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Effect of *Panax ginseng* on Latency of Passive Avoidance Response and Neuronal Damage of Hippocampus

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The effects of crude saponin (SAP) and alkaloid (ALK) fractions of *Panax ginseng* C.A. Meyer on the detrimental effects of electroconvulsive shock (ECS) and scopolamine on passive avoidance response (PAR) were studied in male Sprague-Dawley rats, referring their effects on the neuronal injury and plasticity of hippocampus in response to electrolytic lesion of left entorhinal cortex (ECL). The detrimental ECS effect on PAR was attenuated by pre- and post-treatments with SAP and ALK, respectively, or by pretreatment with aminoguanidine (AG), an inhibitor of diamine oxidase and NO synthase. And the detrimental scopolamine effect on PAR was also inhibited by pre-treatment with ALK or AG, and by post-treatment with SAP or ALK, respectively. On the 7th day after ECL, the brain sections stained by cresyl violet and by acetylcholinesterase (AChE) histochemistry, respectively, showed the chromatolysis and numeral decrease of neurons and the reduction of AChE reactivity in the hippocampus CA1 area and to a lesser extent, in the dentate gyrus. The neuronal cell death of the CA1 area was significantly reduced by SAP, ALK, or AG, and the reduction of AChE reactivity was significantly attenuated by SAP or ALK and to a lesser extent by AG. These results suggests that the protective effect of ginseng SAP and ALK fractions on ECS- or scopolamine-induced impairment of PAR may be ascribed in part to preservation of hippocampal neurons, particularly cholinergic neurons.

Key Words: ECS, Scopolamine, Passive avoidance response, Entorhinal cortex lesion, Ginseng, Aminoguanidine, Cholinesterase, Hippocampus

INTRODUCTION

The population of elderly persons has been gradually increasing. And the prevalence of dementia, a major cause of disability, rises steeply with increasing age. Dementia has a number of causes, such as neurodegenerative disorders, metabolic endocrine disorders, cerebrovascular disturbances, deficiency of nutrients or neurotropic factors, drug toxic reactions, traumas, brain tumors, and so on (Mayeux et al, 1993).

Although Alzheimer's disease, the most common type of dementia characterized by progressive impairments in memory and cognition, shows diffuse atrophy of the brain, many quantitative morphometric studies have suggested that the dementia may be attributable to histopathologic changes in the hippocampus (Ball, 1977; Ball et al, 1985; Barnes, 1994).

The main afferent fibers to the hippocampus are glutamate/aspartate neurons arising from entorhinal cortex and cholinergic neurons from septum (Cotman et al, 1988). So, entorhinal cortex lesion (ECL) may bring about the plasticity of synaptic neurotransmission in the hippocampus (Poirier & Nichols, 1991). In the ECL-induced plasticity, the cholinergic input of hippocampus from septum might be increased to compensate the loss of glutaminergic input from entorhinal cortex (Lynch et al, 1972; Hjorth-Simonsen, 1972; Nadler et al, 1977).

There are many reports suggesting that neuronal

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dysfunction and death produced by brain damages, particularly brain ischemia, may be improved by various pharmacologic agents: antioxidants and free radical scavengers, Ca^{2+} channel blockers, antagonists of N-methyl-D-aspartate (NMDA) receptor, antagonists of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, opioid antagonists, antagonists of platelet activating factor, analogs of thyrotrophin releasing hormone, inhibitors of calpain and other protease, NO synthase inhibitors, and so on (Faden & Saltzman, 1992; Choi, 1995). Unfortunately, any therapeutic drugs have not been clinically proved to be effective in the treatment of brain damage. However, if not satisfactory, a few cholinesterase inhibitors have been tried in control of some types of dementia.

Jeong & Jin (1995) reported the neurotropic activities of ginsenosides obtained from *Panax ginseng*, such as prolongation of neuronal viability, stimulation of axonal development, reduction of ischmic brain injury, and protection of the conditioned avoidance response from its impairment by electroconvulsive shock (ECS), scopolamine, and cycloheximide. These evidences may be considered to be in accordance with the report showing the inhibitory effect of ginseng saponin on hypothermia stress-induced decrease of brain acetylcholine content (Cheng et al, 1986).

