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Protective effect of Daming capsule against chronic cerebral ischemia

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Abstract

Background: Accumulating evidence has shown that chronic cerebral ischemia (CCI) is one of the major causes of vascular dementia (VD) characterized by dysregulated cholesterol homeostasis and lipoprotein disturbances. Positive value of lipid-lowering agents has been widely evaluated for the treatment of VD. In the present study, we investigated whether Daming capsule (DMC) protected against CCI-induced VD and its possible mechanisms of action. DMC is a multi-herbal formula composed of *Rheum palmatum L.*, *Cassia obtusifolia L.*, *Salvia miltiorrhiza*, and *Panax ginseng C.A.*, which has been used to treat hyperlipidemia for years in China.

Methods: A network pharmacology method was established to reveal whether DMC contained any chemical constituent targeting CCI-related proteins. Furthermore, the potential anti-CCI effects of DMC (100 mg/kg or 200 mg/kg) administered for 30 days were investigated *in vivo* on rats that were subjected to permanent bilateral occlusion of the carotid arteries (2-VO). Spatial learning and memory abilities were evaluated using a Morris water maze (MWM) and morphological changes of cerebral cortex and hippocampus were assessed using hematoxylin and eosin staining. Moreover, the lipid peroxidation levels and antioxidative capabilities were measured using biochemical analysis.

Results: Our network pharmacology analysis revealed the existence of multiple CCI-related chemical-target interactions in DMC, suggesting a potential protective effect. An *in vivo* experiment verified that 200 mg/kg DMC improved cognitive deficits of 2-VO rats in the MWM test and attenuated pathological alterations in both the cerebral cortex and the hippocampus. Biochemical assays indicated that DMC decreased malondialdehyde levels and CCI-elevated superoxide dismutase activities, but increased the activities of glutathione peroxidase and catalase.

Conclusions: Our findings suggested that DMC protected against cognitive dysfunction and nerve injuries caused by CCI, which is most likely related to its antioxidant actions.

Keywords: Daming capsule, Chronic cerebral ischemia, Morris water maze test, Antioxidant

Background

Vascular dementia (VD) is the second most common cause of dementia, after Alzheimer's disease, in the elderly population [1]; it includes cognitive function decline and brain structure damages [2]. It has been revealed that hypertension, diabetes, and hypercholesterolemia are crucial risk factors for the pathogenesis and development of VD [3–5]. Increasingly, evidence suggests that the pathogenesis of VD is closely related to cholesterol homeostasis and lipoprotein disturbance [6, 7]. This led to extensive assessment of lipid-lowering agents by many researchers in the field of VD.

Considerable evidence indicates that chronic cerebral ischemia (CCI) is a major cause of VD [8, 9]. Ischemic cerebrovascular diseases, such as CCI, are characterized by vessel lesions. Similar mechanisms of ischemic cardiovascular disease were found in CCI [10]. Among them, oxidative stress and disturbed cellular pro-oxidant—antioxidant status play vital roles in

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CCI-induced cognitive impairment [11]. Attenuating oxidative stress is an important therapeutic strategy for CCI treatment.

Daming capsule (DMC), a multi-herbal formula, has been used clinically to treat hyperlipidemia for years in China; it is composed of four herbs, including *Rheum palmatum L.*, *Cassia obtusifolia L.*, *Salvia miltiorrhiza*, and *Panax ginseng C.A*. DMC was reported to have multiple pharmacological activities, such as preventing cardiac dysfunction [12], resisting free radicals [13, 14], improving lipidemia conditions [15, 16], decreasing blood glucose levels [17], protecting against myocardial ischemia reperfusion injury, reducing myocardial infarctions [18, 14], and restoring aortic endothelial dysfunction [17, 19]. *Rheum palmatum L.*, the monarch component of DMC, was validated to promote blood circulation, dissipate blood stasis, and protect rats with cerebral ischemia [20]. *Cassia obtusifolia L.* attenuates memory impairment induced by scopolamine or permanent bilateral occlusion of the carotid arteries (2-VO) [21]. Moreover, *Salvia miltiorrhiza* [22] and *Panax ginseng C.A.* [23] have shown beneficial effects on many nervous system diseases. Taken together, the above studies strongly suggest therapeutic potential of DMC in CCI.

Recently, network pharmacology has been utilized to virtually evaluate the pharmacological effects of traditional Chinese medicine (TCM) as a whole. By mapping the polypharmacology network, researchers predict the clinical curative effects of TCM and explore the potential drug targets related to the specific disease [24]. Recently, a network pharmacology-based strategy, called "network target, multi-components," was put forward, which was thought to provide a more comprehensive and realistic understanding of TCM [25]. In this study, a network pharmacology method was established to predict the potential therapeutic effects of DMC on CCI by mapping CCI-related polypharmacology networks. Next, a 2-VO rat model of CCI was used *in vivo* to assess the effects of DMC on cognitive deficits, histopathological changes, and oxidative stress after 2-VO injury. Such efforts aim to determine whether DMC has potential therapeutic value for the treatment of CCI.

