보건학석사 학위논문

Strain-dependent responses of subacute exposure to trichloroethylene in a multi-strain panel of mice: An oxidative metabolite and the liver effects

아급성 트리클로로에틸렌 경구노출에 의한 마우스 계통 별 대사 및 간독성 반응 차이

2016년 2월

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이 논문을 보건학석사 학위논문으로 제출함

2015년 11월

서울대학교 보건대학원

환경보건학과 환경보건학 전공

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안영아의 보건학석사 학위논문을 인준함

2015년 12월

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Abstract

Strain-dependent responses of subacute exposure to trichloroethylene in a multi-strain panel of mice: An oxidative metabolite and the liver effects

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Trichloroethylene (TCE), a common industrial material and environmental contaminant, is carcinogenic in kidney to human and can induce liver toxicity in mice; however, there were a few of evidence on liver toxicity in humans. Moreover, recent few studies indicated diverse variability of TCE metabolism and its toxicity in population-based mouse model, but there are still lots of knowledge gaps on subject variability of the health effects. The aim of this study was to identify more susceptible strains and their metabolic determinants and features of liver toxicity using multi-strain of mice. Thus, we measured dose-dependent metabolism and determined liver toxicity of

TCE in seven strains of mice. 129S1/SvImJ, A/J, B6C3F1, BALB/cJ, C3H/HeJ, C57BL/6J and DBA/2J showing diverse genetic background and phenotypes among strains but homogenous traits within a strain were selected and TCE was administered with oral gavage for four weeks at 0, 10, 100 and 1000 mg/kg/day (4 groups per strain; 3 to 5 mice per group). Serum trichloroacetic acid (TCA) as an oxidative metabolite of TCE and various markers of liver weight gain and toxicity were measured at 24 hours after the last dose including the proliferating cellular nuclear antigen-labeling index (PCNA-LI) of liver tissues. There were significant dose- and strain-dependent differences in levels of serum TCA and liver weight gain after TCE exposure for four weeks ($p < 0.05$). Fold change of TCA to control group increased by dosage and some strains were distinguishable; however, those trends for liver weight gain were not maintained in some strains. While DBA/2 and A/J produced TCA noticeably more than others, BALB/c, 129S1/SvImJ and A/J yielded more liver-weight gain by dosage. C57BL/6J was not markedly increase in both TCA and liver weight compared with the others. In general, TCE metabolism (TCA production) had strong correlation with liver-weight gains ($\rho=0.52$, $p<0.05$); however, not all higher TCA producing strains were higher liver-weight gaining group. Both high liver-weight gaining strains (BALB/c, 129S1/SvImJ and A/J) and a low strain (C57BL/6J) showed marginal correlation between PCNA-LI and liver weight gain ($\rho=0.36$, $p=0.068$), but there were significant increases of PCNA-LI in 129S1/SvlmJ and C57BL/6J at TCE 1000 mg/kg relative to control ($p < 0.05$). Cell proliferation (PCNA-LI) could explain high liver-weight gain by dose in 129S1/SvImJ not A/J; interestingly, there were significant increase in PCNA in a low liver-weight gaining strain (C57BL/6J). There was no significant difference of levels of liver injury marker among strains by dosage and necrosis was not detected in all. Suggestively, although it was confirmed that

TCE metabolism attributed to liver weigh gain, strains with high metabolism were not same as in the more liver-weight gain. Further studies are required for causes and the modes of action of the liver-weight gain, but cell proliferation could be explanatory of it in 129S1/SvImJ.

Keywords: Trichloroethylene, dose- and strain-dependent differences, an oxidative metabolite, liver weight gain, a multi-strain panel of mice

Student Number: 2009-23612

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1. Introduction

Trichloroethylene (TCE), a chlorinated compound widely used as a metal degreaser and industrial intermediate, is the ubiquitous environmental contaminant found in groundwater (US EPA, 2011). TCE is thought to cause adverse health effects through multiple organ toxicities. For acute exposure, it has an effect on the nervous system. For chronic exposure, it can cause liver- (Bull, 2000), neuro- (Barton and Clewell, 2000), autoimmune toxicity, as well as kidney cancer to animals and humans (Lash et al., 2006; IARC, 2013). However, it has been difficult to achieve consensus on human risk of TCE, although the information of adverse effects exists (NRC, 2006).

Human exposure to TCE in environment is a health concern primarily because it is classified as a human carcinogen and it can be harmful by all routes of exposure (IARC, 2013). Based on a meta-analysis of 15 independent epidemiologic studies, a causal association between TCE and kidney cancer in human was confirmed . However, TCE risk assessment document published by U.S. EPA described that the weight of evidence for liver cancer was not sufficient to classify TCE as carcinogenic to human. A point of contention is that human relevance of TCE-induced liver cancer was not fully explained, while liver is well established target organ in mice (US EPA, 2011).

Metabolism is thought to be an important requirement for TCE to exert hepatotoxic effects (Buben and O'Flaherty, 1985). Trichloroacetic acid (TCA) and/or dichloroacetate (DCA) through cytochrome P450-dependent oxidation may be responsible for the induction of hepatic tumors or liver toxicity (Lash et al. 2014). DCVG, DCVC and/or NAcDCVC, conjugates mediated by GST after TCE exposure, associates with developing kidney cancer. In short,

organ-specific toxicity ensues from two major pathways for the metabolism. In addition, species differences in both of humans and exist in the relative contribution of each pathway (NRC, 2006).

Specifically for liver toxicity from TCE exposure, several researches have shown inter-species and even inter-strain differences, of which arguments suggested wide range of variability in the health outcomes. Rats and mice showed different susceptibility in liver cancer where the latter produce more oxidative metabolites such as TCA and DCA (Elfarra et al. 1998; Larson and Bull 1992; Lash et al. 2000). Larson and Bull (1992) reported that the metabolic rates of TCE-to-TCA were much higher in mice by dosage although metabolic pathways of TCE were shared with rats qualitatively. Even in a species, there was wide variability in metabolism and health outcomes after the exposure. Recently, a multi-panel of inbred mice model including fifteen strains showed wide variability in metabolism and health outcomes even at the same levels of single exposure to TCE at 2100 mg/kg (Bradford et al. 2011). In repeated doses of TCE at 100 mg/kg and 400 mg/kg for one- and four-week, strain-dependent TCA production in serum and liver was confirmed, where the different profiles of metabolic enzymes among the strains were associated with the results (Yoo et al. 2015). However, there has been no study with longer term of exposure and different dosages at concurrent assay.

Despite variability, metabolic pathways and modes of action of TCE was regarded similar qualitatively across species and strains. As for liver toxicity, oxidative metabolites were reported as associated with hepatomegaly (Bull et al. 2002). Moreover, TCA, known as a peroxisome proliferator, can activate peroxisome proliferator-activated receptors (PPARs) which participates in lipid metabolism, and can induce inflammation and proliferation of both cell

and peroxisome via cellular signaling processes (Chinetti et al. 2000). In general, cell proliferation (increase of cell counts in tissues) is a compensatory to cellular injury or tissue growth, but it is not clear that hepatomegaly followed by TCE treatment was attributed to either cell proliferation or enlargement of cell or tissue size or both (Bull 2000; Yang et al. 2007). Figure 1 illustrates the tentative exposure-health effect continuum.

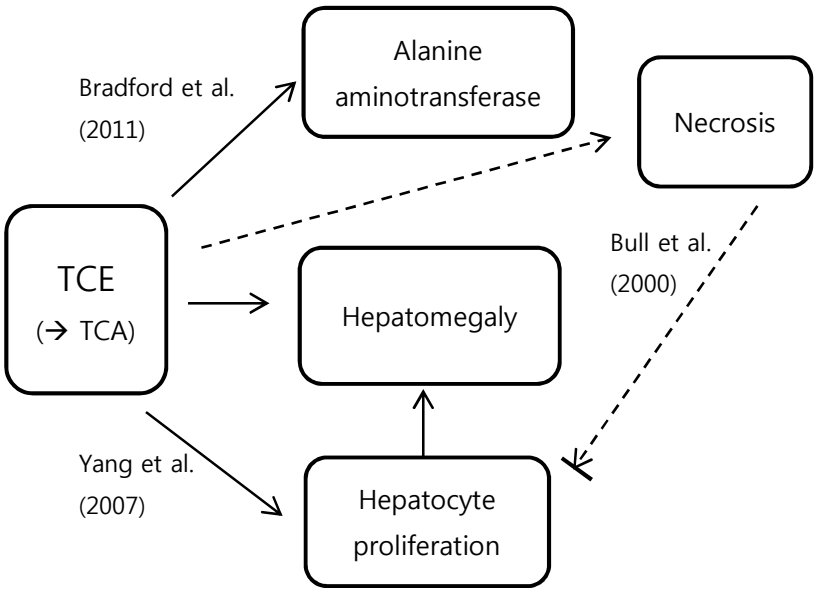


Figure 1. Schematic summary of TCE-induced liver effects found in mice.

