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Anti-stress Effect of Pyroligneous Liquid in SD Rats and ICR Mice

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Abstract – Pyroligneous liquid(PL) is produced by carbonizing Oak in 350~400°C. It is traditionally used for treating stress-related disorder, hepatic disease, immune disorder, G-I disorder and inflammatory disease. The aim of this study is to investigate anti-stress effects of PL. The experiments were performed with the use of young(9 weeks of age) male rats of SD strain and the male ICR mice (20-25 g). Animals of the normal group were not exposed to any stress and the control group were exposed to stress. The rats of the Ginseng, diazepam(BZ) and PL supplementary group were orally administered once a day 100 g of Ginseng extract-kg body weight, 5 mg of BZ/kg body weight and 1 ml of PL/100 g body weight and then exposed to stress. The mice of the Ginseng, BZ and PL supplementary group were given water containing 100 g of Ginseng extract/100 ml potable water, 5 mg of BZ/kg 100 ml of drinking water and 10 ml of PL/100 ml of drinking water and exposed to stress. Animals were given materials for 7 days after stabilizing them, and then were given supplementary materials for 5 days with stress. They were stressed by immobilization for 30 minutes and then the animals were exposed to electroshocks for 5 minutes. We recorded stress-related behavioral changes of experimental animals by stressing them using the Etho-vision system and measured the levels of corticosterone in blood While stress suppressed locomotor activity of animals, PL-supplementation partially blocked the stress effect of locomotion in rats and mice, and also partially blocked stress-induced behavioral changes such as freezing, burrowing, smelling and rearing activity in rats and freezing, grooming, tailing and rearing in mice. The staying time of stressed rats and mice in open area decreased and in closed area it increased relatively in elevated plus maze test. However, these changes also partially were blocked by PL-supplementation. PL- supplementation decreased levels of blood corticosterone increased by stress in rats. These results suggest that PL protects partially the living organism from stress attack in some cases.

Key words ☐ Pyroligneous liquid, Stress, Behavior, Plus-maze, Corticosterone

INTRODUCTION

Good or bad, if it is a change in our life, it is stress as far as our body is concerned(Blazer et al., 1987; Chrousos and Gold, 1992; Seley, 1993). We have known for a long time that overstress, although it was considered as just emotional stress, could cause physical damage to the gastrointestinal tract, endocrine system, skin or cardiovascular system(Hurst *et al.*, 1976; Barsky *et al.*, 1986; Breier *et al.*, 1987; Chrousos and Gold, 1992;). Only recently we have learned that overstress actually causes physical changes in the brain. One of the most exciting medical advances of our decade has been an understanding of how overstress physically affects our brain(Chrousos and Gold, 1992; Dimsdale *et al.*, 2000). We now know that the fatigue, aches and pains, crying spells, depression, anxiety attacks and sleep disturbances are caused by brain chemical malfunction induced by overstress(Dimsdale *et al.*, 2000; Barsky *et al.*, 1986). Many functional foods involving Ginseng extract, DHA and Ginkgo biloba extract, can help a person suffering from overstress to feel healthy again, sleep well, and be free from aches, anxiety, and depression were introduced and produced (Yuan *et al.*, 1989; Takahashi *et al.*, 1992; Ward *et al.*, 2002; Takeuchi *et al.*, 2003). However, we can not approve all of them because it was not confirmed to evaluate overstress and its application in functional food. In this study, we tried to confirm a method to evaluate anti-stress effect of functional foods. We especially intended to apply methods to overstress psychologically experimental animal and tested them to evaluate anti-stress effect of a functional food. We selected simple methods

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that can psychologically expose overstress to animals and control easily the level of stress. Animals were given an electroshock stimulation and a restraint stress. We examine the behavioral changes and the changes of organ weights and blood corticosterone levels induced by overstress.

Pyroligneous liquid is produced through the process of carbonizing oak in 350~400°C. It contains 96% of H_2O, 3% of acetic acid and 1% of organic compound. There are 200 kinds of constituents including minerals, vitamin B-complex and organic acids in it. The organic acids in constituents were presumed as active materials. It is traditionally used for the treatment of stress related disorder, hepatic disease, immune disorder, G-I disorder and inflammatory disease(kim and Kim, 1998; Choi, 2002) but the effects on psychological stress were not proven.

