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Original Article

Targeting Nfr2/ARF signaling is important for mangiferin protecting PC12 cells against hydrogen peroxide-induced apoptosis

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Abstract: Oxidative damage is critically associated with the pathogenesis of Parkinson's disease (PD), implying that screening natural product and pharmacological targeting of the antioxidant machinery need further attention. Mangiferin, a natural flavonoid compound, belongs to polyphenols which are the predominant group of natural antioxidants showing powerful antioxidant activity. Therefore, we aim at investigating the neuroprotective role of mangiferin and to clarify the mechanism of action against H_2O_2-induced neurotoxicity in PC12 cells. Mangiferin treatment caused an obvious increase in nuclear factor E2-related factor 2 (Nrf2) protein expression and protein level of antioxidant response element (ARE) pathway genes in PC12 cells. Exposure of PC12 cells to mangiferin provided protection against H_2O_2-induced oxidative injury that was dependent on Nrf2, since treatment with Nrf2 siRNA failed to block against H_2O_2 neurotoxicity or induce Nrf2-dependent cytoprotective genes in PC12 cells. These results indicated that activation of Nrf2/ARE signaling by mangiferin is strongly associated with its neuroprotective effects against H_2O_2 neurotoxicity and suggested that targeting the Nrf2/ARE pathway may be a promising target for PD therapy.

Keywords: Mangiferin, Nfr2/ARF, ROS, apoptosis, PC12 cell

Introduction

Increasing evidence reports that oxidative stress is critically relevant with the pathogenesis of Parkinson's disease (PD); pharmacological targeting of the antioxidant agent is drawing much attention. PD is characterized by progressive and selective loss of dopaminergic neurons in substantia nigra pars compacta (SNc) [1-3], but the pathogenesis remains unclear. Nuclear factor erythroid-2 related factor 2/antioxidant response element (Nrf2/ARE) pathway is considered as a promising pathway that confers protection to a variety of oxidative stress-related neurodegenerative insults [4-6]. The transcription factor Nrf2 is a key regulator in the coordinated induction of a battery of cytoprotective genes, including those encoding for endogenous antioxidant such as hemeoxygenase (HO-1), glutathione cysteine ligase regulatory subunit (GCLC) and glutathione cysteine ligase modulatory subunit (GCLM) [7, 8].

Polyphenols are the predominant group of natural antioxidants showing powerful antioxidant activity due to inducing gene expression of antioxidant enzymes, free radical scavenging effect, hydrogen donating, singlet oxygen quenching and so on [9-11]. Mangiferin (2-C-β-D-glucopyranosyl-1, 3, 6, 7-tetrahydroxyxanthone) derived commonly from the plants of Anacardiaceae and Gentianaceae families can provide a unique and underutilized source of potential therapeutic agent [12, 13]. This compound is mainly isolated from mango tree (*Mangifera indica* L.) which is widely distributed, and has been taken into use long time. Mangiferin was employed to treat disorders like melancholia, bleeding dysentery and others in different corners of the world [14-16]. Accumulating studies investigated mangiferin's antioxidant [17, 18], antitumor [19, 20], antidiabetic [21, 22] and immunomodulatory activities [23, 24]. The objective of the study is to investigate whether Naringenin can decrease oxidative

