SH@W Safety and Health at Work www.e-shaw.org

Safety and Health at Work 2010;1:80-86

pISSN : 2093-7911
eISSN : 2093-7997

Original Article

A Study of Micronucleus Induction with Methyl Formate and 2-Methylbutane in Bone Marrow Cells of Male ICR Mice

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Objectives: We investigated the genotoxicity of two chemicals, methyl formate and 2-methylbutane, using male ICR mice bone marrow cells for the screening of micronucleus induction. Although these two chemicals have already been tested numerous times, a micronucleus test has not been conducted and the amounts used have recently been increased.

Methods: 7 week male ICR mice were tested at dosages of 250, 500, and 1,000 mg/kg for methyl formate and 500, 1,000, and 2,000 mg/kg for 2-methlybutane, respectively. After 24 hours of oral administration with the two chemicals, the mice were sacrificed and their bone marrow cells were prepared for smearing slides.

Results: 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 two chemicals.

Conclusion: It was concluded that the two 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 two chemicals have no mutagenic potential under each study condition.

Key Words: Mice, Bone marrow, Micronucleus induction, Methyl formate, 2-Methylbutane

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 methyl formate (CAS No. 107-31-3) and 2-methylbutane (CAS No. 78-78-4) for which the definitive information is insufficient. The toxicological information on these two chemicals as gained in this study will be used to promote workers' rights to know and to prepare or update the material safety data sheet (MSDS) for these chemicals.

Methyl formate ($C_2H_4O_2$), with a molecular weight of 60.05, is a colorless liquid [gas above 89°F], and has an agreeable odor [1,2]. It has a boiling point of 31.5°C and a melting point of –99.8°C [3]. It is soluble in ether, chloroform; miscible with ethanol, and shows 230,000 mg/L water solubility at 25°C [4]. It is a flammable liquid, so if mixed with water or flammables an irritating vapor is produced [5]. It is a serious fire hazard when exposed to heat or flames [6]. It is used as a high-boiling refrigerant for household appliances [7], solvent; and is an intermediate for dimethylformamide, and a

Received: May 31, 2010, **Accepted:** July 13, 2010
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fumigant and larvicide for tobacco and food crops.

Its Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit (PEL) for an 8-hr Time Weighted Average (TWA) is 100 ppm (250 mg/m^3) [8], and the 15 min Short Term Exposure Limit (STEL) is 150 ppm [9]. The National Institute of Occupational Safety and Health (NIOSH) Recommended Exposure Limit (REL) for 10 hr TWA is 100 ppm (250 mg/m^3), and for 15 min STEL is 150 ppm (375 mg/m^3). The immediate danger level to life or health is 4,500 ppm [2]. The LD_{50} value is 1,500 mg/kg b.w. (rat, oral). The LC_{50} of rat inhalation is over 5.2 mg/L/4 hr, the LD_{50} of rat dermal is over 4,000 mg/kg b.w., the LD_{50} of rabbit oral is 1,622 mg/kg [6], and the LC_{50} of mouse inhalation is 7.48 mg/L/3 hr [10]. One of the effects among workers occupationally exposed to methyl formate and other solvents (30% methyl formate, in addition to unspecified amounts of ethyl formate and ethyl and methyl acetate), involves visual disturbances (temporary blindness in 1 case), CNS depression, irritation of the mucous membranes, and dyspnea [11]. Exposure to its vapor has been reported to produce retching and CNS depression [12].

The genotoxicity of methyl formate was negative in the Ames test using Salmonella typhimurium strains TA 1535, TA 100, TA 1537, and TA 98 at concentrations of 20-5,000 μg/plate with and without metabolic activation [10].

2-methylbutane (C_5H_{12}, M.W. 72.149) is a volatile liquid or gas, and has a pleasant gasoline-like odor [13]. The boiling point is 27.8°C at 760 mmHg and the melting point is –159.77°C [14]. It is miscible with alcohol and ether [15], soluble in hydrocarbons, oils, while 48 mg is soluble in 1 liter of water at 25°C [16]. Its major uses are as a solvent for manufacturing chlorinated derivatives, a blowing agent for polystyrene, and it is also used to produce amylnaphthalenes and isoprenes [16]. Direct contact of liquid hydrocarbons with lung tissue (aspiration) will result in chemical pneumonitis, pulmonary edema, and hemorrhage [16]. In dogs, 120,000 ppm of 2-methylbutane was required to induce light anesthesia. Acute exposure to 2-methylbutane at levels of 150,000 to 170,000 ppm was lethal to dogs [17]. Exposure to 90,000 ppm for 11 minutes showed light anesthesia. At higher concentrations of 110,000 and 120,000 ppm, the CNS depression effect appeared within 2 and 4 minutes of exposure, respectively [15].

