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Original Article

Effects of resveratrol glucoside on the recovery of motor function after focal cerebral ischemia-reperfusion injury in rats and its underlying mechanism

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Abstract: Objective: To investigate the effects of polydatin in Hu Huang Liniment for burns on the rehabilitation of motor function after focal cerebral ischemia-reperfusion injury in rats. Methods: A total of 80 rats were divided into a model group, control group, polydatin 1 (PD 1) group and PD 2 group; with 20 animals in eachgroup, followed by relevant treatment. Results: Cerebral infarction volume in PD 2 was < control group < PD 1 < model group (P < 0.05). PD 1 and 2 had lower moisture content in their brain tissue compared with the model and control groups, and PD 2 < PD 1 (P < 0.05). The average body weight in the control group gradually decreased from 2-6 days after modeling, and increased from 8 days after modeling, with obvious differences from that before modeling (P < 0.05). The average body weight in PD 1 and 2 decreased from 2-4 days after modeling with obvious differences from that before modeling (*P* < 0.05). Postural reflex scores of the control group and PD 1 and 2 at 2, 4, 6, & 8 days after modeling were lower than that in the model group, and PD 2 < PD 1 < control group (*P* < 0.05). Percentage of limb occupation at 4, 6, & 8 days after modeling gradually rose, among which the percentage in PD 2 was > PD 1 > control group > model group (P < 0.05). MDA, NO and SOD levels in the control & PD 1 and 2 groups were significantly different from those in model group; and the differences between PD 1 and 2 & control groups as well as between PD 1 and 2 groups were significant (P < 0.05). Conclusion: Polydatin in Hu Huang Liniment for burns may relieve the focal cerebral ischemia-reperfusion injury in rats, and effectively reduce the infarct size and edema, so as to improve the recovery of acute injured motor function.

Keywords: Hu Huang Liniment for burns, polydatin, focal cerebral ischemia in rats, reperfusion injury, acute injury, motor function

Introduction

At present, cerebrovascular disease is on the list of major types of diseases that threaten human life. The incidence of cerebrovascular in China has risen gradually, so are the number of deaths caused by it every year [1]. Clinical studies found that the occurrence of cerebrovascular diseases is positively correlated with age. Being preveland mainly in the elderly. Most patients, after timely active treatment, can be saved but may be unfortunately left with different functional disorders that require a long period of time to recover and the quality of rehabilitation varies with each individual [2].

Ischemic cerebrovascular diseases occur more frequently than hemorrhagic ones. In patients with cerebral ischemia, cerebral infarction due to arterial embolism accounts for more than half [3]. In clinical practice, patients with ischemic cerebrovascular disease are generally treated by methods based on the physiopathologic mechanism, yet conventional therapies cannot effectively rebuild the brain tissue structure or restore the neurological function [4]. So far, many studies have shown that plasminogen activator plays an important role in ischemic cerebrovascular disease, but it is only applicable to a small number of patients because of the strict therapeutic time window [5].

Neuroprotectants are crucial drugs for the treatment of cerebral ischemic diseases now. In this paper, polydatin, a stilbene compound contained in Polygonum cuspidatum, in Hu Huang Liniment for burns serves as a Chinese herbal medicinal ingredient and has been proved to play a role in protection of nerves by removing stasis or cold or dampness, and unblocking the meridians, etc [6].

There has been plenty of previous studies on the use of resveratrol in the treatment of nerve injury after cerebral ischemia/reperfusion in mice, and the effect of resveratrol on nerve injury after cerebral ischemia/reperfusion has been confirmed. However, previous studies have focused more on the repair of nerve injury. There are few studies and analyses on oxidative stress and rehabilitation of motor function, and the specific mechanism of resveratrol has not been fully clarified. Based on this, in the present study, 80 rats were used to find the effect of polydatin in Hu Huang Liniment for burns on the recovery of acute injured motor function in rats after focal cerebral ischemia-reperfusion injury, and to explore and analyze the underlying mechanism of actions.

Material and methods

Materials

A total of 80 clean and healthy male rats (about 210 g) provided by the Experimental Animal Center of our city were housed and fed in separate cages with a suitable environment.

