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Genetic Toxicity Test of 1,2-Dibromoethane by Ames, Micronucleus, Comet Assays and Microarray Analysis

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Abstract – 1,2-Dibromoethane(DBE) has been widely used as a soil fumigant, an additive to leaded gasoline and an industrial solvent. In this study, we have carried out in vitro genetic toxicity test of 1,2-dibromoethane and microarray analysis of differentially expressed genes in response to 1,2-dibromoethane. 1,2-Dibromoethane showed mutations in base substitution strain TA1535 both with and without exogenous metabolic activation. 1,2-Dibromoethane showed mutations in frame shift TA98 both with and without exogenous metabolic activation. 1,2-Dibromoethane showed DNA damage based on single cell gel/comet assay in L5178Y cells both with and without exogenous metabolic activation. 1,2-Dibromoethane increased micronuclei in CHO cells both with and without exogenous metabolic activation. Microarray analysis of gene expression profiles in L5178Y cells in response to 1,2-dibromoethane selected differentially expressed 241 genes that would be candidate biomarkers of genetic toxic action of 1,2-dibromoethane.

Keywords □ 1,2-Dibromoethane, Ames test, COMET, MN assay, Microarray, S9 fraction

INTRODUCTION

1,2-Dibromoethane(DBE) has been widely used as a soil fumigant, an additive to leaded gasoline and an industrial solvent (Fishbain, 1980). People exposed for a long time to low levels of DBE suffered from bronchitis, headache, depression and impaired spermatogenesis, whereas intoxication which high doses resulted in severe damage to the liver and kidney, frequently with fatal consequences (Prakash *et al.*, 1999; Singh *et al.*, 2000). Any route of exposure (inhalation, oral or dermal) lead to rapid distribution of DBE within the organism via blood stream, and no effect antidote is as yet known (Humphreys *et al.*, 1999). The long-term effects of DBE include carcinogenicity in experimental animals, but direct epidemiological evidence in man is still not available (Alavanja *et al.*, 1990). According to the International Agency for Research on Cancer DBE is classified as probably carcinogenic to human-group 2A (IARC, 1999). The harmful effects of DBE are usually attributed to products of its bioactivation in the organism (Tezuka *et al.*, 1980), although the teratogenic effects of DBE itself have also been reported (Brown-Woodman *et al.*, 1998).

The genotoxicity of DBE has been extensively investigated in both *in vitro* and *in vivo* assays. In *in vitro* tests in prokaryotic organisms, DBE was generally genotoxic, both with and without exogenous metabolic activation (Zoetemelk *et al.*, 1987), although negative results were obtained in some assays (Buselmaier *et al.*, 1972; Shiau *et al.*, 1980). In cultured mammalian cells, DBE caused forward mutation (Brimer *et al.*, 1982), sister chromatid exchange (Tucker *et al.*, 1984), unscheduled DNA synthesis (Working *et al.*, 1986) and cell transformation (Colacci *et al.*, 1995). *In vivo* assay, DBE induced recessive lethal mutation (Kale and Kale, 1995), gene mutations (Graf *et al.*, 1984) and mitotic recombination (Graft *et al.*, 1984) in drosophila melanogaster. DNA damage was observed in the liver of mice and rat exposed orally on by intraperioneal injection (Storer and Conolly, 1983).

Although the genetic toxicity of 1,2-dibromoethane has been reported, no further study has not been carried out to find out the underlying mechanism of genetic toxic action of 1,2-dibromoethane. In this study, we have tested 1,2-dibromoethane

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using Ames test, in vitro micronuclei assay in CHO cells, single cell gel/comet assay in L5178Y cells, microarray analysis of gene expression profiles in L5178Y cells in order to find out biomarker genes in reponse to genetic toxicity of 1,2-dibromoethane.

