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Study of genetic polymorphism in solvent exposed population and its correlation to *in vitro* effect of trichloroethylene on lymphocytes

Munish Kumar[1], Shikha Tewari[1], Priti Sharma[1], Vipin Kumar Verma[1], L.K.S. Chauhan[2], Sudhir Kumar Agarwal[3], U.N. Dwivedi[3] and Sudhir Kumar Goel*[1]

[1]Petroleum Toxicology Division, [2]Cell Biology Section, Indian Institute of Toxicology Research, Lucknow - 226 001, India
[3]Department of Biochemistry, Lucknow University, Lucknow - 226 007, India

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Abstract: *Trichloroethylene (TCE) is major industrial pollutant that contaminate environment. Its exposure may lead to hepato-renal toxicity along with the cancer progression. Although extensive research is done on its toxicity, still not much is known about its genotoxic potential on humans in relation to genetic polymorphism. Cytochrome P450 (CYP P-450) and glutathione-S-transferases (GSTs) are important in cellular detoxification of TCE. Variations in gene sequences result in population specific regional genetic variations (polymorphism). Genotyping of CYP1A1, GSTM1, GSTT1 and GSTP1 polymorphism was performed in 220 normal and 97 solvent-exposed individuals from northern part of India using real time PCR, PCR and restriction digestion techniques. The parameters examined to study genotoxicity were chromosomal aberration (CA) and cytokinesis block micronucleus assay (CBMN) in lymphocyte culture in vitro. The observed average frequencies for GSTM1 (null) and GSTT1 (null) were 41, 22 and 12.7%, respectively in normal subjects whereas frequencies of CYP1A1/GSTP1 with (ile/ile) or (ile/val) or (val/val) were found to be 76.2/52, 21.4/42.1 and 2.4/5.9% respectively. It was further observed that the frequencies of above genes were found to be similar in solvent exposed groups. The distribution frequencies of GST genes, when compared with other reports from various regions of India show variations. In vitro TCE exposure (2, 4 and or 6 mM) did not show any significant genotoxic effect. TCE may be toxic due to its metabolite.*

Key words: *Genotoxicity, TCE, Lymphocytes, CYP1A1, GSTP1, Real time PCR*
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Introduction

Solvents are a distinct group of substances characterized by their ability to dissolve oils, fats, plastics, rubber and resins. Many of these solvents are known to cause cancer either as such or through their metabolic activation by CYPs and GSTs. The cytochrome P-450 1A1 gene (*CYP1A1*) is part of a super gene family located at human chromosome 15q22-q24 (Hildebrand *et al.,* 1985). To date, at least five polymorphic sites have been identified in the *CYP1A1* gene and two of these have been extensively studied in relation to cancer susceptibility. Substitution of A4889G in exon 7 produces an Isoleucine/Valine (*ile/val*) exchange at codon 462 and a T6235C substitution in the 3' non-coding region (Sobti *et al*., 2004). Based on sequence homology and immunological cross-reactivity, human cytosolic GSTs have been grouped into seven families, designated GST Alpha, Mu, Pi, Sigma, Omega, Theta, and Zeta (Pemble and Taylor, 1992; Board *et al*., 2000). The GSTs have been presumably arisen from a single common ancestor and their substrate specificity and diversity have been reshaped by gene duplication, gene recombination, and an accumulation of mutations (Rebbeck, 1997; Xu *et al*., 1998; Dunning *et al*., 1999). The single GSTP1, which is known as GSTpi, gene located at 11q13 is 2.8 kb long and contains seven exons (Kano *et al*., 1987). Several single nucleotide polymorphisms (SNPs) have been described in the GSTP1 gene, out of these two arise by amino acid substitutions in codons 104 (*ile/val*) and 113 *(ala/val*) at exons 5 and 6. (Ali-Osman *et al*., 1997).