On the other hand, Kim et al (1992) reported that ECS-induced impairment of active avoidance response was reduced by aminoguanidine, an inhibitor of diamine oxidase (Schuler, 1952; Seiler, 1987) and NO synthase (Corbett et al, 1992).

Therefore, this study was tried to ascertain the effects of crude saponin and alkaloid fractions of *Panax ginseng* C.A. Meyer on the impairment of passive avoidance response (PAR) induced by ECS and scopolamine (Glick & Zimmerberg, 1972; Lerer et al, 1986; Squirre, 1977) in comparison to the effects of aminoguanidine, referring their effects on the ECL-induced neuronal death and plasticity of the hippocampus.

METHODS

Animals

Adults male Sprague-Dawley rats (Dae-Han Animal Comp) weighing 250±20 g were used. All rats were kept in light and dark cycle with the light phase from 8:00 a.m.~8:00 p.m. for 7 days, and then PAR analysis and ECL were carried out during the dark phase.

Preparation of ginseng saponin and alkaloid fractions

Ginseng saponin: White ginseng powder of 1000 g was extracted with 95% ethanol of 2 l at 75°C for 6 hours, and then extracted twice with 95% ethanol of 1 l. The ethanol extract (2.8 l) was filtered through Whatman No. 2 paper, and concentrated upto 50 ml by rotary evaporator at 65°C. The concentrate was diluted up to 200 ml by adding DW, and was washed 3 times with 200 ml of diethyl ether to exclude its lipid soluble components. The aqueous layer was extracted three times with water-saturated n- buthanol of 200 ml. The 3 butanol extracts were mixed and concentrated by rotary evaporator at 70°C and dissolved in 100 ml of absolute methanol. The methanol solution was evaporated by Speed vac concentrator, and consequently, crude saponin fraction of 11.15 g was obtained.

Ginseng alkaloid: White ginseng powder of 1000 g was extracted with 75% ethanol of 2 l at 75°C for 12 hours, and then extracted twice with 75% ethanol of 1 l. The ethanol extract (2.9 l) was filtered through Whatman No. 2 paper, and concentrated upto 900 ml by rotary evaporator at 70°C. The concentrate was added with 10% HCl of 900 ml and was washed twice with 1.8 l of diethyl ether. The aqueous layer was ammoniated to pH 9.0, and extracted three times with chloroform of 2 l. Chloroform extract was concentrated by rotary evaporator at 57°C, and dissolved in 100 ml of absolute methanol. The methanol solution was evaporated, and finally, the crude alkaloid fraction of 2.96 g was obtained.

Analysis of passive avoidance response

PAR was studied in a one-trial learning, step- through type situation of a two-way shuttle box (Lafayette), which utilizes the natural preference of rats for a dark environment. The two-way shuttle box was divided by a guillotine door, and each of two compartments has equipped with a halogen bulb of 100 W and with a metal grid floor, through which an inescapable electrical shock (3 mA for 5 sec) could be delivered to the dark room in a learning trial. On day 1, the

rats were habituated to the dark room for 5 min. Immediately after habituation, the room was converted to a bright room by illumination of the bulb positioned 20 cm above the floor, and the rat was allowed to enter the dark room. The latency to cross from the bright room to the dark one was automatically recorded. Such trial was further given once a day for three days. Upon entry the dark room at the end of the last adaptation trial, the rat was given an inescapable electric shock through a metal grid for the first learning of PAR. The rats showing the latency of longer than 15 sec in the last adaptation trial were excluded. 24 Hr after the learning trial, the remained rats were habituated to the dark room for 5 min and then was given one more electric footshock for over-learning of PAR. The latency of the rats was tested 10 min after the over-learning trial, and the rats showing the shorter latency than the cut-off period of 180 sec was excluded. On the next day, retention of PAR was tested and the latency was measured up to a maximum of 180 sec. The eight or more rats were allocated to each of 16 animal groups. The data obtained in this study were shown as mean ±standard deviation and analysed using the one-way ANOVA test.

