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# Centrophenoxine improves chronic cerebral ischemia induced cognitive deficit and neuronal degeneration in rats<sup>1</sup>

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**KEY WORDS** meclofenoxate; brain ischemia; free radicals; maze learning; TXB<sub>2</sub>; 6-keto-PGF<sub>1α</sub>; Bax protein; p53 protein

## ABSTRACT

**AIM:** To study the effects of centrophenoxine (CPH, meclofenoxate) on chronic cerebral hypoperfusion induced deficits in rats. **METHODS:** Chronic hypoperfusion in rats was performed by permanent bilateral ligation of the common carotid arteries. Morris water maze was used to measure spatial memory performance. Spectrophotometrical techniques were used to assay SOD, GPx activities, MDA content, TXB<sub>2</sub>, and 6-keto-PGF<sub>1α</sub> levels. Morphological change was examined by HE staining. The expression of Bax and p53 protein were assayed by immunohistochemistry analysis. **RESULTS:** Chronic hypoperfusion in rats resulted in spatial memory impairments shown by longer escape latency and shorter time spent in the target quadrant. These behavioral dysfunction were accompanied by increase in SOD and GPx activities, the content of MDA, the levels of pro-inflammatory mediators (TXB<sub>2</sub>, 6-keto-PGF<sub>1α</sub>), overexpression of Bax and P53 protein, and delayed degeneration of neurons in cortex and hippocampus. Oral administration of CPH (100 mg/kg, once per day for 37 d) markedly improved the memory impairment, reduced the increase in antioxidant enzyme activities, MDA content and the levels of pro-inflammatory mediators to their normal levels, and attenuated neuronal damage. **CONCLUSION:** The abilities of CPH to attenuate memory deficits and neuronal damage after ischemia may be beneficial in cerebrovascular type dementia.

## INTRODUCTION

With increasing number of elderly in world populations, dementia, characterized by progressive loss of memory and higher cortical functions, has given rise to enormous socioeconomic burden. Apart from Alzheimer's disease, vascular dementia (VD) is usually considered the second most common dementia produced

by ischemia, hypoxia, or haemorrhagic brain lesion. Among the studies on its pathogenic mechanisms, free radicals theory of aging has brought growing interest. Free radicals are highly reactive molecules that initiate radical chain reactions and damage cellular macromolecules, including proteins, DNA, and lipids, ultimately leading to cell death<sup>[1,2]</sup>. It was reported that in patients with VD, hydroxyl radical (OH<sup>-</sup>) levels in blood were significantly higher than that in the control, whereas SOD activity was lower<sup>[3]</sup>. These findings introduced the possibility of using antioxidants as therapeutic intervention in patients with VD.

Centrophenoxine (CPH), an ester of *p*-chloro-

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phenoxyacetic acid (PCPA) and dimethylaminoethanol (DMAE)<sup>[4]</sup>, is a potent nootropic that acts to stimulate glucose uptake, oxygen consumption, and promote energy metabolism in the brain<sup>[5,6]</sup>. Beneficial effects of CPH treatments have been observed in pathological states, such as cerebral atrophy, brain injuries, and chronic alcoholism<sup>[7]</sup>. During the past two decades, it is interesting to note that DMAE, the effective part of the CPH molecule, is an efficient OH<sup>-</sup> radical scavenger<sup>[8]</sup>. It has also been shown that the rates of brain RNA-synthesis of CPH-treated animals were markedly elevated than that of the control<sup>[8]</sup>, and memory deficits in aged subjects and patients with VD were relieved, without any remarkable side effects<sup>[9,10]</sup>. These findings have prompted us to explore whether CPH has beneficial effects in the cerebral ischemia model.

## MATERIALS AND METHODS

**Chemicals** Centrophenoxine<sup>[5,11,24]</sup> (colorless power, purity >98 %) was a product of Shanghai Huaihai Pharmaceutical Co. Assay kits of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) were purchased from Nanjing Jiancheng Bio-Tek Co. Assay kits of thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 6-keto-PGF<sub>1 $\alpha$</sub>  were purchased from Medical School of Soochow University. Rabbit anti-mouse p53 monoclonal IgG (sc-100) and rabbit anti-mouse Bax monoclonal IgG (sc-7480) were purchased from Santa Cruz Biotechnology.

