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Original Research

Protective Effects of Querectin against MPP^+-Induced Dopaminergic Neurons Injury via the Nrf2 Signaling Pathway

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Abstract

Background: Parkinson's disease (PD) is a common selective and progressive neurodegenerative disorder of nigrostriatal dopaminergic (DA) neurons. Quercetin is a bioflavonoid with antioxidant, anti-inflammatory, anti-aging and anti-cancer properties. However, the exact mechanism by which quercetin exerts its protective effect on DAergic neurons remains unclear. **Purpose**: To investigate the underlying molecular mechanism of quercetin's protective effect on DA neurons using 1-methyl-4-phenylpyridinium (MPP^+)-induced PD ferroptosis model *in vitro*. Methods: MPP^+ was used to induce cytotoxicity in SH-SY5Y/primary neurons. Cell viability and apoptosis were assessed by CCK-8 assay and flow cytometry. The expression levels of ferroptosis-related proteins (NCOA4, SLC7A11, Nrf2, and GPX4) were determined by Western blotting. Malondialdehyde (MDA), iron, and GPX4 levels were assesed using corresponding assay kits. Lipid peroxidation was assessed by C11-BODIPY staining. **Results**: In the MPP^+-induced ferroptosis model of SH-SY5Y cells, the expressions of SLC7A11 and GPX4 were inhibited, and the expression of NCOA4 protein was increased, causing the overproduction of MDA and lipid peroxidation. Quercetin can reduce the above changes caused by MPP^+, that is, reduce the protein expression of NCOA4 in SH-SY5Y cells, increase SLC7A11 and GPX4 partially inhibited by MPP^+, and reduce MDA overproduction and lipid peroxidation to protect DA neurons. Nrf2 inhibitor ML385 could inhibit quercetin-induced increase of GPX4 and SLC7A11 protein expression, indicating that the protective effect of quercetin was mediated through Nrf2. **Conclusions**: The results of this study suggest that quercetin regulates ferroptosis through Nrf2-dependent signaling pathways, thereby inhibiting MPP^+-induced neurotoxicity in SH-SY5Y/primary neurons.

Keywords: Parkinson's disease; quercetin; Nrf2-dependent signaling pathway; ferroptosis

1. Introduction

Parkinson's disease (PD) is a common, age-related, chronic, disabling neurodegenerative disease [1]. It is characterized by an abnormal accumulation of α-syn, the formation of Lewy bodies and the eventual loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) [2]. Dopaminergic drugs designed to replace dopamine action in the brain are currently the mainstay of treatment for PD. However, as the disease course of PD progresses, the benefits provided by various PD therapeutic drugs gradually diminish while the side effects of their treatment continue to increase. Moreover, there is no cure for PD, and the existing treatments can only help to relieve symptoms and maintain quality of life [3]. Therefore, there is an urgent need to develop new and more effective drugs for PD treatment.

The pathogenesis of PD has been shown to be related to oxidative stress and lipid peroxidation-induced damage and death of DA neurons [4]. Excess reactive oxygen species (ROS) generated by multiple pathways play an important role in the progression of several neurodegenerative diseases, including PD [5,6]. Ferroptosis is an iron-dependent and caspase-independent form of regulated cell death initiated by intracellular reduced glutathione (GSH) and lipid peroxidation [7]. The process of ferroptosis involves iron-dependent lipid oxidation [8] and GPX (glutathione peroxidase) is the first line of defense against mitochondrial damage and oxidative stress [9], which directly reduces phospholipid hydroperoxides and oxidized lipoproteins in biological membranes [10,11].

Nuclear factor erythroid 2-related factor 2 (Nrf2), expressed in various brain cells including astrocytes, microglia and DA neurons, is a redox-sensitive regulator of antioxidant enzymes. Previous studies have shown that the

Nrf2 signaling pathway plays a role in protecting against neuroinflammation, stroke, and oxidative damage-induced neurodegeneration [12,13], and helps to defend against oxidative and toxic neurological damage [14]. In the resting state (i.e., non-oxidative stress), Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2, leaving it in an inactive state in the cytoplasm, where it is degraded by proteasomal ubiquitination [15,16]. However, under oxidative stress, Keap1 dissociates and translocates Nrf2 from the cytoplasm to the nucleus, where it activates antioxidant response element (ARE)-mediated gene transcription of detoxification and antioxidant enzymes [17] [including glutathione peroxidase (GPX), oxidoreductase (NQO1), and superoxide dismutase (SOD)] to maintain cellular redox homeostasis [18]. In addition to regulating antioxidant enzymes, Nrf2 signaling pathways have anti-inflammatory [19], anti-fibrosis [20], and anti-apoptotic [21] effects. Furthermore, since the development and progression of PD have been associated with ferroptosis [22] targeting Nrf2, an antioxidant transcription factor that inhibits iron degeneration, may be an attractive new option for the treatment of PD [23,24].

Quercetin is a catecholic flavonol that undergoes enzymatic decomposition during plant metabolism [25] into a dimer that with weak antioxidant and antiferritin activities [26,27]. Thus, it can coexist in the same plant with its dimer metabolite quercetin Diels-Alder anti-dimer [28]. Quercetin has several pharmacological effects, including antioxidant, anti-inflammatory, anti-cancer, anti-allergic , and anti-aging [29–32]. For example, studies have shown that quercetin can inhibit ferroptosis, thereby improving the symptoms of kidney damage, type 2 diabetes, and several other diseases [33,34]. Another study showed that the antioxidant properties of quercetin helped prevent ferroptosis in the liver of mice fed a high-fat diet and reduced fat accumulation, iron overload, lipid peroxidation, and inflammation [35]. Thus, quercetin has a broad therapeutic potential and may be useful for the treatment of many different diseases [36–38], as well as being a candidate for preventive medicine. However, its potential application in the treatment of PD still remains unclear.

Prior studies have shown that quercetin has a protective effect on DA neurons through anti-lipoperoxidation, mitochondrial function improvement, and cell death inhibition [39–41]. However, the mechanism underlying its protective effects against cellular lipid peroxidation have not been fully elucidated. Therefore, in the present study, an animal model was used to explore how quercetin achieves anti-lipid peroxidation in PD and we hypothesized that quercetin may protect DA neuronal activity by downregulating ferroptosis.

2. Materials and Methods

2.1 Chemicals

ML385 (Cat.no.HY-100523), RSL3 (Cat.no.HY-100218a) and Ferrostatin-1 (Cat.no.HY-100579) chemicals were purchased from MCE Corporation (Monmouth Junction, NJ 08852, USA). Quercetin (Cat.no.117-39-5) was obtained from Macklin Biochemical Technology Co., Ltd (Shanghai, China), and the molecular structure of quercetin was shown in **Supplementary Fig. 1**.