This study aims to investigate anti-stress effects of Pyroligneous liquid as a candidate of anti-stress-related functional foods comparing its effect to that of Ginseng and Diazepm which were well known for anti-stress activity.

MATERIALS AND METHODS

Animals and Diets

The male Sprague Dawley rats(8-10 weeks of age) and the male ICR mice (20-25 g) used in this study were obtained from Hanlim experimental animal Co. We used pyroligneous liquid(30% PL solution) produced from Choa pharmaceutical Co., Red Ginseng extract(KRG extract) produced by Korean Ginseng Corp. It was well known that Ginseng extract or its constituents such as ginsenosides have antistress effect(Yuan *et al.*, 1989; Takahashi *et al.*, 1992). We chose Ginseng extract as a positive control. All animals were housed in a temperature (22 ± 2°C) and humidity(55 ± 5%) controlled animal room on a 12 hr/12 hr light/dark(6 A.M.-6 P.M.) schedule. They had free access to food and water throughout the experiments. The animals were divided into five groups after stabilizing them for 1 week in our animal room. Animals belonging to the normal group were not exposed to any stress. The rats of the control group were orally administered saline 1ml/ 100g body weight and were exposed to stress, while the mice of control group were given normal water and exposed to stress. The rats of the Ginseng, diazepam(BZ) and PL supplemented group were orally administered once a day 100 g of Ginseng extract/kg body weight, 5 mg of BZ/kg body weight and 1 ml of PL/100 g body weight and exposed to stress. The mice of the Ginseng, diazepam(BZ) and PL supplemented group were given water containing 100 g of Ginseng extract/100 ml drinking water, 5 mg of BZ/100 ml drinking water and 10 ml of PL/100 ml drinking water and exposed to stress. Animals were given only supplementary materials for the first 7 days after stabilizing them, and then were supplemented materials for 5 days with stress.

Induction of Stress

Animals were given supplementary materials before exposing to stress. The mice were usually subjected to restraint stress by keeping them in a well ventilated conical plastic tubes(3 cm in diameter and 7 cm in length) for 30 minutes each day. During the restraining period, the mice did not have any access to food and water. At the end of each restraint stress, the mice were exposed to electroshocks(1 mA intensity; 1sec duration; 20sec intershock interval) for 5 minutes. The rats were usually subjected to restraint stress by keeping them in a well ventilated conical polypropylene tubes(6.2 cm in diameter and 16.5 cm in length) for 30 minutes each day. During the restraining period, the rats did not have any access to food and water. At the end of each restraint stress, the rats were exposed to electroshocks (5 mA intensity; 1sec duration; 20sec intershock interval) for 5 minutes.

Behavioral apparatus

The equipment was located in the animal room allowing the observer to view and observe the animals through a computer outside the room. After inducing terminal stress (in the manner described above), behavioral changes of animals were monitored automatically using a computerized EthoVision system (Noldus IT b.v. , Netherlands). In the locomotor activity and elevated plus-maze tests, the behavioral parameters were analyzed by an automatic videotracking system.

Locomotor activity

The apparatus consisted of 9 black plastic boxes (47×47 cm), and the field was bordered by 42-cm-high side walls. The total moved distance, total movement time and turn angles were monitored 5 minutes after terminal stress for 20 minutes(Noldus *et al.*, 2001).

Elevated plus-maze test

The Elevated plus-maze box and arms were made of Plastic. The apparatus consisted of two open arms (50×10 cm in rats; 30×6 cm in mice), alternating at right angles, with two arms enclosed by high walls of 30 cm in rats and 20 cm in mice. The four arms delimited a central area of 10×10 cm. The whole apparatus was placed 50 cm above the floor. Animals were placed in the central square after measuring stress related activity and were allowed to explore the maze freely for 5 minutes. The parameters measured were the times spent in open and closed areas(Noldus *et al.*, 2001).

Stress related activity tests

After terminal stress, animals were placed alone in individual plastic cage (40×20×18 cm in rats; 26×20×13 cm in mice). The behavioral activities were measured soon after stress. Smelling, feeding, burrowing, freezing, tailing, face washing and grooming time were recorded for 5 minutes (Takeuchi *et al.*, 2003). Rearing frequency was measured using EthoVision system 5 minutes after terminal stress for 20 minutes (Noldus *et al.*, 2001).

