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Protective Effects of Total Flavones of Rhododendra on Cerebral Ischemia Reperfusion Injury

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Abstract: This study was to investigate the protective effects and possible mechanisms of total flavones of rhododendra (TFR) against cerebral ischemia reperfusion injury in rats and mice. Cerebral ischemia/reperfusion injury was induced by occluding the right middle cerebral artery (MCA). Infarct volume, neurological deficit, brain water content, levels of malondialdehyde (MDA), nitric oxide (NO) contents, lactate dehydrogenase (LDH) activity in plasma and brain, levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and mRNA expression of inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) in brain were evaluated 7 or 10 days after treatment. TFR significantly reduced infarct volume, ameliorated the neurological deficit and reduced the brain water content. The activities of SOD, LDH and GPX in brain were enhanced, while the activity of LDH in plasma and the contents of MDA and NO in plasma and brain were decreased. While, the expression of iNOS and nNOS mRNA in brain were down-regulated, the expression of eNOS mRNA in the brain was up-regulated. These results suggest that TFR has protective effects for cerebral injury in rats and mice, which might be associated with its antioxidant properties and ability to regulate the expression of NOS isoforms.

Keywords: Total Flavones of Rhododendra (TFR); Cerebral Ischemia; Reperfusion; Oxidative Stress; Lipid Peroxidation; Nitric Oxide.

Introduction

Cerebrovascular ischemic disease is one of the most common diseases with high disability rate and mortality in clinic. Neuroprotective agents reduce the injuries in ischemic penumbra and promote the recovery of brain function. Finding more effective neuroprotective agents with fewer risks has been a challenge. Some traditional Chinese medicines have been

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proven to be effective in alleviating symptoms that are similar to those induced by cerebral ischemia. It was found that some traditional Chinese medicines, especially those containing flavones, have protective effects against cerebral ischemic injury. Rhododendra is one of the plants that is rich in flavones, such as quercetin, hyperin and rutin (Dai *et al*., 2004; Wang *et al*., 2002), and has been used for treating patients with bronchitis for hundreds of years in China. Recent studies have shown that most flavones have protective effects against cerebral ischemic injury (Guo *et al*., 2002). Also, total flavones of rhododendra (TFR) have protective effects against myocardial ischemic injury by scavenging oxygen free radicals and inhibiting nitric oxide (Yuan *et al*., 2006). However, there is very limited information about the protective effect of TFR on animal models of cerebral ischemic reperfusion injury. We set out to investigate the protective effect of TFR on cerebral ischemic reperfusion injury and the possible underlying mechanism.

Materials and Methods

Drugs and Reagents

TFR (content of flavones over 60%) was provided by the Institute of Nature Medicine, Anhui Medical University (Hefei, China). Ginkgo biloba extract (EGB) was provided by the herb production factory of Ning-bo City (Ningbo, China). Nimodipine (Nim) was provided by Shandong Xinhua Tragacanth Limited Company (Shandong, China). MDA, SOD, GPX, LDH and NO assay kits were purchased from Nanjing Jiancheng Biological Company (Nanjing, China). RT-PCR test kit was purchased from TaKaRa Biotechnology (Dalian) Limited Company (Dalian). Diethyl pyrocarbonate (DEPC) was purchased from Solarbio.

Animals and Treatments

This investigation conforms to the regulations stipulated by Anhui Medical University Animal Care Committee which follows the protocol outlined in *The Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996).

The animals were obtained from the Experimental Animal Center of Anhui Medical University. Seventy healthy Kunming male mice and 56 healthy Wistar male rats were used in the study. The animals were housed in clear plexiglas cages with stainless steel wire lids and filter tops, in a temperature-controlled (20–24°C) room; they were maintained on a 12 hours light/dark cycle (lights on at 7:00 A.M.). Mice and rats were tested during the light phase of the light/dark cycle.

The mice weighing 18 to 22 g were randomly divided into the following 7 groups with 10 mice in each group: sham, NS, TFR 30 mg/kg, TFR 60 mg/kg, TFR 120 mg/kg, EGB 160 mg/kg and Nim 20 mg/kg. They were administrated by gavage once a day for 10 days.

The rats weighing 250~330 g were randomly divided into the following 7 groups with 8 rats in each group: sham, NS, TFR 15 mg/kg, TFR 30 mg/kg, TFR 60 mg/kg, EGB 80 mg/kg and Nim 10 mg/kg. They were administrated by gavage once a day for 7 days.

