Neuroprotective Effects of Hydroxyethylpuerarin against Focal Cerebral Ischemia-Reperfusion in Rats

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Abstract

Our present study was performed to investigate whether hydroxyethylpuerarin (HEP) has a neuroprotective effect on brain injury after focal cerebral ischemia/reperfusion by middle cerebral artery occlusion (MCAO) in adult male Wistar rats. Animals were subjected to one hour of middle cerebral artery occlusion and 48 hours of reperfusion with the pretreatment of drugs (HEP 15, 30, 60 mg/kg or nimodipine 0.4 mg/kg i.v.) or vehicle. The behavioral tests were used to evaluate the damage to central nervous system. The percentage of brain infarct area was assessed in the brain slices stained with 2% solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC). The pathologic histological changes were observed by H&E staining and the occurrence of apoptosis was determined by flow cytometry. The results showed that pretreatment with HEP at doses of 15, 30, 60 mg/kg exhibited significant neuroprotective effects on rats against focal cerebral ischemia-reperfusion injury by markedly decreasing neurological deficit scores and the percentage of infarct area, reducing necrosis and apoptosis of neurons. All these findings suggest that HEP might provide neuroprotection against focal cerebral ischemia/reperfusion injury probably through its antioxidant and anti-inflammatory property.

Key Words: hydroxyethylpuerarin, cerebral ischemia/reperfusion injury, neuroprotection, anti-oxidation, anti-inflammation

Introduction

Stroke is the third leading cause of death and the most important source of disability, among which cerebral ischemia stroke represents about 85% of all (18). Thrombolytic therapy is used to achieve the most important objective in arterial occlusion in order to get quickly restore flow after occurrence of an acute occlusion. But the possibility exists that restoration of blood circulation can result in further damage in the brain. With the expanded use of thrombolytic agents in clinical stroke trials, reperfusion injury is a fact that should be taken into consideration whenever we try to reestablish blood flow to a previously ischemic part of the brain. The theory of reperfusion injury can explain the surprising fact that sometimes permanent ischemia is better tolerated than severe transient ischemia (3, 9). Therefore, it is important to find out neuroprotective drugs against cerebral ischemia/reperfusion injury.

Cell death characterized injury by MCAO model can be divided into two broad categories: early necrotic death of cells in the ischemic core (mainly in the cortex) and delayed death of susceptible neurons in other neighboring regions (mainly in the hippocampus). Hippocampal cell death occurs over an extended time, these neurons have the potential to be rescued by pharmacological agents (5). Moreover, the hippocampal neurons are known to play an important role in learning and memory processes (8). Isoflavine has been demonstrated prossessing the effect of improving learn and memory with some neurodegeneration models (4).

Hydroxyethylpuerarin (HEP, 8-C-β-D-glucopyranosyl- 7,4- dihydroxyethylaxyisoflavone, Compound N-2035, see Fig. 1), was extracted from

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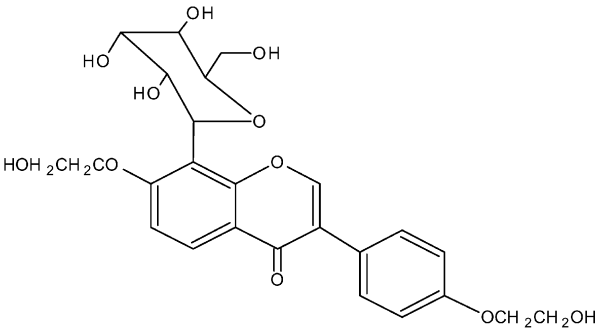

Fig. 1. Chemical structure of hydroxyethylpuerarin (HEP, 8-C-β-D-glucopyranosyl- 7,4- dihydroxyethylaxyisoflavone, MW 504).

the dried root of Chinese traditional medicine, *puerariae radix*, then structurally modified for the purpose of increasing the blood brain barrier (BBB) permeability. Our previous studies have shown that hydroxyethylpuerarin could exhibit significant anti-oxidant and anti-inflammatory effects against central nervous system injury by using animal models and cultured brain cells. (2, 7, 17, 21).

The present study was undertaken to test whether pretreatment of hydroxyethylpuerarin, a kind of isoflavine would prevent or diminish tissue damages of both the cortex and the hippocampus caused by focal brain ischemia/reperfusion in rats.