Since there were identical genotype and phenotype within strain and wide diversity between strains, a multi-strain mouse model was considered as an useful tool to investigate inter-individual variability in human population

exposed to environmental chemicals (Bradford et al. 2011; Chiu et al. 2014; Rusyn et al. 2010; Tryndyak et al. 2012). By utilizing the benefits of multi-strain model, we performed the present study to fill the knowledge gaps as follows:

- TCE exposure for four weeks would affect dose- and strain-dependent formation of an oxidative metabolite and its liver toxicity in appropriate genetically defined mouse model analogous to human population.

The purposes of the present study are:

(1) to gauge the quantitative differences in TCE metabolism in multi-strain of mice

(2) to investigate inter-strain differences in the TCE-induced responses coupled with some mode of actions such as proliferation and injury of liver cells.

2. Materials and methods

2.1. Chemicals

Trichloroethylene (TCE; 99.5+%, Sigma Aldrich, St. Louis, MO, USA) was dissolved in corn oil, and corn oil was used as a vehicle. Zoletil 50 (Virbac, France) and Rompoun (Bayer, Germany) as anesthetic drugs were purchased.

HPLC-grade acetonitrile (ACN), HPLC-grade water, HPLC-grade methanol, spectrophotometric grade diethyl ether, ammonium hydroxide (29%), trichloroacetic acid (TCA, 99+%), ACS grade sulfuric acid (98%), ACS grade acetic acid (100%) and 2-(2-methoxyethoxy)ethanol (2-MEE, 99%) were purchased from Fisher Scientific (Pittsburgh, PA). Dichloroacetic acid (DCA, 99+%), [$^{13}C_2$]DCA (98%), [$^{13}C_2$]TCA (98%), and ammonium formate salt (99.9%) were purchased from Sigma (St. Louis, MO). DCVC, [$^{13}C_5$,^{15}N]DCVC, DCVG and [$^{13}C_4$,^{15}N]DCVG were synthesized by modification of a published method (McKinney et al., 1959), details are available from Dr. Avram Gold. NAcDCVC and [$^{13}C_4$,^{15}N]NAcDCVC were purchased from Toronto Research Chemicals (Toronto ,Ontario, Canada).

2.2. Animals and treatments

Male mice of seven strains were purchased at 8 to 12 weeks of age from Jackson Laboratories (USA): 129S1/SvImJ, A/J, B6C3F1, BALB/cJ,

C3H/HeJ, C57BL/6J, DBA/2J. Based on the data from fifteen strains (Bradford et al. 2011; Rusyn 2014), seven strains were selected, and that represented widely varying degree of formation of oxidative metabolites of TCE and the key enzyme related to metabolism and liver toxicity (Yoo et al. 2015). They were selected They were housed 3 to 5 animals per cage with hardwood chip bedding and maintained on a 12-hour light/dark cycle at a constant temperature of 22±1℃ and humidity of 35-50%. Mice were fed NTP-2000 wafer diet (Zeigler Brothers Inc., Gardners, PA) and had free access to water (*ad libitum*). Doses of 0, 10, 100, and 1000mg/kg TCE with corn oil vehicle (10 ml corn oil/kg body weight of mouse) were administered to mice using a ball-tipped intubation needle for four weeks (five days per week) in compliance with NTP protocol. Each group of dosing TCE consists of three to five mice. Mice were sacrificed at 24-hour after last dosing of TCE. Body weight was recorded right before dosing TCE and sacrificing the mice. After postmortem examination, blood samples and liver tissues were collected. Whole blood samples were separated into serum using an SST tube (Becton-Dickinson, Oxford, United Kingdom) according to the manufacturer's instruction. All serum samples were stored at -70℃ before analysis. After weighing liver, the liver tissues were fixed in in 10% neutral buffered formalin for 15 hours before histological and immunohistological processing. All animal studies were approved by Institutional Animal Care and Use Committee in Seoul National University.

2.3. Determination of TCE metabolites in serum

The levels of TCE metabolites in serum were determined using HPLC-ESI-MS/MS as detailed elsewhere (Kim et al. 2009) with slight modifications

as follows (Figure 2). An aqueous mixture of internal standards (5 µl; [$^{13}C_2$]DCA, [$^{13}C_2$]TCA and [$^{13}C_4$,^{15}N]NAcDCVC; [13C5,15N]DCVC and [$^{13}C_4$,^{15}N]DCVG) was spiked to diluted serum specimen (50 µl) with water (100 µl). Then, serum protein was removed by filter centrifugation (Microcon YM-10, Danvers, MA) at 14,000 × g for 30 min at 25 °C. Subsequently, 2 ml of diethyl ether was added to extract haloacetic acids after acidifying the media with 100 µl of 3% (v/v) sulfuric acid. The ether layer was transferred to another vial and reduced in volume under N_2, and finally transferred to 300 µl glass vial insert containing 5 µl of water for solvent transfer before dryness. The residue was reconstituted in 100 µl of mobile phase: 68.6% ACN, 29.4% 40 mM ammonium formate (pH 9.1) and 2% 2-MEE. The aqueous fraction left after the ether extraction above was neutralized with 5 µl of 28% NH_4OH prior to extraction of DCVG and DCVC through a solid phase extraction (SPE) cartridge (StrataTM X-AW, 30 mg 96-well plate; Phenomenex, CA). After conditioning with 300 µl of methanol, followed by equilibration with 300 µl of water, the samples (～ 300 µl) were loaded, washing with 300 µl water, and finally eluted with 250 µl of basic methanol (pH adjusted at 10.8 by 29% NH_4OH). Light vacuum (up to 50 mmHg) was applied to expedite washing and elution. The final eluent was collected into 300 µl glass vial inserts and dried in a Speed Vac Concentrator (Thermo-Fisher, CA) before reconstitution with 20 µl of 80:20 water/methanol containing 0.1% acetic acid.

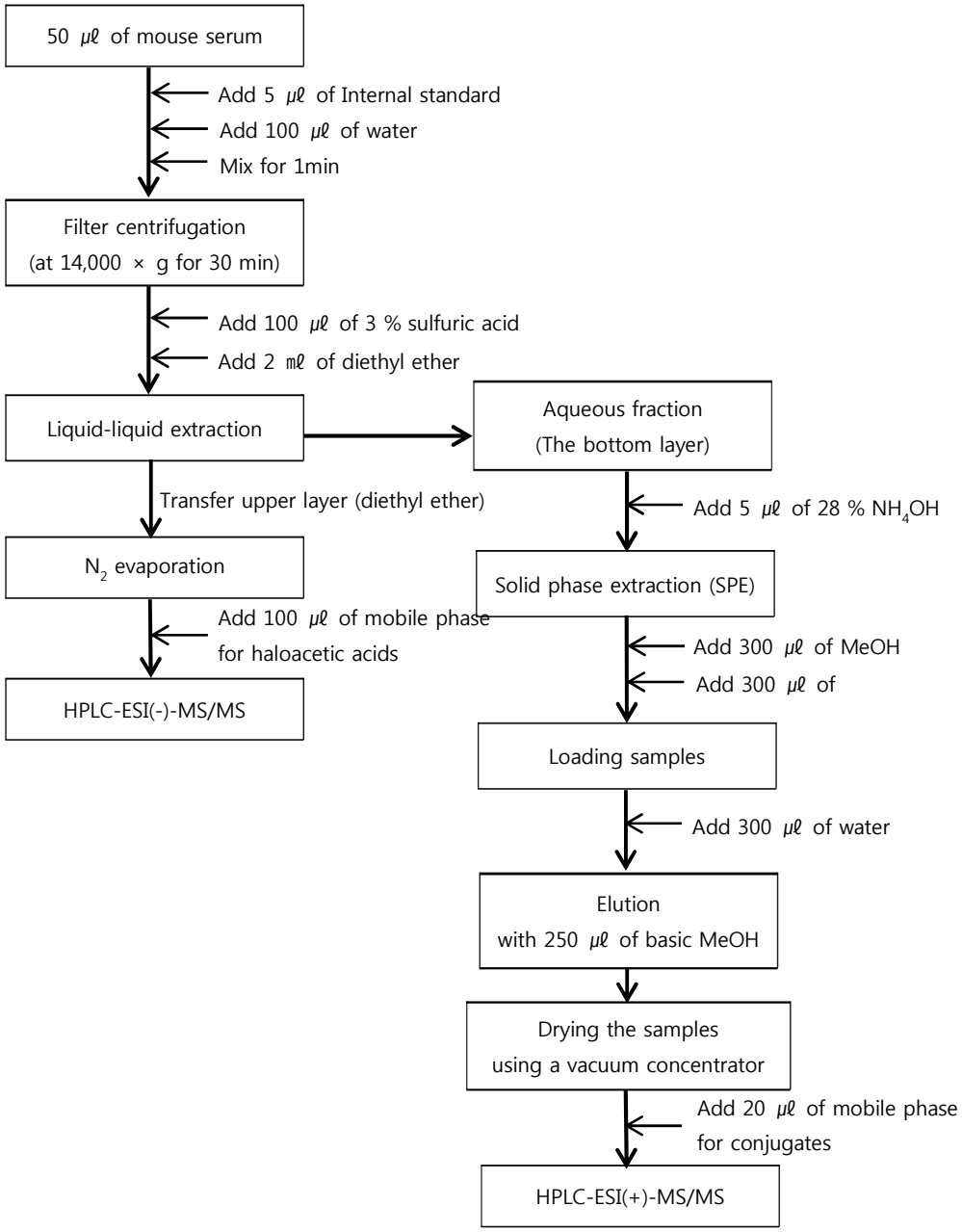


Figure 2. Procedure of sample preparation for TCA in serum.

