

## Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by $\beta$ -amyloid 31-35

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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  $\beta$ -amyloid (A $\beta$ ) 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 A $\beta$ 31-35 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.

**Genistein: Folic acid:  $\beta$ -Amyloid peptide 31-35: Apoptosis: Neuroprotection: Cultured cortical neurons**

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  $\beta$ -amyloid (A $\beta$ ) peptide, oxidative stress and the inflammatory process, and the ApoE4 gene<sup>(1–3)</sup>. A $\beta$  forms extracellular deposits in senile or diffuse plaques and in cerebral vasculatures in the Alzheimer's disease brain. Researchers have found that many A $\beta$  peptides, such as A $\beta$ 1-42, A $\beta$ 1-40, A $\beta$ 25-35 and A $\beta$ 31-35 can induce neurons to undergo apoptosis *in vitro*, which is tightly associated with the process of Alzheimer's disease<sup>(4,5)</sup>.

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<sup>(6)</sup>. Epidemiological studies have suggested that the consumption of oestrogen is associated with a reduced risk of dementia in women<sup>(7)</sup>. 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<sup>(8)</sup>. In an ovariectomised mouse experiment, the researchers found that escape latency was significantly shortened in a group orally administered soyabean isoflavone continuously<sup>(9)</sup>. Recently, Azcoitia *et al.*<sup>(10)</sup> 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<sup>(11)</sup>. 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<sup>(12)</sup>. A randomised, double-blind, placebo-controlled study showed that daily supplementation of 800  $\mu$ g oral folic acid for 3 years could improve cognitive function<sup>(13)</sup>.

Genistein and folic acid prevent neuronal damage from many excitotoxicities; however, the mechanism of their neuroprotective actions has not been fully elucidated. Our previous study indicated that genistein and/or folic acid attenuated the toxic and apoptotic effects of cyclophosphamide in either

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

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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<sup>(14)</sup>. The present study is aimed to evaluate the effects of genistein and folic acid on A $\beta$ 31-35-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  $\mu$ g/ml (100  $\mu$ M) and 40  $\mu$ g/ml (90  $\mu$ M), respectively. A $\beta$ 31-35 was purchased from Sigma Chemical. It was dissolved in sterile bi-distilled water at a concentration of 3 mg/ml and stored at  $-20^{\circ}\text{C}$  until use. Peptides were aggregated by incubation, at 3 mg/ml in sterile bi-distilled water, at  $37^{\circ}\text{C}$  for 4 d and then used as neurotoxin to cultured neurons.

### Cortical neuron culture and neurotoxicity treatments to cultured neurons

The neuron culture protocol was adapted from the established method in our laboratory<sup>(15)</sup> for primary cultured cortical neurons of the newborn (no more than 24 h) Wistar rat. Briefly, rats were killed by rapid decapitation and their brains quickly harvested. After cortical layers were minced and dissociated by mechanical trituration, cortical neurons were dissociated in Dulbecco's modified Eagle's medium supplemented with 10% low-endotoxin horse serum, 10% heat-inactivated fetal bovine serum (v/v), 26 mM-bicarbonate, 25 mM-D-glucose, 25 mM-HEPES, penicillin (10 mg/ml) and streptomycin (10 mg/ml). The single-cell suspension was plated into 35 mm diameter dishes ( $2 \times 10^5$  cells/cm<sup>2</sup>) pre-coated with poly-L-lysine. Cultures were maintained in an incubator at  $37^{\circ}\text{C}$  under a 5% CO<sub>2</sub>-95% air atmosphere. Non-neuronal cell division was halted by exposure to 10 mM-cytosine arabinoside for 1 d and the cells were then transferred into a maintenance medium identical to the plating media.

After 48 h of culture, 2 h before the neurons were exposed to A $\beta$ 31-35 (25  $\mu$ mol/l), genistein and/or folic acid were added. After another 24 h, cell viability assessment and other experiments were performed. Each experiment was repeated at least three times.