ECS and scopolamine treatments

By about 10 sec after the over-learning of PAR, the animals were given ECS (60 mA, 0.9 sec, 200 Hz) via ear-clip electrodes using a ECT unit (Ugo Basile) and scopolamine (1.5 mg/kg, i.p.), respectively.

Treatments with ginseng fractions and aminoguanidine

Three hours before and after ECS or scopolamine treatment, respectively, the rats were intraperitoneally given 0.85% saline (1 ml/kg: SAL) as a control, crude ginseng saponin fraction (40 mg/kg: SAP) and alkaloid fraction (10 mg/kg: ALK), and aminoguanidine (30 mg/kg: AG), respectively. Animal number of each drug-treatment group was 8 or more.

Entorhinal cortex lesion

Under the anesthesia with pentobarbital sodium (50 mg/kg, i.p.), rats were placed in a stereotaxic apparatus (David-Korf) and the electrolytic lesion was made by means of a stainless steel unipolar electrode (0.3 mm in diameter) insulated with polyurethane paint except for 0.5 mm at the tip. An anodal constant current of 1 mA was passed through the electrode for 40 sec. Three electrolytic lesions were made in left hemispheres at 5.52 mm, 6.60 mm, and 7.26 mm below the surface point of 0.83 mm posterior and 2.30 mm lateral to bregma (Paxinos & Watson, 1986; Poirier & Nichols, 1991). Sham-operated rats used as controls were prepared by lowering the electrode into the three points of the cortex without passing any current. Rats were given the first i.p. injection of each compound 3 hr before ECL, and for the following 6 days, single daily i.p. injection of each compound was performed at 10:00~11:00 a.m.

Histological studies of hippocampus

To carry out the histological study, animals were sacrificed on the 7th day after ECL. The five or six rat brains were used for each of five study groups, and their slices were observed as follows.

Cresyl violet staining: Under the anesthesia with pentobarbital sodium (50 mg/kg, i.p.), the brain was fixed by perfusion of 4% paraformaldehyde solution containing 1% glutaraldehyde, and was coronally cut into 6 μm thick slices using a microtome. The brain slices were placed on a slide was sequentially hydrated through 100%, 96%, 90%, 80%, and 70% ethanol for 2 min each, and then placed in 0.3% cresyl violet solution for 10 min. The stained slices were differentiated in 96% ethanol and dehydrated through 70%, 80%, 90%, 96%, and 100% ethanol for 2 min each. And the slices was treated 3 times with xylene for 5 min each, mounted on slides using Canada balsam, and finally photographed with a magnification of ×200 using a Zeiss microscope.

Acetylcholinesterase (AChE) staining: Under the anesthesia with pentobarbital sodium (50 mg/kg, i.p.), the brain was fixed by perfusion of 4% paraformaldehyde solution containing 0.5% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. And the brain was taken out and stored at 4°C. Within 7 days, the brain was frozen and coronally cut into 8 μm thick slices using a cryomicrotome (AO Histostat). The brain slices were stained according to a modified method (Hanker et al, 1973; Tago et al, 1986) originally described by Karnovsky & Roots

(1964), as briefly described below. The brain slice was placed on a slide, washed with 0.1 M acetate buffer (pH 4.7), treated with 0.1 M acetate buffer containing 1 mM acetylthiocholine iodide, and then developed with 3,3'-diaminobenzidine tetrahydrochloride. The stained slice was washed with 0.05 M Tris-HCl buffer (pH 7.6), and dehydrated through 70%, 80%, 90%, 96%, and 100% ethanol for 2 min each. The slice was treated 3 times with xylene for 5 min each, mounted on slides using Canada balsam, and finally photographed with a magnification of × 15.625 using a Zeiss microscope.

RESULTS

Avoidance Latencies of experimental groups before PAR learning trials

As summarized in Table 1, the rats to show the avoidance latency of less than 15 sec in the last adaptation trial, were divided into 16 animal groups, all of which, in the peroid without ECS loading or any drug treatment and before PAR learning, showed the avoidance latencies ranging from 4.5±1.5 sec to 8.6 ±3.3 sec. And the latencies were remeasured 10 min after the over-learning trial, and the rats showing the shorter latency than the cut-off period of 180 sec was excluded in this study (described in the materials and methods).

