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28-day inhalation toxicity of 3-methoxybutyl chloroformate in rats

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ABSTRACT

The 28-day repeated inhalation study was applied for hazard assessment of 3-methoxybutyl chloroformate (3-MBCF) in Sprague Dawley rats. Groups of five rats per sex were exposed 6 h/day, 5 days per week for 4 weeks to test substance concentration (ranging from 3 to 12 ppm) using a whole-body exposure system. At the terminal sacrifice, following blood collection and gross pathological examination, organ weights were determined and fixed organs were examined. The micronucleus test was performed using bone marrow cells. Exposure of 3-MBCF induced mortality at concentrations above 6 ppm. Decreases in body weight and food intake, hematologic alterations, organ weight changes, and gross and microscopic findings were seen even at the lowest concentrations of 3 ppm. Histopathology revealed principal test substance exposure correlated with lesions in the respiratory tract in both male and female rats above 3 ppm. Groups of male rats exposed above 6 ppm show microscopic lesions in spleens, livers, testes and epididymides; however, the micronucleated polychromatic erythrocytes frequency in bone marrow cells was not changed. Based on histopathology of the respiratory tract and other organs, the no observed adverse effect level (NOAEL) of 3-MBCF in the present study was less than 3 ppm.

1. Introduction

South Korea is one of the biggest chemical-producing countries in the world, and the 3rd largest producer in Asia after China and Japan [1]. In addition to producing large amounts of chemicals, hazardous chemicals are often found in workplaces, and the number of such chemicals has been steadily increasing. In South Korea more than 300 chemicals are newly registered each year, and there are presently approximately 43,500 types of chemicals used in Korean workplaces [2].

Recently, EU regulation no.1907/2006 on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) is the main basis for the environmental hazard assessment of industrial chemicals in each country [3]. In South Korea, The Ministry of Environment (MOE) reinforced the Registration, Evaluation, Authorization and restriction of Chemicals (K-REACH) to manage and inform the matters concerning the registration of chemical substances, as well as the review and assessment of the toxicity, hazards, and risks of chemical substances and products containing hazardous chemical substances [4]. MOE published a draft list of 518 existing chemical substances for registration under the Act on the Registration and Evaluation of Chemicals [5]. According to this list, manufacturers and importers who administer new chemicals or chemicals exceeding 1 ton per year should register it within 3 years after the publication date. The amount of ecotoxicological data requested depends on the production or import tonnage: higher tonnages require the provision of more extensive datasets, such as acute and chronic tests with fish and aquatic invertebrates, or reproduction studies.

3-Methoxy butyl chloroformate (3-MBCF; CAS No. 75032-87-0; Synonymes: 3-Methoxybutyl chloroformate; 3-methoxybutyl carbonochloridate; EINECS 278-058-3; AC1MI6SD) is a clear to light-yellow in color, water insoluble, and possesses a severe, pungent odor. 3-MBCF is used as a reactive chemical intermediate, especially for any chemical compound containing carbonate, pyrocarbonate, carbamate, urethane, and others, and may be used in organic chemical and plastics manufacturing. (PubChem Compound Database, 2005). 3-MBCF is a harmful material (Globally Harmonized System of Classification and Labelling

Abbreviations: ANOVA, analysis of variance; CT, computed tomography; EDTA, ethylenediamine tetraacetic acid; GHS, Globally Harmonized System of Classification and Labelling of Chemicals; GLP, Good Laboratory Practice; HCT, hematocrit; HGB, hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MNPCE, micronucleated polychromatic erythrocytes; MOE, The Ministry of Environment; NCE, normochromatic erythrocytes; NOAEL, no observed adverse effect level; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; PLT, platelets; RBC, red blood cell counts; RDW, red cell distribution width; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; SD, Sprague-Dawley; SPF, specific-pathogen-free; WBC, white blood cell counts; 3-MBCF, 3-methoxy butyl chloroformate

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of Chemicals (GHS) Acute toxicity, oral category 4) and causes skin irritation, serious eye irritation, and respiratory irritation (GHS skin corrosion/irritation-category 2; GHS serious eye damage/eye irritation-category 2A; GHS specific target organ toxicity, single exposure; respiratory tract irritation-category 3) (ECHA, 2017)

3-MBCF is included in the 518 existing chemical substances registered for their hazard to humans, animals, and the environment according to K-REACH [5], but there is little information associated with the biological hazard of 3-methoxybutyl chloroformate (3-MBCF). Only a single study reported about the pulmonary effects, including tracheolaryngeal inflammatory edema, bronchial dilation, and alveolar rupture using computed tomography (CT) in laboratory animals [6]. Here we confirmed potential toxicities of 3-MCBF in Sprague-Dawley (SD) rats exposed for 28 days. The study was performed in compliance with the Good Laboratory Practice (GLP) guidelines and the Organization for Economic Cooperation and Development (OECD) [7,8].

