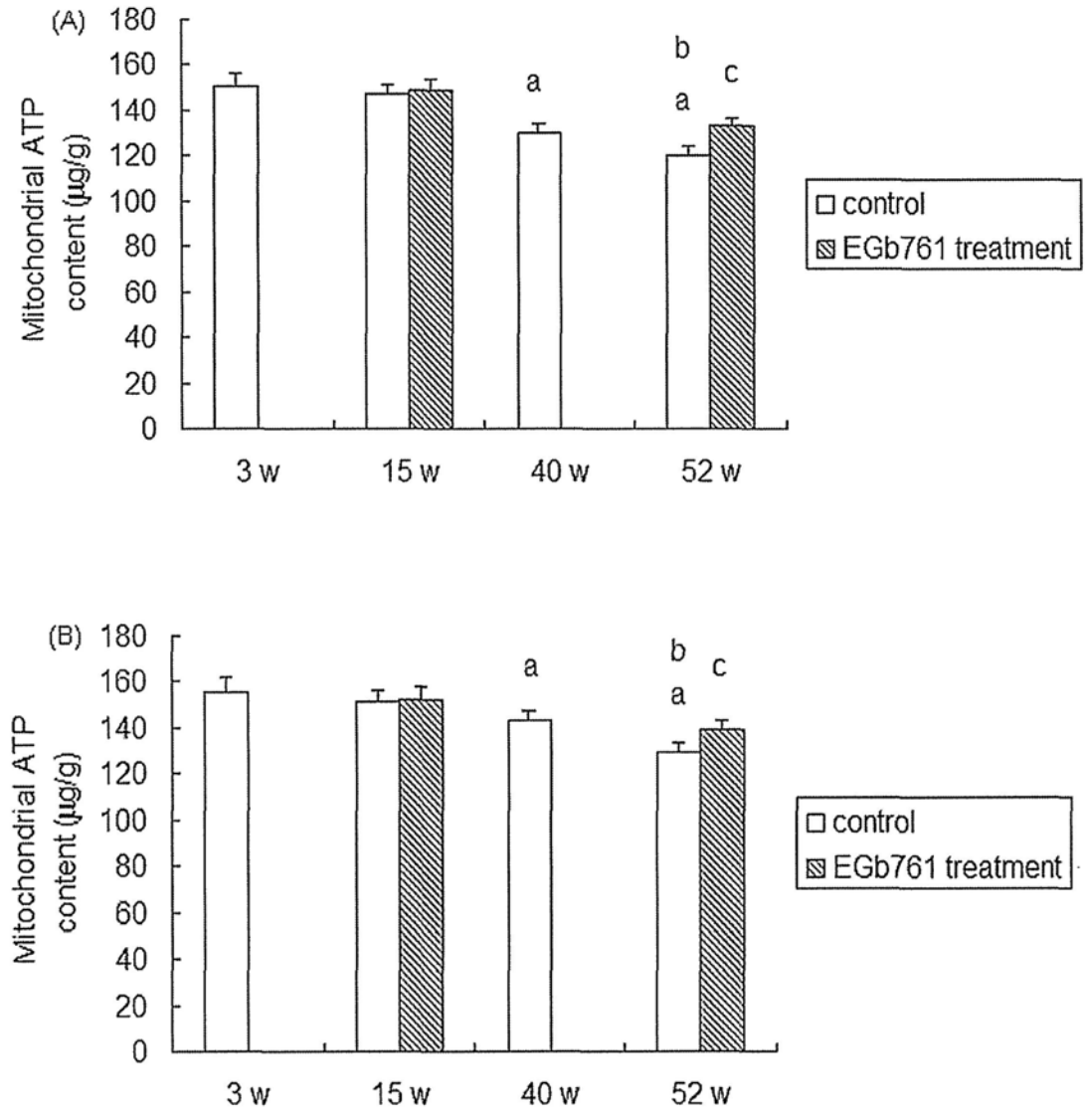


Figure 4.2 Preventive and rescue effects of EGb761 on mitochondrial ATP content in platelets and hippocampi of SAMP8 mice. Two groups of SAMP8 mice aged 3 weeks and 40 weeks, respectively, were given EGb761 at a daily dose of 100 mg/kg. After 12 weeks of EGb761 treatment, mitochondrial ATP content in platelets (A) and hippocampi (B) was measured using a HPLC-based method. Values are presented as mean ± S.D. of five determinations. [a]: compared with 15-week-old controls, $p<0.01$; [b]: compared with 40-week-old controls, $p<0.01$; [c]: compared with 52-week-old controls, $p<0.01$.

