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Prevention of Hippocampal Neuronal Damage and Cognitive Function Deficits in Vascular Dementia by Dextromethorphan

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Abstract Dextromethorphan (DM) is a non-competitive antagonist of NMDA receptors and a widely used component of cough medicine. Recently, its indication has been extended experimentally to a wide range of disorders including inflammation-mediated central nervous system disorders such as Parkinson disease (PD) and multiple sclerosis (MS). In this study, we investigate whether DM treatment has protective effects on the hippocampal neuron damage induced by bilateral occlusion of the common carotid arteries (two-vessel occlusion [2VO]), an animal model of vascular dementia (VaD). Sprague–Dawley (SD) (10 weeks of age) rats were subjected to the 2VO, and DM was injected intraperitoneally once per day for 37 days. Neuron death, glial activation, and cognitive function were assessed at 37 days after 2VO (0.2 mg/kg, i.p., "DM-0.2" and 2 mg/kg, i.p., "DM-2"). DM-2 treatment provided protection against neuronal death and glial activation in the hippocampal CA1 subfield and reduced cognitive impairment induced by 2VO in rats. The study also demonstrates that activation of the Nrf2-HO-1 pathway and upregulation of superoxide dismutase (SOD) play important roles in these effects. These results suggest that DM is effective in treating VaD and protecting against oxidative stress, which is strongly implicated in the pathogenesis of VaD. Therefore, the present study suggests that DM treatment may represent a new and promising protective strategy for treating VaD.

Keywords Vascular dementia · Dextromethorphan · Microglial activation · Oxidative stress

Introduction

Following Alzheimer's disease, vascular dementia (VaD) is the second most common type of dementia. VaD is caused by a range of cardiovascular or cerebrovascular conditions that may lead to ischemic, hypoperfusive, or hemorrhagic brain lesions that are characterized by loss of cognitive functions [1–4]. Considerable evidence indicates chronic cerebral hypoperfusion (CCH) contributes to the development and progression of dementia through pathways of inflammation and oxidative stress in blood vessels and results in damage to learning and memory functions [5, 6].

Oxidative stress is believed to play an important role in the pathogenesis of different neurodegenerative diseases in age-related patients such as those with Parkinson's disease (PD) [7], Huntington's disease [8], and amyotrophic lateral sclerosis [9]. An increasing number of studies have shown that oxidative stress is strongly implicated in the pathogenesis of VaD and particularly nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1(Nox1)-mediated oxidative stress plays an important role in neuronal cell death and cognitive dysfunction in VaD ([10–12], [13, 14], [12, 15]). Several studies have confirmed that antioxidant therapy, such as the use of vitamins, pioglitazone, aliskiren, and curcumin, may play a role in the prevention and treatment of AD and VaD [16–19]. However, antioxidant therapies have not achieved the intended results. New and effective intervention strategies for preventing 2VO-induced neuron death and cognitive impairment need to be further investigated.

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Dextromethorphan (DM) is known as a component of cough medicine that is widely used in clinics. At doses ranging from 10–80 mg/kg, DM inhibits voltage-gated Ca^{2+}-channels, stimulates sigma-1 receptors, and works as a non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptors [20]. For these properties, DM has been confirmed to exert neuroprotection in models of cerebral ischemia, spinal cord injury, and epilepsy [21–23]. Recently, accumulating evidence has demonstrated that DM or other morphinans could inhibit the production of reactive oxygen species (ROS) in activated microglia/macrophages to exert a neuroprotective effect in age-related diseases, such as atherosclerosis, hypertension, PD, and MS [24–28]. Furthermore, DM has been used clinically for decades with a proven safety record and is a small molecule that can be administered orally suggesting that DM is ideal for long-term treatment. Based on these properties of DM, the current study was conducted to test the hypothesis that DM may improve cognitive function in 2VO animals by inhibiting oxidative stress. The results of this study may provide a novel alternative strategy to ameliorate the cognitive dysfunction of VaD patients.

In this study, we explored the neuroprotective effects and mechanisms using the 2VO rat model. We found that DM treatment could significantly ameliorate neuron degeneration in the hippocampal CA1 region and improve cognitive function. The Nrf2-HO-1 pathway and upregulation of SOD were involved in the protection and mitigation of levels of oxidative stress induced by CCH.

Materials and Methods

Animals and Treatment

This animal experiment was conducted under the supervision of Shanghai Jiao Tong University Animal Ethics Committee. Healthy, adult male Sprague–Dawley (SD) rats aged 8 weeks were housed with a 12 h light–dark cycle at room temperature (19 ± 2 °C) and with free access to food and water.

The primary antibody mouse anti-glial fibrillary acidic protein (GFAP), mouse anti-myelin basic protein (MBP), and DM were purchased from Sigma (St. Louis, MO, USA). The rabbit anti-heme oxygenase isoform 1 (HO-1) was obtained from Stressgen (Victoria, BC, Canada), and the rabbit anti-β actin was from Bioss (Beijing, China). SOD and caspase-3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, US). Rabbit anti-NeuN, rabbit anti-MAP-2, rabbit anti-keap1, rabbit anti-TNF-α, and rabbit anti-nuclear factor E2-related factor 2 (Nrf2) were obtained from Abclonal Technology (Cambridge, MA, USA). Mouse anti-4-hydroxy-2-nonenal (4-HNE) and mouse anti-dephosphorylated neurofilament protein (detected using the SMI-32 antibody) were from Abcam (Cambridge, MA, US) and Dako (Carpinteria, CA, US), respectively. Goat anti-Iba-1 was obtained from Millipore (Billerica, MA, US). The secondary antibody Alexa Fluor® 488 donkey anti-mouse or anti-rabbit IgG (H + L) and Alexa Fluor® 555 donkey anti-rabbit and anti-goat IgG (H + L) were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents were of the highest grade available from local commercial sources.

After administration of chloral hydrate (350 mg/Kg, i.p.) for anesthesia, the common carotid artery was isolated from the adjacent vagus nerve and double-ligated with 6–0 silk sutures to perform the two-vessel occlusion (2VO). Sham-operated rats were subjected to the same procedure, except for the carotid ligation. Animals were randomly divided into the following groups: group 1 (sham group with no occlusion), group 2 (control group with 2VO procedure), group 3 (2VO group with DM-0.2 administration), and group 4 (2VO group with DM-2 administration). DM was dissolved in sterile saline and administered by intraperitoneal injection. Adult SD rats received 2VO and were administered saline, DM-0.2 or DM-2, 30 min after surgery and every day until the rats were euthanized.

