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새로운 뇌 위축 동물 모델과 그 모델에서의 고려인삼의 보호 효과

임강현[#*1], 김명규[2], 이세나[1], 김현미[2], 정주호[3]

1: 세명대학교 한의과대학
2: 우석대학교 약학대학
3: 경희대학교 고황의학연구소

Novel animal model for brain atrophy and protective effects of Korean ginseng

Kang-Hyun Leem[#*1], Myung-Gyou Kim[2], Se-Na Lee[1], Hyun-Mi Kim[2], Joo-Ho Chung[3]

1: College of Korean Medicine, Semyung University, Jechon 390-711, Korea
2: College of Pharmacy, Woosuk University, Wanju 565-701, Korea
3: Kohwang Medical Research Institute, Kyung Hee University, Seoul 130-701, Korea

ABSTRACT

Objectives : Anti-oxidants are known to prevent neuronal diseases with pathological and physiological changes such as the brain atrophy and cognitive impairment. This study was designed to investigate the protective effects of Korean ginseng on the oxidative stress induced pathologic changes, and develop new animal model for the brain atrophy. Korean ginseng has anti-oxidant, anti-aging, and protective effects on the brain ischemia.

Methods : The intracerebroventricular (ICV) hydrogen peroxide (H_2O_2) injection into mice was conducted to generate oxidative stress.

Results : The ICV H_2O_2 (1 M, 5 μl) injection did not induce either convulsion or death in the acute phase. At the end of second week, cognitive impairment and pathologic change of the brain were observed. The massive brain atrophy was found in the H_2O_2-injected mice, especially in the hippocampus and thalamus. Treatment with Korean ginseng showed a protective effect against the brain atrophy. The H_2O_2-injected mice revealed cognitive impairment in the passive avoidance test, and Korean ginseng alleviated cognitive impairment.

Conclusion : The results indicate that Korean ginseng has a protective effect on the oxidative stress-induced neuronal damages.

Key words : Brain atrophy, Korean ginseng, oxidative stress, hydrogen peroxide, mouse

#*Correspondence to: Kang-Hyun Leem, O.M.D., Ph. D.
College of Korean Medicine, Semyung University, Jechon 390-711 Korea
· Tel: 82-43-649-1341 · E-mail: lkh@semyung.ac.kr
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INTRODUCTION

Korean ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae), is one of the most popular tonics that have been exploited worldwide including Korea, China, and Japan. Korean ginseng, called "In-Sam" in Korean, has been frequently used in Traditional Korean Medicine (TKM) for various therapeutic applications. The first record of Korean ginseng prescription as a medicinal herb appeared in 'Shinnongbonchogyoung'. The nature and flavor of Korean ginseng are slightly warm, sweet, and bitter. It acts on the spleen, lung, and heart meridians. According to 'Shinnongbonchogyoung', Korean ginseng is not only the primary medicine administered for promoting vitality of the five viscera (Liver, Heart, Lung, Kidney, and Spleen), but also is the therapeutic medicine that helps restore composure, or remove causes of illness of the five viscera. Long term administration of Korean ginseng also improves eyesight and increases longevity[1-3]. Ginseng has been reported to show various pharmacological effects in the central nervous system, such as memory facilitation[4,5] and behaviors[6]. In addition, it has anti-oxidant[7], anxiolytic activities[8], anti-fatigue[9], anti-obese[10], anti-carcinogenic[11], anti-stress, and adaptogenic effects on the acute hypothermia[12]. Korean ginseng contains many active ingredients, e.g., ginsenosides that have beneficial neuroprotective effects[13-15]. This herb stimulates the immune function in the elderly[16], and has a potent recovery effect of impaired brain growth exposed to ethanol in neonatal rats[17].

Brain atrophy is a final outcome of many diseases, such as Alzheimer's disease, non-Alzheimer's dementia, brain ischemia, traumatic brain injury, multiple sclerosis, and alcoholism[18-23]. The neuronal death and decline of cognitive ability are generally found. The neuronal death includes complicated and various mechanisms, e.g., free radicals, mitochondrial dysfunction, calcium, proteases, and cell cycle[24]. Oxidative stress is known to be one of the main mechanisms leading to neuronal death[25,26]. However, pathologic changes due to excessive oxygen free radical in the brain are not well defined. The anti-oxidants, anti-aging effects, and protective effects on the brain ischemia of Korean ginseng have been reported[27-36]. In this study, we performed in vivo experiments to examine the neuroprotective effects of Korean ginseng on the oxidative stress-induced brain atrophy, and to develop the novel animal model for the brain atrophy. Intracerebroventricular (ICV) injection of hydrogen peroxide (H_2O_2) was conducted to make the excessive oxygen free radical. The passive avoidance test was performed to determine the cognitive impairment. Atrophic changes in the brain after H_2O_2 ICV injection were examined using the cresyl violet staining.

