Protective effect of 7-difluoromethoxy-5,4'-Di-hydroxyl isoflavone against the damage induced by glutamate in PC12 cells

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Abstract. 7-difluoromethoxy-5,4'-Di-hydroxyl isoflavone (dFGEN), prepared by the difluoromethylation of genistein, is an active chemical entity. In this study, our main purpose was to investigate whether dFGEN had an effect on glutamate-induced apoptosis in cultured PC12 cells. The PC12 cells were treated with different glutamate concentrations for 24 h *in vitro*. The PC12 cells impaired by glutamate were used as the cell model of excitability. Cells were incubated for 30 min with genistein, dFGEN, vitamin E, and exposed to 10 mM glutamate for 24 h. Cell morphology was observed by light microscopy. The growth and proliferation of PC12 cells were detected by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cell apoptosis was determined by flow cytometry (FCM) with propidium iodide (PI) staining. The activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and the content of malondialdelyde (MDA) were measured by kits, respectively. Acridine orange (AO) staining was used to detect characteristics of cell apoptosis. When PC12 cells were incubated with glutamate for 24 h, cells appeared to have significant changes in shape. The cellular viability was reduced and the apoptotic rate was increased. The levels of LDH and the content of MDA were increased. The activity of SOD was decreased. After PC12 cells were pretreated with dFGEN, dFGEN significantly improved cell morphology, cell growth and proliferation, suppressed apoptosis of cells, reduced the release of LDH, improving SOD activity and decreased MDA content in a concentration-dependent manner. AO staining displayed that apoptosis was decreased. These results suggested that dFGEN has a protective effect against glutamate-induced damage in PC12 cells. dFGEN seemed to have a better protective effect than the lead compound genistein in a concentration-dependent manner. The mechanism of protective effect of dFGEN may be mainly related to its antioxidative activity.

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Introduction

With the improvement of people's living standard, social aging trend will be more obvious, so senile disease rates are on the rise. Neurodegenerative diseases is a progressive disease associated with age in nervous system, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Statistics indicate that thirty-four million people will have AD in the world by the year 2025 (1). It is estimated that the central nervous system (CNS), such as AD, PD, brain cancer, and stroke, will cost trillions of dollars for their treatment (2). Alzheimer's disease is the most common type of dementia. The United States statistics show that AD affects 5.4 million people and ~200,000 people under the age 65 have younger-onset AD (3). The clinical presentation is mainly memory loss and cognitive decline. Currently available drugs for the treatment of AD are purely for symptoms (4) and among these drugs are most the cholinesterase inhibitors (5). Another type of drug available for AD patients is an N-methyl-daspartate (NMDA) receptor antagonist named memantine (6). Many drugs are available to treat AD currently, but the effect is not significant or serious side effect may be occurs. We still lack specially effective drug treatment for the AD patients. Therefore, it is a very important responsibility and obligation for the pharmaceutical industry to study a new central nervous system drugs with high quality, high efficiency and low side effects.

In recent years, a large number of research data show that many acute and chronic neurodegenerative diseases are association with extracellular abnormally gathered glutamic acid in the brain (7). Glutamate, an excitatory amino acid, is one of the major neurotransmitters in the central nervous system (CNS). Glutamate participate in multiple physiological pathology processes. The right amount of glutamate is required to maintain normal cell physiological activities. However, high concentrations of glutamate in the brain can lead to neuronal damage. Glutamate are thought to be involved in the etiology of a number of neurodegenerative disorders including AD, PD, ALS, MS.A large amount of glutamate cause excessive activation of NMDA receptors, leading to calcium overload which can trigger a cascade of events eventually leading to apoptosis or necrosis (8).

Genistein (4',5,7-trihydroxyisoflavone) (Fig. 1), plentiful in soybeans, has estrogenic activity. Physiological levels of estrogen have many significant biological activities which are beneficial for treatment of osteoporosis and menopausal symptoms (9), AD in women after menopause (10), inflammatory response (11,12),

atherosclerosis (13), liver cancer (14), colon cancer (15), and it is neuro-protective (16). Genistein has a strong protective effect on the damage induced by oxidative stress (17). However, the absorption of genistein in the gastrointestinal tract is poor, resulting in low biological activity (18). To overcome this problem, the CHF2 group was introduced into the lead compound genistein, which can change its chemical and physical properties (19). After the introduction of CF_2 into genistein, Fu *et al* (20) design and synthesized a series of difluoromethyl-derivatives of genistein to screen an effective drug with oxidative stress injury model. The results confirmed that 7-difluoromethylyl-genistein is a protective new chemical entity for injury model induced by oxidative stress (21).