Methods

All the animal experiments were carried out at Animal center of Chongqing Sanxia Central Hospital. Focal cerebral ischemia model construction: The rats were anaesthetized with 350 mg/kg chloral hydrate at 10% and fixed in a supine position. The skin was cut in the middle of the neck in order to bluntly separate the right common carotid artery, external carotid artery and internal carotid artery, being careful not to injure the nervi vagus. The common carotid artery and the external carotid artery were ligated followed by the dissociation of the pterygopalatine artery branch flowing through the internal carotid main artery until the extracranial end. A frog heart clip clamped the beginning of the pterygopalatine artery. At the common carotid artery and internal carotid artery branch, a small incision was created. Nylon angling line was covered at one end with polyurethane and wet with sodium heparin solution were put into the common carotid artery from the incision created close to the internal carotid artery and fed through until there was a sense of resistance (the length of about 18 cm). The head of the line slowly went through the area where the middle cerebral artery begins to the near-end where it further went to the thinner anterior cerebral artery creating interruption of blood flow coming from the ipsilateral internal carotid artery, anterior cerebral artery, and posterior cerebral artery. The right middle cerebral artery was occluded. After the rat woke up, the left forelimb showed a circle-drawing action as it bent and moved forward with the tail lifted up indicated the success of right middle cerebral artery occlusion.

Administration: Rats in the model group were given 6 mg/kg normal saline (Specification: 4.5 g:500 ml, Approval by H20113297, Manufactured by Shandong Qidu Pharmaceutical Co., Ltd.). Rats in the control group were given 6 mg/kg Edaravone injection (Specification: 20 ml:30 mg * 2 dose, Approval by H20080056, Manufactured by Sinopharm Group Guorui Pharmaceutical Co., Ltd.). Those in the polydatin group 1 (PD 1) were treated with 10 mg/kg polydatin (polydatin injection, yellow clear liquid, Manufactured by Shenzhen Neptunus Pharmaceutical Co., Ltd.) whilst those in the PD 2 group were treated with 40 mg/kg polydatin. Two hours after modeling, lines were pulled out to restore the ischemia reperfusion, and 1 hour later the rats in all groups were injected with drugs in the intravenously sublingually the day before acess to feeding.

Observed indicators

(1) Cerebral infarction volume: The rats were euthanized and their brains were removed at 24 hours after reperfusion for cryofixation and coronal slices of thickness of 2 mm (**Figure 1**). The brains were placed in 2% of freshly prepared TTC phosphate buffer and were stained for 10 minutes at 37°C. Red indicated normal brain tissue. White indicated infarction. Infarct size was measured by an image analysis system. Percentage of infarct volume: V% = (A1 + A2 + AN)/(B1 + B2 + BN), where A is the infarct

Figure 1. Results of brain tissue sections in the 4 groups. A: Model group; B: Control group; C: PD 1 group; D: PD 2 group.

size of each slice, B is the gross area of slice, and N is the number of slices.

(2) Moisture of brain tissue: The rats were euthanized at 24 hours after ischemia reperfusion to separate the brain tissue for wet weight. Then, they were dried in an oven at 100°C for 1 day before the collecting of dry weight. Moisture of brain tissue (%) = (wet weight - dry weight)/ wet weight * 100%.

(3) Rate of changes in body weight: Rate of changes in body weight = (body weight on that very day - body weight on the first day)/body weight on the first day * 100%.

(4) Posture reflex: At a distance of 1 meter from the ground, the tail of rat was lifted up. Then each rat was given 20 attempts of forelimb extension of which the average was taken. Posture reflex scores: 0, normal with

Table 1. Volume of cerebral infarction after focal cerebral ischemia reperfusion injury in rats ($\bar{x}$ ± s)

Groups	Cases	Volume of cerebral infarction (%)
Model	20	28.31±7.95
Control	20	14.55±5.42
PD 1	20	15.36±5.81
PD 2	20	11.64±4.19

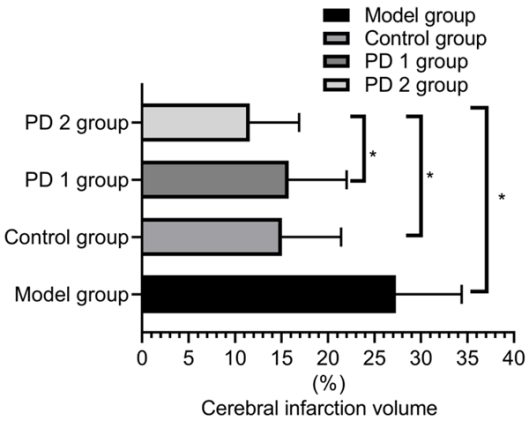

Figure 2. Cerebral infarction volume of rats in the 4 groups. The cerebral infarction volume in model group was the maximal one compared with the other groups ($P < 0.05$). That in PD 2 group was smaller than that in control group ($P < 0.05$) and PD 1 group ($P < 0.05$). * indicated $P < 0.05$ between groups.

both forelimbs fully stretchable; 1, slightly abnormal that the right forelimb is stretched but the left one sticks to the chest; 2, severely abnormal that the upper body curls and the left forelimb sticks to the chest [7].