Morris Water Maze

The Morris water maze (MWM) was used to test the alterations of assess cognitive and behavioral performance between the different groups. On day 31 after 2VO, the rats began the 5-day MWM test. The pool was 2 m in diameter and filled with water and latex liquid at 25 ± 1 °C to a depth of 60 cm. During the acquisition training, the translucent acrylic platform (10 cm in diameter) was located in the center of the northeast quadrant and submerged 1.5 cm below the water surface. The rats were subjected to four trials per day for five consecutive days. In each of the four trials, the rats were gently released into the water by facing the tank wall at four different starting positions equally spaced around the perimeter of the pool. The rats were given a maximum of 90 s to find the hidden platform. Upon reaching the platform, the rats were allowed to stay on it for 15 s. If the rat failed to find the platform within 90 s, the training was terminated and a maximum score of 90 s was assigned. The rat was then guided to the hidden platform by hand and allowed to stay on the platform for 15 s. The latency to escape onto the hidden platform was recorded as the performance of spatial learning. To assess spatial memory, a probe trial was performed 24 h after the last training trial. In this trial, the platform was removed from the tank and the rats were allowed to swim freely for 60 s in the pool before they were removed from the water. The pool was located in the center of a room containing prominent spatial cues and had a recessed video camera mounted on the ceiling to monitor swim paths. The video signal was relayed to a computer running specialized data acquisition software (‘Watermaze’ Actimetrics, Wilmette, IL, USA) that records the path taken by the animal. On day 37 after UCCAO, the rats were euthanized and brain tissues were removed for immunohistochemical staining and western blots.

Immunofluorescence Staining

SD rats were anesthetized with 8 % chloral hydrate (450 mg/Kg, i.p.) and then perfused transcardially with 0.01 % heparin/saline followed by 4 % paraformaldehyde/0.1 mol/L phosphate buffer saline (PFA/PBS, pH = 7.4). The brains were removed and post-fixed in PFA solution for 12 h and then cryoprotected in 20 and 30 % sucrose at 4 °C, respectively. Each brain block containing the CA1 or striatum was cut into 20-μm-thick coronal sections on a freezing microtome. The sections were incubated with 10 % bovine serum albumin and 0.3 % Triton X-100 in PBS for 1 h and then incubated overnight at 4 °C with primary antibodies specific to neurons (NeuN, 1:1000), HO-1 (1:100), GFAP (1:2000;), SOD (1:500), and caspase-3 (1:500) in PBS. Alexa Fluor® 488 goat anti-mouse IgG (H + L) (1:500) and Alexa Fluor® 555 goat anti-rabbit or anti-mouse IgG (H + L) were used as secondary antibodies (1:500; Invitrogen, USA) to visualize the staining. Negative control tests were run without primary or secondary antibodies. Images were acquired with a laser confocal microscope (SP8; Leica Microsystems, Wetzlar, Germany). The images were processed with NIH ImageJ software, and NeuN, Iba-1, and caspase-3-positive cells were counted manually at ×400 magnification. Total cell counts were averaged from at least six sections of each hippocampal CA1 region per animal. The data for GFAP, SMI-32, and SOD are presented as the percentage of the average positive stained area in the individual sections.

Western Blotting

Hippocampal and striatal tissues dissected from the whole brain were lysed with lysis buffer containing 50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (pepstatin 1 g/ml, aprotinin 1 g/ml, leupeptin 1 g/ml). The lysate was centrifuged at 12,000×*g* for 10 min, and the supernatant was used for analysis. Protein concentrations were determined by the Bradford assay kit (Bio-Rad Laboratories, USA). Nuclear extracts were prepared with a commercial kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). A total of 30 μg of protein was separated using 12 % SDS polyacrylamide gels and transferred onto PVDF membranes. After being blocked with 10 % milk for 2 h, membranes were incubated with the primary antibodies against Iba-1 (1:1000), keap1 (1:500), Nrf2 (1:500), HO-1 (1:1 000), NOX-1 (1:1000), and MBP (1:1000) overnight and then incubated with anti-mouse or rabbit secondary antibody conjugated to horseradish peroxidase at a dilution of 1:10,000 for 2 h. β-actin was detected by an anti-β-actin monoclonal antibody (1:8000) using a similar procedure to ensure equal samples of protein. Crossreactivity was visualized using ECL western blotting detection reagents and then analyzed through scanning densitometry by a UVP image system.

Statistical Analysis

Statistical analysis was performed using SPSS version 18.0 software for Windows (SPSS Inc., Chicago, IL, USA). The results are presented as the mean ± SEM. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used to compare differences between mean values for data involving more than two groups. A probability value of $P < 0.05$ was taken to indicate statistical significance. The behavioral data were analyzed by two-way repeated-measures analysis of variance (ANOVA).

Results

Neuronal Loss and Glial Cell Activation in 2VO-Operated Rats Is Attenuated by DM-2 Treatment After 2VO Brain Injury in the CA1 Region of the Hippocampus

Hippocampal neuronal death was determined by NeuN immunostaining in tissue sections from the hippocampus obtained at 37 days after 2VO operation. As depicted in Fig. 1a, b, the number of NeuN-positive cells was significantly reduced to 70 % in the CA1 region of the 2VO-operated rats compared with sham-operated rats ($P < 0.05$, Fig. 1a, b) and the loss of NeuN-positive cells was mitigated by DM-2 treatment. The number of GFAP-positive astrocytes and Iba-1-positive microglia (Fig. 1c–f) in the hippocampal CA1 region of rats were decreased after DM-2 treatment. The protein extracted from total hippocampal tissue was subjected to western blot analysis using anti-MAP2, anti-GFAP, and anti-Iba-1 antibodies. MAP-2, GFAP, and Iba-1 immunostaining in the hippocampal CA1 region indicated that expression of MAP-2 was increased; GFAP and Iba-1 were decreased in the DM-2-treated rats compared with the 2VO-operated rats (Fig. 1g–i).

