



Neuroprotective effect of dimethyl fumarate on cognitive impairment induced by ischemic stroke

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Background: Oxidative damage may contribute to post-stroke cognitive impairment (PSCI), but the underlying mechanisms are not fully elucidated. Dimethyl fumarate (DMF) has been used as an antioxidant in multiple sclerosis and psoriasis patients. We hypothesized that redox state was associated with PSCI, and DMF might exert neuroprotective effect against PSCI via anti-oxidative actions.

Methods: To confirm this hypothesis, we first conducted a clinical study (NCT03519828) that enrolled patients diagnosed with acute ischemic stroke within 48 hours. Data were analyzed based on demographic characteristics, disease history, clinical data and redox state. Logistic regression was used to identify the factors associated with PSCI. Next, a middle cerebral artery occlusion (MCAO) rat model was used to explore the antioxidant capacity and neuroprotective effect of DMF. Furthermore, behavioural experiments, histology and immunostaining, and transmission electron microscopy were also performed.

Results: Higher baseline NIHSS score, lower GSH/GSSG and T-AOC levels were found in the PSCI patients. Better performance in Morris water maze and shuttle box testing, more regular arranged neurons and Nissl bodies, less TUNEL-positive cells and autophagosomes, lower expression of 4-HNE, and higher expression of GCLM and NQO1 were found in the (DMF + MCAO) rats compared with the MCAO rats.

Conclusions: These findings suggest that DMF may alleviate PSCI via neuroprotective actions, providing a new therapeutic strategy for PSCI.

Keywords: Post-stroke cognitive impairment (PSCI); dimethyl fumarate (DMF), neuroprotection; oxidative damage

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Introduction

Stroke is the second leading cause of death in the world, resulting in more disabilities than any other diseases (1,2). Besides physical impairment, cognitive impairment can be observed in many stroke patients. Post-stroke cognitive impairment (PSCI) is a category of syndromes from mild cognitive impairment to dementia induced by stroke (3), and it is one of the most common factors threatening the life quality of patients (4,5). Because acute ischemic stroke (AIS) is the most common type of stroke (6,7), cognitive impairment induced by AIS has attracted worldwide attention.

Ischemic stroke patients are prone to cerebral hypoxia. Prolonging the time of cerebral ischemia will aggravate the degree of hypoxia, which may lead to cognitive impairment. Frontal lobe, thalamus, hippocampus and basal ganglion are closely associated with cognitive ability, so if these brain areas are damaged, the patients are at a high risk of cognitive impairment (8,9). Consequently, the time and location of cerebral ischemia usually affect cognitive ability. However, although the above conditions are similar, the cognitive ability of patients may still be different.

Some researchers suggested that oxidative damage might also contribute to PSCI (10). Sood *et al.* (11) have reported that ischemic stroke may cause oxidative damage, apoptosis and cognitive impairment. Loh *et al.* (12) have demonstrated that the increased reactive oxygen species (ROS) induced by stroke can cause neuronal injury. ROS react with cellular macromolecular through oxidation, leading to cell necrosis and apoptosis. LeDoux *et al.* (13) have showed that a large amount of ROS may result in cell death and neurological dysfunction.

Dimethyl fumarate (DMF), an antioxidant drug, has been approved by the U.S. Food and Drug Administration as a first-line medication for the treatment of multiple sclerosis and psoriasis (14,15). Studies have reported that DMF may play a neuroprotective role through antioxidant pathways (16-18). However, DMF has never been used to treat PSCI as an antioxidant.

In this study, we first conducted a clinical study to evaluate the association between redox state and PSCI. Next, we used a middle cerebral artery occlusion (MCAO) rat model to examine whether DMF can exert neuroprotection against PSCI and explore the possible underlying mechanisms. Morris water maze and shuttle box testing were used to assess learning and memory ability. Hematoxylin and eosin (HE), Nissl dyes, terminal

deoxynucleotidyl transferase-mediated dUTP nick-and labeling (TUNEL), and transmission electron microscopy were used to reflect neuronal injury. Immunofluorescent (IF) and immunohistochemical (IHC) were used to detect oxidative or anti-oxidative effect.

Methods

Standard protocol approvals, registrations, and patient consents

The protocol of our study was registered in ClinicalTrials.gov with the number NCT03519828, and it was reviewed and approved by the Ethics Committee of General Hospital of Northern Theater Command [NO. k (2017) 34]. All participants or their representatives provided written informed consent before the patients were enrolled.

Participants

Participants were gathered from General Hospital of Northern Theater Command. Inclusion criteria included: 40–80 years old, diagnosed with AIS, first stroke onset or past stroke without obvious neurological deficit (mRS ≤ 2), time from onset to enrollment ≤ 48 hours, no obvious aphasia or audio-visual barriers, and signed informed consent by patients or their legally authorized representatives. Exclusion criteria included: intracranial haemorrhage, transient ischemic attack, disturbance of consciousness or language expression, dementia or failure to complete assessment. The study was conducted from March 2018 to December 2018.

According to the Montreal cognitive assessment (MoCA) score, the patients was diagnosed with or without PSCI. PSCI was defined as patients with a MoCA score < 26 . If the patient's education level was middle school or below, the demarcation point was 25 (19).

Data collection

Patient information collected included demographic characteristics, disease history, clinical data and redox state. Reduced glutathione/oxidized glutathione (GSH/GSSG), total antioxidant capacity (T-AOC), catalase (CAT) and glutathione peroxidase (GSH-PX) were used as indicators of redox state in the patients. Blood samples were collected immediately after admission.

Levels of blood GSH and GSSG were measured with

BIOXYTECG GSH/GSSG-412 kit (OxisResearch, Portland, USA). Blood with anticoagulant was immediately mixed with or without 1-methyl-2-vinyl-pyridim trifluoromethane sulfonate for the measurement of GSSG and total glutathione (GSH + GSSG), respectively. $GSH = \text{total glutathione} - (2 \times GSSG)$.

The plasma was obtained after the centrifugation (2,000 rpm, 4 °C, 10 min) of anticoagulant-containing blood samples. Levels of T-AOC, CAT and GSH-PX in the plasma were measured using the corresponding kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instruction.

Animals and housing

Forty-eight male Sprague-Dawley rats were obtained from Liaoning Changsheng Biotechnology Company Limited (Liaoning, China). Since most stroke patients were elderly, rats with 12–14 months of old (weighing 600 ± 50 g) were used. All the rats were housed on a twelve-hour light: twelve-hour dark cycle (6:00 a.m.–6:00 p.m.) in the Animal Experimental Centre of China Medical University, with free access to animal food and tap water ad libitum (specific pathogen-free conditions). All the animal experiments were implemented under the protocol approved by the Ethics Committee of China Medical University, and in line with the relevant guidelines and regulations.

Experimental design

A randomized 4-animal block allocation was employed to assign rats to one of the four experimental groups ($n=12$ animals/group): Sham group, DMF group, MCAO group and (DMF+ MCAO) group. Rats were numbered 1 to 48 such that for each sequential set of 4 animals, one animal in each set was allocated into each experimental group. Rats in the Sham group were pretreated with methyl cellulose (MC) and subjected to sham operation. MC was given intragastrically 12.5 mg/kg twice daily for 10 consecutive days, and 3 days before sham operation. In sham operation, right internal carotid artery (ICA), external carotid artery (ECA) and common carotid artery (CCA) were exposed, but no ligation was performed. Rats in the DMF group were pretreated intragastrically with DMF and subjected to sham operation. Similar to MC, DMF was given 12.5 mg/kg twice daily for 10 consecutive days, and 3 days before sham operation. Rats in the MCAO group were pretreated with MC and subjected to MCAO. MCAO was performed

using a modified method described by Longa *et al.* (20). In brief, right ICA, ECA and CCA were exposed through a midline incision on the neck, and then CCA was ligated and transected. A silicone rubber cylinder was inserted into ICA until the silicone rubber cylinder was 17–19 mm away from CCA bifurcation. The origin of right middle cerebral artery was occluded by tightening the silicone rubber cylinder. Rats in the (DMF + MCAO) group were pretreated with DMF and subjected to MCAO. A flow diagram summarizing the process was shown in *Figure 1A*.

