Forsythiaside A protects against focal cerebral ischemic injury by mediating the activation of the Nrf2 and endoplasmic reticulum stress pathways

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Abstract. Ischemic stroke is a common type of stroke with a high mortality and morbidity rate. Preventing and controlling cerebral ischemic injury is particularly important. Forsythiaside A (FA) has been reported to have anti-inflammatory and antioxidant activities. The aim of the present study was to explore the impact of FA on middle cerebral artery occlusion (MCAO)-induced cerebral ischemic injury in rats. The results indicated that FA markedly increased the percent survival and decreased the neurological deficit score in rats with cerebral ischemic injury. Furthermore, cell apoptosis was significantly inhibited by FA administration, which was accompanied by decreased caspase-3 and caspase-9 expression. A marked increase in the expression levels of nuclear factor-erythroid 2-related factor 2 (Nrf2), NAD(P)H quinone dehydrogenase 1 and glutathione-s-transferase was detected in FA-treated rats. In addition, treatment with FA reduced malonaldehyde expression, and enhanced the expression of superoxide dismutase and glutathione. Furthermore, endoplasmic reticulum (ER) stress was vastly alleviated by FA treatment, as evidenced by the increased expression of B-cell lymphoma 2, apoptosis regulator and the downregulated expression of phosphorylated (phospho)-protein kinase RNA-like ER kinase (PERK)/PERK, phospho-inositol-requiring enzyme 1 (IRE1α)/IRE1α and CCAAT-enhancer-binding proteins homologous protein. Taken together, the present study demonstrated that FA attenuated cerebral ischemic damage via mediation of the activation of Nrf2 and ER stress pathways. These data may provide ideas for novel treatment strategies of cerebral ischemic damage.

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Abbreviations: MCAO, middle cerebral artery occlusion; Nqo1, NAD(P)H quinone dehydrogenase 1; GST, glutathione-s-transferase; ER, endoplasmic reticulum; PERK, protein kinase RNA-like ER kinase; Nrf2, nuclear factor erythroid 2-related factor; IRE1α, inositol-requiring enzyme 1α; PERK, protein kinase RNA-like ER kinase; CHOP, C/EBP homologous protein; SOD, superoxide dismutase; MDA, malonaldehyde; GSH, glutathione

Key words: forsythiaside A, cerebral ischemia injury, Nrf2, oxidative stress, ER stress

Introduction

Acute ischemic stroke, the leading and increasing cause of acquired neurological disability worldwide in adults, has a considerable health and socioeconomic impact (1,2). As the most metabolically active organ, the brain has a higher demand for oxygen and glucose, compared with other organs (3). Ischemic stroke limits oxygen and glucose transport to neurons, and damages the maintenance of ionic gradients across cell membranes (4). Currently, the major therapeutic strategies of ischemic stroke supported by the Food and Drug Administration are intravenous tissue plasminogen activator administration and endovascular thrombectomy (5). Nevertheless, the vast majority of stroke patients are inadequately treated due to the narrow therapeutic window (6). Thus, the discovery of novel therapeutics for ischemic stroke are urgently required.

Forsythiaside A (FA), one of the major active constituents extracted from the air-dried fruits of *Forsythia suspensa*, has been reported to possess a wide range of pharmacological properties, including anti-inflammation and antioxidant activities (7). It has been demonstrated that FA has beneficial effects in various diseases, such as alleviating ovalbumin-induced asthma (8), suppressing influenza A virus infection (9) and mediating cytochrome P450 activity (10). Kim *et al* (11) reported that FA exhibited a neuroprotective effect against transient cerebral global ischemia in gerbils. However, the underlying mechanisms have not been completely elucidated.

The pathophysiology of ischemic stroke is complex and involves irreversible neuronal injury. Cerebral ischemia triggers a cascade of cellular processes that promote neuronal death and neurological dysfunction (12). It is well established that apoptosis and necrosis are the major types of cell death that occur in ischemic brain damage (13). It has been

demonstrated that they are typically induced by oxidative stress, endoplasmic reticulum (ER) stress and inflammation in the development of cerebral ischemia (14). Oxidative stress is one of the most important triggers of epigenetic dysregulation in cerebral ischemia. Furthermore, oxidative stress may lead to the disruption of homeostasis in the ER, known as ER stress (15). Thus, oxidative and ER stress may be major targets in the treatment of cerebral ischemia.

