# Mechanistic Evaluation of Trichloroethene-Mediated Autoimmune Hepatitis-Like Disease In Female MRL+/+ Mice

Shakuntala Kondraganti[a], Rolf König[b], Paul J. Boor[a], Shahnawaz Khan[a], Bhupendra S. Kaphalia[a], M. Firoze Khan[a] and G.A.S. Ansari[a,*]

[a]*Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA*

[b]*Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA*

**Abstract:** Environmental and occupational exposure to trichloroethene (TCE) is associated with autoimmune diseases (ADs). However, the mechanisms of TCE-mediated ADs are not fully elucidated. Previous investigations showed that chronic low dose exposure of autoimmune-prone female MRL+/+ mice to TCE resulted in development of autoimmune hepatitis-like disease (AIHLD). To elucidate the mechanisms involved in the development of AIHLD, we treated female MRL+/+ mice with TCE (0.5 mg/ml) via drinking water for 24, 36 and 48 weeks. Exposure to TCE resulted in increased lymphocytic infiltration and periportal hepatocellular necrosis in the livers of mice exposed for 48 weeks. Significantly increased apoptotic cells were observed in the livers after 24 weeks of TCE exposure as analyzed by TUNEL assay. Staining of Kupffer cells with RM-4 monoclonal antibody showed a decrease in number of Kupffer cells at 36 and 48 weeks of TCE exposure which may cause delayed/reduced clearance of apoptotic bodies. These observations led us to hypothesize that compromised Kupffer cell function may cause impaired clearance of apoptotic bodies, leading to secondary necrosis and inflammation. To test this hypothesis, we treated HepG2 cells with various concentrations of TCE and time periods. TCE treatment of HepG2 cells at 12mM and higher concentrations led to their decreased viability after 24h. When TCE-treated HepG2 cells were co-cultured with untreated RAW cells, the phagocytic function of RAW cells was reduced accompanied by increased secretion of tumor necrosis factor alpha. These results suggest that increased apoptosis and decreased phagocytic function of Kupffer cells may probably lead to accumulation of apoptotic bodies and secondary necrosis and the resulting inflammation could be a plausible mechanism of TCE-induced AIHLD.

**Keywords:** Trichloroethene (TCE), Autoimmune hepatitis, HepG2 cells, RAW cells, Apoptosis, Phagocytosis.

## INTRODUCTION

Trichloroethene (TCE), a chlorinated hydrocarbon, is a widely used industrial chemical and a common environmental pollutant. Environmental exposure to TCE can occur through air or *via* contaminated ground water and soil, whereas occupational exposure results primarily from its use as a degreasing solvent. Chronic TCE exposure in humans has been implicated in inducing a number of autoimmune diseases (ADs) like systemic lupus erythematosus [1], systemic sclerosis [2, 3], dermatitis [4, 5], scleroderma [6-8] and fasciitis [9]. Studies in rodents have also showed that TCE exposure induces/exacerbates an autoimmune response [10, 11] and its long-term exposure causes autoimmune hepatitis-like disease (AIHLD) [11, 12].

Autoimmune hepatitis is an inflammation of the liver characterized by elevated serum IgG levels, antinuclear antibodies (ANA) and T-lymphocytic infiltration. Autoimmune hepatitis is classified into Type 1 and Type 2 associated with ANA and anti-smooth muscle antibodies, whereas Type 2 is associated with anti- microsomal

with the former being the most common. Type 1 is antibodies [13]. Although the exact etiology of autoimmune hepatitis is unknown, viruses, drugs and chemicals are known to contribute to the disease [14, 15]. Case reports also show that occupational exposure to TCE results in generalized skin disorders and accompanying hepatitis with or without jaundice [16-20]. In a case reported, TCE exposure resulted in hepatitis, rash and eosinophilia and the immune mechanisms involved were thought to be similar to halothane-induced hepatitis [21].

We and others have shown that TCE and its metabolites induced various autoantibodies and autoimmune hepatitis-like disease in female MRL+/+ mice [10-12, 22]. Disturbance in T lymphocytes leading to increased antigen-induced antibody production by B cells was seen in patients with autoimmune hepatitis [23]. Similarly, protein adducts formed by the metabolism of drugs like halothane or minocycline may act as neo-antigens, generating auto-antibodies and cell-mediated immune responses resulting in liver injury [24]. A protein adduct of TCE metabolite was tentatively identified in the livers of TCE-treated rats [25]. Albumin haptenized with metabolites of TCE induced both humoral and T cell responses [26]. Interestingly, one such adduct, formyl-albumin induced AIHLD similar to TCE [26]. Also, CD4^{+} T cell activation with mononuclear infiltration in portal regions consistent with autoimmune hepatitis was observed in

*Address correspondence to this author at the Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0438, USA; Tel: (409)772-3655; Fax: (409)747-1763; E-mail: sansari@utmb.edu

autoimmune-prone MRL+/+ female mice treated with TCE via drinking water [12].

Macrophages are mononuclear phagocytes which play a pivotal role in regulation of inflammation and innate immune responses by exerting both pro and anti-inflammatory responses as well as anti-proliferative and cytotoxic activities [27]. Macrophages also act as antigen-presenting cells and are involved in initiating specific T-cell immune responses [28]. Resident liver macrophages (Kupffer cells) have high phagocytotic activity and eliminate microbes and apoptotic cells through phagocytosis. However, persistence of apoptotic cells eventually leads to further cell death through necrosis and induction of proinflammatory responses with concommitant release of TNF-α, IL6 and IFN-γ [28, 29]. In SLE patients, macrophages with atypical morphology and defective adhesion capabilities have been reported, a phenotype which may be responsible for the impaired phagocytosis of apoptotic bodies, thus leading to chronic inflammation and autoimmunity [30].

