Chrysophanol Liposome Preconditioning Protects against Cerebral Ischemia-reperfusion Injury by Inhibiting Oxidative Stress and Apoptosis in Mice

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Abstract: Previous studies have indicated that Chrysophanol Liposomes (Chr-lip) have demonstrated potent neuroprotective effects against Cerebral Ischemia Reperfusion (CIR) injury but supporting evidence at molecular mechanisms is lacking. Two hundred health Kunming male mice were randomly divided into the sham group, model group and Chr-lip treatment (10, 5, and 0.5 mg kg⁻¹) groups. Chr-lip treatment groups intraperitoneally injected with Chr-lip for three successive days, then subjected to brain ischemia induced by MCAO. After reperfusion 24 h, neurological deficits, brain water content, neuronal ultrastructure, histopathological changes, oxidative stress-related biochemical parameters, neuronal apoptosis and apoptosis-related proteins were assessed by the methods of HE staining, Hoechst33258 staining, kits, western blot and real-time quantitative PCR. Chr-lip significantly improved neurological deficits (p<0.05), neuronal ultrastructure and histological deficits, reduced brain water content (p<0.05) and decreased apoptotic cells (p<0.05) after cerebral ischemia. Chr-lip also markedly reduced the level of malondialdehyde (MDA) and enhanced the activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in CIR mice (p<0.05). Further investigation demonstrated that Chr-lip remarkably promoted the expression of Bcl-2 and inhibited the expression of Bax, Cytochrome C (Cyt-C) and Caspase-3 at protein and mRNA levels (p<0.05), respectively. These findings indicate that Chr-lip has protective effects against CIR injury in mice and the neuroprotective effects may be attributed to attenuating oxidative stress and inhibiting neuronal apoptosis.

Key words: Chrysophanol liposomes, oxidative stress, apoptosis

INTRODUCTION

Cerebral ischemia is one of the leading causes of disability and human death across the world and its incidence is speculated to rise with the increase in the number of the aging population (Wang et al., 2012a). There are several pathological mechanisms in the process of cerebral ischemic such as excessive release of excitatory amino acids (Wang et al., 2013a), loss of ionic homeostasis (Shah and Abbruscato, 2013), energy failure (Ten and Starkov, 2012), inflammatory response (Liu et al., 2014), increased oxidative stress (Yan et al., 2014) and apoptosis (Guan et al., 2013). These mechanisms eventually cause irreversible damage of the brain tissue. So recent years, chemical drugs such as calcium ion antagonist and radical scavengers as well as neuroprotective agents have been used for the treatment of CIR injury. However, side effects such as resistance to drugs, cerebral hemorrhage and gastrointestinal irritation may exceed the clinical benefits for long-term therapy (Chen et al., 2014). Furthermore, chemical drugs are generally difficult to achieve significant therapeutic results. Fortunately, clinical applications and experimental reports of traditional Chinese medicines against CIR injury have been descendant (Qi et al., 2009; Zhao et al., 2010; He et al., 2012).

Chrysophanol (Chry), an important anthraquinone compound, come from the dry roots of Rheum palmatum. Whose chemical structure is made of anthraquinone rings (Fig. 1) and it can scavenge O₂⁻ and diphenylpicrylhydrazyl (DPPH) free radicals (Iizuka et al., 2004). Numerous studies have demonstrated that Chry possesses anti-allergic (Kim et al., 2000), antifungal (Agarwal et al., 2000), anti-tumor (Shi et al., 2001) and anti-apoptotic properties (Chen et al., 2002). Our previous studies demonstrated that Chr-lip had the obvious bioavailability and could improve passive avoidance learning and memory after CIR in mice than Chry monomer

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experiments and care were performed in accordance with the procedure approved by the Animal Ethics Committee of the Hebei North University (Chinese License Key Number: SCXK (Ji) 2004-0001). This study was approved by the Local Ethics Committee in May 2012.