PC12 is a cell line derived from a rat adrenal medulla pheochromocytoma. PC12 cells have typical characteristics of nerve cells, which can be widely used as a nerve cell model. There are many mechanisms of nerve cell injury, one of which is the glutamate-induced damage. In this study, we used MTT assay to measure cell growth and proliferation activity, flow cytometry (FCM) with propidium iodide (PI) staining and acridine orange (AO) staining to detect cell apoptosis, assay kits to detect LDH activity, SOD activity and MDA content. Then we investigated the effect of 7-difluoromethoxy-5,4'-Di-hydroxyl isoflvone (dFGEN) on PC12 cells induced by glutamate.

7-difluoromethoxy-5,4'-dihydroxy isoflavone (dFGEN) is a genistein derivative (332 kDa) that had 7-OH group substituted by -$OCHF_2$. Its formula is shown in Fig. 2.

Materials and methods

Reagents. dFGEN (98% pure) was synthesized as reported in our laboratoty (20). Genistein and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT)v were purchased from Sigma (USA). Glutamate, vitamin E and Dimethyl sulfoxide (DMSO) were purchased from Genview (USA). Dulbecco's minimum essential medium (DMEM) were obtained from Hyclone (USA). Fetal calf serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. (Hangzhou, China). Acridine orange was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Cell lysis buffer, trypan blue staining, penicillin and streptomycin were purchased from Beijing Dingguo Changsheng Biotech Co., Ltd. (China). Lactate dehydrogenase (LDH) assay kit, superoxide dismutase (SOD) assay kit, lipid peroxidation (MDA) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay kit and trypsin were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Cell culture plates and cell culture dishes were purchased from Corning Inc. (USA).

Cell culture and treatment. PC12 cells (rat adrenal pheochromocytoma cells) were purchased from Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 incubator. When cells were ~80% confluent, new media with 1% newborn calf serum were added before the drug treatment. Glutamate was added in final concentrations ranging from 1 to 20 mM in the pilot study, and the concentration (10 mM) was selected by determining dose-response curves. When needed, cells were incubated for 30 min with genistein (0.1, 1.0 and 10 μM), dFGEN (0.1, 1.0 and 10 μM), vitamin E (10 μM), and then exposed to 10 mM glutamate for 24 h.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The PC12 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. After 24 h, the cells were treated with various concentrations of genistein, dFGEN, vitamin E for 30 min prior to glutamate (10 mM) treatment for 24 h. Briefly, 20 μl MTT was added to each well at a final concentration of 0.5 mg/ml, and afterwards the cells were cultured for 4 h at 37˚C. The medium was then carefully removed and 150 μl of DMSO was added to each well. The absorbance at 490 nm wavelength (A_{490}) was measured with enzyme-linked immunosorbent instrument (ElX800, Bio-Tek, USA). Experiment was divided into zero setting group, control group, and experimental group. The cell viability was expressed as a percentage of the viability of the control culture. Relative cell proliferation inhibition rate (IR) = (1 - average A_{490} of the experimental group/average A_{490} of the control group) x 100%.

Flow cytometry (FCM) with propidium iodide (PI) staining. The PC12 cells were seeded in 6-well plates at a density of $2x10^5$/ml (2 ml/well) and incubated for 24 h. Cells were treated by drugs in the same way as described above. Cells were treated for 24 h, then colleted and harvested with 0.25% trypsin and made into a single cell suspension, washed with cold PBS twice. Cells were resuspended as a single cell suspension with 50 μl PBS, fixed with l ml of cold 70% ethanol, stained with propidium iodide (PI) and cell apoptosis was detected using flow cytometry (FC 500 American Beckman Coulter Co.).

Morphological observations and acridine orange staining. The PC12 cells were seeded in 6-well plate at a density of $2x10^5$/ml (2 ml/well) and incubated for 24 h. Cells were treated by drugs in the same way as described above. After cells were treated for 24 h, cell morphological changes were observed under an optical microscope. Then the cells were washed two times with cold PBS and incubated with AO (5 μg/ml) at room temperature for 10 min in the dark. The stained cells were observed using fluorescence microscope (Olympus BX41, Japan Olympus Co.) and images were taken.