2.2 SH-SY5Y Cells and MPP^+-Induced PD Cell Model

SH-SY5Y cell line was a kind gift from the Department of Neurology, Tongji Hospital Affiliated with Tongji University. The cells of the human neuroblastoma line (SH-SY5Y) were provided by the Cell Bank/Stem Cell Bank of Type Culture Collection Committee of Chinese Academy of Sciences. The SH-SY5Y cell line was cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (10100147, Gibco, CA, USA) and 1% Penicillin-Streptomycin (P/S). All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere and were mycoplasma-free (Mycoplasma Detection Kit, C0301S, Beyotime Biotechnology, Beijing, China). All cell lines were authenticated shortly before use by the STR technique, carried out by Yanyan Jiang. Mycoplasma testing has been done for the cell lines used. Cells were cultured in T75 cell culture flasks and passaged after reaching 80%–90% confluency. For the MPP^+-induced PD cell model, SH-SY5Y cells were incubated for 24 hours in a culture medium with an MPP^+ concentration of 500 μM.

2.3 Evaluation of Cell Apoptosis

Apoptosis was quantified using the apoptosis detection kit Annexin V FITC/PI (Cat.no.AP101-100-AVF, Linktech, Hangzhou, China). Briefly, SH-SY5Y/primary neuronal cells (3×10^5) were added to a six-well plate. Then, quercetin (final concentration of 10 or 20 μm) was added to each well and incubated for 24 h (5% CO_2, 37 °C). Cell suspensions were collected, mixed with binding buffer, and then mixes with Annexin V/PI (5 μL/well) for 10 minutes at room temperature according to the protocol of Annexin V-FITC/PI kit. Living normal cells (Annexin V^-/PI^-), early apoptosis (Annexin V^+/PI^-), late apoptosis (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+) were then distinguished by flow cytometry. Data were analyzed and stained SH-SY5Y/primary neuronal cells were counted by Beckman flow cytometer.

2.4 Lipid-Reactive Oxygen Species Assay

The C11-BODIPY 581/591 (Thermo Fisher Scientific, Waltham, MA, USA) instrument was used to measure lipid ROS (LPs) levels. Cell medium was added with a final concentration of 2 μM BODIPY (Cat.no. C11 D3861, Invitrogen, Shanghai, China) and cells were incubated for 30 min at 37 °C in the dark, and fluorescence intensity (excita-

tion wavelength of 488 nm and emission wavelength of 520 nm) was measured by flow cytometry. Experiments were performed in triplicate.

2.5 Malondialdehyde (MDA) Detection

A standard curve for MDA was prepared according to the manufacturer's instructions (Cat.no.S0131S, Beyotime Biotechnology, Beijing, China) and target cells were lysed to assess MDA levels. MDA concentration was determined using the Lipid Peroxidation MDA Assay Kit (Beyotime). MDA levels (nmoL/mL) were calculated as follows: [sample optical density (OD) value - blank OD value]/(standard OD value - blank OD value) $\times$ standard concentration (10 nmoL/mL) $\times$ sample dilution before testing.

2.6 Cell Counting Kit-8 (CCK-8)

The CCK-8 assay kit (Cat.no.RM02823, ABclonal Technology, Woburn, Massachusetts, USA) was used to quantify cell viability after treatment. The optical density (OD) of CCK-8 was examined at 450 nm. The cell survival rate of cells was calculated as follows: OD treatment/OD control $\times$ 100.

2.7 Measurement of Intracellular Iron

An iron assay kit (Cat.no.BC5415, Solarbio, Beijing, China) was used to measure intracellular iron concentrations.

2.8 Quantification of Glutathione Peroxidase4 (GPX4) Expression

Cells were fixed with fixative disruptor (eBioscience, Cambridge, UK), stained with 1 mg/mL of GPX4 antibody (Abcam, Cambridge, UK), and then incubated with Alexa Fluor 647-labeled anti-rabbit GPX4 antibody according to the manufacturer's instructions, and rabbit IgG monoclonal (ab172730; Abcam) was used as primary isotype control. Cell sampls were tested on beckmancytoflex flow cytometer (BDBiosciences). Next, GPX4 expression was measured by flowcytometric analysis using FlowJo 10.4.2 software (Ashland, OR, USA).

2.9 Mitochondrial Membrane Potential (MtMP) Measured with JC-1 Staining

Changes in the intracellular MtMP were measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cat.no.C2006, Beyotime Biotechnology, China) according to the manufacturer's instructions, and fluorescence intensity was measured using flow cytometers at wavelengths of 488 nm and 525 nm.

2.10 Western Blot

Cell samples were collected and lysed in 1$\times$RIPA lysis buffer (Cat.no.P0013C, Beyotime Biotechnology, Beijing, China) containing protease inhibitor cocktail. Denatured protein samples were prepared and protein concentrations were assayed according to the manufacturer's instructions. Samples (20 μg per lane) were separated by SDS-PAGE gel electrophoresis and transferred to Polyvinylidene Fluoride (PVDF) membranes, which were blocked with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) buffer containing 5% skimmed milk for 1 hour at room temperature, and then incubated with antibodies against SLC7A11 (Cat.no.A13685, Abconal, Wuhan, China), GPX4 (Cat.no.ab125006, Abcam, Cambridge, UK), Nrf2 (Cat.no.A1244, Abconal, Wuhan, China), NCOA4 (Cat.no.A5695, Abconal, Wuhan, China) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cat.no.5174s, Cell Signaling Technology, MA, USA) overnight at 4 °C. Later, membranes were incubated with secondary antibodies (Cat.no.ab150079, Abconal, Wuhan, China) for 1 hour at room temperature. Finally, membranes were washed with 1$\times$TBST and the protein bands were visualized with Enhanced chemiluminescence (ECL) detection reagents.

2.11 Primary Neuronal Cell Cultures

The preparation of primary cortical cultures was performed as previously described [42]. Briefly, cells were isolated from the cortex of male and female neonatal mice at P0-P1 days postnatally. Primary neuronal cell cultures were prepared as described previously. Microtubule-associated protein (MAP2) was used as a neuronal marker and immunolabelled with rabbit polyclonal MAP2 antibody (Abcam, ab32454, 1:750). MAP2 and 4′,6-diamidino-2-phenylindole (DAPI) were used to stain neurons and nuclei, respectively, see **Supplementary Fig. 2**.

2.12 Statistical Analysis

GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA, www.graphpad.com) statistical software was used to perform the statistical analysis. Means were expressed as standard deviations (SD). Statistical analysis included one-way ANOVAs and the Tukey-Kramer method for multiple comparisons in all experiments. $p < 0.05$ was considered statistically significant ($*p < 0.05$) and significance of $p < 0.001$ was considered highly significant ($***p < 0.001$). All experiments were conducted in triplicate.