The mutagenic activity of 2-methylbutane has been assayed using the Ames test at the concentration of 100,000 ppm. It is not mutagenic both in the presence and absence of a metabolic activating system [15]. The LC_{50} of 2-methylbutane for mouse inhalation is 450 mg/L/2 hr [17], 1,000 mg/L/1 hr (estimated) [15], or 140,000 ppm/2 hr [15] separately. 2-methylbutane is negative in sex-linked recessive lethal assays, in postmeiotic and meiotic germ cells of male Drosophila melanogaster at 26,000 ppm by injection or feeding. The Ames test using Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and TA1538 was performed with and without metabolic activation. The plates were exposed to gas mixtures for 6 hours and incubated at 37°C for an additional 40 to 45 hours. 2-methylbutane was toxic at concentrations of 10% and above. Further studies were carried out at 1, 2, 5, and 8% concentration levels and showed no mutagenicity at these lower concentrations [17].

As per the above, many tests have been conducted other than the micronucleus 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 [18] in the root tips of broad beans, Vicia faba. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, performed in this study with methyl formate and 2-methylbutane.

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.

Materials and Methods

Chemicals and animal feeding conditions

Methyl formate (Sigma, St Louis MO, USA, Lot No. 12890BH, 99%) and 2-methylbutane (Sigma, St Louis MO, USA, Lot No. 01043LH, 99+%) were used as the test chemicals. Olive oil (Sigma, St Louis MO, USA, Lot No. 058K0684) was used as a solvent according to the results of the solubility test. The positive control used mitomycin C (MMC) (Sigma, St Louis MO, USA, Lot No. 028K1815).

Animals and experimental design

The mouse (Mus musculus) bone marrow micronucleus test was carried out according to OECD guidelines (TG 474, 1997) [19]. 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 250, 500, and 1,000 with methyl formate and 500, 1,000, and 2,000 mg/kg dosages with 2-methlybutane, respectively. At 24 hours 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°C (± 3°C) and relative humidity of 50-60%. 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 a 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 (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 2,000 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 ANOVA test and the Kruskal-Wallis tests (α=0.05) using the SigmaStat v. 3.11.

Results

Animal body weights with oral exposure to methyl formate and 2-methylbutane

There were no specific symptoms among animals orally exposed to methyl formate and 2-methylbutane. The ranges of body weights of animals exposed to methyl formate and 2-methylbutane were 35.45-41.14 g and 34.81-39.88 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.

Table 1. Animal body weight in micronucleus tests with oral exposure to methyl formate and 2-methylbutane

Exposure method	Concentration	No. of animals	Average body weight (mean ± SD)
Orally exposed to methyl formate for	Negative control (Olive oil)	6	37.87 ± 1.25 g
24 hours	250 mg/kg b.w.	6	37.89 ± 1.33 g
	500 mg/kg b.w.	6	37.82 ± 1.52 g
	1,000 mg/kg b.w.	6	37.87 ± 1.41 g
	Positive control (MMC, 0.5 mg/kg b.w.)	6	38.16 ± 1.72 g
Orally exposed to 2-methylbutane	Negative control (Olive oil)	6	38.28 ± 1.04 g
for 24 hours	500 mg/kg b.w.	6	38.10 ± 1.28 g
	1,000 mg/kg b.w.	6	37.68 ± 1.61 g
	2,000 mg/kg b.w.	6	38.03 ± 1.00 g
	Positive control (MMC, 0.5 mg/kg b.w.)	6	38.00 ± 1.33 g

Table 2. Results of the main micronucleus test with methyl formate (for 24 hours)

Groups	PCE* observed	MNPCE† observed	MNPCE frequency (%)	(PCE + NCE‡) counted	PCE counted	PCE/ (PCE + NCE) (%)
Negative control	2,038 ± 22.01	3 ± 1.26	0.15 ± 0.06	523 ± 9.72	259.5 ± 93.47	49.70 ± 18.44
250 mg/kg b.w.	2,032 ± 16.89	3.17 ± 1.47	0.16 ± 0.07	522.83 ± 17.22	283.17 ± 45.73	54.04 ± 7.40
500 mg/kg b.w.	2,049.33 ± 42.90	4.17 ± 1.17	0.21 ± 0.06	526.67 ± 13.17	294 ± 33.78	55.94 ± 7.40
1,000 mg/kg b.w.	2,030.5 ± 10.21	3.67 ± 1.03	0.18 ± 0.05	520.33 ± 12.44	255.83 ± 24.33	49.16 ± 4.43
Positive control	2,063 ± 42.91	27 ± 11.37	1.32 ± 0.56	536.33 ± 24.34	279.67 ± 90.38	52.49 ± 18.86

*PCE: polychromatic erythrocyte. †MNPCE: micronucleated polychromatic erythrocyte. ‡NCE: normochromatic erythrocyte.
Compared with negative control $p < 0.05$.
All values are expressed as mean ± SD.

