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Safety Evaluation of Tobacco Substitute (Herbrette); Inhalation Toxicity, Mutagenicity and Immunotoxicity

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ABSTRACT. Inhalation toxicity, mutagenicity, and immunotoxicity tests were performed using a smoke generation system to investigate the safety of Herbrette, a tobacco substitute made with the leaves of *Perilla frutescens*. ICR mice were exposed to nicotine-free Herbrette smoke with concentrations of 0 (control), 4.08 ± 1.32 mg/m^3 (low dose), 7.72 ± 2.14 mg/m^3 (medium dose) and 12.83 ± 1.69 mg/m^3 (high dose) total particulate matters (TPM) for 4 weeks. When compared to the control group, the body weights, organ weights in the exposed groups did not show any significant differences. However, certain change of several serum chemical data and biochemical parameters were observed, however, the changes were within normal physiological ranges. Moreover, no changes in organ weight, and no gross/microscopic changes were observed between the exposed and control groups. *Salmonella typhimurium* reverse mutation, *in vivo* chromosomal aberration and micronucleus assays revealed that Herbrette did not induce mutagenicity. Upon evaluation of peripheral cellular immunity of mice through *in vitro* lymphocyte proliferation assay, no significant difference was observed in mean stimulation index between the exposed and control groups. Taken together, our results strongly suggest that Herbrette may not cause toxicity on mice under current condition.

Keywords: Tobacco substitute, Herbrette, Inhalation toxicity, Mutagenicity, Immunotoxicity.

INTRODUCTION

Worldwide tobacco problem is growing immensely, with the World Health Organization reporting that almost 1 billion men and 250 million women are daily smokers. Although the upward trend of global cigarette consumption is slowing down, the overall consumption is still growing, with approximately 5.5 trillion cigarettes consumed in 2000 (Mackay and Eriksen, 2002; World Health Organization, 2002). The United States National Cancer Institute estimates that almost 50 million Americans are tobacco smokers (National Institutes of Health and National Cancer Institute, 1990). In Korea, more than 17 million men and 1.2 million women were reported to be smokers (Korea Institute for Health and Social Affairs, 1998). Cigarette smoke has been implicated in various degenerative pulmonary and cardiovascular diseases, as well as lung cancer and other malignancies (US Surgeon Generals Report, 1985; Sherman, 1991; Frank, 1993; Bartecchi *et al.*, 1994). Each year, tobacco products kill some 3 million people worldwide, and this number is increasing continuously. WHO estimates that, unless current smoking patterns are reversed, by the decade 2020~2030 tobacco smoke will be responsible for 10 million deaths per year, 70% of them occurring in developing countries. Based on scientific evidences accumulated since early 1950s, over 25 diseases are now known or strongly suspected to be causally related to smoking (Sajjan *et al.*, 2003). However, notwithstanding the campaigns and warnings against the smoking addiction, a large part of the world population continues to smoke. From a health perspective, perhaps the important subsets of smokers

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are those who are unable to quit despite repeated efforts. Traditional smoking cessation programs have shown limited success rates. Therefore, the development of nicotine-free tobacco substitute, which should provide benefits to inveterate smokers to quit smoking, is strongly required.

Herbrette, a tobacco substitute composition, was designed to aid smokers quit smoking, and its chief ingredient is the leaves of Ja-So-Yueb, *Perilla frutescens* BRITTON var. *acuta* KUDO (Labiatae) are found in Hangekoubokuto, Saibokuto, and other traditional Chinese herbal remedies which are primarily used to treat inflammatory diseases, clinical depression, and anxiety-related disorders such as anxiety neurosis and anxiety hysteria (Nakazawa *et al.*, 2003). The leaves of *Perilla frutescens* contained many components such as essential oils, flavones, and phenylpropanoids have been identified. However, the bioactive compounds for the traditional use of the herbal medicine are still unidentified (Nakazawa *et al.*, 2003).

Accordingly, the safety of Herbrette was evaluated through repeated inhalation toxicity, mutagenicity, and immunotoxicity studies using ICR mice. We report here results of the toxicity studies of Herbrette, based on inhalation toxicity, mutagenicity, and immunotoxicity tests performed according to the Regulation of Korean Food and Drug Administration (1999. 12).

MATERIALS AND METHODS

Generation of cigarette smoke

Experiments were performed using an automatic mainstream and sidestream analytical smoking machine (Dusturbo, Seoul, Korea). All technical specifications of the smoking machine fulfill the requirements set forth in the International Organization for Standardization (ISO) standards 4387 and 3308. The smoking machine simultaneously exposes a group of animals to smoke released from the burning end of sidestream and puff of mainstream smoke drawn from a cigarette using a puffer box. The puff volume of each cigarette was adjusted to 35 ml, and the puff was delivered for 2 s at a 1 min interval. The smoke then moved into an exposure chamber (whole body type, 0.5 m^3, Dusturbo).

Construction and composition of Herbrette

Herbrette was designed to match industrial standards and to be representative type of cigarettes sold worldwide. Herbrette, made with the leaves of *Perilla frutescens*, and cellulose acetate filters allowing 30% ventilation, was manufactured using conventional commercial equipment. During the blending and processing of Herbrette, humectants consisting of glycerol and propylene glycol were added to increase the moisture holding capacity and aid in the processing, while flavors were used to complement the subjective characteristics of the smoke. The added ingredients were non-volatile materials such as sugars and licorice, and highly volatile aromatic materials such as menthol. Other types of ingredients used to enhance the flavor of Herbrette smoke include foods such as chocolate and spices such as vanilla.

