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Original Article

Toxicity of Methylcyclohexane and Its Effect on the Reproductive System in SD Rats

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Objectives: There is limited data regarding the toxicity of methylcyclohexane, despite its wide use in rubber adhesives, paint diluents, and cleansing agents. This study aimed to verify the toxicity and influence on the reproductive system of methylcyclohexane after its repeated injection in Sprague Dawley (SD) rats.

Methods: Methylcyclohexane was injected subcutaneously into male and female SD rats once a day, five times a week, for 13 weeks at different doses (0, 10, 100, and 1,000 mg/kg/day) for each group. The toxicity of testing material was verified by observing the change in body and organ weight, hematological change, pathological findings, and effect on the reproductive system at each different concentration.

Results: In the 1,000 mg/kg/day group, there were cases of animal deaths. In animals that survived, hematological changes, including a decrease in the red blood cell count, were observed. A considerable weight gain or loss and pathological abnormalities in the liver, kidney, and other organs were found. However, the 10 and 100 mg/kg/day groups did not cause deaths or other specific abnormalities. In terms of reproductive toxicity, there were changes in hormone levels, including a significant decrease in hormones such as estradiol and progesterone ($p < 0.001$) in male animals. Menstrual cycle change for female animals did not show concentration dependency.

Conclusion: When injected repeatedly for 13 weeks, methylcyclohexane proved to be toxic for the liver, heart, and kidney at a high dose. The absolute toxic dose was 1,000 mg/kg/day, while the no observed adverse effect level was less than 100 mg/kg/day. The substance exerted little influence on the reproductive system.

Key Words: Methylcyclohexane, Reproductive toxicity, Lethal concentration 50, Lethal dose 50

Introduction

This study aimed to assess the toxicity of methylcyclohexane, a substance often used in rubber adhesives, ink and paint diluents, and cleansing agents. When absorbed by the human body for a long period or at a high dose, methylcyclohexane irritates eyes as well as the mucosa and upper respiratory tract. It is also known to induce neurological disorders such as headaches, dizziness, and nausea [1,2].

The half lethal concentration (LC) 50 of methylcyclohexane is known to be 3,750 ppm (15.054 mg/L) [3], which corresponds to a class 4 acute hazardous substance ($10 < LC50 \leq 20$ mg/L) according to the Ministry of Employment and Labor's official notice No. 2009-68, "Standard for classification and labeling of chemical substance and material safety data sheet (MSDS)" [4]. Nevertheless, data are very limited on its toxicity, effect on the reproductive system, and prediction of the main target organ when the human body is exposed for a long period.

Literature reviews show that this substance is a volatile organic compound with a molecular weight (mass) of 98.18.

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Its chemical formula is written as C7H14, pertaining to CAS No. 108-87-2. LD50 (mouse) is 2,250 mg/kg/day, and LC50 (mouse) is 41,500 mg/m^3/2 h. LD50 (rat) is at least 3,200 mg/kg/day, while LC50 in rabbit is 7,613 ppm/4 h [5,6]. As for the effect on the body due to repeated exposure, Lazarew et al. [7] have claimed that mice tend to deteriorate when exposed to the substance at 7,500-10,000 ppm for two hours, while exposure of 10,000-12,500 ppm causes death.

Treon et al. [8] claimed that minor damage to the liver and kidney is induced in rabbits when they are exposed repeatedly to 3,330 ppm for 10 weeks, while there is no effect on monkeys, even at continuous exposure of 370 ppm. He also reported that its toxicity is similar to that of cyclohexane, and the liver and kidney are the most affected organs [5].

In epidemiological studies of humans, a case was reported of peripheral and central neurological symptoms that occurred in a shoe repairman who deals with adhesives and uses methylcyclohexane as a solvent as well as ethylacetate and cyclohexane [9]. The subject's peripheral neurological symptoms seemed to continue for months, even after his withdrawal from exposure by job transposition [9]. Although those reports recognize the substance, quantitative toxicity assessment data such as the no observed adverse effect level by specific dose of exposure or period of exposure are very limited.

The purpose of this study was to provide basic data on classifying the toxicity of a chemical substance and to give information on the MSDS in order to prevent damage to workers' health. This study uses animal testing; animals underwent repeated injections of methylcyclohexane for 13 weeks. This study also examines toxicity of a substance at different doses in the body, such as changes in weight, hematological changes, and effects on the main target organ and reproductive system. That information can be used to predict the effect of exposure, often caused by negligence in handling the substance, on human beings who work in industrial fields. Also, it can provide basic data for the classification of hazardous substance and MSDS.

Materials and Methods

Testing materials

Methylcyclohexane of CAS No. 108-87-2, 500 g, produced with Sigma-Aldrich's (St. Louis, MO, USA) Lot No. 01144TH, was set as the testing substance, while olive oil produced by Sigma-Aldrich (St. Louis, MO, USA) was used as excipient.

Animals and rearing condition

As for the animals, Sprague-Dawley (SD) rats of the specific pathogen free variety that were six weeks old and produced by SLC Japan were purchased from the Central Lab. Animal Inc. Korea. After they were subjected to quarantine and acclimatization for one week at a rearing facility, they were distributed according to the stratified random sampling so that the average weight of each group would be distributed evenly. As such, the rats were classified into a control group and a group with different dose of testing substance injected. They were reared in a polysulfone rearing box (W 235 × L 380 × H 175 mm). The rearing conditions were as follows: temperature of 22 ± 3°C, relative humidity of 30-70%, illumination of 150-300 Lux and for 12 hours. Hard food (LabDiet Picolab 5053 PMI), sterilized with radiation, was supplied by ORIENT BIO Inc. to ensure that the rats could consume the food freely. For drinking water, service water was sterilized with a U.V sterilizer and Micro Filtration. Rats were then fed freely using a water bottle.

