Neuroprotective effects of tetrahydroxystilbene glucoside against rotenone-induced toxicity in PC12 cells

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Abstract: To investigate the mechanism of the protective effect of tetrahydroxystilbene glucoside (TSG) on nerve cells, an injury model induced by rotenone in PC12 cells was constructed. Cell viability was detected by using CCK8 assay. Apoptosis was detected by using flow cytometry. The mitochondrial membrane potential (MMP) was detected by using the fluorescent probe JC-1. Generation of reactive oxygen species (ROS) in PC12 cells was determined using the CM-H$_2$DCFDA probe. Protein expression in PC12 cells was detected using western blotting. The results showed that TSG (20-100 μM) attenuated the cytotoxic effects of rotenone on PC12 cells. TSG pretreatment attenuated the apoptosis rate, the degradation of PARP and the activation of cleaved caspase 3, which was induced by rotenone. TSG can significantly reduce the effect of rotenone on the reduction of MMP and the expression of cytoC in the cytosolic fraction. TSG attenuated rotenone-induced de-phosphorylation and mitochondrial translocation of cofilin, as well as rotenone-induced accumulation of ROS. The western blot results showed that ROT could decrease the expression level of p-GSK-3β and p-AKT, and TSG could weaken these effects of rotenone. In addition, TSG increased the expression level of Nrf2 in the nuclear fraction. These results suggest that TSG could protect PC12 cells against rotenone through multiple pathways. Thus, TSG has the potential to become a novel neuroprotective agent.

Key words: tetrahydroxystilbene glucoside, rotenone, PC12 cells, apoptosis, cofilin, ROS
INTRODUCTION

Neurodegenerative diseases, such as Alzheimer’s disease, are common diseases in middle-aged and elderly people (1). Although the causes of neurodegenerative diseases are complex and the majority of mechanisms are unknown, one common feature of these diseases is that the neurons in the brain and spinal cord are damaged (2). Protection of neuron cells is a common treatment strategy for neurodegenerative diseases, but there are limited drugs available for neuroprotection in the clinic (3). Therefore, it is necessary to develop novel neuroprotective drugs. Previous studies have found that numerous monomer compounds from natural plants have the function of protecting nerve cells and regulating the nervous system (4).

Previous studies have shown that 2,3,5,4’-tetrahydroxystilbene 2-O-β-D-glucoside (tetrahydroxystilbene glucoside, TSG), a monomer derived from Polygonum multiflorum Thunb, has the effects of anti-oxidative, attenuating atherosclerosis and improving the ability of learning and memory (5-7). However, there are few studies on the neuroprotective mechanism of TSG.

The PC12 cell line is a rat adrenal gland pheochromocytoma cell line. Since it has the common characteristics of neuroendocrine cells and can be passaged normally, it is widely used in neurophysiological and neuropharmacology research (8). Rotenone is an isoflavone extracted from the leguminous plants of the genus Rotenone, which is widely used as an insecticide in agriculture. Due to its strong neurotoxicity, it is also widely used as a model drug for nerve cell injury (9).

Multiple studies have shown that rotenone can induce apoptosis and mitochondria damage through a variety of signaling pathways, such as the AKT/GSK-3β signaling pathway (10). Cofilin, a key regulator of actin filament dynamics, has been reported that it is associated with mitochondrial damage (11).
De-phosphorylated cofilin is transferred from the cytoplasm to mitochondria prior to cytochrome c release, leading to mitochondrial damage \(^{12}\). However, whether rotenone causes mitochondrial damage through the cofilin pathway has not been reported to date.

In the present study, an injury model of PC12 cells induced by rotenone was constructed, and the protective effect and mechanism of TSG on neural cell injury were explored.

**MATERIALS AND METHODS**

**Reagents.** TSG (A0219) was purchased from Chengdu Must Bio-Technology Co., Ltd. Rotenone (S2348) was purchased from Selleck Chemicals. Carbonyl cyanide m-chlorophenylhydrazone (CCCP; C2006-4) was purchased from Beyotime Institute of Biotechnology. Antibodies against PARP (13371-1-AP), cleaved caspase 3 (19677;), cytochrome c (10993-1-AP) and proliferating cell nuclear antigen (10205-2-AP) were purchased from ProteinTech Group, Inc. Antibodies against AKT (ab200195), phosphorylated (p)-AKT (Ser272 and 274; ab192623), cofilin (ab42824), p-cofilin (Ser3; ab12866), voltage dependent anion channel 1 (VDAC1;ab14734) and Nrf2(ab137550) were obtained from Abcam. Anti-GAPDH antibody (2118S;) was purchased from Cell Signaling Technology, Inc. Antibodies against GSK-3β (AF1543;) and p-GSK-3β (Ser9; AF1531) were purchased from Beyotime Institute of Biotechnology.

