

5-HMF attenuates striatum oxidative damage via Nrf2/ARE signaling pathway following transient global cerebral ischemia

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Abstract Recent studies have shown 5-hydroxymethyl-2-furfural (5-HMF) has favorable biological effects, and its neuroprotection in a variety of neurological diseases has been noted. Our previous study showed that treatment of 5-HMF led to protection against permanent global cerebral ischemia. However, the underlying mechanisms in cerebral ischemic injury are not fully understood. This study was conducted to investigate the neuroprotective effect of 5-HMF and elucidate the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway mechanism in the striatum after transient global cerebral ischemia. C57BL/6 mice were subjected to bilateral common carotid artery occlusion for 20 min and sacrificed 24 h after reperfusion. 5-HMF (12 mg/kg) or an equal volume of vehicle was intraperitoneally injected 30 min before ischemia and 5 min after the onset of reperfusion. At 24 h after reperfusion, neurological function was evaluated by neurological disability status scale, locomotor activity test and inclined beam walking test. Histological injury of the striatum was observed by cresyl violet staining and terminal deoxynucleotidyl transferase

(TdT)-mediated dNTP nick end labeling (TUNEL) staining. Oxidative stress was evaluated by the carbonyl groups introduced into proteins, and malondialdehyde (MDA) levels. An enzyme-linked immunosorbent assay (ELISA)-based measurement was used to detect Nrf2 DNA binding activity. Nrf2 and its downstream ARE pathway protein expression such as heme oxygenase-1, NAD (P)H:quinone oxidoreductase 1, glutamate-cysteine ligase catalytic subunit and glutamate-cysteine ligase modulatory subunit were detected by western blot. Our results showed that 5-HMF treatment significantly ameliorated neurological deficits, reduced brain water content, attenuated striatum neuronal damage, decreased the carbonyl groups and MDA levels, and activated Nrf2/ARE signaling pathway. Taken together, these results demonstrated that 5-HMF exerted significant antioxidant and neuroprotective effects following transient cerebral ischemia, possibly through the activation of the Nrf2/ARE signaling pathway.

Keywords 5-hydroxymethyl-2-furfural · Transient global cerebral ischemia · Oxidative stress · Neuroprotection · Nrf2

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Introduction

Transient global cerebral ischemia (tGCI), occurs in conditions like cardiac arrest, cardiac surgery, profound hypoxia and severe hypotension, is an acute neurological injury (Harukuni and Bhardwaj, 2006; Peskine et al. 2010; Wiklund et al. 2012). Neuronal cell damage occurs following global ischemia insult in susceptible brain regions, such as the striatum and the hippocampus (Pulsinelli et al. 1982). The hippocampus receives much attention (Soares et al. 2016; Takeuchi et al. 2015), but the striatum has not been well studied. Evidence suggests that the striatal neurons are vulnerable

to tGCI, as are hippocampal CA1 neurons, and tGCI produces consistent striatum injury (Wang et al. 2012; Yoshioka et al. 2011a; Yoshioka et al. 2011b). The neuronal loss shows a close correlation with the deficits in neurological function, which are similar to clinic symptoms caused by neurological disorders, such as stroke (Ernst et al. 2014; Lindvall and Kokaia 2006). Many studies have suggested various factors that contribute to this vulnerability, such as ion imbalance, excitotoxic damage, oxidative stress, and apoptosis, among others (Doyle et al. 2008).

There is abundant evidence that reperfusion-induced oxidative stress following global cerebral ischemia (GCI) plays an important role in inducing selective neuronal cell damage and oxidative damage (Chen et al. 2011; Prentice et al. 2015; Ruszkiewicz and Albrecht 2015). Reactive oxygen species (ROS) generated enormously following GCI cause oxidative damage to many cellular components, including lipids, proteins and DNA that can impair cellular function and lead to the death of the cells. Therefore, the antioxidant therapy for ischemia/reperfusion (I/R) induced damage attracts intense interest (Carbone et al. 2015; Nabavi et al. 2014).

The transcription factor nuclear factor erythroid-2 related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway is considered to be the major cellular defense against oxidative stress (Thompson et al. 2015). There is increasing evidence that induction of the Nrf2/ARE signaling pathway confers protection against cerebral ischemia-reperfusion injury. Under basal nonactivated conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap 1) to form the Keap-1-Nrf2 complex in the cytosol and limits Nrf2-mediated gene expression. Upon activation, the Keap-1-Nrf2 complex is dissociated, Nrf2 translocates into the nuclei to bind ARE and activates ARE-dependent transcription of phase II and antioxidant defense enzymes, including heme oxygenase-1 (HO-1), NAD (P)H:quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase (GCL), to attenuate cellular oxidative stress (Kanninen et al. 2015; Nakka et al. 2016).

5-hydroxymethyl-2-furfural (5-HMF), a five carbon-ring aromatic aldehyde, is the final product of carbohydrate metabolism that exists abundantly in many foods and drinks (Liu et al. 2009; Mesias-Garcia et al. 2010; Wu 2009). 5-HMF is also one of the major active components of some Chinese herbal medicines such as *Cornus officinalis* (Cao et al., 2013; Ding et al. 2010; Wang et al. 2010), *Rehmannia radix* (Lin et al. 2008; Zhang et al. 2014) and *Schisandra chinensis* (Li et al. 2015). 5-HMF has been reported to have several pharmacological effects, including antioxidant activity, the inhibition of red blood cell sickling, the amelioration of hemorheology, and the neuroprotective effect against hypoxia (Hannemann et al. 2014; Li et al. 2011a; Li et al. 2011b; Li et al. 2015; Lin et al. 2008; Villela et al. 2009; Zhang et al. 2015).