DM-2 Treatment Protects White Matter in the Striatum and Optic Tract Area

Immunostaining for neurofilament protein (SMI-32) and western blots for MBP were used to identify axonal and myelin injury in the striatum, respectively. There were many neuronal fibers that intensely expressed SMI-32 in the fiber fascicles of the internal capsule of the striatum in the 2VO-operated rats (Fig. 2a, c), and a decrease of MBP was also observed by western blots (Fig. 2e). However, DM-2 treatment could ameliorate the damage. Chronic cerebral hypoperfusion caused a large increase in microglial activation (sham, $P < 0.001$) in the optic tract (Fig. 2b, d). Following DM-2

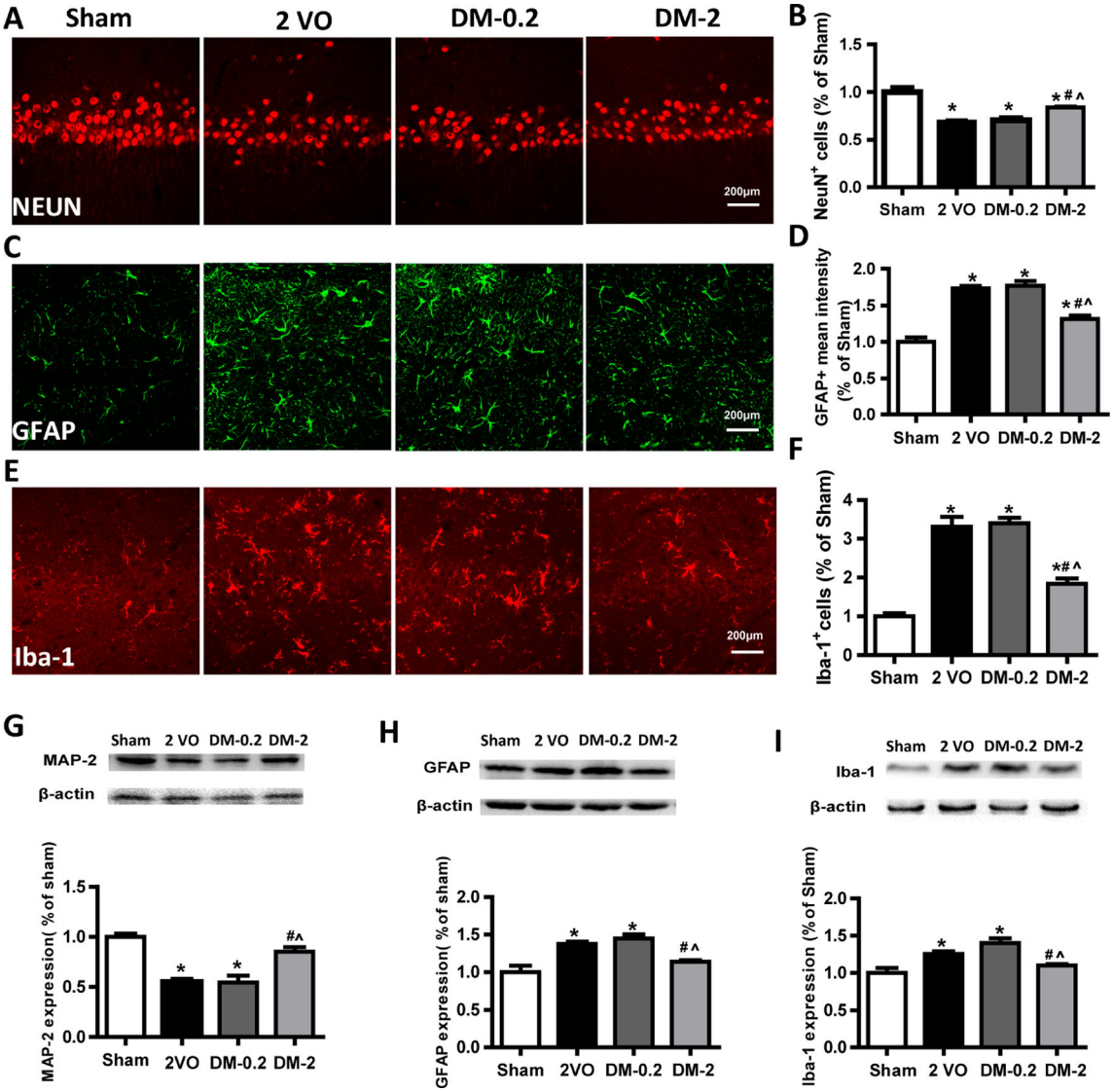

Fig. 1 Hippocampal neuronal loss and glial activation in 2VO-operated rats are attenuated by DM-2 treatment after 2VO brain injury in the hippocampal CA1 subfield. **a**, **c**, **e** Representative photographs of tissue sections stained with NeuN (*red*) antibody, GFAP (*green*) antibody, and Iba-1 (*red*) antibody from rat hippocampal CA1 area taken from the sham group (Sham, $n=4$) and 2VO-operated group ($n=4$) with or without DM (2VO, DM-0.2, DM-2, $n=4$). *Scale bars* = 200 μm. **b** NeuN-positive cell counts in 2VO-operated rats treated with DM-2 were significantly restored in the hippocampal CA1 area ($n=4$, $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2). **d**, **f** GFAP-positive astrocytes and Iba-1-positive microglia were reduced by DM-2 treatment ($n=4$, $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2). **g**–**i** DM-2 treatment significantly reduced neuron loss and glial activation induced by 2VO assessed by western blots in the hippocampus. The data are presented as the ratio of MAP-2, GFAP, and Iba-1 to β-actin protein levels. Each *bar* represents the mean ± SEM of four independent experiments. $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2

treatment, the number of Iba-1-positive microglia in the optic tract was significantly decreased to 71 % compared with the 2VO-operated rats ($P<0.01$) (Fig. 2b, d).