Pretreatment effects on ECS-induced impairment of PAR

The avoidance latency of normal rats not to be exposed to PAR learning was 7.8±3.0 sec. And as described above, the latencies of all drug-treatment groups were controlled to 180 sec, the cut-off time of avoidance. But 24 hr after ECS, the period of saline-treatment (SAL) group was markedly shorten to 13.1±5.2 sec and this ECS-induced shortening effect was observed to be significantly reduced in other drug-pretreatment groups: crude ginseng saponin (SAP)/($F_{(1.15)}$=12.88. $p<0.01$) and alkaloid (ALK)/($F_{(1.15)}$=49.25, $p<0.01$), and aminoguanidine (AG)/($F_{(1.14)}$=17.11, $p<0.01$). And the latency of ALK group was greater than those of SAP and AG groups: SAP/($F_{(1.14)}$=5.24, $p<0.05$) and AG/($F_{(1.13)}$=4.34, $p<0.05$) (Fig. 1).

Posttreatment effects on ECS-induced impairment of PAR

The latency of SAL group measured 24 hr after exposure to ECS, was 13.1±5.2 sec. Like the pretreatment study observed above, this shortening effect of ECS was significantly reduced by posttreatments with SAP/($F_{(1.15)}$=31.29, $p<0.01$) and ALK/($F_{(1.15)}$=11.34, $p<0.01$). And the latency of SAP group was greater than that of AG group/($F_{(1.14)}$=13.71, $p<0.01$) (Fig. 2).

Table 1. The avoidance latency of eight or nine rats, measured before learning of passive avoidance response or any-treatment, which were allocated to sixteen experimental groups, as shown below

Treatments and Medications	ECS		Scopolamine	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
SAL	8.6±3.3	6.2±3.7	4.5±1.5	8.5±3.8
SAP	8.2±2.9	7.2±3.1	7.5±3.3	6.2±1.9
ALK	7.7±3.0	6.5±6.5	8.5±2.6	8.2±0.6
AG	6.8±2.8	6.1±1.5	6.7±2.2	7.8±1.5

Each of sixteen animal groups consist of one treament of transversal line and one medication of vertical line, and all data were measured before any treatment and medication, showing only avoidance latency after adaptation trials for 3 days.

Abbreviations mean as follows: SAL - 0.85% saline, 1 ml/kg, i.p.; SAP - crude ginseng saponin fraction, 40 mg/kg, i.p.; ALK - 10 mg/kg, i.p.; AG - aminoguanidine, 30 mg/kg, i.p..

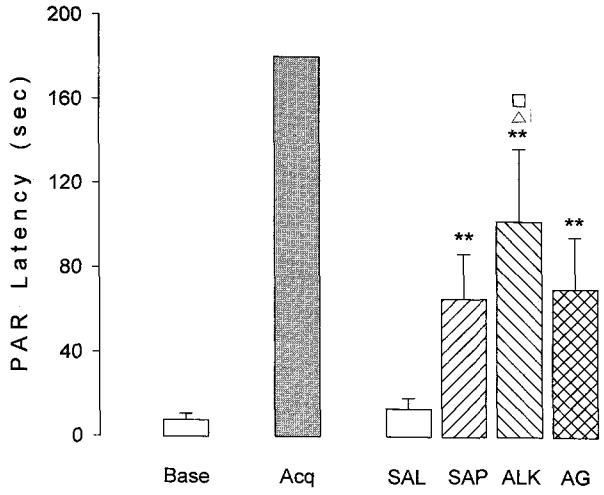

Fig. 1. Pretreatment effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the ECL-induced reduction of PAR latency.
Pretreatments were performed 3 hr before ECS, and each bar represents mean±S.D. of 8 or 9 data.
Abbreviations: Base-latency after the adaptation trials and without foot-shock dependent PAR learning; Acq - cut-off latency after PAR-learning.
** $P<0.01$ (vs SAL); △ $P<0.05$ (vs SAP); □ $P<0.05$ (vs AG)