(5) Experiment on asymmetry in limbs: A transparent glass cylinder of 30 cm in height and 18 cm in diameter was used. Normally, rat stands from time to time in the cylinder with forelimbs touching the wall simultaneously with the same frequency of use of both forelimbs. If the rat had ischemic brain injury, the affected limb will be used less used. A total of 20 tests of asymmetry in the limbs was performed. The 20 records produced an average that was used to calculate the percentage of limb use: = (R + L + B) * 100%, where B is the number of simultaneous use of both forelimbs, L is the number of independent use of the left affected forelimb, and R is the number of independent use of the right healthy limb.

Table 2. Moisture of brain tissue after focal cerebral ischemia reperfusion injury in rats ($\bar{x}$ ± s)

Groups	Cases	Moisture of brain tissue (%)
Model	20	65.86±3.25
Control	20	64.53±2.95
PD 1	20	61.95±2.48
PD 2	20	57.13±1.28

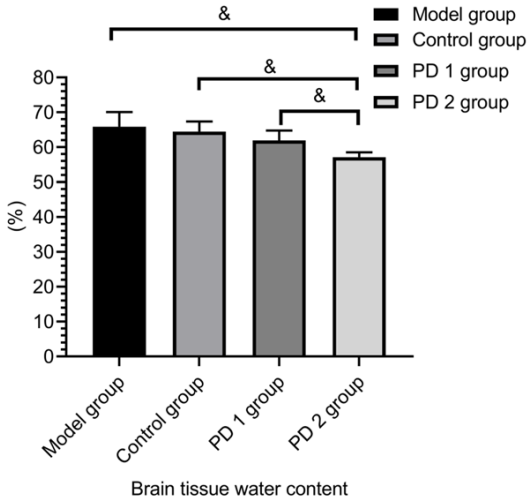

Figure 3. Brain tissue water content of rats in the 4 groups. The brain tissue water content in PD 2 group was smaller compared with that in model group ($P < 0.05$), in control group ($P < 0.05$), and in PD 1 group ($P < 0.05$). & indicated $P < 0.05$ between groups.

(6) Pheochromocytoma PC12 cell culture: PC12 cells were cryopreserved in liquid nitrogen that could be recovered when needed. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% of fetal bovine serum and were grown in conditions of 5% carbon dioxide at 37°C. As the bottom was covered with cell growth, cells were subject to trypsinization before discarding the culture solution and rinsing with PBS. Later, 0.125% of trypsin was used for 1-2 min, and then DMEM culture solution. Scattered cells were collected in centrifuge tubes and centrifuged at 800 rpm for 5 min. The supernatant was removed. From the resuspended cells in DMEM culture solution, a small amount was injected into the counting chamber under an inverted phase contrast microscope. The number of cells was recorded. Then, 5 * 10^5/ml cells were seeded into a 96-well culture plate for continued cultivation until a monolayer was observed.

Table 3. Polydatin 3 changes in body weight of rats with focal cerebral ischemia reperfusion injury ($\bar{x} \pm s$)

Groups	Cases	Variable	Before modeling	2 days after	4 days after	6 days after	8 days after
Model	20	Body weight (g)	210.40±1.15	190.86±1.28	188.62±1.43	186.35±1.12	182.02±2.31
		Rate of change (%)	-	-7.62±5.72	-10.53±10.49	-12.54±12.32	-12.06±13.35
Control	20	Body weight	208.32±1.39	182.13±1.25	185.32±1.46	184.46±1.23	191.46±1.42
		Rate of change	-	-9.59±3.62	-9.13±6.28	-9.45±7.25	-7.52±8.12
PD 1	20	Body weight	209.88±1.62	192.23±1.38	189.62±1.32	194.42±2.38	204.13±1.51
		Rate of change	-	-6.53±8.05	-4.15±11.32	-3.52±13.18	-1.69±14.72
PD 2	20	Body weight	213.75±2.49	193.16±2.19	185.44±2.35	202.32±2.54	211.32±2.49
		Rate of change	-	-8.46±5.37	-12.48±12.82	-13.34±13.89	-10.08±13.28

