한국환경분석학회지 제16권 (제2호) 77~83, 2013
J. of the Korea Society for Environmental Analysis

A Study of Micronucleus Induction with 3 Chemicals in Bone Marrow Cells of ICR Mice: Octadecanamide, 3-chloro-2-hyrdoxypropansulfonic acid sodium salt, 2,4,7,9-tetramethyl-5-decyne-4,7-diol

Kyung-Taek RIM[1,†], Yong-Hyun Chung[1], Hyeon-Yeong Kim[1], and Byeong-Kyu Lee[2]

[1]Center for Chemical Safety and Health, Occupational Safety and Health Research Institute, Korea Occupational Safety and Health Agency, Daejeon, Korea
[2]Biotoxtech, Choongcheongbuk-do, Korea

Received April 19, 2013/Revised May 7, 2013/Accepted June 14, 2013

We investigated the genotoxicity of three chemicals, octadecanamide, 3-chloro-2-hyrdoxypropansulfonic acid sodium salt and 2,4,7,9-tetramethyl-5-decyne-4,7-diol, using male ICR mice bone marrow cells for the screening of micronucleus induction. Although these three chemicals have already been tested numerous times, a micronucleus test has not been conducted. 7 week male ICR mice were tested at dosages of 500, 1,000, and 2,000 mg/kg for the 3 chemcals, respectively. After 24 hours of oral administration with the three chemicals, the mice were sacrificed and their bone marrow cells were prepared for smearing slides. As a result of counting the micronucleated polychromatic erythrocyte (MNPCE) of 2,000 polychromatic erythrocytes (PCE), all treated groups expressed no statistically significant increase of MNPCE compared to the negative control group. There were no clinical signs related with the oral exposure of these three chemicals. It was concluded that these three chemicals did not induce micronucleus in the bone marrow cells of ICR mice, and there was no direct proportion with dosage. These results indicate that the three chemicals have no genotoxic potential under each study condition.

Key words: Mice, Bone marrow, Micronucleus, Octadecanamide, 3-Chloro-2-hyrdoxypropansulfonic acid sodium salt, 2,4,7,9-Tetramethyl-5-decyne-4,7-diol

1. Introduction

The necessity for a chemical hazard assessment has increased because the number of workers exposed to chemicals has risen with the development of many industries. The *in vivo* micronucleus test was performed on mammalian bone marrow cells treated with three chemicals of octadecanamide (CAS No. 124-26-5), 3-chloro-2-hyrdoxypropansulfonic acid sodium salt (CAS No. 126-83-0) and 2,4,7,9-tetramethyl-5-decyne-4,7-diol (CAS No. 126-86-3) for which the definitive information is insufficient. Many toxicological studies have been conducted other than the micronucleus test. But the available genotoxic data on these three 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. The purpose of this micronucleus induction is to screen the cytogenetic damage that results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. Micronuclei were first used to quantify chromosomal damage by Scott and Evans,[1)] and are now recognized as one of the most successful and reliable assays for genotoxic carcinogens, performed in this study with 3 chemicals.

The toxicological information on these three chemicals as gained in this study will be used to promote

[†]To whom correspondence should be addressed.
Tel: 82-42-869-0345, Fax: 82-42-863-9001, E-mail: rim3249@gmail.com

workers' rights to know and to prepare or update the material safety data sheet (MSDS) for these chemicals.

The physico-chemical and toxicological information of these three chemicals are shown in Table 1.

This study will contribute to improving the testing of chemicals by generally used genotoxicity testing methods, as well as investigations on the underlying mechanism and the interpretation of genotoxicity data on hazardous chemicals.

2. Materials and Methods

2.1. Chemicals and animal feeding conditions

Octadecanamide (Aldrich, MO, USA, Lot No. 19827PI, 94.4%), 3-chloro-2-hyrdoxypropansulfonic acid sodium salt (Wako, Tokyo, Japan, Lot No. WTL0528) and 2,4,7,9-tetramethyl-5-decyne-4,7-diol (Aldrich, MO, USA, Lot No. 01901JD, 99.2%) were used as the test chemicals. 0.5% methylcellulose (expressed as "MC 1500cP" in this report, Wako, Lot No. ASQ6053) was used as a solvent in case of the two former, and corn oil (Sigma, MO, USA, Lot No. 065K0077) of the other one according to the results of the solubility test. The positive control used mitomycin C (MMC) (Sigma, MO, USA, Lot No. 075K0478).