Pretreatment effects on scopolamine-induced impairment of PAR

The avoidance latency of normal rats to be selected through the adaptation trials for 3 days, was 6.8±3.0 sec. And as described above, the latencies of all drug-treatment groups were controlled to 180 sec. But 24 hr after scopolamine treatment, the period of SAL group was markedly shorten to 11.5±8.8 sec and this shortening effect of scopolamine was significantly reduced by ALK/($F_{(1.15)}$=38.23, $p<0.01$) and AG/($F_{(1.14)}$=22.34, $p<0.01$). And as compared with the latency of SAP group, the latencies of ALK and AG groups were significantly greater: ALK/($F_{(1.15)}$=32.62, $p<0.01$) and AG/($F_{(1.14)}$=19.60, $p<0.01$) (Fig. 3).

Posttreatment effects on scopolamine-induced impairment of PAR

The latency of SAL was shortened from 180 sec or longer to 13.1±5.2 sec by scopolamine treatment. This shortening effect of scopolamine was moderately reduced by posttreatments with SAP/($F_{(1.14)}$=31.20, $p<0.01$) and ALK/($F_{(1.14)}$=13.86, $p<0.01$), but was

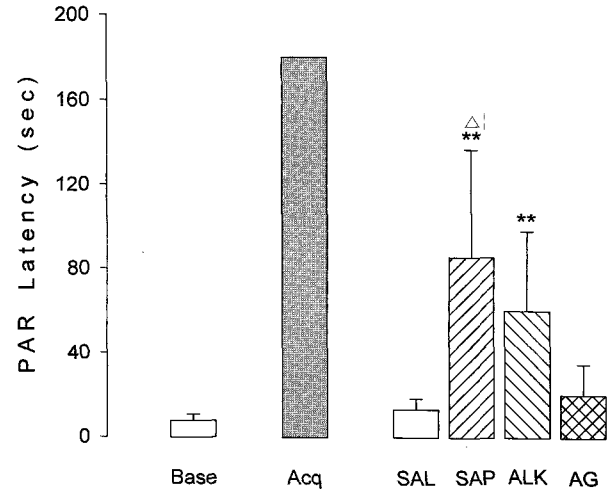

Fig. 2. Posttreatment effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the ECL-induced reduction of PAR latency.
Posttreatments were performed 3 hr after ECS and each bar represents mean±S.D. of 8 or 9 data.
** $P<0.01$ (vs SAL); △ $P<0.01$ (vs AG)

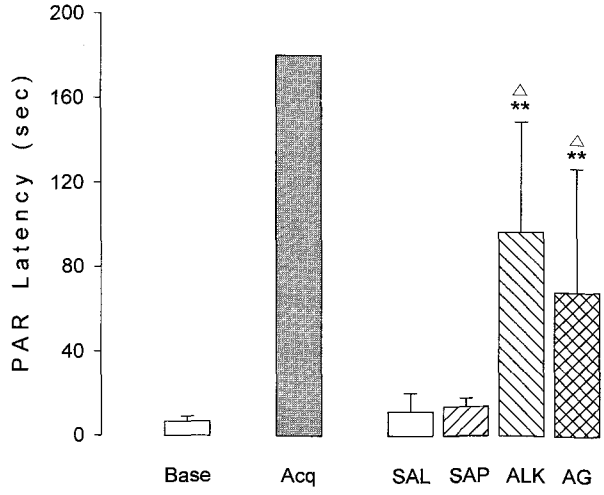

Fig. 3. Pretreatment effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the scopolamine-induced reduction of PAR latency.
Pretreatments were performed 3 hr before scopolamine treatment and each bar represents mean±S.D. of 8 or 9 data.
** $P<0.01$ (vs SAL); △ $P<0.01$ (vs SAP)