3. Results

3.1 Structure and Toxicity of Quercetin on SH-SY5Y and Primary Neuron Cells

The chemical structure of quercetin is shown in **Supplementary Fig. 1**. As previously described, high concentrations of quercetin can induce apoptosis in certain cells. To determine the optimal concentration of quercetin for treating neuronal cells, SH-SY5Y/primary neuron cells were coincubated with different concentrations of quercetin for 24 h, and then cell viability was measured by CCK-8 assay. Quercetin was found to affect the viability of SH-SY5Y/primary neuron cells, as shown in Fig. 1A,B. After

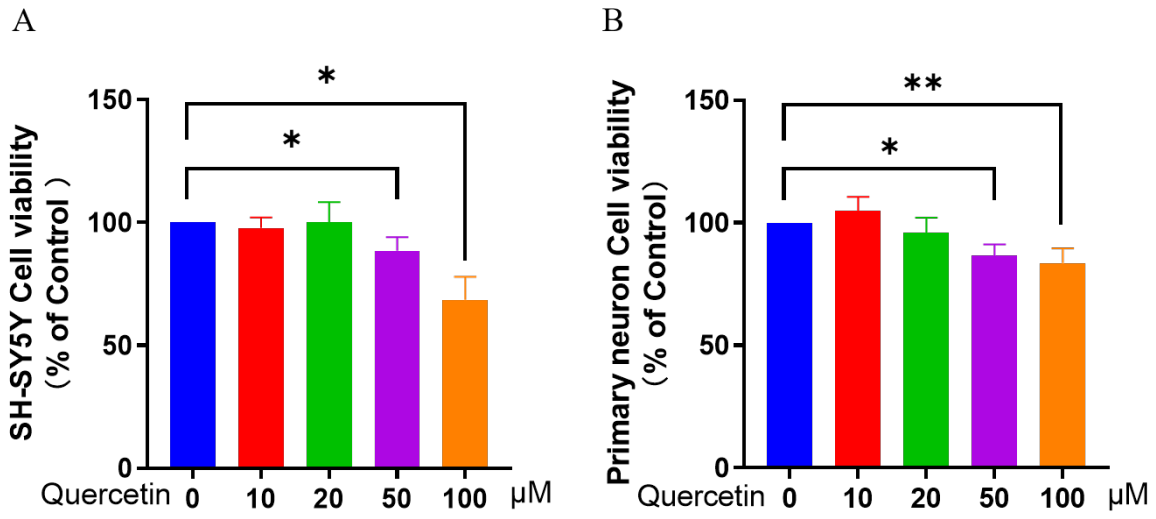

Fig. 1. Effect of different concentrations of quercetin on SY-SY5Y/ Primary neuron cell viability. CCK-8 assay was used to determine neuronal viability. (A) Effect of quercetin on cell viability of SH-SY5Y/Primary neurons. (B) Effect of quercetin on primary neuron cell viability. Three independent experiments were used to calculate the mean ± SD. *$p < 0.05$, **$p < 0.01$.

exposure to lower concentrations of quercetin (<20 μM) for 24 h, no significant cytotoxicity was found. Therefore, quercetin at concentrations of 10 μM and 20 μM was used in subsequent experiments.

3.2 Quercetin Reduced MPP$^+$-Induced Cell Death in SH-SY5Y Cells

Next, the effect of quercetin on neuronal cell apoptosis was examined by flow cytometry (Fig. 2A,B). Compared to control cells, decreased neuronal cell viability and increased cell death were observed in the MPP$^+$-treated group, indicating the successful establishment of a PD cell model. In MPP$^+$-treated SH-SY5Y and primary neuronal cells, quercetin significantly reduced cell death and improved cell viability in a dose-dependent manner, indicating a protective effect of quercetin on DA neurons. Higher doses of quercetin (20 μM) reduced cell death more than the Fer-1 (5 μM), especially in the SH-SY5Y cell line.

3.3 Quercetin Reduces MDA Levels and Iron Content and Increases GPX4 Levels in MPP$^+$-Induced SH-SY5Y Cells

Lipid peroxidation is a key indicator of oxidative stress and plays an important role in the progression of neurodegenerative diseases, including PD [43]. MDA is a commonly used marker of lipid peroxidation. In the present study, the MDA level increased significantly in the MPP$^+$-induced PD cell group compared to the control group (Fig. 3A), indicating a substantial increase in lipid peroxidation levels. However, quercetin decreased the MPP$^+$-induced MDA levels in a concentration-dependent manner, suggesting that it can reduce lipid peroxidation in MPP$^+$-induced SH-SY5Y cells.

Iron is a stimulator of oxidative stress and iron overload that promotes ferroptosis [44]. The iron content of MPP$^+$-treated cells was slightly increased compared to the control group, but quercetin significantly decreased the iron content in a concentration-dependent manner (Fig. 3B). This confirms that quercetin reduces iron accumulation in MPP$^+$-treated SH-SY5Y cells.

GPX4 is an important antioxidant enzyme in the ferroptosis pathway. In the MPP$^+$-induced PD cell group, the protein expression of GPX4 was significantly lower than that in the control group (Fig. 3). Quercetin prevented the further reduction of MPP$^+$-induced GPX4 levels in a concentration-dependent manner. These results suggest that quercetin exerts antioxidant effects on DA neurons by decreasing MDA levels and iron contents as well as increasing GPX4 levels.

3.4 Quercetin Exerts a Protective Effect on DA Neurons by Regulating GPX4

The previous findings suggest that quercetin inhibits ferroptosis through upregulation of GPX4, thereby reducing DA neuron death. To further investigate the underlying mechanism of this regulatory effect, we examined the effect of quercetin on DA neurons after blocking GPX4 expression with RSL3. Compared with the control group, the viability of PD cells was significantly reduced in the RSL3 group, while PD cell death was increased (Fig. 4A). This confirmed the significant iron toxicity observed after RSL3 induction. GPX4 levels were significantly increased in the quercetin and RSL3-treated group compared with the RSL3-treated group. Therefore, a 5 μM concentration of RSL3 was chose to induce ferroptosis and explore the effect on rescuing primary neuron cell viability, as shown in Fig. 4B, and the same general trend of quercetin-dependent, increased anti-apoptotic activity in primary neuron was observed. Compared with the control group, the