**Separation and purification of chrysophanol by HPLC:**
Degreased anthraquinone glycosides were hydrolyzed with dilute sulfuric acid in rhubarb. We extracted aglycone by hot chloroform. Impurity was removed from extracting solution accordingly using different alkaline solution such as rheemic acid and arsenic acid. Chry was precipitated through sodium hydroxide with hydrochloric acid. The precipitate was dried in vacuo. PHPLC (PHPLC, Agilent, USA and California) method was established for purifying the substances of Chry. The column was ZORBAX SB-C18: (21.2 × 250 mm, 7 μm) with a mixture of methanol-0.01% phosphoric acid (85:15) solution as the mobile phase, at a flow rate of 20 mL min⁻¹. The column temperature was at 28°C. The detection wavelength was 254 nm. Fractions were collected based on the peak, the threshold being Min: 2.2. Both Nuclear Magnetic Resonance (NMR) and HPLC were used for the structure and quantitative analysis of Chry. HPLC (Agilent, USA, California) method was established for quantification of Chry. The column was Hypersil ODS2 (4.6×150 mm, 5 μm), the column temperature was at 35°C, the injection volume was 20 μL, the mobile phase was methanol-0.1% phosphoric acid (85:15), the flow rate was 1 mL min⁻¹, the detection wavelength was 254 nm.

**Preparations of Chr-lip:** According to the previous study (Wang et al., 2011) the optimized preparation conditions were as follows: lecithin (30.0 mg), cholesterol (10.0 mg) and Chry (3.0 mg), vitamin E (10.0 mg), PEG2000, dehydrated alcohol (10 mL), Tris damping fluid (10 mL), hydration time was 50 min and hydration temperature was 45°C. The organic solvent was completely evaporated and shaken for 2 h at 40°C. Finally, we obtained high, middle and low-doses of Chr-lip (10.0, 5.0, 0.5 mg kg⁻¹). Chr-lip permeability was 2.8 stored for 15 days at 4°C.

**Surgical procedure:** Mice (n = 40 per group) were anesthetized by ip 3.5% chloral hydrate (10 mL kg⁻¹). Body temperature was regulated at 37°C by homoiothermy bench. The model was similar to that described previously (Wang et al., 2012b). After the skin incision, the External Carotid Artery (ECA), the left Common Carotid Artery (CCA) and the Internal Carotid Artery (ICA) were carefully exposed and dissected away from adjacent nerves. Microvascular aneurysm clips were applied to the ICA and the left CCA. A coated filament...
was introduced into an arteriotomy hole, fed distally into the ICA and advanced 10 mm from the carotid bifurcation. The ICA clamp was removed and focal cerebral ischemia started. After ischemia for 2 h, the filament was gently removed. The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued and the animals were returned to the pre-warmed cages. Sham group underwent the same surgery but not subjected to ischemia-reperfusion.

**Experimental groups:** Following successful surgery the mice were divided into model group, high, middle and low-dose treated group. In the randomized and controlled animal experiment, mice were divided into the sham, model and Chr-lip treatment (10.0, 5.0, 0.5 mg kg⁻¹) groups. Chr-lip was i.p. once a day for three days before ischemia and once at 2 h before the onset of ischemia. While the sham and model groups were given the same volume of saline (mL 200 g⁻¹). Animals were sacrificed and tissues were removed for analysis after 24 h of reperfusion.

**Measurement of neurological deficits:** Neurological deficits of each mouse were determined after 24 h of reperfusion according to Clark et al. (1997) by a single experimenter who was blinded to the experimental group.

**Assessment of the brain water content:** Mice were anesthetized by an intraperitoneal injection of 3.5% chloral hydrate and then quickly perfused with normal saline and 4% paraformaldehyde in PBS. The brain was removed, fixed in 4% paraformaldehyde for 24 h, immersed overnight in 70% ethanol, dehydrated and paraffin embedded. After sectioning, tissues were deparaffinized with xylene two times for 20 min each time, dehydrated with an ethanol gradient and then rinsed with PBS. The sections were incubated with 25 mM Hoechst 33258 (Beyotime Institute of Biotechnology, Beijing, China) for 15 min at 37°C, washed with PBS, mounted onto slides using antifade mounting medium and then examined under a 90i multifunction microscope (Nikon, Tokyo, Japan).