Behavioural experiments

Learning and memory ability and athletic ability of the rats were tested on preoperative day 1, postoperative day 1 and postoperative day 6, respectively. Learning and memory ability was assessed by Morris water maze (MWM) and shuttle box testing. Athletic ability was tested by neurological deficit scoring. MWM testing was performed between 8 and 11 am, and shuttle box testing was performed between 2 and 5 pm.

MWM testing

MWM testing was applied to assess the ability of spatial learning and spatial memory (21). MWM task was performed in a round container (100 cm in diameter and 50 cm in height) (Noldus Inc., Netherlands) filled with water (21.5 ± 0.5 °C). The pool was divided into four quadrants and monitored with a video camera on the top. A platform was placed 1 cm below the water surface in the third quadrant.

MWM testing began 6 days before the operation. Rats underwent 4 training trials daily for 5 continuous days. In each trial, rats were placed in one of the four quadrants to find the platform. They were allowed to search platform and climb on it within 60 s. If the rats failed in 60 s, they would be guided onto the platform and allowed to stay on it for 10 s. After training trials, testing trials were performed with the platform being removed. In testing trials, rats were only placed in the quadrant opposite the platform. Testing trials were performed on preoperative day 1, postoperative day 1 and postoperative day 6, respectively. Escape latency in the target quadrant and trajectory heat map were used as the measures for the learning and memory ability of rats. The process of MWM testing was presented in *Figure 1B*.

Shuttle box testing

Shuttle box testing was run by a two-way shuttle box

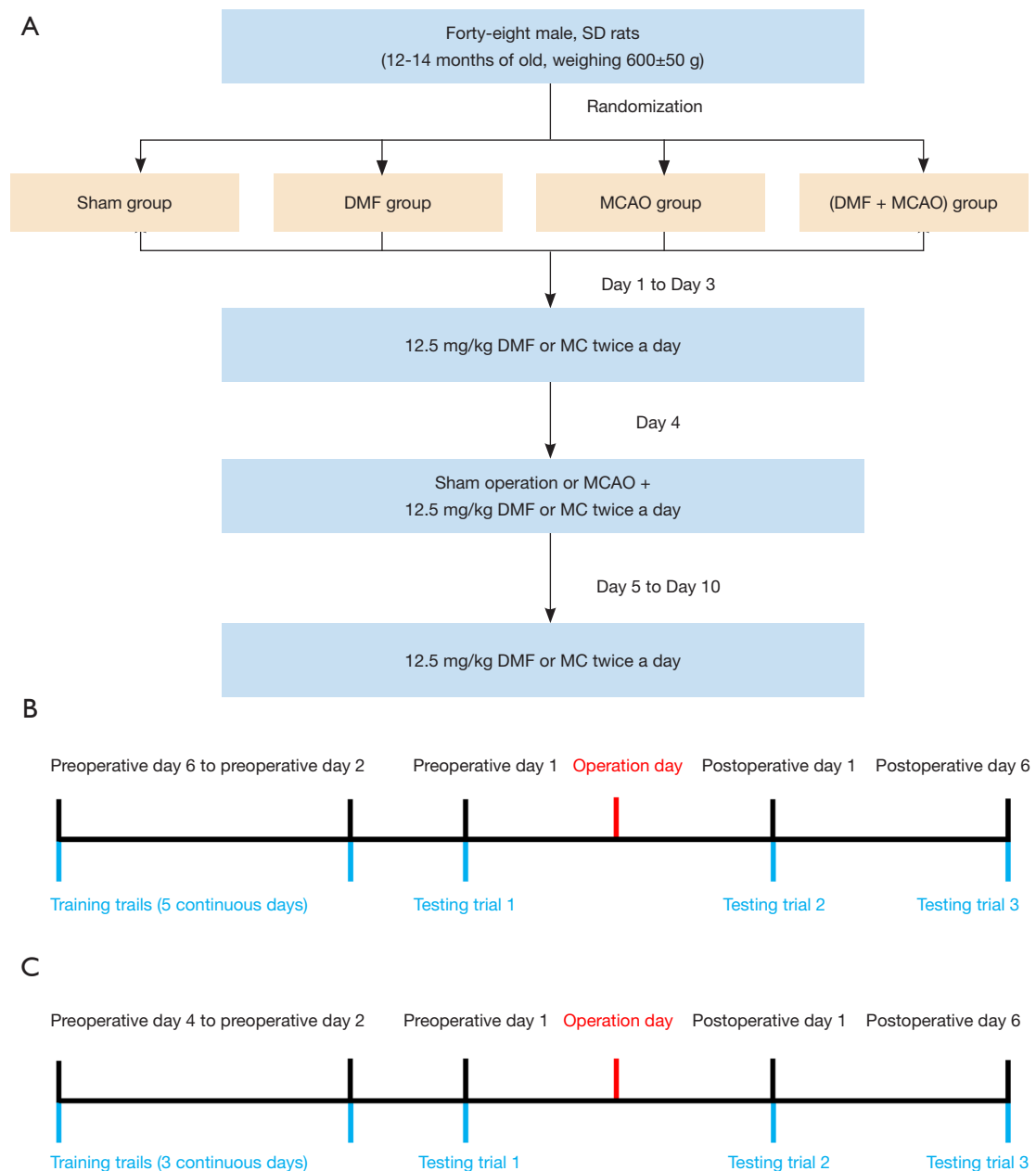


Figure 1 Flow diagram of the animal study. (A) Flow diagram summarizing the grouping and intervention design. Sham group: MC + sham operation. DMF group: DMF + sham operation. MCAO group: MC + MCAO. (DMF + MCAO) group: DMF + MCAO. Rats were pretreated with MC or DMF for 10 consecutive days, and 3 days before sham operation or MCAO; (B) flow diagram of MWM testing; (C) flow diagram of shuttle box testing.

(30 cm × 30 cm × 20 cm; Jinan Yiyuan Co., Shandong, China). Training trials started 4 days before the operation and continued for 3 continuous days. Testing trials were performed on preoperative day 1, postoperative day 1 and

postoperative day 6, respectively. Each rat underwent 20 trials daily after a five-minute adaptation period. Rats were exposed to a 10-second light followed by a 10-second foot shock (30 V, 1.0 mA) in each trial. If the rat moved to the

other chamber with the light was on, the behavior was counted as avoidance and the light was turned off with no exposure to foot shock. If it went to the other chamber with a foot shock, the behavior was counted as escape and the shock was terminated immediately. If it did not change chambers during a trial, the behavior was counted as error and the rat received a 10-second foot shock. In each trial, times of avoidance were used as the measure for the learning and memory ability. The process of shuttle box testing was presented in *Figure 1C*.

Neurological deficit scoring

Neurological deficits of rats were scored on preoperative day 1, postoperative day 1 and postoperative day 6, respectively. The neurological deficits were scored on a five-point scale: a score of 0 indicated no neurological deficit, a score of 1 (failure to extend left forepaw fully) indicated a mild neurological deficit, a score of 2 (circling to the left) indicated a moderate neurological deficit, a score of 3 (falling to the left) indicated a severe neurological deficit, and a score of 4 (being unable to walk spontaneously and with a depressed level of consciousness) indicated a most severe neurological deficit (20).

Plasma redox state

On the postoperative day 7, rats were killed by decapitation after anaesthetized with 2% pentobarbital sodium for approximately 1–2 min. Levels of GSH/GSSG and T-AOC in the plasma were used as indicators of redox state in the rats. Kits from BIOXYTECG (OxisResearch, Portland, USA) and Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were separately used to measure GSH/GSSG and T-AOC levels according to the manufacturer's instruction.