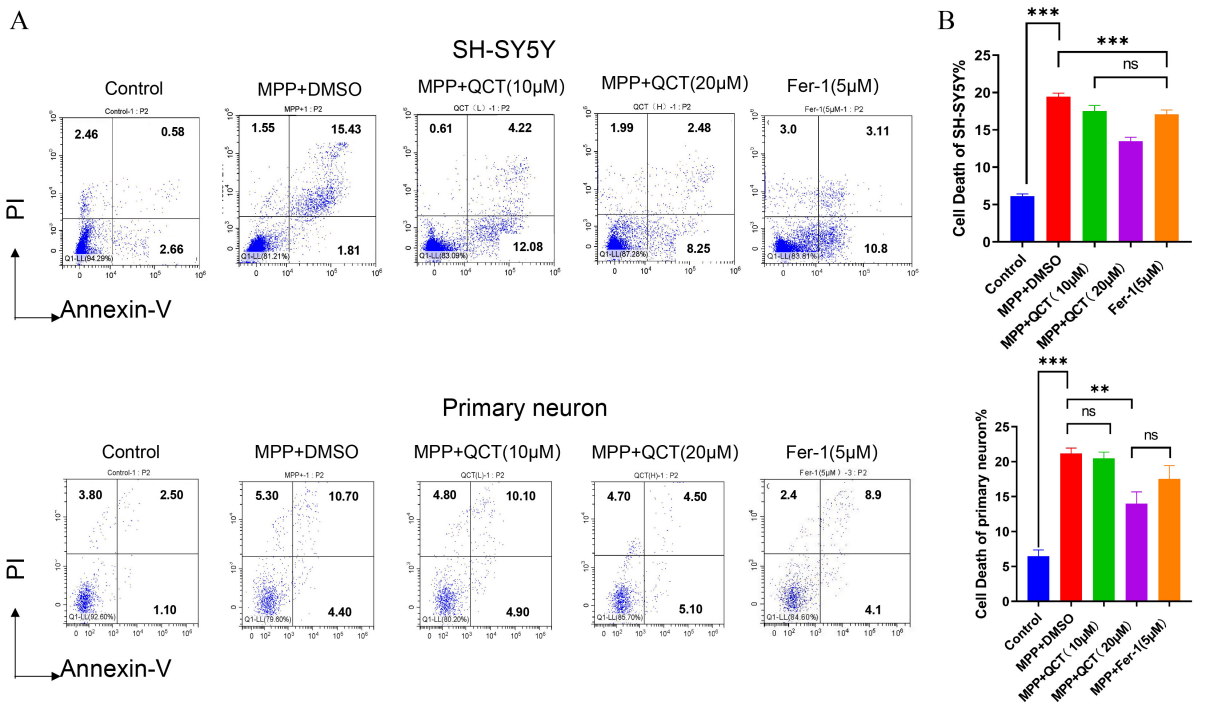

Fig. 2. Quercetin reduces MPP$^+$-induced neuron apoptosis. The effect of quercetin (10/20 μM) on the induction of apoptosis in SH-SY5Y/primary neuron cells after 24 h treatment of MPP$^+$ was assessed using Annexin-PI assay. (A,B) Representative flow cytometry plots are shown on the left panel (Annexin-PI). Annexin V$^-$PI$^-$, annexin V$^+$PI$^-$, annexin V$^+$PI$^+$, and annexin V$^-$PI$^+$ cells were defined as viable, early apoptotic, late apoptotic, and necrotic cells, respectively. The statistical analysis of the total number of dead cells (early, late apoptotic, and necrotic cells) is shown in the right panel. All data shown are the mean ± SD of 5 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. Ferrostatin-1 (Fer-1) is a synthetic selective ferroptosis inhibitor that inhibits cell death by preventing damage to membrane lipids through a reduction mechanism.

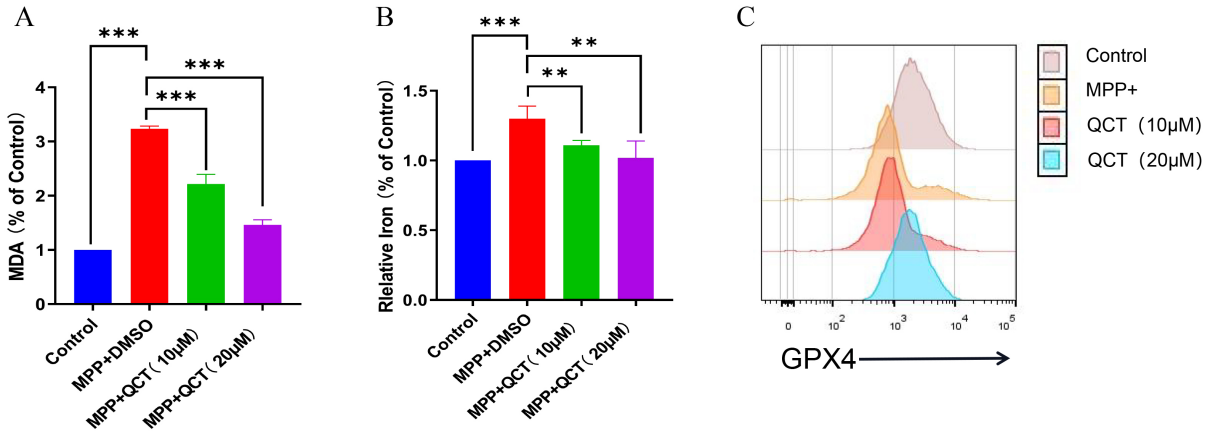

Fig. 3. Quercetin reduces the changes of MDA, iron content and GPX4 levels caused by MPP$^+$ in neuronal cells. (A) The results of MDA levels in SH-SY5Y cells from different treatment groups. (B) Analysis of the intracellular iron content. (C) Flow cytometric analysis of integrin GPX4 protein expression. All data shown based on the mean ± SD of 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

viability of primary neuron cells was significantly reduced in the RSL3 group, while primary neuron death was increased (Fig. 4B), and treatment with quercetin improved cell viability in a dose-dependent manner. Furthermore, The high-dose quercetin group (20 μM) showed better anti-apoptosis effect than the low-dose group (10 μM), particularly in terms of GPX4 expression (Fig. 4C). In conclusion, the induction on PD cell apoptosis by RSL3 was reversed

