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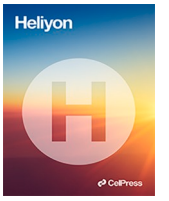

Research article

Dihydroquercetin regulates HIF-1α/AKT/NR2B signalling to improve impaired brain function in rats with metabolic syndrome

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ABSTRACT

Dihydroquercetin (DHQ) is commonly used as a dietary additive, but its activity in improving brain injury with metabolic syndrome (MS) remains known. In present study, the MS rat model was induced using 10 % fructose water. The apoptosis rate of primary brain cells was detected. The HIF-1α/AKT/NR2B signalling pathway, levels of KEAP1/NRF2, HO-1 and NQO-1 were detected. *In vitro* experiments were performed using H_2O_2-stimulated PC-12 cells. The effect of DHQ on rates of cell survival and apoptosis were detected. After silencing HIF-1α, we further elucidate the mechanism of action of DHQ. The results indicated that DHQ reduced the hyperactivity and inhibited oxidative stress via increasing the levels of HIF-1α/AKT/NR2B signalling pathway, whereas regulated KEAP1/NRF2 pathway. *In vitro* experiments showed that the HIF-1α plays an important role in this process. Overall, DHQ may improve impaired brain function in rats with metabolic syndrome by regulating the HIF-1α/AKT/NR2B signalling pathway.

1. Introduction

In recent years, metabolic syndrome (MS) has been regarded as a serious health problem in both developed and developing countries. MS is associated with glucose intolerance, insulin resistance (IR), obesity, dyslipidemia, and hypertension [1]. MS also increases the risk of brain function impairment [2–4]. Accumulating evidence hints at the contribution of MS to the development and progression of cognitive impairment. IR is at the core of MS and likely represents the key link between MS and cognitive impairment [5–7].

Some scholars have reported that hypoxia-inducible factor 1α (HIF-1α) plays a key role in brain injury [8,9]. Up-regulation of HIF-1α can protect nerves and improve brain injury associated with diabetes [10]. Furthermore, it can reduce brain cell apoptosis and oxidative stress in cerebral ischemia injury [11]. Elevated HIF-1α is associated with elevated heat shock protein 70 (HSP70) levels. HSP70 is a downstream molecular chaperone of heat shock factor 1 (HSF1) and regulates phosphoinositide-3-kinase (PI3K)/p-Protein kinase B (AKT) to attenuate apoptosis [12]. The N-methyl-D-aspartic acid receptor 2B (NR2B) subunits are essential for synaptic plasticity and cognitive functions. Some studies suggest that underlying mechanisms of NR2B are associated with PI3K/Akt signalling pathways [13,14].

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The health benefits of natural products have long been recognized. Regular consumption of dietary polyphenols is associated with reduced risk of several chronic diseases. Flavonoids are used as a family of polyphenol compounds that are used as dietary antioxidants and biologically active additives [15–17]. Dihydroquercetin (DHQ) is one of the most important dihydroflavone compounds which is commonly found in *Larix sibirica Ledeb. (Pinaceae)* and *Pseudotsuga taxifolia (Lamb.) Britton (Pinaceae)*. It was reported that DHQ displayed anticancer and neuroprotective properties [18]. However, there are no reports on the effects of DHQ in brain injury associated with metabolic syndrome [19,20]. Our previous study showed that DHQ can significantly reduce the blood pressure and insulin resistance index and improve the blood lipid disorder and renal injury in MS rats by activating the PI3K signalling pathway [21]. However, whether DHQ can also improve the cognitive impairment in MS, remains unknown. Therefore, the present study aimed at investigating the mechanisms underlying DHQ effects on brain dysfunction *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

DHQ (480-18-2) was purchased from Hangzhou Shangjie Chemical Co., Ltd. (Zhejiang, China). The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel preparation kit was purchased from Bio-Rad Laboratories (Hercules, USA). Anti-rabbit and anti-mouse secondary antibodies were purchased from LI-COR (Nebraska, USA). The bicinchoninic acid (BCA) protein concentration determination kit was purchased from Beijing Solarbio Science Technology Institute (PC0020, Beijing, China).

2.2. Animals and treatments

Male SHR and Wistar-Kyoto (WKY) rats (8 weeks old), weighing approximately 190 g, were purchased from Vital River Laboratory Animal Technology (Beijing, China). Animals were kept at 25 °C with 12 h light/dark cycles and housed in groups of four rats per cage. After three days of feeding, the rats were divided into the following groups with the indicated oral administrations (n = 10): 10 mL/kg/day of distilled water for the WKY rats (normal control group WKY); free consumption of 10 % fructose water for SHR (model control group SHR-FW); 25 mg/kg/day and 50 mg/kg/day of DHQ for the SHR with free consumption of 10 % fructose water (SHR-FW + DHQ-L group and SHR-FW + DHQ-H group, respectively). The rats in the SHR-FW + DHQ-L and SHR-FW + DHQ-H groups received 25 mg and 50 mg of DHQ by gavage, and the rats in the other groups received normal saline 1 mL/100 g by gavage for Seven weeks. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Henan University of Chinese Medicine, and experiments were approved by the Animal Ethics Committee of Henan University of Chinese Medicine.

2.3. Open field test

After seven weeks, each rat was placed at the centre of a black acrylic box (50 cm × 50 cm × 40 cm), and the total distance travelled and total movement in 5 min were recorded based on the mid-point of the rat's body. The experimental system (OFT-100) used cameras and data analysis software provided by Chengdu Taimeng Co., Ltd. The behavioural tests were carried out in a soundproof room.

2.4. Accelerated rotarod test

Accelerated rotarod test was performed to test the motor coordination and limb strength of rats. Rats were placed on an on a six-lane accelerating rotarod. When the rotarod was started, the timing was started, and the rotational speed of the rotarod was gradually increased during the period until the rat fell off the rotarod and the timing ended. Before the formal test, all rats were trained for 3 days to adapt the new surroundings. The experimental system (YLS-31B) was provided by Jinan Yiyan Co., Ltd.

2.5. Brain samples

The animals were euthanized with pentobarbital immediately after the open field tests and accelerated rotarod tests. Brains were quickly dissected out. Flow cytometry and Histomorphological examination were performed. The rest of the brains were stored in liquid nitrogen.

2.6. Flow cytometry

Flow cytometry was used to determine the apoptosis of hippocampal primary cells using the fluorescein isothiocyanate-propidium iodide (FITC-PI) Apoptosis Detection Kit (Becton, Dickinson Company). Dissected brain tissues were washed with phosphate-buffered saline (PBS) and chopped with scissors. Cells were collected by centrifugation at 150×*g* for 5 min. Erythrocytes were lysed by adding 1 mL erythrocyte lysis buffer for 5 min, and the debris were removed by centrifugation at 150×*g* for 5 min. Cells were was resuspended in 10 mL PBS and filtered using a 75-micro mesh filter. The filtrate was transferred to centrifuge tubes, centrifuged at 150×*g* for 5 min and brain primary cells were collected. After resuspension in PBS, cells were incubated in 100 μL labelling solution (5 μL of PI, 5 μL of FITC and 90 μL of 10 × binding buffer) in dark at room temperature for 15 min. Subsequently, 400 μL of 10 × binding buffer was added to terminate the staining reaction. FITC positive cells were defined as apoptotic cells and analyzed. Flow cytometry analyses were

performed using Fluorescence-activated cell sorting (FACS) AriaIII (BD Biosciences, USA).