In our previous study, we found that both pretreatment and post-treatment of 5-HMF prolong the survival time on permanent GCI mice subjected to bilateral common carotid arteries occlusion (BCCAO). And the neuroprotective effect may involve the ability of 5-HMF to increase Superoxide dismutase (SOD) activity, and decrease malondialdehyde (MDA) level (Ya et al. 2012). However, the underlying mechanisms of the effect of 5-HMF on tGCI are still not clarified. Based on the previous study, we hypothesized that 5-HMF may confer neuroprotection against tGCI, at least in part, through regulating Nrf2/ARE endogenous antioxidative system. The present study for the first time reveals that 5-HMF might be beneficial in ameliorating ischemic-induced neurological deficits in behavioral tests and mitigating the oxidative stress and histological neuronal damage in the striatum in tGCI animal model by upregulating phase II and antioxidant gene expression via Nrf2 activation.

Materials and methods

Animal and transient global cerebral ischemia model

Adult 8-week old male C57BL/6 mice weighing 20 to 22 g were obtained from the Beijing Vitalriver Experimental Animal Co., Beijing, China and housed under 12/12 h dark/light cycle and specific pathogen-free (SPF) conditions with continuous free access to food and water. Animals were randomly divided into the following three groups: sham-operated group ($n = 48$); vehicle-treated ischemic group ($n = 48$); 12 mg/kg 5-HMF-treated ischemic group ($n = 48$).

Transient global cerebral ischemia model was produced as described previously (Kim and Lee 2014; Ya et al. 2012). Briefly, the animals were fasted overnight from food but allowed free access to water. The mice were anesthetized with 10 % chloral hydrate (0.4 mL/kg, intraperitoneal injection) and allowed to breathe spontaneously. During the surgical procedure, the body temperature of the animal was maintained at 37.0 ± 0.5 °C with a heating pad. A midline incision was made in the neck, and the bilateral common carotid arteries (BCAs) were carefully exposed and separated from the surrounding connective tissue and nerve fibers, then encircled loosely with 4/0 silk threads to enable later occlusion with non-traumatic artery clips. The BCAs were then occluded using artery clips for 20 min. Following the occlusion, the clips were removed to allow complete reperfusion. The sham-surgery mice were subjected to the same procedures except for occlusion of the BCAs. After surgery, the wound was sutured and the animals were placed under heat lamps to maintain body temperature until they fully recovered from anesthesia. The mice were returned to their cages and given free access to water and food. All mice were kept warm using an electric heating blanket before they awoke and were able to

move spontaneously. All experimental procedures were conducted in accordance with the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation, and every effort was made to minimize both the number of animals used and the suffering endured by the animals.

Drug administration and experimental design

5-hydroxymethyl-2-furfural was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile saline and used at dosage of 12 mg/kg. The selection of the dosage in which 5-HMF has the most significant and reproducible neuroprotective effect in the tGCI model was based on our previous study (Ya et al. 2012) and the preliminary experiment in the present study. Mice in the 5-HMF groups were intraperitoneally injected with 5-HMF 30 min before ischemia and 5 min after the onset of reperfusion. The mice in the model and sham-operated groups received an equal volume of 0.9 % normal saline. Behavioral tests were performed 24 h after reperfusion. Mice were then sacrificed 24 h after I/R and their brains were harvested for brain edema, histological analysis, western blotting and oxyblot procedures, and biochemical assays.

Behavioral assessments

Neurological disability status scale

Neurological deficits in mice were assessed 24 h after global cerebral ischemia. This part of experiment was performed as described (Rodriguez et al. 2005). The neurological disability status scale has 10 progressive steps from 0 to 10; the higher the score the greater the neurological dysfunction is. In brief, zero represents normal and 2 represents slight decrease in mobility and the presence of passivity. 4 represents moderate neurological dysfunction. 6 corresponds to more handicapped mice but still able to walk. 8 corresponds to respiratory distress, and total incapacity to move/coordinate. Status 10 refers death. In all cases, where criteria for the precise grade were not met, the nearest appropriate number was utilized: 1, 3, 5, 7 and 9. A person blind to the study was trained to properly identify normal mice behavior and neurobehavioral alterations.

Locomotor activity test

Locomotor activity was measured as described (Wei et al. 2005). The apparatus was a fully computerized multi-box infrared sensitive motion-detection system consisted of four-cylinder boxes (diameter: 25 cm; height: 13 cm) made of transparent Lucite. For each cylinder box, three pairs of sending-receiving photoelectric cells were placed around the side, 1 cm above the bottom of the box, forming three light beams

traveling through the box with the angles of 60 degrees between every two light beams. Horizontal locomotor activity was measured as mice traveling across and then interrupting light beams that produced signals each time to computer for counting the activity. One mouse was placed in each box; four mice were tested simultaneously. At the start of the test trial, mice were allowed to adapt to the box for 2 min. Total signals within 5 min were calculated as the index of locomotor activity. Each box surface was cleaned after one mouse was tested. All mice were subjected to locomotor activity 24 h after tGCI.

Inclined beam walking test

An inclined beam walking test was performed 24 h after global cerebral ischemia. This test was employed to evaluate forelimb and hind limb motor coordination (Gulati and Singh 2014). Each mouse was individually placed on a metallic bar 60 cm long and 1.5 cm wide, inclined at an angle of 60 degrees from the ground. The motor performance of the mouse was scored on a scale ranging from 0 to 4. A grade of 0 was given to mice that could readily traverse the beam and no foot fault; grade 1 was assigned to mice demonstrating foot faults on the beam between 45 and 60 cm; grade 2 was given to mice demonstrating foot faults on the beam between 30 and 60 cm; grade 3 was assigned to mice demonstrating foot faults on the beam between 15 and 60 cm, and grade 4 was given to mice completely unable to walk on the beam.

Determination of brain edema

Animals (12 animals/group) were reanesthetized at 24 h after BCCAO. The brains were removed. The brain samples were immediately weighed to obtain the wet weight. Samples were dried for 72 h at 60 °C and the dry weight was determined. Tissue water content (%) was calculated as $[\text{wet weight} - \text{dry weight}] / \text{wet weight} * 100$.

