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Geniposide protects PC-12 cells against oxygen and glucose deprivation-induced injury by up-regulation of long-noncoding RNA H19

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ABSTRACT

Aims: Hypoxic-ischemic encephalopathy (HIE) is a common brain injury disease in neonates, which can lead to neonatal disability and death. Geniposide (GEN) is a main ingredient of *Gardenia jasminoides*, whose anti-tumor, anti-inflammatory and anti-apoptotic effects have been reported in various diseases. However, the effect of GEN on HIE remains uninvestigated. This study aimed to clarify the protective effect of GEN on PC-12 cells against oxygen and glucose deprivation (OGD)-induced injury.

Main methods: PC-12 cells were subjected to OGD treatment, cell viability, cell cycle-associated factors, apoptosis and apoptosis-associated factors were then determined. The different concentrations of GEN were used to stimulate PC-12 cells, and the effects of GEN on cell proliferation and apoptosis in OGD-treatment cells were assessed. Subsequently, relative expression level of H19 was analyzed in PC-12 cells after treatment with GEN. After this, si-H19 was transfected into PC-12 cells to explore the regulatory effect of H19 on PC-12 cells after treatment with GEN and OGD. Besides, PI3K/AKT and Wnt/β-catenin pathways were examined by western blot assay.

Key findings: OGD significantly inhibited cell viability, decreased CyclinD1, CDK4 and CDK6 expression, induced apoptosis and up-regulated Cleaved-Caspase-9/-7/-3 expression in PC-12 cells. GEN treatment obviously alleviated OGD-induced cell injury. Additionally, H19 expression was up-regulated by GEN, and H19 knockdown reversed the protective effect of GEN on PC-12 cells against OGD-induced injury. Finally, GEN activated PI3K/AKT and Wnt/β-catenin pathways by regulating H19 in OGD-insulted PC-12 cells.

Significance: The findings suggested that GEN protected PC-12 cells against OGD-induced injury by up-regulation of H19.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a brain lesion induced by the disruption of cerebral blood flow which causes hypoxic or ischemic injury [28]. It is commonly occurred in neonates, and is one of the main causes of disability of children after the neonatal period [6,26]. HIE leads to mental retardation, epilepsy, learning impairment, blindness, and cerebral palsy [1]. Owing to difficult to ascertain the time and cause of brain injury induced by HIE, there is still no signal and effective method for the treatment of HIE [11]. Optimal management of HIE involves medication and hypothermia therapy has been proven to be a promising new therapy for HIE [3]. However, treatment of HIE is still a major conundrum in clinic. Therefore, it is necessary to explore a new way to solve the treatment problem of HIE.

Geniposide (GEN) is an iridoid glycoside extracted from the mellow fruits of *Gardenia jasminoides* [7]. It is widely known to exert pleiotropic effects, such as anti-oxidative, anti-tumor, anti-angiogenic and anti-inflammatory activities [14,35]. Additionally, a strong evidence stated that GEN exerted the neuro-protective effect on PC-12 cells, which assisted PC-12 cells against hydrogen peroxide (H_2O_2)-induced cell injury [24]. Further, GEN has been reported to be a novel agonist for glucagon-like peptide-1 (GLP-1) receptor, which exhibits the neuro-trophic property to induce the neuronal differentiation of PC-12 cells [23]. Moreover, one interesting study demonstrated that GEN could prevent PC-12 cells from oxidative damage through regulation of mitogen-activated protein (MAP) kinase pathway [22]. However, whether GEN can protect PC-12 against oxygen and glucose deprivation (OGD)-induced cell injury remains unclear.