Detection of LDH activity. The PC12 cells were seeded in 24-well plates at a density of $1.2x10^5$/ml (1 ml/well) and incubated for 24 h. Cells were treated by drugs in the same way as described above. Cells were treated for 24 h, the culture supernatants were collected to a 1 ml Eppendorf tube, and LDH activity was detected at 450 nm by the assay kit.

Detection of SOD activity and MDA content. The PC12 cells were seeded in 66-mm culture dish at a density of $1x10^6$/ml (4 ml/dish) and incubated for 24 h. Cells were treated by drugs in the same way as described above. Cells were treated for 24 h, then colleted, washed two times with cold PBS, lysed at 4˚C for 30 min in lysate and then centrifuged at 12,000 x g for 10 min at 4˚C. SOD activity and MDA contents were measured according to the direction of the assay kit.

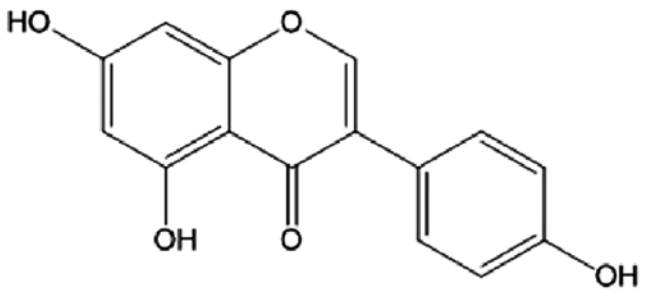


Figure 1. The structure of genistein.

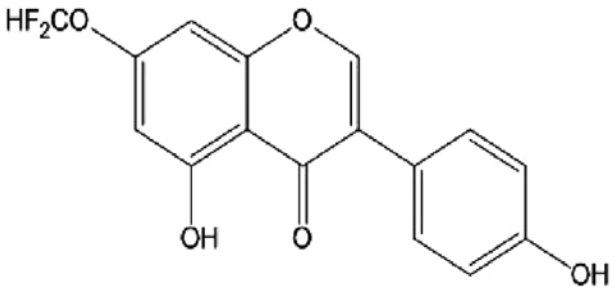


Figure 2. The structure of 7-difluoromethoxy-5,4'-Di-hydroxyl isoflavone (dFGEN).

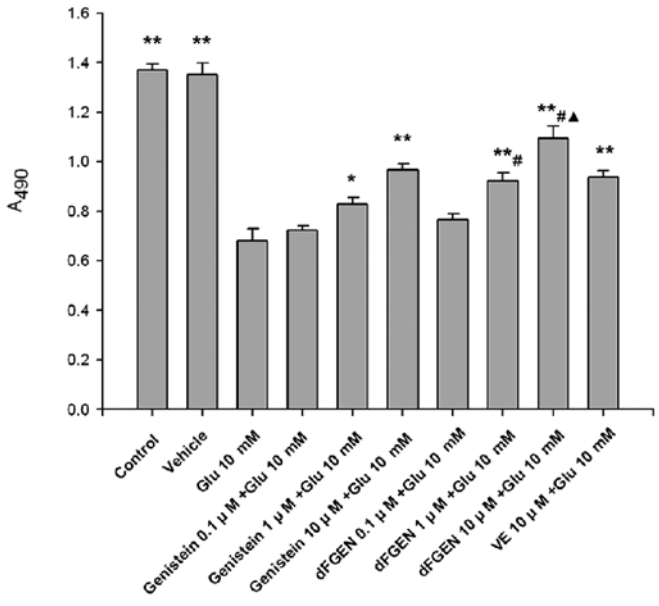


Figure 3. The effect of dFGEN on the proliferation of PC12 cells by MTT assay. The PC12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. The cell inhibitory rate was detected using MTT assay. *p<0.05 vs glutame 10 mM; **p<0.01 vs glutame 10 mM; $^{\#}$p<0.05 vs the same concentration of genistein + glutame 10 mM; $^{\blacktriangle}$p<0.05 vs the same concentration of VE + glutame 10 mM.

Statistical analysis. Data are presented as the mean ± SD. The database were set up with the SPSS 16.0 software package for analysis. The means of multiple groups were compared with one-way ANOVA, the two-two comparisons among the means were performed with LSD t-test. P<0.05 was considered as statistically significant.