2.7. Histomorphological examination

After dissection, the brian were fixed in 4 % paraformaldehyde overnight, embedded in paraffin, sectioned at a thickness of 5 μm, and placed on separate glass slides [22]. After haematoxylin-eosin (HE) staining, the brian sections were routinely observed under light microscopy (NIKON Eclipse ci, Tokyo, Japan).

2.8. Western blotting

The cytosolic proteins from the brain tissue were isolated using a cytoplasmic-membrane protein extraction kit (Beijing Applygen Technologies Institute, Beijing, China). Protein samples (50 μg) from each group were loaded onto 10 % SDS-PAGE, transferred to Polyvinylidene fluoride (PVDF) membranes (Merck Millipore Ltd, Germany), and incubated with primary antibodies against HIF-1α (20960-1-AP, 1:1000, Proteintech, China), HSP70 (ab181606, 1:1000, Abcam, UK), *p*-AKT (ab38449, 1:500, Abcam, UK), AKT (60203-2-Ig, 1:500, Proteintech, China), GTPase dynamin-related protein 1 (Drp1) (ab184247, 1:500, Abcam, UK), Mitochondrial fission factor (MFF) (ab129075, 1:500, Abcam, UK), PI3K (60225-1-Ig, 1:500, Proteintech, China), NR2B (21920-1-AP, 1:500, Proteintech, China), HSF-1(16107-1-AP, 1:1000, Proteintech, China), KEAP1 (A1820, 1:1000, ABclonal, China), NRF2 (A0674, 1:1000, ABclonal, China), HO-1 (A1346, 1:1000, ABclonal, China), NQO-1 (A0047, 1:1000, ABclonal, China) and β-actin (66009-1-Ig, 1:5000, Proteintech, China), overnight at 4 °C. After washing five times with Tris-buffered saline with Tween-20 (TBST, 0.2 %), the blots were incubated with the respective secondary antibodies (1:30000, LI-COR Biosciences, USA) for 1 h at room temperature. The blots were then washed thrice with PBST before detecting antigen-antibody complexes. The Odyssey two-colour infrared fluorescence imaging system was used for imaging. The band fluorescence values in Western blot were normalized to β-actin levels by Image Studio software (LI-COR Biosciences, ME, USA) [23].

2.9. Assessment of oxidative stress and inflammatory factors

Frozen brain tissue samples were homogenized in ice-cold PBS. The homogenate was centrifuged at 4 °C, 15000×*g* for 10 min. Protein quantification was carried out using the BCA Protein Assay Kit. The levels of tumour necrosis factor (TNF)-α (E-EL-R2856c, Elabscience, China) and interleukin (IL)-1β (E-EL-R0012c, Elabscience, China) were detected by ELISA according to the manufacturer's instructions. The activity of glutathione (GSH; A006-2-1, Nanjing Jiancheng Bioengineering Institute, China) and glutathione-peroxidase (GSH-Px; A005-1-2, Nanjing Jiancheng Bioengineering Institute, China) were determined by colorimetric method as per the manufacturer's instructions.

2.10. Cell culture and treatment

The PC-12 cell line (Adrenal pheochromocytoma in male rats, 1101RAT-PUMC000024) was obtained from the Cell Resource Center, Peking Union Medical College (which is part of the National Science and Technology Infrastructure, the National Biomedical Cell-Line Resource, NSTI-BMCR). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (12100-038, Gibco, USA) supplemented with 10 % foetal bovine serum (11011-8611, Tianhang, China), 1 % L-glutamine and a 1 % penicillin-streptomycin mixture. Cells were incubated at 37 °C in humidified air with 5 % carbon dioxide. In the damage model, the cells were treated with 100 μM H_2O_2, for 24 h. Subsequently, they were incubated with 0.1, 0.5, or 1 nm DHQ for 24 h.

To optimize the dose of DHQ, PC-12 cells were randomly divided into five groups: (1) normal control group (NC), (2) model group (M), (3) model + 0.1 nM DHQ group (DHQ-0.1), (4) model + 0.5 nM DHQ group (DHQ-0.5), (5) model + 1 nM DHQ group (DHQ-1).

To further examine the effects of DHQ on HIF-1α signalling pathway, PC-12 cells were randomly divided into 5 groups, namely: (1) normal control group (NC); (2) model group (M); (3) model + 0.5 nM DHQ group (DHQ); (4) model + 0.5 nM DHQ + Si-NC group (DHQ + Si-NC); (5) model + 0.5 nM DHQ + Si–HIF-1α group (DHQ-1).

2.11. Determination of apoptosis, oxidative stress, and cell viability

Apoptosis of PC-12 cells was determined by Annexin V-FITC (Fluorescein Isothiocyanate) or PI (Propidium Iodide) staining as per the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, USA). FITC positive cells were defined as apoptotic cells and analyzed. Flow cytometry analyses were performed using Fluorescence-activated cell sorting (FACS) AriaIII (BD Biosciences, USA). In addition, the levels of reactive oxygen species (ROS) in cells were determined by staining with a 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe. Cell viability was evaluated using the Cell Counting Kit (CCK)-8 assay (Abcam, Cambridge, UK), and absorbance was measured at 450 nm.

2.12. In-cell-western assay in PC-12 cells

PC-12 cells were plated onto 96-well plates (1247971, Thermo, USA) at a density of 3×10^4 cells/mL After drug treatment, the media was discarded and the cells were fixed with 4 % formaldehyde for 20 min. Cells were then permeabilised using 0.1 % Triton for 20 min and blocked with 5 % bovine serum albumin (BSA) at room temperature for 1.5 h. They were then incubated with the primary

antibody, HIF-1α (1:100), HSP70 (1:100), *p*-AKT (1:100), PI3K (1:100), NR2B (1:100), HSF-1(1:100) and β-actin (1:200), overnight at 4 °C. Subsequently, they were incubated with secondary antibody (1:1000, goat antirabbit 925-68071, goat-mouse 925-32210, Li-COR, USA) for 1 h at room temperature, and the protein intensity was quantified using Odyssey (Clx, Li-COR Biosciences, USA).

2.13. Transfection

The specific siRNA against HIF-1α (si–HIF–1α) and its control (si-NC) were synthesized by GenePharma (Shanghai, China). They were transfected into the cells using Lipofectamine 3000 reagent (Invitrogen) for 48 h. After transfection, the treatment of DHQ was administered.

2.14. Detection of the HIF-1α/AKT/NR2B signalling pathway by a high-connotation imaging system

PC-12 cells were seeded in 96-well plates at a uniform density of 2×10^4 cells/mL (E190236X; PerkinElmer, Waltham, MA, USA). After transfection and treatment with DHQ, cells were washed with PBS, fixed with 4 % paraformaldehyde for 20 min, permeabilised with 0.1 % Triton for 20 min, and washed five times with PBS. Treated cells were blocked with 5 % goat serum for 1.5 h at room temperature, incubated overnight at 4 °C with primary antibody, HSP70 (1:100), *p*-AKT (1:100), NR2B (1:100), HSF-1(1:100), and then incubated with secondary antibody (1:500, Cy3 Goat Anti-Rabbit IgG; catalogue number, AS007; ABclonal, Woburn, MA, USA) for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole for 5 min. A high-connotation imaging system (Opera Phenix®; PerkinElmer) was used for scanning. The relative protein expression was normalized against the control group using Harmony 4.8 (PerkinElmer).

