Original Research Article

Protective effect of EGb761 against Aβ1-42 -induced SH-SY5Y cells injury and blood-brain barrier disruption via regulating Akt/Nrf2 signaling pathway

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Abstract

Purpose: Alzheimer’s disease (AD) is a common disease in the world caused by deposition in the brain parenchyma, accumulation of beta amyloid which leads to the blood brain barrier (BBB) disruption. Regardless of enough progress in the treatment of AD, the principal mechanism of BBB injury is yet not clear.

Methods: In this study we examined the impact of EGb761on Aβ 1-42-induced SH-SY5Y cells in vitro model of AD. Cell viability was assessed by using MTT assay, flow cytometry was used to check the rate of cell apoptosis, ROS generation and BBB leakage was assessed by measuring the level of fluorescence in Aβ-induced SH-SY5Y cells using a reactive oxygen species kit assay and BBB permeability assay, and mRNA levels of Bax, Bcl-2, caspase-3 was measured by using RT-qPCR. Furthermore, western blot analysis was used to measure the protein expressions of Akt, Nrf2 and HO-1 in Aβ 1-42-induced SH-SY5Y cells.

Results: The effect of EGb761 was investigated on the cell apoptosis induced by Aβ 1-42 and generation of ROS and we found that EGb761 plays a protective role against cell injury induced by Aβ 1-42. Cell apoptosis and ROS generation in SH-SY5Y cells decreased significantly with the treatment of EGb761. Furthermore, BBB permeability reduced considerably when the cells treated with EGb761 and the expression levels of Caspase-3 and Bax decreased while that of Bcl-2 were markedly increased in the Aβ 1-42-induced SH-SY5Y cells. Also, an increased in expression levels of p-Akt, Nrf2 (nucleus) and HO-1 was observed with the treatment of EGb761 in Aβ 1-42-induced SH-SY5Y cells.

Conclusion: It can be concluded from these results that EGb761 could play a protective role by inhibiting apoptosis and protect Aβ 1-42-induced cell injury in vitro model of AD via activating Akt/Nrf2 signaling pathway. Our study suggested that EGb761 might be a therapeutic agent for the prevention and treatment of AD.

Keywords: EGb761, oxidative stress, Alzheimer’s disease (AD), BBB, Akt/Nrf2

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INTRODUCTION

Alzheimer’s disease (AD) is a common neurodegenerative disease worldwide that leads to memory loss and behavioral deterioration [1]. An essential factor of the pathogenesis of AD is the accumulation of amyloid-beta (Aβ) [2]. Oxidative stress is the leading cause of cell
death, and mostly cellular proteins are damaged because of the overproduction of reactive oxygen species (ROS). The oxidative stress plays a vital role in the stimulation of many neurodegenerative diseases such as Huntington’s disease [3], Parkinson’s disease [4] and AD [5]. The blood brain barrier (BBB) is a physiological barrier which is formed by brain endothelial cells and joined together by numerous junctions [6]. BBB plays a vital role in controlling various molecules and then create a blockage through brain endothelium [7]. The accumulation of Aβ is caused by the leakage of BBB due to cerebrovascular changes, leading to the progression of AD [8]. Although great research has been done to study the pathogenesis of AD, however, the deposition of Aβ in brain and cerebral capillaries is not clear. Moreover, the exact mechanism of BBB leakage is also unknown. Therefore, targeting Aβ-induced blood brain barrier disruption in SH-SY5Y cells might be a promising approach for the AD treatment. Ginkgo biloba leaf extract are used as herbal medicine for many diseases because of its neuroprotective effect and cognitive impairment properties [9]. Flavonoids, bilobalide, ginkgolic acid and ginkgolides are the important compounds found in EGb761 extract [10]. EGb761 has gained much attention because of its therapeutic effects in pharmacology, such as protection of microvascular endothelial cells and especially for the treatment of AD [11]. Furthermore, there is evidence that EGb761 acted as a protective drug in AD treatment [12,13] by limiting neural and vascular damage [12]. However, the exact molecular mechanism essential for this treatment is still unclear. So there is a need to investigate the underlying mechanism by which EGb761 exerts its neuroprotective role in the treatment of AD.