Experiment group and the amount of testing substance injection

Five female and five male animals were classified into one group. A Ministry of Employment and Labor official notice No. 2009-68, "Chemical substance's classification, labeling and standard for classification and labeling of chemical substance and MSDS" [4], globally harmonized system (GHS) "standard for chemical substance classification", research on the related literature [5,6,8], and data on the toxicity of the testing substance from previous studies [3] were used as a reference to provide basic data for methylcyclohexane toxicity assessment and hazardous substance classification according to the dose of injection for testing substance. The concentration levels were set at 0, 10, 100, and 1,000 mg/kg/day, considering study animals injected with low and high doses. The low dose concentration was equivalent to a level that is not regarded as toxicity level to the animal, while the high dose was equivalent to the absolute toxic dose that could be definitely toxic. Injection was made through the subcutaneous layer of the cervicodorsal part of the animals and was conducted daily five days a week for 13 weeks.

Testing method

The Ministry of Employment and Labor's official notice for chemical substance toxicity testing method was used as a reference [10,11]. Death and survival for the animals was based on repetitive injection of the testing substance, clinical symptoms, weight changes, hematological and serum biochemical tests, and all types of tests that assessed the effect on the reproductive system. Pathologic tests and others were conducted as follows.

Observation of clinical symptoms

Death and survival of animals, skin, hair, eye irritation, respiratory system, movement and behavior pattern, and others were observed from the day of injection to the day of autopsy. However, a dead animal was subjected to autopsy immediately after it was discovered after measuring its weight.

Body weight change

The body weight of all the animals was measured on the date when the injection started. In addition, we measured their weights two times per week for the 13 weeks as well as a final time on the date right before the autopsy.

Hematological & Serum biochemical test

After substance injection (for 13 weeks) was completed, blood sampled from abdominal aorta was analyzed by using an automatic blood analyzer (ADVIA 2120, SIEMENS, USA). Moreover, some of the sampled blood was subjected to centrifugation for 10 minutes at 3,000 rpm to make serum, which was then analyzed with an AU400 automatic biochemical analyzer (Olympus, Japan). Electrolytes were measured using an electrolyte analyzer (RAPIDCHEM 744 Analyzer, SIEMENS, USA).

Pathologic test

Organs were extracted and fixated in bouin solvent, then paraffin embedded and cut to 4-5 μm thickness. Hematoxylin & eosin (H&E) dye was used to test the pathologic effect on the experimental group compared to control group using an optical microscope.

Test on the effect on the reproductive system (Spermatogenic cell count and abnormality test)

To study the effect of the substance on the male animals' spermatogenesis, changes in the testis' spermatogenic cell count were tested. Each animal's peritubular hyalinization was classified into four stages according to the spermatogenic cells' characteristics [12]. The sperm generation cycle method [13,14] was used to conduct tests on the germ cell number and normality by each stage of generation within the seminiferous tubule.

Hormone analysis

Whole blood was sampled from the abdominal aorta of the animals. A centrifugal separator (2,000 rpm, 10 minutes) was then used to separate the serum. Hormone levels were quantified using Vitros Eci (Johnson & Johnson, USA) equipment, which is a chemiluminescence immunoassay analysis system that includes reagents such as estradiol (pmol/L), sample for analysis 17-beta-Estradiol ELISA (RE52041, IBL), and sample for regular analysis (Johnson & Johnson, USA, Assay range: Male 19.7-242 pmol/L, female 97.5-592 pmol/L [follicular phase], 685-1,404 pmol/L [pre-ovulatory peak], 120-738 pmol/L [luteal phase], and 19.7-141 pmol/L [post-menopausal female]). Follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and prolactin were also analyzed using that method.

Female animals' menstrual cycle test

Four weeks and 13 weeks after the substance was injected, we observed the vaginal smear, which was extracted from female animals using repetitive pipetting with sterilized saline solution (0.2 mL) through a microscope (Nikon, ECLIPSE 50i, 200X - 400X). We characterized the menstrual cycle changes of the experimental group referenced to control group using a reference method (Adriana, 2007). In general, 4.5 days -5.5 days are considered normal for the SD rats' menstrual cycle, and exceeding this period was considered abnormal.

Statistical analyses

For the statistical analyses of test results, weight, hematologic and blood chemistry, organ weight, estradiol, testosterone, progesterone, LH, FSH, analysis of prolactin, and statistical analyses on the menstrual cycle test were subjected to the analysis of variance (ANOVA) test using SPSS version 18 (SPSS Inc., Chicago, IL, USA) The ANOVA test was verified at a level of significance of $p < 0.01$. When the results of verification, $p < 0.01$, or, in other words, when a significant difference was recognized, Dunnett's or Duncan's multiple range test, a multiple comparison method for the control group and experimental group, was conducted. In addition, statistical significance ($p < 0.01$) between both groups was verified. The results were labeled using average mean and standard deviation.

Results

Observation of clinical symptoms

During the substance injection period, the 10 and 100 mg/kg/day groups, including the control group, did not produce any dead animals. However, movement of the animals slowed down. In the case of the 1,000 mg/kg/day group, four female and male animals died (between the 5th to 60th days after the injection started), and there were no particular abnormalities found in the surviving animals.