**Animal** Adult male Sprague-Dawley rats weighing 180-200 g were used (Shanghai Experimental Animal Center, Chinese Academy of Sciences). They were housed 5 per cage in a temperature and humidity-controlled room (temperature: 22±1 °C, humidity: 60 %) with free access to food and water. The rats were kept on a 12-h light/dark cycle. The rats were acclimatized to housing conditions for at least 4 d before use.

**Surgery** The rats were anesthetized with chloral hydrate (350 mg/kg, ip). Bilateral common carotid arteries were exposed through a midline neck incision, double ligated with 4-0 type surgical silk, and cut between ligation in ischemia rats. The sham-operated rats received the same operation except ligation. During ischemia, body temperature was maintained at 37.5±0.5 °C by means of a heating lamp until the rats recovered thermal homeostasis.

**Water maze task** The rats' spatial memory performance were evaluated using a Morris water

maze<sup>[11]</sup>. Each rat received two trials everyday for 5 consecutive days. Latency to escape onto the hidden platform was recorded. On the fifth training day, each rat was subjected to a 60 s probe trial in which the platform was removed and the time spent in Q3 was recorded. To examine the possibility that the difference in spatial learning between groups, if any, was associated with vision impairment, the escape latency of each rat was determined with the visible platform at 1 cm above water surface after the probe trial. Swimming activities were monitored by a video camera linked to a computer-based image analyzer. Test was performed between 9:30 and 11:00 AM. CPH was administered orally 30 min before the test.

**Biochemical examinations** Rats were killed by decapitation, the cerebral cortex, hippocampus and striatum were separated on ice, and were homogenized with ice-cold saline to be 10 % (w/v) homogenates. The extents of membrane lipid peroxidation in brain were monitored by measuring MDA formation<sup>[13]</sup>. The assay for SOD was based on its ability to inhibit the oxidation of oxyimine by O<sub>2</sub><sup>-</sup> produced from the xanthine/xanthine oxidase system<sup>[14]</sup>. GP $\alpha$  activity was determined according to the method of Daret *et al*<sup>[15]</sup>. TXB<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  were determined, according to the manufacturer's instructions. Protein concentration was measured by the Coomassie blue protein-binding<sup>[16]</sup> using bovine serum albumin (BSA) as standard.

**Morphology** Three or four rats chosen randomly from each group were anesthetized with chloral hydrate (350 mg/kg, ip) after behavior experiment, and then perfused transcardially with normal saline followed by 4 % paraformaldehyde. All brains were then postfixed in the same fixative at 4 °C, dehydrated and then embedded in paraffin blocks. Coronal sections of 8  $\mu$ m were stained with hematoxylin-eosin.

**Immunohistochemistry** Tissue sections were deparaffinized and rehydrated through graded alcohol. Endogenous peroxidase activity was blocked by incubation in 10 % hydrogen peroxide for 10 min. After three times rinses with PBS, the sections were blocked with 1:10 normal goat serum to suppress nonspecific background staining. Then, the primary antibody, rabbit anti-mouse p53 monoclonal antibody and rabbit anti-mouse Bax monoclonal antibody was applied at 1:50 dilution. After incubation for 20 h at 4 °C, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100, 37 °C, 1 h), then followed by SABC kit. The sections were subsequently incubated with diamino-

benzidine 0.5 g/L, observed under light microscope.

**Statistical analysis** Results were presented as mean±SEM. Groups difference in the escape latency in the Morris water maze training task were analyzed with two-way analysis of variance (ANOVA). Data collected from the probe trials and the biochemical studies were analyzed using one-way ANOVA followed by Duncan's test. A value of  $P<0.05$  was considered statistically significant.

