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Full Length Research Paper

A study of micronucleus induction with isopropenyl benzene and trimellitic anhydride in bone marrow cells of Institute for Cancer Research (ICR) mice

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The genotoxicity of two chemicals, isopropenyl benzene and trimellitic anhydride, was evaluated using male Institute for Cancer Research (ICR) mice bone marrow cells for the screening of micronucleus induction. 7 week male ICR mice were tested at dosages of 500, 1000, and 2000 mg/kg for isopropenyl benzene and 475, 950 and 1900 mg/kg for trimellitic anhydride, respectively. As a result of counting the micronucleated polychromatic erythrocyte (MNPCE) of 2000 polychromatic erythrocytes (PCE), all treated groups expressed no statistically significant increase of MNPCE as compared to the negative control group. It was concluded that these two chemicals did not induce micronucleus in bone marrow cells and no direct proportion with dosage.

Key words: Mice, bone marrow, micronucleus induction, isopropenyl benzene, trimellitic anhydride.

INTRODUCTION

The necessity for a chemical hazard assessment has increased, because the number of workers exposed to chemicals has risen with the development of chemical industries. The *in vivo* micronucleus test was performed on mammalian bone marrow cells treated with two chemicals of isopropenyl benzene (CAS No. 98-83-9) and trimellitic anhydride (CAS No. 552-30-7) for which the definitive information is insufficient. Its permissible exposure limit of isopropenyl benzene with Occupational Safety and Health Administration (OSHA) standard is 100 ppm (480 mg/㎥) as a ceiling value. The 8 h time-weighted average (TWA) is 50 ppm; the 15 min short term exposure limit (STEL) is 100 ppm (ACGIH, 2008). As National Institute for Occupational Safety and Health (NIOSH, 1997) recommendations, the 10 h TWA is 50 ppm (240 mg/㎥), the 15 min STEL is 100 ppm (485 mg/㎥) and 700 ppm is immediately dangerous to life or health.

Its major uses are polymerization monomer, especially for polyesters (Lewis, 1997), and a third monomer to raise the glass transition temperature in acrylonitrile-butadiene-styrene rubber-thermoplastic resin (Kirk-Othmer, 1997), and it also uses as a polyvinyl chloride high temperature ABS comonomer, reactive diluent (unsaturated polyester resins) (Ashford, 1994).

The half-lethal dose (LD_{50}) of isopropenyl benzene with rat oral route is 4900 mg/kg (ITII, 1982). The prolonged skin contact with isopropenyl benzene may cause dermatitis and repeated inhalation may result in CNS depression (Clayton, 1993, 1994). Overall, isopropenyl benzene appears to be somewhat less toxic than styrene and vinyl toluene. Generally, isopropenyl benzene is an irritant to the eyes, skin and upper respiratory tract (Clayton, 1993, 1994).

Isopropenyl benzene was evaluated for mutagenicity in Chinese hamster ovary (CHO) cells exposed *in vitro* for 5 h to concentrations of 0 (dimethyl sulfoxide (DMSO) solvent control), 0.05, 0.075, 0.1, 0.125 and 0.15 µl/ml/plate with and without added Aroclor-induced rat liver S9 mix (Amoco Corp, 1991). The concurrent

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cytotoxicity ranging from 0 to 92% in the initial selective assay, no significant substance-related mutagenicity relative to control was observed, either in the presence or absence of metabolic activation (Amoco Corp, 1991).

NIOSH (NOES Survey, 1983) has statistically estimated that 3214 workers are potentially exposed to isopropenyl benzene in the U.S. Occupational exposure to isopropenyl benzene may occur through inhalation and dermal contact with this compound at workplaces where isopropenyl benzene is produced or used.

The second chemical for this study, trimellitic anhydride is used in the production of resins, adhesives, polymers, dyes (O'Neil, 2001) and as a chemical intermediate to make plasticizers (Lewis, 2001) may result in its release to the environment through various waste streams (Lewis, 2001).

The substance is irritating to the skin, the respiratory tract and is severely irritating to the eyes (IPCS, 2005). The occupational exposure standards of trimellitic anhydride are that 8 h TWA is 0.0005 mg/㎥ (inhalable fraction and vapor); 15 min STEL is 0.002 mg/㎥ (inhalable fraction and vapor) (ACGIH, 2008). The NIOSH recommendations are the 10 h TWA is 0.005 ppm (0.04 mg/㎥). From these values, it should be handled in the workplace as an extremely toxic substance (NIOSH, 2005).

