

The Role of 6-Gingerol on Inhibiting Amyloid β Protein-Induced Apoptosis in PC12 Cells

Gao-feng Zeng,^{1,*} Shao-hui Zong,^{2,*} Zhi-yong Zhang,¹ Song-wen Fu,¹
Ke-ke Li,³ Ye Fang,³ Li Lu,¹ and De-qiang Xiao¹

Abstract

Our previous study suggests that ginger root extract can reverse behavioral dysfunction and prevent Alzheimer's disease (AD)-like symptoms induced by the amyloid- β protein ($A\beta$) in a rat model. 6-Gingerol is the major gingerol in ginger rhizomes, but its effect on the treatment of AD remains unclear. In this study, we aimed to determine if 6-gingerol had a protective effect on $A\beta_{1-42}$ -induced damage and apoptotic death in rat pheochromocytoma cells (PC12 cells) and to investigate the underlying mechanisms by which 6-gingerol may exert its neuroprotective effects. Our results indicated that pre-treatment with 6-gingerol significantly increased cell viability and reduced cell apoptosis in $A\beta_{1-42}$ -treated cells. Moreover, 6-gingerol pretreatment markedly reduced the level of intracellular reactive oxygen species (ROS) and malondialdehyde (MDA), the production of nitric oxide (NO), and the leakage of lactate dehydrogenase (LDH) and increased superoxide dismutase (SOD) activity compared with the $A\beta_{1-42}$ treatment group. In addition, 6-gingerol pretreatment also significantly enhanced the protein levels of phosphorylated Akt (p-Akt) and glycogen synthase kinase-3 β (p-GSK-3 β). Overall, these results indicate that 6-gingerol exhibited protective effects on apoptosis induced by $A\beta_{1-42}$ in cultured PC12 cells by reducing oxidative stress and inflammatory responses, suppressing the activation of GSK-3 β and enhancing the activation of Akt, thereby exerting neuroprotective effects. Therefore, 6-gingerol may be useful in the prevention and/or treatment of AD.

Introduction

ALZHEIMER'S DISEASE (AD), THE most common form of dementia, is a progressive neurodegenerative disorder of the brain characterized by progressive memory impairment, disordered cognitive function, and altered behavior.¹ Data have suggested that there are 26.6 million AD patients as of 2009, and this number will quadruple by 2050 if no cure or preventive measure is found.² Currently, no effective anti-AD drugs are available to either stop or reverse the progression of AD, although the development of anti-AD drugs has been slightly successful in aspects of symptomatic improvement, such as the development of acetylcholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) receptor antagonists.^{3,4}

AD is characterized pathologically by deposition of extracellular senile plaques and intracellular neurofibrillary tangles.⁵ Amyloid- β ($A\beta$), the major component of senile plaques, is derived from sequential proteolysis of the amyloid precursor protein by sequential cleavages of β -secretase and

γ -secretase and plays a critical role in the pathophysiology of AD.^{6,7} Despite the abundance of studies that have been designed to investigate the underlying mechanisms of the neurotoxicity induced by $A\beta$, the precise mechanisms still remain unclear. Accumulating evidence suggests that oxidative stress and inflammatory responses are the major mechanisms of $A\beta$ -induced neurotoxicity.^{8,9} For instance, the accumulation of $A\beta$ in primary neurons could induce oxidative stress.¹⁰ $A\beta$ accelerates additional inflammatory pathways by activation of cyclooxygenase-2 in astrocytes via an interaction with interleukin-1 β , tumor necrosis factor- α , and a nuclear factor- κ B (NF- κ B) mechanism in the rat brain.¹¹ Moreover, evidence also indicates that $A\beta$ -induced neurotoxicity is associated with the PI3K/Akt/GSK-3 β signaling pathway.^{12,13} Therefore, investigating novel anti-AD drugs to reduce the $A\beta$ -induced neurotoxicity may aid in AD prevention and/or treatment.

Ginger (*Zingiber officinale* Roscoe), the rhizome of the plant *Z. officinale*, is one of the most popular species and has

¹College of Public Hygiene of Guangxi Medical University, Nanning, Guangxi, P.R. China.

²Department of Osteopathia, the First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, P.R. China.

³Graduate School of Guangxi Medical University, Nanning, Guangxi, P.R. China.

*These authors contributed equally to this work and should be considered as co-first authors.

been utilized in traditional medicines, especially in Asia and Africa.¹⁴ Our preliminary study reveals that ginger root extract could reverse behavioral dysfunction and prevent AD-like symptoms in a rat model of AD.¹⁵ 6-Gingerol, a phenolic compound, is the major gingerol in ginger rhizomes with diverse pharmacological activities, including anti-tumor, anti-inflammatory, and anti-oxidant effects.^{16–19} In a recent study, 6-gingerol exhibited neuroprotective effects against cell apoptosis induced by A β through its anti-oxidative role.²⁰

In this study, we aimed to determine if 6-gingerol exhibited protective effects of 6-gingerol on A β _{1–42}-induced damages and apoptotic death in pheochromocytoma cells (PC12) cells, and not only investigated the role of 6-gingerol in the process of anti-oxidative stress but also proved its role of anti-inflammatory responses and Akt/GSK-3 β signaling pathway to investigate the underlying mechanisms by which 6-gingerol may exert its neuroprotective effects.

