·Original Article·

An inhibition of ceruloplasmin expression induced by cerebral ischemia in the cortex and hippocampus of rats

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Abstract: Objective To explore effects of cerebral ischemia on the ceruloplasmin (Cp) expression in the cortex and hippocampus of rats. **Methods** Male Wistar rats were randomly divided into cerebral ischemia group and control group. Cerebral ischemia was induced by ligating bilateral common carotid arteries and the ischemic rats were further subgrouped according to ischemia time. The control rats received a sham operation. The expression of Cp mRNA in the cortex and hippocampus was measured by reverse transcription polymerase chain reaction (RT-PCR). The Cp expression was shown by immunohistochemistrical (streptavidin peroxidase, SP) method. **Results** In ischemia group, the expression of Cp mRNA in the cortex and hippocampus decreased compared with that in control group ($P < 0.01$); and the longer rats experienced cerebral ischemia, the lower Cp mRNA expressed. By immunohistochemistry, Cp was shown expressed in the neural cells including epithelial cells of choroid plexus, ependymal cells, astrocytes of cortex and hippocampus, and vascular endothelial cells, but not in pyramidal cells and granulosa cells of cortex and hippocampus. Cp levels in the cortex and hippocampus decreased in rats suffering from cerebral ischemia for 3 d, 7 d and 28 d but not in rats exposed to ischemia for 1 d compared with that in control group ($P < 0.05$). Iron concentration correlated negatively with Cp expression in the cortex and hippocampus of rats exposure to ischemia (the cortex, $r = -0.831$, $P < 0.01$; the hippocampus, $r = -0.809$, $P < 0.01$). **Conclusion** Cerebral ischemia inhibited Cp expression in the cortex and hippocampus of rats. The decrease of Cp might be involved in iron deposition in neurons.

Keywords: cerebral ischemia; brain iron concentration; ceruloplasmin

1 Introduction

Ceruloplasmin (Cp) is a ferroxidase that can safely convert toxic ferrous (Fe^{2+}) iron to nontoxic ferric (Fe^{3+}) form[1]. A secreted form of Cp is expressed mainly by liver, and a glycosylphosphatidylinositol (GPI)-anchored form is expressed by astrocytes in the central nervous system(CNS)[2,3]. Null mutations of Cp gene in human (aceruloplasminemia) result in iron accumulation in various organs including the liver and CNS[4]. Iron accumulation and neurodegeneration under this condition usually occurs between 45 and 55 years in human. Mice deficient in Cp ($Cp^{-/-}$) also showed iron accumulation in the liver and other organs[5]. These researches implied that Cp may play an important role in brain iron homeostasis. The oxidation of ferrous iron to ferric iron mediated by Cp is necessary for incorporation of iron into transferrin, since transferrin only binds the ferric form of iron. As a ferroxidase, Cp may also play a role in a transferrin-independent iron uptake system in blood–brain barrier[6].

Iron is an essential metal involved in many metabolic processes and serves as a cofactor for various heme and nonheme proteins, including cytochromes of the mitochondrial oxidative chain for ATP generation, enzymes for DNA repair, and neurotransmitter synthesis. On its divalent state (Fe^{2+}), iron is highly toxic since it produces free radicals resulted from the reaction with hydrogen peroxide and molecular oxygen. Free radicals can induce lipid peroxidation, break DNA strands, degrade biomolecules, and eventually cause

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cell death[7].

Ischemic stroke is the second leading cause of death in the world, and its incidence is expected to rise with the projected increase of aging population. Many factors including excitotoxicity and free radicals are associated with this kind of brain injury. Disturbances of brain iron homeostasis have been proved to link to the acute neuronal injury following cerebral ischemia. Free iron catalyzes the conversion of superoxide and hydrogen peroxide into hydroxyl radicals, which will promote oxidative stress and lead to subsequent cell death/apoptosis. In recent years, considerable experimental evidence has emerged regarding the role of iron neurotoxicity following cerebral ischemia. Clinical studies have also shown the neurotoxicity of iron in stroke patients. Understanding the changes of iron metabolism in the brain and its relationship to the neuronal injuries in ischemic stroke may provide new therapeutic targets and improve the prognosis of stroke patients.

