

## Neuroprotective effects of anti-aging oriental medicine *Lycium barbarum* against $\beta$ -amyloid peptide neurotoxicity

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### Abstract

As aged population dramatically increases in these decades, efforts should be made on the intervention for curing age-associated neurodegenerative diseases such as Alzheimer's disease (AD). Natural plant extracts of *Lycium barbarum* are well-known to exhibit anti-aging effects. We therefore hypothesized that they exhibit neuroprotective effects against toxins in aging-related neurodegenerative diseases. In this study, we aimed to investigate whether extracts from *L. barbarum* have neuroprotective effects against toxicity of fibrillar  $A\beta_{1-42}$  and  $A\beta_{25-35}$  fragments. Primary rat cortical neurons exposed to  $A\beta$  peptides resulted in apoptosis and necrosis. Pre-treatment with extract isolated from *L. barbarum* significantly reduced the release of lactate dehydrogenase (LDH). In addition, it attenuated  $A\beta$  peptide-activated caspases-3-like activity. The extract elicited a typical dose-dependent neuroprotective effect. Effective dosage of this extract was wider than that of a well-known western neuroprotective medicine lithium chloride (LiCl). We have further examined the underlying mechanisms of the neuroprotective effects. In agreement with other laboratories,  $A\beta$  peptides induce a rapid activation of c-Jun N-terminal kinase (JNK) by phosphorylation. Pre-treatment of aqueous extract markedly reduced the phosphorylation of JNK-1 (Thr183/Tyr185) and its substrates c-Jun-I (Ser 73) and c-Jun-II (Ser 63). Taken together, we have proved our hypothesis by showing neuroprotective effects of the extract from *L. barbarum*. Study on anti-aging herbal medicine like *L. barbarum* may open a new therapeutic window for the prevention of AD.

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**Keywords:** *Lycium barbarum*; Neuroprotection; Beta-amyloid; Arabinogalactan-protein

### 1. Introduction

Aging is one of the factors leading to the dysfunction of the normal cellular regulation, affecting both central nervous and immune systems (Kawakami et al., 1999). It is an important risk factor of various chronic diseases,

including cancer, cardiovascular diseases and neurodegenerative diseases such as Alzheimer's disease (AD). Aging accounts for more than 50% cases for sporadic AD (Holliday, 1996). AD is pathologically characterized by extracellular senile plaques, intracellular neurofibrillary tangles and extensive neuronal loss (Selkoe, 1991). Senile plaque is the accumulation of a 39–43 amino acid peptide called beta-amyloid ( $A\beta$  peptide). The characteristics of apoptosis have been found in AD brains (Eckert et al., 2003; Roth, 2001). Also, in vitro studies show that  $A\beta$  peptide causes apoptosis in cultured neurons (Chang et al., 2002a; Lin et al., 2004; Loo et al., 1993; Suen et al., 2003a,b).  $A\beta$ -triggered neuronal cell death has been suggested in the involvement of caspase activation (Allen et al., 2001; Harada and Sugimoto, 1999; Troy et al., 2000), stress kinase

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(such as JNK, p38 MAPK, PKR, etc.) activation (Morishima et al., 2001; Suen et al., 2003b; Tamagno et al., 2003), ion channels formation (Kagan et al., 2002; Lashuel et al., 2002; Lin and Kagan, 2002; Mobley et al., 2004), membrane destabilization (Mason et al., 1999; Mingeot-Leclercq et al., 2002), membrane receptor-mediated response (Li et al., 2004; Tsukamoto et al., 2003), oxidative stress and intracellular calcium imbalance (Canevari et al., 2004). Works in our laboratory has been focused on the development of neuroprotective agent from anti-aging herbs.

The elder population is fast growing all over the world. Recently, caloric restriction and hormonal supplementation are used for anti-aging purpose (Chiba et al., 2002; Mattson, 2000). Current studies suggest that development of anti-aging drugs from Chinese medicinal herbs may be one of the possible interventions (Bastianetto and Quirion, 2002; Chang, 2001; Lei et al., 2003). Oriental herbal medicine has been widely investigated for drug development because it has fewer side effects and it is much safer to be used than that of synthetic compounds (Wong et al., 1994).

The fruits of *Lycium barbarum* (Solanaceae), also called *Fructus Lycii*, has been used for thousands of years in traditional Chinese medicine and is one of the dietetic Chinese medicines that have been officially recognized as being both food and a Chinese medicine. It is believed that *L. barbarum* is effective to be an anti-aging agent as well as nourishment of eyes, livers and kidneys. The polysaccharides isolated from the aqueous extracts of *L. barbarum* have been identified as one of the active ingredients responsible for the biological activities. The anti-aging property of *L. barbarum* has been investigated in different models. Extracts of *L. chinense mill* (grown from different regions but belongs to the same species) have anti-decrepity effect in brain and heart tissues in mice by increasing the activity of superoxide dismutase (SOD) (Xu and Fang, 2000). *L. chinensis* capsule prolongs the life span of drosophila (Xu et al., 2001). Polysaccharides from *L. barbarum* exhibit anti-aging function in fruit flies and mice (Wang et al., 2002a). Apart from anti-aging effect, *L. barbarum* inhibits hydrocortisone-induced apoptosis in dose-dependent manner in cultured rat spleen (Lu et al., 1999). Its polysaccharides also exert protection against time and hyperthermia-induced degeneration in cultured seminiferous epithelium (Wang et al., 2002b).

Since *L. barbarum* possess anti-aging property as well as protective effects in different cell types, it is hypothesized that *L. barbarum* can also exert protection in neurons. In this study, we aim to investigate whether these extracts from the fruit of *L. barbarum* exhibit neuroprotective effects against A $\beta$  peptide neurotoxicity. Our results showed that the aqueous extract is neuroprotective by attenuation of A $\beta$ -activated caspase-3-like and LDH activity. The extract has wider effective dosages than that of the well-known western neuroprotective drug LiCl. Western blot analysis revealed that the neuroprotection mechanism of *L. barbarum* relies

on inhibition of JNK signaling pathway. Given that extracellular accumulation of A $\beta$  peptide is involved in AD pathogenesis, the neuroprotective *L. barbarum* extracts deserve further exploration in the prevention for AD.

## 2. Materials and methods

### 2.1. Materials

*Lycium barbarum L.* was the product of Ning Xia Huizu Autonomous Region, People's Republic of China. Sugar composition was determined by gas chromatography-mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides by acidic methanolysis. It was performed by the Complex Carbohydrate Research Center [University of Georgia, Athens, Ga.; supported in part by the Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DF-FG09-93ER-20097)]. The amino acid composition analysis was determined by post-column ninhydrin detection on a Beckman amino acid analyzer Instruments (Models 6300) performed by the Scientific Research Consortium, Inc. (St Paul, MN, USA).

Materials used for neuronal cell culture were purchased from Gibco BRL (Burlington, Ont., Canada). Other chemicals were obtained from the following companies: A $\beta$  peptides (both 1–42 and 25–35 fragments), 4' -6-Diamidino-2-phenylindole (DAPI), lithium chloride (LiCl), protease inhibitor cocktail and phosphatase inhibitor cocktail from Sigma-Aldrich, Inc. (Saint Louis, USA); LDH cytotoxicity assay kit from Roche Diagnostics (Mannheim, Germany); heparin, JNK inhibitor (SP600125), caspase-3 substrate (Ac-DEVD-pNA) from Calbiochem, Inc. (Darmstadt, Germany); rabbit polyclonal antibodies for JNK, c-Jun and cleaved caspase-3 detection from Cell Signaling Technology (Beverly, MA, USA); anti- $\beta$ -actin monoclonal antibody from Sigma-Aldrich, Inc. (Saint Louis, USA); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies from DAKO (Glostrup, Denmark); PVDF membrane was from Bio-Rad (Richmond, CA, USA); Biomax X-ray film from Kodak (Tokyo, Japan); enhanced chemiluminescence (ECL) detection kit from Amersham (Buckinghamshire, UK); Re-Blot western blot recycling kit from Chemicon Int' 1, Inc. (Temecula, CA, USA).

### 2.2. Preparation of *Lycium barbarum* extracts (LBA)

The dried fruit of *Lycium barbarum* (400 g) was ground into small pieces with a blender and the resultant residue was extracted twice with distilled hot water (6 L) for 3 h. The combined extracts were concentrated and deproteinated by the Sevag method (Sevag, 1934) and the resulting aqueous fractions was extensively dialyzed against running distilled water for 2 days (Molecular Weight Cut Off 6000–

8000 Da, Spectra/Por<sup>®</sup> 1 RC dialysis membrane, Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA)). The retentate was concentrated to 400 ml under reduced pressure and precipitated by addition of three volumes of 95% ethanol. After centrifugation and washing by acetone, the resulting precipitate was vacuum-dried at 40 °C to yield a brown powder (LBA, 1.7% from the material, i.e. 6.8 g).