2. Materials and methods

2.1. Animals

Male and female specific-pathogen-free (SPF) SD rats aged 6–7 weeks were purchased (Orient Bio Inc., South Korea) and acclimatized for 11 days in polycarbonate cage with SPF conditions before the grouping. Exposures were conducted in inhalation chambers (Model No. SIS-20RG, Sibata Co., Japan) with individual wire mesh cages, three for each concentration of 3-MBCF plus a control chamber with HEPA filtered clean air under SPF laboratory conditions. The ambient temperature and relative humidity of the chamber was 22 ± 3 °C and 50 ± 20%, respectively, with a 12:12 h light:dark cycle (lights on at 8:00 am) with 150 ~ 300 lx of illumination. The rats received rodent chow (LabDiet 5002, Purina Mills., St. Louis, MO, USA) and tap water ad libitum. All animal protocols described in this study were approved by the Committee on Animal Research Committee of the Occupational Safety and Health Research Institute.

2.2. Chemicals and inhalation exposure

3-Methoxybutyl chloroformate (99.5% pure, CAS N0.:75032-87-0) was purchased from Sekiatofina Co., LTD. (South Korea, Lot No.3066), and the inhalation facilities are previously described [6] and will be summarized here briefly. The Environmental conditions were monitored every 30 min using equipment (Model No. ICS-21RG, Sibata Co. Ltd., Japan). The concentration-analysis of vaporized 3-MCBF (using a gas generator, Model No. VG-4R, Shbata Co. Ltd., Japan) in the chambers was performed every 15 min during exposure using gas chromatography (Model No. GCS-14BFS, SHIMADZU, Japan) with a flame-ionization detector and a column (15% DC-200; mesh 80/100, AW-DMCS, USA). The maximum concentration of 12 ppm and intermediate exposure concentrations of 3-MBCF were selected based on responses from preliminary studies.

2.3. Experimental design

Twenty rats of each sex were randomly assigned to four groups (n = 5); control (filtered air), 3, 6, and 12 ppm and were exposed to 3-MBCF for 6 h/day, 5 days/week for four weeks. Body weight data were collected twice a week for the first 2 weeks and then at least once per week after. Individual food consumptions were also collected once a week for 4 weeks. Clinical observations were recorded twice a day during exposure periods. At the end of the experiment all animals fasted for 12 h and were then anesthetized with pentobarbital (30 mg/kg, JW pharmaceutical, South Korea). Blood samples were then collected from the abdominal aorta. Then animals were sacrificed by exsanguination from the abdominal aorta and necropsy, including gross findings and organ weight determinations, was performed on all animals.

2.4. Hematology

Hematologic examination was performed using a hematology analyzer (CL-7200, Shimazu, Japan). Whole blood samples were collected in sample tubes containing ethylenediamine tetraacetic acid (EDTA2 K) and examined for the following parameters: white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and platelets (PLT).

2.5. Urinalysis

Three days before the necropsy, the urine samples were collected and analyzed using Uriscan 10 SGL strip and Uriscan S-300 (Youngdong Pharmatics, South Korea). The parameters including blood, bilirubin, urobilinogen, ketones, protein, nitrite, glucose, pH, specific gravity and leucocytes were measured.

2.6. Histopathological assessment

At the necropsy of all animals contained found dead animals, organs including the thymus, heart, testes, ovaries, lung with trachea, kidneys, spleen, liver, pancreas, stomach, and brain were removed and weighed. All organs/tissues were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedding. Tissue slides with 4 μm thickness prepared and stained with hematoxylin and eosin. Histological examination and capture of digital images was performed under the light microscope (Axioscope2, Zeiss, Germany).

2.7. Bone marrow micronucleus test

Bone marrow cells from the right femur of the rats were flushed using fetal bovine serum (FBS, Hyclone, GE Healthcare, IL, USA). The cell suspensions were centrifuged at 1000 rpm for 5 min, and the pellets were re-suspended and smeared on slides. After air-drying, the slides were fixed in methanol and stained with acridine orange solution (40 μg/mL). According to Hayashi et al. [9], the stained slides of bone marrow were observed for micronuclei, and the ratio of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) was counted. The frequency of micronucleated polychromatic erythrocytes (MNPCE) was scored in each 2000 PCE per animal using fluorescence microscope (Nikon, Optiphot-2, Tokyo, Japan).

2.8. Statistical analysis

Data were expressed as the mean ± standard deviation. The SPSS statistical system (Version 10.0; SPSS Inc., IL, USA, USA) was used to analyze parameters including body weight, food consumption, organ weights, hematologic data, and micronucleus assay data, followed by testing for variance homogeneity. Data were analyzed using the one-way analysis of variance (ANOVA) and Dunnett's multiple test or Kruskal-Wallis test with Bonferroni correct for comparison between the control and tested groups (except 12 ppm group). The incidence and severity for histopathologic findings were compared by uses of Fisher's exact probability test and Kruskal-Wallis test with Bonferroni correction. A value of $P < 0.05$ is considered as statistically significant.

