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Effects of *Ginseng Radix* on the ischemia-induced 4-vessel occlusion and cognitive impairments in the rat

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Abstract : Ginseng powerfully tonifies the original Qi. Ginseng used for insomnia, palpitations with anxiety, restlessness from deficient Qi and blood and mental disorientation. In order to investigate whether Ginseng cerebral ischemia-induced neuronal and cognitive impairments, we examined the effect of Ginseng on ischemia-induced cell death in the hippocampus, and on the impaired learning and memory in the Morris water maze and passive avoidance in rats. Ginseng when administered to rat at a dose of 200 mg/kg i.p. water extracts to 0 minutes and 90 minutes after 4-VO, significantly neuroprotective effects by 86.4% in the hippocampus of treated rats. For behavior test, rats were administered Ginseng (200 mg/kg p.o.) daily for two weeks, followed by their training to the tasks. Treatment with Ginseng produced a marked improvement in escape latency to find the platform in the Morris water maze. Ginseng reduced the ischemia-induced learning disability in the passive avoidance. Consistent with behavioral data, treatments with Ginseng reduced ischemia-induced cell death in the hippocampal CA1 area. Oxidative stress is a causal factor in the neuropathogenesis of ischemic-reperfusion injury. Oxidative stress was examined in a rat model of global brain ischemia. The effects of Ginseng on lipid peroxidation (inhibition of the production of malondialdehyde, MDA) in different regions of the rat brain were studied. Ferrous sulfate and ascorbic acid (FeAs) were used to induce lipid peroxidation. The antiperoxidative effect showed 48-72% protection from tissue damage as compared with untreated animals. These results showed that Ginseng have a protective effect against ischemia-induced neuronal loss and learning and memory damage.

Key words : *ginseng*, neupropection, four-vessel occlusion, cognitive impairment

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[1,2]. Global cerebral ischemia results in neuronal damage of selective vulnerable cells, most notably the CA1 cells of the hippocampus[3]. Pyramidal neurons in the CA1 region of hippocampus die 4-7 days following transient forebrain ischemia[4]. The hippocampus bas been shown to be critically involved in learning and memory processes[5].

The root of *Panax ginseng* C.A. Meyer (Araliaceae) has been traditionally used herbal medicines in far eastern countries including China, Korea and Japan. Many kinds of paper have reported its biological activities. In the course of study on the biological activity of ginseng, we found that it had beneficial actions include: ischemia-induced learning disability and neuronal loss in wistar. Ginseng has eight major ginsenoside have been shown to exert an anti-inflammatory[6], anti-oxidant[7], and anti-cancer[8]. *Panax ginseng* reported to have neuroprotective effects on stroke animal models[9,10]. Among its diverse effects on the central nervous system, ginseng is known to improve learning and memory[11]. And also we studied that oxygen free radical-induced lipid peroxidation. It has been strongly suggested to play an important role in the pathogenesis of delayed neuronal damage after global cerebral ischemia[12]. Here, we have analysed whether delayed CA1 neural cell death, as induced by 4-VO, is associated with deficits in learning and memory. The aim

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of present study was to assess the protective effects of ginseng on ischemia reperfusion injury induced global cerebral ischemia in rat hippocampus.

MATERIAL AND METHODS

Cerebral ischemia

Adult male wistar rats 6 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 $37.0 \pm 0.5^{\circ}C$ 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 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 $37.0 \pm 0.5^{\circ}C$ after surgery.

Ginseng Treatment

Ginseng 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 200 μl/200 g distilled water at the same time points. Beinning the day after ischemia induction, some animals were administered 200 μl ginseng extract solution p.o. daily for seven days. Animals retained for behavioral testing were administered ginseng for an additional seven days during the test period. Animnals were injected with scopolamine (1 mg/kg, i.p., Sigma #0929) during Morris water maze and passive avoidance testing as described below. Control animals were injected with an equal volume of physiological saline.

Morris water maze

In the standard use of water maze the rat is placed into the pool at one of several randomly ordered start locations near the wall and swims to a submerged platform in a fixed position(simple task). Escape latency, swim speed and swim distance are the main parameters of these escape trials which provide imformation about the ability of learning and about the motor performance. The commercially available video tracking programme, the Etho-Vision®, from Noldus Information Technologies, can analyse rat behavior in an arena. The maze was a stainless pool (186 cm diameter 50cm in height) filled with clear water($25 \pm 2^{\circ}C$) from which they could escape onto a hidden platform[13]. The platform (10cm diameter 49cm in height) was hidden 1.0cm below the water level. In the probe trial, the platform is removed and the rat is permitted to swim freely about the pool for a given time[14].