Results

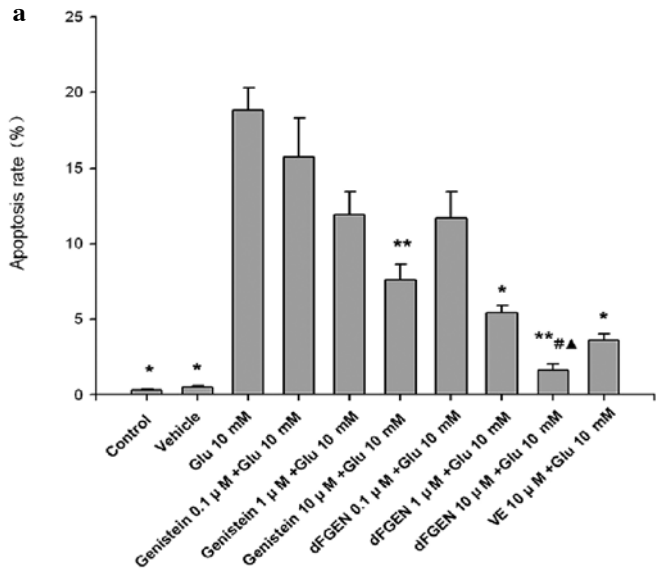
Effects of dFGEN on glutamate-induced PC12 cell proliferation. As shown in Fig. 3, the MTT assay demonstrates that glutamate obviously inhibited proliferation of PC12 cells compared with the control group (p<0.01). Results showed that dFGEN could effectively increased proliferation of PC12 cells in a concentration-dependent manner. Compared with the same concentration of genistein (1.0 and 10 μM), dFGEN (1.0 and 10 μM) could distinctly increase proliferation of PC12 cells (p<0.05). The cell viability of 10 μM dFGEN was >10 μM vitamin E (p<0.05).

Effects of dFGEN on glutamate-induced PC12 cell apoptosis. As shown in Fig. 4, the percentage of apoptotic cells was increased from 0.29±0.06 to 18.82±1.47% after the cells were exposed to 10 mM glutamate for 24 h (p<0.05). dFGEN effectively decreased apoptosis of PC12 cells in a concentration-dependent manner. dFGEN (10 μM) distinctly decreased glutamate-induced PC12 cell apoptosis compared to the same concentration of genistein and vitamin E (p<0.05).

Effects of dFGEN on glutamate-induced PC12 cell morphology and apoptotic morphology. As shown in Fig. 5, glutamate-induced PC12 cells exhibited significantly morphological alterations under microscopy and they became smaller and irregularly shaped. dFGEN obviously improved cell morphology. DNA-binding dye acridine orange (AO) was used to observe the morpgological characteristic of apoptotic cells. As shown in Fig. 6, AO staining displayed karyopyknosis, chromatin condensation and apoptotic bodies by fluorescence microscopy in glutamate treated group. Dense staining yellow-green fluorescence and granules could be seen under the fluorescence microscope. dFGEN evidently decreased the glutamate-induced apoptosis of PC12 cells.

Effects of dFGEN on glutamate-induced PC12 cell LDH activity. As shown in Fig. 7, while glutamate (10 mM) increased the release of LDH to 492.58±8.48 U/l (p<0.01), dFGEN reduced their release in a concentration-dependent manner. Compared with the same concentration of genistein (1.0 and 10 μM), dFGEN (1.0 and 10 μM) distinctly decreased LDH activity of PC12 cells (p<0.01). The LDH activity of 10 μM dFGEN group was lower than in the 10 μM vitamin E group (p<0.01).

Effects of dFGEN on glutamate-induced PC12 cell SOD activity and MDA content. As shown in Fig. 8, glutamate treatment for 24 h induced an increase in MDA formation but decreased SOD enzyme activity in PC12 cells (p<0.01). While dFGEN significantly attenuated lipid peroxidation in a concentration-dependent manner. Compared with the same concentration of genistein (1.0 and 10 μM), dFGEN (1.0 and 10 μM) distinctly improved SOD activity and decreased MDA content (p<0.01). dFGEN attenuated lipid peroxidation (10 μM) compared with 10 μM vitamin E (p<0.01).