Measurement of weight changes

Among results on the measurement of the control group by each substance injection period and by the 10, 100, and 1,000

mg/kg/day groups' weight, in the case of the 1,000 mg/kg/day group, there were significant ($p < 0.001$) changes, namely, a drastic weight decrease (241.98 ± 18.80 g) compared to the control group (288.96 ± 12.99 g) from the day after the injection of the substance in both female and male animals. In male animals, there were animals that started to die from the 23rd day of substance injection. Four out of five died by the 45th day. In female animals, four out of five died from the 5th day of injection to the 60th day. The group of male animals injected with 100 mg/kg/day showed an increase (290.31 ± 04.03 g) in weight compared to the control group (288.96 ± 12.99 g), but the increase was not significant.

Hematological & Serum biochemical test

The results of the hematological test are shown in Tables 1 and 2. There was no category in male animals that showed statistical significance among the 10 and 100 mg/kg/day groups compared to the control group. In female animals, monocytes of the 100 mg/kg/day group decreased (0.84 ± 0.26 103/mm^3) significantly ($p < 0.05$) compared to the control group (1.25 ± 0.26 103/mm^3). In the 1,000 mg/kg/day group, however, the number of surviving animals was small. Thus, it was not possible to assess statistical significance, but the white blood cell count (WBC), neutrophil (NEU) count, eosinophil (EOS) count, and platelet (PLT) count increased drastically compared to the control group as the standard, while the red blood cell count, hemoglobin (HGB), and hematocrit decreased.

Serum biochemical tests showed that the inorganic phosphorus (IP) concentration of the 10 mg/kg/day group in the male animals increased significantly ($p < 0.01$) compared to the control group (5.36 ± 0.32 mg/dL), as shown in Table 3. In the 100 mg/kg/day group, the concentration of total protein (TP), albumin (ALB), and IP increased (6.54 ± 0.11 mg/dL, 4.22 ± 0.04 mg/dL, 6.82 ± 0.30 mg/dL\dL, respectively) significantly ($p < 0.01$) compared to the control group (6.12 ± 0.19 mg/dL, 4.00 ± 0.12 mg/dL, 5.36 ± 0.32 mg/dL, respectively). In female animals, the urea nitrogen in blood (BUN), creatinine, and alkaline phosphatase (ALP) of the 100 mg/kg/day group increased compared to the control group, as shown in Table 4. However, a statistically significant difference was not recognized. In the 1,000 mg/kg/day group, it was not possible to assess statistical significance, since the number of surviving ani-

Table 1. Hematological test results of male rats

Items	Control	10 mg/kg/day	100 mg/kg/day	1,000 mg/kg/day
WBC	6.11 ± 2.51	6.15 ± 0.99	6.7 ± 0.96	12.84
NEU	2.22 ± 1.03	2.57 ± 0.45	2.21 ± 0.45	5.85
LYM	0.90 ± 0.49	0.67 ± 0.16	1.06 ± 0.18	1.16
MON	1.30 ± 0.46	1.22 ± 0.15	1.77 ± 0.28	1.41
EOS	1.68 ± 1.17	1.65 ± 0.40	1.69 ± 0.29	4.07
BASO	0.02 ± 0.02	0.03 ± 0.02	0.01 ± 0.01	0.36
RBC	8.87 ± 0.33	8.57 ± 0.16	9.05 ± 0.21	7.77
HGB	15.38 ± 0.75	14.96 ± 0.36	15.62 ± 0.47	12.2
HCT	44.70 ± 3.10	42.04 ± 1.18	44.58 ± 1.26	34.7
MCV	50.36 ± 1.99	49.04 ± 0.84	49.28 ± 1.68	44.7
MCH	17.36 ± 0.73	17.48 ± 0.36	17.28 ± 0.57	15.7
MCHC	34.48 ± 1.78	35.62 ± 1.32	35.06 ± 1.77	35.2
RDW	18.58 ± 1.47	19.00 ± 0.97	18.38 ± 0.63	24.8
PLT	768.60 ± 93.19	731.80 ± 34.87	736.60 ± 49.03	1,248.0
MPV	6.72 ± 0.49	6.50 ± 0.43	6.84 ± 0.31	6.3

All values are expressed as mean ± standard deviation.
WBC: white blood cell count (10^3/mm^3), NEU: neutrophil (10^3/mm^3), LYM: lymphocyte (10^3/mm^3), MON: monocyte (10^3/mm^3), EOS: eosinophil (10^3/mm^3), BASO: basophil (10^3/mm^3), RBC: red blood cell count (10^6/mm^3), HGB: hemoglobin (g/dL), HCT: hematocrit (%), MCV: mean corpuscular volume (μL), MCH: mean corpuscular hemoglobin (pg), MCHC: mean corpuscular hemoglobin concentration (%), RDW: red blood cell distribution width (μm), PLT: platelet (10^3/μL), MPV: mean platelet volume (μL).