### 2.3. Primary neuronal cultures

Primary neuronal cultures were prepared from embryonic day 17 Sprague–Dawley rats (Laboratory Animal Unit, The University of Hong Kong, Hong Kong) according to our previous published methods (Chang et al., 2002a; Lin et al., 2004; Suen et al., 2003a,b). Cerebral cortices were dissected from the whole brains and mechanically dissociated in phosphate-buffered saline (PBS) with glucose (18 mM). Cells were seeded onto 6-well or 4-well plates ( $2 \times 10^6$  cells/well and  $0.5 \times 10^6$  cells/well, respectively) pre-coated with poly-L-lysine (25 µg/ml). The culture medium consisted of minimum essential medium (MEM) supplemented with 5% heat inactivated fetal bovine serum, glucose (18 mM), L-glutamine (2 mM), insulin (5 µg/ml), progesterone (0.02 µM), putrescine (100 µM), selenium (30 pM), penicillin (50 U/ml) and streptomycin (50 µg/ml). Neurons were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and were cultured for 7 days prior to treatments. Having done the GFAP and OX42 staining for astrocytes and microglial cells, the purity of neurons in our culture was over 98%. Only about 2% cells are GFAP positive and none of them are OX42 positive cells.

### 2.4. Treatment of neurons

To investigate whether LBA exert neuroprotective effects, neurons on 6-well plates were treated with LBA (ranging from  $10^{-4}$  to 100 µg/ml) for 1 h, and then exposed to Aβ<sub>25–35</sub> (25 µM) or Aβ<sub>1–42</sub> (20 µM) for 24 h. The peptides were incubated with autoclaved Milli-Q water at 37 °C for 1 day (Aβ<sub>25–35</sub>) and 7 days (Aβ<sub>1–42</sub>) prior to use. For ‘Wash-out’ experiment, neurons were pretreated with LBA for 1 h. Afterwards, LBA-containing medium was removed and neurons were then washed with  $1 \times$  PBS prior to exposure to Aβ peptide. LDH toxicity and caspase activity assays were performed to assess neuronal apoptosis. The percentage of apoptotic neurons was determined by counting cells with DNA fragmentation or condensation stained by DAPI under the fluorescent microscope (Leica, Wetzlar, Germany).

In order to compare the effectiveness of LBA and other well-known western drugs/inhibitors such as LiCl and a specific JNK inhibitor (SP600125) in neuroprotection, neurons were treated with different concentrations of LBA, LiCl (2–20 mM) or SP600125 (1–20 µM) for 1 h, followed by exposure to Aβ<sub>25–35</sub> (25 µM) for 24 h. Caspase-3-like activity assay was then carried out.

### 2.5. Measurement of general cytotoxicity

Culture medium was collected for LDH activity assay to determine the level of general cytotoxicity. The assay was conducted in accordance with the manufacturer’s instructions. Briefly, the culture medium was incubated with the assay buffer for 30 min in dark. Release of LDH was determined by reading the absorbance at 492 nm. We have examined that the extract per se did not interfere LDH assay. Results were expressed as the percentage of control.

### 2.6. Caspase-3-like activity assay

Caspase-3-like activity was assayed using the colorimetric caspase-3 substrate from Calbiochem, Inc. (Darmstadt, Germany). Neurons were scratched and lysed in lysis buffer containing DTT (5 mM), EDTA (0.1 mM), HEPES (50 mM, pH 7.4) and Triton-X (0.2%). The lysate was then centrifuged at  $20,000 \times g$  for 30 min at 4 °C. 60 µg of protein from each sample was incubated with caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37 °C. The caspase-3-like activity was determined by measuring the absorbance (at 405 nm) of the yellow product (pNA) cleaved from the substrate (i.e. DEVD cleavage). From the absorbance readings, values of specific activity (s.a., unit = pmol/min/µg) were calculated. Caspase-3-like activity of the control group was regarded as 0% toxicity and that of the Aβ-treated group as 100% toxicity, whereas % toxicity is calculated as:  $[(s.a._{of\ A\beta\text{-treated/LBA-treated}} - s.a._{control}) \div (s.a._{A\beta\text{-treated}} - s.a._{control})] \times 100$ . Results were expressed as % of toxicity. The activity assay was counter confirmed by using western-blot analysis of the active fragment of caspase-3.

### 2.7. DAPI staining

To further examine neuronal apoptosis, apoptotic bodies were determined by DNA fragmentation and condensation using DAPI nuclear staining (Nardi et al., 1997; Wyllie et al., 1980). After treatment, neurons were fixed with methanol:acetone (1:1) for 15 min. DAPI (1 µg/ml in PBS) was added to the cells for 5 min, followed by 3 times washing with PBS. Under a fluorescent microscope, the number of apoptotic and normal neurons was counted in five different fields for each well. A total number of about 200 neurons could be counted in each field. Results were expressed as the percentage of apoptotic neurons.

### 2.8. Western blotting

To examine the level of activated caspase-3 as well as the phosphorylated form of JNK and c-Jun, neurons after treatment were scratched and lysed in ice-cold lysis buffer containing Tris–HCl (10 mM, pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> (20 mM), Na<sub>3</sub>VO<sub>4</sub> (2 mM), Triton X-100 (1%), Glycerol

(10%), deoxycholate (0.5%), SDS (0.1%), phenylmethylsulfonyl fluoride (PMSF, 1 mM), protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysate was centrifuged at  $20,000\times g$  for 30 min at 4 °C. Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (50  $\mu\text{g}$  protein/lane) using 10% polyacrylamide gels. Afterwards, the proteins were transferred onto a PVDF membrane, followed by blocking with 5% non-fat dry milk in TBST (TBS containing 0.1% Tween-20). The membrane was then incubated with anti-cleaved caspase-3 (1:1000), anti-phosphorylated JNK (1:1000), anti-total JNK (1:1000), anti-phosphorylated c-Jun-I (1:1000), anti-phosphorylated c-Jun-II (1:1000), anti-total c-Jun (1:1000) or anti- $\beta$ -actin (1:5000) for 2 h at room temperature, followed by the incubation of horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 h at room temperature. Bands were visualized on a Biomax X-ray film using the ECL detection kit. The membranes were then stripped with the use of the Re-Blot western blot recycling kit and re-probed with monoclonal anti- $\beta$ -actin antibody (1:5000) as primary antibody, and goat anti-mouse-HRP (1:2000) as secondary antibody.

## 2.9. Statistical analysis

All values obtained were expressed as means  $\pm$  standard error (SE) from at least 3 independent experiments. The significance of differences among different groups was determined by one-way ANOVA, followed by Student-Newman-Keuls as post-hoc test. To increase the level of confidence,  $*P < 0.001$  was considered statistically significant in all experiments.

## 3. Results

### 3.1. Neuroprotective effects of the aqueous extract LBA

To examine the effects of the aqueous extract LBA from *L. barbarum* on A $\beta$ -peptide-induced neurotoxicity, neurons were pretreated with LBA (ranged from  $10^{-4}$  to  $5 \times 10^2$   $\mu\text{g}/\text{ml}$ ) for 1 h, followed by a 24-hour exposure to A $\beta_{25-35}$  (25  $\mu\text{M}$ ). For the analysis of cytotoxicity, the extracellular concentration of LDH was measured. Fig. 1a shows the percentage of control of LDH activity of various treatment groups. For the control group without exposure to LBA or A $\beta$  peptide, its LDH activity was  $100.0 \pm 0.9\%$  of control. The LDH release was  $152.9 \pm 2.1\%$  of control if neurons were exposed to A $\beta$  peptide per se. LBA ranged from 0.1 to 100  $\mu\text{g}/\text{ml}$  was able to significantly reduce the LDH release triggered by A $\beta$  peptide. The results demonstrated that LBA exhibited cytoprotective effects against A $\beta$ -peptide toxicity. After wash-out procedure of LBA, the LDH activity was similar to that from the normal pre-treatment procedure (Fig. 1b). It implies that cytoprotective effects of LBA would not be due to the binding to A $\beta$  peptide and prevent its interaction to cell membrane to trigger cell death.