HPLC-ESI-MS/MS was Agilent 1100 series (Santa Clara, CA, USA) and Shiseido Nanospace SI-2 (Shiseido, Japan). And AB SCIX API 4000 (Redwood city, CA) was used. Luna Amino HPLC column (150×2.0 mm, 3 μm) and Strata X-AW solid-phase extraction (SPE) Cartridge were purchased were purchased from Phenomenex (Torrance, CA, USA), Microcon centrifugal filter YM-30 (30kDa) from Millipore(Billerica, MA, USA), YMC C18 column (150×2.0 mm, 3 μm) from Waters (Milford, MA, USA) were used.

2.4. *Determination of alanine aminotransferase (ALT) in serum*

Serum alanine aminotransferase (ALT) as a damage biomarker of liver function was analyzed using its standard protocol (ID Labs inc., London, ON, Canada). Microtiter plate was prepared, a 10 μl of serum sample and a 240 μl of Master mix in the assay kit were added to the wells, and then measured the absorbance of each sample at 340nm using spectrofluorophotometer (Tecan, USA)

2.5. Hematoxylin & Eosin staining

After the dissected livers were fixed in 10% formalin, the fixed samples were processed in a series of graded ethanol solutions, cleared in xylene (Sigma) and embedded in paraffin wax. Paraffin sections were cut at 5 μm thickness by rotatory microtome, deparaffinized, rehydrated and stained with 1% cresol fast violet staining solution (15-20 minutes) for histological

examination. The tissue sections were viewed and imaged using a light microscopy.

2.6. Detection of proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen is present in all stages of the cell cycle except G0 and is a well-established marker of cell proliferation used extensively to demonstrate cell growth (Channel et al. 1998). The liver tissues were fixed in 10% neutral buffered formalin for 15 hours, dehydrated in ethanol and Hemo-De (Fisherbrand, Fisher Scientific Pittsburgh, PA), and paraffin embedded at less than 60 ℃ using a Histomatic MVP (Fisher scientific). 4㎛-thick sections were cut, mounted on ChemMate Plus Slides (BioTek Solutions, Santa Barbara, CA), and air dried. Sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to PBS buffer (pH 7.4). Immunohistological process of the liver sections was performed on a Tech-Mate 1000 (BioTek Solutions, Santa Barbara, CA) automated immunostaining system. For antigen retrieval, the sections were microwaved in citrate buffer (BioTek Solutions) and then reacted with hydrogen peroxide (3% for 20 min) and blocked with normal horse serum (BioTek Solutions). Slides were then incubated overnight at room temperature with mouse monoclonal antibody (PCNA-Ab-1, Oncogene Science, Cambridge, MA).

Detection of PCNA-positive nuclei was performed using biotinylated horse anti-mouse (BioTek Solutions) secondary antibody and avidin-biotin compex (ABC kit, BioTek Solutions) (20 min incubation for each, at room temperature), followed by incubation in hydrogen peroxide/diaminobenzidine

(BioTek Solutions) for 21 min. Tissues were counterstained with hematoxylin. The pictures of liver sections were taken in 10 high-power fields (X200 magnification) by a light microscopy. Quantitative image analysis was performed using ImageJ. Images of liver sections immunohistochemically labeled for PCNA were analyzed to detect positive and negative hepatocyte nuclei. Detection criteria were the color-feature identification between brown (positive) and blue (negative) nuclei. All histological assessments were made in a blinded fashion by two investigators. The number of brown-stained nuclei divided by total nuclei was used as a proliferating cell nuclear antigen-labeling index (PCNA-LI).

2.7. Statistical analysis

Each experimental group was consisted of 3 to 5 animals. All Data were analyzed using commercially available statistical software SAS 9.4 and JMP 10 (SAS Institute Inc., Cary, NC USA). For TCA concentration less than LOD (limits of detection), the number equal to LOD divided by square root two (LOD/$\sqrt{2}$) was used as a proxy value. The other metabolites which were less than 21 % of detection rate were censored from statistical analysis. All variables from this study were not normally distributed, so these data were transformed natural log (ln), followed by statistical analysis. Generalized linear models (GLM) were performed to compare data among strains with Tukey's multiple comparison, where the dependent variables were natural logarithms of serum TCA, relative liver weight gain (liver weight difference for four weeks divided by body weight at sacrifice) and PCNA-LI and explanatory variables were administered amounts of TCE, strain and interaction between the dose and strain for each dependent variable. All values

corresponded to the lsmean ± 95% confidence limit of three to five independent experiments. Principle component analysis (PCA) and cluster analysis were performed to classify and cluster the strains tested in this study. In addition, Spearman correlation was used to analyze correlation among TCE-induced responses including a metabolite. For all test, a *p*-value of 0.05 was considered significantly.

2.8. Scheme of study design

Overall, a scheme of study design was illustrated in Figure 3.

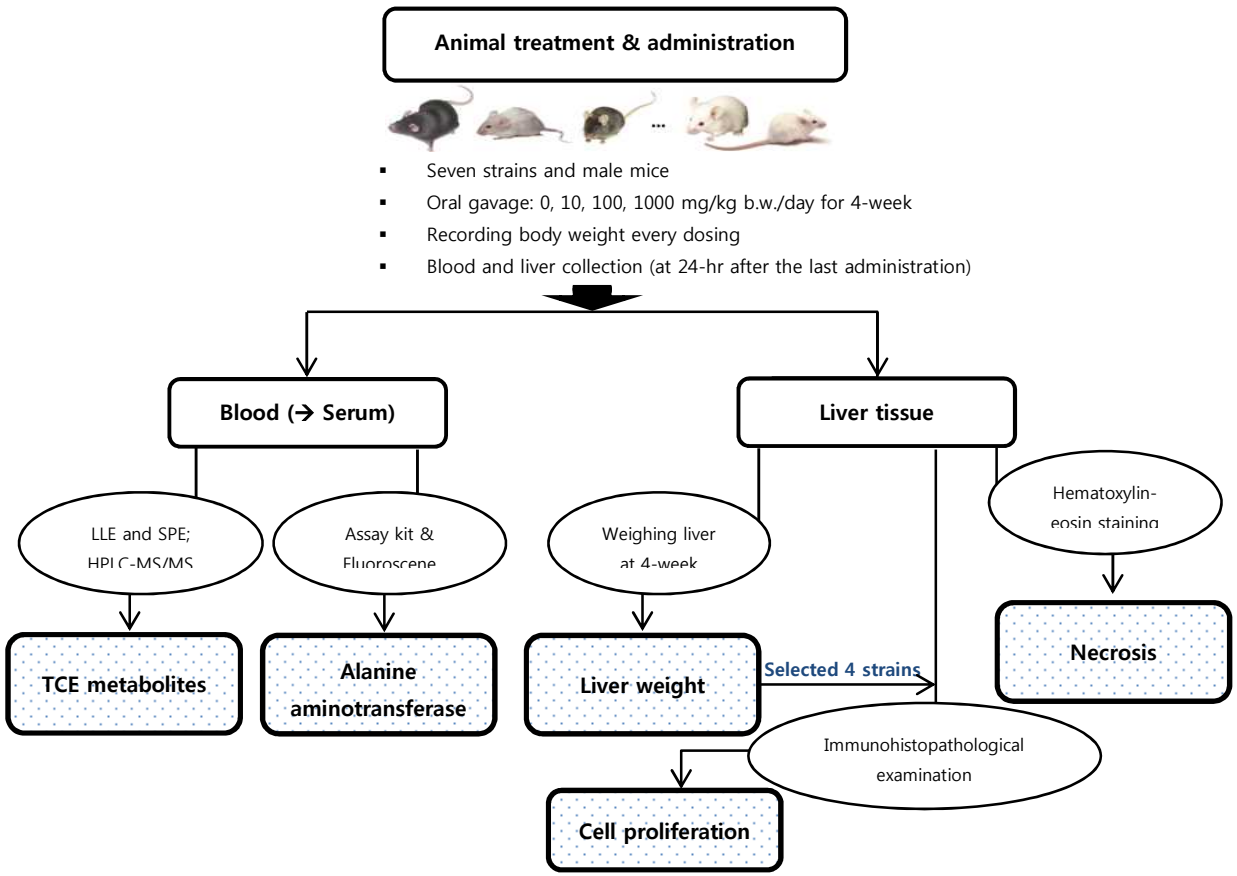


Figure 3. A scheme of study design.

