

# Neuroprotective Effects of Polysaccharides from Wolfberry, the Fruits of *Lycium barbarum*, Against Homocysteine-induced Toxicity in Rat Cortical Neurons

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**Abstract.** Previous clinical and epidemiological studies have suggested that elevated plasma homocysteine (Hcy) levels increased the risk of Alzheimer's disease (AD). Although the underlying mechanisms of its toxicity are elusive, it has been shown that Hcy damages neurons by inducing apoptosis, DNA fragmentation, and tau hyperphosphorylation. Wolfberry (*Lycium barbarum*) is a fruit that is known for its eye-protective and anti-aging properties in Asian countries. Previous studies from our laboratory have demonstrated that polysaccharides derived from wolfberry (LBA) have the ability to protect neurons from amyloid- $\beta$  ( $A\beta$ ) peptide neurotoxicity. We hypothesize that the neuroprotective effects of wolfberry is not limited to  $A\beta$  and can also provide protection against other AD risk factors. In this study, we aim to elucidate the neuroprotective effects of wolfberry against Hcy-induced neuronal damage. Our data showed that LBA treatment significantly attenuated Hcy-induced neuronal cell death and apoptosis in primary cortical neurons as demonstrated by LDH and caspase-3 like activity assay. LBA also significantly reduced Hcy-induced tau phosphorylation at tau-1 (Ser198/199/202), pS396 (Ser396), and pS214 (Ser214) epitopes as well as cleavage of tau. At the same time, we also found that the phosphorylation level of p-GSK3 $\beta$  (Ser9/Tyr 216) remained unchanged among different treatment groups at all detected time points. LBA treatment suppressed elevation of both p-ERK and p-JNK. In summary, our data demonstrated that LBA exerted neuroprotective effects on cortical neurons exposed to Hcy. Therefore, LBA has the potential to be a disease-modifying agent for the prevention of AD.

Keywords: Apoptosis, homocysteine, neuroprotection, tau phosphorylation, wolfberry

## INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino-acid which is involved in the metabolism of methionine. Levels of Hcy are controlled by the remethylation and transsulfuration pathways which in turn are regulated nutritionally [1]. Vitamin B6 and B12 are essen-

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tial in controlling Hcy levels as they act as co-factors for enzymes involved in the two pathways. Under normal condition, the plasma level of total Hcy is between 5 to 15  $\mu\text{mol/L}$ . However, this level is considerably higher among the elderly and even more severe among males [2]. The elevation in Hcy level, called hyperhomocysteinemia, can be caused by many factors. It has been suggested that folate or Vitamin B deficiency is associated with hyperhomocysteinemia, especially in the older populations [3]. Studies have shown that hyperhomocysteinemia is a risk factor for vascular diseases such as stroke [4] and myocardial infarction [5,6]. Moreover, it is also associated with several neurological disorders, including Alzheimer's disease (AD) [7–10], Parkinson's disease (PD) [11,12], and depression among the elderly [13–15]. Results from a prospective, observational study shows that even mild to moderate increases of plasma Hcy level increases the risk of AD. A 5  $\mu\text{mol/liter}$  increment of plasma Hcy level increases the risk of AD by 40% [8]. Folate deficiency and hyperhomocysteinemia precede the onset of dementia [7], further suggesting the causal role of Hcy in AD development. The underlying mechanisms of its toxicity remain elusive, but evidence shows that high levels of Hcy can induce direct toxicity to different types of tissues [16–18]. Some scientists proposed that Hcy-lowering therapy with vitamin B may be beneficial to health [19], while others conduct research on agents with direct protective effects against Hcy toxicity [20].

Wolfberry (the fruits of *Lycium barbarum*) is a food supplement that has recently gained popularity in North America, South Africa, and Europe. This dried fruit has been used as functional food as well as medicinal herbs in Asian countries for more than thousands of years. In *Chinese Materia Medica*, wolfberry is described as a "tonic-herb" and is believed to promote longevity. Wolfberry and its active component, *Lycium barbarum* polysaccharides, have been found to provide beneficial effects against multiple aging-associated conditions [21–28]. In our laboratory, we found that polysaccharides from aqueous extract of wolfberry (*Lycium barbarum*), known as LBA, elicit *in vitro* neuroprotective effects against amyloid- $\beta$  ( $A\beta$ ) toxicity [27]. Our recent data have also suggested that LBA is able to protect neurons against glutamate excitotoxicity in rat cortical neurons [29]. These findings have led to a hypothesis that LBA is able to protect neurons against neurodegeneration in AD through multiple effects. In the present study, we aim to investigate the protective effects of LBA on Hcy-induced toxicity in cultured primary cortical neurons.

## MATERIALS AND METHODS

### *Materials and chemicals*

The dry fruit of wolfberry (*Lycium barbarum*) was purchased from Ning Xia Huizu Autonomous Region. The glycosyl composition and amino acid composition analysis were carried out as previously described in the procedure by Yu and co-workers [27] as we used the same batch of LBA as in the previous report.

Materials for cell culture including minimum essential medium (MEM), penicillin, and streptomycin were purchased from Gibco-BRL (Burlington, Ont., Canada). Other chemicals were obtained from these following companies: D,L-homocysteine, protease inhibitor cocktail, phosphatase inhibitor cocktail, 4',6-diamidino-2-phenylindole (DAPI), and anti- $\beta$ -actin monoclonal antibody were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Lactate dehydrogenase (LDH) cytotoxicity assay kit from Roche Diagnostics (Mannheim, Germany). Caspase-3 substrate (Ac-DEVE-pNA) and caspase-3 inhibitor VII were obtained from Calbiochem, Inc. (La Jolla, CA, USA). Rabbit polyclonal antibodies against phosphorylated-JNK, total JNK, cleaved caspase-3, phosphorylated ERK1/2 (P44/42), total ERK, and phosphorylated GSK-3 $\beta$  (Ser9) were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibody against cleaved-Tau (Asp421) was purchased from Upstate (Lake Placid, New York, USA). Mouse monoclonal antibody GSK-3 $\beta$  (pY216) and Tau-5 against total tau were purchased from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal antibody tau pS396 against tau phosphorylated at Serine 396, tau pS214 against tau phosphorylated at Serine 214, and mouse polyclonal antibody tau-1 against non-phosphorylated tau at Serine 198/199/202 were purchased from BioSource International (Hopkinton, MA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were from DAKO (Glostrup, Denmark). PVDF membrane was from Bio-Rad (Richmond, CA, USA). Biomax X-ray film was from GE Healthcare (New York, USA). Enhanced chemiluminescence (ECL) detection kit was from Amersham (Buckinghamshire, UK).

