Oral administration of *Crataegus* flavonoids protects against ischemia/reperfusion brain damage in gerbils

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Abstract

Stroke is the third leading cause of death as dementia is a main symptom of Alzheimer’s disease. One of the important mechanisms in the pathogeny of stroke is free radical production during the reperfusion period, therefore the effects of a type of natural antioxidant, i.e. *Crataegus* flavonoids (CF), on brain ischemic insults were investigated in Mongolian gerbil stroke model. Results showed that pretreatment of the animals with CF decreased reactive oxygen species (ROS) production, thiobarbituric acid reactive substances content, and nitrite/nitrate concentration in brain homogenate, increased the brain homogenate-associated antioxidant level in a dose-dependent manner. CF pretreatment increased the amount of biologically available NO by scavenging of superoxide anion produced during reperfusion. At same time, in the process of ischemia/reperfusion brain damage, the content of nitrite/nitrate (the end product of NO) increased, and of NO detected by ESR decreased. Oral pretreatment with CF decreased the nitrite/nitrate content in the brain homogenate and increased the biologically available NO concentration in a dose-dependent manner. The increasing effect of antioxidant on NO might be due to its scavenging effect on superoxide anion, which could react with NO into peroxynitrite. iNOS was implied in delayed neuron death after brain ischemic damage and it was found that pretreatment with CF could decrease the protein level of tumor necrosis factor (TNF)-α and nuclear factor-kappa B (NF-κB), and increase the mRNA level of NOS estimated by western blotting and RT-PCR. More neurons survived and fewer cells suffered apoptosis in the hippocampal CA1 region of CF treated animal brain. These results suggest that oral administration of this antioxidant increases the antioxidant level in the brain and protects the brain against delayed cell death caused by ischemia/reperfusion injury.

Keywords: *Crataegus* flavonoid, ischemia/reperfusion brain damage, natural antioxidant, nitric oxide, reactive oxygen species.


Stroke is a major cause of death and disability in the world; it is the third leading cause of death and the primary cause of long-term disability in adult. Therefore, there is a great interest in the basic mechanisms by which ischemia/reperfusion (IR) cause damage and in the prevention of the disease. Reactive oxygen species (ROS) generated from the respiratory chain in mitochondria, ischemia-activated xanthine/hypoxtanthine oxidase and lipid fatty acid metabolism play an important role in the brain IR process (Piantadosi and Zhang 1996; Kuehl and Egan 1980). Due to the high rate of oxidative metabolic activity, high content of polyunsaturated fatty acids, relatively low antioxidant capacity, low repair activity and non-replicating nature of the neuronal cells, the brain is very susceptible to the damage caused by oxygen radicals (Traystman et al. 1991). The burst in production of ROS results in damage to cellular proteins, lipids and DNA. In the cerebral circulation system, the burst in production of ROS damages the endothelium cell and smooth muscle cell, induces blood platelet aggregation and vascular permeability.
changes, and results in edema (Matsuo et al. 1996; Chan et al. 1984).

Crataegus (hawthorn) is one of the oldest medicinal plants and is described by many pharmacopoeias. Crataegus extract (from leaves with flowers) has been used to treat the early stages of congestive heart failure and angina pectoris (Schmidt et al. 1994). Pharmacological studies also demonstrate that Crataegus extract (from fruit and flowers) can decrease the level of cholesterol in serum (Von Eiff 1994) and inhibit platelet conglomeration (Rogers et al. 2000). Evidences show that Crataegus extract (from several parts of the plant including leaves) has antioxidant effects in vitro or in vivo, Crataegus extract scavenges superoxide anion, hydroxyl radical, and hydrogen peroxides, and inhibits lipid peroxidation (Bahorun et al. 1994; Bahorun et al. 1996).

In vivo experiments show that Crataegus extract (from fruit) increases the concentration of α-tocopherol and inhibits the oxidation of human low-density lipoprotein (LDL) (Zhang et al. 2001a). Clinical studies indicate that its antioxidant activity is the mechanism of Crataegus extraction’s cardioprotective benefit (Miller 1998). Though Crataegus had extensive application in the clinic to treat heart-related diseases, no reports about the effects of Crataegus extract on brain related diseases were reported.