Table 3. Results of the main micronucleus test with 2-methylbutane (for 24 hours)

Groups	PCE* observed	MNPCE† observed	MNPCE frequency (%)	(PCE + NCE‡) counted	PCE counted	PCE/ (PCE + NCE) (%)
Negative control	2,020.17 ± 13.21	1.83 ± 0.98	0.09 ± 0.05	520 ± 18.67	254.5 ± 62.10	49.32 ± 13.30
500 mg/kg b.w.	2,036.67 ± 23.61	4.5 ± 2.26	0.22 ± 0.11	526.83 ± 23.12	279.33 ± 41.05	53.34 ± 9.78
1,000 mg/kg b.w.	2,065 ± 39.79	3.33 ± 2.16	0.16 ± 0.10	524.67 ± 15.29	282.17 ± 47.27	53.80 ± 9.16
2,000 mg/kg b.w.	2,028 ± 21.13	4.5 ± 1.52	0.22 ± 0.07	527.67 ± 25.38	230.17 ± 48.22	43.78 ± 9.55
Positive control	2,049 ± 23.78	36.33 ± 10.09	1.78 ± 0.50	547.5 ± 59.15	209.67 ± 61.24	38.97 ± 12.97

*PCE: polychromatic erythrocyte. †MNPCE: micronucleated polychromatic erythrocyte. ‡NCE: normochromatic erythrocyte.
Compared with negative control $p < 0.05$.
All values are expressed as mean ± SD.

The frequencies of erythrocytes with micronucleus induction were 0.15±0.06%, 0.16±0.07%, 0.20±0.06%, and 0.18±0.05% in the negative control group, and 250, 500, and 1,000 mg/kg in the methyl formate treated group, respectively. The ratios of PCEs (polychromatic erythrocytes) within total erythrocytes were 49.70±18.44%, 54.04±7.40%, 55.94±7.40%, and 49.15±4.43% in the negative control group, and 250, 500, and 1,000 mg/kg in the methyl formate treated group, respectively. Statistically significant changes were not observed compared with the negative control group (Table 2).

The frequencies of erythrocytes with micronucleus induction were 0.09±0.05%, 0.22±0.11%, 0.16±0.11%, and 0.22±0.07% in the negative control group, and 500, 1,000, and 2,000 mg/kg in the 2-methylbutane treated group, respectively. The ratio of PCEs (polychromatic erythrocytes) within total erythrocytes were 49.32±13.30%, 53.34±9.78%, 53.80±9.16%, and 43.78±9.55% in the negative control group, and 500, 1,000, and 2,000 mg/kg in the 2-methylbutane treated group, respectively. There were no statistically significant changes observed compared with the negative control group (Table 3).

Discussion

In Korea, roughly 20,000 tons of methyl formate is used every year, around 900 workers deal with it, and about 380,000 tons of 2-methyl butane is used. However, the numbers of workers dealing with it is yet to be fully explored and ascertained. The National Institute of Occupational Safety and Health (NIOSH, NOES Survey 1981-1983) has statistically estimated that 7,738 workers (1,402 of whom are female) are potentially exposed to methyl formate in the US. Monitoring data indicate that the general population may be exposed to methyl formate via the inhalation of ambient air and the ingestion of food and drinking water [20]. The production of methyl formate as a food additive, solvent, and organic synthesis intermediate [17,21] may result in its release to the environment through va-

rious waste streams. Methyl formate's use as a fumigant and larvicide [1,17] will lead to its direct release into the environment. NIOSH has also statistically estimated that 6,605 workers (1,247 of whom are female) may be exposed to 2-methylbutane in the USA. Monitoring data indicate that the general population may be exposed to 2-methylbutane via the inhalation of ambient air and contact with gasoline. They have also statistically estimated that 6,605 workers may be exposed to 2-methylbutane in the USA [22]. Expected ground level concentrations in US urban air ranges from 50 to 350 ppb [23].

Section 8 (a) of the Toxic Substances Control Act (TSCA) requires manufacturers of this chemical substance to report preliminary assessment information concerned with its production, exposure, and use to the United States Environmental Protection Agency (EPA) as cited in the preamble in 51 FR 41329. The section 8 (d) model rule requires manufacturers, importers, and processors of listed chemical substances and mixtures to submit to the EPA copies and lists of unpublished health and safety studies. Methyl formate is included on this list [24].

