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Effects of α-Asarone against Global Cerebral Ischemia in Rats

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Abstract – Based on the use of *Acorus gramineus* SOLAND (AG) for the treatment of stroke in traditional Korean medicine, the present study was carried out to evaluate neuroprotective effects of α-asarone after transient global cerebral ischemia using rat 4-vessel occlusion (4VO) model in rats. α-Asarone (5 mg/kg) administered intraperitoneally significantly protected CA1 neurons against 10 min transient forebrain ischemia as demonstrated by measuring the density of neuronal cells stained with Cresyl violet. α-Asarone significantly reduced hippocampal neuronal cell death by 85.2% where as its isolated single compounds from AG compared with a vehicle-treated group.
Keywords – Stroke; Neuroprotective; α-Asarone; Global cerebral ischemia.

Introduction

Global cerebral ischemia resulting from cardiac arrest, stroke and hypoxia is a problem of increasing clinical significance. A four- vessel occlusion (4-VO) model of rats has been developed the treatments for cerebral ischemic injury. The pattern of neuronal injury in this model similar to that reported in global ischemia in humans, including substantial neuron death in the cerebral cortex and hippocampal CA1 region (Pulsinelli and Brierley, 1979; Ginsberg and Busto, 1989). Global cerebral ischemia results in neuronal damage of selective vulnerable cells, most notably the CA1 cells of the hippocampus (Kirino and Sano, 1984). Pyramidal neurons in the CA1 region of hippocampus die 4-7 days following transient forebrain ischemia (Pulsinelli *et al*., 1982). The hippocampus bas been shown to be critically involved in learning and memory processes (Barnes, 1988).

The volatile oil obtained by the steam distillation of an indigenous Indian plant *Acorus calamus* possesses interesting actions on the central nervous system (Dandiya and Cullumbine, 1959; Dandiya and Sharma, 1962).

The methanol extract and the essential oil from AG exhibited neuroprotective action against the excitotoxicity induced by glutamate (Glu) or N-methyl-D-aspartate (NMDA) in cultured rat cortical cells (Cho *et al*., 2001) Based on the receptor binding studies using a use-dependent channel blocker [3H]MK-801, the neuroprotective action is primarily through the blockade of NMDA receptor function (Cho *et al*., 2000). The essential oil exhibited more potent inhibition of the binding, and consistently, exerted more potent neuroprotective action than the methanol extract.

The drug α-asarone (5 mg/kg body weight, i.p.), one of the active principle components of *Acorus gramineus* SOLAND (AG) aministered intraperitoneally 0 and 90 min after induced ischemia. (Chun *et al*., 2008) has been traditionally used herbal medicines in far eastern countries including China, Korea and Japan. In the course of study on the biological activity of AG, we found that it had beneficial actions include: ischemia-induced learning disability and neuronal loss in wistar. The dose (5 mg/kg) of α-asarone was effective in decreasing scopolamine induced deficit in passive avoidance test. Scopolamine induced dementia by α-asarone in the present study indicates the adaptogenic and anti-amnesic properties of α-asarone. Among its diverse effects on the central nervous system, the present study was carried out to evaluate the neuroprotective effects of α-asarone.

Material and Method

Global cerebral ischemia – Adult male wistar rats 6

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weeks 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 preoperative 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. The rats were placed on a heating pad during recovery from anesthetized to maintain the body temperature at 37.0 ± 0.5 after surgery.

α-Asarone treatment

α-Asarone – was purchased from Aldrich Chemical Co. (Milwaukee, WI). The other chemicals were obtained from Sigma Chemical Co. (St Louis, MO). α-Asarone (5 mg/kg, i.p.) was administered to rats 0 and 90 min after induction of ischemia. Ischemia-only animals were injected i.p. with 180 μl distilled water at the same time points. Beinning the day after ischemia induction, some animals were administered α-asarone (5 mg/kg) p.o. daily for seven days. Animals retained for behavioral testing were administered α-asarone for an additional seven days during the test period. Animals were injected with scopolamine (2 mg/kg, i.p., Sigma #0929) during passive avoidance testing as described below. Control animals were injected with an equal volume of physiological saline.

