Original Article

Protective effect of TSG against oxygen-glucose deprivation in cardiomyoblast cell line H9c2: involvement of Bcl-2 family, Caspase 3/9, and Akt signaling pathway

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Abstract: Objective: This study was designed to investigate the effect of TSG (2, 3, 5, 4'-tetrahydroxystibene-2-O-β-D-glucoside) on ischemic cardiomyopathy (ICM) related cell apoptosis and the mechanism related to it in vitro.

Methods: Rat cardiomyoblast cell line H9c2 was cultured in oxygen-glucose withdrawal medium for 8 hours to establish an in vitro cell model of oxygen-glucose deprivation (OGD). Cells were pretreated with TSG to test the protective effect of it against OGD. Cell viability, apoptosis, mitochondrial transmembrane potential (ΔΨm), and apoptosis related proteins were detected using appropriaed methods. Differences between treatments were analyzed. Results: OGD treatment inhibited cell viability, expression of Akt and Bax, induced loss of ΔΨm, cell apoptosis, and triggered expression of Bcl-2 and Caspase-3/9. TSG pretreatment, on the contrary, suppressed OGD-induced cell apoptosis, ΔΨm loss, Bcl-2 and Caspase-3/9 expression, and promoted OGD-inhibited cell viability, Bax and Akt expression.

Conclusion: We concluded that TSG’s protective effect against OGD-induced in vitro ischemic cell model was associated to Akt/Caspase-3 pathway. TSG might be explored as a therapeutic target for ICM.

Keywords: TSG, oxygen-glucose deprivation, ischemic cardiomyopathy, cell apoptosis, Akt signaling

Introduction

Ischemic cardiomyopathy (ICM), a type of coronary heart disease (CHD), is a disturbance caused by multiple abnormal changes-induced cerebral vasculature occlusion [1, 2]. The occlusion of cerebral vasculature contributed to subsequently decreased delivery of glucose and oxygen to the brain that is oxygen-glucose deprivation (OGD), disruption of the ionic equilibrium (pH value) and calcium (Ca2+) homeostasis, and eventual cell death [2, 3], thus ICM is an important cause of heart failure [4]. Due to the complex causes and multiple disturbances, exploring of therapeutic treatment for ICM is a hard project.

During ICM progresses, or OGD, cerebral and heart cells are in oxygen-deficient environment, facilitating cell apoptosis or inhibiting cell viability, which is not conducive to patient survival and recovery [5]. Cell apoptosis is complex cellular function related to various factors, such as cell apoptosis related proteins Bcl-2 family, Akt and NF-κB signaling pathway, and mitochondrial transmembrane potential (ΔΨm) status [6-8]. For instance, Bcl-2 family, which consists of antiapoptotic proteins including Bcl-2, and pro-apoptotic proteins including Bax, promotes cell apoptosis via increase of Bcl-2 and decrease of Bax [6, 7]. Loss of ΔΨm relates to cell apoptosis [9-11].

During the past decades, medicines and gene therapies applying to ICM treatment in vivo, in vitro, or in clinical practice, had been widely reported, such as icariin, active flavonoid extracted from Epimedium brevicornum Maxim [12], resveratrol [13], tocilizumab [5], tetramethylpyrazine [14], and miR-210 inhibition [15]. All of these medicines had been proved to inhibiting OGD-induced cell apoptosis and increasing cell viability via regulating expression of Bcl-2 family proteins and related
signaling pathway [15], thus promoting recovery of ICM, in vitro by cell models or in vivo by animal models.

TSG (2, 3, 5, 4’-tetrahydroxystibene-2-O-β-D-glucoside), botanical extract ingredients extracted from traditional Chinese medicine Polygonum multiflorum [16], has protective effect on cell proliferation [17], and antioxidation [18]. However, few studies focusing on the TSG protective effect on OGD-induced cell apoptosis.

This study was designed to investigate the effect of TSG on OGD-induced cell apoptosis and the mechanism related to it, in vitro. H9c2 cells were treated with oxygen and glucose withdrawal medium to achieve in vitro OGD cell model. TSG pretreated to cells. Apoptotic related proteins and signaling pathways, cell viability, and apoptosis rates, and ΔΨm were detected with appropriated methods. The effect of TSG on OGD-induced cell apoptosis was evaluated using statistical analysis. The potential of using TSG as the therapeutic treatment for ICM would be discussed.

