Effect of *Cyperus rotundus* on ischemia-induced brain damage and memory dysfunction in rats

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ARTICLE INFO	ABSTRACT
	Objective(s): Global cerebral ischemia-reperfusion injury causes loss of pyramidal cells in CA1 region of hippocampus. In this study, we investigated the possible neuroprotective effects of the ethanol extract of *Cyperus rotundus* (EECR) on a model of global transient ischemia in rat, by evaluating the pathophysiology of the hippocampal tissue and spatial memory. ***Materials and Methods:*** Treatment group (EECR, 100 mg/kg/day) was gavaged from 4 days before, to 3 days after ischemia. Morris water maze test was performed 1 week after ischemia for 4 days. Brain tissue was prepared for Nissl staining. ***Results:*** Our data showed no statistical difference between the treatment and ischemia groups in water maze task. So, treatment of ischemia with EECR cannot improve spatial learning and memory. On the contrary EECR ameliorated the CA1 pyramidal cell loss due to transient global ischemia/ reperfusion injury. ***Conclusion:*** These results suggest that EECR cannot reduce the ischemia-induced, cognitive impairments seen after transient, global cerebral ischemia but can prevent pyramidal cell loss in CA1 region of hippocampus.

Introduction

Global Cerebral ischemia-reperfusion injury (GCIRI) is one of the most important causes of death in the developed countries (1, 2). It is well-known that hippocampus, especially CA1 area, is vulnerable during global cerebral ischemia, therefore, learning and memory impairments may occur in many of these patients due to hippocampal damage (3–7) and despite many efforts, there is no effective approach to prevent and treat this memory and cognitive impairment.

Many studies have demonstrated that, inflammation following cerebral ischemia and reperfusion after brain ischemia, increases these inflammatory reactions, which can exacerbate neuronal injury (8–10). Release of pro-inflammatory cytokines, such as necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, during ischemia/ reperfusion injury following both focal and global cerebral ischemia (11–13) and production of these cytokines can significantly increase the risk and extent of brain injury (14). A cascade of cellular and molecular events during brain ischemia, leads to delayed neuronal death. One of the well-established mechanisms in the pathogenic processes of neuronal death following ischemia is the massive release of glutamate and over-activation of glutamatergic receptors (15). Thus, it is necessary to explore the novel therapeutic strategies, including herbal based drugs for the treatment of stroke. A traditional Indian medicinal herb, *Cyperus rotundus* (family: *Cyperaceae*) is used as nervine tonic and nootropic in the Ayurvedic system of medicine (16). The present study was undertaken to investigate the neuroprotective effect of ethanol extract of *Cyperus rotundus* (EECR), in rat model of global cerebral ischemia/reperfusion. EECR showed marked central nervous system (CNS) depressant action compared to its other extracts in preliminary pharmacological screening (17). However, no work has been reported on the CNS activities of this plant. The present study has investigated the possible neuroprotective effects of EECR on a model of global transient ischemia, by evaluating the pathophysiology of the hippocampal tissue and spatial memory.

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Materials and Methods

Plant material and preparation of crude extract

The rhizomes of *C. rotundus* (herbarium No: PMP-215) were purchased from a local herbal store in Tehran, Iran, and identified by Dr Gholamreza Amin (School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran), the voucher specimen (8005) was deposited in Pharmacy School. For preparation of the hydro-alcoholic extract, 1000 g of the ground rhizomes of *C. rotundus* were macerated in ethanol 70% three times (each time 24 hr). The extract was then filtered and concentrated with vacuum evaporator and the percentage yield was 12%.

