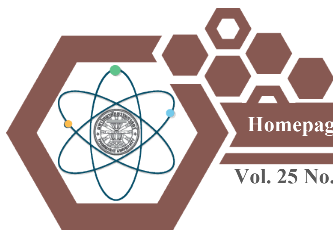

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Original research article

Effects of Trichloroethylene on Lymphocyte Viability, Genotoxicity and Alteration of *c-Myc* and *WT1* Genes

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ABSTRACT

Trichloroethylene (TCE) is a volatile organic compound and chlorine atom in the molecule. It is widely used for degreasing metals which contain environmental and industrial pollutants. It is a toxic chemical substance. The aims of this study were focused on the toxic effect of TCE on human lymphocyte viability in vitro and the impact of TCE toxicity on the expression of *WT1* and *c-Myc* genes. The TCE concentrations of 0.002, 0.004, 0.006, 0.008 and 0.016 mM/L were treated in human lymphocytes for 24 h. Lymphocytes were cultured to study the toxic effect of TCE on lymphocyte viability by MTT assay and gene expression alterations by Quantitative RT-PCR (qRT-PCR) technique. The results showed that, the experimental groups at various TCE concentrations had lower cell viability than the control group ($p<0.05$). Quantitative RT-PCR (qRT-PCR) analysis showed up-regulated expression in the experimental groups comparison with the control groups of *c-Myc* gene [0.008 mM/L (1.4-fold) and 0.016 mM/L (1.7-fold)] and *WT1* gene [0.008 mM/L (1.7-fold) and 0.016 mM/L (2.5-fold)]. It is indicated that TCE can cause cytotoxic effect in human lymphocytes and effects in *c-Myc* and *WT1* genes expression. The expression levels of *c-Myc* and *WT1* increased in humans may lead to the onset of cancer in the future. The knowledge about TCE toxicity will be useful for forensic evidence for the factory workers or the people in the community who stay around the industrial site and face TCE exposure.

Keywords: Trichloroethylene; Toxic lymphocyte viability; *c-Myc* gene; *WT1* gene

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1. Introduction

Trichloroethylene (TCE) is a colorless, volatile organic compound and a chlorine atom in the molecule which typically is used in large quantities as a dissolvent, metal degreaser, chemical intermediate and a component of consumer products [1]. TCE was used as an anesthetic volatile agent. It is widely used in industries as a solvent, as an intermediate chemical for refrigerant manufacturing, metals degreasing, spotting agent in dry cleaning, glue, correction pens, film cleaner, furniture polish and insecticide [2].

A number of health problems arise both in animals or humans, if exposed to TCE. Acute exposure affects the central nervous system and cause headache, drowsiness, dry throat, eye and respiratory system irritation; skin, eyes, and liver damage; coma and death. Ford *et al.* [3] reported on deaths from breathing during its use in degreasing operations of high TCE concentrations. Chronic, repeated or prolonged exposure has resulted in effects to the liver, acute hepatitis, and effects in the respiratory system and the heart and kidneys, while low-level exposure causes impairment of the central nervous system, and cause teratogenesis and carcinogenesis.

The TCE and its metabolites such as trichloroacetic acid (TCA), dichloroacetic acid (DCA) and chloral hydrate (CH) show genotoxicity. There is some evidence that TCE or metabolites bind to DNA and can induce single-strand DNA breaks in hepatic and kidney cells [4]. The alterations in immune responses in mice were observed when given 0.1 mg/mL TCE in drinking water which shows inhibition of humoral and cell-mediated immunity, as well as effects on the function of macrophage and monocyte-granulocyte progenitor cells [5].

The *c-Myc* gene located in human chromosome 8q24 contains three exons and two introns. In exon 2, the smaller 64 kDa polypeptide in translation is initiated at the canonical AUG start codon derived from an internal ribosome entry mechanism and a larger 67 kDa polypeptide is initiated 15 codons upstream of the AUG at a CUG codon at its exon 1. The c-Myc protein of 64 kDa polypeptide, contains 439 amino acids and the 64 kDa polypeptide comprises an N-terminal extension of 14 amino acids. The *c-Myc* gene is proto-oncogene activated in animal and human tumors, such as chromosomal translocations related to *c-Myc* in murine plasmacytoma and human Burkitt's lymphoma, lung carcinoma, breast carcinoma, cervical carcinoma, ovarian carcinoma [6].