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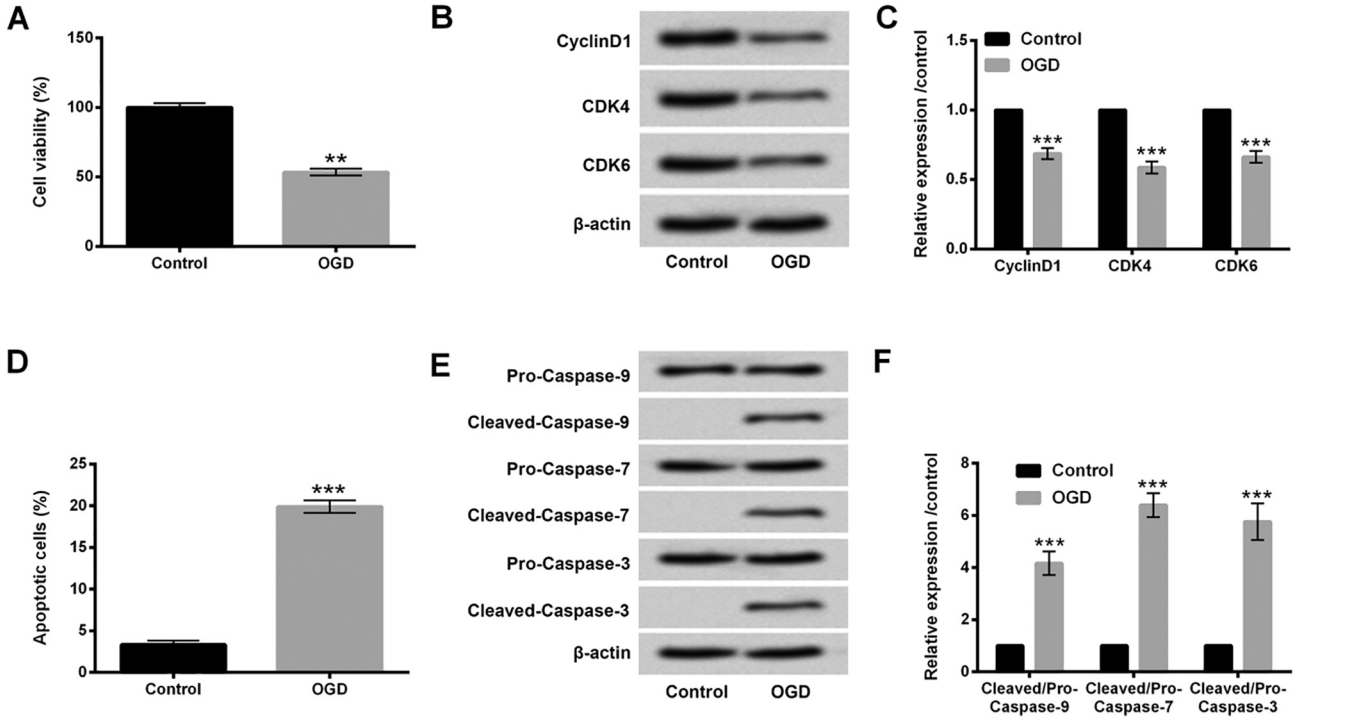

Fig. 1. Effect of OGD treatment on PC-12 cells proliferation and apoptosis
PC-12 cells were subjected to OGD treatment for 24 h, (A) cell viability, (B and C) protein levels of CyclinD1, CDK4 and CDK6 were examined by CCK-8 and western blot assays; (D) cell apoptosis and (E and F) protein levels of pro-Caspase-9/-7/-3 and Cleaved-Caspase-9/-7/-3 were examined by flow cytometry and western blot assays.
OGD: oxygen glucose deprivation; CDK: cyclin-dependent kinase; CCK-8: Cell Counting Kit-8; $^{**}P < 0.01$; $^{***}P < 0.001$.

Long-noncoding RNAs (lncRNAs) are a novel class of non-coding RNAs, which are transcripts with a length > 200 nucleotides, and unable to encode proteins [38]. Recent study demonstrated that lncRNAs play vital roles in neural development and are closely associated with the pathogenesis of diseases [39]. As an important member of lncRNA, H19 has been reported in different diseases, such as cancers [21,42], diabetic cardiomyopathy [43] and cerebral ischemia reperfusion (I/R) injury disease [33]. However, the effect of H19 on HIE is still uninvestigated.

OGD induced cell injury is a complex process accompanying a series of changes, such as reactive oxygen species (ROS), calcium overload and mitochondrial permeability transition pore (mPTP) [8,36]. In immature animals, a great number of studies have confirmed that OGD model is the most commonly used in vitro model of HIE [9,10]. In this study, we constructed OGD cell injury model to mimic HIE, and the regulatory effect of GEN on OGD-injured PC-12 cells were explored. We highlighted the molecular mechanism of GEN protected PC-12 cells against OGD-induced injury, as well as determine the potential influences of H19 in OGD-injured PC-12 cells. These findings might provide a new light for the treatment of HIE.

2. Materials and methods

2.1. Cell culture and OGD treatment

PC-12 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) (LifeTechnologies, Carlsbad, CA, USA) was used to culture PC-12 cells, supplemented with 10% horse serum (HS, LifeTechnologies), 5% fetal bovine serum (FBS, LifeTechnologies) and 1% Penicillin/Streptomycin (P/S, LifeTechnologies) at 37 °C with 5% CO_2. After incubation, the common culture medium was replaced with glucose-free DMEM (without FBS and glucose). Then, these cells were cultured in a hypoxic incubator with 5% CO_2 and 95% N_2 for 2 h. Subsequently, the cell culture medium was refreshed to complete medium before completing the OGD. Cells were then returned into a normal incubator and incubated for 24 h. The normal medium under normoxia served as control.