Phytochemical screening

Phytochemical investigations of the hydro-alcoholic extract of *C. rotundus* rhizomes were carried out using standard methods and tests (18, 19). Chemical tests were carried out on the hydro-alcoholic extract of *C. rotundus* rhizomes to identify their chemical constituents by using standard procedures. The test for tannins was carried out by subjecting 1 g of extract in 2 ml of distilled water; filtered and ferric chloride reagents were added to the filtrate. The extract was subjected to frothing test for the identification of saponins and to Fehling's test for glycosides. Alkaloids were detected in the alkaloid fraction obtained by a classical acid-base extraction procedure for alkaloids and analyzed by TLC in chloroform: methanol: ammonia solution 25% 8:2:0.5 as solvent system, spots were detected after spraying with Dragendorff's reagent. The presence of flavonoids was determined by adding 1% aluminum chloride solution to the extract and the yellow coloration. Another test for flavonoids, by adding dilute ammonia (5 ml) to the extract and then concentrated sulfuric acid (1 ml) was performed. Steroids were detected by adding 1ml of acetic anhydride to 0.25 g methanolic extract of each sample with 1 ml H_2SO_4. The color changed from violet to blue or green indicating the presence of steroids. The test for anthraquinones was performed with 0.5 g of extract boiled with 10 ml sulfuric acid and filtered. Then the filtrate was shaken with 5 ml $CHCl_3$, $CHCl_3$ layer was removed to another tube, and 1 ml of ammonia was added and color change was observed. For coumarins, a piece of filter paper was moistened in NaOH and then kept over a test tube with boiling plant extract solution. If the filter paper later showed any yellow fluorescence under UV light, that was taken to indicate a positive test for coumarins. Detection of terpenoids (triterpenoids) was carried out by adding 2 ml of $CHCl_3$ to 0.5 g of extract and then adding carefully concentrated H_2SO_4 (3 ml) to form a layer and reddish to brown color in interface.

Induction of brain ischemia/reperfusion and drug administration

24 Adult male Wistar rats weighing 250–300 g were randomly divided in 3 groups (control, ischemia, and treatment) and kept individually in a 12 hr light/dark cycle cage with free access to water and food according to the principles and procedures of the National Institutes of Health Guidelines for Animal Experiments of Islamic Azad University. The rats in control and treatment groups were operated on according to the modified global cerebral ischemia model, which can be induced by the occlusion of the two common carotid arteries or two-vessel occlusion (2VO), to induce reversible ischemia for 20 min. In brief, the clamping of the bilateral carotid arteries was ligated with microsurgery clamp under anesthesia using pentobarbital sodium (40 mg/kg). These clamps were withdrawn 20 min after the onset of ischemia. Body temperature was maintained at 37°C via a homoeothermic blanket (20). Control animals were only anesthetized by pentobarbital sodium.

Ischemia group animals underwent all the surgical procedures. 4 days before induction of ischemia and 3 days after the ischemia, the ethanol extract of *C. rotundus* (EECR) was (100 mg/kg), gavaged for treatment group.

All animals were kept in animal house for 1 week after ischemia, then Morris water maze task was performed. Rats were sacrificed 1 day after ending of Morris water maze training. Brains were removed for histological assessment (Nissl method).

Behavioral testing

Training in Morris water maze was carried out 1 week after cerebral ischemia. The apparatus consisted of a circular galvanized steel tank 170 cm in diameter and 45 cm high, which was filled with water (22±1°C) to a depth of 13 cm, which was surrounded by a variety of extra-maze cues. Tank was divided into four quadrants and the escape platform (diameter: 18 cm) was located in the southeast (SE) quadrant which submerged 2 cm below the surface of the water at a fixed position. In the spatial acquisition phase, the rats learned to find a submerged platform using extra-maze cues. Each rat participated in 16 trials, which were organized into daily block of 4 trials for 4 consecutive days. The Rats were allowed to swim freely for a maximum of 60 sec or until the platform was located. If the platform was not located during this time, the rat was guided to the platform and allowed to remain there for 20 sec. After 1 day of testing, animals were sacrificed and their brains examined for hippocampal damage.

Histological procedures

Animals were sacrificed 1 day after water maze testing. For Nissl staining, we used paraffin-embedded brain sections that received transcardiac
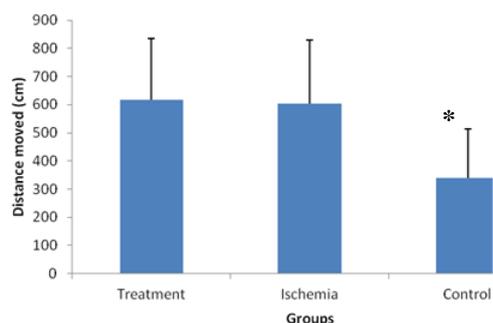
Figure 1. Effect of *Cyperus rotundus* on distance moved in ischemia-induced memory deficit model
*P-value ≤ 0.05, statistically different from ischemia group. Data are mean±SD

Nissl staining

Continuous coronal sections (10 µm in thickness) of rat brain were prepared. These sections were processed with 1.0% cresyl violet, dehydrated, and cover slipped with Entellan. Histological changes of brains were observed under an optical microscope. The diameters and the numbers of the CA1 pyramidal cells of hippocampus were measured in stained sections (3 sections of the hippocampus of each rat) by using light microscope. Photomicrographs were taken at × 400 magnifications with a microscope (Olympus *AX-70*) and analyzed by image tool 2 software.

