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Inhalation Effects of Korean Ginseng and Pine Needle on the Protection from Injury of Mouse Lung by Formaldehyde Exposure

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Formaldehyde (FA) is an important industrial chemical, but it can cause allergic reactions, sick building syndrome and so on. It has also been observed to cause cancer in scientific studies using laboratory animals, and it even causes cancer in humans. Natural products such as ginseng and pine needle containing complicated mixtures of organic chemicals are widely used in the world, because their effective components are responsible for some pharmacological activities including antioxidative effect, anticancer effect. We investigate the effect of Korean ginseng (GE), pine needle extract (PE) and combined GE and PE (cNPE) on mouse lung injury by FA exposure. GE, PE and cNPE was directly transported to pulmonary cells through respiratory organ by nebulizer inhalation. In the case of FA exposure, the pulmonary structure was damaged and its function became abnormal. However, cNPE-FA, GE-FA, and PE-FA treated groups showed similar with the control group compared with FA group. Among them, GE was proved to be more effective than any other extracts. These results demonstrate that natural product extracts could protect pulmonary structure and function against FA exposure.

Key Words: Ginseng, Pine needle, Formaldehyde, Pulmonary, Antioxidant

INTRODUCTION

Formaldehyde is an important industrial chemical used to make other chemicals, building materials, and household products (yu et al., 1998). FA, by itself or in combination with other chemicals, serves a number of purposes in manufactured products. In homes, the most significant sources of FA are likely to be pressed wood products made using adhesives that contain urea-FA (UF) resins (wang et al., 2004). FA is a colorless gas with strong smell. When it present in the air at levels above 0.1 ppm, it can cause watery eyes, burning sensations in the eyes, nose and throat, nausea, coughing, chest tightness, wheezing, skin rashes, allergic reactions and sick building syndrome. It has also been observed to cause cancer in scientific studies using laboratory animals, and it even causes cancer in humans. However, typical exposures to humans are much lower. Therefore, any risk of causing cancer is believed to be small at the level of which humans are exposed (Apte et al., 2000). High concentrations of FA also may trigger attacks in people with asthma. Some people are very sensitive to FA while others may not have any noticeable reaction to the same level. Persons have developed allergic reactions (allergic skin disease) to FA through skin contact with solutions of FA or durable-press clothing containing FA. Others have developed asthmatic reactions and skin rashes from exposure to FA (James et al., 2001).

Natural products from plants are widely used in the world. Herbs contain complicated mixtures of organic chemicals, the levels of which may vary substantially depending upon many factors related to the growth, production, and processing of the herbal product (Capassoa et al., 2000). Herbal products are also commonly used by patients with certain chronic medical conditions, including breast cancer, liver disease, AIDS, asthma, and rheumatologic disorders (Kayser et al., 2003). Although the estimated percentage of adults currently using herbs varies, and it depends upon differences in survey methodology, it is clear that herbs are used by a

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large and growing percentage of the population. Especially, ginseng saponin such as Rb1, Rb2 and Rg1 from the root of *Panax* ginseng have been regarded as the main effective components responsible for the pharmacological and biological activities, such as anti-aging, antidiabetic, anticancer, analgesic effects, and protection from physical and chemical stress, in addition with biological effects on the central nerve system, tranquilizing action and others (Lee, 1992; Gillis, 1997). Rutin and other components in pine needle content have known the effect on ROS elimination as well as paralysis, arteriosclerosis, hypertension, diabetes and other disease (Igor et al., 2001). These natural products were raised immunity about the various pollutants and have studied effective curing agent against various pollutant-induced disease as well, and several results were noticed (Igor et al., 2001; Kitts et al., 2000; Juergens et al., 2003). However, studies on the effects of natural products have examined mainly *in vitro* or through a definite method of oral administration. Therefore, the purpose of the present study was to investigate the inhalation effect of GE, PE, and cNPE by transporting these two extracts directly to pulmonary cells through respiratory organ using nebulizer.