GEN (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and was dissolved in phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, MO, USA). Cells were stimulated with GEN for 1 h at the concentration of 0, 100, 200 or 300 μM before treatment with OGD.

2.2. Cell counting Kit-8 (CCK-8) assay

PC-12 cells were cultured in 96-well plate at a density of 5×10^3 cells/well, and these cells were treated with OGD alone, GEN alone or co-treated with OGD and GEN, cell viability was then assessed by using CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, after treatment, 10 μL of CCK-8 solution was added to each well, and was incubated at 37 °C for 1 h under the conventional culture condition. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.3. Detection of apoptosis

Apoptosis of OGD and GEN treated PC-12 cells was determined by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Briefly, these treated cells were washed twice with PBS (Sigma Chemical Co.) and re-suspended in buffer. The Annexin V-FITC (5 μL) and PI (5 μL) were added to the cell suspension and stained these cells for 15 min at room temperature in the dark. Subsequently, the fluorescence intensities of these stained cells were analyzed by using flow cytometry assay (Beckman Coulter, Fullerton, CA, USA). The data in the experiment were analyzed by using FlowJo

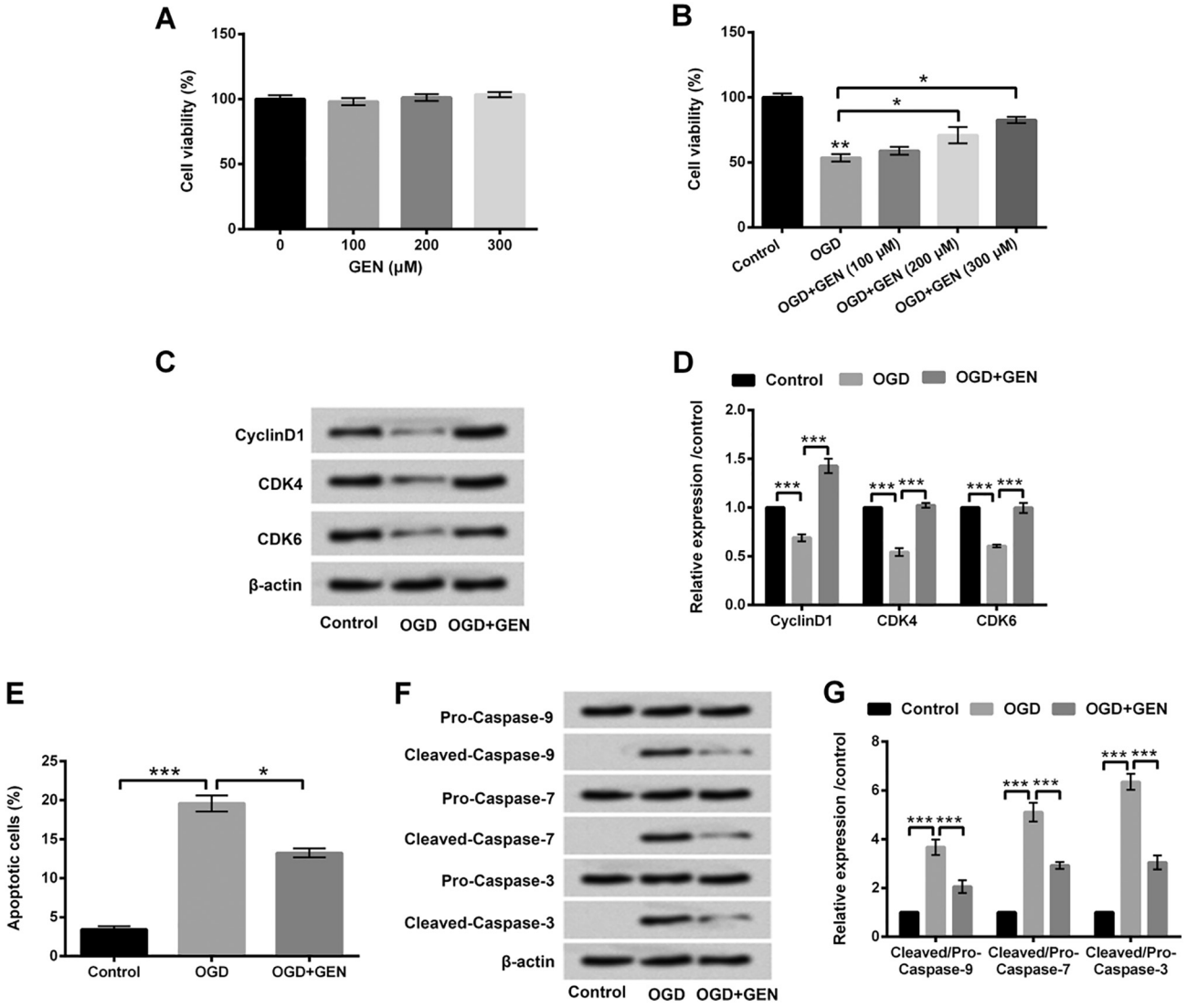

Fig. 2. Effect of GEN on cell proliferation and apoptosis in OGD-treated PC-12 cells.
(A) PC-12 cells were treated with GEN (100, 200 or 300 μM) for 24 h, and cell viability was determined by CCK-8 assay; (B) PC-12 cells were pre-treated with GEN (100, 200 or 300 μM) and then subjected to receive OGD treatment for 24 h, cell viability was determined again; (C and D) protein levels of CyclinD1, CDK4 and CDK6 were examined by CCK-8 and western blot assay after GEN (300 μM) and OGD treatment; (E) cell apoptosis and (F and G) protein levels of pro-Caspase-9/-7/-3 and Cleaved-Caspase-9/-7/-3 were examined by flow cytometry and western blot assays after GEN (300 μM) and OGD treatment.
GEN: geniposide; OGD: oxygen glucose deprivation; CDK: cyclin-dependent kinase; CCK-8: Cell Counting Kit-8; $^{*}P < 0.05$;$^{**}P < 0.01$; $^{***}P < 0.001$.

software (Tree Star, San Carlos, CA, USA).

2.4. Cell transfection