Materials and methods

Materials

The PC12 cell line was obtained from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). 6-Gingerol (C₁₇H₂₆O₄, purity \geq 98%) was purchased from Shanghai Fu life Industry Co. Ltd; A β _{1–42} was purchased from Wuhan Moon Biosciences Co. Ltd; Ham's F-12K (Kaighn's) Medium was purchased from BOSTER (Wuhan, China); fetal bovine serum (FBS) and horse serum were obtained from Gibco (Grand Island, NY); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Amresco (Solon, OH); nerve growth factor 2.5S protein (NGF) was obtained from Merck Millipore (Billerica, MA); the Annexin V-FITC/PI Apoptosis Detection Kit was purchased from KeyGEN BioTECH (Nanjing, China); Hoechst 33258 was obtained from the Beyotime Institute of Biotechnology (Shanghai, China); the reactive oxygen species (ROS) assay kit, nitric oxide (NO) assay kit, lactate dehydrogenase (LDH) assay kit, superoxide dismutase (SOD) assay kit, and cell malondialdehyde (MDA) assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); the phospho-Akt (Ser473) antibody, Akt antibody, GSK-3 β (Ser9) antibody, and GSK-3 β rabbit monoclonal antibody (mAb) were obtained from Cell Signaling Technology (Danvers, MA); and the anti- β -actin rabbit polyclonal antibody was purchased from EarthOx (Millbrae, CA).

Preparation of aggregated A β

A β _{1–42} was dissolved in deionized distilled water at a concentration of 1 mM and incubated at 37°C for 3 days to form aggregated amyloid. After aggregation, the solution was stored at –20°C until use.

Cell culture

PC12 cells were cultured in F12K medium containing 10% horse serum, 5% fetal bovine serum (FBS), and 100 U/mL of penicillin-streptomycin at 37°C with a 5% CO₂ atmosphere in a humidified incubator. PC12 cells were sub-cultured approximately once a week and split 1:3 when the culture was 80–90% confluent.

MTT assay

PC12 cells were harvested from flasks and plated onto poly-D-lysine-coated 96-well plates with approximately 20,000 cells in 100 μ L of serum free F12K medium supplemented with 50 ng/mL of nerve growth factor (NGF) per well. After 5 days of induced differentiation by NGF, the cells were treated with prepared A β _{1–42} (5, 10, and 20 μ M) for 24, 48, and 72 hr, and cell viability was then evaluated with an MTT assay. To determine the protective effects of 6-gingerol on A β _{1–42}-induced cell injury, PC12 cells were treated with 6-gingerol at different concentrations (40, 80, 120, 200, and 300 μ M) for 4 hr, and the cells were then incubated with 10 μ M A β _{1–42} for another 48 hr. Then, the cells were incubated in fresh F12K medium containing 0.5 mg/mL MTT at 37°C for 4 hr. Next, the reaction mixture was carefully removed, and 150 μ L of dimethylsulfoxide (DMSO) was added to each well. The absorption values were read at 570 nm using a Multiskan Multiwell microplate reader (Thermo Scientific, USA), and the experiments were repeated in triplicate. The data were expressed as the mean percent of viable cells versus control.

Nuclear staining for cell apoptosis by Hoechst 33258

To detect the effect of 6-gingerol on A β _{1–42}-induced cell apoptosis, the chromosomal condensation and morphological changes of the cell nucleus were assessed by staining with Hoechst 33258. The differentiated PC12 cells were treated with 6-gingerol at different concentrations (80, 120, and 200 μ M) for 4 hr, after which 10 μ M A β _{1–42} was added, and the cells were incubated for an additional 48 hr. After being washed with phosphate-buffered saline (PBS), the cells were fixed with immunostaining fix solution (Beyotime, Shanghai, China) at room temperature for 15 min. The cells were washed twice with PBS, and then stained with Hoechst 33258 fluorochrome (10 μ g/mL) at 37°C for 10 min in the dark. After three rinses with PBS, the Hoechst-stained nuclei were imaged with a confocal laser scanning microscope (Nikon, Japan).

Flow cytometric analysis of cell apoptosis

Apoptosis was determined using an Annexin V-FITC/PI assay kit. Briefly, cells were harvested after treatment, rinsed twice with cold PBS, and then re-suspended in binding buffer at a density of 1×10^6 cells/mL. After incubation with 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide (PI) working solution for 15 min in the dark at room temperature, the stained cells were analyzed with a flow cytometer (BD Biosciences, USA).

Measurement of intracellular ROS

Intracellular ROS were measured using the dichlorodihydro-fluorescein diacetate (DCFH-DA) method. PC12 cells (1×10^5) were seeded in six-well plates, and differentiation was induced by NGF for 5 days in a CO₂ incubator at 37°C. Then, the cells were treated with 6-gingerol at different concentrations (80, 120, and 200 μ M) for 4 hr, after which 10 μ M of A β _{1–42} was added, and the cells were incubated for another 48 hr. After the medium was removed, the cells were washed twice with serum-free F12K medium and then incubated with serum-free F12K medium containing 10 μ M DCFH-DA in the dark at 37°C for 45 min. The cells were washed twice with PBS, and finally, fluorescence was

measured by photofluorography and quantified with ImageJ software (NIH Image, Bethesda, MD).

NO, LDH, SOD, and MDA assay

PC12 cells were seeded in six-well plates, and differentiation was induced by NGF for 5 days in a CO₂ incubator at 37°C. Then, the cells were treated with 6-gingerol at different concentrations (80, 120, and 200 μM) for 4 hr, after which 10 μM Aβ₁₋₄₂ was added, and the cells were incubated for another 48 hr. At the end of the drug treatment, the culture supernatant and cells were collected separately by centrifugation. Then, the levels of NO, LDH, and SOD activity in the medium were measured using a NO assay kit, LDH assay kit, and SOD assay kit in accordance with the manufacturer's instructions, respectively. The level of intracellular MDA was also detected with an MDA assay kit according to the manufacturer's instructions.