MATERIALS AND METHODS

1. Chemicals and apparatuses

Raw ginseng (*Panax ginseng* C.A Meyer) consisted of dried ginseng (4 years old) cultured in Keumsan, South Korea and pine needle (*Pinus densiflora*) cultured in Chungsong, Kyungsangbukdo were grounded. Hydrogen peroxide (H_2O_2), pyrogallol, NADPH, thiobarbituric acid (TBA), glucose 6-phosphate, glucose 6-phosphate dehydrogenase (G6PD), trichloroacetic acid (TCA), (N-[2-Hydroxyethyl] piperazin-N'-[2-ethanesulfonic] acid (HEPES), ethylenediaminetetra-acetic acid (EDTA) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical (St. Louis, MO, USA). Ammonium persulfate, sodium dodecyl sulfate (SDS), acrylamide, N,N,N',N'-tetramethylene-diamine (TEMED), and Bio-Rad protein assay kit were obtained from Bio-Rad (Hercules, CA, USA). Methanol and Tween 20 were obtained from Merck (Darmstadt, Germany). Antibodies against surfactant protein D was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) western blot detection kit was obtained from Amersham Phamacia Biotech. (Oxford, UK). EDTA tubes were obtained from BD Vacutainer system (Plymouth, UK). Hematoxyline, eosin, xylene and ethanol were obtained from Shandon Inc. (Japan). Autotechnicon and rotary microtome citadel-1000 were obtained from Shandon Inc. (Japan), and Marienfild histobond slide glass was obtained from Superior (Germany).

2. Animals

Eight week-old male ICR mice, weighing 25~30 g, free of respiratory disease, were purchased from Hyo-Chang Science (Daegu, Korea) and quarantined for 1 week before FA exposure. The mice were housed in plastic cages (453×293×247 mm, 19L) with chopped Aspen wood bedding and maintained on a 12 h light/12 h dark cycle. All animals had access to water and laboratory mouse diet, which was purchased from SamYang Co. (Wonjoo, Korea), *ad libitum* before, during and after experiment.

3. Extraction of Korean ginseng and pine needle

The dried samples (1 kg) of raw ginseng and pine needle were extracted at 45℃ in 80% (v/v) methanol (15 L) for 24 h (Ko et al., 1995; Shin et al., 2001; Moon et al., 1999). The extract was then filtered, and the filtrate was concentrated with a rotary evaporator (Laborota 4000, Heidolph, Japan). The extraction system was set up in a same manner that was described briefly in Fig. 1. The concentrate was lyophilized in a freezing-dryer (Neocool, Yamato, Japan) and stored at -80℃. Just before using, the concentrate was filtered using 11 μm filter paper (No 1, Whatman, NJ, USA).

4. Experimental protocol

Animals were fed on standard commercial pellet diet and had free access to water. After an acclimatization of one week, the animals were randomly divided into six groups for the treatment as experimental condition (Table 1). Extracts were inhaled 50 mL per day, for 6 day/week, during 4 weeks, using a nebulizer in a nose and mouth exposure system. The extract dosage was decided according to the results of a preliminary study. Mice were exposed to FA in a nose and mouth exposure system too. Because the experimental condition of FA exposure is in need of sufficient damage (without life of experimental animal), it seems reasonable to conclude that experimental condition of FA exposure is 500 ppm/19 L for further experiments on the grounds that the previous research (Yu et al., 1998).

