Mulberry Extracts Alleviate Aβ25–35-Induced Injury and Change the Gene Expression Profile in PC12 Cells

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1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia in the elderly. AD, characterized by the progressive degeneration of cognition and memory, is correlated with the appearance of neurofibrillary tangles, senile plaques, and loss of neurons in the brain [1–4]. The processing of amyloid precursor protein (APP), a type I transmembrane glycoprotein, plays an important role in the development of AD [5, 6]. In the amyloidogenic pathway, cleavage of APP by β-secretase results in the release of a soluble, 110 kDa N-terminal fragment, sAPPβ, and a 12 kDa membrane-anchored C-terminal fragment, CTFβ. Subsequently, CTFβ is cleaved by γ-secretase and generates a 4 kDa Aβ peptide [7]. Aβ peptide is the major component of senile plaques and has been suggested to play a causal role in the development and progression of AD [8]. Aβ can trigger a cascade of pathogenic events such as culminating of neuronal apoptosis/death, dystrophy of neurites, excitoactivation of glutamate receptors, and induction of oxidation stress [9]. Aβ25–35, a synthetic peptide corresponding to amino acids 25–35 in Aβ1–40 and Aβ1–42, possesses the same β-sheet structure and exhibits large β-sheet fibrils [10, 11]. It retains most physical and biological properties of full-length Aβ, including its toxicity [12]. More importantly, Aβ25–35 is a particularly intractable...
peptide because it aggregates rapidly, unlike the full length Aβ, which requires aging for more than 1 week before it aggregates and becomes toxic [13]. As such, it is often used for the in vitro study.

Anthocyanins are a group of naturally occurring phenolic compounds that are responsible for the brilliant color of blue, red, and purple of leaves, flowers, and fruits [14]. Because of significant property of anthocyanin is antioxidant activity, the neuroprotective effect of anthocyanin has received a lot of attention in the field of nutrition research [4, 15]. Many studies showed that effects of the antioxidant activity and neuroprotection of anthocyanins in vitro. At the same time, in vivo test also confirmed that anthocyanins can reduce the injury area of cerebral ischemic damage in rat [16, 17]. Mulberries (Morus alba) have been used in traditional oriental medicine throughout world and in particular in China and contain high amounts of anthocyanins [14, 17, 18]. Zou et al. optimized the microwave-assisted extraction (MAE) conditions of anthocyanins from mulberry using response surface methodology (RSM). Under these conditions, 54.72 mg anthocyanins were obtained from 1.0 g mulberry powder. Furthermore, 8 anthocyanins were identified by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) in mulberry extract. Among them cyanidin-3-glucoside and cyanidin-3-rutinoside are the major anthocyanins in mulberry [19, 20]. Studies showed that black-colored mulberry fruit extracts contain the highest levels of anthocyanin, total phenolic, and flavonoid as well as strongest antioxidant compared with other colors of mulberry fruit extracts [8, 21]. Mulberry fruits exhibit a variety of biological and physiological effects, such as anti-thrombotic, antioxidant, antimicrobial activity, anti-inflammation, and neuroprotection [4]. In recent years, many papers have been published on the neuroprotective effects of mulberry extracts (ME). Animal studies found that mulberry fruits and their neuroprotective constituent—cyanidin-3-O-D-glucopyranoside (C3G), isolated from the mulberry fruits, can alleviate the cerebral ischemic injury and aging-associated neuronal damage in vivo using a mouse-brain-injury model with a transient middle cerebral artery occlusion (MCAO) [10]. Shih et al. (2010) found that ME, which are rich in phenolics and anthocyanins increased the antioxidant enzymes activities (Glutathione peroxidase, Catalase) and improved learning and memory ability in senescence-accelerated mice (SAMP) [4]. In in vitro experiments, anthocyanins in ME can inhibit Aβ25-35 Spontaneous aggregation into oligomers and their neurotoxicity in human neuronal SH-SY5Y cells and have neuroprotective effects on the PC12 cells exposed to hydrogen peroxide and oxygen glucose deprivation (OGD) [14, 19].

