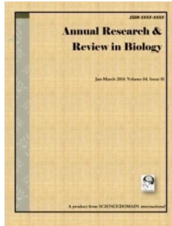

Annual Research & Review in Biology
4(12): 1915-1926, 2014

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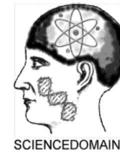

Neuroprotective Effect of *Dracocephalum moldavica* L. Total Flavonoids in Transient Cerebral Ischemia in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors QZ and DW designed the study. Author TL performed the statistical analysis. Author YS wrote the protocol and wrote the first draft of the manuscript. Authors XD and ZJ managed the analyses of the study. Authors ZG and MZ managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 8th January 2014
Accepted 8th February 2014
Published 28th February 2014

ABSTRACT

Aims: The aim of the present study was to investigate the potential neuroprotective effects of *Dracocephalum moldavica* L. total flavonoids (DMTF) on cerebral oxidative stress and damage as well as apoptosis induced by transient cerebral ischemia in rats.
Methodology: Wistar rats were randomly divided into five groups: model group, sham group, DMTF high, middle, low dose groups (50, 25, 12.5 mg/kg). Focal cerebral ischemia/reperfusion was induced by middle cerebral artery occlusion (MCAO) for 2 h, followed by 22 h reperfusion. The reduced glutathione/ oxidized glutathione (GSH/GSSG) ratio, antioxidant enzyme activities, malondialdehyde (MDA), protein carbonyl and 8-hydroxy-deoxyguanosine (8-OHdG) contents were determined. The neuron apoptosis was measured by TdT-mediated dUTP nick end labeling (TUNEL) assay. At last, the

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neurological deficit was observed.
Results: Transient cerebral ischemia led to significant depress in GSH/GSSG ratio, depletion in antioxidant enzyme activities, rise in MDA, protein carbonyl, 8-OHdG contents, neuron apoptosis and neurological deficit scores. Pre-treatment with DMTF obviously attenuated the brain oxidative stress and damage, apoptosis and neurological deficits.
Conclusion: These findings suggested that DMTF has neuroprotective effect on cerebral ischemia/reperfusion injury which might be related to its antioxidant and anti-apoptosis activity.

Keywords: Dracocephalum moldavica L.; total flavonoids; transient cerebral ischemia; oxidative stress; antioxidant; neuroprotection.

1. INTRODUCTION

Stroke, a serious neurological disease, remains one of the leading cause of death in the world and the first cause of permanent disability in adults [1,2]. It occurs due to a critical reduction of cerebral blood flow in discrete regions of the brain which results from the sudden or gradually progressing obstruction of cerebrovascular [3]. The pathophysiology of stroke includes oxidative damage, apoptosis, excitotoxicity, inflammatory and ionic imbalances [4]. The final event is neuronal death which leads to an irreversible loss of neuronal function [5]. Focal cerebral ischemia constitutes a large majority of the clinical cases in stroke pathology with the infarct usually occurring in the middle cerebral artery. Intravenous administration of recombinant tissue-plasminogen activator (rt-PA) which is the only one FDA-approved drug for the treatment of patients with cerebral ischemia is now widely used in the United States and the European Union [6,7]. However, rt-PA must be given within 3 h of cerebral ischemia onset and increases the risk of symptomatic intracranial hemorrhage [8,9]. It is also well established that cerebral ischemia/reperfusion causes over activation of membrane receptors, accumulation of extracellular glutamate and intracellular calcium, which leads to oxidative damage to lipids, proteins, nucleic acids and cell death [10,11].

Flavonoids are the most potent and versatile biologically active compounds in plant and have been known as outstanding antioxidant and neuroprotection [12,13]. Their antioxidant effects were confirmed to stem from the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving reactive oxygen species (ROS) [14]. Flavonoids were also reported to protect neurons against injury induced by neurotoxins, suppress neuro-inflammation and promote memory, learning and cognitive function [15]. Studies are now being focused on to look for flavonoids from different plants that can prevent transient cerebral ischemia [16-18].