**Cell culture.** PRIM-1640 medium (SH30809.01; HyClone; Cytiva) supplemented with 10% fetal bovine serum were used to culture PC12 cells (obtained from ATCC). The culture conditions were 37 °C and 5% CO\(_2\).
**Cell Counting Kit-8 (CCK-8) cell viability assay.** Cells were seeded into 96-well plates at a density of $7 \times 10^3$ cells/well and incubated for 24 h, followed by treatment with TSG or rotenone for 24 h. Subsequently, 10 μl/well CCK-8 (HY-K0301; MedChemExpress) was added and incubated for 2 h. Next, the absorbance at 450 nm was measured with a microplate reader (Agilent Technologies, Inc.).

**Flow cytometric analysis of apoptosis.** Cells were cultured into 6-well plates. Following treatment with TSG or rotenone, the cells were collected and stained with 10 μl PI (5.0 μg/ml) and 5 μl Annexin V-FITC or apoptosis detection. The flow cytometry (Accuri C6) was used to detect the stained cells and the FlowJo 7.6.1 software was used to analyze data.

**Measurement of mitochondrial membrane potential (MMP).** Cells were seeded in 6-well plates and stained with JC-1 probe. After washed twice with PBS, the red and green fluorescence was observed with a fluorescence microscope. Quantification of fluorescence intensity was performed with ImageJ software (Version 1.37C). the relative ratio of red (J-aggregates) to green (monomers) fluorescence was used to represent the level of MMP.

**Measurement of intracellular ROS generation.** Cells were seeded into 6-well plates. After treated with rotenone or TSG, the culture medium was replaced and the cells were incubated with 10 μM CM-H$_2$DCFDA for 30 min at 37°C. After washed twice with PBS, the green fluorescence was observed with a fluorescence microscope. Quantification of fluorescence intensity was performed with ImageJ software (Version 1.37C).
**Western blotting.** PC12 cells were lysed with RIPA buffer to obtain protein. Mitochondrial proteins were isolated using a Cell Mitochondria Isolation kit. Nuclear proteins were isolated using Nuclear and Cytoplasmic Protein Extraction kit. Protein samples were separated via SDS-PAGE and then transferred to PVDF membranes. After being blocked with 5% milk, the membranes were incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h and visualized using an chemiluminescence substrate.

**Statistical analysis.** All experimental data are presented as the mean ± SD of three independent experiments. Comparisons were performed using a one-way analysis of variance and Student’s t-test using SPSS (Version 17.0).

**RESULTS**

*TSG protects PC12 cells against rotenone-induced toxicity.* First, CCK-8 assay was used to determine the effect of TSG and rotenone on PC12 cell activity. TSG at 60-100 μM slightly increased the viability of PC12 cells (Fig. 1A), while 0.5-8 μM rotenone significantly inhibited it’s viability (Fig. 1B). Next, after incubating with rotenone (4 μM) in the presence or absence of TSG (10-100 μM), the viability of PC12 cells was evaluated. As showed in Fig. 1C, co-incubation with TSG (20-100 μM) attenuated the cytotoxic effects of rotenone. These results indicated that TSG could protect PC12 cells against rotenone-induced toxicity.

*TSG attenuates rotenone-induced apoptosis.* The effect of TSG on rotenone-induced apoptosis was evaluated. As showed in Fig. 2A and B, rotenone (4
μM) could induce apoptosis, and the apoptosis rate of the group subjected to TSG treatment combined with rotenone was significantly lower than that of the group subjected to single rotenone treatment (P<0.05). Consistent with these findings, TSG pre-treatment attenuated the degradation of poly (ADP-ribose) polymerase (PARP) and the activation of cleaved caspase 3 induced by rotenone (Fig. 2C and D). These results indicated that TSG could attenuate rotenone-induced apoptosis.

**TSG attenuates rotenone-induced mitochondrial damage.** Mitochondrial damage is an important cause of rotenone induced apoptosis \(^{(13)}\). Thus, the current study determined the effect of TSG on rotenone-induced mitochondrial damage.

The results of JC-1 staining showed that rotenone could reduce the mitochondrial membrane potential, which showed that the green fluorescence increased and the red fluorescence decreased. However, TSG could reduce the effect of rotenone on the reduction of MMP (Fig. 3A and B). The presence of cytochrome c in the cytoplasm also suggests that mitochondria are damaged. The results of western blotting showed that more cytochrome c could be detected in the cytoplasm after rotenone treatment, but TSG could reduce its expression (Fig. 3C and D). These results suggest that TSG could attenuate the mitochondrial damage induced by rotenone.