Histological analysis of striatal injury

Histological injury of the striatum was evaluated 24 h after reperfusion by cresyl violet and terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end labeling (TUNEL) staining using a TUNEL labeling assay kit (Roche Company, Switzerland). The brains were removed, postfixed for 24 h, and coronal sections were cut from bregma +1.1 to -0.1 mm at 30 μm. Sections were grouped into two sets; the first set was stained with 1 % cresyl violet, the second for TUNEL staining.

For cresyl violet staining, damaged neurons exhibited features such as pyknosis and shrunken cell bodies. Semi-quantitative analysis of damaged cells was performed using a grading scale. Briefly, the observed damage was graded as follows: 0 = no neuronal damage; 1 = 1 % – 20 % damage;

2 = 21 % – 40 % damage; 3 = 41 % – 60 % damage; 4 = 61 % – 80 % and 5 = 81 % – 100 %, and both sides of the striatum were examined. The number of damaged cells was counted under a light microscope (200 \times).

For quantification of TUNEL staining, the TUNEL-positive cells in five sub-regions (central, dorsomedial, dorso-lateral, ventromedial, and ventrolateral) on both sides of the striatum were assigned, each consisting of a rectangle of 250 \times 125 μm^2 . Results were averaged and expressed as the number of cells/mm² in the striatum. All histological analysis was performed in a blind fashion.

Western blot

Both sides of the striatum were removed 24 h after 20 min of BCCAO ($n = 4$ per group). The nuclear and cytoplasmic fractions were extracted using a Total Protein Extraction Kit and Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc., Beijing) following the manufacturer's protocols. Cytoplasmic protein was collected for HO-1, NQO1, glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modulatory subunit (GCLM) and the nuclear protein was collected for Nrf2. The protein concentration of the supernatant fluid was quantified by spectrophotometry. Supernatant fluids were held at 95 $^{\circ}\text{C}$ in sodium dodecyl sulfate-loading buffer for 5 min before loading onto gels. Equal amounts of protein (50 μg) were then loaded in each lane of 10 % SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes and then incubated with 5 % skimmed milk for 1 h. Membranes were incubated with rabbit anti-Nrf2 polyclonal antibody (1:500, Abcam Inc., Cambridge, MA), rabbit anti-HO-1 polyclonal antibody (1:400; Santa Cruz, USA), goat anti-NQO1 polyclonal antibody (1:500, Santa Cruz, USA), rabbit polyclonal anti-GCLC antibody (1:200, Abcam Inc., Cambridge, MA); anti-GCLM antibody (1:500; Abcam Inc., Cambridge, MA). The density of each protein blot was compared with that of Histone H3 or β -actin as loading control, and the final results are expressed as fold changes by normalizing the data to the control values.

DNA binding activity assay of Nrf2

Nrf2 DNA binding activity was detected using Active Motif's (Carlsbad, CA, USA) enzyme-linked immunosorbent assay (ELISA)-based TransAM[®] Nrf2 Kit following the manufacturer's protocol. Briefly, 40 μl complete binding buffer was mixed with 10 μl protein sample (containing 10 μg protein) and incubated in a 96-well plate that was coated with oligonucleotide containing a consensus binding site for Nrf2 for 1 h at room temperature with mild agitation. For positive or blank control, 10 μl of the provided nuclear extract or complete binding buffer was used instead of the protein samples. After washing, 100 μl diluted Nrf2 antibody (1:1000) was

added to each well and incubated for 1 h, followed by incubation with 100 μl of diluted HRP-conjugated antibody (1:1000) for another 1 h. Developing solution (100 μl) was added and incubated for 3 min at room temperature before addition of 100 μl of stop solution. Absorbance was read on a spectrophotometer at 450 nm. Binding activity values were expressed as fold changes in absorbance by normalizing the data to the values of sham-operated groups.

Detection of protein oxidation and lipid peroxidation

The extent of protein oxidation was detected by using an OxyBlot Kit. The cytoplasmic supernatant fluids of tissue samples were prepared as described for the western blot analysis. With the use of a commercial kit (#S7150; Millipore), we observed the carbonyl groups as indicators of oxidative protein damage (Conlon et al. 2003). The samples were incubated with 2, 4- dinitrophenylhydrazine (DNP), and the DNP-derivatized carbonyl groups were specifically detected by western blotting with an anti-DNP antibody. The images were scanned and quantified as the western blot analysis.

The extent of lipid peroxidation was determined by measuring the total MDA levels. Twenty-four hours after tGCI, the animals were re-anesthetized. The striatums were removed quickly and homogenized in 0.1 M sodium phosphate buffer (pH 7.4) at a ratio of 1:10 (w/v). Then the homogenate was centrifuged at 3000 $\times g$ at 4 $^{\circ}\text{C}$ for 10 min. Protein concentrations were determined by spectrophotometry, with bovine serum albumin as the standard. MDA level was determined following the kit instructions (MDA Detection Kit were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In brief, MDA content was assessed by measuring levels of thiobarbituric acid reactive substances at 532 nm using a spectrophotometer (WFZ800–3, Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China). MDA was assayed based on the reaction of MDA with thiobarbituric acid to form a pink chromogen. The results from the thiobarbituric acid reactive substances assay were expressed as MDA equivalents. The measurements were performed according to the detailed procedures provided in the manufacturer's instructions.

Statistical analysis

All the data in this study are expressed as mean \pm standard error (SE). Statistical analysis for all the data was done using one-way analysis of variance (ANOVA) followed by Dunnett's test as post hoc analysis. A level of $P < 0.05$ was considered statistically significant.