**Western blot analysis:** Mice were anesthetized by an intraperitoneal injection of 3.5% chloral hydrate and then quickly perfused with normal saline. Brains were immediately removed and stored at -80°C until use for detecting protein expression of Bax, Bcl-2, Cyt-C and Caspase3. Protein concentrations were determined using a BCA kit. Samples loaded at equivalent amounts of total protein (25 µg) were analyzed by 12% SDS-PAGE. After separation, proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane by a semi-dry transfer method which was then incubated with 5% skim milk for 1 h at 37°C. Afterward, the PVDF membrane was incubated with the following primary antibodies: Rabbit polyclonal anti-Bax (1:200), rabbit polyclonal anti-Bcl-2 (1:200), rabbit polyclonal anti-Cyt-C (1:200), rabbit polyclonal anti-caspase3 (1:200) or rabbit polyclonal anti-β-actin (1:200) antibody (Beijing Biosynthesis Biotechnology, Beijing, China) overnight at 4°C. After washing with TBST, the PVDF membrane was incubated with the appropriate secondary antibody (goat anti-rabbit IgG/HRP 1:1500) (Beijing Biosynthesis Biotechnology) for 1 h at 37°C and developed using a DAB kit. The images were quantified using Quantity One software and β-actin served as the internal standard.
**qRT-PCR analysis:** Mice were anesthetized by an intraperitoneal injection of 3.5% chloral hydrate and then quickly perfused with normal saline. Brains were immediately removed and frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using Trizol (Tiangen Biotech Beijing Co., Beijing, China) and reverse transcribed into cDNA using the TIANscript RT Kit (Tiangen Biotech Beijing Co., Beijing, China) according to the manufacturer’s instructions. The newly synthesized cDNA was amplified using the SuperReal PreMix (SYBR Green) PCR kit (Tiangen Biotech Beijing Co., Beijing, China) and detected with the 7300 real time PCR System (ABI, Carlsbad, CA, USA). Primer sequences and PCR product lengths for various transcripts were as follows: Bax, forward 5'-TTTTTCATAGGGGTTTCTCATCCAGG-3', reverse 5'-CCATATTGTGCCTGCTACATCTC-3', 151 bp; Bcl-2, forward 5'-ACCCTCTGATTTTTCTCCACC TA-3', reverse 5'-AAATCATAGCCACACACATGG-3', 119 bp; Cyt-C, forward 5'-ATAGAGCAAACTCAGTACGAC-3', reverse 5'-TACAGACACCTCTCAAAATACCCCA-3', 120 bp; Caspase3, forward 5'-GACCTTCTGACTAATGTCTGCTG-3', reverse 5'-CGCATAGCCAAACCCACCTGAGTA-3', 126 bp; QAPDH, forward 5'-ATGACATCAAGAGGTTGCAAG-3', reverse 5'-GAAAGTGGGAATTTGCTGTTGAA-3', 112 bp. Relative quantification of transcript abundance was carried out using the following equation:

\[ F = 2^{-\left[\frac{Ct(target, test) - Ct(ref, test)}{Ct(target, calibrator) - Ct(ref, calibrator)}\right]} \]

**Statistical analysis:** Statistical analyses were performed using SPSS 10.0 (SPSS, Chicoga, IL, USA). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) and Least Significant Differences (LSD) test Results are expressed as Means±SD. The level of significance was p<0.05.

**RESULTS**

**Purity of Chry and entrapment efficiency in Chr-lip:**

The chemical formula of the product was determined to be \( \text{C}_{12} \cdot \text{H}_{20} \cdot \text{O}_{4} \). 1H-NMR (DMSO-d6): δ: 12.17 (1H, s, -OH), 12.07 (1H, s, -OH), 7.15 (1H, s, -CH=), 7.70 (1H, s, -CH=), 7.87 (1H, d, -CH=), 7.72 (1H, dd, -C6H), 7.32 (1H, dd, -C7-H), 2.51 (3H, s, Ar-CH3). The purity of Chry was 98.9%, the entrapment efficiency of Chr-lip was 88.5% (Fig. 2).

**Neurological scores and the water content of brain:** We found that the neurological scores in sham group were (1.17±0.76) and the neurological scores in model group were increased to (27.33±1.53). Pretreatment with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) significantly reduced the neurological deficits (p<0.01, p<0.01, p<0.05) (Fig. 3a).

As shown in Fig. 3b, was significantly increased in model group compared with sham group (75.05±3.121% vs. 58.67±4.04%, p<0.01). Pretreatment with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) significantly reduced the brain water content (p<0.01, p<0.01, p<0.05).

