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Total flavonoid of *Litsea coreana* leve exerts anti-oxidative effects and alleviates focal cerebral ischemia/reperfusion injury

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Abstract

In this study, we hypothesized that total flavonoid of *Litsea coreana* leve (TFLC) protects against focal cerebral ischemia/reperfusion injury. TFLC (25, 50, 100 mg/kg) was administered orally to a rat model of focal ischemia/reperfusion injury, while the free radical scavenging agent, edaravone, was used as a positive control drug. Results of neurological deficit scoring, 2,3,5-triphenyl tetrazolium chloride staining, hematoxylin-eosin staining and biochemical tests showed that TFLC at different doses significantly alleviated cerebral ischemia-induced neurological deficits and histopathological changes, and reduced infarct volume. Moreover, it suppressed the increase in the levels of nitrates plus nitrites, malondialdehyde and lactate dehydrogenase, and it diminished the reduction in glutathione, superoxide dismutase and catalase activities induced by cerebral ischemia/reperfusion injury. Compared with edaravone, the protective effects of TFLC at low and medium doses (25, 50 mg/kg) against cerebral ischemia/reperfusion injury were weaker, while the protective effects at high dose (100 mg/kg) were similar. Our experimental findings suggest that TFLC exerts neuroprotective effects against focal cerebral ischemia/reperfusion injury in rats, and that the effects may be associated with its antioxidant activities.

Key Words

neural regeneration; total flavonoids of *Litsea coreana* leve; focal cerebral ischemia/reperfusion injury; oxidative stress; neuroprotection; infarct volume; neurological deficit scores; malondialdehyde; glutathione; superoxide dismutase; grants-supported paper; neuroregeneration

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INTRODUCTION

Transient focal cerebral ischemia is a disruption of cerebral blood supply to the brain. Rapid initiation of reperfusion is the most effective treatment for reducing infarct area and behavioral deficits caused by ischemia. However, reperfusion has the potential to introduce additional injury, which is called ischemia/reperfusion injury.

It is well-established that excitotoxicity, oxidative stress, inflammation and apoptosis are the major pathobiological mechanisms of ischemia/reperfusion injury^[1-2]. Among these factors, oxidative stress plays a central role in cerebral ischemia/reperfusion injury^[3]. During cerebral ischemia/reperfusion, reactive oxygen species production is dramatically increased^[4-5] and overwhelms endogenous antioxidant systems including antioxidant enzymes, such as superoxide dismutase and catalase, and small molecule antioxidants, such as glutathione, vitamin C and vitamin E^[6], ultimately leading to oxidative stress^[7]. The brain is very susceptible to oxidative damage owing to its high oxidative metabolic rate, low antioxidant capacity and inadequate ability for neuronal repair^[8]. Consequently, oxidative stress after ischemia/reperfusion induces a number of deleterious events, such as mitochondrial dysfunction, that may lead to apoptosis, inflammation and excitotoxicity^[9].

A number of drugs with potential neuroprotective activity have been used in the treatment of stroke. However, there is no clinically effective therapy for the management of acute stroke except for tissue plasminogen activator^[10] and edaravone^[11]. Because tissue plasminogen activator and edaravone may produce adverse reactions and have inconvenient routes of administration, increasing attention has been given to the neuroprotective properties of traditional medicines^[3, 12-14].

Litsea coreana leve is a traditional Chinese medicine, and is described in the *Compendium of Materia Medica*. It has been tradi-

tionally used as a hypolipidemic drug in southern China for hundreds of years. Moreover, previous studies showed that *Litsea coreana leve* has protective effects on hepatitis and inflammatory disease^[15]. Total flavonoid of *Litsea coreana leve* (TFLC) contains the main bioactive components of this plant, and possess antioxidant properties^[16]. However, the neuroprotective effects of TFLC on cerebral ischemia/reperfusion remained unclear.

The present study aimed to clarify whether TFLC has a neuroprotective effect on cerebral ischemia/reperfusion injury, and whether the protective effect is related to its anti-oxidative properties. A rat model of focal ischemia/reperfusion, similar to clinical cerebral ischemic disease, was used in the present study. Neurological deficit scores were evaluated, infarct volume was assessed and histopathology on brain tissues was performed to investigate the neuroprotective effect of TFLC. Levels of nitrates plus nitrites, malondialdehyde and glutathione, as well as activities of superoxide dismutase, lactate dehydrogenase and catalase were examined to determine whether the protective action of TFLC is related to its antioxidant properties. Edaravone was used in the study as a positive control drug because of its free radical scavenging ability, high antioxidant activity, and its anti-ischemic effect. The positive control drug was designed to assess the robustness of the rat model and to evaluate the efficacy of TFLC.

