

Influence of Cilostazol on Changes in Cyclin D1 Expression in Cerebral Cortex of Rats with Chronic Cerebral Ischemia

Running title: Clinical application of Cilostazol

Ai-Xuan Wei¹, Ming-yu Shao¹, Yang Liu¹, Yu Sun¹, Li-Min Wang¹, Xing-Yu Ma¹, Jing Mang²,
Zhong-Xin Xu²

1. Department of Internal Medicine-Neurology, Jilin Central Hospital, Jilin Province 132011,
China

2. Department of Internal Medicine-Neurology, China Japan Union Hospital of Jilin University
130021, China

*Corresponding author

Ai-Xuan Wei

Department of Internal Medicine-Neurology,

Jilin Central Hospital, Jilin Province

No.4 Nanjing street, Chang yi District

Jilin 130021, China

Tel: +86 0432-63073089

Fax: +86 0432-62066508

E-mail: aixuanweidoc@163.com

Abstract

Background: The influence of cilostazol on learning and memory, and cyclin D1 expression in the cerebral cortex of rats with chronic cerebral ischemia were investigated. **Methods:** A chronic cerebral ischemia model was established using the permanent bilateral common carotid artery occlusion method (2VO), learning and memory capacity was detected using the Morris water maze, and expression changes in apoptosis regulating gene cyclin D1 were tested by RT-PCR. **Results:** Results of the Morris water maze indicated that significant extensions were found in the escape latent period and swimming path of rats in the ischemia group (2VO group), learning and memory results in the cilostazol group was obviously superior compared to the 2VO group ($P<0.05$), and the expression of cyclin D1 was observed to increase in both the ischemia and cilostazol intervention groups at the 9th week of ischemia. A significant difference was observed, compared with the sham operation group ($P<0.05$), the expression level decreased in the ischemia group compared with the cilostazol group, and a significant difference was identified compared with the ischemia group ($P<0.05$). **Conclusion:** Cilostazol can reduce nerve function impairment and improve learning and memory functions by affecting changes in apoptosis regulating genes.

Keywords: Cilostazol, Chronic Cerebral Ischemia, Learning and Memory, Cyclin D1, Apoptosis

Introduction

Cognitive dysfunction caused by chronic cerebral ischemia is a disease that greatly influences quality of life, and its incidence rate has increased annually with the ageing of the population. Chronic cerebral ischemia, such as long-term chronic cerebral hypoperfusion, causes cognitive dysfunction; which often leads to different degrees of cerebral injuries and changes in cerebral functions. Currently, cognitive dysfunction caused by chronic cerebral ischemia has been a focus of various studies.

The pathomechanism of ischemic brain damage is a process that involves multiple pathophysiological links, as well as multiple genes and targets. Cilostazol could inhibit platelet aggregation and dilate blood vessels, improve micro-circulatory functions. Cilostazol is taken orally for 12 weeks, triglyceride levels would be reduced by approximately 15% and high-density lipoprotein levels would be increased by approximately 10%. Latest studies (Zhao *et al.* 2010) indicate that cilostazol can accelerate insulin-like growth factor-1 (IGF-1) growth to improve cognitive dysfunction in the hippocampus of mice. Izumi Yuzawa *et al.* (Yuzawa *et al.* 2008) adopted the acute focal cerebral ischemia model to prove that cilostazol can protect the brain by increasing ischemic semi-silent zone rCBF, reducing the volume of cerebral infarction and improving the score of nervous functions.

In this study, we adopted the chronic cerebral ischemia model to detect the expression of cell cycle regulatory factor cyclin D1. Furthermore, the mechanism in which chronic cerebral ischemia leads to cognitive dysfunction, as well as the influence of cilostazol on cognitive dysfunction caused by chronic cerebral ischemia and its mechanism, were investigated. It would provide a new treatment target for the intervention and treatment of cognitive dysfunction caused by chronic cerebral ischemia and to present new ideas for the prevention and treatment of chronic ischemic brain damage in clinic.

1. Materials and Methods

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Jilin Central Hospital.

1.1 Animal grouping Ninety-six healthy male Wistar rats weighing 320-340 g (provided by the Laboratory Animal Center of Jilin University) were randomly divided into three groups (sham operation group, ischemia group and cilostazol group) after being screened by the Morris water maze experiment. Then, each group was randomly divided into three sub-groups according to ischemia time: three-week group, six-week group and nine-week group.

1.2 Main reagents and instruments Cilostazol (provided by Zhejiang Otsuka Pharmaceutical Co., Ltd.; trade name: Pletal); Morris water maze system (manufactured by Huaibei Zhenghua Bio-instruments Equipment Co., Ltd.).

1.3 Model establishment and sample collection The animal model of chronic cerebral ischemia was established by using the permanent bilateral common carotid artery occlusion method (2VO), according to da la Torre et al. Within 24 hours following the operation for the cilostazol group, 30 mg/kg of cilostazol solution was intragastrically administered per day; while sodium chloride solution at the same quantity was intragastrically administered at the same time for the ischemia group. At three weeks, six weeks and nine weeks, rats in all groups were anesthetized again and guillotined to remove the brain.