HO-1 Plays a Critical Role in DM-Mediated Neuroprotection Against 2VO in Rats

HO-1 and Nrf2 are essential in DM-2-mediated neuroprotection against neuronal injury induced by 2VO. We investigated the cytoprotective mechanisms of DM-2 against the neuronal injury of VaD. The antioxidant enzymes play important roles in cellular self-defense. Once produced, they are effective for long periods of time as they operate catalytically without being consumed. Among these enzymes, heme oxygenase-1 (HO-1) catalyzes the breakdown of toxic heme and generates multiple neuroprotective molecules such as biliverdin and carbon monoxide, the one which is recognized as dynamic sensors of cellular oxidative stress and likely arbiters of tissue redox homeostasis. Nrf2 is the major transcription factor responsible for the upregulation of HO-1; therefore, the levels of

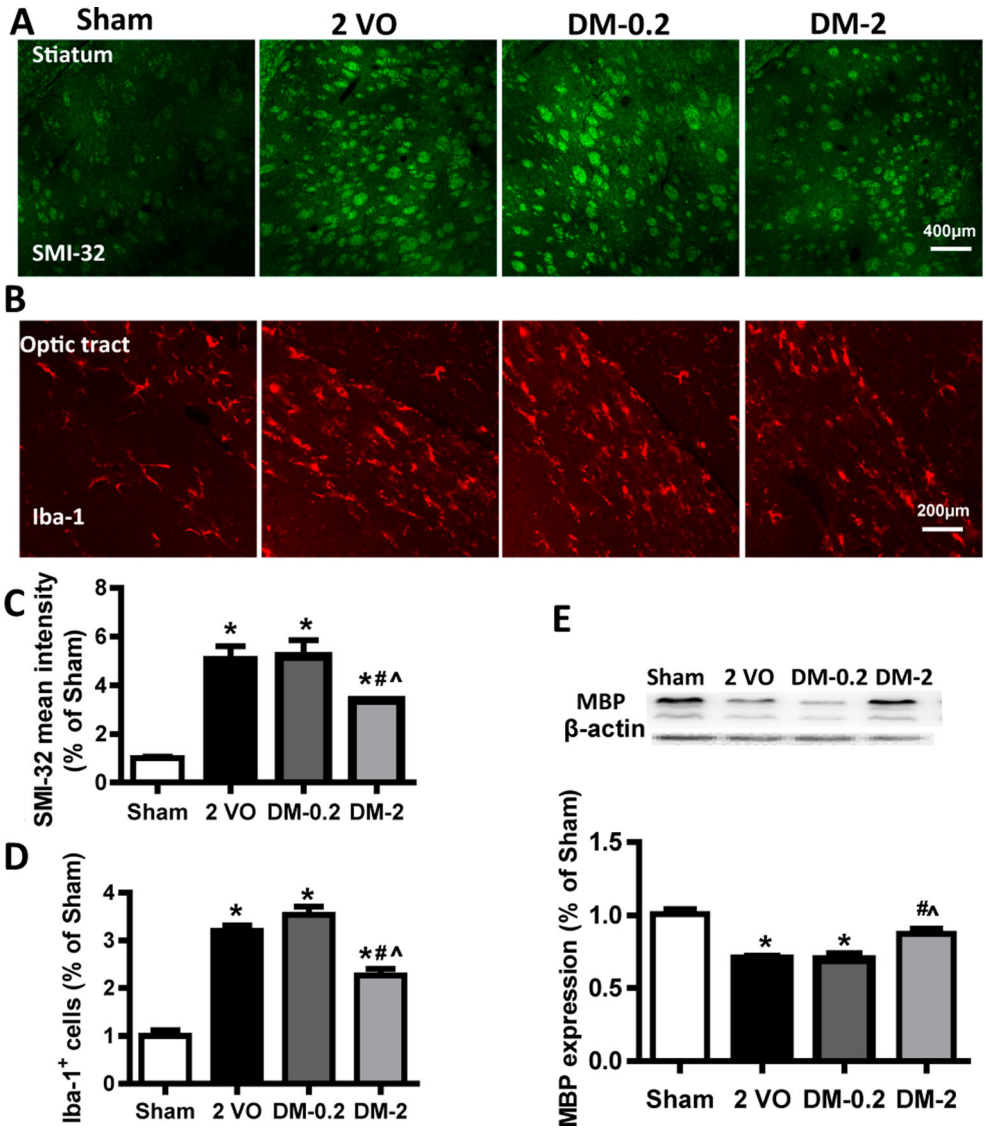

Fig. 2 DM-0.2 treatment protects white matter in the striatum and optic tract area. **a**, **b** Representative immunofluorescent staining for dephosphorylated neurofilament protein (SMI-32) in the striatum and Iba-1 in the optic tract area at 37 days after DM treatment. **c** DM-2 treatment attenuated the white matter impairment in the striatum with lower intensity of SMI-132 ($n=4$, $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2). **d** The microglial activation in the optic tract area was significantly reduced by the DM-2 treatment ($n=4$, $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2). **e** MBP in the striatum is protected by DM-2 treatment as assessed by western blots. The data are presented as the ratio of Iba-1 to β-actin protein levels. Each *bar* represents the mean ± SEM of four independent experiments. $^{*}P<0.05$, compared with Sham, $^{\#}P<0.05$, compared with 2VO, $^{\wedge}P<0.05$, compared with DM-0.2

HO-1 and Nrf2 were assessed in this study. As shown in Fig. 3c, the level of HO-1 was significantly increased in DM-2-treated rats. To verify that Nrf2 mediates the rise in HO-1 in this model, the levels of Nrf2 in the cytoplasm and nucleus were assessed. Western blots showed that there was a significant increase in Nrf2 in the nucleus and a significant decrease in Nrf2 in the cytoplasm (Fig. 3a, b), verifying that the nuclear translocation of Nrf2 mediates the rise of HO-1 in this model. Furthermore, we assessed the level of caspase-3 (a marker of proapoptotic protein) in the hippocampal CA1 area. The data show that caspase-3-positive cells were identified in the 2VO-operated group, whereas few caspase-3-positive cells were observed in the sham-operated rats. However, the number of caspase-3-positive cells was reduced by 25 % in the DM-2-treated 2VO group compared with the 2VO-operated rats ($P<0.05$) (Fig. 3d, e). Together, these results are consistent with the hypothesis that HO-1 and Nrf2 are essential for DM-2 mediated neuroprotection in VaD.