Octadecanamide is off-white beads that have molecular weight 283.49. Both of 3-chloro-2-hydroxypropansulfonic acid sodium salt and 2,4,7,9-tetramethyl-5-decyne-4,7-diol have properties of white solid.

2.2. 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 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, 1,000, and 2,000 mg/kg with three chemicals, respectively. At 24 hours after treatment with these chemicals administered orally there were normally 6 male animals per

Table 1. Physico-chemical and toxicological information of 3 chemicals be tested

	Octadecanamide (CAS No. 124-26-5)	3-Chloro-2-hyrdoxypropansulfonic acid sodium salt (CAS No. 126-83-0)	2,4,7,9-Tetramethyl-5-decyne-4,7-diol (CAS No. 126-86-3)
Uses	Realease agent in plastic or film manufacturing, chemical intermediate in fabric waterproof agent, corrosion restrainer in oil well	Intermediate in anionic detergent (e.g., high grade alkyl ether) and amphipathic chemicals	Anti-humidity & anti-foaming, rinse aid, viscosity reduction, cleanser, penetrant
Physico-chemical properties	m.p. 109°C b.p. 251°C f.p. 207°C solubility - insoluble vapor density 9.78 log Kow 6.70 M.W. 283.30	pH 5.6~6.7 m.p. 256°C b.p. 535.82°C log Kow 1.46 M.W. 197.58	m.p. 42~44°C b.p. 255°C f.p. > 110°C M.W. 226.36
Toxicological Properties	Acute oral LD_{50} > 5,000 mg/kg (Rat) Skin - mild irritation	-	Acute oral LD_{50} > 5,000 mg/kg (Rat) Skin - no irritation (Rabbit)
Environmental Properties	Fish LC_{50} 0.031 mg/L (14 days)	B.C.F. 4.269 Non-degradable	Fish LC_{50} 3.780 mg/L (96 hr)

m.p. melting point; b.p. boiling point; f.p. flash point; log Kow octanol-water partition coefficient; M.W. molecular weight; LD_{50} half-lethal dose; LC_{50} half-lethal concentration; B.C.F. bioconcentration factor.
*Mostly referred from material safety data sheets information in KOSHANET (http://www.kosha.or.kr/bridge?menuID=69). Searches were conducted using keywords chemical name AND/OR CAS number.

group. The experimental animal room was maintained at a temperature of 22.7-23.9 and relative humidity of 44.6-47.9%. The animal studies were approved by an animal ethics committee to ensure that appropriate animal care before the animals was obtained for research (Approval No. 06138, 06139, 06140).

2.3. 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. 5% 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 hours. 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 2,000 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 was visually scored (at least 2,000 cells per mouse) by optical microscopy using a fluorescence microscope (BX51, Olympus, Tokyo, Japan). 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.

2.4. 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 Kastenbaum & Bowman Table (5%), t-test (5%) and the ANOVA test (5%) using the SigmaStat v. 3.11.

3. Results

3.1. Animal body weights with oral exposure to 3 chemicals

There were no specific symptoms among animals orally exposed to octadecanamide, 3-chloro-2-hydroxypropansulfonic acid sodium salt and 2,4,7,9-tetramethyl-5-decyne-4,7-diol. The ranges of body weights of animals exposed to these three chemicals were 34.96 to 35.47 g, 32.88 to 34.14 g, and 33.08 to 36.14 g, respectively (Table 2).

3.2. 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 ratios of erythrocytes with micronucleus induction (MNPCE) were 0.8±0.8, 0.2±0.4, 0.5±0.5 and 0.8±0.8 counts/2,000 PCE cells in the negative control group, 500, 1,000 and 2,000 mg/kg octadecanamide treated group, respectively. Positive control was 155.0± 13.3 counts/2,000 PCE cells. The ratios of PCEs (polychromatic erythrocytes) within total erythrocytes were 0.523±0.040, 0.499±0.037, 0.506±0.035 and 0.502± 0.024 counts in the negative control group, 500, 1,000 and 2,000 mg/kg octadecanamide treated group, respectively. Statistically significant changes were not observed compared with the negative control group (Table 3).