RESULTS

Quantitative analysis of experimental animals

Seventy-two rats were randomly divided into six groups ($n = 12$): sham-surgery, vehicle (ischemia/reperfusion + 0.5% sodium carboxymethyl cellulose *via* intragastric administration), and high-, medium- and low- dose TFLC groups (ischemia/reperfusion + 25, 50 and 100 mg/kg TFLC *via* intragastric administration, respectively), and edaravone group (ischemia/reperfusion + 3 mg/kg edaravone *via* intraperitoneal injection).

All 72 rats were included in the final analysis, with no dropout.

Effects of TFLC on neurological function in rats with cerebral ischemia/reperfusion injury

The neurological deficit scores of rats were evaluated after middle cerebral artery occlusion for 2 hours and reperfusion for 24 hours. The neurological deficit scores were significantly decreased in the high-, medium- and low-dose TFLC groups compared with the vehicle group ($P < 0.05$). Compared with the edaravone group, neurological deficit scores in the low-dose TFLC group were significantly elevated ($P < 0.05$), but there were no significant differences in neurological deficit scores between the medium- and high-dose TFLC groups and the edaravone group ($P > 0.05$; Figure 1).

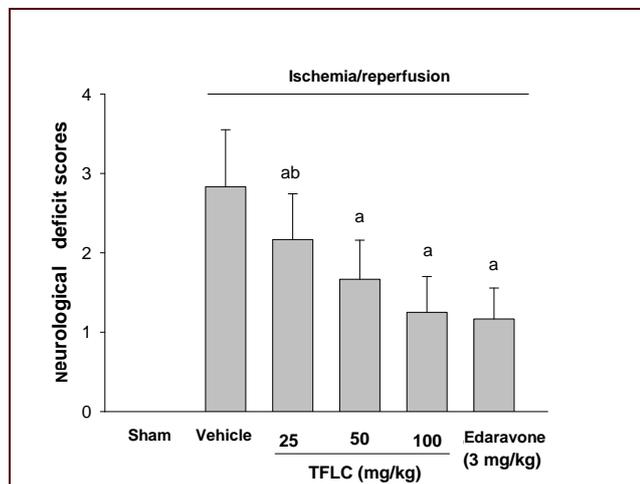


Figure 1 Effect of total flavonoid of *Litsea coreana leve* (TFLC) on neurological function in rats after cerebral ischemia/reperfusion injury.

Neurological deficit scores indicate extent of injury to the nervous system. The neurological deficit scores were evaluated after middle cerebral artery occlusion for 2 hours and reperfusion for 24 hours. The higher the score is, the more severe the injury is. Elevated neurological deficit scores after ischemia/reperfusion were significantly attenuated by TFLC (25, 50 and 100 mg/kg).

^a $P < 0.05$, vs. vehicle group; ^b $P < 0.05$, vs. edaravone group. Data are expressed as mean ± SD of 12 rats per group. Statistical evaluation was performed using one-way analysis of variance followed by multiple comparisons Dunnett's test. Sham: Sham-surgery group.

Effects of TFLC on brain injury in rats with cerebral ischemia/reperfusion injury

The brains were harvested immediately after the neurological deficit scores evaluation and stained with 2,3,5-triphenyl tetrazolium chloride (TTC; Figure 2). The infarct volumes were significantly reduced in the various TFLC groups compared with the vehicle group ($P < 0.05$). Compared with the edaravone group, the infarct volumes

in the low- and medium-dose TFLC groups were significantly elevated ($P < 0.05$), but there was no significant difference in infarct volume between the high-dose TFLC group (100 mg/kg) and the edaravone group. These results are consistent with the histopathological findings (Figure 3), showing that TFLC noticeably reduces ischemia/reperfusion-induced pathological change in the rat brain.