Western blotting

PC12 cells were harvested and washed twice with PBS solution after drug treatment. Then, the cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride [PMSF], sodium orthovanadate, sodium fluoride, EDTA, and leupeptin). Protein concentrations in the supernatants were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer. Before immunoblotting, the cell lysate was boiled in SDS-polyacrylamide gel electrophoresis (PAGE) Sample Loading Buffer for 5 min. The cell lysates were electrophoresed by 8% SDS-PAGE for 2.5 hr at 60 V and then electrotransferred onto a nitrocellulose filter membrane. After blocking for 1 hr in a solution of 5% (wt/vol) bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature, the membrane was subsequently incubated at 4°C overnight with the appropriate amount of primary antibodies against p-Akt (Ser 473), Akt, p-GSK-3β (Ser9), GSK-3β, and β-actin. After washing four times with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) at room temperature for 1.5 hr and again washed four times. The blotted proteins were visualized using the electrochemiluminescence (ECL) western blotting detection reagents, and the band intensities were quantified with ImageJ software (NIH Image, Bethesda, MD).

Statistical analysis

All of the data are expressed as the means ± standard deviation (SD). The significance was determined by one-way analysis of variance (ANOVA), and the least significant difference (LSD) multiple-range tests were used to analyze the differences between groups. The data were analyzed using SPSS 16.0 software, and a value of *p* < 0.05 or *p* < 0.01 was considered to indicate a statistically significant difference.

Results

MTT assay for cell viability

PC12 cells were treated with various concentrations of Aβ₁₋₄₂ (5, 10, and 20 μM) for 24 hr, 48 hr, and 72 hr, and

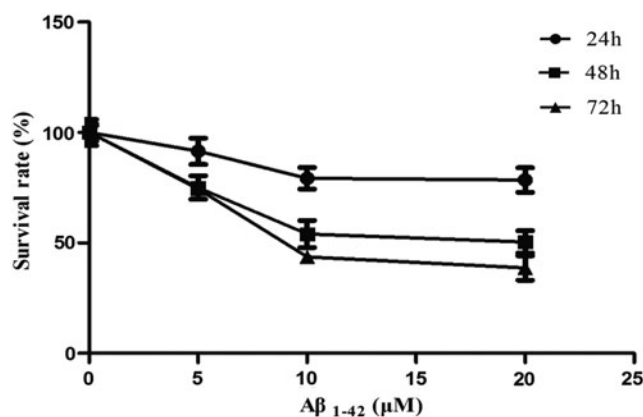


FIG. 1. Effect of amyloid-β (Aβ₁₋₄₂) on PC12 cell viability.

cell viability was evaluated with an MTT assay. Within different time groups, the cell survival rate decreased as dose increased, and the Aβ₁₋₄₂ treatment group of 10 and 20 μM significantly reduced the viability of PC12 cells compared with the control group. The cell survival rate also decreased as time increased, and the viability of the PC12 cells in Aβ₁₋₄₂ treatment groups at 48 hr and 72 hr were significantly reduced compared with the 24-hr groups, as shown in Fig. 1.

Effect of 6-gingerol on Aβ₁₋₄₂-induced cell injury

To verify the protective effect of 6-gingerol on Aβ₁₋₄₂-induced cell injury, the PC12 cells were pretreated with serial concentrations of 6-gingerol (40, 80, 120, 200, and 300 μM) for 4 hr before treatment with Aβ₁₋₄₂ (10 μM) for 48 hr, and cellular viability was detected by MTT assay. As shown in Fig. 2, the Aβ₁₋₄₂ treatment group significantly reduced the cell viability compared with the control group; the groups pretreated with 80, 120, and 200 μM of 6-gingerol

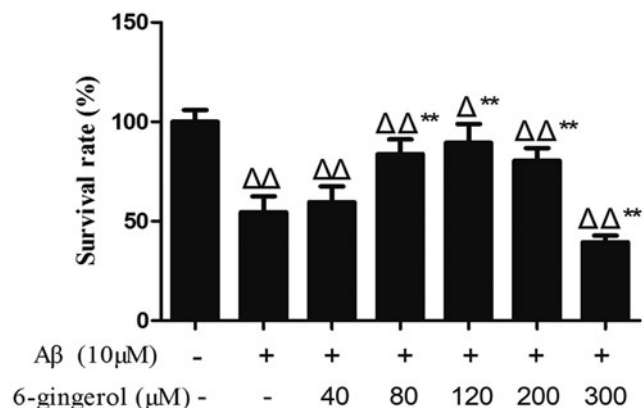


FIG. 2. The MTT assay detects the effect of 6-gingerol on amyloid-β (Aβ₁₋₄₂)-induced cell injury. PC12 cells were pretreated with or without 6-gingerol (40, 80, 120, 200, and 300 μM) for 4 hr before treatment with Aβ₁₋₄₂ for 48 hr. The data are presented as the means ± standard deviation (SD) of four individual experiments. (Δ) *p* < 0.05 compared with the control group; (ΔΔ) *p* < 0.01 compared with the control group; (**) *p* < 0.01 compared with the Aβ₁₋₄₂ treatment group.