Blood sampling and Measurement of Serum Corticosterone

After monitoring locomotor activity, blood samples(rat 3 ml; mouse 1.5 ml in heparinized tubes) were taken through heart puncture between 11:00 A.M - 1:00 P.M, and then adrenal gland in rat and spleen in mouse were dissected and weighed.

The serum corticosterone level was measured by a modified method(Harikai *et al.*, 2003) using HPLC system composed with SI-2 3001 pump, SI-2 3002 UV-Visible detector, SI-2 3004 column oven, separation(Shiseido, Tokyo, Japan), and column Capacell Pak C18 MG 120(5 μm, 3 × 250 mm). Corticosterone and dexamethasone(Sigma, St. Louis, MO, U.S.A) were used as the internal standard. We injected 40 μl of treated sample solution into HPLC column. We used acetonitrile : methanol :sulfuric acid solution(32 : 4 : 64) as the mobile phase with a flow rate of 500 μl/min. We determined corticosterone level as absorbance in wavelength 240 nm using dsCHROM computing program(Shiseido, Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± S.E.M.. ANOVA was used to compare the scores among the groups for one variable. This was followed by post hoc comparisons using the Newman-Keuls test.

RESULTS

Fig. 1 shows that stress affects locomotor activity in both animals of rats and mice. Changes in locomotor behavior, measured as the total moved time and moved distance, were also significantly different between animals exposed to stress and those not exposed to. The stress condition resulted in a signifi-

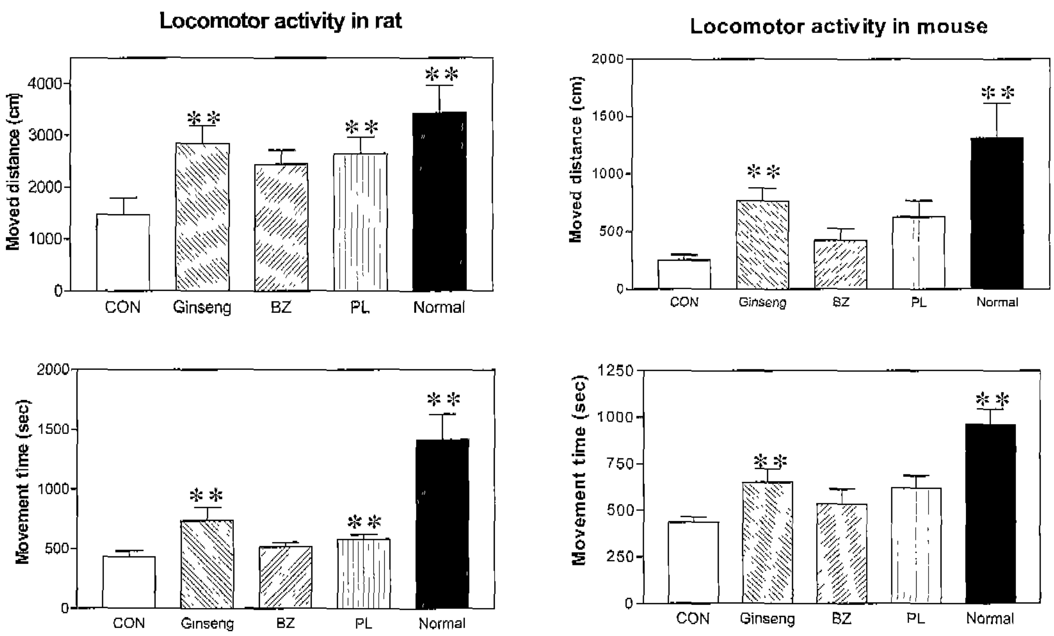

Fig. 1. Effects of Pyroligneous liquid on Locomotor activity test in SD rats(n = 7) and ICR mice(n = 8). Each bar represents mean ± SEM of total moved times(right) and distances(left) for 20 minutes after loading stress. Normal, BZ(Diazepam), Ginseng or PL (Pyroligneous liquid) versus Control, **p<0.01; *p<0.05.

cant decrease in locomotor activity but PL-supplementation blocked this stress-induced suppression of locomotion especially in total moved distance for 20 minutes. This effect was similar to Ginseng's effect.