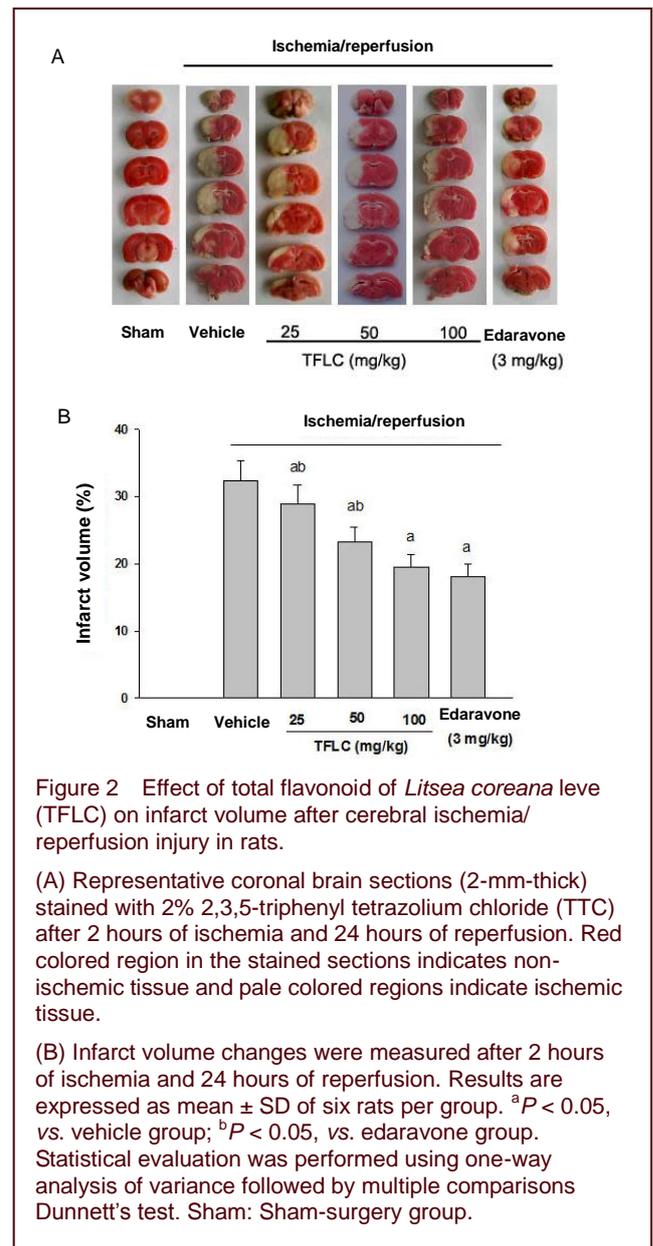


Figure 2 Effect of total flavonoid of *Litsea coreana leve* (TFLC) on infarct volume after cerebral ischemia/reperfusion injury in rats.

(A) Representative coronal brain sections (2-mm-thick) stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) after 2 hours of ischemia and 24 hours of reperfusion. Red colored region in the stained sections indicates non-ischemic tissue and pale colored regions indicate ischemic tissue.

(B) Infarct volume changes were measured after 2 hours of ischemia and 24 hours of reperfusion. Results are expressed as mean ± SD of six rats per group. ^a $P < 0.05$, vs. vehicle group; ^b $P < 0.05$, vs. edaravone group. Statistical evaluation was performed using one-way analysis of variance followed by multiple comparisons Dunnett's test. Sham: Sham-surgery group.

Brain tissues were also stained with hematoxylin-eosin. The right brain tissues (on the ischemic side) were harvested and fixed in 10% formalin, embedded in paraffin, and sliced. As shown in Figure 3, there was no histopathological change observable in the sham-surgery group. In the vehicle group, in contrast, there was visible necrosis, pyknosis of neurons, congestion of blood vessels and neuronal loss.

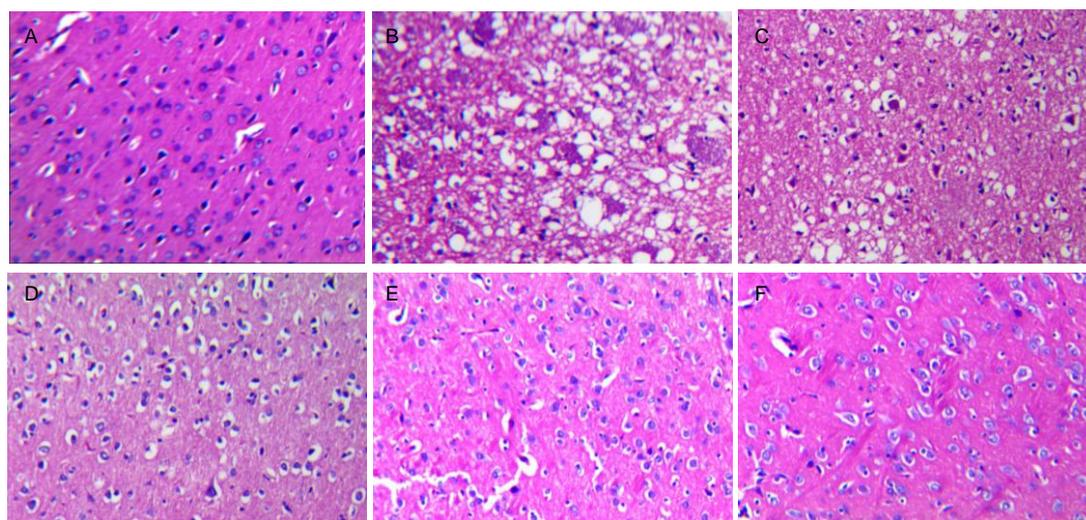


Figure 3 Effect of total flavonoid of *Litsea coreana leve* (TFLC) on histopathological changes in the ischemic regions after cerebral ischemia/reperfusion injury in rats (hematoxylin-eosin staining, $\times 100$).

