Original Article

The mechanism of 3-methoxy puerarin on decreasing the cerebral ischemia-reperfusion injury in rats

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The aim of this study was to explore the mechanism of 3-methoxy puerarin on decreasing the cerebral ischemia-reperfusion injury in rats. Before the model of cerebral ischemia-reperfusion injury was made, the rats in one group (3-methoxy puerarin group, 3-MP group) were pretreated with 3-methoxy puerarin (100mg/kg) by gavageing two times per day for seven days. At an hour before operation, the rats in the 3-MP group were additionally given 3-methoxy puerarin by gavageing once. The level of prostacyclin (PGI_2) and the expression of endothelin-1 (ET-1) mRNA in cerebral tissue, the activity of plasma tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) were measured. Cerebral tissue pathologic changes were also observed. The levels of PGI_2 in cerebral tissue and the activity of plasma t-PA in 3-MP group were significantly higher than those in the group of cerebral ischemia-reperfusion injury (CIRI group) ($p<0.01$). The activity of plasma PAI and the expression of ET-1 mRNA in cerebral tissue in 3-MP group were significantly lower than those in CIRI group ($p<0.01$). The cerebral tissue pathologic changes were significant in CIRI group, which were significantly ameliorated in the 3-MP group. The study showed, in the rat model of cerebral ischemia-reperfusion injury, 3-methoxy puerarin can not only increase the level of PGI_2 in cerebral tissue and the activity of plasma t-PA, but also inhibit the activity of plasma PAI and the expression of ET-1 mRNA in cerebral tissue. Those findings might be the mechanisms behind the protecting effects of 3-methoxy puerarin on the cerebral ischemia-reperfusion injury.

Key Words: 3-methoxy puerarin, cerebral ischemia-reperfusion injury, prostacyclin, endothelin-1

Introduction

Acute ischemic blood-stroke is a disease which severely influences the health of the peoples. Acute ischemic blood-stroke is characterized as higher incidence rate, higher mortality rate. It is very important to treat the disease during the acute and earlier period. Our previous study showed that 3-methoxy puerarin extracted from wild puerarin could decrease cerebral ischemia-reperfusion injury in rats. However, the mechanism of how 3-methoxy puerarin alleviate the injury is still unclear. The rat model of cerebral ischemia-reperfusion injury induced by the method of four-vessel occlusion was investigated by the treatment with 3-methoxy puerarin in the study.

The aims of this study were: (1) To investigate the mechanisms behind the protecting effects of 3-methoxy puerarin on cerebral ischemia-reperfusion injury in rats. (2) To investigate the thrombolytic effects of 3-methoxy puerarin on cerebral ischemia-reperfusion injury in rats.

Methods

Animals

30 healthy and pure male SD rats (10 month old, weighing 300g-350g) were purchased from animal Experimental Animal Center of Zhejiang Province.

Drugs and reagents

3-methoxy puerarin were made by our lab. Prostacyclin (PGI_2) assay kits were purchased from Jiancheng Bioengineering Company, Nanjing, China. Tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) assay kits were purchased from Taiyang Bioengineering Company, Fujian, China. Endothelin-1 (ET-1) gene probe and cardiox labeled probe kits were purchased from Rochs company.

Animal model

The model of cerebral ischemia-reperfusion injury were made according to Wurtman's[1] method of four-vessel occlusion, which were modified in this study. After anesthetized with injected 10% chloral hydrate intraperitoneally injection, the rats were fixed in ventral decubitus.

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Consequently, a sterile occiput incision on the neck was made to expose transverse process pterygoideus foramen of the first cervical vertebra. An electric coagulation needle was used to occlude bilaterally vertebral arteries in the foramen by the way of cauterization. Then, the fixation position of the rats was changed into the dorsum position. Cervical ventro-median incision was made to separate and expose bilateral common carotid arteries. The muscles and skin of neck were seamed after disinfection. The bilateral common carotid arteries were occluded with a miniature bulldog clamp at the next day. The rats in the normal control group (NC group) were treated by using similar operation except arteria vertebralis electric coagulation and common carotid artery occlusion. The criteria of successful model includes: (1) The rats were unconsciousness and quadriplegia. (2) Righting reflex and corneal reflex disappeared. (3) The frequency of EEG slowed down.

Study design

30 male SD rats were randomly divided into three groups: the NC group, the group of cerebral ischemia-reperfusion injury (CIRI group), and the group of cerebral ischemia-reperfusion injury treated with 3-methoxy puerarin (3-MP group). Every group included 10 rats. Before making cerebral ischemia-reperfusion injury, the rats in the 3-MP group were pretreated with 3-methoxy puerarin (100mg/kg) by gavageing two times per day for seven days. The rats in the NC group and CIRI group were treated with the same volume of normal saline by gavageing per day. At an hour before operation, the rats in the three groups were additionally given 3-methoxy puerarin or normal saline by gavaging once. In the CIRI group and 3-MP group, the common carotid artery was occluded 30 minutes, and then the artery clamps were removed for 30 minutes. After that, we took the blood from abdominal artery and immediately harvested the brain tissue. In the NC group, blood and brain tissue were harvested at 60 minutes after the operation.