Here, we report a plausible mechanism by which TCE induces AIHLD. We focused our studies on apoptosis and lymphocytic infiltration in the livers of female MRL+/+ mice chronically exposed to TCE through the drinking water. We observed delayed/reduced clearance of apoptotic bodies and compromised Kupffer cell function in the livers of TCE-treated mice compared to controls. These in vivo observations were complemented by in vitro studies by co-culturing TCE-treated HepG2 (human hepatocellular carcinoma cell line) cells and untreated RAW (mouse leukemic monocyte macrophage cell line) cells. We observed that the phagocytic activity of RAW cells against apoptotic HepG2 cells was compromised and the secretion of TNF-α was increased. Our results suggest that increased apoptosis in the liver and decreased phagocytosis by Kupffer cells may be contributing to TCE-mediated AIHLD.

## MATERIALS AND METHODS

### Animal Studies

Five-week old female MRL+/+ mice, purchased from The Jackson Laboratories (Bar Harber, ME), were housed in plastic cages on a bedding of wood chips at the University of Texas Medical Branch (UTMB) animal house facility maintained at ~22 °C, 50–60% relative humidity, and a 12 h light/dark cycle. The animals were provided standard lab chow and drinking water ad libitum and were acclimated for one week before the treatment. TCE (purity 99+%, Sigma, St. Louis, MO) was dissolved in drinking water containing 1% Alkamuls EL-620 emulsifier (Rhodia Chemicals, Cranbury, NJ). The mice were divided into 6 groups of 5 each and received TCE (0.5mg/ml) or drinking water containing 1% Alkamuls EL-620 emulsifier only (controls) for 24, 36 and 48 weeks. The dose used corresponds to the Occupational Safety and Health Administration's (OSHA) permissible exposure limit of 100 ppm (www.osha.gov/dts/-chemicalsapling/data/CH_273000.html) (~0.4 mg/ml) for an eight-hour exposure. The water was changed twice weekly. The average water intake was ~0.13 ml/g/d for controls and ~0.12 ml/g/d for TCE-treated mice, representing an average daily TCE intake of about ~60 µg/g/d in the treatment groups. After 24, 36 and 48 weeks the mice were euthanized under Nembutal anesthesia (sodium pentobarbital, i.p., 80

mg/kg) and the livers removed immediately and portions fixed in neutral buffered formalin, while the remaining portions were stored at -80°C for further analysis.

### Histopathology

#### *H&E Staining*

The 10% neutral-buffered formalin fixed and paraffin embedded liver sections were stained with Hematoxylin and Eosin (H&E) for morphological evaluation. The histological scoring was done based on lymphocytic infiltration as follows: Grade 0 = no change; Grade 1 = focal periportal lymphocytic infiltration; Grade 2 = infiltration surrounding portal with occasional infiltration around central veins; Grade 3 = diffuse periportal infiltration with consistent infiltration around central vein with areas of hepatocellular necrosis or apoptosis.

#### *CD3 Staining*

To detect T-cells, formalin fixed and paraffin embedded liver sections were deparaffinized, followed by heat induced antigen retrieval at pH 9.0 using target antigen retrieval solution and overnight incubation at 4°C with polyclonal rabbit anti-mouse CD3 antibody (Dako, Carpinteria, CA). Sections were then incubated with secondary antibody followed by peroxidase staining for CD3 expression and images captured with an Olympus 1X71 microscope (Olympus, Hamburg, Germany). Histological grading of CD3 T-cell infiltration was done based on lymphocytic infiltration.

#### *TUNEL Staining*

Formalin-fixed and paraffin-embedded liver sections were stained with DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI). After deparaffinization liver sections were fixed in 4% formaldehyde in phosphate buffer saline for 15 minutes and permeabilized with 100µl of 20µg/ml of Proteinase K solution for 10 minutes. Sections were then equilibrated with 100µl of equilibration buffer for 10 minutes, labeled with TdT reaction mix and incubated at 37°C for 60 minutes. The reaction was stopped with 2X SSC (saline-sodium citrate solution, pH 7.0) and slides mounted with Vectashield mounting medium with DAPI for nuclear staining (Vector Laboratories, Burlingame, CA) and 10 fields counted for apoptotic cells using an Olympus 1X71fluorescent microscope with DP7.1 camera at 40X magnification.

#### *Kupffer Cell Staining*

Formalin fixed, paraffin embedded sections were stained for macrophages (Kupffer cells) using the monoclonal antibody RM-4 (Cosmo Bio Co Ltd, Carlsbad, CA). Tissue sections were deparaffinized and antigen retrieval done with sodium citrate buffer at pH 6.0, followed by blocking with blocking serum for one hour, with overnight primary antibody incubation at 4°C and immunoperoxidase staining with ABC staining kit (Santa Cruz Biotechnology, Santa Cruz, CA) as per the manufacturer's protocol. Images were captured with an Olympus 1X71 microscope at 20X.