Small interfering RNA oligonucleotides targeting H19 (si-H19) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The scrambled siRNA (si-NC) was used as a negative control. PC-12 cells were transfected with si-H19 and si-NC by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After transfection for 48 h, cells were harvested for the following experiments.

2.5. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from treated or transfected cells by using TRIzol reagent (Invitrogen). Reverse transcription was performed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA). RT-qPCR was performed by using the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Basel, Switzerland). The expression level of H19 was normalized to β-actin and was analyzed by using $2^{-\Delta\Delta Ct}$ method [25].

2.6. Western blot assay

The treated PC-12 cells were prepared in RIPA lysis buffer (Beyotime, Shanghai, China) containing with protease inhibitors (Roche Diagnostics). After quantitative analysis of the proteins by using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), proteins were assessed by using western blot assay according to the manufacturer's instructions. Briefly, the equal amounts of proteins were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After this, the proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were then blocked in non-fat milk for 1 h, and were incubated with primary antibodies at 4 °C overnight. The primary antibodies included CyclinD1 (ab16663), CDK4 (ab199728), CDK6 (ab124821), Pro-Caspase-9 (ab135544), Cleaved-Caspase-9 (ab2324), Pro/Cleaved-Caspase-7 (ab32522), Pro-Caspase-3 (ab32150), Cleaved-Caspase-3 (ab2302), t-

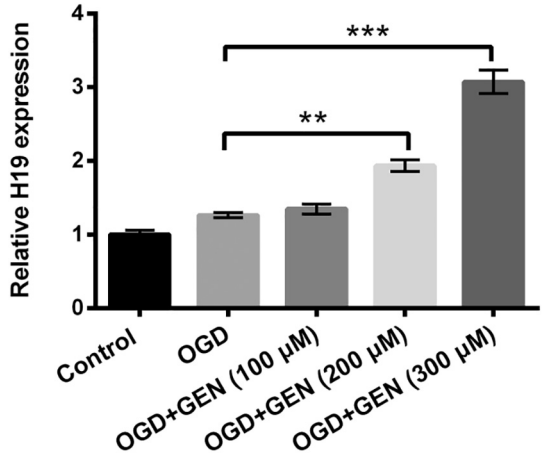

Fig. 3. Effect of GEN on H19 expression level in OGD-treated PC-12 cells.
PC-12 cells were pre-treated with GEN (100, 200 or 300 μM) and then subjected to receive OGD treatment for 24 h. The expression level of H19 was determined by RT-qPCR assay.
GEN: geniposide; OGD: oxygen glucose deprivation; RT-qPCR: reverse transcription-quantitative PCR. $^{**}P < 0.01$; $^{***}P < 0.001$.

PI3K (ab191606), phospho (p)-PI3K (ab182651), t-AKT (ab18785), p-AKT (ab38449), β-catenin (ab32572), and β-actin (ab8227, Abcam, Cambridge, UK). After washing in PBS for three times, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (ab205718, 1:2000, Abcam) for 2 h at room temperature. The blots were visualized by using enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK). The intensity of the bands was quantified by using Image Lab™ Software (Bio-Rad).