Recently, nuclear factor erythroid 2-related factor 2 (Nrf2) has been reported to have crucial function against oxidative stress [14]. Nrf2 is a redox-regulated gene whose level is reduced in the nucleus during neuroinflammation diseases such as AD [14]. Anti-oxidative enzymes such as heme oxygenase-1 (HO-1) are produced during the activation of Nrf2 [15]. Besides, HO-1 induction inhibited the inflammation and oxidative stress signifying the effective function of Akt/Nrf2 in AD [16]. Other studies have revealed that Akt activated expression of HO-1 via the Nrf2 signaling pathway [17]. These studies suggested that Akt/Nrf2 is related to the inflammation and oxidative stress suggesting that Akt/Nrf2 might be a therapeutic target for AD. Therefore, the purpose of this present study was to investigate the effect of EGb761 on the activation of Akt/Nrf2 signaling pathway in Aβ-induced cellular model of AD. In this study, we demonstrated the protective effect of EGb761 on Aβ-induced BBB disorder in SH-SY5Y cells via Akt/Nrf2 signaling pathway.

**MATERIALS AND METHODS**

**Reagent preparation**

As described in previous studies, Aβ 1-42 was prepared by using Lyophilized human Aβ 1-42 [18]. Aβ 1-42 was first dissolved in 1mM of hexafluoroisopropanol (HFIP; PARCHEM, New York, USA) and then into sterilized microcentrifuge tubes. This HFIP was then kept in Speed Vac, and the peptide was stored at a freezing temperature of -20°C. A 2 mM Aβ 1-42 was first dissolved in DMSO (Sigma-Aldrich, St Louis, USA) followed by its dilution into frozen Opti-MEM (Thermo Fisher Scientific, New Jersey, USA). The solution was centrifuged for 5 minutes and incubated for 24 hr at 4°C. EGb761 was dissolved in DMSO (150 mg/ml) and kept at 25°C. The concentrated stock solution was diluted with Opti-MEM to make the required concentrations (25, 50, 100, 250 µg/ml) of EGb761.

**Cell culture**

SH-SY5Y cells (ATCC, Molsheim Cedex, France) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Biocompare, CA, USA) containing 20% FBS (Biological Industries, CT, USA), 100 µg/ml streptomycin and 1% penicillin (Cell Biogics, Chicago, USA). These cells were then kept in a humidified incubator which contains 95% air and 5% CO₂ at 37°C. The medium was replaced and renewed every 3 days.

**Cell transfection**

SH-SY5Y cells were raised to 80-90-% confluence before treatment. Then these cells were washed with PBS twice and then culture medium was replaced with Opti-MEM medium (Sigma-Aldrich, MO, USA). For the treatment of SH-SY5Y cells with EGb761 and Aβ 1-42, these cells were pretreated with EGb761 for 1 h followed by the treatment with Aβ 1-42.

**Cell viability**

MTT assay was used to measure cell viability. 96-well plates were used to seed SH-SY5Y cells and then these cells were treated with different concentrations of EGb761. MTT (Abcam, ON, Canada) reagent (1 x 50ml) stock solution was diluted in PBS (Biocompare, CA, USA) and then
added to each well containing 100 µL of DMSO medium. The medium was removed and replaced after 3hr and 30 min of incubation and 37°C, and these crystals were lysed with 100 µL DMSO medium by moderately shaking the plate. A microplate reader (Abcam Inc., ON, Canada) was used to measure the absorbance at 590 nm. The cell viability was measured as a percentage relative to the control cells.

Flow cytometry

Hoechst-33258 (Cyman, Michigan, USA) was used to check the cell apoptosis in SH-SY5Y cells. Plates (6-well) having density 1 × 10⁵ cells/well were used to culture cells with DMEM for 24 hr till it reached 95% confluence. The culture medium was replaced with DMEM without serum, and these cells were centrifuged for 10 min at room temperature. They were then washed at with PBS for almost 30 min at room temperature and then the apoptotic cells were suspended using an Annexin V-FITC (Biolegend, CA, USA) buffer. Cells were treated with 10 µL of PI and 10 µL of Annexin V-FITC and incubated in the dark for 30 min at room temperature. Finally apoptotic cells were observed on the basis of nuclear morphological changes by using a fluorescent microscope (Biocompare, MA, USA) at × 400 magnification. The ratio of apoptotic cells to total cells was used to calculate the percentage of apoptotic cells. The process was repeated three times.

ROS measurement

A Reactive Oxygen Species Kit (Abcam, ON, Canada) was used to measure the level of cellular reactive oxygen species. In viable cells, H2DCFDA is oxidized by reactive oxygen species to 2′, 7′ – dichlorofluorescein (DCF). This DCF is highly fluorescent compound at 535 nm. Cells were rinsed with PBS three times and then with H2DCFDA. These cells were diluted to a final concentration of 10 µM followed by the incubation in the dark for 40 min at 37°C. A 96-well plate were used to wash stained cells with PBS and then analyzed by using fluorescent microscopy (Abcam, ON, Canada). The fluorescence levels in the cells were measured by a microplate reader (Abcam, ON, Canada) with excitation at 485 nm and emission at 535 nm. The cellular ROS level was expressed as a percentage of the control cells.