Crataegus flavonoids (CF) used in this study is a standard product, extracted from the leaves of the Crataegus Pinnalifida Bge (hawthorn), contained 62.35 ± 3.79% of the total flavonoids, and 27.2 ± 2.93% of proanthocyanidine as measured by HPLC (Chang et al. 2001; Chang et al. 2002). To evaluate the protective effect of CF on central nervous system injury, oral administration of this antioxidant was investigated in Mongolian gerbil stroke model, IR damage.

Materials and methods

Animals and treatments

All animal used in the procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were accepted by the local animal care committee. Male Mongolian gerbils (50–70 g) were housed one per cage under standard conditions (23 ± 1°C and lights on 8 : 00–20 : 00 h) with ad libitum access to food and water. The animals were randomly divided into four groups: the IR group suffered 5 min ischemia after treatment with proper drinking water for 15 days; the sham group suffered the same treatment as the IR group except without arterial occlusion. The low- and high-dose group suffered the same treatment as IR group except that drinking water contained 0.5 mg/mL and 2.5 mg/mL CF, respectively. The amount of water drunk by the animals was determined every day to calculate the amount of drug actually taken by the gerbils. The body weights of the gerbils were determined at the beginning and end of the experiments.

The ischemic operations were performed after the gerbils were anesthetized with ethyl carbamate (1300 mg/mL, intraperitoneal). In the supine position, a midline ventral incision was made in the neck. Both common carotid arteries were exposed, separated carefully from the vagus nerve, and occluded for 5 min with aneurysm clips. This procedure consistently resulted in delayed neuronal death in the CA1 region of the hippocampus (Kirino 1982). The body temperature was monitored with a rectal probe and an incandescent lamp during the operation and in the following 5 h to maintain a body temperature of 37 ± 0.5°C.

One hour after the operation, a number of the anesthetized animals was decapitated. The brain was removed and rinsed with ice-cold physiological saline, then homogenized in 5 : 1 (v/w) ice-cold 0.1 mol/L phosphate buffer pH 7.4. The homogenates were used to investigate the lipid peroxidation, nitrite/nitrate content and the homogenate associated antioxidant level.

Assays for thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was assessed by measuring the TBARS content in the brain homogenate according to previous methods (Ohkawa et al. 1979). An aliquot (100 μL) of the homogenate was added to a reaction mixture containing 100 μL of 8.1% sodium dodecyl sulfate (SDS), 750 μL of 20% acetic acid (pH 3.5), 750 μL of 0.8% thiobarbituric acid and 300 μL distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 4000 × g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm. The TBARS content was expressed as nmol/mg tissue protein.

Nitrite/nitrate content

The nitrite/nitrate level of brain homogenate, end products of NO, were measured as previously described (Grisham et al. 1995). Briefly, an aliquot (100 μL) of the homogenate was first incubated with nitrate reductase (0.2 U/mL), FAD (0.005 mM), and NADPH (0.1 mM) in a total volume of 500 μL at 37°C for 30 min to reduce nitrate to nitrite. Then 5 μL of lactic dehydrogenase (1500 U/mL) and 50 μL of 100 mM pyruvic acid were added and incubated at 37°C for 10 min to oxidize any unreacted NADPH. The nitrite concentration in the samples was then measured by Griess reaction, by adding 0.5 mL 0.2% naphthylethylenediamine and 0.5 mL 2% sulfanilamide in 5% concentrated phosphate buffer. After 10-min incubation at room temperature, the absorbance was determined at 543 nm.

Brain homogenate associated antioxidant level

The scavenging ability of brain homogenates on superoxide anion and hydroxyl radical were assessed by ESR and spin trapping method using DMPO (5,5-dimethyl-1-pyrroline-n-oxide) as spin trapping reagents.

