

# Ginkgolide K protects SH-SY5Y cells against oxygen-glucose deprivation-induced injury by inhibiting the p38 and JNK signaling pathways

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**Abstract.** The purpose of the present study was to explore the protective effect and functional mechanism of ginkgolide K (GK: C<sub>20</sub>H<sub>22</sub>O<sub>9</sub>) on cerebral ischemia. SH-SY5Y cells were exposed to oxygen-glucose deprivation (OGD) to simulate an ischemic model *in vitro*. Cell viability, reactive oxygen species (ROS), nuclear staining with Hoechst 33258 and mitochondrial membrane potential were detected following 4 h of exposure to OGD. Subsequently, the expression levels of the apoptosis-related proteins, caspase-9, caspase-3, Bcl-2, Bax, p53 and c-Jun, as well as the mitogen-activated protein kinases (MAPKs) signaling molecules were detected by western blot analysis. GK significantly elevated the cell viability and decreased the generation of ROS and the number of apoptotic cells in a dose-dependent manner. Furthermore, GK markedly decreased the protein expression levels of p-p38, p-JNK, p-p53, p-c-Jun and the expression levels of Bcl-2, Bax, cleaved caspase-9 and caspase-3. In conclusion, GK demonstrated a neuroprotective effect on the simulated cerebral ischemia *in vitro*, and this effect was mediated through the inhibition of the mitochondria-mediated apoptosis pathway triggered by ROS-evoked p38 and JNK activation.

## Introduction

Cerebral stroke is a leading cause of disability worldwide, and it is estimated that ischemic stroke accounts for approximately 85% of the case (1). Moreover, hospitalizations for ischemic

stroke have shown a year-on-year increase among adolescents and young adults (aged 5-44 years) (2). The pathophysiological processes of ischemic stroke, which trigger neuronal necrosis and apoptosis, are complex and extensive. These include a cascade of bioenergetics failure, loss of cellular ion homeostasis, increased intracellular calcium-induced excitotoxicity, reactive oxygen species (ROS)-mediated toxicity, activation of neuronal and glial cells, cytokine-mediated cytotoxicity and disruption of the blood-brain barrier (3). Currently, intravenous recombinant tissue plasminogen activator (r-TPA) to induce thrombolysis combined with a neuroprotective drug to rescue dying neurons is the common clinical strategy for acute ischemic stroke therapy (3).

Ischemic stroke triggers multiple and overlapping cell signaling pathways that may contribute to cell damage or cell survival. The mitogen-activated protein kinases (MAPKs), which control a broad spectrum of cellular processes including apoptosis, growth, inflammation and stress responses, are important modulators of a variety of diseases. There are also increasing evidences that MAPKs are crucial regulators of hemorrhagic and ischemic cerebral disease, furthermore, raising the possibility that MAPKs may be a drug discovery target for ischemic stroke (4,5). P38 and JNK are two of the main members of the MAPKs signaling group. Thus, emerging evidences suggest that activation of p38 and JNK may play an important role in ischemia-induced neuronal apoptosis. The apoptosis is triggered by the enhanced pro-apoptotic activity of p53 and phosphorylation of the c-Jun regulated by p38 and JNK activities (6).

Ginkgolide K (GK: C<sub>20</sub>H<sub>22</sub>O<sub>9</sub>, as shown in Fig. 1) is a diterpene lactone compound isolated from the leaves of *Ginkgo biloba* which has a long history of therapeutic application as a natural medicine for cardiovascular diseases in humans (7). Recently, GK has been reported to protect the heart against ER stress injury by activating the IRE1 $\alpha$ /XBP1 pathway (8), and also markedly protect PC12 cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity by ameliorating oxidative stress and mitochondrial dysfunction (9). Oxygen-glucose deprivation (OGD) is widely used as an *in vitro* model for stroke due to its similarities with the *in vivo* models of brain ischemia, and it is a simple and highly useful technique, not only for the

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elucidation of the role of key cellular and molecular mechanisms, but also for the development of novel neuroprotective strategies. SH-SY5Y cells exposed to OGD constitute a classical model used to mimic cerebral ischemic injury. In the present study, the neuroprotective effect and functional mechanism of GK on cerebral ischemia were further confirmed by OGD-stimulated SH-SY5Y cells *in vitro*.

## Materials and methods

GK was extracted and separated by Jiangsu Kanion Modern Traditional Chinese Medicine Research Institute with 98% purity. SH-SY5Y cells were purchased from Cell Bank of the Chinese Academy of Sciences (no. CRL-2266) which is imported from the ATCC (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Bestbio Biotechnology (Shanghai, China). The ROS assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit antibodies against p38, p-p38 (Thr180/Tyr182), JNK, p-JNK (Thr183/Tyr185), p53, p-p53 (Ser15), c-Jun, p-c-Jun (Ser73), Bcl-2, cleaved caspase-3, caspase-3, tubulin, actin and the secondary antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit antibodies against Bax and cleaved caspase-9 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). PVDF membrane and ECL western detection reagent were obtained from Bio-Rad Laboratory (Hercules, CA, USA). All other reagents were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) unless otherwise stated.