2.15. RNA extraction and qRT-PCR analysis

RNA was extracted from the brain tissues or PC-12 cells using an RNA Extraction Kit (R1200, Solarbio, China). The RNA purity and concentration were determined by measuring and comparing the absorbance at 260 and 280 nm. Then, 2 μg of total RNA was reverse transcribed into cDNA using the HiScript®II 1st Strand cDNA Synthesis kit (R211-01, Vazyme Biotech, China). Subsequently, PCR was performed using the ChamQ™ Universal SYBR® qPCR Master Mix (Q711-02, Vazyme Biotech, China) [24]. The primer sequences are listed in Table 1.

2.16. Statistical analysis

All data are presented as the mean ± SEM. All experiments were repeated at least three times. The data were analyzed with SPSS 19.0 statistical software. The statistically significant differences between the groups were determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. DHQ improved behaviour in rats with metabolic syndrome

In all the open field tests, the SHR-FW group consistently displayed more hyperactive tendency than the WKY group ($P < 0.01$, Fig. 1). The DHQ-treated group travelled a shorter distance and for a lesser time ($P < 0.01$) than the SHR-FW group (Fig. 1).

3.2. DHQ improved brain cell apoptosis in rats with metabolic syndrome

In the histomorphological examination section, we found that the vertebral cell density and morphology of the hippocampus in the WKY group were normal, while low density and apoptotic cells could be observed in rats with metabolic syndrome. The DHQ-H group

Table 1
Primer sequences.

Target	Forward Primer(5′–3′)	Reverse Primer(5′–3′)
BDNF	TACCTGGATGCCGCAAACAT	TGGCCTTTTGATACCGGGAC
P53	TCACTCCAGCTACCCGAAGA	GTCAGGCCCCACTTTCTTGA
HIF-1α	AAGTCTAGGGATGCAGCACG	AGATGGGAGCTCACGTTGTG
HSP70	CCAGTGCGGCCTTAGTAGAG	CCTCAGACTCCGCCTTGTTT
HSF-1	ATGCCATGGACTCCAACCTG	TCATGTCGGGCATGGTCAC
PI3K	ACACGGGGGCATTCAAAGAT	GTCGTTGTGCCTGTCACCTA
NRF2	TGTAGATGACCATGAGTCGC	TCAGGGGTGGTGAAGACTGA
KEAP1	CTTCGGGGAGGAGGAGTTCT	GGGCAGTCGTATTTGACCCA
HO-1	GCCTGGTTCAAGATACTACCTCT	CTGAGTGTGAGGACCCATCG
NQO-1	ATTGTATTGGCCCACGCAGA	GATTCGACCACCTCCCATCC
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

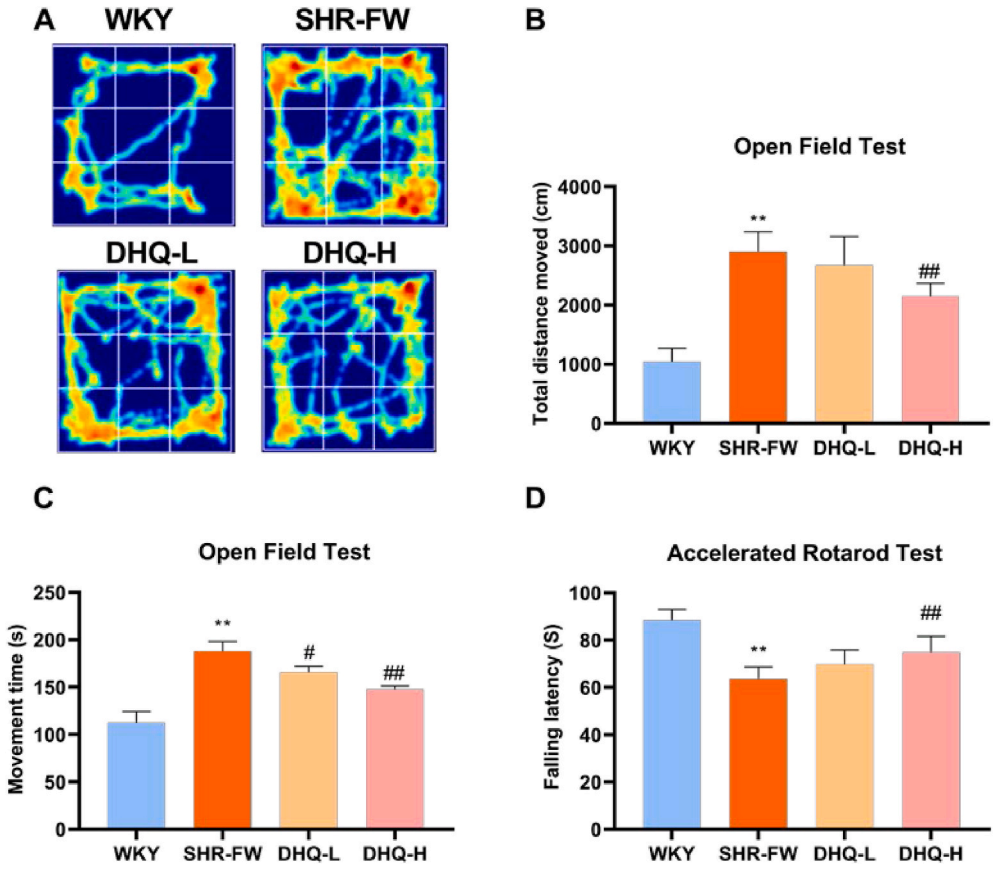

Fig. 1. DHQ improves neurofunction in rats with metabolic syndrome. (A)The trajectories of each group in the open field. (B) (C) The total distance travelled and total movement in 5 min in the open field. (D) The falling latency in the accelerated rotarod tests. Data are shown as mean ± SD (n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the WKY group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* SHR-FW).

had a significantly higher number of hippocampus cells and improved cell arrangement as compared to that in the model group (Fig. 2A).

Flow cytometry showed that the apoptotic levels of brain cells in rats with metabolic syndrome were significantly higher as compared to that of WKY rats, and DHQ treatment could significantly reduce the percentage of apoptotic cells (Fig. 2B).

3.3. DHQ improved impaired brain functions in rats with metabolic syndrome

To evaluate the brain function impairment in rats with metabolic syndrome, we measured the oxidative stress-related indicators (GSH-Px and GSH) [25], inflammatory factors (TNF-α and IL-1β) [26,27], brain nutrition factors (brain-derived neurotrophic factor [BDNF]) [28–30].

Activities of GSH-Px and GSH were significantly lowered in the brains of rats with metabolic syndrome as compared to the WKY group, while the levels of TNF-α and IL-1 were significantly enhanced. The high doses of DHQ lead to significant increase in the levels of GSH-Px and GSH and significantly reduced TNF-α and IL-1β levels as compared to levels in control group for metabolic syndrome (Fig. 3A and B).

The mRNA levels of BDNF in brains of rats with metabolic syndrome were significantly reduced as compared to those in the WKY group, and DHQ treatment could significantly increase the BDNF (Fig. 3C).

3.4. Effect of DHQ on HIF-1α/AKT/NR2B signalling pathway in brains of rats with metabolic syndrome

We measured the expression of HIF-1α/AKT/NR2B signalling pathway-related proteins in the brains of rats in each group by western blotting. The expression of HIF-1α, HSF-1, HSP 70, PI3K, and *p*-AKT in the SHR-FW group were significantly lower as compared to the WKY group. DHQ treatment could significantly increase the expression of these proteins (Fig. 4).