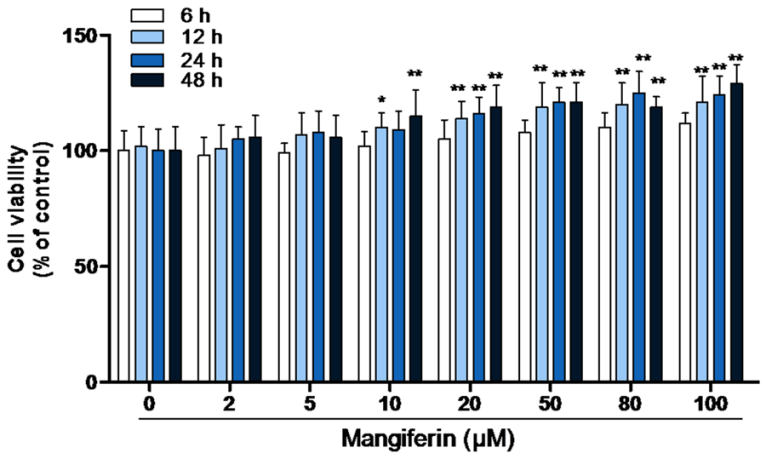


Figure 1. Effect of mangiferin on cell proliferation of PC12 cells. After treated with mangifein (0-100 μM) for 6, 12, 24 and 48 h, CCK-8 assay was employed to identify the cell viability of PC12 cells. Data are expressed as mean ± SD, n = 6, **P* < 0.05; ***P* < 0.01, *versus* control.

damage in PC12 cellular and how Nrf2-ARE pathway is involved.

Materials and methods

Cell viability

Cell viability was detected by Cell Counting Kit (CCK)-8 kit (Zhongze Biotech, Shanghai, China). Briefly, 4 × 10^3 cells were seeded in each 96-well plate, and further incubated for 6, 12, 24 and 48 h, respectively. CCK-8 reagent was added to each well at 1 h before the endpoint of incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader. Experiments were repeated at least three times each time in triplicate.

Apoptosis

With mangiferin (0, 20 and 80 μM) treatment for 2 h, hydrogen peroxide (H_2O_2, 200 μM) insulted PC12 cells were stained with annexin V-fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Detection of Reactive Oxygen Species (ROS)

Detection of ROS was performed by flow cytometric analysis as described previously. In brief, cells (5 × 10^4 cells/well) were cultured in 24-well plate with mangiferin (0, 20 and 80 μM) treatment for 2 h, and hydrogen peroxide (H_2O_2, 200 μM) insulted PC12 cells after a period of exposure (12 h). Cells were washed with PBS and resuspended in complete medium followed by incubation with 0.5 μM dihydrorhodamine 123 (Sigma) for 30 min at 37°C. ROS fluorescence intensity was determined by cytometry with excitation at 490 nm and emission at 520 nm.

miRNA transfection

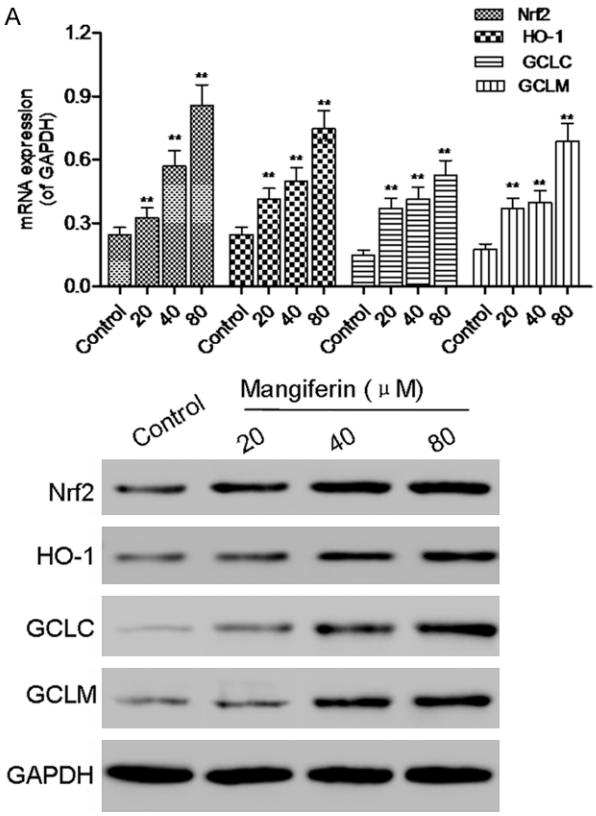
Cells were seeded in antibiotic-free medium the day before transfection. The cells were transfected with 50 nmol/L of Nrf2-siRNA or negative control by using lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the instructions provided by the manufacturer. After 48 h, the transfected cells were collected and processed for quantitative real-time PCR (qPCR) and western blot analysis.