Wilms´ tumor gene (*WT1*) is a tumor suppressor gene coding for a zinc-finger transcription factor. It is located on chromosome 11p13 and consists of 10 exons. Exon 5 consists of an additional 17 amino acids and is specific to mammals, while exons 7-10 consist of 4 zinc-fingers at the C-terminus [7] and the KTS (lysine, threonine and serine) site, which is between zinc-fingers 3 and 4, and produces four isoforms; 17AA(+) KTS(+), 17AA(+) KTS(-), 17AA (-) KTS(+) and 17AA(-) KTS(-), each of which has different functions [8]. The *WT1* 17AA(+) KTS(+) isoform assists the inhibitory effect of *WT1* antisense oligomers on cancer cells [9]. The 17AA(+) KTS(-) isoform shows involvement in tumorigenesis of lymphoid malignancy. The functions of 17AA(-) KTS(+) isoform

remain unclear [10]. The 17AA(-) KTS(-) isoform induces G1 arrest in osteosarcoma cell lines [11] and inhibits G1/S progression [10]. Wilms′ tumor 1 (*WT1*) gene encodes a zinc finger transcription factor WT1 which is crucial for cellular proliferation, promoter regions of DNA and survival [12]. In this way, *WT1* controls proliferation, differentiation, cell cycle and apoptosis and *WT1* is overexpressed in several types of leukemia including chronic myeloid leukemia (CML) and acute lymphoblastic leukemia type (ALL) [13].

Our studies were focused on the cytotoxic effect of TCE on human lymphocyte viability in vitro. We also studied the impact of TCE genotoxicity on the expression of *WT1* and *c-Myc* genes. TCE exposure is harmful to the factory workers or the people in the community who stay around the machine/ electronic industrial sites. In cases where industrial workers or people who stay around industrial sites using TCE die, the knowledge about TCE toxicity in this study will be useful for forensic evidence. This knowledge also helps to control and prevent pollution problems due to TCE toxicity.

2. Materials and Methods

2.1 Chemicals

Trichloroethylene (CAS No. 79-01-6) was purchased from Loba chemie (Mumbai, India). MTT (3-[4.5-dimethy-thiazol-2-yl-2,5-diphenyltetrazolium bromide) was procured from Bio Basic Canada (Ontario, Canada). Dimethylsulphoxide (DMSO) and Giemsa stain was purchased from Sigma-Aldrich (St. Louis, USA.). RPMI medium and chemical for cell culture were purchased from Thermo Fisher Scientific (Massachusetts, USA).

2.2 Specimen collection

Heparinized blood was collected from 20 healthy volunteers (age 20-25 years and no history of hematologic diseases and genetic disorders). Approximately 10 mL of blood was collected from each healthy volunteer. The peripheral blood mononuclear cell (PBMC) isolation was done following Debey *et al.* [14]. Lymphocytes were cultured for a study on the effect of TCE on lymphocyte viability and gene expression alterations. This study was approved by the Thammasat University ethics committee (EC123/2018).

2.3 Study on the effect of TCE on lymphocyte viability

Human lymphocyte cultures from the same subject were separated to be both control (n=20) and experimental (n=20) groups. The TCE (conc. 0.002, 0.004, 0.006, 0.008 and 0.016 mM/L) was added to the experimental groups, respectively.

The $1x10^5$ cells/mL of lymphocyte were added in RPMI 1640 medium and incubated at 37 °C for 48 h. After 48 h, the concentrations of TCE 0.002, 0.004, 0.006, 0.008 and 0.016 mM/L were added in lymphocyte cultures and incubated for 24 h. After 24 h incubation time, an MTT assay was done to analyze the viability of lymphocyte. For the MTT assay, the contents were centrifuged at 1500 rpm for 10 min, and 0.5 mL of 300 μg/mL MTT in phosphate buffer saline solution (PBS) was added to each well and incubated for 4 h at 37°C. The medium was removed and formazan was dissolved in DMSO, and the optical density was measured at 570 nm. using a Bio-assay reader [15].

2.4 Study on the expression of *c-Myc* and *WT1* genes alteration

The effect of TCE on *c-Myc* and *WT1* genes alteration was studied by TCE with the concentrations of 0.004, 0.008 and 0.016 mM/L.