Superoxide anion scavenging ability was determined in a riboflavin system containing 0.3 mM riboflavin, 5 mM EDTA, 0.2 mM DMPO, 2 mM DETAPAC and 1 μL brain homogenate of a 30 μL total volume at pH 7.4 in a capillaries at room temperature. 30 s after light exposure, the amount of adducts (DMPO-OH) was measured by ESR spectroscopy.

The hydroxyl radical generation system contained 0.1 mM DMPO, 0.025 mM iron(II), 0.01% H2O2, 2 μL brain homogenate of a 50 μL total volume of phosphate-buffered saline (PBS) solution at pH 7.4 in a capillaries at room temperature. Seventy seconds after the solution was mixed, the amount of DMPO-OH was measured by ESR spectroscopy. The homogenate that could scavenge more free radicals was processed as a more potent antioxidant.
radicals had a higher antioxidant level in riboflavin and Fenton reaction systems.

**ROS and NO detection**

One hour after the 5 min IR insult, some of the anesthetized animals were decapitated, and the brain was removed, washed with ice-cold physiological saline, and then dissected along the middle line. The left hemisphere was used to detect ROS according to a previously published method with minor modifications (Capani et al. 2001). Briefly, the hemisphere was weighed and homogenized in 1 mL of a spin trap solution containing 100 mM of N-tert-butyl-α-phenylnitro-rone (PBN) and 2 mM diethylenetriamine-pentacetic acid (DPTA) in ice-cold PBS. Then 500 μL of ethyl acetate were added, vortexed for 30 s and centrifuged at 10 000 × g for 5 min. The ethyl acetate phase was transferred to a quartz cell for ESR study at room temperature.

The right brain was used to detect the NO production as previously described with minor modifications (Zhang et al. 2001b), sample preparation was similar with that in detecting ROS except the trapping solution contained 10 mM diethyldithiocarbamate (DETC), 1 mM sulfate iron(II) and 5 mM hyposulfite.

**ESR conditions**

The heights of the second peak from the ESR spectra of DMPO and PBN spin adducts were measured. For the NO signal, the heights of the three peaks were measured and their total was used to analyze the signal intensity. This approach proved to be more precise than previously described (Shen et al. 2000). The Bruker ER-200 X-band ESR spectrometer settings for trapping oxygen radicals were as follows: center field, 3445 G; scan range, 200 G; microwave frequency, 9.47 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 1 G. The typical ESR conditions for detecting NO was as above except for the scan width (400 G), and modulation amplitude (3.2 G).

**Nissl staining and TUNEL experiments**

Six days after IR insult, some of the animals were anesthetized deeply with sodium pentobarbital (50 mg intraperitoneal) and perfused transcardially with phosphate buffer for 10 min followed by phosphate-buffered 4% glutaraldehyde for 15 min. The brains were removed and postfixed in the phosphate-buffered 4% glutaraldehyde solution for 24 h, and then cryoprotected in 30% sucrose for 72 h. Coronal sections (12 μm) were cut at the level of the dorsal hippocampus and alternate sections were stained with cresyl violet. The number of survival neurons in CA1 pyramidal cell layer from three to four sections per animal at dorsal hippocampal level was counted by a blinded observer using a light microscopy. Only whole neurons with visible nucleus were counted. The data were expressed as surviving cell number per mm in CA1 region.

The TUNEL assay was applied to the neighboring cryostat section. The apoptotic cell was identified using a modified end-labeling technique originally described by Gavrieli et al. (1992) (in situ cell apoptosis detection kit, Wuhan Boster Biological Technology Co., Wuhan, China). The sections were rinsed twice for 5 min in Tris-buffered solution (TBS), 10 min in 0.5% peroxide of methanol at room temperature to quench endogenous peroxidase and rinsed thrice for 2 min each in distilled water. Then the sections were permeabilized with proteinase K (2 mg/mL) for 60 s and washed thrice for 2 min each with TBS. After addition of 20 μL labeling buffer, the sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) containing digoxigenin-dUTP (0.3 e.u./μL) at 37°C for 2 h in a humidified chamber, then rinsed three times for 2 min on each occasion in TBS, and incubated with blocking buffer contained bovine serum albumin for 30 min at room temperature in humidified chamber. The sections were then incubated for 30 min with anti-digoxigenin-biotin for 30 min at 37°C, and then rinsed three times for 2 min each. The sections were then incubated with avidin-peroxidase for 30 min at 37°C in humidified chamber and washed four times for 5 min each with TBS. The staining was developed with diaminobenzidine as chromogen. Two controls per assay were performed: incubating sections with DNAase I served as a positive control and omission of the terminal transferase from the reaction mixture served as a negative control.