Methods

Chemicals and reagents

DMC (Drug Approval Number: Z20030085) was provided by Harbin Yida Co., Ltd. (Harbin, China). Hydergine (Batch No. 2K847T) was purchased from Tianjin Huajin Pharmaceutical Co., Ltd. (Tianjin, China). Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other reagents were of analytical grade.

Collection of information about DMC compounds and related targets

The information about DMC compounds was obtained from the TCM Database @Taiwan [26]. After that, the STITCH 3.1 database was used to search for corresponding targets to these constituents [27]. We only retained targets with high confidence scores (>0.7) in *Homo sapiens*. The compounds with many targets (n >50) were discarded to avoid overestimation of the potential effects of DMC on CCI. Next, the HuGE Navigator was applied to obtain experimentally validated CCI-related genes [28].

Construction of CCI-specific target-constituent-herb association networks for DMC

We integrated constituent-herb associations and constituent-target associations and imported it into Cytoscape v2.8.3 [29] to establish four target-constituent-herb association networks, in which targets, chemicals, and herbs were represented as nodes with different shapes. The gene-CCI associations were also imported as node attributes. If a target was not associated with CCI, it was deleted from the network.

Animals and establishment of a 2-VO rat model

Adult male Sprague-Dawley rats (250–300 g) were purchased from the Animal Center of the 2nd Affiliated Hospital of Harbin Medical University (Harbin, China). All animals were housed in a room with a controlled temperature of 23 ± 1 °C and humidity of 55 ± 5 % with a regular 12 h light-dark cycle and provided free access to water and food. All experimental procedures regarding the animals were approved by the ethic committees of Harbin Medical University. The rats were acclimatized to the housing conditions for at least 4 days before use. All behavioral tests were performed between 8:00 a.m. and 17:30 p.m. CCI was induced by 2-VO according to the procedures described before [30]. Rats were anesthetized with 10 % chloral hydrate [350 mg/kg, intraperitoneal injection (i.p.)]. Briefly, the bilateral common carotid arteries of rats were separated through a midline cervical incision, from the cervical sympathetic and vagal nerves, and double ligated with 5–0 silk suture. The sham group received the same operation without the arterial ligation. During the surgery, the rat's body temperature was maintained at 37.5 ± 0.5 °C using a heating pad until they recovered from anesthesia. Then, all the animals were housed in cages with food and water *ad libitum* and used after one week of quarantine and acclimatization.

Drug administration

On the eighth day after surgery, the 2-VO rats were randomly assigned to four groups: (1) the rats in the 2-VO group were administered saline, the rats in the DMCl

group were administered low-dose (100 mg/kg) DMC, (3) the rats in the DMCh group were administered high-dose (200 mg/kg) DMC, and (4) the rats in the HYD group were administered 0.6 mg/kg Hydergine. Rats in the sham group were administered saline. Rats were orally administered the drug once daily at 8:00 am. Hydergine was dissolved in saline and used as a positive control. The common human daily dose of DMC is 1.8 g/60 kg/d of body weight. According to the formula: d/rat = d/human × 0.71/0.11 [31], the corresponding dose of DMC for rats was 187 mg/kg/d. Therefore, 200 mg/kg/d and 100 mg/kg/d were chosen as the high and low doses, respectively. DMC powder was suspended in saline; the dosage volume was 10 mL/kg.

Morris water maze (MWM) test

In order to investigate the effect of DMC on 2-VO-induced learning and memory impairment, the spatial learning and memory performance of rats were tested using the MWM test [32]. The MWM test was conducted after drug administration for 30 days. Escape latency was used to assess learning and the memory of the rats. If the rat failed to locate 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 and allowed to stay on the platform for 30 s before being removed from the water. A computer tracking program was started as soon as the rats were released into the water and stopped when the rats climbed on the platform or did not locate the platform within 90 s. After the last training trial on day 5, the platform was removed from the pool and the rats were allowed to swim for 90 s by the probe trials; the swimming distance in the target quadrant without the platform was used to evaluate leaning ability.

Histopathological examination

Rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) after the behavioral test and were perfused through the left cardiac ventricle with 100 mL saline, followed by 4 % paraformaldehyde in phosphate buffer. Then, the brain of each rat was removed and fixed by immersion in the same fixative at 4 °C. The fixed brains were dehydrated and embedded in paraffin blocks. They were cut into 4-mm thick serial sections. These sections were then stained with hematoxylin and eosin and examined for brain damage under a light microscope (magnification × 200) by an examiner blinded to experimental conditions.