In a previous research, we have shown that cerebral ischemia may lead to increase of iron concentration and abundant iron deposition in neurons in the cortex and hippocampus of rats[8]. The abnormal iron accumulation in the brain can produce free radicals to promote lipid peroxidation, which will aggravate lesions of ischemic brain tissues and induce neurodegenerative diseases[9]. Researches have confirmed that the decrease of ferroportin 1 (FP1) in neurons may be associated with the pathogenesis of ischemic brain injury. However, the mechanism by which iron deposits in neurons during cerebral ischemia was not elucidated yet.

We hypothesized that Cp may be involved in the pathogenesis of ischemic brain injury. In present study, we measured the expression of Cp in the cortex and hippocampus of cerebral ischemia rats, attempting to confirm this hypothesis.

2 Materials and methods

2.1 Animal groups and surgical procedures A total of 60 male Wistar rats weighing 250-300 g were treated following institutional guidelines. Rats were divided into control group (n = 12, receiving a sham operation) and ischemia group (n = 48, bilateral common carotid arteries were ligated). Ischemia group were further subdividedinto 4 groups according to ischemia time (n = 12 for each group). The protocol for cerebral ischemia production has been described previously[10]. Briefly, animals were anesthetized with chloral hydrate (350 mg/kg, i.p.) and allowed spontaneous respiration throughout the surgery. Through a midline cervical incision, both common carotid arteries were exposed and double-ligated with silk sutures. The common carotid arteries of control rats were isolated but not ligated. Cp levels in the cerebral cortex and hippocampus were measured at day 1, 3, 7, and 28, respectively, after the surgery day that the bilateral common carotid arteries were ligated. After the operation, the rats were kept in animal quarters with food and water available.

The fresh brains of six rats in every group were taken out for RT-PCR to measure the levels of Cp mRNA. Other six rats were fixed by perfusion with 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer (PB; pH 7.4) and the brains were removed from the skull and post-fixed in the same fixative.

2.2 RT-PCR Total RNA was purified by using Trizol reagent (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.), and RT-PCR was performed by using Takara two-step RT-PCR kits (Takara Biotechnology, Dalian, Co., Ltd.) following the protocol of manufacturer. According to Chen *et al*.[11], the primers were as follows: GPI-Cp (AF202115) forward 5'-GTA TGT GAT GGC TAT GGG CAA TGA-3', reverse 5'-CCT GGA TGG AAC TGG TGA TGG A-3'; and GAPDH (NM_017008) forward 5'-TCC CTC AAG ATT GTC AGC AA-3', reverse 5'-AGA TCC ACA ACG GAT ACA TT-3' (Takara Biotechnology Co. Ltd., Dalian, China). PCR was performed under the following conditions: Step 1, 2 min at 94 °C for one cycle; and Step 2, 30 s at 94 °C, 30 s at 56 °C (50 °C for GAPDH), 1 min at 72 °C, for 30 cycles.The level of Cp mRHA was expressed as a ratio of Cp to GAPDH.

2.3 Immunohistochemistry Tissue samples were embedded in paraffin, and sections were cut at 5 μm with a microtome and placed on coated slides. Paraffin was removed from the tissue sections with xylene, and the sections were rehydrated in graded ethanol solutions. Antigen retrieval was performed by heating in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. After blocked with 5% normal rabbit serum, sections were incubated with the primary antibody for Cp (mouse anti-rat Cp antiserum at 1:50 dilution; Alpha Diagnostic Intl, Inc., USA) at 37 °C for 2 h. They were rinsed in PBS and incubated with biotinylated goat anti-mouse IgG (Beijing Zhongshan Golden Bridge Biotechnol-

ogy Co., Ltd.), followed by streptavidin peroxidase solution (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Samples were colored in DAB with 0.003% H_2O_2. The tissues were then lightly counterstained with hematoxylin and examined by light microscopy. Control sections were incubated with PBS or pre-immune rabbit serum instead of primary antibody. For each section, ten fields of view were examined; mean density and sum area of objects, and mean density and sum area of field of view were measured by Image-Pro plus 6.0 software. According to Shen[12],Positive Unite (PU) was calculated by the following formula:

$$PU = \frac{(Mean\ Density\ of\ Objects - Mean\ Density\ of\ Field\ of\ View)}{(1 - \frac{Sum\ Area\ of\ Objects}{Sum\ Area\ of\ Field\ of\ View}) \times 255} \times 100\%$$

2.4 Statistical analysis Statistical analysis was performed by SPSS11.5. Values are expressed as mean±SD. Difference between groups was analyzed by one way ANOVA. Values were considered significant at $P < 0.05$.