MATERIALS AND METHODS

1. Animal care and sample preparation

Male ICR mice (20-30 g) were purchased from Samtako Bio (Osan, Korea). Animals were housed at the room temperature of 22 ± 1°C with 12-h light-dark cycle (light on 8:30 a.m. to 8:30 p.m.) with free access to the food and the water. Animals maintained in the same environments for one week prior to the experiment. The experimental procedures were carried out according to the animal care guidelines of National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. H_2O_2 was purchased from Sigma Co. (Seoul, Korea) and diluted to 1 M with saline. Korean ginseng, the roots of 6-year-old "White Ginseng", was purchased from Kumsam drug market in Korea. Dried Korean ginseng (200 g) was heat-extracted twice with distilled water. The filtrates were evaporated with a rotary vacuum evaporator, and lyophilized. The weight of resulting extract powder was 32.3 g. The extract was dissolved in water and administrated daily (100 mg/kg per day, *p.o.*).

2. Intracerebroventricular (ICV) injection

ICV injection was performed as described previously[38]. In brief, mice were anesthetized with ether, then the 2 mm double-needle (tip: 27 gauge × 2 mm and base: 22 gauge × 10 mm) fixed to the 25 μl Hamilton microsyringe was inserted into the bregma. The volume of ICV injection was 5 μl.

3. Determination of concentration of hydrogen peroxide (H_2O_2)

Mice were divided into four groups of eight: the control and three H_2O_2-injected groups (300 mM, 100 mM, and 1 M). The concentrations of H_2O_2 were determined from previous reports[39,40]. The mice in the control group were given saline. The mortality ratio and behavioral changes were observed for two weeks as previously described[41]. In brief, animals were handled by same person, and examined in the familiar and unfamiliar environments. The first observation of animals was made in home cage as a familiar environment. The animal was then removed from its home cage and examined in a more open environment, which is defined as an unfamiliar environment. Behavioral changes while removing from the cage were observed. At the end of second week, the mice (1 M, ICV) only showed sluggish and hesitating behaviors when removed from the cage. Accordingly, we performed next experiments at 1 M H_2O_2 concentration and two week period.

4. Experiments for new animal model of brain atrophy and neuroprotective effects of Korean ginseng

Mice were divided into three groups of eight: saline ICV-injected the control group (n = 8) and 1 M H_2O_2 ICV-injected two experimental groups (n = 8, one group is the H_2O_2 group and the other group is the H_2O_2-KG group). The ICV-injections of saline or H_2O_2 were performed on the first day as described above. Daily administration of Korean ginseng (100 mg/kg, *p.o.*) was executed to the H_2O_2-KG group. The control and H_2O_2 groups were given daily administration of distilled water.

5. Passive avoidance test

On 13 days after H_2O_2 injection, mice were trained on a one-trial step-through passive avoidance task[42]. In brief, the passive avoidance box was divided into two compartments, illuminated and dark, and equipped with a grid floor. During the training trial, each mouse was placed in the lighted compartment; as soon as mouse entered the dark compartment, the door was closed and the mouse received an inescapable shock (0.25 mA, 1 sec). In the testing trial, 24 h after the training trial, the mouse was placed again in the lighted compartment. Re-entry time to the dark compartment was measured (the step-through latency maximum testing limit was 300 sec).

6. Brain tissue preparation and histological quantification of brain atrophy

On the following day of the passive avoidance test, animals were anesthetized with pentobarbital sodium (50 mg/kg). Upon reaching to the state of complete anesthesia, they were perfused and fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). Brains were removed from the cranium, post-fixed for 2 days, and then washed in 0.1 M PB. Finally, they were immersed in the 30% sucrose solution for storage at 4°C prior to sectioning. Brains were frozen using a cryostat and sectioned into 40 μm thick sections, as described previously[43]. Coronal sections at 1.06 mm posterior to the bregma were used as quantification area for atrophy[20,44]. Areas of each structure were retraced by the computer connected digitizing tablet[45]. The following structures were analyzed: the cerebral cortex, the hippocampus, and the thalamus. Figure 3. A depicted the structures in the analysis[46].

7. Statistics

The values were expressed as means ± SEM (n = 8). Statistical analyses between control, H_2O_2 and

H_2O_2-KG-group were performed by one way ANOVA test with Tukey's post hoc test. Significance level was set at $p < 0.05$.

RESULT

1. Determination of concentration of hydrogen peroxide (H_2O_2)

All mice with 5 μl ICV injection (300 mM, 100 mM, and 1 M H_2O_2) did not show convulsion or death in the first week. At the end of second week, mice with the treatment of 1 M H_2O_2 showed slow and hesitating behaviors (Table 1). Accordingly, all next experiments including Korean ginseng administration were executed with 1M H_2O_2 ICV injection for 2 week period.