2.7. Statistical analysis

All results of multiple experiments were shown as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software (IBM, New York, NY, USA). The *P*-values were calculated by using a one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant result.

3. Results

3.1. OGD inhibited cells proliferation and induced apoptosis in PC-12 cells

PC-12 cells were subjected to OGD treatment, and cell viability and the protein levels of cell cycle-associated factors (CyclinD1, CDK4 and CDK6) were determined. Compared with control group, we found that OGD treatment significantly inhibited the viability radio of PC-12 cells ($P < 0.01$, Fig. 1A), and decreased the protein levels of CyclinD1, CDK4 and CDK6 in PC-12 cells ($P < 0.001$, Fig. 1B and C). Then, cell apoptosis and the protein levels of apoptosis-associated factors (Cleaved/Pro-Caspase-9, Cleaved/Pro-Caspase-7 and Cleaved/Pro-Caspase-3) were studied. The results in Fig. 1D–F showed that the percentage of apoptotic cells was induced by OGD treatment ($P < 0.001$), and the protein levels of Cleaved-Caspase-9/-7/-3 were also increased by OGD treatment in PC-12 cells ($P < 0.001$). These results stated that OGD could induce PC-12 cells injury.

3.2. GEN alleviated OGD-induced cell injury in PC-12 cells

We used different concentrations of GEN (100, 200 and 300 μM) to treat PC-12 cells, and cell viability was examined by CCK-8 assay. The results showed that no significant changes in the viability of PC-12 cells were observed after stimulation by GEN (Fig. 2A). These results indicated that GEN at the concentrations of 100–300 μM has no cell toxicity in PC-12 cells. Subsequently, PC-12 cells were treated with GEN (100, 200 and 300 μM), and were subjected to OGD treatment, cell viability was examined again. Results in Fig. 2B displayed that cell viability was significantly increased by GEN in a dose-dependent manner in OGD-insulted PC-12 cells ($P < 0.05$). Therefore, 300 μM GEN was used to treat PC-12 cells in the following experiments. The results in Fig. 2C and D displayed that protein levels of CyclinD1, CDK4 and CDK6 were enhanced in PC-12 cells after co-treatment with OGD and GEN ($P < 0.001$). Additionally, flow cytometry assay results showed that cell apoptosis was reduced by GEN in OGD-insulted PC-12 cells ($P < 0.05$, Fig. 2E), as well as western blot assay revealed that the protein levels of Cleaved-Caspase-9/-7/-3 were also decreased by GEN in OGD-insulted PC-12 cells ($P < 0.05$, Fig. 2F and G). These findings indicated that GEN could alleviate OGD-induced cell injury in PC-12 cells.

3.3. GEN enhanced the expression level of H19 in OGD-insulted PC-12 cells

PC-12 cells were treated with different concentrations of GEN (100, 200 and 300 μM), and then exposed to OGD treatment. After treatment, the expression level of H19 was determined in these treated cells. The results showed that H19 expression level was significantly up-regulated by GEN (200 μM, $P < 0.01$; and 300 μM, $P < 0.001$, Fig. 3) in OGD-insulted cells. These data indicated that GEN could up-regulate the expression level of H19 in OGD-insulted PC-12 cells.

3.4. GEN alleviated OGD-induced PC-12 cells injury via up-regulation of H19

Afterward, the plasmids of si-H19 and si-NC were transfected into PC-12 cells, and the transfection efficiency was presented in Fig. 4A showed that H19 expression level was markedly down-regulated in si-H19-transfected cells compared with that in si-NC-transfected cells ($P < 0.001$). The data indicated that si-H19 was successfully transfected into PC-12 cells to inhibit H19 expression. In the following experiments, we observed that knockdown of H19 significantly suppressed cell viability ($P < 0.01$, Fig. 4B), down-regulated CyclinD1, CDK4 and CDK6 protein levels ($P < 0.001$, Fig. 4C and D), induced apoptosis ($P < 0.001$, Fig. 4E), and increased Cleaved-Caspase-9/-7/-3 protein levels in PC-12 cells after co-treatment with OGD and GEN ($P < 0.001$, Fig. 4F and G). These findings hinted that GEN alleviated OGD-induced PC-12 cells injury might be through up-regulation of H19.

