Glycitein exerts neuroprotective effects in Rotenone-triggered oxidative stress and apoptotic cell death in the cellular model of Parkinson’s disease

Na Dong¹ and Zhilong Yang²✉

¹Department of Neurology, Tianjin Huanhu Hospital, Tianjin, 300350, China; ²Department of Neurology, The Third Affiliated Hospital of Soochow University, Changzhou, 213003, China

Parkinson’s disease (PD) is one of the prevalent neurodegenerative diseases among aging populations. Despite recent advancements, still, no therapy exists for the prevention of PD. Given the remarkable pharmacological properties of flavonoids, this study was designed to investigate the protective effects of a plant-derived flavonoid, glycitein in a cellular model of PD. Rotenone, a prevalently used pesticide, has been employed to study the pathology of PD. The results of the present study showed that Glycitein significantly ($P<0.05$) prevented the rotenone-induced inhibition of cell viability as evident from the MTT assay. Additionally, it was found that the increased ROS levels triggered by rotenone in human SK-N-SH neuroblastoma cells were significantly ($P<0.05$) diminished upon glycitein treatment. Glycitein treatment also restored the mitochondrial membrane potential of the rotenone treated SK-N-SH cells. These effects of Glycitein were mainly due to its ability to trigger the increased activity of the ROS scavenging enzymes such as GSH, SOD and CAT. The ATP estimates showed that the ATP levels were significantly ($P<0.05$) reduced in the rotenone treated SK-N-SH cells. Nonetheless, glycitein treatment could restore the ATP levels of the SK-N-SH cells in a dose-dependent manner. Rotenone has also been shown to induce apoptosis in human cells and the results of the annexin V/PI staining showed that glycitein exhibits remarkable potential to prevent in the rotenone triggered apoptosis in SK-N-SH cells. This was also accompanied by alteration in the expression of Bax, Bcl-2 and caspase-3 expression. Taken together, the study indicates that glycitein exhibits neuroprotective effects by preventing rotenone induce oxidative stress and apoptotic cell death. These findings point towards the use of glycitein in the management of neurodegenerative diseases such as PD.

Keywords: glycitein; parkinson; apoptosis; oxidative stress; flavonoids

Received: 15 October, 2021; revised: 08 November, 2021; accepted: 30 December, 2021; available on-line: 12 June, 2022

✉e-mail: zhilongyang76@gmail.com

Abbreviations: MMP, mitochondrial membrane potential; MTT, MTT 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; ROS, reactive oxygen species

INTRODUCTION

Flavonoids constitute a vast and diverse group of phenolic compounds which are evolutionarily conserved and ubiquitously distributed across the plant kingdom (Ayaz et al., 2019). Throughout evolution, these metabolites have developed a wide array of physiological roles in plants (Bombardi et al., 2018). Human beings have used flavonoid-rich plant-derived extracts for the management of several ailments. The antioxidant, anti-inflammatory and anti-cancer properties are well established in the literature (Gutierrez-Zepeda et al., 2005; Khan et al., 2012). Studies have proved that the properties of flavonoids may be attributed to their ability to scavenge reactive oxygen species (ROS). The production of ROS has been held responsible for the development of several diseases including neurodegenerative diseases (Stephenson et al., 2017). Neurodegeneration is the constant and slow loss of neuronal cells in particular regions of the brain. Studies have shown that neuronal loss is the main pathological characteristic of neurodegenerative diseases such as Parkinson’s disease (PD) (Azad et al., 2011). The aging of the brain, oxidative stress, mitochondrial dysfunction and initiation of apoptosis are some of the main features of neurodegenerative disease (Reddy & Beal, 2008). Although tremendous progress has been made in the field of brain research, the molecular pathogenesis of neurodegenerative diseases is yet to be fully understood. The diagnosis of the disease at the advanced stage and proper therapeutic interventions are essential to prevent the progression of neurodegenerative diseases (Dauer & Przedborski, 2003). Since times immemorial, flavonoid enriched nutraceuticals have been used to improve cognitive function and to prevent the onset of neurodegenerative diseases in human (Lx et al., 2019). Studies have also stressed the usage of flavonoid-rich natural products for the prevention of neurodegenerative diseases including PD (Zhang et al., 2002; Hwang et al., 2012). PD is generally characterized by motor and cognitive impairment and prevalently presents as a sporadic human ailment (Khan et al., 2010). Data from human post-mortem tissues has revealed reactive oxygen species (ROS), and apoptotic activity to be critical in the pathogenesis of PD (Zhang et al., 2002), suggesting that compounds interfering both with ROS and apoptosis might exert protective effects. Given the known antioxidant activity of flavonoids, the present study was designed to evaluate the protective effects of glycitein in cellular mode of PD.