BBB permeability

An in vitro model of BBB co-culturing was established following the previous research [19]. An apical compartment on a polycarbonate membrane Transwell permeable insert (Thomas Scientific, NJ, USA) containing 6.5 mm diameter and 0.4 µm pore size was used to culture SH-SY5Y (5 × 10⁵ cells/m²) cells. After cells reached 90% confluence, a 1.5 ml of HHBS assay buffer (1.26 mM CaCl₂, 0.49 mM MgCl₂, 5.33 mM KCl, 0.44 mM KH₂PO₄, 5.56 mM glucose, 20 mM HEPES, pH = 7) was added to the basolateral compartment. This culture medium was replaced with HHBS assay buffer (0.5 ml). This medium was removed from the basolateral compartment after 30 min and a multiwall plate reader (Bio-Rad, PA, USA) was used to measure the fluorescence in this medium at 490 nm excitation and 535 nm emission.

RT-qPCR

Total RNAs were extracted from the SH-SY5Y cells using the RNA Isolation Kit (Sigma-Aldrich, MO, USA) according to the manufacturer’s protocol. The concentration of RNA and purity were assessed by optical density spectrometry at 260 nm. The total RNA of all samples was first reverse-transcribed into cDNA, followed by the amplification of PCR according to the manufacturer’s instructions of the RT-PCR kit (Biocompare, CA, USA). Gene expression was detected with the following primers given in the Table 1. Results of RT-qPCR were calculated through 2⁻ⁿδδCt methods to qualified expressions of RNAs.

Table 1: Primers used for detection of gene expression

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaxForward</td>
<td>GGCCTGAGTCCAGCTCTTTA</td>
</tr>
<tr>
<td>Bax Backward</td>
<td>GTCTGGAAGACGGGACATC</td>
</tr>
<tr>
<td>Bcl-2 Forward</td>
<td>CCTCGCTGCAACAAATCTCC</td>
</tr>
<tr>
<td>Bcl-2 Backward</td>
<td>TGGAGAGAATGTGTTGGGCTCT</td>
</tr>
<tr>
<td>Caspase-3 Forward</td>
<td>TCCTGCTGGTACAGATGTCG</td>
</tr>
<tr>
<td>Caspase-3 Backward</td>
<td>CTTCAACCAGTGGTACAGATGTCG</td>
</tr>
<tr>
<td>β-actin Forward</td>
<td>CACCATGATCCCTGGCATGG</td>
</tr>
<tr>
<td>β-actin Backward</td>
<td>CCTGCTGTGATCCACACATC</td>
</tr>
</tbody>
</table>

Western blot analysis

Cell extracts were prepared by rinsing the cells twice with PBS and suspended in RIPA lyse buffer (Biocompare, CA, USA) containing phosphatase inhibitors. The protein expressions of samples were examined by using bicinchonin acid protein assay kit (Sigma-Aldrich, MO, USA). These proteins were loaded and subjected to 12% SDS-PAGE. The membranes were blocked with 5% skimmed milk for 2 hrs at 25°C and incubated overnight at 4°C. The primary antibodies used for incubation of these membranes are: anti-Bax (1:1000; NBP1-28566; Novous Biologicals, CO, USA ), anti-Bcl-2...
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(1:1000; NB100-56098; Novous Biologicals, CO, USA), anti-Nrf2 (1:1000; NBP1-32822; Novous Biologicals, CO, USA), anti-HO-1 (1:1000; NBP1-97507; Novous Biologicals, CO, USA), anti-caspase-3 (1:1000; NB100-56708; Novous Biologicals, CO, USA), anti-Akt (1:1000; NB100-56749; Novous Biologicals, CO, USA) and anti-β-actin (1:1000; NB600-501).

Peroxidase-conjugated anti-rabbit or anti-mouse (1:2000; NB100-305; Novous Biologicals, CO, USA) secondary antibodies were incubated for 1h at room temperature. These membranes were depicted by using ECL western detection agent (Thermo Fisher Scientific, MA, USA). Proteins bands were visualized by a ChemiDoc™ MP Imaging System (170-01402; Bio-Rad, MA, USA).

Statistical analysis

All data were analyzed by IBM SPSS 18.0 software (IBM Inc., New York, USA) and expressed as the mean ± SD and experiments were repeated three times. GraphPad Prism 7.0 (Graphpad Software, CA, USA) was used to perform statistical analysis. A student t-test or one-way analysis of variance (ANOVA) was used to find the significant difference in different groups. When the value of p < 0.05, then it was considered statistically significant.

