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Geraniol Attenuates Cerebral Ischemia-Reperfusion Injury by Suppressing PINK1/Parkin Mediated Mitophagy

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ABSTRACT

Introduction

Cerebral ischemia-reperfusion injury (CIRI) leads to brain damage and arises when blood flow to the brain is initially restricted and subsequently restored. Geraniol, a monoterpenoid alcohol presented in essential oil, showed multiple pharmacological activities including antimicrobial, anticancer but its neuroprotection in CIRI via PINK1/parkin mitophagy has not been explored. So this study aim to determine the therapeutic potential of geraniol in CIRI, particularly focus on its ability to suppress PINK1/Parkin-mediated mitophagy.

Methods

The experiment was carried out in PC12 cell line, sourced from American Type Culture Collection (ATCC). The cells were cultured, Oxygen–glucose deprivation/reoxygenation (OGD/R) model was prepared and underwent geraniol treatment. The cell viability assay (at different concentration of geraniol and different reoxygenation time) was performed. Protein expression was analyzed by western blotting, adenosine triphosphate (ATP) content was measured to assess mitochondrial function and apoptosis was determined.

Results

The result of cell viability assay showed that 20 μM geraniol did not significantly affect cell viability compared with the control and cell viability increased with reoxygenation time in both OGD/R as well as OGD/R group treated with geraniol. Western blot result showed that, compared with the control group, OGD/R increased PINK1, Parkin, Beclin1, and LC3B levels, while decreasing TOMM20 and P62. Treatment with 20 μM geraniol reversed these changes, by reducing PINK1, Parkin, Beclin1, and LC3B expression and restoring TOMM20 and P62 levels. Similarly geraniol treatment increased viable cell and decreased apoptosis cell compared to OGD/R model.

Conclusion

Geraniol was found to reduce OGD induced injury by suppressing PINK1/Parkin mitophagy.

Keywords

CIRI; geraniol; mitophagy; PINK1; parkin

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INTRODUCTION

The central nervous system (CNS) is slightly susceptible to oxidative damage and the mitochondria in injured nerve cells in brain serve as major sources of reactive oxygen species (ROS). Cerebral ischemia-reperfusion injury (CIRI), a neurological disorder that occurs when blood flow to the brain is initially reduced or blocked and subsequently restored.[1] During ischemia, lack of oxygen interferes with mitochondrial energy production (adenosine triphosphate, ATP) and resulting various consequences, leading to mitochondrial dysfunction.[2]

Mitochondrial dysfunction can lead to cell death; therefore, damaged mitochondria should be removed from cell via mitophagy process.[3] Impaired mitophagy leads to accumulation of the dysfunctional mitochondria that impairs normal cellular function and ultimately leads to cell death.[4, 5] The PTEN-induced putative kinase 1 (PINK1)/Parkin-mediated pathway is a key mitophagy pathway. [6] Studies have suggested that any disruption of the PINK1/Parkin pathway can impair mitochondrial function, resulting in cellular damage and dysfunction across various tissues, including neurons.[7]

Plant-derived phytochemicals are increasing attention as a source of bioactive compounds and geraniol a monoterpenoid alcohol found in the essential oils of plants, possess a bioactivities.[8] In addition, its lipophilic nature enables it to effectively cross the blood–brain barrier. It was found to possess a wide range of biological activities, including antioxidant, anti-inflammatory, antimicrobial, cardio protective, neuroprotective, and anticancer, primarily through the modulation of multiple signaling pathways.[9, 10] It exerts neuroprotective effect in certain disorders including Parkinsonism and Alzheimer disease, however its role in ischemic stroke remains unexplored. In present study, protective role of geraniol in ischemic stroke was explored via PINK1/Parkin-mediated mitophagy.