Table 2. Hematological test results of female rats

Items	Control	10 mg/kg/day	100 mg/kg/day	1,000 mg/kg/day
WBC	5.88 ± 2.53	4.05 ± 0.60	3.40 ± 0.78	2.98
NEU	2.25 ± 1.09	1.60 ± 0.30	1.31 ± 0.33	1.46
LYM	1.24 ± 1.16	0.65 ± 0.28	0.56 ± 0.06	0.22
MON	1.25 ± 0.26	0.92 ± 0.23	0.84 ± 0.26*	0.57
EOS	1.03 ± 0.25	0.86 ± 0.20	0.68 ± 0.21	0.71
BASO	0.11 ± 0.21	0.03 ± 0.01	0.01 ± 0.01	0.01
RBC	7.90 ± 1.16	8.00 ± 0.48	7.94 ± 0.57	5.57
HGB	15.74 ± 3.89	15.20 ± 0.57	15.54 ± 1.03	9.0
HCT	44.78 ± 7.18	46.52 ± 3.02	46.20 ± 3.05	28.2
MCV	56.62 ± 1.16	58.14 ± 1.13	58.24 ± 1.18	50.7
MCH	19.76 ± 2.25	19.04 ± 0.64	19.58 ± 0.41	16.2
MCHC	34.88 ± 3.43	32.72 ± 1.04	33.64 ± 1.25	31.9
RDW	16.22 ± 0.84	16.38 ± 0.18	16.20 ± 0.69	21.0
PLT	691.80 ± 77.95	639.00 ± 105.47	594.40 ± 225.54	83.0
MPV	6.26 ± 0.48	6.28 ± 0.53	6.24 ± 0.46	6.7

All values are expressed as mean ± standard deviation.
Significant differences as compared to control: *$p < 0.05$.
WBC: white blood cell count (10^3/mm^3), NEU: neutrophil (10^3/mm^3), LYM: lymphocyte (10^3/mm^3), MON: monocyte (10^3/mm^3), EOS: eosinophil (10^3/mm^3), BASO: basophil (10^3/mm^3), RBC: red blood cell count (10^6/mm^3), HGB: hemoglobin (g/dL), HCT: hematocrit (%), MCV: mean corpuscular volume (μL), MCH: mean corpuscular hemoglobin (pg), MCHC: mean corpuscular hemoglobin concentration (%), RDW: red blood cell distribution width (μm), PLT: platelet (10^3/μL), MPV: mean platelet volume (μL).

mals was small. Male animals experienced an increase in ALP, creatine phosphokinase, and IP compared to the control group, while female animals experienced a significant increase in lactate dehydrogenase and ALP and showed a significant decrease in BUN, alanine aminotransferase, and IP.

Weight changes of organs

In male animals in the 1,000 mg/kg/day group, the weight of the thymus, adrenal gland, lungs, kidney, spleen, liver and brain decreased significantly. In female animals in the 1,000 mg/kg/day group, the weight of the thymus, adrenal gland, ovary, lungs, kidney, spleen, and liver decreased significantly. However, compared to the control group, there was neither concentration dependence nor special significance in the 10 or the 100 mg/kg/day groups.

Pathological test

In the liver of the 10 mg/kg/day group, one case each of microgranuloma and bile duct proliferation was observed. In the 100 mg/kg/day group, one case of the hepatic cytoplasmic vacuolation was observed (Fig. 1). In the 1,000 mg/kg/day group, liver microgranuloma (Fig. 2), bile duct proliferation, cardiac hyperemia/hemorrhage (Fig. 3), and myocardial necrosis (Fig. 4) were observed. In some of the animals, renal protein casts (Fig. 5) and hyperemia/hemorrhage (Fig. 6) were observed. When the diameter of the seminiferous tubules and thickness of gametes were measured, the effect resulting from the injection of substance was not recognized.

Female animals of the 100 mg/kg/day group manifested one case of liver microgranuloma and two cases of bile duct proliferation. In the 1,000 mg/kg/day group, bile duct proliferation of the liver, hyperemia, and renal hyperemia/hemorrhage were observed.

Effect on the reproductive system

Testis' spermatogenic cell count and form

Spermatogenic cell count and form in the male animals in each experimental group were observed. There were no changes in the spermatogenic cell count or special changes in the form following repetitive substance injections in the case of the 10, 100,

Table 3. Serum biochemical test results in male rats

Items	Control	10 mg/kg/day	100 mg/kg/day	1,000 mg/kg/day
TP	6.12 ± 0.19	6.30 ± 0.16	6.54 ± 0.11*	6.2
ALB	4.00 ± 0.12	4.12 ± 0.04	4.22 ± 0.04*	3.5
BUN	14.76 ± 2.15	16.30 ± 1.17	16.86 ± 1.11	19.8
CREA	0.64 ± 0.09	0.62 ± 0.04	0.60 ± 0.00	0.5
TBIL	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.1
ALT	54.40 ± 22.33	43.40 ± 6.66	49.40 ± 14.22	41.0
AST	99.60 ± 24.48	81.00 ± 7.81	86.80 ± 15.83	98.0
LDH	268.60 ± 173.38	232.40 ± 122.12	252.20 ± 177.69	239.0
ALP	293.40 ± 31.32	288.20 ± 67.84	325.20 ± 45.07	618.0
GLU	165.00 ± 15.26	177.80 ± 9.93	167.40 ± 11.08	196.0
TCHO	72.00 ± 10.42	63.20 ± 9.01	70.60 ± 14.57	60.0
TG	59.20 ± 12.28	53.20 ± 9.68	53.80 ± 12.72	40.0
CPK	103.00 ± 44.32	98.20 ± 25.75	95.20 ± 34.62	181.0
IP	5.36 ± 0.32	5.92 ± 0.30*	6.82 ± 0.30*	7.0

All values are expressed as mean ± standard deviation.
Significant differences as compared to control: *$p < 0.01$.
TP: total protein (mg/dL), ALB: albumin (mg/dL), BUN: urea nitrogen in blood (mg/dL), CREA: creatinine (mg/dL), TBIL: total bilirubin (mg/dL), ALT: alanine aminotransferase (IU/L), AST: aspartate aminotransferase (IU/L), LDH: lactate dehydrogenase (IU/L), ALP: alkaline phosphatase (IU/L), GLU: glucose (mg/dL), TCHO: total cholesterol (mg/dL), TG: triglyceride (mg/dL), CPK: creatine phosphokinase (IU/L), IP: inorganic phosphorus (mg/dL).

and 1,000 mg/kg/day groups compared to the control group.

