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DNA Single Strand Breaks of Perchloroethylene and Its Bio-degradation Products by Single Cell Gel Electrophoresis Assay in Mammalian Cell System

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Abstract

Perchloroethylene (tetrachloroethylene, PCE), a dry cleaning and degreasing solvent, can enter groundwater through accidental leak or spills. PCE can be degraded to trichloroethylene (TCE), 1, 1-dichloroethylene (DCE) and vinyl chloride (VC) as potential bio-product. These compounds have been reported that they can cause clinical diseases and cytotoxicity. However, only a little genotoxic information of these compounds has been known. In this study, we investigated DNA single strand breaks of PCE, TCE, DCE and VC by single cell gel electrophoresis assay, (comet assay) which is a sensitive, reliable and rapid method for DNA single strand breaks with mouse lymphoma L5178Y cells. From these results, 37.5 μg/ml of PCE, 189 μg/ml of TCE and 56.4 μg/ml of DCE were revealed significant DNA damages in the absence of S-9 metabolic activation system meaning direct-acting mutagen. And in the presence of S-9 metabolic activation system, 41.5 μg/ml of PCE, 328.7 μg/ml of TCE and 949 μg/ml of DCE were induced significant DNA damage. In the case of VC, it was revealed a significant DNA damage in the presence of S-9 metabolic activation system. Therefore, we suggest that chloroethylene compounds (PCE, TCE, DCE and VC) may be induced the DNA damage in a mammalian cell.

Keywords: single cell gel electrophoresis assay (comet assay), DNA single strand breaks, perchloroethylene (tetrachloroethylene, PCE), trichloroethylene (TCE), 1,1-dichloroethylene (DCE), vinyl chloride (VC)

Perchloroethylene (tetrachloroethylene, PCE) and trichloroethylene (TCE) are widely used in industry as degreasers, dry cleaning agents, paint removers, solvents for chemical extraction and components of adhesives and lubricants[1-2]. These compounds can enter ground water through accidental leak or spills. Various pollution by these compounds has occurred[3-4], already.

Exposure of these chlorinated ethene compounds to human often occurs in workplace and/or ambient environment via inhalation and/or direct dermal contact. Also, PCE, TCE and their degradation products can be take in agricultural products which will be consumed by human.

In anaerobic condition, PCE can be degraded to TCE and other chlorinated ethenes as its major and potential bio-products. Degradation of PCE can be carried out by some bacteria strain, which have ability of chlorinated compounds degradation[5]. Some of remediation by those bacteria of sites contaminated with these compounds is critical, because chlorinated ethenes are toxic[6], and natural anaerobic degradation often leads to even more toxic compounds such as vinyl chloride, a well-known carcinogen[7]. TCE, 1, 1-dichloroethylene (1, 1-DCE), cis-DCE, trans-DCE, vinyl chloride (VC) and other chlorinated ethenes constitute a main group of bio-degradation products and environmental pollutants from PCE degradation[8-9].

The major toxic effects from long-term inhalant exposure to a mean of 8 hours Time Weighed Average (TWA) of 15 ppm PCE in humans are neurobiological (headache, impairment of memory and concentration), cardiac (arrhythmia), nephrological and hepatic[10]. Exposure to TCE also can cause a wide array of toxicological effects on animal and human, especially notable at hepatic, renal and neurobiological[11-13]. And it has been reported that exposure to DCE is the cause of liver damage and hepatic toxicity[14-15]. VC is well known as its cytotoxicity and carcinogenicity to human and animals[16]. Moreover, it has been reported that these chlorinated ethene compounds can induce apoptosis, as well as genotoxicity.

Although there are many reports about various toxicity by many types of exposure (*i.e.*, inhalant or epidermal) and clinical diseases, only few reports

have managed genotoxicity of PCE and its degradation products. In the present study, we evaluated genotoxic effects of PCE, TCE, DCE and VC by single cell gel electrophoresis assay (comet assay), which has been known as a sensitive, reliable and rapid method to detect DNA single strand breaks with mouse lymphoma L5178Y cells.