**Electron microscopy**

The electron microscopy experiments were performed as previously described (Sewartz-Bloom et al. 2000). Briefly, 3 days after IR insult, the gerbils were killed by perfusion transcardially with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and stored in the same fixative solution overnight. A 500-μm block (measured along the pyramidal cell body layer) of area CA1 was dissected and fixed in buffered 4% glutaraldehyde for an additional 24 h. Samples were postfixed in 2% (w/v) OsO4/1% (w/v) KFCN of cacodylate buffer and stained en bloc with 1% (w/v) uranyl acetate. After dehydration in ethanol, slices were embedded in Spurr resin. A section (0.5 mm) was stained with toluidine blue to locate areas of interest. Subsequently, 60–70 nm sections were cut and stained with uranyl acetate and Sato lead stain. The sections were examined with a JEM 100CX electron microscope.

**RT-PCR determination of mRNA level of iNOS**

Total RNA was isolated from hippocampus of the animals using RNA isolation Kit (Gibcol BRL), and the mRNA level of iNOS was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis as described previously (Poynter and Daynes 1998). An aliquot of 1 μg of total RNA from each sample was reverse transcribed to cDNA using a First-Strand cDNA Synthesis Kit (Gibcol BRL). A standard 25 μL polymerase chain reaction (PCR) contained 1 μL of the reverse transcribe reaction, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl2, 1 × assay buffer B, 1.5 U Taq polymerase (Promega, Madison, WI, USA), 30 pmol forward primer, 30 pmol reverse primer (Life Technologies, Rockville, MD, USA). The primers of iNOS were designed according to the published sequences: 5′-TTT GAC CAG ACC CAG AG-3′ (upstream) and 5′-TTG GTG GCA AAG ATG AGC TC-3′ (downstream) which correspond to 366–385 and 547–566, respectively, in iNOS cDNA bank. The primers of β-actin were used as an internal standard to normalize the results. Aliquots (10 μL) of the PCR reaction were electrophoresed on a 1.4% agarose gel. PCR products were visualized with ethidium bromide. Amplification of each gene yielded a unique band of the expected size (β-actin 545 bp).

**Western blots**

The hippocampus was homogenized on ice in buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02 mM Na2, 100 μg/mL phenylmethylsulfonyl

fluoride, 1 μg/mL Aprotinin and 1% Triton X-100) at 0°C for 30 min. The lysates were then centrifuged at 12 000 × g for 25 min at 4°C. The supernatant were used for SDS-PAGE and protein content was estimated by the method of Bradford. About 30 μg total proteins were loaded. Proteins were separated on 15% polyacrylamide gels and transferred to a nitrocellulose membrane. Blots were blocked in a blocking buffer, containing 5% bovine serum, 0.1% Tween 20 in 0.1 M PBS (pH 7.4), and incubated with primary antibody [rabbit anti-NF-κB p65 and rabbit anti-TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively]. Consequently the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1 : 5000) for 1 h at room temperature with constant agitation, then washed and stained with 3, 3′-diaminobenzidine tetrahydrochloride.

Statistical analysis
Data were expressed as means ± SD. The data were statistically evaluated using the Microsoft origin program. The Student’s t-test and one-way analysis of variance (ANOVA) were used to analyze the differences between two groups and different groups, respectively, and p-values were considered significant when p < 0.05.

Results
Free radicals trapped in the brain homogenate after IR
As measured by the method of the ESR signal intensities, 1 h following 5 min ischemia, ROS trapped by PBN in the brain homogenate significantly increased by about 36.89% in the IR group comparing with the sham group (Fig. 1, Table 1). ROS significantly decreased by about 17.37 and 31.14%, respectively, in low- and high-dose groups pretreated with CF for 15 days compared with the IR group. ROS in the high-dose group were even lower than that in the sham group.

