Int J Clin Exp Med 2015;8(2):2435-2440
www.ijcem.com /ISSN:1940-5901/IJCEM0003866

Original Article

Efficacy of Rho kinase inhibitor on cognitive impairment induced by chronic cerebral hypoperfusion in rats

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Received November 15, 2014; Accepted January 8, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: This work aims to explore the efficacy of Rho kinase inhibitor Fasudil on cognitive impairment induced by chronic cerebral hypoperfusion in rats. A total of 32 male adult Sprague Dawley (SD) rats were randomly divided into three groups: treatment group, control group and sham-operated group for severe carotid artery stenosis model. After two weeks, 8.35 mg/kg Fasudil and physiological saline were intraperitoneally applied twice per day in treatment group and control group, respectively. Morris water maze test was performed in each group to detect the changes of cognitive function and observe the hippocampal pathomorphology in rats after eight weeks. The average escape latency distinctly shortened ($P < 0.01$) and the percentage of swimming distance in the platform quadrant significantly increased ($P < 0.01$) in treatment group compared with those at corresponding time points in control group. The rate of carotid artery stenosis in rats had no statistical difference between treatment and control groups ($P > 0.05$). Fasudil effectively improved hippocampal pathomorphology. Rho kinase inhibitor obviously ameliorated cognitive impairment induced by chronic cerebral hypoperfusion in rats.

Keywords: Rho kinase inhibitor, mild cognitive impairment, chronic cerebral hypoperfusion

Introduction

Research has demonstrated that carotid artery stenosis is not only an important risk factor for cerebrovascular diseases such as transient ischemic attack and cerebral infarction, but also associates with cognitive impairment [1]. Its underlying mechanism might be that ischemia and hypoxia cause pathological physiology changes, including excess production of free radicals, metabolism disorder of membrane phospholipid in hippocampal neurons, intracellular calcium overload and release of abundant excitatory amino acid, and finally result in the death of cholinergic neurons in the hippocampus, decreased activity of choline acetyltransterase, reduced amount of acetylcholine in brain, and eventual cognitive impairment in patients [2]. Carotid endarterectomy and carotid angioplasty and stenting show a significant therapeutic effect on cognitive impairment [3]. In addition, carotid angioplasty and stenting and cerebral protection system-related complications are very common, such as vasospasm, carotid artery dissection and acute stent thrombosis, etc [4]. However, the incidence of cranial nerve injury was dramatically higher in carotid endarterectomy than that of carotid angioplasty and stenting [5]. High expression and excessive activation of Rho kinase closely correlate with the initiation and development of many cardiovascular and cerebrovascular diseases; while Rho kinase inhibitor provides a new hope for the treatment of cardiovascular and nervous system diseases. Shibuya *et al* have discovered that Rho kinase inhibitor Fasudil has apparent efficacy for cerebral infarction [6], and it might function by inhibiting the migration, transformation infiltration and phagocytosis of inflammatory cells, as well as suppressing inflammatory reaction after cerebral ischemia [7]. Lin *et al*. [8] have revealed that Rho kinase inhibitor improves cognitive impairment induced by chronic cerebral ischemia. Therefore, we utilized Rho kinase inhibitor to treat cognitive impairment induced by chronic cerebral hypoperfusion in rats and observe its efficacy, so as to provide new therapy for the prevention and treatment of vascular cognitive impairment.

Table 1. The changes of the rate of carotid artery stenosis in rats before and after treatment in each group (%, x ± s, n = 12)

Group	Before treatment	After treatment
Sham-operated group	3.12 ± 0.08	3.28 ± 0.12
Control group	94.09 ± 2.45[b]	98.24 ± 2.67[b]
Treatment group	95.04 ± 2.24[b]	93.20 ± 2.21[a,b]

[a]Compared with control group, $P > 0.05$; [b]Compared with sham-operated group, $P < 0.01$.

Materials and methods

Animals and grouping

A total of 32 healthy male Sprague Dawley (SD) rats with body weight of (250 ± 25) g were provided by Laboratory Animal Centre of Medical College in Tongji University and randomly divided into two groups, treatment group and control group. There were 12 rats in each group, and the rest eight rats were in Sham-operated group. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Clinical Medical College.