### *Preparation of wolfberry polysaccharide (LBA) and its chemical analysis*

The wolfberry polysaccharide (LBA) used in this study was prepared from an aqueous extract according

to our previous publication [27]. In brief, the dry fruits of wolfberry were initially crushed into small pieces for better extraction. The residue was boiled in distilled water for 3 h to obtain an aqueous solution, which was further concentrated and deproteinated by the Sevag method [30]. The resulting aqueous solution underwent dialysis against distilled water for 2 days (Molecular Weight Cut Off: 6000–8000 Da). The retentate was concentrated and then precipitated by ethanol. The retentate was then concentrated and dried to provide a brown powder (LBA).

The LBA we used in this study is the same batch as in our previous publication [27], where the details of chemical analysis have been reported. The neutral sugar composition profile was conducted by the Complex Carbohydrate Research Center, The University of Georgia (Athens, GA, USA). In brief, LBA contains about 35% arabinose, 16% galactose, 10% rhamnose, and also some glucose, xylose, glucuronic acid, and manose. The purity of LBA was found to be 61% (w/w carbohydrates).

#### *Primary cortical cultures and experimental treatments*

Primary cortical cell cultures were established from embryonic day 17 Sprague-Dawley rat embryos via the method described previously [31,32]. Briefly, cortical neurons were dissected and seeded into 6-well plates pre-coated with poly-L-lysine (25  $\mu\text{g}/\text{ml}$ ) at a density of  $1.0 \times 10^6$  cells/well. The cultures were kept in culture medium that consisted of MEM supplemented with 5% heat inactivated fetal bovine serum, glucose (18 mM), L-glutamine (2 mM), insulin (5  $\mu\text{g}/\text{ml}$ ), progesterone (0.02  $\mu\text{M}$ ), putrescine (100  $\mu\text{M}$ ), selenium (30 pM), penicillin (50 U/ml), and streptomycin (50  $\mu\text{g}/\text{ml}$ ). Neurons were maintained at conditions of 37°C in a humidified 5% CO<sub>2</sub> atmosphere. All experiments were performed with 7-day old cultures. For pre-treatment experiment, primary cortical neurons were incubated with different dosages of LBA for 1 h and then co-incubated with Hcy [1 mM] for the duration as indicated. Western-blot analysis and examination of neuronal cell death were then performed. For the post-treatment experiment, neurons were incubated with Hcy [1 mM] for 1, 2, or 4 h before the subsequent addition of LBA for co-incubation. The cultures were further allowed to be incubated for 23 or 22 or 20 h, respectively.

#### *Measurement of general cytotoxicity with LDH assay*

Neuronal cell death in the various treatment groups was detected by lactate dehydrogenase (LDH) assay. The assay was conducted as described previously [33, 34]. In brief, culture medium was collected and centrifuged; cell free medium was incubated with the assay reagents for 30 min in dark. The release of LDH was determined by measuring the absorbance with a spectrophotometric plate reader at the wavelength 592 nm. Results were expressed as the fold of control (fold of control was calculated as follow: absorbance of Hcy-treated/LBA-treated/absorbance of control).

#### *Measurement of apoptosis with caspase-3-like activity assay*

Neuronal apoptosis was determined by measuring the caspase-3-like activity. Neurons were harvested and the concentration of the protein content was detected by Bio-Rad DC protein assay carried out according to the manufacturer's instructions. Fifty micrograms of protein from each sample were incubated with caspase-3 substrate (Ac-DEVD-pNA), DTT, and reaction buffer for 2 h at 37°C. Caspase-3-like activity was determined by measuring the absorbance (at 404 nm) of the yellow product (pNA) which cleaved from the substrate (i.e., DEVD cleavage) during the reaction. The values of specific activity (s.a., unit = pmol/min/ $\mu\text{g}$  of protein) were calculated. Results were expressed as fold of control.

#### *Determination of apoptotic nuclei with DAPI staining*

The number of apoptotic cells was determined by DNA staining with DAPI fluorescence probe as published [35]. Apoptotic neurons were characterized by the presence of fragmented or condensed nuclei. After treatment, neurons were fixed with methanol:acetone (1:1) for 20 min, and then stained with DAPI (1  $\mu\text{g}/\text{ml}$  in PBS) for 10 min. Five different fields were counted per treatment in triplicate under a fluorescent microscope. A total amount of approximately 200 neurons were counted in each field. Results were expressed as percentage of apoptotic cells from total cell counted.

#### *Western-blot analysis*

Total lysate was collected as described elsewhere [36]. In brief, neurons were lysed in ice-cold ly-