Histology and immunostaining

On the postoperative day 7, rats were killed by decapitation after anaesthetized with 2% pentobarbital sodium for approximately 1–2 min. Right brain tissues were fixed in 4% paraformaldehyde buffer overnight and then transferred to 70% ethanol. Samples were subsequently embedded in paraffin and cut into 4 μm thick sections.

HE staining, Nissl staining and TUNEL staining

HE and Nissl staining were carried out according to the standard protocols. A high-power optical microscope (magnification, $\times 400$) was used to observe the pyramidal

neurons in the hippocampal CA1 region. Three randomly selected visual fields were used to indicate the histological changes (22).

TUNEL staining was performed using an In Situ Cell Death Detection Kit, TUNEL red (Vazyme Biotech Co., Jiangsu, China), and cell nuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI) staining (Vector, Burlingame, CA, USA). TUNEL-positive cells were detected on 10 randomly selected fields from each tissue. The average number of TUNEL-positive cells in the hippocampal CA1 region per high power field was expressed as apoptosis degree (23).

IF staining and IHC staining

4-hydroxynonenal (4-HNE) was frequently measured as an indicator of oxidative damage. To examine the expression of 4-HNE in hippocampal tissue, IF staining with 4-HNE antibody (1:100; ab46545, Abcam, US) was conducted. Pretreated hippocampal sections were incubated with 4-HNE antibody at 4 $^{\circ}\text{C}$ overnight. After washing with phosphate-buffered saline, the slides were mounted with VECTASHIELD Mounting Medium. The stained sections were observed under fluorescence microscope (Nikon Corporation, Tokyo, Japan) and representative images were captured.

For IHC, rabbit polyclonal antibody against glutamate cysteine ligase modifier subunit (GCLM) (14241-1-AP, Proteintech Group, Hubei, China) and NAD(P)H: quinone oxidoreductase 1 (NQO1) (11451-1-AP, Proteintech Group, Hubei, China) were both used at dilutions of 1:200. To quantify GCLM and NQO1 expression in the tissue, ten random fields per sample were digitally imaged. All the sections were semi-quantitatively analyzed by Image-Pro Plus 6.0. The integrated optical density (IOD) was measured in five fields in each section on the 400-fold magnified images, and the average IOD was used for statistical analysis (24).

Transmission electron microscopy

Hippocampal tissues were fixed in 2.5% glutaraldehyde solution (Sigma-Aldrich, C5882, St. Louis, MO) in 0.1 M phosphate buffer overnight at 4 $^{\circ}\text{C}$. After dehydration through a graded series of ethanol, the tissues were infiltrated with Epon 812 (SPI, USA). Finally, the specimens were cut into 1- μm sections with ultramicrotome (LKB-V, Sweden) and stained with uranyl acetate and lead citrate. Images were captured by a transmission electron

microscope (H-7650; Hitachi, Tokyo, Japan).

Statistical analysis

In the clinical study, for continuous variables that were normally distributed, data were presented as mean and standard deviation. When variables were not normally distributed, data were presented as median and interquartile range. Count data were described with n (%). The Kolmogorov-Smirnov test was used to assess the normality of the data. To compare quantitative variables between the two groups, *t*-test or Wilcoxon rank sum test was used. Chi-square test was used to compare count data. Variables with a marginal association with PSCI ($P < 0.1$) were entered in a multivariable logistic regression model. Multivariate binary logistic regression analyses were performed to identify the factors associated with PSCI. Odds ratio (OR) and 95% confidence interval (CI) were also calculated. A value of $P < 0.05$ in two tails was considered significant. Above data analyses were all performed with SPSS 20.0.

In the animal study, data were expressed as mean \pm standard deviation. Difference between the groups in the behavioural experiments was analyzed by repeated-measures analysis of variance followed by Bonferroni post hoc test. Similarly, a value of $P < 0.05$ in two tails was considered statistically significant. The statistical analyses were performed using GraphPad Prism 6.

Results

Low antioxidant capacity might be associated with PSCI

One hundred participants were enrolled in this study according to the inclusion and exclusion criteria. According to the MoCA score at admission, the participants were divided into two groups, including 61 in the PSCI group and 39 in the control group. Patient characteristics in the two groups were listed in *Table 1*.

The results of the univariate analysis showed that the possible different variables between the two groups were age, stroke history, baseline National Institutes of Health Stroke Scale (NIHSS), Oxfordshire Community Stroke Project (OCSP) type, Trial of Org 10,172 in Acute Stroke Treatment (TOAST) classification, GSH/GSSG, T-AOC and GSH-PX ($P < 0.1$). No significant difference was observed in the other factors between the groups. In addition, the levels of GSH/GSSG, T-AOC, CAT and GSH-PX in the groups were also showed in *Figure 2*.

The variables with marginal difference between the groups ($P < 0.1$) were taken into multivariate logistic regression analysis. Among the variables, baseline NIHSS score, GSH/GSSG and T-AOC levels remained different between the groups ($P < 0.05$). Furthermore, higher baseline NIHSS score, lower GSH/GSSG and T-AOC levels were found in the PSCI patients (*Tables 2-4*).

Multiplicative interaction was performed to test whether interaction existed between age and other variables which might be correlated with PSCI (stroke history, admission NIHSS, OCSP, TOAST classification, GSH/GSSG, T-AOC and GSH-PX). In this analysis, no multiplicative interaction was found between age and the variables ($P > 0.05$) (*Table 5*). Therefore, the results in the logistic regression analysis were credible.

DMF alleviated cognitive impairment of rats subjected to MCAO

In the MWM testing, the escape latency in the training trials was shown in *Figure 3A*. Most rats could find platform quickly and accurately through the five-day training, and there was no significant difference between the groups. The escape latency of the rats on preoperative day 1, postoperative day 1 and postoperative day 6 was shown in *Figure 3B*. The escape latency in the MCAO group was significantly longer than that in the (DMF + MCAO) group and Sham group on postoperative day 1 and postoperative day 6. The trajectory heat map was shown in *Figure 3C*. Multiple searches in the target quadrant were found in the groups on preoperative day 1, and no significant difference between the groups was observed. On postoperative day 1 and postoperative day 6, searches in the target quadrant were rarely found in the MCAO group. Compared with the MCAO group, more searches in the target quadrant in the (DMF + MCAO) group and Sham group were observed.

In the shuttle box testing, the avoidance response in the training trials was shown in *Figure 4A*. An upward trend of the avoidance response was observed, and no significant difference between the groups was found. The avoidance response on preoperative day 1, postoperative day 1 and postoperative day 6 was shown in *Figure 4B*. On postoperative day 1 and postoperative day 6, the avoidance response in the MCAO group was significantly less than that in the (DMF + MCAO) group and Sham group.