3. Results

3.1. Body and liver weight

The effects of the 4-week exposure to different amounts of TCE on body and liver weight in seven strains of male mice aged eight to twelve weeks were presented in Table 1. Body weight was not adversely affected by any dosage of TCE exposure ($p > 0.05$). In groups of mice exposed to only the vehicle and TCE 10mg/kg b.w./day, there was no significant difference of liver weight such as the absolute liver weight and relative liver to body weight. On the other hand, the average absolute liver weight and relative liver to body weight were significantly increased for groups of the mice exposed to 100mg/kg b.w./day and 1000mg/kg b.w./day of TCE, compared to the groups treated by only the corn oil vehicle ($p < 0.01$). The results presented in Table 1 also showed strain-dependent effects of TCE ($p < 0.05$).

Table 1. Body weight, absolute liver weight, and relative liver weight to body weight in seven strains following TCE exposure for four weeks

Strain	Dose of TCE (mg/kg b.w./day)	n	Body weight (g)	Absolute liver weight (g)	Relative liver weight to body weight (%)
129S1/SvlmJ	0	4	21.24 ± 0.85	0.84 ± 0.08	3.95 ± 0.25
	10	4	20.68 ± 1.18	0.84 ± 0.09	4.05 ± 0.29
	100	4	20.70 ± 1.38	0.89 ± 0.09	4.27 ± 0.16
	1000	4	23.29 ± 1.28	1.41 ± 0.07	6.06 ± 0.08
A/J	0	4	23.70 ± 2.62	0.80 ± 0.08	3.39 ± 0.07
	10	3	23.31 ± 0.21	0.89 ± 0.06	3.81 ± 0.24
	100	3	25.26 ± 0.86	0.94 ± 0.06	3.73 ± 0.12
	1000	4	24.97 ± 0.67	1.21 ± 0.04	4.86 ± 0.19
B6C3F1	0	4	25.80 ± 0.81	1.09 ± 0.10	4.21 ± 0.26
	10	4	27.14 ± 0.79	1.15 ± 0.07	4.24 ± 0.16
	100	4	27.08 ± 0.28	1.22 ± 0.07	4.49 ± 0.21
	1000	4	28.10 ± 1.13	1.43 ± 0.09	5.10 ± 0.17
BALB/c	0	4	26.37 ± 1.03	1.25 ± 0.06	4.73 ± 0.24
	10	4	26.06 ± 1.20	1.25 ± 0.03	4.81 ± 0.13
	100	4	26.64 ± 0.94	1.51 ± 0.17	5.67 ± 0.45
	1000	4	26.10 ± 1.79	1.67 ± 0.16	6.38 ± 0.20
C3H/He	0	4	28.19 ± 2.20	1.20 ± 0.08	4.25 ± 0.26
	10	4	29.04 ± 3.34	1.19 ± 0.13	4.09 ± 0.21
	100	4	26.94 ± 0.66	1.22 ± 0.32	4.54 ± 1.22
	1000	3	29.19 ± 1.16	1.55 ± 0.11	5.30 ± 0.17
C57BL/6J	0	5	23.92 ± 0.89	1.02 ± 0.02	4.28 ± 0.17
	10	4	23.30 ± 1.09	0.99 ± 0.05	4.26 ± 0.02
	100	5	23.14 ± 0.60	1.03 ± 0.08	4.47 ± 0.24
	1000	5	21.66 ± 0.37	1.06 ± 0.04	4.88 ± 0.21
DBA/2	0	5	24.83 ± 1.16	1.00 ± 0.11	4.04 ± 0.35
	10	5	24.21 ± 3.28	0.90 ± 0.09	3.73 ± 0.28
	100	5	24.13 ± 2.13	1.01 ± 0.13	4.16 ± 0.18
	1000	5	24.99 ± 0.90	1.28 ± 0.10	5.11 ± 0.35

Note. All values above were presented by mean ± sd.

3.2. *Levels of TCE metabolites in serum*

Only TCA was 100% detected at a level higher than the limit of detection (LOD) in the serum samples. The LOD was defined as three times the standard error of the y-intercept divided by the slope of the calibration curve. The LODs of the five metabolites in mouse serum were as follows: TCA, 0.438 nmol/ml; DCA, 0.081 nmol/ml; NAcDCVC, 0.018 nmol/ml; DCVC, 0.002 nmol/ml; DCVG, 0.001 nmol/ml. Although the LODs were lower than those found in similar studies (Bradford et al. 2011; Kim et al. 2009), the measured levels of DCA, DCVG, DCVC and NAcDCVC were below the LOD or not detected at all. One possible explanation for this is that the other metabolites might have already been excreted in the urine because the sampling was performed 24 hours after the administration of the last dose of TCE.

Figure 4 shows that after exposure for four weeks, TCE dose was positively related with the fold change of serum TCA and that the patterns of the fold change of serum TCA were different among the seven strains of mice ($p<0.01$). In the group of exposure to TCE 10mg/kg b.w./day, C57BL/6J mice were different from 129S1/SvImJ ($\beta=0.71$, SE=0.33, $p<0.05$), A/J ($\beta=2.00$, SE=0.36, $p<0.0001$) and DBA/2 ($\beta=2.98$, SE=0.32, $p<0.0001$) in terms of formation of serum TCA. In the group of exposure to TCE 100mg/kg b.w./day, C57BL/6J mice were different from 129S1/SvlmJ ($\beta=2.16$, SE=0.32, $p<0.0001$), A/J ($\beta=2.75$, SE=0.34, $p<0.0001$), BALB/c ($\beta=0.83$, SE=0.32, $p<0.05$) and DBA/2 ($\beta=3.89$, SE=0.30, $p<0.0001$). In the group of exposure to TCE 1000mg/kg b.w./day, C57BL/6J mice were different from 129S1/SvlmJ ($\beta=1.60$, SE=0.32, $p<0.0001$), A/J ($\beta=4.30$, SE=0.32, $p<0.0001$), B6C3F1 ($\beta=0.97$, SE=0.32, $p<0.01$), BALB/c ($\beta=0.95$, SE=0.32, $p<0.01$), C3H/He ($\beta=0.73$, SE=0.34, $p<0.05$) and DBA/2 ($\beta=4.05$, SE=0.30, $p<0.0001$).

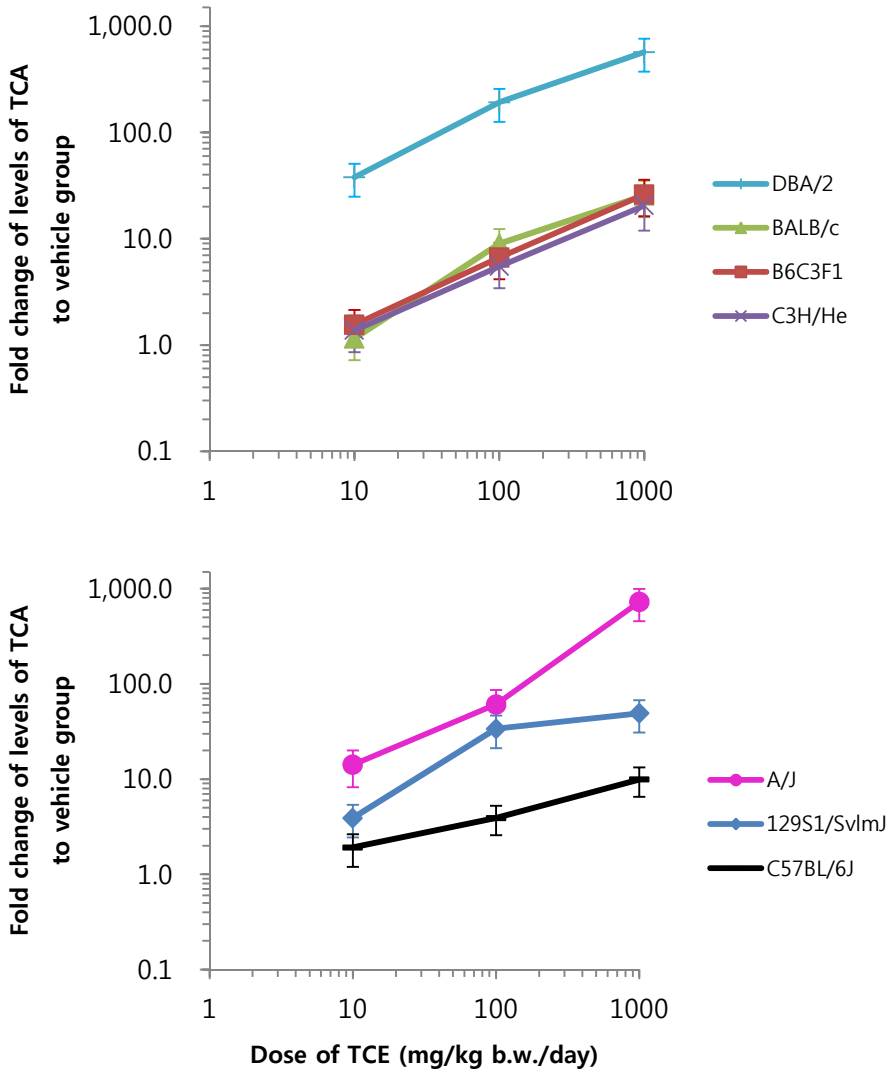


Figure 4. Fold change of TCA levels in seven strain after TCE exposure for four weeks. Data correspond to the least-square means (LSmean) ± 95% confidence limit of LSmean after adjustment for strain, dosage and interaction (strain×dose). Each point included three to five observations.