Biochemical analysis

Rats were decapitated under anesthesia after the MWM test. The brains were rapidly removed, and then the hippocampus and cerebral cortex were removed separately and placed in ice-cold saline, blotted dry, weighed, and then immediately frozen and stored at −80 °C until biochemical assays were performed. Then, tissue samples were cut into pieces and homogenized in ice-cold saline to yield 10 % (w/v) homogenates. The oxidant and antioxidant status of the cerebral cortex and hippocampus of rats subjected to CCI was assessed by measuring the MDA levels and the activities of SOD, GPx, and CAT, according to the manufacturer's instructions. Protein content was determined using the Bradford method [33].

Statistical analysis

All results were presented as mean ± SEM. GraphPad Prism v6.0 software was used for statistical evaluation. One-way analysis of variance followed by the Tukey's test was used to analyze data. The level of significance was established at $p < 0.05$.

Results

Multiple constituents in DMC were associated with CCI

In total, 298 constituents were found in DMC and 55 target-constituent associations related to CCI were identified (Table 1). After integrating experimental evidence from multiple sources, four CCI-specific target-constituent-herb association networks were constructed for DMC, as shown in Fig. 1, in which multiple constituents were found to be associated with CCI-related targets in one herb. Multiple constituents in one herb had common CCI-related targets.

DMC attenuated learning and memory impairment induced by 2-VO

In the MWM test, rats of all groups were efficient at locating the platform after successive trials. As shown in Fig. 2 (a–e), the escape latencies of rats decreased gradually in all groups during the five day test. For the first two days of the training trials, the escape latencies did not differ significantly between groups. However, the 2-VO group exhibited a longer latency to locate the platform than the sham group ($p < 0.05$, Fig. 2c) from

Table 1 Validated CCI-related target-constituent interactions in DMC

Herb	Constituent (*n*)	Gene (*n*)	Target constituent interaction
Rheum palmatum L.	7	9	14
Cassia obtusifolia L.	4	4	4
Salvia miltiorrhiza	11	16	30
Panax ginseng C.A.	6	6	8
Total	25	27	55