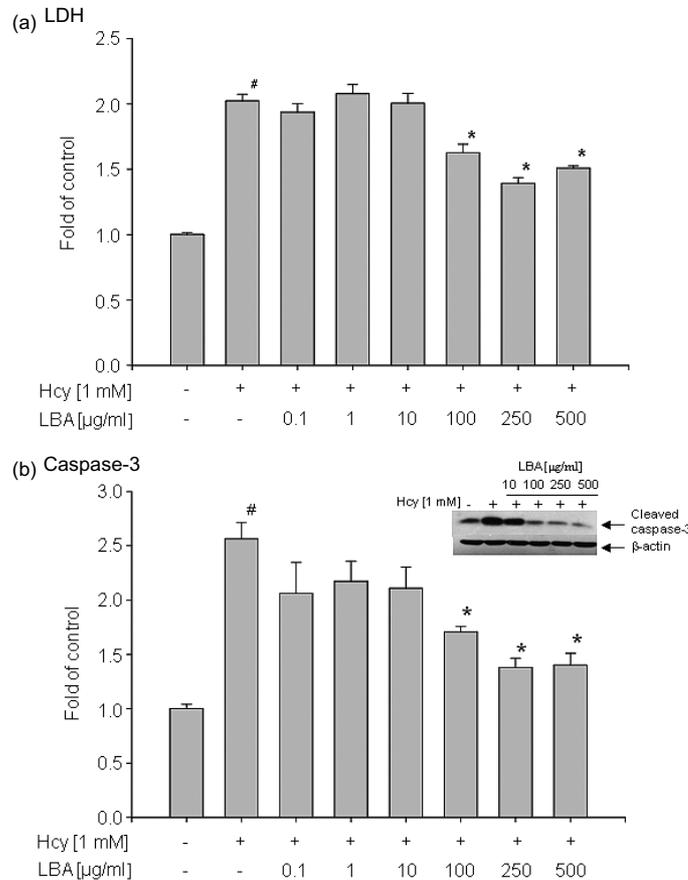


Fig. 1. LBA attenuated Hcy-induced neuronal cell death and apoptosis in primary cortical neurons. Rat cortical neurons were pre-treated with indicated dosages of LBA for 1 h followed by exposure to Hcy [1 mM] for 24 h. (a) LDH assay, which measured the LDH released into culture medium, was performed to determine the level of neuronal cell death. (b) Colorimetric caspase-3 like activity assay was carried out to detect the level of caspase-3 activation and therefore apoptosis. The specific activity of caspase-3 was  $0.21 \pm 0.01$  pmol/min/ $\mu$ g of protein in the control group. Changes in the level of caspase-3 were reconfirmed by Western-blot probed with cleaved caspase-3 antibody. Results were expressed as fold of control. Data represents mean  $\pm$  SE from at least 3 independent experiments. The significance of differences among treatment groups was determined by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test. <sup>#</sup> $p < 0.001$  compared to control. <sup>\*</sup> $p < 0.001$  compared to cultures treated with Hcy [1 mM].

sis buffer (10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 1% Triton-X-100, 10% glycerol, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and phosphatase inhibitor cocktail). After homogenization and centrifugation, the supernatant was collected. The sample were subjected to SDS 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by incubation with 5% w/v non-fat milk in TBST (TBS containing 0.1% Tween-20) for 1 h. Primary antibody were diluted in TBST and incubated with the membrane as described follow: cleaved-caspase 3 (1:2000, 4 h), cleaved-tau (Asp421) (1:1000, overnight), tau pS396

(1:2000, overnight), tau pS214 (1:1000, overnight), tau-1 (1:30,000, 4 h), tau-5 (1:4000, 4 h), phosphorylated ERK (1:2000, 2 h), non-phosphorylated ERK (1:2000, 2 h), phosphorylated JNK (1:1000, 4 h), non-phosphorylated JNK (1:1000, 4 h), GSK-3 $\beta$  (Ser9) (1:4000, 4 h), and GSK-3 $\beta$  (pY216, 1:4000, 4 h). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 h and subsequently developed by using the ECL or ECL-plus Western blotting detection kit. The membranes were then stripped with stripping buffer (50 mM glycine, 2% SDS, pH 2.0) and re-probed with monoclonal anti- $\beta$ -actin antibody (1:10,000, 1 h) and goat anti-mouse HRP secondary antibody. Opti-

cal density of the blots was measured with Image J software (National Institutes of Health, USA).

### *Statistical analysis*

Data were expressed as mean  $\pm$  standard error (SE) from at least 3 independent experiments. The significance of the differences among different groups was determined by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### *LBA protected neurons against Hcy-induced neuronal cell death*

Previous reports have shown that Hcy can induce neurotoxicity [37–39]. The first part of this study was to examine the protective effects of LBA on Hcy-induced neurotoxicity using LDH and caspase-3 like activity assay. Neurons had a normal turnover and baseline level of LDH release of  $12 \pm 0.3\%$  of total lysis. Exposure of cortical neurons to Hcy [1 mM] for 24 h resulted in neuronal cell death. This is reflected by 2-fold increase in LDH release into the culture medium (Fig. 1a). Pre-treatment of neurons with 100, 250, and 500  $\mu\text{g/ml}$  of LBA for 1 h significantly reduced cell death. Cell death was decreased to  $1.6 \pm 0.07$ ,  $1.4 \pm 0.04$ , and  $1.5 \pm 0.01$  fold of control when receiving 100, 250, or 500  $\mu\text{g/ml}$  of LBA, respectively. From our data, 250  $\mu\text{g/ml}$  of LBA led to the greatest reduction in LDH, further increase of LBA dosage did not provide additional protective effects. The effects of LBA on Hcy-induced apoptosis were examined by caspase-3 like activity assay. The basal level of caspase-3 like activity was  $0.30 \pm 0.04$  pmol/min/ $\mu\text{g}$  of protein in the control group. After Hcy treatment, there was a  $2.8 \pm 0.1$  fold increase. 250  $\mu\text{g/ml}$  of LBA attenuated the level of caspase-3 like activity to  $1.4 \pm 0.09$  fold of control (Fig. 1b). According to our previous report, incubation with LBA alone (0.1 to 500  $\mu\text{g/ml}$ ) does not have a significant effect on LDH release and caspase-3 like activity [27]. This suggests that incubation of LBA alone had no effect on the viability of primary cortical cells.

Cytoprotective effects of LBA were further confirmed by counting the number of apoptotic cells in various treatment groups. The upper panel of Fig. 2 shows neurons in each treatment group after DAPI staining.

Under fluorescent imaging, the nuclei appeared blue in color. Arrows indicate apoptotic cells with either condensed or fragmented nuclei. The percentage of apoptotic cells in treatment groups were calculated and presented in the lower panel of Fig. 2. In the control group,  $17.7 \pm 0.8\%$  of the total neurons were found to be apoptotic. Hcy markedly increased the number of apoptotic bodies to  $40.0 \pm 1.2\%$  of the population. Treatment with 500  $\mu\text{g/ml}$  of LBA significantly attenuated the number of apoptotic cells induced by Hcy to  $24.3 \pm 0.6\%$ .

### *Post-treatment of LBA rescued neurons from Hcy-induced toxicity*

Elevation of Hcy levels would increase the risk of developing AD and also induces direct cytotoxic effects to neurons. Neuronal rescue may offer an opportunity of improving disease prognosis. To evaluate the effects of LBA on neuronal rescue, post-treatment experiment was performed. Neurons were incubated with Hcy [1 mM] for 1, 2, 4 h before subsequent addition of LBA for co-incubation. The release of LDH (Fig. 3a) and the activities of caspase-3 (Fig. 3b) were significantly attenuated by LBA (100, 250, and 500  $\mu\text{g/ml}$ ) in all indicated post-treatment time points. From Fig. 1 and Fig. 3, it was suggested that 250  $\mu\text{g/ml}$  LBA was the most effective dosage for pre-treatment and post-treatment experiments. Taking 250  $\mu\text{g/ml}$  of LBA as an example for illustration of post-treatment, there was a  $31.2 \pm 3.2\%$  reduction in caspase-3-like activity even 4 h after exposure to Hcy. Our data suggested that LBA was effective in rescuing neurons from Hcy-induced toxicity.