In the present study, whether FA alleviated focal cerebral ischemic injury was investigated. The underlying molecular mechanisms were also explored *in vivo*. The results demonstrated that focal cerebral ischemic injury was markedly mitigated by regulating the activation of nuclear factor erythroid 2-related factor (Nrf2) and ER stress pathways in cerebral ischemia.

Materials and methods

Animals and ethics. A total of 64 specific-pathogen-free (SPF) mixed sex Wistar rats (male to female ratio, 31:33; age, 10-12 weeks; weight, 250-280 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China) and maintained under SPF conditions, with a maintained temperature of 22˚C and a 12-h light/dark cycle at 60% humidity. All animals were fed a normal diet with free access to food and water until 2 days prior to surgery. All experimental protocols were approved by the Committee for Laboratory Animal Care and Use of Cangzhou People's Hospital (Cangzhou, China).

Middle cerebral artery occlusion (MCAO) and grouping. FA (purity ≥98%) was purchased from ALB Materials, Inc. (Henderson, NV, USA). MCAO was performed according to the intraluminal suture method, as described previously (16,17). Wistar rats (weighing 250-280 g) were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). Body temperature was monitored and maintained at 37.0±0.5˚C during surgery. Briefly, after making an incisions in the midline of the ventral cervical skin, the left common carotid, external carotid and internal carotid were exposed. A 4-0 nylon monofilament coated with polylysine was introduced into the internal carotid artery through the common carotid artery to occlude the origin of the middle cerebral artery. The intraluminal suture was carefully withdrawn to establish reperfusion following 90 min of ischemia to establish a transient MCAO model. Laser Doppler flowmetry (Periflux system 5000; Perimed AB, Datavägen, Sweden) was used to monitor cerebral blood flow. Rats that succumbed to ischemia were excluded from the study. Following MCAO induction for 7 days at 37.0±0.5˚C, rats in each group were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) and were sacrificed by cervical dislocation. Brain tissues and blood samples were collected for the subsequent experiments. The criteria for the successful establishment of the model was that rat local cortical blood flow decreased by 15±5% of the baseline following filament insertion, and could quickly be restored by reperfusion.

Rats were randomly divided into four groups (n=16 per group): i) Control (Ctrl) group (healthy rats); ii) FA group [healthy rats treated with FA (50 mg/kg/day) by intraperitoneal injection for 7 successive days]; iii) MCAO group (rats without treatment were subjected to MCAO); and iv) MCAO+FA group [rats subjected to MCAO were treated with FA (50 mg/kg/day) by intraperitoneal injection for 7 successive days following surgery].

Neurological deficit score. The neurological scores were measured every 24 h from day 1 to 10 following MCAO according to the Zea-Longa neurological deficit scores (18). The scoring criteria were as follows: 0, normal, no neurological signs; 1, cannot completely stretch contralateral forelimbs; 2, contralateral circling when walking; 3, contralateral fall when walking; and 4, cannot walk and lowering of consciousness.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay. Brain tissues isolated from rats were fixed with 4% formaldehyde solution for 40 min at room temperature (25±0.5˚C), embedded in paraffin and cut into 4 μm sections with a microtome. TUNEL staining was conducted using an *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, following dewaxing and rehydration, sections were incubated with 20 μl/ml proteinase K for 15 min at 37˚C. Sections were subsequently immersed in equilibration buffer for 10 min at room temperature (25±0.5˚C) and incubated with TdT and dUTP-digoxigenin, followed by incubation with 2% anti-digoxigenin-peroxidase solution for 1 h at 37˚C. Afterwards, sections were stained with 3% diaminobenzidine-H_2O_2 solution for 10 min at room temperature (25±0.5˚C). The number of positive cells in each section were counted under a light microscope (magnification, x400; Olympus Corporation, Tokyo, Japan).

