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Protective effects of *Polygala tenuifolia* on ischemia-induced 4 vessel occlusion in rats

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SUMMARY

The root of *Polygala tenuifolia* Willd (PT) is known to have neuroprotective effects and as an antidementic herb in Chinese and Japanese traditional medicine. We examined potential neuroprotective effects of PT using the 4-vessel occlusion model in rats. In this study, the efficacy of PT for the prevention of neuronal damage and for the reduction of memory impairment was investigated. The results indicate that PT confers significant neuroprotection especially for ischemic hippocampal neurons.

Key words: *Polygala tenuifolia*; Neuroprotective; Ischemia; Hippocampus

INTRODUCTION

Neuroprotection for ischemic stroke refers to strategies that antagonize the injurious biochemical and molecular events that eventuate in irreversible ischemic injury. Global cerebral ischemia resulting from cardiac arrest, stroke and hypoxia is a problem of increasing clinical significance. Rigorously conducted experimental studies in animal models of brain ischemia provide incontrovertible proof-of-principle that high-grade protection of the ischemic brain is an achievable goal (Ginsberg, 2008).

Dysfunction of mitochondria induced by ischemia is considered to be key event triggering neuronal cell death after brain ischemia (Racay *et al.*, 2008). Several cyclooxygenase (COX) inhibitors have proved to be neuroprotective in stroke models. Chronic cerebral hypoperfusion such as multiple lacune infarctions is related to neurological disorders and contributes to a cognitive decline. Its experimental model in rats is permanent, bilateral common carotid artery occlusion (Institoris *et al.*, 2007). By performing 4-vessel occlusion (4-VO) model of global brain ischemia, neuronal damage of selective vulnerable cells, most notably the CA1 cells of the hippocampus is generated. Global cerebral ischemia results in pyramidal neurons in the CA1 region of hippocampus die 4 - 7 days following transient forebrain ischemia (Pulsinelli and Brierley, 1979). The hippocampus bas been shown to be essentially involved in learning and memory processes (Ginsberg and Busto, 1989).

The methanol fraction of an ethanolic extract from the roots of *Polygala tenuifolia* Willd (PT) showed antagonistic action on neurotoxicity induced by glutamate and serum deficiency in PC12 cells

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(Li *et al*., 2008). PT inhibited potassium cyanide (KCN)-induced hypoxia and scopolamine-induced memory impairment in mice (Karakida *et al*., 2007). Park *et al.* (2006) suggested that both ethanol extract and n-butanol fraction of PT root can reduce brain damage during ischemia and reperfusion and prevent lipid peroxidation. In another study, bioassay-guided fractionation of PT led to the isolation of six new triterpenoid saponins, onjisaponins V - Z, and Vg (1 - 6), together with ten known saponins (7 - 16). Furthermore, compounds 1 - 16 showed neuroprotective effects against serum deficiency (Li *et al.,* 2008).

It has been strongly suggested to play a role in the pathogenesis of delayed neuronal damage after global cerebral ischemia. The authors investigated whether delayed CA1 neuronal cell death, as induced by 4-VO, is associated with deficits in learning and memory. The present study aimed at assessing the protective effects of PT on ischemia reperfusion injury induced global cerebral ischemia in rat hippocampus.

MATERIALS AND METHODS

Cerebral ischemia

Adult male wistar rats 6 w of age (weight of 180 – 200 g at the time of surgery) were used in the study. The animals were initially anesthetized with 3.5% isofluorane and then maintained during operation on 1.5% isofluorane in N_2O: O_2 (70: 30) mixture on the first day and the vertebral arteries were electrocauterized in the alar foramina at the level of the first cervical vertebrae. Bilateral common carotid arteries were exposed and carefully separated from the carotid sheath, cervical sympathetic and vagus nerves through a ventral cervical incision. The rats were placed on a heating pad during recovery from anesthetized to maintain the body temperature at 37.0 ± 0.5 after surgery.

The next day, both common carotid arteries were occluded for 10 min. while the animals awake. It results in damage limited to the hippocampal area. Rats that become unresponsive and loss the righting reflex within 2 min occlusion but show no seizure during and after ischemia are used further experiments. Reperfusion was achieved by releasing the clips at the end of 10 min ischemic period. Animals were that developed post-operative complications such as excessive weight loss (> 20% of pre-operative body weight) and showed evidence of unilateral hippocampal damage were excluded from the study. The rats which received the same operation without carotid arteries ligation served as the sham-operated control. The rats were allowed to survive for 7 days (8 controls, 8 sham rats and 8 ischemia rats) or for 14 days (8 controls, 8 sham rats and 8 ischemia rats). The rats were placed on a heating pad during recovery from anesthetized to maintain the body temperature at 37.0 ± 0.5 after surgery.

The Rotarod test

Rotarod and beam balance were performed to evaluate the behavioral effect of PT on sensory-motor dysfunction after ischemia. The rotarod test involved accelerated rotation of rotarod (Ugo Basile, Italy) from 0 to 25 rpm within 2 min and was conducted at 7 days after ischemia. The latency time for each rat was determined from five separate trials; the lowest and highest outlier data were excluded and the remaining three data were averaged for the final result.