Dracocephalum moldavica L. is an important traditional Chinese medicine which has been used to benefit heart and brain, activate blood circulation, dissipate blood stasis, relieve pain and detoxicate for thousands of years [19]. Flavonoids were known to be major active chemical component isolated from *Dracocephalum moldavica* L. Pharmacological studies showed that DMTF was able to antioxidant, ease angina, improve myocardial ischemia, decrease blood viscosity and inhibit platelet aggregation [20,21]. Based on the anti-cerebral ischemia/reperfusion effect of flavonoids, in addition to its antioxidant activity, it might be possible for DMTF to prevent brain from cerebral ischemia/reperfusion. Thus, we performed

the present experiment to examine the effects of DMTF on cerebral ischemia/reperfusion in order to understand the mechanisms of its potential neuroprotection.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Total flavonoids(purity≥80%) was extracted from shade dried whole plant in Xinjiang Institute of Meteria Medica Research. Hypoxanthine, Xanthine oxidase, catalase, 5, 5'-dithio-bis (2-nitrobenzoic acid), oxidized disulfide, reduced glutathione were purchased from Sigma Chemical Co.(St. Louis, MO). 1,1,3,3-tetramethoxypropane has been obtained from Fluka Chemical Co.(Ronkonkoma, NY). All other chemicals and reagents were of analytical grade.

2.2 Animals

Male Wistar rats weighing 220±20 g were purchased from Vital River Laboratories (Peking, China), sixty rats were randomly divided into five groups, 12 rats in each group. Rats were housed in a room with temperature of 24±1ºC and relative humidity of 50±5%. Animals were fasted for 12 h before surgery but had free access to water. All experimental procedures in this study were performed in accordance with the Institute guidelines on the care and use of experimental animals.

2.3 Drug Treatment and Surgical Operation

In this study, rats were randomly divided into model (distilled water, 10 ml/kg, i.g.), sham (distilled water, 10 ml/kg, i.g.), DMTF (50, 25, 12.5 mg/kg, i.g.) treated group. DMTF was dissolved in distilled water. DMTF and distilled water was administered once a day for five days before operation.

Middle cerebral artery occlusion (MCAO) model was established according to the method described by [22]. In brief, rats were anesthetized through the intraperitoneal injection of 10% chloral hydrate (350 mg/kg) and the left common carotid artery, internal carotid artery and external carotid artery were exposed surgically. A 4-0 monofilament nylon suture with a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. After 2 h of MCAO, the suture was removed to restore blood flow. The sham-operated rats received all surgical operation without the suture inserted. The core body temperatures were maintained at 37ºC during the whole procedure.

2.4 Tissue Preparation for Biochemical Analysis

As soon as animals were sacrificed, ischemic brain tissues were quickly removed and blotted on filter paper. Tissues were homogenized with 10 volumes of ice-cold normal saline and centrifuged at 4624×g for 15 min to assay the ratio of GSH/GSSG, the activities of antioxidant enzymes and the contents of oxidative products. Protein content was assayed according to the method described by Bradford [23].

2.5 Measurement of GSH/GSSG Ratio

Redox status was mearsured in terms of reduced glutathione (GSH) and oxidized disulfide (GSSG) ratio. The ratio of GSH/GSSG was measured according to a previous method [24]. Briefly, T-GSH was detected basing on the 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. GSSG was evaluated by measuring 5-thio-2-nitrobenzoic acid (TNB) which was produced from the reaction of reduced GSH with DTNB. GSH level in brain tissue was calculated as the difference between T-GSH and GSSG.

2.6 Measurement of Antioxidant Enzyme Activities

Superoxide Dismutase (SOD) activity was measured according to the method described previously [25]. One unit of SOD activity was defined as the amount that shows 50% inhibition. SOD activity was expressed as U/mg protein.

The catalase (CAT) activity was estimated by using the method as reported by Campo et al. [26]. One unit of CAT activity was defined as the amount of CAT required to decompose 1 μmol/L of hydrogen peroxide per min. CAT activity was expressed as U/mg protein.

Measurement of Glutathione Peroxidase (GPX) activity was performed as described by Jagetia et al. [27]. One unit of GPX activity was defined as the GPX in 1 mg protein that led to the decrease of 1 μmol/L GSH in the reactive system per minute. GPX activity was expressed as U/mg protein.