3.3. Liver weight gain

To compare to liver weight gain among the seven strains exposed to TCE for four weeks, Figure 5 presents the patterns of fold change of liver to body weight. Increases in fold change of liver to body weight showed strain-dependent effects ($p<0.01$). In the group of exposure to TCE 1000mg/kg b.w./day, 129S1/SvImJ mice showed evident changes of liver weight gain, and are different from B6C3F1 ($\beta=-0.24$, SE=0.05, $p<0.0001$), BALB/c ($\beta=-0.13$, SE=0.05, $p<0.05$), C3H/H3 ($\beta=-0.20$, SE=0.06, $p<0.001$), C57BL/6J ($\beta=-0.28$, SE=0.06, $p<0.0001$) and DBA/2 ($\beta=-0.25$, SE=0.07, $p<0.001$) significantly. A/J mice ($\beta=-0.13$, SE=0.07, $p=0.064$) were marginally different from 129S1/SvImJ mice.

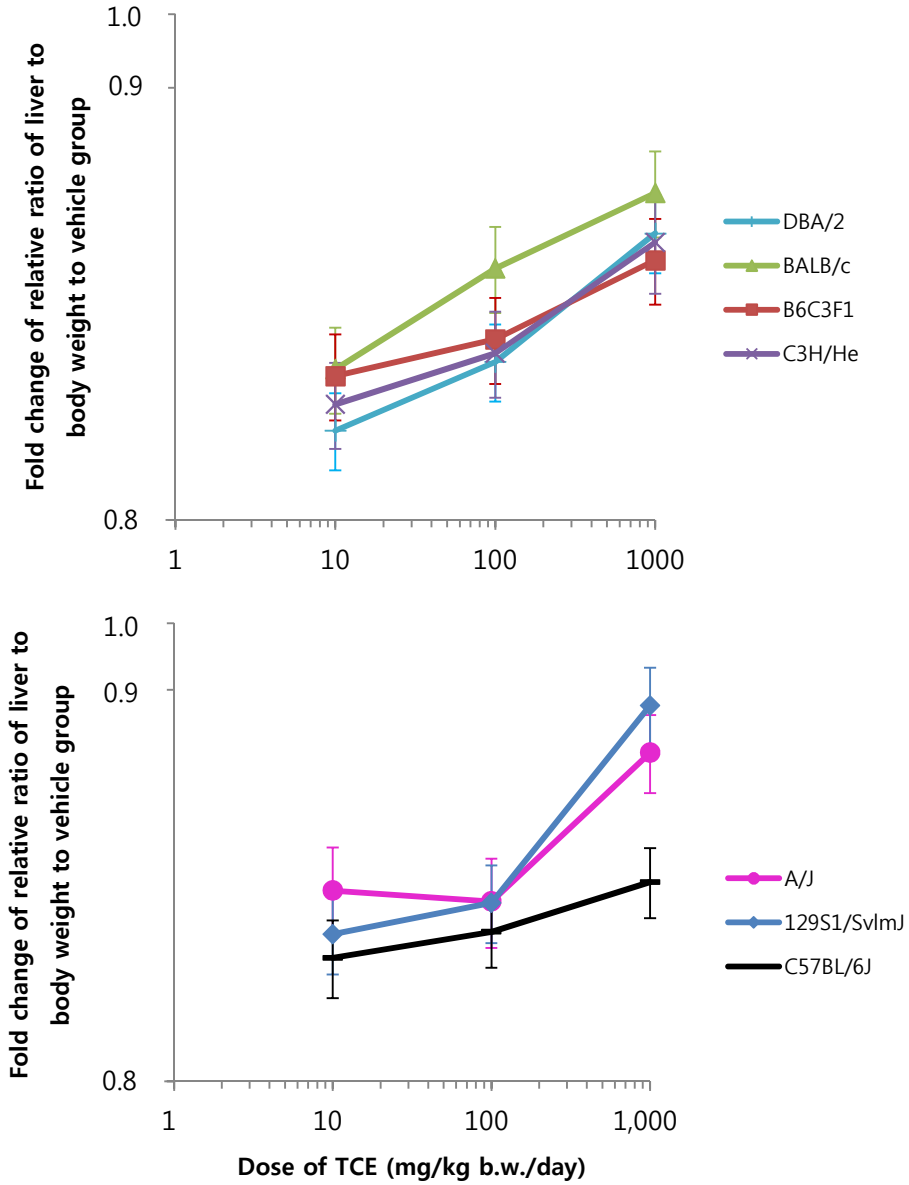


Figure 5. Fold change of relative ratio of liver to body weight in seven strains after TCE exposure for four weeks. Data correspond to the least-square means (LSmean) ± 95% confidence limit of LSmean after adjustment for strain, dosage and interaction (strain×dose). Each point included three to five observations.

3.4. Levels of ALT in serum

Subacute exposure to TCE at 0, 10, 100 and 1000 mg/kg b.w./day had no statistically significant effect on levels of serum ALT as a clinical chemistry indicator of liver injury. The averages of serum ALT among control groups showed the different background levels of ALT in different strains of mice. Nevertheless, there was no strain-dependent effect.

3.5. Necrosis in liver

We examined the necrosis as a marker for irreversible liver injury after TCE exposure for four weeks. There were no occurrences of necrosis in the livers of mice from all seven strains although ‘monocyte infiltration’ was observed in the livers of some of the tested mice from different strains.

3.6. *Hepatocyte proliferation*

Four strains distinguished clearly in Figure 6 were selected for determining PCNA-LI to observe cell proliferation. Oral gavage of TCE 1000mg/kg b.w./day caused a significant increase in PCNA-LI (%) in the liver, compared to the vehicle group ($p<0.05$) (Figure 6). Figure S2 further shows strain-dependent effect on PCNA-LI (%) among tested four strains ($p<0.05$), although necrosis was not observed in any dose and strain groups.

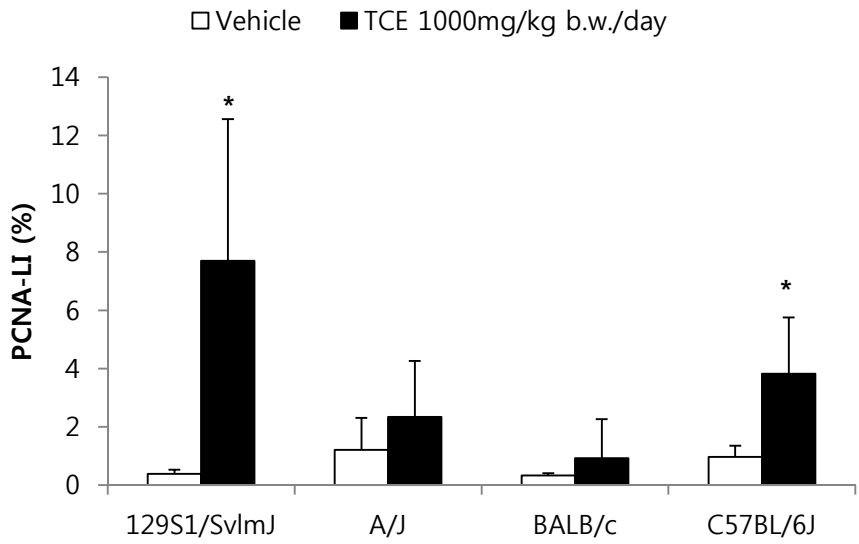
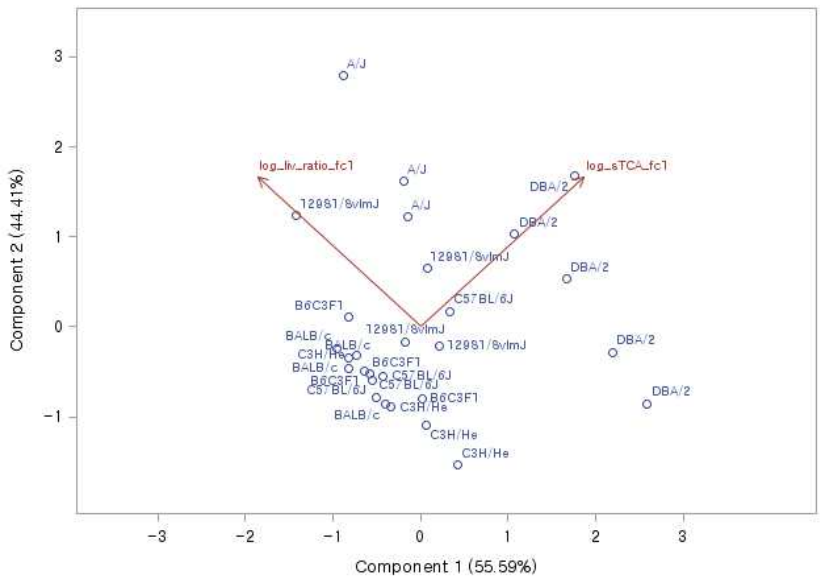


Figure 6. Change of PCNA-LI (%) in mouse liver following exposure to TCE 1000mg/kg b.w./day in selected strains of mice for four weeks. Data are mean ± SD, n=4/group. The symbol (*) indicates significant ($p < 0.05$) difference as compared with vehicle group and other three strains.