MATERIALS AND METHODS

Cell Culture

The SK-N-SH neuroblastoma cells were grown in (DMEM) 12 (1: 1), supplemented with 2 mM glutamine, penicillin (100U/mL), streptomycin (100 U/mL), gentamicin (100 µg/mL), and 10% (vol/vol) heat-inactivated
the cells were subjected to treatment with rotenone, glycitein or a combination of both. For enzyme activity, the cells were subjected to homogenization with extraction buffer followed by centrifugation at 18,000×g for 25 min. Thereafter, the supernatant was harvested and used for the determination of CAT activity. The reaction mixture consisted of 5 mM H₂O₂ in 50 mM PBS buffer (pH 7.0). The reaction was initiated by the addition of 100 µL of protein extract to 900 µL of reaction solution. The decrease in OD₅₇₀ at room temperature was measured by spectroscopy. One unit of CAT was defined as the amount of enzyme that could decompose 1 µmol of H₂O₂ in 1 min at 25°C (extinction coefficient is 0.039 M⁻¹ cm⁻¹ at 240 nm).

For superoxide dismutase (SOD) activity each 3 mL of reaction mixture had 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium salt (NBT), and 0.1 mM EDTA. Small aliquots of this mixture were separately put into small glass tubes and addition of 100 µL of protein extract plus mM of riboflavin was followed. After thorough mixing the tubes were illuminated. In control tube the sample was replaced by 100 µL buffer. Change in absorbance by the formation of the formazan was measured at OD₅₇₀. The increase in the absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per minute. 50% inhibition was taken as equivalent to 1 unit of SOD enzyme.

For GSH activity, an aliquot of 50 µL of protein extract was added to 740 µL of sodium phosphate buffer (0.1 M; pH 7.4), 50 µL of 1 mM of sodium azide, 25 µL of glutathione reductase (1 unit/mL), 5 µL of 0.25 mM of H₂O₂, 25 µL of 1 mM GSH, 50 µL of 1 mM of EDTA and 50 µL of 0.2 mM of NADPH. Oxidation of NADPH (substrate) was determined at 340 nm. Activity of GSH was calculated as amount of NADPH oxidized per min per mg protein.

**Estimation of ATP Levels**

The treated SK-N-SH were collected by centrifugation at high speed. The levels of the intracellular ATP were then determined with the help of ATP Bioluminescence Kit HS II (Roche) as per the guidelines of the manufacturer.

**Western blotting analysis**

After treatment with rotenone or Glycitein doses, the SK-N-SH cells were lysed using a lysis buffer. Protein content within each lysate was quantified with a BCA assay. Afterward, 40 µg cell lysates were resolved through SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, United States). Thereafter, membranes were blotted using primary antibodies (Santa Cruz, CA, USA) with 1:1000 dilution. Then membranes were subjected to secondary antibodies treatment at 4°C overnight. Finally, an enhanced chemiluminescence reagent (ECL) (Amerham, Piscataway, NJ, United States) was utilized to spot the protein (Bax, Bel-2, caspase-3 and actin) signals.