CCI: Chronic cerebral ischemia; DMC: Daming capsule

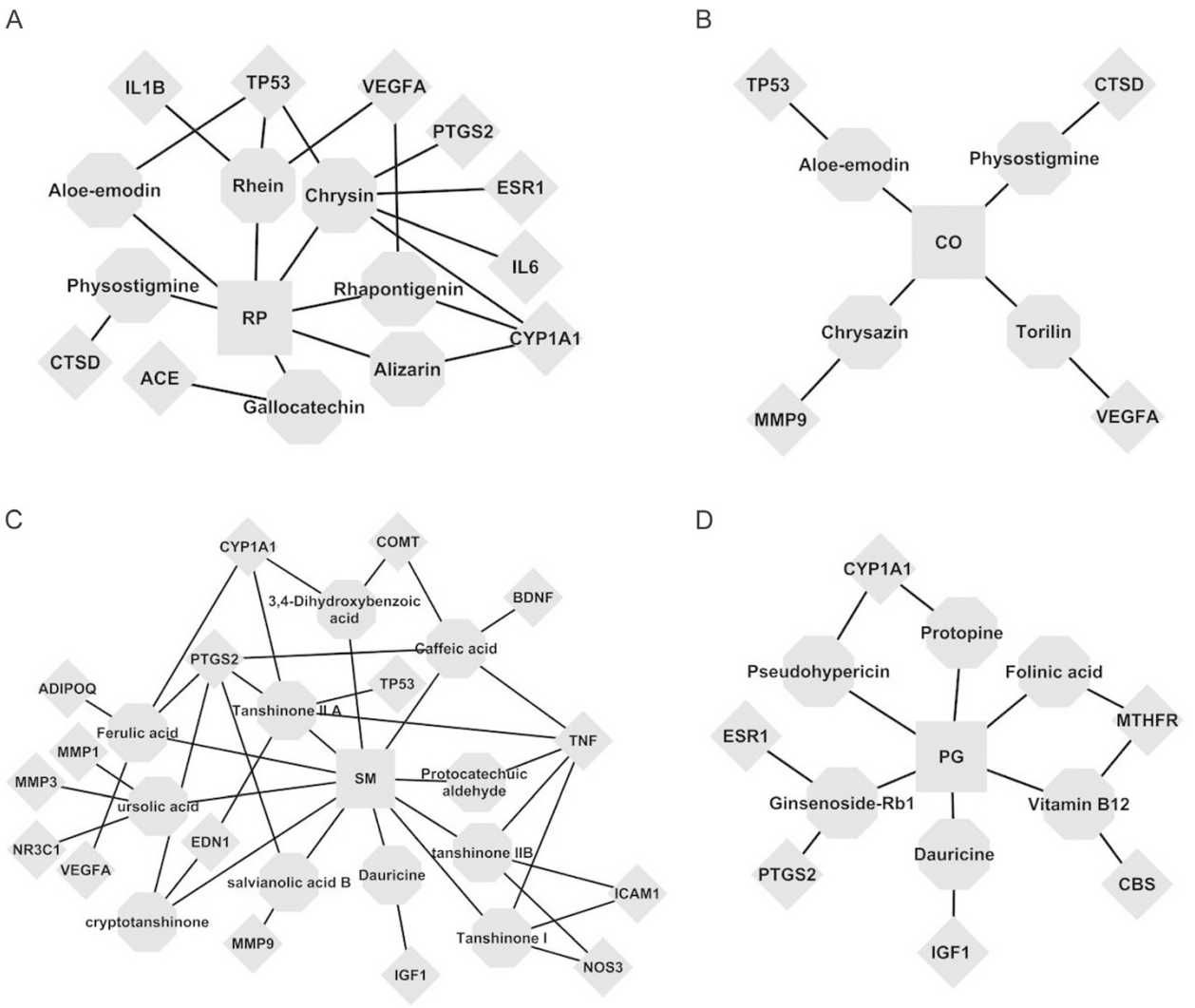

Fig. 1 CCI-specific target-chemical-herb association networks for RP (**a**), CO (**b**), SM (**c**), and PG (**d**) in DMC. Diamond, hexagonal, and rectangular nodes represent targets, chemicals, and herbs, respectively. Edges represent validated associations between chemicals and the target or herbs. CCI: chronic cerebral ischemia; DMC: Daming capsule; RP: *Rheum palmatum L.*; CO: *Cassia obtusifolia L.*; SM: *Salvia miltiorrhiza*; PG: *Panax ginseng C.A*

the third day onwards. These results demonstrated that CCI caused obvious learning and memory impairment in 2-VO rats. The escape latency of the DMCl group was shorter than that of 2-VO group, but not significantly different. The escape latency of the DMCh group significantly decreased on the fifth day ($p < 0.05$), similar to that observed for the 0.6 mg/kg HYD group (Fig. 2e). In the probe trials, the percentage of swimming distance in the target quadrant of the 2-VO group significantly decreased compared to the sham group. Compared to the 2-VO group, the percentage of swimming distance in the target quadrant of the DMCh and HYD groups significantly increased ($p < 0.05$, Fig. 2f). Furthermore, the swimming paths of the 2-VO and DMCl groups were uniformly distributed around the four quadrants, while the swimming paths of the other groups focused on the target quadrant (Fig. 3).

DMC reversed CCI-induced morphological changes of the cerebral cortex and hippocampus

Hematoxylin and eosin staining was used to survey the morphological changes in the cerebral cortex and hippocampus after CCI and the effects of drug treatment. As shown in Fig. 4, prominent morphological changes, such as neuronal cell loss, nuclei shrinkage, glial proliferation, and dark staining of neurons, were visualized in the cerebral cortex and hippocampus of the 2-VO group. DMC (200 mg/kg) prevented CCI-induced histological injuries in the rat brain, similar to that found in the HYD group.