#### *Cell Culture and Treatment*

Human hepatocellular carcinoma (HepG2) cells and mouse leukemic monocyte macrophage (RAW 264.7) cells from the ATCC were cultured in Dulbecco's Modified Eagle

Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco, Carlsbad, CA) in a humidified atmosphere containing 5% $CO_2$ at 37°C. HepG2 cells were treated with different concentrations (1mM – 32mM) of TCE dissolved in 0.8% DMSO for various time periods (1h – 72h). All experiments were done independently three times in triplicates.

### MTT Assay

Cell viability of TCE-treated HepG2 cells was determined by MTT Cell Viability Kit (Biotium Inc, Hayward, CA). Briefly, 1 x $10^4$ HepG2 cells were plated per well into a 96-well culture plate (Fisher Scientific, Pittsburgh, PA) and treated with various concentrations of TCE or DMSO for various lengths of time. Then 10μl of MTT solution was added to each well and the incubation continued for 4h at 37°C, followed by removal of media. DMSO (200μl) was added to each well and mixed by pipetting to dissolve the formazan and the absorbance measured on a Benchmark Plus microplate reader (BioRad, Hercules, CA) at a test wavelength of 570 nm and reference wavelength of 630 nm.

### Cell Death Assays

### TUNEL Assay

Apoptotic cell death in TCE-treated HepG2 cells was determined using the DeadEnd Fluorometric TUNEL System (Promega). HepG2 cells were plated at 5 x $10^3$ cells per well into 8-well Lab-tek chamber slides (Fisher Scientific) and treated with various concentrations of TCE for 24h. The cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, labeled with TdT reaction mix for 1h at 37°C and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Stained cells were observed using an Olympus 1X71 fluorescent microscope and images captured at 20X.

### Flow Cytometry

Apoptosis was quantitated by flow cytometry using the FITC/Annexin V apoptosis detection kit I (BD Biosciences, San Jose, CA). HepG2 cells treated with TCE (1 x $10^5$ in 100μl of 1X binding buffer) were incubated with 5μl FITC/Annexin V and 5μl propidium iodide for 15 min at room temperature. The cells were analyzed by flow cytometry using a Becton–Dickinson FACS Canto (BD Biosciences).

### RAW Cell Culture

RAW cells (1 x $10^4$ per well) were plated in a 96-well plate and treated with TCE. Cell viability was determined as described above. To determine phagocytic activity RAW cells were plated at 2 x $10^5$ cells per well in a 6-well plate and treated with TCE (8mM - 20mM dissolved in 0.8% DMSO). After 24h, latex beads (Sigma-Aldrich, St. Louis, MO) were added (10%, 3-μm size) in fresh medium and the incubation continued for 2h. The medium was decanted and the cells were washed and stained with Diff-Quik stain set (Dade Behring AG, Dudingen, Switzerland) following the manufacturer's protocol. A total of 10 fields of RAW cells and their content of internalized latex beads (phagocytosized

beads) were counted using an Olympus 1X71 fluorescent microscope at 40X magnification.

### Co-Culture of HepG2 and RAW cells

HepG2 cells were treated with TCE for 24h, washed, counted and co-cultured with untreated attached RAW cells at a 2:1 ratio (100,000:50,000) for 24h in a 24-well culture plate. Cells were washed with PBS and stained with Diff-Quik Staining Kit (Dade Behring AG). To quantitate phagocytosis, ten 40X fields of cells were counted for phagocytosis and results expressed as percentage of positive cells/high power field.

### Enzyme-linked Immunosorbent Assay (ELISA)

TNF-α was measured in the culture supernants of TCE-treated HepG2 cells co-cultured with untreated RAW cells using a Biosource ELISA kit (Invitrogen, Carlsbad, CA).

### Statistical Analysis

All data are presented as mean ± SD. For the determination of statistical significance, the data were subjected to the analysis of variance (ANOVA) followed by Student– Newman–Keuls post hoc test. P values ≤ 0.05 were considered to be statistically significant.

## RESULTS

### Effect of TCE Exposure on Liver Morphology

TCE and protein adducts of TCE metabolites such as formyl-albumin induce histopathological changes consistent with AIHLD [11, 12, 26]. In our studies in female MRL+/+ mice, livers stained with H&E showed absent/minimal lymphocytic infiltration after 24wks (Grade 0-1) with predominantly periportal infiltration with occasional infiltration around central vein after 36wks (Grade 2) of TCE treatment. After 48wks of TCE treatment, heavy acute and chronic lymphocytic infiltration was seen. The inflammatory infiltrate was associated with patchy hepatocyte necrosis at the limiting plate of the portal triad (Grade 3) (Fig. **1a**). Lymphocyte infiltration was confirmed by immunohisto-chemistry using a polyclonal antibody against the T-cell specific marker CD3 (Fig. **1b**).

### TCE Induced Apoptosis in the Livers of MRL+/+ Mice

To evaluate TCE-induced apoptosis, untreated and TCE-treated livers were stained using the DeadEnd Fluorometric TUNEL system. A significant increase in apoptosis of two-fold was observed after 24wks of TCE-treatment as compared to respective controls while, no significant increases were observed at 36 and 48 weeks (Fig. **2**). These findings suggest that increased apoptosis may precede lymphocytic infiltration.