Western blot analysis. Brain tissues were sampled and homogenized with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing 1% phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Inc., Waltham, MA, USA) on the ice. Homogenates were centrifuged at 12,000 x g for 20 min at 4˚C and the supernatant was collected. Protein concentration was measured with a bicinchoninic acid protein assay (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Following this, 20 μg protein extracts were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% bovine serum albumin (Bio-Rad Laboratories, Inc.) for 1 h at room temperature, membranes were incubated with the following primary rabbit monoclonal antibodies: Caspase-3 [cat. no. 9662; 1:1,000; Cell Signaling Technology, Inc. (CST)], caspase-9 (cat. no. 9502; 1:1,000; CST), Nrf2 (cat. no. 12721; 1:1,000; CST), glutathione-s-transferase (GST) (cat. no. 2624; 1:1,000; CST), NAD(P)H quinone dehydrogenase 1 (Nqo1) (cat. no. 3187; 1:1,000; CST), protein kinase RNA-like ER kinase (PERK) (cat. no. 5683; 1:1,000; CST), phosphorylated (phospho)-PERK (cat. no. 3179; 1:1,000; CST), phospho-inositol-requiring enzyme 1 (p-IRE1α) (cat. no. ab48187; 1:1,000; Abcam), IRE1α (cat. no. ab37073; 1:200; Abcam), C/EBP homologous protein (CHOP) (cat. no. 2895; 1:1,000; CST), B-cell lymphoma 2 (BCL-2) (cat. no. 15071; 1:1,000; CST) and β-actin (cat. no. 4970; 1:1,000; CST) at

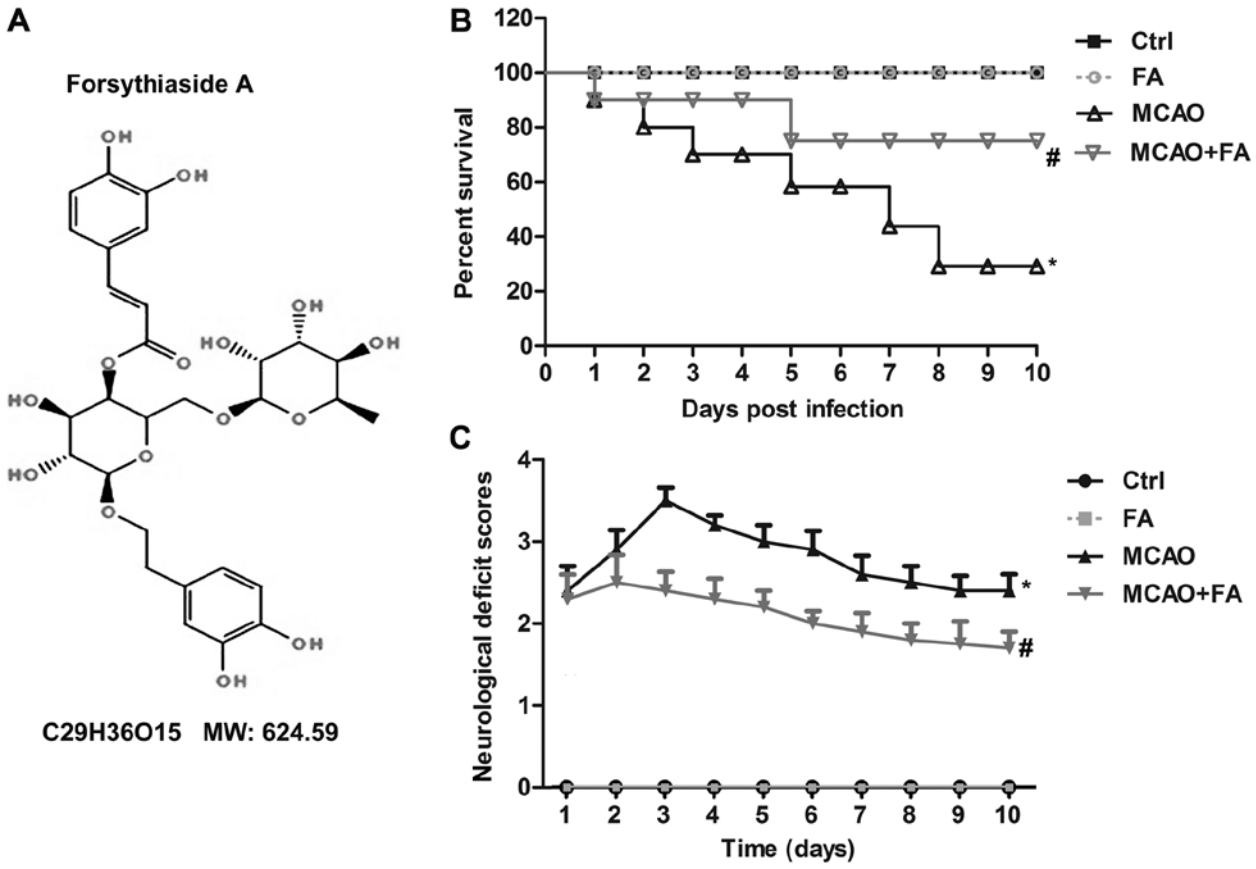

Figure 1. FA alleviates focal cerebral ischemic injury. (A) The structure of FA. (B) Survival rates were recorded every day for 10 days following MCAO. (C) Nerve injury was evaluated using the Zea-Longa neurological deficit scores. Experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl group; #P<0.05 vs. MCAO group. FA, forsythiaside A; MW, molecular weight; MCAO, middle cerebral artery occlusion; Ctrl, control.