3 Results

We investigated the effects of cerebral ischemia on Cp mRNA expression in the cortex and hippocampus of rats. As illustrated in Fig. 1, RT-PCR products yielded a single major band in the cortex and hippocampus of control rats, consistent with the expected band of base pairs for Cp, 449 base pairs. In the cerebral ischemia rats, the expression of Cp mRNA in the cortex and hippocampus decreased compared with that

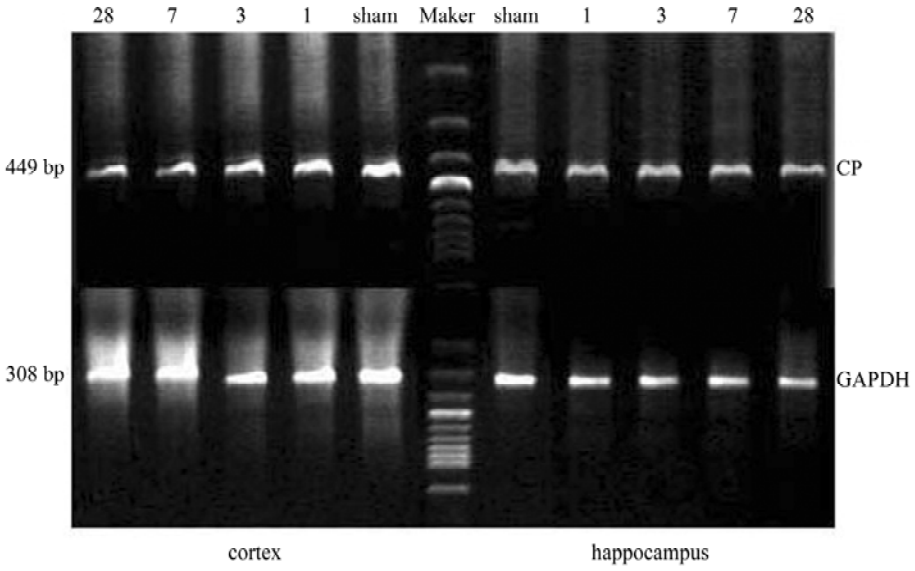

Fig. 1 Expression of Cp mRNA in the cortex and hippocampus of rats seperated by RT-PCR.

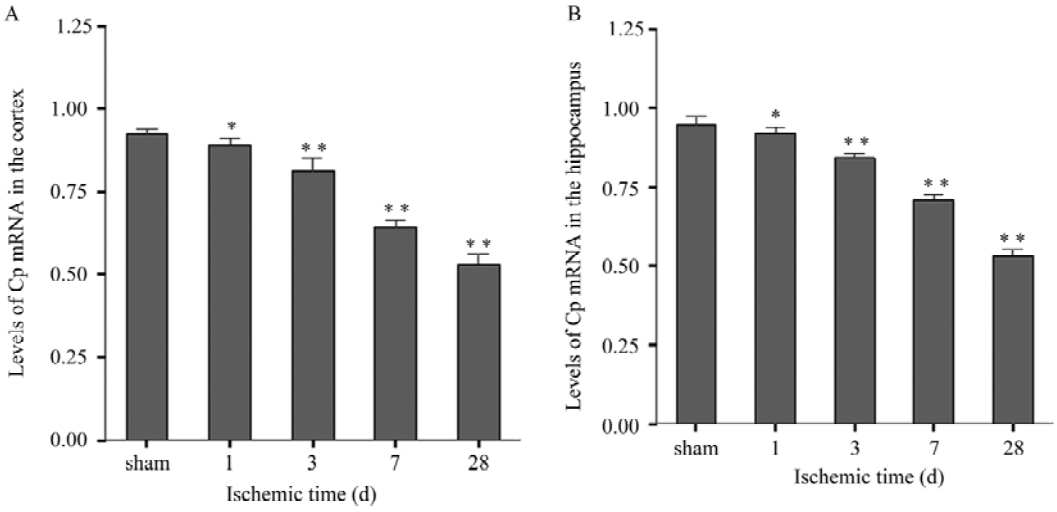

Fig. 2 Cp mRNA levels in the cortex (A) and hippocampus (B) of rats. Data are expressed as the ratio of Cp to GAPDH. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs* sham group.

in sham operation group ($P < 0.01$). As demonstrated in Fig. 2, the longer rats experienced cerebral ischemia, the lower Cp mRNA expressed.