METHODS

Cell culture

Pheochromocytoma (PC) 12 cells were used in cell experiments to replicate the neurons. These cells line were sourced from ATCC (American Type Culture Collection, Manassas, VA, USA). For the experiments, PC12 cells were cultured in standard medium consisting of 89% DMEM (Thermo Fisher, China), 5% FBS (BIOAGRIO, Nanjing, China), 1% penicillin–streptomycin (Beyotime, Shanghai, China), and 5% horse serum (Solarbio, Beijing, China). For the experiment, the cells were cultured under normal conditions, and once they reached the appropriate confluency, they were treated according to the experimental protocol.

Oxygen–glucose deprivation/ reoxygenation model

The oxygen-glucose deprivation and reoxygenation (OGD/R) model was used as an in vitro model of ischemia.[11] Cells were maintained under normal culture conditions, and upon reaching the appropriate confluency, they were subjected to OGD injury for 2 hours, followed by reoxygenation. Following OGD, the glucose-free medium was replaced with the original culture medium containing glucose, and the cells were reoxygenated for 12 hours. Cells in the control group were maintained in standard glucose-containing medium under normoxic conditions at 37°C for the same duration.

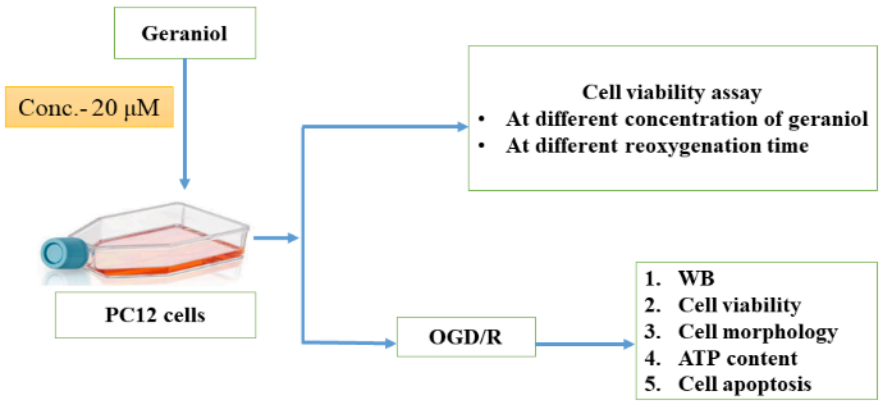

Figure 1. Experimental design

Drug treatment

PC12 cell viability was assessed after treatment with geraniol at various concentrations (5, 10, 20, 40, 80, 160, and 320 µM; Shanghai Yuanyu Biotechnology, China) dissolved in 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Based on cytotoxicity results, 20 µM geraniol (Shanghai Yuanyu Biotechnology, China) had no significant effect on cell viability and was therefore selected for subsequent experiments, added to the culture medium with 0.1% DMSO (Sigma-Aldrich, St. Louis, MO, USA) prior to OGD/R treatment.

Cell viability assay

Cell viability was measured using an MTT assay kit (BioFroxx, Germany). Cells were seeded in 96-well plates and treated according to experimental conditions. Following OGD/R injury, 20 µL/well of MTT was added and incubated at 37 °C for 2 h. Formazan crystals were dissolved with 150 µL DMSO (Sigma-Aldrich, USA), and absorbance was measured at 490 nm using a microplate reader (Bio-RAD, USA).

Western blotting for protein expression analysis

Protein samples from PC12 cells were separated by molecular weight using 12.5% SDS–polyacrylamide gel electrophoresis (Epizyme, Shanghai, China) at 90 V for 30 min, followed by 120 V for 60 min. Proteins were then transferred onto a PVDF membrane (Cell Signaling Technology, USA) and blocked with 5% skimmed milk for 2 h to prevent nonspecific binding. Subsequently, the blocked membranes were washed with tris-buffered saline containing 1% Tween-20 (TBST; Beyotime, China) and incubated with the primary antibodies β-actin (dilution: 1:6,000, Proteintech, #66009-1), LC3II/I (dilution: 1:1,000, Cell Signaling Technology,#12741), TOMM20 (dilution:1:1,000, Abcam, #ab186735), NAF-1 (dilution: 1:1,000, Cell Signaling Technology, #60758), Beclin1 (dilution: 1:1,000, Affinity, #AF5128), p62 (dilution: 1:1,000, Cell Signaling Technology, #5114), PINK1 (dilution: 1:2,000, Affinity, #DF7742), Parkin (dilution: 1:1,000, ABclonal, #A0968) overnight at 4 ◦C. After overnight incubation with the primary antibodies, the membranes were washed with TBST and then incubated with the corresponding secondary antibodies, Goat anti-mouse IgG, (dilution 1:1,000, Proteintech, #SA00001–1) and goat anti-rabbit IgG, dilution (1:1,000, Proteintech #SA00001–2) for 2 hours. Finally, protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA).