Western blot

Cultured or transfected cells were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (Jrdun, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rcf for 10 min at 4°C and the supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Zhongze, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies against Nrf2 (Abcam), GAPDH (Fermentas), HO-1 (Abcam), GCLC (Abcam), GCLM and caspase-3 (CST) and caspase-9 (CST). Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China).

Statistical analysis

All results are presented as the mean ± SD and the data were analyzed by a SPSS 13.0 statistical package (SPSS Inc., Chicago, IL). Data for multiple comparisons were subjected to one-way ANOVA followed by Dunnett's test and a



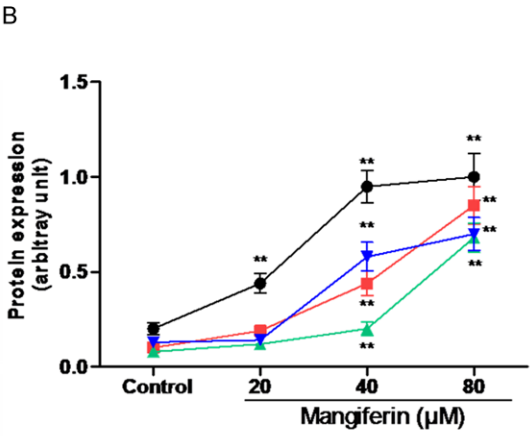


Figure 2. Mangiferin facilitates Nrf2 and its down-stream targets. A. After mangiferin treatment for 3 h, mRNA expression of Nrf2, HO-1, GCLC and GCLM was evaluated by RT-PCR. Data are expressed as mean ± SD, n = 6, $^{**}P < 0.01$, *versus* control. B. After treated with mangifein (0, 20, 40 and 80 μM) for 24 h, western blot was used for determining the protein level of PC12 cells. Data are expressed as mean ± SD, n = 6, $^{**}P < 0.01$, *versus* control.

value of $P < 0.05$ was considered statistically significant.

Results

Mangiferin promotes the proliferation of PC12 cells

We firstly determined the effect of mangiferin (0-100 μM) on the cell viability of PC12 cells by CCK8 assay as previously described. As displayed in **Figure 1**, we found that mangiferin could effectively stimulate the cell proliferation of PC12 cells in a time- and dose-dependent manner. 20 μM of mangiferin could remarkably increase the cell viability compared with the control group at 6, 12, 24 and 48 h. While, there was no significant difference in cell viability between the 80 μM and 100 μM group. Herein, 20, 40 and 80 μM of mangiferin were carried out for the further study.

Mangiferin increased the expression of Nrf2 and its downstream targets

The transcription factor Nrf2 is a key regulator in the coordinated induction of several cytoprotective genes, including those encoding for endogenous antioxidant such as HO-1, GCLC and GCLM. The effect of mangiferin on the expression of Nrf2, HO-1, GCLC and GCLM was determined by RT-PCR and western blot. After mangiferin treatment for 3 h, mRNA expressions of Nrf2, HO-1, GCLC and GCLM were obviously increased compared with the control group (**Figure 2A**). Protein level of Nrf2, HO-1, GCLC and GCLM was determined by western blot after mangiferin intervention for 6 h. The **Figure 2B** exhibited that Nrf2, HO-1, GCLC and GCLM expressions were all improved notably in comparison with that of control group.

Mangiferin protected the PC12 cells against the H_2O_2-induced apoptosis

We further demonstrated the protective effect of mangiferin on H_2O_2-insulted cells. After 24 h of hydrogen peroxide treatment, cell apoptosis was detected by flow cytometry. The **Figure 3** showed that hydrogen peroxide remarkably induced the apoptosis of PC12 cells (40.25 ± 6.3%) compared with the control group (3.12 ± 0.52%). 20 and 80 μM of mangiferin could decrease the apoptosis rate to 33.21 ± 4.2% and 20.17 ± 2.56% respectively.