Concentrations of smokes

The Herbrette smokes in the chamber were sampled with a personal sampler (MSA 484107, USA) that contained mixed cellulose ester filter (0.8 μm pore size 37 mm diameter, Millipore AAWP 03700, USA). Smokes were sampled every 15 min for 6 h with a flow rate 2 l/min. Time weighted average (TWA) of total particulate matters (TPM) of burning 20 cigarettes for 6 hrs was approximately 20 mg/m^3. Since no mortality was observed following 6-h exposure to 40 mg/m^3 TPM, LC_{50} of Herbrette inhalation was considered to be higher than that of 40 mg/m^3 in mice (data not shown). No abnormal clinical signs, gross findings or development of body weights were observed in all mice (data not shown). Therefore, the mice were exposed to 3 different concentrations; 4.08 ± 1.32 (low dose), 7.72 ± 2.14 (medium dose) and 12.83 ± 1.69 mg/m^3 (high dose) TPM.

CO measure and gas chromatography-mass spectrometry (GC-MS) analysis of TPM

Herbrette smoke was maintained at concentrations of 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3, and the smoke samples were separately collected using a personal air sampler. CO concentration was determined five times at concentration of 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3 during the test period using a portable complex gas detector (MSA, BFE-1304-98, USA). The amount of TPMs was measured by weighing the filter before and after the collection of TPMs. For the measurement of several representative PAHs in Herbrette smoke, TPMs were extracted from the cellulose ester filter using 20 ml of *n*-hexane by employing the sonic extraction method. The *n*-hexane extract was re-extracted with 20 ml of ethyl acetate and evaporated. A Hewlett-Packard model 5890A gas chromatograph was operated in conjunction with a VG Analytical model 70-SE mass spectrometer and a VG-11-250J mass data system. An ultra-2 column containing cross-linked 5% phenylmethylsilicon (25 m × 0.2 mm I.D. × 0.33 μm film thickness) was used. Column temperature was programmed at 15°C/min from 230 to 310°C (injec-

tion temperature, 280°C; transfer line temperature, 280°C; helium flow-rate, 0.7 ml/min) (Kim *et al.*, 2003). In addition, Herbrette smoke captured on the membrane filters was analyzed for nicotine.

Study of inhalation toxicity

Five-week-old male and female, specific pathogen-free (SPF) ICR mice, purchased from Biogenomics, Korea, were acclimated to a 12 h lights, 12 h dark cycle with light from 08:00 to 20:00 h. The mice were fed Samyang (Korea) and tap water *ad libitum* for 1 week before the initiation of the experiment. The mice weighting 25.2 ± 2.1 g (male), 22.6 ± 2.4 g (female) were randomly assigned to 4 groups. Four groups of 10 each male and female mice were exposed to smokes at concentration of 0, 4.08 ± 1.32 mg/m^3, 7.72 ± 2.14 mg/m^3, and 12.83 ± 1.69 mg/m^3 for 6 h/day, 5 day/week for 4 weeks in the exposure chamber. The day following the last exposure, the animals were subjected to clinical chemical, hematological and pathological examinations. Blood chemistry was carried out to determine the serum levels of total protein (TPROT), albumin (ALB), total biliruin (TBILI), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), creatinine (CREAT), blood urea nitrogen (BUN), sodium (Na), calcium (Ca), phosphorus (P) and chloride (Cl) using a biochemical autoanalyzer (VITALAB, Merck, The Netherlands). Hematological parameters, which were estimated from 10 mice of each sex per each group, consisting of hematocrit (HCT), erythrocyte (RBC), leucocyte (WBC), neutrophil (NE), lymphocyte (LY), monocyte (MO), eosinophil (EO), basophil (BA), prothrombin time (PT), partial thromboplastin time (PTT), and blood platelet (PLT) were determined using a hematological autoanalyzer (Coulter T540 hematology system, Coulter world Headquarters, USA). Weights of the liver, kidneys, testes, ovaries, spleen, brain, and lungs were recorded before fixation with 10% buffered formalin. Other tissues such as oviducts, pancreas, thyroid, trachea, uterus, and vagina were collected and preserved in 10% formalin and subsequently processed for microscopic examinations.

Data were expressed as mean ± S.D. Multiple variance of analysis and Duncan's multiple range tests were used to compare the blood chemistry and parameters of hematological values of the control with the exposure groups.