2.4.1 RNA extraction

RNA was extracted by UltRNA Column Purification kit (Applied Biological Materials Inc, British Columbia, Canada). RNA was isolated as described in the kit procedure. Then, quality and quantity of RNA concentration were checked with A260/A280 ratio.

2.4.2 Quantitative RT- PCR (qRT-PCR) analysis

The cDNA (RNA 20 ng) was synthesized by RT-PCR reaction using Precision nanoScript 2 Reverse Transcription kit. The RT master mixes were prepared to contain 4X Buffer 5 µl, 10 mM dNTP mix 1 µl, Enzyme 1 µl, 1X RT random primer 1 µl, RNAes/DNAse free water 3 µl and 9 µl of RNA. The total volume in the tube was 20 µl. The tube was loaded using a thermal cycler programmed at 25 °C for 5 min, at 42 °C for 20 min, at 75 °C for 10 min, and at 12 °C for 10 min. The amplification of genes was carried out in a real-time PCR machine using SYBR green-based chemistry. The reaction mixture was prepared to contain 2X SYBR mix 10 µl, 5 µM forward primer 0.8 µl, 5 µM reverse primer 0.8 µl, sterile water 6.4 µl and cDNA template 2 µl. The total volume in the tube was 20 µl. The β-actin gene (*ATCB*) was used as an internal control. The primer sequences used for various genes were as follows: *c-Myc* gene (NM_002467.4) and *WT1* gene (NM_000378.4) shown in Table 1. The thermal cycler was programmed at 95 °C for 2 min, at 95 °C for 10 sec and 60 °C for 45 sec. The cycle time was 58 cycles.

Table 1. The primers used in qRT-PCR analysis.

Gene	Primer Sequence (5′-3′)
ATCB (housekeeping)	Forward: CCATCATGAAGTGTGACGTGG
	Reverse: ATCTTCATTGTGCTGGGTGCC
c-Myc	Forward: GTAGTGGAAAACCAGAAGCCTC
	Reverse: AGAAATACGGCTGCACCGAG
WT1	Forward: GCTGTCCCACTTACAGATGCA
	Reverse: TCAAAGCGCCAGCTGGAGTTT

Table 2. Effect of TCE on human lymphocyte viability in different concentrations.

Concentration of TCE (mM/L)	Optical Density (mean±SE)
Control	0.59±0.06
0.002	0.42±0.03
0.004	0.38±0.03
0.006	0.31±0.01
0.008	0.26±0.01
0.016	0.23±0.01

2.5 Statistics analysis

The toxicity of TCE on lymphocyte viability was tested by ANOVA. The effects of TCE on *c-Myc* and *WT1* genes expressions were analysed by ANOVA [16] and *p* values of less than 0.05 were considered statistically significant.

3. Results

3.1 Effect of TCE on lymphocyte viability

Human lymphocyte cultures were examined by MTT assay to determine the TCE-induced cytotoxicity at concentration of 0.002, 0.004, 0.006, 0.008 and 0.016 mM/L for 24 h. The value of mean absorbance was directly related to the number of the living cells. From our result, lymphocyte viability decreased when the TCE concentrations increased comparing between control and the experimental groups ($p<0.05$) (Table 2). In addition, the 50% inhibitory concentration (IC_{50}) of TCE concentration was 0.0095 mM/L which effected growth or depletion of human lymphocytes.

3.2 Effect of TCE on *c-Myc* and *WT1* alteration

Gene expression analysis of *c-Myc* and *WT1* genes was done by qRT-PCR using beta-actin gene (*ATCB*) as housekeeping. The control group and the experimental groups with different TCE concentrations (0.004 mM/L, 0.008 mM/L and 0.016 mM/L) were studied for gene alteration.

3.2.1 Effect of TCE on c-Myc Expression

The expression level analysis revealed up-regulation of *c-Myc* gene in the experimental groups at 0.008 mM/L (1.4-fold) and 0.016 mM/L (1.7-fold) in comparison to the control as shown in Fig. 1.

The result expression level analysis revealed up-regulation of *c-Myc* gene and showed a statistically significant difference between the control and the experimental groups at 0.016 mM/L ($p<0.05$). Comparing within the experimental groups, the experimental group at 0.016 mM/L was found to have a statistically different significance from the experimental group at 0.004 mM/L ($p<0.05$) as shown in Fig 1.