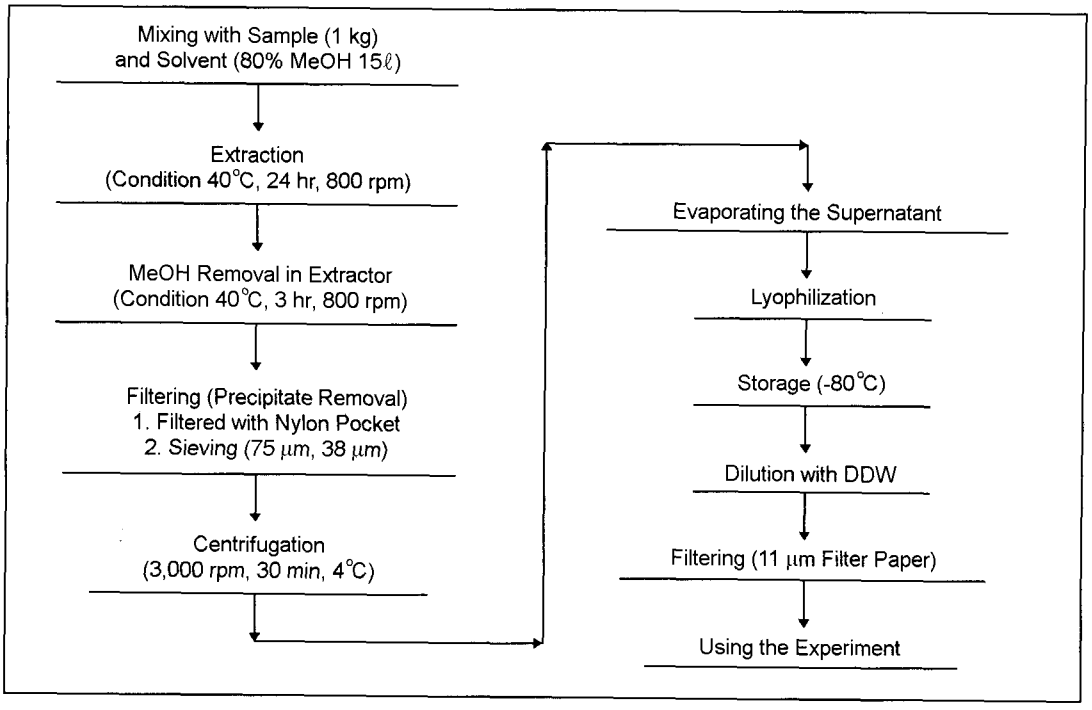

Fig. 1. Schematic view of the natural product extraction system. Extraction methods was used prior study.

Table 1. Experimental animals were divided into six groups as follow

Group	Treatment
Control	no (only fresh air)
Sham	deionized distilled water (50 ml)
cNPE-FA	0.1% ginseng extract (25 ml) + 0.1% pine needle extract (25 ml) + 500 ppm FA exposure
GE-FA	0.1% ginseng extract (50 ml) + 500 ppm FA exposure
PE-FA	0.1% pine needle (50 ml) + 500 ppm FA exposure
FA	500 ppm FA exposure

The procedure continued for 10 h every day in an inhalation chamber.

5. Blood sampling and analysis

1 mL of blood was collected after the cervical dislocation of animals. This blood was collected into chilled tubes containing EDTA, and then stored at 4℃. The analysis is conducted in Green Cross Reference Lab (Green Cross, Korea).

6. Histological assessment

For morphologic evaluation, the left lung was inflation fixed by intratracheal instillation 10% buffered formalin at 25 cm^2 of water pressure for 1 h, and stored in 70% ethanol before processing. Tissues were dehydrated in a series of alcohol steps, transferred into xylene and embedded in paraffin. Four μm sections were stained with hematoxylin-eosin and examined on light microscopy by a pathologist who did not know whether the animal had been exposed to FA. Light microscopy at 100 X and 400 X magnifications was used to grade the stained sections of the lung. The morphological change of pulmonary tissue was examined by the Zeiss Axiovert 200 light microscope (Germany).