RESULTS

EGb761 reduced Aβ1-42-induced cell injury of SH-SY5Y cells

MTT assay was used to check the effect of EGb761 on the cell viability of SH-SY5Y cells. From the results, it was clear that cell viability remained unchanged by the treatment with different concentrations of EGb761. While the cell viability considerably decreased when treated with EGb761 at a concentration of 250 µg/ml. (Figure 1) Consequently, the concentration of EGb761 between 50 and 100 µg/ml was used for further experiments.

The SH-SY5Y cells were treated with different concentrations of EGb761 followed by the treatment with Aβ 1-42. The results revealed that the cell viability decreased significantly when the SH-SY5Y cells treated with Aβ 1-42 as compared to control group. On the other hand, treatment of SH-SY5Y cells with EGb761 considerably enhanced the cell viability in a concentration-dependent manner. (Figure 1).

Figure 1: EGb761 reduced Aβ1-42-induced cell injury of SH-SY5Y cells: MTT assay was used to measure the cell viability in Aβ-induced SH-SY5Y cells after treating with different concentrations of EGb761. P < 0.05

EGb761 prevented Aβ1-42-triggered apoptosis in SH-SY5Y cells

The cells were incubated with EGb761 for 120 min and then treated with Aβ 1-42 for almost one day to study the effect of EGb761 on the cell apoptosis in SH-SY5Y cells. We examined different concentrations of EGb761, and our results showed that the rate of cell apoptosis considerably increased when SH-SY5Y cells were treated with Aβ 1-42 as compared to the control group. Moreover, if the cells were treated with EGb761 before treatment with Aβ 1-42, then there was a decrease in the rate of cell apoptosis in SH-SY5Y cells. (Figure 2).

Figure 2: EGb761 prevented Aβ1-42-triggered apoptosis in SH-SY5Y cells: The rate of cell apoptosis in Aβ-induced SH-SY5Y cells was measured by using caspase-3 assay. P < 0.05

EGb761 attenuated Aβ1-42-induced ROS generation in SH-SY5Y cells

Oxidative stress is a crucial factor in producing cytotoxicity induced by Aβ 1-42. Hence, Reactive Oxygen Species Kit was used to measure the level of cellular oxygen reactive species and check the effect of EGb761 on the generation of ROS in the cells induced by Aβ 1-42. It can be seen from these results that there was a significant increase in the fluorescence level in SH-SY5Y cells signifying higher ROS generation.
in the cells treated with Aβ 1-42 as compared to the control group. In contrast, the level of fluorescence in SH-SY5Y cells reduced when the cells were treated with EGb761 before the treatment with Aβ 1-42 indicating that ROS generation decreased significantly in Aβ-induced SH-SY5Y cells. It can be concluded from these results that EGb761 can significantly reduce the Aβ 1-42-induced ROS generation in SH-SY5Y cells. (Figure 3).

**EGb761 reduced BBB leakage induced by the Aβ1-42**

Previous research has shown that micro vascular injury was associated with leakage of BBB in AD patients [20]. Therefore, we used BBB permeability assay to check BBB leakage in terms of fluorescence levels in Aβ-induced SH-SY5Y cells. Our results showed that fluorescence level in Aβ-induced SH-SY5Y cells was higher indicating that barrier permeability increased in SH-SY5Y cells induced with Aβ 1-42. While the treatment with EGb761 significantly reduced the fluorescence level in SH-SY5Y cells which in turn decreased the barrier permeability in the Aβ 1-42-induced SH-SY5Y cells compared to the control group in a concentration-dependent manner. (Figure 4).

**Effect of EGb761 on protein levels of Caspase-3, Bax and Bcl-2 in Aβ1-42-induced SH-SY5Y cells**

RT-qPCR was used to check the proteins expressions of Caspase-3, Bax, Bcl-2 in Aβ 1-42-induced cells. From the results, it can be seen that the level of proteins expressions of Caspase-3 and Bax significantly increased. In contrast, the expression of Bcl-2 decreased significantly in Aβ 1-42-induced SH-SY5Y cells compared to control group. (Figure 5). Furthermore, treatment with EGb761 significantly reduced the proteins expressions of Caspase-3 and Bax while enhanced the level of proteins expressions of Bcl-2 in Aβ 1-42-induced SH-SY5Y cells in a concentration-dependent manner. (Figure 5).
Figure 6: EGb761 reversed Aβ1-42-induced upregulation of HO-1 expression in SH-SY5Y cells: A-B) Western blot analysis was used to measure the protein expressions of Akt/Nrf2 signaling pathway in Aβ-induced SH-SY5Y cells. P < 0.05