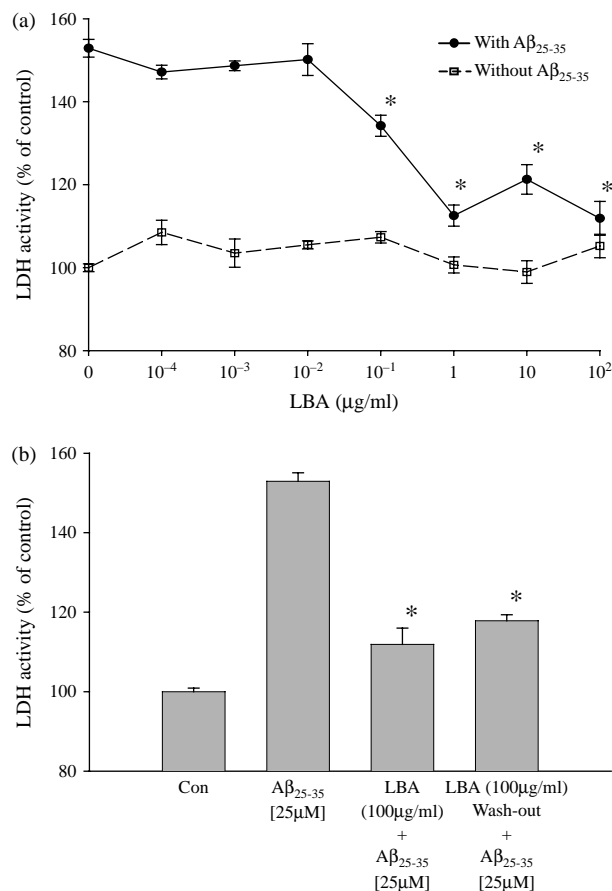


Fig. 1. Effect of the aqueous extract LBA on A $\beta$ -induced cytotoxicity. Neurons were treated with different concentrations of LBA for 1 h, followed by a 24-hour incubation with or without A $\beta_{25-35}$  (25  $\mu\text{M}$ ). LDH activity assay was used to measure the release of LDH in culture medium. (a) LDH activity of LBA ranged from 0 to 100  $\mu\text{g}/\text{ml}$ . (b) Comparison of the cytoprotective effects of LBA with or without the wash-out procedure. Results are expressed as mean  $\pm$  SE from at least three independent experiments.  $*P < 0.001$  vs. the group treated with A $\beta$  peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

In order to evaluate the effects of LBA on A $\beta$ -triggered activation of caspase-3, the colorimetric caspase-3-like activity assay was conducted. As shown in Fig. 2a, a wide range of concentrations of LBA significantly lowered caspase-3 activity triggered by A $\beta$  peptide. LBA at 100  $\mu\text{g}/\text{ml}$  showed the best neuroprotective effect since it could reduce caspase-3 activity to 11.8% when compared with A $\beta$  peptide treated group.

LBA contains both carbohydrates and amino acids, which may account for the protective effects. To further confirm its chemical composition by a biological assay, acidification was carried out to destroy the structure of LBA prior to treatment. Simply, LBA was dissolved in medium containing concentrated HCl (1 M), and then incubated at 80 °C with shaking (40 rpm) for 18 h. Afterwards, the acidified LBA was neutralized with medium containing NaOH (1 M). Neurons were treated with this 'acidified LBA' for 1 hour before a 24-

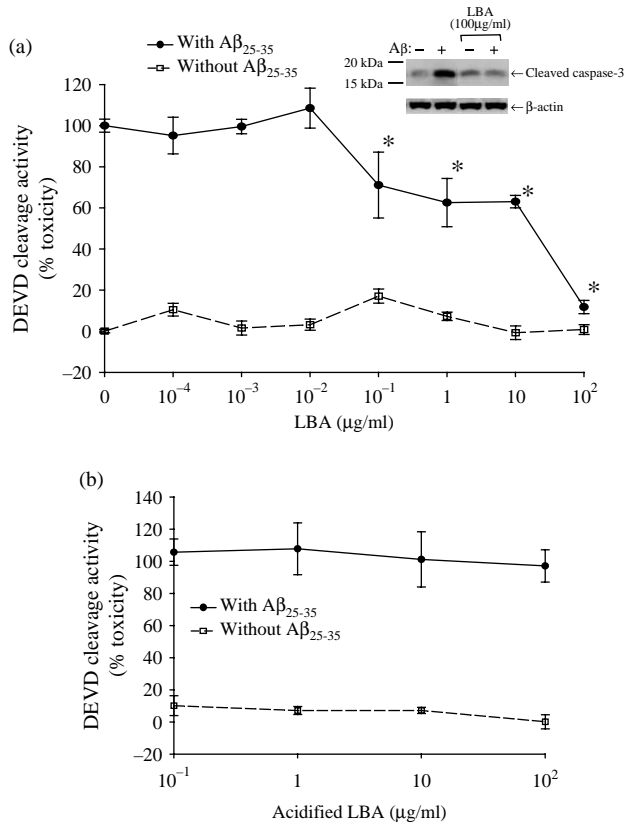


Fig. 2. Neuroprotective effects of the aqueous extract LBA. Neurons were treated with different concentrations of LBA for 1 h, followed by a 24-hour incubation with or without Aβ<sub>25-35</sub> (25 μM). After treatment, neuronal apoptosis was determined by the colorimetric caspase-3-like activity assay by measuring the absorbance (at 405 nm) of the yellow product (pNA) cleaved from the substrate. DEVD cleavage activity was expressed as % toxicity. Percentage toxicity is calculated as: [(s.a. of Aβ-treated/LBA-treated - s.a. control) ÷ (s.a. Aβ-treated - s.a. control)] × 100. (a) Caspase-3-like activity assay. Western blot analysis showed the level of activated caspase-3. (b) Caspase-3-like activity assay after treatment with the 'acidified LBA' and Aβ<sub>25-35</sub> peptide. Results are expressed as mean ± SE from at least three independent experiments. \**P* < 0.001 vs. the group treated with Aβ peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

hour incubation of Aβ<sub>25-35</sub> (25 μM). Caspase-3-like activity assay was done and Fig. 2b shows that LBA lost its protective effects against Aβ toxicity after acidification.

### 3.2. LBA also attenuates Aβ<sub>1-42</sub> peptide-triggered apoptosis

The neuroprotective effects of LBA were re-confirmed by using Aβ<sub>1-42</sub> peptide as it is a toxic factor in AD brain. DAPI staining was performed to distinguish normal and apoptotic bodies with a fluorescent microscope. As shown in Fig. 3a, Aβ<sub>1-42</sub> increased the percentage of apoptotic neurons to 22.4 ± 1.3% from 11.8 ± 1.0% in control. LBA at 100 μg/ml effectively attenuated the Aβ<sub>1-42</sub> peptide neurotoxicity to 13.5 ± 0.9%. Colorimetric caspase-3-like activity assay was also performed. LBA (100 μg/ml)

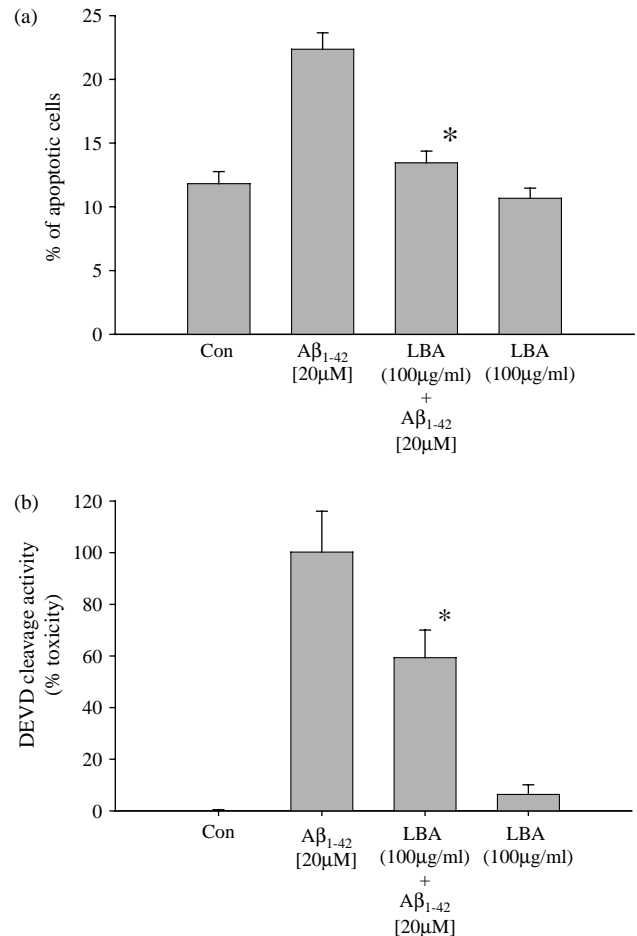


Fig. 3. LBA inhibits Aβ<sub>1-42</sub>-triggered neuronal cell death. Neurons were treated with 100 μg/ml LBA for 1 h, and then subjected to Aβ<sub>1-42</sub> (20 μM) for 24 h. (a) DAPI staining was performed to distinguish normal and apoptotic cells under fluorescent microscopy. Percentage of apoptotic cells was calculated as: (no. of apoptotic neurons/no. of total neurons) × 100. (b) Colorimetric caspase-3-like activity assay. DEVD cleavage activity was expressed as % toxicity. Percentage toxicity was calculated as: [(s.a. of Aβ-treated/LBA-treated - s.a. control) ÷ (s.a. Aβ-treated - s.a. control)] × 100. Results are expressed as mean ± SE from at least three independent experiments. \**P* < 0.001 vs. the group treated with Aβ peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

significantly reduced Aβ<sub>1-42</sub> peptide-stimulated caspase-3-like activity to 59.3 ± 10.7% (Fig. 3b).