Trichloroethylene (TCE) is a chlorinated aliphatic hydrocarbon used extensively in industrial dry cleaning, for the production of printing ink, paint and textile printing. It is also used in the food extraction process as well as a metal degreaser in industries such as watch factory and lock industries *etc*. Due to its use in various processes, it became a common environmental contaminant. Main site of its contamination is ground water and water (Spencer *et al*., 2006). TCE is major environmental contaminant and is known for many toxic effects such as neurotoxicity and cancers. Occupationally exposed workers were having chromosomal abnormalities, specifically breaks, gaps, deletions, inversions, translocations and hyperploidy (ATSDR, 1997). Another study reported that both male B6C3F1 mice and Sprague-Dawley rats exhibited hepatic cell DNA damage in the form of single-strand breaks after oral exposure to TCE (Nelson and Bull, 1988). Exposed up to 5,000 ppm TCE for 6 hr showed no aberrations in sister chromatid exchange or induction of micronuclei in erythrocytes (Kligennan *et al*., 1994). Cigarette smoking and TCE exposure may act synergistically to increase the rate of sister chromatid exchange (Seiji *et al*., 1990). In a similar investigation of sister chromatid

* **Corresponding author:** sudhirg2k2@rediffmail.com

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exchange, negative results were obtained for both smokers and non-smokers exposed to TCE (Nagaya *et al*., 1989). Present study was planned to see the genetic polymorphic status in population consisting of control (not exposed to any solvents) and workers from laundry and metal degreaser industry (exposed to solvents). Further, to understand how the polymorphic status of individuals might affect the toxic/genotoxic potential of TCE, we planned *in vitro* study for assessing the chromosomal aberration (CA) and micronucleus (MN) induction by selecting the null genotype individuals, and incubating their blood with different concentrations of TCE.

Materials and Methods

The Institutional Ethical Committee approval was obtained to conduct the present study and, the selection of individuals was made as per the standard questionnaire in consultation with the statistician at IITR, Lucknow. Blood (in EDTA vials) was collected from all the subjects for the study.

Selection of normal individuals: The study group consisted of 220 young normal individuals who were resident of north India and knowingly unexposed to any kind of solvents. The criteria of selection was based on the fact that persons were asked whether they had any exposure to any type of solvent in lifetime or not or worked in any laundry, factory where solvents were used. The consumption of tobacco in any form (cigarette/bidi smoking, chewing tobacco in beetle leaf, pan-masala/gutka), use of alcohol, was noted through a detailed questionnaire.

Selection of solvent exposed individuals: The study group consisted of 97 individuals in the city of Lucknow from laundry and industry where TCE is being used as metal degreaser. Ethnic origins of all the subjects examined were same as compared to the control subjects. Criteria for the selection was based again on the questionnaire. All the subjects were put to medical checkup, their length of service in the laundries and metal degreaser workshop were taken as per questionnaire.

CYP1A1 gene polymorphism: Real time PCR using fluorescent probes as described earlier (Basham *et al*., 2001), analyzed CYP1A1 polymorphism. Isolated DNA (25 ng) was amplified in a total volume of 20 µl reaction mixture containing 6 µM of each of the primers and 150-300 nM of probes.

Forward primer 5'-GCATGGGCAAGCGGAA-3'
Reverse primer 5'- GCCAGGAAGAGAAAGACCTCC-3'
FAM-TCGGTGAGACCATTGCC CG-BHQ1
HEX-CCGTGAGACCGTTGCCGC-BHQ1

Each set of reaction included both positive DNA for allele A labeled with Hexachloro-6-Carboxy-fluorescine (FAM) and allele B labeled with 6-carboxy-x-Rhodamine (HEX) fluorescent dye. The quencher in both probes used was BHQ1. PCR reaction was carried out in Real time PCR machine (Stratagene, Mx3000 p). PCR cycling conditions were: initial denaturation at 94°C for 10 min. followed by 40 cycle of denaturation at 92°C for 15 sec., annealing and extension at 60°C for 1 min.

GSTM1 and GSTT1 gene polymorphism: Analysis for GSTM1 and GSTT1 gene polymorphism was done by multiplex PCR as described earlier (Abdel-Rehman *et al*., 1996). Isolated DNA (100 ng) was amplified in a total volume of 25 µl reaction mixture containing 10 pmol of each of the following primers.

GSTM1: Forward, 5'-GAACTCCCTGAAAAGCTAAAGC-3'
Reverse, 5'-GTTGGGCTCAAATATACGGTGG-3'

GSTT1: Forward, 5'-TTCCTTACTGGTCCTCACATCTC-3'
Reverse, 5'-TCACGGGATCAT GGCCAG CA- 3'

Exon 7 of CYP1A1 genes were co-amplified and used as an internal control using following primers

Forward, 5'-GAACTGCCACTTCAGCTGTCT-3' and
Reverse, 5'CAGCTGCATTTGGAAGTGCTC-3'.