The expression of Cp was shown in the neural cells including epithelial cells of choroid plexus, ependymal cells, astrocytes of the cortex and hippocampus, and vascular endothelial cells, but not in pyramidal cells and granulosa cells of the cortex and hippocampus. The brown staining in neuronal cells was the positive staining, present predominantly in the cytoplasm and the membrane. There was a close correlation between the expression level of Cp and the ischemia time. A strong expression of Cp was shown in the cerebral cortex (Fig. 3E) and hippocampus (Fig. 3A, C) in control rats. In the rats exposed to ischemia for 1 d, Cp expression in the

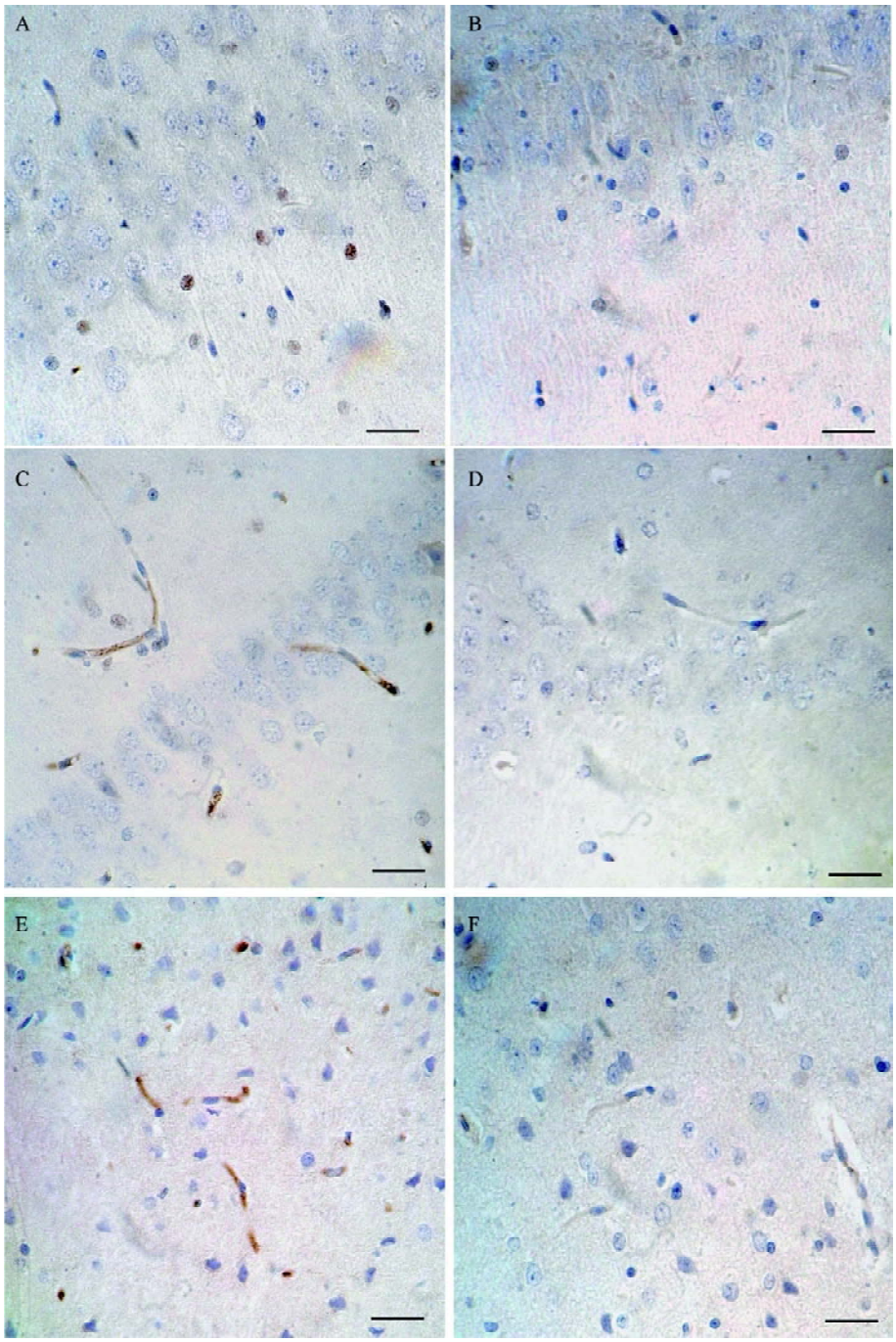

Fig. 3 Expression of Cp detected by immunohistochemistry. A, C: Hippocampus of control rats; B, D: hippocampus of 28-d ischemia rats; E, F: cortex of control rats and 28-d ischemia rats, respectively. Scale bar, 25 μm in A and B; 50 μm in C-F.