Results

Cytotoxicity of PCE and Its Degradation Products

To determine the optimal concentration, cytotoxicity of each compound was assessed by exposure to 0-5,000 μg/ml with 2-fold serial dilution for 2 hours using trypan blue exclusion assay. However, exposure of VC was performed at 0-20 μg/ml, because of the highest available concentration within 20 μg/ml. All measurements of cytotoxicity were performed in the presence and absence of S-9 metabolic activation systems. Based on results of cytotoxicity assay, 20% inhibitory concentration (IC_{20}) of each compound was calculated (Table 1). PCE showed the highest cyto-

Table 1. IC_{20} values of perchloroethylene, trichloroethylene, 1, 1-dichlroethylene and vinyl chlroide

Chemical	IC_{20} values	
	+S-9	−S-9
PCE	83.0 μg/ml	150.0 μg/ml
TCE	1,314.8 μg/ml	378.0 μg/ml
DCE	1,898.0 μg/ml	225.6 μg/ml
VC	>20.0 μg/ml	>20.0 μg/ml

PCE: perchloroethylene, TCE: trichloroethylene, DCE: 1, 1-dichloroethylene, VC: vinyl chloride

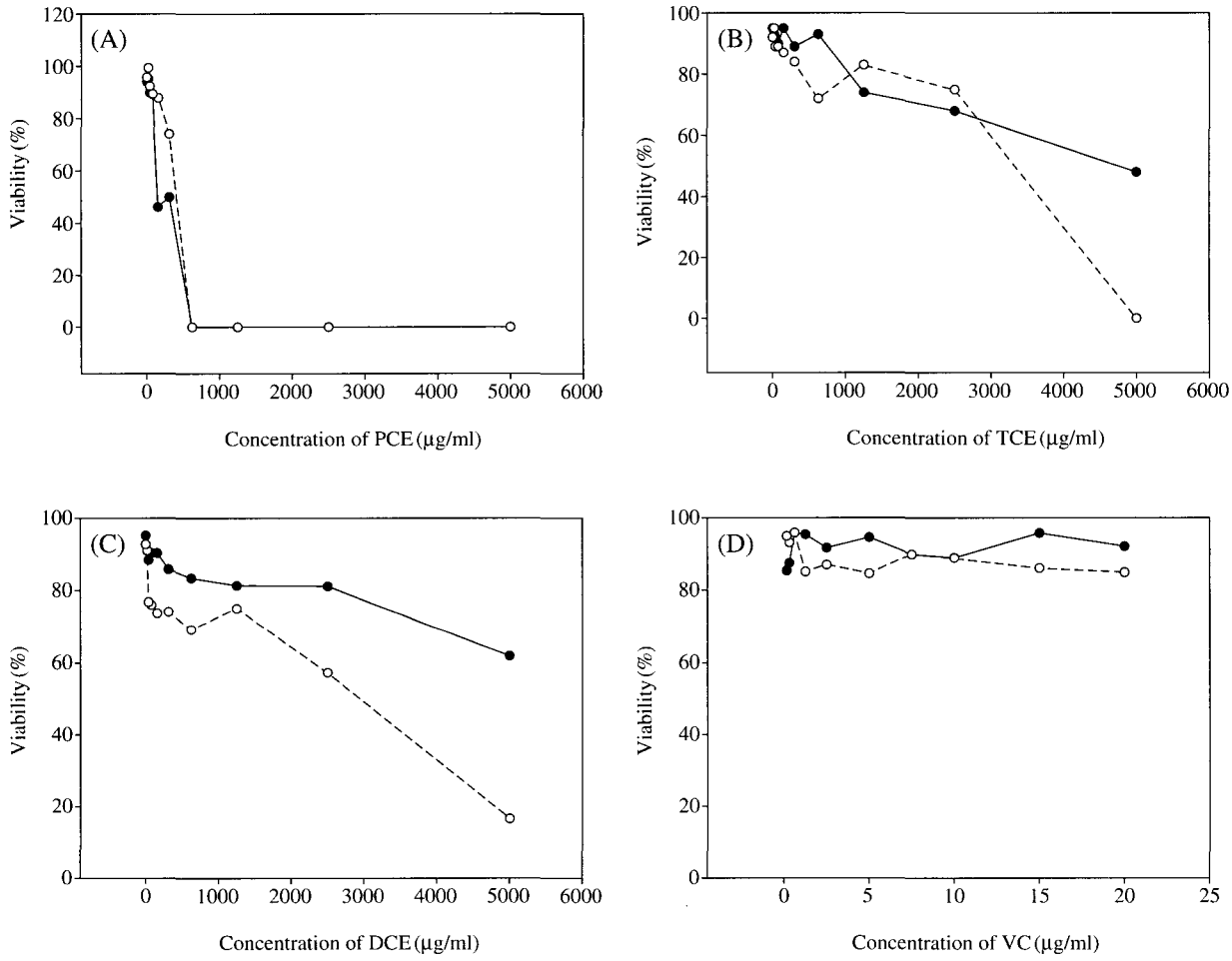

Fig. 1. Cytotoxicity of each compounds in L5178Y cells: (a) perchloroethylene (PCE) (b) trichloroethylene (TCE) (c) 1, 1-dichloroethylene (DCE) (d) vinyl chloride (VC) : a solid line; in presence of S-9 metabolic activation system, a dashed line; in absence of S-9 metabolic activation system.