Mouse Middle Cerebral Artery Occlusion (MCAO) Model

The mouse cerebral ischemic injury was induced by a middle cerebral artery occlusion (MCAO) (Zhang *et al*., 1997). Mouse was anesthetized with 3% chloral hydrate (300 mg/kg, ip) and fixed on a heated operation table to maintain body temperature at 36.5 to 37.5°C. The right external and internal carotid arteries (ECA and ICA, respectively) were dissected from the surrounding connective tissue through a midline neck incision, and the ECA was ligated, two microvascular clips were placed across the common carotid artery and the ICA. A fish thread at a diameter of 0.185 mm, a length of 5 cm with a round tip was gently introduced into the ICA via the common carotid artery until it passed the MCA origin (approximately 15 mm). Two hours after the completion of MCA occlusion, reperfusion was allowed by withdrawal of the thread until the tip cleared the internal carotid artery. The mouse was then allowed to wake-up with free access to food and water in the cage. The mice were examined neurologically before they were sacrificed, 24 hours after recirculation of MCA, according to an established scoring system (Bederson *et al*., 1986). Briefly, 5 neurological scales of mice were evaluated after reperfusion for 24 hours as follows; The mouse had no sign or only narrowed unilateral ocular slit, scaled 0; The mouse flexed and adducted the contralateral limbs when its tail was lifted, scaled 1; The muscle resistance of the mouse was weakened when being pushed to the right side, scaled 2; The mouse rotated to the contralateral side when it crawled, scaled 3; The mouse was unconscious and could not crawl, scaled 4. In the sham group, the thread did not reach the MCA origin (no more than 15 mm) and MCA was not occluded.

The mice were sacrificed after 24 hours of reperfusion. The blood samples were collected and the brains were harvested.

Measurement of NO and MDA Level and LDH Activity

Blood was collected and centrifuged at 2000 g for 10 min. Serum was stored at −20°C until assay. Plasma nitrite, a product of NO was estimated by using Griess method at 550 nm and plasma MDA was measured by thiobarbituric acid method by spectrophotometry at 550 nm. LDH activity was measured at 440 nm by spectrophotometer.

Determination of Water Content of Brain

The right brain was removed and the water blotted on the surface with filter paper. The wet weight of the brain was determined with the Electronic Analysator. The brain was placed into an oven and baked at 80°C for 24 hours. The dried brain was weighed. The following formula was used to calculate the water weight of the brain. Water content of brain (%) = (wet weight of brain − dry weight of brain)/wet weight of brain × 100%.

Rat MCAO Model

The experimental procedure was identical to the aforementioned method used in mouse study. Rats were subjected to 2 hours MCAO followed by 24 hours of reperfusion. However, the rats were anesthetized with 10 % chloral hydrate (300 mg/kg, ip). The diameter of thread was about 0.235 mm, and the thread was inserted from the common carotid artery to the right ICA until it passed the MCA origin (approximately 20 mm) (Longa *et al*., 1989). After reperfusion for 24 hours, the rats were sacrificed and the brains were harvested. The cerebral infarct volume was evaluated by using TTC staining.

A second set of Wistar rats was randomly divided into 7 groups, 8 rats per group. The dosage schedule and procedures were repeated as described in the above test. Right brains were harvested and weighed. The brain tissues from right hemispheric fronto-parietal lobe cortex tissues were separated and stored in liquid nitrogen for the measurement of NOS mRNA. Other specimen was homogenized in ice-cold normal saline by 1:9 (w/v) for the assays of biochemical indexes.

Measurement of Infarct Volume

The rats were given an overdose of chloral hydrate and decapitated. Brains were quickly removed, rinsed in cold normal saline, placed at −80°C for 3 min and sliced into 2 mm-thick coronal sections. Tissue sections were incubated for 30 min at 37°C in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline in the dark. The borders of the infarct in each brain slice were outlined and the area quantified using NIH image software. To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. Infarct volume was calculated by integration of infarct areas for all slices of each brain (Swanson *et al*., 1990).

Measurement of NO and MDA Level and LDH, SOD and GPX Activities

The homogenized of brain tissue was centrifuged (1760 g × 10 min), the supernatant was collected for measuring NO and MDA content and activities of LDH, GPX and SOD. The aforementioned methods were used to measure NO and MDA levels and LDH activity. GPX activity was measured at 412 nm by spectrophotometer, SOD activity was measured at 550 nm by xanthine oxidase method.