### 3.3. Comparison of protective effects between LBA and Lithium chloride

Lithium chloride (LiCl) has been shown to protect neurons against Aβ peptide toxicity (Alvarez et al., 1999; Wei et al., 2000). It is also known for the treatment of manic-depressive illness (Chuang et al., 2002). Having demonstrated the neuroprotective effects of LBA against Aβ peptide neurotoxicity, we further compared pharmacological responses of LBA with LiCl. Neurons were pretreated with either LiCl (2–20 mM) or LBA (0.1–100 μg/ml) for 1 h, and subsequently exposed to Aβ<sub>25-35</sub> peptide (25 μM)

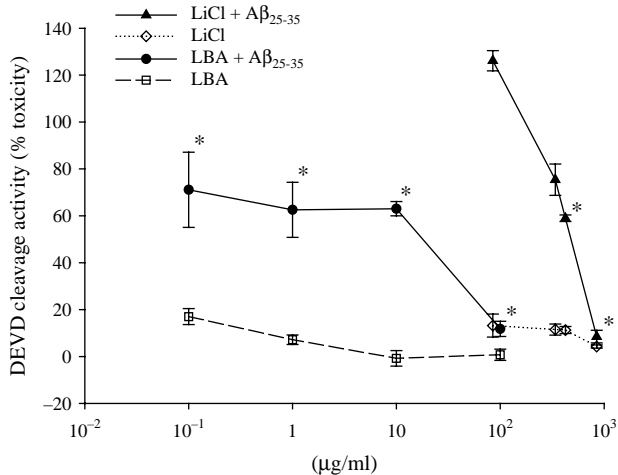


Fig. 4. Comparison of protective effects between LBA and LiCl. Neurons were pretreated with either LiCl (2–20 mM) or LBA (0.1–100 µg/ml) for 1 h, then, incubated with or without Aβ<sub>25–35</sub> (25 µM) for 24 h. After treatment, colorimetric caspase-3-like activity assay was done to examine the level of apoptosis. DEVD cleavage activity was expressed as % toxicity. Percentage toxicity is calculated as:  $(\text{s.a. of } \beta\text{-LBA/LiCl-treated} - \text{s.a. control}) / (\text{s.a. } \beta\text{-treated} - \text{s.a. control})$ . Results are expressed as mean  $\pm$  SE from at least three independent experiments. \* $P < 0.001$  vs. the group treated with Aβ peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

for 24 h. Fig. 4 shows the caspase-3-like activity after LBA and LiCl treatment. The unit of concentration of LiCl was converted from mM to µg/ml for the purpose of comparison with LBA. LiCl at 85 µg/ml (2 mM) exhibited toxicity. The toxicity decreased when the concentration of LiCl increased. LiCl could effectively attenuate Aβ peptide-stimulated caspase-3 activity in the range of 340–850 µg/ml (i.e. 8–20 mM). In addition, it exhibited maximum neuroprotection ( $8.5 \pm 2.7\%$ ) at 850 µg/ml (20 mM). For LBA, it exerted protective effects on neurons against Aβ peptide neurotoxicity from 0.1 to 100 µg/ml. The toxicity was significantly reduced to  $11.8 \pm 3.2\%$  when LBA was used at 100 µg/ml. From the result, the effective dosage for neuroprotective LBA (0.1–100 µg/ml) was much wider than that of LiCl (340–850 µg/ml). Also, LBA has about two orders lower working concentration than that of LiCl.

#### 3.4. Comparison of neuroprotective effects of LBA and Heparin

Heparin has a well-defined structure of highly sulfated polysaccharides that has been demonstrated to attenuate Aβ-peptide neurotoxicity (Bergamaschini et al., 2002). Since the composition of LBA contained polysaccharides, we used heparin to make a comparison on their protective effects. Neurons were pre-incubated with heparin (1 µM, i.e. 5 µg/ml) or LBA (100 µg/ml) for 1 h following by a 24-hour exposure to Aβ<sub>25–35</sub> peptide (25 µM). Morphology of neurons after different treatments was shown in Fig. 5. In control group (a), neurons were in good condition as

revealed by the fine neuronal network and the round cell bodies. Neurons treated with Aβ<sub>25–35</sub> for 24 h underwent apoptosis (Fig. 5b), as shown by the destruction of the fine neuritis network as well as the shrinkage of cell body. LBA per se did not alter the morphology of neurons (Fig. 5c). Pretreatment with LBA could protect neurons against Aβ toxicity as shown by the fine morphology in Fig. 5d. Neurons exposed to heparin exhibited elongated neuritis (Fig. 5e). This type of morphological changes could not be revealed by simple biochemical assay of caspase-3-like activity. Therefore, results of morphological changes were shown instead of caspase-3-like activity. Heparin exerted neuroprotective effects against Aβ peptide neurotoxicity as shown by intact neuritis (Fig. 5f). Caspase-3-like activity was also markedly reduced when neurons were pre-treated with heparin (Fig. 5g). By examining the morphology of neurons, it was suggested that the modes of neuroprotection elicited by LBA were different from that of heparin.

#### 3.5. Western-blot analysis of JNK and c-Jun in neurons treated with LBA

c-Jun N-terminal kinase (JNK) is one of the stress kinases involved in Aβ peptide neurotoxicity. Upon Aβ peptide stimulation, JNK is activated by phosphorylation, and in turn phosphorylates its substrate, c-Jun (Morishima et al., 2001). In order to investigate whether there was any relationship between the JNK signaling pathway and the neuroprotective effects of LBA, protein extracted after treatment were subjected to western blotting to detect the phosphorylated form of JNK and c-Jun. The levels of phospho-JNK-1, phospho-c-Jun-I and phospho-c-Jun-II were increased in neurons exposed to Aβ<sub>25–35</sub> peptide (Fig. 6). Pretreatment with LBA at 100 µg/ml markedly attenuated the levels of these three phosphorylated proteins. The protein levels of total JNK, total c-Jun and β-actin were unchanged after treatment of LBA or LiCl.

#### 3.6. Comparison of neuroprotective effects between the specific JNK inhibitor SP 600125 and LBA

Western-blot analysis results suggested that neuroprotection mechanism of LBA might rely on inhibition of the JNK signaling pathway. The specific inhibitor of JNK, SP600125, has been shown to exert neuroprotection against Aβ peptide toxicity (Hashimoto et al., 2003). We then compared SP600125 with LBA to examine their effectiveness in neuroprotection. Fig. 7 shows the caspase-3-like activity after SP600125 and LBA treatment. The unit of concentration of SP600125 was converted from mM to µg/ml for the purpose of comparison. While SP600125 could significantly reduce the toxicity to  $27.6 \pm 10.0\%$  at 1.1 µg/ml (5 µM), low concentration (1 µM or 0.22 µg/ml) of SP600125 could not attenuate Aβ-stimulated caspase-3 activity. Neuroprotective effects of SP600125 at 4.4 µg/ml (20 µM) could reduce Aβ peptide neurotoxicity to