**Cell viability assay.** SH-SY5Y cells were cultured in RPMI-1640 medium supplemented with 10% FBS in a 5% CO<sub>2</sub>, 37°C incubator. The SH-SY5Y cells of logarithmic growth were seeded in 96-well plates (2x10<sup>4</sup> cells/well) and cultured overnight. For OGD and reoxygenation model, the culture medium of SH-SY5Y cells was first replaced with RPMI-1640 medium containing no glucose, and then the plates were placed in a hypoxia chamber aerated with 95% N<sub>2</sub> and 5% CO<sub>2</sub> for 4 h in a 37°C incubator. Afterwards, the plates were transferred to the 5% CO<sub>2</sub>, 37°C incubator with reoxygenation for 1 h.

After OGD 4 h, SH-SY5Y cells treated with GK at a dose of 25 µg/ml were cultured with reoxygenation for different times (1, 2, 4 and 6 h). The CCK-8 assay, a sensitive colorimetric assay for determination of the number of viable cells, was used in the cell proliferation and cytotoxicity analysis. WST-8 (10 µl) was added to each well, and then the cells were cultured for an additional 2 h to allow for the reaction of WST-8. Furthermore, WST-8 is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Finally, the absorbance at 450 nm was measured using a microplate reader. OD450 nm values were converted to a percentage and all groups were compared to the control group (100%).

In addition, SH-SY5Y cells were treated with different concentrations of GK (12.5, 25 and 50 µg/ml) followed

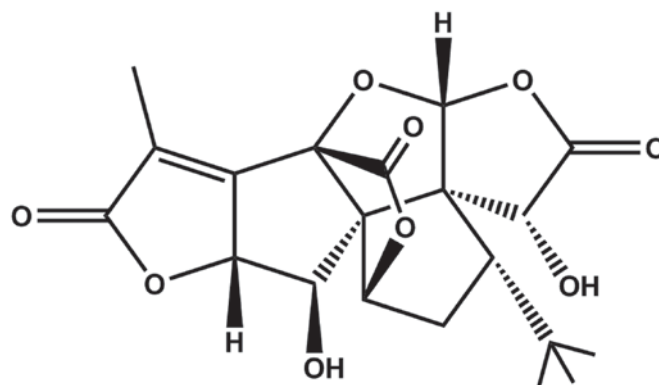


Figure 1. Chemical structural formula of GK. GK, ginkgolide K.

by reoxygenation for 1 and 24 h respectively. Relative cell viability was also measured by CCK-8 assay.

**Nuclear staining by Hoechst 33258.** To observe the nuclear changes occurring during OGD, the chromatin-specific dye, Hoechst 33258, was used to stain the nuclei. After treatment with different concentrations of GK (12.5, 25 and 50 µg/ml) and reoxygenation for 1 h, cells were washed with PBS and fixed in 4% paraformaldehyde at 4°C overnight, and then cells were permeabilized with 0.3% Triton X-100 at room temperature for 30 min. The cells were subsequently incubated with 10 ng/ml Hoechst 33258 in the dark for 10 min. After two additional rinses with PBS, cells were photographed under a fluorescent microscope at 350 and 460 nm (Leica Microsystems GmbH, Wetzlar, Germany) with x200 magnification.

**Detection of ROS and mitochondrial membrane potential.** After exposure to OGD for 4 h, followed by treatment with different concentrations of GK (12.5, 25 and 50 µg/ml) and reoxygenation for 1 h, cells were incubated with 10 mM DCFHDA in the dark at 37°C for 20 min, and washed twice with PBS. Then the fluorescence intensity of DCF was measured with a microplate reader.

Additionally, GK-treated cells were incubated with 1 µM rhodamine-123 in the dark at 37°C for 20 min. After two additional rinses with PBS, rhodamine-123 intensity was determined by flow cytometry. Cells with reduced fluorescence (less rhodamine-123) were counted as the collapse of mitochondrial membrane potential.

**Western blot analysis.** After exposure to OGD for 4 h, SH-SY5Y cells (5x10<sup>6</sup>/dish of 100-mm<sup>2</sup> size) treated with GK and reoxygenation for 1 h were collected on ice, and optimal cell lysis solution was added to completely release the proteins for 2 h. The supernatants were collected after centrifuging at 14,000 x g at 4°C for 10 min, and then protein concentrations were assayed with a BCA kit. After SDS-PAGE electrophoresis, proteins were transferred to a PVDF membrane. The transferred membranes were blocked with 5% nonfat milk for 2 h at room temperature, and incubated with primary antibodies at 4°C overnight. After three rinses with TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, after three additional rinses with TBST, the immune complexes were detected using ECL western

