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Impact of Trichloroethylene Exposure on Micronucleus Frequency as a Result of Industrial Exposure

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ABSTRACT

Trichloroethylene (TCE) is one of the important volatile organic compounds that have been used in industry throughout the world. It is a well-known rodent carcinogen and potent mutagen in humans. Workers occupationally exposed to TCE at variable concentrations are at risk of chromosomal damage. Micronucleus formation in human lymphocytes is widely used as a biomarker for monitoring genetic instability in humans. The purpose of this study was to assess the related risk via the micronucleus frequency through trichloroethylene (TCE) exposure in 64 occupationally exposed workers and 63 normal healthy individuals. The micronucleus frequency was counted in the smear of peripheral blood lymphocytes using the cytokinesis-block micronucleus (CBMN) test. Urinary trichloroacetic acid (TCA), a biological marker of TCE exposure, was subjected to gas chromatography-electron capture detection (GC-ECD) using the modified headspace technique. The effect of donor gender, age, alcohol consumption, smoking and duration of exposure on the micronucleus frequency was studied. Occupational exposure to TCE resulted in significantly increased levels of TCA in urine. The mean urinary TCA concentration of exposed workers was 16.10 ± 3.57 $mg.L^{-1}$ compared to the control value of 6.21 ± 4.87 $mg.L^{-1}$. The micronucleus frequency was significantly higher ($P < 0.01$) in TCE exposed workers (5.53 ± 0.71 per 1,000 cells) than those of controls (3.65 ± 0.34 per 1,000 cells). There was no correlation between the micronucleus frequency and gender, smoking or alcohol consumption in both exposed workers and controls. However, significant correlations were observed between micronucleus induction and the urinary TCA concentration, the duration of exposure and the age of workers. These results indicated that increased micronucleus frequency is associated with occupational trichloroethylene exposure.

Keywords: micronucleus, occupational exposure, trichloroethylene, genotoxicity

INTRODUCTION

Trichloroethylene (TCE), is one of the most widely used industrial solvents as a dry-cleaning and metal degreasing agent (Bakke *et al*., 2007). Consequently, due to it use in many processes in both factories and laboratories, most trichloroethylene is released into the environment following use and has been reported as a major industrial pollutant that has been identified as

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an environmental contaminant of groundwater, surface water and soil (Spencer *et al.*, 2006; Seo *et al.*, 2011). TCE is known for its many toxic effects such as cardiac effects, pulmonary toxicity, neurotoxicity and probably genotoxicity (Lock and Reed 2006). The target organs for the systemic effects of this chemical are the liver and kidneys (Environmental Protection Agency, 1979) Inhalation is the main route of exposure to trichloroethylene while less common routes are ingestion and dermal contact (Seo *et al.*, 2011). Many studies of trichloroethylene in experimental animals indicated the induction of cancer in several organs such as the kidneys, liver, lung, testes, and lymph nodes (Fisher and Allen, 1993). Although several studies have demonstrated the toxicity of TCE in a variety of systems, the occurrence of genotoxic and carcinogenic effects in humans is still arguable (Kumar, 2009; Karami *et al.*, 2012; Hansen *et al.*, 2013; Vlaanderen *et al.*, 2013). The increased incidence of chromosome abnormalities such as breaks, gaps, deletions and hyperploidy have been observed in the lymphocytes of exposed workers (Lavin, 2000). The results of other studies also suggested the absence of sister chromatid exchanges when the subjects were exposed to trichloroethylene (Brandom *et al.*, 1990; Seiji *et al.*, 1990). In contrast, according to the study of Nagoya *et al.* (1989), there was no incidence of sister chromatid exchanges in TCE-exposed workers.