Discussion

Aging is companied by many neurodegenerative diseases. A consistent number of neurons undergo apoptosis in the aged brain, which may lead to neurodegeneration in the long-term (22). Glutamate is the major excitatory neurotransmitter in the CNS. In many neurodegenerative diseases, glutamate causes over-excited glutamate receptors, causing glutamate excitotoxicity, which can trigger a cascade of events eventually leading to apoptosis or necrosis (23). Data suggest that glutamate excitotoxicity is an apoptotic process (24), while some data suggest that the excitotoxicity death is only due to the mechanism of necrosis (25). Although mechanism of glutamate-induced excitotoxicity is not clear, a large number of data suggest that the mechanism is related to glutamate-induced oxidative stress (26,27). In this study, we used glutamate (10 mM) to establish PC12 cell injury

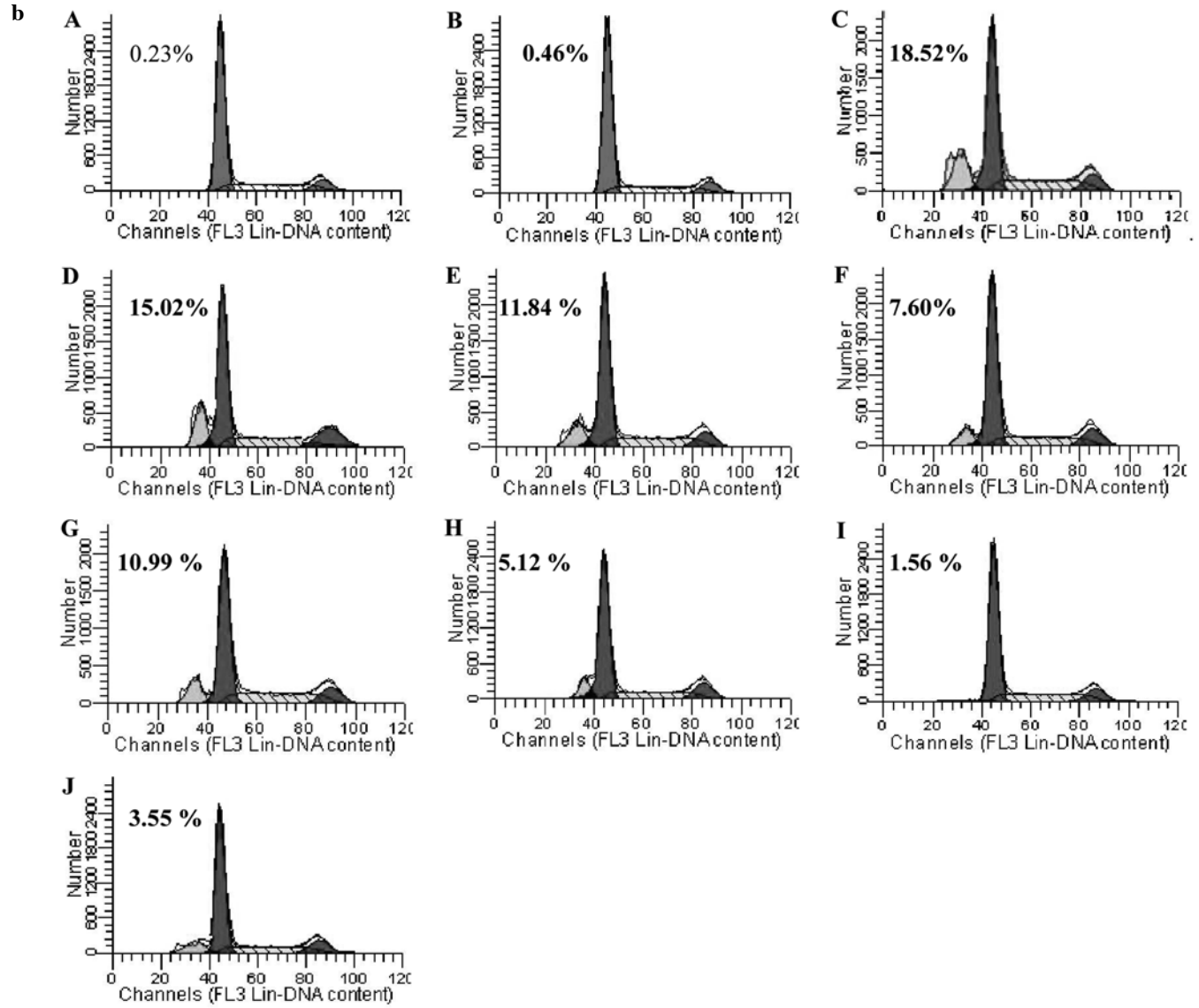


Figure 4. The effect of dFGEN on glutamate-induced PC12 cell apoptosis. The PC12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. Cell apoptosis was detectd by FCM with PI staining. *p<0.05 vs