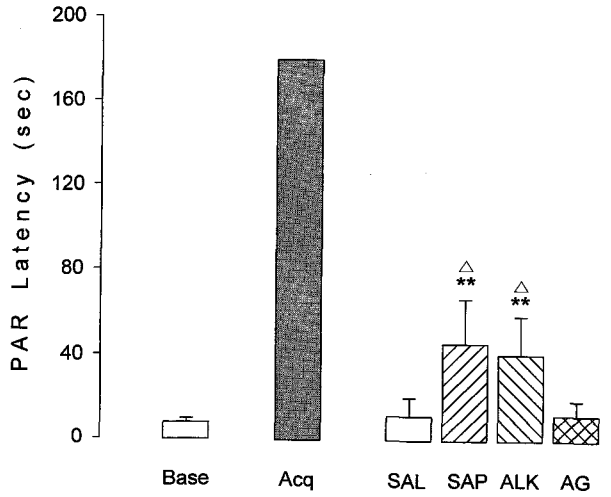

Fig. 4. Posttreatment effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the ECL-induced reduction of PAR latency.
Posttreatments were performed 3 hr after scopolamine treatment and each bar represents mean±S.D. of 8 or 9 data.
** $P<0.01$ (vs SAL); △ $P<0.05$ (vs AG)

not affected by posttreatment with AG. And the latencies of SAP and ALK groups was greater than that of AG group: SAP/($F_{(1.14)}$=26.07, $p<0.01$) and ALK/($F_{(1.14)}$=12.16, $p<0.01$) (Fig. 4).

Effects on ECL-induced damage of Hippocampal neurons

After staining with cresyl violet, the microscopic brain findings (×200) of the sham-operated rat, showed the intact and well arranged neurons of the hippocampal complex. However, unilateral ECL of the left-hand entorhinal cortex induced the marked neuronal damage of the hippocampus of either side, as shown in Fig. 5, which is the findings of the ipsilateral (left) hemisphere, showing little difference from those of the right one. Furthermore, the ECL effect was prominent in the CA1 area and to a significantly lesser extent, in the dentate gyrus. The treatments with SAP and ALK produced the considerable protection of the hippocampus from its neuronal damage by ECL, and to a lesser extent, AG showed the moderately protective effect (Fig. 5).