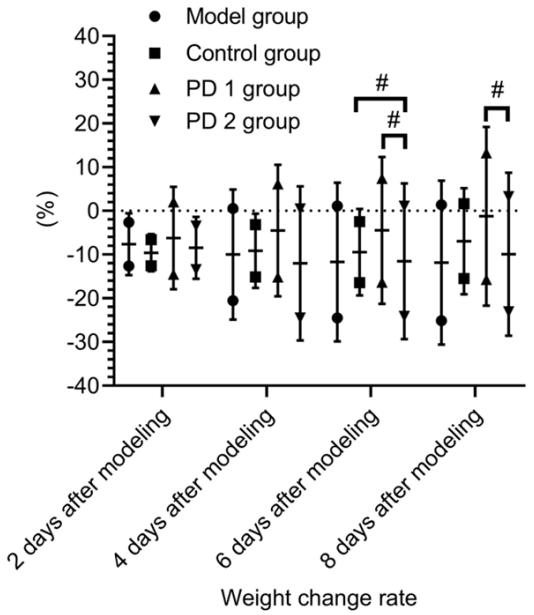

Figure 4. Rate of changes in body weight of rats in the 4 groups. At 6 days after the modeling, the rate of change in PD 2 group was superior to that in control group ($P < 0.05$) and PD 1 group ($P < 0.05$). At 8 days after modeling, the rate of change in PD 2 group was still higher than that in PD 1 group ($P < 0.05$). # indicated $P < 0.05$ between groups.

(7) Oxidative stress: Rats were reoxygenated for 24 hours and the culture medium was collected. Levels of malon dialdehyde (MDA), superoxide dismutase (SOD) as well as nitric oxide (NO) were detected following the instructions as specified in Kit User Guide.

Statistics

SPSS 22.0 was used for statistical analysis. Measurement data was expressed in mean ± standard deviation. *t* test was used for comparisons among groups. Enumeration data was showed by [n (%)]. Comparisons of results among groups were subject to chi-squared test, while within group was subjected to ANVOA, F test. $P < 0.05$ indicated a statistically significance.

Results

Cerebral infarction volume

Average volumes of cerebral infarction in the control, PD 1 and PD 2 groups were separately smaller than that in the model group ($P < 0.05$). There were little difference between PD 1 and control group ($P > 0.05$). Volumes in PD 2 were minor compared with that in control or PD 1 ($P < 0.05$) (**Table 1**; **Figure 2**).

Moisture of brain tissue

Little differences were found between model group and control group in terms of moisture in brain tissues ($P > 0.05$). Those in PD 1 and 2 were lower than the same in model or control group ($P < 0.05$). Between PD 1 and PD 2, the latter reported less moisture in brain tissue (**Table 2**; **Figure 3**).

Rate of changes in body weight

Before the modeling, there were little differences among the four groups in terms of body weight of the rats. Since 2 days after the modeling, the body weight of rats in model group notably decreased compared with that before the modeling until 8 days after modeling ($P < 0.05$). Body weight in control group also decreased until 6 days later but increased again as reported 8 days after modeling; both were significantly different from that before the modeling ($P < 0.05$). Those in PD 1 and 2 decreased until 4 days after with obvious differ-

Table 4. Postural reflex scores of rats with focal cerebral ischemia reperfusion injury ($\bar{x} \pm s$, points)

Groups	Cases	2 days after	4 days after	6 days after	8 days after
Model	20	1.42±0.49	1.37±0.34	1.30±0.50	0.90±0.31
Control	20	1.31±0.32	1.12±0.32	1.09±0.46	0.86±0.19
PD 1	20	1.28±0.46	1.07±0.30	1.03±0.64	0.82±0.43
PD 2	20	1.02±0.38	0.79±0.43	0.70±0.29	0.51±0.38