### Cell viability

Cell viability was assessed using a modified 3-[4,5-dimethylthiazol-2]-2,5 diphenyltetrazolium bromide (MTT) assay. The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal, which is accumulated within healthy cells, and the number of surviving cells is directly proportional to the level of the formazan product created. The colour can

then be quantified using a simple colorimetric assay. Briefly, the cultured cortical neurons were pre-incubated with genistein and/or folic acid for 2 h before being exposed to 25  $\mu$ M-A $\beta$ 31-35 for 24 h. Then 20  $\mu$ l MTT stock solution (5 mg/ml) was added to the culture medium and the solution was incubated for another 4 h at  $37^{\circ}\text{C}$ . The resulted MTT formazan was extracted with 200  $\mu$ l 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.*<sup>(1)</sup>. Briefly, neurons were incubated for 30 min at  $37^{\circ}\text{C}$  in the presence of the dye (10  $\mu$ g/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 A $\beta$ 31-35 treatment, cells were fixed with 4% paraformaldehyde and stained in Hoechst 33342 DNA-binding dye (10 mg/l) for 15 min at  $37^{\circ}\text{C}$  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 low-melting-point agarose (0.5% in PBS) and deposited on conventional microscope slides (initially dipped in 1% agarose and dried) precoated with normal agarose (0.8% in PBS). The slides were then put in a lysis solution composed of 2.5 M-NaCl, 0.1 M-EDTA, 10 mM-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl (Tris-HCl) at pH 10, extemporarily added with 10% DMSO and 1% Triton X-100, for 1 h at about  $4^{\circ}\text{C}$ . 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  $4^{\circ}\text{C}$  (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  $\mu$ g/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 ( $\mu\text{m}$ ), 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  $\beta$ -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: denature at  $94^{\circ}\text{C}$  for 5 min for the first cycle, and then cycles consisting of  $94^{\circ}\text{C}$  for 30 s, annealing at  $54^{\circ}\text{C}$ ,  $53^{\circ}\text{C}$  or  $59^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  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 comparisons were performed by one-way ANOVA. The acceptable level of significance was set at  $P < 0.05$ .

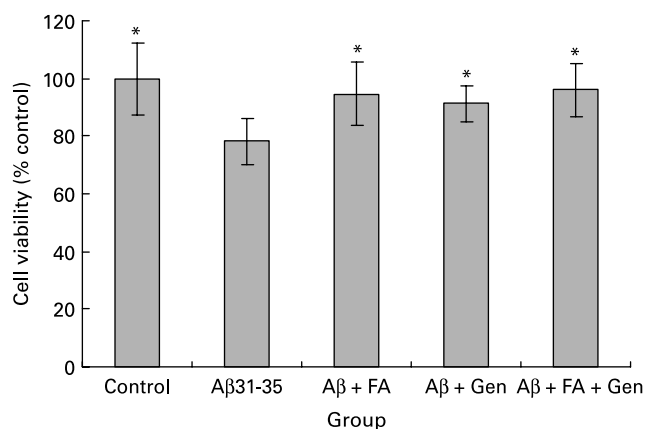
## Results

#### Cell viability

Viabilities of neurons in the control were 1.038 (SD 0.125) (vehicle). After treatment with A $\beta$ 31-35, neuron viability significantly decreased to 0.811 (SD 0.083). However, both folic acid and genistein increased neurons' viability when given 2 h before they were exposed to A $\beta$ 31-35. Though the neurons' viability was slightly increased, there were no statistically significant differences when genistein was co-administrated with folic acid, compared with genistein or folic acid singly administrated as indicated in Fig. 1.

#### Mitochondrial membrane potential

To analyse the alteration in MMP that follows A $\beta$  apoptotic stimulation, we incubated cells treated with  $10\ \mu\text{M}$ -A $\beta$  with the cationic dye rhodamine 123, and subsequently analysed the cells using a flow cytometer. Neurons treated with A $\beta$



**Fig. 1.** Effect of  $\beta$ -amyloid (A $\beta$ ) 31-35 on neuron viability, protection of genistein (Gen) and folic acid (FA) and the protective effect of the co-administration of FA and Gen. Neurons were exposed to vehicle (control), A $\beta$ 31-35, A $\beta$  + FA, A $\beta$  + Gen or A $\beta$  + FA + Gen. FA and/or Gen was added 2 h before the neurons were exposed to A $\beta$ 31-35. Cell survival was quantified at 24 h by assaying 3-[4,5-dimethylthiazol-2]-2,5 diphenyltetrazolium bromide (MTT). Values are means, with standard deviations represented by vertical bars. \* Mean value was significantly different from that of the A $\beta$ 31-35 group ( $P < 0.05$ ).