The levels of Drp1, MFF, and NR2B in the SHR-FW group were significantly higher as compared to the WKY group, and treatment with DHQ abolished this trend (Fig. 4).

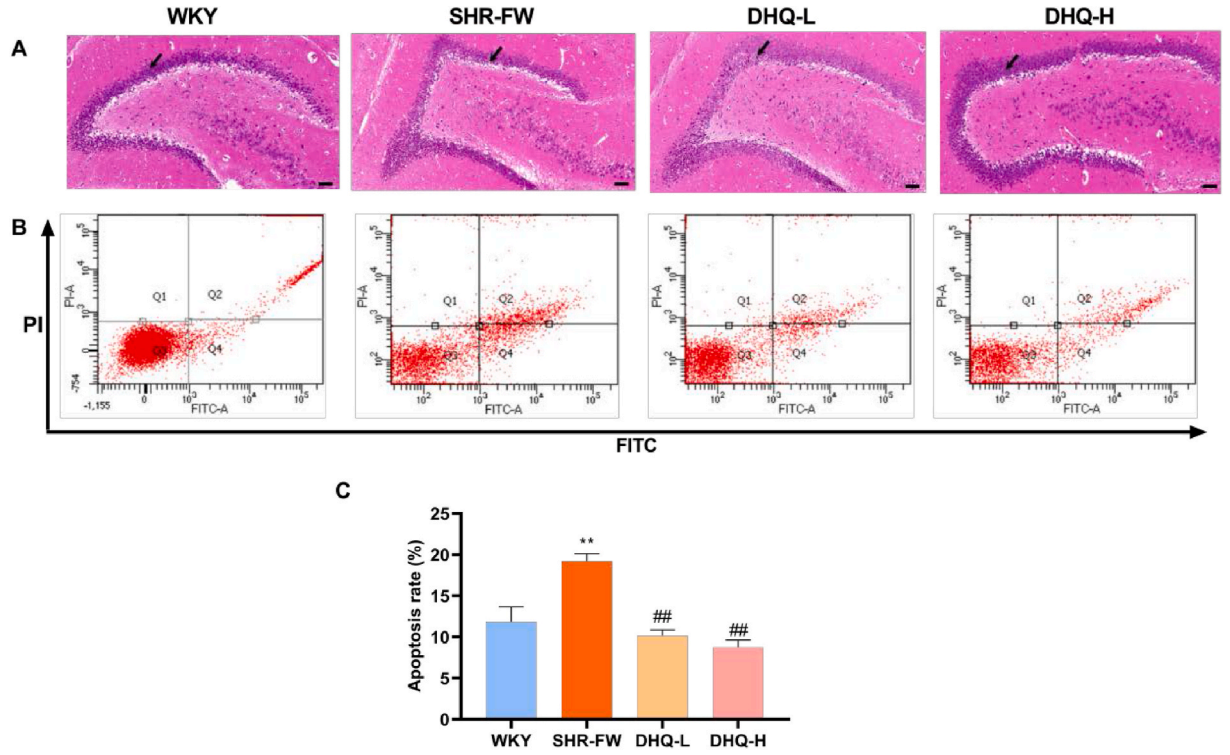

Fig. 2. Apoptosis rate are alleviated by dihydroquercetin (DHQ) in the brains of rats with metabolic syndrome. (A) Representative histopathological images for the haematoxylin and eosin (H&E) of the hippocampus for different groups (scale bars: 50 μm). The arrow represents the vertebral cell of the hippocampus. (B) (C) Flow cytometry scatter plot shows that apoptosis is inhibited by DHQ in the hippocampal of rats with metabolic syndrome. Data are shown as mean ± SD (n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the WKY group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* SHR-FW).

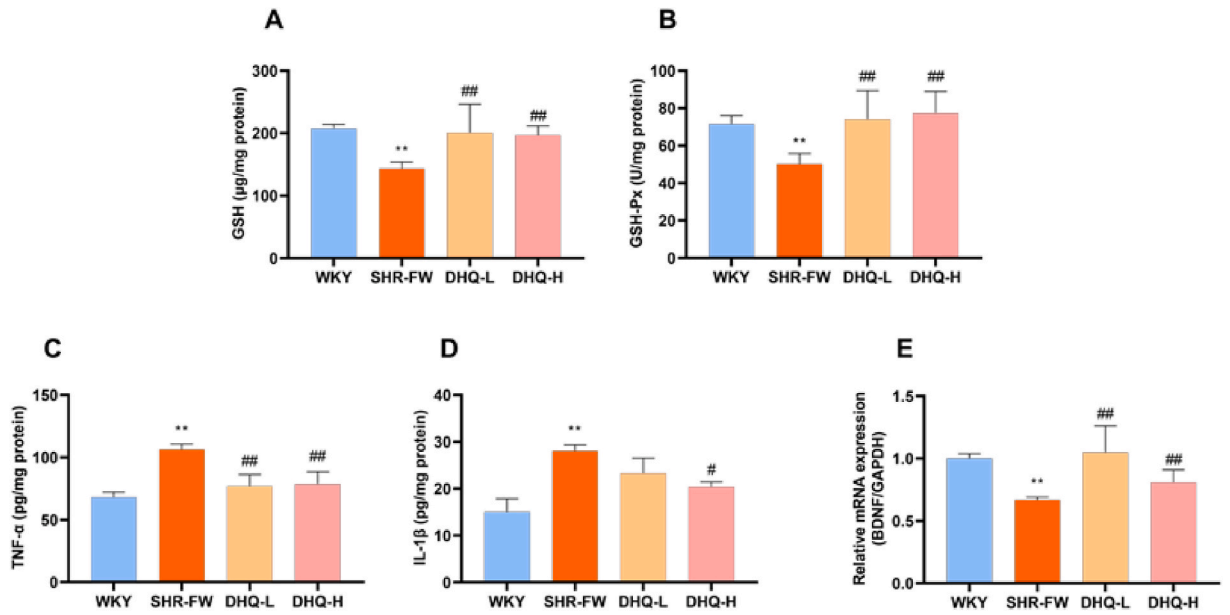

Fig. 3. DHQ improves the impaired brain functions in rats with metabolic syndrome. mRNA levels of (A) GSH, (B) GSH-Px, (C)TNF-α and (D) IL-1β in rats with metabolic syndrome, (E)The brain nutrition factors (BDNF) measured by qRT-PCR. Data are shown as mean ± SD (n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the WKY group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* SHR-FW).