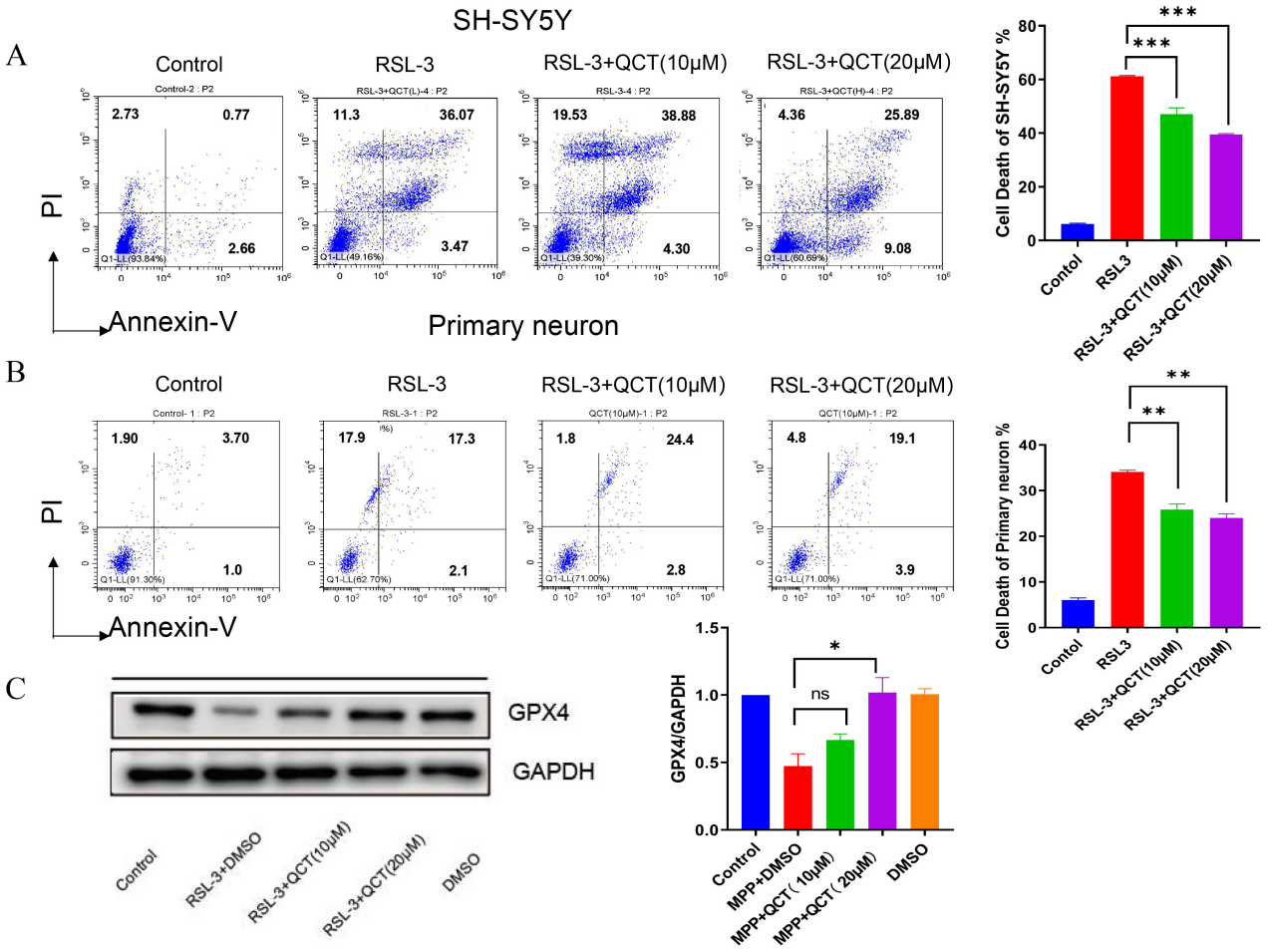

Fig. 4. Quercetin promotes cell viability and GPX4 expression which inhibited by RSL3 induction. (A) Cell viability was assessed by Annexin-PI assay used to assess apoptosis in SH-SY5Y neurons treated with RSL3 followed by treatment with quercetin (10/20 μM) for 24 h. (B) Cell viability was assessed by Annexin-PI assay used to assess apoptosis in primary neurons treated with RSL3 followed by treatment with quercetin (10/20 μM) for 24 h. (C) Western blotting was used to analyze GPX4 levels. All data are expressed based on the mean $\pm$ SD based on 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ compared to control.

by quercetin, and the protective effect of quercetin in PD cell models and primary neuron was achieved by activating the GPX4 pathway.

3.5 Quercetin Inhibits Ferroptosis by Activating Nrf2/GPX4 Axis in SH-SY5Y Cells

Previous studies showed that NCOA4 acts as a mediator of ferritin phagocytosis, causing iron accumulation and ferroptosis. In the current study, NCOA4 levels were significantly upregulated in the MPP^+-treated group compared with the control group, which is consistent with the previous observations on intracellular iron levels. After quercetin treatment, the MPP^+-induced NCOA4 levels gradually decreased in a dose-dependent manner (Fig. 5). This indicates that inhibition of NCOA4 may be one of the pathways by which quercetin inhibits MPP^+-induced ferroptosis in SH-SY5Y cells.

The Nrf2/GPX4 axis is a key factor in the inhibition of ferroptosis, and Nrf2/GPX4 expression is significantly reduced in MPP^+-induced cells. NRF2 is a transcription target for GPX4; therefore, sustained NRF2 activation may lead to increased GPX4 expression and thus higher resistance to ferroptosis-induced cell death. Quercetin increased Nrf2/GPX4 expression levels in a concentration-dependent manner, suggesting that it could inhibit ferroptosis by activating Nrf2/GPX4 and inhibiting NCOA4 in an MPP^+-induced PD cell model.

3.6 Nrf2/GPX4 Plays an Important Role in the Protective Effect of Quercetin

The results of the aforementioned study showed that Nrf2 protein expression in the PD cell model increased with increasing quercetin concentration. Next, the ability of quercetin was evaluated to induce cytoprotective/antioxidant protein expression in Nrf2-driven SH-SY5Y cells. Cells were pretreated with quercetin and the specific Nrf2 inhibitor ML385 for 2 hours, and then treated with MPP^+ for 24 hours and the protein expression lev-