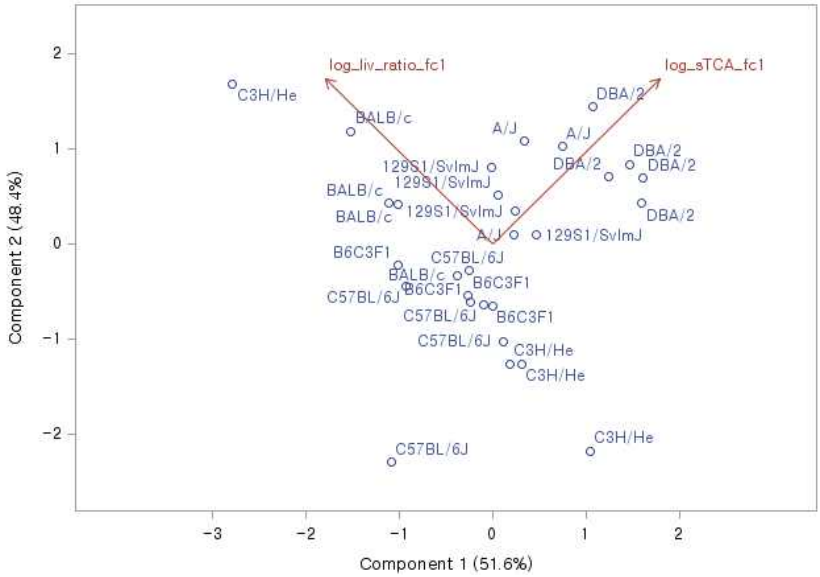
3.7. Principal component analysis of TCA and Liver-weight gain

Figure 7 showed principal component analysis which is to classify mouse strains using major two variables (TCA and liver weight) at each dose of TCE exposure supported. Component 1 consists of both TCA and liver weight and component 2 explains the other variances excluding component 1. In TCE 1000 mg/kg/day, strain-dependent effect was mainly explained by 69.69 % of component 1 while each component was about 50 % respectively in the groups of TCE 10 and 100 mg/kg/day (Figure 7). It suggested that DBA/2 and A/J might be susceptible to producing TCA and 129S1/SvImJ and BALB/c might be susceptible to increases in liver weight at the highest TCE exposure in this study. Meanwhile, C57BL/6J mice were far from these groups. To confirm the explanation, cluster analysis was carried out (Figure S 1). It corresponded with that DBA/2 and A/J were close each other indeed and they were apart from 129S1/SvImJ at TCE 1000 mg/kg b.w./day.

(a)



(b)



(c)

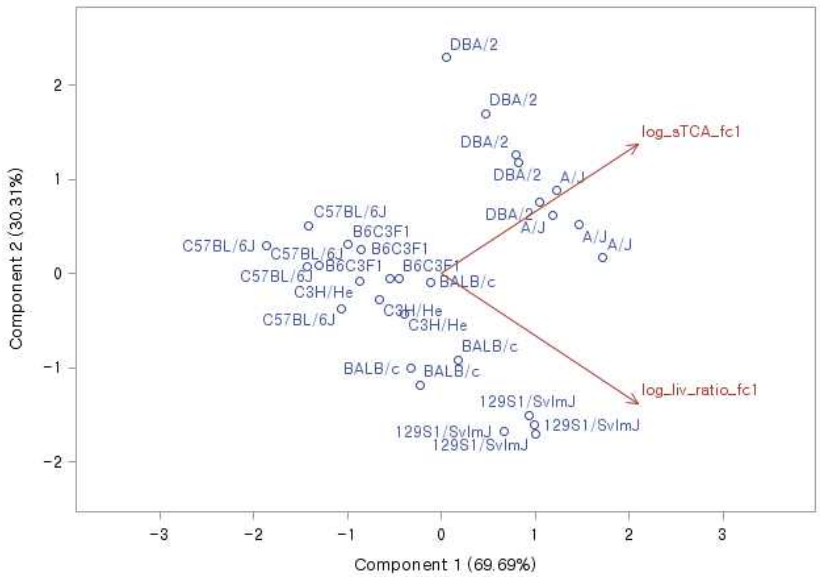


Figure 7. Principal component analysis using two variables (levels of serum TCA and liver weight gain) to classify mouse strains according to dose of TCE exposure: (a), 10 mg/kg b.w/day; (b), 100 mg/kg b.w./day; (c) 1000 mg/kg b.w./day.

4. Discussion

TCE toxicity is linked to its reactive metabolites and it has been believed that liver toxicity is more associated with oxidative metabolites such as DCA and TCA. Among these, TCA is abundant and has longer biological half-time relative to other metabolites (Kim et al 2009; Chiu et al 2014). In the present study, we successfully detected serum TCA 24-hr after the last dosage, which could be useful as both exposure biomarker and measures of metabolic activity of TCE. Also, there was wide variability of TCA production by dosage across test strains (Figure 4). As shown in Figure 3, we tried to find more susceptible strains showing high production of TCA and liver-weight gain by the levels of dosage with hope of strain-dependent variability could be maintained in the pipeline in the continuum of "exposure → metabolism → liver-weight gain", which could be explained with cell-proliferation in the liver according the previous studies (Bradford et al. 2011; Yoo et al 2015). We could confirm our hypothesis as well as the strain-dependent variability in the continuum seemed not to be maintained.

Serum TCA, biomarker of TCE metabolism, was dose-dependent and wide variability among strains. For instance, DBA/2 and A/J produced more TCA but C57BL/6J less compared to others (Figure 4), which was supported by previous reports focusing inter-strain variability in the metabolism using a multi-strain of mice (Bradford et al. 2011; Chiu et al. 2014; Yoo et al. 2015). The formation of TCA is mediated by cytochrome P450 (Lash et al. 2000; Lash et al. 2006). Variability in metabolic profiles and rates seemed determined by genetic polymorphisms governing key metabolic enzymes of TCE and corresponding cellular signaling processes (Kim and Ghanayem 2006; Xu et al. 2012). For example, C57BL/6J mice represented lower levels

of CYP450-dependent oxidation of TCE (Yoo et al. 2015). More than ten-fold difference was found in CYP-mediated metabolism of TCE among 15 strains of mice, which was associated with strain-specific differences in gene expression (Bradford et al. 2011). Briefly, activity or extents of CYP in the liver seemed determining factors in TCE metabolism and related biological effects in the liver across the various strains.

Dose- and strain-dependent effects were also found in liver-weight gain (Figure 5). Furthermore, all seven strains of TCE 1000 mg/kg b.w./day showed apparent differences from each control group in the present study (Table 1). Specifically, BALB/c, 129S1/SvImJ and A/J was more susceptible to liver weight gain, while C57BL/6J was less than them. Likewise, 129S1/SvImJ among inbred mice exposed to TCE 600 mg/kg/day for a week showed significant liver weight gain, and this associated with the key enzymes metabolizing TCE to TCA in mouse liver (Yoo et al. 2015). Liver weight gain has known as one of liver effects induced by subchronic exposure to TCE and TCA in mice (Evans et al. 2009; Kjellstrand et al. 1981; Laughter et al. 2004). TCA might not fully explain the liver weight gain in that it was accounted for only about only 20% to 50% of the degree of hepatomegaly produced by TCE (Chiu 2011). It was required to investigate the determinants of liver weight gain and the effect would have parallels with the different amounts of TCA among strains

The present study further showed that correlation between PCNA-LI and liver weight were statistically marginal (Figure S2; C). The result suggested that liver weight gain can be linked to increased cell numbers induced by TCE exposure. Increases in cell number that may result from either increased proliferation or decreased cell death could be a factor in the process of hepatomegaly (Kumar et al., 2010). In addition, cell and peroxisome

proliferation were likely to be responsible for TCA that is a peroxisome proliferator and activate PPARα, as well as inflammatory reaction through the interaction with various enzymes and proteins (Chinetti et al. 2000). Interestingly, cell numbers in C57BL/6J significantly increased but the strain was a low liver weight gain. Meanwhile, A/J mice was not statistically different from the control group although the levels of TCA and relative liver weight were high at TCE 1000mg/kg b.w./day (Figure 5 and 6). The results indicated that TCA may not be directly responsible for cell proliferation following exposure to TCE in several mouse strains although TCA was strongly associated with liver weight gain and PCNA-LI (Figure S2; A and B). A possible explanation is that TCE exposure elevated nuclear factor-κB (NF-κB) p52 mRNA and protein associated with inflammation in all mice regardless of PPARα, which can be activated by TCA, and hepatic lipid accumulation was observed in PPARα-null and hPPARα mice (Ramdhan et al. 2010).