**Effects of Chr-lip on neuronal ultrastructural changes:** As shown in Fig. 4, in the sham group, chromosomes were distributed uniformly and the nuclear membrane was clearly seen. The structures of the abundant mitochondria and the Golgi apparatus were distinct. In the model group, the cell nucleus was swollen and the nuclear membrane was disrupted or disintegrated. The chromatin was marginalized and vacuoles were observed in the cytoplasm. Mitochondrial cristae and membranes had mostly disappeared while degranulation of the rough endoplasmic reticulum and apicoloplastic bodies were apparent. In the treatment groups, the chromosome distribution was relatively uniform and the nuclear membrane was clear. The mitochondria were reduced to some extent, but swelling of the rough endoplasmic reticulum was mild.

**Effect on histopathology in mice with MCAO-induced CIR injury:** As seen in Fig. 5a, the sham group showed normal neurons with no pathological change. On the other hand, in the model group, neuronal loss was severe and dying neurons showed shrunken cytoplasm and pyknotic nuclei and cellular edema (Fig. 3b). Chr-lip markedly reduced these ischemia/reperfusion induced histopathological changes (Fig. 5c-e).

**Effects of chr-lip on MDA content and antioxidant enzymes activities:** As shown in Table 1, the MDA level of model group rats was enhanced to

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol mg⁻¹ pro⁻¹)</th>
<th>SOD (U mg⁻¹ pro⁻¹)</th>
<th>GSH-Px (U mg⁻¹ pro⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.92±0.55</td>
<td>258.9±15.92</td>
<td>44.67±2.51</td>
</tr>
<tr>
<td>Model</td>
<td>6.22±1.22</td>
<td>102.8±6.97</td>
<td>23.3±1.52</td>
</tr>
<tr>
<td>Chr-lip (0.5 mg kg⁻¹)</td>
<td>4.95±0.98**</td>
<td>142.3±4.910*</td>
<td>33.3±1.15*</td>
</tr>
<tr>
<td>Chr-lip (5.0 mg kg⁻¹)</td>
<td>4.11±0.93*</td>
<td>189.9±4.688*</td>
<td>33.3±1.15*</td>
</tr>
<tr>
<td>Chr-lip (10.0 mg kg⁻¹)</td>
<td>3.44±0.97*</td>
<td>210.8±4.789**</td>
<td>37.0±2.00*</td>
</tr>
</tbody>
</table>

*p<0.01 vs. sham group; *p<0.01, **p<0.05 vs. model group
Fig. 2(a-b): (a) HPLC Chromatograms and (b) $^1$H-NMR of chrysophanol: 1 chrysophanol
Fig. 3(a-b): (a) Chr-lip preconditioning protected neurological function (n = 5-7) and (b) Reduced brain edema (n = 5 per group), following CIR. *p<0.01 compared with sham group, **p<0.01, ***p<0.05 compared with model group.

Fig. 4(a-e): Transmission electron microscopic images of cerebral neuronal ultrastructure after 24 h of reperfusion (12,000×). (a) Sham group, (b) Model group, (c) Chr-lip-treated group (0.5 mg kg⁻¹), (d) Chr-lip-treated group (5.0 mg kg⁻¹) and (e) Chr-lip-treated group (10.0 mg kg⁻¹)
6.22±1.22 nmol mg⁻¹ pro⁻¹ compared with that of sham group 0.92±0.55 nmol mg⁻¹ pro⁻¹. The treatment with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) could diminish the MDA content significantly (p<0.01, p<0.01, p<0.05).

Lower levels of SOD and GSH-Px were detected in model group (102.80±6.97 and 25.33±1.52 U mg⁻¹ pro⁻¹) compared with those of sham group (258.91±15.92 and 44.67±2.51 U mg⁻¹ pro⁻¹) (p<0.01). Treatment with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) could up-regulate the levels of SOD obviously (p<0.01 vs. model group), meanwhile, increase the GSH-Px activity (p<0.01, p<0.01, p<0.05).