glutamate 10 mM; **p<0.01 vs glutamate 10 mM; $^{\#}$p<0.05 vs the same concentration of genistein + glutamate 10 mM; $^{\blacktriangle}$p<0.05 vs the same concentration of VE + glutamate 10 mM. (b) A, Control; B, Vehicle; C, Glu 10 mM; D, genistein 0.1 μM + Glu 10 mM; E, genistein 1 μM Glu 10 mM; F, genistein 10 μM + Glu 10 mM; G, dFGEN 0.1 μM + Glu 10 mM; H, dFGEN 1 μM + Glu 10 mM; I, dFGEN 10 μM + Glu 10 mM; J, VE 10 μM + Glu 10 mM.

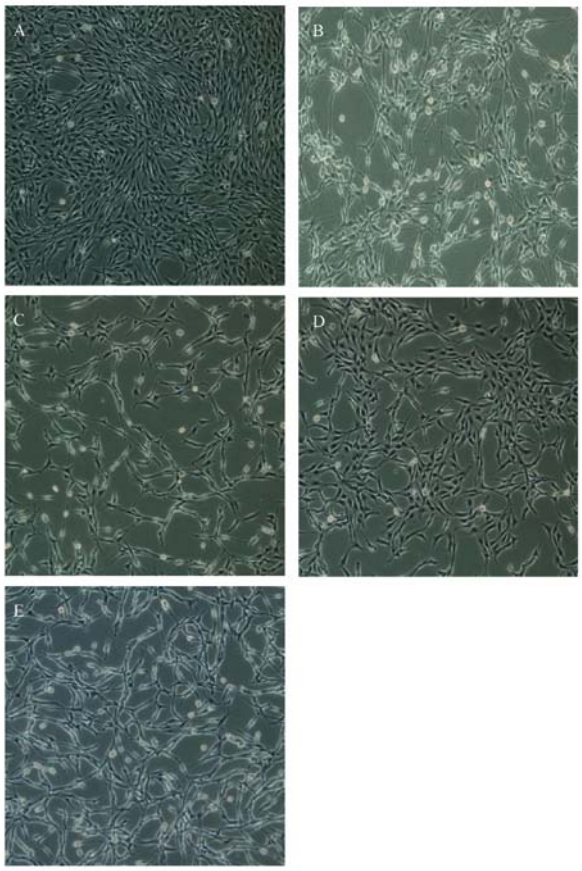


Figure 5. The effect of dFGEN on glutamate-induced PC12 cell morphology (10x10). The PC12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. (A) Control; (B) 10 mM Glu; (C) 10 μM genistein; (D) 10 μM dFGEN; (E) 10 μM VE.

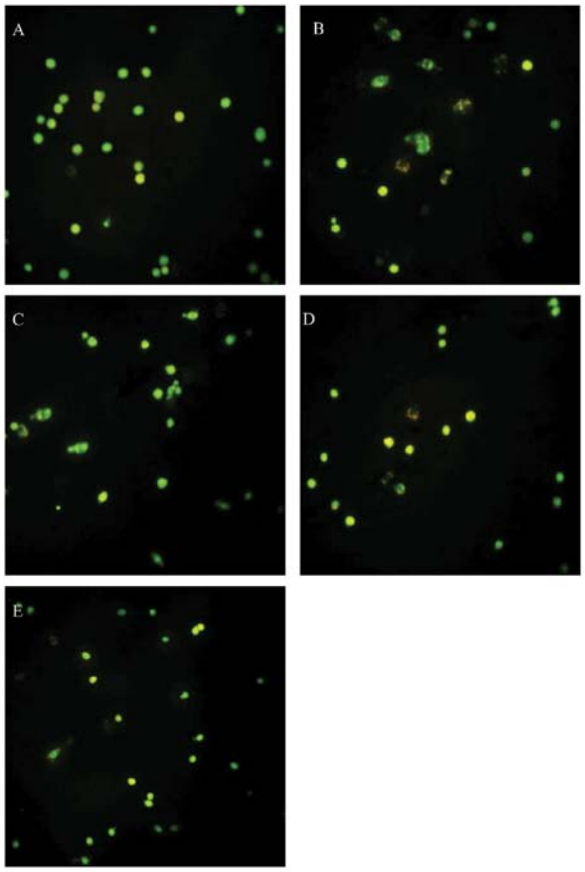


Figure 6. Morphological assessment of apoptosis by AO staining. a fluorescence microscope (10x10) images. The PC12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. (A) Control; (B) 10 mM Glu; (C) 10 μM genistein; (D) 10 μM dFGEN; (E) 10 μM VE.

model, and then to explore whether dFGEN have protective effect on damaged nerve cells compared with genistein and vitamin E.