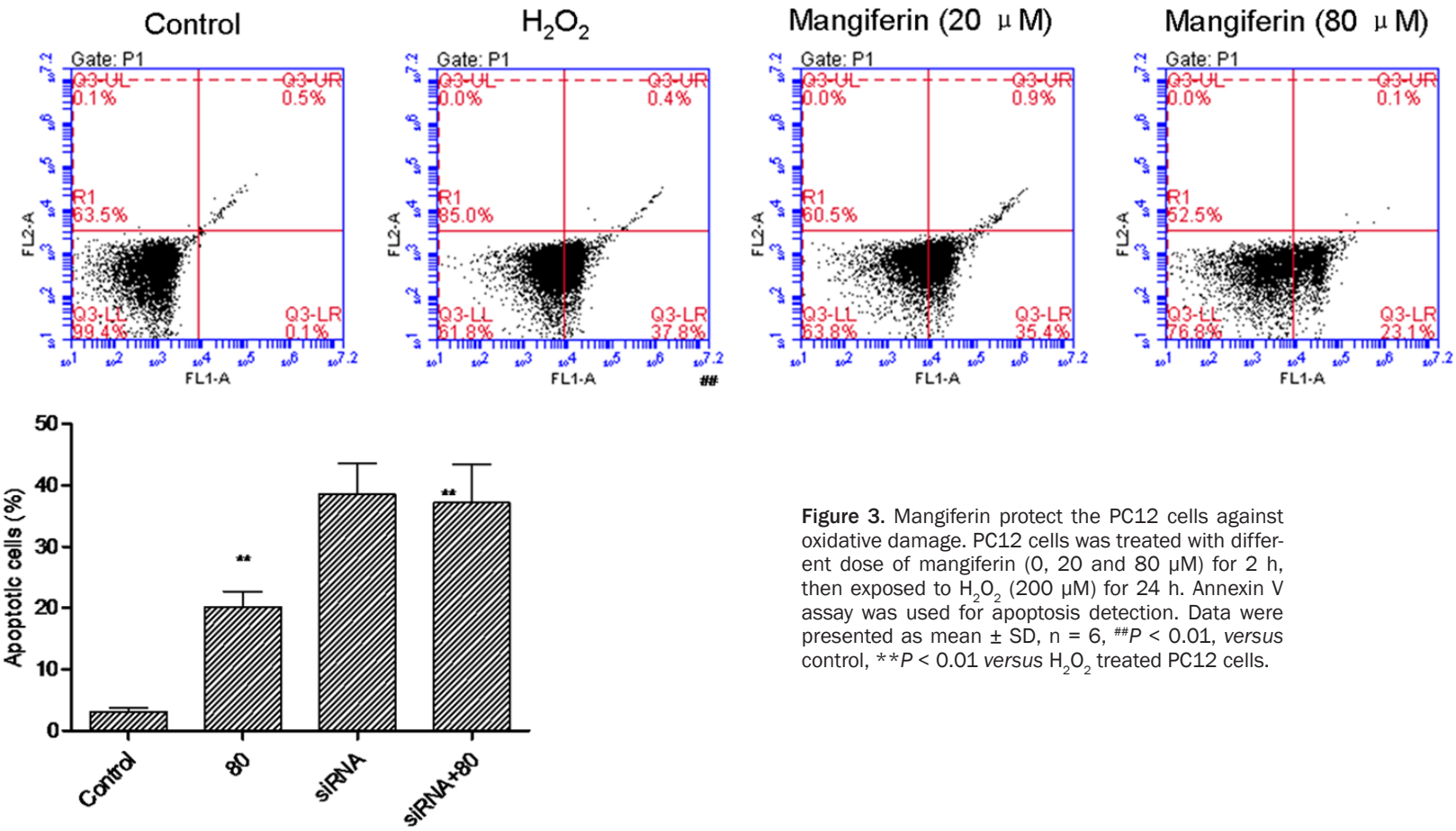


Figure 3. Mangiferin protect the PC12 cells against oxidative damage. PC12 cells was treated with different dose of mangiferin (0, 20 and 80 μM) for 2 h, then exposed to H_2O_2 (200 μM) for 24 h. Annexin V assay was used for apoptosis detection. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control, $^{**}P < 0.01$ *versus* H_2O_2 treated PC12 cells.