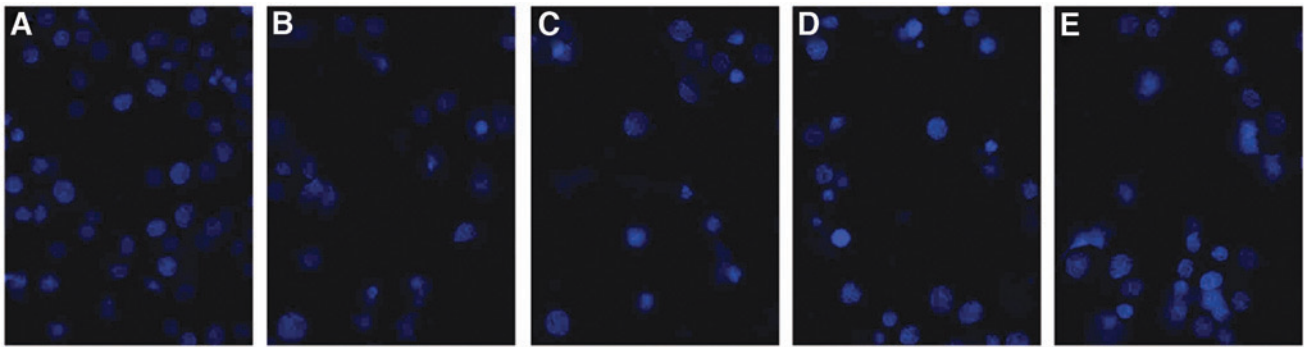


FIG. 3. Hoechst 33258 staining measures the effect of 6-gingerol on amyloid- β ($A\beta_{1-42}$)-induced apoptosis in PC12 cells. Confocal microscope images (magnification, 400 \times): (A) Blank control group; (B) $A\beta_{1-42}$ (10 μ M) group; (C) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (80 μ M) group; (D) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (120 μ M) group; (E) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (200 μ M) group. Color images available online at www.liebertpub.com/rej

exhibited significantly higher cell survival rates compared with the $A\beta_{1-42}$ treatment group. However, the cell survival rate in the 300 μ M 6-gingerol-pretreated group was lower than the $A\beta_{1-42}$ treatment group. The results revealed that 6-gingerol could prevent PC12 cells from $A\beta_{1-42}$ -induced cell death and apoptosis. However, higher concentrations of 6-gingerol (300 μ M) also had cytotoxic effects on PC12 cells. Therefore, we selected the optimal concentrations of 6-gingerol at 80, 120, and 200 μ M for the following studies.

Hoechst 33258 staining for cell apoptosis

Hoechst 33258 dye was used to assess the changes in nuclear morphology because it can diffuse through intact membranes of cells and stain their chromatin. We measured the effects of 6-gingerol on $A\beta_{1-42}$ -induced cell apoptosis through Hoechst 33258 nuclear staining. The nuclei of PC12 cells in the control group were uniformly stained and appeared to have a regular conformation except for a few

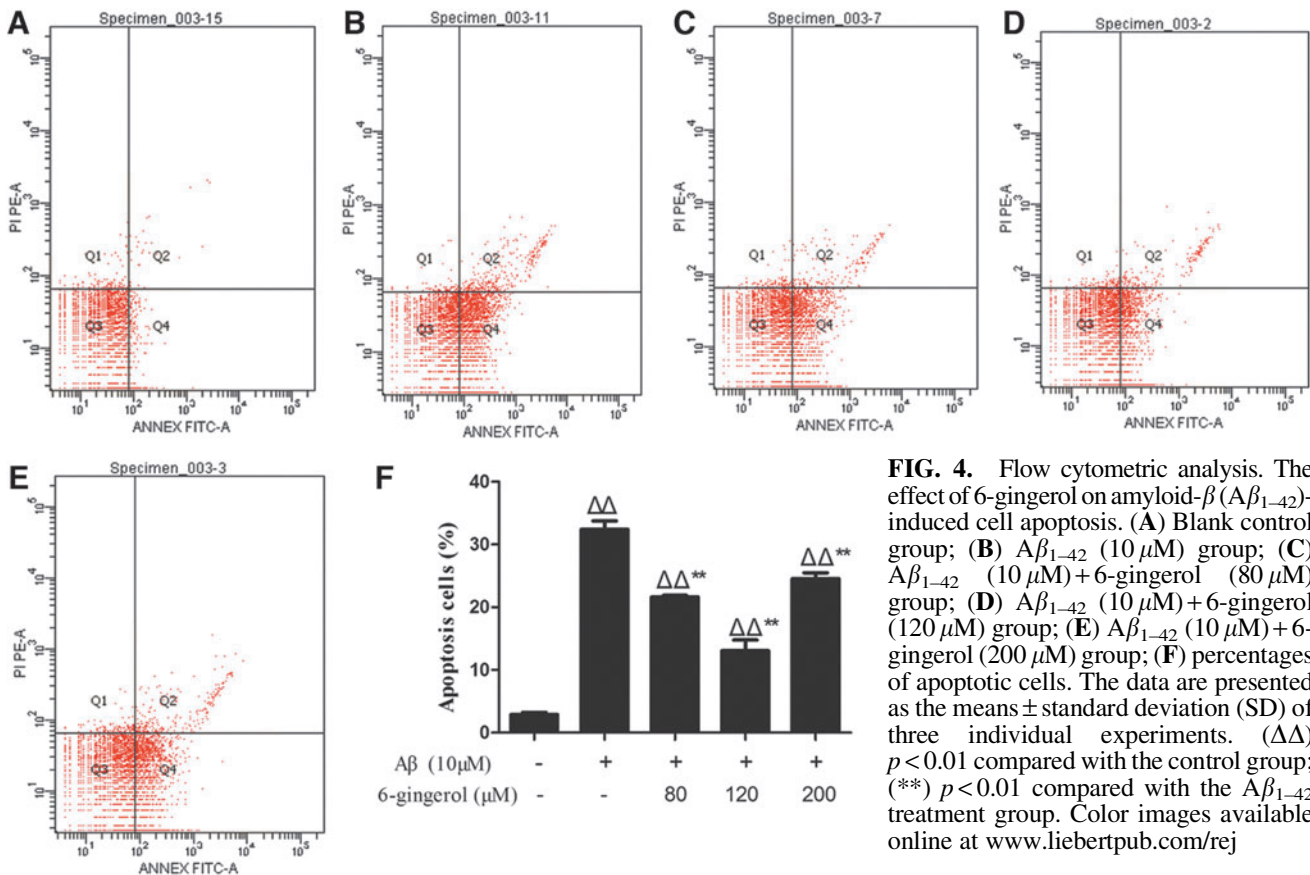


FIG. 4. Flow cytometric analysis. The effect of 6-gingerol on amyloid- β ($A\beta_{1-42}$)-induced cell apoptosis. (A) Blank control group; (B) $A\beta_{1-42}$ (10 μ M) group; (C) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (80 μ M) group; (D) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (120 μ M) group; (E) $A\beta_{1-42}$ (10 μ M) + 6-gingerol (200 μ M) group; (F) percentages of apoptotic cells. The data are presented as the means \pm standard deviation (SD) of three individual experiments. ($\Delta\Delta$) $p < 0.01$ compared with the control group; (**) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group. Color images available online at www.liebertpub.com/rej