Results

5-HMF ameliorated neurological deficits in behavioral tests after tGCI

Behavior tests were performed 24 h after tGCI. Compared with the sham-operated group, vehicle-treated ischemic mice showed worse neurological disability score, significant increase of photobeam interruption in locomotor activity and obvious increase in foot fault along the inclined beam along with more severe impaired ability to traverse through the entire length of the beam ($P < 0.01$). Behavioral outcomes were better in 5-HMF-treated mice compared with those in vehicle-treated mice ($P < 0.05$, Fig. 1).

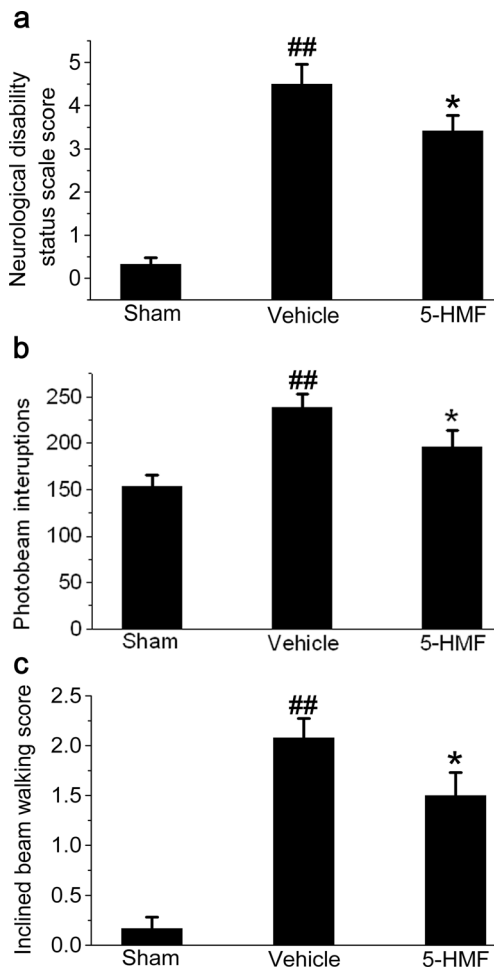


Fig. 1 Behavioral assessments in mice of sham-operated or vehicle-treated or intraperitoneally injected with 5-hydroxymethyl-2-furfural (5-HMF) 30 min before global cerebral ischemia and 5 min after the onset of reperfusion. (a) Neurological disability status scale; (b) Locomotor activity test; (c) Inclined beam walking test. Results are presented as means \pm SE, $n = 12$ for each group. ^{##} $P < 0.01$, vehicle-treated ischemic as compared with sham-operated group. ^{*} $P < 0.05$, 5-HMF-treated ischemic as compared with vehicle-treated ischemic group

5-HMF reduced tGCI-induced brain edema

BCCAO induced brain edema in both hemispheres. We observed brain water content at 24 h after tGCI using the standard wet–dry method. Figure 2 showed that 5-HMF could reduce the brain water content in the brains. In the sham group, brain water content was 79.21 ± 0.54 %. In 5-HMF-treated mice, the brain water content were reduced significantly (80.09 ± 1.12 %) compared with in vehicle-treated ischemic group (81.4 ± 1.5 %, $P < 0.05$, Fig. 2).

5-HMF attenuated neuronal injury in ischemic striatum after tGCI

Histological injury was assessed 24 h after tGCI in the striatum. Cresyl violet staining revealed remarkable neuronal damage in striatum in vehicle-treated group (3.42 ± 0.36 vs. 0.33 ± 0.14 in sham-operated mice, $p < 0.01$). Neurons showed damage such as pyknosis and shrunken cell bodies. With 5-HMF administration, striatal neuronal damage was reduced compared with vehicle-treated group (2.25 ± 0.33 vs. 3.42 ± 0.36 , $p < 0.01$); the neuroprotective effect of 5-HMF was evaluated for statistical significance using semi-quantitative analysis. Neuronal cell damage was seldom observed in the sham-operated group. (Fig. 3a).

The protective effect of 5-HMF against cerebral ischemic damage was further confirmed by TUNEL staining. The striatum was uniformly injured, and there were no significant differences in severity among the five subregions (central, dorsomedial, dorsolateral, ventromedial, and ventrolateral). Figure 3b showed TUNEL staining in samples collected 24 h after reperfusion. The apoptotic cells were characterized by a round and shrunken shape with strong staining in the nucleus. Few TUNEL-positive cells (50 ± 10 cells/mm²) were identified in the Sham

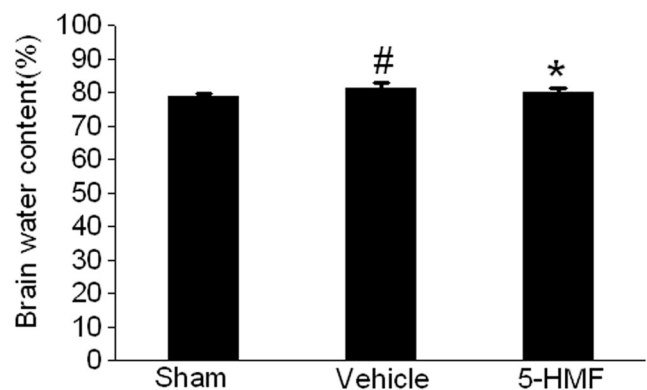


Fig. 2 Edema formation as measured by the brain water content in mice of sham-operated or vehicle-treated or intraperitoneally injected with 5-hydroxymethyl-2-furfural (5-HMF) 30 min before global cerebral ischemia and 5 min after the onset of reperfusion. Results are presented as means \pm SE, $n = 12$ for each group. [#] $P < 0.05$, vehicle-treated ischemic as compared with sham-operated group. ^{*} $P < 0.05$, 5-HMF-treated ischemic as compared with vehicle-treated ischemic group