Statistical analysis

All histological data were analyzed with a one-way ANOVA. *Post hoc* comparisons between independent groups were made with the Tukey test.
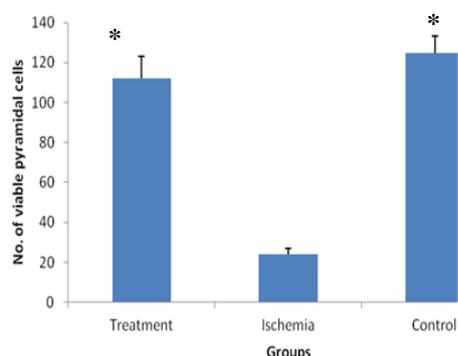

Figure 3. Effect of *Cyperus rotundus* on number of CA1 pyramidal cells in ischemia-induced memory deficit model
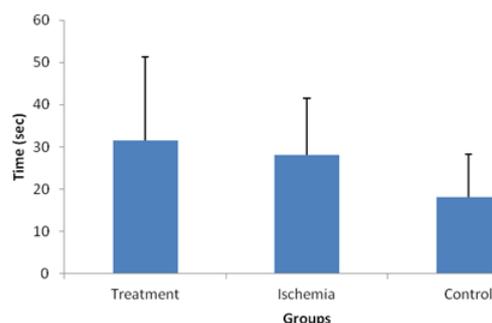*P-value ≤ 0.05, statistically different from ischemia group. Data are mean±SD

Figure 2. Effect of *Cyperus rotundus* on latency time in ischemia-induced memory deficit model
*P- value ≤ 0.05, statistically different from ischemia group. Data are mean±SD

In all cases, the acceptable level for statistical significance was *P*-value ≤ 0.05.

Results

1- Data analyses from Morris water maze test showed that there was statistically significant difference between control and ischemia groups but no statistical difference was seen between ischemia and treatment groups in distance and time (Figure 1, 2).

2- Data of Nissl staining showed that 20 min of bilateral common carotid occlusion caused marked CA1 cell loss. But no statistically significant difference was seen between control and treatment groups (*P*-value= 0.105) it means that EECR can prevent CA1 cell loss due to ischemia/ reperfusion injury (Figure 3, 4)**.**

3- Data which were collected from cell diameters showed that 20 min of bilateral common carotid occlusion caused marked reduced cell diameters in CA1 region of hippocampus. There was statistically significant difference between control group versus ischemia group (*P*-value=0.010). But there was no statistical significant between control and treatment groups (*P*-value= 0.299) (Figure 5).
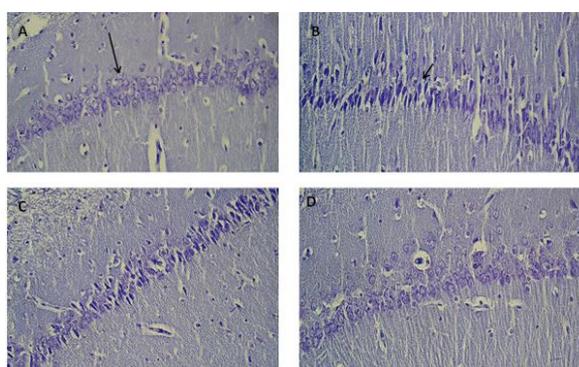

Figure 4. Photomicrographs of coronal sections of CA1 region in hippocampus. A) control, B) ischemia, C) vehicle and D) experimental groups. Long arrow shows an intact neuron and short arrow shows a degenerated neuron (Nissl staining, scale bar= 30 µm)
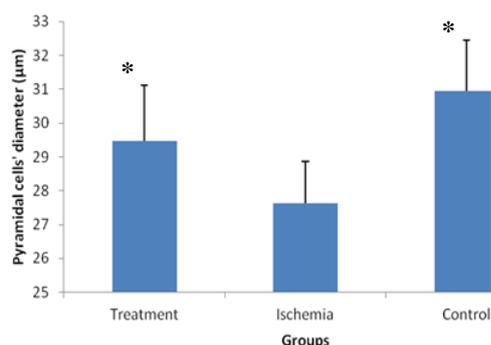
Figure 5. The effect of *Cyperusrotundus* on pyramidal CA1 cells diameters (μm) after transient global ischemia
*P-value ≤ 0.05, statistically different from ischemia group. Data are mean±SD

Discussion

Transient global cerebral ischemia is a clinical outcome of cardiac arrest and other situations that decrease the amount of oxygen in brain during a short period which can lead to CA1 neuron loss in hippocampus (21–23). Degeneration of the CA1 pyramidal neurons is associated with severe hippocampal dysfunction, including spatial learning and memory deficits (24).