7. Preparation of lung tissue

Lung tissue was homogenized in 2 mL of homogenized solution (50 mM Tris-/HCl, pH 7.5, 20 mM HEPES, 1 mM EDTA, 2 mM PMSF, 1% Triton-X 100) with a micro-homogenizer, followed by sonication with an ultrasonic generator (US-50, Nissei, Tokyo, Japan) for 20 s in an ice-cold water bath. Each homogenized solution was centrifuged at 15,000 g at 4℃ for 15 min. The supernatant were stored at -80℃ until use.

8. Measurement of lipid peroxidation

As an index of lipid peroxidation we used the formation of thiobarbituric acid-reactive species (TBARS) during an

acid-heating reaction as previously described (Buege et al., 1978). Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid, subsequently heated in boiling water for 15 min. The TBARS were determined by absorbance at 535 nm using a Shimadzu UV-2401 PC spectrophotometer (Japan) with the sample compartment maintained 25℃.

9. Measurement of enzyme activity

Superoxide dismutase (SOD) activity was determined spectrophotometrically using the pyrogallol assay procedure (Marklund et al., 1974), where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%. Activity of catalase (CAT) was measured in terms of decomposition of H_2O_2, which was followed directly by the decrease in absorbance at 240 nm (Beers et al., 1952). G6PD activity was measured in a buffer (55 mM Tris-HCl, pH 7.8, 3.3 mM $MgCl_2$, 6 mM NADPH and 0.1 M Glucose-6-phosphate) by rate of NADPH reduction at 340 nm (Bautista et al., 1992).

10. SDS-PAGE and Western blotting

Electrophoresis was performed on 12% SDS-polyacrylamide gels. Samples were treated by heating to 100℃ for 5 min with sample loading buffer. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes. Membrane were blocked with 5% nonfat milk in Tris buffered saline (TBS) and then incubated for 2~24 h. The membranes were incubated for 24 h in the presence of primary antibody (polyclonal SP-D antibody, 1:500, 50 ng/ml). Blots were washed with TBST (TBS with 0.1% Tween 20) and then incubated for 2 h in the presence of a 1:1000 secondary HRP-conjugate rabbit anti-mouse IgG in TBS. The membranes were washed and developed with ECL kit.

11. Nebulizing

The aerosol of GE, PE and cNPE was generated by a one-jet nebulizer (MIDAS, H-30, Mega Medical, Korea). The nebulizer produces wet droplets with a diameter of 0.5~5 μm. The average particle size of droplets were 2.659 μm.

RESULTS

1. Change of weight

Fig. 2 shows the weight change of each mice group. The body weight of animals showed a tendency to increase from week 0 to 4 in control and sham groups. On the contrary, body weight of cNPE-FA, GE-FA, PE-FA and FA group were decreased significantly compare with control group from week 0 to 4. But the body weight reduction of cNPE-

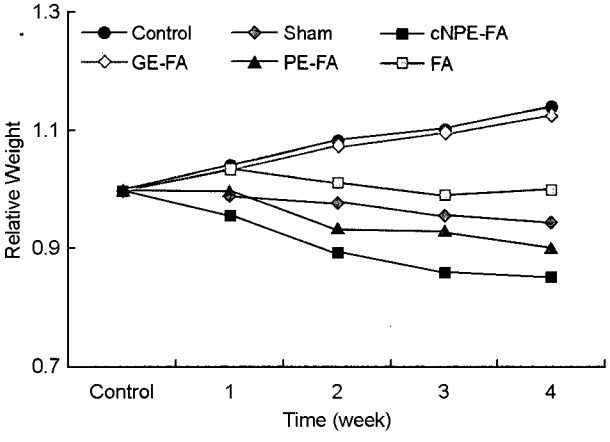

Fig. 2. After experiment, the weight of each mouse was measured at every week with the same time. The ordinate in figure is the relative value which means the ratio of the control value to each group. Result represents the mean ± SD of 5~7 mice per group.