The pool was situated in a room measuring 10cm with different markers on three of the walls as cues. So the water was made opaque by the addition of milk powder. The maze was divided conceptually into four quadrants(1-4) and three concentric annuli. A counter area, twice the size of the platform in diameter was used as a measure of search accuracy.

Place learning Each rat underwent four trials per day for five consecutive days. For each trial, the rat was placed in the water facing the pool wall at one of eight around the tank. Latency to finding the hidden platform (escape latency) was recorded. Each rat was allowed a maximum of 180 second to find a hidden platform and remain on it for 10 second. If a rat did not finded platform after 180 seconds, rat was gently put on it by the investigator. Once the rat located the platform (or was put on it) it was permitted to remain there for 10 second. At the end of the five trials the rat was dried with paper towels.

Probe trial. On the sixth day of lerarning test, the platform was withdrawn and the time the rat swam in each of the four quadrants of the tank was record for 180 second. Learning was defined as at spending a time significantly longer than 75s in the quadrant where the platform was located (training quardrant).

Passive avoidance

A shuttle box containing two compartments (260x 200x

150 mm) separated by a guillotine-type door (90 x 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)[15]. Rats were tested in groups of n=8(scopolamine), n=8(GS extracts), 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(training). The muscarinic receptor antagonist scopolamine, which is known to disrupt learning and memory functions in humans and passive avoidance retention in mice[16]. Training was initiated by placing the rat individually in the illuminated compartment (start box) of the apparatuses.

We checked the number of trials, the inter-trial intervals, the adaptation period and the shock intensity. After 60s a door opened giving access to the adjacent and similar, but dark, compartment (goal box). Rat not entering into the goal box within 180 second were discarded. Upon entering into the goal box, the door closed and an inescapable electric foot shock (1 mA for 2s) was delivered through a grid floor.

Tiobarbituric acid reactive species (TBARS) measurement

The lipid peroxidation level of the hippocampus portion was measured as malondialdehyde(MDA) which is the end product of lipid peroxidation, and reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm[17] 3 ml phosphoric acid (1%) and 1 ml TBA(0.6%) was added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 60 min in a boiling water bath after cooling, 4 ml of n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 20,000 rpm for 20 min. The organic layer was transferred to fresh tube and its absorbance was measured at 532 nm. The standard curve of MDA was constructed over the concentration range of 0-40 μM[18].

RESULTS

Cognitive-Enhancing Activity of ginseng extract following ischemia/reperfusion

The water maze reveals an impairment in spatial learning and memory which can be easily quantified. To compare spatial learning of rat, we tested them on the hidden platform and testing 1 week after induced ischemia. Seven days following ischemia induced by 4-VO, rats were trained in the water maze for 5 days and their mean latency times (±SEM) were measured (Fig. 1). During the escape trials all rats were able to find the hidden platform.. With increasing number of trials the escape latency decreased in both groups. Latency times for the 4-VO rats were consistently longer than times for either the sham-operated or ginseng extract-treated animals, as was the time to reach the platform region during the probe trial on day 6. Ischemic animals receiving ginseng extract took slightly longer to swim to the platform than sham operated animals, but performed just as well in the probe trial. During the probe trial, sham-operated rats spent about 30% of the 180s trial duration swimming within the quadrant in which the platform had previously been located (data not shown). This finding suggests that these animals remembered the platform position better than ischemic animals, which spent significantly less time in that quadrant (data not shown).

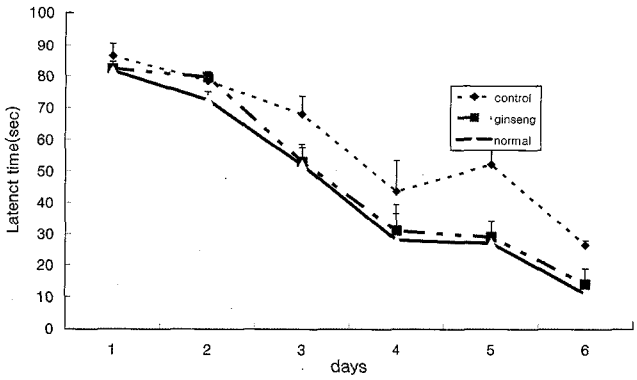

Fig. 1. Effects of ginseng on Morris water maze performance deficits induced by 10 min cerebral ischemia in rat (n= 8-13). Oral administration of ginseng (200 mg/kg twice per day) for 14 days. Mean daily latencies of escaping from the start point onto the hidden platform. Each rat was subjected to two trials per day for 5 consecutive days. Data represent means±SEM.