4˚C overnight. Following rinsing with Tris buffered saline with 5% Tween 20 (TBST) three times, membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (cat. no. ab6721; 1:2,000; Abcam) for 1 h at room temperature. The immunoreactive bands were observed using an enhanced chemiluminescence reagent kit (Bio-Rad Laboratories, Inc.) and analyzed using ImageJ software (version 1.42; National Institutes of Health).

Evaluation of oxidative stress in serum. Following centrifugation at 3,000 x g for 15 min at 4˚C, serum was collected in order to measure the degree of oxidative stress. Superoxide dismutase (SOD), malonaldehyde (MDA) and glutathione (GSH) expression in serum were detected using corresponding commercial detection kits (SOD assay kit, cat. no. 20170316; MDA assay kit, cat. no. 20170314; and GSH assay kit, cat. no. 20170310; all purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The level of GSH and glutathione disulfide (GSSG), and the GSH:GSSG ratio was measured by High-performance liquid chromatography (HPLC) as previously described (19,20). The experiment was conducted strictly according to the manufacturer's instructions.

Statistical analysis. All data are expressed as the mean ± standard deviation. Comparisons of data among groups were performed by two-way analysis of variance followed by Tukey's post-hoc test, using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

FA alleviates focal cerebral ischemic injury. The structure of FA was illustrated in Fig. 1A. To explore the effects of FA on focal cerebral ischemic injury, survival rate and neurological scores were measured. As presented in Fig. 1B, the survival rate was markedly elevated in the MCAO+FA group compared with MCAO group. At the end of the study, 4 (out of 16) and 11 rats (out of 16) succumbed in the MCAO+FA and MCAO group, respectively. No difference was observed in the FA treatment group when compared with the Ctrl group. However, the neurological deficit scores were elevated in the MCAO model group, and FA administration significantly decreased the neurological deficit scores at 3-10 days in MCAO rats (Fig. 1C). These results demonstrated that focal cerebral ischemic injury was alleviated by FA treatment.

FA attenuates cell apoptosis. Apoptotic cells in brain tissues were analyzed by TUNEL staining (Fig. 2A). The results indicated that no significant levels of apoptosis were detected in rats treated with FA alone. An increased proportion of apoptotic cells was detected in the MCAO group when in comparison with the Ctrl group. However, FA administration significantly