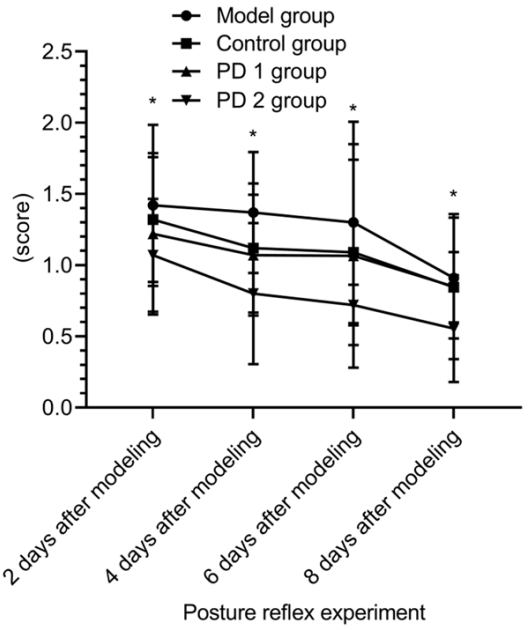

Figure 5. Postural reflex scores of rats in the 4 groups. PD 2 group showed significant lower scores of postural reflex than model or PD 1 group ($P < 0.05$ for both) at 2, 4, 6 & 8 days after modeling. Compared with the scores in model group, the other groups all reported inferior results 2, 4, 6 & 8 days after modeling ($P < 0.05$). * indicated $P < 0.05$ among groups.

ence from that before modeling ($P < 0.05$), but 6 days after modeling until 8 days after it increased instead. It was reported that at 6 days after modeling there was a visible difference from that before modeling ($P < 0.05$) in contrast to the results 8 days after modeling ($P > 0.05$) (**Table 3**; **Figure 4**).

Postural reflex tests

Postural reflex scores of control group and PD 1 and 2 groups at 2, 4, 6, & 8 days after modeling were smaller than that in model group ($P < 0.05$). Scores in PD 2 were also inferior to those in control and PD 1 groups at 2, 4, 6, & 8 days ($P < 0.05$). The differences between control group and PD 1 were not apparent ($P > 0.05$) (**Table 4**; **Figure 5**).

Experiments on asymmetry in limbs

Experiments on asymmetry in limbs showed little difference in percentage of limb use at 2 days after modeling among the 4 groups ($P > 0.05$). Percentage of limb use at 4, 6 & 8 days after modeling separately and gradually rose but the numerical value in the PD 1 and 2 and control groups were higher than that in model group ($P < 0.05$). Both PD 2 & 1 reported higher values than the control group also ($P < 0.05$). The number in PD 2 was superior to that in PD 1 ($P < 0.05$) (**Table 5**; **Figure 6**).

PC12 cell culture

Cells in a normal state grew fast. Two-3 days after inoculation, PC12 cells were found adherent by observation with an inverted microscope and most were trilateral or shuttle-shaped with clear boundaries. Plenty of cytoplasm was visible. Synapses connected with cells all thoughout into a strong network (**Figure 7A**). On the contrary, cells from the cerebral ischemia-reperfusion injury model had reduced dioptric strength and they shrank in size and disconnected (**Figure 7B**). PC12 cells from the model group reported similar cellular damage and morphological expression from pharmaceutical protection. The higher the strength of Polydatin, the less the cellular damage was, suggesting some morphological protection from the drugs.

Oxidative stress level

MDA and NO levels in the control & PD 1 and 2 groups were lower than those in model group but the SOD levels were higher ($P < 0.05$). MDA and NO levels in PD 1 and 2 groups were inferior to that in control group but the SOD levels were superior ($P < 0.05$). MDA and NO levels in PD 2 were reported as less than that in PD 1, but there was more SODs ($P < 0.05$) (**Table 6**).

Discussion

Several factors contribute to the pathological and physiological processes of focal cerebral ischemia by multiple actions, pathways and/or

Table 5. Experiments on asymmetry in limbs of rats with focal cerebral ischemia reperfusion injury ($\bar{x} \pm s$, %)

Groups	Cases	2 days after	4 days after	6 days after	8 days after
Model	20	26.45±3.16	28.75±2.15	30.34±1.75	31.43±3.29
Control	20	24.37±3.15	35.54±1.64	38.15±3.26	40.34±1.29
PD 1	20	26.35±2.12	42.51±2.13	46.16±2.48	48.16±2.35
PD 2	20	25.18±3.12	49.49±2.13	53.34±1.79	59.43±1.19