### TCE Compromised Macrophage Function in the Livers of MRL+/+ Mice

Macrophages play a crucial role in clearing apoptotic cells without inducing inflammation. When the phagocytic activity of macrophages is compromised, clearance of apoptotic cells is delayed and secondary necrosis results
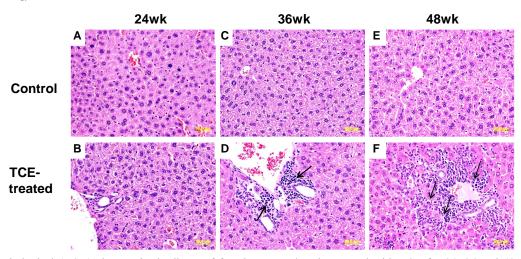
**Fig. (1a).** Histopathological (H&E) changes in the livers of female MRL +/+ mice treated with TCE for 24, 36 and 48 weeks. A. 24wk control; B. 24wk TCE-treated showing absent/minimal lymphocyte infiltration; C. 36wk control; D. 36wk TCE-treated: arrows representing diffuse periportal and occasional central vein infiltration; E. 48wk control; F. 48wk TCE-treated: arrows representing heavy acute and chronic lymphocytic infiltration associated with hepatic necrosis at limiting plate of portal triad (20X magnification). n=5.



**Fig. (1b).** CD3 staining of liver confirming infiltration of T lymphocytes (increased CD3 positive T cells) at 36wk and 48wk of TCE exposure. The staining was confined mostly around periportal and central veins. (20X magnification). No changes were observed at 24wk (data not shown). n=5.
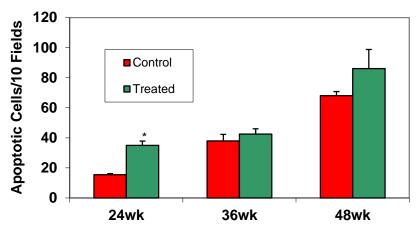


**Fig. (2).** TUNEL assay in the livers of MRL+/+ mice treated with TCE for 24, 36 and 48 weeks. A two-fold increase in apoptotic cells was observed at 24wk, while no statistically significant difference was found at 36wk and 48wk of TCE-exposure as compared to controls. * p < 0.05 compared to controls, n=5.
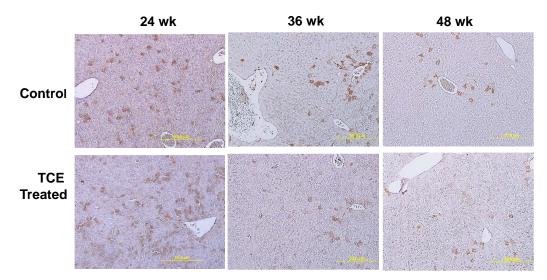
**Fig. (3).** Immunohistochemistry of Kupffer cells in liver sections immunostained with RM-4 antibody show decreased number of stained Kupffer cells at 36 and 48wk compared to 24wk TCE treated mice indicating that Kupffer cell function may be compromised. (20X magnification). n=5.
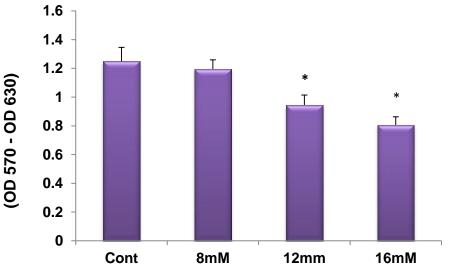


**Fig. (4).** MTT cell viability assay for HepG2 cells shows a significant decrease in cell viability at 12mM and 16mM TCE concentration, compared to controls. * $p < 0.05$ compared to controls. n=3.

followed by the release of proinflammatory cytokines, eventually leading to inflammation and autoimmunity [31]. In TCE-treated livers immunostained with the macrophage and dendritic cell specific antibody RM-4, which recognizes an endolysosomal membrane protein [32], the number of Kupffer cell positively stained with the antibody decreased after 36wks and 48wks compared to 24wks (Fig**. 3**). Even though reduced Kupffer cell staining was evident in the livers of all five TCE-treated mice after 36wks and 48wks, the extent of staining differed and did not attain the statistical significance. This could be due to low number of mice per group and variability in staining. This necessitates further evaluation of the functionality of Kupffer cells by more sensitive techniques such as flow cytometry using cell tracking probes [33]. These results suggest that TCE reduces the number of Kupffer cells and/or the functions of Kupffer cells are compromised, which may result in delayed clearance of apoptotic cells and development of secondary necrosis and inflammation.

**TCE Decreased Cell Viability of HepG2 Cells**

HepG2 cells were initially evaluated for TCE toxicity using concentrations from 1mM to 32mM and for durations ranging from 1h to 72h. A significant decrease in cell viability was observed at 12mM and higher TCE concentrations after 24h of incubation period (Fig. **4**) and hence we used 8mM, 12mM and 16mM concentrations for our studies. The viability of DMSO treated HepG2 cells was similar to the control cells (data not shown).