Group differences in the probe trials and passive avoidance were evaluated with one-way ANOVA followed by Duncan's multiple range testing, using a computerized statistical package. As shown Fig. 1. the mean latency in finding the platform declined progressively during the training period. Rats after ischemia take longer than sham-operated rats. Thus prolongation of latency was markedly shortened by ginseng at a dose of 200 mg/kg. In the probe trials, the swimming time spent in Q3 was used

to estimate retention performance.

Neuroprotective Effect of ginseng extract on global cerebral ischemia in vivo

To examine the neuroprotective effect of ginseng 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 200 μl/200 g. 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[19]. 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 ginseng extract 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 striatum pyramidal was weakly stained, showing occurrence of neuronal cell damage within the CAI subfield (Fig. 2C); Figure 4D 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 ginseng extract had a significantly reduced number of damaged pyramidal neurons in the CAI field (Fig. 2, E and F). There was no significant difference in body temperature between ischemic and ginseng extract treated groups at any time point recorded indicating that neuroprotective effects of ginseng 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. 3). In the ischemic group the viable cell density was 20.3 ± 3.4 cells/mm^2, which is far lower than that in the sham group, 338 ± 10.6 cells/mm^2. In the group injected with ginseng extract, viable cells were measured to be 294.5 ± 10.2 cells/mm^2. Thus ginseng extract rescued 86.4% of the ischemic neurons.

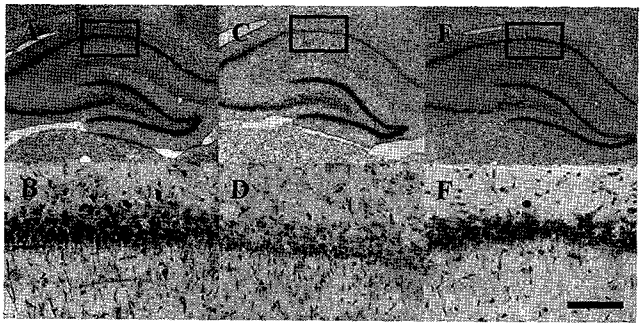

Fig. 2. Representative photomicrographs of cresyl violet-stained hippocampal regions of either sham-operated animals (A, B) or animals that had been subjected to 10 min ischemia followed by the treatment with either saline (C, D) or 200mg/kg of ginseng (E, F). Boxed regions in A, C, and E are shown in 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, ginseng treatment conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (E, F). Scale bar is 100 mm. The male Wistar rats were 6 weeks.

Passive avoidance analysis

Differences were observed in the passive avoidance test among sham-operated, ischemic, and ginseng reated ischemic rats at last time. 7days after 4-V0, there was a significant increase in the retention latency as compared to the initial latency, demonstrating that there was no memory impairment in the 4-V0 rats.

The retention latency in the sham-operated rats ($17,3 \pm 3.2$ s) was high and the 4-VO rats (8.3 ± 2.36 s) was significantly low compared to the initial latency. The retention acquisition in the 4-V0 rats was significantly high as compared the initial latency (Fig. 4).

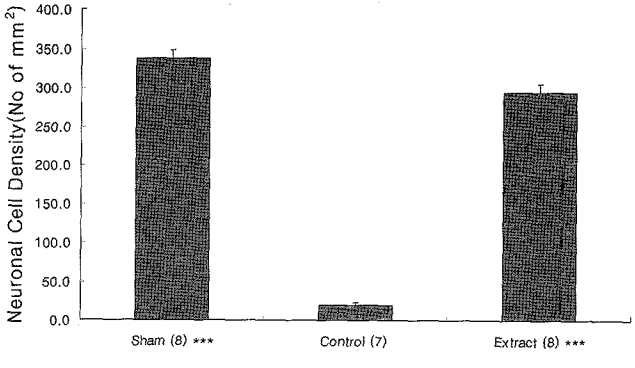

Fig. 3. Neuroprotective effects of ginseng(200 mg/kg). Either saline of ginseng was injected in i.p. into the animals following 10 min ischemia. Seven days later, neuronal cell density in CA1 neurons. Statistically significant differences from saline-treated group (***$p<0.001$). Sham, sham-treated animals (n=8); control, saline-treated animals following ischemia (n=7). ginseng treated animals following ischemia (n=8 for 200 mg/kg). The male Wistar rats were 6 weeks.