Nitric oxide (NO) trapped by the diethyldithiocarbamate (DETC)–iron(II) complex significantly decreased by about 19.17% in the IR group compared with the sham group (Fig. 2, Table 1). Compared with the IR group, NO significantly increased about 44.67% and 77.56% in low- and high-dose groups pretreated with CF for 15 days, respectively. Those groups were also significantly higher than the concentration of the sham group.

TBARS and nitrite/nitrate content in the brain homogenate
IR insult significantly increased the TBARS content in the brain homogenate about 74.04% compared with that of the sham group. While pretreatment with CF decreased the elevation of lipid peroxidation caused by IR in a dose-dependent manner (Table 1), a decrease of 24.25 and 47.39% was observed in low- and high-dose groups, respectively. The TBARS level in the high-dose group was even lower than that in the sham group.

![Fig. 1 ESR spectra of ROS trapped by PBN in brain homogenates 1-h after 5-min ischemic damage. Spectra are from (a) sham group; (b) IR group; (c) low-; and (d) high-dose group, respectively.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>ROS (au)</th>
<th>TBARSs (nmol/ mg/tissue proteins)</th>
<th>Superoxide signal (au)</th>
<th>Hydroxyl signal (au)</th>
<th>NO(au)</th>
<th>Nitrite/nitrate (µM)</th>
<th>Cell viability (cells/mm)</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>12.2 ± 1.5</td>
<td>0.782 ± 0.131</td>
<td>10.4 ± 1.1</td>
<td>18.5 ± 2.7</td>
<td>19.3 ± 2.2</td>
<td>20.301 ± 1.314</td>
<td>270 ± 30</td>
</tr>
<tr>
<td>IR</td>
<td>16.7 ± 0.7†</td>
<td>1.361 ± 0.130†</td>
<td>14.1 ± 2.1†</td>
<td>24.0 ± 5.0†</td>
<td>15.6 ± 1.3†</td>
<td>45.111 ± 4.970†</td>
<td>12 ± 7†</td>
</tr>
<tr>
<td>Low dose</td>
<td>13.0 ± 0.8†</td>
<td>1.031 ± 0.185†</td>
<td>11.1 ± 1.8</td>
<td>21.7 ± 3.4†</td>
<td>22.1 ± 1.0†</td>
<td>36.028 ± 1.820†</td>
<td>129 ± 64†</td>
</tr>
<tr>
<td>High dose</td>
<td>11.5 ± 0.9*</td>
<td>0.716 ± 0.137*</td>
<td>7.5 ± 2.1†</td>
<td>16.8 ± 4.5*</td>
<td>27.7 ± 6.7*</td>
<td>29.499 ± 2.209†</td>
<td>254 ± 35*</td>
</tr>
</tbody>
</table>

Each group represents the mean ± SD of six-nine animals. *p ≤ 0.05 versus IR; †p < 0.05 versus sham. The percentage is compared with the sham group, except those marked with †, which are compared with the IR group. *au, any unit.
The nitrite/nitrate content in the brain homogenate was determined using the Griess method (Grisham et al. 1995). The IR insult significantly increased the nitrite/nitrate content by about 122.21%, and pretreatment with CF significantly decreased the elevation of nitrite/nitrate content by about 20.13 and 34.61% in low- and high-dose groups, respectively. The antioxidant level from the high-dose group was even significantly higher than that of the sham group.

The group subjected to IR had weaker hydroxyl radical scavenging ability than that of sham group about 29% ($p < 0.05$) (Table 1). Comparing with the IR group, pretreatment with CF increased the scavenging ability of the hydroxyl radical by about 9.5% for low-dose ($p > 0.05$) and 30% for high-dose ($p < 0.05$), respectively. The antioxidant level of the high-dose group was even significantly higher than that of the sham group.