Table 1: Mortality of Mice with an Intracerebroventricular Injection of Hydrogen Peroxide (H_2O_2)

Conc. Of H_2O_2	No. of Animals	Cumulative No. of death			Mortality (%)
		Start day	First week	Second week	
1 M	8	0	0	0	0
300 mM	8	0	0	0	0
100 mM	8	0	0	0	0
0 (control)	8	0	0	0	0

Conc., concentration; No., number; H_2O_2, hydrogen peroxide; Mortality was calculated as the cumulative number of death for two weeks divided by the total number of animals.

2. Passive avoidance test

Passive avoidance test was performed to measure the cognitive function. As shown in Figure 1, the H_2O_2-group exhibited markedly reduced step-through escape latency (78.3 ± 22.1 sec) compared to the control group (183.8 ± 38.9 sec). The H_2O_2-KG group, however, showed less decreased step-through escape latency (107.5 ± 25.9 sec), indicating Korean ginseng could protective effect on the memory impairment induced by H_2O_2 (Figure 1).

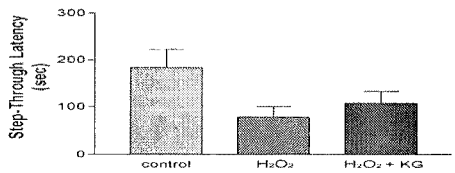

Figure 1. Effect of an intracerebroventriculr (ICV) injection of hydrogen peroxide (H_2O_2) and oral administration of Korean ginseng (KG) on the passive avoidance performance in mice. After 13 days of post-injection, training trial was performed. Testing trial was conducted on 14th day of post-injection. The data are expressed as mean ± SEM (n = 8).

3. Histological quantification of brain atrophy

After ICV injection of H_2O_2, extensive brain atrophy and expansion of the lateral ventricle were clearly observed (Figure 2. B). Saline ICV injected control mice did not show any atrophic changes in the brain (Figure 2. A). Marked shrinkage of the hippocampus and thalamus were demonstrated as well. Korean ginseng effectively protected the atrophic changes induced by H_2O_2 (Figure 2. C).

The area measurements for each brain structure were calculated as shown in Figure 3. A. The cortical area of the control group was 6.95 ± 0.23 mm^2. The cortical area of the H_2O_2 group was 6.92 ± 0.36 mm^2. Korean ginseng administrated group showed the cortical area of 7.05 ± 0.30 mm^2 (Figure 3. B). There was no significant difference in the cortical area among three groups. However, the thalamus and hippocampus showed the massive shrinkage after injection of H_2O_2. The thalamic area of the H_2O_2 group (3.30 ± 0.17 mm^2, $p < 0.05$) showed significant atrophic changes compared with the area of the control group (3.91 ± 0.18 mm^2, Figure 3. C). The hippocampal area of H_2O_2 group (1.27 ± 0.17 mm^2, $p < 0.05$) was significantly decreased compared with the area of control group as well (2.28 ± 0.08 mm^2, Figure 3. D). The administration of Korean ginseng significantly protected the atrophic changes in both the thalamic and hippocampal areas compared with the control

group (3.99 ± 0.06 mm^2, $p < 0.05$ in Figure 3. C and 2.21 ± 0.14 mm^2, $p < 0.05$ in Figure 3. D, respectively).

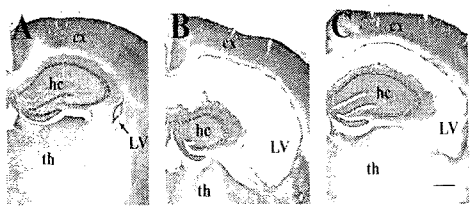

Figure 2. Cresyl violet-stained sections showing brain atrophy after an intracerebroventricular hydrogen peroxide-administration. (A) Brain section from saline-injected control group. (B) Brain section from hydrogen peroxide-injected group. (C) Brain section from Korean ginseng-administrated and hydrogen peroxide-injected group. LV-lateral ventricle, cx-cortex, hc-hippocampus, th-thalamus. Bar = 500 μm.

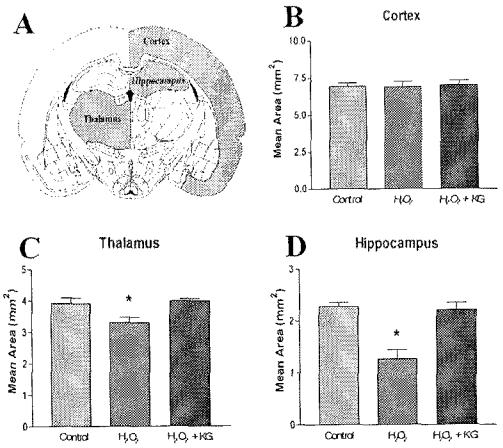

Figure 3. Area measurements for brain atrophy. (A) Illustration of representative bregma level (1.06 mm posterior from the bregma) from which structures were drawn. Figure was adapted from Paxinos and Watson[16]. (B) Mean cortical area of control group, hydrogen peroxide (H_2O_2) group, and (H_2O_2) + Korean ginseng (KG) group. (C) Mean thalamic area of control group, H_2O_2 group, and H_2O_2 + KG group. (D) Mean hippocampal area of control group, H_2O_2 group, and H_2O_2 + KG group. The data are expressed as mean ± SEM ($n = 8$). $^*p < 0.05$.