Semi-Quantitative RT-PCR of NOS mRNA

Total RNA was isolated according the method described previously, briefly, brain tissue was homogenized by using a polytron system in the presence of Trizol (TaKaRa, Japan), chloroform was added, the aqueous phase was collected, and RNA was precipitated with isopropanol. The quantity and RNA integrity were assessed routinely by absorbance

(A260/A280) and ethidium bromide fluorescence of RNA separated electrophoretically on 1% formaldehyde-containing agarose gels.

A standardized semi-quantitative PCR method was used on amplification of the target genes and a constitutively expressed gene, β-actin (reference gene). The following sequence-specific primers were used (ShangHai Sangon Co., China). nNOS forward: 5′-CCG GAA TTC GAA TAC CAG CCT GAT CCA TGG AA-3′, reverse 5′-CCG AAT TCC TCC AGG AGG GTG TCC ACC GCT G-3′, 617 bp. iNOS forward: 5′-CTA CCT ACC TGG GGA ACA CCT GGG G-3′, reverse: 5′-GGA GGA GCT GAT GGA GTA GTA GCG G-3′, 442 bp. eNOS forward: 5′-CTG CTG CCC GAG ATA TCT TC-3′, reverse: 5′-AAG TAA GTG AGA GCC TGG CGC A-3′, 433 bp. β-actin forward: 5′-GTG GGC CGC TCT AGG GAC CAA-3′, reverse: 5′-CTC TTA GTG TCA CGC ACG ATT TC-3′, 540 bp.

Specimen RNA 1 μl (< 500 ng total RNA) was reversely transcribed in 10 μl of a solution containing 2 μl of 25 Mm $MgCl_2$, 1 μl of 10 × RT buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl], 3.75 μl of RNase free dH_2O 1 μl of dNTP mixture (10 mM each), 0.25 μl of RNase inhibitor, 0.5 μl of AMV reverase transcriptase (5 U/μl), 0.5 μl of random 9 mers (50 pmol/μl). Reverse transcription was performed in following conditions: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, 5°C for 5 min. The cDNA was then amplified by using Taq polymerase (Takara, Shiga, Japan) with each pair of primers. The condition for the amplification was as follows: 1 cycle of 94°C for 2 min, and 40 cycles of 94°C for 45 sec (nNOS) or 30 sec (iNOS and eNOS), 62°C (nNOS) or 65°C (iNOS) or 50°C (eNOS) for 1 min (nNOS) or 30 sec (iNOS and eNOS), 72°C for1 min, and 1 cycle of 72°C for 5 min. After amplification, 5 μl of each PCR product was separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light with a multiimage light cabinet. The expression of NOS isoforms were normalized to that of β-actin.

Statistic Analysis

Data are presented as mean ± SD. One-way ANOVA was used to analyze comparison in different groups. $p < 0.05$ was considered to be significant.

Results

Effect of TFR on Score of Functional Disturbance in Mice

Neurologic impairment as indicated by the neurological scales in the untreated mice of the control group (2.2 ± 0.79) was significantly severe compared with the sham group (0.0 ± 0.00) ($p < 0.01$). TFR 30, 60, 120 mg/kg markedly reduced the neurological score (1.5 ± 0.53, 1.4 ± 0.7 and 1.3 ± 0.48) ($p < 0.05$ or $p < 0.01$), suggesting that TFR could improved the neurological disorder induced by cerebral ischemia-reperfusion. 160 mg/kg EGB or 20 mg/kg Nim had a similar effect as in the TFR treated group (Table 1).

Table 1. Effect of TFR on Functional Disturbance and Brain Water Content in Mice

Group	Dose (mg/kg)	Neurological Score	Water Content (%)
NS		2.2 ± 0.79$^{\#\#}$	88.44 ± 2.29$^{\#\#}$
Sham		0.0 ± 0.00	74.25 ± 7.00
TFR	120	1.3 ± 0.48**	78.70 ± 9.95**
	60	1.4 ± 0.7*	76.44 ± 6.63**
	30	1.5 ± 0.53*	79.06 ± 6.58**
Nim	20	1.5 ± 0.53*	79.16 ± 2.67**
EGB	160	1.4 ± 0.52*	78.15 ± 3.13**

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NS control. Each group consists of 10 mice, values are the mean ± SD.