### *LBA attenuated Hcy-induced tau phosphorylation at several epitopes*

Previous reports have shown that Hcy can induce tau phosphorylation, a possible link to the development of AD [37,40,41]. In this part of the study, we aim to investigate the effect of LBA on Hcy-induced tau phosphorylation. As shown in Fig. 4a, the immunoreactivity of Tau-1 (to detect non-phosphorylated tau at Ser 198/199/202) significantly decreased after incubation with Hcy for 4 h. In LBA-treated groups, the immunoreactivity of Tau-1 nearly recovered to the normal level as found in the control group. There was no significant difference in the band intensity of Tau-1 between LBA *per se* and the control (Fig. 4a). Hcy also induced tau phosphorylation at Ser 214; and LBA

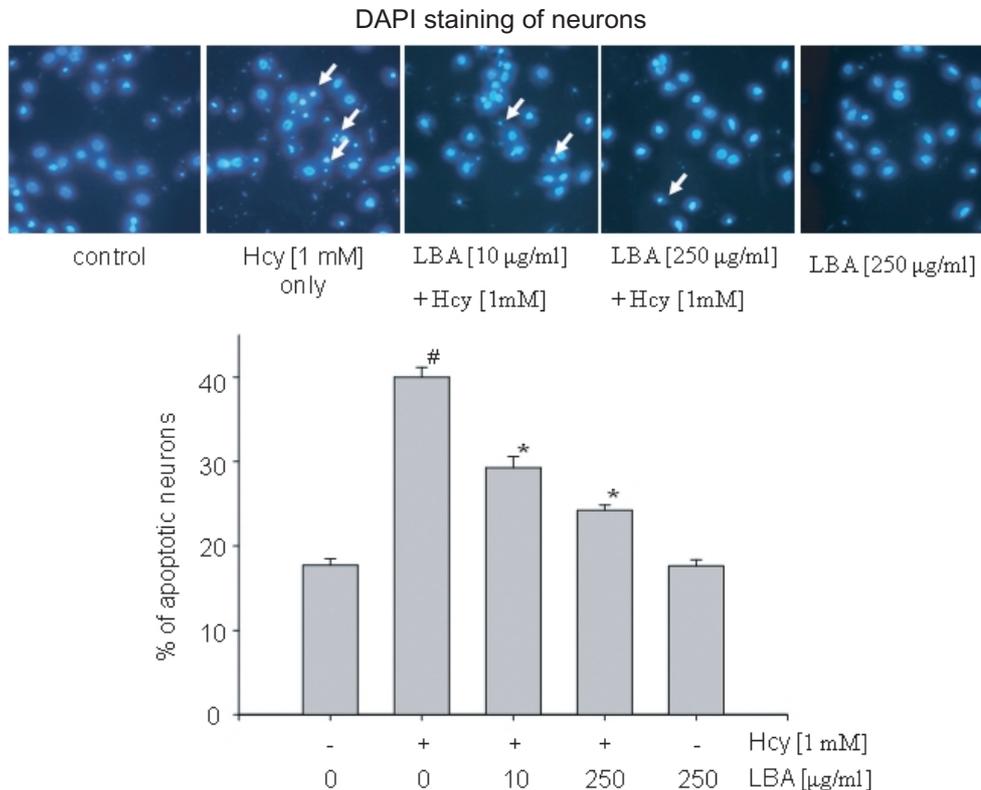


Fig. 2. Treatment of LBA reduced the number of Hcy-induced apoptotic neurons. Neurons were pre-treated with the indicated dosages of LBA for 1 h followed by exposure to Hcy [1 mM] for 24 h. The nuclei were stained with DAPI, which shows a blue color. Arrows indicated condensed or fragmented apoptotic nuclei. Five different fields were counted per treatment in triplicate under a fluorescent microscope. Results were expressed as the percentage of apoptotic cells. Data represent mean  $\pm$  SE from at least 3 independent experiments. The significance of differences among treatment groups was determined by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test. # $p < 0.001$  compared to control. \* $p < 0.001$  compared to cultures treated with Hcy [1 mM].

at 1 or 250  $\mu\text{g/ml}$  significantly attenuated this elevation (Fig. 4b). The increase in tau phosphorylation at Ser 396 was also reduced by LBA treatment (Fig. 4c). The level of total tau probed by Tau-5 did not show significant change (Fig. 4d).

#### *LBA attenuated Hcy-induced tau cleavage*

Proteolytic cleavage of tau protein at the C-terminus (truncation) has recently been identified as a possible link to the pathogenesis of AD [42,43]. Caspases can cleave tau at a highly conserved aspartate residue (Asp421) in its C terminus. We investigated the protective effects of LBA in preventing cleavage of tau. Fig. 5a shows the effects of LBA on Hcy-induced tau cleavage. After incubation with Hcy for 4 h, there was an increase in the immunoreactivity of cleaved-tau (Asp421). Pretreatment with 250  $\mu\text{g/ml}$  LBA for 1 h significantly inhibited the cleavage of tau induced by

Hcy and LBA *per se* did not affect the immunoreactivity of this protein. Tau cleavage at Asp421 site is believed to be caspase-3 dependent. We confirmed this by using caspase-3 inhibitor VII. The highest non-toxic dosage was found to be 20 nM (data not shown). With application of the inhibitor, the immunoreactivity of cleaved-tau was significantly reduced (Fig. 5b).