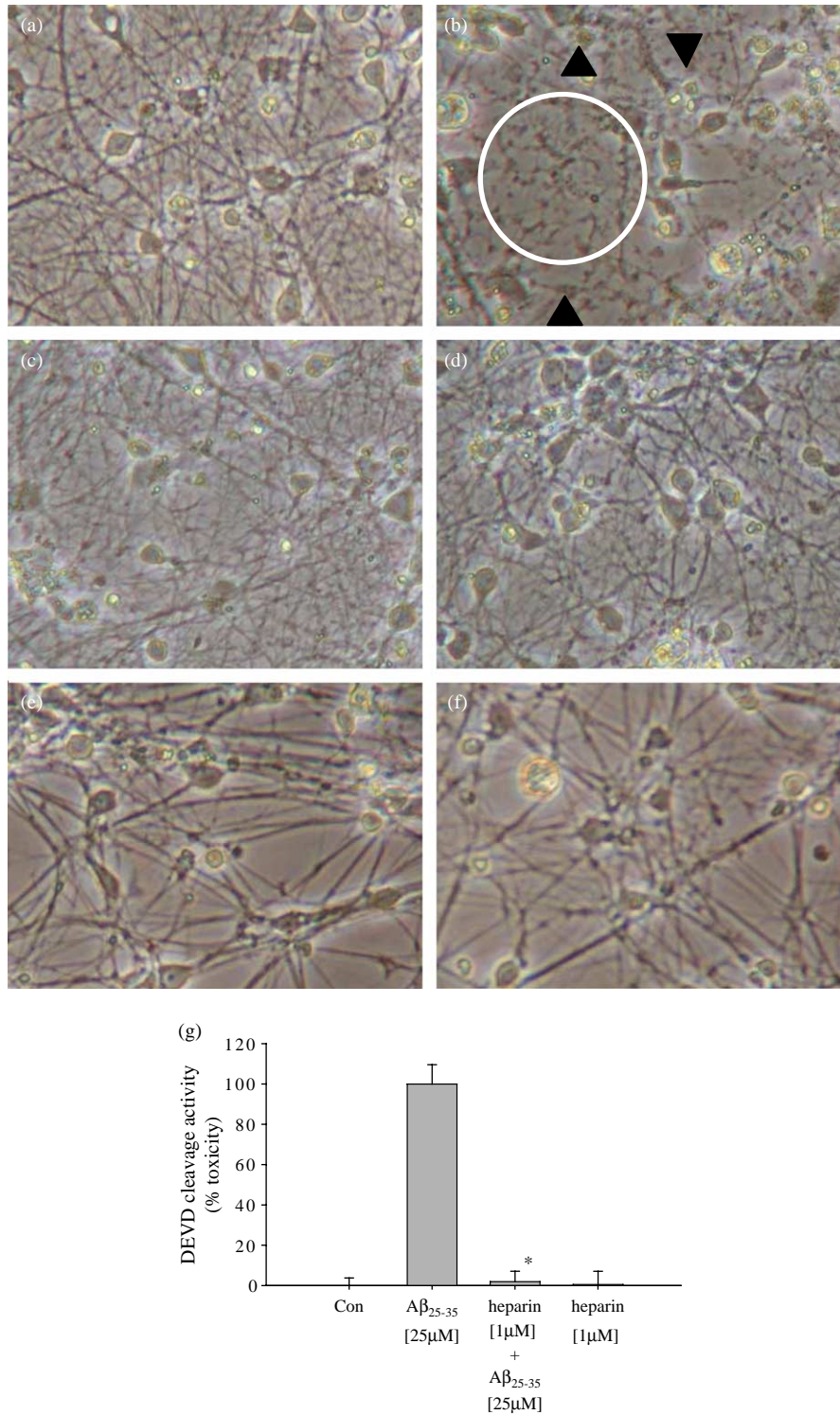


Fig. 5. Comparison of neuroprotective effects of LBA and heparin. Neurons were pre-incubated with either heparin (1  $\mu$ M) or LBA (100  $\mu$ g/ml) for 1 h, followed by a 24-hour exposure to  $A\beta_{25-35}$  (25  $\mu$ M). Morphology of neurons after different treatments was shown. (a) Control, (b)  $A\beta_{25-35}$  (25  $\mu$ M) ( $\rightarrow$  indicates shrunken cell body and the circled area indicates the destroyed neurite network), (c) LBA (100  $\mu$ g/ml), (d) LBA (100  $\mu$ g/ml) +  $A\beta_{25-35}$  (25  $\mu$ M), (e) heparin (1  $\mu$ M) and (f) heparin (1  $\mu$ M) +  $A\beta_{25-35}$  (25  $\mu$ M). (g) Caspase-3 like activity was determined after treatment with heparin (1  $\mu$ M) and  $A\beta_{25-35}$  (25  $\mu$ M). DEVD cleavage activity was expressed as % toxicity. Percentage toxicity is calculated as:  $(S.A.A\beta/heparin-treated - S.A.control) \div (S.A.A\beta-treated - S.A.control)$ . Results are expressed as mean  $\pm$  SE from at least three independent experiments. \* $P < 0.001$  vs. the group treated with  $A\beta$  peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

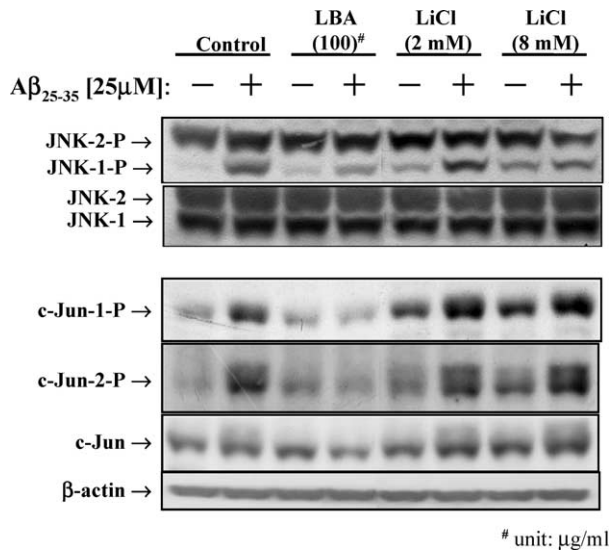


Fig. 6. Western-blot analysis of phospho-JNK and phospho-c-Jun in neurons treated with LBA and LiCl. Neurons were pretreated with LBA (100 μg/ml) or LiCl (2 mM and 8 mM) for 1 h, followed by a 4-hour exposure to Aβ<sub>25-35</sub> peptide (25 μM). Proteins extracted were subjected to western-blot analysis to detect the level of phospho-JNK, phospho-c-Jun, total JNK, total c-Jun and β-actin. β-actin was used as the internal control. Results are expressed from at least three independent experiments.

70.7 ± 4.6% of control value only (Fig. 7). SP600125 at concentration higher than 20 μM was found to exhibit neurotoxicity (data not shown). Comparing the effective dosages between SP600125 and LBA, the therapeutic window for SP600125 was much narrow compared to that of LBA.

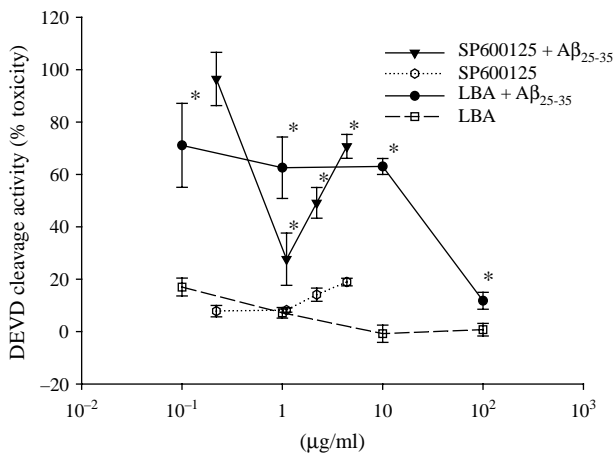


Fig. 7. Comparison of neuroprotective effects between the specific JNK inhibitor SP 600125 and LBA. Neurons were pretreated with either SP600125 (1–20 μM) or LBA (0.1–500 μg/ml) for 1 h, then, incubated with or without Aβ<sub>25-35</sub> peptide (25 μM) for 16 h. After treatment, colorimetric caspase-3-like activity assay was done to examine the level of apoptosis. DEVD cleavage activity was expressed as % toxicity. Percentage toxicity is calculated as: (S.a.of Aβ/LBA-/SP-treated - S.a.control) ÷ (S.a.Aβ-treated - S.a.control). Results are expressed as mean ± SE from at least three independent experiments. \*P < 0.001 vs. the group treated with Aβ peptide only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

Table 1  
Some properties of LBA isolated from *Lycium barbarum*

	LBA
% Yield <sup>a</sup>	1.7
Protein <sup>b</sup>	6.2
Galacturonic acid <sup>b</sup>	23.9
Neutral sugars <sup>b</sup>	61
Arabinose <sup>c</sup>	35.1
Galactose <sup>c</sup>	16.0
Rhamnose <sup>c</sup>	10.0
Xylose <sup>c</sup>	4.0
Glucuronic acid <sup>c</sup>	1.3
Mannose <sup>c</sup>	0.8
Glucose <sup>c</sup>	8.9

<sup>a</sup> % Yield to the dried fruit.

<sup>b</sup> % Dry-weight basis.

<sup>c</sup> Mol %.

### 3.7. Chemical analysis of the aqueous extracts LBA

The chemical composition of the aqueous extracts of the *L. barbarum* was investigated without further purification. The neutral sugar and amino acid composition of the aqueous extract (LBA) was determined. According to the isolation procedure used, LBA contained of 61% (w/w) carbohydrates and 6% (w/w) protein. The protein content was determined from the amino acid analysis. LBA contained arabinose (35%), galactose (16%), rhamnose (10%) in addition to glucose (9%) and galacturonic acid (24%) (Table 1). According to the amino acid analysis the protein consists of 14% (mol%) alanine, 14% cystine and 10% aspartic acid as main amino acids (Table 2). The amino acid analysis also revealed the presence of 0.7% methionine.