Passive avoidance test – The rats were subjected to single trial passive avoidance test as described by A shuttle box containing two compartments (260 × 200 × 150 mm) separated by a guillotine-type door (90 × 115 mm) was used (Gemini Avoidance System, San Diego Instruments, USA). The system is designed to administer a series of trials in which the animal may receive several stimuli (light, sound signal, electric footshock) (Ader *et al*., 1972).

Rats were tested in groups of n = 8 (scopolamine), n = 8 (α-asarone), divided into two experiments with eight rat per group run on separate days and data subsequently vehicle or a dose of drug were injected scopolamine (Sigma Chemical Co., St. Louis, MO) in dose of 2 mg/kg 30 min prior to the training trial. The muscarinic receptor antagonist scopolamine, which is known to disrupt learning and memory functions in humans and passive avoidance retention in mice (Senda *et al*., 1997).

Training was initiated by placing the rat individually in the illuminated compartment (start box) of the apparatuses. After an acclimatization period of 30 s, the guillotine door automatically opens and the animal is subjected to a trial of 270 s. An entry into the dark compartment automatically shuts of the door and the subject is punished with a single low intensity foot shock (1 mA; 2s). Infra red sensors monitor the transfer from one compartment to another, which is recorded as transfer latency (TL) in seconds. TL was recorded for 1st trial (acquisition) and next day 2nd trial (retention). The criterion for successful learning and memory activity was taken as an increase in TL on second trial (retention) as compared to first trial (acquisition). We checked the number of trials, the inter-trial intervals, the adaptation period and the shock intensity. Rats not entering into the goal box within 270 s were discarded.

Results

Neuroprotective effect of α-asarone on global cerebral ischemia *in vivo* – The selection of the doses for cerebral ischemia of α-asarone was guided by the results obtained with individual concentration (1, 3, 5, and 10mg/kg). The doses (5 mg/kg) used was the best neuroprotective effects.

To examine the neuroprotective effect of α-asarone, a dose of 5 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 dose of 180 ul per rat. 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 . In this 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 α-asarone treated group (Fig. 2, A to F). Fig. 2A shows the track of CA1 pyramidal neurons in the sham-operated group; most of these neurons have an unchanged (normal) staining pattern (Fig. 2B). In the ischemic group, the stratum pyramidal was weakly stained, showing occurrence of neuronal cell damage within the CAl subfield (Fig. 2C); Figure 2D shows that pyramidal neurons have undergone coagulative cellular changes typical of apoptosis and were damaged with characteristic apparent gliosis. Compared to the ischemic rats, animals administered α-asarone had a significantly reduced number of damaged pyramidal neurons in the CAl field (Fig. 2, E and F). There was no significant difference in body temperature between ischemic and α-asarone treated groups at any time point recorded indicating that neuroprotective effects of α-asarone 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. 3). In the ischemic group the viable cell density was 42.3 ± 6.8 cells/mm^2, which is far lower than that in the sham group, 306 ± 9.2 cells/mm^2. In the group injected with α-asarone, viable cells were measured to be 267 ± 5.2 cells/mm^2. Thus α-asarone rescued 85.2% of the ischemic neurons.

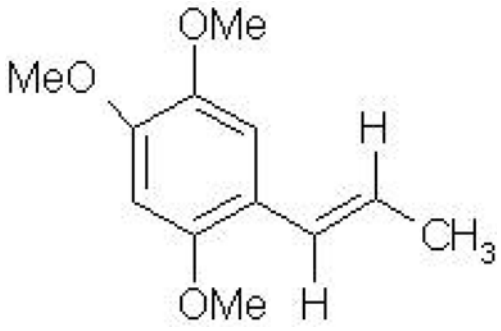

Fig. 1. Structure of α-asarone.