2.7 Measurement of Oxidative Product Contents

Malondialdehyde (MDA) content was determined by the method of Cao et al. [28]. 1,1,3,3-tetramethoxypropane was used as standard and the level of MDA was expressed as nmol/mg protein.

Carbonyl content was assayed with the method decribed by Levine et al. [29]. Carbonyl level was calculated by using the extinction coefficient of 22000 M-1cm-1/mg protein and expressed as nmol/mg protein.

8-hydroxyl-deoxyguanosine (8-OHdG) immunohistochemistry was performed according to the method described by Wang et al. [30]. Rats were overdosed with anesthetic and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH=7.4). The brains were removed and further fixed in 4% paraformaldehyde at 4ºC for 24 h and then cut into equally spaced blocks. Paraffin-embedded blocks were cut into a series of 5-μm-thick slices. And deparaffinized brain sections were immunostained with 5 μg/ml mouse anti-8-OHdG antibodies followed by 0.5% goat anti-mouse IgG labeled with horseradish peroxidase.

2.8 Measurement of Neuronal Apoptosis

In situ nick end labeling was performed with use of a commercially available kit (in Situ Apoptosis Detection Kit, Roche, USA) according to manual. In brief, brain sections were deparaffinized and rehydrated followed by incubating with 20 μg/ml proteinase K in 0.01 M Tris-HCl (pH=7.4) and permeabilized in a solution which contains 0.1% Triton-X 100 and

0.1% sodium citrate. Then these sections were incubated in TUNEL-reaction mixture which contains terminal deoxynucleotidy transferase.

2.9 Measurement of Neurological Deficit Scores

Neurological deficit scores were measured according to the method described by Longa et al. [31]. No neurological deficit=zero, failure to extend right paw fully=one, circling to right=two, falling to right=three, did not walk spontaneously and semi conscious=four.

2.10 Statistic Analysis

Results were expressed as means ± SEM. The significant differences among various groups were determined by one-way analysis of variance (ANOVA) and followed by Duncan's test. The statistically significant was regarded at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Effect of DMTF on GSH/GSSG Ratio

As shown in Fig. 1, compared with sham group, transient cerebral ischemia significantly decreased the ratio of GSH/GSSG. After treatment with DMTF, GSH/GSSG ratio was obviously increased in 50 mg/kg and 25 mg/kg, compared with model group.

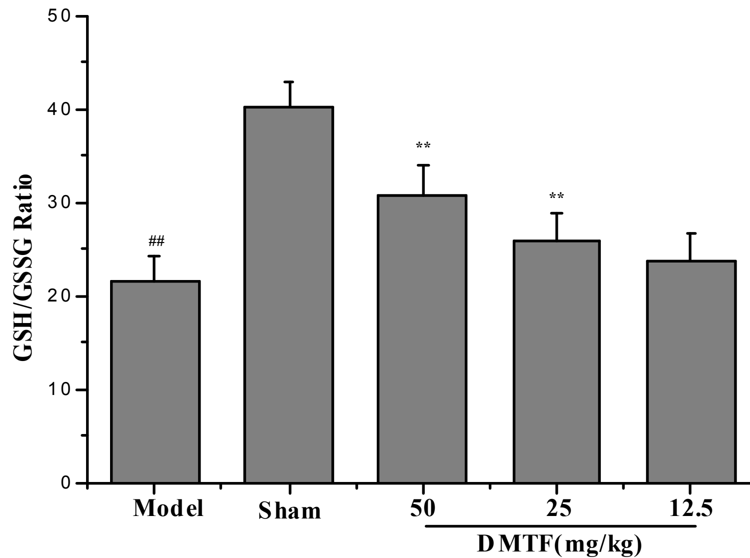

Fig. 1. Effect of DMTF on GSH/GSSG ratio in rats subjected to transient cerebral ischemia

Results were presented as means±SEM (n=8). $^{\#}P<0.05$ compared with sham group. $^{\#\#}P<0.01$ compared with sham group. $^{*}P<0.05$ compared with sham group. $^{**}P<0.01$ compared with sham group

3.2 Effect of DMTF on Antioxidant Enzyme Activities

Compared with the sham group, the SOD activity, the GPX activity and the CAT activity of the model group significantly decreased in brain tissue, while DMTF (50, 25 mg/kg) produced marked increase (Table 1).