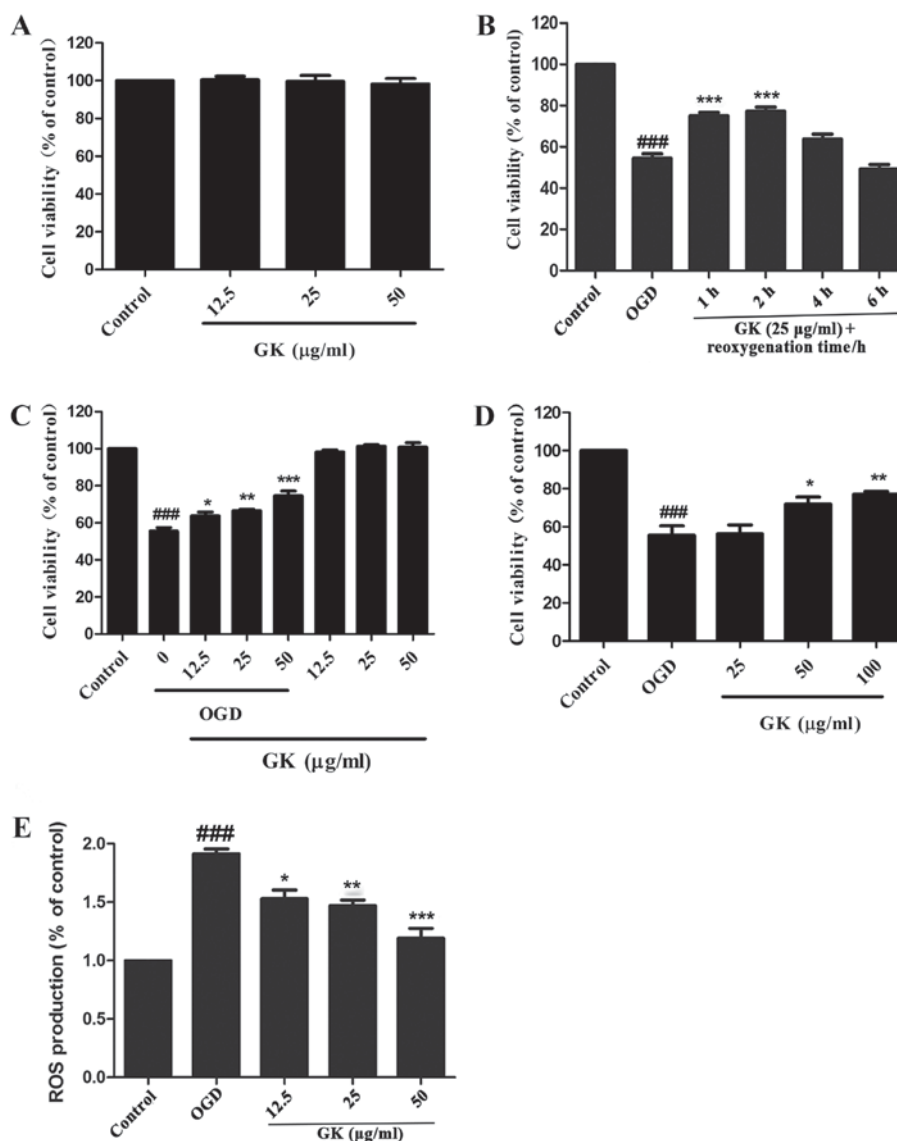


Figure 2. GK increased cell viability and ROS generation of SH-SY5Y cells damaged by OGD. (A) Cells were treated with 12.5, 25 and 50  $\mu\text{g/ml}$  of GK for 24 h and cell viability was measured with the CCK-8 assay. (B) After 4 h OGD, the SH-SY5Y cells were treated with GK and reoxygenation with various times (1, 2, 4 and 6 h). Cell viability was assessed by CCK-8 assay. (C) After 4 h OGD, the SH-SY5Y cells were treated with GK at different concentrations (12.5, 25 and 50  $\mu\text{g/ml}$ ) and reoxygenation for 1 h. Cell viability was assessed by CCK-8 assay. (D) After 4 h OGD, the SH-SY5Y cells were treated with GK at different concentrations (25, 50 and 100  $\mu\text{g/ml}$ ) and reoxygenation for 24 h. Cell viability was assessed by CCK-8 assay. (E) ROS generation was quantified by DCFHDA assay at excitation 488 nm and emission 525 nm. Data are represented as mean  $\pm$  SD from six experiments. ### $P$ <0.001 vs. control group. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. OGD group. GK, ginkgolide K; ROS, reactive oxygen species; CCK-8, Cell Counting Kit-8; OGD, oxygen-glucose deprivation.

detection reagents and photographed with ChemiDoc<sup>TM</sup> XRS+ software to calculate gray value statistics.

**Statistical analysis.** All data are presented as the mean  $\pm$  SD. Data were analyzed with one-way ANOVA analysis followed by Tukey's post hoc test using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**GK increases OGD-damaged SH-SY5Y cells viability.** We first examined the effect of GK on the proliferation of SH-SY5Y cells. The CCK-8 assay showed that GK did not affect the viability of SH-SY5Y cells at concentrations of 12.5, 25 and 50  $\mu\text{g/ml}$  (Fig. 2A).