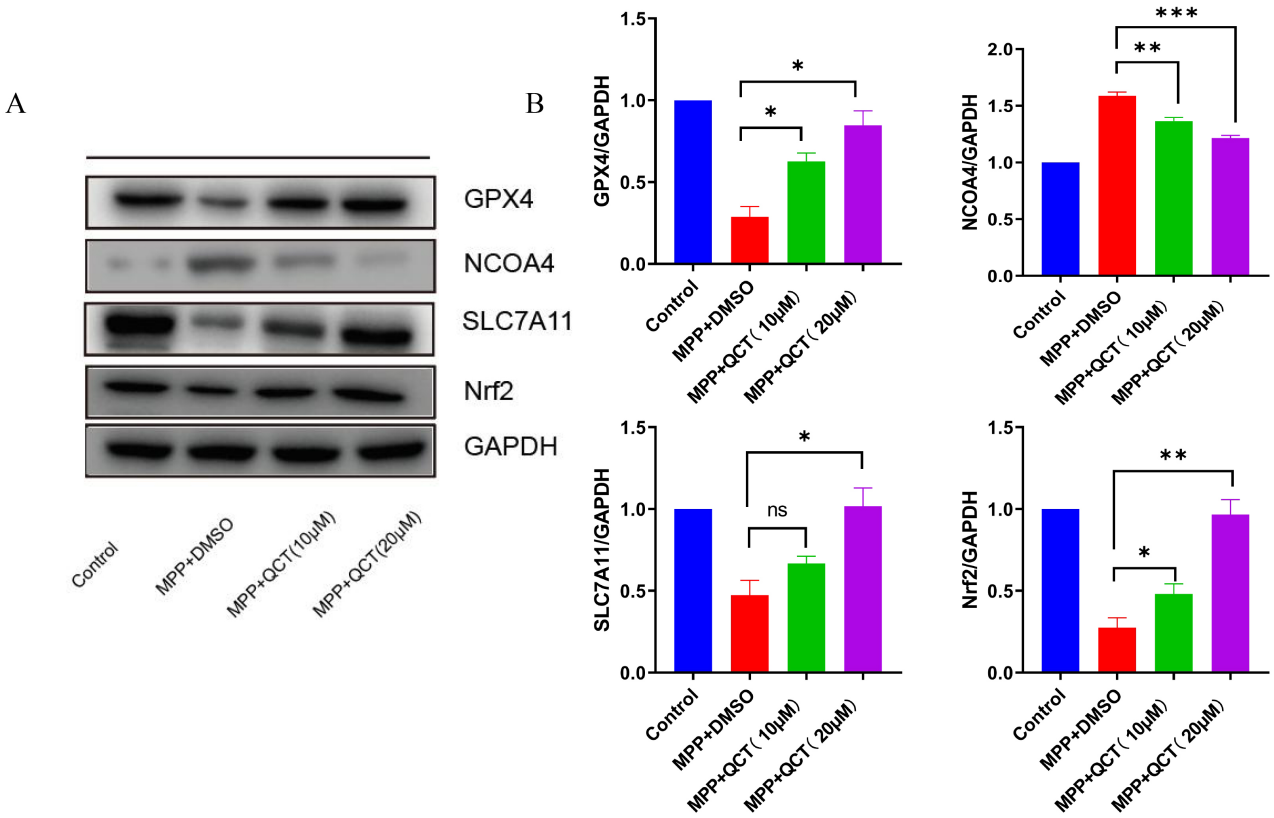

Fig. 5. The effect of quercetin on ferroptosis-related protein levels. (A) Representative WB images are shown on the left. (B) The corresponding relative grayscale statistics of protein levels are shown on the right. The results of five independent experiments are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control.

els for Nrf2, SLC7A11, and GPX4 were determined by Western blotting. The Nrf2 signal was significantly increased in nucleus after quercetin treatment, thus indicating its translocation to the nucleus, as shown in **Supplementary Fig. 3**. Compared to the MPP^+ group, ML385 significantly increased the expression of Nrf2/GPX4-related proteins (Nrf2, SLC7A11, SLCA11A7, and GPX4), suggesting that Nrf2/GPX4 is inhibited by ML385. The results showed that the expression of Nrf2/GPX4-related proteins (Nrf2, SLC7A11, SLCA11A7 and GPX4) was significantly reduced in ML385-treated cells compared with the MPP^+ group, indicating that Nrf2/GPX4 was inhibited by ML385 (Fig. 6A). Pretreatment with 10 μM ML385 significantly reduced the protein expression levels of Nrf2, SLC7A11 and GPX4, whereas MPP^+-treated SH-SY5Y cells were induced by quercetin and the protein expression levels of Nrf2, SLC7A11 and GPX4 were significantly rebounded (Fig. 6B). ML385 also significantly upregulated the MPP^+-induced increase in MDA and lipid peroxidation levels (Fig. 6C,D). Interestingly, the cytoprotective effect of quercetin was blocked by pretreatment with ML385 (Fig. 6E).

Subsequently, JC-1 staining revealed a similar mitochondrial membrane potential signature, which is consistent with the trend of ferroptosis, suggesting that quercetin protects against ferroptosis through the Nrf2-mediated pathway and that blocking the Nrf2 pathway inhibits the protective effect of quercetin.

4. Discussion

Quercetin, a phenolic compound widely distributed in the plant kingdom, is an antioxidant that helps to maintain oxidative homeostasis [45]. Tumor cells can undergo apoptosis in the presence of high concentrations of quercetin [46]. In this study, we investigated the effect of different concentrations of quercetin on the anti-apoptotic capacity of neuronal cells, and the graphic abstract was shown in Fig. 7. SH-SY5Y cells were used as a cell model for *in vitro* study of PD in this study because they have some properties of DA neurons. Our study showed that SH-SY5Y neuronal cells and primary cultured neurons exhibited similar sensitivity to quercetin. In this study, we found that quercetin inhibits MPP^+-induced neurotoxicity in SH-SY5Y/primary neuron cells by regulating ferroptosis via an Nrf2-dependent signaling pathway.

ROS-induced lipid peroxidation plays a key role in cell death, including apoptosis and ferroptosis. Unlike other cell death modes such as autophagy and apoptosis, ferrop-