Yet few studies have used PC12 cells as Aβ25-35-induced injury model to investigate cytoprotective and neuroprotective effects of ME in vitro. Furthermore, mechanisms that ME pretreatment might inhibit development of AD have not been elucidated clearly. To explore mechanisms involved, we use Aβ25-35 treated PC12 cell as an in vitro model to investigate the role of ME and use the genomic techniques to quickly and accurately quantify vast numbers of potential gene expression changes after ME pretreatment. This study could thus have a great nutrigenomics interest and bring new and important light in the field of Alzheimer's disease intervention.

2. Materials and Methods

2.1. Preparation of Black Mulberry Extracts. Mature mulberry fruits (Morus nigra L.) were harvested from a local orchard in turfan depression, Xinjiang Uygur Autonomous Region in China, and purchased from Xinjiang Bencaoang Traditional Chinese Herbal Decoction Pieces Co. Ltd. (Lot NO.: 10121805, Tel.: +86 991 4639388). Mulberry extracts (ME), without any of the amino acids and vitamins, were prepared in the laboratory as described below. Blended fresh mulberry fruits were extracted with 60% alcohol and dehydrated in a freeze dryer (FD-I, USA) for 48 h in a vacuum freezer (~50°C). Total anthocyanin content in black mulberry extracts was 6.8% (0.068 mg/mL) as measured using a full wavelength UV spectrophotometer scanning (wavelength 282 nm). ME stock solution was prepared by dissolving ME in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) (20 mg/mL) and sterilized through a 0.22 μm filter. At the time of treatment, ME stock solution was further diluted with culture medium to a concentration of 200 μg/mL.

2.2. Cell Culture and Treatment. PC12 cell line (rat adrenal pheochromocytoma, The Shanghai Institute of Biochemistry and Cell Biology, SIBCB, China) was grown in high glucose DMEM medium supplemented with 5% (v/v) fetal bovine serum (FBS, HyClone, USA), 10% heat-inactivated horse serum (HS, HyClone, USA), and 1% penicillin/streptomycin (Sigma, USA) in a 5% CO2 incubator at 37°C and saturated humidity. To induce neuronal differentiation, cells were treated with 50 ng/mL of nerve growth factor (NGF, Peprotech, USA). When cells were at about 80% confluence, the medium was replaced with DMEM medium containing 1% FBS, 1% penicillin, and 1% streptomycin with NGF (50 ng/mL). At the treatment, cells were divided into four groups: (1) control group: no treatment, (2) Aβ25-35 group: cells were treated with 20 μmol/L Aβ25-35 for 24 h, (3) ME plus Aβ25-35 group: cells were pretreated with 200 μg/mL ME for 24 h, and then the medium was discarded and switched to that containing 20 μmol/L Aβ25-35 for another 24 h, and (4) ME group: cells were treated with 200 μg/mL ME alone for 24 h [22]. Before the experiment, the Aβ25-35 Peptide solution was incubated at 37°C for a week to produce the conformation of fibril or aggregation before adding to PC12 cells.

2.3. Cell Viability. Cell viability was determined by MTT assay. Briefly, PC12 cells were plated at a density of 1 × 10⁴ cells/well in 96-well plates and treated with ME, Aβ25-35, or ME plus Aβ25-35 at indicated concentration, respectively. At the time of assay, cells were washed with PBS and incubated with MTT reagent (5 g/L, 10% v/v, Sigma, USA) at 37°C for 4 hours. The resulting MTT-formazan crystals were solubilized by dimethylsulfoxide (DMSO, 150 μL) for 10 min at room temperature. Optical density was measured at 570 nm using a microtiter plate reader (uQuant). Results were expressed as
the percentage of MTT reduction as compared with control group.