Each set of reaction included both positive and negative controls. The multiplex PCR method was used to detect the presence or absence of the GSTT1 and GSTM1 genes in the genomic DNA samples. Genomic DNA (150 ng) was added to a PCR mixture, composed of 10 pmol of each primer, 200 µmol dNTPs, 1.5 units of Taq polymerase and 2.5 µl of 10X PCR buffer in a total volume of 25 µl. PCR reaction was carried out in PTC-100 thermal cycler (MJ Research, USA) and cycling conditions were as follows: Initial denaturation: 94°C for 5 min, followed by denaturation 94°C for 1 min, annealing 59°C for 1 min and extension 72°C for 1 min for 35 cycles and final extension 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. DNA from samples positive for GSTM1 and GSTT1 genotypes yielded bands of 215 and 480 bp respectively while the internal positive control (CYP1A1) PCR product corresponded to 315 bp.

GSTP1 gene polymorphism: Polymorphism was analyzed using a previously described PCR-RFLP method (Harries *et al*., 1997). Briefly, amplification was conceded using primers:

Forward, 5'-ACCCCAGGGCTCTATGGGAA-3'
Reverse, 5'-TGAGGGCACAA GAAGCCCCT-3'.

PCR cycling conditions were as follows: Initial denaturation: 95°C for 5 min, followed with denaturation 94°C for 30 second, annealing 58°C for 45 sec for 35 cycles and final extension 72°C for 10 min. The 176-bp amplified product was digested with *Alw261* (Fermentas, USA) for overnight at 37°C and electrophoresed in 3% agarose gel. Presence of restriction site resulted in two fragments (91 and 85 bp) indicating mutant allele (*val/val*) (G/G), and if A/G (*ile/val*) polymorphism incurred then it resulted in three fragments of 176, 91 and 85 bp respectively.

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Lymphocyte cultures and chromosome aberration assay: The method for peripheral human lymphocyte culture was followed as described earlier (Verma and Babu, 1995). To begin with 0.5 ml blood (collected in heparin) obtained by veni-puncture was added in 4.5 ml of complete media RPMI-1640. To stimulate the cell division 100 µl of PHA-(M) was also added (10 µg ml^{-1}). Culture tubes were left in CO_2 incubator (5%) fixed at 37 ^{0}C. After 24 hr cultures were exposed to test compound. TCE at 2,4 and 6 mM for 48 hr. The cultures were treated with colchicine (6 µg ml^{-1}) at 70 hr and harvested at 72 hr by centrifuging the cells at 1000 x rpm for 10 min. Pellets were dispersed in KCl (0.56%) for 20 min at 37°C for hypotonic treatment and culture vials were re-centrifuged. Obtained pellet was fixed gently in freshly prepared Carnoy's solution. Washed 2-3 times till pellet becomes white and left it at 4°C overnight. Slides were prepared by air-dry method and stained with Geimsa (10%). The slides were analyzed at 100 x magnification using a Nikon Elipse, (50i, Japan) microscope, and minimum of 100 well spread metaphases were scored from each subject. Ethyl methanesulfonate (EMS), a mutagen was used as positive control.

Cytokinesis-block micronucleus assay in human lymphocytes: CBMN assay was followed as described by Fenech *et al.* (2003). In brief, blood samples collected in heparinized syringes and the cultures were set up as described for CA. Cytochalasin-B (6 µg ml^{-1}) was added to the culture tubes at 44 hr. After 72 hr of incubation, cells were centrifuged and pellet was dispersed in 0.56% KCl and left for hypotonic shock at room temperature (25±2°C) for 5 min. The scoring was carried out at 40 x magnifications in Nikon Elipse (50 i Japan) microscope. Cells from mononucleated to polynucleated were scored to determine cytokinesis-block proliferation index (CBPI). CBPI is expressed as percentage of dividing cells in the cells population.

Statistical analysis: Statistical analysis was done with SPSS software 12.00. Binary Logistic Regression Model assessed difference in genotype prevalence and association between case and control group. Uni-variant and multi-variant analysis, correlation coefficient, odds ratio (OR) and its 95% confidence interval (CI) were used to describe the strength of association. Odds ratios and its 95% confidence interval were obtained by summarizing data over two habit strata (tobacco users/ non-users) and four age strata in Logistic Regression Model. p-value <0.05 was considered as statistically significant.