Effect of α-asarone on memory – In passive avoidance task vehicle treated group has shown significant increase in TL (19.4 ± 4.8s) on second trial compared to first trial

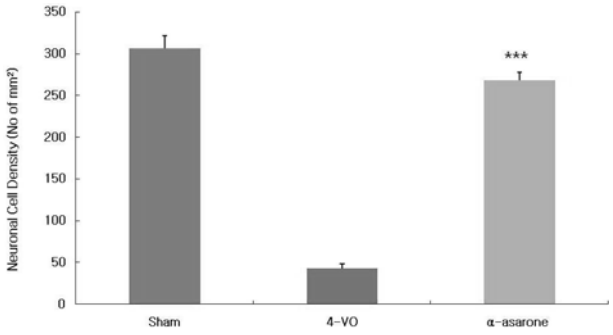

Fig. 3. Neuroprotective effects of α-asarone (5 mg/kg). Either saline of á-asarone was injected in i.p. into the animals following 10 min ischemia. Seven days later, neuronal cell density in CA1 neurons. Statistically significant was differences from saline-treated group (***$p < 0.001$). Sham, sham-treated animals ($n = 8$); control, saline-treated animals following ischemia ($n = 7$). α-asarone treated animals following ischemia ($n = 8$ for 5 mg/kg). The male wistar rats were 6 weeks.

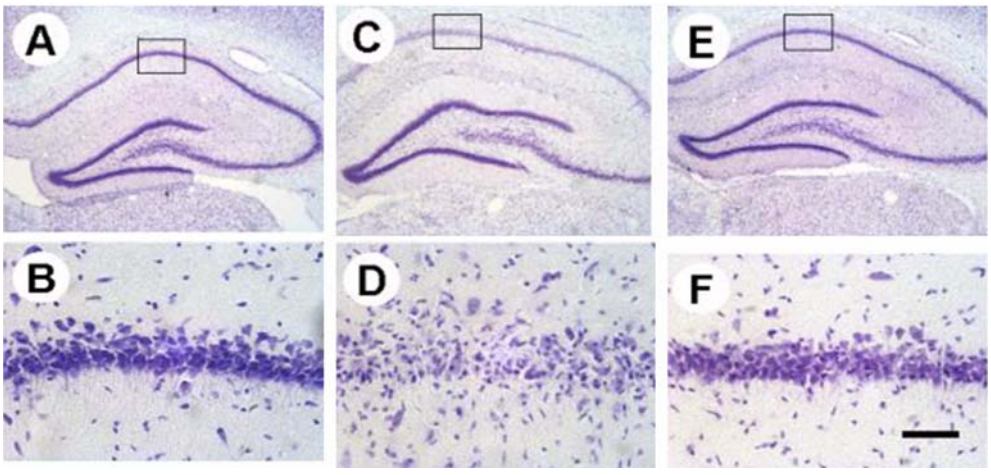

Fig. 2. Representative photomicrographs of cresyl violet-stained hippocampal regions of either sham-operated animals (A, B) or animals that had been subjected to 10 min ischemia followed by the treatment with either saline (C, D) or 5 mg/kg of α-asarone (E, F). Boxed regions in A, C, and E are shown in B, D, and F, respectively. The 10 min ischemia caused selective and delayed neuronal cell loss in the hippocampal CA1 region (C, D). In contrast, α-asarone treatment conferred neuroprotective by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (E, F). Scale bar is 100 ㎛. The male wistar rats were 6 weeks.

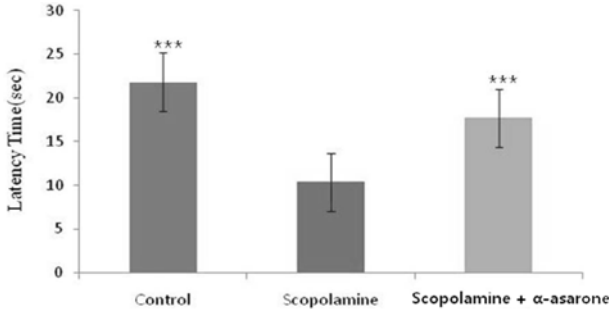

Fig. 4. Effect of α-asarone on scopolamine induced memory deficits in the passive avoidance test. At 30 min after trainining trials, scopolamine (2 mg/kg i.p.) or the same volume of saline was administered to rats. At 30 min after scopolamine injection, the rats were treated with α-asarone (5 mg/kg). Acquisition trials were carried out 30 min after α-asarone treatment. Data represents mean ± SEM (n = 8). Significantly different was from the vehicle control group (one-way ANOVA). The male wistar rats were 7 weeks.