#### *Hcy did not change the phosphorylation state of GSK-3 $\beta$*

GSK-3 $\beta$  phosphorylates tau at large number of sites and is most implicated in abnormal hyperphosphorylation of tau in AD brain (for review, see [44]). The activity of GSK-3 $\beta$  can be regulated through serine and tyrosine phosphorylation. Phosphorylation at Ser 9 leads to inactivation of GSK-3 $\beta$  kinase activity while phosphorylation at Tyr 216 stimulates kinase activity. We had examined any changes in level of GSK-3 $\beta$  phos-

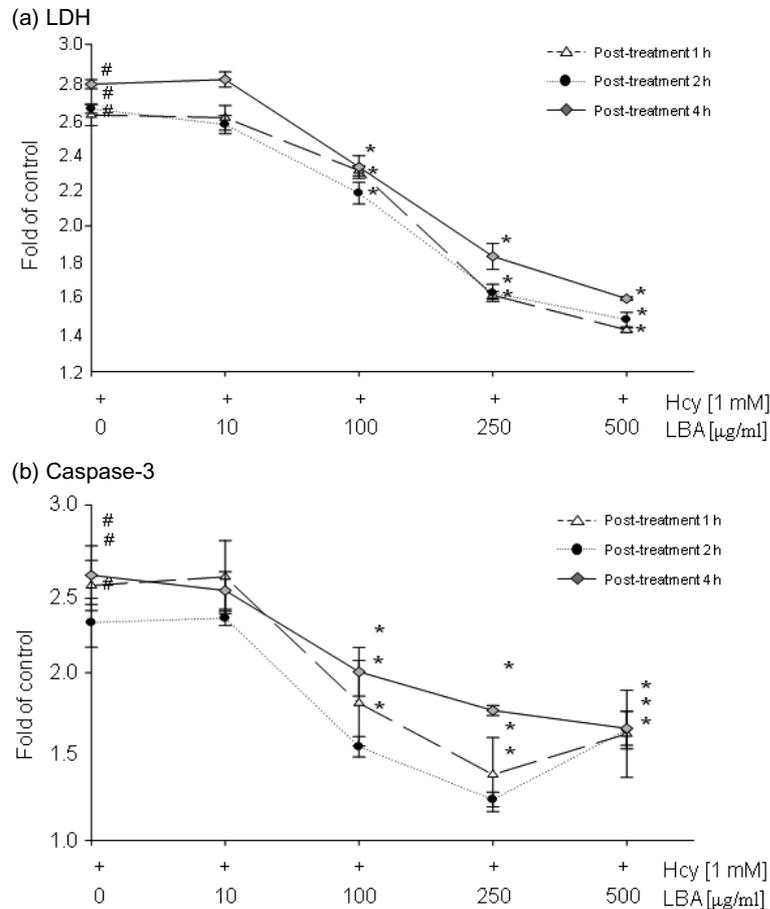


Fig. 3. Post-treatment of LBA attenuated neuronal cell death and apoptosis induced by Hcy. Neurons were exposed to homocysteine for 1 or 2 or 4 h prior to LBA for another 23 or 22 or 20 h respectively. (a) Release of LDH and (b) the level of caspase-3 like activity were measured. The value of control group in both (a) LDH and (b) the level of caspase-3 like activity were 1.0 fold of control. Data were analyzed by one-way ANOVA for multiple comparisons, followed by Student Newman-Keuls as post-hoc test. # $p < 0.001$  compared to control. \* $p < 0.001$  compared to cultures treated with Hcy [1 mM].

phorylation at different time points after Hcy-treatment (Fig. 6). Our data showed that there was no significant change of band intensity of phospho-GSK3 $\beta$  in Western-blot analysis for both Ser 9 and Tyr 216 epitopes at all measured time points. Even after 8 h of Hcy incubation, the band intensity of phospho-GSK3 $\beta$  remained relatively unchanged. Our results suggest that it is unlikely for LBA to protect neurons through modulating the activity of GSK-3.

#### *LBA attenuated Hcy-induced elevation of p-ERK-1 and p-JNK-1*

Besides GSK-3 $\beta$ , tau can be phosphorylated by certain members of the mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinas-

es (ERK1, ERK2), c-Jun N-terminal kinases (JNKs), and p38 [45–47]. ERK can be activated by phosphorylation at Thr180 or Tyr182 while JNK can be activated by phosphorylation at Thr 183/Tyr 185. We had examined any change of these two kinases after Hcy treatment. Our preliminary data showed that the band intensity of phospho-ERK1/2 in Western-blot analysis increased after 1 h of Hcy incubation and reached a peak at 4 h of incubation (data not shown). LBA (1 or 250  $\mu$ g/ml) significantly reduced the band intensity of phospho-ERK1/2 in neurons (Fig. 7a). The band intensity of phospho-JNK-1 was also examined. After Hcy-treatment, its band intensity in Western-blot increased gradually and reached a peak at 2 h (data not shown). Neurons treated with LBA (1, 100, or 250  $\mu$ g/ml) showed significant reduction of phospho-JNK-1 (Fig. 7b).