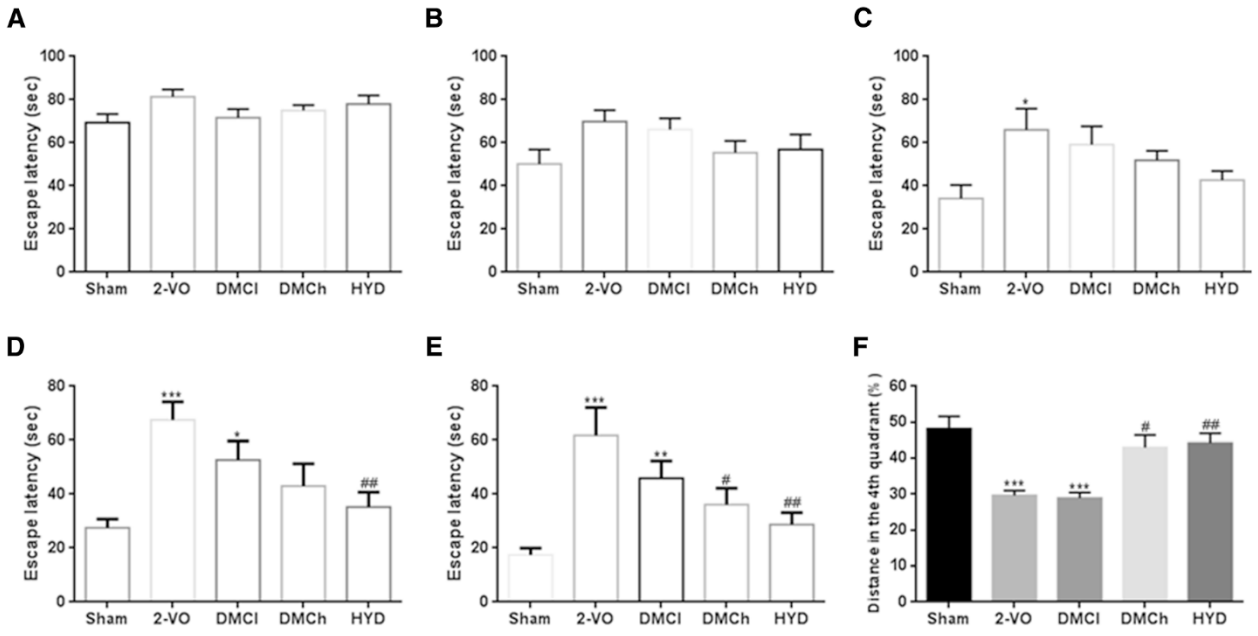

Fig. 2 Effect of DMC on CCI-induced cognitive deficits as measured by MWM test ($n = 8$). **a**–**e** Escape latency from day 1 to day 5. **f** The percentage of distance in the 4th quadrant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the Sham; # $p < 0.05$, ## $p < 0.01$ compared to the 2-VO group. CCI: chronic cerebral ischemia; DMC: Daming capsule

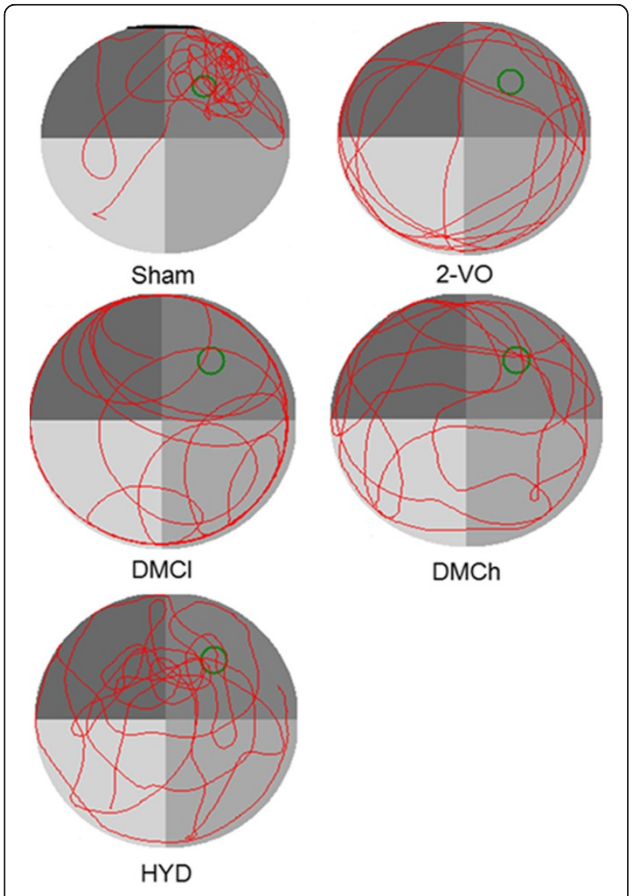

Fig. 3 Measurement of the swimming paths

Effects of DMC on MDA levels, the activities of SOD, GPx and CAT in the cerebral cortex and hippocampus

The MDA levels in the cerebral cortex and hippocampus and the SOD activity in cerebral cortex significantly increased in the 2-VO group compared to the sham group ($p < 0.05$, Fig. 5a–d). After repeated administration of 200 mg/kg DMC, the content of MDA in the two brain regions and the SOD activity in the cerebral cortex significantly decreased compared to the corresponding values in the 2-VO group ($p < 0.05$), whereas administration of 100 mg/kg DMC had modest effects. The SOD activity in the hippocampus did not differ across the groups and a slight increase in the SOD activity was observed only in the 2-VO group. Both the GPx and CAT activities significantly decreased in the cerebral cortex and hippocampus in the 2-VO compared to the sham group ($p < 0.05$, Fig. 5e–h). The activities of GPx and CAT in the DMCh group were significantly higher than that observed in the 2-VO group in the two brain regions ($p < 0.05$, Fig. 5e–h).