Measurements and observations

Cerebral tissues of left hemisphere were taken for analysis. The level of PGI_2 in cerebral tissue, the activity of plasma t-PA and PAI were measured according to the instructions of the commercially available kit. The expression of ET-1 mRNA was assayed with the method of hybridization in situ staining. The main steps include: Right cerebral tissue were harvested and coronal sections (5μm thick) were made along the plane of optic chiasm. The sections were digested with proteolytic enzyme K at 37℃, pre-hybridized at 37℃ for 3h, and hybridized at 41℃ for 24h. And then, the sections were incubated respectively with confining liquid for 30 minutes at 37℃, with rat-anti-cardiox of biotinylation for 60 minutes at 37℃, and with peroxidase of biotinylation for 20 minutes at 37℃. After that DAB coloration reagent were added for coloration. Negative control sections were hybridized with 0.5mol/L PBS instead of the hybrid liquid containing probe. Five visual field observed at 400 amplification times of light microscopy were randomly selected. Leica Qwin image analysis apparatus (Leica, Germany) were used to evaluate positive reactants produced by hybridization in situ. Cerebral tissue pathologic changes were also evaluated. Left frontal region cerebral tissues were taken for making pathologic slides. After fixation and dehydration, the cerebral tissues were embedded with paraffin. 2μm thick pathologic slides of cerebral tissue were prepared and stained with HE. Cerebral tissue pathologic changes were evaluated by 400 amplification times of light microscopy.

Statistical analyses

All data were expressed as Mean ± SD ($\bar{x} \pm s$). Single analysis of variance was used and Newman-keuls method (SPSS 10.0) was adopted for comparisons between two data among groups. p values <0.05 were considered as significant.

Results and discussion

Table 1 shows the change of every parameter in the three groups. The levels of PGI_2 in cerebral tissue in CIRI group were significantly lower than those in NC group (p<0.01). The levels of PGI_2 in cerebral tissue in 3-MP group were significantly higher than those in CIRI group (p<0.01). There are no significant difference between the levels of PGI_2 in NC group and in 3-MP group (p>0.05). The activity of plasma t-PA in CIRI group were significantly lower than those in NC group (p<0.01). The activity of plasma t-PA in 3-MP group were significantly higher than those in CIRI group (p<0.01). The activity of plasma PAI in CIRI group were significantly higher than those in NC group (p<0.01). The activity of plasma PAI in 3-MP group were significantly lower than those in CIRI group (p<0.01). The expression of ET-1 mRNA in CIRI group were significantly higher than those in NC group (p<0.01). The expression of ET-1 mRNA in 3-MP group were significantly lower than those in CIRI group (p<0.01).

Table 1. Changes of PGI_2 the expression of ET-1 mRNA in cerebral tissue, plasma PAI and t-PA in three groups (mean ± SD)

Groups	n	PGI_2 (pg/ mg.protein)	t-PA (IU/ ml)	PAI (IU/ml)	ET-1 mRNA
NC	10	54.2±3.7	0.84±0.04	0.69±0.02	213±34
CIRI	10	22.3±2.2#	0.44±0.06#	0.93±0.03#	577±64#
3-MP	10	43.1±3.3*	0.69±0.04*	0.77±0.06*	429±52*

NC= normal control group, CIRI = the group of cerebral ischemia-reperfusion injury, PGI_2= prostacyclin, t-PA = tissue-type; plasminogen activator, PAI= plasminogen activator inhibitor, ET-1= endothelin-1; # p<0.01 compared with NC group; * p< 0.01 compared with CIRI group

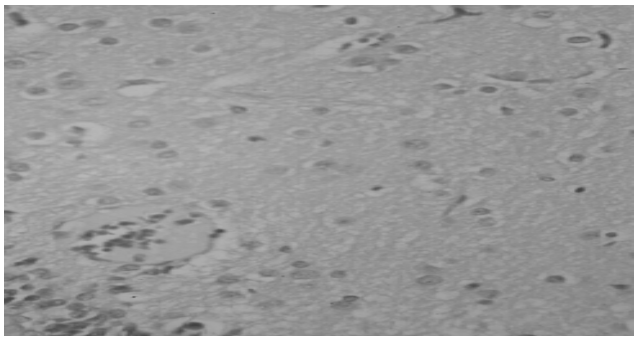

Figure 1. HE staining for the cerebral tissue from rats of NC group (×400)