The ratios of erythrocytes with micronucleus induction were 1.2±0.4, 1.7±0.5, 0.7±0.8 and 1.2±1.0 counts/ 2,000 PCE cells in the negative control group, 500, 1,000 and 2,000 mg/kg 3-chloro-2-hydroxypropansulfonic acid

Table 2. Animal body weight in micronucleus tests with oral exposure to three chemicals

Exposure method	Concentration	No. of animals	Average body weight (mean±SD)
Orally exposed to octadecanamide for 24 hours	Negative control (MC 1500cP)	6	34.96±3.23 g
	500 mg/kg b.w.	6	35.47±1.87 g
	1,000 mg/kg b.w.	6	35.33±1.53 g
	2,000 mg/kg b.w.	6	35.29±1.69 g
	Positive control (MMC, 2 mg/kg b.w.)	6	35.21±1.50 g
Orally exposed to 3-chloro-2-hydroxypropansulfonic acid sodium salt for 24 hours	Negative control (MC 1500cP)	6	34.14±1.79
	500 mg/kg b.w.	6	33.74±1.55
	1,000 mg/kg b.w.	6	33.87±1.11
	2,000 mg/kg b.w.	6	33.92±1.61
	Positive control (MMC, 2 mg/kg b.w.)	6	32.88±1.29
Orally exposed to 2,4,7,9-tetramethyl-5-decyne-4,7-diol for 24 hours	Negative control (MC 1500cP)	6	33.08±2.10
	500 mg/kg b.w.	6	33.46±1.77
	1,000 mg/kg b.w.	6	33.66±2.33
	2,000 mg/kg b.w.	6	36.14±2.37
	Positive control (MMC, 2 mg/kg b.w.)	6	33.41±1.40

Table 3. Results of the main micronucleus test with octadecanamide (for 24 hours)

Groups	Counted *PCE	Counted ‡NCE	PCE/(PCE+NCE)	†MNPCE/PCE
Negative control	261.5±19.98	238.5±19.98	0.523±0.040	0.8±0.8
500 mg/kg b.w.	249.67±18.40	250.33±18.40	0.499±0.037	0.2±0.4
1,000 mg/kg b.w.	253.17±17.38	246.83±17.38	0.506±0.035	0.5±0.5
2,000 mg/kg b.w.	250.83±11.82	249.17±11.82	0.502±0.024	0.8±0.8
Positive control	247.5±15.57	252.5±15.57	0.495±0.031	155.0±13.3

*PCE: polychromatic erythrocyte. ‡NCE: normochromatic erythrocyte. †MNPCE: micronucleated polychromatic erythrocyte. All values are expressed as mean±SD.

Table 4. Results of the main micronucleus test with 3-chloro-2-hydroxypropansulfonic acid sodium salt (for 24 hours)

Groups	Counted *PCE	Counted ‡NCE	PCE/(PCE+NCE)	†MNPCE/PCE
Negative control	244.67±12.48	255.33±12.48	0.489±0.025	1.2±0.4
500 mg/kg b.w.	243.17±3.31	256.83±3.31	0.486±0.007	1.7±0.5
1,000 mg/kg b.w.	255.67±27.29	244.33±27.29	0.511±0.055	0.7±0.8
2,000 mg/kg b.w.	250.83±13.89	249.17±13.89	0.502±0.028	1.2±1.0
Positive control	240±13.94	260±13.94	0.480±0.028	147.7±8.5

*PCE: polychromatic erythrocyte. ‡NCE: normochromatic erythrocyte. †MNPCE: micronucleated polychromatic erythrocyte. All values are expressed as mean±SD.

treated group, respectively. Positive control was 147.7±8.5 counts/2,000 PCE cells. The ratio of PCEs within total erythrocytes were 0.489±0.025, 0.486±0.007, 0.511±0.055 and 0.502±0.028 counts in the negative control group, 500, 1,000 and 2,000 mg/kg 3-chloro-2-hydroxypropansulfonic acid treated group, respectively. There were also no statistically significant changes observed compared with the negative control group (Table 4).

