Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by β-amyloid 31-35

Huan-Ling Yu, Li Li, Xiao-Hong Zhang, Li Xiang, Jie Zhang, Jin-Fang Feng and Rong Xiao*

School of Public Health and Family Medicine, Capital Medical University, Beijing 100069, China

(Received 30 May 2008 – Revised 13 October 2008 – Accepted 9 December 2008 – First published online 31 March 2009)

Genistein and folic acid have been reported respectively to protect against the development of cognitive dysfunction; however, the underlying mechanism(s) for this protection remain unknown. In this report, the mechanism(s) contributing to the neuroprotective effects of genistein and folic acid were explored using rat cortical neuron cultures. We found that genistein and folic acid, both separately and collaboratively, increased cell viability and mitochondrial membrane potential in β-amyloid (Aβ) 31-35-treated neurons. Furthermore, reduced percentage of comet cells and shortened tail length were observed in the neurons treated with genistein or folic acid. A more significant reduction in tail length of the comet neurons was observed in the co-administered neurons. RT-PCR analysis of the cultured cortical neurons showed down-regulated expression of p53, bax and caspase-3, but up-regulated expression of bcl-2 in the three neuroprotective treatment groups compared with neurons from the Aβ31-35 solo-treated group. In a nuclear dyeing experiment using Hoechst 33342, we found that both genistein and folic acid prevent neuronal apoptosis. Collectively, these findings suggest that the mechanism underlying the neuroprotection of genistein and folic acid singly or in combination observed in cultured cortical neuron studies might be related to their anti-apoptotic properties.


Alzheimer’s disease, characterised by intracellular and extracellular deposits of filamentous proteins, progressive cognitive impairments and neuronal loss, is the most common form of dementia. Many biological factors appear to be involved in the development of Alzheimer’s disease, such as β-amyloid (Aβ) peptide, oxidative stress and the inflammatory process, and the ApoE4 gene(1–3). Aβ forms extracellular deposits in senile or diffuse plaques and in cerebral vasculatures in the Alzheimer’s disease brain. Researchers have found that many Aβ peptides, such as Aβ1-42, Aβ1-40, Aβ25-35 and Aβ31-35 can induce neurons to undergo apoptosis in vitro, which is tightly associated with the process of Alzheimer’s disease(4,5).

At present, Alzheimer’s disease affects millions of people worldwide and cannot be diagnosed by a valid clinical method or a biomarker before the onset of disease, and there is no cure. Scientists have found that in addition to non-modifiable genetic risk factors, potentially modifiable factors including environmental exposure and diet-related chronic diseases have been identified as risk factors for Alzheimer’s disease(6). Epidemiological studies have suggested that the consumption of oestrogen is associated with a reduced risk of dementia in women(7). But oestrogen simultaneously contributes to the development of some oestrogen-dependent cancers, such as breast cancer and prostate cancer.

Genistein, a kind of phyto-oestrogen abundant in many plant-based diets, is the predominant isoflavone form in soya food and supplements. Many researches have showed that soya phyto-oestrogens influence cognitive function and behaviour, especially in postmenopausal women(8). In an ovariectomised mouse experiment, the researchers found that escape latency was significantly shortened in a group orally administrated soyabean isoflavone continuously(9). Recently, Azcoitia et al. (10) reported that at high doses (10 mg/kg), genistein showed neuroprotective effects in a rat model.

Folic acid is not only essential for the maintenance of normal brain function, but may also be a potential source for brain therapeutics against excitotoxicity(11). In general, cerebrospinal fluid (CSF) folic acid levels are three or four times higher than blood folic acid levels. Under normal conditions, CSF folic acid concentrations do not vary with age, while late-onset Alzheimer’s disease patients have significantly lower CSF folic acid levels. Folic acid deficiency is the most common cause of hyperhomocysteinaemia that has been suggested as a risk factor of dementia or cognitive impairment(12). A randomised, double-blind, placebo-controlled study showed that daily supplementation of 800 µg oral folic acid for 3 years could improve cognitive function(13).

