NEUROPROTECTIVE EFFECT EXPERIMENTAL STUDY OF CYSTATIN C PRECONDITIONING ON CEREBRAL ISCHEMIA-REPERFUSION INJURY IN MICE

YONGPING LIANG[1,*], JIANNING ZHANG[1], BAOMIN LI[2], JUN WANG[2]
[1]Department of Neurosurgery, Sixth Medical Center of PLA General Hospital, Beijing, PR China - [2]Department of Neurology, First Medical Center of PLA General Hospital, Beijing, PR China

ABSTRACT

Objective*: This article explores the neuroprotective effects of serum cystatin C (Cys C) preconditioning on cerebral ischemia-reperfusion injury in mice.*

Methods: *Forty-four healthy male ICR mice were randomly divided into 11 sham operation groups and 33 model groups (for transient middle cerebral artery occlusion surgery). The model group was randomly divided into an IR group and an IR + Cys C dose group (mice treated with a 100 ng or 200 ng dose of Cys C, respectively), with 11 mice each. The IR + Cys C dose group mice were injected with Cys C 30 min before cerebral ischemia. Cys C expression levels, cerebral infarction area, neurological deficit scores, grasping time, first fall time, escape latency, and number of crossing platforms were compared in each group of mice.*

Results: *After 24 h, the expression of Cys C in the IR group was significantly higher than in the sham operation group (P<0.05). The area of cerebral infarction in the IR group and the Cys C dose group was significantly higher than in the sham operation group (P<0.05). The cerebral infarction area in the IR group and the IR + 200 ng Cys C group was significantly higher than in the IR + 100 ng Cys C group (P<0.05). The neurological deficit scores of the IR group and the IR + 100 ng Cys C group were significantly higher than in the sham operation group (P<0.05). The neurological deficit scores of mice in IR + 100 ng Cys C group were significantly lower than in the IR group (P<0.05). The grip time and first drop time of the IR group and IR + 100 ng Cys C group were significantly shorter than that of the sham operation group (P<0.05). The grip time and first drop time of the IR + 100ng Cys C mice were significantly longer than that of the IR group (P<0.05). After 21 d, the escape latency of the IR group and IR + 100ng Cys C group mice was significantly longer than that of the sham operation group, and the number of times crossing the platform was significantly lower than that of the sham operation group (P<0.05). The escape latency of the mice in the IR + 100ng Cys C group was significantly longer than in the IR group, and the number of times crossing the platform was significantly higher than that in the IR group (P<0.05).*

Conclusion: *The Cys C expression level in mice was significantly increased after cerebral ischemia-reperfusion injury. The pretreatment of mice with cerebral ischemia-reperfusion injury by Cys C can effectively reduce the area of cerebral infarction, promoting the recovery of neurobehavioral function, and improving long-term memory and learning functions, and thus plays a neuroprotective role.*

Keywords: *Cys C, preconditioning, cerebral ischemia-reperfusion injury, neuroprotection.*

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Introduction

Ischemia-reperfusion injury can lead to damage to the heart, brain, kidney, multi-tissue organs, and may even cause irreversible damage, which is the main cause of fatal diseases[(1)]. The damage from cerebral ischemia-reperfusion injury has attracted considerable attention from clinical scholars. Brain tissue is very poorly tolerated after ischemia, and can lead to irreversible neurological damage in a short time[(2)]. It is widely acknowledged that factors such as the massive release of oxygen free radicals and the inflammatory response to intracellular calcium overload play an important role in the development of cerebral ischemia-reperfusion injury, which seriously damages neurons and glial cells, and then triggers irreversible nerve damage[(3)]. Therefore, it is particularly important to prevent and control the risk factors of ischemia-reperfusion injury, and to explore effective interventions that reduce the mortal-

ity of ischemia-reperfusion injury. Cystatin C (Cys C) is a cysteine protease inhibitor encoded by the CST3 gene. Its serum content does not change with age, gender, or C reactive protein (CRP) levels[4]. Clinical studies have confirmed that Cys C is more accurate than serum creatinine for assessing renal function[5]. It has been reported that Cys C synthesis and release can be induced by pathophysiological processes such as tumours and human immunodeficiency virus (HIV) infection, and the mechanism of action in these processes is extremely complicated[6]. Previous studies have confirmed that wild-type Cys C neuroprotective functions are extremely strong[7]. Therefore, this study uses Cys C to treat the mouse ischemia-reperfusion injury model in order to explore the neuroprotective effect of Cys C on mouse cerebral ischemia-reperfusion injury.