Effects of chr-lip on neuronal apoptosis: Neuronal injury in the ischemic hemispheres was analyzed by Hoechst33258 staining. Apoptotic cells were sparsely detected in the sham group, while the apoptotic rate was significantly increased in the model group compared with the sham group (p<0.01). The treatment with Chr-lip
Fig. 6(a-f): Chr-lip protect neuronal cell against cerebral ischemia-induced apoptosis (magnification, 400×), (a) Sham group, (b) Model group, (c) Chr-lip-treated group (0.5 mg kg⁻¹), (d) Chr-lip-treated group (5.0 mg kg⁻¹) and (e) Chr-lip-treated group (10.0 mg kg⁻¹)

(10.0, 5.0 or 0.5 mg kg⁻¹) effectively attenuated the neuronal apoptosis caused by CIR injury, as indicated by significant reduction of apoptotic rate (p<0.01, p<0.01, p<0.05) (Fig. 6).

**Western blot analysis:** As seen in Fig. 7, the expression of Bax was significantly increased in model group compared with the sham group from 0.32±0.08 to 1.22±0.06 (p<0.01). In the treatment groups with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) group, could decrease the overexpression of Bax levels to 0.60±0.05, 0.82±0.05, 1.02±0.03 (p<0.01) compared with the model group. In the treatment groups with Chr-lip (10.0, 5.0 or 0.5 mg kg⁻¹) group, increased the Bcl-2 levels to 1.09±0.06, 0.93±0.03, 0.72±0.04 (p<0.01, p<0.01, p<0.05) compared with the model group. There was a significant exaltation of Cyt-C in model group which the relative ratio of Cyt-C/actin increased to 1.90±0.07 (p<0.01) compared with sham group (0.22±0.01). In the
Fig. 7(a-b): Western blot analyses of protein levels of Bax, Bcl-2, Cyt-C and Caspase3 (a) Western blot bands of Bax, Bcl-2, Cyt-C and Caspase3 expression in the ischemic hemispheres at 24 h after reperfusion, (b) Effect of Chr-lip (10.0, 5.0 or 0.5 mg kg^{-1}) on the Bax, Bcl-2, Cyt-C and Caspase3 expression in ischemic hemispheres cortex at 24 h after reperfusion. *p<0.01 compared with sham group; **p<0.01, ***p<0.05 compared with model group.
treatment groups with Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) group, expression of Cyt-C was decreased to 0.74±0.06, 1.37±0.04, 1.76±0.05 (p<0.01, p<0.01, p<0.05). There was a significant elevation of Caspase3 in model group which the relative ratio of Caspase3/actin increased to 1.66±0.07 (p<0.01) compared with sham group (0.17±0.01). In the treatment groups with Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) group, expression of Caspase3 was decreased to 0.69±0.04, 1.10±0.03, 1.48±0.05 (p<0.01, p<0.01, p<0.05).

**Quantitative real-time PCR (qRT-PCR) analysis:** There existed an excellent linear correlation (r = 0.993 for Bax, r = 0.995 for Bel-2, r = 0.990 for Cyt-C, r = 0.996 for caspase-3 and r = 0.997 for GAPDH) between the Ct value and the logarithm of the DNA copy number. Figure 8 showed that the mRNA expression of Bel-2 in the ischemic hemispheres was markedly decreased from 4.90±0.30 to 1.53±0.25 (p<0.01, n = 6) than that of sham group. Furthermore, administration of Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) increased the mRNA expression of Bel-2 to 3.67±0.15, 2.73±0.15, 2.17±0.49 (p<0.01, p<0.01, p<0.05). In addition, caspase-3 mRNA in the ischemic hemispheres of model group was expressed much higher from 5.80±0.26 to 0.39±0.020 (p<0.01, n = 6) compared with the sham group. However, treatment with Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) to the ischemic mice significantly decreased the mRNA expression of caspase-3 to 3.20±0.40, 4.00±0.43, 5.16±0.20 (p<0.01, p<0.01, p<0.05, n = 6), respectively, vs. the model group. Bax mRNA in the ischemic hemispheres of model group was expressed much higher from 6.90±0.36 to 0.70±0.15 (p<0.01, n = 6) compared with the sham group. However, treatment with Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) to the ischemic mice significantly decreased the mRNA expression of Bax to 3.50±0.20, 4.36±0.32, 5.90±0.40 (p<0.01, n = 6), respectively, vs. the model group. Cyt-C mRNA in the ischemic hemispheres of model group was expressed much higher from 3.40±0.30 to 0.15±0.26 (p<0.01, n = 6) compared with the sham group. However, treatment with Chr-lip (10.0, 5.0 or 0.5 mg kg\(^{-1}\)) to the ischemic mice significantly decreased to 1.43±0.15, 2.33±0.15 and 2.96±0.40 (p<0.01, p<0.01, p<0.05, n = 6), respectively, vs. the model group.