toxicity, and DCE showed the lowest cytotoxicity among all compounds tested in this study. VC was not observed cytotoxicity at the concentrations tested in this experiment.

Single Cell Gel Electrophoresis (comet) Assay

It is well known that carcinogenicity is the most serious effect of toxic chemicals in human health. One of the mechanisms of carcinogenicity, induction of DNA damage can be determined by comet assay, which is widely used for the detection and measurement of DNA strand breaks[17-21]. In this respect, to investigate whether PCE and its bio-degradation products induce DNA strand breaks, the comet assay was performed with PCE, TCE, DCE and VC in L5178Y mouse lymphoma cells, following guideline recommended by IWGTP[22].

Comet assay was carried out at IC_{20} values of each compound as maximum concentration. Fig. 2 (a-d) shows the tail moment of PCE and its degradation products in L5178Y cells. From the results, PCE and its degradation products were observed statistically significant differences of tail moment values compared with negative control ($P<0.05$), except for VC in absence of S-9 metabolic activation systems (S-9).

In detail, the DNA damaging effects of PCE were assessed at the concentration from 20.7 to 83.0 μg/ml in presence of S-9 metabolic activation systems (+S-9) and from 37.5 to 150.0 μg/ml in −S-9. PCE was induced DNA damage at 41.5-83.0 and 37.5-150.0 μg/ml in the +S-9 and −S-9, respectively ($P<0.05$) (Fig. 1a).