Mutagenicity

Ames test. TPMs were extracted from the cellulose ester membrane filter with 20 ml of *n*-hexane using sonic extraction method. The *n*-hexane extract was re-extracted with 20 ml of ethyl acetate and evaporated. One each milligram of *n*-hexane and ethyl acetate extract was diluted with 3 ml dimethyl sulfoxide (DMSO, Sigma, USA) and treated with 100 µl per plate. The *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537, histidine-requiring mutants kindly provided by Dr B.N. Ames (University of California, Berkeley, CA, USA) were maintained as described by Maron and Ames (1983). Genotypes of the test strains were checked routinely for the histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation), and the presence of R factor. S9-mix (0.5 ml) contained 0.05 ml of S9 fraction (1.2 mg of protein concentration) and 0.45 ml of the cofactor solution (Cofactor I^{TM}, Oriental Yeast, Japan). S9-mix contained 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH, and 100 mM sodium phosphate (pH 7.4). Mutagenicity was assayed by the standard Ames test with a modification to the pre-incubation procedure and was performed with/without the metabolic activation system. Saline was used as a negative control, and the positive controls were sodium azide (SAZ), 2-amino-fluoren (2-AF) and ICR191. Briefly, a mixture containing the test compound in 0.1 ml of saline, 0.1 ml of culture in the early stationary phase of the tester strain, and 0.5 ml of S9-mix (or 0.05 M phosphate buffer, pH 7.4 for nonmetabolic activation set) was incubated for 20 min at 37°C in a test tube while shaking. After incubation, 2 ml of 0.05 mM L-histidine-0.05 mM biotin moiten top agar was added to the test tube, mixed, and poured onto a plate of minimal glucose agar medium. Triplicate assays were carried out for each determination. The plate was incubated for 48 h at 37°C, and *his*$^+$ revertant colonies were counted. Similar studies were performed on Keum-Yeon-Cho containing no histidine to check the potential effects of histidine moiety on the mutagenicity of *Salmonella* strain.

Statistical significance of the differences in *his*$^+$ revertant colonies among various groups was checked by the method of Kim and Margolin (1999), and Kim *et al.* (2003).

Chromosomal aberration assay. After exposure, five male and female mice per each group were euthanized by intraperitoneal injection of sodium pentobarbital overdosage. Spleens were dissected out from each animal, and splenocytes were collected by goring the spleen through a sterilized nylon filter and washing the filter to collect the cells in phosphate buffered solution (PBS). The cell suspension was then gently layered to the same volume of Histopaque 1077 (Sigma, USA) and centrifuged at 800 ×g for 15 min. The interface was carefully collected, resuspended in PBS, and centrifuged at 200 ×g for 15 min. The cell pellet was

once more resuspended in PBS and centrifuged at 200 ×g for 10 min. Finally, the cell pellet was resuspended in 1 ml PBS. The cell concentration was calculated using a haemocytometer. Splenocyte cultures were initiated at a concentration of 1×10^6 cells per ml in complete medium. Growth medium consisted of RPMI 1640 (Gibco, UK) supplemented with 15% fetal bovine serum (FBS) (Life Technology, Sweden), 2 mM L-glutamine, and antibiotics. Concanavalin A (Con A, Sigma, USA) at a final concentration of 5 μg/ml was used as a mitogen. At least two separate cultures were prepared from each animal for each experimental point. Cultures were allowed to grow at 37°C in a 5% CO_2 atmosphere with 95% humidity. Seventy-two hours later, mitotic cells were blocked with colcemid, and the cells were harvested by centrifugation at 400 ×g for 10 min. The supernatant was removed, and the cell pellet was resuspended in 10 ml prewarmed hypotonic KCl solution (0.075 M). After 20 min at 37°C, the tubes were centrifuged for 8 min at 400 ×g. The supernatant was removed, and the cell pellet was resuspended in 10 ml fixative (acetic acid:methanol, 1 : 3, V : V). The fixative was changed four times and the cells were resuspended in 0.3~0.5 ml fixative prior to slide preparation. Samples of the cell suspension were added to precleaned slides and air-dried. Chromosomes were stained with diluted Giemsa (1 : 20) and evaluated by a single observer. A total 100 well-spread metaphase cells (50 cells per tube) with 40 ± 2 chromosomes per animal were scored for gaps, breaks, exchanges, and chromatid breaks and exchange. Chromosome and chromatid aberrations were scored separately, and the total percentage of abnormal cells was expressed for statistical analysis. Gaps were recorded but not included in the total chromosome aberration frequency. Student *t*-test was performed to observe the significant difference in various types of chromosome aberrations between the exposed- and control groups.

Micronucleus test. Four groups of five each male and female mice were exposed to concentrations of 0, 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3 for 6 h/day, 5 day/week for 4 weeks. Mitomycin C (MMC, Sigma, USA) was used as a positive control. Micronucleus test was performed according to the method of Schmid (1975). Briefly, the animals were sacrificed by cervical dislocation 24 h after injection for preliminary screening. The femurs of each animal were dissected out, and the bone marrow was flushed out with FBS. The suspension was centrifuged, into which a few drops of FBS were added, and the cell pellet was mixed thoroughly. The bone marrow of the femur was then smeared on glass slides, which were air dried, fixed with absolute methanol, and stained with Giemsa staining solution. Four slides were prepared from each animal, and 1000 polychromatic erythrocytes (PCE) were examined in each slide for the presence of micronucleus. The results were expressed as the average numbers of micronucleated cells per 1000 cells. Polychromatic and normochromatic erythrocyte ratios [PCE/(PCE + NCE) ratio] were also determined. Statistical significance of the differences in micronucleus occurrences among various treated groups was checked by the method of Kim *et al.* (2003).