3.5. GEN activated PI3K/AKT and Wnt/β-catenin signaling pathways via regulation of H19 in OGD-insulted PC-12 cells

Finally, the major signaling pathways of PI3K/AKT and Wnt/β-catenin were determined to uncover the underling mechanism of GEN protecting PC-12 cells against OGD-induced cells injury. As shown in Fig. 5A and B, the results revealed that OGD treatment notably induced the inhibition of p-PI3K and p-AKT in PC-12 cells ($P < 0.01$ or $P < 0.001$). After pre-treatment with GEN, the protein levels of p-PI3K and p-AKT were increased in OGD-insulted PC-12 cells ($P < 0.001$). Additionally, we found that H19 knockdown reversed the promoting effect of GEN on p-PI3K and p-AKT in PC-12 cells ($P < 0.001$). Meanwhile, in Fig. 5C and D, we observed that OGD treatment down-regulated the protein level of β-catenin ($P < 0.05$); GEN treatment obviously up-regulated the protein level of β-catenin in OGD-insulted PC-12 cells ($P < 0.05$); H19 knockdown decreased β-catenin protein level in OGD and GEN co-treated cells ($P < 0.05$). These results indicated that GEN could activate PI3K/AKT and Wnt/β-catenin signaling pathways by up-regulating H19 in OGD-insulted PC-12 cells.