Materials and Methods

Drugs and Reagents

Hydroxyethylpuerarin used in this study was provided by Professor. Chunxu Zuo (Institute of Medica, Shandong Academy of Medical Science, Jinan, Shandong, P.R. China, Lot: 020604). Firstly, puerarin, which served as one of the marker compounds to characterize the *pueraria radix* isoflavone was extracted from *Puerariae radix*, one of the earliest used Chinese traditional drug. Then hydroxyethylpuerarin was obtained by modifying on the basic structure of puerarin. The purity of hydroxyethylpuerarin is more than 97% determined by HPLC. The powder was dissolved in normal saline (NS, pH7.4) by heated to 70-80°C. Nimodipine was from Bayer Company (Bayer AG, Wuppertal, Germany). All chemicals used in this experiment were of analytical reagent grade.

Animals

Adult male Wistar rats weighing 300 ± 30 g purchased from Medical Experimental Animal Center, Shandong University (Jinan, Shandong, P.R. China, clean grade, Certificate No: SCXK 2001003), were housed under a 12-h light/dark cycle (lights on from 7:00 to 19:00) in an air-conditioned (temperature 23 ± 2°C) with the relative humidity of 50% ± 10% animal breeding room. All animals had free access to food and water. Rats were randomly divided into 6 groups, sham group (sham-operation + vehicle, A), FCIR group (focal cerebral ischemia/reperfusion + vehicle, B), hydroxyethylpuerarin groups (FCIR + hydroxyethylpuerarin 15, 30, 60 mg/kg; C, D and E) and nimodipine group (FCIR + nimodipine 0.4 mg/kg as positive control (20), F). Rats received drug treatments or vehicle (normal saline) *via* tail-vein injection approximately 30 min before the onset of ischemia.

Focal Cerebral Ischemia-Reperfusion Model

Animals were fasted overnight but were allowed free access to water before surgery. Transient focal cerebral ischemia was induced by using the filament model as previously described by Longa (6) with a little modification. In brief, each rat was anesthetized with an injection of chloral hydrate in normal saline (350 mg/kg) intraperitoneally and placed in dorsal recumbency. The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) of the rat were carefully isolated through a midline incision in the neck. A nylon suture (diameter 0.24 mm) with its tip rounded by heating over a flame (enlarge the diameter to 0.35 mm) was inserted from the lumen of the ECA to that of the ICA until a mild resistance was felt (about 18 to 19 mm from the bifurcate of CCA). Thereby the origin of the left middle cerebral artery (MCA) was occluded. The neck incision was closed, and the rats were allowed to recover. After one hour of MCA occlusion (MCAO), the suture was carefully removed to restore blood flow. During and after the surgery, a heating pad and a heating lamp were used to maintain the rectal temperature between 36.5°C to 37.5°C until the complete recovery of the animal from the anesthesia. The animals were housed individually until completely palinesthesia. All animals had free access to food and water after awaking from anesthesia. The focal cerebral ischemia/reperfusion model was induced by this procedure. In the sham group, animals were prepared in the same procedure except for the insertion of the nylon suture into the left internal carotid artery.

Neurological Evaluation

Neurological deficit scores were performed in both the vehicle and drug-treated groups after 48 hours of reperfusion by a single experimenter, who was blinded to the experimental treatment groups, according to Longa method (6). Neurological findings were scored on a 5-point scale. The criterion of the

Table 1. Effects of hydroxyethylpuerarin on neurological score in rats injured by cerebral ischemia/reperfusion

	groups					
	A	B	C	D	E	F
Median	0	3	1	1	1	1
Interquartile range	0-0	2-3.25$^{\#\#}$	1-2**	1-2**	1-2**	1-2**

The neurological deficits were recorded after 1 h ischemia followed by 48 h of reperfusion. Neurological deficit scores are expressed as medians and inter-quartile ranges (25th to 75th percentiles).
A (sham-operated received vehicle, i.v.),
B (focal cerebral ischemia-reperfusion (FCIR) received vehicle. i.v.),
C (FCIR received hydroxyethylpuerarin 15 mg/kg, i.v.),
D (FCIR received hydroxyethylpuerarin 30 mg/kg, i.v.),
E (FCIR received hydroxyethylpuerarin 60 mg/kg, i.v.)
F (FCIR received nimodipine 0.4 mg/kg, i.v.)
$^{\#\#}P < 0.01$ for comparison with A analyzed by Kruskal-Wallis H nonparametric test followed by Mann-Whitney test;
$^{**}P < 0.01$ for comparison with B analyzed by Kruskal-Wallis H nonparametric test followed by Mann-Whitney test.

evaluation was as follows: no observable neurological deficit = 0, failure to extend right paw fully = 1, circling to right when walking = 2, falling to right = 3, couldn't walk spontaneously and had depressed levels of consciousness = 4. Animals were sacrificed by rapid decapitation under pentobarbital sodium (30 mg/kg i.p.) anesthesia after neurological evaluation. The brains are immediately removed for other analysis.