Table 2  
The amino acid composition of LBA isolated from *Lycium barbarum*

Amino acid	LBA (Mol %)
Asp	10.4
Glu	1.4
Ser	9.3
Gly	4.9
His	2.8
Arg	9.0
Thr	5.8
Ala	13.6
Pro	4.5
Tyr	3.2
Val	2.9
Met <sup>a</sup>	0.7
Cys <sup>a</sup>	13.6
Ile	2.3
Leu	3.5
Phe	1.5
Lys	4.6
Ammonia	6.1
Total	100.00

<sup>a</sup> Separate analysis using performic acid pre-oxidation treatment.

#### 4. Discussion

In the present study, we had isolated the aqueous extract from the fruits of *Lycium barbarum*, named as LBA, which could significantly protect neurons against A $\beta$  peptide toxicity at microgram level. We had used different parameters to examine the neuroprotective effects of LBA including the LDH cytotoxicity assay; caspase-3-like activity assay and DAPI staining for apoptosis measurement. In agreement to our previous studies (Chang et al., 2002a; Lin et al., 2004; Suen et al., 2003a,b), A $\beta$  peptides induced neuronal cell death as shown by the increase of both LDH and caspase-3-like activity. Our results showed that LBA could attenuate the caspase-3-like activity as well as the LDH cytotoxicity triggered by A $\beta$  peptide. Pretreatment with LBA markedly reduced the number of apoptotic neurons as revealed by DAPI staining. LBA has wide effective dosages when compared with the well-known western neuroprotective drug LiCl. Also, LBA could suppress the A $\beta$ -triggered JNK signaling pathway. Since A $\beta$ -induced apoptosis results in neurodegeneration (Zhang and Herman, 2002), our data suggest that aqueous extract purified from the fruits of anti-aging *Lycium barbarum* are potential neuroprotective agents against neuronal degeneration.

In traditional Chinese medicine, many plants and their active constituents have been widely used for their reputed cognitive-enhancing or anti-aging effects. These herbs include *Lycium barbarum*, *Ginkgo biloba* L., *Lycoris radiata*, *Magnolia officinalis* and *Polygala tenuifolia* Willd (Blasko et al., 2005; Howes and Houghton, 2003). Galantamine is an alkaloid isolated from *Lycoris radiata*. At present, it is the most effective acetylcholinesterase inhibitor which has been launched in the market to treat the cognitive impairment associated with AD (Allain et al., 2003). However, this type of treatment can only ameliorate the symptoms of AD. Increasing lines of evidence have shown that neurodegenerative diseases result from multiple causes such as apoptosis, excessive levels of reactive oxygen species, deficits in neurotransmission, dysfunction of neurons and progressive neuronal loss (Waldmeier and Tatton, 2004). Thus, effort should be made to explore potential candidates that can prevent or delay apoptosis and neuronal dysfunctions found in the diseases. In addition, the idea of anti-amyloid therapy (e.g. inhibition of A $\beta$  aggregation, vaccination with A $\beta$  antibodies and modulation of A $\beta$  production) is also possible (Citron, 2004; Gandy et al., 2003). Current treatment solely based on the acetylcholine receptor may not be sufficient to prevent neuronal death in AD. Owing to the fact that various botanical herbs have been used for many years to prevent aging in oriental world, it is possible to open up the potential for using anti-aging drugs from herbal medicine to treat AD. Recent findings showed that natural plant extracts have the potential not only prevent A $\beta$  toxicity, but also prevent the production of A $\beta$ . Ginkgo biloba extract EGB761 effectively

attenuated A $\beta$ -triggered apoptosis and inhibited A $\beta$  aggregation in vitro (Luo et al., 2002). Curcumin could bind small A $\beta$  peptides to block A $\beta$  aggregation as well as fibril and oligomer A $\beta$  formation (Yang et al., 2005). In present study, we found that the extract LBA can protect neurons against A $\beta$  toxicity, however, we cannot exclude the possibility that LBA may also have the potential to prevent A $\beta$  production and further investigation is required in this direction.

LiCl has been used to treat manic depression for decades (Chuang et al., 2002). Previous studies demonstrated that LiCl protects neurons against different insults in both animal and cellular models (Hashimoto et al., 2002; Nonaka and Chuang, 1998). LiCl can protect neurons by direct inhibition on GSK3 $\beta$ , thus providing protection from caspase-3 activation (King et al., 2001). LiCl can also prevent stress-induced c-Jun protein activation and downstream apoptotic events (Hongisto et al., 2003). Other studies illustrated that LiCl can attenuate A $\beta$ -induced neuronal cell death as shown by the calcein acetoxymethyl ester (calcein-AM) and propidium iodide (PI) staining (Alvarez et al., 1999) as well as MTT analysis (Wei et al., 2000). Since LiCl protects neurons via inhibition on caspase-3 activity and other pro-apoptotic pathways, we have attempted to compare the effectiveness of LiCl with LBA (Fig. 6). In contrast to LiCl, LBA exhibits neuroprotective effects ranging from 0.1 to 100  $\mu$ g/ml, suggesting that LBA will be much flexible for the future development in vivo. Nevertheless, LiCl has been shown to attenuate GSK3 to produce A $\beta$  peptides (Sun et al., 2002). Other aspects of beneficial effects of LiCl cannot be neglected.

Heparin (a naturally occurring polysaccharides with defined chemical structure) has been shown to exert protection for neurons against A $\beta$  toxicity (Bergamaschini et al., 2002). Owing to its chemical nature of polysaccharides, it is viable to compare its effect with LBA on A $\beta$  peptide toxicity. Neurons treated with heparin (5  $\mu$ g/ml) could significantly reduce the A $\beta$ -stimulated caspase-3 activity (Fig. 5f). Nevertheless, the morphology of neurons after heparin treatment is totally different from that of LBA (Fig. 5). Neurons exposed to heparin exhibit elongated neurites (Fig. 5e). The intact neuritis shown in Fig. 5f indicates that heparin can protect neurons from A $\beta$  peptide-induced cell death. Heparin has been shown to interact with growth factors forming heparin-binding growth factors which will then bind to specific membrane receptors (Kan and Shi, 1990; Shah et al., 2004). Also, heparin has been reported to bind A $\beta$  peptides (Brunden et al., 1993). The elongated neurites shown in Fig. 5e may result from the binding of heparin to the membrane proteins, thus altering the normal cell membrane structure. This may further prevent the interaction between A $\beta$  peptide and its target receptors such as tumor necrosis factor type I receptor (TNFRI) (Li et al., 2004), Fas and p75 neurotrophin receptor (Tsukamoto et al., 2003). As stated, physical interaction between heparin and A $\beta$  peptides and membrane proteins has also been reported. This may be a possible mechanism

for heparin to protect neurons against A $\beta$  peptide toxicity. The differences in morphology of neurons after treatment with LBA or heparin suggest that the mechanisms of neuroprotection elicited by them are mediated by different pathways. A $\beta$  peptides have been shown to possess membrane destabilizing properties that may lead to cell death (Mason et al., 1999; Mingeot-Leclercq et al., 2002). From our results, pretreatment with LBA significantly protects neurons from A $\beta$ -induced apoptosis. Whether LBA has the stabilizing effects on A $\beta$  peptides may be one possible explanation accounts for its neuroprotective effects.

Activation of stress kinases such as the c-Jun N-terminal kinase (JNK), p38 MAP kinase and double-strand RNA dependent protein kinase (PKR) has been demonstrated to be associated with A $\beta$ -triggered apoptosis in vitro (Chang et al., 2002a; McDonald et al., 1998; Morishima et al., 2001; Suen et al., 2003b). In AD brain sections, increased immunoreactivity of these activated stress kinases has also been found when compared to the age-matched control (Chang et al., 2002b; Hensley et al., 1999; Onuki et al., 2004; Peel and Bredesen, 2003; Xie et al., 2000). c-Jun is a substrate of JNK and it has been identified to be activated in AD brains (Anderson et al., 1994) as well as A $\beta$ -treated neurons (Anderson et al., 1995). Since the JNK–c-jun signaling pathway is closely linked to A $\beta$  peptide neurotoxicity, we have hypothesized that this signaling pathway is involved in the neuroprotective mechanism of LBA. Western blot analysis (Fig. 6) shows that LBA at 100  $\mu$ g/ml can significantly reduce the level of A $\beta$ -stimulated phospho-JNK, phospho-c-jun-I and phospho-c-jun-II. These results demonstrate that LBA suppresses A $\beta$  toxicity by the inhibition on the JNK–c-jun signaling pathway.

From the western blotting, it is possible that the neuroprotective mechanism of LBA relies on inhibition of the JNK signaling pathway. SP600125, a specific inhibitor of JNK, has been shown to provide protection for neurons against A $\beta$  peptide toxicity (Hashimoto et al., 2003). Comparing SP600125 and LBA, the effective dosage of LBA to resist A $\beta$  toxicity is much wider than that of SP600125 (Fig. 7). The results suggest that LBA may inhibit other pro-apoptotic signaling pathways other than JNK. We are examining other stress kinases such as p38, PKR or ASK1.