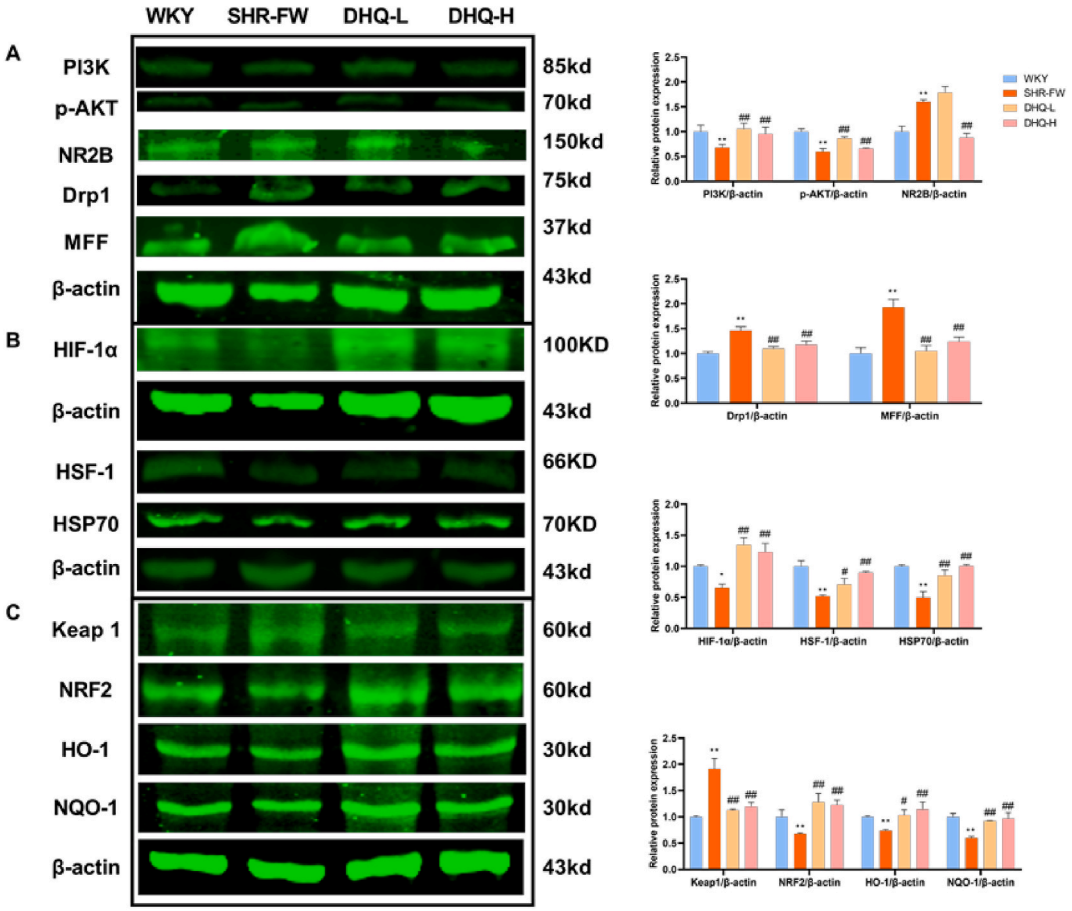

Fig. 4. DHQ regulates the expression of HIF-1α/AKT/NR2B and KEAP1/NRF2 signalling pathway-related factors. Western blot analyses of (A) PI3K, *p*-AKT, NR2B, Drp1 and MFF, (B) HIF-1α, HSF-1, and HSP70, (C) KEAP1, NRF2, HO-1 and NQO-1 in the different groups. Data are shown as mean ± SD (n = 3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the WKY group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* SHR-FW).

3.5. Effects of DHQ on the survival, percent apoptosis, and ROS in PC-12 cells damaged by H_2O_2

Treatment with 100 μM H_2O_2 (M) significantly elevated the percentage of apoptosis (Fig. 5), reduced the percentage of cell survival, and increased the ROS levels in PC-12 cells compared to the NC group; DHQ treatment was able to significantly increase the percentage of cell survival and decrease the percent apoptosis and ROS levels in PC-12 cells (Fig. 5).

3.6. Effect of DHQ on the HIF-1α/AKT/NR2B signalling pathway in H_2O_2 treated PC-12 cells

To further validate the optimal dose of DHQ for the PC-12 cells, the effects of DHQ treatment on the HIF-1α/AKT/NR2B signalling pathway in PC-12 cells damaged by H_2O_2 were evaluated by qRT-PCR. Compared with the NC group, mRNA expressions of the HIF-1α, HSF-1, HSP 70, PI3K and BDNF in the M group were significantly lower, and DHQ treatment increased them significantly (Fig. 6). In addition, compared with the NC group, the mRNA levels of P53 and NR2B were significantly higher; DHQ treatment could significantly reverse these effects. 0.5 nM DHQ was the optimised dose for subsequent experiments (Fig. 6).

The role of HIF-1α in the H_2O_2-induced damaging effect was further examined. Specific siRNA against HIF-1α was used in the subsequent experiments to knock down HIF-1α (Fig. 7A–C). We found that the effect of DHQ on the expression of HSF-1, HSP70, *p*-AKT, and NR2B in H_2O_2-stimulated cells was partly rescued by si–HIF–1α (Fig. 7D–E). Additionally, the effects of DHQ on the mRNA expression of HIF-1α signalling pathway-related proteins (HSF-1, HSP70 and NR2B) (Fig. 7F) were attenuated by si–HIF–1α. We also detected the proteins expression of KEAP1, HO-1, NQO-1, and NRF2 proteins using a high-connotation imaging system and qRT-PCR. The effects of DHQ on the proteins and mRNA expression of KEAP1, HO-1, NQO-1, and NRF2 were attenuated by si–HIF–1α, suggesting that DHQ inhibited H_2O_2-induced damage in PC-12 cells by up-regulating HIF-1α (Fig. 7G–I).