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).

#### DNA structure

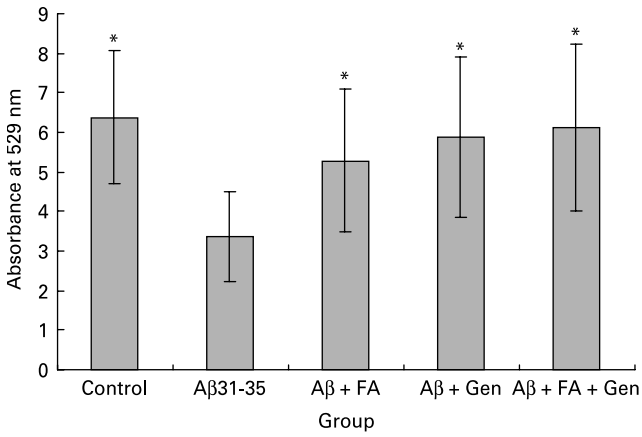
Compared with the control, comet cells and DNA tail length were significantly increased in the A $\beta$ 31-35-treated group (Fig. 3). The comet cells and DNA migration length in three experiments were significantly decreased. It was exciting to find that the DNA migration length in the genistein co-administrated 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, A $\beta$ 31-35 induced neuronal shrinkage, nucleus pycnosis, chromatin

**Table 1.** Primers of BAX, BCL2, caspase-3 (CASP3), TP53 and  $\beta$ -actin (ACTB)

Gene	Primer (5'–3')	Temperature ( $^{\circ}\text{C}$ )	Length (bp)	Exon position
BAX	Forward 5'-TGCAGAGGATGATTGCTGAC-3'	54	173	Base position 141 to 160 of exon 3 Base position 11 to 30 of exon 5
	Reverse 5'-GATCAGCTCGGGCACTTTAG-3'			
BCL2	Forward 5'-CAGCTGCACCTGACGCCCTT-3'	54	195	Base position 579 to 598 of exon 1 Base position 793 to 813 of exon 1
	Reverse 5'-CCCAGCCTCCGTTATCCTGGA-3'			
CASP3	Forward 5'-GGCACACGGGACTTGGAAAG-3'	60	135	Base position 1428 to 1447 of exon 7 Base position 1539 to 1592 of exon 7
	Reverse 5'-TGACTCAGCACCTCCATGATTAAG-3'			
TP53	Forward 5'-GACAGCCAAGTCTGTTATGTG-3'	53	429	Base position 259 to 279 of exon 4 Base position 201 to 218 of exon 6
	Reverse 5'-GGAGTCTTCCAGCGTGAT-3'			
ACTB	Forward 5'-TGGAAATCCTGTGGCATCCATGAAAC-3'	59	348	Base position 186 to 210 of exon 5 Base position 359 to 384 of exon 5
	Reverse 5'-TAAACGCAGCTCAGTAACAGTCCG-3'			