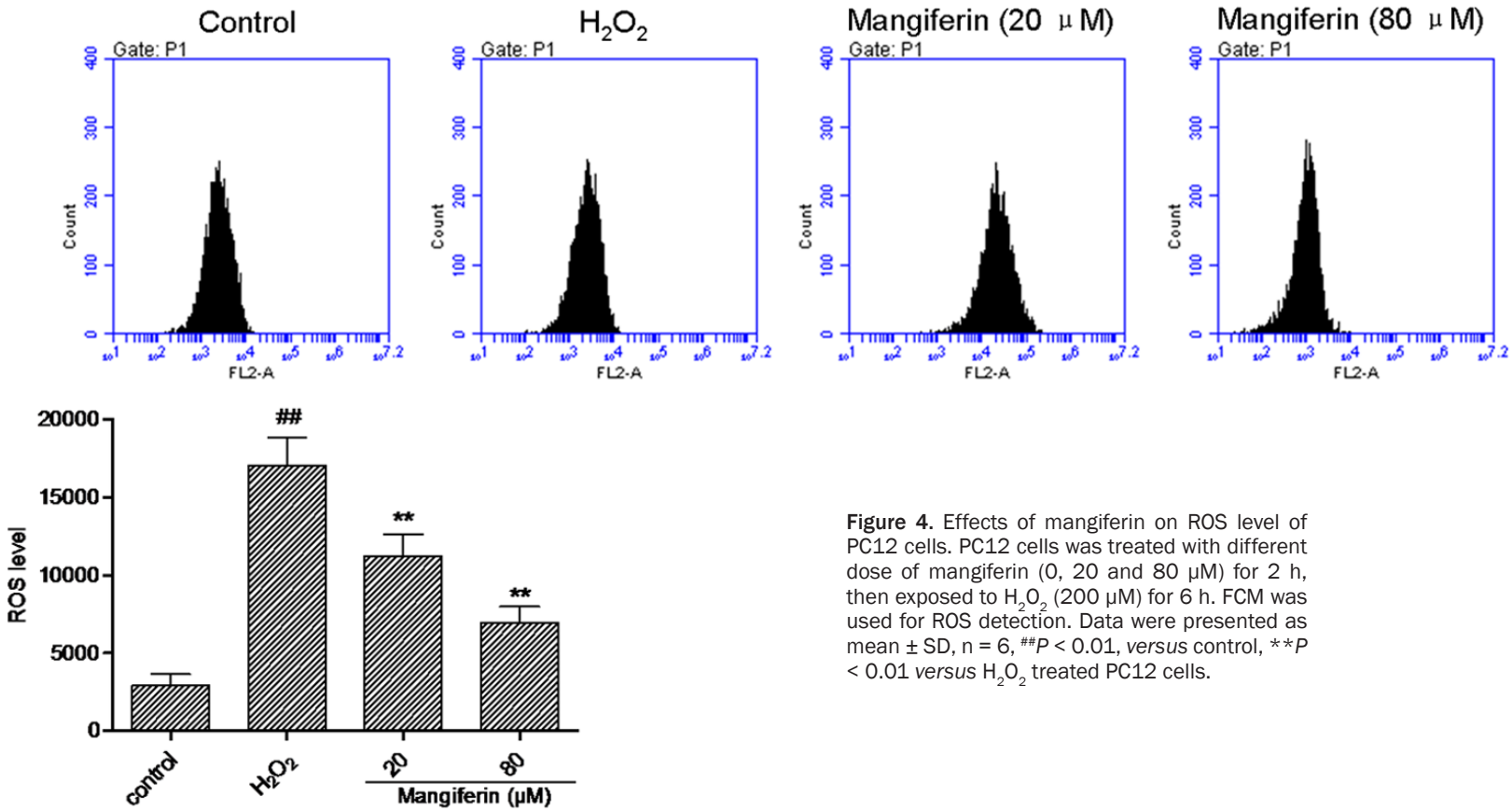


Figure 4. Effects of mangiferin on ROS level of PC12 cells. PC12 cells was treated with different dose of mangiferin (0, 20 and 80 μM) for 2 h, then exposed to H_2O_2 (200 μM) for 6 h. FCM was used for ROS detection. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control, $^{**}P < 0.01$ *versus* H_2O_2 treated PC12 cells.