The micronucleus is the small nucleus that arises from acentric chromosome fragments or whole chromosomes which are not incorporated into one of the daughter cells during mitotic cellular division (Zolzer *et al.*, 2011). A micronucleus test could be used as a powerful tool for the detection and quantification the genotoxic risk as this method is faster and easier than metaphase analysis and it can be used either *in vitro* or *in vivo* (Kirsch-Volders *et al.*, 2011; Sudha *et al.*, 2011). The micronucleus frequency in peripheral blood lymphocytes is determined using cytokinesis-block micronucleus assay (CBMN) relying on the observation that cells that have completed nuclear division have their cytokinesis blocked with cytochalasin-B. The most important aspect of this method is the identification of cells that have completed one round of nuclear division and are capable of expressing chromosome damage as micronuclei. This assay has been shown as a reliable and sensitive cytogenetic biomarker for monitoring genetic instability in humans. The purpose of this study was to determine the micronucleus frequency using CBMN assay in workers occupationally exposed to trichloroethylene and in normal healthy subjects. The exposure to trichloroethylene was determined by urinary trichloroacetic acid concentrations. The association of confounding factors such as age, smoking, and alcohol consumption was also analyzed.

MATERIALS AND METHODS

Chemicals

Cytochalasin B, phosphate buffer saline and dimethyl sulfoxide were purchased from Sigma Aldrich, USA. Giemsa stain, RPMI 1640 medium, phytohemagglutinin, L-glutamine, streptomycin, penicillin and fetal bovine serum were purchased from Gibco Invitrogen Corporation, USA. Potassium chloride (KCL), methanol and glacial acetic acid were purchased from Lab-Scan, Poland.

Population study

A cross sectional survey for cytogenetic monitoring was carried out in the watch industry using three factories, located in Bangkok, Thailand where trichloroethylene was the only organic solvent that the factory workers were exposed to. This chemical was used as a degreaser for cleaning the surface of metal parts. The study population consisted of 127 individuals classified according to their exposure characteristics into two groups. The first group consisted of 64 workers occupationally exposed to TCE. The second group consisted of 63

non-exposed control employees, working mainly in office jobs in the same factories. All subjects provided their informed consent before the study and the Ethical Review Committee for Research, Department of Disease Control approved the study. Each participant in the exposed group and the control was personally informed of the study's aims and interviewed with a standardized questionnaire to provide personal data and to determine each person's history of smoking, drinking, recent illness and medical treatment. The characteristics of the exposed subjects and those in the control (gender, age, duration of exposure, smoking and alcohol consumption) are shown in Table 1.

Sample collection

A sample of 5 mL of venous blood was obtained from each subject during an 8 hr work shift, using a heparinized vacuum blood tube. The samples were taken avoiding possible bias and to assure subject confidentiality and were stored at 4°C. Where possible, specimens were sent to the laboratory within a few hours and were processed immediately. Urine samples (about 15-20 mL) were obtained from each subject at the end of the work shift using sterilized containers and stored at -20°C until analyzed within 1 mth.

Sample analysis

The blood samples were analyzed using CBMN assay according to Fenech and Morley (1985). A lymphocyte culture was set up by adding 0.5 mL whole-heparinized blood in 5 mL of RPMI 1640 supplemented with 10% fetal bovine serum, 2% L-glutamine, 1% antibiotics (100 $\mu g.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin) and 3% phytohemagglutinin. The cultures were incubated at 37°C for 44 hr, then cytochalasin B (final concentration 3 $mg.mL^{-1}$) was added for inducing binucleated cells. At 72 hr incubation, the cultures were harvested by centrifugation at 1,000 rpm for 10 min. After centrifugation, cultures were briefly treated with a hypotonic solution (0.075 M KCl). In further processing, cells were then fixed twice in cold fixative solution (3:1 absolute methanol:glacial acetic acid) for 20 min at room temperature, and after this step, the cell pellet was gently resuspended in a few drops of fresh fixative solution.

For microscopic observation, the slides were prepared by carefully dropping cell suspension onto clean and cold slides. Finally, the cells were air dried and stained with Giemsa. To determine the frequency of binucleated cells with micronuclei, 1,000 binucleated lymphocytes with clearly visible cytoplasm were scored under a light microscope for each subject. Micronuclei were evaluated according to the criteria of Fenech *et al.* (2003), using 400 × magnification for detection and 1,000 × magnification for confirmation.