ATP Content determination

Cellular ATP levels were measured using an ATP assay kit (Beyotime, China). Cells were washed and lysed with 200 µL lysis buffer, followed by pipetting to ensure complete disruption. Lysates were centrifuged at 12,000 × g for 5 min at 4 °C, and the supernatant was collected. ATP concentration was measured via chemiluminescence [12] using a microplate reader (Thermo Fisher Scientific, USA) and calculated from a standard curve.

Flow cytometry analysis

Apoptotic cells were detected and assessed using an apoptosis detection kit (CHAMOT, Shanghai, China). Cells were cultured to the desired density and treated according to the experimental protocol. For flow cytometry, cells were collected, washed with cold PBS (Proteintech, China), and resuspended in 100 µL binding buffer containing 5 µL Annexin V-FITC and 5 µL propidium iodide (PI). After 15 min incubation at room temperature in the dark, apoptosis was analyzed by flow cytometry. [13]

Statistical analysis

Data analysis was performed using Graph Pad Prism software (version 6.0) and results are presented as mean ± SD. Comparisons between groups were conducted using one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Geraniol increases cell viability in normal cells

Among the concentrations tested, 20 µM geraniol did not significantly affect cell viability compared with the control and was therefore selected for subsequent experiments (Fig 2a). Cell viability at different reoxygenation times (6, 12, and 24 hours) was assessed in OGD/R alone and OGD/R with geraniol treatment. Cell viability was significantly higher in the OGD/R + geraniol group compared with OGD/R alone (Fig. 2b). Moreover, cell viability increased as the reoxygenation time increased in OGD/R group as well as the OGD/R group treated with geraniol.

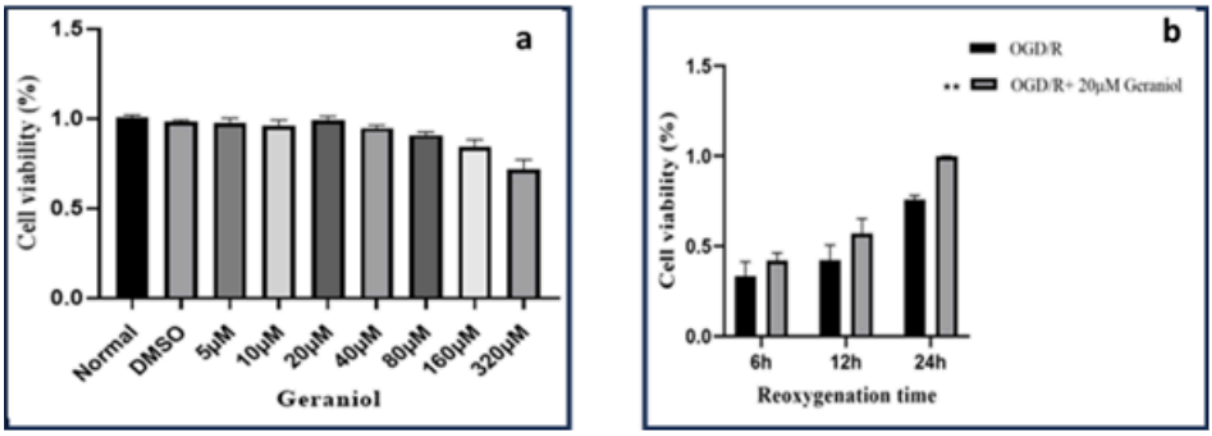

*Figure 2. Geraniol improves cell viability in cells. (a) Cell viability assay at different concentrations of geraniol by using MTT (n=4). (b) Cell viability assay at different reoxygenation times by using MTT (n=4). The data are expressed as mean ± SD. (b) **p < 0.01, vs OGD/R group. (1 unit in Y axis=100%)*