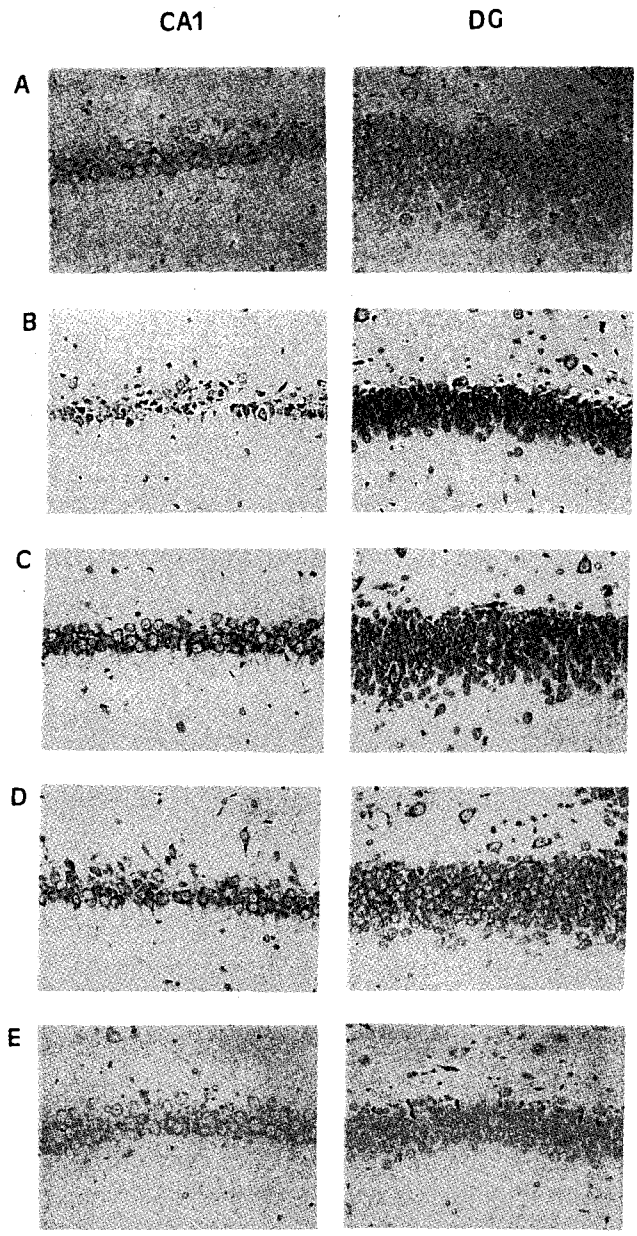

Fig. 5. Effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the microscopic findings (×200) of the ipsilateral hippocampus CA1 area (left) and dentate gyrus (right) to be stained with cresyl violet, after electrolytic lesion of the left-hand entorhinal cortex. Abbreviations: A, sham-operated; B, control, 0.85% saline; C, crude ginseng saponin fraction; D: crude ginseng alkaloid fraction; E: aminoguanidine. The dosage regimen of each drug was described in the materials and methods.

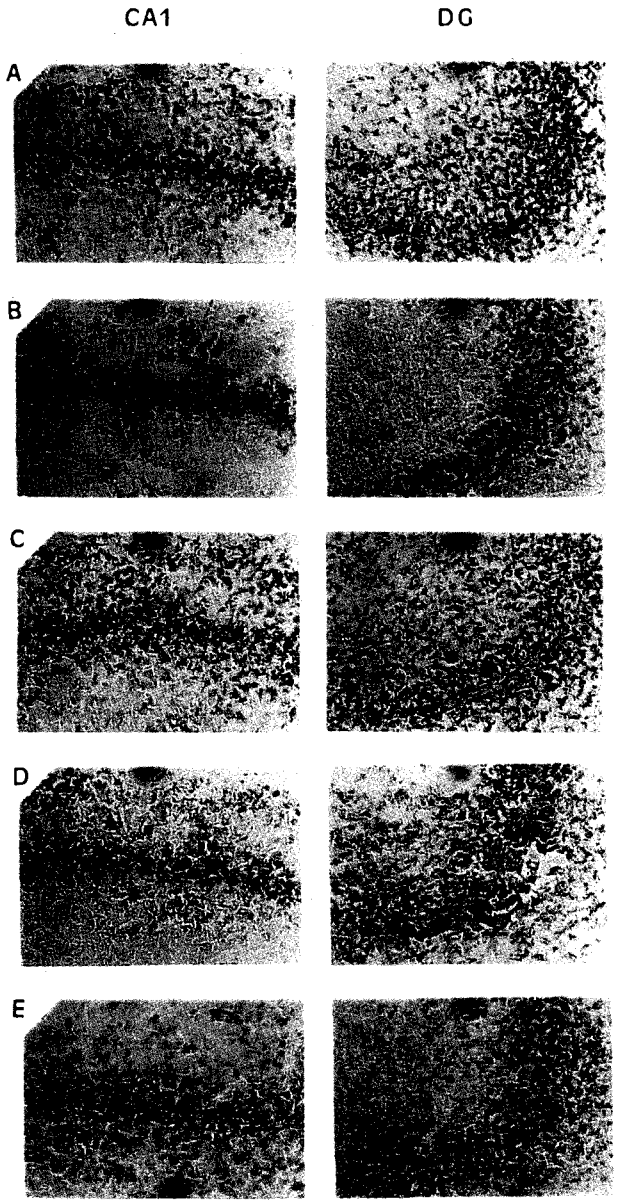

Fig. 6. Effects of crude ginseng saponin and alkaloid fractions and aminoguanidine on the histochemical findings (×15.625) of the ipsilateral hippocampus CA1 area (left) and dentate gyrus (right) to be stained to acetylcholinesterase, after electrolytic lesion of the left-hand entorhinal cortex.

Effects on ECL-induced change of hippocampal acetylcholinesterse

In the histochemical findings of the brain acetylcholinesterase, the hippocampus CA1 and the dentate gyrus areas of the sham-operated rats showed the prominent reactivity to AChE. And as appeared in the cresyl violet stained hippocampus, unilateral ECL of the left-hand entorhinal cortex induced the marked reduction of AChE reactivity of the hippocampus of either side, as shown in Fig. 6, which is the findings of the ipsilateral hemisphere, showing little difference from those of the right one. Furthermore, in accordance with the neuronal damage visualized by cresy violet, the ECL-induced reduction of hippocampal AChE reactivity was prominent in the CA1 area and to a lesser extent, in the dentate gyrus. SAP, ALK, and AG considerablely protected the hippocampal AChE reactivity from its reduction by ECL (Fig. 6).