Table 2. The effects of formaldehyde and cNPE, GE and PE treatment on white blood cell

	Lymphocyte		Neutrophile		Monocyte		Eosinophile	
	Value	STD	Value	STD	Value	STD	Value	STD
Control	1.00	±0.11	1.00	±0.05	1.00	±0.20	1.00	±0.43
Sham	1.04	±0.07	1.09	±0.01	0.97	±0.20	0.67	±0.58
cNPE-FA	2.04	±0.08	2.15	±0.10	1.86	±0.25	2.00	±1.00
GE-FA	1.73	±0.08	1.47	±0.07	1.40	±0.35	0.67	±0.58
PE-FA	2.00	±0.11	1.85	±0.19	2.06	±0.43	3.00	±1.00
FA	2.46	±0.16	2.13	±0.25	2.63	±0.30	3.50	±1.32

The values is the relative value which means the ratio of the control value to each group. Result represent the mean ± SD of five separate experiment

FA, GE-FA and PE-FA groups were less than FA group. Howevere, decreased weight of these groups was not recovered again throughout the experimental period. This result indicated that cNPE, GE and PE were attenuated the weight loss by FA exposure.

2. Alteration of white blood cell (WBC)

Table 2 shows the effects of the FA and natural product extract treatment on WBC. The FA exposure induced increase in the number of WBC. Sham group showed similar pattern with control group. And GE-FA group showed slight increase in WBC numbers except eosinophile. In contrast, the WBC content of cNPE-FA, PE-FA and FA-group was significantly increased compared with control group. These results indicated that natural product extracts attenuated the inflammation and damages by FA inhalation. And GE was the most effective than cNPE and PE in inflammation caused by FA.

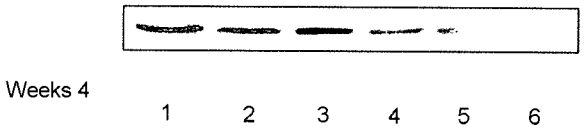

Fig. 3. The SP-D protein from isolated lung tissue. Samples were separated by SDS-PAGE and protein band were visualized by ECL. 1: Control, 2: Sham, 3: cNPE-FA, 4: GE-FA, 5: PE-FA, 6: FA.

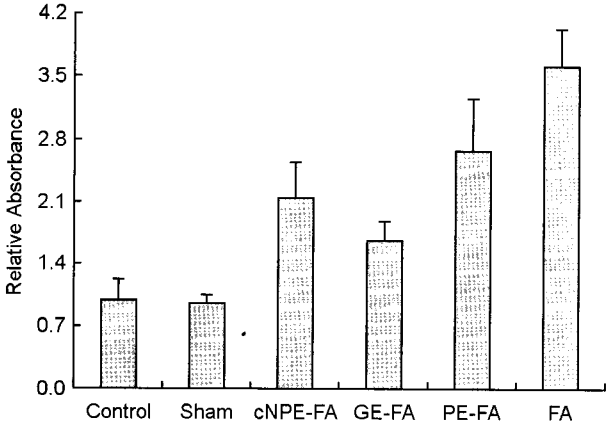

Fig. 4. The effect of formaldehyde according to each condition on mouse lung MDA concentration. Experimental group inhaled according to each condition for 10 hr a day during experimental period. The samples were analyzed by analysis method as described in the Materials and Methods section. The ordinate in figure is the relative value which means the ratio of the control value to each group. Result represents the mean ± SD of five separate experiment.