MATERIALS AND METHODS

Materials

1,2-dibromoethane, 2-aminofluorene, 2-nitrofluorene, sodium azide, methane-sulfonic acid methyl ester, benzo(a)pyrene and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, MO, USA). The S9 fraction was purchased from Moltox® S9 (Canbiotech, U.S.A.).

Ames test

The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using Salmonella typhimurium strains TA98 and TA1535. The tester strains were cultured overnight in nutrient broth medium at 37°C. To the 0.1 ml of bacterial suspension, 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 ml of S9 was added and then 0.1 ml of 1,2-dibromoethane (0.33, 3.3, 10, 33.3, 100 μg/plate) or positive control chemicals such as 2-aminofluorene, 2-nitrofluorene and sodium azide were added and incubated for 20 min at 37°C. After incubation, 2.0 ml of top agar was added to mix and the mixture was poured onto a minimal glucose agar plate. 48 Hours after the incubation at 37 °C, the numbers of revertant colonies were counted (Kasamatsu et al, 2005).

Comet assay

Comet assay was carried out according to Singh et al. (Singh et al., 1988) with slight modification. L5178Y mouse lymphoma cells were grown at 37°C in a 5% CO_2 incubation. L5178Y mouse lymphoma cells were seeded in 12 well plates ($1X10^6$ cells/ml) and were exposed to 25, 50, 100 μg/ml 1,2-dibromoethane for 2 h. Positive controls were 150 μM methyl methanesulfonate (MMS) in the absence of S9, 50 μM benzo(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 μl of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5h at 4°C and then for 20min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 μg/ml). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK).

In vitro Cytokinesis block micronucleus assay

The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders *et al.*, 2003). CHO-K1 cells were grown in 24-well plates and treated with 1,2-dibromoethane (50, 100, 200 μg/ml) or cyclophosphamide (2.5, 5, 10 μg/ml) for 4 h with or without S9. After the treatment, cells were washed with PBS and further incubated for 20 h in the medium containing 3 ìg/ml cytochalasin B. Cells were harvested and spread on glass slide, and fixed with 100% methanol for 5 min and stained with 0.24 mM acridine orange in 1/150 M phosphate buffer (pH 6.8) for 3 min. Micronuclei were scored under the fluorescence microscope at 1000 magnification.

Microarray

The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2000) with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate. After 2 h of treatment with 1,2-dibromoethane (100 μg/ml), cells were resuspended in media without 1,2-dibromoethane and cultured for 20 h. Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by a RNeasy mini kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was amplified using the Affymetrix one-cycle cDNA synthesis protocol. For each array, 15 μg of amplified biotin-cRNAs was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) for 16 h at 45°C in a rotating hybridization oven. Slides were stained with streptavidin/phycoerythrin and washed for antibody amplification. Arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037).

Statistical analysis

All numerical data were expressed as the average of the values obtained ± S.D. and their significance determined by conducting a paired Student's *t*-test

RESULTS

1,2-dibromoethane induced gene mutations in both TA98 and TA1535 strains.