Arabinogalactan-protein (AGPs) is proteoglycans which are widely distributed in the plant kingdom. The chemical composition of AGPs consists of a high proportion of galactose and arabinose residues with usually less than 10% protein (Brillouet et al., 1996; Fincher et al., 1983). Recent studies suggest that AGPs is one of the major ingredients of the aqueous extract of *Lycium chinense* Mill and can be responsible for the variety of biological functions (Qin et al., 2001). As composition of LBA is similar to AGP, we will further examine whether there is

any define composition of AGP in protection of neurons against A $\beta$  peptide.

Taken together, our findings suggest that LBA purified from the fruits of *L. barbarum* exhibit neuroprotective effects against extracellular A $\beta$  peptide-induced apoptosis. The neuroprotective mechanism of LBA may depend on the inhibition of the JNK signaling pathway. Future effort will focus on the isolation and the elucidation of the neuroprotective compounds from this extract. In conclusion, extracts from *L. barbarum* represent a potential neuroprotective agent which deserved to be further explored to prevent neurodegeneration in AD.

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## References

- Allen, J.W., Eldadah, B.A., Huang, X., Knoblach, S.M., Faden, A.I., 2001. Multiple caspases are involved in beta-amyloid-induced neuronal apoptosis. *J. Neurosci. Res.* 65, 45–53.
- Allain, H., tue-Ferrer, D., Tribut, O., Gauthier, S., Michel, B.F., Drieu-La, R.C., 2003. Alzheimer's disease: the pharmacological pathway. *Fundam. Clin. Pharmacol.* 17, 419–428.
- Alvarez, G., Munoz-Montano, J.R., Satrustegui, J., Avila, J., Bogonez, E., az-Nido, J., 1999. Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. *FEBS Lett.* 453, 260–264.
- Anderson, A.J., Cummings, B.J., Cotman, C.W., 1994. Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. *Exp. Neurol.* 125, 286–295.
- Anderson, A.J., Pike, C.J., Cotman, C.W., 1995. Differential induction of immediate early gene proteins in cultured neurons by beta-amyloid (A beta): association of c-Jun with A beta-induced apoptosis. *J. Neurochem.* 65, 1487–1498.
- Bastianetto, S., Quirion, R., 2002. Natural extracts as possible protective agents of brain aging. *Neurobiol. Aging* 23, 891–897.
- Bergamaschini, L., Donarini, C., Rossi, E., De L, A., Vergani, C., De Simoni, M.G., 2002. Heparin attenuates cytotoxic and inflammatory activity of Alzheimer amyloid-beta in vitro. *Neurobiol. Aging* 23, 531–536.
- Blasko, I., Kemmler, G., Krampla, W., Jungwirth, S., Wichart, I., Jellinger, K., Tragl, K.H., Fischer, P., 2005. Plasma amyloid beta protein 42 in non-demented persons aged 75 years: effects of concomitant medication and medial temporal lobe atrophy. *Neurobiol. Aging* 26, 1135–1143.

- Brillouet, J.M., Williams, P., Will, F., Muller, G., Pellerin, P., 1996. Structural characterization of an apple juice arabinogalactan-protein which aggregates following enzymic daarabinosylation. *Carbohydr. Polym.* 29, 271–275.
- Brunden, K.R., Richter-Cook, N.J., Chaturvedi, N., Frederickson, R.C., 1993. pH-dependent binding of synthetic beta-amyloid peptides to glycosaminoglycans. *J. Neurochem.* 61, 2147–2154.
- Canevari, L., Abramov, A.Y., Duchon, M.R., 2004. Toxicity of amyloid beta peptide: tales of calcium, mitochondria, and oxidative stress. *Neurochem. Res.* 29, 637–650.
- Chang, I.M., 2001. Anti-aging and health-promoting constituents derived from traditional oriental herbal remedies: information retrieval using the TradiMed 2000 DB. *Ann. N.Y. Acad. Sci.* 928, 281–286.
- Chang, R.C., Suen, K.C., Ma, C.H., Elyaman, W., Ng, H.K., Hugon, J., 2002a. Involvement of double-stranded RNA-dependent protein kinase and phosphorylation of eukaryotic initiation factor-2alpha in neuronal degeneration. *J. Neurochem.* 83, 1215–1225.
- Chang, R.C., Wong, A.K., Ng, H.K., Hugon, J., 2002b. Phosphorylation of eukaryotic initiation factor-2alpha (eIF2alpha) is associated with neuronal degeneration in Alzheimer's disease. *Neuroreport* 13, 2429–2432.
- Chiba, T., Yamaza, H., Higami, Y., Shimokawa, I., 2002. Anti-aging effects of caloric restriction: involvement of neuroendocrine adaptation by peripheral signaling. *Microsc. Res. Tech.* 59, 317–324.
- Chuang, D.M., Chen, R.W., Chalecka-Franaszek, E., Ren, M., Hashimoto, R., Senatorov, V., Kanai, H., Hough, C., Hiroi, T., Leeds, P., 2002. Neuroprotective effects of lithium in cultured cells and animal models of diseases. *Bipolar Disord.* 4, 129–136.
- Citron, M., 2004. Strategies for disease modification in Alzheimer's disease. *Nat. Rev. Neurosci.* 5, 677–685.
- Eckert, A., Marques, C.A., Keil, U., Schussel, K., Muller, W.E., 2003. Increased apoptotic cell death in sporadic and genetic Alzheimer's disease. *Ann. N.Y. Acad. Sci.* 1010, 604–609.
- Fincher, G.B., Stone, B.A., Clarke, A.E., 1983. Arabinogalactan-protein-structure, biosynthesis and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 34, 47–70.
- Gandy, S., Martins, R.N., Buxbaum, J., 2003. Molecular and cellular basis for anti-amyloid therapy in Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* 17, 259–266.
- Harada, J., Sugimoto, M., 1999. Activation of caspase-3 in beta-amyloid-induced apoptosis of cultured rat cortical neurons. *Brain Res.* 842, 311–323.
- Hashimoto, R., Hough, C., Nakazawa, T., Yamamoto, T., Chuang, D.M., 2002. Lithium protection against glutamate excitotoxicity in rat cerebral cortical neurons: involvement of NMDA receptor inhibition possibly by decreasing NR2B tyrosine phosphorylation. *J. Neurochem.* 80, 589–597.
- Hashimoto, Y., Tsuji, O., Niihara, T., Yamagishi, Y., Ishizaka, M., Kawasumi, M., Chiba, T., Kanekura, K., Yamada, M., Tsukamoto, E., Kouyama, K., Terashita, K., Aiso, S., Lin, A., Nishimoto, I., 2003. Involvement of c-Jun N-terminal kinase in amyloid precursor protein-mediated neuronal cell death. *J. Neurochem.* 84, 864–877.
- Hensley, K., Floyd, R.A., Zheng, N.Y., Nael, R., Robinson, K.A., Nguyen, X., Pye, Q.N., Stewart, C.A., Geddes, J., Markesbery, W.R., Patel, E., Johnson, G.V., Bing, G., 1999. p38 kinase is activated in the Alzheimer's disease brain. *J. Neurochem.* 72, 2053–2058.
- Holliday, R., 1996. The urgency of research on ageing. *Bioessays* 18, 89–90.
- Hongisto, V., Smeds, N., Brecht, S., Herdegen, T., Courtney, M.J., Coffey, E.T., 2003. Lithium blocks the c-Jun stress response and protects neurons via its action on glycogen synthase kinase 3. *Mol. Cell Biol.* 23, 6027–6036.
- Howes, M.J., Houghton, P.J., 2003. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. *Pharmacol. Biochem. Behav.* 75, 513–527.
- Kagan, B.L., Hirakura, Y., Azimov, R., Azimova, R., Lin, M.C., 2002. The channel hypothesis of Alzheimer's disease: current status. *Peptides* 23, 1311–1315.
- Kan, M., Shi, E.G., 1990. Fibronectin, not laminin, mediates heparin-dependent heparin-binding growth factor type I binding to substrata and stimulation of endothelial cell growth. *In Vitro Cell Dev. Biol.* 26, 1151–1156.
- Kawakami, K., Kadota, J., Iida, K., Shirai, R., Abe, K., Kohno, S., 1999. Reduced immune function and malnutrition in the elderly. *Tohoku J. Exp. Med.* 187, 157–171.
- King, T.D., Bijur, G.N., Jope, R.S., 2001. Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3beta and attenuated by lithium. *Brain Res.* 919, 106–114.
- Lashuel, H.A., Hartley, D., Petre, B.M., Walz, T., Lansbury Jr., P.T., 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 418, 291.
- Lei, H., Wang, B., Li, W.P., Yang, Y., Zhou, A.W., Chen, M.Z., 2003. Anti-aging effect of astragalosides and its mechanism of action. *Acta Pharmacol. Sin.* 24, 230–234.
- Li, R., Yang, L., Lindholm, K., Konishi, Y., Yue, X., Hampel, H., Zhang, D., Shen, Y., 2004. Tumor necrosis factor death receptor signaling cascade is required for amyloid-beta protein-induced neuron death. *J. Neurosci.* 24, 1760–1771.
- Lin, M.C., Kagan, B.L., 2002. Electrophysiologic properties of channels induced by Abeta25-35 in planar lipid bilayers. *Peptides* 23, 1215–1228.
- Lin, K.F., Chang, R.C., Suen, K.C., So, K.F., Hugon, J., 2004. Modulation of calcium/calmodulin kinase-II provides partial neuroprotection against beta-amyloid peptide toxicity. *Eur. J. Neurosci.* 19, 2047–2055.
- Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J., Cotman, C.W., 1993. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc. Natl Acad. Sci. USA* 90, 7951–7955.
- Lu, X., Xian, X., Lu, W., Wu, X., Gu, H., 1999. The regulation of Lycium barbarum on apoptosis of rat spleen in vitro. *Zhong Yao Cai* 22, 250–251.
- Luo, Y., Smith, J.V., Paramasivam, V., Burdick, A., Curry, K.J., Buford, J.P., Khan, I., Netzer, W.J., Xu, H., Butko, P., 2002. Inhibition of amyloid-beta aggregation and caspase-3 activation by the Ginkgo biloba extract EGb761. *Proc. Natl Acad. Sci. USA* 99, 12197–12202.
- Mason, R.P., Jacob, R.F., Walter, M.F., Mason, P.E., Avdulov, N.A., Chochina, S.V., Igbavboa, U., Wood, W.G., 1999. Distribution and fluidizing action of soluble and aggregated amyloid beta-peptide in rat synaptic plasma membranes. *J. Biol. Chem.* 274, 18801–18807.
- Mattson, M.P., 2000. Emerging neuroprotective strategies for Alzheimer's disease: dietary restriction, telomerase activation, and stem cell therapy. *Exp. Gerontol.* 35, 489–502.
- McDonald, D.R., Bamberger, M.E., Combs, C.K., Landreth, G.E., 1998. Beta-amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. *J. Neurosci.* 18, 4451–4460.
- Mingeot-Leclercq, M.P., Lins, L., Bensliman, M., Van B, F., Van Der S, P., Peuvot, J., Schanck, A., Bresseur, R., 2002. Membrane destabilization induced by beta-amyloid peptide 29–42: importance of the amino-terminus. *Chem. Phys. Lipids* 120, 57–74.
- Mobley, D.L., Cox, D.L., Singh, R.R., Maddox, M.W., Longo, M.L., 2004. Modeling amyloid beta-peptide insertion into lipid bilayers. *Biophys. J.* 86, 3585–3597.
- Morishima, Y., Gotoh, Y., Zieg, J., Barrett, T., Takano, H., Flavell, R., Davis, R.J., Shirasaki, Y., Greenberg, M.E., 2001. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *J. Neurosci.* 21, 7551–7560.
- Nardi, N., Avidan, G., Daily, D., Zilkha-Falb, R., Barzilai, A., 1997. Biochemical and temporal analysis of events associated with apoptosis