Test on serum levels of sex hormone

After substance injection was completed, male serum was used to analyze hormone levels such as estradiol, prolactin, testosterone, FSH, progesterone, and LH, and the results are shown in Table 5 and Figs. 7 and 8. Female data are not shown, because the hormone levels in females were dramatically changed in accordance with the estrous cycle. In the case of estradiol, we discovered that the 10 and 100 mg/kg/day groups showed a statistically significant ($p < 0.001$) increase (1.85 ± 0.04 and 1.95 ± 0.03 pg/mL, respectively) compared to the control group (1.74 ± 0.03 pg/mL) and progesterone relative to the control group (15.67 ± 6.64 ng/mL) to the significant ($p < 0.05$, $p < 0.01$) reduction in the (4.89 ± 2.57, 2.87 ± 1.42 ng/mL), respectively.

Testis pathologic test

After the substance injection was completed, pathologic examination was conducted on the testis of the male animals in the 10, 100, and 1,000 mg/kg/day groups and in the control group. The examination showed that there were no special abnormalities in the control group and group injected with substance. Moreover, the diameter of the testis seminiferous tubules diameter and thickness of gametes were measured, and a special effect from the substance injection was not recognized.

Effect on menstrual cycle

To observe the female animals' menstrual cycle changes that may have been caused by substance injection, body fluid was extracted from the female animals' vagina on the 4th and 13th week of the substance injection. The fluid was then studied using a microscope to observe menstrual cycle changes. The results are shown in Table 6, which shows that the menstrual cycle period increase was significant ($p < 0.05$) in the 4th week in case of the 10 mg/kg/day group, but there was no concentration relationship. After the 13th week, it was demonstrated that there was no effect on the menstrual cycle changes, since there were no significance and concentration reliability.

Table 4. Serum biochemical test results in female rats

Items	Control	10 mg/kg/day	100 mg/kg/day	1,000 mg/kg/day
TP	6.62 ± 0.28	6.90 ± 0.16	6.92 ± 0.29	6.1
ALB	4.40 ± 0.16	4.60 ± 0.12	4.48 ± 0.13	3.4
BUN	15.28 ± 0.97	16.68 ± 1.15	52.00 ± 83.18	50.1
CREA	0.60 ± 0.00	0.60 ± 0.00	3.30 ± 6.04	0.6
TBIL	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.1
ALT	53.00 ± 17.42	40.20 ± 10.55	379.00 ± 768.70	26.0
AST	96.60 ± 27.59	78.00 ± 6.52	309.20 ± 527.75	93.0
LDH	217.00 ± 100.63	232.80 ± 74.76	356.00 ± 431.8	492.0
ALP	138.40 ± 14.24	158.60 ± 25.11	876.20 ± 1626.15	666.0
GLU	159.60 ± 10.57	163.80 ± 7.76	132.60 ± 73.08	152.0
TCHO	90.60 ± 2.30	80.80 ± 14.67	97.80 ± 23.89	43.0
TG	34.00 ± 8.37	36.60 ± 4.93	33.60 ± 7.13	37.0
CPK	88.40 ± 26.44	188.60 ± 217.03	85.60 ± 23.47	89.0
IP	6.24 ± 0.46	6.12 ± 0.39	5.40 ± 1.88	0.7

All values are expressed as mean ± standard deviation.
TP: total protein (mg/dL), ALB: albumin (mg/dL), BUN: urea nitrogen in blood (mg/dL), CREA: creatinine (mg/dL), TBIL: total bilirubin (mg/dL), ALT: alanine aminotransferase (IU/L), AST: aspartate aminotransferase (IU/L), LDH: lactate dehydrogenase (IU/L), ALP: alkaline phosphatase (IU/L), GLU: glucose (mg/dL), TCHO: total cholesterol (mg/dL), TG: triglyceride (mg/dL), CPK: creatine phosphokinase (IU/L), IP: inorganic phosphorus (mg/dL).

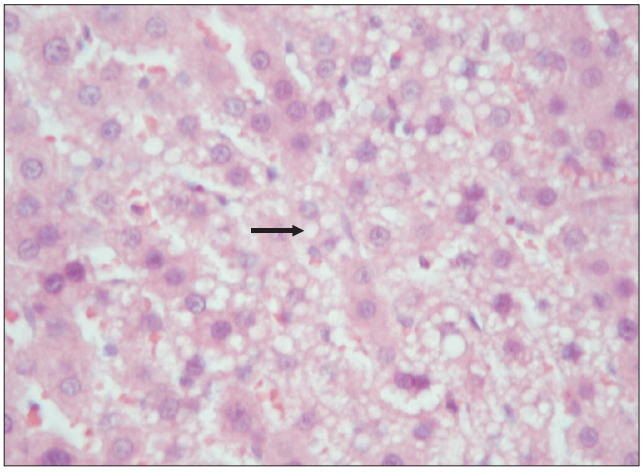

Fig. 1. Representative photograph of liver from the 100 mg/kg/day group, showing cytoplasmic vacuolation.