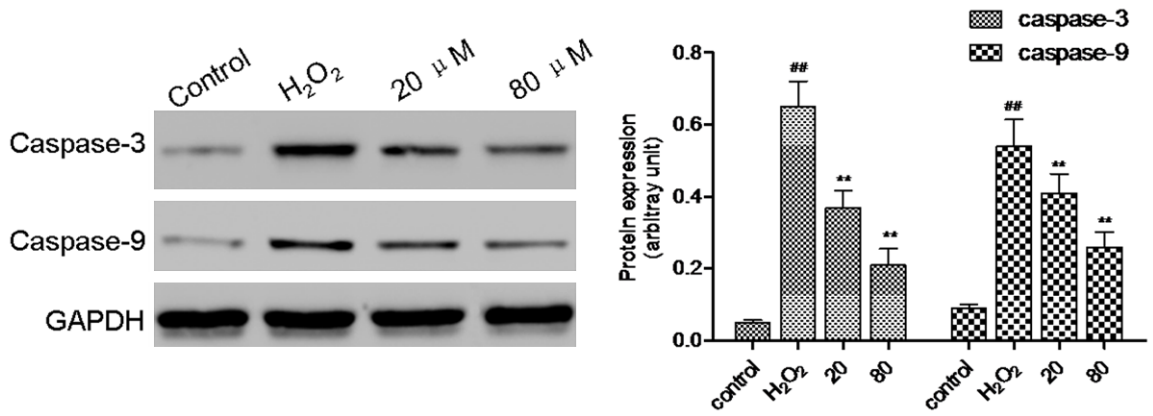


Figure 5. Mangiferin suppressed casapase-3 and caspase-9 expression in H_2O_2 insulted cells. PC12 cells was treated with different dose of mangiferin (0, 20 and 80 μM) for 2 h, then exposed to H_2O_2 (200 μM) for 24 h. Western blot assay was used for protein expression detection. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control, $^{**}P < 0.01$ *versus* H_2O_2 treated PC12 cells.

Mangiferin decreased the ROS level in H_2O_2-insulted cells

ROS overproduction probably results in mitochondria apoptosis. The breakdown of the mitochondrial membrane potential is an early stage of the apoptotic process. FCM was used to determine the levels of ROS production in PC12 cells. As shown in **Figure 4**, H_2O_2 significantly caused an increase level of ROS production, while mangiferin (20 and 80 μM) could effectively decrease the ROS level compared with the H_2O_2 treated group.

Effect of mangiferin on caspase-3 and caspase-9 expression

Caspase-3 is a key downstream effecter of the cysteine protease family that is involved in both the mitochondrial apoptotic pathway and the death receptor pathway. Caspase-3 and caspase-9 are regarded as apoptosis-related proteins. Protein level of caspase-3 and caspase-9 was evaluated by western blot assay. H_2O_2 significantly caused an increase of caspase-3 and caspase-9 in PC12 cells compared with the control groups. Pretreated with the mangiferin (20 and 80 μM) for 2 h effectively revise the increase of caspase-3 and caspase-9 in PC12 cells (**Figure 5**).

Mangiferin protected the PC12 cells against the H_2O_2-induced apoptosis via Nrf2 signaling

With the aim of determining the protective effect of mangiferin against oxidative damage via Nrf2 signaling, we then detected the protective effect of mangiferin on siRNA-Nrf2 cells and normal cells. As shown in **Figure 6A**, mRNA of Nrf2 was expression low in PC12 cells after RNA interference treated for 48 h. Normal and siRNA-Nrf2 cells were pretreated with mangiferin (80 μM) for 2 h and then exposed to H_2O_2 for 24 h. As shown in **Figure 6B**, 80 μM of mangiferin dramatically increased the protein level of Nrf2 and its downstream protein in non siRNA cells compared with the control group. On the other hand, protein level of Nrf2 and its downstream protein in mangiferin (80 μM) pretreated siRNA-Nrf2 cells was decreased, and there is no significant difference of protein level between mangiferin (80 μM) pretreated siRNA-Nrf2 cells and siRNA-Nrf2 cells.