TTC Staining

After frozen at -20°C for 30 min, each brain was sliced into six 3-mm-thick coronal sections. Each slice was incubated in the dark at 37°C for 30 min in a solution of 2% 2, 3, 5 - triphenyl tetrazolium chloride (TTC, Sigma Chemical Co, St. Louis. MO., USA) which was dissolved in phosphate buffer solution (PBS, pH 7.4) and then photographed by a digital camera (Olympus, Tokyo, Japan) (19). Infarct brain was identified as an area of unstained tissue (white) while the living tissue was red. The percentage of infarct cross-sectional area of the whole ipsilateral hemisphere was analyzed by a person who was unaware of the treatments, using a medical morphology image analysis system (Jiangsu Jieda Science and Technology Development Co. Nanjing, Jiangsu, P.R. China).

Flow Cytometric Analysis

The hippocampus was removed and then made into single-cell-suspension. After fixed in 75% ethanol at 4°C overnight, cells were stained with 10 μg/ml propidium iodide (PI, Sigma Chemical Co, St. Louis. MO., USA) solution and incubated at 37°C for 30 min in the dark. Using FACScan flow cytometry (Becton Dickinson, San Joes, CA, USA), 2×10^4 cells were counted and the percentage of apoptotic cells was determined with a computerized analysis system. (ModiFit, Becton Dickinson).

Pathologic Histological Analysis

Forty-eight hours after reperfusion, animals of each group were anesthetized with pentobarbital sodium (30 mg/kg i.p.) and transaortically perfused with 200 ml NS, followed by 4% paraformaldehyde dissolved in PBS (pH 7.4, 4°C). The left hemispheres were isolated and then fixed in the same fixative for 24 to 48 h. After being cut into successive coronal sections (4 μm) by paraffin embedded sectioning, brain tissue was stained with hematoxylin and eosin (H&E). The pathologic histological changes of the pyramidal cells in the CA_1 region of hippocampus were observed through a light microscope (Olympus, Tokyo, Japan) at 200 × magnification and photographed.

Statistical Analysis

Statistical analysis was performed with SPSS 11.0 for windows. Neurological deficit scores were reported as medians and interquartile ranges (25th to 75th percentiles). The neurological scores were analyzed with the Kruskal-Wallis test when >2 groups were analyzed and then with the Mann-Whitney test when 2 groups were compared. Other data are expressed as mean ± SD and analyzed by one-way ANOVA. *P* value of less than 0.05 was accepted as denoting a statistically significant difference.