**TCE induced Apoptosis of HepG2 Cells**

Apoptosis was determined in HepG2 cells treated with TCE by staining with TUNEL assay kit. An increase in apoptotic cells was observed after incubation for 24h with 12mM and higher concentration of TCE (Fig. **5**). Quantification of TCE- induced apoptosis of HepG2 cells using flow cytometry also showed significantly increased apoptosis after incubation with 12mM or higher concentrations compared to controls (Fig. **6**). The percentage
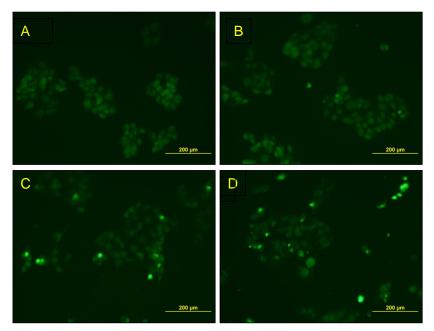
**Fig. (5).** TUNEL staining of HepG2 cells showing increased number of TUNEL positive (apoptotic) after exposure to 12mM (C) and 16mM (D) TCE compared to control (A) and 8mM (B) TCE-treated cells. (20X magnification). n=3.
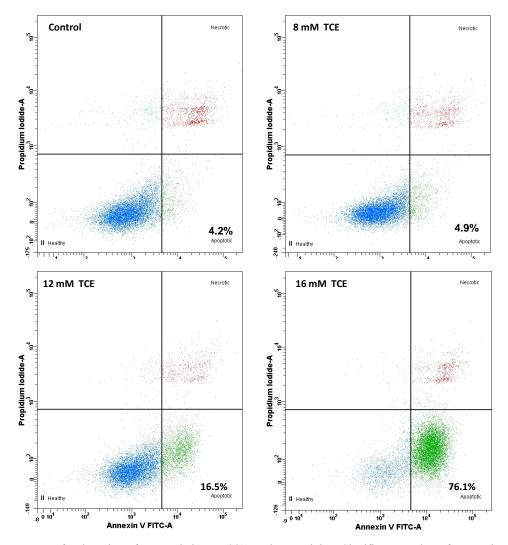


**Fig. (6).** Flow cytometry for detection of apoptosis by FITC/Annexin V staining. Significant number of apoptotic cells were found when HepG2 cells were exposed to 12mM or 16mM TCE compared to controls and 8mM TCE-treated cells. n=3.
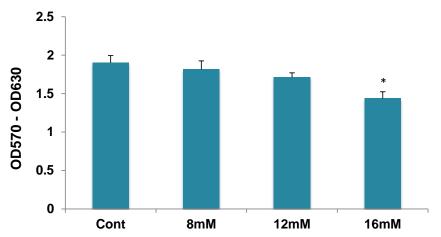
**Fig. (7a).** MTT assay showing significant decrease in cell viability of RAW cells at 16mM TCE concentration compared to controls. * p < 0.05 compared to controls. n=3.
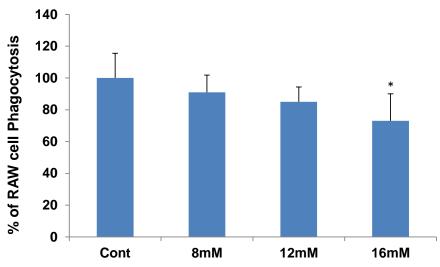


**Fig. (7b).** Phagocytotic assay for latex bead uptake by RAW cells. A significant reduction in phagocytotic activity was seen in cells treated with 16mM *p < 0.05 compared to controls TCE and n=3.

of apoptosis observed in controls, 8mM, 12mM and 16mM TCE concentrations was (4.55 ± 0.49), (5.3 ± 0.56), (18.25 ± 2.47) and (77.15 ± 1.48), respectively.

**TCE Reduced Cell Viability and Phagocytosis of Raw Cells**

RAW cells were treated with TCE at concentrations from 1mM to 32mM and for durations ranging from 1h to 72h. After 24h of incubation, a significant decrease in RAW cell viability was observed at 16mM and higher concentrations of TCE (Fig. **7a**). Also, a significant decrease in RAW cell phagocytic activity (uptake of latex beads) was observed after incubation for 24h with 16mM concentrations of TCE (Fig. **7b**).

**Reduced Phagocytosis of TCE-treated HepG2 Cells Co-Cultured with Untreated RAW Cells**

Since macrophages play an important role in clearing the apoptotic cells through phagocytosis, we co-cultured TCE-treated HepG2 cells with untreated attached RAW (macrophage) cells. A significant decrease in phagocytosis of HepG2 cells was observed at 12mM and higher concentrations of TCE (Fig. **8**). These findings support our

*in vivo* data, which shows that the function of macrophages could be compromised by TCE, probably resulting in delayed/incomplete clearance of apoptotic cells. We also used different combinations of co-culture studies with HepG2 and RAW cells and found that only attached RAW cells engulfed HepG2 cells.

**Effect of TCE on Cytokine Production**

Significant increase in TNF-α levels were observed in the supernatants of TCE-treated HepG2 cells co-cultured with untreated RAW cells at 12mM and higher concentrations (Fig. **9**) indicating that TNF-α may be contributing to pro-inflammatory response in vitro.