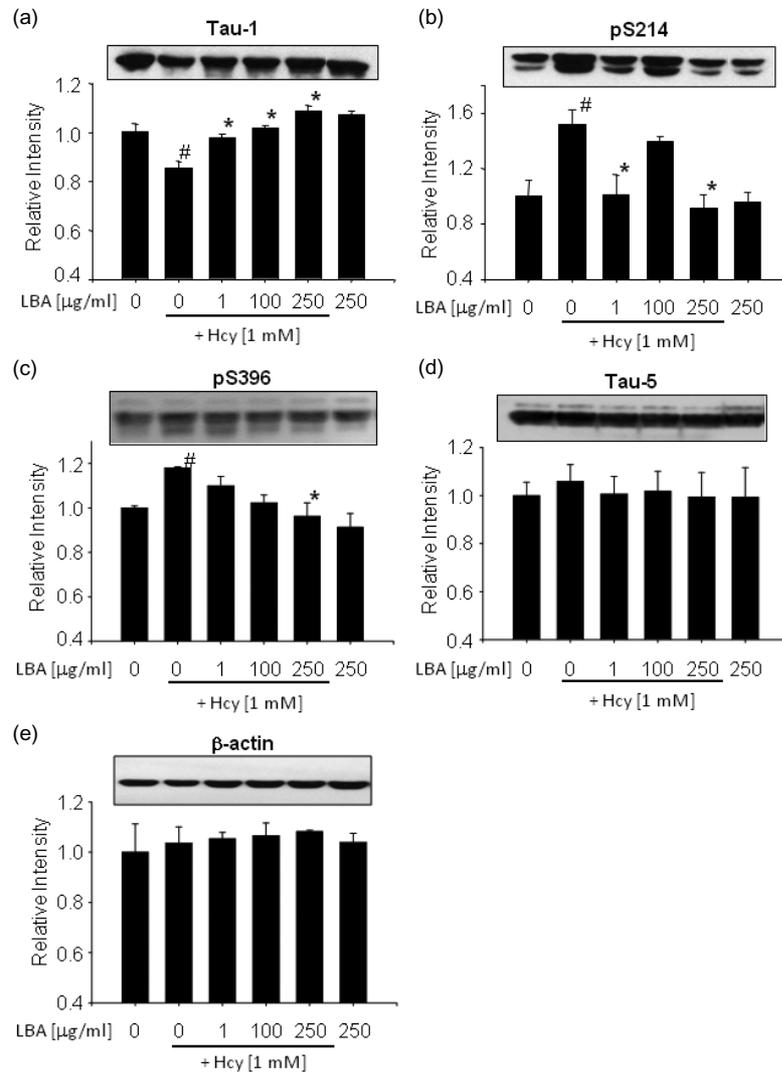


Fig. 4. LBA reduced Hcy-induced tau phosphorylation. Neurons were treated with indicated dosages of LBA for 1 h prior to the exposure to homocysteine for another 4 h. Cells were harvested for Western-blot analysis and tau phosphorylation was detected by Tau-1 (reacts with dephosphorylated tau at Ser 198/199/202), pS396 (reacts with phosphorylated tau at Ser 396) and pS214 (reacts with phosphorylated tau at Ser 214). Total tau was detected with the antibody Tau-5.  $\beta$ -Actin was used as internal control. Data represent mean  $\pm$  SE from at least 3 independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test. #  $p < 0.05$  compared to control. \*  $p < 0.05$  compared to cultures treated with Hcy [1 mM].

## DISCUSSION

Hyperhomocysteinemia has been considered to be associated with a number of neurological disorders including AD. *In vivo* experiments demonstrate possible linkage between Hcy and AD-like pathology. In a mouse model of amyloidosis, it has been found that the cystathionine-beta-synthase (CBS) mutation, which causes hyperhomocysteinemia, may lead to increased levels of  $A\beta_{40}$  and  $A\beta_{42}$  in the brain [48]. Wang and colleagues have also reported that intravenous injection

of Hcy for 2 weeks would induce tau hyperphosphorylation, increase amyloid- $\beta$  protein precursor (APP) cleavage and  $A\beta$  overproduction in rat brain hippocampus [40,49]. Hcy could also induce direct excitotoxicity to cultured cortical neurons by inducing calcium influx through NMDA receptor activation and therefore apoptosis [50]. Hcy could sensitize hippocampal neurons to  $A\beta$  toxicity in APP mutant transgenic mice [51]. Hcy may also indirectly increase the chance of developing AD by enhancing neurovascular ischemic diseases such as stroke [52,53]. Apart from animal ex-

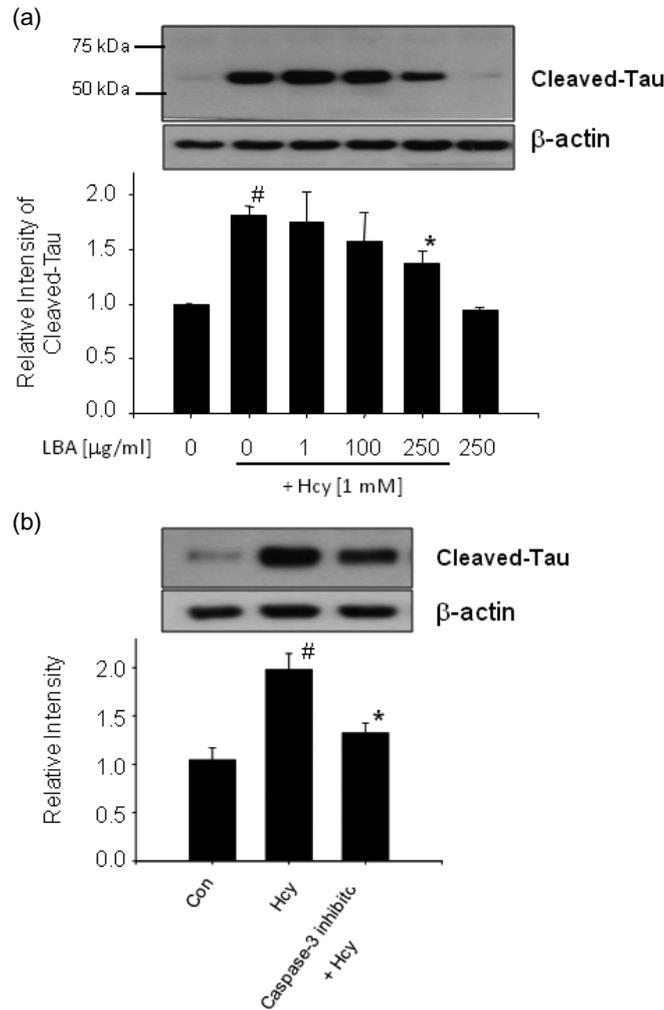


Fig. 5. LBA attenuated Hcy-induced tau cleavage. Neurons were treated with (a) indicated dosages of LBA or (b) caspase-3 inhibitor VII [20 nM] for 1 h prior to the exposure to homocysteine for another 4 h. Cells were harvested for Western-blot analysis and tau cleavage at Asp421 was detected.  $\beta$ -Actin was used as internal control. The images of Western blots films were scanned and quantified with Image J. Data represent mean  $\pm$  SE from at least 3 independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test. # $p < 0.05$  compared to control. \* $p < 0.05$  compared to cultures treated with Hcy [1 mM].

periment, clinical studies suggest that Hcy level is associated with a decline in cognitive functions leading to development of AD. The level of total Hcy in plasma at baseline and follow-up study is found to be high among elderly with memory deficit in 'The Hordaland Homocysteine Study' [54]. In another study, it is found that hyperhomocysteinemia is strongly associated with dementia, AD, and mild cognitive impairment. The study also suggests that high Hcy and low folate may precede dementia onset, which may indicate a causal relation between Hcy and AD [55].