Neurological deficit scoring was performed in all the groups and shown in *Figure 5*. The neurological deficits of the rats were scored 0 in all the groups on preoperative

Table 1 The characteristics of the participants

Variables	Case (n=61)	Control (n=39)	P
Demographic characteristics			
Age (years), mean \pm SD	63.9 \pm 10.7	58.7 \pm 13.6	0.033*
Males, n (%)	45 (73.8)	27 (69.2)	0.622
Educational level, n (%)			0.121
Primary school degree or below	25 (41.0)	10 (25.6)	
Middle school degree	35 (57.4)	26 (66.7)	
Bachelor degree or above	1 (1.6)	3 (7.7)	
Current smoker, n (%)	27 (44.3)	23 (59.0)	0.151
Current drinker, n (%)	13 (21.3)	9 (23.1)	0.835
Disease history, n (%)			
Hypertension	42 (68.9)	32 (82.1)	0.142
Diabetes	17 (27.9)	12 (30.8)	0.755
Coronary heart disease	7 (11.5)	1 (2.6)	0.145
Atrial fibrillation	2 (3.3)	2 (5.1)	0.642
Stroke history	21 (34.4)	7 (17.9)	0.073*
Hyperlipemia	26 (42.6)	22 (56.4)	0.178
Clinical data			
Admission NIHSS, median (IQR)	3 [2–5]	1 [0–3]	0.001*
SBP (mmHg), mean \pm SD	155.0 \pm 22.0	153.0 \pm 20.1	0.645
DBP (mmHg), mean \pm SD	90.5 \pm 12.0	88.1 \pm 12.3	0.333
UA (μ mol/L), median (IQR)	303.0 (275.5–381.0)	329.0 (295.3–394.5)	0.116
Hcy (μ mol/L), median (IQR)	13.4 (10.9–19.3)	13.8 (10.5–17.9)	0.643
GLU (mmol/L), mean \pm SD	6.2 (5.5–7.9)	6.7 (5.5–9.4)	0.393

Table 1 (continued)**Table 1** (continued)

Variables	Case (n=61)	Control (n=39)	P
hsCRP (mg/L), median (IQR)	2.2 (1.2–6.6)	2.3 (1.5–4.0)	0.736
OAT (hours), median (IQR)	24.0 (9.8–25.0)	23.0 (9.0–26.0)	0.989
OCSP type, n (%)			0.028*
TACI	1 (1.6)	1 (2.6)	
PACI	16 (26.2)	7 (17.9)	
POCI	18 (29.5)	4 (10.3)	
LACI	26 (42.6)	27 (69.2)	
TOAST classification, n (%)			0.003*
LAA	16 (26.2)	2 (5.1)	
CE	3 (4.9)	1 (2.6)	
SVO	14 (23.0)	21 (53.8)	
UE	28 (45.9)	15 (38.5)	
Redox state, mean \pm SD			
GSH/GSSG	14.5 \pm 6.0	18.5 \pm 7.6	0.004*
T-AOC (U/mL)	55.6 \pm 25.1	67.4 \pm 20.1	0.012*
CAT (U/mL)	19.0 \pm 7.3	17.9 \pm 7.7	0.477
GSH-PX (U/mL)	102.1 \pm 62.2	78.9 \pm 51.0	0.055*

*, $P < 0.1$. CAT, catalase; CE, cardioembolism; DBP, diastolic blood pressure; GLU, glucose; GSH/GSSG, reduced glutathione/oxidized glutathione; GSH-PX, glutathione peroxidase; Hcy, homocysteine; hsCRP, high sensitivity C reactive protein; IQR, interquartile range; LAA, large artery atherosclerosis; LACI, lacunar infarction; NIHSS, National Institutes of Health Stroke Scale; OCSP, Oxfordshire Community Stroke Project; OAT, onset-to-admission time; PACI, partial anterior circulation infarction; POCI, posterior circulation infarction; SBP, systolic blood pressure; SD, standard deviation; SVO, small vessel occlusion; TACI, total anterior circulation infarction; T-AOC, total antioxidant capacity; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; UA, uric acid; UE, undetermined etiology.

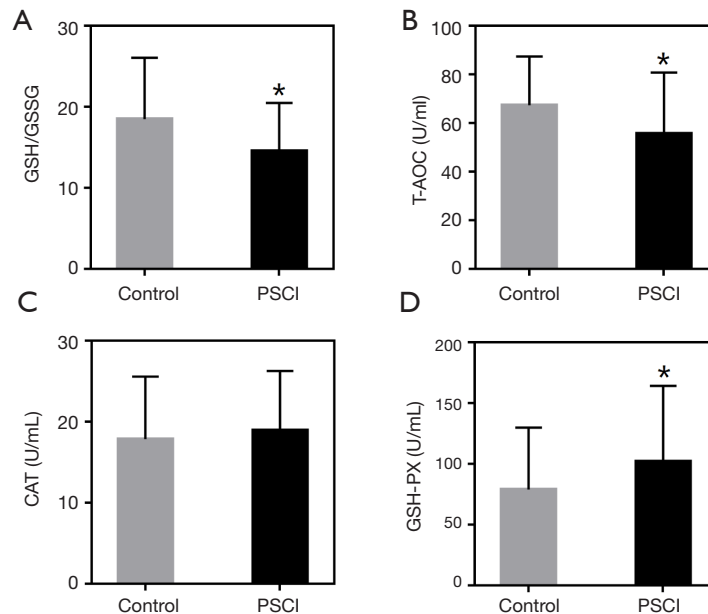


Figure 2 Redox state in the patients. Values are expressed as mean \pm SD. *, $P < 0.05$, compared with the Control group. (A) Ratios of GSH to GSSG (GSH/GSSG); (B) T-AOC level; (C) CAT level; (D) GSH-PX level.

day 1. On postoperative day 1 and postoperative day 6, the neurological deficit scoring in the Sham group and DMF group was still 0. There was no significant difference in the neurological deficit scoring between the MCAO group and (DMF + MCAO) group on postoperative day 1 and postoperative day 6.

DMF alleviated neuronal injury in hippocampal CA1 region of rats

HE staining was performed to monitor neuronal morphology and pyramidal cells in hippocampal CA1 region (Figure 6A). A normal neuronal morphology with well-arranged pyramidal cells was observed in the Sham group. However, irregular neuronal arrangement with karyopyknosis and dark staining in some neuron nuclei was shown in the MCAO group. Compared with the MCAO group, more regular arranged neurons were found in the (DMF + MCAO) group.

Neuronal structure in hippocampal CA1 region was detected by Nissl staining (Figure 6B). A complete neuronal structure with clearly visible nucleoli, lightly stained cytoplasm and neat arrangement was shown in the Sham group. Nevertheless, significant cell shrinkage, irregular cell morphology, deeply stained nuclei and decreased Nissl bodies were exhibited in the MCAO group. Compared

with the MCAO group, more regular cell morphology and increased Nissl bodies were shown in the (DMF + MCAO) group.

To detect the number of apoptotic cells in hippocampal CA1 region, TUNEL staining was conducted (Figure 6C). Limited TUNEL-positive cells were observed in the Sham group (10.2 ± 2.3), while dramatically more TUNEL-positive cells were found in the MCAO group (37.8 ± 5.4 ; $P < 0.05$). Compared with the MCAO group, the number of TUNEL-positive cells in the (DMF + MCAO) group was less (16.2 ± 3.4 ; $P < 0.05$; Figure 7).

Transmission electron microscopy imaging was also utilized to assess neuronal structure in hippocampal CA1 region (Figure 8A). None of autophagosomes was found in the Sham group. However, autophagosomes were found in the MCAO group and (DMF + MCAO) group. Compared with the MCAO group, the number of autophagosomes in the (DMF + MCAO) group was less (Figure 8B).

DMF exerted anti-oxidative effect on hippocampal CA1 region of rats

Levels of GSH/GSSG and T-AOC in the plasma were measured by the corresponding kits. There is no significant difference in GSH/GSSG and T-AOC levels between the Sham group and MCAO group. However, compared with

Table 2 Multivariate logistic regression analysis of GSH/GSSG influence on cognitive impairment after stroke

Variables	β	OR (95% CI)	P
Age	0.004	1.004 (0.959–1.051)	0.863
Admission NIHSS	0.256	1.292 (1.027–1.626)	0.029**
Previous stroke	0.978	2.660 (0.685–10.329)	0.158
TOAST classification			0.230
UE	–	1 (Reference)	–
LAA	1.061	2.891 (0.521–16.024)	0.224
CE	0.547	1.728 (0.114–26.271)	0.694
SVO	–0.790	0.454 (0.135–1.522)	0.201
OCSF type			0.486
LACI	–	1 (Reference)	–
TACI	–0.973	0.378 (0.012–12.122)	0.582
PACI	–0.041	0.960 (0.234–3.939)	0.954
POCI	0.986	2.681 (0.587–12.250)	0.203
GSH/GSSG	–0.089	0.915 (0.842–0.995)	0.037**

***P*<0.05. CE, cardioembolism; CI, confidence interval; GSH/GSSG, reduced glutathione/oxidized glutathione; LAA, large artery atherosclerosis; LACI, lacunar infarction; NIHSS, National Institutes of Health Stroke Scale; OCSF, Oxfordshire Community Stroke Project; OR, odds ratio; PACI, partial anterior circulation infarction; POCI, posterior circulation infarction; SVO, small vessel occlusion; TACI, total anterior circulation infarction; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; UE, undetermined etiology.