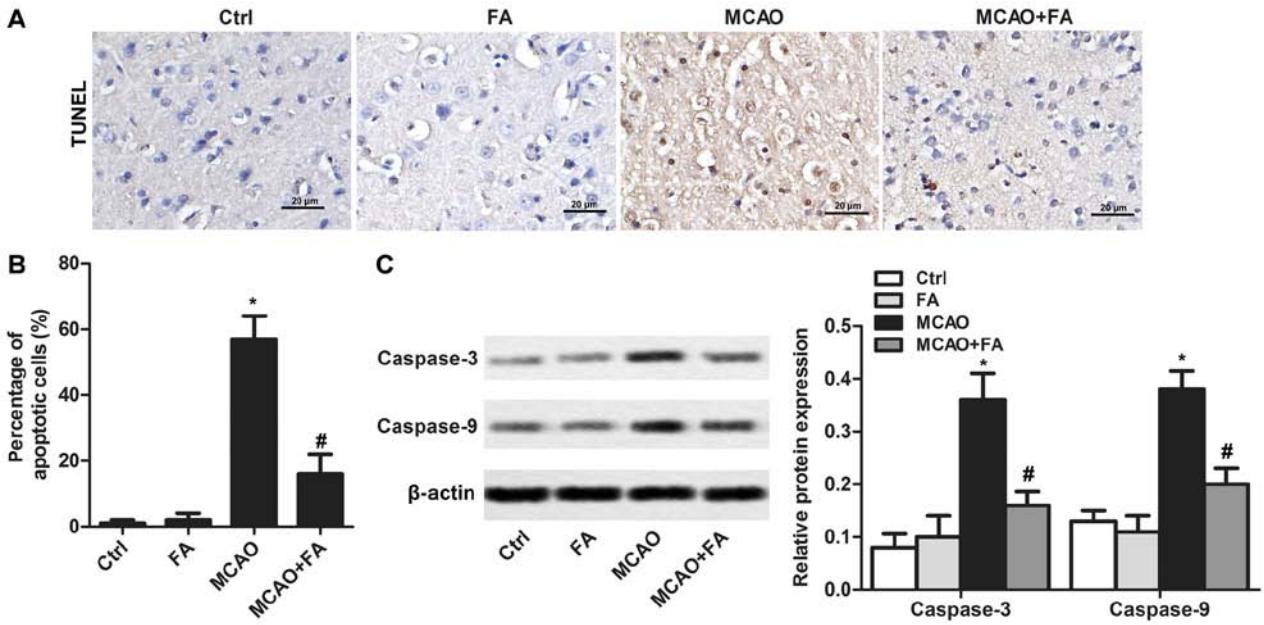

Figure 2. FA attenuates cell apoptosis. (A) Cell apoptosis was measured in brain tissues with TUNEL staining. Original magnification, x400; scale bars, 20 μm. (B) The percentage of apoptotic cells was calculated from the proportion of TUNEL positive cells. (C) The expression levels of caspase-3 and caspase-9 were assessed by western blotting. Experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl group; #P<0.05 vs. MCAO group. TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; Ctrl, control; FA, forsythiaside A; MCAO, middle cerebral artery occlusion.