- induced by lowering the extracellular potassium concentration in mouse cerebellar granule neurons. *J. Neurochem.* 68, 750–759.
- Nonaka, S., Chuang, D.M., 1998. Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. *Neuroreport* 9, 2081–2084.
- Onuki, R., Bando, Y., Suyama, E., Katayama, T., Kawasaki, H., Baba, T., Tohyama, M., Taira, K., 2004. An RNA-dependent protein kinase is involved in tunicamycin-induced apoptosis and Alzheimer's disease. *EMBO J.* 23, 959–968.
- Peel, A.L., Bredesen, D.E., 2003. Activation of the cell stress kinase PKR in Alzheimer's disease and human amyloid precursor protein transgenic mice. *Neurobiol. Dis.* 14, 52–62.
- Qin, X., Yamauchi, R., Aizawa, K., Inakuma, T., Kato, K., 2001. Structural features of arabinogalactan-proteins from the fruit of *Lycium chinense* Mill. *Carbohydr. Res.* 333, 79–85.
- Roth, K.A., 2001. Caspases, apoptosis, and Alzheimer disease: causation, correlation, and confusion. *J. Neuropathol. Exp. Neurol.* 60, 829–838.
- Selkoe, D.J., 1991. The molecular pathology of Alzheimer's disease. *Neuron* 6, 487–498.
- Sevag, M.G., 1934. Deproteinization and removal of capsular polysaccharides. *Biochem. Z.* 273, 419–423.
- Shah, B.H., Farshori, M.P., Catt, K.J., 2004. Neuropeptide-induced transactivation of a neuronal epidermal growth factor receptor is mediated by metalloprotease-dependent formation of heparin-binding epidermal growth factor. *J. Biol. Chem.* 279, 414–420.
- Suen, K.C., Lin, K.F., Elyaman, W., So, K.F., Chang, R.C., Hugon, J., 2003a. Reduction of calcium release from the endoplasmic reticulum could only provide partial neuroprotection against beta-amyloid peptide toxicity. *J. Neurochem.* 87, 1413–1426.
- Suen, K.C., Yu, M.S., So, K.F., Chang, R.C., Hugon, J., 2003b. Upstream signaling pathways leading to the activation of double-stranded RNA-dependent serine/threonine protein kinase in beta-amyloid peptide neurotoxicity. *J. Biol. Chem.* 278, 49819–49827.
- Sun, X., Sato, S., Murayama, O., Murayama, M., Park, J.M., Yamaguchi, H., Takashima, A., 2002. Lithium inhibits amyloid secretion in COS7 cells transfected with amyloid precursor protein C100. *Neurosci. Lett.* 321, 61–64.
- Tamagno, E., Robino, G., Obbili, A., Bardini, P., Aragno, M., Parola, M., Danni, O., 2003. H<sub>2</sub>O<sub>2</sub> and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK. *Exp. Neurol.* 180, 144–155.
- Troy, C.M., Rabacchi, S.A., Friedman, W.J., Frappier, T.F., Brown, K., Shelanski, M.L., 2000. Caspase-2 mediates neuronal cell death induced by beta-amyloid. *J. Neurosci.* 20, 1386–1392.
- Tsukamoto, E., Hashimoto, Y., Kanekura, K., Niikura, T., Aiso, S., Nishimoto, I., 2003. Characterization of the toxic mechanism triggered by Alzheimer's amyloid-beta peptides via p75 neurotrophin receptor in neuronal hybrid cells. *J. Neurosci. Res.* 73, 627–636.
- Waldmeier, P.C., Tatton, W.G., 2004. Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? *Drug Discov. Today* 9, 210–218.
- Wang, J., Wang, H., Zhang, M., Zhang, S., 2002a. Anti-aging function of polysaccharides from *Fructus lycii*. *Acta Nutrimenta Sinica* 24, 189–194.
- Wang, Y., Zhao, H., Sheng, X., Gambino, P.E., Costello, B., Bojanowski, K., 2002b. Protective effect of *Fructus Lycii* polysaccharides against time and hyperthermia-induced damage in cultured seminiferous epithelium. *J. Ethnopharmacol.* 82, 169–175.
- Wei, H., Leeds, P.R., Qian, Y., Wei, W., Chen, R., Chuang, D., 2000. Beta-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment. *Eur. J. Pharmacol.* 392, 117–123.
- Wong, C.K., Leung, K.N., Fung, K.P., Choy, Y.M., 1994. Immunomodulatory and anti-tumour polysaccharides from medicinal plants. *J. Int. Med. Res.* 22, 299–312.
- Wyllie, A.H., Kerr, J.F., Currie, A.R., 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68, 251–306.
- Xie, J., Guo, Q., Zhu, H., Wooten, M.W., Mattson, M.P., 2000. Protein kinase C iota protects neural cells against apoptosis induced by amyloid beta-peptide. *Brain Res. Mol. Brain Res.* 82, 107–113.
- Xu, C., Fang, Y., 2000. Experimental study on the anti-decrepit effect of *Lycium Chinese* Mill. *J. Ji Ning Med. Coll.* 23, 19–22.
- Xu, M., Zhang, X., Xu, S., 2001. Anti-aging effect of *Lycium Chinensis* capsule on *Drosophila*. *J. Tong Ji Univ. (Med. Sci.)* 22, 143–147.
- Yang, F., Lim, G.P., Begum, A.N., Ubeda, O.J., Simmons, M.R., Ambegaokar, S.S., Chen, P.P., Kaye, R., Glabe, C.G., Frautschy, S.A., Cole, G.M., 2005. Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J. Biol. Chem.* 280, 5892–5901.
- Zhang, Y., Herman, B., 2002. Ageing and apoptosis. *Mech. Ageing Dev.* 123, 245–260.