After exposure to OGD for 4 h, SH-SY5Y cells were treated with a moderate dose of GK (25  $\mu\text{g/ml}$ ) and reoxygenation for different durations (1, 2, 4 and 6 h). To evaluate the effect of GK, cells viability was measured using the CCK-8 method. The results showed that cell viability significantly decreased after the exposure to OGD. However, GK treatment at a dose of 25  $\mu\text{g/ml}$  for 1 and 2 h significantly increased the cells viability respectively (Fig. 2B). While the cell viability decreased after reoxygenation and GK treatment for 4 and 6 h, this was considered to be caused by increased damage due to prolonged glucose deprivation. Considering the time-dependent nature of this effect, we selected reoxygenation and GK treatment for 1 h as the optimal duration in the subsequent experiments.

Next, we examined the effects of GK treatment at different concentrations (12.5, 25 and 50  $\mu\text{g/ml}$ ). The assay

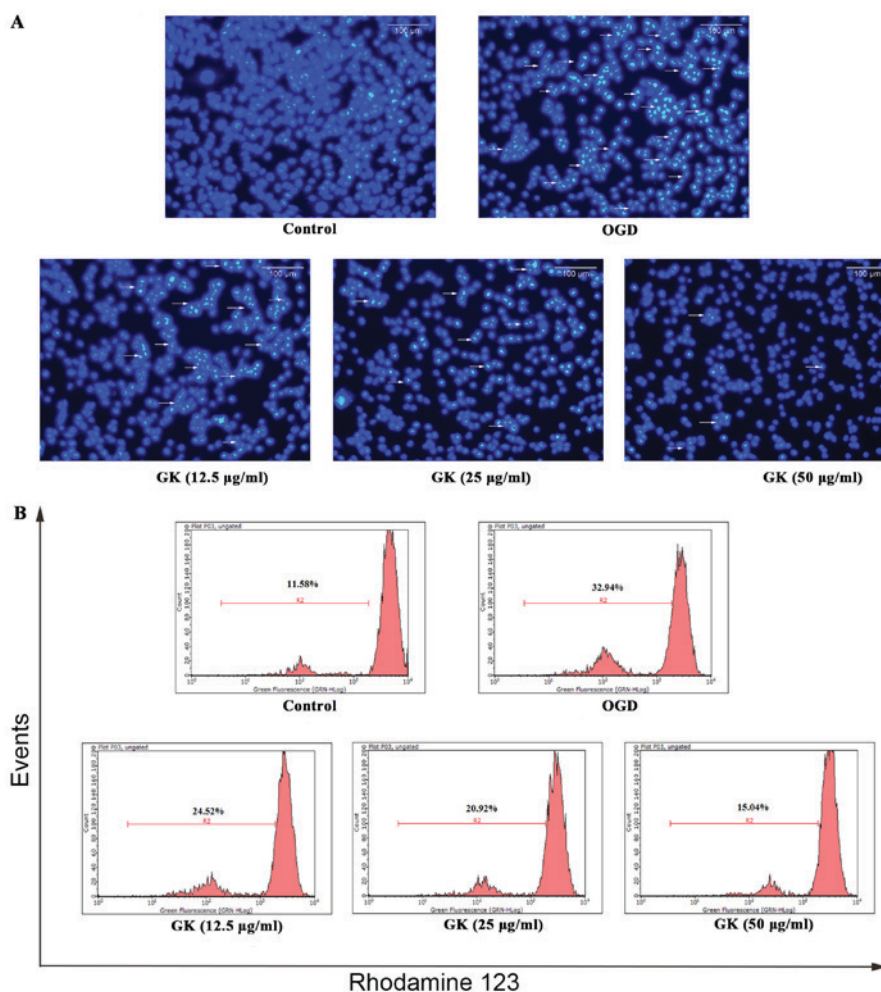


Figure 3. GK reduced apoptosis of SH-SY5Y cells damaged by OGD. After 4 h OGD, the SH-SY5Y cells were treated with GK at different concentrations (12.5, 25 and 50  $\mu\text{g/ml}$ ) and reoxygenation for 1 h. (A) Morphological analysis of nuclear chromatin was evaluated by Hoechst 33258 staining. Scale bar=100  $\mu\text{m}$ . (B) Rhodamine123 single staining was performed to measure dissipation of mitochondrial membrane potential by flow cytometry. The rate indicated the percentage of cells with dissipation of mitochondrial membrane potential. GK, ginkgolide K; OGD, oxygen-glucose deprivation.

demonstrated that GK significantly increased the cell viability in a dose-dependent manner (Fig. 2C). In general, GK plays a neuroprotective role in OGD-damaged SH-SY5Y cells, in a dose-dependent manner. We also assayed reoxygenation after OGD stimulation and GK treatment at different concentrations (25, 50 and 100  $\mu\text{g/ml}$ ) for 24 h to verify whether GK has protective effect on cell damage or not. As predicted, GK significantly suppressed cell death following OGD for 4 h and reoxygenation for 24 h, although 25  $\mu\text{g/ml}$  of GK had no marked effect (Fig. 2D).