**Statistical analysis**

Experiments were done with three biological replicates and the values are shown as mean ± standard deviation (S.D.). Student’s t-test (for comparison between two samples) and one way analysis of variance followed by Tukey’s test (for comparison between more than two samples) were used for statistical analysis using Graph-
Glycitein in the cellular model of Parkinson’s disease

RESULTS

Glycitein prevents rotenone-induced decrease in cell viability

The results of the MTT cell viability assay showed that rotenone caused a significant (P<0.05) depletion of the cell viability of the human SK-N-SH neuroblastoma cells. These effects of rotenone were found to be dose-dependent. The IC<sub>50</sub> of rotenone against human SK-N-SH cells was found to be 100 nM (Fig. 1A). Next, the SK-N-SH cells were treated with 0 or 100 nM rotenone plus different doses of glycitein (0, 2.5, 5, 10 and 20 µM). The results showed that glycitein caused a significant (P<0.05) restoration of the rotenone-induced depletion of SK-N-SH neuroblastoma cell viability (Fig. 1B).

Glycitein inhibits the rotenone-induced ROS formation and MMP loss

Next, the ROS levels of the rotenone treated (100 nM) and untreated cells were determined by the DCFH-DA fluorescence probe. It was found that rotenone caused a significant (P<0.05) increase of the ROS levels in SK-N-SH cells. The ROS levels increased to around 215% relative to the untreated control. Nonetheless, the ROS levels decreased significantly (P<0.05) and dose-dependently upon glycitein treatment as revealed by the intensity of the fluorescence (Fig. 2). The fluorescence probe JC-1 was used to examine the MMP levels of the untreated and rotenone treated SK-N-SH cells. The results showed that rotenone caused a significant (P<0.05) decline in the MMP levels of the SK-N-SH cells relative to the untreated control. The MMP levels in the rotenone treated SK-N-SH cells decreased to around 20%. Nonetheless, glycitein treatment restored the mitochondrial function by increasing the MMP levels in rotenone-treated cells. The MMP levels were 55% and 67% at 5 and 10 µM doses of Glycitein (Fig. 3).

Glycitein increases the activity of the antioxidant enzymes

The effects of glycitein on the rotenone-induced suppression were assessed by standard biochemical assay. The results showed significant (P<0.05) depletion of the GSH, SOD and CAT proteins upon rotenone treatment of the SK-N-SH cells relative to untreated cells. However, glycitein treatment of human SK-N-SH cells caused a significant (P<0.05) and dose-dependent increase of the GSH, SOD and CAT (Fig. 4A–C).

Glycitein prevents rotenone induce ATP depletion

The ATP levels of the rotenone administrated cells were estimated by standard assay, and it was found that treatment of the SK-N-SH cells with rotenone caused a significant decrease of the ATP levels. The ATP levels of the SK-N-SH cells decrease to less than 40% of the untreated control. However, administration of glycitein to rotenone treated SK-N-SH cells could significantly (P<0.05) and dose dependently restore the ATP levels (Fig. 5).

Figure 1. Glycitein prevents the rotenone-induced cell viability. (A) Effects of rotenone on the viability of SK-N-SH neuroblastoma cells (B) Effects of Glycitein on the rotenone-induced inhibition of cell viability. The experiments were performed in triplicate and expressed as mean ± S.D. (*P<0.05).

Figure 2. Glycitein inhibits the rotenone-induced ROS levels in neuroblastoma cells. The experiments were performed in triplicate and expressed as mean ± S.D. (*P<0.05 and **P<0.01).
Glycitein prevents rotenone-induced apoptosis

Annexin V/PI staining was performed to assess the effects of glycitein on the rotenone-induced apoptosis. The results showed that rotenone caused apoptosis of the SK-N-SH cells. The apoptotic SK-N-SH cell percentage was 21% relative at 100 nM dosage of rotenone. Glycitein at 5 µM treatment reduced the rotenone-induced apoptosis to 13%. At 10 µM dosage of the glycitein, the apoptosis decreased to 5.7% (Fig. 6A). The western blot analysis showed that glycitein treatment caused a decrease in a rotenone triggered upregulation of Bax and caspase-3 expression (Fig. 6B). This was concomitant with an increase in Bcl-2 expression indicative of the anti-apoptotic effects of glycitein on the SK-N-SH cells.

