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Studies on DNA Single Strand Break of Seven Phthalate Analogues in Mouse Lymphoma L5178Y Cells

Jae-Chun Ryu*, Hyung-Tae Kim and Youn-Jung Kim

Toxicology Laboratory, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Korea

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ABSTRACT : **Phthalate analogues are a plasticizer and solvent used in industry and were reported to be a potential carcinogen classified in the category of suspected endocrine disruptors. Most common human exposure to these compounds may occur with contaminated food. They may migrate into food from plastic wrap or may enter food from general environmental contamination. Since these substances are not limited to the original products, and enter the environment, they have become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health. To determine whether seven phthalate analogues i.e. diallyl phthalate, diisodecyl phthalate, di-n-nonyl phthalate, butyl benzyl phthalate, di-n-octyl phthalate, di-tridecyl phthalate, and dibutyl phthalate, can induce DNA strand breakage that is one of the various factors related to the mechanism of carcinogenicity, the comet assay which has been widely used for the detection and measurement of DNA strand breaks, was conducted in L5178Y mouse lymphoma cells. From these results, seven phthalates revealed dose-dependent decrease of cell viability, however, no remarkable cytotoxicity was observed even at high concentration of 100 μg/ml phthalates. And also, the results showed that the induction of DNA strand breaks by seven phthalates was not significantly different from the control in this study.**

Keywords: DNA break, Comet, Phthalate, Mouse lymphoma L5178Y cell

Introduction

Among the many synthetic chemicals used in chemical reaction processes in industry, phthalates are well used as plasticizers and softeners to increase the flexibility and workability of high-molecular-weight polymers. The annual world wide production of phthalates approximates 3 million metric tons (Bauer and Herrmann, 1997). Phthalates are well used in plastic goods (e.g., in children's toys, paints, lacquers, cosmetics, as well as food wrappings) and many medical items, such as blood bags, tubes, and filtering membranes. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health.

The establishment of toxicity and detection of synthetic chemicals that may pose a genetic hazard in our environment is subjects of great concern at present (WHO, 1971). Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified. Concern about their use has been mounting. It has been suggested that substances present in the environment may contribute to the development of hormone-dependent cancers and comprise reproductive capacity in humans and wildlife (Colborn *et al.*, 1993; Davis *et al.*, 1993; Eubanks, 1997). Phthalates are often mentioned as suspected endocrine disruptors, i.e., some phthalates are blamed for causing damage to the testes and decreasing sperm production (Gray *et al.*, 1982; Hardell *et al.*, 1997) and are reported to be a potential carcinogen. The influence of phthalates on hepatocarcinogenesis was documented in animal models (Huber *et al.*, 1996; Richmond *et al.*, 1996).

Generally, the carcinogenicity of chemicals including endocrine disrupting chemical is one of the potential toxicity that may consider for the human health. As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by a comet assay, which is widely used for the detection and measurement of DNA strand breaks.

Although many phthalates has been well used in industry, there are few reports on the genotoxicity at present. Our laboratory had also been involved in toxicity evaluation, especially in genotoxicity (Ryu *et al.*, 1993, 1994a,b, 1996a,b,

*To whom all correspondence should be addressed

1998a,b,c, 1999a,b,, 2000, 2001a,b,c, 2002; Kim *et al*, 2001). In this study, we aim to investigate the induction of DNA damage of these seven phthalate analogues, diallyl phthalate, diisodecyl phthalate, di-n-nonyl phthalate, butyl benzyl phthalate, di-n-octyl phthalate, di-tridecyl phthalate, and dibutyl phthalate, by using comet assay (Ryu *et al*, 1997, 2001d) in L5178Y mouse lymphoma cell line followed by guideline of Tice *et al.* (2000).

Materials and Methods

Chemicals

Methyl methanesulfonate (MMS) were obtained from Sigma-Aldrich Co. (St. Louis, USA). MMS was dissolved and further diluted in distilled water. Diallyl phthalate (CAS No. 131-17-9), benzyl n-butyl phthalate (CAS No. 85-68-7), di-n-octyl phtalate (CAS No. 117-84-0), ditridecyl phthalate (CAS No. 119-06-2) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Diisodecyl phthalate (CAS No. 26761-40-0) and di-n-nonyl phthalate (CAS No. 84-76-4) were obtained from Merck (Darmstadt, Germany). Dibutyl phthalate (CAS No. 84-74-2) were obtained from Sigma-Aldrich Co. (St. Louis, USA). They were dissolved in ethanol immediately before use. The final concentration of ethanol used in the medium was below 1%.