(A) Sham-surgery group; (B) vehicle group; (C) low-dose TFLC group (25 mg/kg); (D) medium-dose TFLC group (50 mg/kg); (E) high-dose TFLC group (100 mg/kg); (F) edaravone group (3 mg/kg). Fields of views ($\times 100$) were randomly selected from the ischemic region of each section, and cell number, cell morphology and nuclear morphology were observed under a light microscope. Sham: Sham-surgery group.

Administration of TFLC markedly attenuated these pathological and morphological changes.

Effects of TFLC on nitrates plus nitrites, malondialdehyde and glutathione levels in the ischemic (right) brain tissue of rats with cerebral ischemia/reperfusion injury

To provide further insight into cerebral ischemia/reperfusion-induced oxidative damage and the effects of TFLC, a number of oxidative stress-related biochemical markers were measured. In the vehicle group, the level of nitrates plus nitrites was significantly increased compared with sham-operated animals ($P < 0.05$). The nitrates plus nitrites levels were significantly reduced in the TFLC groups (25, 50 and 100 mg/kg) compared with the vehicle group ($P < 0.05$). Compared with the edaravone group, the nitrates plus nitrites level in the low-dose TFLC group was significantly elevated ($P < 0.05$), but there were no significant differences in the nitrates plus nitrites levels between the medium- and high-dose TFLC groups and the edaravone group ($P > 0.05$; Figure 4A). In vehicle-treated rats that experienced 2 hours of ischemia and 24 hours of reperfusion, the level of malondialdehyde in brain tissue was significantly increased compared with the sham-operated rats ($P < 0.05$). In the TFLC groups, the levels of malondialdehyde were significantly decreased compared with the vehicle group ($P < 0.05$). Compared with the edaravone group, the malondialdehyde level in the low-dose TFLC group was significantly elevated ($P < 0.05$), but there were no sig-

nificant differences in malondialdehyde level between the medium- and high-dose TFLC groups and the edaravone group ($P > 0.05$; Figure 4B).

In brain tissue, the glutathione level in vehicle-treated rats was significantly decreased compared with the sham-surgery group ($P < 0.05$). The glutathione levels were significantly increased in the TFLC groups (25, 50 and 100 mg/kg) compared with the vehicle group ($P < 0.05$). Compared with the edaravone group, the glutathione level in the low-dose TFLC group was significantly decreased ($P < 0.05$), but there were no significant differences in glutathione level between the medium- and high-dose TFLC groups and the edaravone group ($P > 0.05$; Figure 4C).

Effects of TFLC on lactate dehydrogenase, catalase and superoxide dismutase activities in brain tissues of rats with cerebral ischemia/reperfusion injury

As shown in Table 1, compared with the sham-surgery group, the activity of lactate dehydrogenase in brain tissues in the vehicle group was significantly elevated by 106% ($P < 0.05$). TFLC (25, 50 and 100 mg/kg) reduced the activity of lactate dehydrogenase by 10%, 21% and 36%, respectively, compared with vehicle-treated rats ($P < 0.05$). Compared with the edaravone group, lactate dehydrogenase activity in the low- and medium-dose TFLC groups was significantly elevated ($P < 0.05$), but there was no significant difference in activity between the high-dose TFLC group and the edaravone group ($P > 0.05$).

