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# Trichloroethylene induced cutaneous irritation in BALB/c hairless mice: Histopathological changes and oxidative damage

Tong Shen[a,b], Qi-Xing Zhu[a,b,c], Sen Yang[a], Chang-Hao Wu[d], Hong-Fu Zhang[e], Chen-Fan Zhou[c], Xue-Jun Zhang[a,*]

[a] *Institute of Dermatology, Anhui Medical University, 81 Meishan Road, Hefei, Anhui 230032, PR China*
[b] *Department of Toxicology, School of Public Health, Anhui Medical University, Hefei, Anhui, PR China*
[c] *Department of Occupational health and Environmental Health, School of Public Health, Anhui Medical University, Hefei, Anhui, PR China*
[d] *Department of Physiology, Division of Medicine, Royal Free & UCL Medical School, University College London, Rowland Hill Street, London NW3 2PF, UK*
[e] *Department of Pathology, The First Affiliated Hospital, Anhui Medical University, Hefei, Anhui, PR China*

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ABSTRACT

Trichloroethylene (TCE), a colorless and volatile organic solvent, has long been a major chemical hazard during occupational exposure because of its extensive use in industry. Exposure to TCE can cause significant skin lesions, but the effect of TCE on skin irritation has received little attention. We therefore investigated the effect of TCE on skin irritation and oxidative stress using hairless mice. BALB/c hairless mice were subjected to acute and cumulative topical TCE treatment. Skin reactions were evaluated by visual inspection, histopathology examined by microscopy and oxidative stress assessed by measurement of malondialdehyde (MDA) levels, superoxide dismutase (SOD) activities and nitric oxide (NO) production. Under acute and cumulative TCE irritation, the skin developed erythema and edema, and the predominant histopathological features were hyperkeratosis, spongiosis and inflammatory cell infiltrates. In parallel to these morphological changes, acute TCE irritation also concentration-dependently increased MDA levels and inhibited SOD activities of the skin. However, in cumulative irritation, the MDA levels and SOD activities were initially elevated with increased TCE concentrations, but thereafter reduced with further concentration increments; the linear dose–response relationship was not preserved. TCE also concentration-dependently increased NO production both in acute and cumulative irritation. These results suggest that TCE is capable of producing skin irritation effect in vivo, with histopathological changes characterized by hyperkeratosis, spongiosis and inflammatory cell infiltrates. Moreover, oxidative stress may be associated with the clinical manifestations and histopathological abnormalities in TCE-induced skin irritation.

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## 1. Introduction

Trichloroethylene (TCE), a colorless and volatile organic solvent, is widely used in industry as metal degreasing and dry cleaning agent for various cleaning operations, such as computer chips and electronic product, and has been a major chemical hazard during occupational exposure (ATSDR, 1995; IARC, 1995). Under current technological and economical conditions, direct skin contact with TCE still often occurs and is inevitable during the process of various operations. TCE has been shown to produce a broad spectrum of toxicological effects in the skin. With the development of industry, an increasing number of reports on skin injuries have been implicated with TCE in recent years. TCE-caused dermatitis by occupational exposure has become a new and urgent problem to be solved in the field of public health in fast-developing countries including China (Huang et al., 2002; Nakajima et al., 2003; Chiu et al., 2006).

Evidence suggests that TCE-elicited dermatitis involves complex immune response and is regarded as a delayed-type hypersensitivity (DTH) response (Griffin et al., 2000; Kaneko et al., 2000; Chen et al., 2006). As an allergen, the skin sensitization potential of TCE has been studied in the past (Tang et al., 2002). As the patch test was negative in TCE-caused dermatitis in some of the patients, the DTH did not fully explain the mechanisms underlying TCE-induced skin lesions. Skin inflammatory reactions are under the control of a network of cytokines (Corsini and Galli, 2000; Effendy et al., 2000).

*Abbreviations:* LPO, lipid peroxidation; MDA, malondialdehyde; NHEK, normal human epidermal keratinocyte; NO, nitric oxide; NRU, neutral red uptake; PBS, phosphate buffered saline; S.D., standard deviation; SOD, superoxide dismutase; TCE, trichloroethylene.