Immunotoxicity. Four groups of five each male and female mice were exposed to concentrations of 0, 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3 for 6 h/day, 5 day/week for 4 weeks. Mouse spleen cells were separated as described for the chromosome aberration assay. Cellular immunity was assessed through *in vitro* lymphocytes proliferation assay (Smialowicz, 1995). lymphocytes were plated in 96-well, round bottom, microplates (200 μl per well) containing concanavalin A (Con A, sigma, USA) and lipopolysaccharide (LPS, Sigma, USA) in triplicates at 37°C, 10% CO_2 for 3 days. To determine immunotoxicity of Herbrette, the cells were pulsed with 1 μCi of [^{3}H] thymidine by incorporating tritium thymidine into the newly synthesized cellular DNA. Glass fiber filters were counted using a liquid scintillation counter (Wallac, Finland), and triplicate values were averaged. Lymphocyte stimulation index was determined as the ratio of lymphocyte counts from stimulated and unstimulated samples. Student's *t*-test was used to evaluate differences in stimulation index (SI) between treated and control groups.

RESULTS

CO measurement and gas chromatography-mass spectrometry (GC-MS) analysis of TPM

CO concentration was each measured five times during the test period. The mean CO at concentration of 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3 were 5.67 ± 2.42, 9.17 ± 1.64, and 14.52 ± 2.56 ppm (mean ± S.D., n=5), respectively. Although several PAHs such as naphthalene and fluorine were detected in Herbrette, their concentration levels were considered negligible (ppb level, data not shown). In addition, the analysis of Herbrette smoke indicated no nicotine.

Four-week inhalation study

Mice exposed to the Herbrette smokes showed no statistically significant changes in body weight during the 4-week experiment (data not shown). In addition, no

Table 1. Biochemical data for male mice in the 4-week inhalation study of Herbrette

Items	Herbrette exposure level (concentration: mg/m^3)			
	Control (0)	Low (4.08 ± 1.32)	Medium (7.72 ± 2.14)	High (12.83 ± 1.69)
Number of animal	10	10	10	10
TPROT (g/dl)	7.48 ± 1.36	8.44 ± 1.59	7.50 ± 0.38	8.20 ± 1.04
ALB (g/dl)	4.20 ± 0.29	4.02 ± 0.73	4.70 ± 0.79	4.24 ± 0.31
TBILI (mg/dl)	0.45 ± 0.05	0.40 ± 0.02	0.50 ± 0.09	0.46 ± 0.10
AST (μ/l)	58.00 ± 8.16	63.40 ± 3.90	57.48 ± 5.21	59.60 ± 13.79
ALT (μ/l)	26.95 ± 5.76	36.17 ± 11.74	34.20 ± 10.59	41.20 ± 3.12**
GLU (mg/dl)	62.52 ± 17.24	61.20 ± 9.12	55.40 ± 12.33	58.23 ± 8.24
CREAT (mg/dl)	1.02 ± 0.38	0.96 ± 0.08	0.88 ± 0.24	0.98 ± 0.11
BUN (mg/dl)	21.35 ± 3.50	20.64 ± 1.18	25.60 ± 3.87	27.96 ± 2.72*
Na (mEq/l)	165.28 ± 27.26	149.66 ± 32.18	172.36 ± 51.14	169.82 ± 18.46
Ca (mg/dl)	12.58 ± 1.36	13.30 ± 1.07	12.98 ± 2.5	12.62 ± 1.24
P (mg/dl)	7.42 ± 1.86	8.12 ± 0.68	98.32 ± 2.20	9.28 ± 2.82
Cl (mEq/l)	129.80 ± 18.41	122.25 ± 12.81	124.40 ± 37.39	153.22 ± 18.83**

Data are mean ± S.D.
*Significantly different from control at $P<0.05$.
**Significantly different from control at $P<0.01$.
TPROT, total protein; ALB, albumin; TBILI, total bilirubin; AST, aspartate amino-transferase; ALT, alanine aminotransferase; GLU, glucose; CREAT, creatinine; BUN, blood urea nitrogen; Na, sodium; Ca, calcium; P, phosphorus; Cl, chloride.

Table 2. Biochemical data for female mice in the 4-week inhalation study of Herbrette

Items	Herbrette exposure level (concentration: mg/m^3)			
	Control (0)	Low (4.08 ± 1.32)	Medium (7.72 ± 2.14)	High (12.83 ± 1.69)
Number of animal	10	10	10	10
TPROT (g/dl)	7.74 ± 0.61	8.27 ± 1.26	7.72 ± 2.06	8.14 ± 0.71
ALB (g/dl)	4.44 ± 0.57	4.94 ± 0.50	4.40 ± 1.20	4.88 ± 0.42
TBILI (mg/dl)	0.52 ± 0.16	0.49 ± 0.03	0.45 ± 0.05	0.60 ± 0.07
AST (μ/l)	31.80 ± 5.21	36.03 ± 3.10	51.80 ± 8.38*	65.80 ± 6.33**
ALT (μ/l)	30.40 ± 9.66	27.44 ± 10.10	35.80 ± 12.07	33.22 ± 6.80
GLU (mg/dl)	55.19 ± 9.76	59.02 ± 3.62	58.52 ± 16.06	56.20 ± 15.2
CREAT (mg/dl)	1.12 ± 0.36	0.88 ± 0.40	1.06 ± 0.28	1.37 ± 0.13*
BUN (mg/dl)	21.90 ± 3.70	22.42 ± 2.81	24.30 ± 3.59	24.88 ± 6.58
Na (mEq/l)	124.40 ± 45.57	135.84 ± 19.48	129.61 ± 44.42	132.58 ± 23.26
Ca (mg/dl)	12.04 ± 0.83	13.70 ± 7.54	11.66 ± 2.15	13.64 ± 2.21
P (mg/dl)	6.96 ± 1.91	8.00 ± 0.44	8.82 ± 1.19	8.58 ± 1.33
Cl (mEq/l)	114.80 ± 26.74	127.36 ± 34.67	118.20 ± 14.38	122.42 ± 24.03