**DISCUSSION**

Chry has multiple pharmacological effects, including anti-aging, anti-inflammatory and anti-cancer activities. It has demonstrated the ability to inhibit growth of some
human cancer cells, such as by inducing cell death (necrosis) in human lung cancer A549 cells and necrosis in J5 cells (Kim et al., 2010; Lu et al., 2010; Ni et al., 2012a, b). However, we found that Chr-lip had the obvious bioavailability and could improve passive avoidance learning and memory after CIR in mice (Li and Zhang, 2011). As the new formulation, it has the potential ability to provide an opportunity for management neurological abnormalities in dementia conditions. In the present study, we showed abundance evidences that Chr-lip exhibited neuroprotective activity against CIR damage in a middle cerebral artery occlusion group. The neurological deficits, brain water content, neuronal ultrastructure and histopathological characterization reveal the neuronal necrosis and degeneration (Hriteu et al., 2012). Neurological deficits were found to be followed by neuronal degeneration and necrosis. The present study showed that pretreatment of Chr-lip improved the neurological deficit and neuronal degeneration. The data also revealed that pretreatment with Chr-lip could reduce brain water content, improving neurological, neuronal ultrastructure and histological deficits in CIR mice. These observations indicated that Chr-lip could efficiently prevent the brain injury induced by CIR.

CIR leads to diverse structural changes in neural cells. In the experiment, we observed the nuclear membrane was disrupted or disintegrated, swelling mitochondrial, decreased rough endoplasmic reticulum, disorganization of polyribosomes and Golgi apparatus in postischemic ischemic hemisphere neurons which is consistent with the studies before (Li et al., 2012). Mitochondrial swelling is one of the initial postischemic changes. In mild ischemia, swollen mitochondria recover their normal shape soon. But, in severe ischemia, in neurons a large number of organelles show condensation, increased matrix density and deposits of an electron-dense material followed by the fracture of mitochondria (Petito and Pulsinelli, 1984). In the experiment, we proved that the cell nucleus was swollen and the nuclear membrane was disrupted or disintegrated, the chromatin was margilated and vacuoles were observed in the cytoplasm, mitochondrial cristae and membranes had mostly disappeared while degradation of the rough endoplasmic reticulum and apoptotic bodies were apparent in model group. However, we also demonstrated slightly shrunken nucleus and complete nuclear membrane in the Chr-lip pretreatment group compared with the significantly aggregated chromatin toward the nuclear membrane in the model group.

During the period of CIR (Wang et al., 2013a), multiple pernicious processes including free radicals accumulation, inactivation of detoxification systems, overproduction of oxidants and consumption of antioxidants have been reported in many researches (Chao et al., 2013; Yun et al., 2013). These changes disorder the normal antioxidative defense system in brain tissue (Chan, 2001). Then free radical will attack biofilm structure which further affect the stability of cell membrane, finally changes in the structure and function of the cell, aggravate cell injury and brain dysfunction. As the final product of lipid peroxidase, MDA is highly reactive and responsible for cytotoxic effects and neuronal death; its content can reflect the extent of lipid peroxidation and indirect react cell damage degree (Patockova et al., 2003). In the normal state of human body, endogenous antioxidant enzymes such as SOD, GSH-Px and GSH play important roles in the maintenance of redox homeostasis in the tissue (Wang et al., 2013b). Therefore, the body’s ability to remove oxygen free radicals can use the activity of GSH-Px, SOD. In the present experiment, the findings have revealed, there was a significant decrease in the SOD and GSH-Px levels, with increase in the levels of MDA, in the brain homogenate of model group compared to sham group. In contrary, the elevated level of MDA was markedly decreased by treatment with Chr-lip, indicating that the neuroprotection conferred by Chr-lip may be attributed to attenuating lipid peroxidation following transient global cerebral ischemia. In addition, Chr-lip was found to be effective in stimulating the activities of SOD and GSH-Px. We demonstrated that when pretreatment with Chr-lip, MDA levels were obviously decreased and activities of SOD and GSH-Px were significantly increased which indicated that Chr-lip has a strong anti-oxidative effect on CIR injury.