4. Discussion

In the current study, we constructed an OGD injury model and

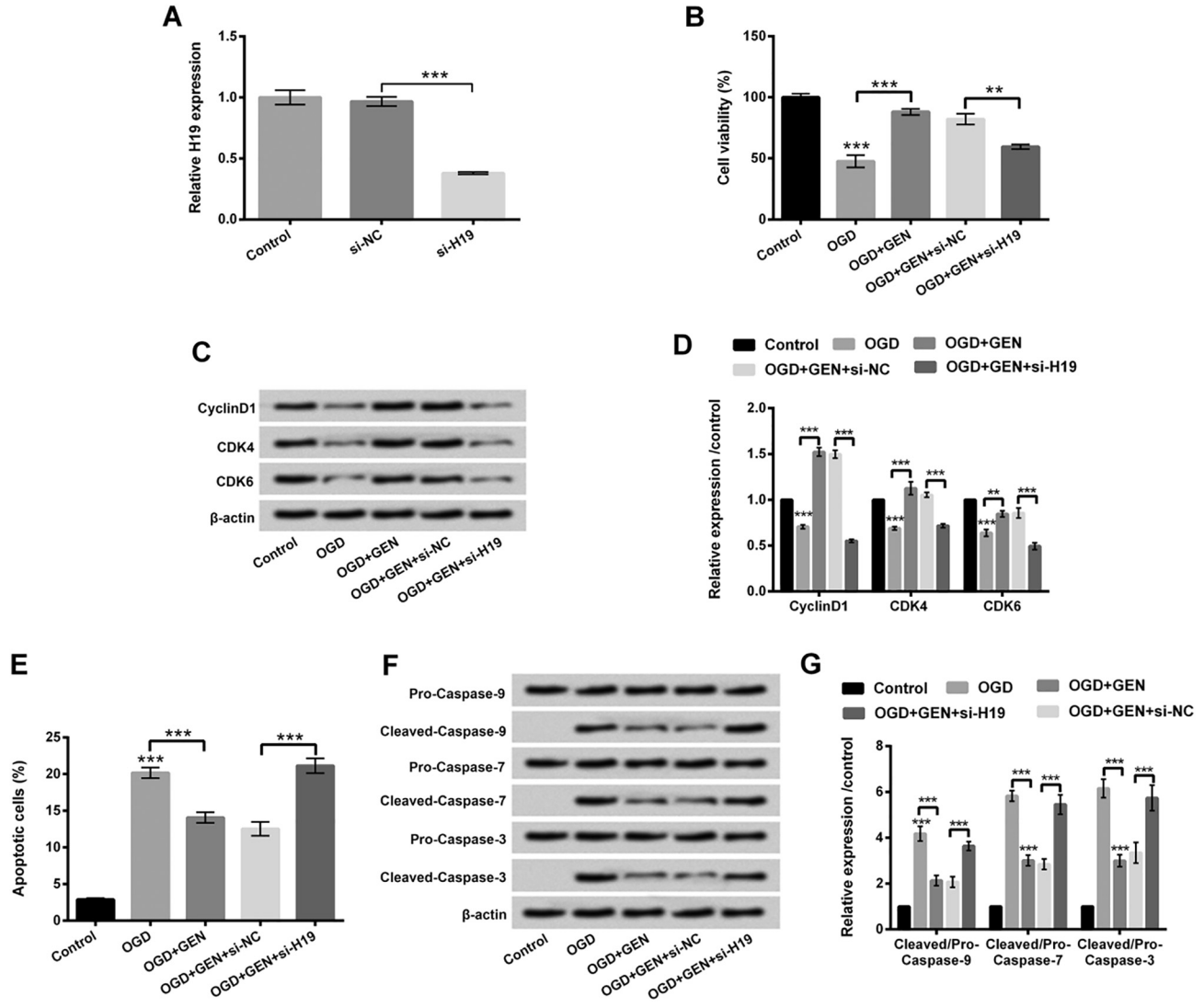

Fig. 4. Effect of H19 on cell proliferation and apoptosis in OGD and GEN co-treated PC-12 cells.
PC-12 cells were transfected with si-NC and si-H19, and the transfection efficiency was analyzed by RT-qPCR assay; PC-12 cells transfected with si-NC and si-H19 were treated by OGD alone or co-treated OGD and GEN, then (B) cell viability, (C and D) protein levels of CyclinD1, CDK4 and CDK6 were examined by CCK-8 and western blot assays; (E) cell apoptosis and (F and G) protein levels of pro-Caspase-9/-7/-3 and Cleaved-Caspase-9/-7/-3 were examined by flow cytometry and western blot assays.
OGD: oxygen glucose deprivation; GEN: geniposide; RT-qPCR: reverse transcription-quantitative PCR; CDK: cyclin-dependent kinase; CCK-8: Cell Counting Kit-8; $^{**}P < 0.01$; $^{***}P < 0.001$.

studied the effect of GEN on OGD-insulted PC-12 cells. The results uncovered that GEN alleviated OGD-induced cells injury by increasing cell viability, up-regulating CyclinD1, CDK4 and CDK6 expression, reducing apoptosis and down-regulating Cleaved-Caspase-9/-7/-3 expression in OGD-treated PC-12 cells. Additionally, we found that GEN increased the expression level of H19 in a dose-dependent manner. Knockdown of H19 reversed the protective effect of GEN on OGD-insulted PC-12 cells. Further, GEN activated PI3K/AKT and Wnt/β-catenin signaling pathways via mediating H19 expression in OGD-insulted PC-12 cells.

HIE is a common and severe brain injury disease in neonatal, which is induced by perinatal suffocation [32]. The pathogenesis of HIE is complex, which can be influenced by neuro-inflammation, oxidative stress and several growth factors [30,31]. OGD model has been widely used to mimic hypoxic and ischemic diseases *in vitro* or *in vivo* [5,18]. In several previous studies, OGD model was constructed to investigate the protective effect of ginsenoside Rg1 and ethyl pyruvate (EP) on PC-12 cells [13,19]. Similarly, we established an OGD-induced cell injury model in PC-12 cells to mimic HIE. We found that OGD significantly inhibited cell proliferation and induced apoptosis in PC-12 cells, indicating the construction of the cell injury model was successful. The results provided the basic research model for the subsequent experiments.

Recent study demonstrated that the traditional Chinese medicine monomer and its active ingredients have protective effect on nerve cells [29]. Study from Chang et al. demonstrated the protective effects of aloin on OGD-injured PC-12 cells [4]. Li et al. found that baicalin could attenuate OGD-induced injury by inhibiting oxidative stress-mediated 5-lipoxygenase activation in PC-12 cells [16]. GEN is one of effective active ingredients of *Gardenia jasminoides* [41]. Previous study stated that GEN could alleviate neuronal cell death in OGD-exposed rat hippocampal slice culture [15]. However, whether GEN shows the neuroprotective effect on OGD-insulted PC-12 cells remains unclear. The present study showed that GEN alleviated OGD-induced cell injury in PC-12 cells. These data indicated that GEN could protect PC-12 cells against OGD-induced cell injury.