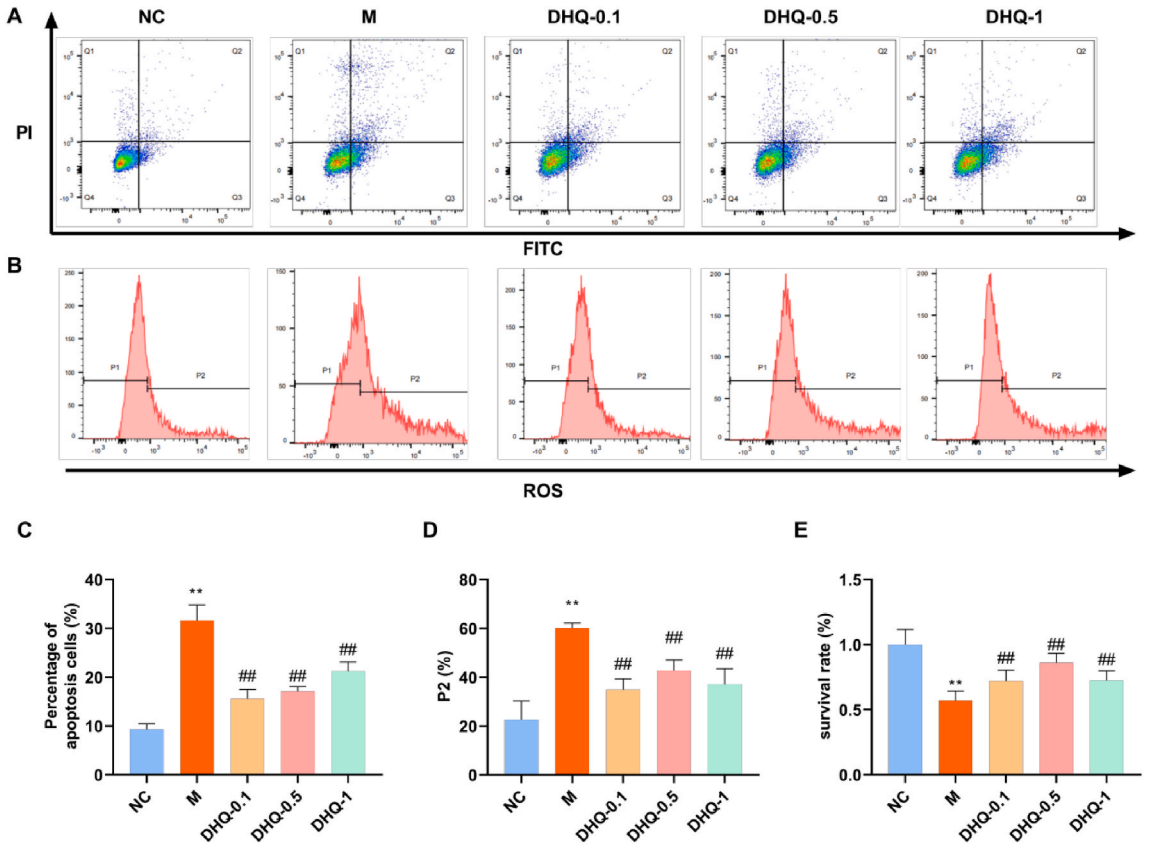

Fig. 5. DHQ treatment increases the percentage of cell survival and decreases the percent apoptosis and ROS levels in PC-12 cells. Effect of DHQ on (A) (C) percent apoptosis, (B) (D) ROS levels and (E) cell survival in PC-12 cells damaged by H_2O_2 treatment. Data are shown as mean ± SD (n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. NC, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. M.).

4. Discussion

MS is a cluster of disease risk factors including hypertension, abdominal obesity, elevated triglyceride levels, low HDL cholesterol levels, and marked resistance to insulin [31–33]. The prevalence of MS increases with increasing weight and age. The overall standardized prevalence of the MS is 24.2 % (24.6 % in men and 23.8 % in women) [34]. In addition to the increased rates of diabetes mellitus and cardiovascular disease, MS is also correlated substantially with brain impairment [33,35,36].

In our previous study, we found that DHQ could significantly reduce the blood pressure and insulin resistance index in MS rats, and also improve the blood lipid disorders [21]. We found that DHQ can improve the cognitive function of rats through open field test, rotarod test. Furthermore, we used flow cytometry to detect the apoptosis levels of primary brain cells, and we observed the changes of hippocampal cells in HE slices. The results showed that DHQ could reduce the percentages of apoptotic brain cells, and increase the cell density in the hippocampus of MS rats. The blood-brain barrier regulates the material exchange between blood and brain tissue and maintains the stability of the internal environment of the brain. Studies have shown that DHQ can improve LPS-induced brain injury and has good fat solubility [37,38]. Therefore, we speculate that DHQ has a certain blood-brain barrier permeability. When the level of brain cell apoptosis increases, it is often accompanied by increased levels of oxidative stress and inflammatory factors [39]. It has been recently reported that excessive mitochondrial fission correlates with brain dysfunction [40,41]. DHQ can significantly reduce the levels of oxidative stress and inflammatory factors in the brain of MS rats, inhibit the excessive activation of mitochondria. Thus, these results provide a preliminary indication that DHQ may improve the impaired brain function in MS rats.

To further examine the mechanism underlying the effects of DHQ in improving brain function in MS rats, we speculated that the insulin signalling pathway may have a role in the process. Inhibition of the NR2B subunits can rescue secondary brain injury and behavioural cognitive impairment [42]. PI3K-AKT signalling can regulate the expression of NR2B to protect from hypoxic-ischemic brain damage [43–45]. In our previous study, we found that DHQ could significantly activate the PI3K signalling pathway in the kidney, and thus, whether it exerted a similar effect in the brain tissue, piqued our interest [21]. We measured the protein levels of PI3K and *p*-AKT by western blotting. We found that the PI3K signalling pathway was significantly inhibited in the brain tissues of MS rats, and DHQ treatment could still significantly rescue the activation of the PI3K signalling pathway. PI3K/Akt, a multifunctional signalling pathway that is associated with cell proliferation, anti-apoptosis and cellular defense, has been reported to regulate KEAP1/NRF2 signal pathway [46]. The KEAP1/NRF2 signal pathway is verified to be critical in cells anti-oxidative stress for its protective effects.

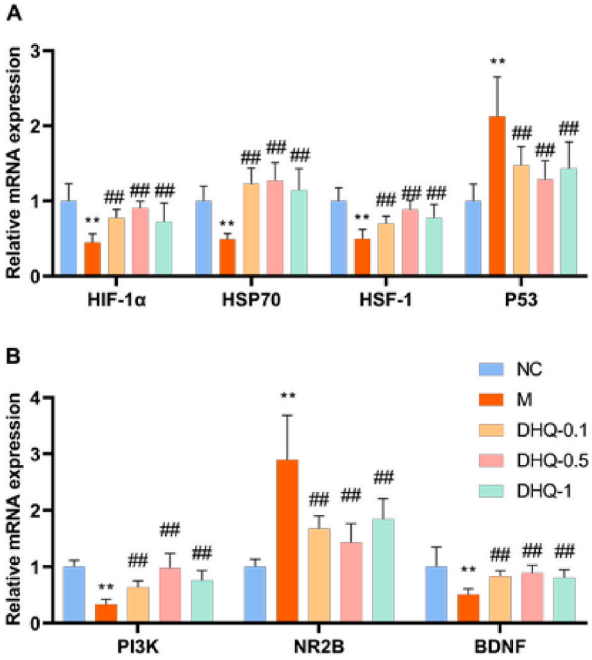

Fig. 6. Effect of DHQ on the HIF-1α/AKT/NR2B signalling pathway in H_2O_2 treated PC-12 cells. (A) (B) mRNA expression in HIF-1α/AKT/NR2B signalling pathway in PC-12 cells visualized by qRT-PCR. Data are shown as mean ± SD (n = 3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. NC, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. M.).

Under normal physiological conditions, NRF2 is anchored in the cytoplasm by binding to KEAP1, which is attached to the actin cytoskeleton, in order to maintain the cytoprotective enzyme and antioxidants at the basic expression levels and the cells in a stable state. When the production of free radicals is increased, KEAP1 is converted to KEAP1 thiol and/or NRF2 is phosphorylated by protein kinase and released from KEAP1 and translocates to the nucleus, thus activating the expression of antioxidant enzymes [47]. It means that activated NRF2 can protect cells against oxidants and electrophiles. Accordingly, NRF2 has been demonstrated to regulate the expression of downstream antioxidant enzymes, including NQO1, HO, catalase, SOD, glutathione reductase, and GSH-Px, in order to keep a normal REDOX state and reduce tissue damage by electrophilic reagent [48]. DHQ can regulate the KEAP1/NRF2 pathway to increase HO-1, NQO-1, GSH and GSH-px.

Transcriptional control of HIF-1α on HSF-1 levels is a regulatory mechanism for sensitisation of the heat shock pathway [49]. A growing body of evidence indicates that HSF-1 is the transcriptional regulator of HSPs. Under stress conditions, HSF-1 can induce the expression of HSPs, including HSP70 and HSP90 [50,51]. Up-regulation of HIF-1α can improve brain injury, reduce brain cell apoptosis, and oxidative stress [10,11].We evaluated the levels of HIF-1, HSF-1, and HSP70 using western blotting. Compared with WKY rats, the levels of HIF-1α, HSF-1, and HSP70 in the brain tissues of MS rats were significantly lower, and DHQ treatment could significantly promote their expression.