The influence of PL-supplementation on the stress behaviors induced immobilization and electroshock was assessed for 5 minutes and 20 minutes. As shown in Fig. 2, the stress exposure resulted in a significant increase of time spent in freezing, grooming, facewashing and burrowing behaviors of rats for 5 minutes and a decrease of time spent in smelling behavior for 5 minutes and rearing frequency of rats for 20 minutes but PL-supplementation partially blocked these stress-induced changes of behavior such as freezing, burrowing, smelling and rearing. Fig. 3 shows that the changes of stress behavior in mice were similar to that of rats. The stress condition resulted in a significant increase of time spent in freezing, grooming, facewashing and tailing behaviors of mice for 5 minutes and a decrease of rearing frequency in mice for 20 minutes but PL-supplementation partially blocked this stress-induced changes of behavior such as freezing, grooming, tailing and rearing. These effects

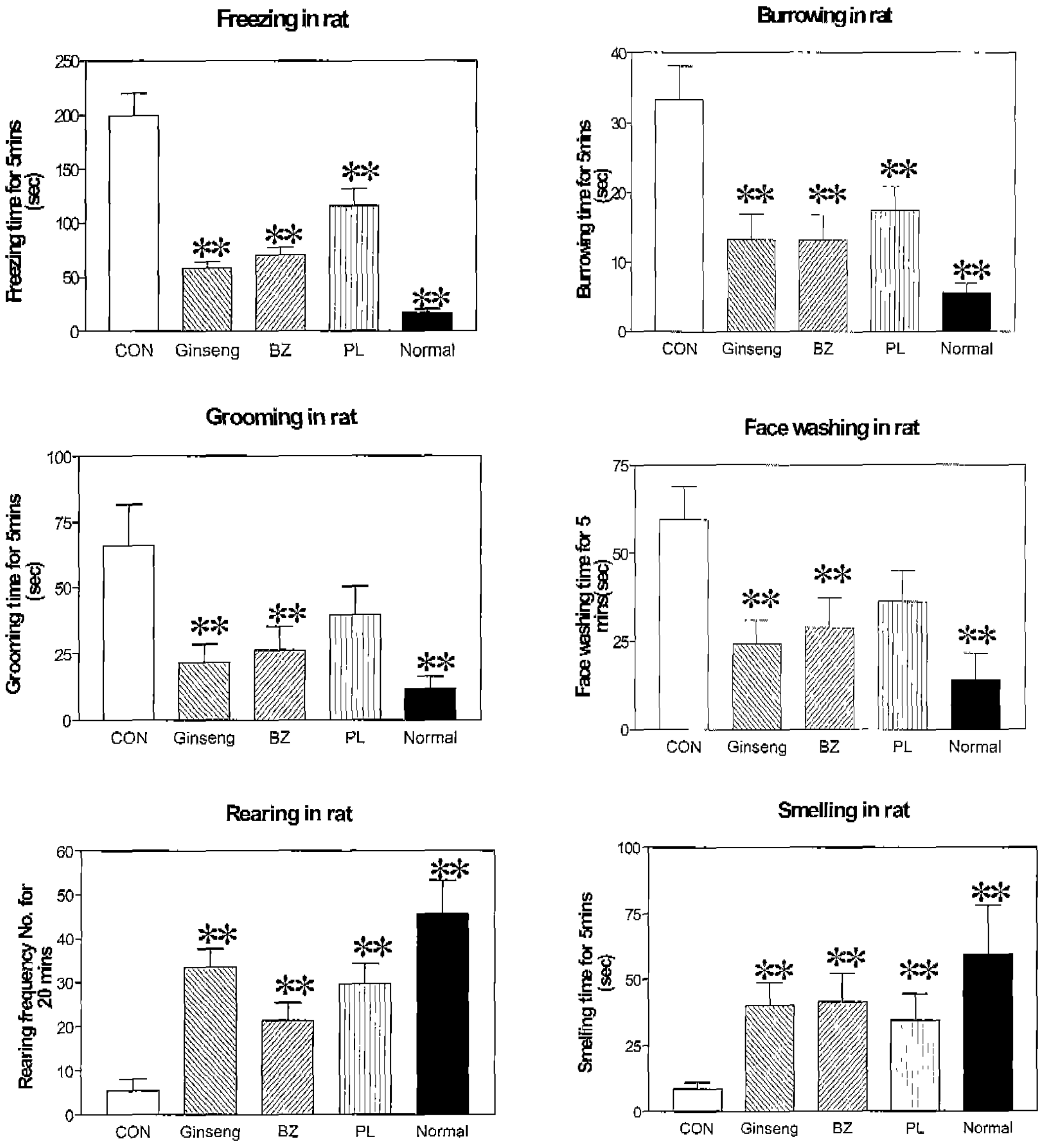

Fig. 2. Effects of Pyroligneous liquid on Stress related activity test in SD rats(n=7). Each bar represents mean ± SEM of total activity times for 5 minutes or frequency numbers for 20 minutes after loading stress. Normal, BZ(Diazepam), Ginseng or PL(Pyroligneous liquid) versus Control, $^{**}p<0.01$; $^{*}p<0.05$.