Model preparation

The rat model of severe carotid artery stenosis was established by constructing the stenosis in carotid artery using syringe needle improved from the degree controllable carotid stenosis rat model by Zhou *et al*. [9]. Using open ether inhalation anesthesia, the rats were placed in a large glass jar sealed, and a few cotton balls soaked in ether were placed. After the rats were anesthetized, they were taken out and worn a special half-open ether inhalation mask. The amount of ether was adjusted according to the breathing and heart rate of rats. The sutures were bathed in dexamethasone (DXM) solution for 10 min before the surgery. The rat was fixed after ether anesthesia, and neck midline incision was performed. The skin and muscle were isolated and the bilateral common carotid artery (CCA) was dissociated. A syringe needle with diameter of 0.45 mm and CCA were tied into a slipknot at 0.5 cm from the bifurcation of internal and external carotid artery of the proximal part of CCA using #0 surgical suture. The end of surgical suture was remained outside and the needle was pulled out carefully. Small amount of DXM was dropped at the surgical field as well as penicillin dissolved in saline. Then the wound was sewed up. In sham-operated group, only bilateral CCA of the rats were dissociated. The rate of severe stenosis in rat model was 70%~99%.

Two weeks after rat model was successfully established, 8.35 mg/kg Fasudil (Tianjin Chase Sun Pharmaceutical Co., Ltd., Tianjin, China) suspended in saline was intraperitoneally administrated to the rats in treatment group twice per day for consecutive two weeks. The same volume of physiological saline was injected into the rats in control group.

Stenosis rate

Four rats from each group were anesthetized by excessive chloral hydrate and their CCAs were isolated and fixed with 4% paraformaldehyde. The stenosis of each CCA was cut out to prepare the cross-sectional slices. Three different sections were selected from the stenosis and the area stenosis rate of each section was analyzed with computer image analysis system. Then the mean value of area stenosis rate for each rat was calculated.

Morris water maze behavioral test

A Morris water maze test modified from previous study [10] was performed in rats of each group before and two weeks after corresponding intervention. ① Navigation test: On the first day, the rat swam freely for 2 min to adapt the environment and being held or other operation. From the second day, the rat was put into the water and faced to the pool wall at the four starting positions of the water pool. The computer monitored and recorded the route and time (escape latency) from being into water till they found and climbed onto the transparent platform, which was considered as one training program. Such training was repeated four times with each rat at 8:00 am and at 2:00 pm of each day. If any rat failed to locate the platform in 2min, the latency was noted as 2 min. The interval of two trainings was 60 seconds and the total training time was for five days. ② Spatial Search test: On the fifth training on the sixth day, the platform was removed; the rat was put into the water and faced to the pool wall at a random starting position. The swimming distance between quadrants and the trac-

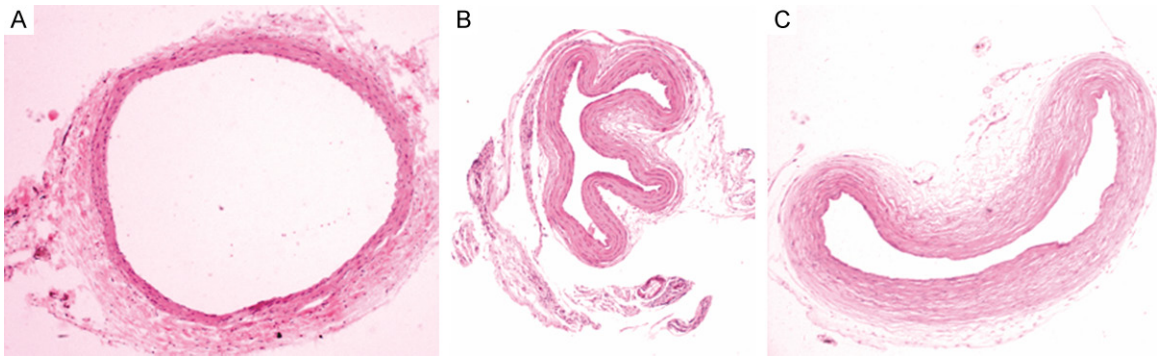

Figure 1. Cross tissue section of rat arteria carotis communis (HE staining × 40). A: Sham-operated group; B: Control group; C: Treatment group.

Table 2. Comparison of the percentage of swimming distance after treatment in each group (%, x ± s, n = 12 in treatment group and control group, n = 8 in Sham-operated group)

Group	Before treatment	After treatment
Sham-operated group	67.75 ± 2.78	68.56 ± 2.32
Control group	36.05 ± 2.67[a]	30.34 ± 2.46[a]
Treatment group	39.24 ± 3.67[b]	46.68 ± 3.67[b]

[a]Compared with control group, $P < 0.01$; [b]Compared with sham-operated group, $P < 0.01$.