Table 2. Effect of TFR on the Activity of LDH and the Contents of MDA and NO in Mice

Group	Dose (mg/kg)	MDA (nmol/ml)	LDH (U/ml)	NO (μmol/L)
NS		23.23 ± 3.15$^{\#\#}$	6691.57 ± 286.65$^{\#\#}$	132.37 ± 27.58$^{\#\#}$
Sham		17.60 ± 3.43	5266.67 ± 559.99	84.12 ± 33.56
TFR	120	19.03 ± 4.34*	5654.62 ± 591.31**	82.89 ± 30.37**
	60	19.21 ± 4.24*	5822.49 ± 307.69**	96.49 ± 36.36*
	30	16.13 ± 2.82**	5763.86 ± 532.08**	97.11 ± 41.63*
Nim	20	18.66 ± 3.19**	5702.01 ± 269.72**	110.10 ± 17.51**
GEB	160	18.80 ± 2.39**	5762.25 ± 211.23**	84.74 ± 35.45*

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NS control. Each group consists of 10 mice, values are the mean ± SD.

Effect of TFR on Brain Water Content in Mice

The occlusion and recirculation of MCA induced severe brain edema as indicated by the measurement of brain water content in untreated mice of the control group. TFR treatment for 10 days markedly decreased the brain water content in 30 mg/kg group (79.06 ± 6.58) and in 60 mg/kg group (76.44 ±6.63) and in 120 mg/kg group (78.70 ± 9.95) compared with the control group (88.44 ± 2.29). Treatment with 160 mg/kg EGB and 20mg/kg Nim had similar effects in reducing brain water content (Table 1).

Effect of TFR on Serum MDA and NO Contents and LDH Activity in Mice

There was a significant increase of serum MDA and NO contents and LDH activity after 24 hours of reperfusion in the control group. Treatment with TFR (30, 60 and 120 mg/kg) markedly inhibited the increases of serum MDA and NO contents and LDH activity. Similar to TFR groups, EGB (160 mg/kg) and Nim (20 mg/kg) treated mice also had lower MDA and NO contents and LDH activity compared to the control group (Table 2).

Effect of TFR on Cerebral Infarct Volume in Rats

An ischemic zone, distinguished as a distinct pale stained area, was consistently identified in the cortex and striatum of the left cerebral hemisphere in rats subjected to cerebral ischemia reperfusion. There was a statistically significant difference between the sham group and the control group in the cerebral infarct volume. In the range of 15~60 mg/kg, TFR significantly attenuated the cerebral infarct volume. EGB (160 mg/kg) and Nim (20 mg/kg) had similar effects (Table 3).

Effect of TFR on Rat Brain LDH Activity and MDA and NO Contents

There were significant decreases of brain LDH activity and increases of MDA and NO contents in the control group. Treatment with TFR (15, 30 and 60 mg/kg) markedly inhibited the decrease of brain LDH activity and increases of MDA and NO contents. Similar to TFR groups, 160 mg/kg EGB and 20 mg/kg Nim also lowered MDA and NO contents and LDH activity in rat brain (Tables 3, 4).

Table 3. Effect of TFR on Cerebral Infarct Volumes Percent, the Content of MDA and NO in Rats

Group	Dose (mg/kg)	Infarct Volume Percent (%)	MDA (nmol/mg Protein)	NO (μmol/L)
NS		25.98 ± 5.19	2.41 ± 0.36$^{\#\#}$	220.89 ± 59.58$^{\#\#}$
Sham		—	1.55 ± 0.23	117.18 ± 42.87
TFR	60	16.55 ± 7.43*	1.89 ± 0.35*	135.42 ± 44.75**
	30	18.40 ± 6.95*	1.85 ± 0.30**	160.09 ± 38.49*
	15	19.72 ± 3.29*	1.62 ± 0.25**	158.75 ± 30.68*
Nim	10	16.04 ± 2.99**	1.53 ± 0.55**	120.02 ± 40.67**
GEB	80	15.49 ± 4.08**	1.32 ± 0.32**	134.94 ± 41.82**

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NS control. Each group consists of 8 rats, values are the mean ± SD.