($p < 0.001$). Scopolamine treatment (amnesia) could not produce a significant increase in TL on second trial (7.2 ± 2.6 s) as compared to first trial. α-asarone treated group exhibited a significant increase in TL (16.4 ± 8.5 s) on 2nd trial in comparison to first trial as well as compared to amnesia group ($p < 0.05$) (Fig. 4).

Discussion

In the present study, the efficacy of α-asarone (5 mg/kg) for the prevention of neuronal damage and for the reduction of memory impairment was studied in wistar rat model of transient global ischemia and in a murine scopolamine model. Based on the use of AG in traditional medicinal herb for the treatment of CNS dysfunction, we tested potential neuroprotective effects of α-asarone using the 4-VO model in rats. The results indicate that α-asarone confers significant neuroprotection against 10 min of ischemia induced by 4-VO. The results indicate that α-asarone confers significant neuroprotection. This is reasonable because AG radix is effective in the prevention and repair of cerebral ischemia (Xuejiang *et al*., 1999). It has also been shown that α-asarone, AG component, protects ischemic hippocampal neurons (Watanabe, 1997).

The aim of anesthesia was to avoid adverse post-operative side effects (e.g. seizures) observed at this duration in non- anesthetized rats. In our experiment there was no difference in cell loss or behavioral performance in the passive avoidance test among the groups. The mode of cell death (necrosis or apoptosis) would be depend on severity of ischemic injury (Choi, 1996). There are several useful techniques to detect apoptosis. Using the electron microscope, we have shown that delayed neuronal death following ~14 min of forebrain ischemia induced by the 4VO is necrotic in the majority of the rat. A complete and skillful electrocoagulation of the vertebral arteries is essential to the success of reproducible ischemia.

The hippocampus is a brain region that demonstrates selective vulnerability to ischemic damage. Hippocampus is a structure directly involved in learning and memory processes. CA1 pyramidal cells, whereas some of those are undergoing apoptosis (Zeng *et al*., 2000). Cell deaths in the hippocampal CA1 region due to transient cerebral ischemia do not occur immediately after completion of ischemia; hippocampal morphology remains normal until four days after ischemia and cell deaths begin four or five days after ischemia (Mori *et al*., 1998). Such cell deaths are referred to as delayed neuronal deaths (Kirino, 1982). The importance of the delayed neuronal death lies in the fact that neurons are not destroyed instantaneously and directly at the end of the period of ischemia. Rather, the cells have a normal appearance early after ischemia because they are still viable. And they are not irreversibly committed to be destroyed. Thus, the immediate post ischemic period represents the "therapeutic window" during which interventions could prevent the delayed neuronal death (Colbounce and Corben, 1994).

The α-asarone was found most effective in the 5 mg/kg i.p. and 10 mg/kg p.o. dose, respectively. Therefore these doses were selected to study the neuroprotection of the α-asarone. The study by other authors have also used α-asarone at a dose of 10 mg/kg p.o. for study its neuroprotective effects against Aβ(25-35)-caused neurotoxicity by inhibiting the effects of NO overproduction in the hippocampus and temporal cortex (Ilhuicamina *et al*., 2009).

There is substantial clinical evidence that muscarinic receptor blockade by drugs like scopolamine results into disruptions of behavioral inhibition, working (short-term) memory, retrieval from reference (long term memory), attention and decisional processes movement and strategy selection and altered sensory processing (Fibiger, 1990).

In the present study, control rats showed significant increase in TL on second trial compared to first trial (acquisition) which shows a successful memory response. Scopolamine treated rats failed to show increase in TL on second trial (retention) indicating deficit in memory (amnesia). However, in rats pretreated with α-asarone, even after scopolamine treatment, a significant increase in TL was observed on second trial (retention). Prevention of scopolamine induced amnesia by α-asarone demonstrated

the potential anti-amnesic effect of α-asarone.

To summarize, α-asarone is characterized cognition-enhancing drug in improving the cognitive impairment caused by global cerebral ischemia. The effect of α-asarone is influenced by many factors (e.g. dose, duration of administration, animal learning models etc). Further studies are required to elucidate mechanisms of its effects on learning and memory.

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