**GK decreases the intracellular ROS content.** The intracellular ROS levels in the presence of oxidative stress induced by OGD were measured by a DCFHDA assay. The results demonstrated that treatment with GK at concentrations of (12.5, 25 and 50  $\mu\text{g/ml}$ ) for 1 h had significantly decreased the ROS levels by  $1.56\pm 0.07$ ,  $1.45\pm 0.07$  and  $1.24\pm 0.05\%$  compared with untreated SH-SY5Y cells, respectively (Fig. 2E).

**GK protects OGD-induced SH-SY5Y cells from apoptosis.** Hoechst-33258 staining (blue) was used to observe the morphology of the nuclei, in order to demonstrate apoptosis in OGD-induced SH-SY5Y cells. By comparison with the

control group, cell nuclear pyknosis, chromatin condensation, chromosome fragmentation, the formation of apoptotic bodies, and other apoptotic changes were observed in OGD-induced SH-SY5Y cells. By contrast, we observed that GK treatment decreased appearance of these morphological features, indicating the attenuation of apoptosis by GK treatment (Fig. 3A).

In addition, we also observed the collapse of mitochondrial membrane potential in OGD-induced SH-SY5Y cells with rhodamine-123 staining. The value was measured by flow cytometry. As shown in Fig. 3B, after exposure to OGD for 4 h, the quantity of SH-SY5Y cells with dissipation of mitochondrial membrane potential was increased from 11.58 to 32.94%. However, mitochondrial membrane potential was dose-dependently decreased in SH-SY5Y cells treated with GK at 12.5, 25 and 50  $\mu\text{g/ml}$  concentrations. Taken together, these data suggest that GK inhibited the apoptosis of SH-SY5Y cells induced by sustained OGD damage.

**GK suppresses p38 and JNK activation in OGD-induced SH-SY5Y cells.** To investigate the mechanism through which GK prevents cellular apoptosis in response to OGD, we next examined the effects of GK on the p38 and JNK signaling via western blot. The results showed that p-p38



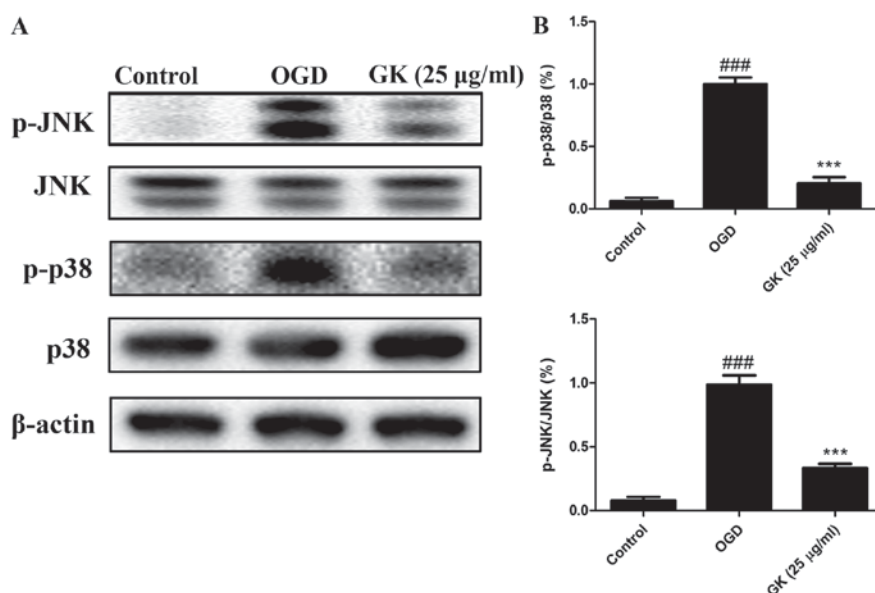


Figure 4. Suppression of p38 and JNK activities with GK treatment in OGD-induced SH-SY5Y cells. (A) Western blot analysis of the protein expression levels of p-p38, p38, p-JNK, JNK in SH-SY5Y cells with 25  $\mu\text{g/ml}$  GK treatment and reoxygenation for 1 h. (B) Quantity one software was used for quantitative analysis of p-p38/p38 and p-JNK/JNK. The results present averages of three independent experiments (mean  $\pm$  SD). <sup>###</sup> $P < 0.001$  vs. control group, <sup>\*\*\*</sup> $P < 0.001$  vs. OGD group. GK, ginkgolide K; OGD, oxygen-glucose deprivation.