Data are mean ± S.D.
*Significantly different from control at $P<0.05$.
**Significantly different from control at $P<0.01$.
TPROT, total protein; ALB, albumin; TBILI, total bilirubin; AST, aspartate amino-transferase; ALT, alanine aminotransferase; GLU, glucose; CREAT, creatinine; BUN, blood urea nitrogen; Na, sodium; Ca, calcium; P, phosphorus; Cl, chloride.

clinical signs of toxicity were observed during or after exposure throughout the study period nor were any clinical pathology changes noted (data not shown). At terminal sacrifice, major points of statistically significant changes in clinical chemistry parameters were observed; however, the changes were within normal ranges and not considered to be exposure-related. Biochemical parameters, ALT, BUN, and Cl, were greatly increased compared to the control group, particularly in male mice exposed to high concentration of Herbrette (Table 1). In female mice, AST level of medium and high concentration groups were higher than those of the control. In addition, CREAT level of high concentration group were higher than that of the control values (Table 2). Hematology parameters, WBC, NE (medium and high) and PLT, of high concentration-exposed male mice were significantly increased compared to the control group (Table 3). On the other hand, NE, LY (medium), and PLT (medium and high) levels were statistically different from the control values in all exposed female groups (Table 4). Moreover, no abnormal changes in organ weight, and no adverse gross/microscopic changes were observed between the exposed and control groups (data not shown).

Table 3. Hematology data for male mice in the 4-week inhalation study of Herbrette

Items	Herbrette exposure level (concentration: mg/m^3)			
	Control (0)	Low (4.08 ± 1.32)	Medium (7.72 ± 2.14)	High (12.83 ± 1.69)
Number of animal	10	10	10	10
WBC (× 10^3 per μl)	2.44 ± 0.92	2.65 ± 0.28	2.51 ± 0.68	5.60 ± 1.04**
NE (× 10^3 per μl)	0.46 ± 0.13	0.33 ± 0.21	0.62 ± 0.20*	0.88 ± 0.24**
NE (%)	14.32 ± 4.06	18.65 ± 8.78	19.52 ± 2.34*	20.29 ± 3.27*
LY (× 10^3 per μl)	2.95 ± 1.26	3.13 ± 1.43	4.45 ± 2.59	6.38 ± 1.79**
LY (%)	78.32 ± 12.24	81.97 ± 13.20	79.83 ± 9.67	87.90 ± 24.80
MO (× 10^3 per μl)	0.10 ± 0.06	0.08 ± 0.05	0.07 ± 0.03	0.08 ± 0.07
MO (%)	3.73 ± 1.57	3.35 ± 2.03	3.63 ± 1.72	3.48 ± 2.11
EO (× 10^3 per μl)	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
EO (%)	1.42 ± 0.33	1.06 ± 0.76	1.15 ± 0.64	1.77 ± 0.98
BA (× 10^3 per μl)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
BA (%)	0.37 ± 0.12	0.27 ± 0.14	0.32 ± 0.26	0.42 ± 0.28
RBC (× 10^3 per μl)	6.47 ± 2.35	6.02 ± 3.08	6.95 ± 3.65	5.88 ± 4.10
HCT (%)	37.52 ± 10.46	38.02 ± 13.46	35.22 ± 3.88	42.56 ± 9.87
PLT (× 10^3 per μl)	764.75 ± 244.48	886.47 ± 188.63	820.63 ± 231.52	1244.49 ± 169.71**

Data are mean ± S.D.
*Significantly different from control at $P<0.05$.
**Significantly different from control at $P<0.01$.
WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocyte; EO, eosinophil; BA, basophil; RBC, red blood cell; HCT, hematocrit; PLT, platelet.

Table 4. Hematology data for female mice in the 4-week inhalation study of Herbrette

Items	Herbrette exposure level (concentration: mg/m^3)			
	Control (0)	Low (4.08 ± 1.32)	Medium (7.72 ± 2.14)	High (12.83 ± 1.69)
Number of animal	10	10	10	10
WBC (× 10^3 per μl)	1.71 ± 1.00	1.87 ± 0.66	2.10 ± 0.52	2.38 ± 1.19
NE (× 10^3 per μl)	0.68 ± 0.24	0.64 ± 0.18	0.76 ± 0.27	1.44 ± 0.19**
NE (%)	16.40 ± 5.45	21.42 ± 10.60	20.92 ± 7.85	22.30 ± 8.86
LY (× 10^3 per μl)	4.79 ± 1.31	4.08 ± 1.59	7.18 ± 1.53*	5.60 ± 1.88
LY (%)	79.65 ± 4.52	81.52 ± 3.00	80.23 ± 4.86	83.24 ± 6.62
MO (× 10^3 per μl)	0.17 ± 0.07	0.14 ± 0.08	0.18 ± 0.09	0.16 ± 0.11
MO (%)	4.64 ± 1.95	3.98 ± 0.85	3.94 ± 1.04	5.67 ± 3.43
EO (× 10^3 per μl)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.02 ± 0.02
EO (%)	1.19 ± 0.92	0.84 ± 0.67	1.11 ± 0.36	1.14 ± 1.00
BA (× 10^3 per μl)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
BA (%)	0.43 ± 0.31	0.39 ± 0.18	0.38 ± 0.23	0.37 ± 0.19
RBC (× 10^3 per μl)	5.99 ± 1.73	6.57 ± 2.47	7.72 ± 2.68	7.57 ± 2.39
HCT (%)	41.50 ± 15.40	43.30 ± 17.80	38.40 ± 14.42	43.99 ± 15.59
PLT (× 10^3 per μl)	652.02 ± 216.70	816.98 ± 273.96	1088. ± 209.12*	1287.11 ± 139.90**