**RESULTS**

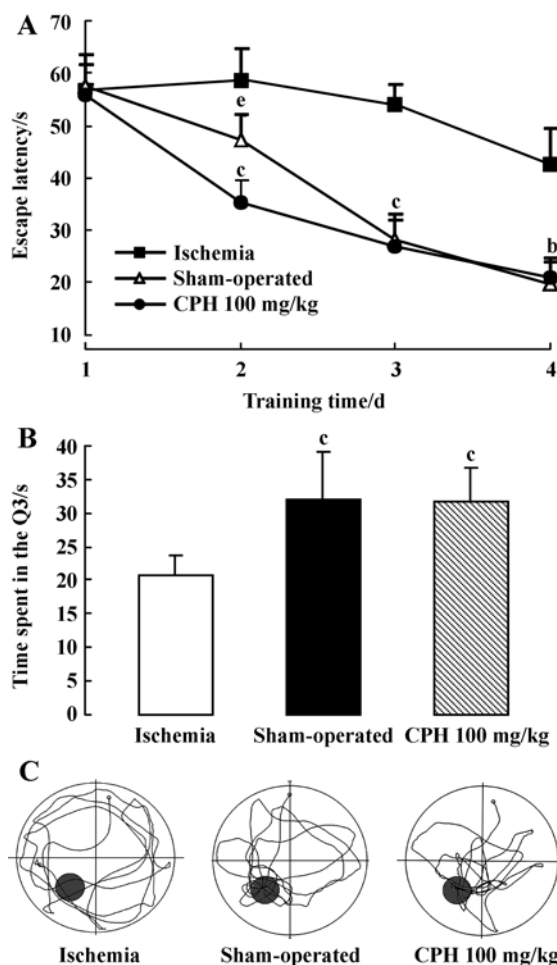
**Effects of CPH on water maze learning** The rats with chronic hypoperfusion took longer to find the platform than did sham-operated rats [ $F(3,72)=5.848$ ;  $P<0.01$ ]. This prolongation of latency was shortened by CPH 100 mg/kg, [ $F(3,72)=5.051$ ;  $P<0.01$ , Fig 1A].

In the probe trials, the swimming time in Q3 was used to evaluate the retention performance. Sham-operated group and CPH-treated group swam longer in Q3 than ischemic group ( $P<0.01$ , Fig 1B). The typical swimming tracks indicated that ischemic rats often searched for the platform in an inappropriate way resulting in the longer latency to locate the platform (Fig 1C). All rats reached the platform in a short period of less than 30 s tested with a visible platform task, and there was no difference between any two groups, indicating that the difference in spatial performance between groups is unlikely to be associated with vision impairments.

**Effects of CPH on SOD, GPx activities, and MDA content** In ischemia rats, the activity of SOD (nU/mg protein), GPx (nU/mg protein), and MDA content (nmol/mg protein) were  $1.42\pm0.07$ ,  $2.81\pm0.02$ ,  $6.25\pm0.3$  in the hippocampus;  $0.91\pm0.04$ ,  $7.41\pm0.25$ ,  $7.05\pm0.4$  in the cortex; and  $1.05\pm0.04$ ,  $4.48\pm0.54$ ,  $5.02\pm0.26$  in the striatum, respectively. Chronic hypoperfusion resulted in significant increase in SOD, GPx activities, and MDA content compared to sham-operated group ( $P<0.01$ ). CPH 100 mg/kg significantly attenuated the increase in SOD, GPx, and MDA in the cortex, hippocampus, and striatum compared to ischemia group (Fig 2).

**Effects of CPH on TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> levels** In saline-treated ischemic rats, marked increase in the levels of TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> in the cortex were observed. CPH 100 mg/kg significantly attenuated the increase of TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> (Tab 1).

**Effects of CPH on ischemia-induced neuronal damage** In the cortex and hippocampus, marked



**Fig 1.** Effects of centrophenoxine(CPH) on water maze performance deficits caused by permanent bilateral ligation of the common carotid arteries in rats. (A) Mean daily latencies of escaping from the start point onto the hidden platform. (B) The time spent in the target quadrant (Q3) in 60 s probe trial (no platform). (C) Typical swim-tracking path in probe trial on the fifth training day.  $n=8-10$ . Mean±SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs ischemia group.

**Tab 1.** Effects of centrophenoxine (CPH) on TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> contents in cortical region of rats chronically hypoperfused after ligation of the bilateral carotid arteries. Mean±SEM.  $n=5$ . <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs ischemia group.