According to the findings of PCA, interstrain differences seemed to be clear at high dose of TCE (Figure 7; C). It suggested that DBA/2 and A/J might be susceptible to producing TCA and 129S1/SvImJ and BALB/c might be susceptible to increases in liver weight at the highest TCE exposure. Meanwhile, C57BL/6J mice were far from these groups. Quantitative differences in the metabolites and liver weight gains may appear to be innate species differences in susceptibility to the effects on producing the metabolites (Bull 2000). In this perspective, the approach finding genetic factors and genetic variants are required to elucidate specific determinants of inter-strain differences in the process of hepatocellular and peroxisome proliferation in further study.

5. Conclusions

As shown in this study, subacute exposure to TCE (1000mg/kg/day) was not sufficient to induce the changes of serum ALT and necrosis in mouse liver. It may be because of the limitation that bioavailability of TCE is low by using corn oil vehicle. Nevertheless, this study elucidates that subacute TCE exposure resulted in strain-dependent effects on the formation of TCA and the increase in liver weight and hepatic cell proliferation among genetically diverse mouse strains. Over all, there was significant difference in the extent of TCE-induced responses according to dosage of TCE exposure and type of strain. Furthermore, the increase in the formation of TCA was correlated to the increase in liver weight and hepatic cell proliferation.

This study suggested that serum TCA could be suitable for a marker of TCE metabolism although the sampling time of blood was at 24 hour after last exposure to TCE. Plus, the experimental data on inter-strain variability obtained through the use of genetically diverse mice may be a key in order to find out the genetic variants of susceptibility to TCE-induced hepatomegaly and the other liver toxicities in further research.

References

Bradford BU, Lock EF, Kosyk O, Kim S, Uehara T, Harbourt D, et al. 2011. Interstrain differences in the liver effects of trichloroethylene in a multistrain panel of inbred mice. Toxicol Sci 120:206-217.

Bull RJ. 2000. Mode of action of liver tumor induction by trichloroethylene and its metabolites, trichloroacetate and dichloroacetate. Environ Health Perspect 108 Suppl 2:241-259.

Bull RJ, Orner GA, Cheng RS, Stillwell L, Stauber AJ, Sasser LB, et al. 2002. Contribution of dichloroacetate and trichloroacetate to liver tumor induction in mice by trichloroethylene. Toxicol Appl Pharmacol 182:55-65.

Channel SR, Latendresse JR, Kidney JK, Grabau JH, Lane JW, Steel-Goodwin L, et al. 1998. A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male b6c3f1 mouse liver. Toxicol Sci 43:145-154.

Chinetti G, Fruchart JC, Staels B. 2000. Peroxisome proliferator-activated receptors (ppars): Nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm Res 49:497-505.

Chiu WA. 2011. Trichloroacetic acid: Updated estimates of its bioavailability and its contribution to trichloroethylene-induced mouse hepatomegaly. Toxicology 285:114-125.

Chiu WA, Jinot J, Scott CS, Makris SL, Cooper GS, Dzubow RC, et al. 2013.

Human health effects of trichloroethylene: Key findings and scientific issues. Environ Health Perspect 121:303-311.

Chiu WA, Campbell JL, Jr., Clewell HJ, 3rd, Zhou YH, Wright FA, Guyton KZ, et al. 2014. Physiologically based pharmacokinetic (pbpk) modeling of interstrain variability in trichloroethylene metabolism in the mouse. Environ Health Perspect 122:456-463.

Elfarra AA, Krause RJ, Last AR, Lash LH, Parker JC. 1998. Species- and sex-related differences in metabolism of trichloroethylene to yield chloral and trichloroethanol in mouse, rat, and human liver microsomes. Drug Metab Dispos 26:779-785.

Evans MV, Chiu WA, Okino MS, Caldwell JC. 2009. Development of an updated pbpk model for trichloroethylene and metabolites in mice, and its application to discern the role of oxidative metabolism in tce-induced hepatomegaly. Toxicol Appl Pharmacol 236:329-340.

Kim D, Ghanayem BI. 2006. Comparative metabolism and disposition of trichloroethylene in cyp2e1-/-and wild-type mice. Drug Metab Dispos 34:2020-2027.

Kim S, Collins LB, Boysen G, Swenberg JA, Gold A, Ball LM, et al. 2009. Liquid chromatography electrospray ionization tandem mass spectrometry analysis method for simultaneous detection of trichloroacetic acid, dichloroacetic acid, s-(1,2-dichlorovinyl)glutathione and s-(1,2-dichlorovinyl)-l-cysteine. Toxicology 262:230-238.

Kjellstrand P, Kanje M, Mansson L, Bjerkemo M, Mortensen I, Lanke J, et al.

1981. Trichloroethylene: Effects on body and organ weights in mice, rats and gerbils. Toxicology 21:105-115.

Larson JL, Bull RJ. 1992. Species differences in the metabolism of trichloroethylene to the carcinogenic metabolites trichloroacetate and dichloroacetate. Toxicol Appl Pharmacol 115:278-285.

Lash LH, Fisher JW, Lipscomb JC, Parker JC. 2000. Metabolism of trichloroethylene. Environ Health Perspect 108 Suppl 2:177-200.

Lash LH, Putt DA, Parker JC. 2006. Metabolism and tissue distribution of orally administered trichloroethylene in male and female rats: Identification of glutathione- and cytochrome p-450-derived metabolites in liver, kidney, blood, and urine. J Toxicol Environ Health A 69:1285-1309.

Lash LH, Chiu WA, Guyton KZ, Rusyn I. 2014. Trichloroethylene biotransformation and its role in mutagenicity, carcinogenicity and target organ toxicity. Mutat Res Rev Mutat Res 762:22-36.

Laughter AR, Dunn CS, Swanson CL, Howroyd P, Cattley RC, Corton JC. 2004. Role of the peroxisome proliferator-activated receptor alpha (pparalpha) in responses to trichloroethylene and metabolites, trichloroacetate and dichloroacetate in mouse liver. Toxicology 203:83-98.

Ramdhan DH, Kamijima M, Wang D, Ito Y, Naito H, Yanagiba Y, et al. 2010. Differential response to trichloroethylene-induced hepatosteatosis in wild-type and pparalpha-humanized mice. Environ Health Perspect 118:1557-1563.

Rusyn I, Gatti DM, Wiltshire T, Kleeberger SR, Threadgill DW. 2010.

Toxicogenetics: Population-based testing of drug and chemical safety in mouse models. Pharmacogenomics 11:1127-1136.

Tryndyak VP, Latendresse JR, Montgomery B, Ross SA, Beland FA, Rusyn I, et al. 2012. Plasma microrna s are sensitive indicators of inter-strain differences in the severity of liver injury induced in mice by a choline- and folate-deficient diet. Toxicol Appl Pharmacol 262:52-59.

Xu XY, Liu YF, Lu LW, Ke YB, Mao JY, Mao KL. 2012. Altered expression of hepatic metabolic enzyme and apoptosis-related gene transcripts in human hepatocytes treated with trichloroethylene. Hum Exp Toxicol 31:861-867.

Yoo HS, Bradford BU, Kosyk O, Shymonyak S, Uehara T, Collins LB, et al. 2015. Comparative analysis of the relationship between trichloroethylene metabolism and tissue-specific toxicity among inbred mouse strains: Liver effects. J Toxicol Environ Health A 78:15-31.

Supplementary information

Strain-dependent responses of subacute exposure to trichloroethylene in a multi-strain panel of mice: An oxidative metabolite and the liver effects

Figure S1. Cluster analysis with two principle components considering the levels of TCA and the relative ratio of liver to body weight following subacute exposure to TCE 1000mg/kg b.w./day in seven strains.

Figure S2. Correlations between serum TCA and relative ratio of liver to body weight (a); serum TCA and PCNA-LI (b); PCNA-LI (b and relative ratio of liver to body weight (c).