3. Results

3.1. Environmental state and inhalation study

Data regarding the actual 3-MBCF concentrations and other conditions monitored in the exposure chambers are presented in Table 1. Briefly, the mean (± SD) concentrations measured during study were 3.3 (± 0.24), 6.1 (± 0.88), and 12.0 (± 1.48) ppm for nominal

Table 1
Changes of concentration in inhalation chambers during the experiment.

Groups	Concentration (ppm)			
	Establishment	Upper	Lower	Mean ± SD
Control	0	0.0	0.0	0.0 ± 0.00
Group 1	3	3.77	2.87	3.3 ± 0.24
Group 2	6	7.77	4.48	6.1 ± 0.88
Group 3	12	16.09	9.98	12.0 ± 1.48

Table 2
Unscheduled mortality rates of the 3-methoxybutyl chloroformate exposed SD rats.

Groups	Survival rates (death/total animal)	
	Male	Female
Control	5/5	5/5
3 ppm	5/5	5/5
6 ppm	2/5	3/5
12 ppm	0/5	1/5

concentrations of 3, 6 or 12 ppm for 6 h/day exposure, respectively.

3.2. In-life parameters

During the exposure, difficulty in respiration, epistaxis, and conditioned gaping (a reflection of nausea) were observed in 6 and 12 ppm groups of rats. On the second exposure day, one male animal exposed to 12 ppm was found dead; at the end of the exposure, all male rats and four female rats were dead. In the 6 ppm group, three male and two female rats were found dead from the seventeen day to the end of the study (Table 2). The mucous exudate from trachea, congestion and discoloration of lung and excess intestinal gas were noted at the dead animals and the observations in respiratory organs with dyspnea were thought to be linked to death of animals. Mean body weights of both male and female rats in 3-MBCF treated groups were generally reduced during 3-MBCF exposure compared to those in the control group (Fig. 1) until exposure Day 26. The body weights showed slight increase on Day 28 in all 3-MBCF inhaled rats. Food consumptions showed more reduction in 3-MBCF exposed rats compared to the control group than in female rats (Table 3, Data of 12 ppm groups not shown due to the high mortality).

3.3. Hematology

Data for hematology was summarized in Table 4 (Data of 12 ppm groups not shown due to the high mortality). In male rats there were dose-dependent, significant decreases of WBC and PLT and an increase of HGB. In female rats, dose-dependent increases of HGB and RDW were found. In addition, significant increase of RBC and RDW were observed in the 6 ppm female group.

3.4. Urinalysis

Only the ketone bodies were increased in male groups compared to control males, but were not in a dose-related manner. No other changes of urinalysis parameters were observed in male and female rats (data not shown).

3.5. Organ weights

Data for absolute and relative organ weights are shown in Tables 5 and 6 (Data for 12 ppm groups not shown due to the high mortality). In male rats, absolute weights of thymus, heart, testes, kidneys, spleen, and liver decreased and lung weights increased in a dose-dependent manner. But the relative weights shown the increased weight of some organs such as heart, testes, lung, kidneys and brain with a dose-dependent manner. In female rats, absolute weights of thymus, heart, kidneys, spleen, and liver decreased and lung weights increased in a dose-dependent manner. As well as, the relative weights were shown the increase of weight in some organs such as heart, lung, kidneys, liver and brain in a dose-dependent manner.

3.6. Histopathological findings

Microscopically, there were pathological changes in lung, trachea, spleen, testes, epididymis, kidney and liver (Fig. 2). In males, inflammatory exudate and enlargement of the alveolar septum, pulmonary edema, phagocyte infiltration, and obstructive broncheitis in the lung were observed in test substance inhaled groups. The incidence and severity of lung lesions were increased with dose-dependent manner compared to control group. In addition, the incidence of trachea-bronchial lesions – including epithelial hyperplasia, epithelial cell atrophy, and squamous metaplasia – was increased compared to control group. Also congestion of the spleen, atrophy of seminiferous tubules, exfoliation of germ cells of testes, and degeneration of germ cells in epididymal ducts were observed in the 6 and 12 ppm groups with increased incidence and dose-dependent manner. Additional observations included obstructive bronchitis in the lung, centrilobular cell necrosis in the liver and one case of myocardial degeneration of 12 ppm group (Table 7). In female rats, lesions were found in the respiratory tracts – similar to those in males – with dose-responsive incidence and severity; however, unlike in male rats, there were no liver lesions. Only minimal to mild congestion of the kidneys was noticed in 6 and 12 ppm groups. Also, the pathologic findings of unscheduled dead rats, from expose day