Its major uses are in preparation of resins, adhesives, polymers, dyes, printing inks (O'Neil, 2001), plasticizer for polyvinyl chloride, high-temp plastics, wire insulation, gaskets and automotive upholstery (Lewis, 2001). Trimellitic anhydride is irritating to the skin, the respiratory tract and is severely irritating to the eyes (IPCS, 2005).

The LD_{50} in mouse with oral route is 1900 mg/kg (Lewis, 2004), the LD_{50} in rat with dermal is 5.6 g/kg (Rom, 1992), the LD_{50} in rat (female) with oral is 2030 mg/kg, in male is 3340 mg/kg and the LC_{50} in rat with inhalation is over 2330 mg/㎥/4 h (OECD, 2002). The LD_{50} in rabbit with dermal is over 2000 mg/kg (European Chemicals Bureau, 2000).

Occupational inhalation of trimellitic anhydride (TMA) apparently results in an antibody response with specificity for unique new antigenic determinants which arise from the coupling of TMA with autologous respiratory tract proteins (Zeiss et al., 1980). NIOSH (2006) has statistically estimated that 17744 workers (5236 of these are female) are potentially exposed to trimellitic anhydride in the U.S. Occupational exposure to trimellitic anhydride may occur through dermal contact with this compound at workplaces where trimellitic anhydride is produced or used. The general population may be exposed to trimellitic anhydride via dermal contact with products containing trimellitic anhydride (NIOSH, 2006).

It was reported that isopropenyl benzene have a positive result at *in vitro* sister-chromatid exchange (SCE) test with human lymphocytes (Tucker et al., 1993). Trimellitic anhydride was found to be negative when tested for mutagenicity using the *Salmonella*/microsome preincubation assay, using the standard protocol approved by the National Toxicology Program (NTP). Trimellitic anhydride was tested as many as 5 *Salmonella typhimurium* strains (TA1535, TA1537, TA97, TA98 and TA100) in the presence and absence of rat and hamster liver S9, at the highest ineffective dose tested in any *S. typhimurium* strain was 10 mg/plate. At this dose, slight clearing of the background bacterial lawn was observed (Mortelmans et al., 1986). In CHO cells, trimellitic anhydride concentrations as high as 2000 mg/L failed to produce an increase in either HGPRT mutations or chromosomal aberrations in the presence and absence of a metabolic activation system (rat liver S9) (OECD, 2002).

As mentioned earlier, many studies have been conducted other than the micronucleus test. But the available genotoxic data on these two chemicals are still controversial with and without mammalian metabolic activation (S9). So, it was necessary for further study according to Good Laboratory Practice (GLP) guideline to secure quality assurance of the test.

MATERIALS AND METHODS

Chemicals and animal feeding conditions

Isopropenyl benzene (Sigma, MO, USA, Lot No. MKBD0620V, 99%) and trimellitic anhydride (Sigma, MO, USA, Lot No. A0261408, 97%) were used as the test chemicals. Olive oil (Sigma, MO, USA, Lot No. BCBD1085) was used as a solvent according to the results of the solubility test. The positive control used mitomycin C (MMC) (Sigma, MO, USA, Lot No. 010M0665).

The molecular weight of isopropenyl benzene is 118.18 (Lide, 2000). It is a colorless liquid which has characteristic (NIOSH, 1997) or sharp aromatic odor (Bingham et al., 2001). The boiling point is 165.4℃, melting point is -23.2℃, the critical temp erature is 384℃, the density/specific gravity is 0.9106 (Lide, 2000) and the critical pressure is 4.36 MPa (Kirk-Othmer, 1997). It is soluble in alcohol, benzene, chloroform and all proportions in acetone, carbon tetrachloride (Weast, 1988-1989) and also soluble in water 116 mg/L at 25℃ (Yalkowsky and Dannenfelser, 1992).

Trimellitic anhydride is colorless crystals that has a boiling point of 390℃, melting point of 161 to 163.5℃ and soluble at 25℃: 0.002 g/100 g carbon tetrachloride; 0.06 g/100 g ligroin; 0.4 g/100 g mixed xylenes; 15.5 g/100 g dimethyl formamide; 49.6 g/100 g acetone and 21.6 g/100 g ethyl acetate (O'Neil, 2001).