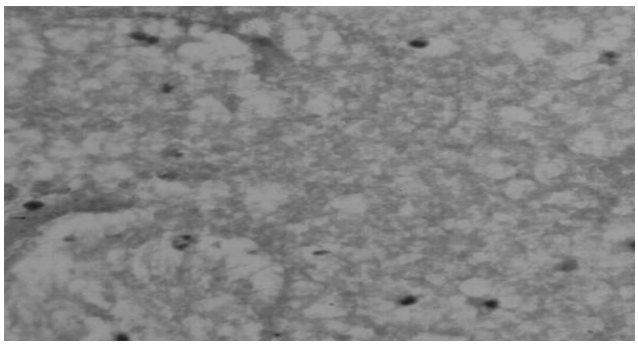

Figure 2. HE staining for the cerebral tissue from rats of CIRI group (×400)

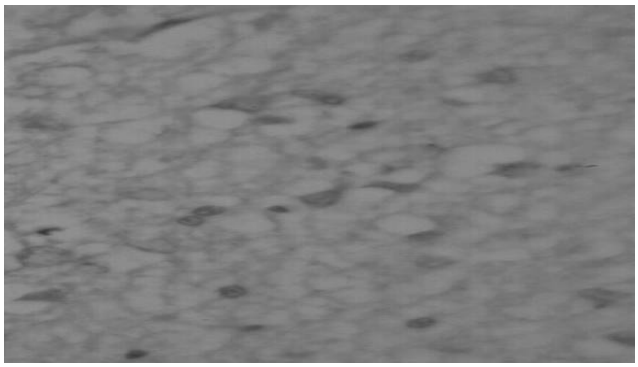

Figure 3. HE staining for the cerebral tissue from rats of 3-MP group (×400)

Figure 1, 2 and 3 showed the pathologic changes of cerebral tissue in the three groups. The pathological results of cerebral tissue by HE staining in NC group showed normal morphologic change. Compared to the normal control group, the pathological results in CIRI group demonstrated significantly ischemic appearance and increased presence of pyknosis neuroepithelial cell. The abnormalities above-mentioned were significantly alleviated in 3-MP group.

3-methoxy puerarin is disassociated and extracted from wild puerarin. 3-methoxy puerarin belongs to the osajin compounds. Recent reports have indicated that cerebral ischemia and reperfusion can lead to the changes of cerebral functions, metabolism, ultrastructure. In the course of cerebral ischemia-reperfusion injury, there are notable changes such as toxicity of free radicals, the level of cerebral tissue PGI_2, the activities of plasma t-PA and PAI etc. Our study investigated the effects of 3-methoxy puerarin on the above-mentioned indexes and analyzed the mechanism of 3-methoxy puerarin on decreasing the cerebral ischemia-reperfusion injury in rats.

The modern studies demonstrated that PGI_2 have the abilities to inhibit platelet agents and thrombogenesis. In the course of cerebral ischemia-reperfusion injury, PGI_2 is relatively deficient, which can cause spasm of cerebral vessel and the changes of vascular permeability, aggravate the cerebral ischemia, lead to cerebral edema and delayed ischemia-reperfusion injury. [2] Our study showed that 3-methoxy puerarin can increase the level of PGI_2. It indicates that 3-methoxy puerarin has the effect of resisting spasm of cerebral vessel and improving vascular permeability.

Plasma t-PA can initiate the physiologic plasminogen and eliminate the deposition of vascular wall. It has higher fibrin specificity and can clear away the unfitted thrombosis, which results in resisting and preventing disease. PAI is the quick inhibitor of t-PA. PAI has the dynamic equilibrium between active condition form and inactive condition form in plasma. PAI in active condition and free t-PA in the plasma can constitute the compound at the proportion of 1:1, which can inhibit the activity of plasminogen. PAI can regulate the activity of t-PA in the circulation. The activity of PAI can increase in the condition of hypercoagulation state and thrombotic disease.[3] That is to say, the disequilibrium of PAI/t- PA can lead to thrombotic disease or make our body in the hypercoagulation state and low plasminogen. Our study showed that 3-methoxy puerarin can markedly inhibit the activity of plasma PAI and increase the activity of plasma t-PA. It indicates that 3-methoxy puerarin has the effect of anticoagulants and antiplatelet agents.

ET-1 which was found in recent years is the regulated factor of blood flow in local organ.[4] It plays an important role in the thrombosis. Our study results show that the expression of ET-1 mRNA was significant increased in CIRI group. 3-methoxy puerarin can significant inhibit the expression of ET-1 mRNA. It indicates that 3-methoxy puerarin has the ability to regulate contraction and relaxation of vessel, inhibit thrombosis and alleviate ischemia-reperfusion injury.

This study showed that, in the rat model of cerebral ischemia-reperfusion injury, 3-methoxy puerarin can not only increase the level of PGI_2 in cerebral tissue and the activity of plasma t-PA, but also inhibit the activity of plasma PAI and the expression of ET-1 mRNA in cerebral tissue. Those findings might be the mechanisms behind the protecting effects of 3-methoxy puerarin on the cerebral ischemia-reperfusion injury in rats.

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