**TSG attenuates rotenone-induced de-phosphorylation and mitochondrial translocation of cofilin.** Recent studies have shown that de-phosphorylation and mitochondrial translocation of cofilin is critical for mitochondrial damage \(^{(11)}\). We detected the effect of rotenone and TSG on the expression of p-cofilin and cofilin in whole cell lysate (WCL) and in mitochondrial fraction. As showed in Fig. 4A and B, the expression of p-cofilin in WCL decreased and the expression of cofilin in
mitochondrial increased after rotenone treatment. TSG itself had no effect on the mitochondrial translocation or de-phosphorylation of coflin, but could attenuate the effect of rotenone on coflin (Fig. 4A and B). These results suggest that TSG could attenuate de-phosphorylation and mitochondrial translocation of coflin induced by rotenone.

**TSG attenuates rotenone-induced accumulation of ROS.** Previous studies have shown that rotenone-induced mitochondrial damage are associated with the accumulation of ROS\(^{(14)}\). The effect of rotenone and T(SG on ROS generation in PC12 cells was explored using CM-H\(_2\)DCFDA probe. The results showed that green fluorescence was enhanced in cells treated with rotenone, while TSG attenuated the rotenone-induced green fluorescence (Fig. 5A and B). These results suggest that TSG could attenuate the rotenone-induced accumulation of ROS. Fig. 5A and B,

**TSG attenuates the effect of rotenone on the AKT/GSK-3\(\beta\)/Nrf2 signaling pathway.** Rotenone can cause cell damage through the AKT/GSK-3\(\beta\)/Nrf2 pathway\(^{(15)}\). The present study explored the effect of rotenone and TSG on that signaling pathway in PC12 cells. As showed in Fig. 6A and B, rotenone decreased the expression level of p-AKT and p-GSK-3\(\beta\), while TSG could reduce the effect of rotenone on the expression of p-AKT and p-GSK-3\(\beta\). In addition, the expression level of Nrf2 increased in the nuclear and decreased in the cytoplasm after TSG treatment. These results suggested that TSG could protect PC12 cells against rotenone through the AKT/GSK-3\(\beta\)/Nrf2 signaling pathway.
DISCUSSION

The present study provided the first evidence to suggest that TSG can protect PC12 cells from rotenone-induced cytotoxicity through multiple pathways. In terms of the mechanism, TSG inhibited rotenone-induced ROS generation, de-phosphorylation and mitochondrial translocation of cofilin, and protected PC12 cells against rotenone-induced cytotoxicity through the AKT/GSK-3β/Nrf2 signaling pathway.

Rotenone, an isoflavone extracted from the leguminous plants, has been widely used as a model drug for nerve cell injury. In the construction of the rotenone-induced injury model in PC12 cells, the treatment concentration of rotenone ranged from 0.5 to 5 μM according to previous reports (16-18). The current study successfully established an injury model using rotenone at 4 μM for 24 h.

Apoptosis is a form of cell death, and it is also the main cause of rotenone damage to PC12 cells (19). PARP is an important DNA repair enzyme, which can specifically recognize and bind to the broken ends of DNA. When apoptosis occurs, PARP can be cleaved and degraded by caspase 3 and lose its DNA repair function (20). Caspase 3 plays a critical role in the process of apoptosis. In an important step of apoptosis, caspase 3 catalyzes the degradation of PARP through activation of its own cleavage (21). The present study confirmed that rotenone could induce cell apoptosis, and promote the activation of PARP degradation and caspase 3 cleavage, while TSG could attenuate these effects of rotenone.

Mitochondrial damage can cause apoptosis, and the decrease in MMP is a sign of mitochondrial damage (22). The results of JC-1 probe staining showed that rotenone could significantly reduce MMP, while TSG could significantly inhibit the decrease in MMP induced by rotenone. Previous studies have shown that when MMP decreases,
cytochrome c is released from mitochondria into the cytoplasm, thus activating the
caspase pathway, resulting in a cascade reaction, and eventually leading to apoptosis
(23). The present study found that rotenone could increase the expression of
cytochrome c in the cytoplasm, while TSG could attenuate that effect of rotenone.

Cofilin is an evolutionarily conserved protein existing in all eukaryotic cells, and
plays an important role in the dynamic reorganization of the actin skeleton (24). Recent
studies have shown that mitochondrial translocation and de-phosphorylation of cofilin
play an important role in mitochondrial damage (25). Ser3 is the key site of cofilin
phosphorylation regulation. When Ser3 de-phosphorylation occurs, cofilin can be
activated and translocated to mitochondria, leading to mitochondrial division (26). The
present study also found that rotenone could decrease the phosphorylation level of
cofilin in whole cells and increase the expression of cofilin in mitochondria. However,
TSG could significantly reduce the effect of rotenone on the phosphorylation and
mitochondrial translocation of cofilin. These results indicated that the mechanism of
TSG attenuating rotenone-induced mitochondrial damage may be that it blocks the
mitochondrial translocation of cofilin caused by rotenone. However, it is not clear
whether TSG directly affects mitochondrial function, which remains to be further
studied.