Materials and methods

Experimental objects and groups

Forty-four healthy male ICR mice were purchased from Changzhou Cavins Experimental Animal Co., Ltd., and their weight was (25±3) g.

The mice were randomly divided into the sham operation group (11 mice), and the model group with transient middle cerebral artery occlusion (33 mice). The model group was randomly divided into the IR group, IR + Cys C dose group (Cys C-treated mice with doses of 100 ng and 200 ng respectively, 11 mice each). All mice were fed adaptively one week before the study.

Thirty minutes before ischemia, Cys C was injected into the lateral ventricle of the mice in the IR + Cys C dose (100 ng, 200 ng) group.

Main reagents and instruments

Reagent

Chloraldehyde hydrate was purchased from Zhengzhou Anlu Environmental Protection Equipment Co., Ltd. Hardeners were purchased from manufacturers of wholesale chemical raw materials. Silicone was purchased from Dongguan Kexin Silicone Hardware Products Co., Ltd. Proteinase K was purchased from Shanghai Jizhi Biochemical Technology Co., Ltd. Paraformaldehyde was purchased from Jinan Chuangshi Chemical Co., Ltd. SDS was purchased from Fuinde Technology Co., Ltd. Sodium chloride was purchased from Beijing Yimao Biotechnology Co., Ltd. Nitrocellulose membrane was purchased from Suzhou Reynolds Biotechnology Co., Ltd. Cys C and Tubulin antibodies were purchased from Wuhan Biotech Biotechnology Co., Ltd.

Instrument

Vernier callipers were purchased from Cisco North Biotechnology Co., Ltd. Doppler flowmeter purchased from Senxi Technology Co., Ltd. The ice maker was purchased from Beijing Xinhua Lvyuan Technology Co., Ltd. A high-speed four-degree centrifuge was purchased from Xi'an Zhongtuan Biological Technology Co., Ltd. An ultrasonic crusher was purchased from Nanjing Cyber Biotech Co., Ltd. The film transfer instrument was purchased from Guangzhou Huijun Biological Technology Co., Ltd. The electrophoresis apparatus was purchased from Nanjing Puyang Scientific Instrument Research Institute.

Methods

• The Cys C protein expression levels of mice in each group were detected by Western blotting after 24 h.

• The cerebral infarction area of the mice in each group was calculated using Alpha Ease software.

• The Longda method was used to score the neurobehavioural function of the mice in each group. The normal mouse activity was 0 points. The contralateral forelimb flexion of the mouse was 1 point. The rear-end rearing phenomenon was 2 points. The opposite side of Qingdao was 3 points when crawling.

• The grab test and rotarod test were used to detect the grip time and first drop time of the mice in each group.

• The Morris water maze test was used to detect the learning and memory function of mice in each group after 21 d.

Statistical methods

SPSS 24.0 statistical software was used to analyse all data. The Cys C expression and cerebral infarction area in each group of mice were expressed by ($\bar{x}\pm s$). Comparisons between two groups were tested by t-test. Comparisons between groups were tested by one way-ANOVA. A $P<0.05$ was considered statistically significant.

Results

Cys C expression comparison after cerebral ischemia-reperfusion injury in mice of each group

After 24 h, the expression of Cys C in the IR group was significantly higher than in the sham operation group ($P<0.05$), as shown in figure 1.