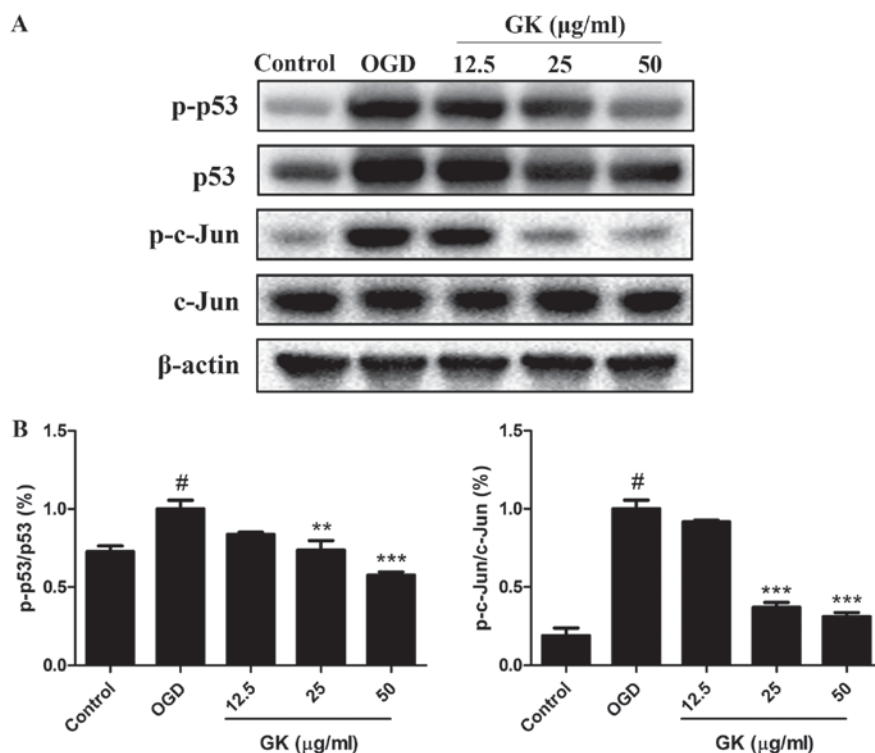


Figure 5. Suppression of the transcription factor activity of p53 and c-Jun with GK treatment in OGD-induced SH-SY5Y cells. (A) Western blot analysis of the protein expression levels of p-p53, p53, p-c-Jun and c-Jun in SH-SY5Y cells with different concentrations of GK treatment (12.5, 25 and 50  $\mu\text{g/ml}$ ) and reoxygenation for 1 h. (B) Quantity one software was used for quantitative analysis of p-p53/p53 and p-c-Jun/c-Jun. The results present averages of three independent experiments (mean  $\pm$  SD). <sup>#</sup> $P < 0.05$  vs. control group, <sup>\*\*</sup> $P < 0.01$  and <sup>\*\*\*</sup> $P < 0.001$  vs. OGD group. GK, ginkgolide K; OGD, oxygen-glucose deprivation.

(Thr180/Tyr182) and p-JNK (Thr183/Tyr185) expressions were notably increased after OGD for 4 h (Fig. 4A). However, after treatment with GK, p-p38 (Thr180/Tyr182) and p-JNK (Thr183/Tyr185) proteins were significantly down-regulated compared with the non-treated control (Fig. 4B), suggesting that GK could suppress the p38 and JNK pro-apoptotic

signaling pathways to protect OGD-damaged SH-SY5Y cells.

*GK reduces p53 and c-Jun transcription factor activation in OGD-induced SH-SY5Y cells.* After activation by intracellular and extracellular stimuli, JNK and p38 can directly enhance the