The present study demonstrates the neurotrophic effect of EECR in rat model of total ischemia–reperfusion. Various pathophysiological mechanisms such as excitotoxicity, inflammation, and apoptosis are involved in ischemic neuronal death (25). We induced the neurological deficit by common carotid occlusion followed by reperfusion.

Our findings revealed that pyramidal cells of CA1 region were damaged and spatial memory deficit was seen in rats that were subjected to 20 min bilateral common carotid occlusion.

The mechanism of ischemia/reperfusion (IR) remains unclear; it seems that reactive oxygen species (ROS) are one of the most important factors that induce neuronal death in IR insult (26).

After IR injury, damaging free radicals such as nitrogen oxide (NO), superoxide (O^{2-}), and peroxynitrite increase (27). Expressions and activities of nitrogen oxide synthases (NOS) are increased in the experimental mouse model of cerebral IR injury. Nitrogen oxide and oxidative products are involved in the pathology of brain ischemia injuries (28).

There is expanding interest in neuroprotective agents that may ameliorate the damage of ROS and NO due to the oxidative mechanism of ischemia-induced cell death and injury (29).

C. rotundus is a traditional herbal medicine that has recently found applications in food industries. In this current study, the neuroprotective effects of *C. rotundus* rhizome extract *(CRE)*, through its anti-oxidant, free radical scavenging activities that play a major role in protection of neurodegenerative disorders, have been explored (30).

Seo *et al* reported that the methanol extract of rhizomes of *C. rotundus* could modulate NO and O^{2-} production due to suppression of inducible nitric oxide synthase (iNOS) in protein, as well as iNOS mRNA expression, and also reduced the production of O^2 (31).

Lee *et al* reported that the alcoholic extract of *C. rotundus* has anti-oxidant property (32) and some studies showed highly significant anti-inflammatory activity of this herb against the exudative and proliferative phases of inflammation in two animal models (33, 34).

Supported by histological data, the demonstration that a drug can ameliorate the behavioral impairment due to IR is a very important finding in clinical settings.

The Stroke Therapy Academic Industry Roundtable (STAIR, 1999) emphasizes that behavioral measurements are very important in the preclinical evaluation of neuroprotective effects of drugs before the beginning of clinical experiments. In the present study, EESR could not reduce the effect of ischemia on spatial and learning memory.

Finally, there was no correlation between any of the behavioral parameters analyzed and the extent of pyramidal cell loss in the CA1 sector of hippocampus in the group treated with EECR. Attempts to establish a quantitative correlation between hippocampal pyramidal cell loss and ischemia-induced behavioral deficits have led to contradictory results:

The close correlation found the number of preserved, intact appearing pyramidal cells alone, which may not be indicative of behavioral changes, and other intra- and/or extra-hippocampal effects may determine cognitive disruption by ischemia.

In our study, no correlation was found between the behavioral deficit measured in the water maze and the extent of pyramidal cell loss in the CA1 sector of hippocampus. The relationship between hippocampal cell loss and cognitive deficits caused by ischemia was reviewed by Bachevalier and Meunier (35), who concluded that a combination of at least three factors may operate, i.e., the role of intra- and extra-hippocampal damage, the nature of the task employed, and the particular memory process taxed. Thus, we cannot exclude the possibility that other brain structures besides the hippocampus may have been damaged by ischemia. Considering the relationship between structure and function, it is also important to emphasize that it is not clear to what extent methodological limitations such as histological assessment and/or random behavioral factors may influence the results of a correlation analysis (36).

Conclusion

The present study showed that EECR cannot reduce the ischemia-induced, cognitive impairments seen after transient, global cerebral ischemia as measured in the Morris water maze.

To the best of our knowledge this is the first report on the effect of EECR against the cognitive outcome of transient, global ischemia in rat. Further studies are necessary to better characterize the effect of EECR in functional improvement after ischemia–induced brain damage.

Acknowledgment

Conflict of interests

The authors report no conflict of interests.

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