2.4. RNA Isolation and cDNA Synthesis. Total RNA was extracted from various groups using Trizol reagent (Invitrogen, Life Technologies, USA.) following instruction. The extracted RNA was further purified using RNasey columns (QIAGEN RNasey Mini Kit, Germany). The quantity and quality of the RNA was determined by Abs260/Abs280 ratio (≥1.80), Abs260/Abs230 ratio (≥1.50), and ethidium bromide fluorescence of RNA resolved in 1% agarose gels. cDNA was synthesized using an oligo dT-T7 promoter primer (Roche Molecular Biochemicals, Mannheim, Germany) and used as a template for in vitro transcription.

2.5. Microarray Analysis. Fluorescently labeled probes for oligo microarray analysis were prepared using Amino allyl Message Amp aRNA kit (Applied Biosystems, Foster City, CA, USA) as instructed. Labeled probes were hybridized to a Phalanx Rat OneArray containing 24358 rat specific probe sets (Phalanx Biotech Group, Inc., China Taiwan) at 50°C for 16 hrs. Slides were washed with 2 x SSC/0.2% SDS at 42°C for 5 min, 2 x SSC at 42°C for 5 min, 2 x SSC at 25°C for 5 min, and then ten times with 0.2 x SSC. Then spin dried slides were scanned using an Affymetrix Gene Array scanner (USA) and analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA) to obtain gene expression ratios. Logged gene expression ratios were normalized by Lowess regression [23]. Rosetta Resolver System (Rosetta Biosoftware) was used for data preprocess and differential gene expression analysis. Cluster 3.0 and Tree View (http://rana.lbl.gov/EisenSoftware.htm) were employed to clustering analysis.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared as mentioned above using the same samples. PrimeScript RT Master Mix Perfect Real Time kit (Takara) was used to synthesize first strand cDNA as described by the manufacturer. Specific DNA sequences were amplified with a PCR mixture (TIANGEN Biotech, China) and resolved on a 2% agarose gel. PCR primers were indicated as follows: Apaf 1, 5’-ATGTTATCCTGTGGGAGAG- TGG-3’ (sense) and 5’-CACCAACTAACAGACGAGCAG-3’ (antisense); Bace 2, 5’-TGTGGACACCCGGAAGCTAA-3’ (sense) and 5’-CCTCAAAGGCCCTTGAGTGGTA-3’ (antisense); Pclb 4, 5’-GCCCATACCTCAGATTCTCCT-3’ (sense), 5’-TACACTGCTTCTCGTGAGT-3’ (antisense); β-actin, 5’-CACCCGGAGTACACCCTTC-3’ (sense) and 5’-CCCATACCCACCATCACCC-3’ (antisense).

2.7. Statistics. Data are expressed as the mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) and post hoc Bonferroni/Dunn test. Values of P less than 0.05 were considered statistically significant.

![Figure 1: Cytoprotective effects of mulberry extracts in Aβ25-35-induced PC12 cells. PC12 cells were pretreated with or without ME (200 μg/mL) for 24 h and exposed to Aβ25-35 (20 μmol/L) for 24 h. The cytotoxicity was measured by MTT assay. The viability of the untreated cells was set to 100%. The values represent mean (% ± S.D.) of three different cultures. (#) Significantly different from the control group (P < 0.05). (*) Significantly different from the Aβ25-35 group (P < 0.05).

### Table 1: Up- (≥2-fold) and down- (<2-fold) regulation in gene expression in PC12 cells after exposure to Aβ25-35 or pretreatment with ME for 24 h.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Downregulated</th>
<th>Upregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ25-35 versus control</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>(ME + Aβ25-35) versus Aβ25-35</td>
<td>98</td>
<td>55</td>
</tr>
</tbody>
</table>

3. Results

3.1. Effect of ME on Viability in Aβ25-35. Treated PC12 Cells. Aβ25-35 treatment resulted in a significant decrease [(38.3 ± 6.9)%] in cell viability in PC12 cells as compared with the control group (P < 0.05, n = 8). ME treatment alone did not significantly affect cell viability; ME pretreatment inhibited Aβ25-35-induced cell death by (29.8 ± 8.7)% (Figure 1). The result shows that ME attenuated Aβ25-35-induced cell injury in PC12 cells.