The mutant frequency(MF) was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to different concentrations of 1,2-dibromoethane (Fig. 1). In TA98 strain, the MF of 1.0 μg 2-nitrofluorene treated bacteria in the absence of S9 was 58.00±6.00 and the MF of cells exposed to 10 μg 2-aminofluorene in the presence of S9 was 114.00± 16.52. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in revertant. The MF of solvent control bacteria were 27.33±4.51 in the absence of S9 and 26.00±9.54 in the presence of S9. The MF of 1,2-dibromoethane (0.33, 3.3, 10, 33.3, 100 μg) treated bacteria were 11.00±5.29, 46.00±7.00, 40.33±8.02, 49.67±2.89, 47.67±6.66 in the absence of S9, 31.33±0.58, 29.67±2.52, 30.67±5.51, 37.67±3.21, 3267±5.69 in the presence of S9, respectively. 1,2-dibromoethane treatments statistically significant increased in revertant numbers in TA98 with or without S9. In TA1535 strain, the MF of 1.5 μg sodium azide treated cells in the absence of S9 was 339.33±18.56 and the MF of bacteria exposed to 10 μg 2-aminofluorene in the presence of S9 was 273.00±18.33. The positive control chemicals, sodium azide and 2-aminofluorene resulted large increases in revertant numbers. The MF of solvent control bacteria were 23.67±4.51 in the absence of S9 and 31.33±7.37 in the presence of S9. The MF of 1,2-dibromoethane (0.33, 3.3, 10, 33.3, 100 μg) treated bacteria were 42.66±8.33, 46.67±3.21, 58.00±2.00, 45.00± 6.24, 204.33±31.72 in the absence of S9, 39.00±8.33, 43.00± 9.90, 45.00±12.29, 85.00±9.17, 170.67±55.73 in the presence of S9, respectively. 1,2-dibromoethane treatments statistically significant increased in revertant numbers in TA1535 with or without S9. These 1,2-dibromoethane dose-dependent increases were therefore considered to have provided clear evidence of mutagenic activity of 1,2-dibromoethane in both TA98 and TA1535.

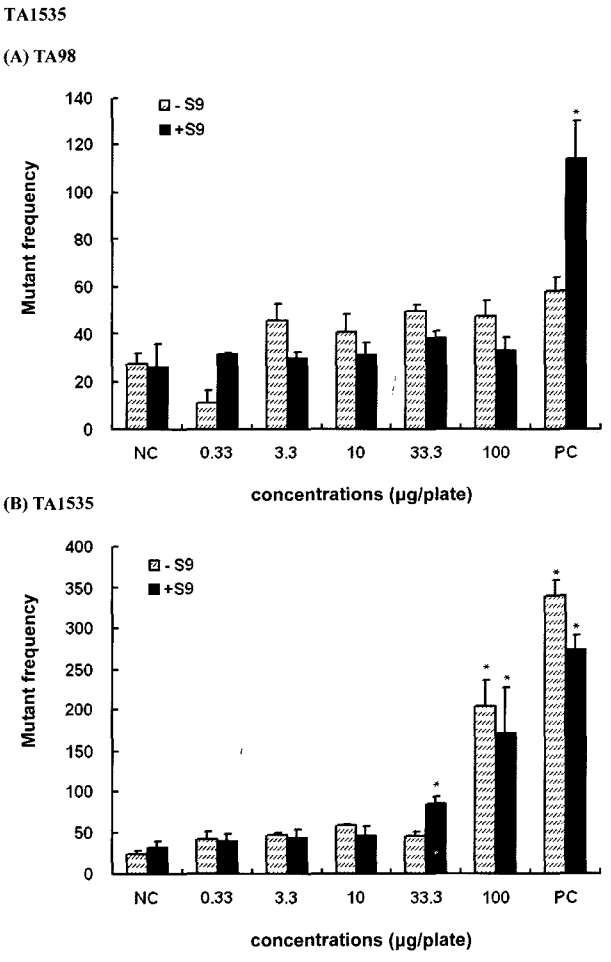

Fig. 1. The mutagenicity of 1,2-dibromoethane tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method (Gatehouse *et al.*, 1994) with or without metabolic activation using Salmonella typhimurium strains TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. PC: positive control (-S9: 1.5 μg/plate sodium azide, +S9: 10 μg/plate 2-aminofluorene)

1,2-dibromoethane induced DNA damage in L5178Y cells

The Olive Tail Moment (OTM) was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lymphoma cells exposed to different concentrations of 1,2-dibromoethane (25-100 μg/ml) for 2 h (Fig. 2). The OTM of MMS-treated cells (150 μM, positive control in the absence of S9) was 29.79±3.84 and the OTM of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 46.75±9.76. The OTM of control cells was 0.21± 0.30 in the absence of S9 and 1.56±1.66 in the presence of S9. Cells were exposed to 25, 50, 100 μg/ml 1,2-dibromoethane for 2 h. OTMs induced by 1,2-dibromoethane were 29.77±4.97, 39.94±4.29, 43.86±9.10 in the absence of S9 and 26.87±6.41, 39.30±6.56, 63.05±9.97 in the presence of S9, respectively. It thus caused a significant increase in DNA damage in comparison to the solvent control.