As for the apoptosis between normal cells and siRNA-Nrf2 cells, the apoptosis rate of H_2O_2 treated siRNA-Nrf2 group was decreased compared with the control group. Mangiferin (80 μM) effectively attenuated the apoptosis of H_2O_2-insulted normal cells. However, mangiferin (80 μM) pretreatment didn't make remarkable decrease in H_2O_2-insulted siRNA-Nrf2 group (**Figure 6C**).

Discussion

Apoptosis of neurocytes directly promotes the level of oxidative stress, which is regarded as one of the most sensitive biological markers, caused by the imbalance between ROS production and antioxidant system [25-27]. In the present study, we evaluated the cytotoxic effects of H_2O_2 and the protective effects of mangiferin by CCK8 assay. It is reported that

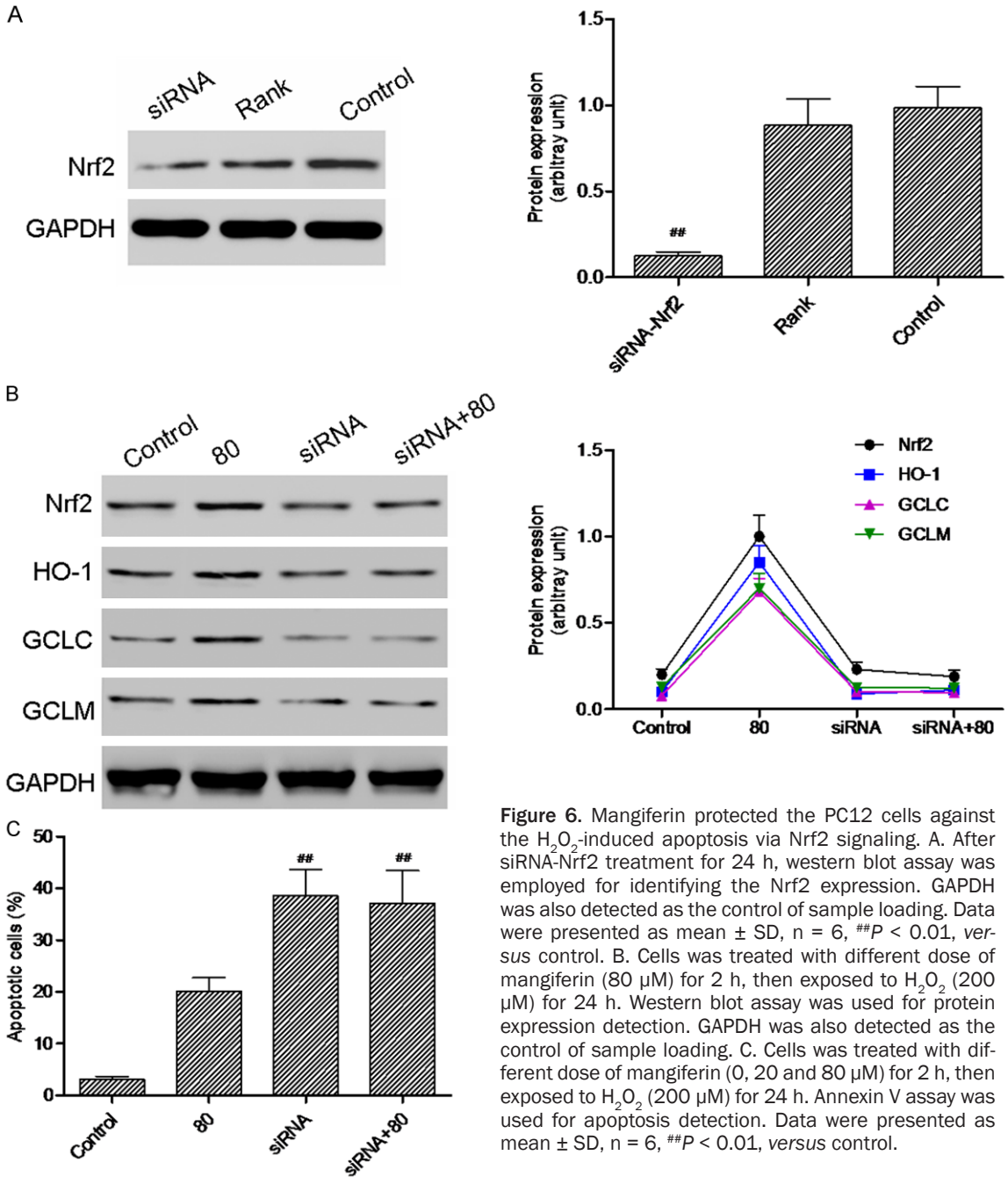


Figure 6. Mangiferin protected the PC12 cells against the H_2O_2-induced apoptosis via Nrf2 signaling. A. After siRNA-Nrf2 treatment for 24 h, western blot assay was employed for identifying the Nrf2 expression. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control. B. Cells was treated with different dose of mangiferin (80 μM) for 2 h, then exposed to H_2O_2 (200 μM) for 24 h. Western blot assay was used for protein expression detection. GAPDH was also detected as the control of sample loading. C. Cells was treated with different dose of mangiferin (0, 20 and 80 μM) for 2 h, then exposed to H_2O_2 (200 μM) for 24 h. Annexin V assay was used for apoptosis detection. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control.

mangiferin can be indicated as a therapeutic agent in oxidative stess-mediated renal toxicity [28]. Mangiferin has been reported to attenuate diabetic nephropathy by reducing oxidative injury mediated cell signaling, TNF-α related and mitochondrial dependent apoptotic pathways in streptozotocin-induced diabetic rats [29]. Mangiferin also can attenuate methylmercury induced cytotoxicity against human neuroblastoma cells by decreasing oxidative stress and suppressing the level of free radicals [30]. Mitochondria plays an important part in the process of cell apoptosis and the increasing level of ROS induced by oxidative stress could cause the mitochondrial membrane potential loss, which subsequent contribute to open mitochondrial permeability transition pore [31]. Increased mitochondrial permeability gives rise to the release of cytochrome c from the mitochondria [32]. The release of cytochrome c trig-