Results

Neurological Evaluation

All animals had scores of zero prior to anesthesia

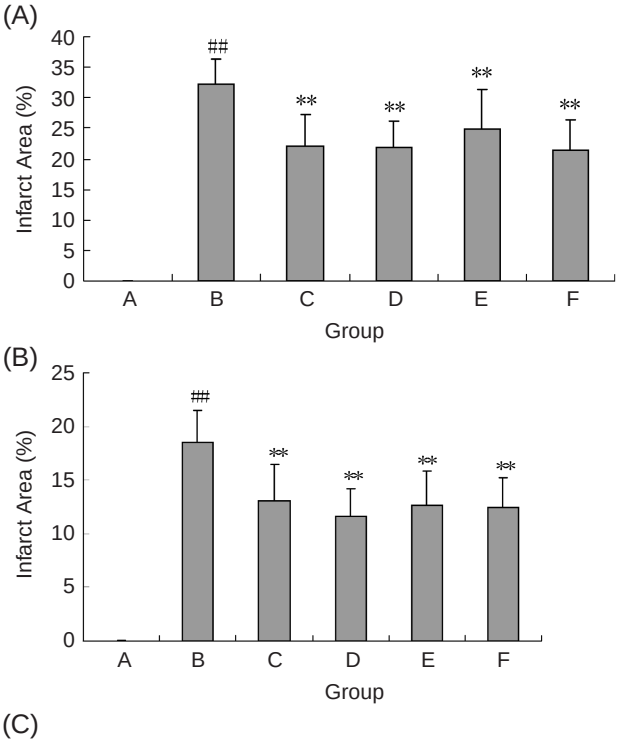

Fig. 2 (A) Effects of hydroxyethylpuerarin on percentage of brain infarct area in rats injured by cerebral ischemia/reperfusion. (B) Effects of hydroxyethylpuerarin on percentage of cortex infarct area in rats injured by cerebral ischemia/reperfusion. (C) Representative TTC-stained brain sections of focal cerebral ischemia/reperfusion rats received hydroxyethylpuerarin or vehicle. From top to bottom, each row represents the typical series of brain slices arranged rostral to caudal. Values are expressed by mean ± SD; $^{\#\#}P < 0.01$ for comparison with A analyzed by one way ANOVA; $^{**}P < 0.01$ for comparison with B analyzed by one way ANOVA. A (sham-operated received vehicle, i.v.), B (focal cerebral ischemia/reperfusion (FCIR) received vehicle, i.v.), C (FCIR received hydroxyethylpuerarin 15 mg/kg, i.v.), D (FCIR received hydroxyethylpuerarin 30 mg/kg, i.v.), E (FCIR received hydroxyethylpuerarin 60 mg/kg, i.v.), F (FCIR received nimodipine 0.4 mg/kg, i.v.)

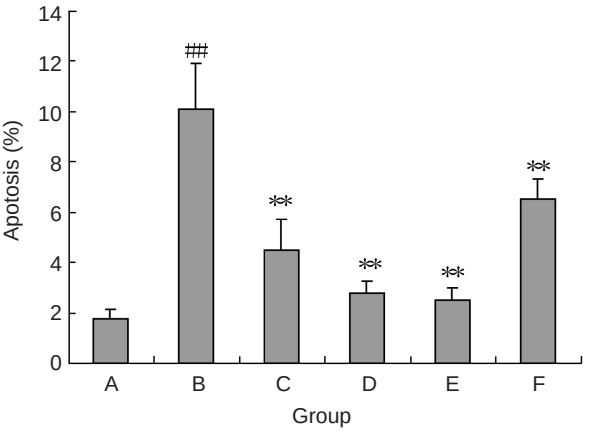

Fig. 3. Effects of hydroxyethylpuerarin on percentage of apoptosis of hippocampal cells in rats injured by cerebral ischemia/reperfusion. Values are mean ± SD; $^{\#\#}P < 0.01$ for comparison with A analyzed by one way ANOVA; $^{**}P < 0.01$ for comparison with B analyzed by one way ANOVA. A (sham-operated received vehicle, i.v.), B (focal cerebral ischemia-reperfusion (FCIR) received vehicle, i.v.), C (FCIR received hydroxyethylpuerarin 15 mg/kg, i.v.), D (FCIR received hydroxyethylpuerarin 30 mg/kg, i.v.), E (FCIR received hydroxyethylpuerarin 60 mg/kg, i.v.), F (FCIR received nimodipine 0.4 mg/kg, i.v.)

or MCAO. After one hour of ischemia followed by forty-eight hours of reperfusion, the range and frequencies of neurological scores for different groups are shown in Table 1.

Percentage of Infarct Area

Using computer-assisted image analysis, the percentages of infarct area of both the whole brain and the cortex significantly increased in FCIR group when compared with sham group. They were decreased in all drug-treated groups while compared to FCIR group (as shown in Fig. 2, A and B). The typical serial brain slices stained with TTC are demonstrated in Fig. 2C.

Flow Cytometric Analysis

After one hour of ischemia followed by 48 hours of reperfusion, hippocampal cells showed a higher apoptosis rate compared with that of the sham group. Hydroxyethylpuerarin can significantly decrease the percentage of apoptotic cells compared with FCIR group (See Fig. 3).

Pathologic Histological Evaluation

Sections stained by H&E demonstrated that one hour ischemia followed by 48 h of reperfusion caused neural cell damage in the hippocampal CA_1 region. In