abnormal nuclei (Fig. 3A), whereas most of the nuclei in the $A\beta_{1-42}$ treatment group exhibited pyknosis and karyorrhexis (Fig. 3B), and this phenomenon was decreased in the 6-gingerol-pretreated group (Fig. 3C–E). The results suggested that 6-gingerol could attenuate $A\beta_{1-42}$ -induced cell apoptosis.

Effect of 6-gingerol on $A\beta_{1-42}$ -induced cell apoptosis by flow cytometry

The anti-apoptotic effects of 6-gingerol in PC12 cells were further studied using an Annexin V-FITC/PI assay. As shown in Fig. 4, flow cytometry detected that 2.87% of the cells were apoptotic in the control group, and cell apoptosis was significantly increased to 32.4% after treatment with 10 μM $A\beta_{1-42}$. After pretreatment with 6-gingerol (80, 120, and 200 μM); however, apoptosis was significantly reduced to 21.67%, 13.07%, and 24.57%, respectively ($p < 0.01$).

Effect of 6-gingerol on $A\beta_{1-42}$ -induced ROS production

To evaluate the effect of 6-gingerol on $A\beta_{1-42}$ -induced ROS, PC12 cells were pretreated with serial concentrations of 6-gingerol (80, 120, and 200 μM) for 4 hr before treatment with $A\beta_{1-42}$ (10 μM) for 48 hr, and cellular viability was detected by fluorescence assay. As shown in Fig. 5, $A\beta_{1-42}$ (10 μM) treatment for 48 hr induced a significant increase of the DCFH-DA fluorescence intensity in cells compared with the control group. After pretreatment with 6-gingerol (80, 120, and 200 μM), however, all of the DCFH-DA fluorescence intensity was decreased significantly. The results indicated that 6-gingerol could attenuate $A\beta_{1-42}$ -induced ROS production.

Effect of 6-gingerol on $A\beta_{1-42}$ -induced NO generation and LDH and SOD activity in the medium and intracellular MDA production

PC12 cells treated with $A\beta_{1-42}$ for 48 hr showed a significant increase in NO level in the supernatant compared with the control group ($6.23 \pm 0.35 \mu\text{M}$ versus $2.58 \pm 0.53 \mu\text{M}$, $p < 0.01$). After pretreatment with 6-gingerol (80, 120, and 200 μM), however, the levels of NO were decreased to $3.95 \pm 0.43 \mu\text{M}$, $2.95 \pm 0.28 \mu\text{M}$, and $4.08 \pm 0.16 \mu\text{M}$, respectively (Fig. 6A).

As shown in Fig. 6B, a significant increase ($p < 0.01$) of LDH activity was found in the medium in the $A\beta_{1-42}$ treatment group compared with the control group. After pretreatment with 6-gingerol (80, 120, and 200 μM) for 4 hr, the LDH activity was significantly decreased compared with the $A\beta_{1-42}$ treatment group.

As shown in Fig. 6C, SOD activity in the medium was significantly decreased in the $A\beta_{1-42}$ treatment group compared with the control group. Pretreatment with 6-gingerol (120 and 200 μM) inhibited the decrease of SOD activity caused by $A\beta_{1-42}$.

As shown in Fig. 6D, treatment with $A\beta_{1-42}$ (10 μM) for 48 hr led to a significant increased production of MDA in PC12 cells, which was decreased by pretreatment with 6-gingerol (120 and 200 μM).

Effect of 6-gingerol on $A\beta_{1-42}$ -induced phosphorylation of Akt and GSK-3 β

To determine the effects of 6-gingerol on $A\beta_{1-42}$ -induced activation of Akt and GSK-3 β in PC12 cells, phosphorylated Akt (p-Akt) and phosphorylated GSK-3 β (p-GSK-3 β) were examined by western blot analysis using phospho-specific antibodies. The results showed that treatment with 10 μM