Data are mean ± S.D.
*Significantly different from control at $P<0.05$.
**Significantly different from control at $P<0.01$.
WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocyte; EO, eosinophil; BA, basophil; RBC, red blood cell; HCT, hematocrit; PLT, platelet.

Mutagenicity

Reverse mutation tests using *S. typhimurium* TA98, TA100, TA102, TA1535, and TA (concentrations of Herbrette smoke extracts was 33, 16.5, and 8.25 μg per plate, respectively) were performed with/without metabolic activation system. No increases in revertants were observed with/without metabolic activation. However, the positive control (2-AF, SAZ, ICR191) showed significant increase in the number of mutation colony compared to the negative control (Tables 5~8).

In vivo chromosome aberration was determined with splenocytes of male and female mice exposed to concentrations up to 12.83 ± 1.69 mg/m^3 per day. No statistically significant increases in aberrations were observed at all concentrations tested, an indication that Herbrette does not have any mutagenicity in terms of chromosome aberration (Table 9).

In the micronucleus study, male and female mice

Table 5. Reverse mutation test on extracts of Herbrette smoke particles captured on the membrane filter by *n*-hexane in S. *typhimurium* with S9 mix

Group	Number of revertant per plate				
	TA98	TA100	TA1535	TA1537	TA102
Negative control	43.0 ± 13.5	195.7 ± 36.4	14.3 ± 6.8	15.7 ± 6.5	306.3 ± 59.7
High (33.00 μg/plate)	53.7 ± 17.0	192.3 ± 29.8	13.7 ± 7.6	18.0 ± 6.2	298.3 ± 59.6
Medium (16.50 μg/plate)	48.3 ± 24.1	205.7 ± 43.3	16.7 ± 4.5	13.3 ± 4.1	345.3 ± 62.2
Low (8.25 μg/plate)	37.7 ± 15.4	224.3 ± 42.8	15.7 ± 8.0	14.0 ± 2.7	323.3 ± 71.81
2-AF (10.00 μg/plate)	346.3 ± 78.5*	NT	NT	NT	306.3 ± 309.9*
SAZ (1.50 μg/plate)	NT	1072.3 ± 321.1*	185.7 ± 30.1*	NT	NT
ICR 191 (1.00 μg/plate)	NT	NT	NT	242.0 ± 66.4*	NT

Data are mean ± S.D.
NT, not tested.
*Significantly different from control at $P<0.05$.

Table 6. Reverse mutation test on extracts of Herbrette smoke particles captured on the membrane filter by *n*-hexane in *S. typhimurium* without S9 mix

Group	Number of revertant per plate				
	TA98	TA100	TA1535	TA1537	TA102
Negative control	50.7 ± 6.5	198.3 ± 18.1	16.7 ± 6.7	17.7 ± 4.2	307.0 ± 29.5
High (33.00 μg/plate)	57.1 ± 10.8	178.7 ± 24.7	18.7 ± 7.6	18.3 ± 7.0	303.0 ± 85.1
Medium (16.50 μg/plate)	48.0 ± 15.2	194.3 ± 26.5	20.2 ± 6.1	14.7 ± 5.5	285.7 ± 42.6
Low (8.25 μg/plate)	49.0 ± 16.7	208.7 ± 36.4	19.8 ± 5.6	21.7 ± 7.0	325.0 ± 38.4
2-AF (10.00 μg/plate)	354.8 ± 76.2*	NT	NT	NT	1185.7 ± 271.4*
SAZ (1.50 μg/plate)	NT	1265.0 ± 349.6*	204.3 ± 84.9*	NT	NT
ICR 191 (1.00 μg/plate)	NT	NT	NT	352.7 ± 58.5*	NT

Data are mean ± S.D.
NT, not tested.
*Significantly different from control at $P<0.05$.

Table 7. Reverse mutation test on extracts of Herbrette smoke particles captured on the membrane filter by ethyl acetate in *S. typhimurium* with S9 mix

Group	Number of revertant per plate				
	TA98	TA100	TA1535	TA1537	TA102
Negative controlHigh	44.6 ± 16.4	202.3 ± 36.2	18.4 ± 5.2	20.2 ± 4.8	278.9 ± 64.3
(33.00 μg/plate)Medium	39.8 ± 12.5	214.6 ± 21.8	17.3 ± 6.5	22.0 ± 5.8	302.2 ± 45.6
(16.50 μg/plate)Low	41.8 ± 8.6	219.5 ± 28.4	20.4 ± 6.1	18.5 ± 4.2	299.7 ± 50.6
(8.25 μg/plate)2-AF	46.5 ± 15.4	188.1 ± 34.4	19.0 ± 3.7	16.6 ± 7.2	318.7 ± 68.8
(10.00 μg/plate)SAZ	331.2 ± 92.2*	NT	NT	NT	1424.5 ± 334.5*
(1.50 μg/plate)ICR 191	NT	1115 ± 272.2*	210.2 ± 42.5*	NT	NT
(1.00 μg/plate)	NT	NT	NT	312.5 ± 48.4*	NT

Data are mean ± S.D.
NT, not tested.
*Significantly different from control at $P<0.05$.

were exposed to Herbrette smoke at concentrations of 0, 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3. No significant differences in the ratios of numbers of micronucleated erythrocytes per 2000 PCE were observed at all concentrations except positive control (Mitomycin C) of both male and female mice (Table 10).