The ratios of erythrocytes with micronucleus induction (MNPCE) were 0.7±0.5, 0.3±0.5, 0.7±0.5 and 0.5±0.8 counts/2,000 PCE cells in the negative control group, 500, 1,000 and 2,000 mg/kg 2,4,7,9-tetramethyl-5-decyne-4,7-diol treated group, respectively. Positive control was 137.3±25.0 counts/2,000 PCE cells. The ratios of PCEs (polychromatic erythrocytes) within total erythrocytes were 0.468±0.023, 0.464±0.017, 0.475±0.022 and 0.473±0.020 counts in the negative control group, 500, 1,000 and 2,000 mg/kg 2,4,7,9-tetramethyl-5-decyne-4,7-diol treated group, respectively. Statistically significant changes were also not observed com-

Table 5. Results of the main micronucleus test with 2,4,7,9-tetramethyl-5-decyne-4,7-diol (for 24 hours)

Groups	Counted *PCE	Counted ‡NCE	PCE/(PCE+NCE)	†MNPCE/PCE
Negative control	234±11.61	266±11.61	0.468±0.023	0.7±0.5
500 ㎎/㎏ b.w.	231.83±8.45	268.17±8.45	0.464±0.017	0.3±0.5
1,000 ㎎/㎏ b.w.	237.33±10.89	262.67±10.89	0.475±0.022	0.7±0.5
2,000 ㎎/㎏ b.w.	236.33±10.09	263.67±10.09	0.473±0.020	0.5±0.8
Positive control	229.33±8.64	270.67±8.64	0.459±0.017	137.3±25.0

*PCE: polychromatic erythrocyte. ‡NCE: normochromatic erythrocyte. †MNPCE: micronucleated polychromatic erythrocyte. All values are expressed as mean±SD.

pared with the negative control group (Table 5).

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

4. Discussion

In Korea, huge tons of these three chemicals are used every year. However, the numbers of workers dealing with it is yet to be fully explored and ascertained. With GHS classification, the octadecanamide is classified with skin corrosion/skin irritation category 2, so it must wash hands after handling and gently wash plenty of soap and water if on skin, wear protective gloves, give the specific treatment on the information label with reference to supplemental first aid instruction, also manufacturer/supplier or competent authority may specify a cleansing agent if appropriate. If skin irritation occurs, get medical advice/attention, and take off contaminated clothing and wash before reuse.[2)]

The other two chemicals have no hazards according to GHS classification. Also all of three chemicals have no genotoxic information with any tests. So we performed the micronucleus induction assays to evaluate the genotoxicity of these three chemicals.

The effects of the simple carboxylic amides such as octadecanamde are marked by a lack of apparent serious hazard by any type of contact, and lack of cumulative or toxic effect is probably explained by their relatively rapid hydrolysis to corresponding acid or in some cases their excretion in urine unchanged, and the decomposition products are sometimes irritating.[3)] Relative lack of irritant effect on skin & mucous membranes indicates that hydrolysis is probably not occurring there to any extent; otherwise lower molecular weight acids produced would cause local damage. Skin sensitization also appears to be rare in simple aliphatic carboxylic amides.[4)]

3-chloro-2-hydroxypropansulfonic acid sodium salt is used in many cleaning products, as emulsifiers and in chemical manufacturing. The exposures are very common, but significant effects are rare. There have been reports of serious toxicity in young children after inadvertent ingestion of products containing concentrated laundry detergent packaged in small, single-use packets. There have been several cases in which young children rapidly developed profuse vomiting, CNS depression, aspiration and respiratory distress requiring endotracheal intubation and mechanical ventilation after swallowing or biting into these packets. Occupational asthma has been reported. Aspiration may result in upper airway edema and considerable respiratory distress. Difficulty in breathing has been described in animals inhaling high concentrations of anionic surfactants.[5)]