Geraniol improves OGD injury via suppressing PINK1/Parkin mitophagy in cells

Cells were treated with 20 μM geraniol and subjected to OGD/R injury. Western blotting was performed to compare the levels of mitophagy marker proteins among the control, OGD/R, and OGD/R + geraniol groups. Compared with the control group, OGD/R treatment led to increased levels of PINK1, Parkin, Beclin1, and LC3B, while TOMM20 and P62 levels decreased. In contrast, the OGD/R + geraniol treated group exhibited a significant reduction in PINK1, Parkin, Beclin1, and LC3B expression, along with increased TOMM20 and P62 levels relative to the OGD/R group (Fig 3).

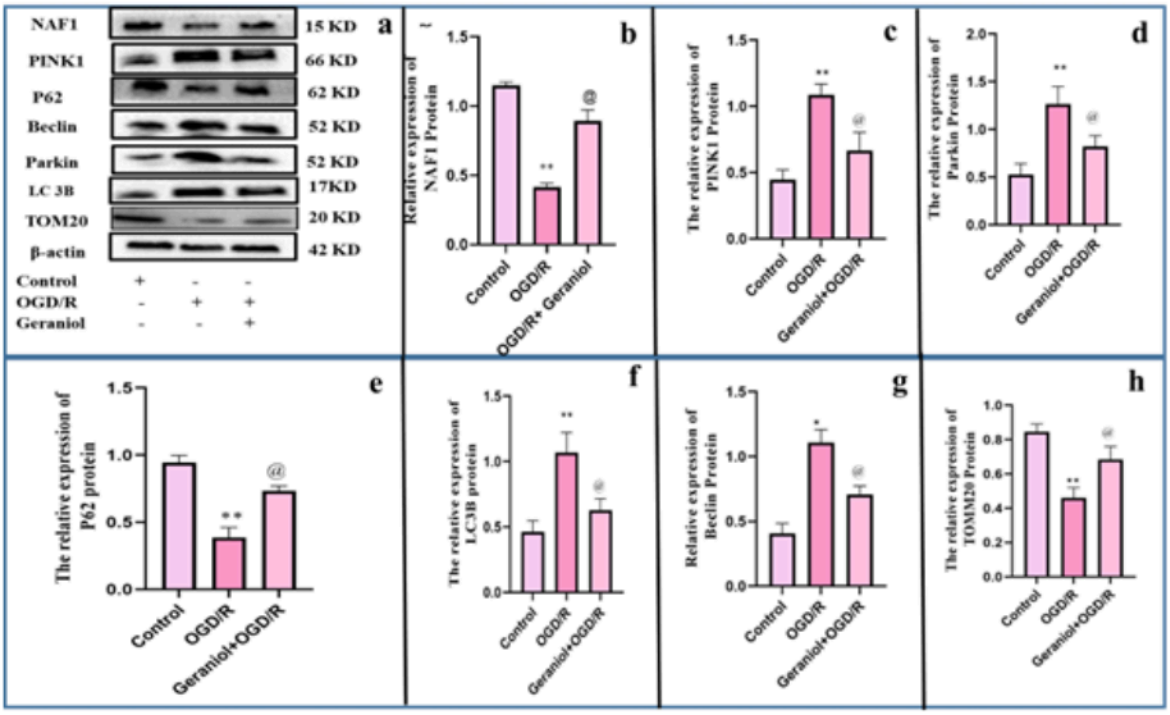

*Figure 3. Geraniol improves OGD injury via suppression of PINK1/Parkin dependent mitophagy. (a) The proteins expressed were evaluated through western blot analysis (n = 4). (b-h) WB analysis of proteins, NAF1, PINK1, P62, TOM20, Beclin, LC3B and Parkin expressed in cells. The geraniol was used at concentration of 20 μM. The data are presented as mean ± SD. *p < 0.05, **p < 0.01, vs. control; @p < 0.05, @@p < 0.01, vs. the OGD/R group.*