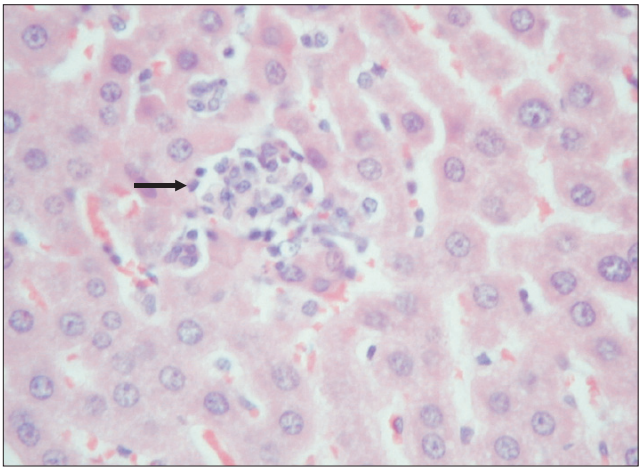

Fig. 2. Representative photograph of liver from the 1,000 mg/kg/day group, showing microgranuloma.

Discussion

Methylcyclohexane is very volatile and offers high solvency. Likewise, it is used often in rubber adhesives, paint diluents, and during the metal cleansing process. In Korea, approximately 2,592 tons are used every year, with 600 workers [3] handling the substance. However, data on the negative effect on human beings, especially on the toxicity by each concentration level or by the period of exposure, are lacking. Thus, this research sought to study the effect on toxicity and the reproductive system by each concentration level by using animals subjected to repetitive long-term injections (13 weeks). A literature review

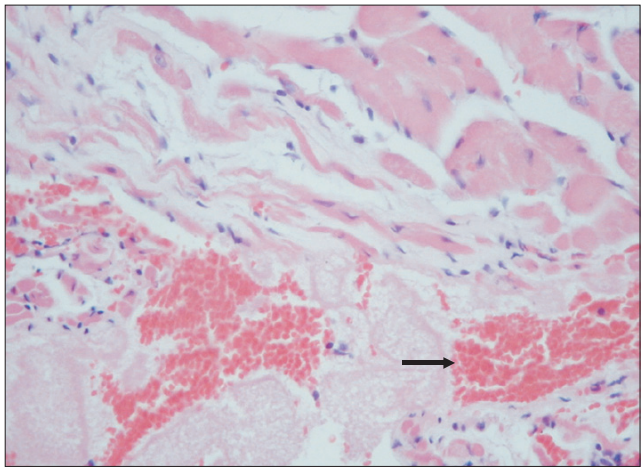

Fig. 3. Representative photograph of the heart from the 1,000 mg/kg/day group, showing congestion.

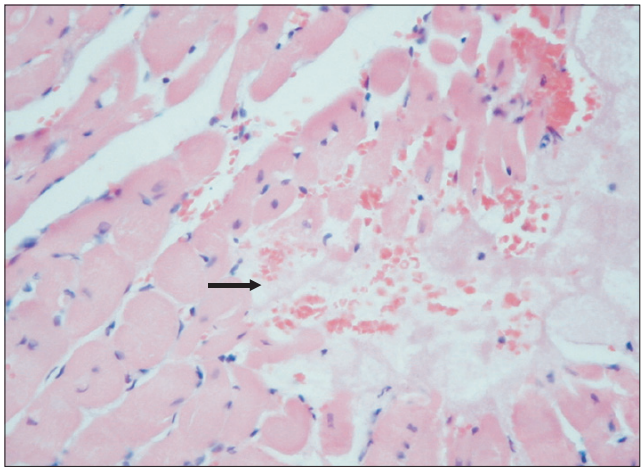

Fig. 4. Representative photograph of the heart from the 1,000 mg/kg/day group, showing myocardial necrosis.

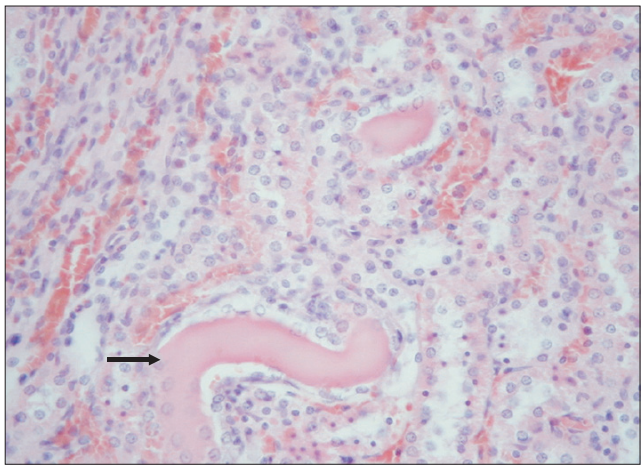

Fig. 5. Representative photograph of the kidney from the 1,000 mg/kg/day group, showing protein cast.