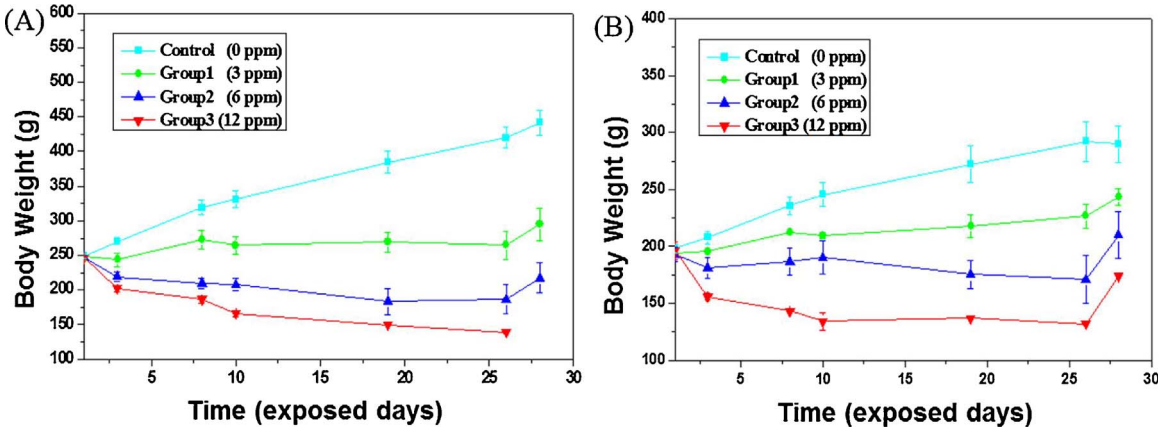

Fig. 1. Mean body weight of SD rats inhaling 3-MBCF for 28-days. (A) male rats; (B) female rats.

Table 3
Total mean food consumptions of the SD rats with inhaled with 3-methoxybutyl chloroformate.

Sex	Groups	Food consumption (g/day/rat)			
		1 week	2 weeks	3 weeks	4 weeks
Male	Control	27.17 ± 1.37 (5/5) [a]	29.07 ± 0.97 (5/5)	28.75 ± 2.6 (5/5)	31.78 ± 2.21 (5/5)
	3 ppm	14.85 ± 4.28 (5/5)	14.26 ± 2.03 (5/5)	11.76 ± 1.75 (5/5)	8.83 ± 4.98 (5/5)
	6 ppm	8.50 ± 3.17 (5/5)	11.11 ± 3.06 (5/5)	11.25 ± 3.87 (4/5)	4.92 ± 4.19 (2/5)
Female	Control	21.30 ± 3.40 (5/5)	22.44 ± 2.04 (5/5)	26.08 ± 3.45 (5/5)	17.71 ± 0.71 (5/5)
	3 ppm	16.50 ± 5.83 (5/5)	15.48 ± 2.43 (5/5)	14.01 ± 1.76 (5/5)	13.24 ± 4.80 (5/5)
	6 ppm	10.65 ± 4.52 (5/5)	12.02 ± 2.17 (5/5)	11.94 ± 4.03 (4/5)	6.63 ± 5.94 (3/5)

Data were expressed as mean ± S.D.
[a] Numbers in parenthesis are the numbers of alive/total animals.

2, were more severe and frequently compared to live animals.

3.7. Bone marrow micronuclues test of 3-MBCF

Incidence of micronucleus formation, distributions of micronuclei found in PCE and NCE, and the ratio of PCE/NCE in femurs of male and female rats are shown in Table 8 (Data for 12 ppm groups not shown due to the high mortality). The MNPCE frequency was not changed significantly in 3 or 6 ppm groups of male rats, but increased in 3 and 6 ppm groups of female rats. However, decreases in the proportion of PCEs to total erythrocytes were observed in males, and were significant in female rats.

4. Discussion

The inhalation of chemicals in many industrial workplaces can be a trigger of respiratory tract disorders as well as systemic diseases, depending on the type of inhaled substance and exposure time [10,11]. Among industrial chemical substances, chloroformate derivatives used as intermediates in the synthesis of materials a higher probability of inhalation exposure than other means, such as oral or dermal routes. These chemicals, such as methyl chloroformate and butyl chloroformate, induce cough, lacrimation, cocking, headache, and nausea in human subjects [12]. In animal studies, the LC_{50} of methyl chloroformate was less than 92 ppm in male F344 rats or 13 ppm in male SD rats, and main target organ was known as nasal turbinate, trachea and lung in animal studies [13,12]. Similarly, butyl chloroformate induced respiratory distress including dyspnea, pulmonary emphysema and edema with hydrothorax in acute inhaled studies. The oral LD_{50} values were known as higher than 1325 mg/kg in rats. Due to the increase in distribution and prevalence in industrial use, 3-MBCF, one of the chloroformate derivatives, has been examined in order to obtain more toxicological data for heath management of industrial workers, but little data have been released to date.

In the present study we confirmed the clear evidence of toxicity of 3-MBCF through the mortality, clinical observations, food intake, body weight loss, hematological data, and pathological observation using laboratory animals. In preliminary inhalation studies for substance level determination, 3-MBCF levels of 10, 30, 90, and 180 ppm were used on using SD rats (Fig. 3); a level of 10 ppm was considered the highest substance level required for the main study due to the observed fatality. In both males and females, body weight gain was significantly reduced in all groups of rats that inhaled 3-MBCF; half of the animals died in the 6 ppm group and only one rat survived in the 12 ppm group. The extreme decrease in food consumption is considered to be associated with the pain and stress caused by irritation of the skin, mucus membranes, and/or eyes. Also, some chloroformate derivatives induced respiratory distress such as dyspnea, which is considered to be associated with low food consumption [12].