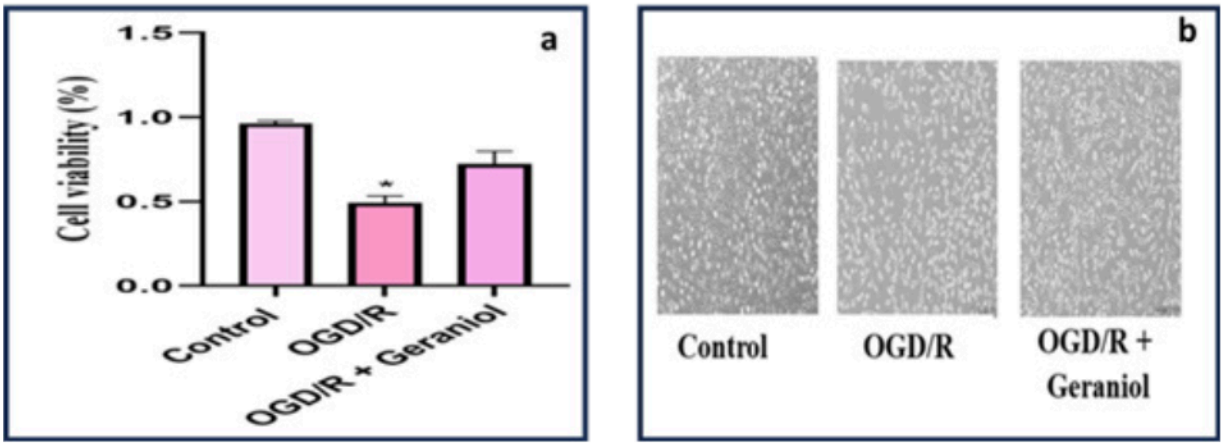

*Figure 4a. Cell viability was assessed by using MTT assay (n = 3). The geraniol was used at concentration of 20 μM. The data are expressed as mean ± SD. **p < 0.05 vs control. 1 unit in Y axis=100%. Figure 4b. Microscopic observation of the morphology of PC12 cells (100×). The geraniol was used at concentration of 20 μM.*

Geraniol increases cell viability

The MTT assay demonstrated that OGD/R markedly reduced cell viability compared with the control group. In contrast, treatment with geraniol significantly improved cell survival in OGD/R-exposed cells, indicating a protective effect of geraniol against OGD/R-induced cytotoxicity (Fig 4a). The cell morphology was studied by visual inspection under inverted phase-contrast microscope (Olympus, Japan). Cells in the control group, maintained extensive branching and dense networks. In contrast, cells in the OGD/R group were spaced apart and showed an increase in dead cells. Geraniol treatment markedly improved cell morphology (Fig. 4b), indicating a protective role of geraniol against OGD-induced injury.

Geraniol reduce cell apoptosis

The results showed higher proportion of apoptotic cells in the OGD/R group compared with the control group. In contrast, treatment with 20 μM geraniol markedly reduced apoptosis in the OGD/R + geraniol group relative to the OGD/R group (Fig.5a). Intracellular ATP levels were further evaluated and found that ATP content was markedly decreased in OGD/R group. Notably, geraniol treatment restored ATP levels, suggesting that geraniol helps preserve mitochondrial function under OGD/R (Fig. 5c) indicating a cytoprotective effect of geraniol against OGD/R.