Materials and methods

H9c2 cell culture and in vitro cell model establishment

A rat cardiomyoblast cell line H9c2 was purchased from ATCC (American Type Culture Collection, CRL-1446, Rattusnorvegicus, Rockville, MD, USA), cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplementing with 10% FBS (Fetal bovine serum, Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin (Invitrogen).

OGD cell model was established to mimic ischemia by transferring cells to serum-glucose withdrawal DMEM in a InVivo 500 chamber (Ruskin Life Science) supplemented anoxia air with 94% N2/5% CO2/1% O2 at 37°C for 8 hours as described [19]. For TSG treatment, cells were pretreated with series TSG at 1, 2, 5, 10, 20, and 50 µM.

Model H9c2 cells pretreated with TSG or not and control cells were seeded in 24-well plates for series time points of 0, 6, 24 and 48 h. Subsequently, cells were additionally treated with solution of Cell Counting Kit 8 (CCK-8) assay (Sigma, St Louis, Mo, USA) for 2 h for absorbance at 450 nm (A450, Microplate spectrophotometer, Bio-Rad Labs, Hercules, CA, USA). For each experiment, three triplicates were performed and averaged value was calculated and reported as mean ± S.E (Standard Error) of three separate experiments.

Annexin V apoptosis assay

For cell apoptosis determination, cells were seeded in 6-well plates for 24 h and treated as experiments, and harvested by trypsin. Apoptotic cell and necrotic cell percentage determination was performed with Annexin V-FITC (Ann V) and propidium iodide (PI, Beckman Coulter, Fullerton CA, USA) staining assay. A total of 10,000 harvested cells were dark-labeled with AnnV and PI for 30 min followed by flow cytometry analysis (Becton Dickinson FACSCalibur™; FITC 530/30 and PI 585/42 nm).

Analysis of mitochondrial transmembrane potential (ΔΨm)

To analyse ΔΨm, a fluorescent dye of lipophilic cationic probe JC-1 (5, 5’, 6, 6’-tetrachlоро-1, 1’, 3, 3’-tetraethylbenzimidazolcarbocyanine iodide, Molecular Probes, Eugene, OR, USA) was used and the JC-1 monomer/JC-1 dimer ratio was assessed as described [20]. In brief, 2×10^5 harvested H9c2 cells were adjusted to 1 mL volume with pre-warmed (37°C) culture medium, gently suspended with 2.5 μL of JC-1 dye (5 mg/ml in stock) and dark-incubated for 10 min at room temperature. After staining, cells were washed twice and resuspended with 1× PBS, and ΔΨm collapse was examined and expressed as FL1-FITC fluorescence (JC-1 monomers, at 535 nm) versus FL2-PE fluorescence (JC-1 aggregates, at 590 nm).

Western blot analysis

Cellular proteins were extracted using RIPA lysis buffer (Solarbio, Beijing, China), measured using a micro BCA kit (Thermo Fisher Scientific Inc., MA, USA), separated by on 10% or 12% SDS-PAGE (Solarbio) and electro-transferred to polyvinylidine difluoride (PVDF) membranes (Invitrogen Corp., Carlsbad, CA, USA), blocked with 5% non-fat dry milk (BD Biosciences, Franklin Lakes, NJ, USA) for 1 h. Then, membranes were probed with the specific primary antibody overnight at 4°C (Caspases 9,
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Figure 1. TSG attenuates oxygen-glucose deprivation-inhibited cell viability. CCK8 assay was used for cell viability determination. H9c2 cells were treated with TSG or OGD stimulus plus TSG pretreatment as described in experiments. All data were expressed as mean ± SE (Standard Error) of three separate experiments. * and ** represents significant differences at P < 0.05 and P < 0.01, vs. control, respectively. # and ## represents significant differences at P < 0.05 and P < 0.01, vs. OGD, respectively.

Figure 2. Detection of apoptosis cell was performed with Annexin V-FITC and PI staining assay. Cells were harvested by trypsin, labeled with Annexin V-FITC and PI and examined by flow cytometry. A total of 10,000 cells were analyzed from each sample. Percentages of early apoptosis cell (Annexin V+/PI-), late apoptosis cell (Annexin V+/PI+), necrosis cell (Annexin V-/PI+), and normal active cell (Annexin V-/PI-) were divided into four quadrants. A: Flow cytometry images; B: Statistical figure graphed using GraphPad Prism 6.0. Data were expressed as mean ± SE (Standard Error) of three separate experiments. * and ** represents significant differences at P < 0.05 and P < 0.01, vs. control, respectively. # and ## represents significant differences at P < 0.05 and P < 0.01, vs. OGD, respectively.