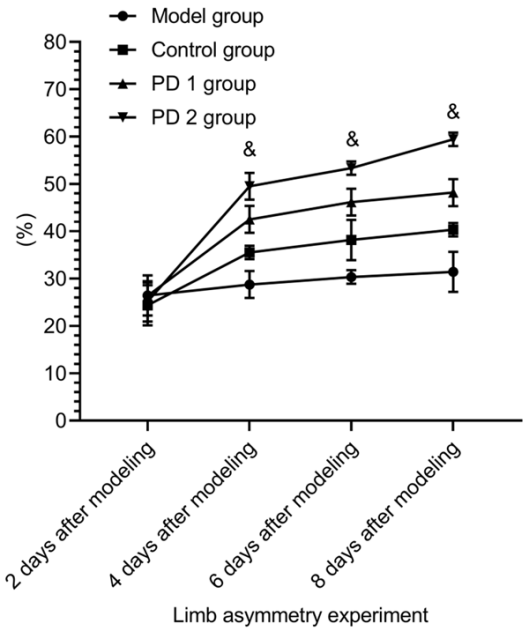

Figure 6. Experiment on asymmetry in limbs of rats in the 4 groups. At 4, 6 & 8 days after modeling, PD 1, PD 2 and control groups revealed superior percentage of limb use ($P < 0.05$) compared with model group, percentages in PD 1 & 2 groups were higher than that in control group ($P < 0.05$), and percentage in PD 2 group overtopped that in PD 1 group ($P < 0.05$). & indicated $P < 0.05$ among groups.

links. The middle cerebral artery is the most common position where cerebral ischemia occurs. Hence, study on the middle cerebral artery occlusion is of practical significance for the treatment of focal cerebral ischemia [8, 9]. In this paper, rats were used to build cerebral ischemia models because of the fact that they have similar cerebrovascular structures to humans in that the internal carotid artery and the vertebrobasilar system join in the arterial ring at the base and then delivers blood through its branches to the brain [10, 11]. The rat models were created by the thread approach which did not require craniotomy or bring obvious trauma and is highly applicable to the cerebral ischemia-reperfusion model building. In this method, it simulates the pathological process of ischemic cerebrovascular disease with better goodness of fit; it also helps evaluation of the cerebral protection of drugs used after cerebral ischemia-reperfusion [12, 13].

As the results showed, both infarct volume and moisture in brain tissue of rats in PD 1 & 2 groups were smaller than those in the model group, in addition to those values in the PD 2 group being less than that in PD 1 ($P < 0.05$), indicating that polydatin reduced the infarct size and cerebral edema in rats after focal cerebral ischemia-reperfusion injury, and that the extent of reduction increased with the dosage, which hinted a dosage association of polydatin with the effects on infarct size. The higher the dosage is, the more obvious the reduction of infarct volume and cerebral edema became. All the effects above have confirmed that resveratrol has a neuroprotective effect, and the mechanism of action in this aspect is similar to that of ischemic preconditioning, and the neuroprotective effect can be achieved by activating the SIRT1 signaling pathway. Moreover, preconditioning induced by resveratrol can activate the signal transduction pathway of neuroprotection, which can help prevent the recurrence of the fatal damage caused by cerebral ischemia. The body weight of rats in model group decreased after the modeling. Body weight of rats in the other groups decreased for a while and increased, where it was much more noticeable of the increase in PD 1 & 2 groups of which the latter reported the maximum increase. These results indicated that polydatin controlled the weight loss of rats after cerebral ischemia-reperfusion injury, and the higher the dosage was, the more specific the control on weight loss would be. Regarding postural reflex tests, the results showed that scores in the model group were superior to those in the other three groups at 2, 4, 6, & 8 days after modeling, being contrary to that in percentage of limb use. Scores in PD 2 at 2, 4, 6, & 8 days after were inferior to those in PD 1 but percentage of limb use was contrary superior ($P < 0.05$). These explained that polydatin improved the limb function of rats with focal cerebral ischemia reperfusion injury, and the higher the dosage was, the better the improvement in limb