Several changes were observed in hematology and urinalysis (data not shown), and the WBC counts were significantly decreased in males but not in females. Increased levels of HGB and HCT were observed in both genders. Hematological indices including HGB and HCT are influenced by various factors. Several studies indicated that pulsed electric field exposure [14], herbal extracts [15,16], and chemicals [17] can influence hematological parameters, but in this study, alteration ranges were minimal and within the normal range, thus it is difficult to judge directly by test substance [18]. Generally, increased RDW indicates the presence of cell of different sized and related with iron deficiency anemia if accompanied by a decrease of MCV. The significant increase of RDW in females was not considered to be substance-related change because it was minimal and shown no other hematological changes related. Also, WBC and PLT were significantly decreased in male rats compared to control groups, but these alterations, observed only male

Table 4
Hematological data in SD rats inhaled with 3-methoxybutyl chloroformate.

Parameters	Male			Female		
	Control	3 ppm	6 ppm	Control	3 ppm	6 ppm
WBC	4.58 ± 0.91	2.64 ± 0.70*	1.60 ± 0.74*	2.50 ± 0.59	2.61 ± 0.55	3.10 ± 1.16
RBC	7.48 ± 0.27	7.55 ± 0.63	8.20 ± 0.23	7.03 ± 0.15	6.80 ± 0.20	7.74 ± 0.26*
HGB	14.30 ± 0.31	15.30 ± 0.42*	16.2 ± 0.71*	13.82 ± 0.52	14.12 ± 044	15.03 ± 0.38*
HCT	41.92 ± 1.14	41.28 ± 3.18	45.8 ± 1.56	40.18 ± 1.15	39.36 ± 1.64	44.97 ± 2.78*
MCV	56.04 ± 0.52	54.72 ± 0.92	55.9 ± 0.35*	57.16 ± 1.57	57.84 ± 1.43	58.07 ± 1.76
MCH	19.14 ± 0.88	20.36 ± 1.59	19.8 ± 0.35	19.66 ± 0.43	20.78 ± 1.12	19.43 ± 1.12
MCHC	34.14 ± 1.32	37.20 ± 2.58	35.4 ± 0.35	34.40 ± 1.41	35.92 ± 1.89	33.53 ± 2.78
RDW	14.94 ± 0.40	15.48 ± 0.89	16.0 ± 0.50	13.86 ± 0.30	15.14 ± 0.55*	15.30 ± 0.26*
PLT	916.6 ± 100.80	693.8 ± 52.47*	680.5 ± 17.68*	833.0 ± 102.24	778.6 ± 54.09	953.3 ± 86.12

All values are expressed as mean ± SD.
HCT, hematocrit (%); HGB, hemoglobin (g/dl); MCH, mean corpuscular hemoglobin (pg); MCHC, mean corpuscular hemoglobin concentration (%); MCV, mean corpuscular volumne (u^3); PLT, platlet ($10^3/u^3$); RBC, red blood cell count ($10^6/mm^3$); WBC, white blood cell count ($10^3/mm^3$); RDW, redcell volum distribution.
Significant differences as compared with control: * $p < 0.05$.

Table 5
Absolute organ weights in SD rats inhaled with 3-methoxybutyl chloroformate.

Organ Weights (mg)	Male			Female		
	Control	3 ppm	6 ppm	Control	3 ppm	6 ppm
Thymus	606.4 ± 128.9	305.6 ± 110.8*	198.5 ± 146.4	567.6 ± 63.3	460.8 ± 91.4	258.0 ± 82.2*
Heart	1432.6 ± 70.3	1024.2 ± 144.3**	907.5 ± 72.8*	960.4 ± 101.1	837.6 ± 54.0	878.3 ± 55.8
Testis (Ovary)	3237.2 ± 154.8	2938.0 ± 232.8	2392.0 ± 115.0	154.8 ± 18.6	166.6 ± 38.9	115.0 ± 24.3
Lung	1240.2 ± 130.8	1757.6 ± 243.6*	1681.0 ± 181.0	1069.8 ± 41.5	1282.8 ± 243.6	2027.3 ± 320.1
Kidney	2664.4 ± 174.9	1949.8 ± 111.1*	1587.5 ± 204.4	1797.2 ± 56.8	1561.0 ± 125.4*	1443.7 ± 53.3**
Spleen	671.8 ± 95.2	447.2 ± 126.2*	266.0 ± 53.7**	510.0 ± 47.6	426.0 ± 69.2	259.0 ± 48.6**
Liver	12093.6 ± 609.1	6681.8 ± 431.4***	5830.0 ± 166.9***	7679.6 ± 776.9	6156.0 ± 441.7**	6651.3 ± 716.0
Brain	1495.4 ± 72.2	1747.6 ± 63.9	1579.0 ± 76.4	1771.6 ± 117.1	1715.4 ± 90.0	1715.4 ± 90.0

All valves are expressed as mean ± SD.
Significant differences as compared with control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 6
Relative organ weights in SD rats inhaled with 3-methoxybutyl chloroformate.