Genistein and folic acid prevent neuronal damage from toxic and apoptotic effects of cyclophosphamide in either

**Abbreviations:** Aβ, β-amyloid; DMSO, dimethyl sulfoxide; MMP, mitochondrial membrane potential; MTT, 3-[4,5-dimethylthiazol-2]-2,5 diphenyltetrazolium bromide.

*Corresponding author:* Dr Rong Xiao, fax +86 10 83911512, email xiaor22@ccmu.edu.cn
rats with neural tube defects or cultured cortical neurons and we also found that the protective effects of genistein co-administered with folic acid were more pronounced (14). The present study is aimed to evaluate the effects of genistein and folic acid on Aβ31-35-induced apoptosis in cultured cortical neurons of rats and their joint functions.

**Materials and methods**

**Materials**

Specific pathogen-free newborn Wistar rats were provided by the Laboratory Animal Center (Capital Medical University, Beijing, China). Genistein and folic acid were purchased from Sigma (St Louis, MO, USA) and genistein was dissolved at 40 mM in dimethyl sulfoxide (DMSO), while folic acid was dissolved in neuron culture medium. The final concentrations of genistein and folic acid were 27 μg/ml (100 μM) and 40 μg/ml (90 μM), respectively. Aβ31-35 was purchased from Sigma Chemical. It was dissolved in sterile bi-distilled water at a concentration of 3 mg/ml and stored at −20°C until use. Peptides were aggregated by incubation, at 3 mg/ml in sterile bi-distilled water, at 37°C for 2 h before being exposed to 25 μM-Aβ31-35 for 24 h. Then 20 μl MTT stock solution (5 mg/ml) was added to the culture medium and the solution was incubated for another 4 h at 37°C. The resulted MTT formazan was extracted with 200 μl DMSO and the absorbance was recorded at 570 nm by microtiter plate reader (Tecan Sunrise Microplate Reader; Tecan Group Ltd, Männedorf, Switzerland). The sample solution was freshly prepared in which DMSO concentration was lower than 0.1%.

**Mitochondrial membrane potential**

The fluorescent dye rhodamine 123 (Molecular Probes, Eugene, OR, USA) was used as a measure of mitochondrial membrane potential (MMP), as described by Almeida et al. (1). Briefly, neurons were incubated for 30 min at 37°C in the presence of the dye (10 μg/ml) and then washed twice with PBS solution. Fluorescence signals were captured using a flow cytometer at 529 nm emission wavelength, corresponding to the fluorescence peak of the monomer and that of the aggregate.

**Measurements of apoptosis**

Hoechst 33342, a fluorescent stain for labelling DNA, was used to observe the apoptotic neurons. Briefly, to visualise nuclear morphology following Aβ31-35 treatment, cells were fixed with 4% paraformaldehyde and stained in Hoechst 33342 DNA-binding dye (10 mg/l) for 15 min at 37°C in the dark, then washed with PBS three times and the nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Single cell gel electrophoresis**

The single cell gel electrophoresis assay was used to assess cells whose DNA was fragmented to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates more slowly and remains within the confines of the nucleoid. Briefly, neurons were trypsinised, centrifuged (2 min; 1000 rpm) and cell pellets were suspended in a pre-warmed DMSO and 1% Triton X-100, for 1 h at about 4°C. DNA was allowed to unwind for 20 min in fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) and the electrophoretic migration was then performed at 4°C (24 min, 20 V, 300 mA, 25 min). The slides were bathed twice for 5 min in neutralising solution (0.4 M-Tris-HCl, pH 7.5) and dried for conservation with 95% ethanol for 5 min. DNA was stained with propidium iodide (2.5 μg/ml in PBS) just before slide examination with a fluorescence microscope (TE2000-E; Nikon, Melville, NY, USA). At least 100 images per dose were analysed using the Comet Assay IV software (Perceptive Instruments, Haverhill, Suffolk, UK). Two parameters, the number of comet cells,
defined as the number of neurons with a comet tail, and DNA migration length (μm), were used to evaluate the extent of DNA damage in individual cells.