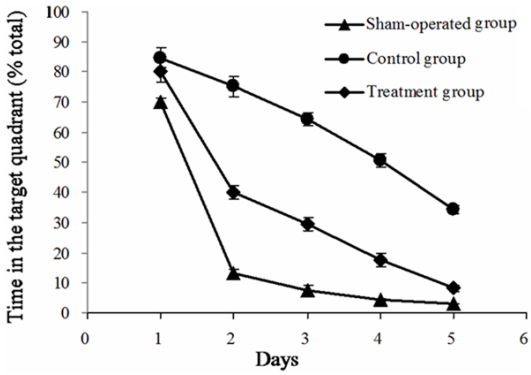

Figure 2. Changes of average escape latency after treatment in each group.

es of searching platform within 2 min were recorded.

Pathological test

After the water maze test, the rat was anesthetized by excessive chloral hydrate. The skull was opened to expose the hippocampus. The hippocampus was isolated and fixed with 4% paraformaldehyde for 48 hours, followed by dehydration, paraffinization, embedding, hematoxylin staining and mounting. The sections were observed under light microscope.

Statistical analysis

SPSS11.0 statistical software was used to analyze the data. The data was presented as mean ± SD. The comparison of two mean values employed t-test and $P < 0.05$ was considered significant.

Results

Stenosis rate

No carotid artery stenosis was observed in rats of sham-operated group. The rate of carotid artery stenosis did not significantly declined after treatment than before treatment ($P > 0.05$), which was shown in **Table 1** and **Figure 1**.

Morris water maze test

The average escape latency markedly lengthened in control group compared with that of sham-operated group ($P < 0.01$), and distinctly shortened in treatment group than that of control group ($P < 0.01$) (**Table 2**; **Figure 2**).

The percentage of swimming distance in the platform quadrant significantly decreased in control group compared with that of sham-operated group ($P < 0.01$), and dramatically enhanced two weeks after treatment than that of control group ($P < 0.01$).

The experiment revealed that the percentage of swimming distance in the platform quadrant in total swimming distance was biggest in sham-operated group, while the percentage of control group obviously decreased and the number

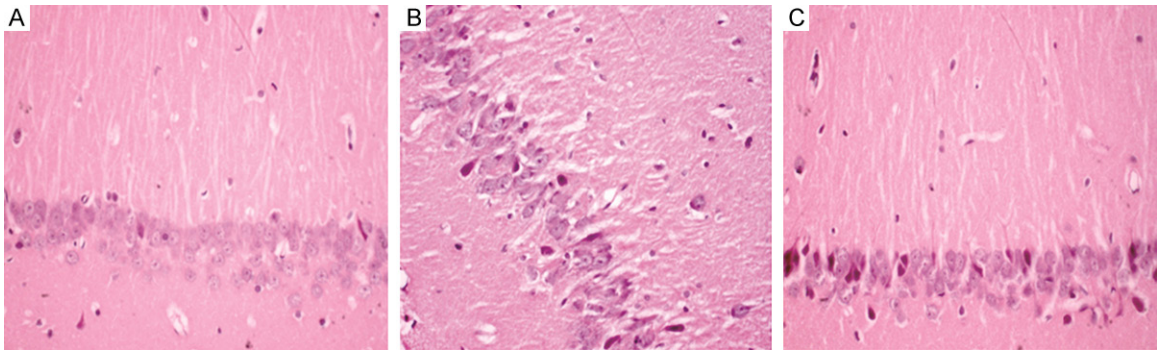

Figure 3. Pathological test (HE staining × 40). A: In Sham-operated group, the morphology, number and distribution of hippocampal neurons were normal, with orderly arrangement. The cell nuclei were large and round, with clear nucleoli; B: In control group, the hippocampal neurons were scattered and arranged disorderly. The pyknosis occurred in some cell nuclei, and the nucleolus disappeared, with unclear cell structure; C: In treatment group, the morphology, distribution and arrangement of hippocampal neurons were significantly improved compared with control group.

into contralateral quadrant increased. After Fasudil treatment, the percentage of swimming distance in the platform quadrant in total swimming distance significantly elevated.

Pathological changes

In sham-operated group, rat hippocampal neurons in alignment had normal morphology, quantity and distribution with big and round nuclei, as well as clear nucleolus, while rat hippocampal neurons in control group arranged in disorder with condensed nuclei, disappeared nucleolus and unclear structure in part of neurons. Rat hippocampal neurons markedly ameliorated in treatment group compared with those at corresponding time points in control group (**Figure 3**).