Table 1. Effect of DMTF on antioxidant enzyme activities in rats subjected to ransient cerebral ischemia

Group	Dose (mg/kg)	SOD (U/mg protein)	GPX (U/mg protein)	CAT (U/mg protein)
Model	/	56.16±5.13##	730.00±66.94##	5.53±0.46##
Sham	/	72.73±4.32	1003.51±50.18	7.03±0.43
DMTF	50	65.29±5.39**	915.16±58.88**	6.61±0.48**
	25	62.28±4.90*	877.64±60.33**	6.30±0.50**
	12.5	58.32±5.27	812.06±60.56	5.91±0.45

*Results were presented as means±SEM (n=8). #P<0.05 compared with sham group. ##P<0.01 compared with sham group. *P<0.05 compared with sham group. **P<0.01 compared with sham group.*

3.3 Effect of DMTF on Oxidative Product Contents

Figs. 2 and 3 showed that the MDA, carbonyl and 8-OHdG contents of ischemia/reperfusion rats were significantly increased compared to those of sham rats. Treatment with DMTF (50, 25 mg/kg) led to obvious decrease in these oxidative product contents.

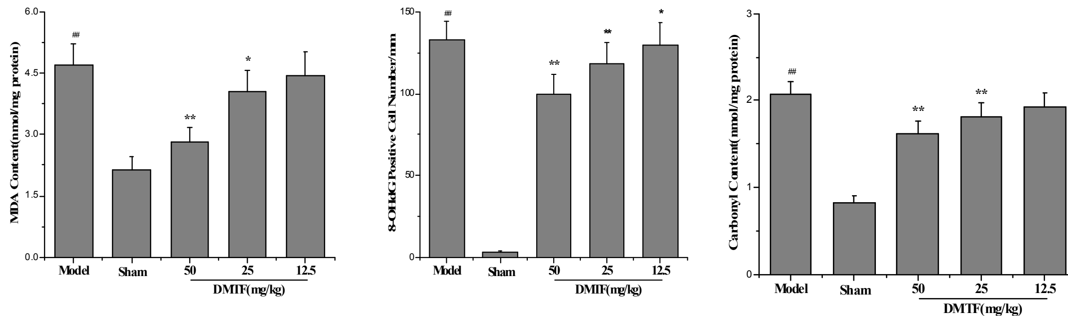

Fig. 2. Effect of DMTF on oxidative product contents in rats subjected to transient cerebral ischemia

*Results were presented as means±SEM (n=8). #P<0.05 compared with sham group. ##P<0.01 compared with sham group. *P<0.05 compared with sham group. **P<0.01 compared with sham group*

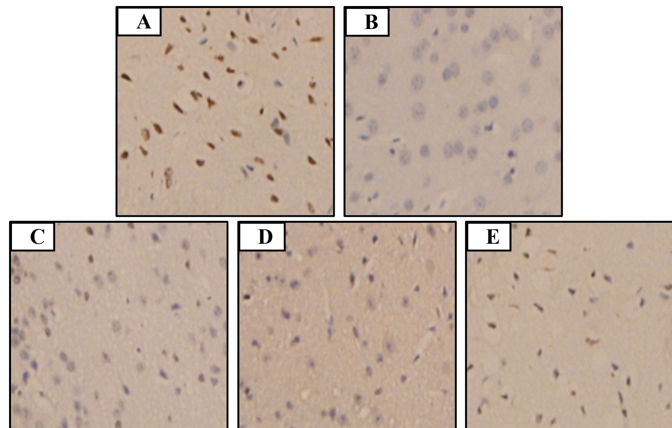

Fig. 3. Effect of DMTF on 8-OhdG level in rats subjected to transient cerebral ischemia. The nucleus of positive cell is brown colored

(A) Model group; (B)Sham group; (C) DMTF-treatment group (50 mg/kg); (D) DMTF-treatment group (25 mg/kg); (E) DMTF-treatment group (12.5 mg/kg).

3.4 Effect of DMTF on Neuronal Apoptosis

TUNEL-positive cells were scarcely observed in the ischemia region of sham-operated rats. In model group, most of the morphologically damaged neurons were positive TUNEL staining. Treatment with DMTF reduced the number of TUNEL-positive cells (Figs. 4 and 5).