Animals and experimental design

The mouse (*Mus musculus*) bone marrow micronucleus test was carried out according to OECD guidelines, TG 474 (OECD, 1997). Groups of specific pathogen free (SPF) male Institute for Cancer Research (ICR) mice were treated with the test substance at three dosage levels, the highest dosage level being the estimated maximum tolerated dose or the standard limit dose for the micronucleus test, whichever is least. Concurrent negative and positive control groups were also treated. It was performed using 7 week-old male ICR mice at 500, 1000, and 2000 with isopropenyl benzene, and 475, 950, and 1900 mg/kg dosages with trimellitic

Table 1. Animal body weight in micronucleus tests with oral exposure to isopropenyl benzene and trimellitic anhydride.

Exposure method	Concentration	No. of animals	Average body weight (mean ± SD)
Orally exposed to isopropenyl benzene for 24 h	Negative control (olive oil)	6	38.94 ± 1.08 g
	500 mg/kg b.w.	6	38.55 ± 1.37 g
	1,000 mg/kg b.w.	6	39.01 ± 1.65 g
	2,000 mg/kg b.w.	6	37.84 ± 1.18 g
	Positive control (MMC, 0.5 mg/kg b.w.)	6	36.44 ± 1.16 g
Orally exposed to trimellitic anhydride for 24 h	Negative control (olive oil)	6	38.47 ± 2.66
	475 mg/kg b.w.	6	39.83 ± 1.50
	950 mg/kg b.w.	6	39.08 ± 1.69
	1,900 mg/kg b.w.	6	38.66 ± 1.59
	Positive control (MMC, 0.5 mg/kg b.w.)	6	37.86 ± 2.22

b.w.: Body weight; SD: standard deviation.

anhydride, respectively. At 24 h after treatment with the two chemicals administered orally, there were normally 6 male animals per group. The experimental animal room was maintained at a temperature of 22℃ (±3℃) and relative humidity of 50 to 60%. The animal studies were approved by an animal ethics committee to ensure that appropriate animal care before the animals was obtained for research.

Bone marrow preparation and micronucleus test

Bone marrow cells were obtained from the femurs immediately following sacrifice. Immature erythrocytes could be differentiated using a variety of staining techniques that rely on their relatively high content of residual DNA. 4% Giemsa was used for mouse bone marrow/peripheral blood and stained immature erythrocytes blue, while the mature erythrocytes with low nucleic acid content appeared pinkish orange. Based on the cell cycle and maturation times of the erythrocytes, the bone marrow was harvested after 24 h. The bone marrow was flushed from the femurs and spread onto slides. The slides were air-dried, fixed and stained with a fluorescent DNA specific stain that easily illuminates any micronuclei that may be present. The 2000 polychromatic erythrocytes (PCEs, reticulocytes; immature erythrocytes) were scored per animal for the frequency of micronucleated cells in each of the 6 animals per dosage group. In addition, the percentage of PCEs among the 500 erythrocytes in the bone marrow was scored for each dosage group as an indicator of chemical-induced toxicity.

The presence of micronucleated polychromatic erythrocytes (MNPCEs) was visually scored (at least 2000 cells per mouse) by optical microscopy using a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) with a BA-2 filter. Cells were considered to be micronucleated when they neatly contained defined chromatin corpuscles with a diameter of less than one-third the diameter of the cell nucleus and stained equal or lighter than the nucleus of the cell from which the micronucleated cell was developed.

Evaluation and data analysis

Data were presented as the mean number of micronucleated cells per 2000 cells for each treatment group. The final conclusion for a micronucleus test was determined in consideration of the results of the statistical analyses. The experimental and control micronucleus frequency for each specimen within and between the different mice strains were compared with the one-way analysis of variance (ANOVA) ($P < 0.001$) test and the Dunnett's method ($P < 0.05$) using the SigmaStat V. 3.11.

RESULTS

Animal body weights with oral exposure to 2 chemicals

There were no specific symptoms among animals orally exposed to isopropenyl benzene and trimellitic anhydride. The ranges of body weights of animals exposed to isopropenyl benzene and trimellitic anhydride were 36.44 to 39.01 g and 37.86 to 39.83 g, respectively (Table 1).