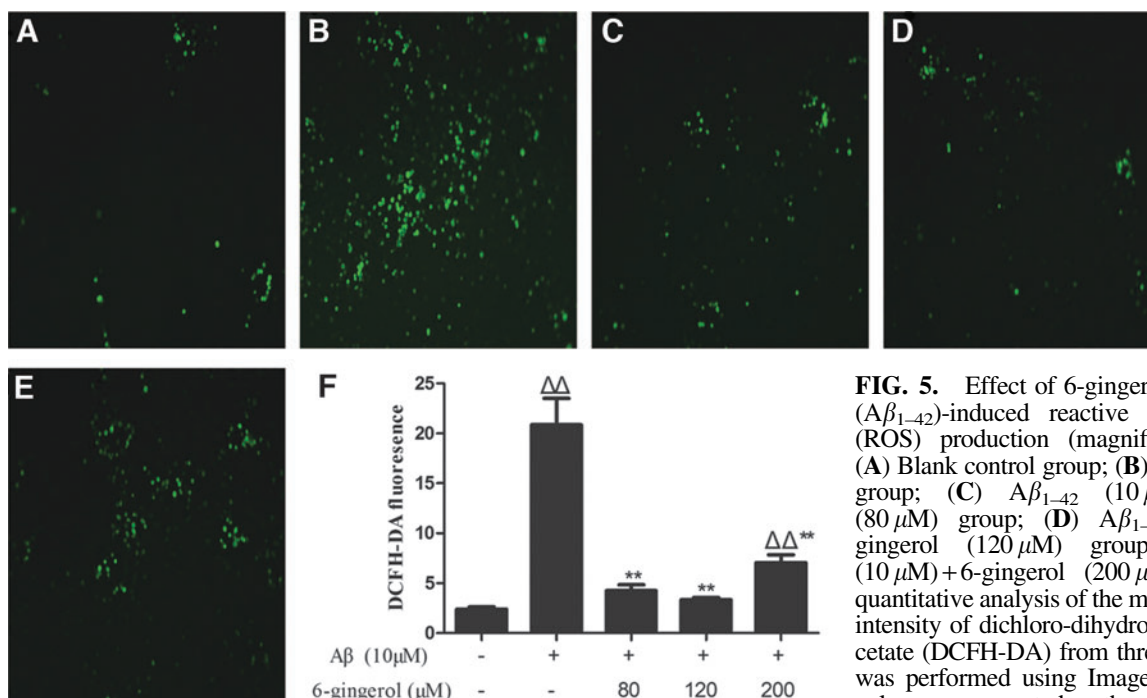


FIG. 5. Effect of 6-gingerol on amyloid- β ($A\beta_{1-42}$)-induced reactive oxygen species (ROS) production (magnification, 100 \times). (A) Blank control group; (B) $A\beta_{1-42}$ (10 μM) group; (C) $A\beta_{1-42}$ (10 μM) + 6-gingerol (80 μM) group; (D) $A\beta_{1-42}$ (10 μM) + 6-gingerol (120 μM) group; (E) $A\beta_{1-42}$ (10 μM) + 6-gingerol (200 μM) group; (F) quantitative analysis of the mean fluorescence intensity of dichloro-dihydro-fluorescein diacetate (DCFH-DA) from three random fields was performed using ImageJ software. The values are presented as the means \pm standard

deviation (SD). (Δ) $p < 0.05$ compared with the control group; ($\Delta\Delta$) $p < 0.01$ compared with the control group; (**) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group. Color images available online at www.liebertpub.com/rej

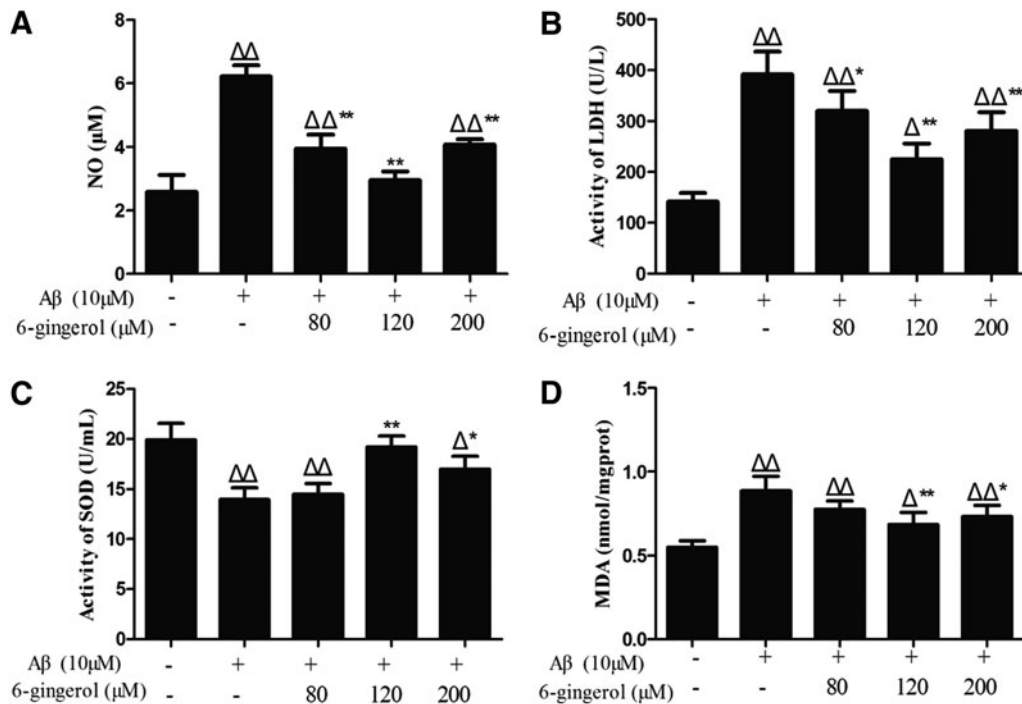


FIG. 6. PC12 cells were pretreated with 6-gingerol (80, 120, and 200 μM) for 4 hr followed by exposure to 10 μM amyloid- β ($A\beta_{1-42}$) for 48 hr. The data are presented as the means \pm standard deviation (SD) ($n=3$). (Δ) $p < 0.05$ compared with the control group; ($\Delta\Delta$) $p < 0.01$ compared with the control group; (*) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group; (**) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group.

$A\beta_{1-42}$ for 48 hr significantly decreased the phosphorylation of Akt and GSK-3 β compared with the control group, whereas pretreatment with 6-gingerol (80 and 120 μM)

markedly inhibited the decrease in the expression of p-Akt and p-GSK-3 β caused by $A\beta_{1-42}$ (Figs. 7 and 8).