Immunotoxicity

Cellular immune status of mice exposed to Herbrette smoke for 4-week of concentrations of 0, 4.08 ± 1.32, 7.72 ± 2.14, and 12.83 ± 1.69 mg/m^3 were compared. No significant differences were observed between the Herbrette smoke exposure and control group (Tables 11~12).

DISCUSSION

Previous toxicity studies have shown Keum-Yeon-Cho (NosmoQ-made with the leaves of *E.ulmoides*) smoke

Table 8. Reverse mutation test on extracts of Herbrette smoke particles captured on the membrane filter by ethyl acetate in *S. typhimurium* without S9 mix

Group	Number of revertant per plate				
	TA98	TA100	TA1535	TA1537	TA102
Negative control	52.2 ± 10.3	211.6 ± 22.5	22.0 ± 5.4	23.6 ± 4.0	292.6 ± 47.4
High (33.00 μg/plate)	46.7 ± 14.2	191.3 ± 43.3	20.6 ± 3.2	21.5 ± 3.7	312.5 ± 38.5
Medium (16.50 μg/plate)	51.5 ± 8.6	224.2 ± 37.2	23.4 ± 4.7	19.2 ± 5.6	334.3 ± 72.1
Low (8.25 μg/plate)	44.8 ± 14.5	200.8 ± 24.8	18.3 ± 6.9	23.1 ± 6.4	266.4 ± 63.2
2-AF (10.00 μg/plate)	328.7 ± 92.2*	NT	NT	NT	1237.2 ± 362.6*
SAZ (1.50 μg/plate)	NT	1266.5 ± 336.4*	258.2 ± 57.5*	NT	NT
ICR 191 (1.00 μg/plate)	NT	NT	NT	387.4 ± 59.4*	NT

Data are mean ± S.D.
NT, not tested.
*Significantly different from control at $P<0.05$.

Table 9. Effect of chromosome aberration induced in ICR mice lymphocytes exposed with Herbrette

Herbrette exposure level (concentration: mg/m^3)	Sex	Total number of examined metaphases	Number of metaphases with different types of chromosomal aberrations						Frequency of cells with chromosome aberration (%)
			Chromatid gap	Chromatid break	Chromatid exchange	Chromosome gap	Chromosome break	Chromosome exchange	
Control (0)	Male	500	7	8	0	1	1	0	1.80 ± 1.10
	Female	500	7	11	0	0	1	0	2.40 ± 1.82
Low (4.08 ± 1.32)	Male	500	5	10	0	2	1	1	2.40 ± 0.55
	Female	500	5	11	0	1	1	0	2.40 ± 0.89
Medium (7.72 ± 2.14)	Male	500	5	8	1	3	1	1	2.20 ± 0.45
	Female	500	4	12	0	0	1	0	2.60 ± 1.14
High (12.83 ± 1.69)	Male	500	8	8	0	2	1	0	1.80 ± 1.30
	Female	500	7	13	1	0	1	0	3.00 ± 1.22

Result are for 5 animals/group (1000 cells/animal). Total chromatid and chromosome gaps/1000 cells at each concentration were recorded but not included as aberrations.

Table 10. Results of micronucleus test in ICR mice exposed with Herbrette

Herbrette exposure level (concentration: mg/m^3)	Sex	Route	Number of animal	Number of MNPCE % (Mean ± S.D./2000 PCE)	PCE (%, Mean ± S.D./500 Erythrocyte)
Negative control (0)	Male	Inhalation	5	0.12 ± 0.057	48.04 ± 2.480
	Female	Inhalation	5	0.12 ± 0.076	49.68 ± 2.175
Low (4.08 ± 1.32)	Male	Inhalation	5	0.14 ± 0.074	48.16 ± 1.899
	Female	Inhalation	5	0.13 ± 0.045	48.34 ± 1.982
Medium (7.72 ± 2.14)	Male	Inhalation	5	0.15 ± 0.079	47.28 ± 2.893
	Female	Inhalation	5	0.15 ± 0.050	49.40 ± 2.506
High (12.83 ± 1.69)	Male	Inhalation	5	0.13 ± 0.055	48.76 ± 3.207
	Female	Inhalation	5	0.14 ± 0.065	49.00 ± 1.463
Positive control	Male	Inhalation	5	6.50 ± 0.408	52.64 ± 0.780
(Mitimycin C, 0.25 mg/kg)	Female	Inhalation	5	6.32 ± 0.284	51.76 ± 1.992

MNPCE, micronucleus polychromatid erythrocyte. PCE, polychromatid erythrocyte.