* Corresponding author. Tel.: +86 551 5161002; fax: +86 551 5161016.
*E-mail addresses:* ayzxj@vip.sina.com, ayzxj@mail.hf.ah.cn (X.-J. Zhang).

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The primary irritation caused by irritants is believed to condition the development and severity of allergic contact dermatitis, due to the initiation of local inflammatory reaction by the release of primary cytokines. The release of cytokines leads to the attraction of inflammatory cells and to the destruction of keratinocytes by cell-mediated cytotoxicity (Levin and Maibach, 2002; Bonneville et al., 2007). Previous studies in our laboratory have demonstrated that TCE could cause cytotoxicity and induce apoptosis associated with oxidative stress in vitro using cultured normal human epidermal keratinocytes (NHEK), and determined the $NR_{50}$ values (used to predict the toxicity in vivo) to be 4.53 mM, a concentration encountered in the workplace, for TCE from NHEK by neutral red uptake (NRU) assay (Zhu et al., 2005a,b; Shen et al., 2007). These results indicated that TCE was not only an allergen, but also an irritant. However, the effect of TCE on skin irritation has received little attention.

The mechanisms underlying skin irritation are complex. In recent years, there has been considerable interest in oxidative stress as a potential mechanism in the pathogenesis of skin disease. It has been observed that oxidative stress has been implicated in the pathogenesis of various conditions including some inflammatory skin diseases such as atopic dermatitis, psoriasis vulgaris, and vitiligo (Fuchs et al., 2001; Okayama, 2005; Bickers and Athar, 2006). Oxidative stress resulting from the formation of excessive reactive oxygen species (ROS) and nitric oxide (NO) species, may damage cell membranes through production of lipid peroxides (LPO), as well as molecules such as nucleic acids, proteins and carbohydrates (Briganti and Picardo, 2003; Kuchel et al., 2003; Nishigori et al., 2004; Sezer et al., 2007). Whether TCE would exert a potent irritant effect by topical application in vivo is yet to be answered: if so, could this be mediated by increased oxidative stress? The aim of the present study was therefore to test the above hypothesis by evaluating the effect of TCE on skin irritation and histopathological alterations in hairless mice, and in parallel experiments, oxidative stress was determined by measuring malondialdehyde (MDA) levels, superoxide dismutase (SOD) activities and NO production.

## 2. Materials and methods

### 2.1. Chemicals

All agents including TCE (99.5% purity, analytical grade or the highest commercial grade available) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Concentrations of TCE used in this study were: 20%, 40%, 80%, and 100% (v/v dissolved in olive oil). Olive oil and distilled water were used as vehicle and blank control.

### 2.2. Animals

Eight- to ten-week-old female BALB/c hairless mice, were purchased from Shanghai Shilaike Experimental Animal Co. Ltd., China, and housed in groups of 3 in stainless steel wire-mesh cages, and had free access to diet and tap water. The animal room was maintained on a 12-h light/12-h dark cycle, temperature and relative humidity were kept at 20–25 °C and 55 ± 5%, respectively. The animals were acclimated for 1 week prior to the treatment, those healthy and weighing 20–30 g selected for experiments. The mice were randomly assigned to treatment or control groups of 5 animals each. All animal study protocols were conducted in accordance with the guidelines for humane treatment set by the Animal Care and Use Committee of Anhui Medical University.

### 2.3. Induction of acute and cumulative irritation

In order to enhance adherence, the dorsal skin of the animals about 2.5 $cm^2$ size area were de-lipidized with ethanol-wetted cotton pads. After air-drying, 50 μl of TCE dissolved in olive oil or control agents were painted topically on de-lipidized site, and then covered with sterile plastic film, which was fixed with non-irritant adhesive tape. The film was removed 4 h post-application (as an occupational hazard, 4 h of application represent a maximum continuous exposure in a working session at a workplace) and the treated area was gently wiped with normal solution to remove any residual liquid from the skin surface. One hour later, skin reactions were observed visually. This treatment was given twice daily (at 3 h interval) for 1 or 14 days in acute or cumulative irritation, respectively.