Urinary TCA determination was performed by gas chromatography with an electron capture detector (GC-ECD; HP 6890; Hewlett Packard; Palo Alto, CA, USA) using a modified HS technique (Christensen *et al.*, 1988). A sample of 1.0 mL of urine was added to a glass-capped bottle, then put in a head-space sampler which was already set to the optimum program and injected into the GC at 250°C with an electron capture detector at 300°C (column: HP-FFAP polyethylene glycol TPA, split mode 10:1) and then exposed to an oven temperature of 80°C for 12 min. The quantity of urinary TCA was analyzed under the relative intensity of the chromatographic signal for 12 min. The limit of detection of TCA was 0.139 $mg.L^{-1}$and the average coefficient of determination (R^2) was 0.99972.

Statistical analysis

The data were analyzed using the SPSS 11.0 program for Window (SPSS Inc.; Chicago, IL, USA). The results were recorded as the mean and standard error for each group. Student's *t*-test for independent samples was used to detect the possible differences in the mean of the micronucleus assay between the exposed and control groups. Correlation analysis was

performed using Pearson's correlation test with the test level for significance set at $P < 0.05$.

RESULTS AND DISCUSSION

The frequency of micronuclei based on different parameters such as gender, age, alcohol consumption and smoking was studied in 64 TCE exposed workers and in 63 normal, healthy controls. The main characteristics of the two groups studied and the results of urinary TCA are presented in Table 1. The age, gender, alcohol consumption and smoking status distributions were similar among the exposed workers and controls. The exposed workers had a mean duration of employment of 14.27 ± 12.49 yr (range 1–39 yr). The level of exposure to TCE was assessed by a biomarker of exposure (urinary TCA). The mean urinary TCA concentration of the exposed group and the control group was 16.10 ± 3.57 and 6.21 ± 4.87 $mg.L^{-1}$, respectively. A highly significant difference between the exposed workers and controls with regard to the TCE biomarker was observed ($P = 0.001$).

The average micronucleus frequency in exposed workers and controls was 5.53 ± 0.71 and 3.65 ± 0.34 per 1,000 cells, respectively. The results (Table 2) showed that exposed workers had a highly significant induction of micronuclei when compared with controls ($P = 0.01$).

Among the smokers, a higher frequency of micronuclei was found in the exposed workers with respect to controls (3.73 ± 0.16 versus 2.33 ± 0.84, respectively), but this difference was not significant ($P = 0.298$). The mean micronucleus frequency observed in non-smokers was significantly higher in exposed workers than in controls (5.91 ± 0.83 versus 3.79 ± 0.35, $P = 0.03$). The average micronucleus frequency in the exposed and control groups with regard to drinking was 6.31 ± 1.29 and 3.33 ± 0.57, respectively. The comparison of micronucleus frequencies between these two groups revealed a significant difference ($P = 0.05$). However, when the micronucleus frequency was compared between the alcoholic and nonalcoholic groups in both the exposed (6.31 ± 1.29 versus 5.12 ± 0.85, respectively) and control groups (3.33 ± 0.57 versus 3.81 ± 0.42, respectively), the results showed no significant difference. In order to examine the effect of age, exposed workers and controls were stratified into two age groups of less than 40 yr and 40 yr and

Table 1 General characteristics of the exposed and control subjects.