Discussion

PC12 cells are clonal cells originating from a transplantable rat pheochromocytoma and exhibit the characteristics of neuronal cells in morphological, physiological, and biochemical aspects.²¹ Although undifferentiated PC12 cells do not have neurites or synapses, the cells respond to NGF with a dramatic change in phenotype and could acquire a number of properties characteristic of sympathetic neurons.²² In our experiment, PC12 cells were differentiated using NGF (50 ng/mL) for 5 days and were then used as the *in vitro* cell models to investigate the protective effects of 6-gingerol on $A\beta$ -induced neurotoxicity.

Extensive studies into the apoptotic and necrotic processes induced by $A\beta$ in neuronal cell lines have been performed.²³⁻²⁵ However, it is still unknown about the exact molecular mechanisms of $A\beta$ -mediated neuronal apoptosis. Thus, our study was first started from the two aspects of cellular viability and survival rate. Figures 1 and 2 showed that the cell viability was decreased in the $A\beta_{1-42}$ treatment group, whereas the cellular survival rate was markedly increased when pretreated with 6-gingerol (80, 120, and 200 μM). With Hoechst 33258 staining and flow cytometric analysis (Figs. 3 and 4), the apoptosis rate was significantly decreased in 6-gingerol-pretreated group (80, 120, and 200 μM) compared with the $A\beta_{1-42}$ analysis group. These results revealed that 6-gingerol significantly attenuates $A\beta_{1-42}$ -induced neurotoxicity by preventing cell damage.

Because the neuropathology of AD is widely associated with many factors such as inflammatory response and

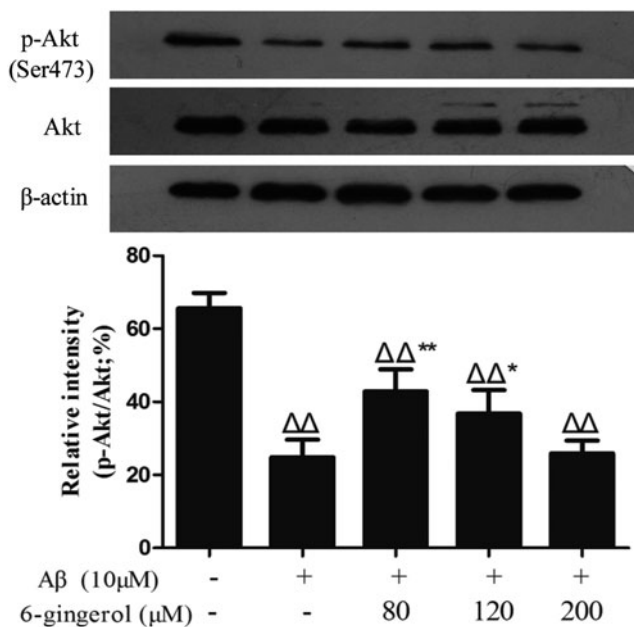


FIG. 7. Effect of 6-gingerol on amyloid- β ($A\beta_{1-42}$)-induced activation of Akt in PC12 cells. The data are presented as the means \pm standard deviation (SD) of three individual experiments. ($\Delta\Delta$) $p < 0.01$ compared with the control group; (*) $p < 0.05$ compared with the $A\beta_{1-42}$ treatment group; (**) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group.

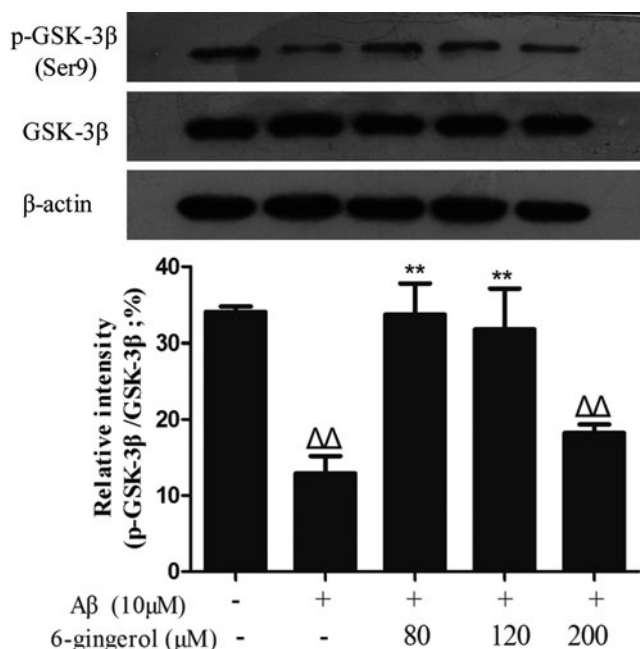


FIG. 8. Effect of 6-gingerol on amyloid- β ($A\beta_{1-42}$)-induced activation of GSK-3 β in PC12 cells. The data are presented as the mean \pm standard deviation (SD) of three individual experiments. ($\Delta\Delta$) $p < 0.01$ compared with the control group; (***) $p < 0.01$ compared with the $A\beta_{1-42}$ treatment group.

oxidative stress, we focused our study on whether 6-gingerol had the role of anti-inflammatory, anti-oxidative damage in PC12 cells induced by $A\beta_{1-42}$. Studies have indicated that NO can generate a high level of pro-inflammatory cytokines to strengthen neurotoxicity, and this increase is consequently related to the development of AD.²⁶ $A\beta$ blocks the normally reparative effects of up-regulated vascular endothelial growth factor and nitric oxide synthases (NOS) and may accelerate *in vivo* vascular pathophysiology in AD.²⁷ Excessive NO generated by NOS could strengthen the neurotoxicity because of the inhibition of glutamate reuptake, hence contributing to neuronal death and injury.²⁸ In the study, it showed that 6-gingerol significantly reduced the levels of NO (Fig. 6A), indicating that 6-gingerol may have anti-inflammatory effects of attenuating the cytotoxicity of $A\beta_{1-42}$ in PC12 cells.