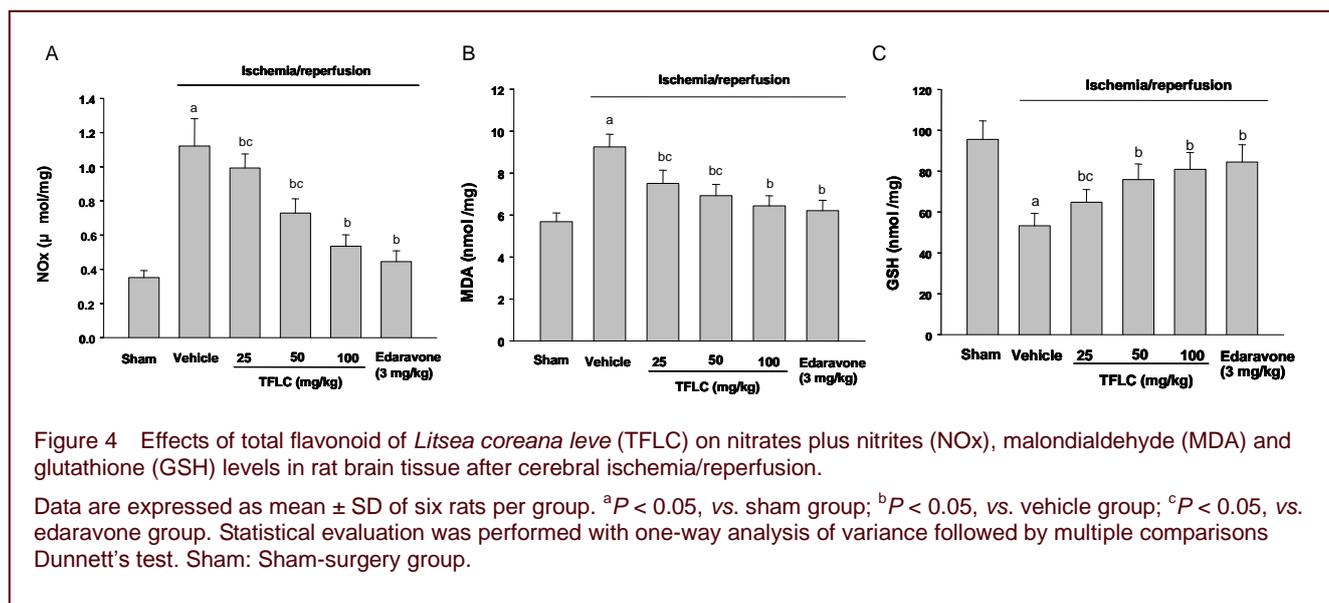


Table 1 Effects of total flavonoid of *Litsea coreana leve* (TFLC) on lactate dehydrogenase (LDH), catalase and superoxide dismutase (SOD) activities in rat brain tissue after cerebral ischemia/reperfusion

Group	Dose (mg/kg)	LDH (mU/mg)	Catalase (U/mg)	SOD (U/mg)
Sham	–	2.22 \pm 0.28	35.7 \pm 4.5	8.26 \pm 0.65
Vehicle	–	4.58 \pm 0.43 ^a	19.8 \pm 2.2 ^a	4.32 \pm 0.48 ^a
TFLC	25	4.13 \pm 0.34 ^{bc}	24.8 \pm 2.4 ^{bc}	6.28 \pm 0.56 ^{bc}
TFLC	50	3.60 \pm 0.31 ^{bc}	27.9 \pm 2.2 ^b	6.85 \pm 0.54 ^b
TFLC	100	2.95 \pm 0.43 ^b	29.3 \pm 2.4 ^b	7.23 \pm 0.53 ^b
Edaravone	3	2.74 \pm 0.23 ^b	31.1 \pm 2.8 ^b	7.45 \pm 0.69 ^b

Data are expressed as mean \pm SD of six rats per group. Statistical evaluation was performed with one-way analysis of variance followed by multiple comparisons Dunnett's test. ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. vehicle group; ^c $P < 0.05$, vs. edaravone group. Sham: Sham-surgery group.

Furthermore, the activities of catalase and superoxide dismutase in vehicle-treated rats were significantly reduced by 45% and 48%, respectively, compared with sham-operated animals ($P < 0.05$). TFLC (25, 50 and 100 mg/kg) mitigated the ischemia/reperfusion-induced reduction in superoxide dismutase activity (which was increased by 46, 56 and 67%, respectively) in comparison with the vehicle group ($P < 0.05$). TFLC (25, 50 and 100 mg/kg) also curtailed the ischemia/reperfusion-induced reduction in catalase activity (which was increased by 25%, 41% and 48%, respectively) in comparison with the vehicle group ($P < 0.05$). Compared with the edaravone group, the superoxide dismutase and catalase activities in the low-dose TFLC group were significantly increased ($P < 0.05$), but there was no significant difference in superoxide dismutase or catalase activity between the medium- and high-dose TFLC groups and the edaravone group ($P > 0.05$).