The potential of Hcy-lowering therapy has been investigated. A recent report shows a reduction of Hcy

with folic acid and vitamin B supplement reduces the risk of overall stroke in participants with a history of cardiovascular diseases [19]. Although this therapeutic strategy could reduce the chance of developing AD risk factor, its use for AD treatment or prevention is still under inquiry. A randomized and double-blind controlled clinical trial shows that a high-dose of Vitamin B supplement for 18 months does not provide any benefit in cognitive functions for individuals with mild to moderate AD. Indeed, adverse effects such as depression were found in the active treatment group [56]. In another cohort study, it has been found that Hcy-reducing treatment with pyridoxine, cobalamin, and folic acid

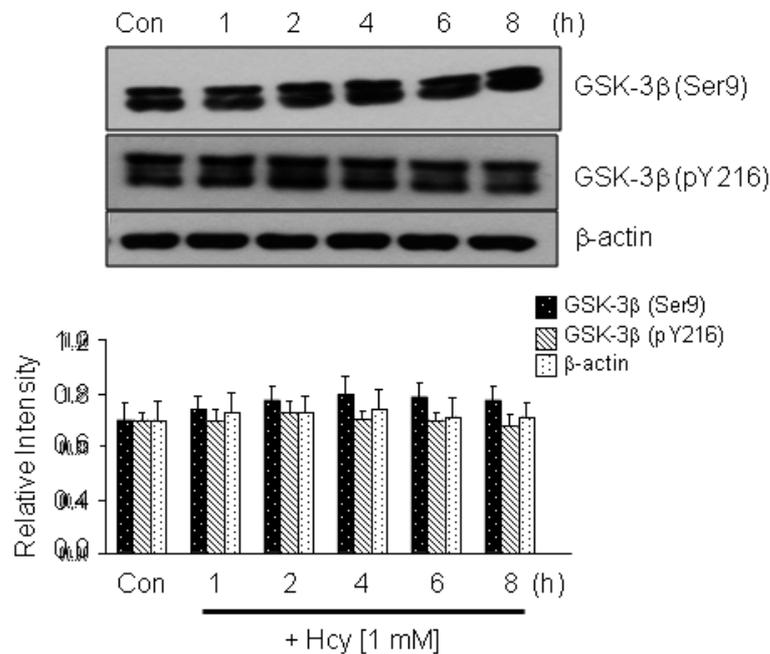


Fig. 6. The level of phosphorylated GSK-3 $\beta$  was not altered in neurons treated with Hcy for the different increments of time. Neurons were exposed to Hcy [1 mM] for the indicated duration of time, and harvested for Western-blot analysis. The level of phosphorylated GSK-3 $\beta$  (Ser9) and GSK-3 $\beta$  (pY216) were detected.  $\beta$ -Actin was used as internal control. Data represent mean  $\pm$  SE from at least 3 independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Student Newman-Keuls as post-hoc test.

does not change the level of plasma A $\beta$ . However, the group also does not find association between plasma A $\beta$  level and cognitive function in this study [57]. Nevertheless, there are agents or medication that provides direct neuroprotective effects against Hcy toxicity. A previous study suggested that neuroprotective compounds against Hcy toxicity can be found in natural herbs. Ginsenoside Rg3, the active compound in *Panax ginseng*, is able to reduce Hcy-induced apoptosis in rat hippocampal neurons [20]. It is therefore possible to develop other potential neuroprotective agents from herbs.

We used primary culture of cortical neurons to examine the protective effects of LBA. In this study, 1 mM Hcy were applied into the culture to induce toxicity. This dosage is higher than that found in AD patients (21.9  $\mu$ mol/liter compared to 12.2  $\mu$ mol/liter in age-matched controls) [10]. However, laboratory experiments often use higher dosages of Hcy in their models. In animals, the concentration of Hcy can increase from 2 to 10 fold comparing to the control [1]. In cell culture experiments, depending on the types of cells, Hcy dosages can be as high as 5 mM. In cell culture models, neurons are only exposed to Hcy for a short period of time (from hours to a few days); but in animals and human, the exposure time is much longer (months to

years). This may explain why a high dosage of Hcy is required to induce toxicity *in vitro*. Before we started our study, we performed trials for the experimental conditions. We found that 1 mM of Hcy is the minimum dosage to induce consistent and significant cell damage. We therefore used this dosage through our study.

In our study, we found that LBA is able to protect neurons against Hcy neurotoxicity. A number of mechanisms have been proposed to explain Hcy-induced toxicity on neurons (for review, see [58]). One such mechanism suggests that Hcy induces excitotoxicity through the activation of NMDA receptor, thereby triggering calcium influx [59,60]. It is able to activate several kinases such as ERK1/2 [60]. Our previous findings show that LBA can protect neurons against glutamate excitotoxicity probably through suppression of stress kinase activation [29]. Since glutamate and Hcy share similarities in their toxicity mechanisms, it is possible that LBA protects neurons against Hcy by modulating the NMDA receptor pathways. Further examination is required to clarify the effects of LBA on NMDA receptor activation and calcium influx.

The second proposed mechanism is the induction of oxidative stress. Previous studies have suggested that Hcy increases the generation of reactive oxygen species