**DISCUSSION**

The etiology of ADs is often unknown. However, both environmental and genetic factors are involved. Many intrinsic factors (e.g., age, sex and genetics) and extrinsic factors (e.g., drugs, chemicals and environmental toxicants) have been implicated in the initiation of ADs [34]. TCE, a halogenated ethene, is known to cause AIHLD in humans and animal models [11, 12, 19]. To define the role of environmental and genetic factors in the induction of
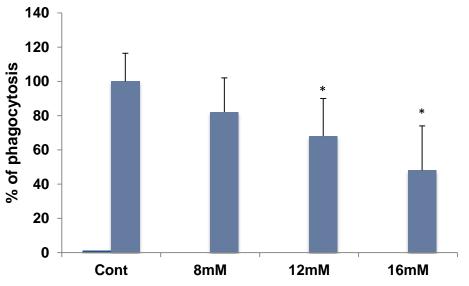
**Fig. (8).** Co-culture of HepG2 cells with RAW cells for phagocytosis. A significant decrease in phagocytosis of TCE-treated HepG2 by RAW cells was observed at 12mM and 16mM concentration compared to controls. * $p < 0.05$ compared to controls. n=3.
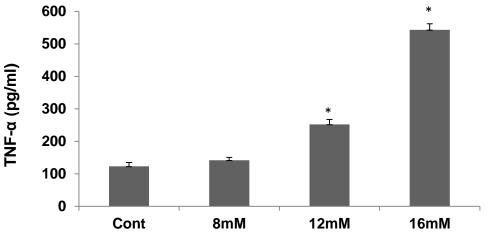


**Fig. (9).** TNF-$\alpha$ in the culture supernants of TCE-treated HepG2 cells co-cultured with RAW cells. Increases in TNF-$\alpha$ levels were observed at 12mM (2 fold) and 16mM (4.5 fold) as compared to controls. * $p < 0.05$ compared to controls. n=3.

AIHLD, we chose TCE as the environmental factor and selected autoimmune-prone female MRL+/+ mice as the intrinsic factor.

Autoimmune hepatitis is characterized by increased serum IgG, ANA and lymphocytic infiltration. The mechanisms for such changes are only partially understood. Previously, we reported that chronic exposure of female MRL+/+ mice to TCE at occupationally relevant doses results in AIHLD [11]. In addition, albumin haptenized with formyl, a metabolite of TCE, induced immunogenic responses and AIHLD in MRL+/+ mice [26].

Here, we report increased T-lymphocytic infiltration in the livers of MRL+/+ mice after 36 weeks of treatment with TCE, consistent with earlier reports of AIHLD [11, 12]. In addition, diffuse periportal and central vein infiltration by T cells with areas of focal hepatocellular necrosis appeared after 48 weeks of treatment with TCE. We observed increased apoptosis in the livers of MRL+/+ mice treated for 24 weeks preceding extensive lymphocytic infiltration. Reduced Kupffer cells subsequently at later time points may be responsible for delayed clearance of apoptotic bodies by resident liver macrophages, causing secondary necrosis and

leading to chronic inflammation. Immunohistology of livers with the macrophage and dendritic cell-specific monoclonal antibody RM-4 against endolysosomal membrane protein [32] indicated an impaired Kupffer cell function. Since, apoptosis and deranged removal of apoptotic cells play a role in autoimmunity [35, 36], our data supports the hypothesis that TCE causes apoptosis and impaired removal of apoptotic cells due to delayed/reduced clearance by macrophages leading to AIHLD. Our previous studies [11] and studies by [37] demonstrated increased secretion of IFN-$\gamma$ in TCE-treated MRL+/+ mice which may lead to an up-regulation of inflammatory genes in MRL+/+ mice and thus, contribute to an inflammatory response.

To study TCE-induced apoptosis and macrophage dysfunction, we used an in vitro approach involving HepG2 cells and RAW cells. TCE treatment induced apoptosis of HepG2 cells which were co-cultured with untreated RAW cells representing macrophages. Our in vitro studies showed a significant decrease in cell viability in TCE-treated HepG2 cells. Studies by [38] elucidated that TCE-induced apoptosis in normal human epidermis keratinocytes. Our findings showed significant increase in apoptosis by TUNEL assay

which was also confirmed by flow cytometry. A decrease in cell viability and phagocytosis was also observed in TCE-treated RAW cells corresponding to an impaired macrophage cell function in TCE-treated mice. We also demonstrated decreased phagocytosis with elevated levels of TNF-α (pro-inflammatory cytokine) in co-culture studies. It is known that phagocytic ingestion of apoptotic cells results in production of anti-inflammatory cytokines and suppression of inflammatory cytokines, whereas failed clearance of apoptotic cells results in the production of pro-inflammatory cytokines like TNF-α [39,40]. Similarly, in chemical-induced hepatotoxicity, increased levels of TNF-α were reported preceding the onset of hepatic parenchymal cell injury [41,42]. Also, hepatocytes may influence functions of Kupffer cells or endothelial cells to express cytokines like IFN-γ or TNF-α [43].

In this study, we observed an early increase in apoptosis followed by necrosis and increased T lymphocytes in the livers of TCE-treated mice. Apoptosis, in addition to maintaining tissue homeostasis, is also essential for maintaining self-tolerance by deletion of autoreactive T and B cells [35]. Elimination of apoptotic cells by macrophages is rapid and immunologically silent [44]. The defective clearance of apoptotic cells results in secondary necrosis due to increased number of apoptotic cells, with potential consequences of inflammation and ADs [45, 46]. Hepatocyte apoptosis induced by auto-reactive T cells throughout liver parenchyma was observed in murine models [47]. It was also reported that defective liver X receptor or peroxisome proliferator-activated receptor δ are known to produce a shift of cytokine production from anti-inflammatory to pro-inflammatory responses [48, 49]. Similarly, the process of removal of apoptotic cells by macrophages is complex and involves numerous factors. Morphological and adhesional defects in macrophages have been reported to cause impaired phagocytosis resulting in chronic inflammation [30]. In addition, serum factors like complement component 1, q and DNase I are necessary for phagocytosis in the degradation of chromatin by macrophages [50, 51]. Macrophages deficient in DNase I produced inflammatory cytokine TNF-α, which activated synovial cells to produce various cytokines leading to chronic polyarthritis [52]. The above reports support our findings that TCE induces autoimmunity by an increase in pro-inflammatory response, as seen in elevated TNF-α in our in vitro experiments.