1,2-dibromoethane induced micronuclei in CHO-K1 cells

CHO-K1 cell cultured RPMI medium and treated with cyclophosphamide(CPA) in the presence of S9. As expected,

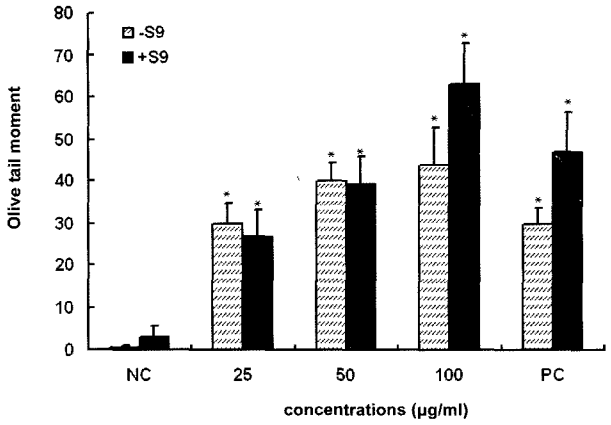

Fig. 2. Olive tail moments by 1,2-dibromoethane in L5178Y mouse lymphoma cells. Oive tail moments were measured using comet assay according to Singh et al. (Singh *et al.*, 1988) with slight modification as described in methods. Olive tail moments of L5178Y mouse lymphoma cells exposed to 25, 50, 100 μg/ml 1,2-dibromoethane for 2h. Negative control was medium. Positive controls were MMS (150 μM) in the absence of S9 and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. NC: negative control. PC: positive control, Data are means ± S.D. (n=15)

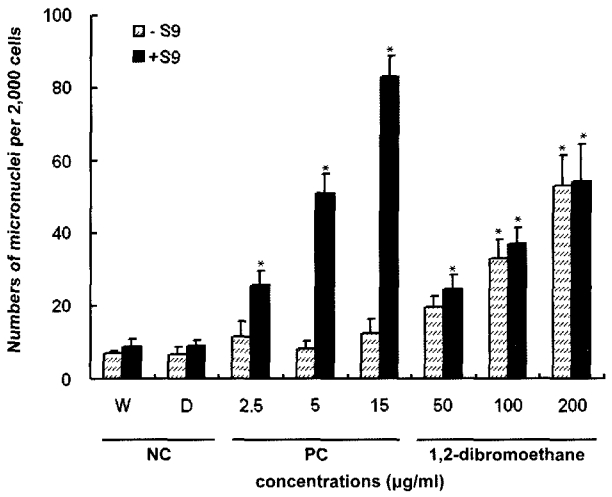

Fig. 3. micronucleus formation by 1,2-dibromoethane in cho-k1 cells. the cbmn (cytokinesis-block micronucleus) assay was performed according to fenech (fenech, 2000) with modification, and the recommendation of the 3rd international workshop on genotoxicity testing (kirsch-volders *et al.*, 2003) as described in methods. cho-k1 cells were grown in 24-well plates and treated with 1,2-dibromoethane (50, 100, 200 μg/ml) or cyclophosphamide (2.5, 5, 10 μg/ml) for 4 h with or without s9. cells were stained with 0.24 mm acridine orange and micronuclei were scored under the fluorescence microscope at 1000 magnification. data are means±s.d. n=3 *: statistically different from concurrent control at $p<0.05$. nc: negative control. pc: positive control (cyclophosphmide 2.5, 5 and 10 μg/ml).