Discussion

In the present study, we demonstrated for the first time that DMC improved cognitive deficits and alleviated neuronal injury induced by CCI in rats. This therapeutic benefit was partly related to the anti-oxidative properties of DMC.

A network pharmacology method was employed for in silico predicting potential pharmacological action of DMC on CCI by constructing CCI-specific target-constituent-herb

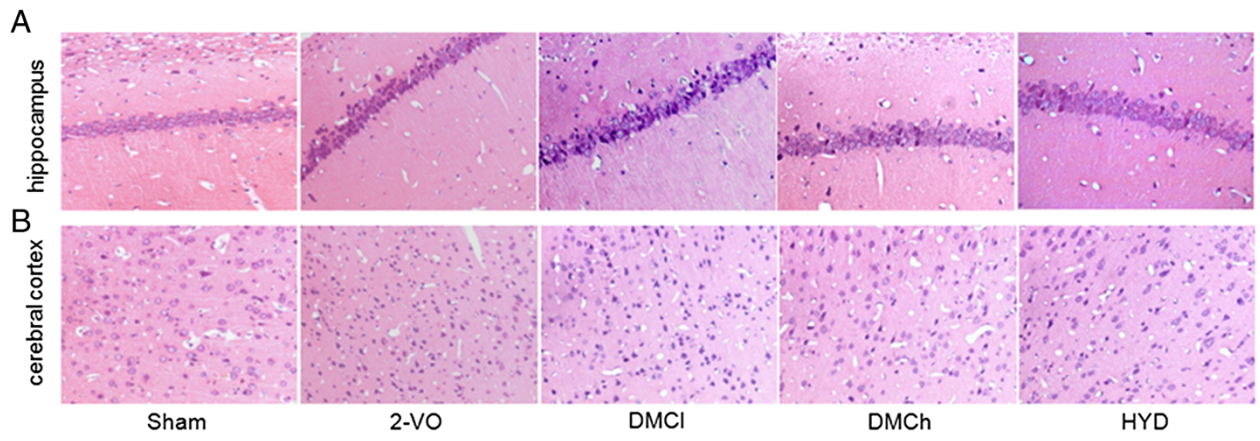

Fig. 4 Representative photomicrographs of the rat hippocampus (**a**) and cerebral cortex (**b**) after CCI. Magnification × 200. CCI: chronic cerebral ischemia; DMC: Daming capsule

association networks for each herb in DMC. Network pharmacology was widely applied as an effective tool to uncover new potential clinical applications for TCM. Our results revealed potential therapeutic effects of DMC on CCI. Multiple CCI-related chemical-target interactions were found in each of the networks. This finding was in-line with the results of previous functional studies that reported that DMC showed multiple effects against ischemia because it contained many chemicals influencing the phenotype of ischemia. Studies on DMC validated its antioxidant properties against lipid peroxidation and free radical scavenging in myocardial ischemia rats [13, 14]. Oxidative stress was associated with apoptosis [34] and the activities of matrix metalloproteinases (MMPs) [35]. Four proteins, p53, MMP1, MMP3, and MMP9, are involved in the networks. Moreover, DMC could abate myocardial cell inflammation [18] and reduce vascular endothelial injury [19]. Consistently, the related proteins, such as IL6, IL1B, TNF, ACE, EDN1, VEGFA, and ICAM1, could be seen in our networks. It has been reported that DMC has lipid-lowering actions [16]. This evidence could be reflected the existence of ADIPOQ in the network. Additionally, two proteins, NOS3 and IGF1, were present in the network. This is in-line with the protective effects of DMC against endothelial dysfunction of the aorta in high fat diet rats via upregulation of NOS3 expression [19] and the beneficial effects on diabetic symptoms [17]. Taken together, we hypothesized that DMC is a protective agent against CCI. The following *in vivo* experiments validated our hypothesis.

CCI could lead to severe memory and learning deficit, which closely resembles the reduced cerebral blood flow that occurs with dementia [8]. The 2-VO surgery was used to build an experimental model mimicking the pathophysiology of learning and memory deficits associated with CCI. The MWM test is the recognized method for evaluating spatial learning and memory [32]. Compared to the sham rats, the 2-VO rats showed an inferior performance in the training and probe trials, indicating impaired spatial learning and memory after CCI. Administration of a high dose of DMC to 2-VO rats improved the scores on the behavioral test, indicating benefits of DMC treatment.

CCI caused lesions on the cerebral cortex and hippocampus, which have a direct correlation to severe memory loss and learning deficit [36]. Consistent with the previous studies, our histological staining results confirmed that CCI induced severe pathological changes in the brain. Neurons in the cerebral cortex and hippocampus were degenerated after CCI. Administration of a high dose of DMC attenuated the 2-VO induced neuronal damage. This observation was consistent with the behavioral test results.