Previous studies have demonstrated that apoptosis plays an important role in neuronal death following CIR (Li et al., 2009b). As we all know, The Bel-2 families of proto-oncogenes encodes specific proteins such as Bax and Bel-2 that critically regulate apoptosis (Abas et al., 2010). The anti-apoptotic effect of Bel-2 occurs by prevention of Cyt-C release into the cytoplasm (Martinou and Youle, 2011). Activated Bax, the pro-apoptotic protein, promotes cell death, unless it is bound by either Bel-2 or Bel-XL (Kim et al., 2011). Compared with the sham group, the Bel-2 expression was markedly decreased and Bax expression was markedly increased in the model group as reported in previous studies (Zhou et al., 2010; Chen et al., 2013). Xi et al. (2011) reported that propofol improved neurobehavioral outcome of CIR rats by the increasing expression of Bel-2 while decreasing expression of Bax with CIR-induced rats at 24 h after ischemia. Wang et al. (2012a) demonstrated
that esculetin exerted its anti-apoptotic activity through up-regulating the expression of Bel-2 and down-regulating the expression of Bax, two apoptosis-related proteins. The current results indicate that treatment with Chr-lip after reperfusion significantly up-regulated the expression of Bel-2 and down-regulated that of Bax in comparison to that observed in the CIR mice, suggesting that Chr-lip inhibited cerebral apoptosis after CIR and offered ideal therapeutic approach to CIR injury. However, caspase is a kind of protease family leading to apoptotic disintegration and executes the critical process of apoptosis in the apoptotic pathway (Degterev et al., 2003; Sung et al., 2012). Caspase3 is an established member of the caspase family known to be involved in the final execution phase of apoptosis and is one of the common down stream effect or sinvaried death receptor-mediated apoptosis pathway (Harrison et al., 2000; Teschendorf et al., 2008). A large number of evidences have testified that the Caspase3 has been up-regulated after CIR. But, previous studies (Torri et al., 2008) suggest that Caspase3 activation depends on Cyt-C release from the mitochondria via regulation by Bel-2 and Bax, i.e., increased Bax expression promotes Cyt-C release and Bel-2 plays an indirect role on Cyt-C release. Neohesperidin (Wang and Cui, 2013) inhibited MCAO-induced upregulation of Bax, Cyt-C and Caspase3, as well as the downregulation of Bel-2. After leaurine treatment (Qi et al., 2009), there showed a marked decrease in the expression of Bax and an increase of Bel-2. The attenuation of mitochondrial membrane swelling and content of Cyt-C in mitochondria isolated from ischemic cortex could also be observed in leaurine treated group. Besides, CoI (Dohare et al., 2008) suppressed the raised Bax protein level and assisted mitochondrial translocation and activation of Bel-2 by changed mitochondrial membrane potential. It also inhibits the cytosolic release of Cyt-C, inhibits the activation of Caspase3 ultimately inhibiting apoptosis. In the present study, western blot and qRT-PCR analysis showed that a marked decrease in the expression of Bax and an increase of Bel-2 in the treatment groups with Chr-lip; moreover, Chr-lip treatment decreased Cyt-C and Caspase3 expression. This suggested that suggesting that Chr-lip inhibited cerebral apoptosis after CIR and offered ideal therapeutic approach to ischemia cerebral injury.

CONCLUSION

In conclusion, we demonstrated that Chr-lip protected against CIR injury by reducing brain water content, improving neurological, neuronal ultrastructure and histological deficits and these beneficial effects were associated with inhibition of oxidative stress or neuronal apoptosis-related pathways, such as elevation of SOD and GSH-Px activities, reduction of the MDA content and the number of apoptotic cells, promotion of Bel-2 expression, inhibition of Bax expression and Cyt-C release and suppression of Caspase3 activation. So, the protective mechanisms of Chr-lip against CIR injury might be involved to its anti-oxidant activities and anti-apoptotic.

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