**Reverse transcriptase polymerase chain reaction**

Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using an RT kit (no. A3500; Applied Promega, Madison, WI, USA). mRNA encoding bax, bcl-2, caspase-3, p53 and β-actin (used as an invariant control) were analysed by RT-PCR. The primer sequences are listed in Table 1. PCR was carried out as follows: denaturation at 94°C for 5 min for the first cycle, and then cycles consisting of 94°C for 30 s, annealing at 54°C, 53°C or 59°C for 30 s, and extension at 72°C for 30 s. Thirty-five cycles were performed for all the genes. Amplification products underwent electrophoresis on a 2.0% agarose gel and the relative quantity of mRNA was estimated by densitometry scanning with X-rays (Gel Doc XRTM; Bio-Rad, Hercules, CA, USA).

**Statistical analysis**

Data are shown as mean values and standard deviations. Statistical analysis was performed by one-way ANOVA. The acceptable level of significance was set at P<0.05.

**Results**

**Cell viability**

Viabilities of neurons in the control were 1.038 (SD 0.125) (vehicle). After treatment with Aβ31-35, neuron viability significantly decreased to 0.811 (SD 0.083). However, both folic acid and genistein increased neurons’ viability when given administrated with folic acid group was much shorter than in the singly treated group (Fig. 2). Both genistein and folic acid demonstrated potential protective activity. Though there were no significant differences between the genistein and folic acid singly treated and co-administrated groups, mitochondrial potential was much higher than that in the singly treated group (Fig. 2).

**DNA structure**

Compared with the control, comet cells and DNA tail length were significantly increased in the Aβ31-35-treated group (Fig. 3). The comet cells and DNA migration length in three experiments were significantly decreased. It was exciting to find that the DNA migration length in the genistein co-administrated with folic acid group was much shorter than that in the genistein or folic acid solo-treated groups.

**Apoptosis analysis**

Neuron apoptosis was observed by Hoechst 33342 nuclear dyeing. Compared with the control group, Aβ31-35 induced neuronal shrinkage, nucleus pycnosis, chromatid displayed significant decrease in mitochondrial potential.

**Mitochondrial membrane potential**

To analyse the alteration in MMP that follows Aβ apoptotic stimulation, we incubated cells treated with 10 μM-Aβ with the cationic dye rhodamine 123, and subsequently analysed the cells using a flow cytometer. Neurons treated with Aβ31-35 displayed significant decrease in mitochondrial potential. Both genistein and folic acid demonstrated potential protective activity. Though there were no significant differences between the genistein and folic acid singly treated and co-administrated groups, mitochondrial potential was much higher than that in the singly treated group (Fig. 2).