We found that the antioxidant effect of DHQ is related to the regulation of NRF2. It is not clear whether the antioxidant effect of DHQ is related to the regulation of HIF-1α. To further evaluate if HIF-1α was a key target of DHQ, we performed *in vitro* experiments. H_2O_2 can cause oxidative damage to cells. Then, we used H_2O_2 treatment to establish an injury model in PC-12 cells. We found that DHQ could significantly inhibit cell apoptosis, reduce oxidative stress, increase cell survival rate, regulate the HIF-1α/AKT/NR2B signalling pathway, and increase the levels of brain nutrition factor. Subsequently, we silenced HIF-1α. Using in-cell-western blotting assay and PCR, we found that the regulation of HIF-1α/AKT/NR2B signalling pathway, inhibition of apoptosis, and KEAP1/NRF2 pathway protein, and increase of brain nutritional factors by DHQ was blocked by the Si–HIF-1α. Furthermore, the levels of proteins downstream of HIF-1α, HSP70, HSF-1, *p*-AKT, and NR2B, were validated by the high content imaging system, and these results were consistent with the findings from in-cell-western blotting assay and PCR. Up-regulation of HIF-1 can alleviate brain injury, while HIF-1 has little research on cognitive impairment. Through our experiments, we found that DHQ can regulate HIF-1 and improve cognitive impairment, which may provide new targets and new ideas for the development of clinical drugs.

In summary, dihydroquercetin could improve impaired brain function in rats with metabolic syndrome by regulating the HIF-1α/AKT/NR2B signalling pathway (Fig. 8). These findings provide new insight and experimental evidence for the treatment of metabolic syndrome. In the future, we will conduct other types of brain dysfunction experiments with DHQ, such as cerebral ischemia-reperfusion injury, acute infectious brain injury, traumatic brain injury and so on.

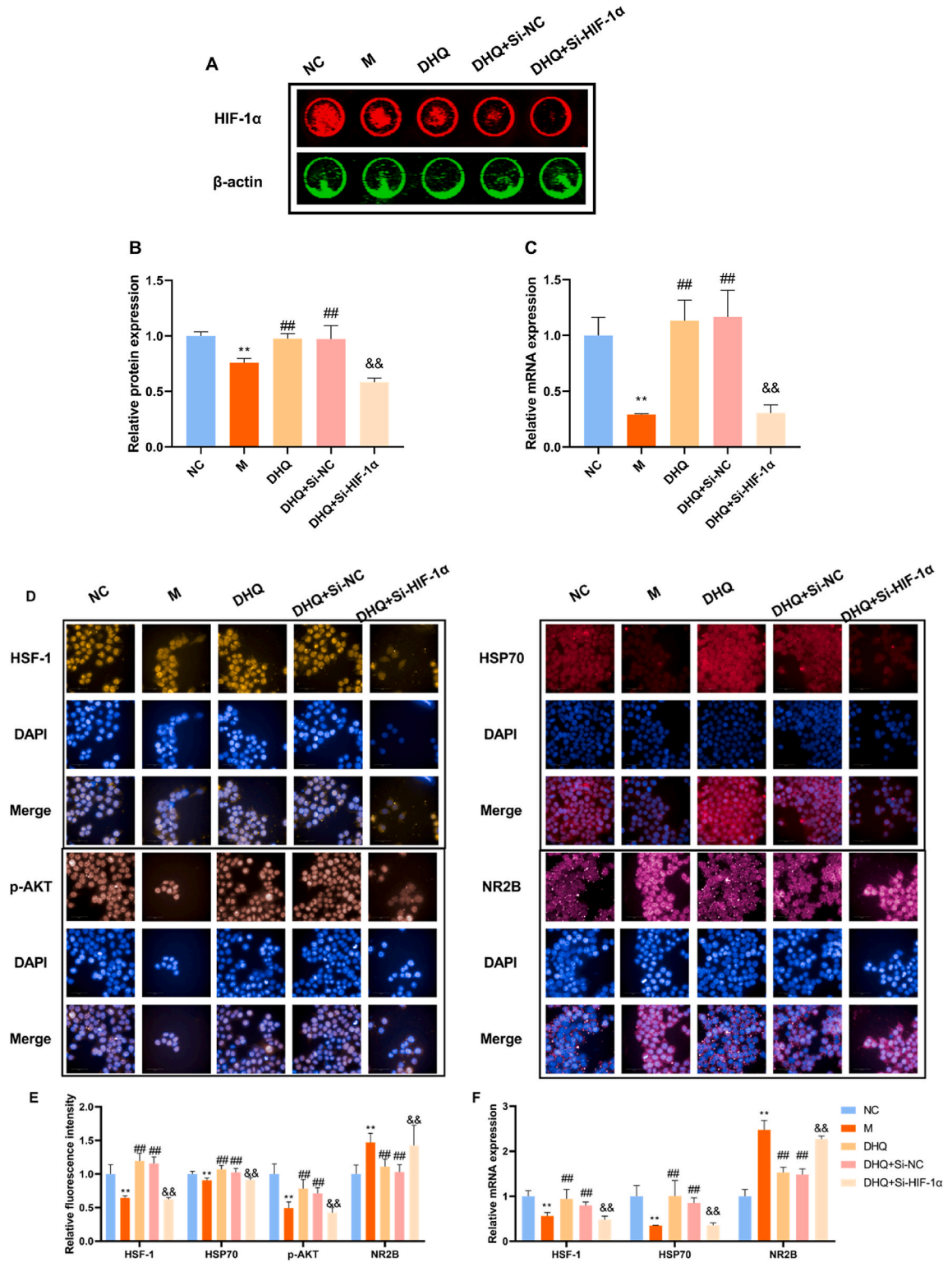

Fig. 7. DHQ rescues H_2O_2-induced injury in PC-12 cells by up-regulating HIF-1α. (A) (B) (C) Protein and mRNA expression of HIF-1α visualized by In-cell Western assay and qRT-PCR in PC-12 cells. (D) (E) Protein expression of HSF-1, HSP70, *p*-AKT, and NR2B visualized by high-connotation imaging system in PC-12 cells. (F) The mRNA expression of HSF-1, HSP70 and NR2B visualized by qRT-PCR. (G) (H) (I) Protein and mRNA expression of KEAP1, HO-1, NQO-1, and NRF2 visualized by high-connotation imaging system and qRT-PCR in PC-12 cells. Data are shown as mean ± SD (n = 3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. NC, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. M.).