Table 3 Multivariate logistic regression analysis of T-AOC influence on cognitive impairment after stroke

Variables	β	OR (95% CI)	P
Age	0.001	1.000 (0.955–1.046)	0.989
Admission NIHSS	0.272	1.313 (1.047–1.648)	0.019**
Previous stroke	1.335	3.798 (0.913–15.799)	0.066
TOAST classification			0.293
UE	–	1 (Reference)	–
LAA	0.611	1.842 (0.311–10.897)	0.501
CE	–0.006	0.994 (0.077–12.772)	0.996
SVO	–0.933	0.394 (0.118–1.307)	0.128
OCSF type			0.374
LACI	–	1 (Reference)	–
TACI	–0.949	0.387 (0.012–12.137)	0.589
PACI	0.087	1.091 (0.257–4.636)	0.906
POCI	1.235	3.439 (0.713–16.591)	0.124
T-AOC	–0.024	0.976 (0.954–0.999)	0.038**

***P*<0.05. CE, cardioembolism; CI, confidence interval; LAA, large artery atherosclerosis; LACI, lacunar infarction; NIHSS, National Institutes of Health Stroke Scale; OCSF, Oxfordshire Community Stroke Project; OR, odds ratio; PACI, partial anterior circulation infarction; POCI, posterior circulation infarction; SVO, small vessel occlusion; TACI, total anterior circulation infarction; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; UE, undetermined etiology.

the Sham group and MCAO group, the T-AOC level of the (DMF + MCAO) group was significantly higher (*Figure 9A*). Although no significant difference was found between the groups in GSH/GSSG, a reasonable trend was shown (*Figure 9B*).

The expression of 4-HNE was evaluated by IF staining. As shown in *Figure 10A*, limited expression was observed in the Sham group, while the expression was higher in the MCAO group. Besides, the expression of the protein was significantly lower in the (DMF + MCAO) group compared with the MCAO group.

The expression of GCLM and NQO1 was evaluated by IHC and shown in *Figure 10B* and *C*. Compared with the Sham group, the expression was higher in the MCAO group and (DMF + MCAO) group. Additionally, the average IOD of the (DMF + MCAO) group (GCLM: 76.3±18.7; NQO1: 11.6±3.9) was higher than the MCAO group

(GCLM: 44.5±13.2; NQO1: 7.5±2.3; *P*<0.05), indicating the expression in the (DMF + MCAO) group was higher.

Discussion

To our knowledge, this is the first study to simultaneously conduct a clinical research and an animal study to explore the effect of antioxidation on cognitive impairment induced by ischemic stroke. First, we found that low antioxidant capacity might contribute to PSCI, which was consistent with the previous findings (25–27). Next, we found that DMF might exert neuroprotective effect against PSCI via anti-oxidative actions. Although DMF has previously been reported to be effective in the treatment of ischemic stroke (28,29), our study first reported the application of DMF in cognitive impairment induced by ischemic stroke.

In the clinical study, higher baseline NIHSS score, lower

Table 4 Multivariate logistic regression analysis of GSH-PX influence on cognitive impairment after stroke

Variables	β	OR (95% CI)	P
Age	0.001	1.001 (0.957–1.046)	0.978
Admission NIHSS	0.268	1.308 (1.039–1.647)	0.022**
Previous stroke	0.950	2.585 (0.703–9.510)	1.153
TOAST classification			0.161
UE	–	1 (Reference)	–
LAA	1.093	2.983 (0.536–16.581)	0.212
CE	0.412	1.510 (0.117–19.520)	0.752
SVO	–0.904	0.405 (0.123–1.338)	0.138
OCSF type			0.647
LACI	–	1 (Reference)	–
TACI	–0.888	0.411 (0.008–20.386)	0.656
PACI	–0.019	0.981 (0.240–4.004)	0.979
POCI	0.848	2.336 (0.497–10.981)	0.283
GSH-PX	0.006	1.006 (0.997–1.014)	0.204

***P* < 0.05. CE, cardioembolism; CI, confidence interval; GSH-PX, glutathione peroxidase; LAA, large artery atherosclerosis; LACI, lacunar infarction; NIHSS, National Institutes of Health Stroke Scale; OCSF, Oxfordshire Community Stroke Project; OR, odds ratio; PACI, partial anterior circulation infarction; POCI, posterior circulation infarction; SVO, small vessel occlusion; TACI, total anterior circulation infarction; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; UE, undetermined etiology.

GSH/GSSG and T-AOC levels were found in the PSCI patients. NIHSS score is a reliable, valid and responsive tool for measuring stroke severity (30). Previous studies have reported that cognitive ability was closely related to the severity of stroke (31,32), which was consistent with our findings. GSH/GSSG and T-AOC levels are both recognized as indicators of antioxidant capacity (33–36). In the present study, low antioxidant capacity was considered to be associated with PSCI. Due to 20% of the body's oxygen consumption and insufficient antioxidant defense, the brain is vulnerable to oxidative damage (37). Some studies have shown that oxidative damage could cause neuronal cells death, leading to PSCI (38,39), which may explain our conclusion. Previous studies have reported that age may be an independent risk factor for PSCI (40–42). In our study, the univariate analysis results showed age was a possible risk factor for PSCI, but the multivariate logistic regression analysis results suggested no significant association between

Table 5 Logistic regression analysis of interaction of age and other factors

Interactive variables	β	OR (95% CI)	P
Age and stroke history			
Age	0.022	1.023 (0.983–1.064)	0.266
Stroke history	–3.058	0.047 (0.000–91.865)	0.429
Age-stroke history interaction	0.056	1.057 (0.942–1.187)	0.344
Age and admission NIHSS			
Age	0.014	1.014 (0.965–1.067)	0.580
Admission NIHSS	–0.193	0.825 (0.265–2.570)	0.740
Age-admission NIHSS interaction	0.007	1.007 (0.989–1.026)	0.432
Age and OCSF			
Age	–0.064	0.938 (0.776–1.133)	0.507
OCSF	–2.079	0.125 (0.005–3.388)	0.217
Age-OCSF interaction	0.027	1.027 (0.976–1.082)	0.306
Age and TOAST classification			
Age	0.105	1.110 (0.988–1.248)	0.080
TOAST classification	0.981	2.667 (0.443–16.050)	0.284
Age-TOAST classification interaction	–0.018	0.982 (0.955–1.011)	0.222
Age and GSH/GSSG			
Age	0.001	1.001 (0.891–1.124)	0.990
GSH/GSSG	–0.262	0.770 (0.491–1.207)	0.254
Age-GSH/GSSG interaction	0.002	1.002 (0.996–1.009)	0.471
Age and T-AOC			
Age	0.097	1.102 (0.970–1.252)	0.137
T-AOC	0.031	1.032 (0.921–1.156)	0.589
Age-T-AOC interaction	–0.001	0.999 (0.997–1.001)	0.347
Age and GSH-PX			
Age	0.080	1.083 (0.989–1.186)	0.087
GSH-PX	0.163	1.177 (0.885–1.565)	0.263
Age-GSH-PX interaction	–0.002	0.998 (0.993–1.002)	0.300

CI, confidence interval; GSH/GSSG, reduced glutathione/oxidized glutathione; GSH-PX, glutathione peroxidase; NIHSS, National Institutes of Health Stroke Scale; OCSF, Oxfordshire Community Stroke Project; OR, odds ratio; T-AOC, total antioxidant capacity; TOAST, Trial of Org 10,172 in Acute Stroke Treatment.