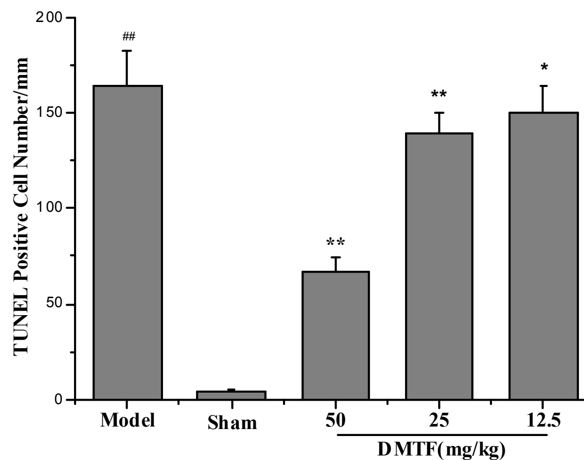

Fig. 4. Effect of DMTF on TUNEL positive cell number in rats subjected to transient cerebral ischemia

*Results were presented as means±SEM (n=8). $^{\#}P<0.05$ compared with sham group. $^{\#\#}P<0.01$ compared with sham group. *P<0.05 compared with sham group. **P<0.01 compared with sham group.*

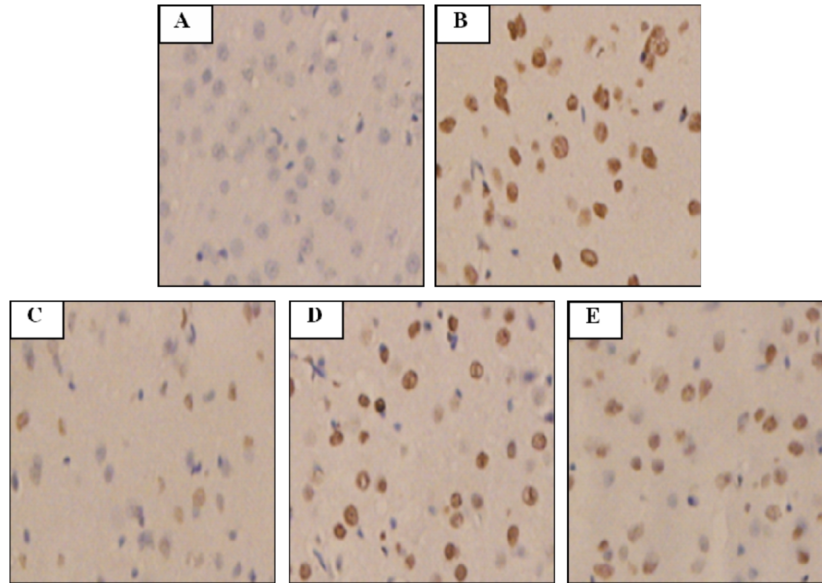

Fig. 5. Effect of DMTF on neuronal apoptosis in rats subjected to transient cerebral ischemia. The nucleus of positive cell is brown colored

(A) Model group; (B)Sham group; (C) DMTF-treatment group (50 mg/kg); (D) DMTF-treatment group (25 mg/kg); (E) DMTF-treatment group (12.5 mg/kg)

3.5 Effect of DMTF on Neurological Deficit Scores

Neurological deficit scores in cerebral ischemia/reperfusion rats were significantly higher than it of the sham control rats after operation, indicating that the model had been effectively set up. Treatment with DMTF (50, 25 mg/kg) significantly decreased neurological deficit scores compared with the model group (Fig. 6).