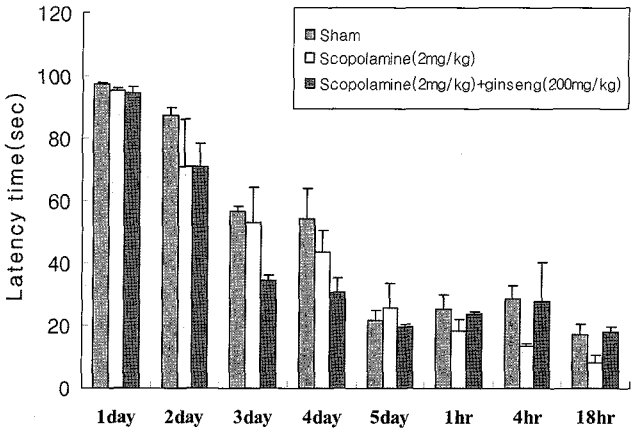

Fig. 4. Effect of ginseng on scopolamine induced memory deficits in the passive avoidance test. At 30 min after trainining trials, scopolamine (2 mg/kg i.p.) or the same volume of saline was administered to rats. At 30 min after scopolamine injection, the rats were treated with ginseng (200 mg/kg). Acquisition trials were carried out 30 min after ginseng treatment. At 5 days after acquisition trials, the test trials were carried out. Data represents mean ± SEM (n=8). Significantly different from the vehicle control group (one-way ANOVA). The male wistar rats were 6 weeks.

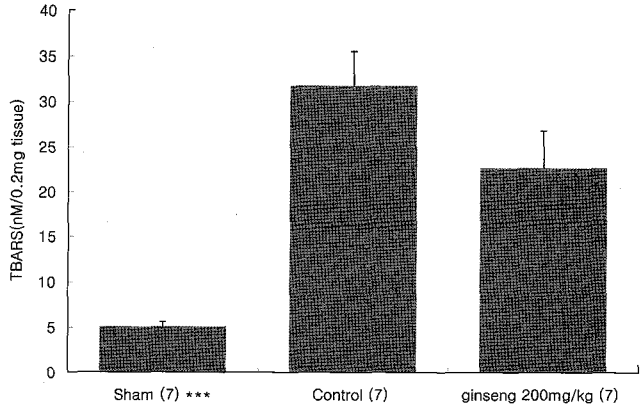

Fig. 5. Effect of ginseng on lipid peroxidation following global cerebral ischemia. MDA levels were measured in 10% homogenates of hippocampus portion from rats subjectecd to 10 min of ischemia. All drugs were administrated intraperitonealy 0 and 90 min after reperfusion. The antiperoxidative effect showed 69.69 % protection from tissue damage as compared with untreated animals. Values are mean ± SEM(n=7) ***p<0.001 as compared with vehicle(normal saline) treated animals (one-way ANOVA followed by Tukey test).

Lipid peroxidation data

The degree of free radical damage following ischemia reperfusion injury was measured as MDA levels. There was an increase 69.69% in the MDA levels following ischemia reperfusion insury as compared with sham-operated animals (5.1 ± 0.5 nmol/g tissue, ***p<0.001) Ginseng post treatment resulted in a significant and dose-dependently reduction in the free radical-mediated lipid peroxidation as indicated by a decrease in the MDA levels, at various dose levels. In ginseng -post treated groups with doses 200 mg/kg, TBARS levels were 5.1 ± 0.5 nmol/g tissue TBARS respectively (Fig. 5).

DISCUSSION

In the present study, the efficacy of ginseng (200 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 ginseng in traditional mdicine for the treatment of CNS dysfunction, we tested potential neuroprotective effects of ginseng using the 4-VO model in rats. The results indicate that ginseng confers significant neuroprotection against 10 min of ischemia induced by 4-VO. The results indicate that ginseng confers significant neuroprotection. This is reasonable because ginseng radix is effective in the prevention and repair of cerebral ischemia[20]. It has also been shown that ginsenoside, a ginseng component, protects ischemic hippocampal neurons[21].

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 imvolved in learning and memory processes.

The aim of anaesthesia was to avoid adverse post-operative side effects(e.g. seizures) observed at this duration in non- anaesthetized rats. In our experiment there was some difference in cell loss or behavioural performance in the watermaze among thegroups. But there was little difference in lipid peroxidation assay.

The mode of cell death (necrosis or apoptosis) would be depend on severity of ischemic injury[22]. 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 CA1 pyramidal cells, whereas some of those are undergoing apoptosis[23]. 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[24]. Such cell deaths are referred to as delayed neuronal deaths[25]. 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[26].

The ginseng was found most effective in the 200 mg/kg i.p. and 200 mg/kg p.o. dose, respectively. Therefore these doses were selected to study the anti-oxidative activities and neuroprotection of these extracts. The study by other authors have also used ginseng at a dose of 200-500 mg/kg p.o. for study its adaptogenic activity[27]. The previous studies with the ginseng for its learning and memory effects have also shown significant facilitatory effects at 100 and 200 mg/kg p.o..[28]

To summarize, ginseng extract is characterized cognition-enhancing drug in improving the cognitive impairment caused by global cerebral ischemia. The effects of these extracts 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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