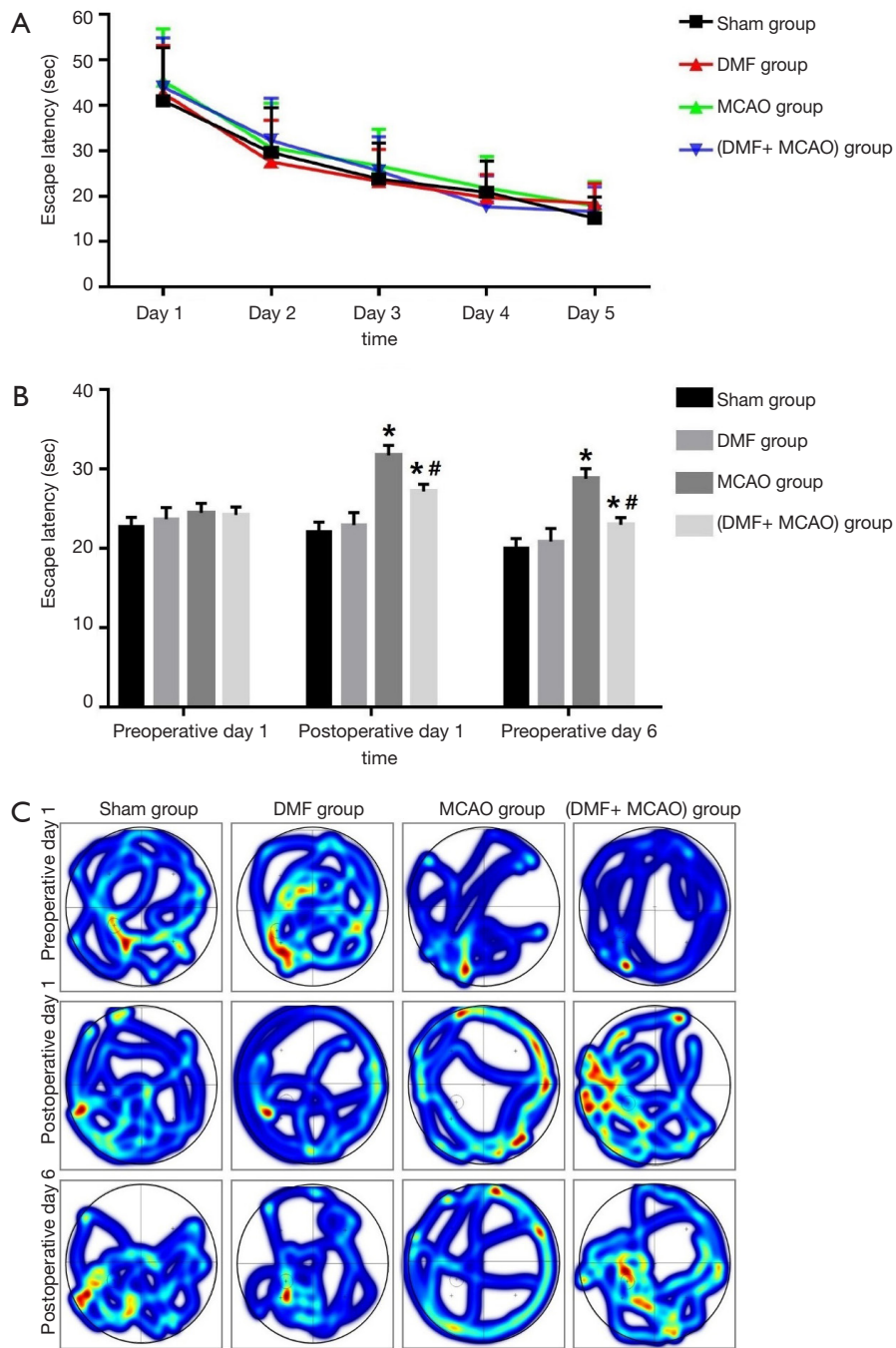


Figure 3 Escape latency and trajectory heat map of the rats in the MWM testing. Values are expressed as mean \pm SD. *, $P < 0.05$, compared with the Sham group; #, $P < 0.05$, compared with the MCAO group. $n = 8-10$. (A) The escape latency for 5 continuous days in the training trials; (B) the escape latency on preoperative day 1, postoperative day 1 and postoperative day 6; (C) trajectory heat map on preoperative day 1, postoperative day 1 and postoperative day 6.

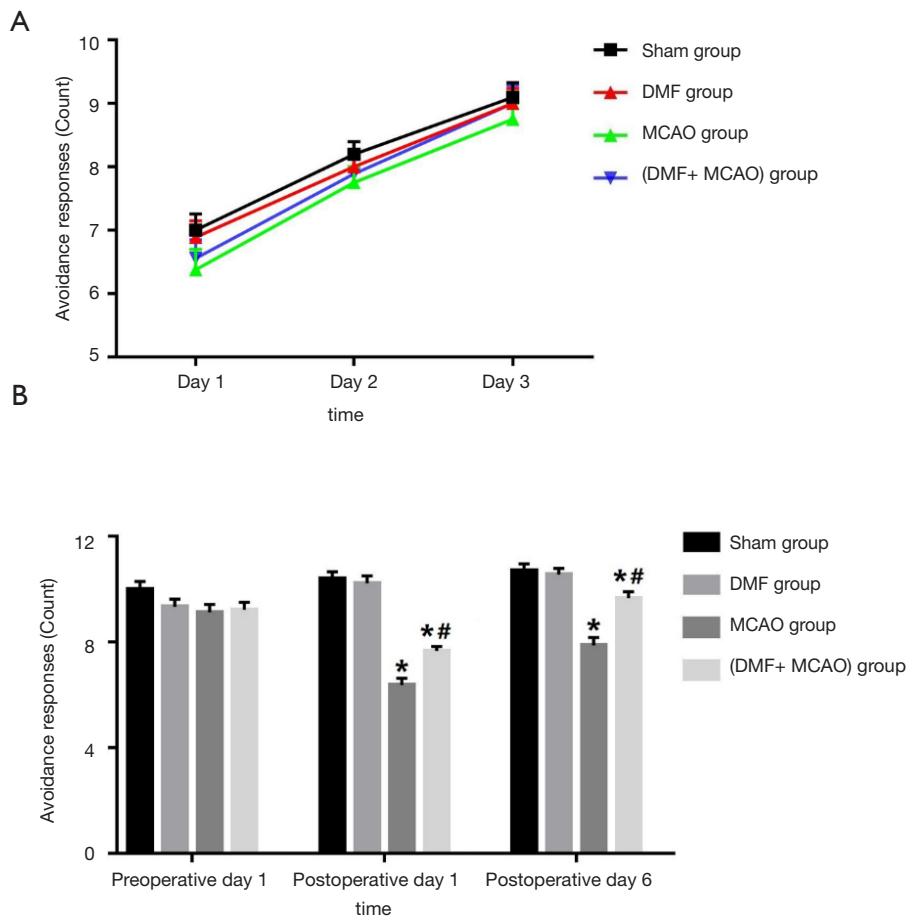


Figure 4 Avoidance responses of the rats in the shuttle box testing. Values are expressed as mean \pm SD. *, $P < 0.05$, compared with Sham group; #, $P < 0.05$, compared with MCAO group. $n = 8-10$. (A) Avoidance responses for 3 continuous days in the training trials; (B) avoidance responses on preoperative day 1, postoperative day 1 and postoperative day 6.