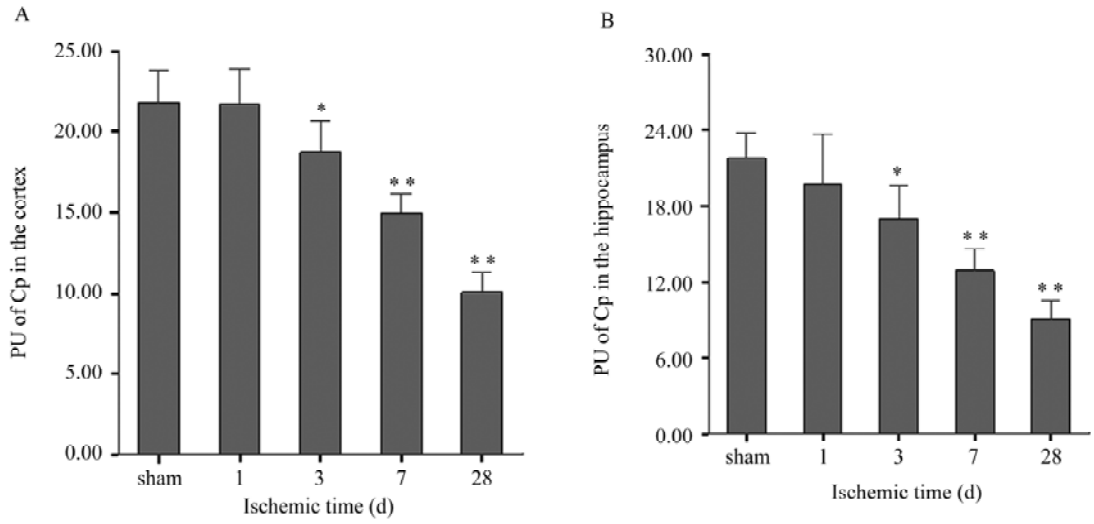

Fig. 4 Positive Unite (PU) of Cp in the cortex (A) and hippocampus (B) of rats. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs* sham group.

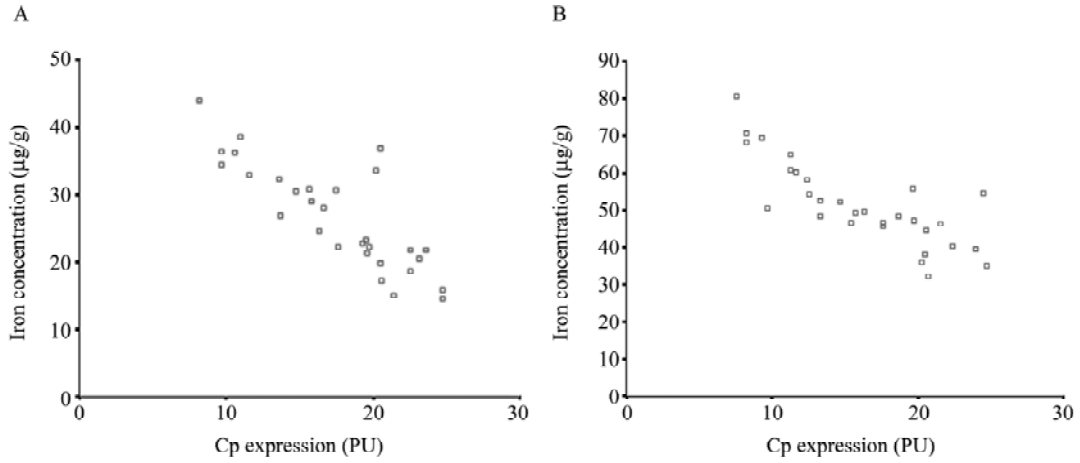

Fig. 5 Correlation analysis between iron concentration and Cp expression in the cortex (A) and hippocampus (B) of rats. A: Cortex, $r = -0.831$, $P < 0.01$; B: hippocampus, $r = -0.809$, $P < 0.01$.

hippocampus and cerebral cortex had no significant change compared with that in control rats. In the rats exposed to ischemia for 3 d, however, the Cp expression was found decreased significantly. The Cp expression decreased further following a delay of ischemia. Few Cp positive staining was shown in rats whose bilateral common carotid arteries were ligatged for 7 d and 28 d (Fig. 3B, D, F).