Organ Weights (%)	Male			Female		
	Control	3 ppm	6 ppm	Control	3 ppm	6 ppm
Thymus	136.8 ± 27.4	101.9 ± 30.1	88.4 ± 58.5	195.9 ± 20.2	189.5 ± 38.0	123.6 ± 41.7
Heart	323.7 ± 20.6	347.6 ± 41.1	417.7 ± 8.3*	330.7 ± 17.6	344.0 ± 13.5	420.5 ± 22.4*
Testis (Ovary)	733.3 ± 56.6	1020.0 ± 113.5**	1091.6 ± 167.9	53.6 ± 7.4	68.7 ± 17.4	55.0 ± 11.3
Lung	281.1 ± 35.5	601.5 ± 113.9*	781.1 ± 161.4	370.2 ± 28.4	527.5 ± 74.9*	964.4 ± 58.1**
Kidney	603.7 ± 56.6	663.5 ± 44.4	729.0 ± 21.1*	620.7 ± 16.5	641.5 ± 47.5	693.4 ± 69.1
Spleen	151.5 ± 17.6	150.0 ± 31.4	121.7 ± 12.5	176.2 ± 16.8	175.3 ± 29.2	124.6 ± 28.7
Liver	2733.9 ± 99.2	2272.3 ± 138.2**	2690.6 ± 192.4	2645.4 ± 156.1	2530.1 ± 173.5	3174.0 ± 41.5**
Brain	339.1 ± 154.3	595.0 ± 35.0*	731.5 ± 108.3	612.1 ± 41.3	705.1 ± 34.2*	899.1 ± 61.1*

All valves are expressed as mean ± SD.
Significant differences as compared with control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

rats, were considered to be due to extreme body weight loss and reduced feed intake rather than specific organ toxicity by the test substance directly.

The weight reductions identified in absolute weights of organs such as heart, testes and kidney were inconsistent with the relative weights of organs and thought be linked with body weight changes. But the increase of absolute and relative lung weights in male and female rats were considered to be related to the inflammation of lung confirmed in