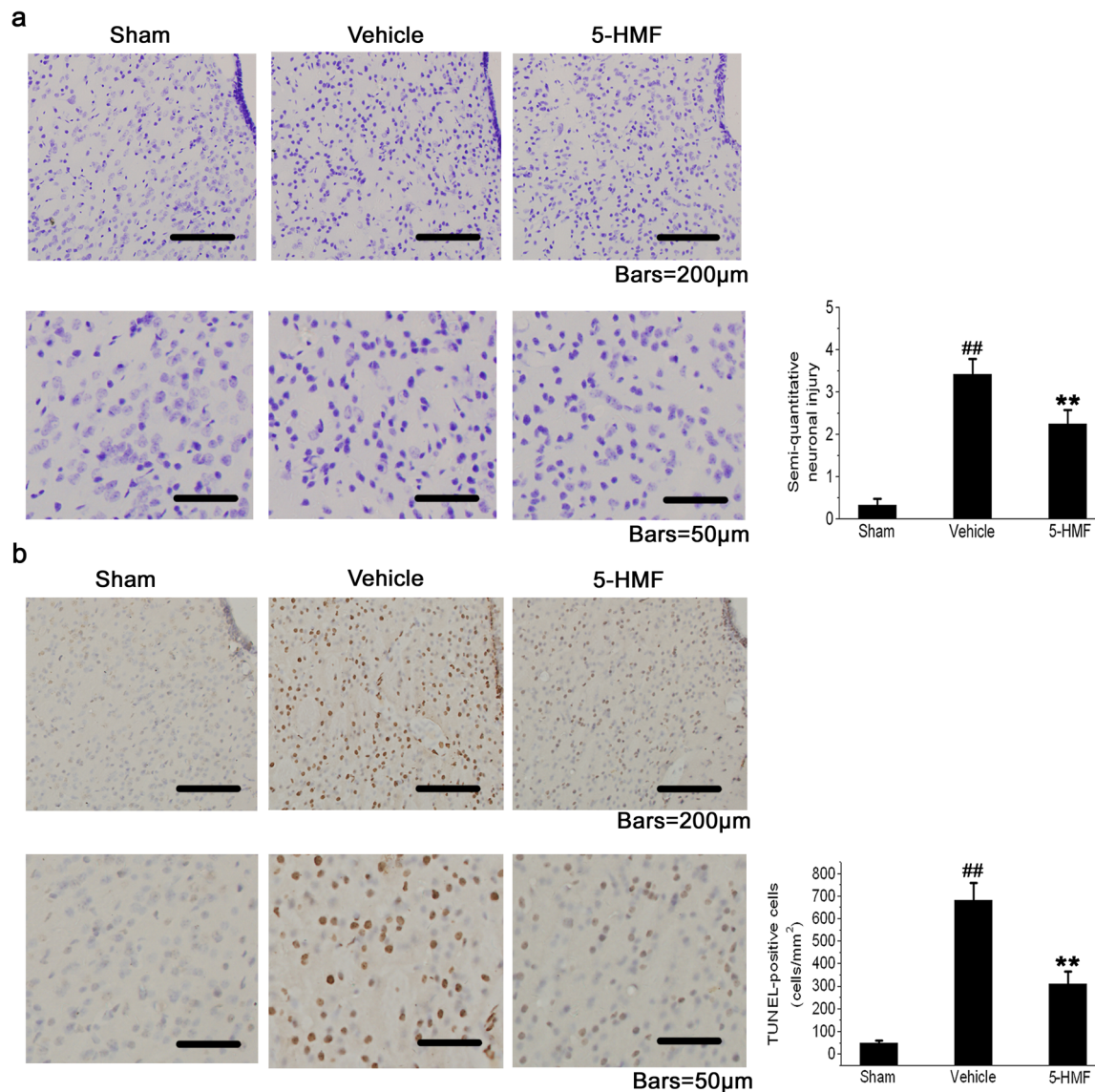


Fig. 3 Representative photomicrographs of cresyl violet and TUNEL staining in the striatum in mice of sham-operated or vehicle-treated or intraperitoneally injected with 5-hydroxymethyl-2-furfural (5-HMF) 30 min before global cerebral ischemia and 5 min after the onset of

reperfusion. (a) Cresyl violet staining; (b) TUNEL staining. Results are presented as means \pm SE, $n = 4$ for each group. ^{##} $P < 0.01$, vehicle-treated ischemic as compared with sham-operated group. ^{**} $P < 0.01$, 5-HMF-treated ischemic as compared with vehicle-treated ischemic group

group. Compared with vehicle-treated group (680 ± 80 cells/mm²), number of TUNEL-positive cells (310 ± 55 cells/mm²) was significantly reduced with 5-HMF treatment ($P < 0.01$, Fig. 3b).

5-HMF alleviated oxidative injury in ischemic striatum following tGCI

To evaluate oxidative injury, we detected the carbonyl groups introduced into proteins and the products of lipid peroxidation with MDA levels. The level of the carbonyl groups significantly increased 24 h after tGCI in the

vehicle-treated ischemic mice compared with the sham-operated mice ($P < 0.01$). The increases in the carbonyl groups significantly decreased in the 5-HMF-treated ischemic mice compared with the vehicle-treated mice ($P < 0.05$, Fig. 4a, b).

The tGCI injury increased MDA level significantly in the striatum 24 h after reperfusion in vehicle-treated ischemic group (379.86 ± 10.62 vs. 332.14 ± 16.46 in sham-operated mice, $P < 0.01$). After 5-HMF treatment, the MDA levels were decreased significantly compared with vehicle-treated ischemic group at 24 h after reperfusion (334.42 ± 10.48 vs. 379.86 ± 10.62 , $P < 0.05$, Fig. 4c).