Table 4. Effect of TFR on the Activities of LDH, SOD and GPX in Rat Cerebrum

Group	Dose (mg/kg)	LDH (U/g Protein)	SOD (U/mg Protein)	GPX (U/mg Protein)
NS		1710.32 ± 110.31$^{\#\#}$	72.68 ± 3.45$^{\#}$	12.31 ± 1.69$^{\#\#}$
Sham		1986.12 ± 237.72	83.01 ± 10.99	17.49 ± 3.23
TFR	60	2019.14 ± 84.5**	82.44 ± 6.54**	16.30 ± 0.85**
	30	1872.41 ± 151.71*	81.18 ± 8.01*	17.89 ± 1.48**
	15	1847.85 ± 114.06**	80.31 ± 8.55*	14.30 ± 1.45*
Nim	10	1911.37 ± 211.51*	80.89 ± 9.98*	15.63 ± 3.04*
GEB	80	1883.05 ± 144.90*	88.97 ± 9.52**	16.12 ± 3.60*

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NS control. Each group consists of 8 rats, values are the mean ± SD.

Effect of TFR on SOD, GPX Activities in Rat Cerebrum

Significant decreases of rat cerebral SOD and GPX activities were detected in the control group. Like EGB or Nim, 30, 60 and 120 mg/kg TFR markedly enhanced the activities of SOD and GPX in rat cerebrum ($p < 0.01$) (Table 4).

Effect of TFR on Expression of NOS mRNA in Rat Cortex

As shown in Fig. 1, the mRNA RT-PCR amplification products of 3 NOS isoforms, eNOS, nNOS and iNOS, were observed in rat cortex. Both eNOS and nNOS mRNAs were present in rat cortex of sham group, but iNOS mRNA was at undetectable level. MCA ischemia reperfusion not only induced iNOS mRNA in the control group, but also significantly increased the expression of eNOS and nNOS mRNAs compared to the sham group ($p < 0.05$ or $p < 0.01$). Thirty and 60 mg/kg TFR markedly increased eNOS mRNA expression, and decreased nNOS and iNOS mRNAs expression compared to the control group ($p < 0.05$ or $p < 0.01$). Nim or EGB had a similar effect.

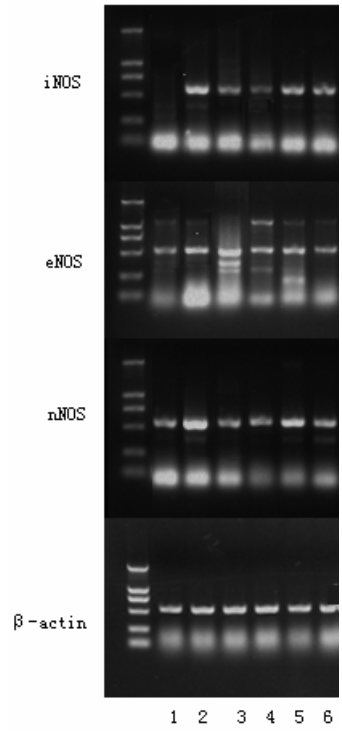

Figure 1. RT-PCR analysis of the expression of iNOS , eNOS, and nNOS RNA in rat brain. PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide. The housekeeping gene β–actin was expressed at equal levels in all samples. Lane 1: sham; lane 2: NS; lane 3: 60 mg/kg TFR; lane 4: 30 mg/kg TFR; lane 5: Nim; lane 6: EGB.

Table 5. Effect of TFR on the Changes of Three NOS Gene Expression in Rat Brain

Group	Dose (mg/kg)	eNOS/β–Actin (OD)	nNOS/β–Actin (OD)	iNOS/β–Actin (OD)
NS	—	$0.51 \pm 0.16^{\#}$	$1.14 \pm 0.12^{\#\#}$	1.13 ± 0.15
Sham	—	0.32 ± 0.11	0.59 ± 0.05	0.00 ± 0.00
TFR	60	$0.75 \pm 0.22^{*}$	$0.85 \pm 0.14^{*}$	$0.77 \pm 0.17^{**}$
	30	$0.67 \pm 0.25^{*}$	$0.91 \pm 0.15^{*}$	$0.81 \pm 0.22^{*}$
Nim	10	$0.77 \pm 0.18^{*}$	0.93 ± 0.26	$0.81 \pm 0.08^{**}$
GEB	80	0.62 ± 0.12	$0.87 \pm 0.18^{*}$	$0.83 \pm 0.16^{*}$

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NS control. Each group consists of 6 rats in eNOS, 5 rats in iNOS, and 4 rats in nNOS, values are the mean ± SD.

Discussion

In the present study, we have found that: 1) TFA has protective effects on cerebral ischemia reperfusion injury; 2) The neuroprotective effect of TFA is involved in scavenging oxygen free radicals; 3) The regulating of NO release and expression of NOS isoforms mediates the neuroprotective effect of TFA.