Oxidative stress is closely associated to neuronal degeneration, which is involved in the pathogenesis of numerous neurodegenerative diseases [37]. Permanent cerebral hypoperfusion produced abnormal changes in free radicals in the brain [38]. Excessive generation of reactive oxygen species (ROS) results in neuronal cellular damage due to the oxidation of membrane lipids, essential cellular proteins, and DNA [39]. MDA is a product of lipid peroxidation that is used to assess the degree of cell destruction by free radicals. We discovered that the MDA content significantly increased in both the cerebral cortex and hippocampus of the 2-VO group. The SOD activity increased in the 2-VO group. This was a compensatory rise in antioxidant activity in response to increased free radical generation [40]. Our results were consistent with previous reports [41]. Activation of SOD did not provide neuronal protection because SOD catalyzes the conversion of superoxide anions to hydrogen peroxide, which is usually considered more cytotoxic than the oxygen-derived free

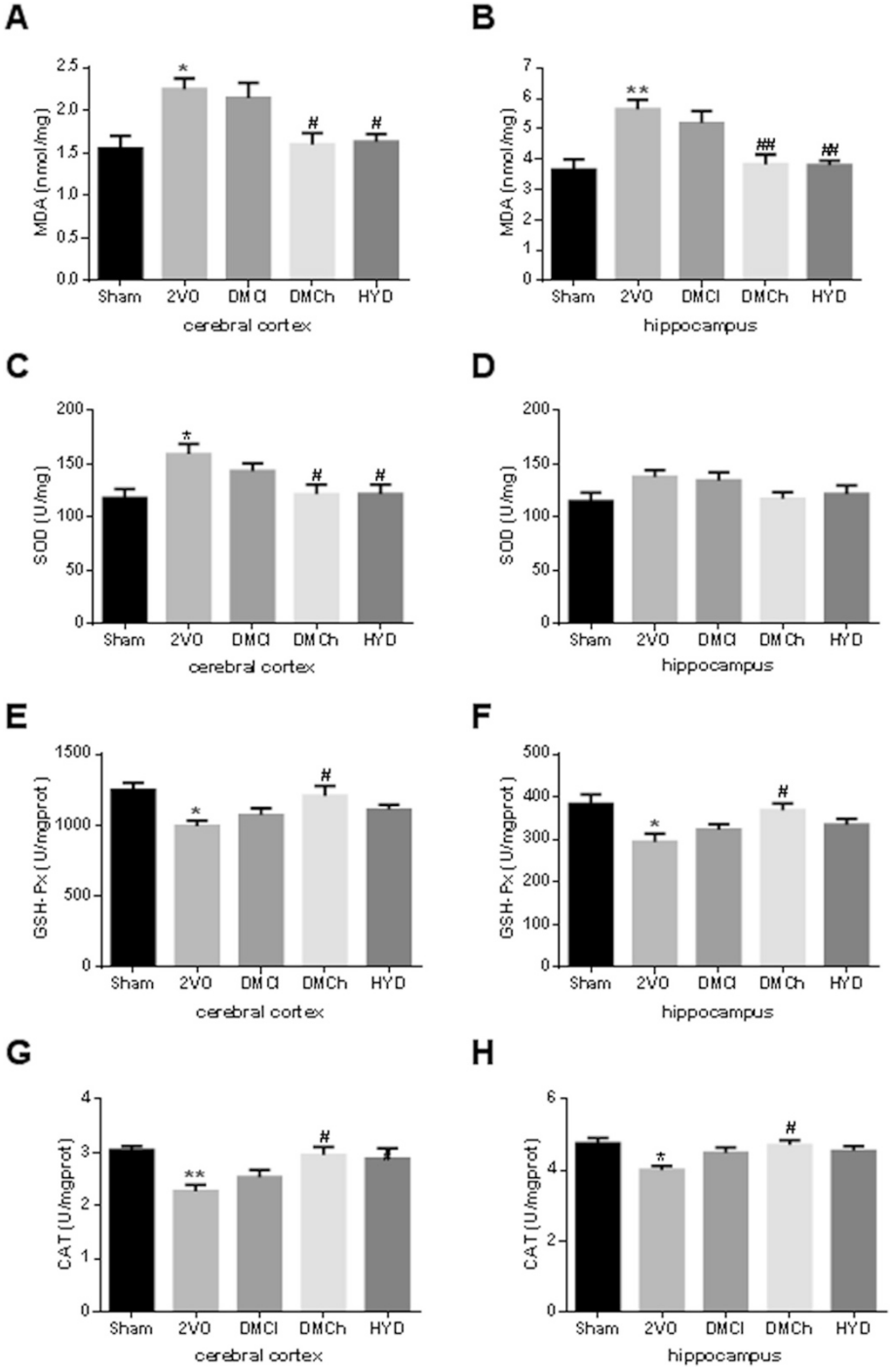

Fig. 5 Effect of DMC on the status of oxidative stress and the activities of SOD, GPx, and CAT in the cerebral cortex and hippocampus of rats with CCI ($n = 5$). DMC decreased the MDA levels in the cerebral cortex (**a**) and hippocampus (**b**). * $p < 0.05$, ** $p < 0.01$ compared to the Sham group; # $p < 0.05$, ## $p < 0.01$ compared to the 2-VO group. **c** DMC reduced the SOD activity in the cerebral cortex of rats with CCI. * $p < 0.05$ compared to the Sham group; # $p < 0.05$ compared to the 2-VO group. **d** No significant change in the SOD levels was found in the hippocampus of rats with CCI. DMC enhanced GPx activity in the cerebral cortex (**e**) and hippocampus (**f**) of rats with CCI. * $p < 0.05$ compared to the Sham group; # $p < 0.05$, compared to the 2-VO group. DMC elevated CAT activity in the cerebral cortex (**g**) and hippocampus (**h**). * $p < 0.05$, ** $p < 0.01$ compared to the Sham group; # $p < 0.05$, compared to the 2-VO group. CCI: chronic cerebral ischemia; DMC: Daming capsule

radicals. GPx and CAT are the main scavengers of hydrogen peroxide [42]. The elimination of hydrogen peroxide is critical in reducing oxidative stress that is induced by CCI. GPx and CAT activity significantly decreased in the cerebral cortex and hippocampus in the 2-VO group. The increase in the MDA levels and the SOD activity is indicative of an increase in the oxidative stress process, whereas a decrease in the GPx and CAT activities indicated insufficient endogenous antioxidant ability. Compared to the 2-VO group, administration of a high dose of DMC diminished MDA production, reversed abnormal SOD activity in the cerebral cortex, and enhanced the GPx and CAT activities. These results suggested that DMC may reverse the abnormality of free radicals and compensate antioxidant abilities following CCI insult. This finding is consistent with previous results that suggest that DMC showed antioxidant properties against lipid peroxidation and free radical scavenging in myocardial ischemia rats [13, 14].