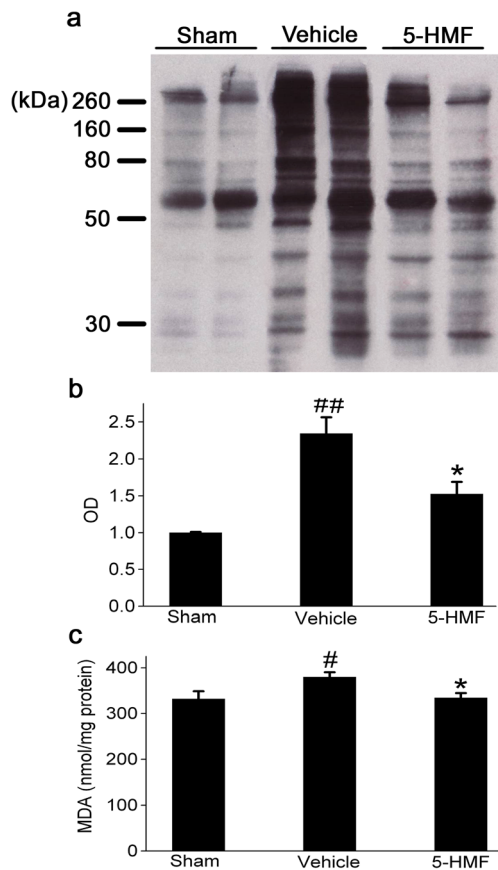


Fig. 4 Protein oxidation detected by the carbonyl groups and the formation of malondialdehyde (MDA) as an index for lipid peroxidation in the striatum in mice of sham-operated or vehicle-treated or intraperitoneally injected with 5-hydroxymethyl-2-furfural (5-HMF) 30 min before global cerebral ischemia and 5 min after the onset of reperfusion. (a) Representative blot of protein oxidation; (b) Quantitative evaluation of optical density of the carbonyl groups bands; results are presented as means \pm SE, $n = 4$ for each group; (c) Bar graph of measurement of MDA, results are presented as means \pm SE, $n = 12$ for each group. # $P < 0.05$, ## $P < 0.01$, vehicle-treated ischemic as compared with sham-operated group. * $P < 0.05$, 5-HMF-treated as compared with vehicle-treated ischemic group

5-HMF activated the Nrf2/ARE pathway in ischemic striatum following tGCI

Nrf2 is the major transcription factor responsible for the up-regulation of antioxidant and phase II detoxifying enzymes. To investigate whether 5-HMF enhanced the nuclear translocation of Nrf2, we extracted the nuclear fraction and analyzed the protein levels of Nrf2 by western blot analysis. Western blots showed that nuclear Nrf2 increased about 1.5-fold after tGCI compared with sham-operated group, while in the 5-HMF treatment group, its accumulation increased significantly (about 3-fold of the sham-operated group) compared with vehicle-treated group ($P < 0.01$, Fig. 5a). Because 5-HMF enhanced the nuclear translocation of Nrf2, we next examined whether 5-HMF treatment leads to enhanced transcriptional

activity of Nrf2. DNA binding activity of Nrf2 in striatal samples at 24 h after tGCI was measured using a commercial TransAM® Nrf2 Kit. As shown in Fig. 5b, DNA binding activity of Nrf2 was significantly increased by 5-HMF, as compared with the vehicle-treated group ($P < 0.01$).

We next determined whether 5-HMF also induced the expression of endogenous ARE-regulated genes. As shown in Fig. 5c-g, mice treated with 5-HMF exhibited an obvious increase in the protein levels of HO-1, GCLC, GCLM and NQO1 in the ischemic striatum compared to vehicle-treated group ($P < 0.05$ or $P < 0.01$).

Discussion

In this study, we investigated the hypothesis that 5-HMF is neuroprotective against striatal neuronal oxidative damage via activation of the Nrf2/ARE pathway after tGCI insult.

We adapted a model of global cerebral ischemia that permits timely and precise BCCAO and reperfusion. This model is relevant to brain insults due to cardiac arrest or other transient ischemic attacks to study its impact on numerous morphological and biochemical alterations in the brain (Doyle et al. 2008), as well as functional changes that can be revealed in behavioral studies (Yan et al. 2007). Using this tGCI mouse model, we assessed the neuroprotective effects of 5-HMF. At the behavioral level, motor functions were evaluated by neurological disability status scale, locomotor activity and inclined beam-walking test. Cerebral ischemia is documented to impair motor ability and provoke pronounced motor hyperactivity, which is most prominent on the first postischemic day after ischemic damage (Araki et al., 2001; Janać et al. 2006; Kuroiwa et al. 1991; Rauš et al., 2012). Our findings are in accordance with the already published data. In the present study, BCCAO for 20 min followed by reperfusion shown significant neurological function impairment as seen in a worse neurological function state in neurological disability status scale, increased motor activity in locomotor activity, and higher motor incoordination score in inclined beam walking test. In our study, the impaired neurological functions were partially restored by treatment with 5-HMF. These results contribute to a better insight into the effects of 5-HMF on cerebral postischemic functional damage.

Transient global cerebral ischemia can produce selective neuronal injury to the brain (Olsson et al. 2003; Pulsinelli et al. 1982). Neuronal cell death is restricted to sensitive areas of brain, such as the striatum (Wang et al. 2012; Yoshioka et al. 2011a; Yoshioka et al. 2011b). In this study, Cresyl violet and TUNEL staining carried out to detect brain damage revealed that the C57BL/6 mice model showed remarkable neuronal damage in the striatum after 24 h of reperfusion and 5-HMF significantly ameliorated pathological injury of the striatum. These data provide direct evidence that 5-HMF can