DISCUSSION

Reperfusion has the potential to introduce additional injury to ischemic brain tissue^[6]. In this study, we evaluated the neuroprotective effect of TFLC in a rat model of focal cerebral ischemia/reperfusion for the first time. Functional damage to the central nervous system was evaluated by measuring neurological deficit scores, infarct volume was analyzed with TTC staining, and histopathological changes were assessed with hematoxylin-eosin staining. The results revealed that treatment with TFLC significantly decreased neurological deficit scores, infarct volume and histological damage in a dose-dependent manner.

It is well known that oxidative metabolism is essential for the survival of the brain, but it is also associated with the generation of reactive oxygen species. Normally, there is a balance between the generation of reactive oxygen species and endogenous antioxidant systems^[17-19]. As an endogenous antioxidant, intracellular glutathione is important for limiting oxidative stress-induced neuronal injury^[6, 20]. Depletion of reduced glutathione may be attributable to the scavenging of reactive oxygen species in animals with ischemia/reperfusion injury^[21]. In the present study, TFLC treatment restored the glutathione content significantly compared with vehicle group in a dose-dependent manner, likely due to its reactive oxygen species scavenging ability. It has previously been reported that endogenous antioxidants, such as superoxide dismutase and catalase, are crucial for protection against oxidative challenge. Superoxide dismutase degrades the superoxide produced in ischemia/reperfu-

sion injuries, and may limit the resulting DNA damage in rat models of cerebral ischemia^[12, 21]. In addition, inadequate catalase activity leads to an increase in the levels of H₂O₂, which can lead to the production of the more reactive hydroxyl radical in ischemia/reperfusion injuries^[22]. In the present study, the TFLC groups displayed a significant increase in the levels of superoxide dismutase and catalase. This protection of antioxidant enzyme activity conferred by TFLC is indicative of its strong reactive oxygen species scavenging ability.

The effects of ischemia/reperfusion on catalase activity are still controversial. Most studies have shown that ischemia/reperfusion in rats lowers catalase activity^[3, 23]. However, some reports indicate that catalase activity does not significantly change during the course of ischemia/reperfusion^[24], or that it significantly increases after ischemia^[25]. In our experiments, the mean catalase activity was significantly decreased in the ischemia/reperfusion group, and this reduction was less severe in the TFLC groups.

Lipid peroxidation can be enhanced following the depletion of endogenous antioxidant systems. Recent studies show that levels of malondialdehyde, a lipid peroxidation product, are significantly increased during cerebral ischemia/reperfusion injury in animal models^[3, 26]. In the present study, we found that the levels of malondialdehyde in the brains of vehicle-treated rats were elevated, and that TFLC had a beneficial effect by inhibiting this increase. In ischemic brain tissues, ischemia causes lactate and protons to accumulate from anaerobic metabolism, and it leads to an increase in lactate dehydrogenase activity, which metabolizes the lactate^[27]. TFLC was able to reduce this increase in lactate dehydrogenase activity, suggesting a beneficial effect of the extract.

In some pathological conditions, such as ischemic stroke, the overproduction of superoxide and nitric oxide can trigger brain damage^[6, 28]. It has been reported that nitric oxide levels in the brain increase from 10 nmol to 1 μmol within 3–24 minutes during brain ischemia^[29]. Previous studies have implicated nitric oxide in the neuronal damage early in acute ischemia^[30]. The production of nitric oxide and oxygen radicals by neurons during reperfusion may contribute to cerebral injury. Nitric oxide reacts with the superoxide radical produced during reperfusion to form peroxynitrite, which is a potent cytotoxic radical that reduces neuronal survival^[28]. In the present study, TFLC significantly suppressed the increase in nitrite concentration after ischemia/reperfusion injury.

Numerous studies have investigated the role of anti-oxidative mechanisms in neuroprotection against ischemic damage. It has been reported that TFLC plays a positive role in hepatic steatosis in rats, which may be related to its antioxidative capacity. In rats with hepatic steatosis, TFLC attenuates lipid peroxidation and reduces the generation of malondialdehyde induced by oxidative damage^[16].

In the present study, we investigated the protective effects of oral administration of TFLC in the focal cerebral ischemia/reperfusion model of stroke in rats. This is the first report to clearly show that TFLC exhibits neuroprotective activity against cerebral ischemia/reperfusion injury after middle cerebral artery occlusion. Our study demonstrates that TFLC significantly decreases neurological deficit scores, cerebral infarct size and histological damage, and that it ameliorates biochemical changes after 2 hours of ischemia and 24 hours of reperfusion. The present study is the first to demonstrate that TFLC can attenuate cerebral ischemia/reperfusion injury, and suggests that this neuroprotective effect is related to the anti-oxidative properties of the traditional medicine.