### Table 1. Primers of BAX, BCL2,caspase-3 (CASP3), TPS3 and β-actin (ACTB)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>Temperature (°C)</th>
<th>Length (bp)</th>
<th>Exon position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX</td>
<td>Forward 5’-TGCAGAAGGATGGATGCTGAC-3’ Reverse 5’-GTACGACTGCGGACACTTAG-3’</td>
<td>54</td>
<td>173</td>
<td>Base position 141 to 160 of exon 3 Base position 11 to 30 of exon 5</td>
</tr>
<tr>
<td>BCL2</td>
<td>Forward 5’-CAGCTGCAGCTGCAGCCTT-3’ Reverse 5’-CCCAGCCCTCCATCTCTGG-3’</td>
<td>54</td>
<td>195</td>
<td>Base position 579 to 598 of exon 1 Base position 793 to 813 of exon 1</td>
</tr>
<tr>
<td>CASP3</td>
<td>Forward 5’-GGCAAGCCGGGTCCCTGAAG-3’ Reverse 5’-TGACTCGACCTGGATGATGAAG-3’</td>
<td>60</td>
<td>135</td>
<td>Base position 1428 to 1447 of exon 7 Base position 1539 to 1592 of exon 7</td>
</tr>
<tr>
<td>TPS3</td>
<td>Forward 5’-GACAGCAAGGCTCTGGATGAG-3’ Reverse 5’-GGAGTCTCCACGCGTGTG-3’</td>
<td>53</td>
<td>429</td>
<td>Base position 201 to 218 of exon 6 Base position 259 to 279 of exon 4</td>
</tr>
<tr>
<td>ACTB</td>
<td>Forward 5’-TGGATCTGGTGGGATCCATGAAAC-3’ Reverse 5’-TAAAAGCGCACTGAGTCCG-3’</td>
<td>59</td>
<td>348</td>
<td>Base position 186 to 210 of exon 5 Base position 359 to 384 of exon 5</td>
</tr>
</tbody>
</table>
margination or crescent-shaped and apoptotic body formation. The percentage of apoptotic neurons was significantly increased when treated with Aβ31-35 compared with that in the control group, while neurons pretreated with genistein and/or folic acid showed a significant but incomplete prevention of apoptosis (Fig. 4).

**mRNA levels of bax, bcl-2, caspase-3 and p53 genes**

Compared with the control group, the expressions of bax, caspase-3 and p53 genes were all up-regulated and bcl-2 genes were down-regulated when the neurons were treated with Aβ31-35. The expression of bax was down-regulated when the neurons treated with Aβ31-35 were incubated with folic acid, genistein and genistein co-administered with folic acid. The expressions of p53 and caspase-3 were down-regulated, while the expression of bcl-2 was up-regulated when the neurons treated with Aβ31-35 were incubated with genistein and genistein co-administered with folic acid (Table 2; Fig. 5).
Discussion

Aβ31-35, the core sequence and active centre of the β-amyloid peptide, induces cell apoptosis in PC 12 cells\(^5\) and rat cerebellar granule cells\(^16\). The mechanisms under the Aβ31-35-induced apoptosis include bax mRNA expression up-regulation\(^17\), caspase activation, DNA fragmentation\(^18\) and protein kinase A activation\(^19\). We found that Aβ31-35 not only poisoned the neurons directly (cell viability decreased), but also decreased MMP, damaged the integrity of nuclear DNA, up-regulated the expression of bax, caspase-3 and p53 genes, down-regulated the expression of the bcl-2 genes, and finally led to neuronal apoptosis. The present results from the MTT assay (Fig. 1), MMP, comet assay and apoptotic analysis (Hoechst 33342 staining and determining expression of apoptosis-related genes) introduced the evidence that genistein and folic acid could protect cultured cortical neurons against Aβ31-35 toxicity.

Genistein, with potentially beneficial health effects such as anti-carcinogenic qualities, has been identified as having anti-prolific and pro-apoptotic effects on various malignant cell types derived from solid and non-solid tumours\(^20\). However, the anti-apoptotic effects of genistein in pancreatic β-cells\(^21\), primary neurons\(^22\) and human mononuclear cells\(^23\), induced by cytokines\(^21\), glutamate\(^22\) and methylglyoxal\(^23\), have been reported widely. These studies always concluded that the anti-apoptotic effects of genistein were dose-dependent\(^21 – 23\). Another study found that cells pre-treated with 50 μM-genistein could significantly prevent HCN1-A cells from cell death induced by 100 μM- and 1 mM-tertiary butylhydroperoxide\(^24\). In the present study, we did not find toxicity of 100 μM-genistein on the cultured cortical neurons treated with Aβ31-35. On the contrary, when administered 2 h before Aβ31-35 was added, genistein protected the neurons from the damage induced by Aβ31-35. These discrepancies may be due to differences in experimental conditions, such as the neural cell type and culture medium (for example, the toxic agent used)\(^25\). So, it is important to study the effective level of genistein, which is toxic or protective to primary cultured cortical neurons damaged by Aβ31-35.