Mitochondria is considered to be very important during cell apoptosis. Mitochondrial activity can be measured with MTT assay. In this study, pretreatment of dFGEN significantly improved cell survival and reduced the rate of proliferation inhibition in a concentration-dependent manner, suggesting that dFGEN could effectively maintain mitochondrial structure and function. dFGEN antagonized the effect of glutamate on cell proliferation inhibition rate, which is more effective than genistein and vitamin E. This study provided the experimental basis for inhibition of apoptosis and antioxidant function of dFGEN.

Propidium iodide (PI) is a nuclear fluorescent dye used for staining DNA. PI can not pass through viable cell membrane, but it can pass through the damaged membrane, used in cell apoptosis detection, which can be used for detection of cell apoptosis. Our study showed that dFGEN decreased the cell apoptosis rate in a concentration-dependent manner. Acridine orange (AO) staining showed that dFGEN evidently decreased the glutamate-induced apoptosis of PC12 cells. We proposed that dFGEN may play a positive role in the process of glutamate-induced PC12 cell apoptosis.

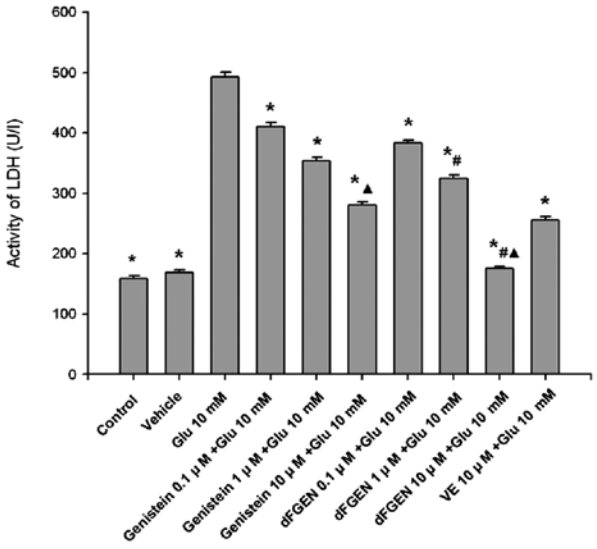


Figure 7. The effect of dFGEN on glutamate-induced LDH activity in PC12 cells. The PC 12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. The cell culture supernatant LDH activity was detected by LDH assay kit. $^{*}p<0.01$ vs glutamate 10 mM; $^{\#}p<0.01$ vs the same concentration of genistein + glutamate 10 mM; $^{\blacktriangle}p<0.01$ vs the same concentration of VE + glutamate 10 mM.