numbers of micronuclei were induced to be 2.5, 5, 10 μg/ml CPA 25.67±4.04, 51.00±5.29, 83.00±5.57, respectively. Cells were exposed 50, 100, 200 μg/ml 1,2-dibromoethane for 4 h. Numbers of micronuclei of 1,2-dibromoethane treated cells were 19.67±3.06, 33.33±5.13, 53.00±8.72 in the absence of S9 and 24.67±3.79, 37.00±4.58, 54.33±10.26 in the presence of S9, respectively. Increase in the numbers of micronuclei with 1,2-dibromoethane treatment was statistically significantly and concentration-dependent (Fig. 3).

Microarray analysis of differentially expressed genes with 1,2-dibromoethane treatment in L5178Y cells

Differentially expressed genes from L5178Y cells treated with 1,2-dibromoethane (100 μg/ml) were analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays. 2237 genes were 1,2-dibromoethane specifically regulated and their fold of change were greater than Log 2. Among them 241 genes were selected after the Welch's T-test and Volcano plot analysis (Fig. 4). Figure 5 showed the results of clustering analysis of 1,2-dibromoethane regulated genes. Table I showed genes which expression were increased with

Table I. Results of gene ontology analysis

Accession No	Gene symol	Gene description	Fold change
NM_009616	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta)	7.9
NM_009465	Axl	AXL receptor tyrosine kinase	7.54
NM_033268	Actn2	actinin alpha 2	4.1
NM_010581	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	3.82
NM_008367	Il2ra	interleukin 2 receptor, alpha chain	3.65
NM_183027	Aplsl3	adaptor-related protein complex AP-I, sigma 3	3.49
NM_021515	Akl	adenylate kinase 1	3.47
NM_001002896	Bfsp2	beaded filament structural protein 2, phakinin	3.4
NM_009943	Cox6a2	cytochrome c oxidase, subunit VI a, polypeptide 2	3.4
NM_025359	Tspanl3	tetraspanin 13	3.31

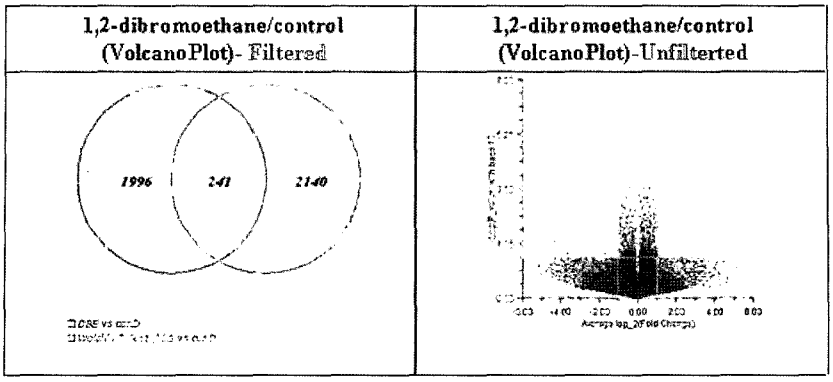

Fig. 4. Numbers of 1,2-dibromoethane regulated genes in L5178Y cells. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with 1,2-dibromoethane (100 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). Profiles were analyzed by VennDiagram (A) and Volcano Plot (B).

1,2-dibromoethane treatment. If these genes expression would be related to genetic toxicity of 1,2-dibromoethane, it would need further study.

DISCUSSION

Little information on the toxicity of ingested DBE in humans was identified. It was estimated that 200 mg/kg body weight is lethal to humans, based on the death of a 60 kg woman who had ingested 12 g (Alexeeff *et al.*, 1990). There were no significant increases in mortality due to neoplastic or non-neoplastic causes in two studies of populations exposed to DBE in the workplace (Ott *et al.*, 1980), although these studies were limited by the small size of the stady population, lack of accounting for possible confounding factors such as smoking or exposure to other substances and inadequate data on exposure. No differences in the frequency of sister chromatid exchange or chromosomal aberrations were observed in 60 workers at six papaya packing plants exposed to a mean DBE concentration of 0.68 mg/m^3 for an average of 5 years compared with a group of 40 controls, even when only workers with long-term exposure (> 5 years) or those with highest peak exposure were considered (Steenland *et al.*, 1987).