Morphometric analysis showed that the expression of Cp in the cerebral cortex and hippocampus decreased 14.03% and 24.66% (both $P < 0.05$), 31.27% and 40.60% (both $P < 0.01$), and 53.74% and 58.16% (both $P < 0.01$), respectively, in rats exposed to ischemia for 3 d, 7 d, and 28 d, as compared with control rats. (Fig. 4)

Correlation analysis showed that iron concentration correlate negatively with Cp expression in the cortex and hippocampus of ischemic rats (Fig. 5A, cortex, $r = -0.831$, $P < 0.01$; Fig. 5B: hippocampus, $r = -0.809$, $P < 0.01$).

4 Discussion

Previous researches showed that cerebral ischemia induced iron concentration increse in the brain and iron deposition in neurons[13-15]. The abnormal increase of iron in the brain can produce free radicals and thus promote lipid peroxidation, which will aggravate the lesion of ischemic brain tissues and induce neurodegenerative diseases[9]. Our previous study has confirmed that the decrease of FP1 in neurons may be involved in the pathogenesis of ischemic brain injury[8]. However, the mechanism

of iron deposition in neurons induced by cerebral ischemia was not elucidated yet. In the present study, we demonstrated that the mRNA and protein levels of Cp decreased in the cortex and hippocampus of rats following cerebral ischemia.

The expression of Cp mRNA in the cortex and hippocampus in control rats was consistent with the result of Klomp *et al*.[16,17]. In rats suffering from cerebral ischemia, the Cp mRNA decreased compared with that in control group; and the longer rats experienced cerebral ischemia, the lower Cp mRNA expressed. By immunohistochemistry, we found that Cp was expressed in the neural cells including epithelial cells of choroid plexus, ependymal cells, astrocyte of the cortex and hippocampus, and vascular endothelial cells, but not in pyramidal cells and granulosa cells of the cortex and hippocampus, which revealed that Cp may play an important role in iron transport in astrocytes and blood–brain barrier. The expression of Cp in the cortex and hippocampus decreased in rats suffering from cerebral ischemia for 3 d, 7 d and 28 d as compared with that in control group, but has no change in rats suffering from cerebral ischemia for 1 d.

Cp is a copper binding glycoprotein found mainly in plasma and also present in several other tissues such as retina and brain[18]. Cp could protect brain from oxidative stress by reducing reactive oxygen species. By oxidizing iron, Cp converts the ferrous form of iron to the safer ferric form[19]. Ferrous iron can generate highly reactive hydroxyl and superoxide free radicals in the presence of hydrogen peroxide or molecular oxygen. Recent work done with Cp knockout mice showed that $Cp^{-/-}$ neural cells had an increased susceptibility to oxidative stress[5]. The decrease of Cp in the brain during ischemia can result in oxidative stress and brain damage.

In the present study, we showed the negative relationship between augment of iron concentration and decrease of Cp expression in ischemia brains, which is resemble with the signs of aceruloplasminemia, a hereditary deficiency of Cp. Patients with aceruloplasminemia exhibit symptoms and signs of CNS degeneration resulted from iron overload in the brain in their fourties or fifties. There are two contrary outlooks about the roles of Cp in the CNS so far. According to Jeong *et al*.[20], glycosylphosphatidylinositol-anchored Cp is required for iron efflux from cells in the CNS via the activity of ferroxidase. However, Qian *et al*.[21-23] considered that Cp might play more important roles in iron uptake than in iron release for neuronal cells.