Edaravone, a free radical scavenger, has an inhibitory effect on lipid peroxidation, and was first commercialized in Japan for the treatment of acute ischemic stroke. Edaravone has a neuroprotective effect on focal cerebral ischemia, and it clinically improves neurological deficits and diminishes brain edema after ischemia in adult patients^[6, 31]. The present study shows that the neuroprotection conferred by TFLC (100 mg/kg) is similar to that provided by edaravone. Furthermore, TFLC can be given *via* oral administration, which is more convenient than that for edaravone.

In summary, oral administration of TFLC reduces infarct volume, and improves the neurobehavioral, neurochemical and histological changes in a rat model of focal cerebral ischemia/reperfusion injury. Taken together, our findings suggest that dietary TFLC may protect the brain from ischemic damage induced by stroke.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

The experiment was performed at the Laboratory of Experimental Center, Department of Pharmacy, Bengbu

Medical College, China, from March to November in 2011.

Materials

A total of 72 healthy male Sprague-Dawley rats, about 8 weeks old, weighing 280–320 g, were purchased from the Experimental Animal Center of Anhui Medical University, China, with animal license No. SCXK (Wan) 2011-002.

The experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[32].

Methods

Preparation of TFLC

The dried leaves of *Litsea coreana* leve were obtained from Ningguo Medicinal Materials Company (Anhui Province, China). TFLC was extracted from the dried leaves^[16]. Quality control indexes were analyzed as previously described^[33]. The flavonoid content was 59.5%, indicating that TFLC mainly consists of these compounds, which are likely responsible for its bioactivities^[16].

All doses given are the gram-weight of the administered TFLC powder in 0.5% sodium carboxymethyl cellulose (CMC-Na) solution. Selection of the doses in this study was based on the dosages used in other disease models^[15-16] and the results of preliminary experiments.

Preparation of middle cerebral artery occlusion model

After overnight fasting, rats were anesthetized with chloral hydrate (350 mg/kg), and subjected to middle cerebral artery occlusion as described previously^[34], with minor modifications.

Briefly, the right common carotid artery, internal carotid artery and external carotid artery were exposed and dissected distally. After the internal carotid artery was isolated, a 4-0 silicone-coated nylon suture was inserted through the external carotid artery stump and gently advanced to occlude the middle cerebral artery. After 2 hours of middle cerebral artery occlusion, the suture was removed to restore blood flow (reperfusion). The incision was sutured, and the rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ with a heating lamp.

Rats were allowed to recover and housed individually, so that reperfusion was allowed for 24 hours. At the end of the reperfusion period, assessment of neurological defi-

cits was performed (criteria for successful modeling was that the neurological deficit score reached or exceeded 1). Then rats were decapitated, the brains were rapidly dissected out, cleaned by rinsing with chilled saline, and divided into two sets. One set of brains (six brain specimens from each group) were used for infarct volume measurements and the other set of brains (the other six brains from each group) were divided into two halves: one for histopathological studies and the other was stored at -80°C for biochemical analysis.

Drug intervention

Rats were given a normal diet for 1 week and acclimated to housing conditions, and then randomly divided into six groups as described above. Vehicle group received only 0.5% CMC-Na solution (Lowa Pharmaceutical Co., Ltd., Liaocheng, Shandong Province, China), and the administration method was similar to that for the TFLC groups.

TFLC groups: TFLC was dissolved in 0.5% CMC-Na solution and intragastrically administered at doses of 25, 50 or 100 mg/kg, once a day for 7 days before middle cerebral artery occlusion. Edaravone group: Edaravone (Jiangsu Simcere Pharmaceutical Co., Ltd., Nanjing, Jiangsu Province, China; positive control), 3 mg/kg, intraperitoneally administered once a day for 7 days. One hour after the last administration of TFLC and 0.5% CMC-Na solution, or 30 minutes after the last administration of edaravone, the middle cerebral artery occlusion model was prepared (occlusion for 2 hours and reperfusion for 24 hours).

Assessment of neurological deficit

Neurological deficit scores ($n = 12$ rats per group) were assessed by an observer blinded to the groups 24 hours after reperfusion.

Evaluation of neurological deficit scores was based on a modified 5-point scale as previously reported^[35]. Normal (0), no observable neurologic deficit; mild (1), flexion of the contralateral torso and the forelimb upon lifting of the animal by its tail; moderate (2), circling to the contralateral side, but normal posture at rest; severe (3), leaning to the contralateral side at rest; and very severe (4), no spontaneous motor activity.

Measurement of infarct volume in brain tissue

Cerebral infarct volumes were assessed with TTC staining as described previously^[36]. Briefly, after the brains were placed briefly in cold saline, brain specimens ($n = 6$ per group) were cut into 2-mm-thick coronal sections and

immediately immersed in 2% TTC (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes at 37°C, followed by overnight immersion in 4% paraformaldehyde.