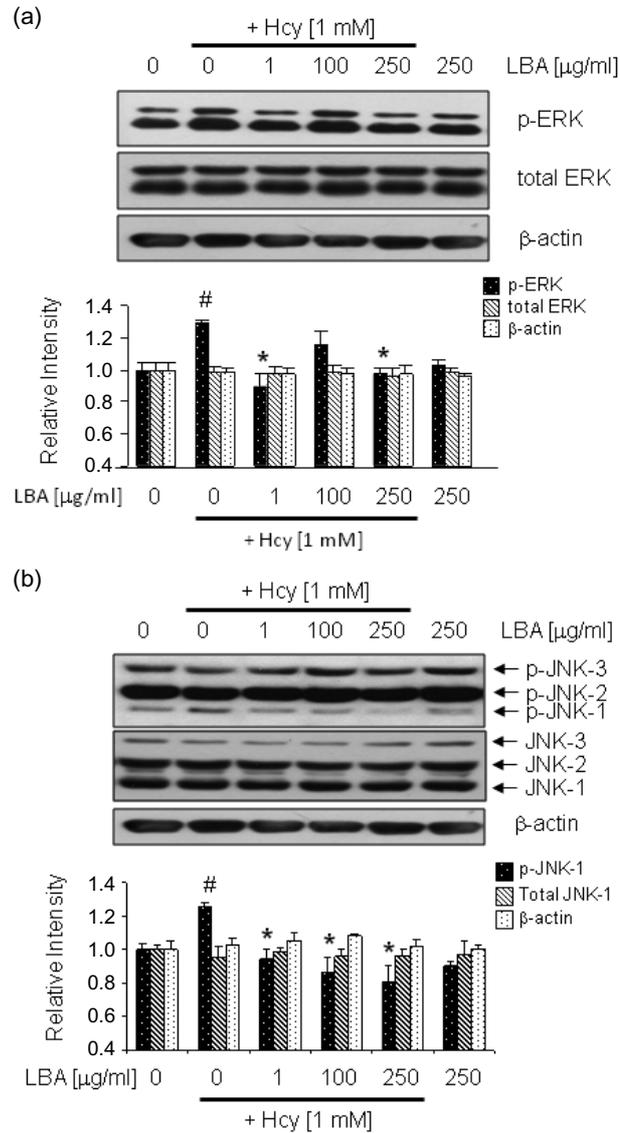


Fig. 7. LBA suppressed Hcy-induced elevation of phosphorylated ERK1/2 and phosphorylated JNK1. Neurons were pretreated with the indicated dosages of LBA for 1 h before they were exposed to Hcy [1 mM]. After 2 or 4 h of Hcy incubation, neurons were harvested for Western-blot analysis of (a) p-JNK or (b) p-ERK1/2. The non-phosphorylated forms of these kinases (JNK and ERK1/2) were also detected.  $\beta$ -Actin was used as internal control. Data represent mean  $\pm$  SE from at least 3 independent experiments. Statistical analysis was performed with one-way ANOVA, followed by Student Newman-Keuls as post-hoc test. <sup>#</sup> $p < 0.05$  compared to control. <sup>\*</sup> $p < 0.05$  compared to cultures treated with Hcy [1 mM].

(ROS), and treatment with antioxidant can reduce Hcy toxicity [38,61,62]. In order to find out if LBA could act as an antioxidant, nitroblue tetrazolium (NBT) reduction assay were carried out to detect the level of intracellular ROS in treatment groups (data not shown). Our data showed that LBA treatment did not reduce the level of ROS, although there was a 40% increase in the level of ROS in Hcy-treated group. The findings suggested that LBA was unlikely an antioxidant. Simi-

lar findings from our previous experiment also showed that LBA was unlikely to reduce glutamate-induced generation of ROS [29].

Another possible link of Hcy and AD is the induction of tau hyperphosphorylation. The levels of hyperphosphorylated tau (p-Tau) are significantly elevated in the brains of individuals suffered from AD compared to the normal control [63]. They can accumulate as intraneuronal tangles of paired helical filament,

which is a pathological hallmark of AD. Hcy is able to induce tau hyperphosphorylation *in vivo* and *in vitro* [37,49,50]. Activation of GSK3 $\beta$  and MAP kinases (JNK and ERK), and also the inactivation of protein phosphatase 2A (PP2A, which can dephosphorylate p-Tau), are proposed to play important roles during tau hyperphosphorylation [40,45,46,50,64]. In our study, we found that LBA attenuated Hcy-induced elevation of phospho-Tau at several epitopes. However, LBA at 100  $\mu$ g/ml could not attenuate S214 phosphorylation (Fig. 4b). This biphasic effects suggest that LBA may consist more than one active component. LBA is not a single molecule but contains groups of polysaccharides from *L. barbarum*. Similar phenomenon was observed before in our previous findings [24]. For natural products, it is common that further purification of one active component may unavoidably lead to loss of some activity of overall beneficial effects. We therefore did not further purify LBA.

We further examined any possible change of JNK, ERK1/2, and GSK3 $\beta$ . Similar to a previous report [40], we did not find any change of band intensity of phospho-GSK3 $\beta$  in Western-blot analysis. Although the report suggested an inhibition of PP2A activity in the hippocampus of Hcy-injected rats, we did not obtain consistent results for the elevation of phospho-PP2A<sub>C</sub> (Try 307) (the inactivated form of PP2A) level in Western-blot analysis (data not shown). This difference may be due to varying experimental procedures and methods employed. It would be beneficial to understand whether LBA can modulate PP2A activity; hence tau phosphorylation. We found that LBA-treated groups had a lower band intensity of phospho-ERK1/2 and phospho-JNK1 in Western-blot analysis after Hcy incubation. To confirm if LBA protected neurons through the suppression of MAP kinase activation, further experiment with the use of specific inhibitors may be required.

In addition to hyperphosphorylation, we observed truncation of tau at the C-terminal at Asp421 site. It has been proposed that tau truncation is an early event in the formation of neurofibrillary tangles. Caspase-cleaved tau can be found in AD brain [43,65]. and can cause neuronal apoptosis [65]. A recent report shows that activated JNK can induce caspase-cleavage of tau [67]. We found that LBA attenuated tau cleavage and at the same time suppressed caspase-3 activity and JNK-1 phosphorylation. Our data suggested that LBA might intervene at an early stage to reduce Hcy-toxicity.

As polysaccharides are macromolecules, one may raise the possibility if they can pass through blood brain

barrier (BBB) in order to reach the brain. The ability of LBA to be permeable to BBB is beyond the scope of this study. However, there is evidence demonstrating that some polysaccharides are indeed able to pass through the BBB and protect the brain from various pathological factors [68,69]. Future study on animal models will assist in clarifying this concern. As LBA is not a single compound but contains groups of polysaccharides, it is not easy to identify their exact molecular structures, hence detail information about its bioavailability in the GI tract. However, previous findings from our laboratory [21] show that oral feeding of LBP, which is a very similar extract to LBA, can have protective effects on retinal ganglion cells. As LBP have effects in the central nervous system (the eye), this provide hints that LBA may be able to maintain its bioavailability after absorption in the GI tract. We are conducting animal studies to confirm this.

In this study, we demonstrated that incubation of Hcy could induce direct cytotoxicity and AD-like pathology, including neuronal apoptosis, tau phosphorylation, and tau cleavage in primary cortical neurons. Our data showed that treatment with LBA, which are natural polysaccharides found in wolfberry, attenuated these pathological changes. At the same time, LBA suppressed Hcy-induced elevation of phospho-ERK-1 and phospho-JNK-1. We have already demonstrated the protective effects of LBA on A $\beta$  and glutamate neurotoxicity, and our data provide further support an idea that LBA has a potential to function as a disease-modifying agent for therapeutic intervention of AD.

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