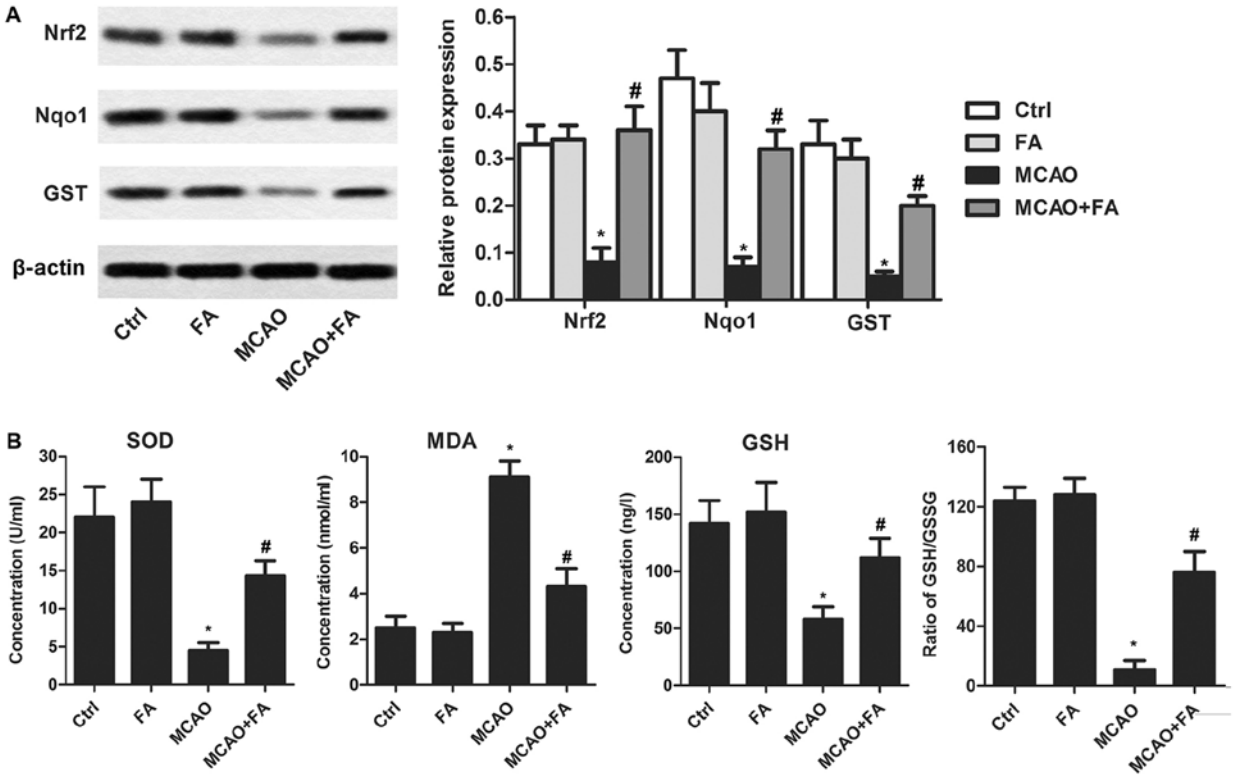

Figure 3. FA ameliorates oxidative stress via the Nrf2 signaling pathway. (A) The expressions of Nrf2, Nqo1 and GST were evaluated by western blotting. (B) Serum levels of SOD, MDA, GSH and the GSH:GSSG ratio were detected using commercial kits. Experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl group; #P<0.05 vs. MCAO group. FA, forsythiaside A; MCAO, middle cerebral artery occlusion; Ctrl, control; Nrf2, nuclear factor erythroid 2-related factor; Nqo1, NAD(P)H quinone dehydrogenase 1; GST, glutathione-s-transferase; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; GSSG, glutathione disulfide.

reduced the percentage of apoptotic cells (Fig. 2B). To further confirm the effects of FA in alleviating the cell apoptosis caused by MCAO, the expression levels of the apoptosis markers caspase-3 and -9 were measured (Fig. 2C). The results