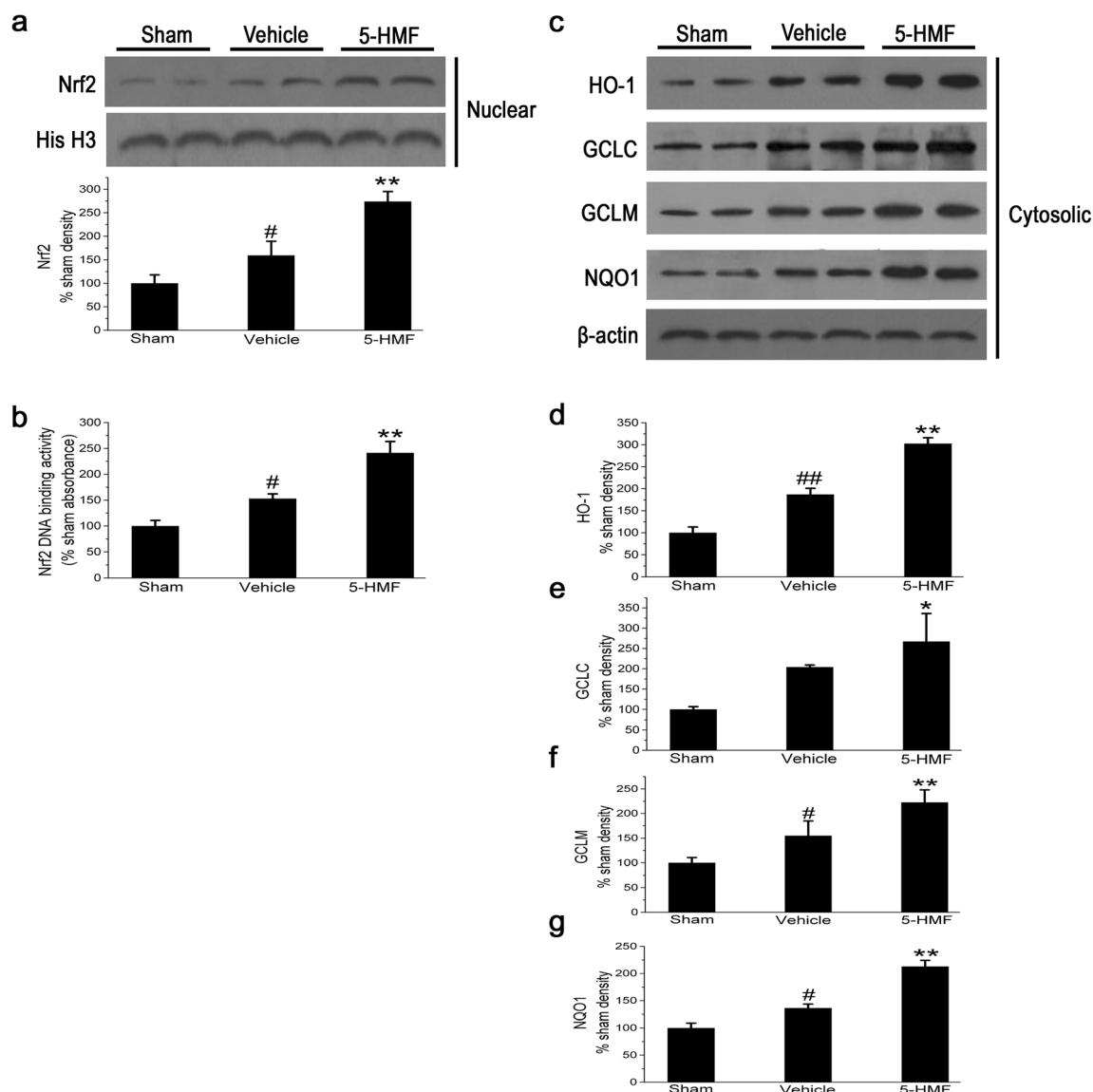


Fig. 5 Nuclear expression of nuclear factor erythroid 2-related factor 2 (Nrf2), Nrf2 DNA binding activity and cytoplasm expression of antioxidant response element (ARE)-regulated genes in the striatum in mice of sham-operated or vehicle-treated or intraperitoneally injected with 5-hydroxymethyl-2-furfural (5-HMF) 30 min before global cerebral ischemia and 5 min after the onset of reperfusion. (a) Representative western blots illustrating expression of nuclear Nrf2. Histone H3 is used as a nuclear marker and as a protein loading control. Bar graph illustrates quantitation of striatal expression of Nrf2 for sham-operated controls (lane 1 and 2) and for mice treated with vehicle (lane 3 and 4) and with 5-HMF (lane 5 and 6); (b) Bar graph of measurement of

nuclear Nrf2 DNA binding activity; (c) Representative western blots illustrating expression of cytoplasmic HO-1, GCLC, GCLM and NQO1 respectively, normalized with the β -actin blots. Bar graphs illustrate quantitation of striatal expression of HO-1 (d), GCLC (e), GCLM (f) and NQO1 (g) for sham-operated controls (lane 1 and 2) and for mice treated with vehicle (lane 3 and 4), with 5-HMF (lane 5 and 6). Results are expressed as percent of the sham-operated control (set at 100), are representative of three similar experiments, and are presented as means \pm SE, $n = 4$ for each group. # $P < 0.05$, ## $P < 0.01$, vehicle-treated ischemic as compared to the sham-operated group. * $P < 0.05$, ** $P < 0.01$, 5-HMF-treated ischemic as compared to the vehicle-treated ischemic group

confer marked histopathological protection against tCGI. Furthermore, ischemia reperfusion is commonly accompanied by edema formation in the brain. A reduction in the degree of edema formation following ischemia is beneficial to the outcome of the injury (Hillard 2008). Thereafter, we evaluated the water contents of brain in mice with or without 5-HMF treatment. The water content in the brains increased markedly

in vehicle-treated mice, whereas this increase was ameliorated by treatment with 5-HMF.