Just recently, a UPLC-ESI-Q-TOF-MS/MS method was applied by us to identify 31 compounds in DMC, among which 6 anthraquinones including chrysophanol, emodin, aloe-emodin, rhein, emodin-O-glucoside and aurantio-obtusin were revealed as potential lipid-lowering bioactive components in DMC [43]. By enhancing brain tissue hypoxia tolerance, chrysophanol was validated to protect neuron damage and improve learning and memory abilities [44, 45]. Emodin exhibited ameliorating effects on cycloheximide-induced memory consolidation impairment in rats [46] and antioxidative stress damage of rat cortical neurons induced by $A\beta_{25-35}$ [47]. Aloe-emodin showed protective effects on the scopolamine-induced memory impairment in mice and hydrogen peroxide-induced cytotoxicity in PC12 cells [48]. Rhein protected against oxidative stress-related endothelial cell injury [49]. Emodin-O-glucoside improved the learning and memory of both normal and intelectual deficit mice induced by scopolamine [50]. There is no evidence indicating that aurantio-obtusin has protective effect against CCI. However, it has been proved of lipid-regulating effect [51]. Taken together, we suppose that the first 5 compounds including chrysophanol, emodin, aloe-emodin, rhein and emodin-O-glucoside be the material basis of the anti-CCI effect of DMC that was shown herein.

Conclusions

In summary, the present study predicted the potential pharmacological actions of DMC on CCI using the network pharmacology method and experimentally demonstrated that DMC inhibited cognitive decline and neuronal damage induced by CCI in rats. The protective effects of DMC on CCI were closely associated with multiple bioactive components and its antioxidant action. The results suggested that DMC might be effective for managing VD.

Abbreviations

VD: Vascular dementia; DMC: Daming capsule; CCI: Chronic cerebral ischemia; MWM: Morris water maze; MDA: Malonic dialdehyde; 2-VO: Permanent bilateral occlusion of carotid arteries; SOD: Superoxide dismutase; TCM: Traditional Chinese medicine; GPx: Glutathione peroxidase; CAT: Catalase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XS and ZD designed the experiments; XS, WZ, RA and YL performed the experiments; XS and WZ analyzed the data; XS, WZ and ZD wrote the paper. All authors read and approved the final manuscript.

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