DISCUSSION

Oxidative stress, loss of mitochondrial membrane potential and initiation of apoptotic cell death are some important triggers in the development of PD (Moon et al., 2005). Rotenone also triggers these events in the neuroblastoma cells and rotenone treated neuroblastoma cells were thus used to act as the cellular model of PD as has been done previously (Tamilselvam et al., 2013). The present findings show that rotenone decreases the viability of the SK-N-SH cells which is consistent with previous studies (Lee et al., 2010). Glycitein is a plant-derived flavonoid with tremendous pharmacological potential. It has been shown to exert protective effects against human glioma (Zang et al., 2019). Zhang et al., showed that glycitein induces apoptosis in human gastric cancer cells (Kang et al., 2007). In another study, Kang and others (Kang et al., 2007) showed that glycitein exerts protective effects on the hydrogen peroxide induced cell damage by scavenging reactive oxygen species. In yet another study, glycitein has been shown to inhibit the proliferation of breast cancer cells (Zhang et al., 2015). Nonetheless, in the present study, we for the first time show that a natural flavonoid glycitein attenuates the toxic effects of rotenone. Rotenone has been shown to cause the production of ROS in human cells (Wang et al., 2005; Radad et al., 2006) and this was further validated by our fin-
Glycitein in the cellular model of Parkinson’s disease

Rotenone triggers the accumulation of the electrons by blocking the respiratory complex 1. These electrons are transferred to the oxygen molecules leading to the formation of $O_2^-$ (Przedborski et al., 2005). The oxidative stress is mainly manifested in the mitochondrial membrane, leading to the disruption of mitochondrial membrane potential. Herein, we found that glycitein could efficiently restore the mitochondrial membrane potential of the rotenone treated neuroblastoma cells. The decrease in the ROS and increase in the MMP levels in the neuroblastoma cells could also be attributed to the capacity of the glycitein to increase the activities of GSH, CAT and SOD. These enzymes efficiently scavenge ROS (Przedborski et al., 2005), leading to a decrease in oxidative stress and consequently restoration of the mitochondrial membrane potential. All these findings are in agreement with previous studies wherein flavonoids such as hesperidin have attenuated the rotenone that triggered oxidative stress (Tamilselvam et al., 2013). Additionally, a previously carried out study has shown that glycitein could attenuate amyloid-induced toxicity and oxidative stress (Khan et al., 2012). ATP is considered essential for the induction of apoptosis (Eguchi et al., 1999). However, rotenone caused depletion of ATP levels so that the induction of apoptosis by rotenone in the neuroblastoma cells could be due to some other mechanism such as oxidative stress. Glycitein on the other hand prevented the rotenone-induced apoptosis via reduction of oxidative stress and restoration of MMP. The reduction of MMP causes the discharge of cytochrome c which in turn leads to the activation of the caspase-3 favouring apoptotic cell death. Additionally, apoptosis is also accompanied by the enhancement of Bax and a decrease of the Bcl-2 expression levels (Jiang & Wang 2004). Herein, we found that glycitein inhibited apoptosis by reducing the expression of Bax, increasing the expression of Bcl-2 and by inhibiting the activation of caspase-3.

CONCLUSION

Taken together, the findings indicate that glycitein attenuates the rotenone-induced neuronal damage by scavenging ROS and thereby preventing oxidative stress and mitochondrial damage. Moreover, glycitein was found to prevent the induction of the apoptotic cell death in rotenone treated cells. These results point towards the applicability of glycitein in the prevention of PD via scavenging of ROS. Although the present study suggests the therapeutic potential of glycitein in the management of the PD, the results are yet preliminary and therefore in vivo studies are urgently required to further validate the neuroprotective effects of glycitein.

Competing interests

The authors declare no competing interests.

REFERENCES