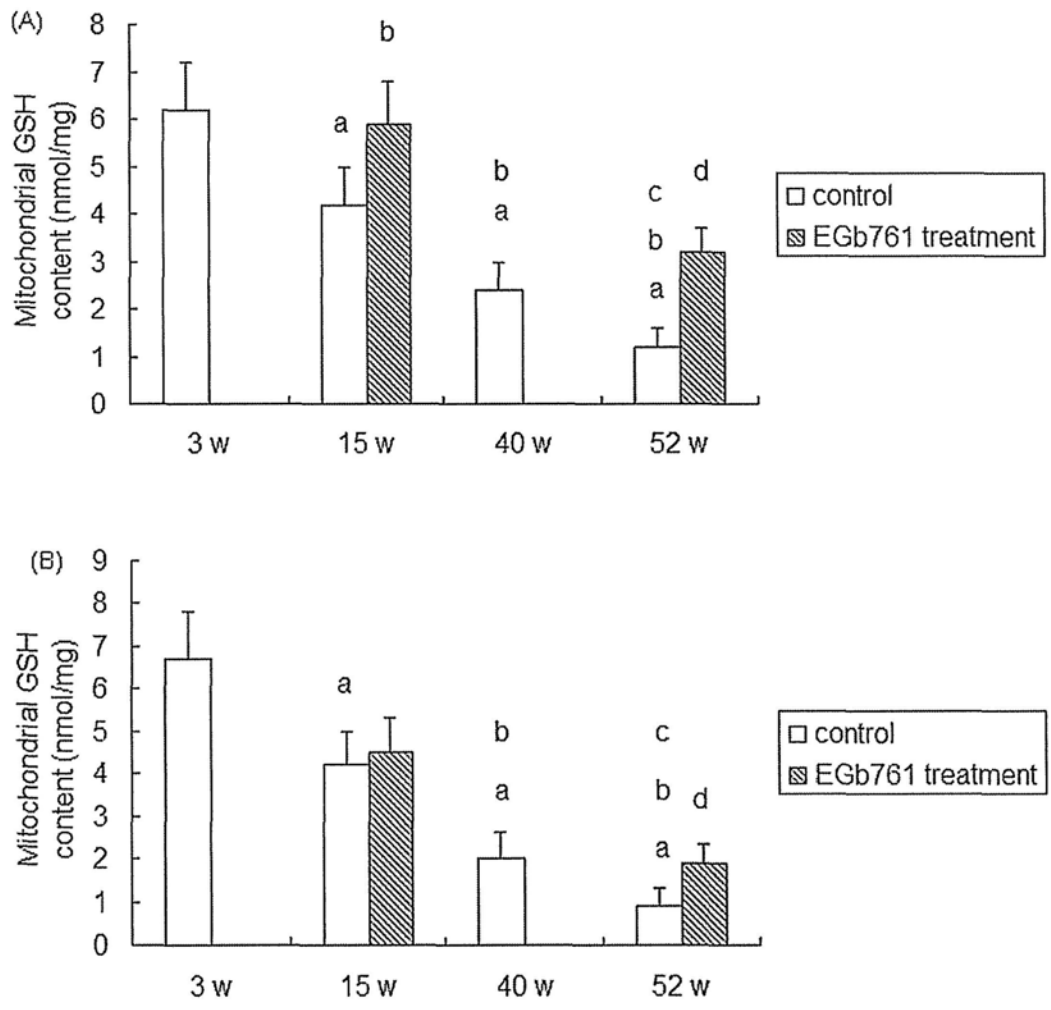


Figure 4.3 Preventive and rescue effects of EGb761 on mitochondrial GSH content in platelets and hippocampi of SAMP8 mice. Two groups of SAMP8 mice aged 3 weeks and 40 weeks, respectively, were given EGb761 at a daily dose of 100 mg/kg. After 12 weeks of EGb761 treatment, mitochondrial GSH content in platelets (A) and hippocampi (B) was tested by using Glutathione Assay Kit. Values are presented as mean ± S.D. of four determinations. [a]: compared with 3-week-old controls, $p<0.01$; [b]: compared with 15-week-old controls, $p<0.01$; [c]: compared with 40-week-old controls, $p<0.01$; [d]: compared with 52-week-old controls, $p<0.01$.

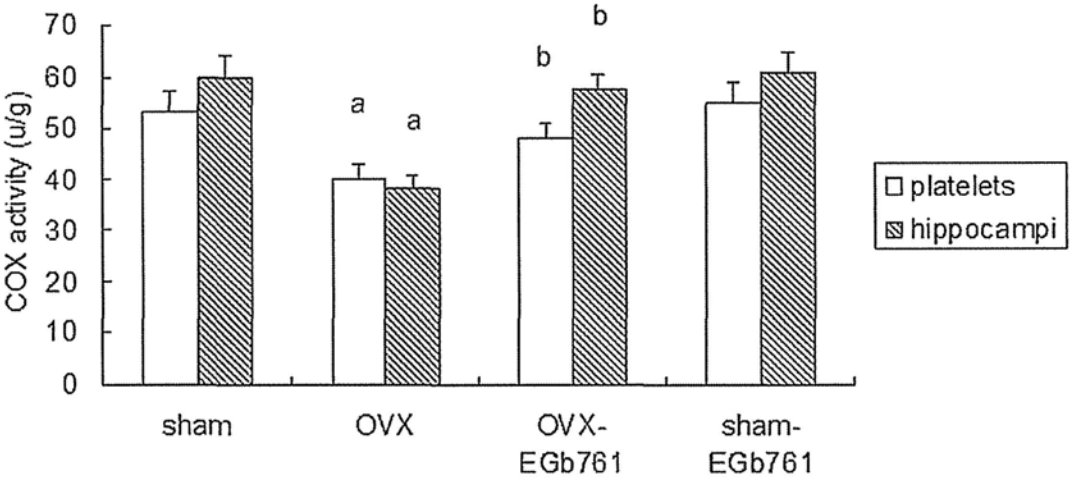


Figure 5.2 Protective effects of EGb761 on COX activity in platelets and hippocampi of ovariectomized and sham-operated rats. Nine-month-old rats were treated as described under *Methods*. COX activity in platelets and hippocampi was then tested by using cytochrome c oxidase assay kit. Values are presented as mean ± S.D. of five determinations. [a]: compared with the sham-operated group, $p<0.01$; [b]: compared with the OVX group, $p<0.01$.