3. Alteration of surfactant protein (SP)

Alterations of SP-D protein designate the abnormality of lung. As shown in Fig. 3, any significant change of SP-D protein from isolated lung tissue was not observed in control, sham, GE-FA and PE-FA group, whereas slight decrease was seen in NPE-FA group. In contrast, FA group showed the significant decrease of SP-D protein compared with the control group. The data indicated that inhalation of

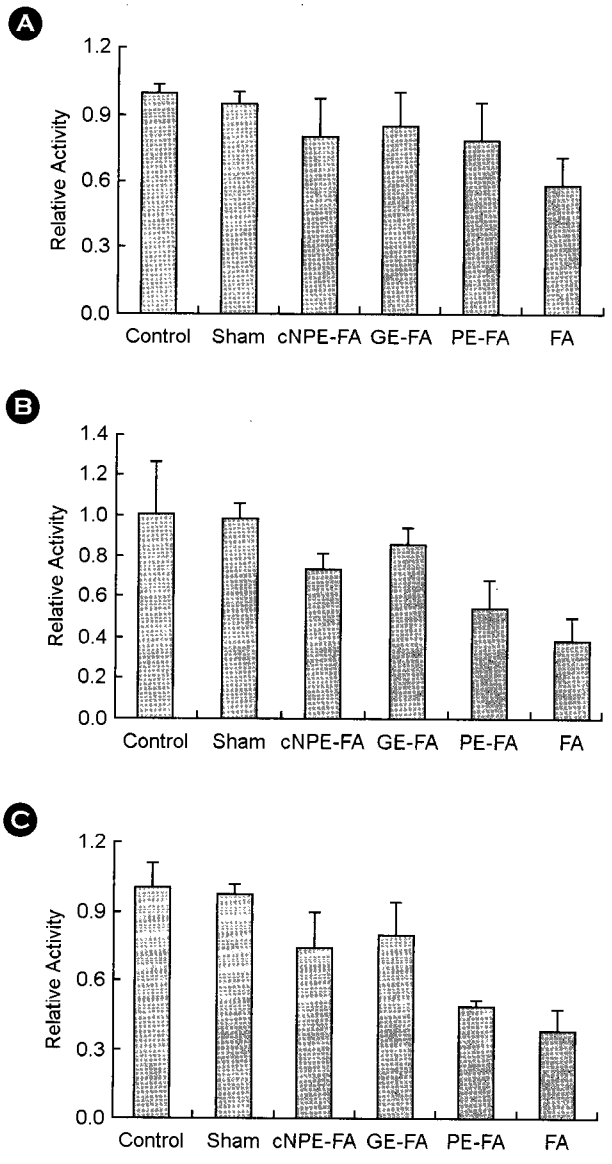

Fig. 5. Antioxidant enzyme activity in tissues of mice treated with formaldehyde. Experimental group inhaled according to each condition for 10 hr during 4 weeks. The ordinate is the relative value which means the ratio of the control value to each group. **(A)** CAT activity **(B)** SOD activity **(C)** G6PD activity. Result represents the mean ± SD of five separate experiments.

cNPE, GE and PE played an important role in SP-D homeostasis during FA inhalation

4. FA-induced lipid peroxidation (LPO)

The effect of FA exposure on malondialdehyde (MDA) content in mouse lung is depicted in Fig. 4. MDA content of FA group was significantly increased as much as 3.5 times of control group. Comparatively, 2.2 times higher content of MDA was found in cNPE-FA group. 1.5 times higher in GE-FA group and 3.0 times higher in PE-FA group. The result clearly showed that cNPE, GE and PE could attenuate lipid peroxidation by FA exposure.

5. Alteration of antioxidative enzyme activity

1) CAT activity

As shown in Fig. 5-A, all groups showed the decreased activity of catalase compared with control group. Especially, the catalase activities of PE-FA and FA groups were significantly decreased, whereas cNPE-FA and GE-FA groups showed slightly decreased activity.