Cell culture and cytotoxicity

The mouse lymphoma cell line L5178Y (*TK*+/- 3.7.2c) was used for this experiment. Cells were cultivated in 90% RPMI-1640 (Life Technologies, MD, USA) with 1 mM sodium pyruvate and 0.1% pluronic, supplemented with 10% heat-inactivated horse serum and antibiotics in a humidified incubator at 37°C with 5% CO_2. For the determination of cell viability, about 10^6 cells were treated for 2 hr with the chemical. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability of treated cultures was related to controls that were treated with the solvent. All experiments were repeated twice in an independent test.

Single cell gel electrophoresis (comet) assay

Preparation of L5178Y cells

For the comet assay, 8×10^5 of cells were seeded into 12 wells plate and then treated as described in the toxicity tests. After 2 hr, cells were centrifuged for 5 min at 100×g, and gently resuspended with PBS. 100 μl of the cell suspension was immediately used for the test. Cells were mixed with 100 of low melting point agarose (LMPA: 1%) and added to fully frosted slide which had been covered with a bottom layer of 100 μl of 1% normal melting agarose. The cell suspension was immediately covered with coverglass and the slides were then kept at 4°C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of 100 μl of 0.5% LMPA by using a coverglass and then the slide were again kept cold at 4°C for 5 min.

Alkaline unwinding/alkaline electrophoresis

The procedure used follows the method described by Singh *et al.*, (1988) with minor modification (Ryu *et al.*, 1997, 2001d). The cells embedded in the agarose on slides were lysed for 1.5 hr in reaction mixture of 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl (pH 10), and 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH and 1 mM Na_2EDTA (pH approximately 13) for 20 min to unwinding of DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

Measurement of DNA damage

After the electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 5 min. The slides were stained by 50 μl of ethidium bromide in distilled water solution on each slide, and then covering the slide with a coverglass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells per slide. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

Results and Discussion

It is well known that carcinogenicity is the most serious effect of toxic chemicals in human health. As one of the mechanisms of carcinogenicity, it has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). Recently, single cell gel electrophoresis (comet assay) introduced to determine the DNA damages in cell level (Singh *et al.*, 1988; Tice *et al.*, 1991; Fairbairn *et al.*, 1996; Anderson *et al.*, 1998 and Speit and Hartmann, 1999) and is widely used for the detection and measurement of DNA strand breaks and cell cycle mechanism including our laboratory (Ryu *et al.*, 2001a,d; Seo *et al*, 1999a, b). In this respect, to investigate whether phthalate analogues not listed in IARC monographs induce DNA strand breakage, the comet assay was performed with seven phthalate analogues in L5178Y mouse lymphoma cells following guideline recommended by IWGTP (Tice *et al.*, 2000).

Cell viability measured 2 hr after treatment by the trypan blue exclusion method in mouse lymphoma L5178Y cells (Fig. 1). Seven phthalates revealed dose-dependent decrease of cell viability, however, no remarkable cytotoxicity was observed even at high concentration of 100 μg/ml phthalates.

The mean tail moments of seven phthalates in the concentration ranges from 20 to 100 μg/ml on L5178Y

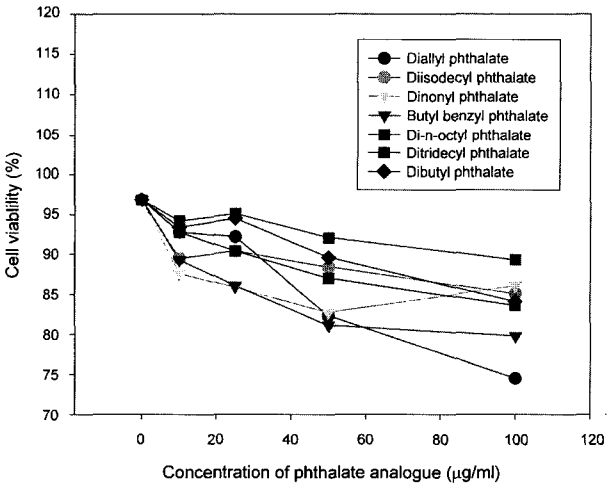

Fig. 1. Cell viability of mouse lymphoma L5178Y cells measured 2 hr after treatment by trypan blue dye exclusion method.