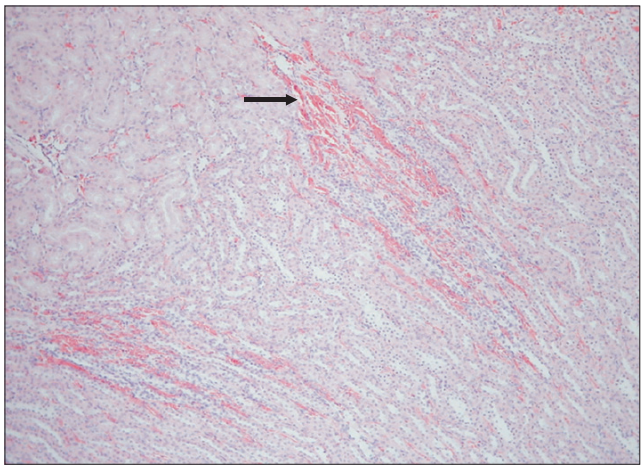

Fig. 6. Representative photograph of the kidney from the 1,000 mg/kg/day group, showing congestion.

Table 5. Serum hormone levels of male rats treated with methylcyclohexane for 13 weeks

Parameter	Sex	Test group		
		Control	10 mg/kg/day	100 mg/kg/day
Estradiol (pg/mL)	Male	1.74 ± 0.03	1.85 ± 0.04*	1.95 ± 0.03*
Prolactin (ng/mL)	Male	11.4 ± 6.33	9.07 ± 3.55	10.63 ± 1.95
Testosterone (ng/mL)	Male	4.34 ± 1.01	4.97 ± 1.39	5.33 ± 1.93
FSH (ng/mL)	Male	1.52 ± 0.59	2.22 ± 0.83	2.09 ± 0.87
Progesterone (ng/mL)	Male	15.67 ± 6.64	4.89 ± 2.57†	2.87 ± 1.42*
LH (ng/mL)	Male	2.52 ± 1.88	4.64 ± 3.55	1.09 ± 1.03

Significant differences as compared to control: *$p < 0.001$, †$p < 0.01$.
FSH: Follicle stimulating hormone, LH: luteinizing hormone.

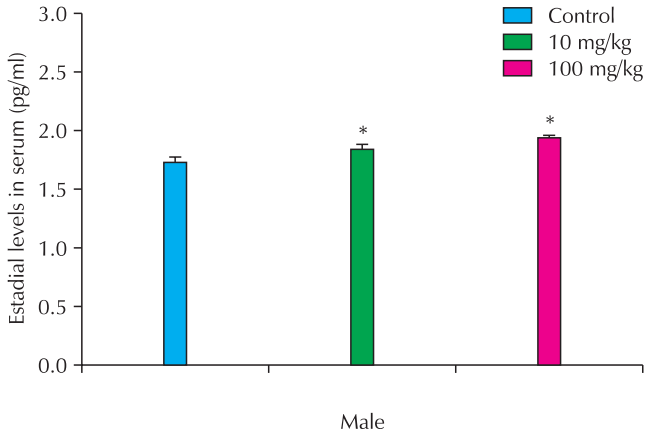

Fig. 7. Estradiol levels in serum of male rats treated with methylcyclohexane for 13 weeks. Significant differences compared to control group: *p < 0.001.

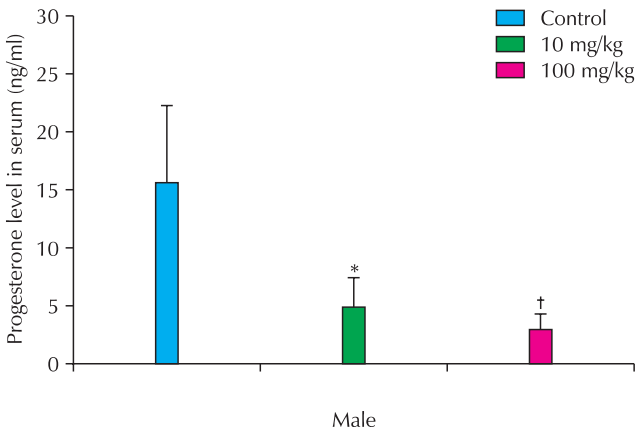

Fig. 8. Progesterone levels in serum of male rats treated with methylcyclohexane for 13 weeks. Significant differences compared to control: *p < 0.01, †p < 0.001.

showed that the LD50 is 2,250 mg/kg/day (mouse) and 3,200 mg/kg/day (rats), while the median lethal concentration LC50 based on absorption is 41,500 mg/m^3/2 h (mouse) and 7,613 ppm/4 h (rabbit) [5,6]. Steam has caused strong symptoms of the eyes and mucosa, and one study reported that narcosis symptoms occurred in addition to congestive changes that were manifested in various organs, including the liver and kidney [15]. The median lethal concentration LC50 based on the exposure via absorption using rats applies to the GHS' hazardous substance type 4 with 3,750 ppm (15.054 mg/L). In the subacute inhalation toxicity test, 400 ppm category, weight decrease and movement increase manifested at 1,600 ppm, and there was a decrease in HGB and increase in cholesterol as well. However, a report showed that there was no special abnormality upon pathologic testing [3]. In this research, there were no deaths in the 10 or 100 mg/kg/day groups. However, death of animals was observed in the 1,000 mg/kg/day group in both female and male animals. Given the decrease in clinical symptoms when it comes to movement and, given denaturation of organs, it has been suggested that toxicity occurs throughout the entire body. When blood tests were conducted on surviving animals, there was no statistically significant change in the male animals in the 10 and 100 mg/kg/day groups compared to the control group, as shown in Table 1. However, in the group of female animals, the 100 mg/kg/day group manifested concentration dependency in monocytes, as shown in Table 2; there was a significant ($p < 0.05$) decrease. The level of the effect was not assessed with the toxic effect, since the blood tests from rats showed that the values were within the normal range [16]. However, in 1,000 mg/kg/day group, WBC, NEU, EOS, and PLT in female and male animals increased or decreased significantly compared to the control