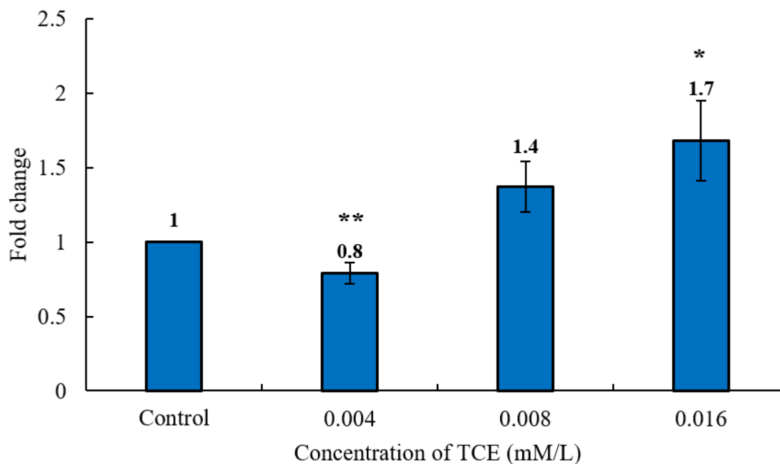

Fig. 1. Effect of TCE concentrations on *c-Myc* gene expressions by qRT-PCR comparing the different concentrations of TCE and each gene expression.
*$p<0.05$, p value compared with control group.
**$p<0.05$, p value compared within the same experimental groups.

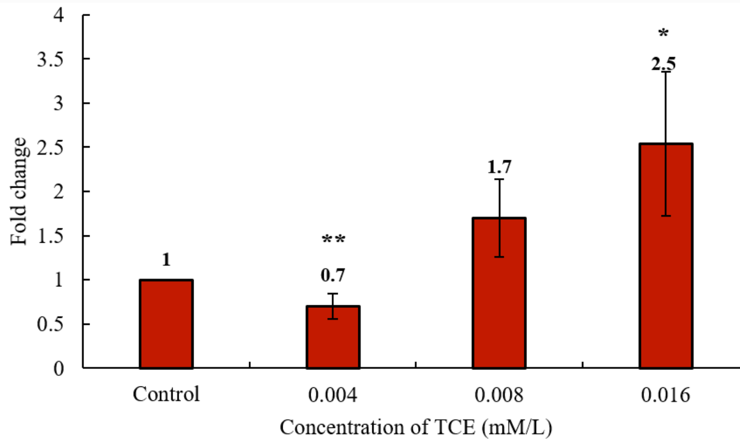

Fig. 2. Effect of TCE concentrations on *WT1* gene expressions by qRT-PCR comparing different concentrations of TCE and each gene expression.
*$p<0.05$, p value compared with control group.
**$p<0.05$, p value compared within the same experimental groups.

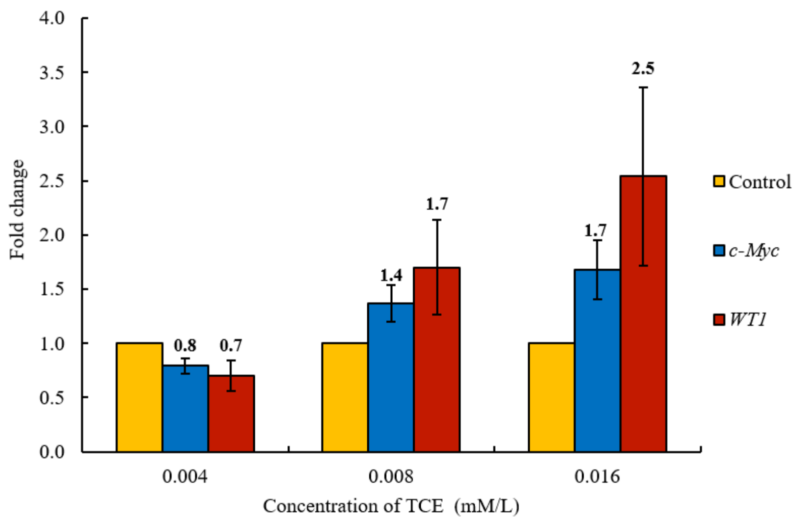

Fig. 3. Effect of TCE concentrations on *c-Myc* and *WT1* genes expressions by qRT-PCR comparing between the same TCE concentrations and the two genes expression.