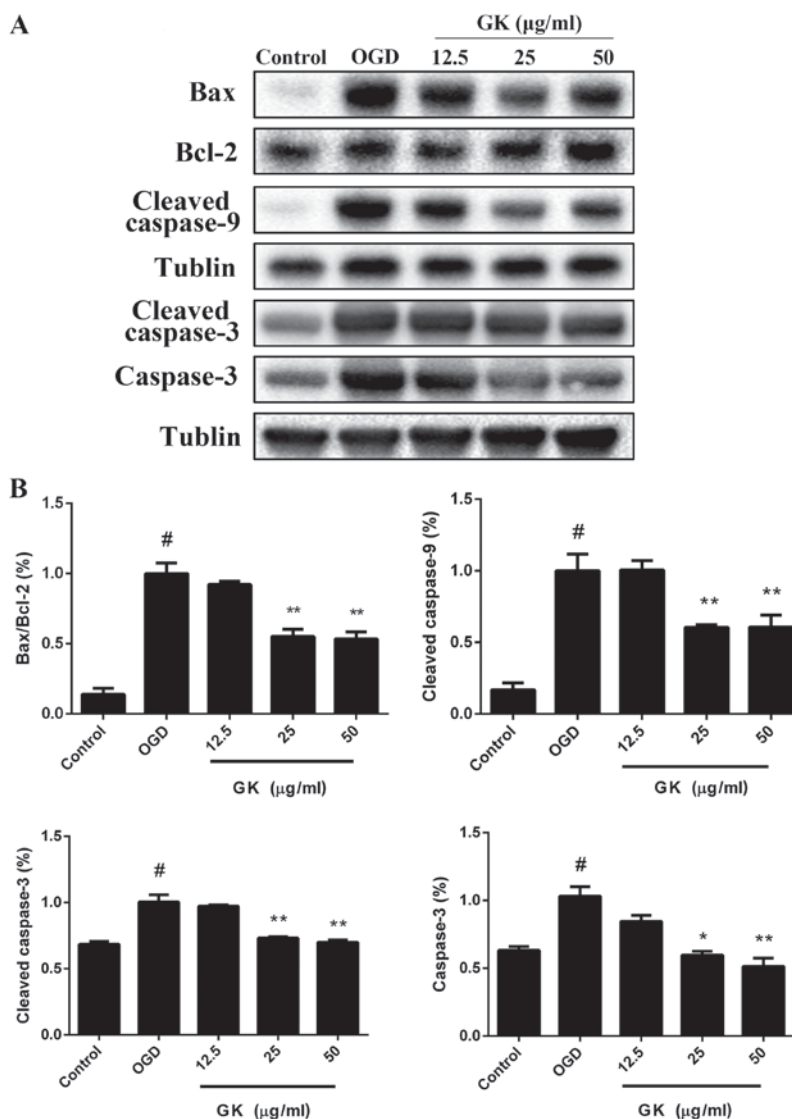


Figure 6. Suppression of Bax/Bcl-2 ratio and cascade protein activities with GK treatment in OGD-induced SH-SY5Y cells. (A) Western blot analysis of the protein expression levels of Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3 and caspase-3 in SH-SY5Y cells with different concentrations of GK treatment (12.5, 25 and 50  $\mu\text{g/ml}$ ) and reoxygenation for 1 h. (B) Quantity one software was used for quantitative analysis of Bax/Bcl-2 and optical density ratio of cascade protein bands normalized to tubulin. The results present averages of three independent experiments (mean  $\pm$  SD). # $P$ <0.001 vs. control group, \* $P$ <0.05 and \*\* $P$ <0.01 vs. OGD group. GK, ginkgolide K; OGD, oxygen-glucose deprivation.

pro-apoptotic activity of p53 and the phosphorylation of the c-Jun to induce apoptosis (10,11). Thus, the activities of p53 and c-Jun were analyzed by western blotting. As shown in Fig. 5A, B, OGD treatment increased the phosphorylation levels of p53 (ser15) and c-Jun (ser73) compared with the control. However, by comparison with the OGD group, GK treatment significantly decreased the levels of p-p53 (ser15) and p-c-Jun (ser73) in a dose-dependent manner, indicating the inhibition of p53 and c-Jun activities by GK in OGD-induced SH-SY5Y cells.

*GK decreases the mitochondrial-related Bax/Bcl-2 ratio to rescue caspase-dependent apoptosis in OGD-induced SH-SY5Y cells.* Activations of p53 and c-Jun immediately trigger the expression of a number of apoptosis regulatory proteins, such as Bax and Bad, but reverse the anti-apoptotic function of Bcl-2 (12). The Bcl-2 family proteins are localized on the mitochondrial outer membrane and to initiate mitochondria-mediated apoptosis. Therefore, we next examined

the effects of GK on the protein levels of the caspase and Bcl-2 families. Western blot analysis showed that treatment with GK reduced the protein level of Bax and increased the level of Bcl-2, thus decreasing the ratio of Bax/Bcl-2 following OGD-induced apoptosis (Fig. 6A, B). Moreover, cleaved caspase-9 and cleaved caspase-3 were reduced after treatment with 12.5, 25 and 50  $\mu\text{g/ml}$  GK compared with OGD group (Fig. 6A and B). In addition, GK also decreased the expression of total caspase-3 in a dose-dependent manner. Collectively, these data demonstrate that GK significantly repressed Bcl-2 family protein-regulated caspase activity in OGD-induced SH-SY5Y cells.

## Discussion

*Ginkgo biloba* extracts, especially ginkgolides mainly including ginkgolide A, B and C have been reported to possess potent protective properties by antagonizing platelet activating factor (PAF), thereby inhibiting platelet aggregation to protect