ROS play an important role in cell proliferation and differentiation as oxygen
free radicals. Excessive accumulation of ROS can cause oxidative stress and
mitochondrial dysfunction. Rotenone-induced apoptosis and mitochondrial damage
are associated with the accumulation of ROS (27). By using CM-H$_2$DCFDA probe
detection, the current study found that rotenone could induce the accumulation of
ROS in PC12 cells, but TSG could significantly reduce the effect of rotenone on the
accumulation of ROS.
AKT has a wide range of functions and is associated with numerous intracellular signaling pathways (28). GSK-3β is a key enzyme involved in liver glucose metabolism. However, it has been found that GSK-3β can regulate apoptosis by affecting mitochondrial permeability (29). Nrf2 is a transcriptional activator, which could regulate the expression of oxidative stress response genes. Certain neuroprotective agents can activate Nrf2 to transfer to the nucleus, enhance the transcription of protective genes and play a protective role (30). The present study found that rotenone could reduce the expression levels of p-AKT and p-GSK-3β, which is consistent with relevant reports, while TSG could attenuate the effect of rotenone on the expression of p-AKT and p-GSK-3β. In addition, it was also found that TSG could activate Nrf2 for nuclear transfer. These results suggest that TSG may protect PC12 cells against rotenone through the AKT/GSK-3β/Nrf2 signaling pathway, and Nrf2 may be one of the targets of TSG. However, it is not clear whether TSG directly affects AKT/GSK3β, which remains to be further studied.

**Conclusion**

In conclusion, TSG can attenuate rotenone-induced apoptosis and mitochondrial damage through multiple pathways, and has the potential to become a novel neuroprotective agent. The present study also provided theoretical data support for the further research and development of TSG.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary materials

The online version of this article contains supplementary materials.
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Figure 1. Protective effect of TSG on the viability of PC12 cells. Cell viability was determined using a CCK-8 assay (n=3). (A) PC12 cells were treated with TSG for 24 h. (B) PC12 cells were treated with rotenone for 24 h. (C) PC12 cells were pre-treated with TSG for 2 h and then treated with rotenone for 24 h. *P<0.05, **P<0.01 vs. 0 μM group or rotenone group in Fig1C. TSG, tetrahydroxystilbene glucoside; CCK-8, Cell Counting Kit-8.
Figure 2. *TSG attenuates rotenone-induced apoptosis.* PC12 cells were pre-treated with TSG (60 μM) for 2 h and then treated with rotenone (4 μM) for 24 h. (A and B) Apoptosis was detected using flow cytometry (n=3). (C and D) Expression levels of apoptotic protein using western blotting (n=3). *P<0.05, **P<0.01. TSG, tetrahydroxystilbene glucoside; PARP, poly (ADP-ribose) polymerase.
Figure 3. TSG attenuates rotenone-induced mitochondrial damage. (A and B)

PC12 cells were pre-treated with TSG (60 μM) for 2 h and then treated with rotenone (4 μM) for 24 h. CCCP (50 μM, 30 min) was positive control. The MMP was detected using JC-1 probe staining. (C and D) Expression levels of cytochrome c in cytoplasm (n=3). *P<0.05, **P<0.01. TSG, tetrahydroxystilbene glucoside.; Rot, rotenone; CCCP, Carbonyl Cyanide m-Chlorophenylhydrazone. MMP; mitochondrial membrane potential

(Color figure can be accessed in the online version.)
Figure 4. TSG attenuates rotenone-induced de-phosphorylation and mitochondrial translocation of cofilin. PC12 cells were pre-treated with TSG (60 μM) for 2 h and then treated with rotenone (4 μM) for 24 h. (A and B) The expression levels of phospho-cofilin and cofilin was detected using western blotting. *P<0.05, **P<0.01. TSG, tetrahydroxystilbene glucoside; Rot, rotenone; p-, phospholation.
Figure 5. TSG attenuates rotenone-induced accumulation of ROS. PC12 cells were pre-treated with TSG (60 μM) for 2 h and then treated with rotenone (4 μM) for 24 h. (A and B) ROS generation was detected using a CM-H$_2$DCFDA probe. *P<0.05, **P<0.01). TSG, tetrahydroxystilbene glucoside; ROS, reactive oxygen species. (Color figure can be accessed in the online version.)
Figure 6. TSG attenuates the effect of rotenone on the AKT/GSK-3β/Nrf2 signaling pathway. PC12 cells were pre-treated with TSG (60 μM) for 2 h and then treated with rotenone (4 μM) for 24 h. (A and B) The expression levels of proteins were determined using western blotting. *P<0.05, **P<0.01. TSG, tetrahydroxystilbene glucoside; p-, phosphorylated; Nrf2, nuclear factor erythroid 2-related factor 2. PCNA, proliferating cell nuclear antigen.