4. Histomorphologic study

Brain atrophy was clearly observed in hippocampus and thalamus after H_2O_2 stress (Figure 2). Control tissue shows normal structures of cerebral cortex, thalamus, and hippocampus (Figure 4. A, D, G). Even if the atrophy of cerebral cortex was not shown, the number of neuronal cells were decreased in the area of secondary motor cortex and cingulum. The brain tissue was damaged and glial cells were shown in the former neuronal area as well (Figure 4. B). The neuronal cell death were prominent in thalamus. Consequently, an increase in the number of glial cells is associated with dilated pericascular space (Figure 4. E). Vascular swelling as well as an increase of glial cells were also apparent in the stratum radiatum of hippocampus (Figure 4. H). The brain tissues of Korean ginseng-administrated group showed the protective effects in pathologic changes in all of cerebral cortex, thalamus, and hippocampus induced by H_2O_2 (Figure 4. C, F, I).

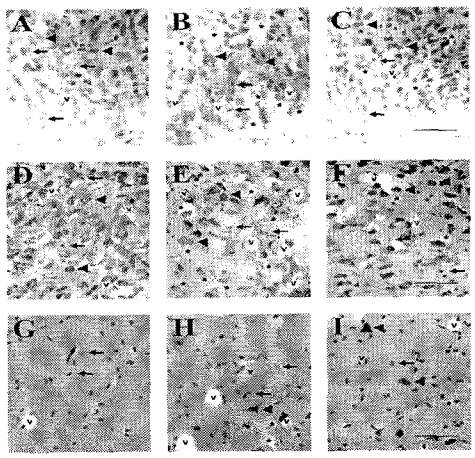

Figure 4. Photomicrographs of cresyl violet stained brain sections. (A) Cerebral cortex (Area of secondary motor cortex and cingulum) of control group. (B) Cerebral cortex of hydrogen peroxide (H_2O_2) group. (C) Cerebral cortex of (H_2O_2) + Korean ginseng (KG) group. (D) Thalamus (Area of latero-dorsal thalamic nucleus) of control group. (E) Thalamus of H_2O_2 group. (F) Thalamus of H_2O_2 + KG group. (G) Hippocampus (The stratum radiatum in hippocampus which lies between CA1 of hippocampus and hippocampal fissure) of control group. (H) Hippocampus of H_2O_2 group. (I) Hippocampus of H_2O_2 + KG group. Arrow head-neuronal cell; Arrow-glial cell; V-blood vessel; *-injured tissue. Bar = 500 μm.

DISCUSSION

Although numerous in vitro studies implicated excessive reactive oxygen species (ROS) in neuronal death, there were few in vivo studies to examine the role of ROS in the pathophysiology of neurodegenerative disorders[47,48]. However, there are many reports concerning the protective effect of anti-oxidants on neuronal death[25,49,50]. Considering the pathologic mechanisms of several neurological diseases including Alzheimer's disease, Parkinson's disease, and Huntington disease, the oxidative stress is one of the main pathologic mechanisms[49,51-54]. In recent kinetic analyses, it was known that the oxidative stress might constitute a rather early event in pathogenesis of Alzheimer's disease and Down syndrome[25,55-57]. The relationship between hippocampal atrophy and brain function was also reported by the clinical studies[58,59].

In recent years, there have been several studies that point to the effects of anti-oxidant and neuroprotective effects of Korean ginseng. Wide pharmacological actions of ginseng by a free radical reaction-inhibition mechanism were observed in 1996[60]. Korean ginseng was known to protect the neuronal loss in the hippocampus, decrease cortical contusion volume, and improve neurological deficits[61]. Korean ginseng extract also protected human neuronal SK-N-MC cells from the apoptosis induced by 2,2',5,5'-tetrachlorobiphenyl[62].

In this study, we observed that H_2O_2 could induce the brain atrophy in vivo. Especially, the hippocampus and thalamus showed excessive atrophic changes. The cognitive change was shown simultaneously. The results suggested the importance of ROS in the brain atrophy and cognitive changes, and this method can provide the novel animal model for the brain atrophy. Moreover, Korean ginseng could protect the brain atrophy induced by excessive ROS.

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