DM-2 Treatment Decreases Oxidative Stress in the Hippocampal CA1 Area

Given the crucial role of oxidative stress in the pathogenesis of VaD, biomarkers of oxidative stress, including endogenous antioxidant enzymes and lipid oxidation, were assessed. As shown in Fig. 4a–c, the activity of endogenous antioxidant enzymes such as SOD was increased (Fig. 4a, b $P<0.05$) and 4-HNE as a measure of protein oxidation (Fig. 4c, $P<0.001$) and the inflammation mediator level of TNF-α (Fig. 4e $P<0.05$) was significantly decreased in the hippocampus after the DM-2 treatment compared with the 2VO-operated rats. Recently, it was reported that ROS produced by NOX-1 as a novel molecular source of ROS induced hippocampal neurodegeneration, damaged nuclear DNA, and caused cognitive impairment in chronic cerebral hypoperfused rats. Therefore, the level of NOX-1 was assessed. As shown in Fig 4d, DM-2 treatment reduced the level of NOX-1 in the

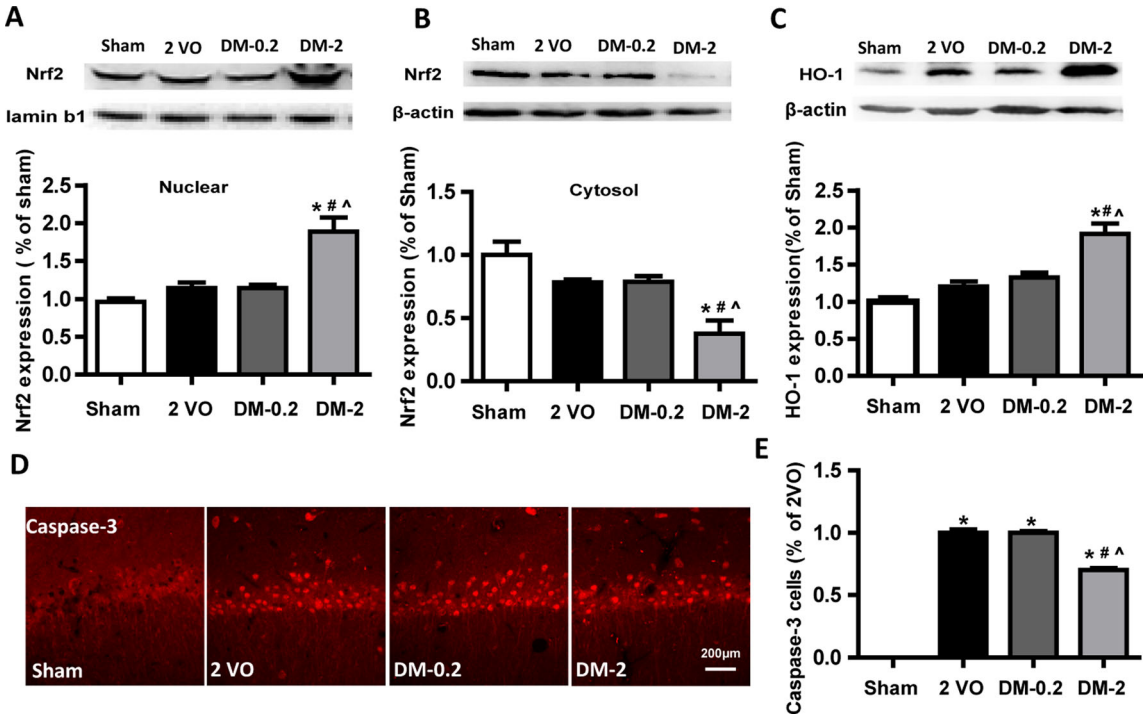

Fig. 3 HO-1 upregulation plays a role in DM-mediated neuroprotection against 2VO in rats. The level of Nrf2 in the nucleus was increased (**b**) and the level in the cytoplasm was decreased (**b**) with the DM-2 treatment. The results suggest that DM-2 treatment mediates nuclear translocation of Nrf2. The expression of HO-1 was significantly increased in the hippocampus (**c**). **d** Representative photographs of tissue sections stained with caspase-3 (*red*) antibody taken from the hippocampus CA1 area of different groups. **e** Caspase-3 cell counts were decreased with DM-2 treatment ($n = 4$, $^{*}P < 0.05$, compared with Sham, $^{\#}P < 0.05$, compared with 2VO, $^{\wedge}P < 0.05$, compared with DM-0.2)

hippocampus compared with the 2VO-operated rat. These data indicate that DM-2 treatment could decrease oxidative stress in the hippocampal CA1 area.

DM-2 Reduces Chronic Cerebral Hypoperfusion-Induced Morris Water Maze (MWM) Performance Deficits in Rats

To evaluate the effect of DM on the recovery of cognitive function after 2VO, spatial memory was evaluated by assessing learning and memory retention using the Morris water maze (MWM) test. To determine whether the group differences observed in escape latencies were due to differences in swimming, especially between the sham-operated group and the 2VO group, swimming speeds were calculated for each group. No differences in swimming speed were observed among the four groups (repeated-measures one-way analysis of variance (ANOVA), Fig. 5a). In the acquisition trials, the sham-operated animals gradually learned the location of the hidden platform, which was evidenced by shorter latencies throughout the test period. CCH induced by 2VO resulted in a significant impairment in spatial learning compared with sham-operated controls (repeated-measures two-way ANOVA, $P < 0.001$; Fig. 5b). DM-2-treated 2VO animals exhibited shorter mean session latencies in locating the platform compared with the 2VO and DM-0.2 group (repeated-measures two-way ANOVA, $P < 0.05$; Fig. 5b). In the probe trial, the time spent in the target quadrant was different between the groups. The analysis showed that the percentage of time and distance spent in the target quadrant was significantly decreased in 2VO-operated rats ($P < 0.001$) compared with sham-operated rats (Fig. 5c, d) and was increased in DM-2-treated rats ($P < 0.001$) compared with 2VO-operated rats (Fig. 5c, d). The difference in platform site crossings was even more significant in the probe trial test (Fig. 5e).

Discussion

In the present study, we demonstrated the following: (1) DM treatment could prevent neuronal death in the hippocampal CA1 region, white matter lesions and glial cell activation, especially microglial activation caused by 2VO-induced VaD; (2) DM treatment could increase HO-1 upregulation by activating Nrf2 in the hippocampus to protect the neuron in the CA1 area, at least in part; and (3) DM treatment could significantly decrease the level of oxidative stress by increasing levels of SOD in the hippocampus resulting in improvement in cognitive function.