The images of the stained sections were captured with a digital camera (Canon PowerShot A520, Canon, Tokyo, Japan) and transferred to the computer (Lenovo V450, Suzhou, Jiangsu Province, China). The infarct area was demarcated and analyzed using Image J software (Image NIH, Philadelphia, USA). The infarct volume was calculated by summation of the infarct area in each slice multiplied by the thickness of the slice and presented as a percentage of infarct volume:

Infarct volume (%) = $(S_1 + S_2 + \dots + S_n) H / (S_1' + S_2' + \dots + S_n')$ H%, where S_n' represents the area of each piece of brain tissue, S_n represents area of each piece of infarct brain tissue, H represents the thickness of each slice.

Histopathological examination of ischemic brain tissue

The brains from control and experimental groups were fixed with 10% formalin and embedded in paraffin wax. Tissues were sectioned at a thickness of 5 μ m according to the standard procedure. The sections were deparaffinized and hydrated gradually, and then stained with hematoxylin and eosin.

Fields of views (100 \times magnification) were randomly selected from the ischemia region in each section, and cell number, cell morphology and nuclear morphology were observed with light microscopy (Olympus, Tokyo, Japan).

Measurement of biochemical indicators in brain tissues

The brain tissue was minced into small pieces, homogenized in cold PBS (0.05 mol/L, pH 7.4) and centrifuged at 10 000 \times g for 15 minutes at 4°C. The supernatant was separated for biochemical determination. Total amount of nitric oxide end products, nitrates plus nitrites, were assayed using the cadmium reduction method^[37]. The levels of malondialdehyde^[38] and glutathione^[3], and enzyme activities of lactate dehydrogenase^[39], superoxide dismutase^[40] and catalase^[41] were determined using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, Jiangsu Province, China). Total protein concentration was determined using a colorimetric method^[42] with a commercially available kit (Jiancheng Institute of Biotechnology) according to the instructions of the manufacturer.

The content or activity of each indicator was calculated using the absorbance value of each tube detected with a colorimetric assay and an ultraviolet spectrophotometer (TU-1900, Beijing Purkinje General Instrument Co., Ltd., Beijing, China). The levels of the indicators were calculated as follows: $X = (A_m - A_0) / (A_s - A_0) \times C_s \div C_m$ (X , level of indicator measured; A_m , optical density of the experimental tube; A_0 , optical density of the blank tube; A_s , optical density of the standard tube; C_s , the protein content of the standard sample; C_m , the protein content of the experimental sample).

Statistical analysis

Data were analyzed using SPSS 11.5 software (SPSS, Chicago, IL, USA) and expressed as mean \pm SD. Statistical evaluation was performed with one-way analysis of variance followed by multiple comparisons Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Research background: Rapid initiation of reperfusion is the most effective treatment for reducing infarct area and alleviating neurological impairments caused by ischemia. However, reperfusion has the potential to produce additional injury, which is called ischemia/reperfusion injury. Oxidative stress plays a very important role in cerebral ischemia/reperfusion injury. Flavonoids found in *Litsea coreana leve* are major components of the plant. The antioxidant properties of a total flavonoid extract of TFLC were identified during a study of its protective effect on liver injury. However, the neuroprotective effects of the medicine on cerebral ischemia/reperfusion injury remained unclear.

Research frontiers: It is well known that excitotoxicity, oxidative stress, inflammation and apoptosis are the major pathophysiological mechanisms underlying ischemia/reperfusion injury. Among these factors, oxidative stress plays a central role in cerebral ischemia/reperfusion injury.

Clinical significance: This is the first report showing that total flavonoids of *Litsea coreana leve* exhibits neuroprotective activity in a rat model of cerebral ischemia/reperfusion, which suggests that dietary TFLC may protect the brain from ischemic damage produced by stroke.

Academic terminology: Total flavonoids of *Litsea coreana leve* are the main components of *Litsea coreana leve*, which is a traditional Chinese medicine.

Peer review: This study demonstrates the protective effect of TFLC on cerebral ischemia/reperfusion injury in rats, and the route of administration is intragastric, which simulates oral administration. The absorption of TFLC is sufficient for efficacy. Compared with intravenous or intraperitoneal injection of drugs such as edaravone, the administration route of TFLC is more convenient. Previous toxicity studies show that total flavonoids

of *Litsea coreana* leve has no significant toxicity or side effects, even at a high dose of 5 g/kg, which indicates few adverse reactions.

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