In addition, oxidative stress is often defined as an imbalance between the cellular production of ROS and the ability of cells to efficiently defend against them.²⁹ Studies suggest that $A\beta$ exerts neuronal toxicity through the generation of excessive ROS following mitochondria superoxide accumulation.³⁰ Oxidative stress can cause cellular damage because the ROS oxidizes vital cellular components, including lipids and nucleic acids, and consequently contributes to the pathophysiology of neurodegenerative diseases such as AD.³¹ The ROS can destroy the integrity of the neuronal cell membrane because of lipid oxidation, resulting in the release of bioactive substances into the extracellular space, such as LDH. Thus, LDH activity in the medium reflects the damage of cell membrane lipids. What's more, SOD, an endogenous anti-oxidant, has an important role in the reduction of oxidative stress and prevention of lipid damage.³² MDA is the degradation product

of the oxygen-derived free radicals and lipid oxidation, and its levels are reflective of the overall levels of oxidative stress.¹⁵ In this study, it was first shown that 6-gingerol not only reduced the production of ROS, LDH, and MDA, but also increased the levels of SOD (Fig. 5 and Fig. 6B–D). This demonstrated that 6-gingerol may have a protective effect on PC12 cells induced by $A\beta_{1-42}$ from two aspects via reducing the release of superoxide and the level of superoxide accumulation in mitochondria.

Recently, the notion that GSK-3 β is associated with the neuropathology of AD has been generally accepted. Research has demonstrated that the hyper-activation of GSK-3 β plays important roles in tau hyper-phosphorylation.³³ Studies have also indicated that GSK-3 β expression by $A\beta$ relates to abnormal amyloid precursor protein (APP) processing and synaptic failure.³⁴ The inhibition of GSK-3 β reduces the β -site APP cleaving enzyme 1-mediated cleavage of APP through a NF- κ B signaling-mediated mechanism, which subsequently reduces $A\beta$ neuropathology and alleviates memory deficits in AD model mice.³⁵ In addition, the pathological activation of GSK-3 β has been reported to induce apoptosis and is inhibited by phosphorylation.³⁶ Therefore, the up-regulation of p-GSK-3 β inhibits its kinase's activity, and this may confer a protective effect on AD. Griffin RJ et al. (2005) indicated that the activation of Akt, shown by an increase in the expression of p-Akt, exhibited neuroprotective effects in AD.³⁷ In addition, evidence suggests that the inhibition of the PI3K/Akt signaling pathway increases GSK-3 β activity, resulting in tau protein hyperphosphorylation.³⁸ In this experiment, our data showed that treatment with $A\beta_{1-42}$ significantly suppressed the expression of p-GSK-3 β (Ser9) and p-Akt (Ser473), whereas pre-treatment with 6-gingerol (80 and 120 μ M) significantly inhibited the decreased expression of p-GSK-3 β and p-Akt caused by $A\beta_{1-42}$ (Figs. 7 and 8). These results suggested that 6-gingerol may enhance neuroprotection through up-regulating the phosphorylation levels of Akt and GSK-3 β .

In physiological and pathological physiology, various processes are related to the AKT/GSK-3 β pathway, including metabolic control, embryogenesis, cell death, and oncogenesis.³⁹ Hyper-phosphorylated Tau is the major component of the paired helical filaments that accumulate in degenerating neurons in AD and other neurodegenerative diseases.⁴⁰ Thus, the AKT/GSK-3 β pathway seems to be vital for AD because it regulates Tau hyper-phosphorylation in cells. Our study indicated that 6-gingerol could activate the AKT/GSK-3 β pathway through activation of AKT and inhibition of GSK-3 β , indicating that 6-gingerol exerted neuroprotective effects through the AKT/GSK-3 β pathway.

In summary, this study focused on three aspects to investigate the protective effects of 6-gingerol on inhibiting $A\beta_{1-42}$ -induced apoptosis in PC12 cells. First, the decreased levels of NO showed its anti-inflammatory role. Second, the results of ROS, MDA, SOD, and LDH assays reflected the anti-oxidative effects of 6-gingerol. Last, we clarified the molecular mechanisms of 6-gingerol on $A\beta_{1-42}$ -induced apoptosis in PC12 cells via Akt/GSK-3 β signaling pathway in our experiments.

Ginger is generally considered a safe herbal medicine, and is on the FDA's Generally Recognized As Safe (GRAS) list.^{41,42} Moreover, ginger is found in a large variety of foods and can be ingested in considerable amounts (250 mg to 1 gram) daily in

the human diet.⁴³ Although some adverse effects of ginger have been reported in pregnant rats, no maternal toxicity was observed.⁴⁴ As the major gingerol in ginger rhizomes, 6-gingerol might be reasonable in the treatment of AD patients without having any serious side effects.

Conclusions

In summary, our study revealed that 6-gingerol may exhibit a protective effect on the apoptosis induced by $A\beta_{1-42}$ in PC12 cells, possibly by reducing oxidative stress and inflammatory responses, suppressing the activation of GSK-3 β , and enhancing the activation of Akt through the AKT/GSK-3 β pathway. Thus, 6-gingerol may be a valuable candidate drug for the treatment of AD. In addition, further studies are needed to investigate the underlying molecular mechanism concerning how 6-gingerol affects the progress of AD *in vivo* before clinical trials can be done.

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Author Disclosure Statement

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Address correspondence to:

Gao-feng Zeng
College of Public Hygiene
of Guangxi Medical University
No.22 Shuangyong Road
Nanning, Guangxi, 530021
P.R. China

E-mail: fengfeng_388@126.com

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