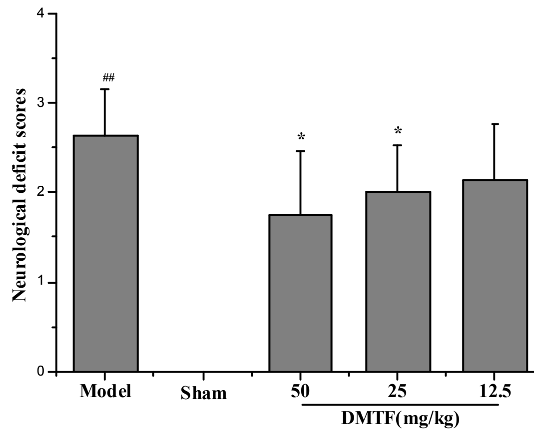

Fig. 6. Effect of DMTF on neurological deficit scores in rats subjected to transient cerebral ischemia

*Results were presented as means±SEM (n=8). #P<0.05 compared with sham group. ##P<0.01 compared with sham group. *P<0.05 compared with sham group. **P<0.01 compared with sham group*

A lot of different flavonoid compounds, such as tilianin, agastachoside, acacetin, apigenin, luteolin, kaempferol, isorhamnetin, syringaresinol, were identified from the whole plant of *Dracocephalum moldavica* L [32,33]. Blood micromolar concentrations of kaempferol efficiently were considered to protect against nitrosative-oxidative stress, and also afforded strong protection against apoptosis after cerebral ischemia/reperfusion [34]. Apigenin was reported to protect neuronal cells from injury in a middle cerebral artery occlusion-induced focal ischemia mice model [35]. Luteolin was pointed out to prevent cerebral ischemia through a rebalancing of pro-oxidant-antioxidant status [36]. The present study demonstrated that Dracocephalum moldavica L. total flavonoids educed neuroprotection by attenuating oxidative stress and apoptosis in focal transient cerebral ischemia, the neuroprotective effect of MMTF is doses-dependent, the 50 mg/Kg dose was found to be the most effective, whereas the lower doses (12.5 mg/Kg) is ineffective.

GSH (glutathione) a tripeptide comprised of glutamate, cysteine and glycine, plays key roles as antioxidant and neuromodulator in the central nervous system [37]. The reduced glutathione/ oxidized glutathione (GSH/GSSG) ratio serves as an index of redox state [38]. In the present study, decreased in GSH/GSSG ratio after transient cerebral ischemia indicates that ischemia/reperfusion altered redox state in brain tissue. DMTF ameliorated the GSH/GSSG ratio suggesting that it might modulate brain antioxidant defense by elevating tissue redox status.

When ROS production exceeds antioxidant defense system capacity, oxidative stress occurs. Primary antioxidant enzymes, such as SOD, CAT and GPX, work in parallel with nonenzymatic antioxidants to protect tissue from oxidative damage induced by ROS [39]. Our study showed that pretreatment with DMTF could increase antioxidant enzyme activities after transient cerebralischemia.

MDA, carbonyl and 8-OHdG have been proposed as the most frequently monitored markers of oxidative damage during ischemia/reperfusion injury. Consistent with earlier reports, we observed obvious increase in oxidative damage, as evident from increased MDA, carbonyl and 8-OHdG contents in model group [40]. DMTF treatment significantly reduced these oxidative damage product contents indicating that DMTF was able to protect brain from oxidative injury.

As mentioned above, ROS damage macromolecules, such as lipids, proteins, DNA and lead to brain injury. Besides their direct injuries, ROS are also involved in cell death/survival signaling pathways lead to neuronal apoptosis after cerebral ischemia [41]. Our study showed that the number of TUNEL-positive neurons was decreased by DMTF which suggested that DMTF was effective to lighten neuronal apoptosis after reperfusion.

Behavioral studies have demonstrated that neuronal damage can lead to neurological disorders, such as language difficulties, vision loss, coordination loss and swallowing difficulty [42]. Our results for neurological deficit scores suggested that treatment with DMTF improved neurological function.

4. CONCLUSION

In conclusion, the present study showed the potential of DMTF in regulating oxidative stress and ROS-induced neuronal apoptosis in transient cerebral ischemia and *Dracocephalum moldavica* L. is a valuable neuroprotective agent in preventing and curing cerebral ischemia.

Further studies are also needed to investigate whether other mechanisms play roles on DMTF's neuroprotective effect.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

ACKNOWLEDGEMENTS

This work was supported by grants from the Funding Project for Academic Human Resources Development in Institutions of Higher Learning Under the Jurisdiction of Beijing Municipality, PHR(IHLB) [PHR20090513]; Funding Project for Academic Human Resources Development in Institutions of Higher Learning Under the Jurisdiction of Beijing Municipality (PHR201107150，PHR201108422).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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