TCE was assessed at the concentration from 328.7

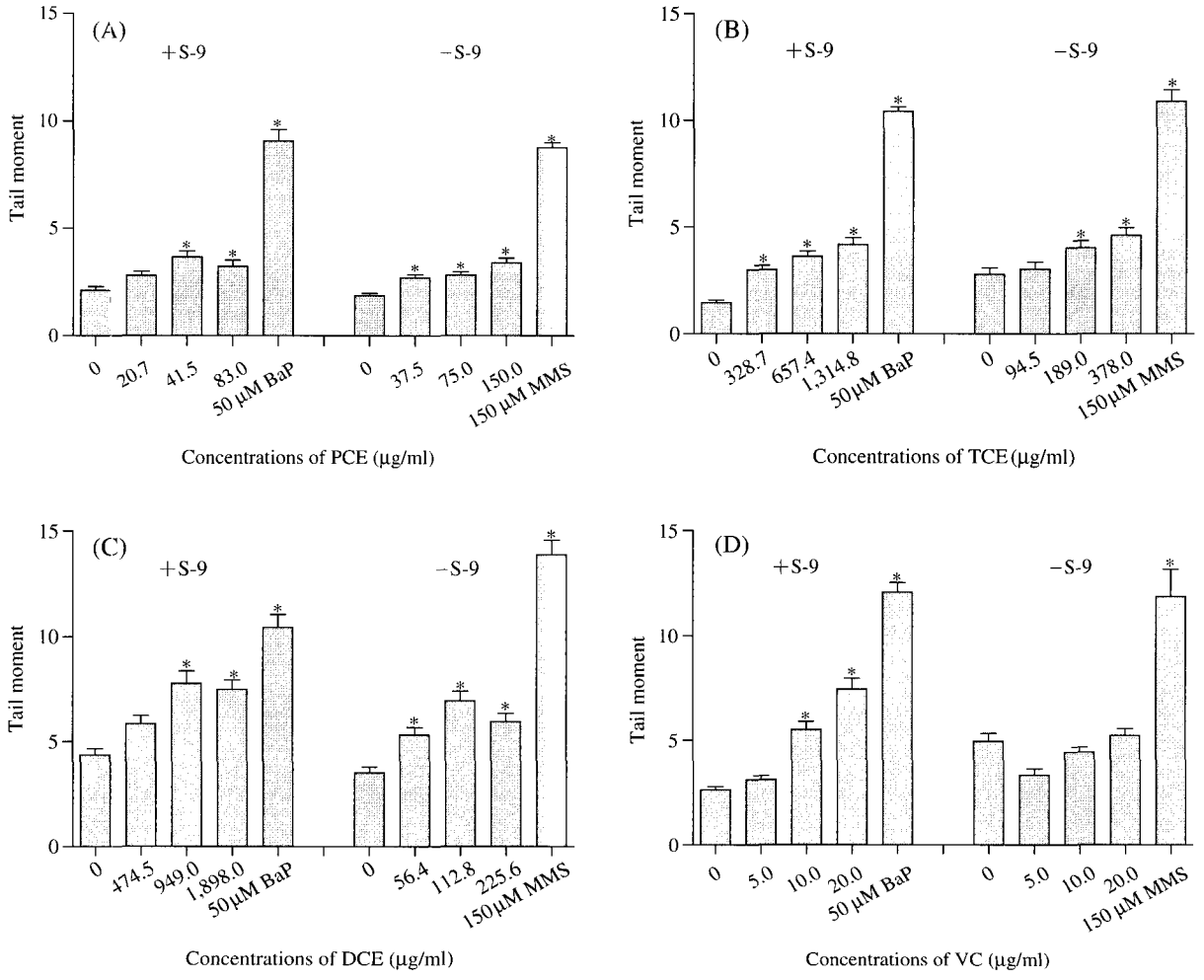

Fig. 2. Comet assay with each compounds in L5178Y cells (+/−S-9). (a) perchloroethylene (PCE) (b) trichloroethylene (TCE) (c) 1, 1-dichloroethylene (DCE) (d) vinyl chloride (VC). Values are means±SE from four experiments. In each experiment the tail moment index had been assessed from 200 separately calculated cells.

to 1,314.8 μg/ml in +S-9 and from 94.5 to 378.0 μg/ml in −S-9. TCE that tested at all concentrations was revealed significant difference of tail moment compared to negative control in +S-9. In −S-9, TCE at 189.0 and 378.0 μg/ml was induced a significant DNA damage ($P<0.05$) (Fig. 2b).

Also, DCE was performed at the concentration from 474.5 to 1,898.0 μg/ml in +S-9 and from 56.4 to 225.6 μg/ml in −S-9. DCE was observed a significant increase of tail moment at 949.0-1,898.0 and 56.4-225.6 μg/ml in +S-9 and −S-9, respectively ($P<0.05$) (Fig. 2c).

In the case of VC, the highest concentration availability in commercial available did not revealed any cytotoxicity when applied as maximum concentration. So the DNA-damaging effects of VC were equally assassed at 5.0 to 20.0 μg/ml (within the highest available concentration) in +S-9 and −S-9. VC at 10.0 and 20.0 μg/ml showed significant DNA damage in +S-9. In −S-9, however, significant DNA damage was not shown ($P<0.05$) (Fig. 2d).

Discussion

Usually, PCE and other chlorinated compounds are exposed to humans and animals at workplace. But, in ecological environment, most of PCE and its degradation products (TCE, DCE, VC and others), which are on surface of the ground or the side of a river, are undergone evaporation to the atmosphere. In the ground (anaerobic condition), low level of PCE and its products can undergo degradation, several times, then those compounds, which are remained under the ground can be absorbed in plants or aquatic animals. Therefore, it is necessary to confirm the toxic effects of PCE and its degradation products.

As shown in Fig. 1, cytotoxicity of PCE and its degradation products were appeared a concentration-dependant decrease in the mammalian cells. Especially, cell viability of PCE was shown a rapidly decrease, less than 1,000 μg/ml in both +S-9 and −S-9. Also, TCE and DCE were shown the cell viability of over 50% even at the maximum concentration (5,000 μg/ml) in +S-9 and 2,500 μg/ml in −S-9. According to above results, cytotoxicity of these compounds in +S-9 is lower than in −S-9. So, we can guess that cytotoxicity of these compounds is decreased through metabolic activation system. Therefore, cytotoxicity of Parent Compound (PCE) and its products were revealed the tendency to decrease in the mammalian cells when PCE and its products were bio-degradated.