2) SOD activity

As shown in Fig. 5-B, control and sham group showed

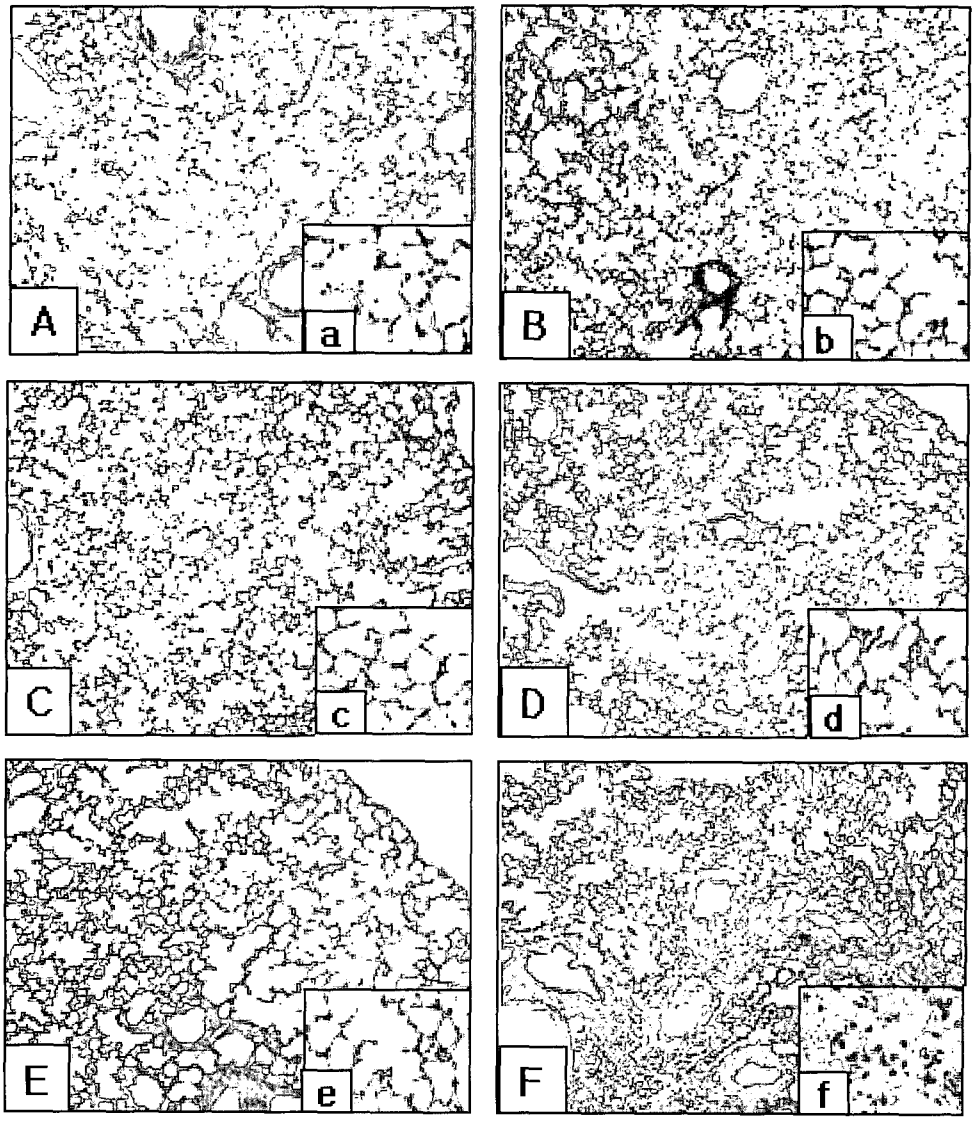

Fig. 6. The H&E stain of pulmonary structure during 4 weeks. Experimental group inhaled according to each condition for 10 hr. The samples were analyzed by analysis method as described in the Materials and Methods section. **A, a**: Control group, **B, b**: Sham group, **C, c**: cNPE-FA group, **D, d**: GE-FA group and **E, e**: PE-FA group **F, f**: FA group. Capital letter: 100×, minuscule: 400×.

the similar SOD activity, but the other groups showed the slightly decreased activity. Especially, a half of enzyme activity in control group was found in FA group.