1.4 Method of etiology detection The Morris water maze experiment was used to detect space learning and memory capacity. This experiment composed of a round pool, an automatic video camera and a computer analysis system. The round pool (institute of materia medica, Chinese academy of medical science) was 170 cm in diameter and 40 cm in height. The automatic video camera (Olympus, Tokyo) tracked the rats. The spatial resolution is up to 640x480 pixels and the temporal resolution is up to 25 frames per second. Data acquisition and image analysis were conducted by automatic image monitoring and processing system. The trial procedure mainly

includes two parts: place navigation trial and space exploration trial. The place navigation trial lasted several days. The rats were put into the water from four entry points facing the wall of the pool several times a day, and the time (escape latency) of finding the platform hidden under the water surface was recorded. Spatial exploration trial is to remove the platform after place navigation trial, and then put the rats into the pool at any entry point to record their swimming track for a certain period of time to investigate the rats' memory of the original platform.

1.5 RT-PCR inspection Three weeks, six weeks and nine weeks, after the model was established, three rats were randomly taken from the sham operation, ischemia and cilostazol groups, respectively, to implement the primer design. Primers of cyclin D1 and β -actin in rats were designed according to the nucleotide sequence in the GenBank. Premier 5.0 software was used to design the primer, and was synthesized by Shanghai Sangon Biotech Service Co., Ltd. (Table 1)

1.6 Data processing All data were expressed as $x \pm SD$. SPSS 13.0 statistical software was used to analyze the results. Analysis of variance (One-Way ANOVA) was used for comparison between groups. Linear correlation analysis was performed and calculate the correlation coefficient r value. $P < 0.05$ was considered statistically significant.

2. Results

2.1 Influence of cilostazol on learning and memory capacity of rats with chronic cerebral ischemia After the operation, the longer the time the rats took to find the platform, and the longer the pathway the rats walked during this period; suggesting the learning and memory capacity of these rats were weak and severely damaged. At three weeks, six weeks and nine weeks of ischemia, the learning and memory results of rats in the ischemia and cilostazol groups were obviously reduced, compared with the sham operation group ($P < 0.05$). However, learning and memory results at all time points in the cilostazol group (ischemia + treatment) obviously

increased, compared with the ischemia group (ischemia only) ($P<0.05$). At the same time, with the increase in training frequency, the space memory capacity of rats increased and the time to find the platform was shortened; indicating that learning exercise can increase memory capacity, but the learning and memory capacity of rats in all groups were different (Tables 2 and 3).

2.2 Influence of cilostazol on cyclin D1 mRNA

A weak-positive mRNA expression of cyclin D1 was found in the sham operation group. At nine weeks of ischemia, the expression of cyclin D1 increased in the ischemia (ischemia only) and cilostazol groups (ischemia + treatment); and a significant difference was observed, compared with the sham operation group ($P<0.05$). Furthermore, expression levels decreased in the cilostazol group (ischemia + treatment), compared with the ischemia group (ischemia only); and the difference was statistically significant when compared with the ischemia group ($P<0.05$; Table 4 and Figure 1).

3. Discussion

Studies have indicated that the learning and memory capacity of rats decreases under a hypoperfusion and low metabolic status (O'Brien, 2006). In this study, the Morris water maze was used to detect the learning and memory function of rats with chronic cerebral ischemia caused by bilateral common carotid artery ligation. Results indicate that significant extensions of escape latency and swimming pathway was found, changes were observed in learning and memory functions, and such phenomena was aggravated as time passed. A significant difference was observed compared with the sham operation group, indicating that rats experienced cognitive dysfunction caused by chronic cerebral ischemia.

Cyclin D1 is a key factor to regulate the G1/S phase. Cyclin D1 is coded by the CCND1 gene of 11q13, composed of 295 amino acids, and combines with cyclin dependent protein kinase 4/6 (CDK4/6). Furthermore, the cyclin D1-CDK4/6 compound can upregulate the cell

cycle to promote cell cycle progression. Cyclin D1 was considered as an important element, when other mechanisms of neuron death cannot make neurons reach the threshold of death(Copani *et al.* 2001). Results of this experiment revealed that the expression of cyclin D1 mRNA can be found in the cortex of the frontal lobe of rats in all groups. After nine weeks in the ischemia group, a weak-positive expression was observed in the sham operation group; and the highest expression was found in the ischemia group. Expression levels in the cilostazol group were lower than in the ischemia group ($P<0.05$).

Cilostazol is a new type of anti-platelet drug. Choi (Choi *et al.* 2002) has reported that the PDE3 inhibitor, cilostazol, can act on focal cerebral ischemia to significantly reduce the volume of cerebral infarction after ischemia-reperfusion injury in rats. In addition, in separated human neuroblastoma and human cortical neurons, cilostazol (Park *et al.* 2006) can suppress TNF- α growth, reduce DNA fragmentation degradation, and reduce neuronal necrosis. Lee (Lee *et al.* 2006) adopted a bilateral common carotid artery ligation model and found that cilostazol can reduce alba vacuolization and rarefaction by reducing the generation of alba TNF- α and the quantity of caspase-3 positive cells, and subsequently possesses a protective effect against nerve cell apoptosis and oxidative cell death. Cilostazol can also improve reperfusion of the ischemic region by continuously increasing cerebral blood flow and speed of the blood flow around the ischemic region, and reduce damage to the grey matter and substantia alba of the ischemic region (Honda *et al.* 2006).