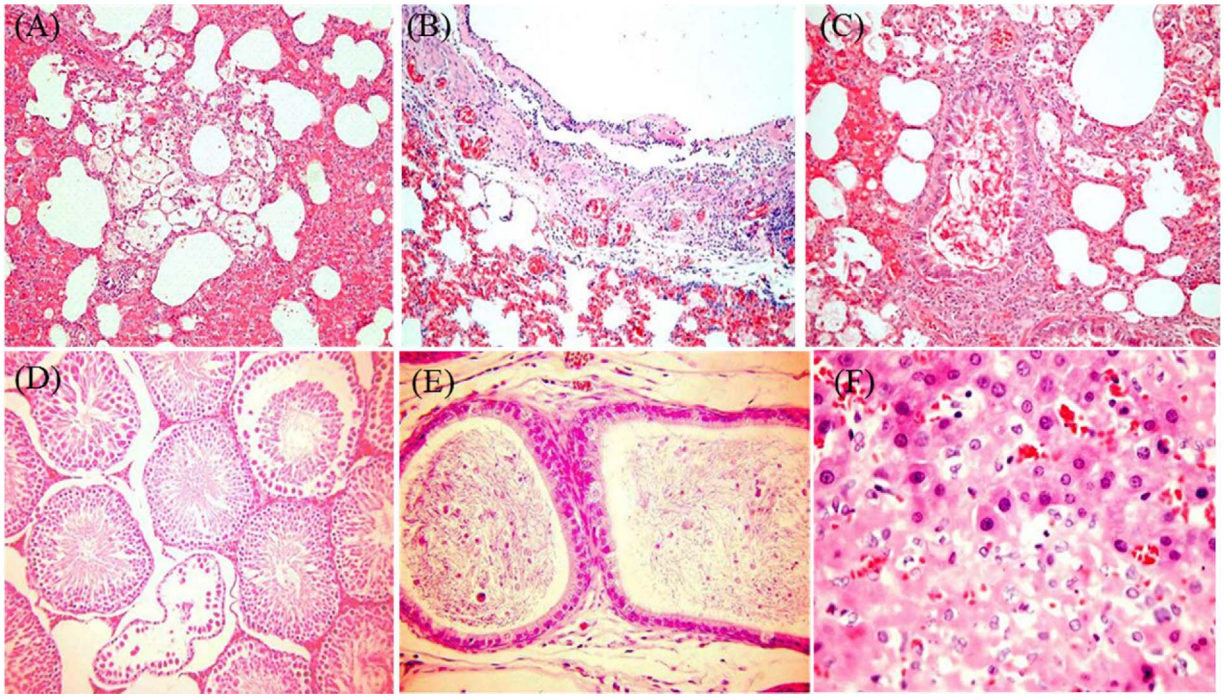

Fig. 2. Light micrograph of lower respiratory tract, testis, epididymis, and liver sections in rats that inhaled 6 or 12 ppm of 3-MBCF for 4 weeks. (A) Alveolar edema and mononuclear cell infiltration in lung of female rat that inhaled 6 ppm of 3-MBCF; (B) Atrophy and exfoliation of epithelial cells in bronchus of male rat that inhaled with 6 ppm of 3-MBCF; (C) Obstructive bronchitis in lung of male rat that inhaled 12 ppm of 3-MBCF; (D,E) lesions in male reproductive organs including germ cell atrophy after inhalation of 6 ppm of 3-MBCF; (F) Centrilobular liver cell necrosis in a male rat that inhaled 12 ppm of 3-MBCF. Hematoxylin and eosin stain (A-F), x100(A–D), x200(E), x400(F).

Table 7
Histopathological findings for SD rats inhaled with 3-methoxybutyl chloroformate.

		Groups	Males				Females			
			control	3 ppm	6 ppm	12 ppm	control	3 ppm	6 ppm	12 ppm
Organs and findings		No. of animals	5	5	5(3)	5(5)	5	5	5(2)	5(4)
Lung	Inflammatory exudate	Total	0	5$^{**,\#}$	5(3)$^{**,\#}$	5(5)$^{**,\#}$	0	4**	5(2)$^{**,\#}$	5(4)$^{**,\#}$
		±		4				3	1	
		+		1	3(1)			1	2	
		+ +			1(1)	3(3)			2(2)	3(2)
		+ + +			1(1)	2(2)				2(2)
	Enlargement of alveolar septum	Total	0	1	4(3)**	5(5)$^{**,\#}$	0	0	4(2)**	5(4)$^{**,\#}$
		±		1	2(1)				3(1)	
		+			2(2)	1(1)			1(1)	
		+ +				2(2)				2(2)
		+ + +				2(2)				3(2)
	Phagocyte infiltration	Total	0	4**	5(3)$^{**,\#}$	5(5)$^{**,\#}$	0	3*	4(2)**	5(4)$^{**,\#}$
		±		3		1(1)		1	1	2(2)
		+		1	2(1)	2(2)		2	2(1)	1(1)
		+ +			2(1)	2(2)			1(1)	2(1)
		+ + +			1(1)					
	Edema	Total	0	3*	4(3)*	5(5)$^{**,\#}$	0	3*	5(2)$^{**,\#}$	5(4)$^{**,\#}$
		±		3	1			3	3	2(2)
		+			1(1)	1(1)			2(2)	2(1)
		+ +			2(2)	2(2)				1(1)
		+ + +				2(2)				
	Obstructive broncheitis	Total	0	0	0	4(4)$^{**,\#}$	0	0	0	2(2)
		±				1(1)				1(1)
		+				2(2)				1(1)
		+ +				1(1)				
		+ + +								
Trachea with bronchi	Epitheilial hyperplasia		0	0	2(1)	4(4)**	0	0	3(2)*	2(2)
	Epitheilial cell atrophy		0	0	2(2)	3(3)	0	0	1(1)	2(2)
	Tracheitis		0	0	4(3)**	5(5)**	0	0	2(2)	5(4)**
	Squamous metaplasia		0	1	4(3)**	5(5)**	0	1	2(2)	3(2)*
Spleen	Congenstion		0	0	3(3)*	5(5)**	0	0	2(2)	4(4)**
Testes	Atrophy, seminiferous tubules		0	0	4(3)**	4(4)**	–	–	–	–
	Exfoliation, germ cell		0	0	1(1)	4(4)**	–	–	–	–
Epididymides	Degeneration, germ cell		0	0	2(2)	4(4)**	–	–	–	–
Liver	Necrosis, centrilobular, ± /+/+ +		0	0	0	5(5)** 1/3/1	0	0	0	0
Heart	Degeneration, myocyte		0	0	0	1(1)	0	0	0	0
Kidneys	Congenstion		0	0	0	0	0	0	1(1)	1(1)

Values in basket are numbers of un-scheduled dead rats with lesions. Grade of change; ± , minimal; +, mild; + +, moderate; + + +, severe.
-: Not evaluated. Significant differences as compared with control: * $p < 0.05$, ** $p < 0.01$ by Fisher's exact test for incidence; #$p < 0.05$ by Krustal-Wallis test for severity).

Table 8
Incidence (%) of micronucleated polychromatic erythrocyte (MCPCE) and PCE/NCE (polychromatic/normochromatic erythrocyte) ratio in SD rats inhaled with 3-methoxybutyl chloroformate.