Increasing evidence indicates that inflammatory-related microglia and astrocyte are considered to be involved in the

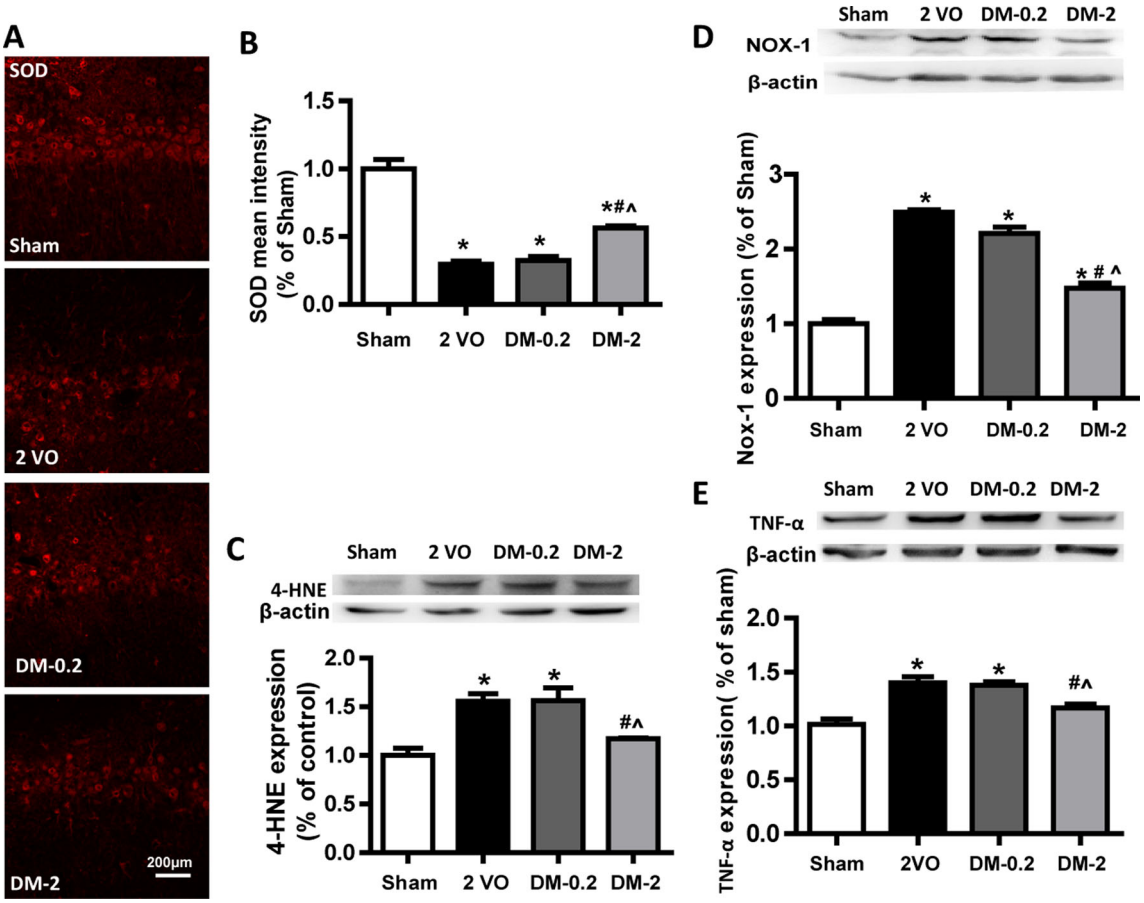

Fig. 4 DM-2 treatment decreases oxidative stress in the hippocampal CA1 area. **a** Representative photographs of tissue sections stained with SOD (*red*) antibody taken from the hippocampus CA1 area of different groups. **b** DM-2 treatment increased the level of SOD ($n = 4$, $^{*}P < 0.05$, compared with Sham, $^{\#}P < 0.05$, compared with 2VO, $^{\wedge}P < 0.05$, compared with DM-0.2). **c** DM-2 treatment decreased the expression of 4-HNE in the hippocampus. The data are presented as the ratio of 4-HNE to β-actin protein levels. Each *bar* represents the mean ± SEM of four independent experiments ($^{*}P < 0.05$, compared with Sham, $^{\#}P < 0.05$, compared with 2VO, $^{\wedge}P < 0.05$, compared with DM-0.2). **d** DM-2 treatment inhibits expression of NOX-1 in the hippocampus. The data are presented as the ratio of NOX-1 to β-actin protein levels. **e** DM-2 treatment inhibits expression of TNF-α in the hippocampus. The data are presented as the ratio of TNF-α to β-actin protein levels. Each *bar* represents the mean ± SEM of four independent experiments ($^{*}P < 0.05$, compared with Sham, $^{\#}P < 0.05$, compared with 2VO, $^{\wedge}P < 0.05$, compared with DM-0.2)

cognitive impairment in CCH. In the brain, microglia are a robust source of oxidative stress where extracellular ROS is generated from several enzymes including iNOS, NADPH oxidase, COX-2, and myeloperoxidase. These enzymes are responsible for inflammatory processes mediated by oxidative stress. It is reported that DM exerts protective effect in PD through the inhibition of microglial cell production of NO, secretion of cytokines such as TNF-α, and release of superoxide free radicals at doses of 10 mg/kg and exert protective effects on MS at dose of 0.1 mg/kg. Based on these researches, two different doses of DM-treatment, 0.2 or 2 mg/kg, were used in the study. In the study, DM-2 treatment alleviated microglial activation accompanied by oxidative stress in the hippocampus (Fig, 1e, f, i) and the effect was even more significant in the optic tract area (Fig. 2b, d), which is more sensitive to CCH. These data suggest that DM treatment could inhibit the microglia activation to exert an antioxidant effect.

Accumulating evidence indicates that DM treatment could target the expression and activity of NADPH oxidase-2 present in microglia to exert neuroprotective effects in atherosclerosis, hypertension, PD, and MS [29]. However, our results show that expression of NOX-2 does not change in the hippocampus in 2VO-induced VaD (data not shown). These results are consistent with the previous report that NOX-1, rather than NOX-2, plays a key role in the pathophysiology of CCH-induced cognitive decline and brain injury [11]. Based on the vital role of NOX-1 in VaD, we assessed the level of NOX-1 in the hippocampus and the results show that DM treatment significantly decreased the level of NOX-1 (Fig. 4d). The result further supports the hypothesis that DM treatment has therapeutic potential for 2VO-induced brain damage.