DISCUSSION

During stroke, the restoration of blood flow is the main strategy for treatment, however it can trigger secondary neuronal injury due to oxidative stress, inflammation, and mitochondrial dysfunction which ultimately leads to cell death.[14] Therefore the treatment of CIRI is challenging despite the advancement in therapeutic strategies.

Various phytochemicals are presented in plants, which make them the primary source of natural bioactive compounds. Geraniol, has been widely recognized for its diverse pharmacological activities. [15] It was found to decrease the inflammatory cytokines and oxidative stress while increases the activity of glutathione peroxidase in a dose dependent manner. Similarly, it also increased the activity of superoxide dismutase. [9] Geraniol influences inflammatory responses not only by inhibiting the NF-κB signaling pathway but also by downregulating pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. [16] Moreover, some studies have reported that geraniol possesses anthelmintic properties and also act as an insect repellent. [17]

In this study, we investigated the effects of geraniol, on mitophagy through the PINK1/ Parkin signaling pathway in vitro. The cell

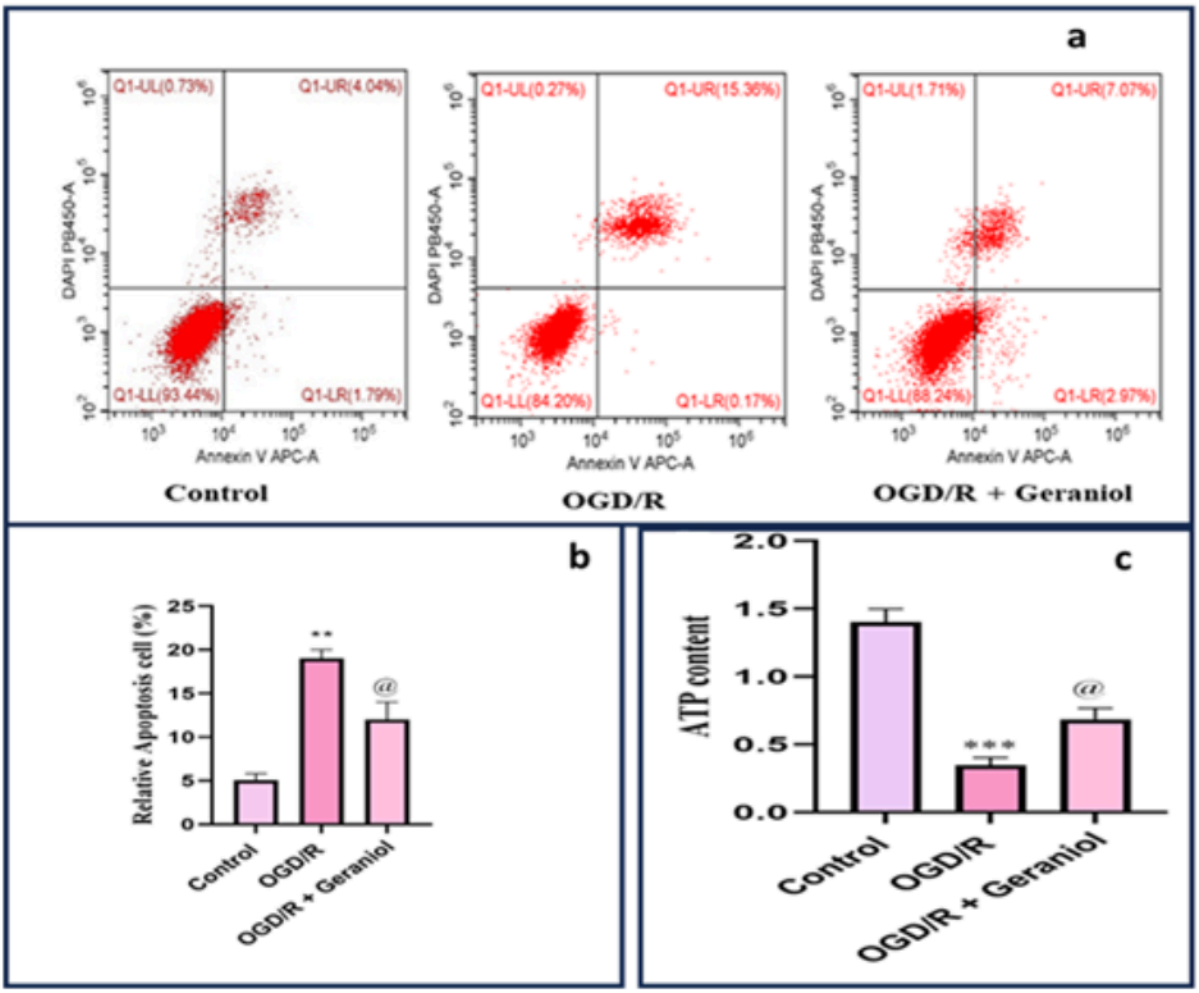

*Figure 5 (a,b). Cell apoptosis was assessed by flow cytometry and statistical analysis (n=4).The geraniol was used at concentration of 20 μM. The data are presented as mean± SD. **p < 0.01, vs. control; @p < 0.05, vs. the OGD/R group. Figure 5c. ATP content was quantified via an ATP assay kit (n=4). The geraniol was used at concentration of 20 μM. The data are presented as mean± SD. ***p < 0.001, vs. control; @p < 0.05 vs. the OGD/R group.*

viability results showed that geraniol at 20 μM did not induce significant cytotoxicity and this concentration was well tolerated by the cells. Accordingly, 20 μM geraniol was closed as a suitable concentration in subsequent experiments for evaluating neuroprotective activity. Our findings showed that the extent of OGD/R-induced cell injury was reduced as the reoxygenation time increased till 24 hrs. A 12-hour reoxygenation interval was selected in the experiments. Insufficient recovery was observed at shorter reoxygenation intervals. After 12 hours of reoxygenation, cells treated with geraniol showed a clear increase in viability compared to those undergoing OGD/R alone.