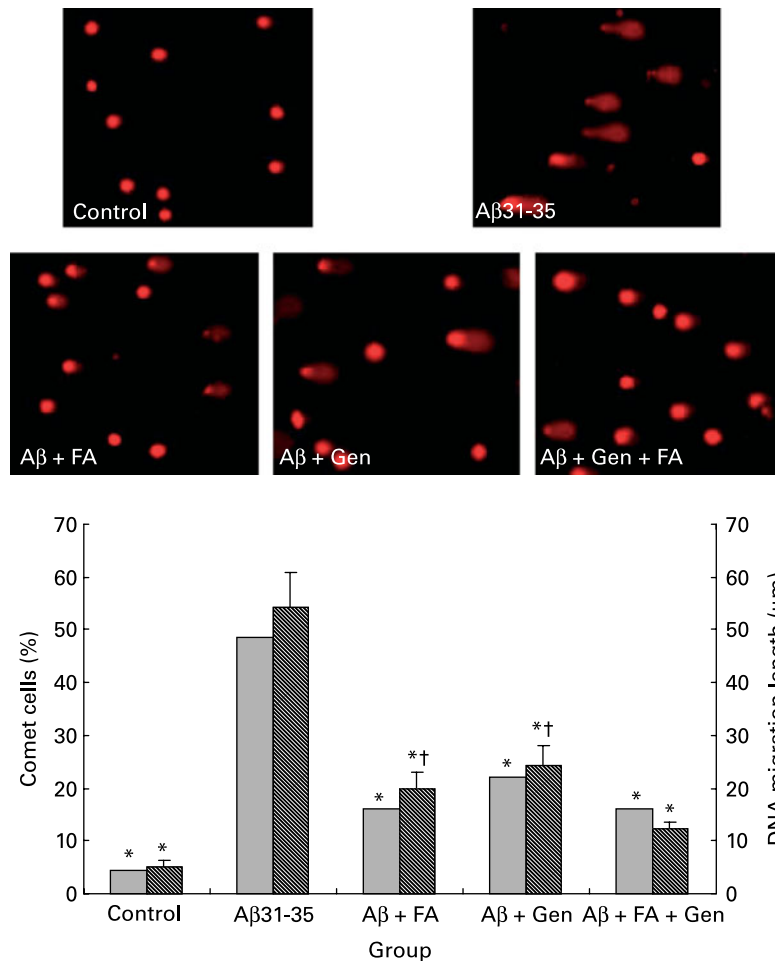


**Fig. 2.** Effect of  $\beta$ -amyloid (A $\beta$ ) 31-35 and protection of genistein (Gen) and folic acid (FA) on mitochondrial membrane potential (MMP) as assessed by fluorescence change of rhodamine 123 at 529 nm. Neurons were stained using the fluorescent probe rhodamine 123 to measure changes in MMP. Values are means, with standard deviations represented by vertical bars. \*Mean value was significantly different from that of the A $\beta$ 31-35 group ( $P < 0.05$ ).

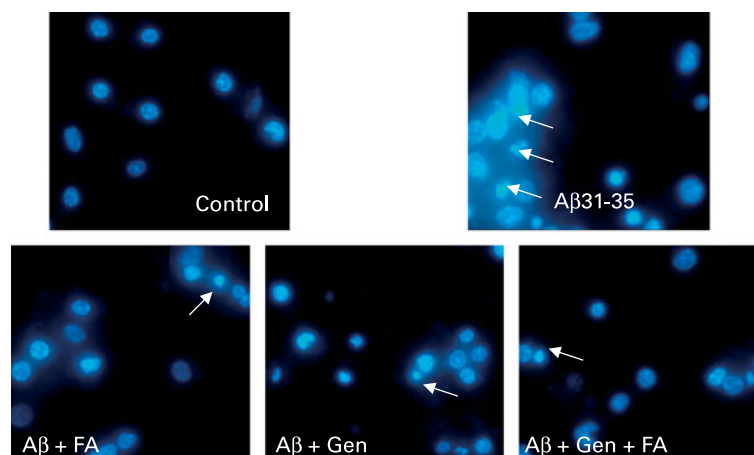
margination or crescent-shaped and apoptotic body formation. The percentage of apoptotic neurons was significantly increased when treated with A $\beta$ 31-35 compared with that in the control group, while neurons pretreated with genistein and/or folic acid showed a significant but incomplete prevention 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 A $\beta$ 31-35. The expression of bax was down-regulated when the neurons treated with A $\beta$ 31-35 were incubated with folic acid, genistein and genistein co-administrated 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 A $\beta$ 31-35 were incubated with genistein and genistein co-administered with folic acid (Table 2; Fig. 5).