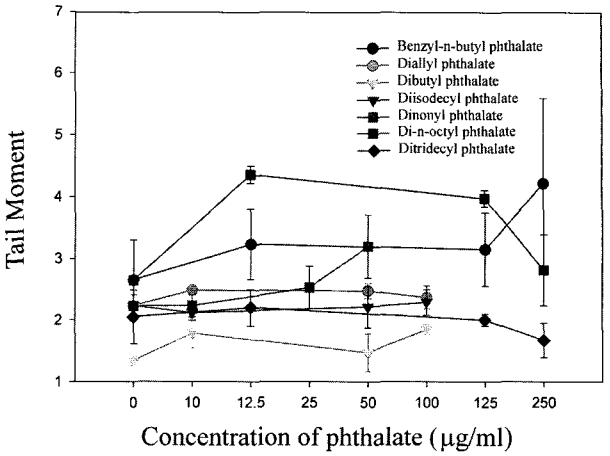

Fig. 2. The results of mean tail moment of seven phthalates. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells. There were no significant differences from controls.

cells in the comet assay were shown in Fig. 2. As a result, no statistically significant differences of tail moment values of seven phthalates were observed compared with control values.

Among several phthalate analogues we subjected, recently, Kleinsasser *et al.* (2000) reported that 354 mM dibutyl phthalate (DBP) induced DNA damage in the comet assay on human mucosa of the upper aerodigestive tract. However, in our result (Fig. 2), DBP did not revealed DNA strand breaks with no statistical significance. Also, it was reported that DBP was a weak direct-acting mutagen in a forward mutation assay in *Salmonella typhimurium* (Seed *et al.*, 1982) and was mutagenic in the mouse lymphoma forward mutation assay only in the presence of metabolic activation (Barber *et al.*, 2000). DBP also showed some evidence of clastogenic activity in Chinese hamster fibroblasts (Ishidate and Odashima, 1977).

To clarify these controversial results for genotoxic and mutagenic effects of DBP, we re-carried out comet assay with wide range of concentrations (10-500 μM) in mammalian cell line. It is observed that DBP showed a cytotoxicity in the high concentration ranges of 250-500 μM (Fig. 3). In the 10-200 μM concentration ranges showing no cytotoxicity, DBP did not induce the DNA damages with statistical significance, as our previous result (Fig. 4).

In summary, the result of comet assay for seven phthalate analogues that genotoxic effects are negative for ability to produce DNA damage in L5178Y cell line. Especially, we confirmed that DBP, one major type

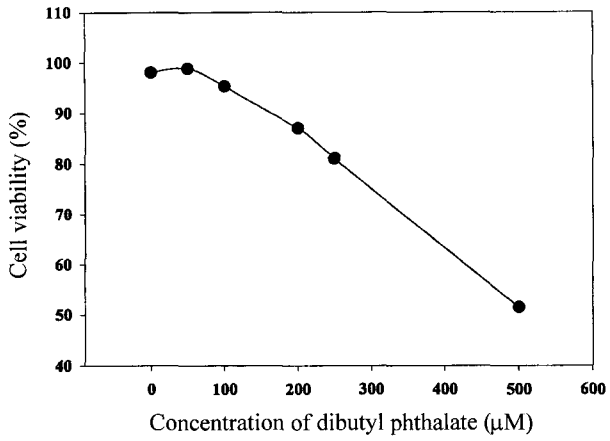

Fig. 3. Cell viability of mouse lymphoma L5178Y cells exposed to dibutyl phthalate for 1 hr by trypan blue dye exclusion method.

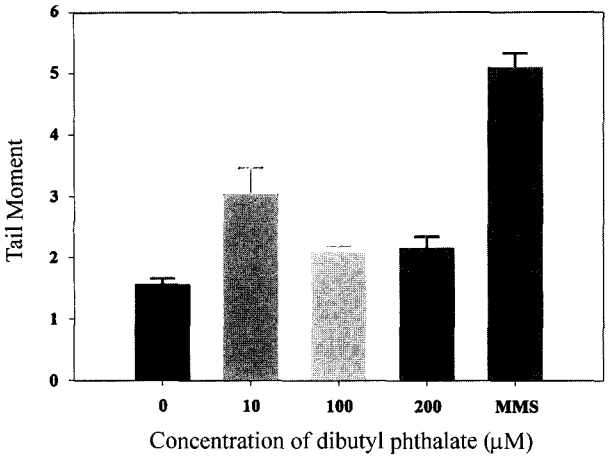

Fig. 4. Response of L5178Y cells to increasing concentration of dibutyl phthalate in comet assay. Mean tail moment indicates DNA damage of cells. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells. MMS represent methyl methanesulfonate as positive control.

of phthalate analogues, did not induce DNA single strand breakage at concentrations showing no cytotoxicity in L5178Y mouse lymphoma cell line.

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