In addition, the methods of assessment have been developed to provide indirect evidence of potential DNA damage or protective activities; for example, antioxidant effects can be monitored by measuring the lipid peroxidation and by measuring the formation of oxidized macromolecules. The DNA reactivity of compounds can further be measured in vitro in experiments with individual DNA bases or with isolated DNA. Further, enzyme measurements may provide information on processes that are associated with DNA protection [25]. The major advantage of methods that detect primary DNA damage is that no cell divisions are required. Therefore, such measurements can be carried out by not dividing or slowly dividing cells in culture or with primary cells isolated from various inner organs. UDS experiments measure "unscheduled" DNA synthesis caused by repair processes, and are based on the determination of the incorporation of ^{3}H-labeled thymidine into DNA using autoradiography [26]. This method has been largely replaced with single-cell gel electrophoresis (SCGE) or "comet" assays intervention trials. In this approach, the formation of "comets" formed due to the migration of DNA in an electric field is used as a measure of DNA damage. The procedure allows for the detection of single- and double-strand breaks, apurinic sites, and conformational alterations of the DNA. Furthermore, lesion-specific enzymes of microbial origin (endonuclease III and formamidopyrimidine glycosylase) can be used to monitor the endogenous formation of oxidized bases [27]. More recently, protocols were developed that enable examiners to study any modifications of repair processes and alterations of sensitivity toward dietary carcinogens and DNA repair [28].

At present, attempts are being made to validate and standardize this relatively new technique that is also increasingly being used in antimutagenicity studies. For a description of the guidelines see Tice et al. [29]; a review describing recent developments in regard to dietary human intervention trials can be found in reference [30]. In gene mutation assays with bacteria (e.g., in the salmonella/microsome assay), the induction of amino acid auxotrophy (e.g., $his^{-} \rightarrow his^{+}$) or resistance toward antibiotics is used as an end point, whereas in experiments with mammalian cells, resistance toward antimetabolites that interfere with the synthesis of DNA bases is used for the detection of gene mutations (e.g., TG^{r}, HPRT). Chromosomal aberrations (CAs), both structural and numerical, can be detected microscopically in metaphase cells. By using fluorescence in situ hybridization (FISH), individual chromosomes or chromosomal regions can be distinguished, and alterations such as translocations that are missed with conventional techniques can be detected. 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. Some authors have described sex as an important variable in the micronucleus test [31], 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 [32].

It was reported that alkaline (pH 13) SCGE assay is highly effective in detecting base oxidation and DNA single-strand breakage (SSB) with 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 [33,34]. Based on this study, we suggest that future studies be directed toward chronic inhalation, carcinogenic tests, and so on. It is suggested that further investigations, such as Fragment Length Analysis using Repair Enzymes (FLARE) assay, Comet assay with repair enzyme as Fpg, Endo III, and real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and other developing tools, be performed. Further, performing these tests with many other chemicals would be useful as a biomarker for chemical risk assessment.

Acknowledgments

This work was supported by the Korea Occupational Safety and Health Agency, the Ministry of Labor, Republic of Korea, and Grant-in-Aid for chemical hazard assessment, 2009.