If Cp plays a role in the iron efflux of neural cells, the most acceptable explanation is that the absence of Cp makes iron unable to be released from cells, which then result in iron deposition in neuron. Obviously, this is not a complete answer. First of all, the iron deposition following cerebral ischemia occurs in neurons while the decrease of Cp takes place in astrocytes. In addition, if excessive iron accumulation in neuronal cells resulted from the absence of Cp, for the same reason, iron (Fe^{2+}) might unable to cross the abluminal membrane of the blood–brain barrier and move from the cytoplasm of brain capillary endothelial cells to the cerebrospinal fluid and interstitial fluid. The fact that brain iron increased abnormally during cerebral ischemia does not support this possibility. It seems impossible for Cp to play a role only in the iron release from neuronal cells but not in that from blood–brain barrier cells. Qian *et al*.[16] offered a new possible explanation that "uptake" is the major role of Cp in the iron balance in brain cells, while "release" is its minor role. Absence of iron efflux is not the major cause for excessive iron accumulation in the neuronal cells in aceruloplasminemia, as suggested traditionally. Probably, the excessive intracellular iron is mainly due to the increase of non-transferrin-bound iron (NTBI) uptake and partly due to decrease of iron release. Under physiological conditions, brain cells obtain iron mainly from transferrin (transferrin-bound Fe^{3+}). Most Fe^{2+}, after transport across the blood–brain barrier, will be oxidized to Fe^{3+} by the ferroxidase activity of Cp and/or spontaneous oxidization. Fe^{3+} then bind to transferrin and be captured by neurons or other brain cells. However, under pathological circumstances, the ferroxidase activity loss of Cp will make it impossible for most ferrous iron to be oxidized to ferric iron. Accordingly, the amount of ferric iron and transferrin-bound Fe^{3+} will decrease; while NTBI such as citrate-Fe^{2+}, ascorbate-Fe^{2+} and free ferrous iron will increase. As a result, the NTBI uptake (mainly in the form of ferrous iron) in neuronal cells or other brain cells will increase abnormally. Within the cells, ferrous iron will unable to be loaded into ferritin owing to absence of Cp[24,25]. In addition, intracellular ferrous iron might not be released even when its concentration increased, because the increase of extracellular ferrous iron concentration will lead to the decrease of ferrous iron concentration differ-

ence between inside and outside brain cells (a gradient for the cellular efflux of ferrous iron). All of these factors will result in excessive intracellular iron accumulation.

Resemble with aceruloplasminemia, the relationship between the increase of iron concentration and the decrease of Cp expression in the ischemic brain can be elucidated by the explanation above. In cerebral ischemia, the increase of NTBI uptake resulted from absence of Cp expression in the blood–brain barrier may cause iron deposition in neurons. But the proof for this explanation is still poor, especially for the increase of NTBI uptake. This study still has defects, and the further research, such as measuring the change of Cp expression at the protein level by immunoblotting and elucidating the relationship among iron deposition, Cp decrease and neuron death during cerebral ischemia is necessary.

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脑缺血对大鼠皮层及海马铜蓝蛋白表达的影响

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摘要：目的 探讨脑缺血对大鼠皮层及海马中铜蓝蛋白(Ceruloplasmin，Cp)表达的影响。**方法** 雄性Wistar大鼠60只，随机分为脑缺血1、3、7、28 d组和假手术对照组，每组各12只。实验组结扎双侧颈总动脉造成大鼠脑缺血，假手术对照组仅分离出双侧颈总动脉但不结扎。采用反转录聚合酶链反应(RT-PCR)检测皮层及海马组织中Cp mRNA的表达，免疫组织化学观察皮层及海马组织中Cp的表达。**结果** 大鼠皮层和海马均表达Cp mRNA。皮层和海马Cp mRNA的表达随缺血时间的延长逐渐降低，缺血1、3、7、28 d组表达均低于假手术组($P < 0.01$)。脑组织脉络丛细胞、室管膜细胞、皮层和海马的星形胶质细胞、血管内皮细胞均表达Cp；而皮层和海马的锥体细胞和颗粒细胞均不表达Cp。缺血1 d组皮层及海马Cp表达与对照组差异不显著($P > 0.05$)；缺血3 d组皮层和海马Cp表达低于假手术组($P < 0.05$)；缺血第7、28 d组Cp表达减少极为显著($P < 0.01$)。脑缺血大鼠皮层和海马中铁含量与Cp的表达呈负相关，相关系数分别为-0.831($P < 0.01$)和-0.809($P < 0.01$)。**结论** 脑缺血可诱导大鼠皮层及海马中Cp表达降低。脑缺血后Cp表达减少可能参与了脑缺血引起的铁含量升高及神经元铁沉积的过程。

关键词：脑缺血；脑铁含量；铜蓝蛋白