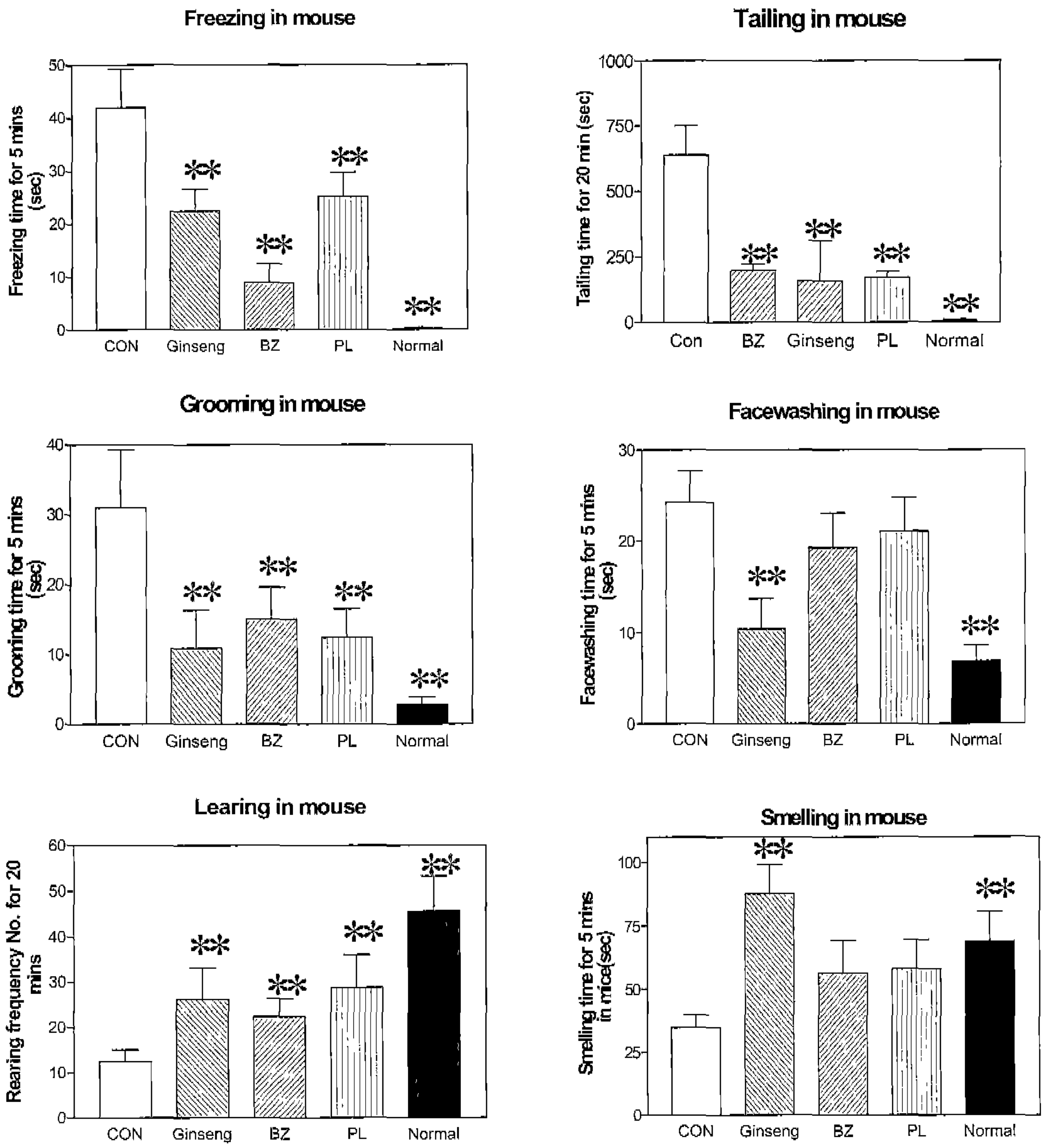

Fig. 3. Effects of Pyroligneous liquid on Stress related activity test in ICR mice(n=8). Each bar represents mean ± SEM of total activity times for 5 minutes or frequency numbers for 20 minutes after loading stress. Normal, BZ(Diazepam), Ginseng or PL(Pyroligneous liquid) versus Control, $^{**}p<0.01$; $^{*}p<0.05$.

were also similar to Ginseng's effects.

The time spent in the open or closed arm for 5 minutes significantly differed between the animals exposed to stress and the unexposed animals as shown in Fig. 4. The animals exposed to stress spent less of their time in the open arm than the unexposed animals. Furthermore, the animals exposed to stress spent more time in the closed arm than unexposed the animals. Stress exposure decrease the total turned degree of the animals. PL-supplementation significantly reversed this stress-induced response in Elevated plus maze test. These effects were also similar to Ginseng's effects.

As shown in Fig. 5, the stress condition resulted in a significant increase of wet weight of adrenal gland but a decrease in spleen. This changes were partially blocked by PL-supplementation. This effect was also similar to Ginseng's effect. Fig. 6 shows that stress affects blood corticosterone levels of rats. The stressed animals have higher corticosterone levels than the stress free animals. PL-supplementation partially blocked this stress-induced increase of blood corticosterone level. This effect was also similar to Ginseng's effect.