Parameter	TCE-exposed workers	Control subjects	*P*–value
Number of subjects	64	63	
Gender			
Male	18 (28.1%)	21 (33.3%)	0.528
Female	46 (71.9%)	42 (66.7%)	
Age (yr)			
(Mean±SD)	35.27±12.25	35.22±12.01	0.985
Duration of exposure (yr)			
(Mean±SD)	14.27±12.49	-	
Smoking status			
Smoking	11 (17.2%)	6 (9.5%)	0.902
Non-smoking	53 (82.8%)	57 (90.5%)	
Alcohol			
Drinking	22 (34.4%)	21 (33.3%)	0.208
Non-drinking	42 (65.6%)	42 (66.7%)	
Urinary TCA($mg.L^{-1}$)	16.10±3.57	6.21±4.87	0.001

TCE = Trichloroethylene; TCA = Trichloroacetic acid.

older (Table 2). An age-dependent increase in the micronucleus frequency was noted in the exposed group (4.23 ± 0.60 versus 7.56 ± 1.49, $P < 0.05$).

Pearson's coefficient correlation was used to investigate the association of age, gender, smoking status, alcohol consumption, duration of work and urinary TCA in determining the frequency of micronuclei in exposed workers. Correlation analyses (Table 3) pointed to a significant positive association of urinary TCA with the level of micronuclei among the exposed workers *(*correlation coeeficient (*r*) = 0.682, *P* = 0.001). In addition, the results suggested a weak positive correlation between the micronucleus frequency and age, and the duration of exposure of workers *(r* = 0.263, *P* = 0.036 *and r* = 0.251, *P* = 0.045, respectively). No significant correlation was evident with any of the other confounding factors studied,.

In an occupational setting, workers directly involved in manufacture, especially in the degreasing process, may be exposed to

Table 2 Micronucleus frequency in lymphocytes of exposed and control subjects.

Parameters	TCE-exposed workers	Control subjects	*t*–test[b] (*P*–value)
Average of micronuclei[a]	5.53±0.71	3.65±0.34	0.010
Gender			
Male	5.61±0.85	3.15±0.55	0.040
Female	5.50±0.94	3.90±0.41	0.210
Age			
< 40	4.23±0.60	3.48±0.39	0.299
≥40	7.56±1.49	4.00±0.61	0.034
Smoking status			
Smoking	3.73±0.16	2.33±0.84	0.298
Non-smoking	5.91±0.83	3.79±0.35	0.030
Alcohol drinking habit			
Drinking	6.31±1.29	3.33±0.57	0.050
Non-drinking	5.12±0.85	3.81±0.42	0.230

[a] = Number of cells with micronuclei per 1,000 binucleated cells.

[b] = Comparison of average micronucleus frequency between exposed and control groups.

TCE = Trichloroethylene.

Table 3 Pearson's correlation coefficient for micronucleus frequency with age, gender, smoking status, alcohol consumption and urinary trichloroacetic acid (TCA) in exposed workers.

Parameter	Micronucleus frequency	
	Correlation coefficient (r)	*P*–value
Gender	0.009	0.945
Age	0.263	0.036[a]
Duration of work	0.251	0.045[a]
Smoking status	0.145	0.252
Alcohol consumption	0.101	0.428
Urinary TCA	0.682	0.001[b]

[a] = Correlation is significant at the 0.05 level (2-tailed).

[b] = Correlation is significant at the 0.01 level (2-tailed).

considerably higher levels of TCE than the general population. The toxicity of TCE in occupational settings has been essentially focused on the metabolite forms of TCE which are dichloroaceic acid (DCA) and trichloroacetic acid (Guha*et al.*, 2012). The International Agency for Research on Cancer has evaluated TCE solvent as a potential carcinogenic agent to humans, though the risk involved in chronic exposure is uncertain. The metabolite form of TCE which occurs in the human body after inhalation of TCE may directly react with the genetic material and has also been shown to generate oxidative damage *in vitro* (Toraason *et al*., 1999)

A cytogenetic biomarker such as a micronucleus assay is one of the preferred methods used to study the impact of environmental, occupational and medical factors on genomic stability and is used to evaluate the end point in human biomonitoring. Analysis of lymphocytes according to the CBMN method could provide evidence of many nuclear abnormalities such as binucleate cells (presence of two nuclei in a cell), karyorrhexis (Revazova *et al.*, 2001; Fenech, 2007; nuclear fragmentation) chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), necrosis and apoptosis (Çelik *et al*., 2003). This method has received increased attention as a simple, sensitive and rapid assay for assessing chromosome damage in various cell types (Ceppi *et al.*, 2010; Bull*et al.*, 2011; Sudha *et al.*, 2011).