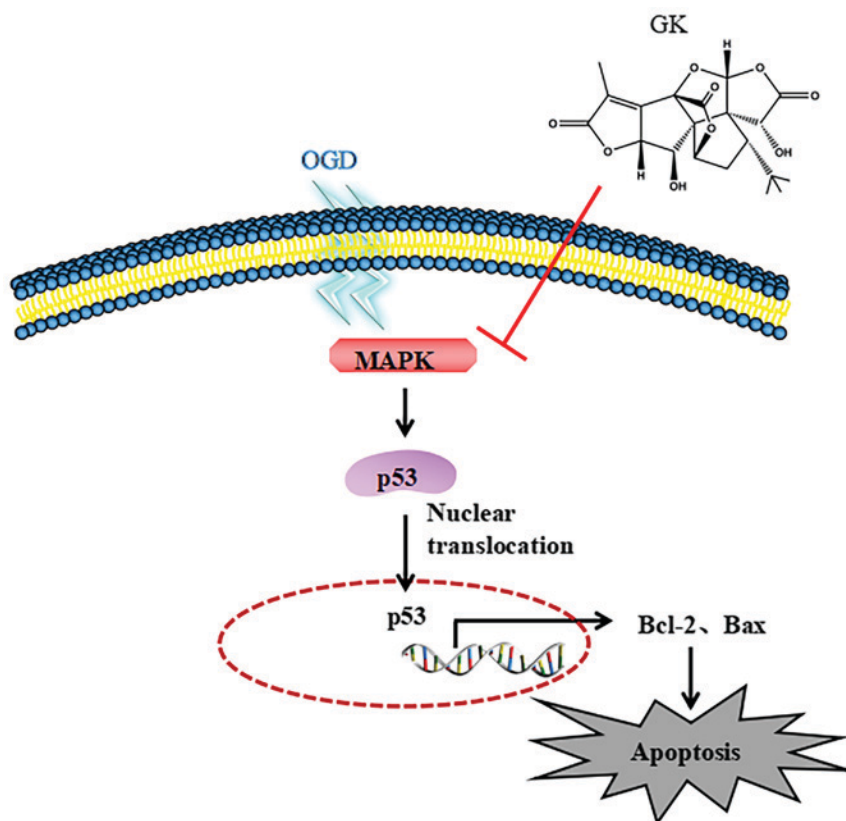


Figure 7. Schematic diagram of the beneficial effects and potential mechanisms of GK against ischemic insults. GK dose-dependently decreased OGD-induced ischemic neuronal damages, which may be mediated by downregulation of MAPK pathway, as well as suppression of pro-apoptotic proteins Bax and p53. GK, ginkgolide K; OGD, oxygen-glucose deprivation; MAPK, mitogen-activated protein kinase.

against ischemic stroke (1,13,14). In this study, we established that GK, a newly isolated compound in ginkgolide family, protected SH-SY5Y cells against OGD-induced apoptosis. The selective inhibition of the p38 and JNK pathways play a crucial role in the neuroprotective effect of GK on cerebral ischemia. These results indicated that GK conferred profound neuroprotection in response to ischemic stroke.

The mitochondrial apoptotic pathway may play an important role in neuronal cell death after cerebral ischemia. When neuronal ischemic injury occurs, there are at least three factors that induce mitochondrial pore channels: the overload of calcium ions in the mitochondria, the oxidative damage to the mitochondrial membrane and the decline of energy levels (6). After death stimuli, the permeability of the mitochondria may increase, which causes the release of Apaf-1, cytochrome c and procaspase-9 from the mitochondria to cytosol. Subsequently, cytochrome c binds to Apaf-1 and leads to the formation of cytochrome c/Apaf-1 multimeric complex. Procaspase-9 gets recruited to the multimeric complex in a 1:1 ratio through the interaction between Apaf-1 and caspase-9. Thus, the procaspase-9 molecules are activated by auto cleavage. Moreover, caspase-3 is activated by caspase-9 to trigger the further downstream apoptotic processes (15-18). In addition, the Bcl-2 family proteins play a crucial role in regulating the mitochondrial permeability after cerebral ischemia (19). The protein levels of Bax and translocation from the cytosol to the mitochondria have been observed to increase after ischemic injury. Furthermore, Bax promotes the release of procaspase-9 and the cytochrome c from the mitochondria coincides to cytosolic through interacting with the

voltage-dependent anion channel and the mitochondrial adenine nucleotide translocator (12). On the other hand, the protein levels of Bcl-2 have been reported to decrease in ischemic rats (20). It was previously demonstrated that the anti-apoptotic effects of Bcl-2 were accompanied by decreased cytochrome c release and reduced activation of caspase-3 (21). In the present study, our results demonstrated that GK exerted a dose-dependent inhibitory on Bcl-2 down-regulation, Bax up-regulation and decreased the caspase-9 and caspase-3 activities in OGD-induced SH-SY5Y cells. These results suggested that GK conferred a neuroprotective effect in the simulated cerebral ischemia *in vitro* by inhibiting the mitochondria-mediated death pathway.

p38 and JNK are two of the main members of the MAPKs signaling group, which are crucial regulators of hemorrhagic and ischemic cerebral disease. The activation of p38 can promote p53 phosphorylation at Ser15 residues to inhibit the ubiquitination and degradation of the p53 (22,23). Similarly, JNK phosphorylates c-Jun at Ser63 and Ser73 regions to activate the pro-apoptotic effects of c-Jun (24,25). Both activated p53 and c-Jun bind to the specific sites on the promoters of the Bcl-2 family proteins, such as Bcl-2 and Bax, to increase the Bax/Bcl-2 ratio (26). In this study, we observed the decreases in the phosphorylation of p53 and c-Jun that may be due to the down-regulation of p38 and JNK activity, as a result of inhibiting the p38 and JNK pathways with GK treatment.

In summary, GK reduced the activities of p38 and p-JNK, decreased the phosphorylation of p53 and c-Jun, inhibited the mitochondria-mediated apoptosis pathway and protected against OGD-induced apoptosis in SH-SY5Y cells (Fig. 7).

Taking into account the above results, GK may be a potential compound for rescuing neurons from ischemic stroke-induced apoptosis, however, its underlying molecular mechanisms must be further explored.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

QL, XL, LL, ZX, JZ and WX designed the study. QL, XL and LL performed the experiments. QL, XL, LL, ZX and JZ analyzed data and drafted the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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