Results and Discussion

Table 1 shows a comparative chart of different genes (CYP1A1, GSTM1, GSTT1 and GSTP1) showing percentage frequency in control and solvent exposed population of northern part of India. The distribution of frequencies for GSTM1/GSTT1 (null genotypes) and GSTP1 genotype was determined both in males and females. The frequencies of GSTM1 (null) genotypes were found to be 40.93 and 41.35% whereas, GSTT1 (null) 21.47 and 22.45% in males and females, respectively. However, frequencies of homozygous deletions for both the genes (GSTM1 and GSTT1) were found to be 13.42 and 12.06% in males and females, respectively. Further, the frequency of GSTP1 with (*ile/ile*), (*ile/val*) or (*val/val*) was 51.9, 43.2 or 6.2% in males and 52.1, 41.1 or 5.7% in females respectively. In case of solvent exposed where 97 samples were used for genotyping the frequencies of GSTs were found to be similar to the control subjects, however, the frequency of GSTM1 (null) and GSTT1 (null)) were 39.2 % and 21.6 % respectively. The frequency of GSTP1 with (*ile/ile)*, (*ile/val)* or (*val/val*) was 54.6, 40.2 and 5.2 % respectively. The frequency of CYP1A1 in control population with (*ile/ile*), (*ile/val)* or (*val/val*) was 76.2, 21.4 and 2.4% respectively. Solvent exposed subjects have frequency of 83.5, 13.4 and 3.15% respectively.

The results of MI and CA analysis in the lymphocytes exposed *in vitro* to TCE are summarized in Table 2. Treatment groups showed inhibition of the MI. Individuals with GSTM1, GSTT1 and GSTP1 (*ile/ile)* genotypes revealed no differences in MI compared to GSTM1/GSTT1 (null) and GSTP1 (*val/val).* TCE exposure does not induce any significant CA in any of the treatment groups compared to positive compound (EMS) (Fig. 1,2). However, some CA in rare case was obtained at higher concentration treatment of TCE. Micronucleus induction observed in the lymphocytes exposed to TCE is summarized in Table 3. All the test samples, including control group, showed 35-41% binucleate cells. Induction of micronucleus formation was not found to be significant in both the groups irrespective of genotypes. In few cases, MN was induced at higher concentration of TCE but it is statistically not significant (Fig. 3, 4). The subjects with GSTM1/GSTT1 and GSTP1 (*ile/ile)* genotypes did not show any significant change in frequency of MN comparison to GSTM1/GSTT1 (null) and GSTP1 (*val/val*) genotypes. The CBPI index was found to be constant in all the treatment group of TCE.

The distribution frequency of CYP1A1 genotype was found to be similar to previous report from north India (Sobti *et al*., 2003). The distribution frequencies of GST genes, when compared with

Table - 1: Comparative chart of different genes (CYP1A1, GSTM1, GSTT1and GSTP1) showing percentage frequency from control (220) and solvent (97) exposed subjects of northern part of India

Genotype	Control (%)	Solvent (%)
CYP1A1 (ile)	76.2	83.5
CYP1A1 (ile)	21.4	13.4
CYP1A1 (Val)	2.4	03.1
GSTP1 (ile)	52	54.6
GSTP1 (ile)	42.1	40.2
GSTP1 (ile/Val)	05.9	05.2
GSTM1 (null)	41.1	39.2
GSTT1 (null)	21.9	21.6

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Table - 2: Mitotic index and chromosome aberrations in lymphocytes of individual with GSTM1, GSTT1 and GSTP1 genotypes following 48 hr *in vitro* exposure of trichloroethylene