In conclusion, our studies demonstrate that TCE induces apoptosis which precedes lymphocytic infiltration leading to AIHLD. This could be due to defective clearance of apoptotic bodies by Kupffer cells, resulting in chronic inflammation. Our future studies will be focused on determining different factors and signaling pathways involved in delayed phagocytosis of apoptotic bodies leading to AIHLD.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## ACKNOWLEDGEMENTS

## REFERENCES

[1]     Kilburn KH, Warshaw RH. Prevalence of symptoms of systemic lupus erythematosus (SLE) and of fluorescent antinuclear antibodies associated with chronic exposure to trichloroethylene and other chemicals in well water. Environ Res 1992; 57: 1-9.

[2]     Lockey JE, Kelly CR, Cannon GW, Colby TV, Aldrich V, Livingston GK. Progressive systemic sclerosis associated with exposure to trichloroethylene. J Occup Med 1987; 29: 493-6.

[3]     Brasington Jr RD, Thorpe-Swenson AJ. Systemic sclerosis associated with cutaneous exposure to solvents: case report and review of the literature. Arthritis Rheum 1991; 34: 363-631.

[4]     Phoon WH, Chan MOY, Rajan VS, Tan KJ, Thirumoorthy T, Goh CL. Steven–Johnson syndrome associated with occupational exposure to trichloroethylene. Contact Derm. 1984; 10: 270-6.

[5]     Xu X, Yang R, Wu N, *et al.* Severe hypersensitivity dermatitis and liver dysfunction induced by occupational exposure to trichloroethylene. Ind Health 2009; 47: 107-12.

[6]     Reinl W. Scleroderma caused by trichloroethylene? Bull Hyg 1957; 32: 678-9.

[7]     Saihan EM, Burton JL, Heaton KW. A new syndrome with pigmentation, scleroderma, gynecomastia, Raynaud's phenomenon and peripheral neuropathy. Br J Dermatol 1978; 99: 437-40.

[8]     Flindt-Hansen H, Isager H. Scleroderma after occupational exposure to trichloroethylene and trichloroethane. Acta Dermatol Venereol 1987; 67: 263-4.

[9]     Waller PA, Daniel C, Cupps T, Metcalf JS, Richard MS, Leroy EC. Fascitis (not scleroderma) following prolonged exposure to organic solvents (trichloroethylene). J Rheumatol 1994; 21: 1567-70.

[10]    Khan MF, Kaphalia BS, Prabhakar BS, Kanz MF, Ansari GAS. Trichloroethene-induced autoimmune response in female MRL+/+ mice. Toxicol Appl Pharmacol 1995; 134: 155-60.

[11]    Cai P, König R, Boor PJ, *et al*. Chronic exposure to trichloroethene causes early onset of SLE-like disease in female MRL +/+ mice. Toxicol App Pharmacol 2008; 228: 68-75.

[12]    Griffin JM, Gilbert KM, Lamps LW, Pumford NR. CD4(+) T-cell activation and induction and induction of autoimmune hepatitis following trichloroethylene treatment in MRL+/+ mice. Toxicol Sci 2000; 57: 345-52.

[13]    Vergani D, Mieli-Vergani G. Autoimmune hepatitis. Curr Opin Gastroenterol 2001; 17: 562-7.

[14]    Vento S, Cainelli F, Renzini C, Concia E. Autoimmune hepatitis type 2 induced by HCV and persisting after viral clearance. Lancet 1997; 350: 1298-9.

[15]    Björnsson E, Talwalkar J, Treeprasertsuk S, *et al*. Drug-induced autoimmune hepatitis:clinical characteristics and prognosis. Hepatology 2010; 51: 2040-8.

[16]    Hisanaga N, Jonai H, Yu X, *et al*. Stevens-Johnson syndrome accompanied by acute hepatitis in workers exposed to trichloroethylene or tetrachloroethylene. Sangyo Eiseigaku Zasshi 2002; 44: 33-49.

[17]    Chae HJ, Lee SK, Lee KJ, *et al*. Exfoliative Dermatitis and Toxic Hepatitis Associated with Occupational Exposure to Trichloroethylene. Korean J Occup Environ Med 2003; 15: 111-7.

[18]    Anagnostopoulos G, Sakorafas GH, Grigoriadis K, *et al.* Hepatitis caused by occupational chronic exposure to trichloroethylene. Acta Gastroenterol Belg 2004; 67: 355-7.

[19]    Pantucharoensri S, Boontee P, Likhitsan P, Padungtod C, Prasartsansoui S. Generalized eruption accompanied by hepatitis in two Thai metal cleaners exposed to trichloroethylene. Ind Health 2004; 42: 385-8.

[20]    Kamijima M, Hisanaga N, Wang H, Nakajima T. Occupational trichloroethylene exposure as a cause of idiosyncratic generalized skin disorders and accompanying hepatitis similar to drug hypersensitivities. Int Arch Occup Environ Health 2007; 80: 357-70.

[21]    Bond GR. Hepatitis, rash and eosinophilia following trichloroethylene exposure: a case report and speculation on mechanistic similarity to halothane induced hepatitis. J Toxicol Clin Toxicol 1996; 34: 461-6.