	Sex	0 ppm	3 ppm	6 ppm
MNPCE (%)	Male	0.16 + 0.08	0.13 + 0.10	0.17 + 0.15
	Female	0.04 + 0.05	0.08 + 0.09	0.08 + 0.10
PCE/PCE + NCE (%)	Male	59.38 + 7.61	57.49 + 9.28	53.91 + 7.39
	Female	60.28 + 9.75	48.75 + 6.99	41.49 + 3.91*

All valves are expressed as mean ± SD.
Significant differences as compared with control: * $p < 0.05$.

histopathology. In the histopathology of respiratory tract indicated that the fatal relation of findings with dose dependent manner between control and substance inhaled groups in both gender. Other findings of spleens, testes, epididymides, and livers also showed test substance-related lesions including epithelial damage with inflammation of tracheobronchial lesions, degeneration and atrophy of male reproductive organs and congestions and necrosis of the spleen and liver, respectively. The germ cell degeneration is common in spermatogenesis, but increased degenerating germ cells that were slugged, unable to determine the stage, were observed in epididymides. Also atrophy of testes is known to be accompanied with reduction of organ weight according to the severity, but the relative weights of testes were increased in our study. It is thought to be associated with the minimal to mild severity of atrophy including absence of germ cells and segmental distribution in some testicular tubules in testes. Also there were a limit to determine the organ weight of accessory glands such as prostate and seminal vesicle and dead animals.

Bone marrow micronucleus assays, which measure substance-related chromosomal or mitotic damage in vivo, provide more insight into factors for genotoxicity of chemicals in vitro [19,20]. In the present study, with little modification from OECD guideline 474 [7,8], we show the results of a genotoxicity test of 3-MBCF. At the middle and high substance levels mortality was elevated: at 6 ppm, three male and two female rats died, while at 12 ppm all male and four out of five female rats died. Substance levels over the tested doses were unavailable for the micronucleus test. In addition, there was no positive control material that induced DNA damage (such as mitomycin C or cyclophosphamide; [21]. Although the MNPCE frequency was increased in 3-MBCF inhaled female groups, 3-MBCF was not considered to be a clastogen because MNPCE frequency was not altered in male rats and there were no dose-related increase. Nevertheless the PCE/PCE + NCE ratio was decreased in male and female rats, suggesting that 3-MBCF may be cytotoxic in bone marrow. Further in vivo or in vitro experiments to confirm the genetic toxicity of 3-MBCF are required.

5. Conclusion

The number and amount of chemicals used in workplace is increasing, and more information is needed to protect workers from

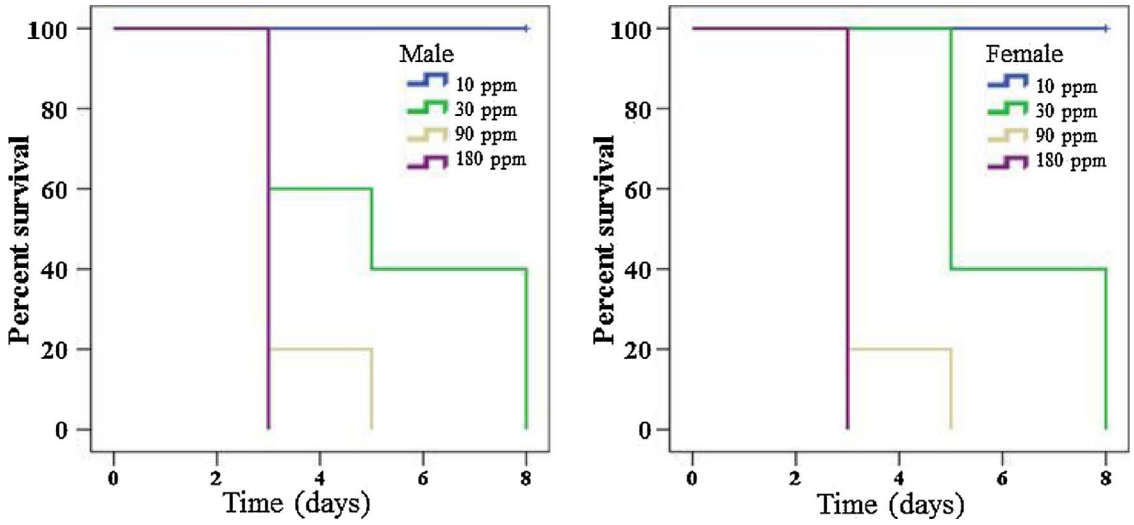

Fig. 3. Lethality of SD rats inhaled with 3-MBCF for 5-days. These preliminary experiments were performed with 5 rats per group in both gender of male and female.

occupational disease. In light of this, we focused on the inhalation toxicity of 3-MBCF using experimental rats. In summary, mortality occurred at 6 and 12 ppm of 3-MBCF during a 28-day repeated inhalation study, but the genotoxicity of 3-MBCF was not confirmed via bone marrow micronucleus test. Based on the body weight decreases, organ weight changes of lung, and incidence of histopathological findings of lung, liver, and testes of male, and lung lesions of female, the low observed adverse effect level (LOAEL) of 3-MBCF was estimated to 3 ppm and the no observed adverse effect level (NOAEL) of 3-MBCF is proposed to be less than 3 ppm following 28-day repeated inhalation to male and female SD rats. Further studies should be considered to confirm the specific organ toxicity mechanism or genotoxicity and their human relevance.

Conflicts of interest

The authors declare that there is no conflict of interest.

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