3.2.2 Effect of TCE on WT1 expression

The expression level analysis revealed up-regulation of *WT1* gene in the experimental groups at 0.008 mM/L (1.7-fold) and 0.016 mM/L (2.5-fold) in comparison to the control as shown in Fig. 2. The result expression level analysis revealed up-regulation of *WT1* gene showed a statistically significant difference between the control and the experimental groups at 0.016 mM/L ($p<0.05$). Comparing within the experimental groups, the experimental group at 0.004 mM/L was statistically significant from the experimental group at 0.016 mM/L ($p<0.05$) as shown in Fig. 2.

When we compared the effect of TCE concentrations between these two genes expression, it was shown that the expression levels of *c-Myc* and *WT1* genes, in the control group and the same TCE concentration group, were statistically insignificant as shown in Fig. 3.

4. Discussion

TCE is a lipophilic compound that readily crosses biological membranes and gets absorbed quickly into the bloodstream and distributes to all compartments within the body. In humans, TCE is metabolized through at least two distinct pathways: oxidative metabolism via the CYP mixed-function oxidase system and GSH conjugation.

From our analysis, the numbers of human lymphocyte viability were decreased when the concentration of TCE increased in the experimental groups and the 50% inhibitory concentration (IC_{50}) of TCE concentration was 0. 0095 mM/ L. That corresponded to the research of Rasmussen *et al.* [17] which specified TCE be the cause of chromosomal abnormalities like breaks, gaps, deletions, inversions, translocations, and hyperdiploidy. Additionally, the frequency of sister chromatid exchange was increased in the peripheral lymphocytes of TCE exposed workers [18] . TCE and its metabolites, such as Trichloroacetic acid (TCA) , Dichloroacetic acid (DCA) and Chloral hydrate (CH) , could bind to DNA and induce single-strand DNA breaks in both hepatic and kidney cells which indicates that TCE was able to induce cytotoxic and genotoxic effects in lymphocyte cells [19].

TCE affects DNA methylation in whole-liver preparations at exposures with increased expression of the proto-oncogenes such as *c-Myc*. The recent data showed that TCE increased in hypomethylation of the promoter regions of *c-Jun* and *c-Myc* in whole-liver DNA [20] . In this study, the expression of *c-Myc* gene was increased due to TCE exposure. The *c-Myc* gene in exon 2 at the N-terminal region induces cell proliferation, cell differentiation, apoptosis, activation of target genes and transformation [21] . TCE exposure may affect *c-Myc* functions about cell proliferation, cell differentiation, apoptosis, activation of target genes and transformation. It was correspondent with previous reports on TCE and its metabolites that DCA and TCA, which are TCE metabolites, could induce hypomethylation and promoted liver tumor in mice and DCA decreased methylation and increased gene expression of *c-Myc* gene [20, 22] . TCE exposure caused abnormal function in *c-Myc* gene. Abnormal *c-Myc* function may cause abnormal process in cell proliferation, cell differentiation, apoptosis and lead to the diseases in human.

The expression of *WT1* gene in exon 6-9 having zinc-fingers in the C-terminal and involved in cell proliferation and survival. Shrestha *et al.* investigated prenatal exposure to air toxins and risk of Wilms′ tumor in children 0-5 years old. They reported that the children prenatally exposed to formaldehyde, polycyclic aromatic hydrocarbons, perchloroethylene, or acetaldehyde in the third trimester have increased odds of Wilms′ tumor per interquartile increased in concentration [23]. These toxins have similar properties like TCE and they affect the *WT1* gene function. Our results showed that TCE exposure affected the expression of the *WT1* gene. TCE increased *WT1* expression and may affect *WT1* function which is involved in controlling proliferation, differentiation, cell cycle and apoptosis. Abnormal *WT1* function was incurred by TCE exposure in this study. Our study reported that the expression of *c-Myc* and *WT1* genes were increased by TCE exposure leading to altered gene functions and subsequent cell death. The *c-Myc* gene was found to be up-regulated by the *WT1* gene [24]. The *WT1* gene controls or stimulates the expression of the *c-Myc* gene. Thus, TCE decreased viability of lymphocytes, increased expression of *c-Myc* and *WT1* genes that may lead to formation of disease such as human cancer in the future.

5. Conclusion

TCE causes human lymphocyte cytotoxicity and increases the expression levels of *c-Myc* and *WT1* genes in humans. People should avoid direct skin contact and avoid inhaling vapors of TCE. TCE contamination within the environment may cause genetic abnormalities and lead to the onset of cancer in the future.

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