3.2. Effect of ME on Gene Expression Profile of Aβ25-35 Treated PC12 Cells. Gene expression profile was analyzed by microarray method using 2-fold change (P < 0.05) as cut point. Of the 24358 genes on the chip, the expression levels of 5 genes (0.02%) were increased and those of 16 genes (0.07%) were decreased in cells treated with Aβ25-35 as compared with control. Pretreatment with ME resulted in increased expression of 55 genes (0.2%) and decreased expression of 98 genes (0.4%) as compared with Aβ25-35 group. Changes in gene expression reflect the influence of ME-supplement on the cells (Table 1).

3.3. Cluster Analysis and Gene Ontology (GO) Classification. For advanced data analysis, all biological replicates were pooled and calculated to identify differentially expressed genes based on the threshold of fold change and P value.
Table 2: Statistical results of GO classification in ME pretreatment group.

<table>
<thead>
<tr>
<th>Term</th>
<th>Count</th>
<th>Up</th>
<th>Upregulated</th>
<th>Down</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion</td>
<td>11</td>
<td>3</td>
<td>Pcdhb9, Igfals, Pcdh9</td>
<td>8</td>
<td>Ptprm, Dsg2, Dpdm, Trpm7, Icam2, Lmo7, Postn, Spp1</td>
</tr>
<tr>
<td>Peptidase activity</td>
<td>12</td>
<td>4</td>
<td>Acr, Em12, Cndp1, Xpnpep3</td>
<td>8</td>
<td>Mmp10, Bace2, Serpinel, Serpinb2, Fgl2, Apaf1, Mysml, Mmp12</td>
</tr>
<tr>
<td>Regulation of angiogenesis</td>
<td>4</td>
<td>1</td>
<td>Figf</td>
<td>3</td>
<td>Col4a2, Ptprm, Serpinel</td>
</tr>
<tr>
<td>Cytokine activity</td>
<td>5</td>
<td>1</td>
<td>Il33</td>
<td>4</td>
<td>Il23a, Ccl2, Ccl27, Spp1</td>
</tr>
<tr>
<td>Ion binding</td>
<td>36</td>
<td>14</td>
<td>Cndp1, Pcdhb9, Trim50, Cacnb2, Necab2, Vwde, Scd, Tmem38b, Ng35, Cyp17a1, Pcdh9, Arsg, Xpnpep3, Cyp4f1</td>
<td>22</td>
<td>Syt4, Lmo7, Plcb4, Pak3, Acsl4, Zc3h14, Mical2, Trpm7, Csrp3, March11, Mmp12, Tmem38b, Mmp10, Plekhf2, Bazla, Trim33, Dsg2, Prdm6, Hvep2, Zfp281, Scl5a7, Prkd3</td>
</tr>
<tr>
<td>Multicellular organism reproduction</td>
<td>9</td>
<td>4</td>
<td>Acr, Avprla, Micalcl, Pcdhgal1</td>
<td>5</td>
<td>Ccl2, Cnd2, Acsl4, Vgf, Prl8a2, Col4A2, Cd2, Loc363458, Postn, Vgf, Mmp12, Mmp10, L23a, Adm, Wasi, Serpinb2, Sema3c, Spp1, Ccl27, Serpinel, Prl8a2, Ptprm, Dpdm, Trpm7, Gas7, Col4A3bp, Serpin</td>
</tr>
<tr>
<td>Others</td>
<td>31</td>
<td>9</td>
<td>Matn3, Igfals, Il33, Metrn, Figf, Avprla, Slc30a8, Scl, Penk</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>36</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Upregulated means upregulated genes, Downregulated means downregulated genes.