Similarly in cell model, geraniol treatment suppressed PINK1/parkin mitophagy, as shown by limiting the excessive mitophagy and evidenced by diminishing the expression of mitophagy-related proteins (PINK1, Parkin, LC3B, P62, Beclin and TOMM20). Additionally, geraniol-treatment increased cell viability, restored ATP levels, and reduced cell apoptosis. Rekha et al. reported the neuroprotective effects of geraniol, showing that cells pretreated with geraniol exhibited increased P62 levels and reduced LC3-I and LC3-II levels following rotenone-induced oxidative stress compared with the control group.[18]

The present finding are consistent with those of Rekha et al., supporting the notion that geraniol protects nerve injury resulting from mitochondrial damage due to inhibition of mitophagy. [18] A similar finding was reported by Yang et al. who investigated the role of geraniol in human brain microvascular endothelial cells underwent OGD/R treatment (HBMECs). Yang et al. found that geraniol at concentrations below 80 μM, had no role in cell viability, which is higher than the effective concentration observed in our study. Additionally the maximal effect was observed at 24 hours of reoxygenation.[19] The study conducted by Wu et al. demonstrated the neuroprotective effect of geraniol; however, the signaling pathways and organelles they investigated were different from those in our study. Their results showed that geraniol protect neural damage following CIRI and reduced apoptosis through the ER-stress-associated PERK-ATF4-CHOP signaling pathway. [20]

CONCLUSION

The protective effects of geraniol in CIRI via PINK1/Parkin-mediated mitophagy was explored in an OGD/R model using western blotting, cell viability assays, apoptosis assays and ATP content measurements. It was found that geraniol treatment suppresses mitophagy marker proteins, increases cell viability, reduces cell apoptosis and restores ATP content, thus exerts protective effect in CIRI by suppressing PINK1/Parkin-mediated mitophagy. The study was conducted in in-vitro model, however findings from animal models and detail studies such as immunofluorescence analysis need to be performed.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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