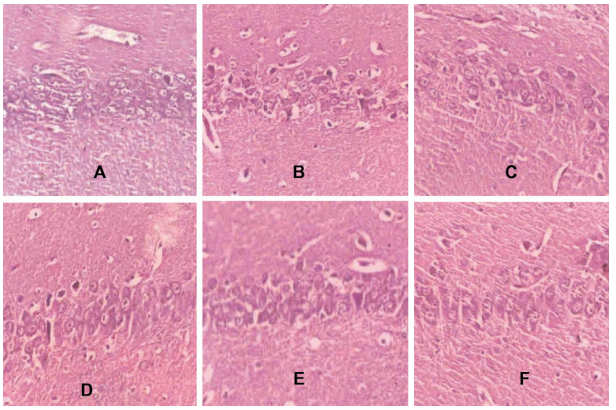

Fig. 4. Representative photomicrographs of H&E stained hippocampal CA_1 regions of focal cerebral ischemia/reperfusion rats received hydroxyethylpuerarin or vehicle. After 60 min of focal cerebral ischemia followed by 48 h of reperfusion, significant cell loss could be seen in hippocampal CA_1 regions of FCIR group. Hydroxyethylpuerarin and nimodipine could diminish the cell loss (H&E × 200). A (sham-operated received vehicle, i.v.), B (focal cerebral ischemia-reperfusion (FCIR) received vehicle, i.v.), C (FCIR received hydroxyethylpuerarin 15 mg/kg, i.v.), D (FCIR received hydroxyethylpuerarin 30 mg/kg, i.v.), E (FCIR received hydroxyethylpuerarin 60 mg/kg, i.v.) F (FCIR received nimodipine 0.4 mg/kg, i.v.)

contrast, hydroxyethylpuerarin treatment conferred neuroprotection by markedly reducing the numbers of damaged neural cells in the CA_1 subfield. The typical sections stained with H&E are demonstrated in Fig. 4.

Discussion

Cerebral ischemia/reperfusion leads to brain injury through a complex series of pathophysiological events leading to neuronal death and subsequent neurological dysfunction. Among which, reactive oxygen species (ROS) and inflammatory theory are well accepted nowadays. There is considerable evidence in favor of the roles of oxygen free radicals and inflammation as important contributors to cell damage in ischemic/reperfusion brains (1, 12). Acceptance of the ROS and inflammatory theory has contributed to an increasing interest in the use of free radical scavengers and anti-inflammatory agents as potential neuroprotective agents.

Hydroxyethylpuerarin is a new member of the isoflavone family. Isoflavones are a group of naturally occurring compounds with a wide range of biological and pharmacological properties including anti-oxidation (16), anti-inflammation (11), anti-cancer (14) and anti-osteoporosis (10) effects. In recent years, special attention is paid to their cardiovascular effects (15). Moreover, recent evidence from clinical and experimental studies supports that isoflavone can play an important role on neurodegenerative diseases (4).

In this study, we used a model of transient focal cerebral ischemia in rats as a way of reproducing clinical situations since reperfusion can occur spontaneously through resolution of an embolus or clinical intervention, and of evaluating pharmacological neuroprotection against the deleterious effect of reperfusion injury.

There are several measurements for the evaluation of brain damage after focal ischemia/reperfusion. In this study, behavioral test was used to evaluate the damage in central nervous system, and TTC staining was used to get morphological evidence of cellular death. In addition, the pyramidal neurons in the CA_1 subfield of the hippocampus which are known to be the most vulnerable were observed to cerebral ischemia. The neural apoptosis of the hippocampus was detected by flow cytometric analysis for the evidence that in cerebral ischemia situation of rat models, apoptotic cell death may occur in addition to necrosis (13). All these measurements show that compared with sham group, animals suffered with an obvious brain damage after one hour of cerebral ischemia and 48 hours of reperfusion. And this phenomenon can be significantly reversed by hydroxyethylpuerarin pretreatment. These data suggested that intravenous pre-administration of hydroxyethylpuerarin resulted in a significant reduction of the neural damage in a MCAO model, with one hour ischemia and 48 h of reperfusion.

Our previous study demonstrated that hydroxyethylpuerarin could exhibit significantly anti-oxidant effect against cerebral ROS injury either *in vivo* or *in vitro*. The results showed that hydroxyethylpuerarin could protect both cultured bovine cerebral microvascular endothelial cells and rats' brain astrocytes from damages induced by hydrogen peroxide (2, 21) and can exhibit significant anti-oxidant effects after focal brain ischemia/reperfusion injury in rats using a reversible MCAO model (17). Lou *et al*. also found that HEP could inhibit neutrophil-mediated inflammatory response after brain ischemia reperfusion in rats (7).

These findings may partly explain the mechanisms of its neuropretective effects. The exact mechanism by which hydroxyethylpuerarin can prevent the cerebral ischemia and reperfusion-induced injury remains to be determined by further study.

It is concluded that the results of our studies indicate that intravenous pre-administration of hydroxyethylpuerarin exhibits neuroprotective effects against focal cerebral ischemia/reperfusion injury in rats. The mechanism of effect is possibly contributed to its effective anti-oxidative and anti-inflammatory activity. These present findings may have an important implication in the future treatment of stroke patients.

Acknowledgments

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