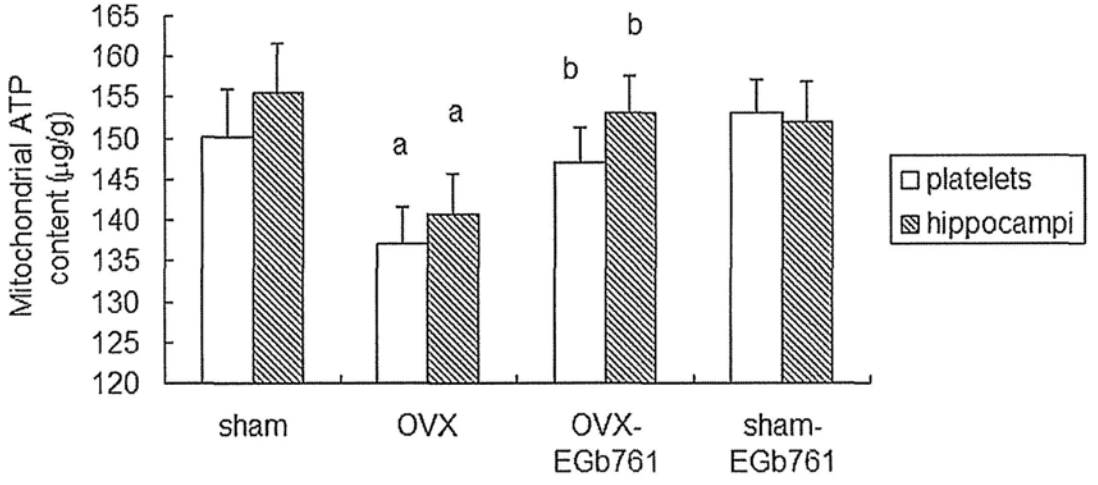


Figure 5.3 Protective effects of EGb761 on mitochondrial ATP content in platelets and hippocampi of ovariectomized and sham-operated rats. Nine-month-old rats were treated as described under *Methods*. Mitochondrial ATP content in platelets and hippocampi was then measured using a HPLC-based method. Values are presented as mean ± S.D. of five determinations. [a]: compared with the sham-operated group, $p<0.01$; [b]: compared with the OVX group, $p<0.01$.

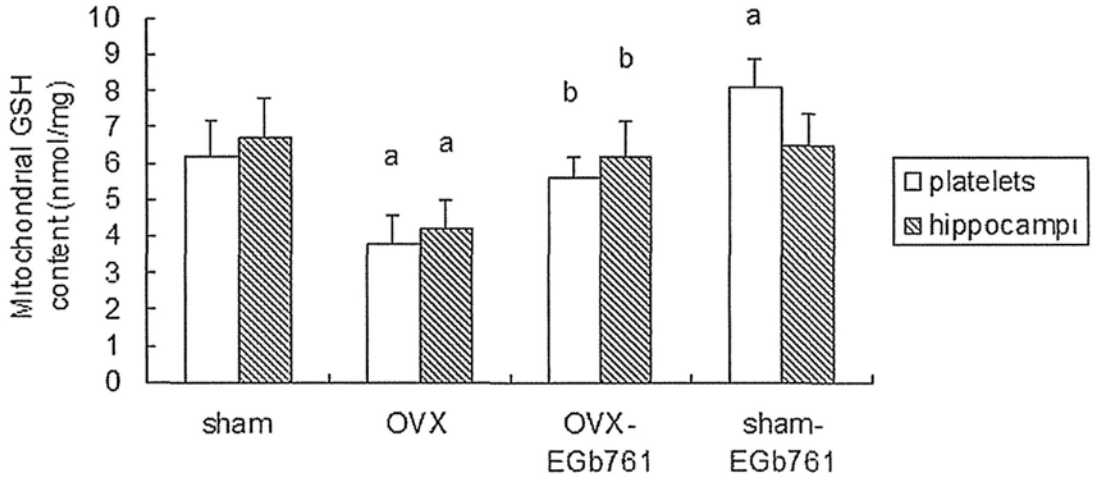


Figure 5.4 Protective effects of EGb761 on mitochondrial GSH content in platelets and hippocampi of ovariectomized and sham-operated rats. Nine-month-old rats were treated as described under *Methods*. Mitochondrial GSH content in platelets and hippocampi was then tested by using Glutathione Assay Kit. Values are presented as mean ± S.D. of five determinations. a: compared with the sham-operated group, $p<0.01$; b: compared with the OVX group, $p<0.01$.