Frequencies of micronucleus induction and cytotoxicity

The preliminary tests were performed as a limit test to determine the maximum dosage. The inhibition of proliferation in the bone marrow cells was not observed in these tests for the two chemicals. The frequencies of erythrocytes with micronucleus induction (MNPCE) were 0.08±0.07, 0.06±0.05, 0.06±0.06 and 0.20±0.15% in the negative control group, and 500, 1000 and 2000 mg/kg isopropenyl benzene treated group, respectively.

Positive control was 0.62 ± 0.62%. The ratios of PCEs within total erythrocytes were 57.21±9.77, 56.24±9.75, 57.31±6.80 and 49.95±7.56% in the negative control group, and 500, 1000 and 2000 mg/kg isopropenyl benzene treated group, respectively. Statistically significant changes were not observed when compared with the negative control group (Table 2).

The frequencies of erythrocytes with micronucleus induction were 0.02±0.03, 0.02±0.03, 0.04±0.04 and 0.10±0.03% in the negative control group, and 475, 950 and 1900 mg/kg trimellitic anhydride treated group, respectively. Positive control was 0.39 ± 0.07%. The ratio of PCEs within total erythrocytes were 47.98 ± 4.77,

Table 2. Results of the main micronucleus test with isopropenyl benzene (for 24 h).

Group	PCE* observed	MNPCE† observed	MNPCE frequency (%)	(PCE + NCE‡) counted	PCE counted	PCE /(PCE + NCE) (%)
Negative control	2,059 ± 59.50	1.67 ± 1.51	0.08 ± 0.07	508.83 ± 5.19	290.83 ± 48.31	57.21 ± 9.77
500 mg/kg b.w.	2,042 ± 49.42	1.17 ± 0.98	0.06 ± 0.05	508 ± 10.15	285 ± 47.29	56.24 ± 9.75
1,000 mg/kg b.w.	2,042 ± 35.56	1.17 ± 1.17	0.06 ± 0.06	512 ± 10.71	298 ± 40.33	57.31 ± 6.80
2,000 mg/kg b.w.	2,028 ± 21.36	4 ± 2.97	0.20 ± 0.15	515 ± 12.95	258 ± 41.41	49.95 ± 7.56
Positive control	2,074 ± 51.96	12.83 ± 13.09	0.62 ± 0.62	512 ± 4.56	184 ± 63.18	35.87 ± 12.44

*PCE: polychromatic erythrocyte; †MNPCE: micronucleated polychromatic erythrocyte; ‡NCE: normochromatic erythrocyte; b.w.: body weight. All values are expressed as mean ± SD. Compared with the One Way ANOVA ($P < 0.001$) test and the Dunnett's method ($P < 0.05$).

Table 3. Results of the main micronucleus test with trimellitic anhydride (for 24 h).

Group	PCE* observed	MNPCE† observed	MNPCE frequency (%)	(PCE + NCE‡) counted	PCE counted	PCE /(PCE + NCE) (%)
Negative control	2,066 ± 47.83	0.50 ± 0.55	0.02 ± 0.03	511 ± 5.20	245 ± 24.59	47.98 ± 4.77
475 mg/kg b.w.	2,047 ± 47.00	0.33 ± 0.52	0.02 ± 0.03	580 ± 147.81	283 ± 31.41	51.01 ± 12.18
950 mg/kg b.w.	2,035 ± 55.13	0.83 ± 0.75	0.04 ± 0.04	510 ± 7.83	238 ± 58.29	46.63 ± 11.17
1900 mg/kg b.w.	2,086 ± 75.74	2.17 ± 0.75	0.10 ± 0.03	512 ± 9.33	287 ± 39.86	56.11 ± 7.72
Positive control	2,041 ± 43.06	8 ± 1.55	0.39 ± 0.07	505 ± 4.46	219 ± 24.02	43.42 ± 4.68

*PCE: polychromatic erythrocyte; †MNPCE: micronucleated polychromatic erythrocyte; ‡NCE: normochromatic erythrocyte; b.w.: body weight. All values are expressed as mean ± SD. Compared with the One Way ANOVA ($P < 0.001$) test and the Dunnett's method ($P < 0.05$).