gers the activation of caspase-9 and caspase-3, and activated caspase-3 directly induces cell apoptosis [33]. In our study, we found that mangiferin (20 and 80 μM) effectively reduced the ROS production and decreased the caspase-3/-9 expression compared with the H_2O_2 treated cells, which is the evidence that mangiferin protect PC12 cell against oxidative injury.

Mangiferin also stimulates Nrf2 transcription and its downstream antioxidant pathways. Nrf2/ARE pathway is considered as a promising pathway that confers protection to a variety of oxidative stress-related neurodegenerative insults. We showed that mangiferin activated Nrf2 pathway in PC12 cells by western blot. The protective effect of mangiferin against H_2O_2 insulted injury in PC12 cells could be blocked by knockdown using Nrf2 siRNA. As shown in **Figure 6B** and **6C**, expression of HO-1, GCLC and GCLM were all decreased in siRNA-Nrf2 cells compared with the control group. There is no significant difference between siRNA group and siRNA + mangiferin (80 μM) group. As a result, targeting the Nrf2/ARE pathway is a favorable approach for PD therapy.

In conclusion, this study suggested that mangiferin could attenuate H_2O_2 induced oxidative injury in PC12 cells, which was realized by its ability of increasing the cell viability and reduce ROS overproduction. The effectiveness of mangiferin against H_2O_2-induced oxidative stress due to activate Nrf/ARE signaling. Taken together, our findings demonstrated that mangiferin can exhibit neuroprotective effect against oxidative stress induced neurodegenerative disorders, and targeting the Nrf2/ARE signaling is a favorable treatment for PD treatment. Further study is needed to determined the activity of mangiferin and clarify the underlying mechanisms involved in vivo.

Disclosure of conflict of interest

None.

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