The beam balance test

The beam balance test was conducted at 7 days after ischemia using a modification of a previously described method. (Alexandre, 2006) The beam used was a wooden square bar (width, 2.5 cm; length, 122 cm; height, 42 cm). After placing the rat on the middle of the beam, the score was rated as follows from 0 to 6: 0, if the rat was unable to stay on the beam; 1, if the rat was able to stay on the beam but made no movements; 2, if the rat attempted to turn left or right on the beam; 3, if the rat turned left or right and walked on the beam,

and the affected hind limb showed more than 50% foot slips; 4, if the rat traversed the beam with more than one but less than 50% foot slips; 5, if the rat traversed the beam with only one foot slip of the hind limb; and 6, if the rat traversed the beam without any foot slips of the hind limb.

PT treatment

PT extract solution (200 mg/kg, i.p.) was administered to rats 0 and 90 min after induction of ichemia. Ischemia-only animals were injected i.p. with 180 μl/kg distilled water at the same time points. Beinning the day after ischemia induction, some animals were administered 200 μl PT extract solution p.o. daily for seven days during behavior test.

RESULTS

To examine the neuroprotective effect of PT extract, a dose of 200 mg/kg was injected i.p. into rats 0 and 90 min after the induction of cerebral ischemia. For the ischemia group, 0.89% physiological saline was injected at a volume of 1.8 ml/kg. When reperfusion is conducted after cerebral ischemia caused by 4-VO, pyramidal neurons in the hippocampus CA1 subfield are the most susceptible to the ischemia and start undergoing cell death 72 h after reperfusion[1]. In the present study, rats were sacrificed 7 days after reperfusion, the time point by which all signs of neuronal cell damage have become manifest. Dorsal hippocampal tissue sections were stained with cresyl violet to visualize CA1 neurons in the ischemic group, the sham-operated group, and the PT extract treated group (Fig. 1, A to C).

Fig. 1A shows the track of CA1 pyramidal neurons in the sham-operated group; most of these neurons have an unchanged (normal) staining pattern (Fig. 1a). In the ischemic group, the stratum pyramidal was weakly stained, showing occurrence of neuronal cell damage within the CAl subfield (Fig. 1B); Fig. 1b shows that pyramidal neurons have undergone coagulative cellular changes typical

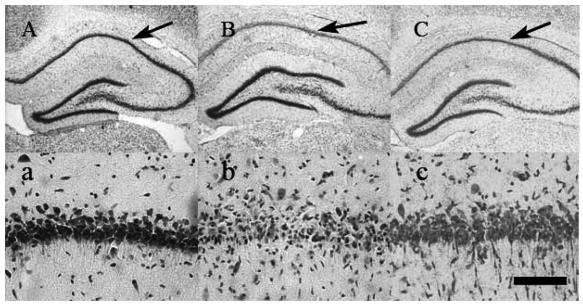

Fig. 1. Representative photomicrographs of cresyl violet-stained hippocampal regions of either sham-operated animals (A, a) or animals that had been subjected to 10 min ischemia followed by the treatment with either saline (B, b) or 200 mg/kg of PT (C, c). Boxed regions in A, B, and C are shown in a, b, and c, respectively. The 10 min ischemia caused selective and delayed neuronal cell loss in the hippocampal CA1 region (B, b). In contrast, PT treatment conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (C, c). Scale bar is 100 um. The male wistar rats were 6 w.

of apoptosis and were damaged with characteristic apparent gliosis. Compared to the ischemic rats, animals administered PT extract had a significantly reduced number of damaged pyramidal neurons in the CAl field (Fig. 1C and c). There was no significant difference in body temperature between ischemic and PT extract treated groups at any time point recorded indicating that neuroprotective effects of PT extract were not due to a decrease in body temperature. Normal CA1 pyramidal neurons from three hemispherical sections each having a size of 1 × 1 mm, were counted and averaged (Fig. 2). In the ischemic group the viable cell density was 101.0 ± 10.7 cells/mm^2, which is far lower than that in the sham group, 340.0 ± 8.6 cells/mm^2. In the group injected with PT extract, viable cells were measured to be 247.0 ± 13.9 cells/mm^2. Thus PT extract rescued 61.1% of the ischemic neurons.

In the rotarod test, the vehicle-treated group (11.9 ± 0.9 s, $P < 0.5$) showed a deficit when compared with the sham operated group (83.7 ± 6.2 s). The 200 mg/kg PT treated groups showed latency time of 42.2 ± 7.8 s, respectively. Significant improvement

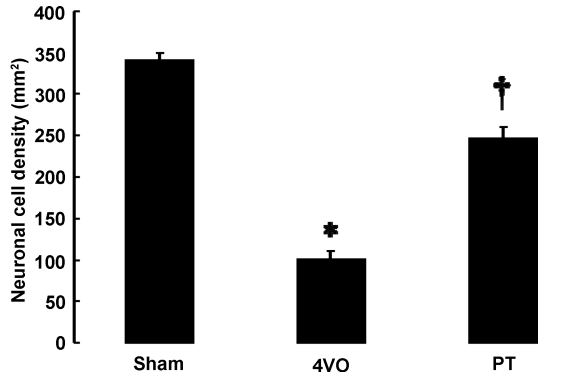

Fig. 2. Neuroprotective effects of PT (200 mg/kg). Either saline of PT was injected in i.p. into the animals following 10 min ischemia. Seven days later, neuronal cell density in CA1 neurons. Statistically significant differences from saline-treated group (***$P < 0.001$). Sham, sham-treated animals (n = 10); control, saline-treated animals following ischemia (n = 7). PT, 200 mg/kg of PT treated group after ischemia (n = 6). The male wistar rats were 6 w.