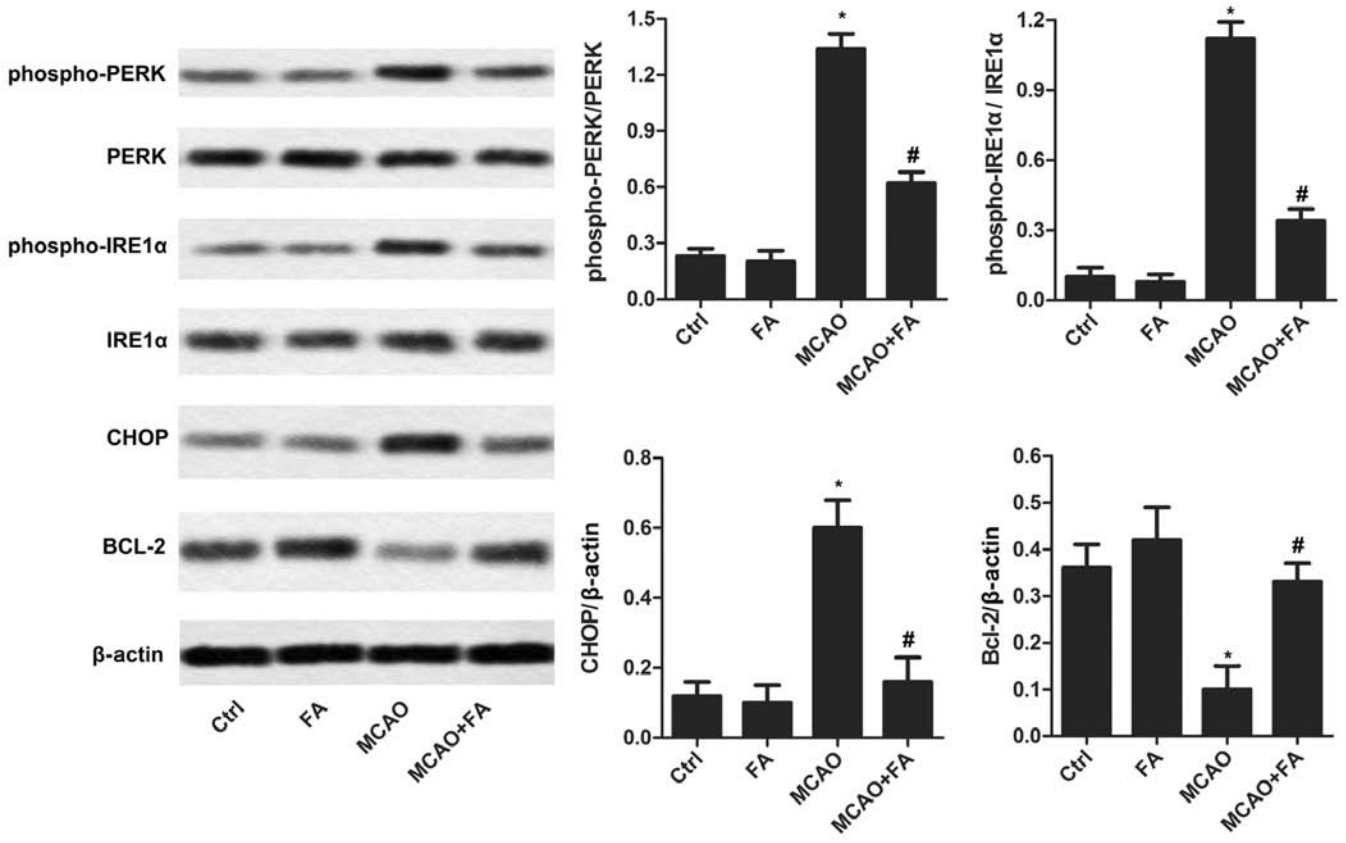

Figure 4. FA reduces endoplasmic reticulum stress. The expression levels of phospho-PERK, PERK, phospho-IRE1α, IRE1α, CHOP and BCL-2 were measured via western blot analysis. Experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl group; #P<0.05 vs. MCAO group. FA, forsythiaside A; MCAO, middle cerebral artery occlusion; Ctrl, control; phospho-, phosphorylated; PERK, protein kinase RNA-like endoplasmic reticulum kinase; IRE1α, inositol-requiring enzyme 1α; CHOP, C/EBP homologous protein; BCL-2, B-cell lymphoma 2.

demonstrated that the elevated expression of caspase-3 and -9 induced by MCAO was significantly suppressed by FA treatment (Fig. 2C).

FA ameliorates oxidative stress via the Nrf2 signaling pathway. To further determine whether FA was directly involved in oxidative stress, Nrf2 signaling pathway proteins were detected by western blotting. As illustrated in Fig. 3A, FA significantly attenuated the MCAO-induced decrease in Nrf2, Nqo1 and GST expression. In addition, FA administration markedly reduced the serum levels of MDA, and significantly elevated SOD and GSH level in the MCAO rat model when compared with the untreated MCAO group (Fig. 3B). Furthermore, the GSH:GSSG ratio was decreased to 10:1 in the MCAO model group, compared with the Ctrl group. FA treatment notably counteracted the decrease caused by MCAO and increased the ratio by 7.5 times (Fig. 3B).

FA reduces ER stress. To detect MCAO-mediated ER stress, ER stress markers, including PERK, phospho-PERK, phospho-IRE1α, IRE1α, CHOP and BCL-2, were measured in brain tissues by western blot analysis (Fig. 4). Levels of phospho-PERK/PERK, phospho-IRE1α/IRE1α and CHOP were significantly reduced following FA treatment in the MCAO rat model when compared with the untreated MCAO group. In addition, Bcl-2 expression was markedly elevated in the MCAO+FA group compared with the untreated MCAO group (Fig. 4).

Discussion

It is well known that FA possesses a broad spectrum of pharmacological applications. However, the impact of FA on focal cerebral ischemic injury remains unclear. In the present study, it was demonstrated that FA reduced focal cerebral ischemic injury by activating the Nrf2 and ER stress pathways.

Brain exposure to hypoxia induces a series of adaptive responses that may result with the reestablishment of cellular homeostasis (21). However, severe insults lead to failed physiological function restoration and cell death. There are also many alternative pathways that result in cellular demise, including apoptosis (22). Apoptosis is a type of programmed cell death that participates in the pathogenesis of ischemic stroke (23). The activation of caspase-3 and caspase-9 has a vital role in regulating cell apoptosis (24). According to previous reports, FA may reduce cell apoptosis in various diseases (22,23). For example, FA inhibited cell apoptosis by reducing levels of caspase-9 by 40% and caspase-3 by 53% in an androgenic alopecia mouse model (25). Song *et al* (26) observed that FA effectively inhibited the replication of bovine viral diarrhea virus, as well as apoptosis induced by bovine viral diarrhea virus in bovine peripheral blood mononuclear cells (26). Similarly, in the present study, cell apoptosis was markedly

inhibited by FA treatment, and its function was accompanied by the reduced expression levels of caspase-3 and caspase-9.