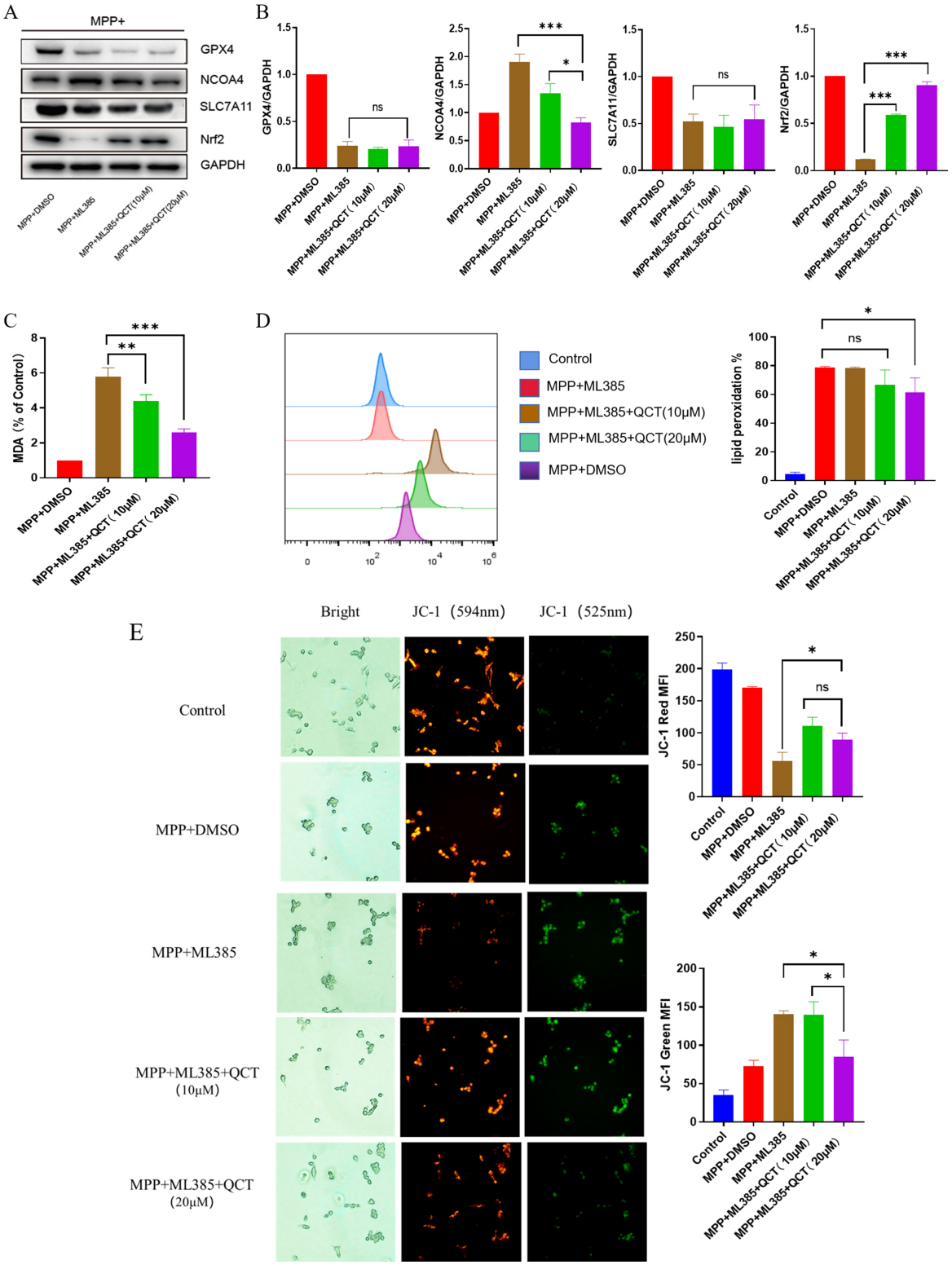

Fig. 6. Nrf2 is protective in the effect of quercetin against MPP^+-induced cytotoxicity. (A,B) Western blot detection of Nrf2 and several ferroptosis-related proteins (NCOA4, SLC7A11, and GPX4). (C) MDA levels in SH-SY5Y cells. (D) Lipid peroxidation in SH-SY5Y cells was determined by C11-BODIPY staining. (E) Fluorescent staining JC-1 of SH-SY5Y cells. QCT: 10μM or 20μM quercetin was added as indicated. ML385 is a specific inhibitor of Nrf2. Each experiment was performed three times independently. Data are expressed as mean $\pm$ SD. *$p < 0.01$, **$p < 0.01$, ***$p < 0.001$.

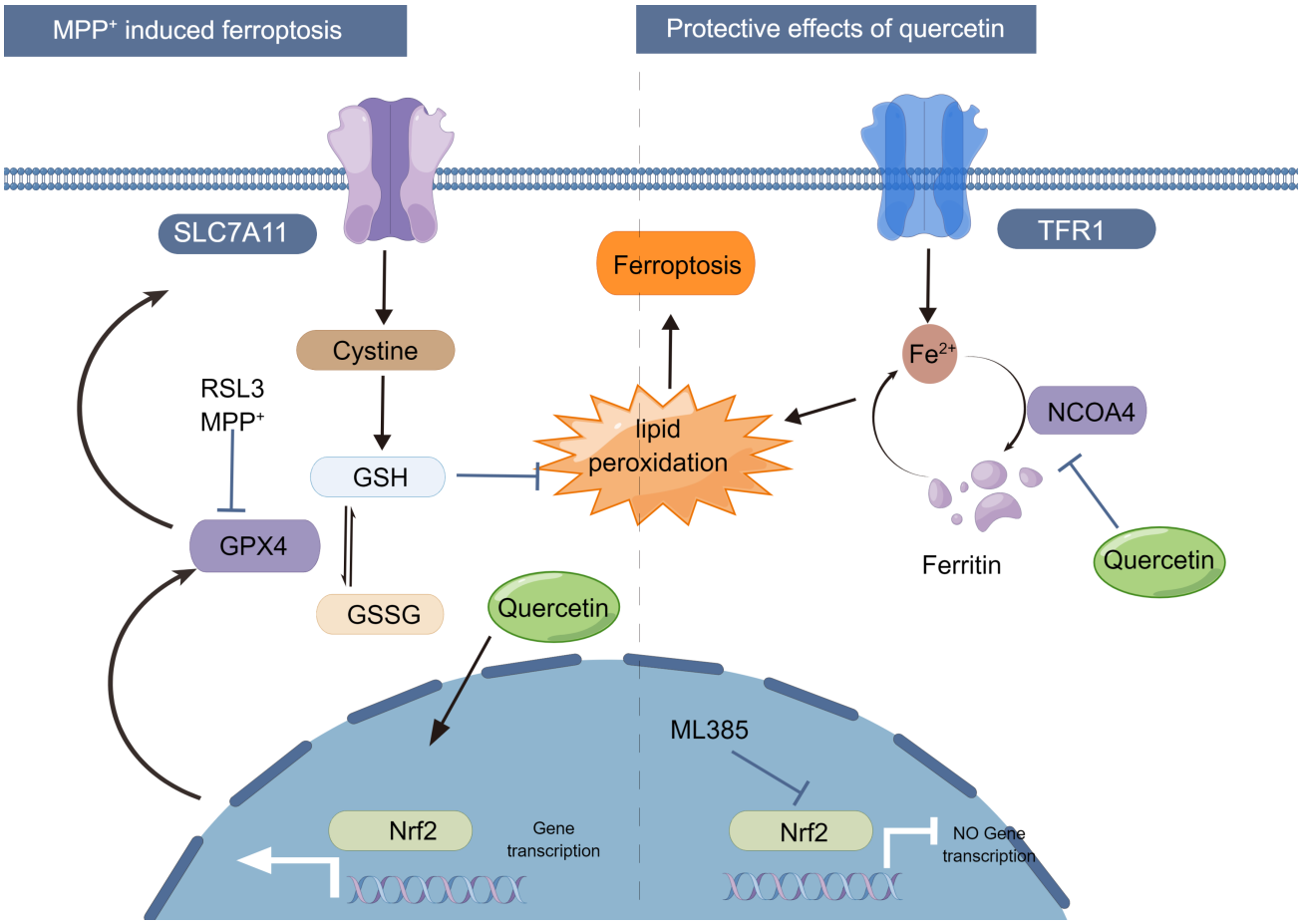

Fig. 7. Proposed mechanism for the protective effect of quercetin on MPP^+-induced ferroptosis.

