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RESEARCH ARTICLE

Cell Death in Mouse Brain following Early Exposure to Trichloroethane (TCE)

Mohamed A. Al-Griw[1*], Naser M. Salama[1], Soad A. Treesh[2], , Lubna N. Algadi[1], Abdul hakim Elnfati[1]

1.Division of Developmental Biology, Zoology Department, Faculty of Science, University of Tripoli, Libya

2.Department of Histology and Medical Genetics, Faculty of Medicine, University of Tripoli, Libya

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Corresponding Author

Mohamed A. Al-Griw

Abstract

Exposure to chemicals has been shown to adversely affect CNS health in rodents and humans. The objective was to evaluate, *in-vivo*, the effects of trichloroethane (TCE), a ubiquitous environmental contaminant, on the integrity of neural cells. A group of albino mice was injected intraperitoneally twice weekly for three weeks with TCE (100 and 400 µg/kg). Animals were followed up for signs of toxicity and death. Alterations in neural tissues have also been investigated by histopathology The results showed a large number of degenerative neural cells (pyknosis of nuclei, DNA fragmentation, chromatin condensation) in the 100 and 400 µg/kg TCE-treated groups comparing to controls. Although there were no significant effect on the neural cell counts, the pattern of increased degenerative cells in TCE-treated groups was higher compared to controls. The results also showed that TCE led to a significant increase in the percent of degenerative neurons. There was also a significant reduction in the percent of neurons. These results correlated with the increase in the percent of glia. This study indicates that TCE exposure had detrimental impact on neural cells, and that neurons are more vulnerable to TCE than glia in this *in-vivo* mouse model.

1. INTRODUCTION

Increasing evidence suggests that neuropathies are multifactorial, and could involve genetic, hormonal and environmental influences. In addition to bacteria and viruses, other environmental factors including such chemical as 1,1,1,1-trichloroethene (TCE) is documented to contribute to the induction and/or acceleration of numerous pathological conditions [1-3]. TCE, a volatile organic compound is widely used as an industrial solvent and a degreasing agent, is implicated in the development of neurotoxicity both in human and animal studies [1-2, 4].

TCE is primarily used for cold-cleaning dip-cleaning, and bucket-cleaning of metal ions to remove grease, oil and wax [5]. Recently, it has been replaced by other solvents due to its damaging effect on the ozone layer; however, acute and chronic reactions associated with TCE use have also been reported in many studies [2, 6]. The CNS is the main target of acute exposure to TCE. The various cellular elements of CNS are separately under injury/attack [7]. It has been demonstrated that neurons are more sensitive to injury than glia, and that glia respond rapidly to injury [8]. Previous studies of TCE toxicity in experimental models have only evaluated CNS activities such as those that have been evaluated: behavior [9], vestibular function [10], and locomotor activity [11].

There are a growing literatures suggest that exposure to environmental chemical contaminants may be associated with an increased risk of numerous neurological disorders, such as autism, drug addiction, schizophrenia, disorders of attention, mood, and anxiety [8, 12]. It has been reported that large increases in neurons and glia are found in humans diagnosed with autism [13]. This suggests that increases in neural cell number are not necessarily

advantageous. It is speculative to suggest that the effects found here could be an indication of a predisposition toward autism [14].

In-vivo studies are essential to assess environmental toxicants with adverse effects on human health. The *in-vivo* study of TCE is of great importance because animal systems are extremely complicated, and the interaction complex cell relationships and physiological interplay between the CNS elements are the most promising approach. However, to the best knowledge we have, no study had designed to study the effects of TCE exposure on the brain cellular elements. Therefore, we conducted an *in-vivo* toxicity study of TCE in albino mice. The current study was designed to examine injury responses of neural cells in mice exposed to TCE, with a range comparable to that in humans [15]. Groups of mice were given TCE injections, and the markers of cell injury and their association with brain damage were evaluated. Our data provide an evidence for a role of TCE in neural cell death, which depending on the intensity of the TCE exposure.

2. MATERIALS AND METHODS

2.1 Animals and Housing

A total of thirty six albino mice aged between three and four weeks and weighing between 21 and 24g were used during the course of the present study. Mice were inbred and housed in plastic cages on a bedding of wood chips at Department of Zoology animal house. The facility maintained at ~ 22 °C, 50 – 60% relative humidity, and a 12 hours light/dark cycle. The animals were provided with standard lab chow and drinking water ad libitum and were acclimated for one week prior to the treatment.

2.2 Study Design and Dosing Procedure

TCE (Baxter International) was suspended in corn oil. Mice were divided into four groups of six mice each and were treated with conditions of sham control group, vehicle control group or TCE (100 or 400 µg/kg) treatment groups. The doses were calculated and delivered in 80-100 µl corn oil based on their body weights [15-16]. Vehicle controls were received an equal volume of corn oil only. The sham controls were not received any treatment. Intraperitoneally (i.p.) administration occurred at a defined time (10:00 am), every 3th day. TCE at 100 and 400 µg/kg was selected because it is the EPA referenced safe dose [17]. The treatment window was selected because this is the critical development window in the mouse [18].