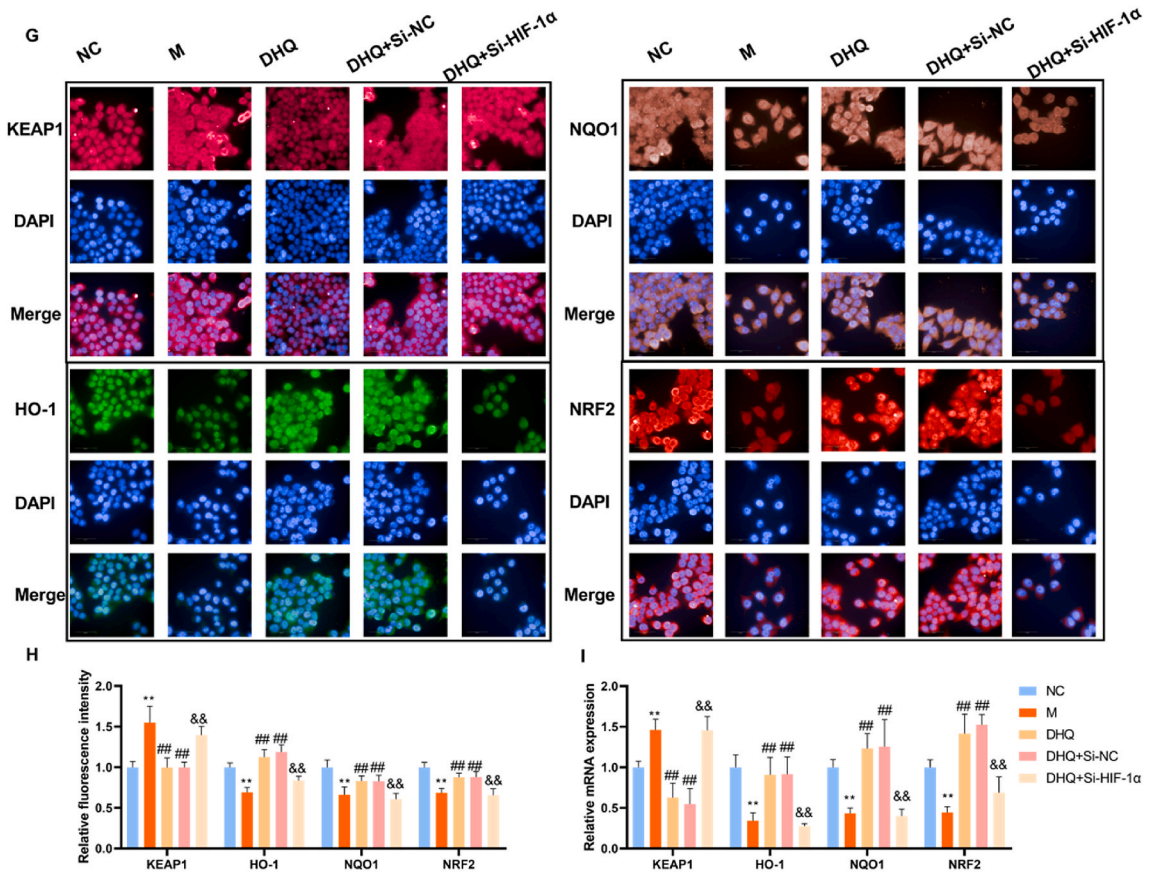

Fig. 7. (*continued*).

5. Conclusion

In summary, dihydroquercetin could improve impaired brain function in rats with metabolic syndrome by regulating the HIF-1α/AKT/NR2B signalling pathway. These findings provide new insight and experimental evidence for the treatment of metabolic syndrome. We would suggest that dihydroquercetin may potentially be utilized as an adjunct for brain injury in metabolic syndrome.

Ethics statement

The study was conducted in accordance with the Experimental Animal Administration regulations issued by the State Committee of Science and Technology of the People's Republic of China. The present study was approved by the Institutional Animal Care and Use Committee, and all study protocols complied with the Guide for the Care and Use of Laboratory Animals (Approval No. DWLL20188003). All the procedures for the care of the rats were in accordance with the institutional guidelines for animal use in research.

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Data availability statement

Data will be made available on request.

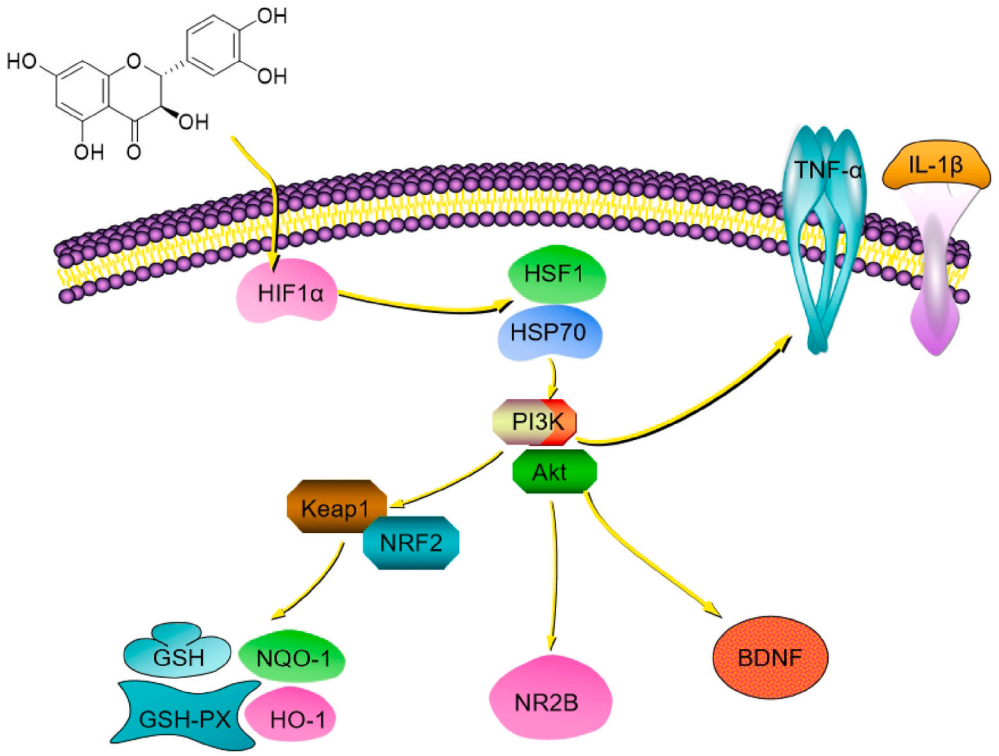

Fig. 8. Dihydroquercetin regulates HIF-1α/AKT/NR2B signalling to improve impaired brain function in rats with metabolic syndrome.

CRediT authorship contribution statement

Yang Fu: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **PeiPei Yuan:** Writing – review & editing, Methodology. **Mengnan Zeng:** Writing – review & editing, Methodology. **Qi Zhang:** Data curation, Formal analysis. **Ying Hou:** Data curation, Formal analysis. **Liyuan Gao:** Data curation, Formal analysis. **Yaxin Wei:** Data curation, Formal analysis. **Yajuan Zheng:** Data curation, Formal analysis. **Weisheng Feng:** Project administration, Resources, Software, Supervision, Validation, Visualization, Funding acquisition. **Xiaoke Zheng:** Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AKT	Protein kinase B
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CCK	Cell Counting Kit
DAPI	4′,6-Diamidino-2-phenylindole
DCFH-DA	2′,7′-Dichlorodihydrofluorescein diacetate
DHQ	dihydroquercetin
DMEM	Dulbecco's modified Eagle's medium
Drp1	GTPase dynamin-related protein 1
ELISA	Enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GPX	Glutathione peroxidase
GSH	glutathione
HE	Haematoxylin-eosin
HIF-1α	hypoxia-inducible factor 1α

HO-1	hemoxegenase-1
HSF1	heat shock factor 1
HSP70	heat shock protein 70
IL	Interleukin
IR	insulin resistance
KEAP1	Kelch-like ECH-associated protein 1
MFF	Mitochondrial fission factor
MS	metabolic syndrome
NQO-1	NADPH quinone oxidoreductase-1
NR2B	N-methyl-d-aspartic acid receptor 2B
NRF2	nuclear factor erythroid 2-related factor 2
NT-3	neurotrophin type 3
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.1 % Tween® 20 detergent
PI	Propidium iodide
PI3K	phosphoinositide-3-kinase
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RT-PCR	Quantitative real time polymerase chain reaction
SHR	spontaneous hypertensive rats
TBST	Tris-buffered saline with 0.1 % Tween® 20 detergent
TNF	tumour necrosis factor α
WB	Western blotting
WKY	Wistar-Kyoto

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