Group	TXB <sub>2</sub> /ng·L <sup>-1</sup>	6-keto-PGF <sub>1α</sub> /ng·L <sup>-1</sup>
Ischemia	57.2±8.4	20.0±2.2
Sham-operation	30.6±4.5 <sup>f</sup>	6.7±2.8 <sup>f</sup>
Ischemia+CPH (100 mg/kg)	32.2±3.8 <sup>f</sup>	10.5±3.3 <sup>e</sup>

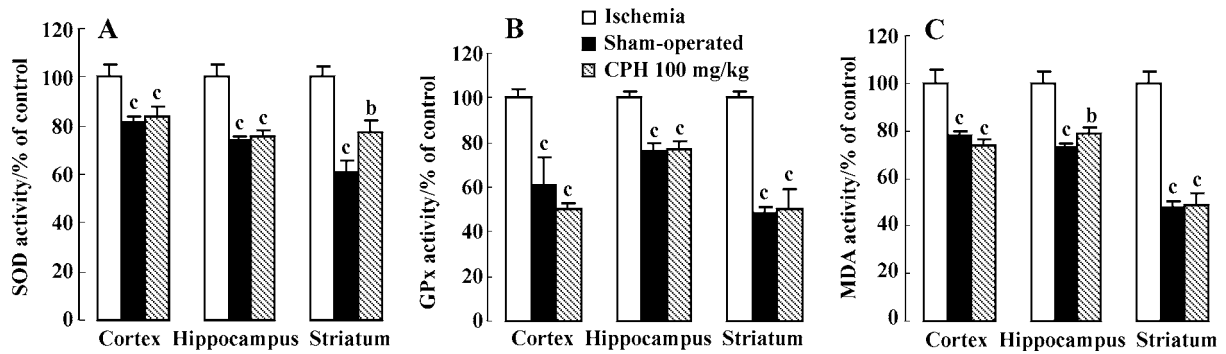


Fig 2. Effects of centrophenoxine (CPH) on superoxide dismutase (SOD), glutathione peroxidase (GPx) activity, and MDA content in rats chronically hypoperfused after ligation of the bilateral carotid arteries.  $n=8-10$ . Mean $\pm$ SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs ischemia group.

morphological changes were detected in ischemic group: neuronal cells loss, glial proliferation, nuclei shrinkage, and dark staining of neurons were observed in hippocampal CA1 region and cortex. Co-treatment with CPH (100 mg/kg) markedly reduced these pathological changes (Fig 3).

**Effects of CPH on the expression of Bax and P53 protein** Photomicrographs of immunohistochemi-

cal localization of Bax and p53 protein in cortical neuronal cells were shown in Fig 4. Chronical hypoperfusion rats exhibited strongly increased Bax and p53 reactivity. However, in the CPH-treated group, no obvious abnormalities in the intensity or localization of immunostaining for the two pro-apoptotic proteins could be found. No significant difference on Bax and p53 expression was observed among three treatments