Table 3: Significant genes based on microarray data in AD pathway.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>log₂ (ratio)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>78963</td>
<td>Apaf1</td>
<td>Apoptotic peptidase activating factor 1</td>
<td>−1.0464</td>
<td>0.00267</td>
</tr>
<tr>
<td>288227</td>
<td>Bace2</td>
<td>Beta-site APP-cleaving enzyme 2</td>
<td>−1.0448</td>
<td>0.00001</td>
</tr>
<tr>
<td>25031</td>
<td>Plcb4</td>
<td>Phospholipase c, beta 4</td>
<td>−1.0673</td>
<td>0.00381</td>
</tr>
</tbody>
</table>

The correlation of expression profiles between biological replicates and treatment conditions was demonstrated by unsupervised hierarchical clustering analysis. For this microarray project, the number of genes clustered was 144 (Figure 2). According to biological process ontology analysis, GO classification items enriched in the difference-expression genes were mainly related to cell adhesion, peptidase activity, cytokine activity, ion binding activity, and angiogenesis regulation in ME pretreatment group \((P < 0.05)\) (Table 2, Figure 3).

3.4. Pathway Analysis and Verification. Based on NCBI database, the screened differentially expressed genes Apaf1, Bace2, and Plcb4 were enriched in the “Alzheimer’s disease-reference pathway” \((P < 0.01)\) (Figure 4) and meanwhile significantly downregulated in \(\Delta \beta_{25-35}\)-injured PC12 cells after ME intervention as compared with \(\Delta \beta_{25-35}\) group \((P < 0.05)\) (Table 3). RT-PCR method was used to verify the changes of Apaf1, Bace2, and Plcb4 mRNA expression. Figure 5 showed that mRNA levels of Apaf1, Bace2, and Plcb4 were upregulated in \(\Delta \beta_{25-35}\) treated PC12 cells as compared with control group \((P < 0.05)\), increased by \((61.5 ± 13.2)\)% \((33.9 ± 4.3)\)% and \((43.1 ± 9.3)\)% respectively, which was consistent with the microarray analysis. These results indicated that ME pretreatment could substantially downregulate Apaf1, Bace2, and Plcb4 mRNA expression levels in \(\Delta \beta_{25-35}\)-injured PC12 cells.

4. Discussion

There is an increasing interest in the beneficial effects of nutritional antioxidants on health via the delay of aging and age-related diseases \([24–29]\). The observed protection may be the result of the antioxidant and anti-inflammatory properties of the polyphenolic compounds found in these fruits and vegetables \([30]\). Our previous studies found that pretreatment of PC12 cells with 200 \(\mu\)g/mL ME could almost completely reverse \(\Delta \beta_{25-35}\)-induced neuronal injury, counteract ROS formation, and inhibit apoptosis. The results suggested that ME could alleviate \(\Delta \beta_{25-35}\)-induced injury in PC12 cells, which might be associated with the antioxidative and antiapoptosis effects \([22]\). In this study, we further investigate the possible mechanisms involved.

To explore the molecular mechanisms of neuroprotective effect of ME, the transcription of 24,358 genes was analyzed by gene chips. Combined with bioinformatics analysis,
Figure 2: Hierarchical clustering of PC12 cells analyzed with the microarray chip. PC12 cells were pretreated with or without ME for 24 h and exposed to Aβ25–35 (20 μmol/L) for 24 h. Data are representative of three different experiments. Up- and downregulated genes are represented in red and green colors, respectively.
Figure 3: The results of GO classification in ME pretreatment group.