3) G6PD activity

Activity of G6PD was measured in each group and the results are shown in Fig. 5-C. cNPE-FA, GE-FA and PE-FA groups showed relative decrease tendency, whereas control group and sham groups showed the similar activity. The lowest activity was also observed in FA group. These results suggest that natural product extracts are effective in keeping antioxidative enzyme in lung.

6. Alteration of morphology

As shown in Fig. 6, the pulmonary structure in FA group was deleteriously altered in the morphology, but control and sham groups maintained in its original structure. The pulmonary structure of FA group was atypical, irregular, attached and fused compared with control group. In contrast, cNPE-FA and GE-FA groups showed the similar structure as control group. Furthermore, the pulmonary tissue of FA group was thicker than control group. PE-FA group had thicker tissues than cNPE-FA and GE-FA, but not similar to FA group. These data indicated that treatment of cNPE or GE could keep the pulmonary structure from FA exposure.

DISCUSSION

A multiform of aromatics is used for the relief of neuropsychosis syndromes as a tranquilizer or a headache mitigative. Unfortunately, it seems that aroma therapy using a specific perfume is limited to this field (Bachinger et al., 2000; Baker et al., 2004). In this study, the functional abilities of natural product extracts have been examined using nebulizer. Simultaneous inhalation of natural product extracts with FA exposure showed little weight loss, whereas FA exposure without treatment of natural product extracts resulted in significant weight loss. This indicates that inhalation of natural product extracts can minimize weight loss by FA-induced damage. In hematological analysis, neutrophile, lymphocyte, monocyte and eosinophile were significantly increased by FA exposure. Even though the increasing tendency of white blood cells was also observed in GE-FA, PE-FA and cNPE-FA groups, the treatment of cNPE, GE or PE minimized the increase of white blood cells by damage from FA exposure. This indicates that inhalation of natural product extracts can reduce inflammation and damage in lung by FA exposure. In particular, the number of eosinophile, an index of allergy (Freedman et al., 1996; Schwartz et al., 1993), was more increased than other subtypes of blood cells, which indicates that FA can induce allergy and other similar symptoms.

FA exposure resulted in significant reduction of SP-D protein in lung, but simultaneous treatment of natural product extracts did not produce any significant reduction of SP-D. This means that inhalation of natural product extracts can maintain homeostasis of SP-D protein by preventing damage of the pulmonary tissues (type II cell).

FA exposure caused the increase of lipid peroxidation as high as 3 times of control group. On the other hand, inhalation of natural product extracts with FA exposure reduced significantly the amount of lipid peroxidation compared with FA exposure only. Among them, GE inhalation showed the highest reduction rate of lipid peroxidation, even though it was still higher than control group. As a result, it was confirmed that inhalation of natural product extracts (especially GE) could reduce the amount of lipid peroxidation increased by FA exposure.

In analysis of antioxidative enzymes, sham, cNPE-FA, GE-FA and PE-FA groups showed the similar values of SOD and CAT activity with control group, but it was still higher than FA group. In case of G6PD, a little reduction of enzyme activity in cNPE-FA, GE-FA and PE-FA groups was observed when compared with control and sham groups, but half of enzyme activity was detected in FA group. Among them, GE-FA group had higher activity than cNPE-FA or PE-FA groups, which means GE contains more effective substances. In the result, inhalation of natural product extracts can contribute to maintain the the function of antioxidative enzymes in mouse lung.

FA exposure caused pulmonary structure damaged to be abnormal. However, cNPE-FA, GE-FA, and PE-FA groups kept more similar pulmonary structure as control group rather than FA exposure group. In this experiment, GE was proved to be more effective than PE or cNPE.

Therefore, it should be concluded that natural product extracts have pharmacological effects on pulmonary structure and function against damage by FA exposure. Although direct inhalation into the lung using a nebulizer instead of oral administration and injection is expected to give effective and fast results, more precise experiments should be con-

ducted with appropriate concentration of FA competent to the condition of living environment.

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