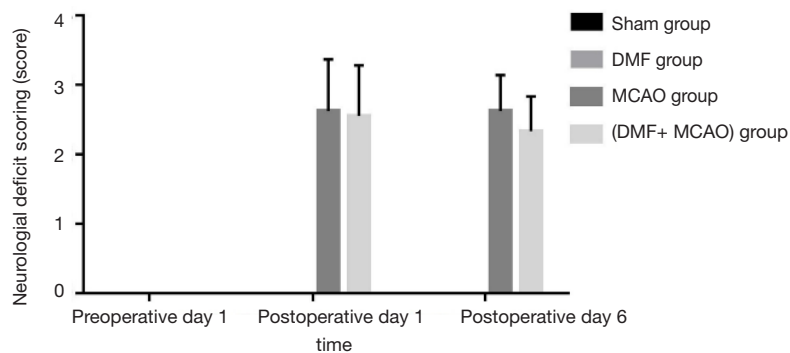


Figure 5 Neurological deficit scoring of the rats. Values are expressed as mean \pm SD. $n = 8-10$. The neurological deficits were scored 0 in the four groups on preoperative day 1. On postoperative day 1 and postoperative day 6, the neurological deficits in the Sham group and DMF group were still 0, while neurological deficit scoring increased in the MCAO group and (DMF + MCAO) group.

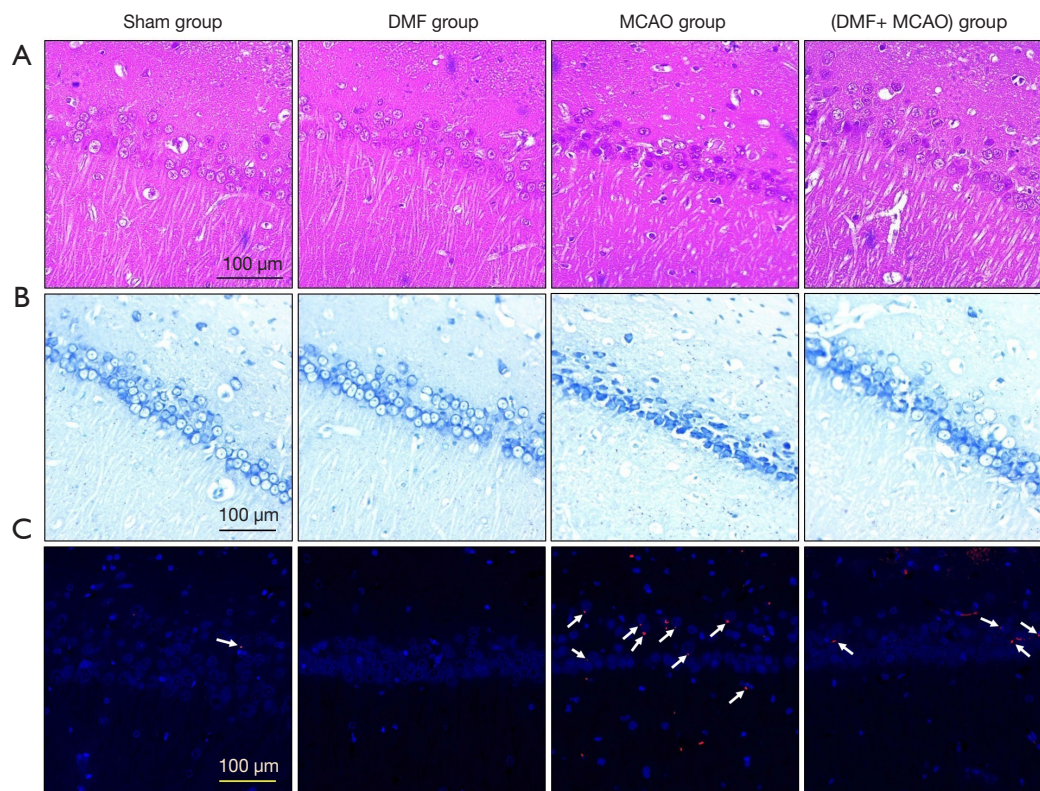


Figure 6 DMF alleviates neuronal injury in hippocampal CA1 region. n=4–6. (A) H&E staining and (B) Nissl staining were conducted to observe neuronal morphology (magnification $\times 400$); (C) TUNEL staining was conducted to observe apoptotic cells (magnification $\times 200$).

age and PSCI. To reach a more accurate conclusion, studies with larger sample size are needed in the future.

Corresponding to the elderly stroke patients in the clinical study, Sprague-Dawley rats (12–14 months of old) were used in the animal study. In the MWM and shuttle box testing, better performance was found in the (DMF + MCAO) rats compared with the MCAO rats. Combined with the similar neurological deficit scoring in the two groups, DMF was considered to improve the learning and memory ability of the MCAO rats. Histopathological morphology of the neurons in the hippocampal CA1 region was observed by HE and Nissl staining. Compared with the MCAO rats, more regular arranged neurons and Nissl bodies were observed in the (DMF + MCAO) rats. Furthermore, the less number of the TUNEL-positive cells and autophagosomes in the (DMF + MCAO) rats suggested DMF might reduce cell apoptosis and autophagy. Overall, DMF might play a neuroprotective role in the cognitive impairment induced by ischemic stroke.

4-HNE is a major end-product of oxidation of

polyunsaturated fatty acids, and it is usually considered as an indicator of oxidative damage. Lipid peroxides and their decomposition products can directly or indirectly affect many functions necessary to maintain homeostasis in cells and organs (43,44). In the present study, the expression of 4-HNE was lower in the (DMF + MCAO) rats compared with the MCAO rats, which suggested that DMF might alleviate oxidative damage in the MCAO rats. Nuclear factor erythroid 2-related factor 2 (Nrf2) is the major regulator of the cellular defense machinery against oxidative stress, and it regulates the expression of the downstream genes through the antioxidant response element (ARE) (45). Antioxidant enzymes (such as GCLM) and phase II drug metabolizing enzymes (such as NQO1) are both encoded by the downstream genes (46). The expression of GCLM and NQO1 was higher in the MCAO group and (DMF + MCAO) group compared with the Sham group. That was because the rats subjected to MCAO suffered from oxidative damage, resulting in the increase of anti-oxidative effect. Furthermore, the expression of GCLM and NQO1 was

higher in the (DMF + MCAO) group compared with the MCAO group, suggesting DMF might exert anti-oxidative effect via the ARE of Nrf2.

Although our study shows DMF may play a neuroprotective role in the patients with PSCI, the side effects of DMF should also be taken seriously. DMF is an alkylating agent, which can modify nucleophilic groups in proteins (such as cysteine thiol) nonspecifically and covalently (47,48). As a result, DMF may have serious side effects. For instance, a 30% decline in lymphocytes has occurred after taking DMF, which may lead to infection

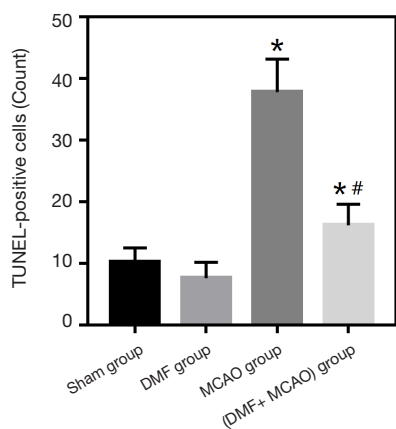


Figure 7 Quantitation of TUNEL-positive cells. Values are expressed as mean \pm SD. *, $P < 0.05$, compared with the Sham group; #, $P < 0.05$, compared with the MCAO group. $n = 4-6$.

(49,50). Furthermore, since rebound phenomena after discontinuation of DMF for multiple sclerosis have previously been described (51), further researches should be conducted to determine the long-term anti-oxidant effect on PSCI. Metabolite monomethyl fumarate (MMF) is the major metabolite of DMF. The ester's structure of MMF enables it rapidly come across the blood-brain barrier to implement direct anti-oxidant effects on nerve cells (52,53). In recent years, because the electrophilicity of MMF is weaker than that of DMF, researchers began to pay attention to MMF with the hope of developing a safer drug than DMF (54,55).