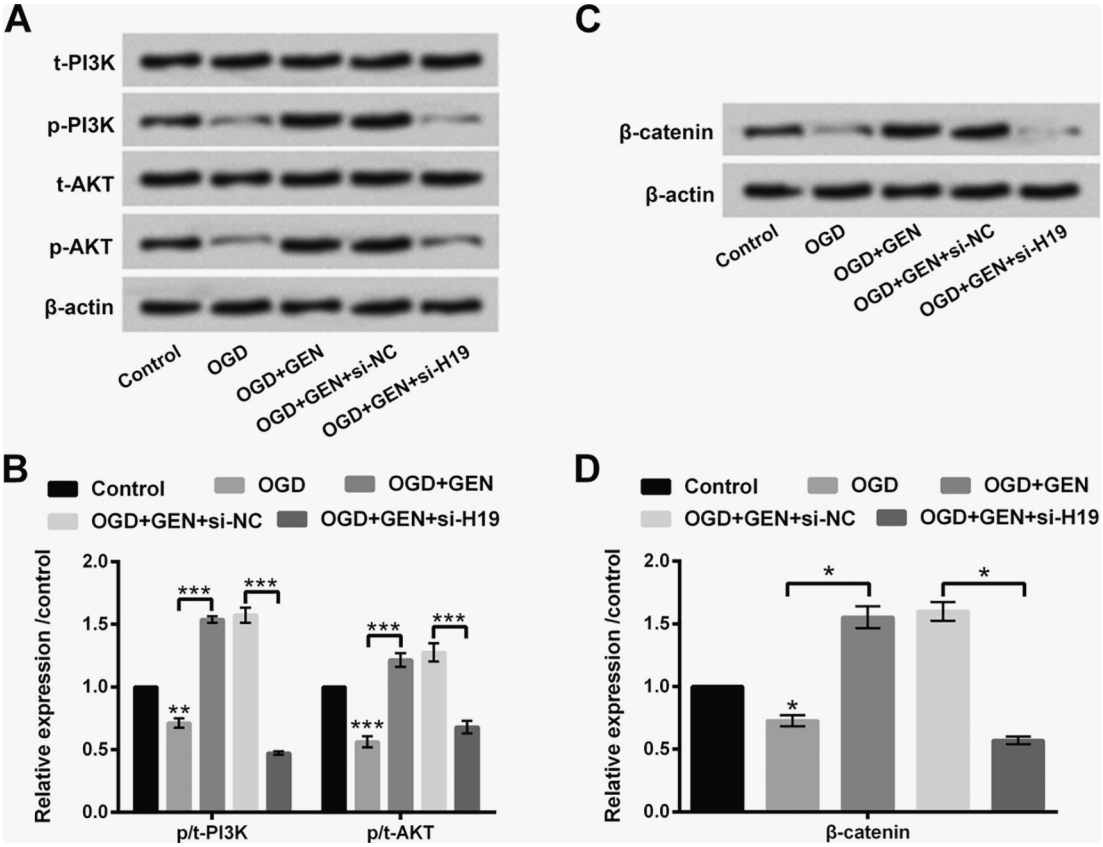

Fig. 5. Effect of GEN on PI3K/AKT and Wnt/β-catenin signaling pathways in OGD-treated PC-12 cells.
PC-12 cells transfected with si-NC and si-H19 were treated by OGD alone or co-treated OGD and GEN, (A and B) protein levels of p/t-PI3K and p/t-AKT, and (C and D) protein level of β-catenin were examined by western blot assay.
OGD: oxygen glucose deprivation; GEN: geniposide; PI3K: phosphatidylinositol 3-kinase; AKT: protein Kinase B; $^{*}P < 0.05$;$^{**}P < 0.01$; $^{***}P < 0.001$.

Increase evidence has proven the vital regulatory role of lncRNAs in neural cells [2]. For example, lncRNA BC088414 has been reported to aggravate OGD induced neural cell injury [40]. Additionally, knockdown of NONRATT021972 protected PC-12 cells against OGD-induced cell injury [17]. H19 is an important lncRNA, which has the capacity to mediate fundamental cellular biological processes [27]. However, whether H19 affects the neuro-protective effect of GEN on OGD-insulted PC-12 cells remains unclear. In this study, results showed that GEN significantly up-regulated H19 expression in PC-12 cells. Further interesting study demonstrated that the protective effect of GEN on OGD-insulted PC-12 cells was abolished by H19 knockdown. These findings hinted that H19 might be involved in regulating the neuro-protective effect of GEN in PC-12 cells.

PI3K/AKT and Wnt/β-catenin pathways are important signaling transduction pathways, which are closely related to the proliferation and apoptosis of nerve cells [12]. One study reported that nobiletin protected PC-12 cells from endoplasmic reticulum stress (ERS)-induced apoptosis in OGD/R injury by activating PI3K/AKT signaling pathway [20]. Another study demonstrated that puerarin protected differentiated PC-12 cells from H_2O_2-induced apoptosis via regulation of PI3K/AKT signaling pathway [37]. Wnt/β-catenin signaling pathway has reported to be involved in the neuro-protective effect of amyloid-beta protein (Aβ) on PC-12 cells [34]. Based on these previous studies, we explored the effect of GEN on PI3K/AKT and Wnt/β-catenin pathways in OGD-insulted PC-12 cells. We found that GEN activated PI3K/AKT and Wnt/β-catenin signaling pathways by up-regulating H19 expression in OGD-insulted PC-12 cells. These data demonstrated the possible molecular mechanism of GEN protecting PC-12 cells against OGD-induced injury.

5. Conclusion

According to the above results, we concluded that GEN could protect PC-12 cells against OGD-induced injury by up-regulation of H19. The molecular mechanism might be through activation of PI3K/AKT and Wnt/β-catenin signaling pathways. These findings indicated that GEN might be a useful therapeutic drug for the treatment of HIE. Further studies are still necessary to explore the wider applications of GEN in clinic.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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