Table 6. Analysis of the estrous cycle and amenorrhea in female rats treated with methylcyclohexane for 13 weeks

Time (day)	Control	10 mg/kg/day	100 mg/kg/day
4-week	4.26 ± 0.64	5.40 ± 0.55*	5.00 ± 0.71
13-week	4.50 ± 0.87	4.10 ± 0.89	4.40 ± 1.67

Significant differences as compared to control: *p < 0.05.

group; those results were considered abnormal and to result from substance injection. Serum biochemical tests showed that the increase in TP and ALB observed in the male animals of the 100 mg/kg/day group was significant, as shown in Tables 3 and 4, but the degree of increase was subtle, and there was no concentration dependency. Moreover, given that similar data was not recognized in female animals, this was considered a random effect that was not related to the substance injection. As for the increase in the IP observed in the 10 and 100 mg/kg/day groups in male animals, it increased to concentration dependency. However, this was not manifested in female animals. Moreover, given that there has been no opinion regarding hypoparathyroidism, renal failure, or excessive Vitamin D related to the IP increase, it was not possible to confirm a correlation with substance injection. Pathologic test results showed that there were abnormalities in the liver and kidney in the 10 and 100 mg/kg/day groups, but concentration dependency was not manifested. Histological abnormalities of the liver observed in the female and male animals did not occur often. Those were common results [3,17-19] that were also observed often in the normal rats. Thus, it was considered a spontaneous finding that was not related to exposure to the substance. However, in the 1,000 mg/kg/day group, microgranuloma of the

liver, bile duct proliferation, cardiac hyperemia/hemorrhage, and myocardial necrosis were observed in male animals, as shown in Figs. 1-6. In some livers, non-special macrophage aggregation and galvanic hyperemia/hemorrhage were manifested, while the kidney showed protein casts and hyperemia/hemorrhage. Female animals manifested liver bile duct proliferation and hyperemia, while renal hyperemia/hemorrhage was not observed in normal rats. Given that the level of abnormality was severe, it was suggested that this was caused by substance injection. Accordingly, it was confirmed when rats were injected with methylcyclohexane repeatedly for 13 weeks, and that caused abnormalities in the liver, heart, and kidney in the 1,000 mg/kg/day group. When exposed to a high concentration level, target organs were liver, heart and kidney, which is similar to results that Kinkead et al. [20] came up with based on a study of the existing literature. Kinkead et al. [20] reported on a curtailing weight increase and observed a nephropathy toxic effect [21] that pertained to the observation of tubular nephrosis. Recently, research on the toxicity of aromatic compounds centered on toluene and xylene reported on the antigonadotropic effect that curtails the function of reproductive system stimulating hormones that significantly decreases the concentration of estradiol and testosterone, which are sex hormones. It was reported that, if exposed for a long period, endocrine system disruption [22] and a serum testosterone curtailing effect and others occurred [23]. Thus, this research also sought to check whether or not substance injection is related to the toxicity of the reproductive system. Study was conducted after the animals were exposed to the substance repeatedly during 13 weeks and showed that the concentration dependency or significance by experimental groups compared to the control group in the spermatogenic cell count and form from the male animals' testicles was not recognized. In the serum hormonal test, there was no change or concentration reliability based on substance injection compared to the control group in prolactin, testosterone, FSH, and LH levels, as shown in Table 5. In the case of estradiol, however, a significant ($p < 0.001$) increase and substance injection concentration dependency was manifested in male animals compared to the control group. In the male testis pathologic test, there was no special lesion resulting from substance injection. In the menstrual cycle test, the 10 mg/kg/day group manifested a significant ($p < 0.05$) increase in terms of the menstrual cycle in the 4th week, but there was no concentration dependency. When that was factored in, toxicity of the reproductive system was assessed as very low. Accordingly, the above mentioned diverse tests were conducted, and the results showed that the acute toxicity LC50 of the methylcyclohexane corresponds to the type 4 of acute toxicity hazardous substance using the hazardous substance classification of the Ministry of Employment and Labor's official notice 2009-68 as the standard, since it was 3,750 ppm. This study that injected the substance in SD rats repeatedly once per day, five days a week, for 13 weeks, showed that the absolute toxic dose was 1,000 mg/kg/day, while the amount of non-toxic substances corresponded to less than 100 mg/kg/day. Likewise, the GHS and Ministry of Employment and Labor's official notice No. 2009-68's "Standard on the chemical substance classification · labeling and MSDS" standard for the hazardous substance (10 < capacity = 100 mg/kg/day, 90 days) did not apply. However, this study was limited in the sense that the injection took place for only 13 weeks using SD rats, which were injected subcutaneously. In the longer run, it would be necessary to conduct detailed research on the effect of long-term injection (repetitive injection for two years) on the body through chronic toxicity and cancer-causing tests in order to assess the effect when exposed for a long time at low concentration. That study is needed to check chronic toxicity, to determine whether or not it causes cancer, and to ensure safety through the assessment of general toxicity through a health malfunction dynamic study for workers who handle methylcyclohexane.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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