Discussion

Vascular cognitive impairment (VCI), firstly put forward by Hachinski and Bowler in 1993, is a term to define a large class of cognitive impairment syndromes from mild cognitive impairment to dementia induced by cerebrovascular disease risk factors (such as hypertension, diabetes and hyperlipidemia, etc.) and obvious (such as cerebral infarction and cerebral hemorrhage, etc.) or not obvious (such as leukoaraiosis and chronic cerebral ischemia) cerebrovascular disease (including hereditary cerebrovascular disease) [11]. Laszlo *et al*. [12] have confirmed that carotid artery stenosis can cause cognitive impairment as an independent risk factor, which mainly results from thrombosis and hypoperfusion. Hojo *et al*. [13] have demonstrated that ligation of bilateral common carotid arteries distinctly damages cognitive function of rats, and also causes demyelination in white matter, reactive hyperplasia of glial cells and neuronal apoptosis, which are similar to the brain pathologic changes in patients with VCI. Our research displayed that the cross-sectional area stenosis of rats in control group was 92.09 ± 2.31%, chronic cerebral ischemia induced by severe carotid artery stenosis significantly lengthened escape latency of rats, markedly shortened percentage of swimming distance in the platform quadrant in total swimming distance, as well as apparently damaged learning and memorial function of rats, all of which aggravated gradually along with time, indicating that severe carotid artery stenosis led to cognitive impairment and neuronal ischemia in rats.

Rho associated kinase (ROCK) is a crucial enzyme involved in a series of cell life phenomenon, including mitotic cells adhesion, cytoskeleton regulation, tumor cell invasion and muscle cell contraction, etc [14]. Phosphorylated adducing-substrate of ROCK has been observed in ischemic brain tissue by Yagita *et al* using immunohistochemical staining, which indicates that ROCK is active in the ischemic brain tissue [15]. Research has demonstrated that activation of ROCK might be involved in cognitive impairment induced by chronic cerebral hypoperfusion. Its underlying mechanism is possible that abnormally activated ROCK has adverse effects on ischemic brain tissue

through the following pathways: 1) causing vasospasm [16]; 2) promoting thrombosis, affecting reconstruction and restoration of the neural network, leading to oxidative stress and promoting cell apoptosis, etc [17]. Fasudil is a powerful ROCK inhibitor that strongly reduces ROCK activity [18]. A number of studies have shown that Rho kinase inhibitor plays an important role in vascular remodeling, improvement of blood circulation and blood pressure regulation, and is directly involved in the treatment of cardiovascular and cerebrovascular diseases through the interaction with vasoactive substances, such as angiotensin II and endothelin-1, etc [19]. Our research result found that Fasudil obviously ameliorated cognitive function and alleviated cognitive impairment induced by chronic cerebral hypoperfusion caused by carotid artery stenosis in rats, which was consistent with the conclusion of previous studies. Several studies have revealed that Rho kinase inhibitor prevents the formation of atherosclerosis, and the restenosis after angioplasty shares similar pathological characteristics with atherosclerosis. Therefore, Rho kinase inhibitor is also able to suppress restenosis [20, 21]. The cognitive function was improved in treated rats. Its mechanism may be that, Rho kinase inhibitor can indirectly promote the nitric oxide production, playing a role in antagonizing oxygen free radicals. It can block the metabolism of brain neurotransmitter, prevent certain harmful metabolites on neuronal metabolic toxicity, promote the neurite outgrowth, and inhibit the neuronal apoptosis [22]. However, drug therapy is only a short-term relief of clinical symptoms, which cannot stop the further development of pathological changes.

Recently, clinical research has shown that carotid artery stent placement significantly improves cerebrovascular disease and cognitive impairment induced by carotid artery stenosis, but various postoperative complications, such as thrombosis, cerebral ischemia and restenosis, as well as hyperperfusion and hypoperfusion need to be solved, especially restenosis after stent placement seriously hinders the further development of the technique.

Our study demonstrated that there was no statistical change of rate of carotid stenosis in treatment group. Simulteneously, Morris water maze test suggested that the indicators of rats were markedly different after treatment from those of control group, which indicated that Fasudil could distinctly ameliorate the cognitive impairment induced by chronic cerebral hypoperfusion in rats. In conclusion, Rho kinase inhibitor showed a significant therapeutic effect on cognitive impairment induced by chronic cerebral hypoperfusion.

Disclosure of conflict of interest

None.

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