Concentrations of TCE (mM)	Genotype codes	Mitotic index[a]	Fragments	Chromatid break	Gaps[b]	Aberrations (Aberrant cells)	% Aberrations (Aberrant cells)
DMSO (0.3%)	A	6.3±0.42	- -	2(2)	2(1)	2(2)	1.36(1.36)
	B	6.1±0.85	- -	2(2)	2(2)	2(2)	1.26(1.26)
	C	5.9±0.17	- -	1(1)	1(2)	1(1)	0.82(0.82)
	D	5.7±0.5	- -	2(2)	2(2)	2(2)	1.31(1.31)
2	A	5.8±0.11	1(1)	1(1)	- -	2(2)	1.23(1.23)
	B	5.6±0.25	1(1)	1(1)	- -	2(2)	1.31(1.31)
	C	5.9±0.42	- -	- -	1(1)	1(1)	0.60(0.6)
	D	5.8±0.20	- -	1(1)	2(2)	1(1)	0.53(0.53)
4	A	6.1±0.23	1(1)	1(1)	3(3)	2(2)	1.43(1.43)
	B	6.4±0.31	- -	1(1)	- -	1(1)	1.25(2.12)
	C	6.3±0.06	- -	2(2)	2(2)	2(2)	1.56(1.53)
	D	5.8±0.09	1(1)	- -	- -	1(1)	0.86(0.86)
6	A	5.6±0.29	- -	2(2)	2(2)	2(2)	1.16(1.16)
	B	5.5±0.04	2(2)	- -	1(2)	2(2)	1.23(1.23)
	C	5.9±0.42	- -	1(1)	1(2)	1(1)	0.79(0.82)
	D	5.7±0.24	1(1)	- -	1(1)	1(1)	0.80(0.80)
EMS (240 µg ml^{-1})		1.2±0.21***	4(5)	8(6)	9(7)	12(11)	9.45***

[a]Expressed as mean ± SE, two-way ANOVA; ***p<0.001 compared to DMSO, chromatid gaps scored but excluded from total aberrations, A and B represent GSTM1, GSTT1 present and GSTP1 (ile) genotype, C and D represent GSTM1, GSTT1 null and GSTP1 (Val/Val) genotype (comparison between groups and genotype showed no significance), EMS = Ethyl methanesulfonate

Table - 3: Micronucleus induction in lymphocytes of individuals with GSTM1, GSTT1 and GSTP1 genotypes following 48 hr *in vitro* exposure to trichloroethylene

Concentrations of TCE (mM)	Genotype codes	% BN cells	MNBN/ BN	% MNBN	MNPN/ PN	% MNPN	CBPI
DMSO (0.3%)	A	40.3	12/1234	0.97	3/1165	0.26	1.86
	B	38.9	8/1052	0.76	2/1036	0.19	1.83
	C	37.1	9/1098	0.82	4/1209	0.33	1.94
	D	37.6	10/1131	0.88	3/1198	0.25	1.91
2	A	39.2	25/1225	2.04	06/1151	0.52	1.87
	B	38.5	31/1315	2.35	06/1087	0.55	1.83
	C	37.3	26/1191	2.18	07/1208	0.58	1.82
	D	39.3	23/1093	2.10	05/1031	0.49	1.79
4	A	41.2	25/1212	2.06	4/1050	0.38	1.83
	B	39.1	23/1102	2.08	07/1189	0.58	1.82
	C	37.7	30/1293	2.32	05/1108	0.58	1.87
	D	39.1	24/1093	2.19	08/1315	0.61	1.88
6	A	38.1	32/1185	2.70	07/1126	0.62	1.78
	B	38	32/1119	2.86	09/1398	0.62	1.77
	C	36.5	35/1362	2.57	09/1284	0.70	1.81
	D	35.6	29/1243	2.34	06/1066	0.56	1.83
EMS		23.8	134/1089	12.3***	39/1023	3.8***	1.21

A and B represents GSTM1, GSTT1 present and GSTP1 (ile) genotype, C and D represent GSTM1, GSTT1 null and GSTP1 (Val/Val) genotype MNBN Micronucleated binucleate cells, MNPN - Micronucleated polynucleate cells, PN - Polynucleate cells, BN - Binucleate cells, CBPI - Cytokinesis-block proliferating index, EMS = Ethyl methanesulfonate. ***p<0.001 compared to DMSO (Comparison between groups and genotype showed no significance)

other reports from various regions of India show variations. The frequency of homozygous deletions (null genotype) for GSTM1 and GSTT1 was found to be 22.4 and 17.6% in southern part whereas 54 and 13% in eastern part respectively (Vettriselvi *et al*., 2006; Ghosh *et al*., 2006). The frequency of GSTP1 (*val/val)* has been reported to be 3.1% in south Indians and 2.7% in eastern part of India. Present study revealed 41% GSTM1 (null), 21.5% GSTT1 (null) and 5.9% GSTP1 (*val/val)* determined in 220 subjects of north