Oxidative stress is considered to be one of the key mechanisms involved in the development of stroke (27). As a result of increased reactive oxygen species (ROS) and reactive nitrogen species production, oxidative stress damages all components of the cell, including DNA, lipids and proteins (28). Nrf2 is a master regulator of cellular stress responses, inducing the expression of antioxidant and detoxification enzymes, and preventing oxidative stress-induced cell injury (29). Research has demonstrated that higher levels of ROS induce Nrf2 to translocate to the nucleus and regulate SOD, GSH and MDA levels (30). Emerging evidence suggests that FA may alleviate oxidative stress in various diseases. Wang *et al* (31) observed that the expression of Nrf2 and heme oxygenase-1 was markedly elevated in lipopolysaccharide-induced BV2 microglia cells (31). Furthermore, a significant decrease in MDA activity and an increase in SOD levels were detected in the brain homogenates of FA-treated mice in comparison with aged senescence accelerated mice (32). In addition, in PC12 cells, the nuclear levels of Nrf2 and antioxidant enzymes (Mn/SOD and catalase) were significantly elevated by FA treatment, indicating that the anti-oxidative effects of FA may be closely associated with the activation of the Nrf2 signaling pathway (33). According to a previous report, in response to oxidative and electrophilic stress, Nrf2 translocates to the nucleus and interacts with anti-oxidant response elements to induce the transcription of cytoprotective genes (34). Oxidative stress may lead to Nrf2 activation, which in turn acts as an autoregulatory feed-forward loop that dampens the increased ROS levels, thereby maintaining homeostasis following tissue or cellular injury (34,35). A similar result was concluded in the present research, where FA treatment markedly activated the Nrf2 pathway, and its function was accompanied by decreased MDA expression, as well as increased SOD and GSH expression. In addition, according to the research of Tanaka *et al* (36), increased levels of Nrf2 were observed 2-8 h following MCAO. This was contrary to the results of the present study; this difference may be explained by the treatment time. In Tanaka's research, Nrf2 expression levels were detected 2-8 h following MCAO (36). However, in the present study, cerebral ischemia was induced by 75 min of MCAO with an intraluminal filament, followed by 24 h of reperfusion. Furthermore, brain tissues and blood samples were collected following treatment for 7 days. Other studies have also reported a decrease in Nrf2 expression in a MCAO model (37,38), which was similar to the results of the present study. Considering that different treatment durations may affect the expression levels of Nrf2, the effect of FA on Nrf2 expression following MCAO will be investigated within 24 h in our future research.

Notably, GSH is considered to be one of the most important scavengers of ROS, and its ratio with GSSG may be used as a marker of oxidative stress (39). The ratio of reduced to oxidized glutathione within cells is often used as a marker of cellular toxicity (40,41). In a resting cell, the molar GSH:GSSG ratio exceeds 100:1, but in various models of oxidative stress, this ratio has been demonstrated to decrease to values of 10:1 and even 1:1 (42). The ratio in the present study was decreased to 10:1 in the MCAO model, and FA treatment counteracted this decrease caused by MCAO. These results further suggested the protective effect of FA in cells under oxidative stress.

Sustained ER stress is associated with numerous neurological diseases (43). A variety of factors may induce ER homeostasis disruption, including hypoxia and oxidative stress. ER stress stimulates the activation of the unfolded protein response (UPR). A previous study demonstrated that the UPR participates in a large number of neurological disorders, including cerebral ischemic injury (44). In mammals, UPR signaling is mediated by three ER transmembrane protein sensors: Activating transcription factor 6, IRE1 and PERK (45). Additionally, ER stress regulates the expression of the pro-apoptotic proteins CHOP and BCL-2, which serve an important role in the induction of cell death by stress (46). In the present study, the MCAO-induced decrease in BCL-2 expression, along with the increase in p-PERK/PERK, p-IRE1α/IRE1α and CHOP expression was markedly suppressed by FA treatment. These results suggested that ER stress was mitigated by FA administration.

In conclusion, the present study demonstrated that FA effectively alleviated neurological damage induced by MCAO. The protective effect of FA may be associated with the Nrf2 and ER stress signaling pathways. FA markedly reduced cell apoptosis, as well as the expression of caspase-3 and caspase-9. Furthermore, oxidative stress was significantly suppressed by FA treatment via the activation of the Nrf2 pathway. Thus, the present study provided experimental evidence supporting the clinical application of FA to treat focal cerebral ischemic injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TM interpreted the data regarding the MCAO model and TUNEL assay. YS was involved in western blot analysis, the oxidative stress assay and statistical analysis. YW was responsible for the design of the study and drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments in the present study were approved by the Animal Care and Research Committee of Cangzhou

People's Hospital (Cangzhou, China). All experiments were performed in compliance with relevant laws and guidelines. In addition, all experiments were conducted following the institutional guidelines of Cangzhou People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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