We know that a high degree of oxidative stress could trigger endogenous protective antioxidant systems, such as SOD

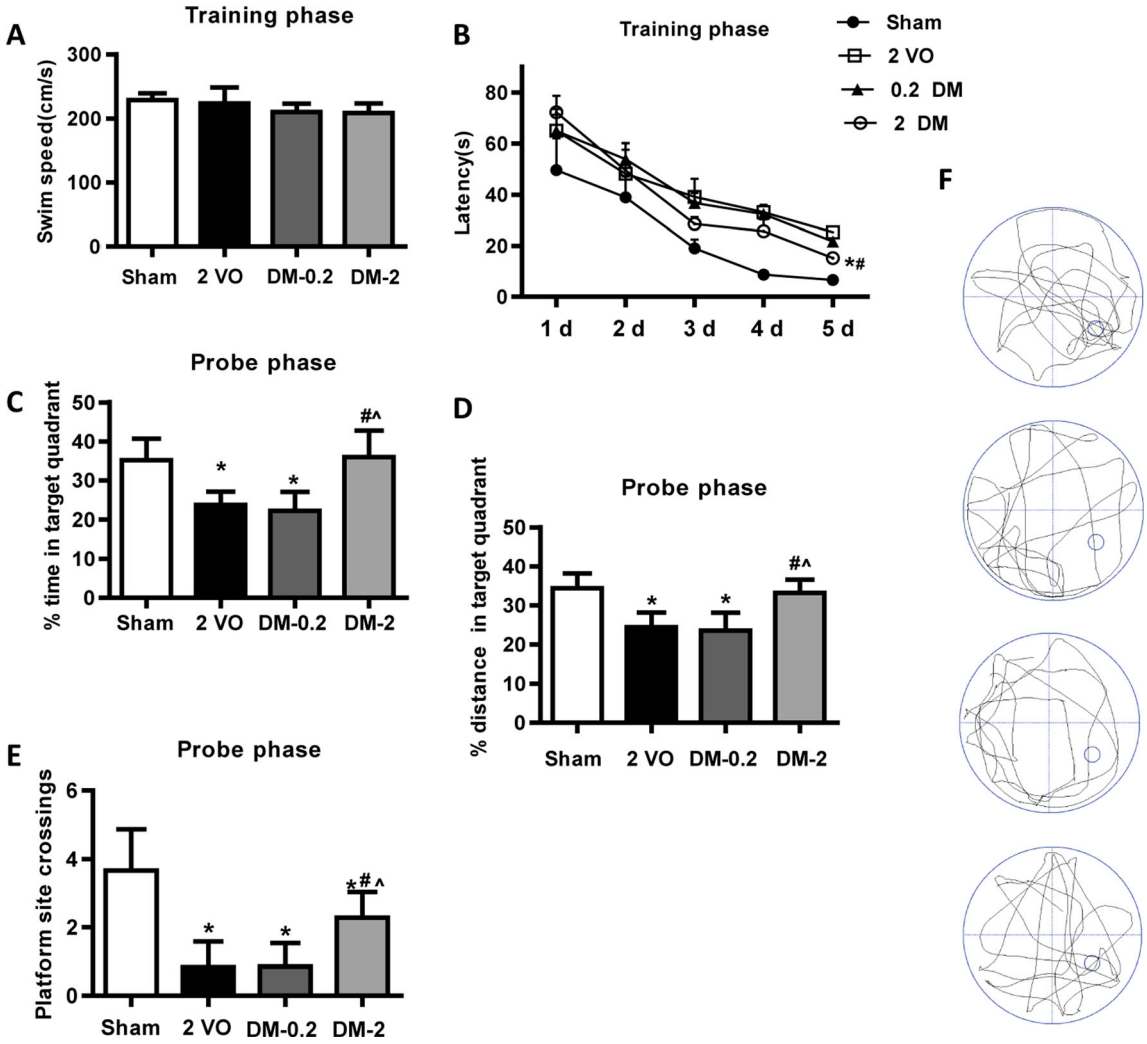

Fig. 5 DM-2 reduces chronic cerebral hypoperfusion-induced Morris water maze (*MWM*) performance deficits in rats. Spatial memory evaluation using swimming speed (**a**), time latency (**b**), % time in target quadrant (**c**), % distance in target quadrant (**d**), and platform site crossings (**e**) in the probe trial test ($n = 6$/group, $^{*}P < 0.001$, compared with Sham, $^{\#}P < 0.001$, compared with 2VO, $^{\wedge}P < 0.001$, compared with DM-0.2). Sham and 2VO rats treated with vehicle; DM-0.2 and DM-2 treated with DM (0.2 mg/kg, i.p., "DM-0.2" and 2 mg/kg, i.p., "DM-2", respectively, for 37 days). **f** Representative swim paths from the probe phase

and HO-1, which are important defenses against oxidative stress [30]. In this study, DM treatment increased the level of SOD in 2VO-induced VaD. The result is in agreement with the results of previous reports that DM increased SOD, reduced thiobarbituric acid reactive substances in the hippocampal and striatal regions of monosodium glutamate-induced neurodegeneration in rats, and improved neuroprotection [31]. Among the antioxidant enzymes, HO-1 is particularly noteworthy not only because it degrades toxic heme but also because it produces multiple neuroprotective molecules, including the antioxidant biliverdin and carbon monoxide [32]. Recent studies have also demonstrated that brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) expression was upregulated following the upregulation of HO-1 in dopaminergic neurons [33–35]. In our study, we found that DM treatment enhanced the nuclear translocation of Nrf2 to upregulate the activation of HO-1. These results suggest that DM exerts its protective effect by activating the Nrf2-HO-1 pathway.

In summary, our study provides novel insight that DM could protect neurons against the damage induced by VaD. The present study is the first demonstration of the mechanistic involvement of these neuroprotective effects. Thus, this research provides a promising therapeutic strategy for the treatment of VaD.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