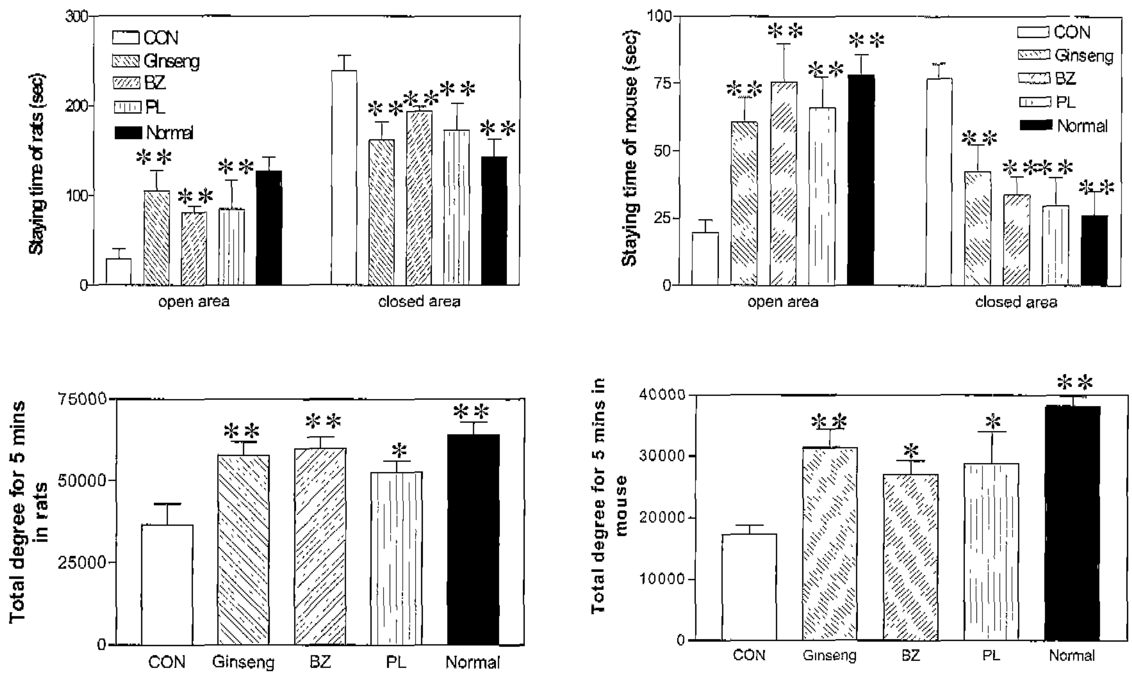

Fig. 4. Effects of Pyroligneous liquid on Elevated plus maze test in SD rats(n=7) and ICR mice(n=8). Each bar represents mean ± SEM of times spent in open or closed area and total turn angles for 5 minutes after loading stress. Normal, BZ(Diazepam), Ginseng or PL(Pyroligneous liquid) versus Control, **p<0.01; *p<0.05.

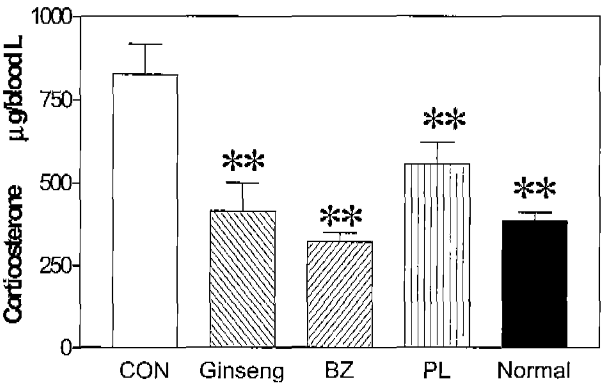

Fig. 5. Effects of Pyroligneous liquid on Changes of organ weights in SD rats and ICR mice. Each bar represents mean ± SEM of wet weights of adrenal glands(rats) and spleen(mice) after loading stress. Normal, BZ(Diazepam), Ginseng or PL(Pyroligneous liquid) versus Control, **p<0.01; *p<0.05.

DISCUSSION

We applied a modified method to induce stress response in rats and mice. We chose the Ginseng extract and diazepam as a positive control in testing antistress effect of functional food. It was well known that Ginseng extract or its constituents such as ginsenosides have antistress activity on animals subjected to stressful stimuli such as footshock, cold and heat (Yuan *et al.*, 1989; Takahashi *et al.*, 1992; Kaneko et al., 1996; Kim *et al.*, 2003b; Choi *et al.*, 2003) and diazepam also has antistress activity on acute and chronic stress (Ida *et al.*, 1985; Finlay *et al.*, 1995; Beck and Fibiger, 1995). Changes in locomotor behavior, stress behavior, plus maze test, plasma corticosterone level and organ weight induced by stress condition which we established in this study were similar to the results of the other studies(Glass, 1977; Fanselow, 1980; Morimoto *et al.*, 1993; Djordjevic *et al.*, 2003; Kim *et al.*, 2003a; Takeuchi *et al.*, 2003). Thus changes were reversed or blocked by supplementation of Ginseng extract. Our results were also similar to theirs. Therefore, this result indicates that the method of stress exposure applied in this study was established properly in testing antistress effect of functional food and Ginseng was properly used as a positive control.

In order to test antistress effect of PL, behavioral reactivity was assessed using three parameters and six sub-parameters in rats and mice. These reflect levels of psychological stress in rats and mice. PL-supplementation blocked stress-induced suppression of locomotion in rats and mice, and also partially blocked stress-induced behavioral changes such as freezing, burrowing, smelling and rearing behavior in rats and freezing, grooming, tailing and rearing in mice. The animals exposed to stress spent less time in the open arm and more time in the