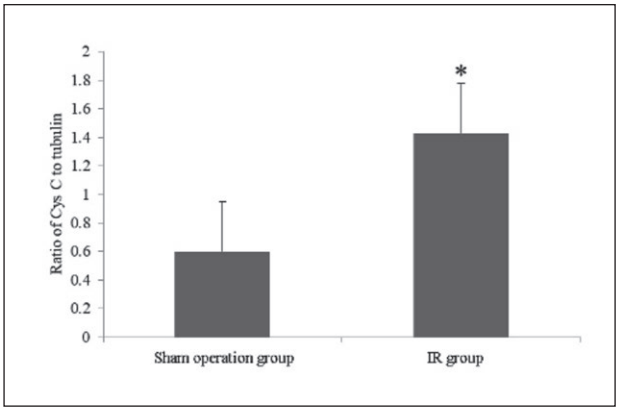

Figure 1: Comparison of Cys C expression after cerebral ischemia-reperfusion injury in mice of each group.
*Note: *indicates that compared with the sham operation group, P<0.05.*

Effect of different doses of Cys C on cerebral infarction area of mice in each group

The cerebral infarction area in the IR group and Cys C dose group was significantly higher than in the sham operation group (P<0.05).

The cerebral infarction area in mice in IR group and IR + 200 ng Cys C group was significantly higher than in the IR + 100 ng Cys C group (P<0.05). Cys C had the best protective effect on brain tissue at 100 ng, as shown in Table 1.

Group	n	Cerebral infarction area (%)
Mock surgical group	11	0
IR Group	11	55.43±6.78*#
IR+100 ng Cys C Group	11	39.23±5.23*
IR+200 ng Cys C Group	11	44.85±6.54*#

Table 1: Effect of different doses of Cys C on cerebral infarction area of mice in each group ($\bar{x}\pm s$).
*Note: *indicates P<0.05 compared with sham operation group; #indicates P<0.05 compared with IR + 100 ng Cys C group.*

Effect of Cys C on neurobehavioral function of mice in each group

The neurological deficit scores of the IR group and IR + 100 ng Cys C group were significantly higher than those of the sham operation group (P<0.05). The neurological deficit scores of mice in the IR + 100 ng Cys C group were significantly lower than those in the IR group (P<0.05), as shown in Table 2.

Group	n	Neurological deficit score (points)
Mock surgical group	11	0.19±0.09
IR Group	11	2.91±0.36*
IR+100 ng Cys C	11	1.61±0.33*Δ

Table 2: Effect of Cys C on neurobehavioral function of mice in each group ($\bar{x}\pm s$).
*Note: *means compared with sham operation group, P<0.05; Δmeans compared with IR group, P<0.05.*

Effect of Cys C on grip time and first drop time of mice in each group

The grip time and first drop time of the IR group and IR + 100 ng Cys C group were significantly shorter than that of the sham operation group (P<0.05). The grip time and first drop time of the IR + 100 ng Cys C mice were significantly longer than that of the IR group (P<0.05), as shown in Table 3.

Group	n	The grip time (s)	The first drop time (s)
Mock surgical group	11	50.89±6.74	189.24±35.76
IR Group	11	8.23±1.01*	82.54±17.37*
IR+100 ng Cys C	11	20.45±3.58*#	164.79±26.54*#

Table 3: Effect of Cys C on grip time and first drop time of mice in each group ($\bar{x}\pm s$).
*Note: *means compared with sham operation group, P<0.05; Δmeans compared with IR group, P<0.05.*

Effect of Cys C on learning and memory function of mice in each group

After 21 d, the escape latency of the IR group and the IR + 100 ng Cys C group was significantly longer than that of the sham operation group, and the times crossing the platform was significantly less than that of the sham operation group (P<0.05). The escape latency of mice in the IR + 100 ng Cys C group was significantly longer than that of the IR group, and the times crossing the platform was significantly higher than the IR group (P<0.05), as shown in Table 4.