Oxidative stress has an important role in neuronal injury during reperfusion following ischemia (Niizuma et al. 2010). We investigated oxidative stress status in the striatum and found higher protein carbonyls, one of the most commonly used markers of oxidative stress after tGCI; relative band

densities in brain tissues from vehicle-treated ischemic mice were higher than in sham operated mice. In addition, the levels of MDA, a product of lipid peroxidation, increased 24 h after tGCI. These results suggest that susceptibility to oxidative stress could be one of the mechanisms of ischemic vulnerability in striatal neurons. Our study showed that 5-HMF treatment significantly reduces oxidative damage. The results are consistent with our previous experiments, reporting that 5-HMF inhibited lipid peroxidation, as shown by decreased MDA content in the BCCAO mouse model (Ya et al. 2012). These findings suggest that reduced oxidative damage accounted for the 5-HMF neuroprotective effect in tGCI mice.

Both previous reports and our study showed that 5-HMF treatment inhibits oxidative stress and has neuroprotective effect for cerebral I/R injury, but it remains unclear how 5-HMF effectively protects neurons against oxidative stress induced toxicity. The Nrf2/ARE signaling pathway has been gaining recognition as a key contributor to the cellular response to neuronal injury *in vitro* and *in vivo* (Calkins et al. 2005; Satoh et al. 2008; Shih et al. 2005). Nrf2 is a key regulator in cell survival mechanisms, and its activation can coordinately upregulate expression of several antioxidative enzymes such as HO-1, GCLC, GCLM and NQO1, which play important roles in combating oxidative stress. Activation of Nrf2 can protect against cerebral I/R injury (Wang et al. 2013). However, relatively little is known about the relationship between the neuroprotection of 5-HMF and Nrf2/ARE signaling pathway after cerebral ischemia. The current results showed that after treatment with 5-HMF, the Nrf2 nuclear translocation, the binding activity of Nrf2 to AREs, and the cytosolic expression of HO-1, GCLC, GCLM and NQO1 significantly increased, which suggest that the Nrf2/ARE signaling pathway was activated. Activation of Nrf2 with 5-HMF results in upregulation of antioxidative HO-1, GCLC, GCLM and NQO1 and reduction of oxidative damage in the cerebral ischemia. These findings provide compelling data that 5-HMF prevents oxidative stress by activating Nrf2/ARE signaling.

Many previous studies examined the time course of oxidative injury in striatum following tGCI, of which demonstrated that there were significant changes at 24 h post-stress in most markers of oxidative stress, and much of the changes were maximal at 24 h post-stress (Candelario-Jalil et al. 2001; Yoshioka et al. 2013; Yoshioka et al., 2011a, b). Results from our previous study and preliminary experiments also indicated 5-HMF had neuroprotective effects at 24 h after global I/R (Ya et al. 2012). In the present study, we chose 24 h after tGCI as the endpoint based on literature data and our previous study that had been published (Ya et al. 2012). The observation of the current study strengthens the notion that treatment with 5-HMF exerted significant antioxidant and neuroprotective effects within 24 h of imposition of I/R. Further studies are planned to determine the therapeutic time-window of 5-HMF, and extend observation periods after ischemic events to establish long-lasting neuroprotective effects of 5-HMF.

The structure-activity relationship offers preliminary insight into various mechanisms of antioxidant activity of 5-HMF. Oxidative stress, or electrophilic compounds that mimic oxidative stress (Nrf2 inducers), can modify key sulfhydryl group interactions in the Keap1-Nrf2 complex, allowing dissociation and nuclear translocation of Nrf2 (Dinkova-Kostova et al. 2002; Shih et al. 2005). A variety of Nrf2 inducers have the common chemical property of reacting with the sulfhydryl groups of cysteine residues in Keap1 (Kobayashi et al. 2006). Structurally the 5-HMF molecule contains an α,β -unsaturated carbonyl group that can directly interact with cysteine and cysteine containing peptides via the Michael addition reaction. The interaction of 5-HMF and Keap1 through cysteine may trigger Nrf2 nuclear translocation and antioxidant gene transcription, which may be one of the mechanisms accounts for the antioxidative effect of 5-HMF. Many studies have confirmed the role of 5-HMF in antioxidation (Ding et al. 2010; Wang et al. 2010; Zhao et al. 2014). An *in vitro* study systematically investigating the antioxidant activity of 5-HMF scavenging reactive oxygen species (ROS) and the expressions of myeloperoxidase (MPO), glutathione (GSH), and superoxide dismutase (SOD), suggested that the bio-chemical nature of 5-HMF may be explained by its structure (Li et al. 2009).

Overall, our findings add novel perspectives in the interpretation of the mechanisms underlying the neuroprotective effect of 5-HMF in tGCI, suggesting that Nrf2 activation may contribute to beneficial effects of 5-HMF. In this regard, our study lacks mechanistic insight into activation of the Nrf2/ARE pathway by 5-HMF, and the association between the increased Nrf2 level/activity and its downstream targets. The results are descriptive and the increases in HO-1, GCLC, GCLM and NQO1 expression are only correlative. Interference experiments using pharmacological and/or genetic tools are required, but not performed in our current work. And the extent of Nrf2 translocation from the cytosol to nucleus was not completely investigated because cytoplasmic Nrf2 expression was not measured in the present study. There are some limitations to this study in its present stage, but these will be overcome by further work.

In conclusion, we demonstrated for the first time that 5-HMF affords neuroprotection against tGCI via activation of the Nrf2/ARE pathway. The results of the present study have demonstrated that the treatment of tGCI mice by 5-HMF markedly ameliorated neurological function deficit, improved histopathologic properties and antioxidant status and activated Nrf2/ARE signaling pathway. Nrf2/ARE pathway may not be the only mechanism through which 5-HMF protect cells (Zhang et al. 2015). Several issues related to the antioxidant effects of 5-HMF still need to be clarified. Our finding is important for understanding the neuroprotective mechanism of 5-HMF and further studies may help to develop a new therapeutic strategy for stroke treatment.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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