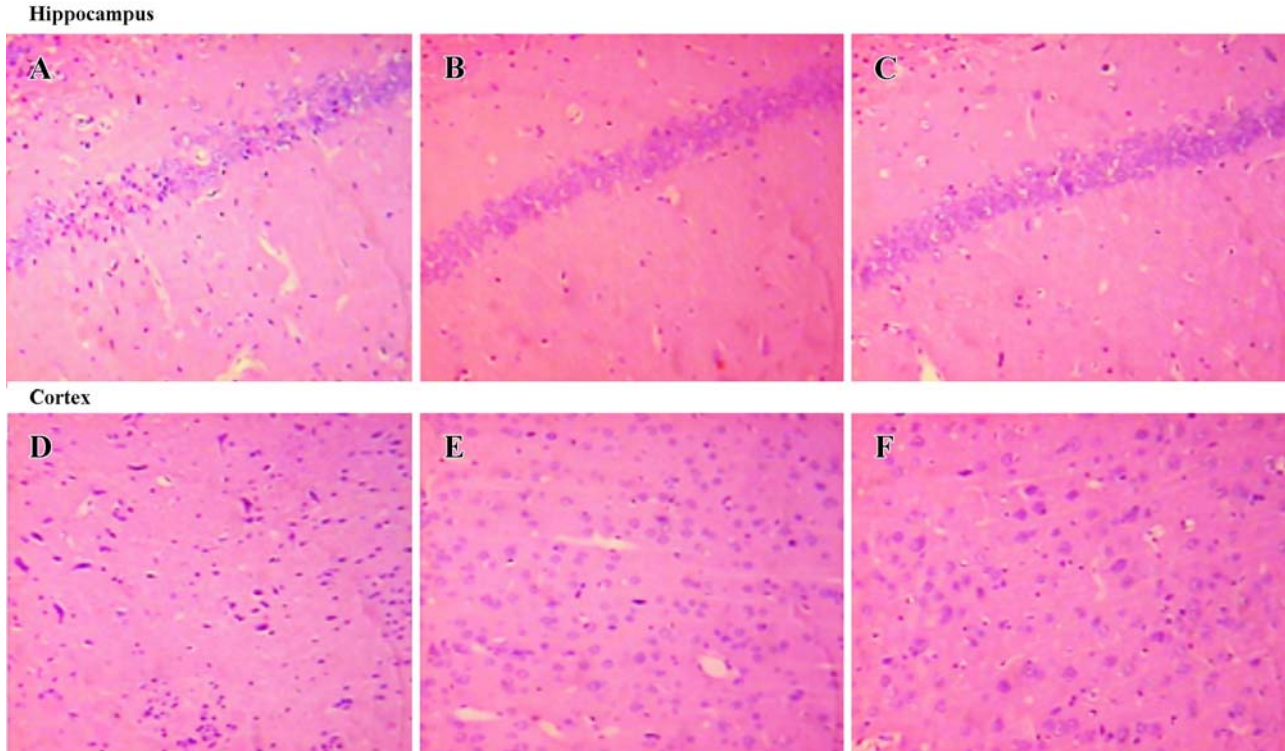
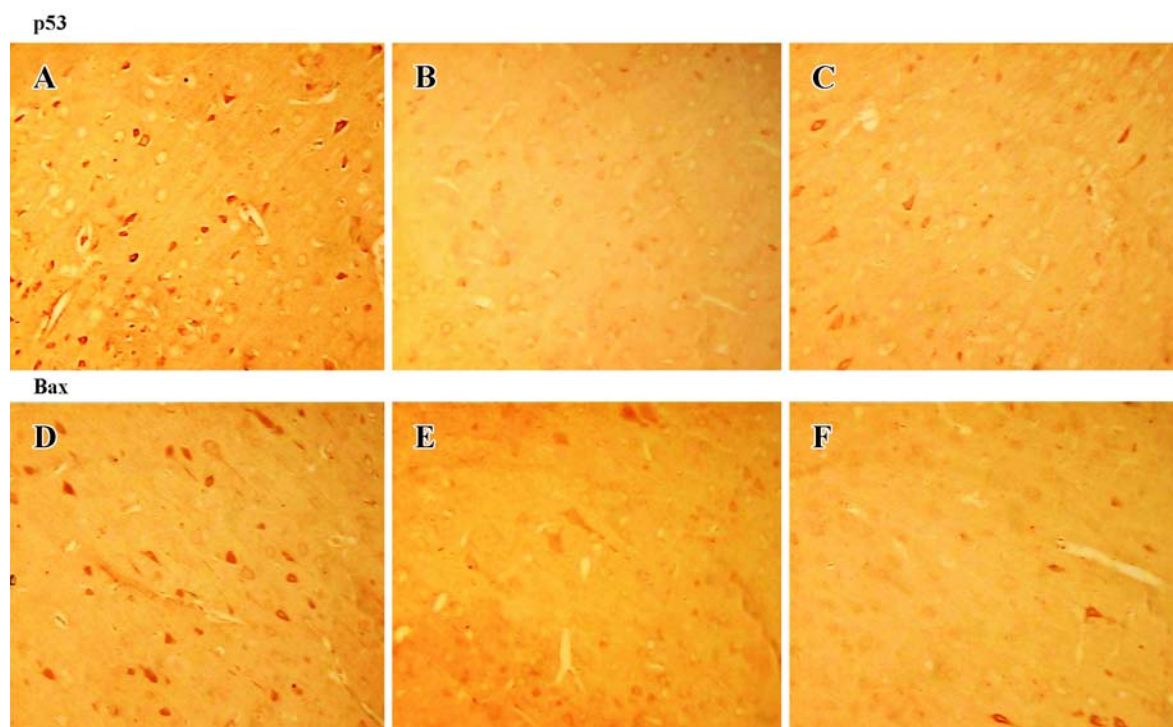


Fig 3. Effects of centrophenoxine (CPH) on morphologic changes in rats' hippocampus and cerebral cortex induced by chronic ischemia. Sections from three or four rats in each group were examined. A and D: Ischemia group; B and E: Sham-operated; C and F: CPH 100 mg/kg. HE stain.  $\times 100$ .