Bcl-2, p-Akt, Akt, and GAPDH) or at room temperature for 120 min (activated Caspase 3, and Bax), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000) for 60 min at room temperature. Anti-activated Caspase 3 (1:500) and Caspase 9 (1:500)
1000) antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). Anti-Bcl-2 (1:300) and Bax (1:300) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other three antibodies anti-p-Akt (1:1000), Akt (1:1000) and GAPDH (1:2000) were obtained from Cell Signaling Technology (CST, Beverly, MA, USA). Blots were subjected to the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ, USA), and quantitative analysis for band intensity was performed by densitometry software QuantiScan (Biosoft Ferguson, MO, USA).

All statistical figures were analyzed and graphed using GraphPad Prism 6.0 (GraphPad Software Inc. La Jolla, CA) for windows, and all data were expressed as means ± SE of three separate experiments. The differences between two groups or more groups were analyzed using Student’s unpaired t-test and one-way ANOVA, respectively. \( P < 0.05 \) was considered statistically significant.

**Results**

**TSG attenuates OGD-inhibited cell viability**

To study the effects of OGD stimulus or TSG therapy on cell viability, CCK8 assay was performed on cell line H9c2, exposed to culture conditions with oxygen-glucose withdrawal alone or together with TSG. **Figure 1A** shows that TSG administration alone markedly promoted cell viability with respect to the control cells \( (P < 0.05) \), in agreement with previous reports [21]. An opposite trend was found when cells were stimulated with OGD, showing a marker reduction in cell viability **Figure 1B**. However, we could notice TSG triggered significant changes in cell viability of OGD cell model in a dose- and time-dependent manner \( (P < 0.05) \). These data suggested that OGD-simulated cell growth inhibition could be attenuated by TSG stimulation.

**TSG inhibits OGD-triggered cell apoptosis**

Since OGD inhibited cell viability as experiments showed, and some experimental mo-
dels revealed OGD activated cell death [13, 22], we tested the ability of OGD to induce apoptosis of H9c2 cells. We determined the maker increase in apoptotic cell percentage (Ann V+/PI-, early apoptosis and Ann V+/PI+, late apoptosis) of cells with oxygen-glucose withdrawal than controls (P < 0.05, Figure 2).

Moreover, we observed decreases in apoptotic percentage of H9c2 cells with oxygen-glucose withdrawal together with TSG exposure, in TSG dose-dependent manner. These results revealed TSG protected cells against OGD activated cell death.

TSG blocks OGD stimulated changes on ΔΨ

As reported, loss or damage of ΔΨ is a precipitating factor for cell apoptosis [23, 24], we speculated OGD damaged ΔΨ and TSG’s protective effect might benefit to blocking this damage. To address this speculation, we detected ΔΨ using the fluorescent lipophilic cationic probe JC-1 and ΔΨ collapse was expressed as FL1-FITC (JC-1 monomers)/FL2-PE (JC-1 aggregates) fluorescence. Results in Figure 3 showed OGD markedly upregulated the JC-1 monomers/JC-1 aggregates fluorescence to over 11 times the control level (P < 0.01, Figure 3). OGD induced apoptotic cells showed a significant loss of ΔΨ, as a reduction in JC-1 aggregates accumulation (FL2 fluorescence) and a concomitant increase in JC-1 monomers accumulation (FL1 fluorescence). We next examined the effect of TSG protection on OGD-induced ΔΨ damage. Results showed that TSG pretreatment of ischemic H9c2 cells blocked OGD-induced ΔΨ damage, in a dose-dependent way, in an opposite trend to OGD stimulus showing an increase in JC-1 aggregates accumulation and a concomitant reduction in JC-1 monomers accumulation. These results sug-
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Suggested TSG protected cells on OGD damaged ΔΨ_m, and this might benefit TSG's protective effect on cell apoptosis [24].