An elevated micronucleus frequency in the lymphocytes of TCE-exposed workers compared to the controls was found in the present study which could be the consequence of continuous absorption of chemical genotoxic substances. Wang *et al*. (2001) reported an increase in the frequency of micronuclei in the CHO-K1 cells after exposure to vaporous TCE. Similarly, micronucleus induction observed in lymphocytes exposed to TCE was also reported by Kumar *et al.* (2002). However, the conclusive data on the genotoxicity of TCE in humans is still conflicting in many reports, as according to the study of micronucleus frequency in populations occupationally exposed to genotoxic agents, the influence of smoking and drinking habits on micronucleus production has differing results, as some studies have shown evidence of an association (Dittberner *et al.*, 1997; El-Zein *et al.*, 2006; Nefi *et al.*, 2013) but other studies have not (Bolognesi *et al.*, 2002; Lima *et al.*, 2010; Sellappa *et al.*, 2010). The current study showed that workers with a history of smoking and drinking and exposure to TCE have significantly higher frequencies of micronucleus induction compared to the controls, with revealed evidence of cytogenetic damage in these individuals. Several studies have reported age as the most demographically variable factor involved in the enhanced frequency of micronuclei, especially in workers exposed to chemicals (Wojda*et al.*, 2007; Sudha *et al.*, 2011; Gentile *et al.*, 2012). The current data with respect to the number of individuals providing increased numbers of micronuclei associated with increased age because the aging process is related to a progressive elevation in spontaneous chromosome instability and the loss of efficiency in DNA repair mechanisms, resulting in the accumulation of genetic lesions (Wojda *et al.*, 2007).

The current study determined urinary TCA as a biomarker of exposure and the frequency of micronuclei in lymphocytes was counted as an indicator of chromosome and DNA damage in terms of the CBMN test. A high, positive correlation was found between the micronucleus frequency and urinary TCA concentration ($R = 0.682$, $P = 0.001$) of exposed workers, indicating that DNA damage is directly proportional to TCE exposure. In addition, the current findings on urinary TCA showed highly significant ($P = 0.001$) and greater levels in exposed workers than in the control. This could be due to the fact that the workers were directly exposed to TCE via different routes such as ingestion and dermal absorption, but

the most likely route of exposure is by inhalation (Bakke *et al*., 2007). It is possible that this could have long term effects on human health as upon entering blood circulation it exerts cytotoxic and genotoxic properties.A weak positive correlation was found between the duration of occupational exposure to TCE (years of exposure) and micronucleus frequency *(r* = 0.251, *P* = 0.045). A similar result was observed in dry cleaning workers who were exposed to perchloroethylene (Everatt *et al*., 2013).

CONCLUSION

The study revealed an increased micronucleus frequency in lymphocytes of TCE-exposed workers compared to the controls. There was no correlation of the micronucleus frequency with gender, smoking and alcohol consumption. However, significant correlations between micronucleus induction and urinary TCA concentration, the duration of working in the factory and the age of exposed workers were observed. These results indicated that long term exposure to a high dose of TCE may induce chromosome breakage and DNA damage. The association of these cytogenetic effects with TCE exposure gives important information to the risk assessment process and may also be used to assess health risks for exposed groups. In conclusion, the detection of chromosomal damage induced by TCE and its metabolites in peripheral lymphocytes by means of CBMN assay offers a potential biomarker applicable in occupational health investigations.

Further research to measure the level of environmental contamination of TCE in different factory settings is needed in order to accurately evaluate workers' exposure to these chemical agents. Moreover, preventive and protective measures must be applied to reduce occupational exposure to TCE, and subsequently, to prevent adverse effects on workers' health.

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