**Survival of pyramidal cells in the CA1 area of hippocampus accessed by Nissl staining**

The CA1 region of the hippocampus is susceptible to transient ischemic insults. We assessed cell survival in the CA1 region of the hippocampus with Nissl staining. Figure 3 represents the photographs of coronal sections containing the hippocampal CA1 region obtained 6 days after IR insult. In the CA1 region from the IR group, the pyramidal cells have almost completely disappeared as shown in Fig. 3(b) and Table 1. There were $12 \pm 7$ cells/mm in the CA1 region from the IR group, while there were $270 \pm 30$ cells/mm in the CA1 region from the sham group. In contrast to the IR group, pretreatment with CF significantly increased the number of survival pyramidal cells in the CA1 region (Fig. 3c,d) in a dose-dependent fashion ($129 \pm 64$ cells/mm for low-dose.
group, 254 ± 35 cells/mm for high-dose group in Table 1). There was no significant difference between the sham group and high-dose group.

DNA damage in the CA1 area of hippocampus accessed by TUNEL
The pyramidal cell of the CA1 region from the sham group was TUNEL-negative (Fig. 4a), while most of the pyramidal cells of CA1 region from IR group were TUNEL-positive (Fig. 4b). As shown in Fig. 4(b), the neuronal dendrites of some pyramidal cells were TUNEL-positive stained, which suggested the transportation of the fragmented DNA from nuclei to neuronal fiber terminals, and apoptosis accompanied in the transient ischemic neuronal damage (Hara et al. 1999). Pretreatment with CF decreased the TUNEL-positive cell in a concentration-dependent manner (Fig. 4c,d).

IR damage assessed by transmission electron microscopy
The effect of transient ischemic insult was also examined by transmission electron microscopy (TEM) after ischemic insults for 3 days. As shown in Fig. 5(a), the nuclei of the pyramidal cells in the CA1 region from the sham group are normal, and DNA is dispersed evenly throughout the nucleus. While most of the nucleus of the corresponding region from the IR group shrunk, and the DNA condensed around the nucleus membrane (Fig. 5b). Some of the pyramidal cells of the CA1 region from the low-dose group suffered the same process as IR group, i.e. nuclei shrunk and the DNA condensed. Some of the cells are better, with the DNA evenly distributed in the nucleus [except in the rough endoplasmic reticulum connecting with the nuclear membrane swells as shown in Fig. 5c]. The cells from the high-dose group are similar to those of the sham group (Fig. 5d), i.e. DNA is distributed evenly throughout the nucleus. The results suggest that pretreatment with CF protects the pyramidal cells in the CA1 region from IR damage.

Effect of CF on mRNA levels of iNOS
A single band was detected in RT-PCR products from RNA of the iNOS (Fig. 6). It can be found that levels of mRNA in the sham group are very weak; IR enhanced the levels and pretreatment with CF decreased the levels in hippocampus. This suggests that enhanced NO formation in the hippocampus after IR may be due to enhanced iNOS and that the decreased levels of radicals in the pretreated animal may come partly from the inhibitory effect of CF on iNOS.

Effect of CF on expression of NF-κB p65 and TNF-α measured by western blot
As shown in Figs 7 and 8, it can be found that the expression of NF-κB p65 is enhanced by about 75% by IR, and that
pretreatment of CF decreased the expression in a dose-dependent manner. Similar results were toward for TNF-α, the enhancement of TNF-α reached about 50% and pretreatment by CF significantly decreased the expression of TNF-α especially in the higher dose group.

**Discussion**

This study has shown that a natural antioxidant, CF, has protective effect on the transient brain IR insult, probably through an ROS-NO and TNF-α-NF-κB related pathway.

The pronounced protective effect of CF suggests that this ancient medicine usually used for healing heart failure also has clinical value for treating the brain-related diseases.

The drinking water and animal body weight were controlled during the experiments. Calculated from the water intake, the actual doses taken by the animals were 32.5 ± 10.0 mg/kg/day, and 167.0 ± 49.0 mg/kg/day, respectively, in the low- and high-dose group. This is lower than previously reported, in which either 2% in diet or 600 mg/kg/day were used (Fehri et al. 1991; Al Makdessi et al. 1999). There were few side-effects, the maximal tolerated dose in the mouse was 13.6 g/kg/day (Shanxi Gold Armature Pharmaceutical Co. Ltd, Shaxi, China), and therefore the dose used in this study was safe. There were no significant differences in the changes of body weight among these four groups. In addition, there were no side-effects observed after treatment with CF for 15 days.