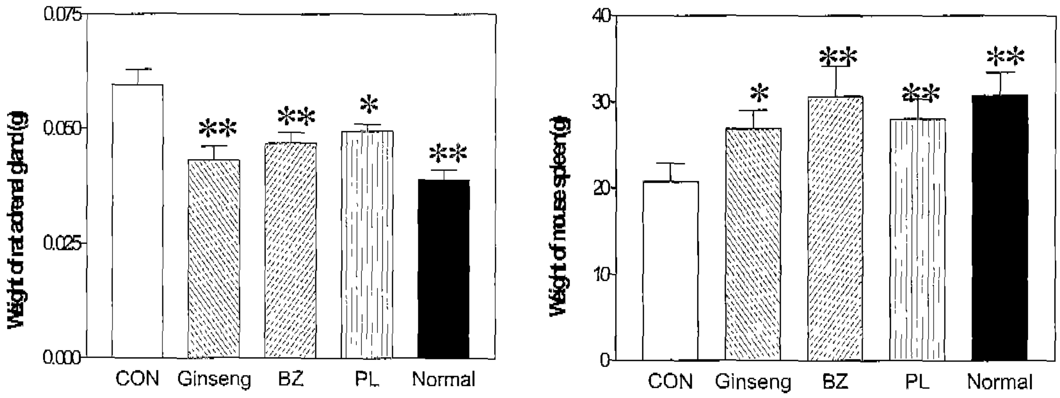

Fig. 6. Effects of Pyroligneous liquid on Blood corticosterone levels in SD rats. Each bar represents mean ± EM of serum corticosterone levels after loading stress. Normal, BZ(Diazepam), Ginseng or PL(Pyroligneous liquid) versus Control, $^{**}p<0.01$; $^{*}p<0.05$.

closed arm than the animals not exposed to stress. PL-supplementation significantly reversed stress-induced response in elavated plus maze test. These effects were also similar to Ginseng's effects. This result indicates that PL can partially protect from psychological stress.

Stress was thought to be a non-specific response to stressors always including the activation of adrenal glucocorticoid and catecholamine release (Ida *et al.*, 1985; Finlay *et al.*, 1995; Beck and Fibiger, 1995; Djordjevic *et al.*, 2003). The hypothalamic-pituitary-adrenal (HPA) axis is one of the hormonal systems mediating the stress response (Kim *et al.*, 2003a). The main regulation of stress-related activity of the HPA axis occurs at the level of parvicellular subdivision of the hypothalamic paraventricular nuclei, and the majority of these neurons secrete corticotropin releasing hormone and vasopressin which synergistically stimulate ACTH secretion by the pituitary corticotropic cells (Djordjevic *et al.*, 2003). ACTH then enters the systemic circulation, stimulating corticosterone synthesis from the cholesterol and its release from the adrenal cortex and enlargement of adrenal gland (Djordjevic *et al.*, 2003). The stress condition resulted in a significant increase of wet weight of adrenal gland in rats and a decrease of spleen in mice in this study. These changes were partially blocked by PL-supplementation. PL-supplementation partially blocked stress-induced increase of blood corticosterone level. These effects were also similar to Ginseng's effects. It was well known that stress induces activation of adrenal gland resulting to the enlargement of adrenal gland and increase of corticosterone secretion(Glass, 1977; Schwartz *et al.*, 1988; Morimoto *et al.*, 1993; Park *et al.*, 1996; Djordjevic *et al.*, 2003; Kim *et al.*, 2003a; Takeuchi *et al.*, 2003).

Pyroligneous liquid contains 96% of H_2O, 3% of acetic acid and 1% of organic compound. There are 200 kinds of constituents including minerals, vitamin B-complex and organic acids contained in this liquid (Kim and Kim, 1998; Choi, 2002). The organic acids were presumed as active materials but we don't know exactly what the active material is. We will study on this subject in the next experiment.

In conclusion, while stress suppressed locomotor activity of animals, PL-supplementation partially blocked the stress effect of locomotion in rats and mice, and also partially blocked stress-induced behavioral changes such as freezing, burrowing, smelling and rearing activity in rats and freezing, grooming, tailing and rearing in mice. The staying time of stressed rats and mice in open area decreased while in closed area it increased relatively in elevated plus maze test. But these changes also partially were blocked by PL-supplementation. PL- supplementa-tion decreased levels of blood corticosterone increased by stress in rats. These results suggest that PL protects partially the living organism from stress attack in some cases and have a possibility to use as a functional food in order to alleviate stress response.

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