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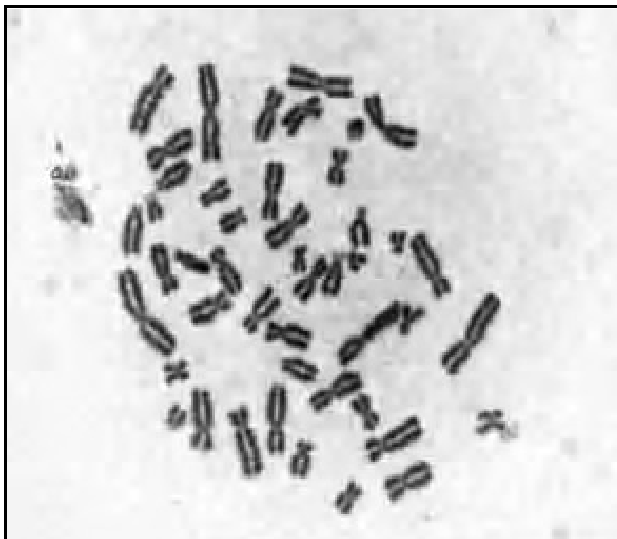

Fig. 1: Metaphase chromosomes of lymphocyte exposed to TCE (2 mM) showing no structural changes

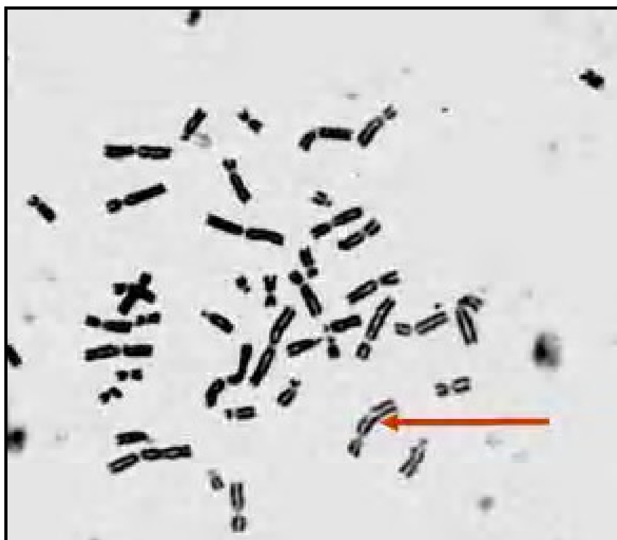

Fig. 2: Chromosome break in lymphocyte treated with TCE (4 mM)

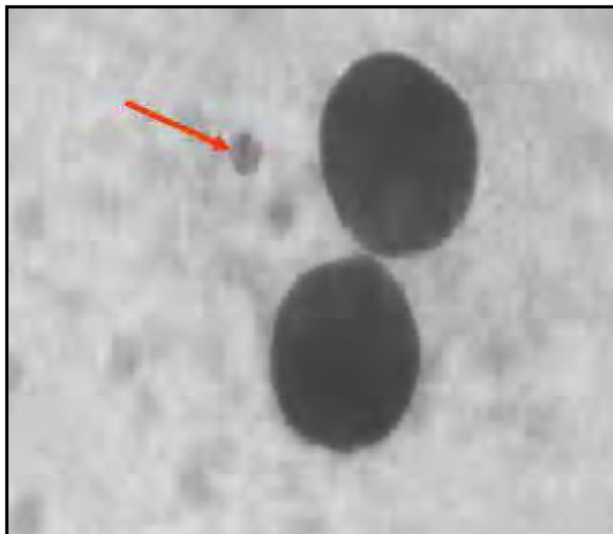

Fig. 3: Induction of micronucleus in human lymphocyte treated with TCE (4 mM)

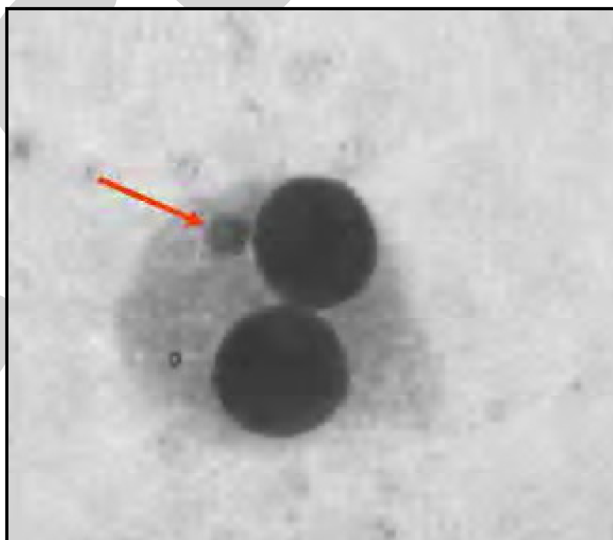

Fig. 4: Induction of micronucleus in human lymphocyte treated with TCE (6 mM)