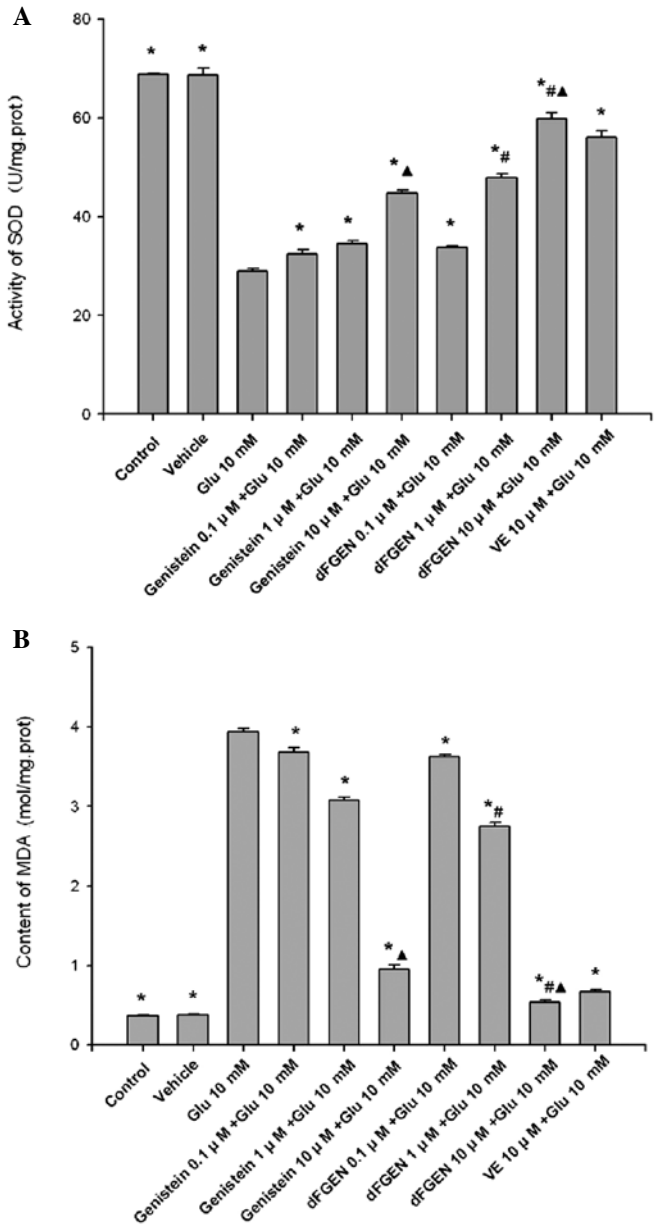


Figure 8. The effect of dFGEN on glutamate-induced SOD activity and MDA content in PC12 cells. The PC12 cells were treated with various concentrations of dFGEN (0.1, 1.0 and 10 μM), genistein (0.1, 1.0 and 10 μM) or vitamin E (10 μM) for 30 min followed by the addition of glutamate to a final concentration of 10 mM, and incubated for 24 h. The activity of SOD and the content of MDA were detected by SOD assay kit and MDA assay kit. *p<0.01 vs glutamate 10 mM; #p<0.01 vs the same concentration of genistein + glutamate 10 mM; ▲p<0.01 vs the same concentration of VE + glutamate 10 mM.

LDH activity is a classic indicator that reflects cell function. The more LDH release into extracellular space the greater damage to cells. Schreihofer and Redmond (28) reported that phytoestrogen genistein can reduce LDH release of cortical cells induced by glutamate. The results in this study showed that genistein, dFGEN and vitamin E could reduce LDH release of PC12 cells to different degrees. Compared with the same concentration of genistein and vitamin E, dFGEN could distinctly decrease LDH activity of PC12 cells and reduce the degree of cell damage. Our results indicated that dFGEN has a protective effect better than the lead compound genistein and vitamin E in a dose-dependent manner.

Oxidative stress is an important factor in a neurodegenerative disease. The loss of redox system balance will lead to oxidative damage of the body. SOD activity and MDA content are the indicators reflecting free radical damage. Our study showed that dFGEN increase SOD activity and decrease MDA content more than the same concentrations of genistein and vitamin E. The results suggested that dFGEN could protect PC12 cells against oxidative stress by increasing capacity of scavenging the free radicals, reducing the damage of free radicals on the cells, inhibiting changes in cell membrane permeability. This might represent one of the mechanisms for dFGEN against glutamate induced damage.

The process of glutamate-induced PC12 cell injury involves multiple signal transduction pathways, and the mechanism is very complex. Numerous studies show that Bcl-2 gene family (29) and caspase proteinase family (30) play important roles in the apoptotic process. Some reports have shown that apoptosis of PC12 cells may be related to the ERK1/2 and P38 signaling pathway (31). Our present preliminary study shows that dFGEN protects PC12 cells against glutamate toxicity. Its anti-apoptotic mechanism and *in vitro* experiments still need further research, so that ultimately our goal for dFGEN applied to neurodegenerative disease treatment can be achieved.

In conclusion, dFGEN can improve cell morphology, improve cell viability, suppress the apoptosis of cells, reduce the release of LDH, improve SOD activity and decrease MDA content in a concentration-dependent manner. These data demonstrated that dFGEN provide better protection against PC12 cell injury caused by glutamate than the lead compound genistein in a concentration-dependent manner. The mechanism of protective effect of dFGEN may be mainly related to its antioxidative activities.

Acknowledgements

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