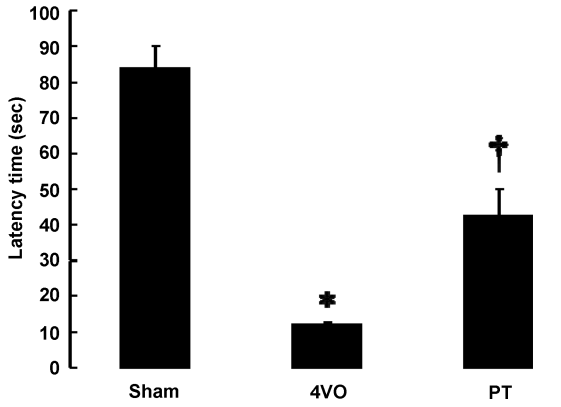

Fig. 3. Effects of PT on rotarod test after ischemia in rat. Oral administration of PT (200 mg/kg once a day) for 7 days. **$P < 0.01$ significantly different from the Control group. Sham, sham operated group (n = 5). 4VO, vehicle-treated group after ischemia (n = 5). PT, 200 mg/kg of PT treated group after ischemia (n = 6). Data represent means ± S.E.

was observed in all PT-treated groups ($P < 0.05$, Fig. 3).

In the beam balance test, the vehicle-treated group (1.8 ± 0.4 points, $P < 0.001$) showed a deficit when compared with the sham-operated group (5.2 ± 0.4 points). The 200 mg/kg PT-treated groups scored 3.8 ± 0.7 points, respectively. Significant

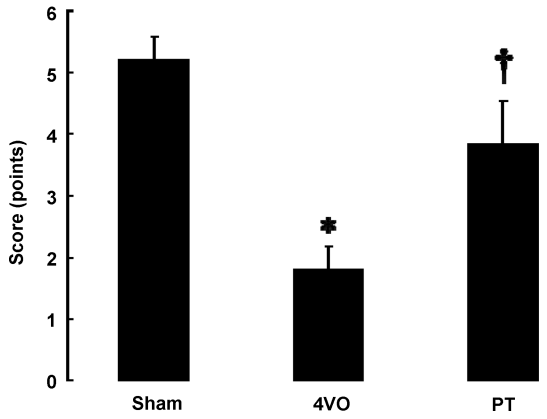

Fig. 4. Effects of PT on beam balance test performance deficits induced by 10 min cerebral ischemia in rat (n = 5 - 6). Oral administration of PT (200 mg/kg once a day) for 7 days. **$P < 0.01$ significantly different from the control group. Sham, sham operated group (n = 5). 4-VO, vehicle-treated group after ischemia (n = 5). PT, 200 mg/kg of PT treated group after ischemia (n = 6). Data represent means ± S.E.

improvement was observed in the 200 mg/kg PT-treated group (Fig. 4, $P < 0.05$).

DISCUSSION

In the present study, the efficacy of PT for the prevention of neuronal damage and for the reduction of motor impairment was investigated. We examined potential neuroprotective effects of PT using the 4-VO model in rats. The hippocampus in brain is known to demonstrate selective vulnerability to hypoxic and ischemic damage. The hippocampal morphology remains normal until three days after ischemia and cell deaths begin four or five days after ischemia (Ginsberg, 1989). A recent review article showed a close survey of the most extensively evaluated neuroprotective agents and classes. Among the agent-classes considered are calcium channel blockers; glutamate antagonists; GABA agonists; antioxidants/radical scavengers; phospholipid precursor; nitric oxide signal-transduction down-regulator; leukocyte inhibitors; hemodilution; and a miscellany of other agents. Park *et al.* (2002) investigated the effects of PT BT-

11 on neurotoxicity induced by glutamate and toxic metabolites of amyloid precursor protein (APP) such as amyloid beta protein (Ab) and C-terminal fragment of APP (CT) in primary cultured neurons of rat.

In the present study, the two sensory motor function tests were mainly designed to determine whether PT could ameliorate the postischemic dysfunction with regard to motor coordination, sensory motor integration, spontaneous locomotion, forelimb responses to senses, and hindlimb motor functions in rodents (Gerasimov, 2001).

The results indicate that 200 mg/kg PT treated groups showed significant improvement in the postischemic sensory motor dysfunction when compared with the vehicle-treated group as observed in the rotarood, and beam balance tests.

In conclusion, we demonstrated that the orally treatments with PT at the occlusion and reperfusion reduced the cell death and improved the neurological outcome following transient global cerebral ischemia in rats.

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