Folic acid deficiency induces neurotoxicity by multiple routes. It was reported that folic acid deprivation increased cytosolic Ca and reactive oxygen species (ROS) and impaired mitochondrial function\(^26\). Moreover folic acid was remarkably neuroprotective against glutamate and N-methyl-d-aspartic acid cytotoxicity in a dose- and time-dependent manner\(^27\). In the present study, folic acid supplementation decreased MMP and the percentage of comet cells that had been increased by Aβ31-35 in cultured cortical neurons. It has been reported that folic acid deprivation induced...

### Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>BAX Mean (SD)</th>
<th>BCL2 Mean (SD)</th>
<th>CASP3 Mean (SD)</th>
<th>TP53 Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.069* (0.018)</td>
<td>0.329* (0.070)</td>
<td>0.072* (0.000)</td>
<td>1.656* (0.085)</td>
</tr>
<tr>
<td>Aβ31-35</td>
<td>0.122 (0.052)</td>
<td>0.118 (0.073)</td>
<td>0.190 (0.014)</td>
<td>2.451 (0.273)</td>
</tr>
<tr>
<td>Aβ + FA</td>
<td>0.035* (0.012)</td>
<td>0.170 (0.063)</td>
<td>0.144 (0.056)</td>
<td>1.532 (0.222)</td>
</tr>
<tr>
<td>Aβ + Gen</td>
<td>0.044* (0.023)</td>
<td>0.269* (0.063)</td>
<td>0.099* (0.013)</td>
<td>0.890* (0.117)</td>
</tr>
<tr>
<td>Aβ + FA + Gen</td>
<td>0.048* (0.017)</td>
<td>0.250* (0.045)</td>
<td>0.073* (0.030)</td>
<td>1.244* (0.536)</td>
</tr>
</tbody>
</table>

* Mean value was significantly different from that of the Aβ31-35 group (P<0.05).
neurodegeneration changes typical of those observed in Alzheimer’s disease, including increased cytosolic Ca, ROS, phosho-tau and the apoptotic process; an increase in glutathione and reduction in ROS levels were observed following supplementation of folic acid-deprived cultures(28). Substantial evidence has indicated that the neuronal damage caused by Aβ is mediated through oxidative stress(29,30). So, the mechanism of folic acid supplementation alleviating the damage of mitochondria and DNA induced by Aβ may be related to the alteration of oxidative stress conditions.

Mitochondria are integrated in a number of signalling pathways, including cell death cascades, thus controlling cellular homeoestasis in multiple ways(31). Mitochondria undergo two major alterations during apoptosis. The first is the permeability of the outer mitochondrial membrane. This event is tightly regulated by members of the Bcl-2 family and involves the conformational change of pro-apoptotic family members such as Bax. Second, the electrochemical gradient that is normally present across the inner mitochondrial membrane is lost (membrane depolarisation)(32). An array of evidence suggests that alteration of mitochondrial function is critically involved in the apoptotic process. Dysfunctional mitochondria bear the risk of futile ATP hydrolysis and enhancement of oxidative stress. Furthermore, extensive mitochondrial damage may lead to the dissipation of the membrane potential across the inner membrane and induce cell death by the release of pro-apoptotic proteins(33). It is reported that in response to adverse stimuli, the mitochondrial permeability transition pore is actively opened, resulting in the collapse of MMP and release of multiple pro-apoptotic proteins such as Smac, Cyto C, AIF, Omi, etc, from the impaired mitochondria to cytosol(34) and these proteins in turn trigger a cascade of events and eventually lead to cell apoptosis. We found that MMP markedly decreased after exposure to Aβ31-35. However, the reduction of MMP was attenuated after genistein and/or folic acid treatment, which indicated that the neuroprotective effect of genistein and/or folic acid might be related to maintaining the structure and function of mitochondria.