**Fig. 3.** Effect of  $\beta$ -amyloid (A $\beta$ ) 31-35 on DNA structure and protection of genistein (Gen) and folic acid (FA). The y axes represent the number of cells with a comet tail (%) (□) and the DNA migration length ( $\mu\text{m}$ ; ▨). Values are means, with standard deviations represented by vertical bars. \* Mean value was significantly different from that of the A $\beta$ 31-35 group ( $P < 0.05$ ). † Mean value was significantly different from that of the A $\beta$  + FA + Gen group ( $P < 0.05$ ).



**Fig. 4.** Neurons were stained with the DNA-binding fluorochrome Hoechst 33342. Fluorescence micrographs are of cortical cell nuclei from untreated cells (control), cells exposed to 25  $\mu\text{M}$ - $\beta$ -amyloid (A $\beta$ ) 31-35, cells exposed to folic acid (40  $\mu\text{g}/\text{ml}$ ) 2 h before 25  $\mu\text{M}$ -A $\beta$ 31-35 (FA), cells exposed to genistein (27  $\mu\text{g}/\text{ml}$ ) 2 h before 25  $\mu\text{M}$ -A $\beta$ 31-35 (Gen), and cells co-incubated with genistein plus folic acid (Gen + FA). For explanation, see the Apoptosis analysis section.

## Discussion

A $\beta$ 31-35, the core sequence and active centre of the  $\beta$ -amyloid peptide, induces cell apoptosis in PC 12 cells<sup>(5)</sup> and rat cerebellar granule cells<sup>(16)</sup>. The mechanisms under the A $\beta$ 31-35-induced apoptosis include bax mRNA expression up-regulation<sup>(17)</sup>, caspase activation, DNA fragmentation<sup>(18)</sup> and protein kinase A activation<sup>(19)</sup>. We found that A $\beta$ 31-35 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 A $\beta$ 31-35 toxicity.

Genistein, with potentially beneficial health effects such as anti-carcinogenic qualities, has been identified as having anti-proliferative and pro-apoptotic effects on various malignant cell types derived from solid and non-solid tumours<sup>(20)</sup>. However, the anti-apoptotic effects of genistein in pancreatic  $\beta$ -cells<sup>(21)</sup>, primary neurons<sup>(22)</sup> and human mononuclear cells<sup>(23)</sup>, induced by cytokines<sup>(21)</sup>, glutamate<sup>(22)</sup> and

methylglyoxal<sup>(23)</sup>, have been reported widely. These studies always concluded that the anti-apoptotic effects of genistein were dose-dependent<sup>(21–23)</sup>. Another study found that cells pre-treated with 50  $\mu\text{M}$ -genistein could significantly prevent HCN1-A cells from cell death induced by 100  $\mu\text{M}$ - and 1 mM-tertiary butylhydroperoxide<sup>(24)</sup>. In the present study, we did not find toxicity of 100  $\mu\text{M}$ -genistein on the cultured cortical neurons treated with A $\beta$ 31-35. On the contrary, when administered 2 h before A $\beta$ 31-35 was added, genistein protected the neurons from the damage induced by A $\beta$ 31-35. 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)<sup>(25)</sup>. So, it is important to study the effective level of genistein, which is toxic or protective to primary cultured cortical neurons damaged by A $\beta$ 31-35.

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<sup>(26)</sup>. Moreover folic acid was remarkably neuroprotective against glutamate and *N*-methyl-D-aspartic acid cytotoxicity in a dose- and time-dependent manner<sup>(27)</sup>. In the present study, folic acid supplementation decreased MMP and the percentage of comet cells that had been increased by A $\beta$ 31-35 in cultured cortical neurons. It has been reported that folic acid deprivation induced

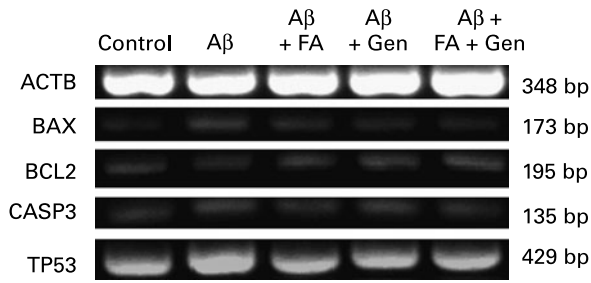
**Table 2.** Effect of genistein (Gen) and/or folic acid (FA) on BAX, BCL2, caspase-3 (CASP3) and TP53 mRNA levels in the brain