After pretreatment with CF for 15 days, compared with the IR group, the number of surviving neurons significantly increased by about 43.34 and 89.63% in low- (32.5 ± 10.0 mg/kg/day) and high-dose (167.0 ± 49.0 mg/kg/day) groups, respectively. Cell apoptosis were verified by the TUNEL and TEM experiments. As showed by TUNEL (Fig. 4), apoptotic cell decreased in a concentration dependent manner in the CF pretreated group compared with the IR group. The result of the TEM experiments also supported the protective effect of CF on ischemic insult (Fig. 5). It was also found that pretreatment with CF for 15 days decreased ROS production, inhibited the TBARS generation, and improved the brain homogenate associated antioxidant level in a dose-dependent manner. These results are consistent with the antioxidant activity of CF previously reported in vitro or in vivo (Bahorun et al. 1996; Bahorun et al. 1994), and suggest that CF could cross the blood–brain barrier and function as an antioxidant with which to treat the brain reactive oxygen insult.

NO is also involved in the mechanism of brain ischemic insult (Iadecola 1997). The results (Table 1) showed different trends after IR and pretreatment with CF. Nitrite/nitrate content increased after IR operation and was decreased by pretreatment with CF, while NO decreased by IR and increased by pretreatment with CF in a concentration dependent manner. NO is an unstable free radical, and rapidly reacts with the superoxide anion to form the very toxic product, peroxynitrite, which further decomposes into a hydroxyl radical. Peroxynitrite and hydroxyl radicals are the most highly toxic ROS in living body (Beckman and Koppenol 1996). Under ischemic conditions when the L-arginine concentration was rate limiting, the electron transfer became uncoupled from NO production, NOS also produced a mixture of superoxide anion and NO that directly reacted to form peroxynitrite and resulted in cytotoxicity (Xia and Zweier 1997). Because the formation of ROS increased in the ischemic insult (Fig. 1), more superoxide anion was...
available to react with NO to form peroxynitrite. The available NO trapped by the DETC–iron(II) complex decreases as shown in Table 1. Pretreatment with CF decreased the available ROS that might react with NO, as a result the nitrite/nitrate contents decreased, while NO trapped by DETC–iron(II) increased (as shown in Table 1). The role of NO in the mechanism of IR brain damage has been debated (Iadecola 1997). However, there is no doubt at present that the toxic reactions of NO resulted in part from its rapid reaction with superoxide anion, leading to peroxynitrite and peroxynitrous acid formation (Beckman and Koppenol 1996). These reactive species initiate lipid peroxidation, oxidize protein and non-protein sulphydryls, hydroxylate and nitrate aromatic compounds. Our results suggest that part of the protective effect of CF on ischemic insult might be through inhibiting the formation of peroxynitrite (Shutenko et al. 1999). They found that the NO levels in ischemic brain were increased by treatment with quercetin, but they did not measure nitrite/nitrate content. In some literature, nitrite/nitrate content was directly expressed as the NO production, however, ROS, nitrite/nitrate and NO should all be considered in this kind of study. NO functions as endothelium-derived relaxing factor (EDRF) in the circulatory system after pretreatment with CF, thus improving levels of NO derived relaxing factor (EDRF) in the circulatory system. NO and mRNA level of iNOS, indicating that the protecting effect of CF on the brain against IR damage may pass through the regulation of the pathway of NF-κB and TNF-α.

It has been observed that Crataegus extract can inhibit platelet aggregation and 5-hydroxytryptamine (5-HT) release (Rogers et al. 2000), can decrease LDH levels (Al Makdessi et al. 1999), and can suppress the inflammatory response (Holubarsch et al. 2000). Other than ROS and nitric oxide, one or more other mechanisms might function in the protective effects of Crataegus extract on IR insult.

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References