[22]    Khan MF, Kaphalia BS, Ansari GAS. Time-dependent autoimmune response of dichloroacetyl chloride in female MRL+/+ mice. Immunopharmacol Immunotoxicol 1997; 19: 265-77.

[23]    O'Brien CJ, Vento S, Donaldson PT, *et al.* Cell-mediated immunity and suppressor-T-cell defects to liver-derived antigens in families of patients with autoimmune chronic active hepatitis. Lancet 1986; 1: 350-3.

[24]    Liu ZX, Kaplowitz N. Immune-mediated drug-induced liver disease. Clin Liver Dis 2002; 6: 755-74.

[25]    Halmes NC, McMillan DC, Oatis Jr JE, Pumford NR. Immunochemical detection of protein adducts in mice treated with trichloroethylene. Chem Res Toxicol 1996; 9: 451-6.

[26]    Cai P, König R, Khan MF, Kaphalia BS, Ansari GAS. Different immune responses to albumin adducts of reactive intermediates of trichloroethene in MRL+/+ mice. Toxicol Appl Pharmacol 2007; 220: 278-83.

[27]    Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G, *et al.* Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. Immunobiology 2006; 211: 487-501.

[28]    Laskin DL. Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. Chem Res Toxicol 2009; 8: 1376-85.

[29]    Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. J Pathol 2008; 214: 161-78.

[30]    Muñoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat Rev Rheumatol 2010; 5: 280-9.

[31]    Gaipl US, Voll RE, Sheriff A, Franz S, Kalden JR, Herrmann M. Impaired clearance of dying cells in systemic lupus erythematosus. Autoimmun Rev 2005; 4: 189-94.

[32]    Iyonaga K, Takeya M, Yamamoto T, Ando M, Takahashi K. A novel monoclonal antibody, RM-4, specifically recognizes rat macrophages and dendritic cells in formalin-fixed, paraffin-embedded tissues. Histochem J 1997; 29: 105-16.

[33]    Brouckaert G, Kalai M, Krysko DV, *et al.* Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. Mol Biol Cell 2004; 15; 1089-100.

[34]    Pollard KM, Hultman P, Kono DH. Toxicology of autoimmune diseases. Chem Res Toxicol 2010; 23: 455-66.

[35]    Lorenz HM, Herrmann M, Winkler T, Gaipl U, Kalden JR. Role of apoptosis in autoimmunity. Apoptosis 2000; 5: 443-9.

[36]    Dieker JW, Van der Vlag J, Berden JH. Deranged removal of apoptotic cells: its role in the genesis of lupus. Nephrol Dial Transplant 2004; 19: 282-5.

[37]    Gilbert KM, Przybyla B, Pumford NR, *et al.* Delineating liver events in trichloroethylene-induced autoimmune hepatitis. Chem Res Toxicol 2004; 22: 626-32.

[38]    Shen T, Ma T, Ye LP, Wang LJ, Zhu QX. Mechanisms of apoptosis induced by trichloroethylene in normal human epidermis keratinocytes. Zhonghua Yi Xue Za Zhi 2009; 1: 3101-5.

[39]    Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature 1997; 390: 350-1.

[40]    Fadok VA, McDonald PP, Bratton DL, Henson PM. Regulation of macrophage cytokine production by phagocytosis of apoptotic and post-apoptotic cells. Biochem Soc Trans 1998; 26: 653-6.

[41]    Roy A, Soni GR, Kolhapure RM, Banerjee K, Patki PS. Induction of tumour necrosis factor alpha in experimental animals treated with hepatotoxicants. Indian J Exp Biol 1992; 30: 696-700.

[42]    Yee SB, Ganey PE, Roth RA. The role of Kupffer cells and TNF-alpha in monocrotaline and bacterial lipopolysaccharide-induced liver injury. Toxicol Sci 2003; 71: 124-32.

[43]    Russwurm S, Stonans I, Stonane E, *et al.* HepG2 hepatocytes express IFN-$\gamma$, TNF-$\alpha$, TGF-$\beta$, M-CSF, oncostatin-M, ICAM-1, IL-4, IL-5, IL-7, IL-10, IL-11, IL-12 and IL-6 receptor genes in vitro. Crit Care 1998; 2(Suppl 1): P005.

[44]    Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature 2000; 407: 784-8.

[45]    Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum 1998; 41: 1241-50.

[46]    Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol 2002; 2: 965-75.

[47]    Lapierre P, Béland K, Alvarez F. Pathogenesis of autoimmune hepatitis: from break of tolerance to immune-mediated hepatocyte apoptosis. Transl Res 2007; 149: 107-13.

[48]    A-Gonzalez N, Bensinger SJ, Hong C, *et al.* Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. Immunity 2009; 31: 245-58.

[49]    Mukundan L, Odegaard JI, Morel CR, *et al.* PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. Nat Med 2009; 15: 1266-72.

[50]    Taylor PR, Carugati A, Fadok VA, *et al.* A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J Exp Med 2000; 192: 359-66.

[51]    Gaipl US, Franz S, Voll RE, Sheriff A, Kalden JR, Herrmann M. Defects in the disposal of dying cells lead to autoimmunity. Curr Rheumatol Rep 2004; 6: 401-7.

[52]    Kawane K, Ohtani M, Miwa K, *et al.* Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. Nature 2006; 443: 998-1002.