**Fig 4.** Effects of centrophenoxine (CPH) on apoptotic related protein expression in cortical region of rats chronically hypoperfused after ligation of the bilateral carotid arteries. **A and D:** Ischemia group; **B and E:** Sham-operated; **C and F:** CPH 100 mg/kg.  $\times 100$ .

in hippocampus (data not shown).

## DISCUSSION

Permanent bilateral ligation of the common carotid arteries in rats is a chronic cerebral hypoperfusion model, which results in significant reduction of cerebral blood flow<sup>[17]</sup> and causes learning and memory impairments and neuronal damage resembling those in cerebrovascular diseases<sup>[18]</sup>. The present studies showed that chronic cerebral ischemia induced marked amnesic effects along with signs of neurodegeneration, including: (1) spatial learning and memory deficits shown by longer escape latency and shorter time spent in the target quadrant; (2) significant neuronal loss and nuclei condensation in the cortex and hippocampus especially in CA1 region. These results, in accordance with previous reports<sup>[19]</sup>, indicate that cerebral ischemia is related to cognitive impairment, hypofunction of cholinergic neurons, and neuronal death. It has been well documented that chronic hypoperfusion induced marked cholinergic abnormalities in rats<sup>[20]</sup>. DMAE moiety of CPH can enter the choline synthesis cycle and consequently improve the brain's acetylcholine supply<sup>[21,22]</sup>. It seems likely that the beneficial effects of CPH on the ischemia-

induced cognitive deficits were due primary to its effects of acetylcholine supplement.

There are massive evidences showing that free radicals are capable of mediating neuron degeneration and death, and are possibly involved in the pathogenesis of neuron death in neurodegenerative diseases such as VD<sup>[23]</sup>. The present data showed that MDA content was markedly increased in the three brain areas, suggesting that during cerebral ischemia there is an important production of free radicals. Meanwhile, the activities of antioxidant enzymes (SOD and GPx) were also elevated in the three brain regions. It is possible that a compensatory rise occurs in response to heightened oxidative stress after the ischemia insult. Chronic treatment with CPH reversed the abnormality of the free radicals system in ligated rats. It is thought that the beneficial effect of CPH results from DMAE which is phosphorylated and remains in the membrane as phosphatidyl-dimethylamino-ethanol. DMAE can act as an OH<sup>-</sup> free radical scavenger. The antioxidant function of DMAE could stabilize brain biomembrane and is a basis for the fixation of traces of neuronal circle which is thought to be the biological structure of memory<sup>[24]</sup>.

The present finding implicated that the neuropro-

protective effects of CPH may proceed through anti-apoptotic pathway. It is well documented that oxidative stress, mitochondria dysfunction, and  $\text{Ca}^{2+}$  overload are involved in basic molecular and biological process leading to apoptosis<sup>[25]</sup>. In our experiment, HE staining showed that cortical and hippocampal neurons in ligated rats revealed apoptotic-like morphologic changes. Further immunohistochemical analysis demonstrated that Bax and p53 were up-regulated during chronic hypoperfusion. CPH attenuated apoptotic-like changes and neuron losses and reversed the shift in the expression pattern apoptosis-related proteins (Bax and p53) induced by ischemia in the cortex. One of the possible explanations of the neuroprotection is that CPH ameliorates the mitochondria dysfunction, stimulates brain RNA-synthesis, and reduces free radicals-mediated cell death.

Excessive  $\text{TXA}_2$  release during and after cerebral ischemia can trigger strong vasoconstriction and platelet aggregation, lead to cerebral blood flow secondary decrease<sup>[26]</sup>. CPH was effective to ameliorate the cerebral circulation of the ischemic brain in experimental animals<sup>[27]</sup>. Our results indicated that CPH reduced  $\text{TXA}_2$ -induced cytotoxicity by inhibition of  $\text{TXA}_2$  synthesis and acceleration of  $\text{PGI}_2$  synthesis may be involved in the improvement of cerebral blood flow in ischemic brain.

Our findings well suggested that CPH had potential therapeutic and neuroprotective effects based on a multi-target mechanism, and may be helpful in the treatment of vascular dementia.

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