Since the pathophysiological mechanisms of post chronic cerebral ischemic hypoperfusion damage are complicated, and the expression of the cyclin D1 gene is regulated by many interactional factors, further research should be conducted to investigate whether cilostazol plays a role in the direct pathway or other indirect pathways of suppression of the cyclin D1 gene expression in the cortex of the frontal lobe of rats in the chronic cerebral ischemic hypoperfusion damage model. Results of this study provide a reliably theoretical basis for the

treatment of chronic ischemic cerebral damage with the clinical application of cilostazol.

Conclusion

CyclinD1 was highly expressed in the frontal cortical area after chronic cerebral ischemia and the anti-apoptosis effect of cilostazol on neurons may be related to it inhibiting the expression of core apoptosis regulation gene CyclinD1, resulting in reducing nerve function impairment and improving learning and memory functions.

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Conflict of interest

All authors have no conflict of interest regarding this paper.

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Figure Legend

Figure 1 Expression of CyclinD1mRNA at Nine Weeks after Cilostazol Intervention. A Sham operation group; B Nine weeks of ischaemia; C Nine weeks of intervention.

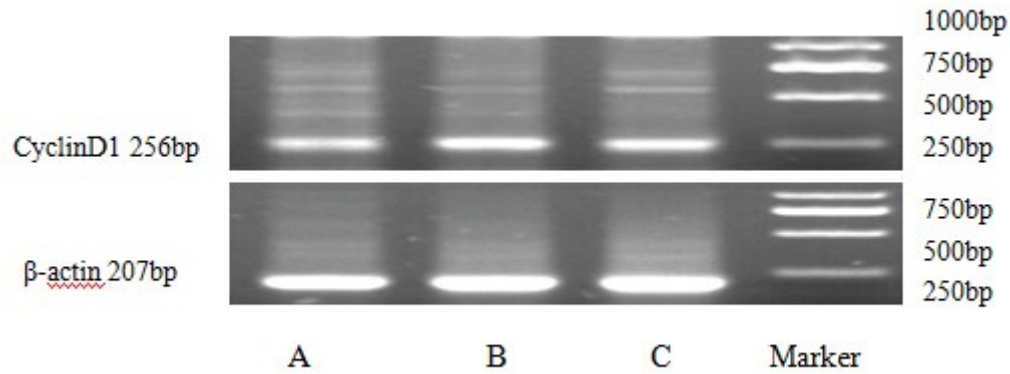


Table 1: Primer sequence, PCR amplification production fragment and annealing temperature.

The purpose gene	Upstream and downstream primer sequences 5'-3'	Product fragment	Annealing temperature
CyclinD1	Upstream primer 5'-GGTGAACCTT GGTCGGTGTCAACG-3' downstream primer 5'- GAGCCC TT CCACGATGCCAA -3'	256bp	55°C
β -actin	Upstream primer 5'- CACCCGCGAGTACAACCTTC-3' downstream primer 5'- CCC ATA CCC ACC ATC ACACC A-3'	207bp	60°C

Table 2 Escape Latency of Rats at Different Ischaemia Times (s).

Group	3 Weeks	6Weeks	9Weeks
Sham operation group	13.29±4.76	14.02±3.86	15.45±4.06
Ischemia group	29.16±6.43*	32.24±5.12*	36.65±7.85*
Cilostazol group	18.23±2.45* Δ	20.35±2.57* Δ	23.58±2.32* Δ

* $P < 0.05$ Compared with sham operation group; $\Delta P < 0.05$ Compared with ischemia group; Values expressed as mean \pm SD.

Table 3 Swimming path of Rats at Different Ischaemia Times (cm).

Group	3 Weeks	6 Weeks	9 Weeks
Sham operation group	648.25±97.54	423.72±56.31	235.68±32.48
Ischemia group	1345.61±246.28*	1531.72±198.63*	1892.45±302.41*
Cilostazol group	896.51±102.27* Δ	1043.39±103.58* Δ	1039.85±185.30* Δ

*P<0.05 Compared with sham operation group; Δ P<0.05 Compared with ischemia group; Values expressed as mean \pm SD.

Table 4 Expression of CyclinD1 mRNA at Nine Weeks after Cilostazol Intervention.

Compared with the control group *P<0.05 Compared with ischemic group Δ P<0.05 Values expressed as mean

Grouping	The number of cases	The ratio of OD (CyclinD1mRNA/ β -actin)
Sham operation group	10	0.2515±0.0417
Ischemia 9 weeks	10	0.5518±0.0785*
Cilostazol intervention group	10	0.4271±0.0493* Δ

\pm SD.