Figure 4: Screened differentially expressed genes were rich in Alzheimer’s disease-reference pathway (http://www.genome.jp/kegg-bin/show_pathway?rno05010). Note. The gene in red box represents downregulated expression after ME pretreatment in Alzheimer’s disease-reference pathway.
the gene expression profiles in samples were significantly affected. Further analysis shows that in ME pretreatment group, the downregulated genes were mainly related to cell adhesion, cytokine activity, and angiogenesis regulation, and upregulated genes were mostly related to ion binding activity and multicellular organism reproduction. Based on NCBI database, the screened differentially expressed genes Apaf1, Bace2, and Plcb4 were enriched in the “Alzheimer’s disease-reference pathway.” That is, these genes not only play an important role in the development of AD, but also their essential for the normal development of the brain [38] and function of Apaf1 is associated with the development of many human diseases [37]. The previous result found that ME preincubated cells can effectively reduce the rate of cell apoptosis [22]. The gene chip results and further validation found that Apaf1, Bace2 and Plcb4 mRNA levels were normalized to β-actin mRNA level and presented as relative value. The values represent mean ± S.D. of the each group (n = 6) of three independent experiments. (#) Significantly different from the control group (P < 0.05). (∗) Significantly different from the Aβ25-35 group (P < 0.05).

Figure 5: mRNA expression of Apaf1, Bace2, and Plcb4 genes measured by RT-PCR. Cells were pretreated with or without ME for 24h and exposed to Aβ25-35 (20 μmol/L) for 24 h. (a) Typical mRNA bands of Apaf1, Bace2, Plcb4 and β-actin from control group, 200 μg/mL ME treatment alone group, pretreatment group with 200 μg/ml ME, and treatment group with Aβ25-35 alone. (b) Apaf1, Bace2 and Plcb4 mRNA levels were normalized to β-actin mRNA level and presented as relative value. The values represent mean ± S.D. of the each group (n = 6) of three independent experiments. (#) Significantly different from the control group (P < 0.05). (∗) Significantly different from the Aβ25-35 group (P < 0.05).
overexpressed in Aβ25–35 group than those in the control group, while 200 μg/mL ME preincubated for 24 h could significantly inhibit Aβ25–35-induced upregulation of Bace2 mRNA expression. Therefore, we speculate that BACE2, not BACE1, as another therapeutic target, is expected to become a promising way to treat AD.

Translation products of Plcb4 gene are PLC-β 4, one of the isozymes of phospholipase C-β (PLC-β). Phospholipase C (PLC), as an important enzyme, is widespread in various cells, having species and cell specificity in the basic biochemical characteristics, function, and subcellular distribution [43, 44]. PLC is the phospholipid component of cellular membrane and also participates in the apoptosis signal transduction in various cells, such as neurons [45, 46]. Other studies have found that PLC is involved in the regulation of oxidative stress caused by oxidative glutamate toxicity induced neuronal cell death in immature cortical neurons and hippocampal neurons [47]. Our previous experimental results and other literature [20] found that ME pretreatment alleviated the damage of membrane structure of cells or organelles, especially preserved the mitochondrial membrane integrity and inhibited the decrease of mitochondrial membrane potential (see Supplementary Figure in supplementary materials available online at http://dx.doi.org/10.1155/2014/150617). In the present research, we noted that Plcb4 mRNA in PC12 cells were obviously more overexpressed in Aβ25–35 group than those in the control group, which can be inhibited by ME preincubation. The results indicated that ME could inhibit apoptosis by the way of suppressing gene expression of Plcb4 in PC12 cells.

5. Conclusions

In summary, the results suggested that ME pretreatment could substantially alleviate the cell injury induced by Aβ25–35, which may be related to the antioxidative and antiapoptotic properties of ME (or anthocyanins). ME may negatively regulate the expression of Apaf1, Bace2, and Plcb4 genes, thereby delaying the development of AD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Nan Song and Hongpeng Yang contributed equally to this work. Yugang Jiang, Chuan Qin, and Fuzhi Lian contributed equally to the supervision this work.

Acknowledgments

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