Table 11. Representative mitogen-stimulated splenic lymphocyte response data of male mice exposed to Herbrette

Herbrette exposure level (concentration: mg/m^3)	Number of animal	Mitogen				
		Cells only	Con A	Stimulation index (Con A, % of cell only)	LPS	Stimulation index (LPS, % of cell only
Control (0)	5	2419.6 ± 404.2	52706.5 ± 1807.9	2218.5 ± 372.5	16514.4 ± 3563.4	722.5 ± 143.6
Low (4.08 ± 1.32)	5	2460.1 ± 240.7	53956.0 ± 1927.8	2209.4 ± 349.1	16208.3 ± 1898.2	699.3 ± 107.1
Medium (7.72 ± 2.14)	5	2572.3 ± 282.6	52804.3 ± 1040.5	2125.8 ± 204.3	17526.8 ± 2318.9	702.6 ± 119.1
High (12.83 ± 1.69)	5	2674.0 ± 234.6	54477.8 ± 2128.6	2098.3 ± 396.7	18761.8 ± 2168.5	714.7 ± 81.9

Data are mean cpm ± S.E.M.

Table 12. *Representative mitogen-stimulated splenic lymphocyte response data of female mice exposed to Herbrette*

Herbrette exposure level (concentration: mg/m^3)	Number of animal	Mitogen				
		Cells only	Con A	Stimulation index (Con A, % of cell only)	LPS	Stimulation index (LPS, % of cell only
Control (0)	5555	2203.1 ± 520.4	52583.4 ± 1299.8	2397.7 ± 259.0	21500.3 ± 3563.4	1073.4 ± 167.7
Low (4.08 ± 1.32)		2298.6 ± 299.8	53431.8 ± 1238.9	2372.5 ± 253.8	21980.6 ± 1898.2	976.2 ± 118.8
Medium (7.72 ± 2.14)		2296.1 ± 265.8	52910.7 ± 1431.5	2324.3 ± 292.3	17526.8 ± 2318.9	878.3 ± 109.9
High (12.83 ± 1.69)		2774.7 ± 487.2	53816.4 ± 2583.4	1989.5 ± 393.9	18761.8 ± 3168.5	812.1 ± 124.2

Data are mean cpm ± S.E.M.

has no toxic effect in inhalation toxicity, mutagenicity and immunotoxicity (Kim *et al.*, 2003). In continuation with the above study, toxicity of Herbrette, a newly developed tobacco substitute made with the leaves of *Perilla frutescens*, was evaluated.

In the 4 weeks repeated inhalation toxicity test, exposure to various concentrations of the Herbrette smoke significantly increased the enzyme activities (AST, ALT, CREAT), as well as other hematological parameters (CI, NE, LY, PLT, WBC) of mice compared to the control group ($P<0.05$, $P<0.01$). However, these changes were not consistent nor showed concentration-dependency, although some parameters were concentration-dependent and differences were observed between the sexes. Moreover, organ weight and adverse gross or microscopic changes were not observed between the exposed and control groups (data not shown).

We performed *S. typhimurium* reversion assay with/without histidine moiety, *in vivo* chromosomal aberration assay and *in vivo* micronucleus assay using mouse bone marrow cells to examine the mutagenicity of Herbrette. In *S. typhimurium* reversion assay, no responses were observed in all tester strains with/without metabolic activation (Tables 5~8). However, the positive control showed significant increase in the number of mutation colony compared with the negative control. These results strongly suggest that Herbrette does not have any mutagenic potential on Salmonella strains. The results of *in vivo* chromosomal aberration and *in vivo* micronucleus assay are similar to those obtained from *S. typhimurium* reversion assay. All tested groups did not show significant increases in chromosome aberration and frequencies of micronucleated reticulocytes compared with the control group in both sexes (Tables 9~10). This result suggests that Herbrette does not induce any mutational changes during erythrocyte development. Therefore, in view of the results so far achieved, Herbrette has no mutagenic potentials under these experimental conditions.

Peripheral cellular immunity of mice exposed with Herbrette was evaluated by the *in vitro* lymphocyte proliferation assay. No significant differences were observed between the Herbrette smoke exposure groups and control group (Tables 11~12). In the study of Singh *et al.* (2000), acute nicotine treatment also suppressed the Con A-induced T cell proliferation. However, our study suggests that Herbrette does not have a systemic effect nor does it suppress the peripheral cellular immunity.

The combustion of tobacco is a complex dynamic process. Cigarette smoke has been reported to contain more than 4000 constituents, some of which have been identified as human and/or animal carcinogens, or to be associated with the principle chronic health effects of smoking (IARC, 1986; Kim *et al.*, 2003). In this study, the mean CO concentrations were measured five times during the test, during which several PAHs were detected; however, the concentration levels were negligible. And the analysis of Herbrette indicated no nicotine.

A complete smoke analysis and a subsequent toxicological risk assessment for each of these compounds are obviously impossible, with analytical difficulties such as the low smoke levels and the smoke background levels aggravating the problem. Several compounds that are considered to be highly biologically active, such as some of the PAHs, which have very strong mutagenic potency, occur at extremely low concentrations in cigarette smoke that current methology does not allow quantification with sufficient accuracy (Kataoka *et al.*, 1998; Kim *et al.*, 2003), and thus, require more progressive and precise analytical methods.

In conclusion, at the concentration of 12.83 ± 1.69 mg/m^3 (about 20 cigarettes) per day, which was the highest dose in the present study, Herbrette did not produce exposed-related toxic effects in the 4-week inhalation toxicity, mutagenicity and immunotoxicity tests using the mice.

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