DISCUSSION

The present study revealed the neuroprotective effects of EGb761 against Aβ-induced SH-SY5Y cells injury and blood brain barrier disruption in vitro model of AD. Previous research demonstrated that inflammation, ROS and oxidative stress are the critical factors for the progression of AD. Also, the deposition of Aβ enhanced oxidative stress and ROS production, which leads to memory impairment and neurodegeneration diseases including AD [21]. Although the exact Pathogenic mechanism of BBB leakage is not clear. However, it has been proved from various animal models that Aβ deposition in vessels leads to the disruption of the BBB [22]. In the present study we cultured SH-SY5Y cells with Aβ to make a BBB model in AD and determined the protective effect of EGb761 on this cell model of BBB. The cell viability of SH-SY5Y cells first decreased when these cells were induced with Aβ while the rate of cell apoptosis increased in Aβ-induced SH-SY5Y cells. Also, there was an increase in ROS generation in Aβ-induced SH-SY5Y cells. On the other hand, Treatment with EGb761 significantly increased cell viability and decreased the rate of cell apoptosis and the production of ROS in Aβ-induced SH-SY5Y cells.

EGb761 has been extensively used for the treatment of many neurodegenerative diseases, such as impaired cognition function in AD [23]. Previous research revealed that activation of caspase-3 could stimulate apoptosis in neurons by Aβ-induced inflammation injury while treatment with EGb761 and HBO reversed the effect of apoptosis [24]. Also, the treatment with EGb761 significantly enhanced the protein expressions of Bcl-2 while decreased the activation of caspase-3. These findings suggested that EGb761 could improve antiapoptotic protein expressions by reducing the apoptosis of neurons induced by oxidative stress [25]. In the animal model of AD, the apoptosis was regulated by decreasing the expression of anti-apoptotic proteins and increasing the expressions of pro-apoptotic proteins, suggesting that the ratio of Bax/Bcl-2 is vital in neuronal apoptotic index [26]. Furthermore, Bcl-2 also plays a critical role in the regulation of cell death caused by oxidative stress [27]. Therefore, in this study we investigated the role of these anti-apoptotic and pro-apoptotic proteins on a cellular model of AD. Our results indicated that proteins expressions of Bcl-2 were significantly increased in Aβ-induced SH-SY5Y cells when treated with EGb761, while protein expressions of Bax and caspase-3 markedly decreased. These results indicated that treatment with EGb761 decreased the Bax and caspase-3 protein expressions while increased Bcl-2 proteins expressions, thus inhibited cell apoptosis in Aβ-induced SH-SY5Y cells.

Nrf2 signaling pathways, are considered as anti-oxidant stress pathway, and played a vital role in response to oxidative stress [28]. In oxidative stress, the downstream antioxidant gene and activation of Nrf2 was controlled by PI3K/Akt signaling pathways [29]. Previous studies have demonstrated that Nrf2 could activate anti-oxidative enzymes such as HO-1 and NAPDH and can protect against oxidative stress in various neuroinflammation diseases such as ischemia. Also, the level of ROS and oxidative stress is increased by Nrf2 in a mice model of stroke disease [30]. The activation of anti-oxidant gene such as Nrf2 and HO-1 might be involved in the neuroprotective mechanism in vivo and vitro model of AD [31]. In the present study the protein expressions of Nrf2, Akt and HO-1 was assessed to find the mechanism of EGb761 to protect Aβ-induced injury in SH-SY5Y cells. Our results showed that the protein expressions of p-Akt, nuclear Nrf2 and HO-1 reduced in Aβ-induced SH-SY5Y cells while treatment with EGb761 significantly enhanced the protein expressions of these signaling pathways. Also, EGb761 activated p-Akt followed by the upregulation of HO-1 expression, which in return protected Aβ-induced injury in SH-SY5Y cells.
CONCLUSION

EGb761 efficiently prevented Aβ-induced cell injury in SH-SY5Y cells by increasing cell viability and decreasing cell apoptosis and ROS generation. Moreover, EGb761 had a protective effect on reduction of BBB leakage. Furthermore, the protein expressions of Bax and caspase-3 significantly decreased while the expression level of Bcl-2 increased in the cells treated with EGb761. Also, the expressions of HO-1 were upregulated by the treatment with EGb761 via activation of Akt/Nrf2 pathway leading to the reduction in inflammatory injury induced by Aβ in SH-SY5Y cells. These results suggested that EGb761 might be a therapeutic drug for the treatment of AD.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Liling Wang and Jianhua Mi contributed equally to the study.

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