References

1. O'Neil MJ. The merck index: an encyclopedia of chemicals, drugs, and biologicals. 13th ed. Whitehouse Station, NJ: Merck and Co., Inc., 2001:1084.
2. NIOSH. NIOSH Pocket Guide to Chemical Hazards & Other Databases CD-ROM. Department of Health & Human Services, Center for Disease Prevention & Control, National Institute for Occupational Safety and Health. DHHS (NIOSH) Publication No., 2004-103, 2003. Publication No., 2004-103.
3. Lewis RJ. Sax's dangerous properties of industrial materials. 10th ed. New York: John Wiley & Sons Inc., 1999:2449.
4. Lide DR. CRC handbook of chemistry and physics. 81st ed. Boca Raton: CRC Press LLC, 2000:3-167.
5. Fire protection guide to hazardous materials. 13th ed. Quincy, MA: National Fire Protection Association, 2002:49-99.
6. Lewis RJ. Sax's dangerous properties of industrial materials. 9th ed. New York: Van Nostrand Reinhold, 1996:2243.
7. Snyder R. Ethyl browning's toxicity and metabolism of industrial solvents. 2nd ed. New York: Elsevier, 1992:321.
8. US Government, 29 CFR 1910.1000; U.S. National Archives and Records Administration's Electronic Code of Federal Regulations: http://www.gpoaccess.gov/ecfr. at Accessed [May 14, 2010].
9. American Conference of Governmental Industrial Hygienists TLVs and BEIs. Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices; 2008. Cincinnati, OH. 40.
10. European Chemicals Bureau, IUCLID Dataset, Methyl formate (107-31-3) (2000 CD-ROM edition): http://ecb.jrc.it/esis/esis.php. at Accessed [May 14, 2010].
11. American Conference of Governmental Industrial Hygienists. Documentation of Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices for 2001. Cincinnati, OH. 2001. Cincinnati, OH. 1.
12. EPA. Office of Pollution prevention and toxics; High production Volume (HPV) Challenge program's Robust summaries and Test Plans: http://www.epa.gov/chemrtk/viewsrch.htm. at Accessed [May 14, 2010].
13. Bingham E, Cohrssen B, Powell CH. Patty's toxicology. 5th ed. New York: John Wiley & Sons, 2001:4-26.
14. IPCS, CEC; International Chemical Safety Card on 2-methylbutane. 1994 April: http://www.inchem.org/documents/icsc/icsc/eics1153.htm. at Accessed [May 14, 2010].
15. Lide DR. CRC handbook of chemistry and physics. 86th ed. Boca Raton, Florida: CRC Press, 2005:3-30.
16. Lewis RJ. Hawley's condensed chemical dictionary. 14th ed. New York: John Wiley & Sons, Inc., 2001:627.
17. European Chemicals Bureau; IUCLID Dataset, 2-Methylbutane (78-78-4) (2000 CD-ROM edition). 2009 October: http://ecb.jrc.it/esis/esis.php.at Accessed [May 14, 2010].
18. Scott D, Evans HJ. X-ray-induced chromosomal aberrations in vicia faba: changes in response during the cell cycle. Mutat Res 1967;4:579-99.
19. Organization for Economic Operation and Development. Guidelines for Testing of Chemicals; Mammalian Erythrocyte Micronucleus Test (TG 474). Paris, 1997:1-10.
20. NIOSH. International Safety Cards. Methyl Formate. 107-31-3: http//www.cdc.gov/niosh/ipcs/nicstart.html.at Accessed [May 14, 2010].
21. Bingham E, Cohrssen B, Powell CH. Patty's toxicology. 5th ed. NY: John Wiley & Sons Inc., 2001:548-552.
22. NIOSH. NOES. National Occupational Exposure Survey conducted from 1981-1983. Estimated numbers of employees potentially exposed to specific agents by 2-digit standard industrial classification: http://www.cdc.gov/noes.at Accessed [May 14, 2010].
23. Verschueren K. Handbook of environmental data on organic chemicals. 4th ed. New York: John Wiley & Sons, 2001:1340.
24. US Government, 40 CFR 716.120; U.S. National Archives and Records Administration's Electronic Code of Federal Regulations; 2005 June: http://www.gpoaccess.gov/ecfr.at Accessed [May 14, 2010].
25. Knasmüller S, Nersesyan A, Misík M, Gerner C, Mikulits W, Ehrlich V, Hoelzl C, Szakmary A, Wagner KH. Use of conventional and -omics based methods for health claims of dietary antioxidants: a critical overview. Br J Nutr 2008;99(E Suppl 1): ES3-52.
26. Kramer PJ. Genetic toxicology. J Pharm Pharmacol 1998;50: 395-405.
27. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004; 26:249-61.
28. Hoelzl C, Knasmüller S, Misík M, Collins A, Dusinská M, Nersesyan A. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: recent results and trends. Mutat Res 2009;681:68-79.
29. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 2000;35:206-21.
30. Nersesyan A, Hoelzl C, Ferk F, Misik M, Knasmueller S. Use of single cell gel electrophoresis assays in dietary intervention trials. In: Dhawan A, Anderson D, editors. The comet assay in toxicology. cambridge: Royal Society of Chemistry, 2010.
31. Fenech M, Neville S, Rinaldi J. Sex is an important variable affecting spontaneous micronucleus frequency in cytokinesis-

blocked lymphocytes. Mutat Res 1994;313:203-7.
32. Vanparys P, Vermeiren F, Sysmans M, Temmerman R. The micronucleus assay as a test for the detection of aneugenic activity. Mutat Res 1990;244:95-103.
33. Collins AR, Duthie SJ, Dobson VL. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis 1993;14:1733-5.
34. Cavallo D, Ursini CL, Setini A, Chianese C, Piegari P, Perniconi B, Iavicoli S. Evaluation of oxidative damage and inhibition of DNA repair in an in vitro study of nickel exposure. Toxicol In Vitro 2003;17:603-7.