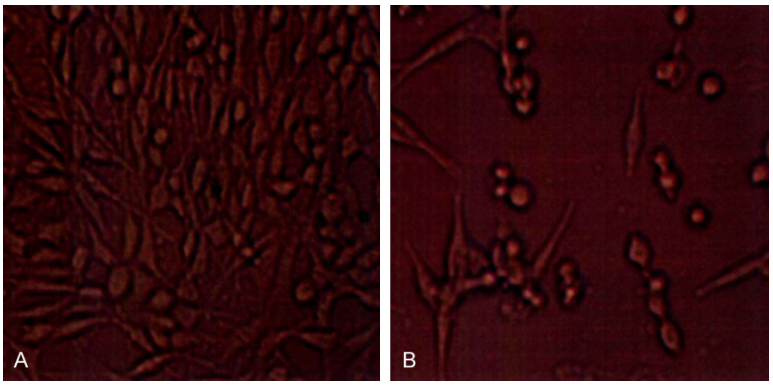

Figure 7. Results of PC12 cell culture. A: Observations under microscope 3 days after PC12 cell inoculation; B: Observations under microscope after oxygen and glucose deprivation of PC12 cells.

Table 6. Oxidative stress after oxygen and glucose deprivation in PC12 cells ($\bar{x} \pm s$)

Groups	Cases	MDA (nmol/mg)	SOD (U/mg)	NO (μmol/g)
Model	20	11.21±1.13	16.72±0.32	42.16±0.92
Control	20	9.46±0.53	17.45±0.23	31.26±2.18
PD 1	20	7.66±0.75	18.54±0.36	25.49±1.18
PD 2	20	7.10±0.49	19.60±0.18	22.43±2.14

function was, that is, polydatin may improve the physical impairment of these rats on a dose-dependent basis.

Antioxidase SOD catalyzes the formation of superoxide anion free radicals and induces disproportionation reaction which blocks the toxic actions of free radicals that offers favorable protection of cells [14, 15]. Oxygen radicals damage the cell membrane structures and release a large amount of lipid peroxides leading to the final product of MDA. MDA levels reflect the extent to which lipids are peroxided [16, 17]. In cerebral ischemia-reperfusion injury, NO plays roles in protection of the nerves and safeguards cytotoxicity [18]. Following the occurrence of cerebral ischemia, MDA and NO increase remarkably whilst SOD level decreases instead. Controlling to these indicators of oxidative stress would be beneficial to conditions of cerebral ischemia-reperfusion injury [19]. In this paper, MDA and NO in PD 1 and 2 were less than those in control group, contrary to that SOD in PD 1 and 2 higher than that in control group ($P < 0.05$). Besides, there were significant differences between the PD 1 and PD 2 ($P < 0.05$). These suggested polydatin modified oxidative stress after focal cerebral ischemia-reperfusion injury, and the higher the dosage was, the more obvious the improvement could be. Regarding the anti-oxidation mechanism of polydatin, analysis shows that the mechanism includes neurohormonal pathways and pretreatment pathways. These pathways include cell signal transduction. When these pathways are activated, the antioxidant capacity can be improved to elevate cell tolerance, improve neurotrophic factor levels, thus playing a good neuroprotective function. Polydatin is extracted from the dried rhizomes of Polygonum cuspidatum, a perennial herbaceous plant of Polygonum, Polygonaceae. It is white powder and should be called a styrylbenzenol compound similar to resveratrol as for its biological activity. It can be broke down into resveratrol in the intestinal canal by glycosidases. In view of the fact that polydatin is composed of 3 phenolic hydroxyl groups, it has been looked upon by some scholars as a oxygen free-radical scavenger and is often used in traditional Chinese medicine for invigorating the blood circulation to remove blood stasis [20, 21]. While in modern pharmacological studies, polydatin offers other efficacies such as conditioning female hormones, antioxidants, cancer, cell apoptosis, and inflammation [22]. From other research on polydatin, the impact on the cardiovascular system is considered to be the most crucial. It was found to be capable of inhibiting abnormal expression of tissue factors and the cytoplasm in vascular cells, lowering the risk of cardiovascular diseases, as well as contributing to the protection of cardiovascular function by actions to lipid metabolism [23].

In summary, polydatin in Hu Huang Liniment for burns can distinctly ameliorate the behavioral symptoms caused by cerebral ischemia-reperfusion injury in rats, reduce cerebral edema and infarct size on the affected side, and improve motor function. Nevertheless, the resu-

Its in this study are based on animal experiments may not be representative considering the nature of animal experiments, only a few groups were established and only 2 schemes of dosage were used. More clinical studies using more groups and more schemes of dosage to confirm the values of polydatin in Hu Huang Liniment for burns in the treatment of focal cerebral ischemia-reperfusion injury are warranted.

Disclosure of conflict of interest

None.

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