**TSG suppresses OGD-simulated cell apoptosis via Bcl-2 family proteins and Akt signaling pathway**

Bcl-2 family proteins, including Bcl-2 and Bax, play crucial roles in cell fate decision with active impact on caspasers [3]. Akt takes vital part in cell survival and apoptosis via related signaling pathways such as P13K [25, 26]. Here we investigated the expression of Bcl-2 family proteins responded to ischemia in vitro in OGD cell model. We determined that H9c2 cells starved from oxygen and glucose increased the expression of activated Caspase 3, 9 and Bcl-2 to about 2~3 times the control level, and decreased the expression of Bax and p-Akt to one-third or one-fourth of the control (Figure 4). An opposite trend were observed in TSG pretreated cells, as TSG pretreatment inhibited OGD-triggered increase in Caspase-3, 9 and cell apoptosis, inhibited anti-apoptotic protein Bax and cell viability, suggesting the proapoptotic effect of OGD treatment. Moreover, TSG pretreatment annealed OGD-induced cell apoptotic related cellular dysregulation, revealing the antiapoptotic characteristics of TSG pretreatment and the therapeutic potential on ICM treatment. This was in accordance with results from other papers showing TSG's antiapoptotic activity [30].

Akt is a substrate of P13K/Akt antiapoptotic cascade. OGD decreased Akt phosphorylation [31]. Inhibitions of Akt signaling pathway and Caspase-3 cleavage are critical for cell apoptosis promotion [1]. In this study, we conformed that OGD induced inhibition of Akt and upregulation of Caspase-3 in H9c2 cells could be reversed by TSG pretreatment, revealing OGD promoted cell apoptosis via P13K/Akt/Caspase-3 signaling pathway and TSG's antiapoptotic activity was associated to P13K/Akt/Caspase-3 signaling pathway.

Cell apoptosis itself and OGD-induced cell apoptosis relates to damage of cell membranes and loss of ΔΨ_m [9-11]. Loss or damage of ΔΨ_m is a precipitating factor for cell apoptosis [23, 24]. ΔΨ_m is widely recognized as a mitochondrial functional indicator [32]. OGD treatment triggered reactive oxygen species (ROS) production, early increase in intracellular Ca^{2+} uptake, which damage cellular membranes and related to cell apoptosis closely [33, 34]. Abnormal accumulation of ROS and intracellular Ca^{2+} challenged cellular ion structure balance, thus the intensity and permeability of cell cyto and plasma membranes, as well as mitochondri-
drial permeability transition (MPT) and loss of \( \Delta \psi_m \) [35]. Moreover, opening of MPT pore (MPTP) is recognized critical determinant of ischemic-reperfusion injury, MPT and collapse of \( \Delta \psi_m \) by decreasing JC-1 aggregates and accumulating JC-1 monomers [36, 37]. The opening of MPTP and promotes its secondary effect on accelerating ROS production and ROS-induced damage on DNA fragmentation and cell necrosis [38]. OGD induce cell death and collapse of \( \Delta \psi_m \) were conformed by our research group and presented in this present study, this was in accordance with the reported results from other researches [10, 11]. Furtherly, we demonstrated TSG's protective ability against collapse of \( \Delta \psi_m \) by blocking OGD-induced decrease in JC-1 aggregates and increase of JC-1 monomers, suggesting TSG repaired OGD-destroyed intensity of cellular membranes.

**Conclusion**

In conclusion, our results clearly demonstrated that OGD-induced *in vitro* ischemic cell model apoptosis included decrease in cell viability, loss of \( \Delta \psi_m \), upregulation of Caspase-3, Caspase-9, and proapoptotic protein Bcl-2, and downregulation of antiapoptotic Bax and p-Akt. On the contrary, TSG's pretreated cells showed conversed results of these changes. TSG pretreatment increased cell viability, \( \Delta \psi_m \), downregulated Caspase-3, Caspase-9 and Bcl-2 expression, and upregulated Bax and p-Akt expression, in comparison to OGD treatment. This suggested that TSG's protective effect against OGD-induced *in vitro* ischemic cell model were associated to Akt/Caspase-3 pathway. In addition, these might shade light on TSG's potential therapeutic target for ICM.

**Disclosure of conflict of interest**

None.

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**References**


[12] Mo ZT, Li WN, Zhai YR, Gong QH. Icariin attenuates OGD/R-induced autophagy via Bcl-2-dependent cross talk between apoptosis and autophagy in PC12 cells. Evid Based


[22] Yan W, Chen Z, Chen J, Chen H. Isoflurane pre-conditioning protects rat brain from ischemia reperfusion injury via up-regulating the HIF-1α expression through Akt/mTOR/s6K activation. Cell Mol Biol (Noisy-le-Grand, France) 2016; 62: 38-44.


[33] Cao ND and Kim JY. Exposure to electromagnetic field attenuates oxygen-glucose deprivation-induced microglial cell death by reduc-
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