It was observed that 30, 60 and 120 mg/kg TFR markedly reduced the neurological score in mouse. This suggested that TFR could improve mouse neurological disorder induced by cerebral ischemia-reperfusion. Our data also demonstrated that 15, 30 and 60 mg/kg of TFR markedly reduced ischemia reperfusion-induced cerebral infarct volume in rat. LDH serves as a very important metabolic enzyme in brain cells and is released into the blood stream from injured brain cells. Thus, LDH level in serum or brain cell is a reliable index to evaluate cerebral ischemic injury. Our result showed that treatment with TFR significantly decreased the LDH activity in mouse blood serum and increased it in rat cerebrum. Aforementioned results indicated that TFR had a significant protective effect against cerebral ischemia reperfusion injuries.

There is evidence that excess of oxygen free radicals are generated and released during cerebral ischemic reperfusion injury, which react with lipids, proteins and nucleic acids, resulting in the peroxidization of lipids and impairing of cell functions (Schmidley, 1990; Evans, 1993). The level of MDA reflects the production of oxygen free radicals. SOD, one of free radical scavenging enzymes, can scavenge oxygen free radicals originating from active oxygen, protect cell membrane structure from the damage of oxygen free radicals, and serve as an important index of anti-oxidatization (Satoh *et al*., 2002; Mizuno *et al*., 1998). GPX is another important free radical scavenging enzyme that performs several vital functions; it not only functions by removing H_2O_2 formed after the SOD-catalyzed dismutation reaction but also detoxifies the lipid hydroperoxides (Love, 1999; Chan, 1996). Our results showed that TFR significantly increased the activities of SOD and GPX, and reduced the level of MDA in rat cerebrum. It suggested that TFR could improve the oxygen free radicals scavenging ability, which may be one of the mechanisms for the neuroprotective effect of TFA on cerebral ischemia perfusion injury.

Nitric oxide is a transient gaseous second messenger molecule functioning in vascular regulation, immunity, and neurotransmission. NO levels are associated directly with the

development of brain injury in strokes and other neuropathological disorders in humans (Naka *et al*., 2000; Veltkamp *et al*., 2002). Our results showed that cerebral ischemia reperfusion significantly induced the increases of total NO levels in both rat cerebrum and mouse serum. However, NO content markedly decreased in brain tissues of rats after treatment with TFR at doses of 15, 30 and 60 mg/kg and in serum of mice at doses of 30, 60 and 120 mg/kg, indicating the decrease of NO level may be, at least partially, involved in the protective effect of TFR against cerebral ischemia reperfusion.

Following acute ischemic or hypoxic injury to the brain, over-entry of Ca^{2+} into cells causes the activation of nitric oxide synthase (NOS), which catalyzes an enzymatic reaction, leading to the synthesis of nitric oxide (Yan *et al.*, 2004; Dimmeler *et al*., 2000; Zollner *et al*., 2000). Three distinct NOS isoforms have been identified, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO can be neuroprotective or neurotoxic during cerebral ischemia, depending on the NOS isoform involved. eNOS produces NO with beneficial effects for promoting collateral circulation and microvascular flow (Moncada *et al*., 1991), whereas NO overproduction by nNOS or iNOS during ischemia is cytotoxic by disrupting DNA mitochondrial respiration due to inhibition of aconitase and the mitochondrial electron transport complex I and II (Dawson *et al*., 1992; Knowles *et al*., 1990; Gross *et al*., 1995). Based on these findings, it was supposed that the NO-synthases could be attractive targets for treating cerebral ischemia-induced neuronal damage (Gajkowska *et al*., 1999; Santizo *et al*., 2000). In view of the detrimental and beneficial roles of NOS isoforms in ischemic brain injury, further investigations about the effect of TFR on the expression of NOS isoforms in rat cortex was performed in the present study. We found that expression of 3 NOS isoforms in rat cortex markedly increased after cerebral ischemia reperfusion in the control group, a similar finding as previous studies (Nakatomi *et al*., 2002; Raber *et al*., 2004; Zhu *et al*., 2003). Treatment with TFR (30 or 60 mg/kg) markedly decrease iNOS and nNOS mRNAs expression, whereas increase eNOS mRNA expression. These results suggest that TFR offered protection against ischemia-reperfusion injury by modulating iNOS, nNOS or eNOS expressions.

In summary, the present study was the first to show that TFR has protective effects on cerebral ischemia reperfusion injuries through scavenging oxygen free radicals and regulating the NO release and expression of NOS isoforms.

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