Some limitations should be addressed. First, the sample size is relatively small in the clinical study. To reach a more definitive conclusion, studies with larger sample size are needed in the future. Second, because depression, anxiety, and poor sleep quality have not been measured, their relationship with PSCI could not be assessed. In order to obtain more comprehensive factors affecting PSCI, these factors need to be measured. Third, patients in the study received similar routine treatments such as antiplatelet, anticoagulant and statin according to the Chinese stroke guideline. However, we did not measure those redox levels after treatment in the present study, thus the dynamic effect of those redox levels has not determined. Also, we did not follow up the patients in the present study, so we only measured the cognitive ability at admission and used the MoCA score at admission to reflect the cognitive ability after stroke. In order to explore the long-term relationship between redox level and PSCI, follow-up should be

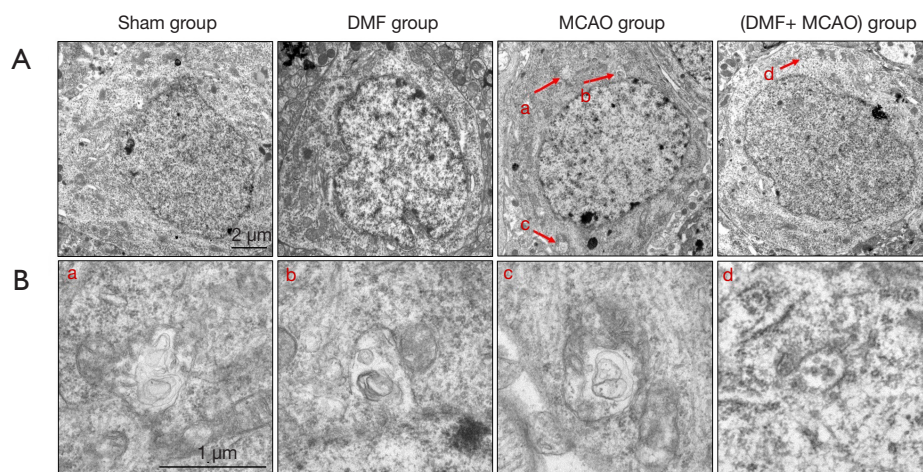


Figure 8 Autophagosomes observed by transmission electron microscopy. Small letters in *Figure 8A* and *B* indicate autophagosomes and the corresponding amplified autophagosomes, respectively. (A) Transmission electron microscopy was used to observe autophagosomes (magnification $\times 10,000$); (B) the amplified autophagosomes in (A) (magnification $\times 25,000$).

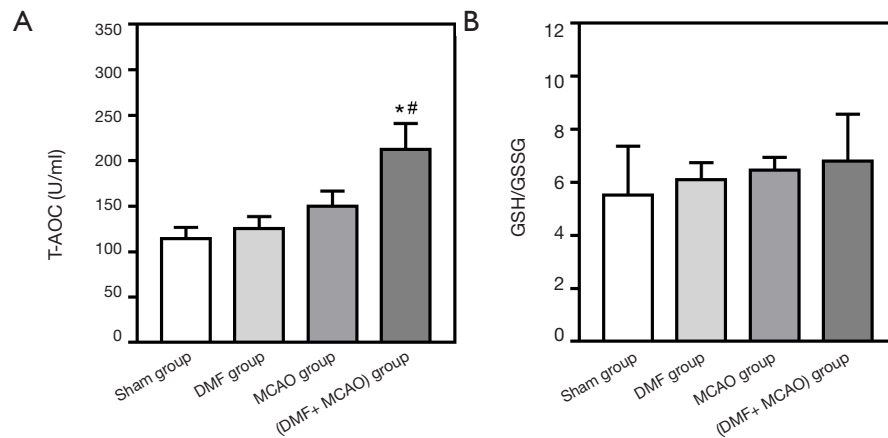


Figure 9 Redox state in the rats. Values are expressed as mean \pm SD. *, $P < 0.05$, compared with the Sham group; #, $P < 0.05$, compared with the MCAO group. $n = 8-10$. (A) T-AOC level; (B) ratios of GSH to GSSG (GSH/GSSG).

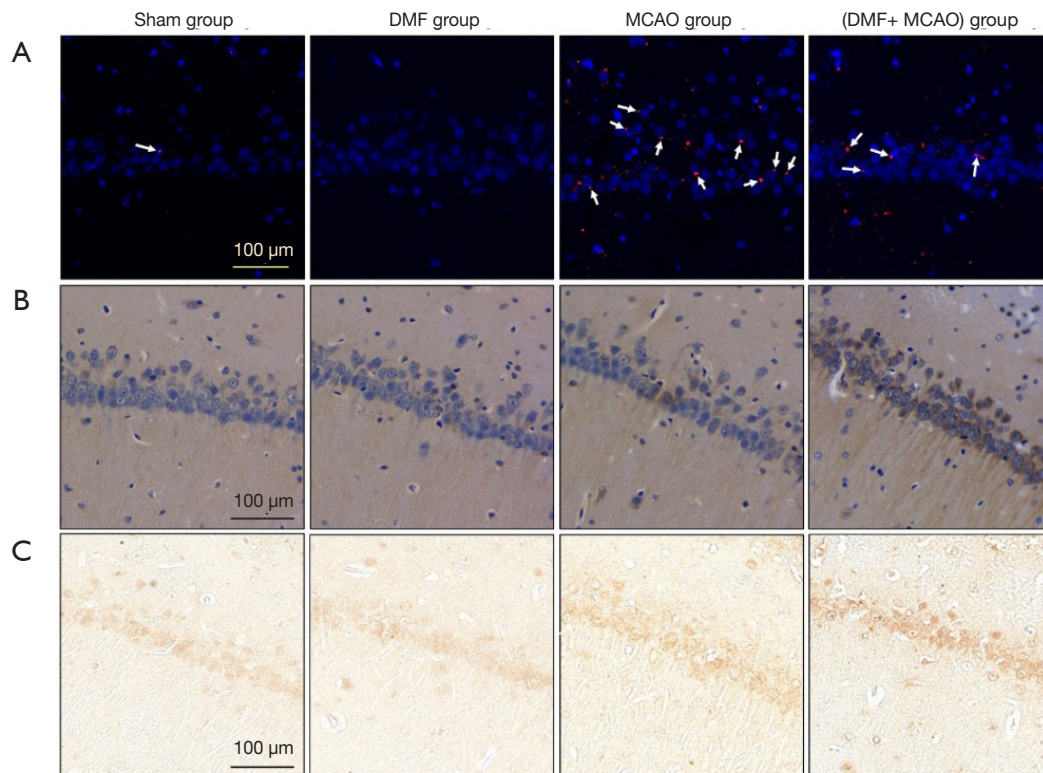


Figure 10 DMF exerts anti-oxidative effect on hippocampal CA1 region. $n = 4-6$. (A) The expression of 4-HNE was evaluated by IF staining (magnification $\times 200$); (B) the expression of GCLM was evaluated by IHC (magnification $\times 400$); (C) the expression of NQO1 was evaluated by IHC (magnification $\times 400$).

conducted. Finally, DMF may also play a neuroprotective role through other mechanisms, such as anti-inflammatory response, so the specific mechanism needs to be further explored.

In summary, our study suggested that low antioxidant capacity might be associated with PSCI, and DMF might alleviate PSCI via neuroprotective actions, providing a new therapeutic strategy for PSCI. Further researches are needed to explore the underlying mechanisms.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The clinical study was reviewed and approved by the Ethics Committee of General Hospital of Northern Theater Command [NO. k (2017) 34]. Written informed consent was obtained from all patients. The animal study was approved by the Ethics Committee of China Medical University.

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