Bcl-2 family members are the arbiters of the mitochondrial apoptotic pathway, which is subdivided into two classes including anti-apoptotic members such as Bcl-2, Bcl-xl, Bcl-w and Mcl-1, which protect cells from apoptosis, and pro-apoptotic members such as Bax, Bak, Bad, Bid and Bim, which induce cell apoptosis. The stoichiometry of pro-v. anti-apoptotic Bcl-2 family members in the cell determines whether the cell lives or dies. This fine balance between anti-apoptosis and pro-apoptosis is regulated at the transcriptional or post-translational level in response to various cellular cues. Our earlier study found that the suppression of bcl-2 gene expression was the early signal in the neuron’s programmed death when exposed to an apoptotic agent(14,35). When the bax:bcl-2 ratio was > 1, bax regulated mitochondrial cytochrome c release in vivo and in vitro(36). In the present study, the expression of the bcl-2 gene was significantly down-regulated while the expression of the bax gene was significantly up-regulated and the ratio of bax:bcl-2 was greater than 1 in Aβ31-35-treated neurons, which indicated the ongoing of apoptosis. If pretreated with genistein or genistein co-administered with folic acid, the situation could be reversed (Table 2).

It has been demonstrated that Aβ1-42 and Aβ25-35 may induce caspase-dependent apoptosis. Apoptotic cell death associated with the activation of caspases has been found in several neuronal cell types exposed to Aβ. Activation of caspase-3 is a key event in the execution of the apoptotic cascade in central nervous system disorders including Alzheimer’s disease. Aβ31-35 can also induce apoptosis in the cortical and hippocampal neurons as Aβ25-35 does and further study showed that apoptosis was mediated by caspase-dependent pathways(17-35). In the present study, we gave evidence that genistein or genistein co-administered with folic acid could alleviate the up-regulation of the caspase-3 gene induced by Aβ31-35, which indicated that the effect of anti-apoptosis by genistein was achieved possibly via caspase-dependent pathways.

Recently, involvement of p53 in neuronal death occurring in Alzheimer’s disease(37) has been detected. Cell culture studies have established strong relationships between p53 expression and neuronal death induced by DNA-damaging agents and glutamate(38). From these results, we could suggest that the expression of p53, bax, bcl-2 and caspase3 were changed by Aβ31-35, which induced the neurons to undergo apoptosis and genistein or genistein co-administered with folic acid could intervene this apoptotic progress.

In the study, the protective effects of genistein co-administered with folic acid to DNA integrity were significantly different from that of genistein or folic acid, although there were no significant differences for cell viability and MMP. The reason may be related to the isolated protective effects of genistein and folic acid that were strong enough so that the combination was not prominent, while the protective effect was enhanced when the isolated effects were not sufficient. On the other hand, the antioxidative activity of genistein has been reported widely(23,24,39-41). The anti-apoptotic effect of folic acid and genistein may be mainly related to their antioxidative activity; common physiological mechanisms may be affected, such that there was no enhanced protection in some variables examined.

The present results suggest that genistein and/or folic acid protected the neurons from the damage of Aβ31-35 by maintaining mitochondrial function and DNA integrity and regulating the apoptosis-related genes. The cooperation effects of genistein and folic acid were significantly displayed when the single effects were not strong enough. The molecular mechanisms of the anti-apoptosis function of genistein in cultured cortical neurons and if there is an effective genistein concentration spectrum that is beneficial to Aβ-treated neurons will be the next goals in further studies.
Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (no. 30771802 and 30571560) and the Beijing Municipal Commission of Education Science and Technology Developmental Plan Foundation (no. KM200610025010 and KZ200710025011). H.-L. Y contributed to the drafting of the paper and the Hoechst 33342 nuclear dyeing; J.-F. F. did the data analysis; L. L., X.-H. Z, J. Z., and L. X. contributed to the cell culture, RT-PCR, single cell gel electrophoresis, MMP and MTT analyses; R. X. designed the study and was the final principal of the study.

There is no conflict of interest associated with the present study.

References