2,4,7,9-tetramethyl-5-decyne-4,7-diol as detergents are used in many cleaning products, as emulsifiers and in chemical manufacturing. The general structure is a hydrocarbon chain linked to an ionic group (anionic or cationic) or an alcohol group (nonionic). Soaps are salts of a fatty acid made by the action of alkali or natural fats and oils. Detergents dissolve lipid layers in tissue and produce local irritation and injury. The most common effects are skin, mucosal and eye irritation. Aspiration can cause upper airway irritation and respiratory distress, most often in young children. Rarely, ingestion can cause caustic injury to the GI tract. Significant corneal injury is rare, but has been reported after ocular exposure. There have been reports of serious toxicity in young children after inadvertent ingestion of products

containing concentrated laundry detergent packaged in small, single-use packets. There have been several cases in which young children rapidly developed profuse vomiting, CNS depression, aspiration and respiratory distress requiring endotracheal intubation and mechanical ventilation after swallowing or biting into these packets. Skin irritation has been reported after prolonged occupational dermal contact. Skin dryness, irritation, and contact dermatitis have been reported after varying degrees of exposure to detergents. Eczema resulted from occupational exposure to surfactants.[5)]

The micronucleus assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens because the 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.[6)]

Some authors have described sex as an important variable in the micronucleus test,[7)] 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.[8)]

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 extracted, and preparations made and stained.[9)]

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, sex, and treatment regimen to be used in the main study.[10)]

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[11)] or smear preparations are made and then stained. The use of a DNA specific stain [e.g., acridine orange[12)] or Hoechst 33258 plus pyronin-Y[13)]] 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[14)]] can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory, and the systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

From this study, we suggest that future studies be directed toward chronic inhalation, carcinogenic tests, and so on. These tests with many other chemicals would be useful as a biomarker for chemical risk assessment.

Acknowledgements

This work was performed by Biotoxtech with supported by the Korea Occupational Safety and Health Agency (KOSHA), the Ministry of Employment and Labor, Republic of Korea and Grant-in-Aid for chemical hazard assessment.

References

1. D. Scott and H. J. Evans, *Mutat Res*, **1967**, 4, 579-599.
2. Korea Occupational Safety and Health Agency. Material Safety Data Sheet. KOSHANET (http://www.kosha.or.kr/bridge?menuID=69), Incheon, Republic of Korea. **2013**.
3. Patty, F. (ed.). Industrial Hygiene and Toxicology: Volume II: Toxicology. 2nd ed. New York: Interscience Publishers, **1963**, pp. 1829.
4. Patty, F. (ed.). Industrial Hygiene and Toxicology: Volume II: Toxicology. 2nd ed. New York: Interscience Publishers, **1963**, pp. 1832.
5. Rumack BH POISINDEX(R) Information System Micromedex, Inc., Englewood, CO, 2013; CCIS Volume 156, edition expires May, 2013. Hall AH & Rumack BH (Eds): TOMES(R) Information System Micromedex, Inc., Englewood, CO, **2013**; CCIS Volume 156, edition expires May, 2013.

6. S. J. Kim, K. T. Rim, M. G. Kang, J. K. Kim, Y. H. Chung and J. S. Yang, *Saf Health Work*, **2010**, 1, 80-86.
7. M. Fenech, S. Neville and J. Rinaldi, *Mutat Res*, **1994**, 313, 203-207.
8. P. Vanparys, F. Vermeiren, M. Sysmans and R. Temmerman R, *Mutat Res*, **1990**, 244, 95-103.
9. K. H. Mavournin, D. H. Blakey, M. C. Cimino, M. F. Salamone and J. A. Heddle, *Mutat Res*, **1990**, 239, 29-80.
10. R. J. Fielder, J. A. Allen, A. R. Boobis, P. A. Botham, J. Doe, D. J. Esdaile, D. G. Gatehouse, G. Hodson-Walker, D. B. Morton, D. J. Kirkland and M. Richold, *Mutagenesis*, **1992**, 7, 313-319.
11. M. Hayashi, T. Morita, Y. Kodama, T. Sofuni and M. Jr. Ishidate, *Mutat Res*, **1990**, 245, 245-249.
12. M. Hayashi, T. Sofuni and M. Jr. Ishidate, *Mutat Res*, **1983**, 120: 241-247.
13. J. T. MacGregor, C. M. Wehr and R. G. Langlois, *Mutat Res*, **1983**, 120, 269-275.
14. F. Romagna and C. D. Staniforth, *Mutat Res*, **1989**, 213, 91-104.