1. Aggarwal NT, Decarli C (2007) Vascular dementia: emerging trends. Semin Neurol 27:66–77
2. Erkinjuntti T, Roman G, Gauthier S, Feldman H, Rockwood K (2004) Emerging therapies for vascular dementia and vascular cognitive impairment. Stroke 35:1010–1017
3. Jellinger KA (2008) Morphologic diagnosis of "vascular dementia"—a critical update. J Neurol Sci 270:1–12
4. Roman GC (2003) Vascular dementia: distinguishing characteristics, treatment, and prevention. J Am Geriatr Soc 51:S296–304
5. de la Torre JC (2010) Vascular risk factor detection and control may prevent Alzheimer's disease. Ageing Res Rev 9:218–225
6. Lin MS, Chiu MJ, Wu YW et al (2011) Neurocognitive improvement after carotid artery stenting in patients with chronic internal carotid artery occlusion and cerebral ischemia. Stroke J Cereb Circ 42:2850–2854
7. Yan MH, Wang X, Zhu X (2013) Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic Biol Med 62:90–101
8. Ayala-Pena S (2013) Role of oxidative DNA damage in mitochondrial dysfunction and Huntington's disease pathogenesis. Free Radic Biol Med 62:102–110
9. Faes L, Callewaert G (2011) Mitochondrial dysfunction in familial amyotrophic lateral sclerosis. J Bioenerg Biomembr 43:587–592
10. Aliev G, Smith MA, Obrenovich ME, de la Torre JC, Perry G (2003) Role of vascular hypoperfusion-induced oxidative stress and mitochondria failure in the pathogenesis of Azheimer disease. Neurotox Res 5:491–504
11. Choi DH, Lee KH, Kim JH et al (2014) NADPH oxidase 1, a novel molecular source of ROS in hippocampal neuronal death in vascular dementia. Antioxid Redox Signal 21:533–550
12. Liu H, Zhang J (2012) Cerebral hypoperfusion and cognitive impairment: the pathogenic role of vascular oxidative stress. Int J Neurosci 122:494–499
13. Bennett S, Grant MM, Aldred S (2009) Oxidative stress in vascular dementia and Alzheimer's disease: a common pathology. J Alzheimer's Dis JAD 17:245–257
14. Gackowski D, Rozalski R, Siomek A, Dziaman T, Nicpon K, Klimarczyk M, Araszkiewicz A, Olinski R (2008) Oxidative stress and oxidative DNA damage is characteristic for mixed Alzheimer disease/vascular dementia. J Neurol Sci 266:57–62
15. Casado A, Encarnacion Lopez-Fernandez M, Concepcion Casado M, de La Torre R (2008) Lipid peroxidation and antioxidant enzyme activities in vascular and Alzheimer dementias. Neurochem Res 33:450–458
16. Chaves M, Toral A, Bisonni A et al (2014) Treatment with vitamin D and slowing of progression to severe stage of Alzheimer's disease. Vertex 25:85–91
17. Masaki KH, Losonczy KG, Izmirlian G, Foley DJ, Ross GW, Petrovitch H, Havlik R, White LR (2000) Association of vitamin E and C supplement use with cognitive function and dementia in elderly men. Neurology 54:1265–1272
18. Yang FB (2011) Protective effects of curcumin on vascular endothelial injuries induced by radiation. Zhonghua lao dong wei sheng zhi ye bing za zhi. Zhonghua laodong weisheng zhiyebing zazhi. Chin J Ind Hyg Occup Dis 29:846–848.
19. Zhang C, Browne A, Child D, Tanzi RE (2010) Curcumin decreases amyloid-beta peptide levels by attenuating the maturation of amyloid-beta precursor protein. J Biol Chem 285:28472–28480
20. Werling LL, Lauterbach EC, Calef U (2007) Dextromethorphan as a potential neuroprotective agent with unique mechanisms of action. Neurologist 13:272–293
21. Britton P, Lu XC, Laskosky MS, Tortella FC (1997) Dextromethorphan protects against cerebral injury following transient, but not permanent, focal ischemia in rats. Life Sci 60:1729–1740
22. Kim HC, Pennypacker KR, Bing G, Bronstein D, McMillian MK, Hong JS (1996) The effects of dextromethorphan on kainic acid-induced seizures in the rat. Neurotoxicology 17:375–385
23. Topsakal C, Erol FS, Ozveren MF, Yilmaz N, Ilhan N (2002) Effects of methylprednisolone and dextromethorphan on lipid peroxidation in an experimental model of spinal cord injury. Neurosurg Rev 25:258–266
24. Zhang W, Wang T, Qin L et al (2004) Neuroprotective effect of dextromethorphan in the MPTP Parkinson's disease model: role of NADPH oxidase. FASEB J Off Pub Fed Am Soc Exp Biol 18: 589–591
25. Chechneva OV, Mayrhofer F, Daugherty DJ, Pleasure DE, Hong JS, Deng W (2011) Low dose dextromethorphan attenuates moderate experimental autoimmune encephalomyelitis by inhibiting NOX2 and reducing peripheral immune cells infiltration in the spinal cord. Neurobiol Dis 44:63–72
26. Liu SL, Li YH, Shi GY et al (2009) Dextromethorphan reduces oxidative stress and inhibits atherosclerosis and neointima formation in mice. Cardiovasc Res 82:161–169
27. Liu Y, Qin L, Li G, Zhang W, An L, Liu B, Hong JS (2003) Dextromethorphan protects dopaminergic neurons against inflammation-mediated degeneration through inhibition of microglial activation. J Pharmacol Exp Ther 305:212–218
28. Wu TC, Chao CY, Lin SJ, Chen JW (2012) Low-dose dextromethorphan, a NADPH oxidase inhibitor, reduces blood pressure and enhances vascular protection in experimental hypertension. PLoS ONE 7:e46067
29. Lee JH, Park SY, Shin YW, Hong KW, Kim CD, Sung SM, Kim KY, Lee WS (2006) Neuroprotection by cilostazol, a phosphodiesterase type 3 inhibitor, against apoptotic white matter changes in rat after chronic cerebral hypoperfusion. Brain Res 1082:182–191
30. Fukai T, Ushio-Fukai M (2011) Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal 15:1583–1606
31. Ramanathan M, Sivakumar S, Anandvijayakumar PR, Saravanababu C, Pandian PR (2007) Neuroprotective evaluation of standardized extract of Centella asciatica in monosodium glutamate treated rats. Indian J Exp Biol 45:425–431
32. Imuta N, Hori O, Kitao Y, Tabata Y, Yoshimoto T, Matsuyama T, Ogawa S (2007) Hypoxia-mediated induction of heme oxygenase type I and carbon monoxide release from astrocytes protects nearby cerebral neurons from hypoxia-mediated apoptosis. Antioxid Redox Signal 9:543–552
33. Hung SY, Liou HC, Kang KH, Wu RM, Wen CC, Fu WM (2008) Overexpression of heme oxygenase-1 protects dopaminergic neurons against 1-methyl-4-phenylpyridinium-induced neurotoxicity. Mol Pharmacol 74:1564–1575
34. Hung SY, Liou HC, Fu WM (2010) The mechanism of heme oxygenase-1 action involved in the enhancement of neurotrophic factor expression. Neuropharmacology 58:321–329
35. Motterlini R, Foresti R (2014) Heme oxygenase-1 as a target for drug discovery. Antioxid Redox Signal 20:1810–1826