51.01 ± 12.18, 46.63 ± 11.17 and 56.11 ± 7.72% in the negative control group, and 475, 950 and 1900 mg/kg trimellitic anhydride treated group, respectively. There were also no statistically significant changes observed when compared with the negative control group (Table 3).

It concluded that these two chemicals did not inhibit the bone marrow cell proliferation in all treated groups, and did not make the micronucleus induction.

DISCUSSION

The micronucleus assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, so it was used in this study with isoprenyl benzene and trimellitic anhydride. Micronucleus (MN) formation results either from chromosome breakage (clastogenicity) or aneuploidy. By using pancentromeric probes, it is possible to draw conclusions if MN is formed as a consequence of chromosomal breakage (clastogenicity) or aneuploidy (Kim et al., 2010).

Some authors have described sex as an important variable in the micronucleus test (Fenech et al., 1994), with males generally more sensitive to the induction of micronuclei than females. However, other studies have shown no sex-related differences in micronucleus test results (Vanparys et al., 1990). When a bone marrow erythroblast develops into a PCE, the main nucleus is extruded. Any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualisation of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of MNPCEs in treated animals is an indication of induced chromosome damage.

The bone marrow of rodents is routinely used in this test since PCEs are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow is extracted, and preparations were made and stained (Mavournin et al., 1990). When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained (MacGregor et al., 1990; The Environmental Mutagen Society of Japan, 1995). The test may be performed in two ways:

(1) Animals are treated with the test substance once. Samples of bone marrow are taken at least twice, starting not earlier than 24 h after treatment, but not extending beyond 48 h after treatment with appropriate interval(s) between samples. The use of sampling times earlier than

24 h after treatment should be justified. Samples of peripheral blood are taken at least twice, starting not earlier than 36 h after treatment, with appropriate intervals following the first sample, but not extending beyond 72 h. When a positive response is recognized at one sampling time, additional sampling is not required.
(2) If 2 or more daily treatments are used (e.g. two or more treatments at 24 h intervals), samples should be collected once between 18 and 24 h following the final treatment for the bone marrow and once between 36 and 48 h following the final treatment for the peripheral blood (Higashikuni and Sutou, 1995). Other sampling times may be used in addition, when relevant.

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain and sex, and treatment regimen to be used in the main study (Fielder et al., 1992).

Commonly, cells are removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally (Hayashi et al., 1990; The Environmental Mutagen Society of Japan, 1995) or smear preparations are made and then stained. The use of a DNA specific stain [e.g. acridine orange (Hayashi and Ishidate, 1983) or Hoechst 33258 plus pyronin-Y (MacGregor et al., 1983)] can eliminate some of the artifacts associated with using a non-DNA specific stain.

This advantage does not preclude the use of conventional stains (e.g. Giemsa). Additional systems [e.g. cellulose columns to remove nucleated cells (Romagna and Staniforth, 1989) can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood (Gollapudi and McFadden, 1995). All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (Richold et al., 1990; Lovell et al., 1989). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

The results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results in the micronucleus test indicate that a substance induces micronuclei which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

With the advantage of micronucleus induction assay as the above, it was reported that alkaline (pH 13) single cell gel electrophoresis (SCGE) assay is highly effective in detecting base oxidation and DNA single-strand breakage (SSB) with reactive oxygen species (ROS). It is applied as a study with cellular repair activity with DNA segments for revealing or amplifying the genotoxic effects and for measuring the repair activity with DNA repair enzymes or the inhibition of DNA damage by antioxidants, as well as for quantitative measurements of the specific oxidative base (Collins et al., 1993; Cavallo et al., 2003). We suggest that future studies be directed toward chronic inhalation, carcinogenic tests, etc. These tests with many other chemicals would be useful as a biomarker for chemical risk assessment.

Based on this study, we suggest that the preventive efforts that contaminated protective clothing should be segregated so that there will be no direct personal contact by personnel who handle, dispose, or clean the clothing. Quality assurance to ascertain the completeness of the cleaning procedures should be implemented before the decontaminated protective clothing is returned for reuse by the workers. Contaminated clothing should not be taken home at the end of shift, but should remain at employee's place of work for cleaning. The worker should immediately wash the skin when it becomes contaminated (NIOSH, 2005).

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