Indian population showing variation in Indian population residing in different parts of the country (Mishra *et al*., 2005; Vettriselvi *et al*., 2006; Ghosh *et al*., 2006; Naveen *et al*., 2004; Jain *et al*., 2006).

The frequency of GSTP1 (*val/val*) genotype observed in our study, was comparable to the earlier report published from north India (Mishra *et al*., 2005). Sample size, life style, origin and environmental conditions may contribute to such variations in the frequencies of CYP1A1 and GST genes in different geographical regions.

Investigations on the genotoxicity of TCE in humans have not been conclusive as data regarding its genotoxicity are conflicting. Positive results were observed for chromosomal aberrations and hyper diploid cells, but the results were negative for chromosomal non-disjunction (Rasmussen *et al*., 1988). A few negative results were also reported regarding TCE induced genotoxicity. There was no evidence for DNA damage in mice, Fischer 344 rats (Mirsalis *et al*., 1989) or CD-2 mice (Doolittle *et al*., 1987) following oral TCE exposure or in male Sprague-Dawley rats following intraperitoneal injection (Mirsalis *et al*., 1989).

The present investigation does not show any genotoxic effects of TCE irrespective of genotype (Bruning *et al*., 1997, 2003). It is further observed (unpublished observation) by our group that it is the metabolite of TCA which gives DNA adducts in mice using the technique of P^{32} post labeling of DNA adducts. It is also important to note that GSTs are playing important role in the metabolism of secondary metabolites of TCE like DCVC

(McGoldrick *et al*., 2003; Clay, 2008). However, there was no direct evidence for DNA adduct formation by TCE, covalent binding to DNA and RNA from various organs in rats and mice after intra-peritoneal injection (Mazzullo *et al*., 1992).

TCE has led to increase in the incidence of renal cancers in persons. Clinical and epidemiological data identified a mode of action that supported the renal carcinogenicity of TCE particularly at a high-dose (Bruning and Bolt, 2001; Harth *et al*., 2005). According to the concept, reactive metabolites of TCE exert genotoxicity on the proximal tubule of the human kidney, with particular involvement of locally generated DCVC (Dichlorovinyl cysteine) (McGoldrick *et al*., 2003). The second type of toxicity by TCE has been ascribed by oxidative metabolites of TCE, involving folate deficiency and increased excretion of formic acid as intermediate steps (Green *et al.,* 2003). The concept that the local generation of reactive metabolites from TCE involves (a) an initial glutathione conjugation of TCE to *S*-dichlorovinyl-glutathione, (b) splitting of the terminal glutathione amino acids [glu and gly] to *S*-DCVC and (c) the final formation of reactive thioketenes by β-lyase. *N*-Acetylation of *S*-DCVC to *S*-dichlorovinyl-mercapturic acid, which is excreted in the urine, may be viewed as a step of detoxification (Goeptar *et al.,* 1995; McGoldrick *et al*., 2003). Individual differences in susceptibility to TCE induced nephrocarcinogenicity may be conferred by genetic polymorphisms of GSTs (Bruning *et al*., 1997; Kumar *et al*., 2002).

Present study on TCE does not impart any genotoxicity on peripheral blood lymphocytes either through CA or through CBMN assay in control or solvent exposed lymphocytes. It was further confirmed that the genetic polymorphism in GSTs are not affecting by the *in vitro* treatment of TCE on peripheral blood lymphocytes. In general, the genetic polymorphism potentially important for biomarker response largely depends on the exposing agent, biological material examined and ethnicity of the population under study. Individual exposure level may also be variable factor, and a reliable estimate of the exposure may be supportive to interpret genotype toxicant exposure interaction.

To our knowledge, this is the first study that shows the genotoxicity of TCE and role of genetic polymorphism in northern part of Indian subcontinent. Further study is required to see the genotoxic effect of various metabolites of TCE in relation to genetic polymorphism.

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