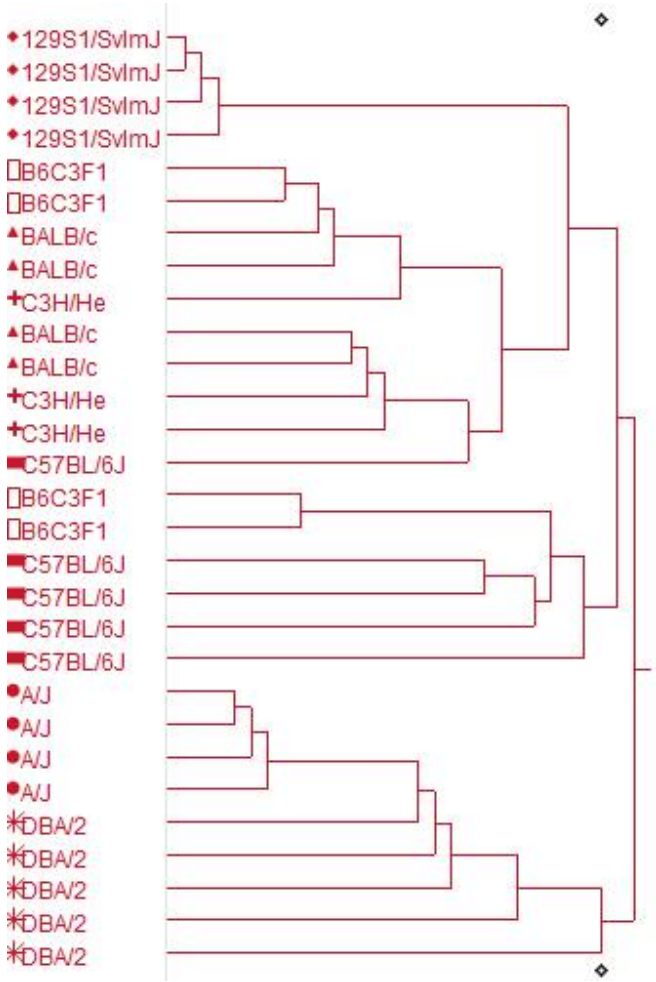
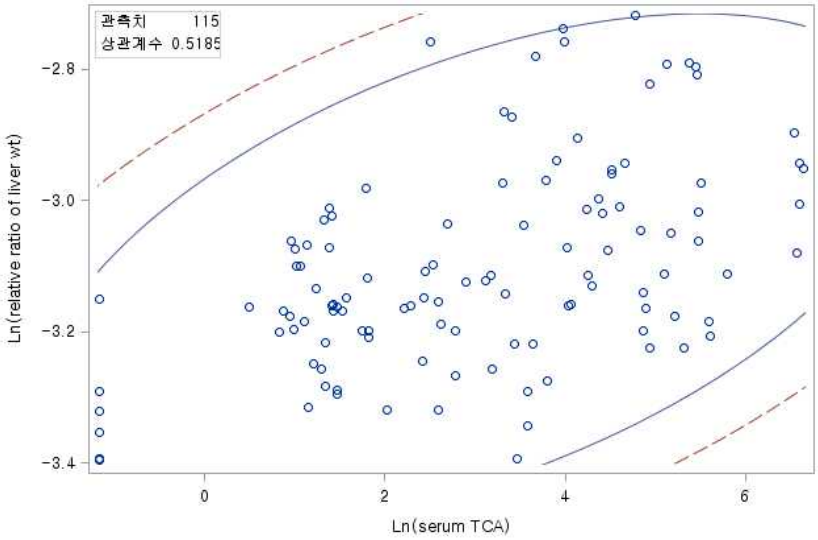


Figure S1. Cluster analysis with two principle components considering the levels of TCA and the relative ratio of liver to body weight following subacute exposure to TCE 1000mg/kg b.w./day in seven strains.

(a)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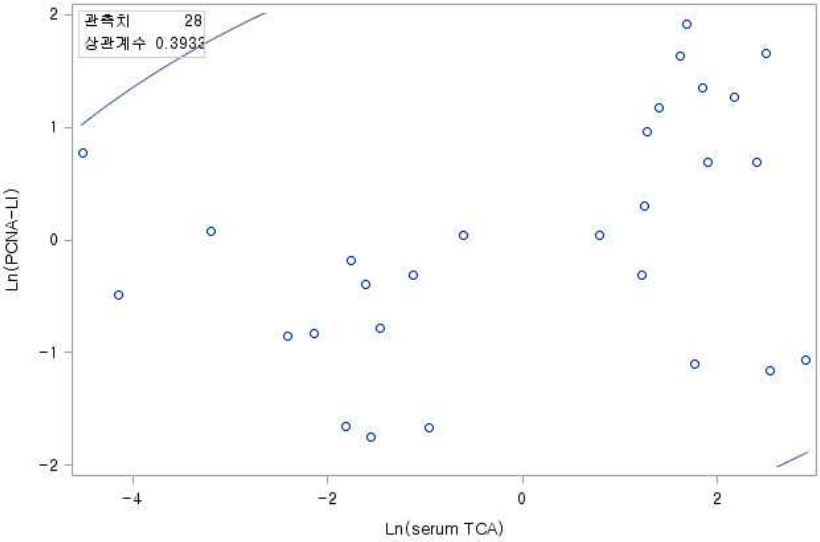
상관계수 0.5185
-2.8
-3.0
-3.2
-3.4
Ln(relative ratio of liver wt)
0
2
4
6
Ln(serum TCA)

(b)


관측치 28
상관계수 0.3933
2
1
0
-1
-2
Ln(PCNA-LI)
-4
-2
0
2
Ln(serum TCA)

(c)

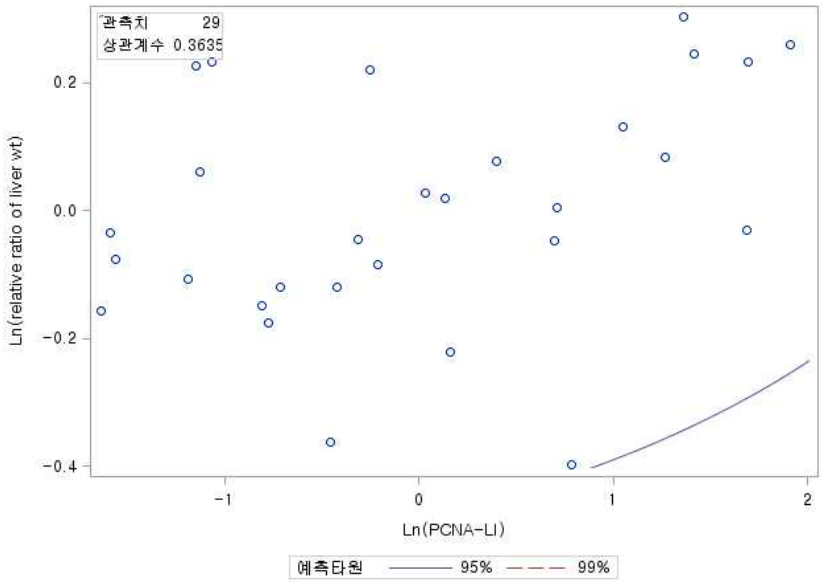


Figure S2. Correlations between serum TCA and relative ratio of liver to body weight (a); serum TCA and PCNA-LI (b); PCNA-LI (b and relative ratio of liver to body weight (c).

국문초록

아급성 트리클로로에틸렌 경구노출에 의한 마우스 계통 별 대사 및 간독성 반응 차이

서울대학교 보건대학원 환경보건학과

안 영 아

트리클로로에틸렌 (Trichloroethylene, TCE)은 인체발암물질로 확정되었지만, 신장 이외의 표적장기에 대해 비발암성 독성기전에 대해 충분한 근거가 없다. 동물실험 연구들을 살펴보면 종 간 표적장기가 다를 뿐만 아니라, 계통 간에서도 독성반응의 차이를 보이고 있으며, 여전히 TCE 대사와 독성기전에 관한 결정인자는 구체적으로 밝혀지지 않았다. 따라서 본 연구에서는 유전적으로 다양한 마우스 모델을 이용하여 아급성 TCE 노출 후 체내 대사산물과 간독성에 대한 계통 간 반응 차이를 정량적으로 비교하고자 하였다.

일곱 계통 (seven strains)의 마우스 (3~5개체/그룹)에게 TCE를 corn oil에 섞어 0, 10, 100, 1000 mg/kg b.w./day로 4주 동안 경구노출 시킨 후 생체시료를 수집하였다. 혈청시료에서 TCE 대사산물과 간손상 지표를 측정하였으며, 간무게를 체중으로 보정하여 나타내었다. 또한 간괴사를 관찰하기 위해 hematoxylin-eosin staining을 수행하였고, 면역화학적 염색으로 세포증식을 proliferating cell nuclear antigen-labeling index (PCNA-LI, %)로 표현하였다.

아급성 TCE 노출은 투여용량과 마우스 계통에 따라 트리클로로아세트산 (Trichloroacetic acid, TCA) 생성량과 간무게 증가에 대해 모두 통계적으로 유의한 차이를 나타냈다 ($p < 0.05$). 정량적으로 볼 때 DBA/2는 TCA 증가에 민감한 계통이었으며, 129S1/SvImJ는 간무게 증가에 민감한 계통으로 관찰되었다. 반면에, C57BL/6J는 TCA 생성과 간무게 증가에 상대적으로 둔감하게 반응하는 것으로 보였다. 혈청 ALT 증가와 간괴사는 나타나지 않았으나, TCE 고노출군에서 간무게 증가와 관련된 세포증식 여부를 확인할 수 있었다. 특히 129S1/SvImJ는 TCE 고노출군에서 유의하게 세포증식이 관찰되었으며($p < 0.05$), 간무게 증가와 관련 있는 결정인자 중 하나로 볼 수 있었다.

TCE의 4주 노출을 통해 대사적 차이와 간무게 증가에 대한 계통 간 차이를 정량적으로 비교할 수 있었다. 또한 이러한 반응들에 대한 계통 간 차이와 관련된 요인 중 하나로 세포증식을 들

수 있었다. 그 외 설명되지 않은 계통 간 차이는 계통이 가진 고유한 유전적 특성으로 사료되며, 본 연구는 TCE 노출에 대한 실험적 자료를 제공함으로써 차후 독성영향의 유전적 결정인자를 연구하는데 유용한 자료가 될 것이다.

주요어: 트리클로로에틸렌, 마우스 모델, 계통 간 차이, 산화적 대사 산물, 간무게 증가

학 번: 2009-23612