tosis is a non-apoptotic cell death characterized by lipid peroxidation and iron overload [47]. MPP^+-induced PD cells are characterized by mitochondrial contraction. Mechanistically, MPP^+ induced low expression of GPX4 and high expression of transferrin receptor 1 (TfR1) in PD cells, and desferrioxamine (DFO) could protect neurons *in vitro* by blocking the production of iron-assisted oxidative free radicals and inhibiting ferroptosis [48]. Several iron chelators are clinically approved by the US Food and Drug Administration (FDA) for the treatment of anemia and iron overload diseases. MPP^+ can cause lipid peroxidation, oxidative stress, and excessive production of ROS, eventually triggering cell death and leading to damage or loss of DA neurons. Nonetheless, MPP^+-induced cell death could be rescued by Fer-1. The results of the present study suggest that the ferroptosis phenotype is involved in MPP^+-induced cell death and that cell death can be rescued by quercetin at concentrations of 10 μM and 20 μM. Interestingly, quercetin was more effective in SH-SY5Y cells than in primary neurons. Furthermore, treatment with 20 μM quercetin resulted in better anti-apoptotic outcomes compared to treatment with 5 μM Fer-1, suggesting that quercetin may also affect cell viability through the ferroptosis pathway.

Previous studies have shown that MDA and Fe^{2+} overload lead to ferroptosis [49,50]. In this study, MPP^+ induction significantly increased the intracellular MDA and iron concentrations, and this regulation was reversed by quercetin. GPX4 is a key protein that regulates lipid peroxidation. The present study showed that induction of MPP^+ significantly reduced intracellular GPX4 expression, leading to lipid peroxidation and promoting an altered ferroptosis phenotype.

To further evaluate the effect of quercetin against ferroptosis, the iron atrophy agonist ML385 was used to treat SH-SY5Y cells to induce a classical ferroptosis phenotype. Again, it was found that quercetin could counteract this process and exert a neuroprotective effect on cell viability. Therefore, we suggest that quercetin may protect neuron cells by modulating the ferroptosis phenotype. Many studies have also shown that the flavonoid quercetin exerts antioxidant, anti-inflammatory, and anti-proliferative properties [51]. These may be exerted through several mechanisms, including direct effects on signal transduction pathways [52]. Previous studies have shown that quercetin can enhance the antioxidant capacity of the inherent antioxidant gene *Nrf2* and its downstream GPx, reduce the excessive production of MDA, and maintain cellular redox proper-

ties [53]. Studies have also shown that quercetin can reduce inflammation and oxidative stress by activating the Nrf2 signaling pathway to promote cell proliferation, upregulate SLC7A11, and reduce ferroptosis [54,55]. Therefore, SLC7A11 might be a potential target for a single pathway via Nrf2 [56–58].

In this study, MPP^+ induced NCOA4, one of the contributors to ferroptosis, and reduced Nrf2. SLC7A11 (SLC7A11 solute carrier family 7 member 11, also known as xCT) is the 11th member of the solute carrier family 7 and belongs to the cystine/glutamate reverse transporter proteins, which are mainly involved in amino acid transport across the plasma membrane. Our protein expression assay of SLC7A11 was designed to show that the mechanism of cell protection by QCT may involve multiple antioxidant proteins downstream of NRF2, and activation of NRF2 can upregulate SLC7A11, thus protecting metastatic cells from ferroptosis. These observations suggest that MPP^+ can regulate the ferroptosis-related signaling pathway. Nrf2 is an essential transcription factor that participates in antioxidant and anti-inflammatory activities by interacting with various signaling pathways [59,60]. In the present study, we found that MPP^+ treated SH-SY5Y cells showed reduced Nrf2 protein expression. Moreover, a dose-dependent increase in Nrf2 expression was observed with increasing concentrations of quercetin, which is consistent with the protective effect of quercetin. Despite treatment with ML385, low levels of Nrf2 expression were observed. One of the aims of this study was to determine whether Nrf2 was essential for quercetin to exert its neuronal protection. The results showed that pretreatment with ML385 had inhibitory effects on anti-ferroptotic and antioxidant effects of quercetin. Therefore, NRF2 activity is essential to defend against damage to MPP^+-SH-SY5Y cells caused by oxidative stress and ferroptosis.

In conclusion, the Nrf2/GPX4 pathway was found here to regulate lipid ROS levels, leading to ferroptosis. Quercetin was able to reduce the effect of MPP^+ on SLC7A11 and Nrf2 expression levels in a dose-dependent manner, thereby inhibiting ferroptosis. Previous studies have shown that promotion of Nrf2 by NRF2 agonists or natural drugs prevents neurodegeneration [61,62]. On this basis, the present study proposes that quercetin can act as an agonist of the Nrf2/GPX4 pathway to resist the process of lipid peroxidation, thus exerting a protective effect on cells, which may be a new treatment strategy for PD.

The present study has a few limitations. First, no *in vivo* studies were conducted. In addition, since the brain is protected by the blood-brain barrier (BBB), which acts as a vital boundary between neural tissue and the circulating blood, new drugs proposed for the treatment of PD need to penetrate the blood-brain barrier and the blood–spinal cord barrier. Our *in vitro* experience also suggests that different concentrations of drugs are needed to mimic any potential *in vivo* effects.

5. Conclusions

The current study has shown that quercetin treatment reduces oxidative stress and inhibits ferroptosis in an MPP^+-induced cell model of PD, and that the Nrf2/GPX4 pathway played a key role in the protective effect exerted by quercetin on MPP^+-treated SH-SY5Y cells. The present findings may provide new research strategies for treating PD.

Abbreviations

PD, Parkinson's disease; DA neuron, dopaminergic neuron; α-syn, α-Synuclein; MPP^+, 1 - methyl - 4 - phenylpyridine; Nrf2, nuclear factor erythroid-2-related factor 2; GPX4, glutathione peroxidase 4; ARE, antioxidant response element; ELISA, enzyme-linked immunosorbent assay; WB, Western blotting; FITC, fluorescein isothiocyanate; CCK-8, Cell Counting Kit-8; MtMP, Mitochondrial membrane potential; GSH, glutathione; MDA, malondialdehyde; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 member 11; NCOA4, Nuclear receptor coactivator 4; ML385, a Nrf2 inhibitor; RSL3, an inhibitor of GPX4 (ferroptosis activator).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

DH is the recipient of fundings and supervise the present project, as well as reviewed and edited the manuscript; FY administrated the project, wrote and reviewed the manuscript; WY performed formal analysis, data curation and supervision; HX made the investigation and visualization; YJ performed the methodology, data resources collection, data validation, and writing the original draft; GX performed the data validation and resources collection; softwares application was in charge of AA. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2803042.

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