DBE is metabolized by two main routes. Microsomal oxidation mediated by cytochrome P450 2E1 represents the first pathway (Wormhoudt et al, 1996), leading to formation of bromoaldehyde capable of binding to proteins and non-protein thiols (Hill *et al.*, 1978). The second pathway give rise to a highly reactive episulfonium ion via a glutathione-S-transferase (GST) mediated conjugation of DBE with cellular glutathione. This

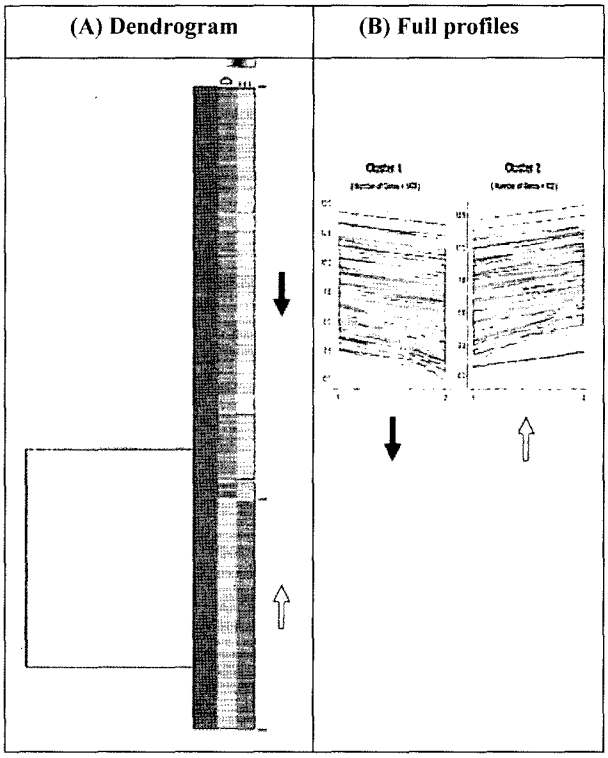

Fig. 5. Results of hierarchical clustering by 1,2-dibromoethane. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with 1,2-dibromoethane (100 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). The data were analyzed by hierarchical clustering, and green represents down regulation of the transcripts; black, no change; red, up regulation of the transcript.

episulfonium ion reacts with DNA and forms N^7-guanine adducts (Koga *et al.*, 1986) and, to a lesser extent, N^1-adenine adducts (Ballering *et al.*, 1993). Treatment of isolated rat liver mitochondria with DBE induces formation of DNA adducts, even in the mitochondrial DNA, and the process is accompanied by glutathione depletion, decreased ATP levels and inhibition of respiratory enzymes (Thomas *et al.*, 2001). Our results of DBE on Ames test, Comet assay, micronuclei assay , and microarray analysis would help to gain genetic toxic effect of DBE which would be useful for the risk assessment. Owing to the numerous adverse effects of DBE in the organism, the US Environmental Protection Agency (EPA) recommended to stop its use in agriculture more than 25 years ago. However, this prohibition has not been obeyed on a world-wide scale (Singh *et al.* 2000) and industrial applications of DBE still lead to the exposure of a vast number of workers in central and eastern europe (Fiabianova *et al.*, 1999) and in spain (Gonzles and Agudo, 1999). In addition, DBE contaminates soil and ground water (Xia and Rice, 2001) and it has recently been shown in Minnesota, USA, that its concentration in the environment is higher than previously assumed based on mathematical modeling (Pratt *et al.*, 2000).

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