DISCUSSION

There are many evidences suggesting that the hippocampus may be either a waystation for long- term memory or a facilitation system that is essential for the storage of memories elsewhere in the brain, presumably the cerebral cortex (Amaral et al, 1990; Kupfermann & Kandel, 1995). And in the hippocampus glutaminergic afferent neurons from the entorhinal cortex participate in learning and memory via activation of NMDA receptors (Levisohn & Isacson, 1991). In addition, cholinergic neurons from the septum are known to be the other main excitatory afferent projections to hippocampus (Nitsch et al, 1992). Therefore, the lesioning of entorhinal cortex has been well proved to induce the hippocampal plasticity of glutaminergic neurons raising from the contralateral entorhinal cortex and of cholinergic neurons from septum (Lynch et al, 1972; Nadler et al, 1977; Nitsch et al, 1992).

Petkov & Mosharrof (1987) and Chepurnov et al (1994) reported that ginseng saponin might facilitate learning and reduce the latency of active avoidance response, and Benishin (1992) suggested the ginseng saponin- dependent activation of central cholinergic transmissions, which had been proved to have a major role in the acquisition process (Bartus et al, 1982; Davies, 1985).

An earlier study of ours (Kim et al, 1992) demonstrated that CDP-choline and aminoguanidine appeared to recover the impaired active avoidance response.

In this study, therefore, the effects of saponin and alkaloid on the impairment of PAR learning and

retention induced by ECS and scopolamine were investigated, in comparison to the effect of aminoguanidine, referring their effects on the hippocampal neuronal injury and plasticity of cholinergic neurons appeared on the 7th day after unilateral ECL.

The impairment of PAR learning by ECS and scopolamine was reduced by pretreatment or posttreatment of a crude ginseng alkaloid fraction. The ECS-induced impaired PAR was reduced by pretreatment or posttreatment of a crude ginseng saponin fraction, but the impairment of PAR by scopolamine was reduced by posttreatment with the saponin fraction, but little affected by posttreatment with it. Finally, the impairments of PAR induce by ECS and scopolamine, respectively, were reduced by pretreatment of the alkaloid fraction, but not affected by posttreatment with aminoguanidine.

These results seem to be in accordance with the report of Chepurnov et al (1994) which showed the beneficial actions of ginseng fractions on learning and memory.

Therefore, after partial ablation of glutaminergic input into the hippocampus by unilateral ECL, we evaluated the effects of crude ginseng saponin and alkaloid fractions on the neuronal injury and plasticity of cholinergic neurons in the hippocampus CA1 area and dentate gyrus. ECL produced the marked neuronal cell death and reduction of AChE reactivity in the CA1 and dentate gyrus of the hippocampus of either side. Consequently, the ginseng saponin and alkaloid fractions produced the significant protection of the hippocampal neurons and AChE reactivity from their damages by ECL. Meanwhile, as compared to those effects of ginseng fractions, aminoguanidine, to a lesser extent, showed the beneficial effect on those ECL injuries. Thses results obtained in the histochemical AChE study seem to be contrast to the plasticity reaction of the hippocampal cholinergic neurons, which was proposed to compensate the ECL-induced deficit of glutaminergic inputs (Lynch et al, 1972). Therefore, this results seem to suggest that the glutaminergic input into the hippocampus may play as an impotant neutropin on the cholinergic neuron in the hippocampus, particularly in the CA1 area, and that the hippocampal cholinergic function might be preserved or enhanced by aminoguanidine as well as crude ginseng saponin and alkaloid fractions. And these data seem to be accodance with the previous evidences (Benishin, 1992; Kim et al, 1992).

So, the protective effects of crude ginseng saponin and alkaloid fractions as well as aminoguanidine on ECS- and scopolamine-induced impairment of PAR might be ascribed in part to their beneficial activities on the hippocampal neurons, particularly cholinergic neurons.

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