### 2.4. Skin reaction inspection and histopathological examination

Skin response to TCE treatment at each application site was visually assessed after each application. Reactions were graded as negative, mild (erythema alone), moderate (erythema with edema), or severe (erythema, edema, and vesiculation).

At the end of treatment, the animals were anesthetized and sacrificed by cervical dislocation. Skin biopsies from treated sites were taken and fixed in 10% buffered formaldehyde solution for at least 48 h, embedded into paraffin blocks and processed according to routine protocols. Sections of 5-μm thickness were cut from each sample, stained with hematoxylin-eosin (H&E). All sections were examined with a light microscope (BH-2, Olympus, Japan), by a pathologist unaware of the treatment of the samples. Histological parameters of the skin were evaluated using PAS-9000 pathological image analysis system (Logene Biological & Medical Engineering Co. Ltd.), and included hyperkeratosis (thickening of the stratum corneum), parakeratosis, spongiosis, exocytosis and dermal infiltration; these were scored as follows: (−) absent, (±) equivocal, (+) mild, (++) moderate and (+++) severe.

### 2.5. Homogenate preparation of skin tissue and quantification of protein content

The skin samples were excised promptly and rinsed in ice-cold saline, then homogenized in 4 ml phosphate buffered saline (PBS, pH 7.8) using a Polytron homogenizer. Homogenates were centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was collected and used for assay. The protein content in the homogenate was determined according to the method of Lowry et al. (1951). Bovine serum albumin served as standard.

### 2.6. LPO and SOD activities measurement

The levels of MDA, served as an indicator of LPO, were determined using the thiobarbituric acid-reactive substances (TBARS) method and performed according to the procedures described by Heath and Packer (1968) with slight modifications. 0.1 ml of the homogenate supernatant was transferred into a test tube containing 0.2 ml of 8% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA) and vortexed. The mixture was incubated for 40 min in boiling water. After cooling, an *n*-butanol and pyridine mixture (15:1, v/v) was added and centrifuged at 1000 × *g* for 10 min. Absorbance was determined at 532 nm, 1,1,3,3-tetra-methoxypropane was used as a standard. The results were expressed as mmol/mg protein.

The anti-oxidative enzyme-SOD activities was determined according to the method of Nishigori et al. (1989), which is based on inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine oxidase system as a superoxide generator. 50 μl of the homogenate supernatant was added in the reaction mixture containing 985 μl of 100 mM PBS (pH 7.4), 0.3 mM $K_2H_2$-EDTA, 0.5 mM NBT, and 0.1 mM xanthine. The mixture was preincubated for 3 min at 25 °C. Then 10 μl of 0.02 U/ml xanthine oxidase was added and centrifuged. Absorbance was recorded at 550 nm. SOD activities was calibrated from a standard curve of percentage inhibition of NBT reduction and expressed as units/mg protein. One unit of SOD was defined as the amount causing 50% inhibition in the NBT reduction rate.

### 2.7. Determination of NO production

Since NO rapidly degrades to nitrate and nitrite in aqueous solution, the total nitrate and nitrite levels were estimated as an index of NO production. This test used a spectrophotometric method based on the Griess reaction (Green et al., 1982). To measure nitrite plus nitrate levels, 0.1 ml supernatant was mixed with Griess reagent (consisting of one part 1% sulfanilamide in 5% orthophosphoric acid) at room temperature for 10 min, and the absorbance was then measured at 550 nm. The concentration of nitrite plus nitrate was calculated according to a $NaNO_2$ standard linear curve. Results were expressed as μmol/mg protein.

### 2.8. Statistical analysis

Throughout the text, data were expressed as mean ± standard deviation (S.D.). All statistical analysis was performed by ANOVA followed by Student–Newman–Keuls test, with SPSS 12.0 software package. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. TCE induced skin irritation and histopathological changes in hairless mice

In acute irritation test, TCE treatment for 1 day resulted in mild to moderate irritation, and the skin developed erythema and edema

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