PCE and its degradation products induced concentration-dependently DNA damage as analyzed with comet assay. The comet assay, as a high throughput toxicity screening tool (HTTS) for detection of DNA damage (single-strand breaks) in mammalian cells, was already assigned as a reliable measure, revealing a dependable dose response relationship with various genotoxicants[23-29]. The positive response was observed at the concentrations that caused no or only minor decrease in survival after the exposure (survival was in all cases >80% compared to that of the negative control). The lowest concentration that induced DNA damage in comet assay was comparable for PCE (41.5 μg/ml), TCE (328.7 μg/ml), DCE (949.0 μg/ml) and VC (10 μg/ml) in +S-9. Except for VC that is already known to have a high toxicity, DNA damage levels were increased with decreasing chlorine elements from parent compound to degradation products (VC>PCE>TCE>DCE). Different pattern appeared in −S-9. The lowest concentration that inducing DNA damage in comet assay was comparable for PCE (37.5 μg/ml), TCE (189.0 μg/ml) and DCE (56.4 μg/ml) in −S-9. DNA damage levels were shown in the order of PCE>DCE>TCE. Exceptionally, at 20 μg/ml VC, may not induce DNA damage.

In the case of VC, although VC has shown a little cytotoxcity in the very low concentration, it was observed the significant DNA damage in presence of S-9 metabolic activation system. Accordingly, it is necessary to perform the comet assay at higher concentration than the concentration that is being used in this study.

In the previous studies, PCE has been reported to cause reverse mutation in the Ames test using, *Salmonella typhimurium*[30] and was not revealed mutagenic effect in the mouse lymphoma forward mutation assay[31]. In comet assay and performed in this study, however, PCE induced DNA damage. Also, TCE was mutagenic in the mouse lymphoma forward mutation assay only in +S-9 and was not caused reverse mutation in the Ames test[32]. But, comet assay of TCE was induced DNA damage, also effect of DCE on lymphoma forward mutation assay in mouse were not reported in the mouse lymphoma forward mutation assay. It was not caused reverse mutation in the Ames test[33]. As well, VC caused reverse mutation in the Ames test[34], and induced DNA damage in liver cells and lymphocyte of rats with dose-response relationship in the comet assay[35] that is consistent with our data. These results show that there are differences in the potency of genotoxicity among the four compounds by the applied assay. Consequently, the comet assay appeared to be the most sensitive for detecting the genotoxicity of

the PCE and its degradation products.

Methods

Chemicals

Methyl methanesulfonate (MMS) and benzo[a] pyrene (BaP) were obtained from Sigma-Aldrich Co. (St. Louis, USA). MMS was dissolved and further diluted in distilled water, and BaP was dissolved and further diluted in dimethyl sulfoxide (DMSO). PCE (CAS No. 127-18-4) and TCE (CAS No. 79-01-6) with a 99.5% purity (the highest commercial grade available) and 1, 1-DCE (>99% purity) were obtained from Sigma-Aldrich Co. (St. Louis, USA). VC (concentration of 2,000 μg/ml in methanol, the highest commercial grade available) was obtained from SUPELCO Co. (USA). PCE, TCE and DCE were dissolved in DMSO immediately before the use. VC was diluted in methanol immediately before the use. The final concentration of DMSO and methanol used in the medium was below 1%.

Cell Culture and Cytotoxicity Tests

The mouse lymphoma cell line L5178Y was employed for a comet assay. 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 hours with the chemicals. After the staining of 0.4% trypan blue (Life Technologies, MD, USA), 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 duplicated in an independent test.

Single Cell Gel Electrophoresis Assay (comet assay)

Preparation of L5178Y cells for comet assay. 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 hours, 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 μl 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.*[19], with minor modification. 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.

Evaluation 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 5 μg/ml ethidium bromide solution on each slide, and then covering the slide with a coverglass. Image of 200 randomly selected cells (50 cells from each of four replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis Komet 5.5 (Kinetic Imaging Limited, Liverpool, UK), determining the mean tail moment (percentage of DNA in the tail times tail length) of the 200 cells. The comparison of each test group with negative and positive control was analyzed with one way of analysis of variance (ANOVA) followed by Dunn's test. $P < 0.05$ was considered statistically significant.

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