Group	n	Escape latency (s)	Number of crossing platforms (times)
Mock surgical group	11	19.51±5.01	4.02±0.62
IR Group	11	38.61±8.73*	1.53±0.46*
IR+100 ng Cys C	11	25.84±4.58*#	2.89±0.58*#

Table 4: Effect of Cys C on learning and memory function ($\bar{x}\pm s$).
*Note: *means compared with sham operation group, P<0.05; Δmeans compared with IR group, P<0.05.*

Discussion

The content of Cys C in cerebrospinal fluid is extremely rich, and it is mainly synthesized in the central nervous system. Experiments related to degenerative diseases of the nervous system have confirmed that Cys C shows a great neuroprotective effect in stimulating neural cell differentiation and resisting amyloid production[8]. Clinical studies have shown that serum Cys C content is closely related to ischemic stroke[9]. Some scholars have found that the risk of the disease was positively correlated with

the level of Cys C by measuring the content of Cys C in the serum of 199 patients with ischemic stroke[10]. Studies related to liver failure have shown that the serum Cys C content of patients after plasma exchange treatment is significantly lower than before treatment[11]. Huang[12] detected serum Cys C content in 45 patients with gastric cancer before and after treatment, and found that the serum Cys C content of gastric cancer patients after treatment was significantly lower than before treatment. Cys C plays an important role in the pathogenesis of various diseases. Previous research has confirmed that Cys C has strong antibacterial and antiviral effects. In addition, Cys C can also participate in inflammatory reactions and exert neuroprotective effects against brain damage, such as in early brain damage. Increased Cys C release can reduce nerve damage. After the mouse Cys C gene was disabled, the degree of local cerebral ischemia further increased[13]. Jin[14] and other researchers have found that as the cerebral ischemia-reperfusion time increased, the level of Cys C in the brain tissue of mice showed a significant increase and then decrease, which may be related to the compensatory increase of Cys-C in the brain tissue that could not match the consumption. Clinical studies have confirmed that increased Cys C content can be used as an independent risk factor for cerebral ischemia-reperfusion injury[15].

In this study, by using the transient middle cerebral artery model, it was found that the expression level of Cys C in mice with cerebral ischemia-reperfusion injury was significantly higher than in the sham operation group, suggesting that Cys C may exert an endogenous protection effect in acute stress injury. The mice were pretreated with Cys C 30 min before the cerebral ischemia. The results showed that the cerebral infarction area of the IR group and the IR + 200 ng Cys C group was significantly higher than in the IR + 100 ng Cys C group ($P<0.05$). The neurological deficit scores of mice in IR + 100 ng Cys C group were significantly lower than those in the IR group ($P<0.05$). It is suggested that Cys C pretreatment can significantly reduce the cerebral infarction area in mice with cerebral ischemia-reperfusion injury, and that the neuroprotective function is strong. At the same time, the neuroprotective effect of low-dose Cys C is stronger than high-dose Cys C. High-dose Cys C can further aggravate the degree of cerebral infarction. The grip time and first drop time of the IR + 100 ng Cys C mice were significantly longer than those of the IR group ($P<0.05$). The escape latency of mice in the IR + 100 ng Cys C group was significantly longer than in the IR group, and the times crossing the platform was significantly higher than that in the IR group ($P<0.05$). It is suggested that Cys C pretreatment is beneficial to the recovery of limb function and learning and memory ability.

In summary, Cys C expression levels in mice were significantly increased after cerebral ischemia-reperfusion injury. The pretreatment of mice with cerebral ischemia-reperfusion injury by Cys C can effectively reduce the area of cerebral infarction, promote the recovery of neuroethological functions, improve long-term memory and learning function, and play a neuroprotective role. A Cys C dose of 100 ng was the best for protecting brain tissue. Due to the limited study time, the intrinsic mechanism of Cys C has not been fully elucidated, and a large number of multicentre samples need to be collected for further exploration.

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Corresponding Author:
Yongping Liang
Email: nqdgv0@163.com
(China)