(Mean values and standard deviations)

Group	BAX		BCL2		CASP3		TP53	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0.069*	0.018	0.329*	0.070	0.072*	0.000	1.656*	0.085
A $\beta$ 31-35	0.122	0.052	0.118	0.073	0.190	0.014	2.451	0.273
A $\beta$ + FA	0.035*	0.012	0.170	0.063	0.144	0.056	1.532	0.222
A $\beta$ + Gen	0.064*	0.023	0.269*	0.063	0.099*	0.013	0.890*	0.117
A $\beta$ + FA + Gen	0.048*	0.017	0.250*	0.045	0.073*	0.030	1.244*	0.536

A $\beta$ ,  $\beta$ -amyloid.

\* Mean value was significantly different from that of the A $\beta$ 31-35 group ( $P < 0.05$ ).



**Fig. 5.** Effect of  $\beta$ -amyloid (A $\beta$ ) 31-35 and protection of genistein (Gen) and folic acid (FA) on the mRNA levels of  $\beta$ -actin (ACTB), BAX, BCL2, caspase-3 (CASP3) and TP53 genes in cortical neurons.

neurodegeneration changes typical of those observed in Alzheimer's disease, including increased cytosolic Ca, ROS, phospho-tau and the apoptotic process; an increase in glutathione and reduction in ROS levels were observed following supplementation of folic acid-deprived cultures<sup>(28)</sup>. Substantial evidence has indicated that the neuronal damage caused by A $\beta$  is mediated through oxidative stress<sup>(29,30)</sup>. So, the mechanism of folic acid supplementation alleviating the damage of mitochondria and DNA induced by A $\beta$  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 homeostasis in multiple ways<sup>(31)</sup>. 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)<sup>(32)</sup>. An array of evidence suggests that alteration of mitochondria 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<sup>(33)</sup>. 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<sup>(34)</sup> 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 A $\beta$ 31-35. 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-x1, 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- 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<sup>(14,35)</sup>. When the bax:bcl-2 ratio was  $>1$ , bax regulated mitochondrial cytochrome c release *in vivo* and *in vitro*<sup>(36)</sup>. 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 A $\beta$ 31-35-treated neurons, which indicated the ongoing of apoptosis. If pretreated with genistein or genistein co-administrated with folic acid, the situation could be reversed (Table 2).

It has been demonstrated that A $\beta$ 1-42 and A $\beta$ 25-35 may induce caspase-dependent apoptosis. Apoptotic cell death associated with the activation of caspases has been found in several neuronal cell types exposed to A $\beta$ . Activation of caspase-3 is a key event in the execution of the apoptotic cascade in central nervous system disorders including Alzheimer's disease. A $\beta$ 31-35 can also induce apoptosis in the cortical and hippocampal neurons as A $\beta$ 25-35 does and further study showed that apoptosis was mediated by caspase-dependent pathways<sup>(17-19,35)</sup>. In the present study, we gave evidence that genistein or genistein co-administrated with folic acid could alleviate the up-regulation of the caspase-3 gene induced by A $\beta$ 31-35, 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<sup>(37)</sup> has been detected. Cell culture studies have established strong relationships between p53 expression and neuronal death induced by DNA-damaging agents and glutamate<sup>(38)</sup>. From these results, we could suggest that the expression of p53, bax, bcl-2 and caspase3 were changed by A $\beta$ 31-35, which induced the neurons to undergo apoptosis and genistein or genistein co-administrated with folic acid could intervene this apoptotic progress.

In the study, the protective effects of genistein co-administrated 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<sup>(23,24,39-41)</sup>. 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 A $\beta$ 31-35 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 A $\beta$ -treated neurons will be the next goals in further studies.

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

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