2.3 Clinical Assessment

During the experimental procedure, mice were observed twice per day for any adverse clinical signs or abnormal behavior that may result from toxicity. Mice were also assessed for morbidity and mortality twice daily, midmorning and late afternoon. In addition, deaths occurred overnight were recorded the next morning. Two independent observers confirmed the criterion for killing. Body and brain for four groups were weighted until the end of experimental course.

2.4 Histological Studies

After the dissected brains were fixed in 10% formalin, the fixed samples were processed in a series of graded ethanol solutions, cleared in xylol (Sigma) and embedded in paraffin wax. Paraffin sections were cut at 5-7 µm thickness by rotatory microtome, deparaffinized, rehydrated and stained with 1% cresyl fast violet staining solution (15-20 minutes) for histological examination. Tissue sections were viewed and imaged using light microscopy (Leica, Germany).

2.5 Neural Cell Count

Neurons and glia were determined as described previously [19-22]. On cresyl fast violet staining, neurons were distinguished from glia by their morphology, staining pattern, and, to some extent, by their size. In the sampling section and lookup sections we did, in fact, count cells as neurons if they were large and possessed a stellate shape and a darkly stained cytoplasm, even if their nucleus and nucleolus were not clearly distinguishable (although in most cases they were) (Figure 1). The diameter of the smallest cells counted as neurons was approximately 10 µm. However, we did count cells as glia if they were characterized by their oval shape, homogeneously dark nuclear staining, and consistently small diameter (~5–8 µm) (Figure 1). Under these conditions, we considered astrocytes, oligodendrocytes, and microglia to represent a single type of glia. Other non-neural cells such as pericytes and endothelial vascular cells were carefully excluded [19].

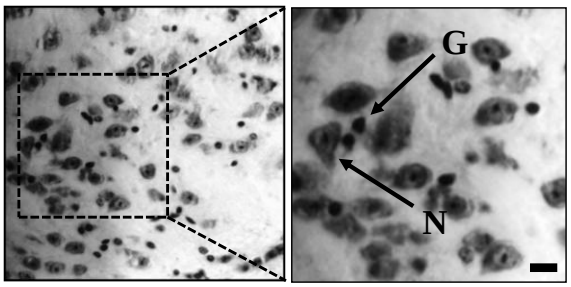

Figure 1. A photograph of cresyl-fast-violet section showing a neuron (N) and glia (G) at high magnification used for counting. Note distinguishing characteristics in shape and size. Scale bar: 10 µm.

2.6 Microscopy and Cell Scoring

Cell numbers were counted in 10 high-power fields (X100 magnification) using ImageJ software (version 1.45). We counted only those neurons whose nucleoli were clearly visible and those glia with a well-outlined nucleus [19, 23]. All histological assessments were made in a blinded fashion by two investigators.

2.7 Statistics

The statistical analysis were performed using SPSS software, version 20. The parametric one-way ANOVA followed by the post hoc Bonferroni multiple comparison test was used to assess the difference among groups. If the data were not normally distributed, the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn's multiple comparison test was performed. *P*-value ≤ 0.05 was considered significant.

3. RESULTS

3.1 Injury Responses of Neural Cells

To examine injury response of neural cells, we counted and compared the number of degenerative cells in cresyl-fast violet-stained brain sections of controls and TCE-treated groups. The results showed that the counts degenerative neural cells in sham groups was similar ($P = 0.831$) to those in vehicle groups (Figure 2A). Statistical analysis revealed that the total number of degenerative neural cells was higher in TCE treatment groups compared to controls. Specifically, mice treated with 100 µg/kg TCE had more degenerative cells (72.33 ± 7.49%; $P = 0.002$) compared to controls (34.17 ± 4.64%; Figure 2A). However, mice treated with TCE 400 µg/kg had more degenerative cells (94.33 ± 9.42%; $P = 0.00042$) compared to controls (14.67 ± 1.28%; Figure 2A). Critically, treatment with TCE had no significant effect on the total number of neural cells compared to controls (Figure 2B). No significant sex differences were detected when males and females were compared.

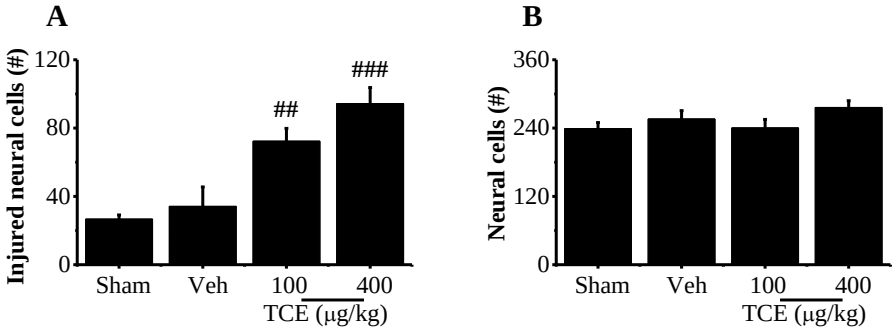

Figure 2. Injury response of neural cells. Evaluation was performed in high-power fields (X100) in control and TCE-treated mice. Quantification of degenerative neural cells. (B) Quantification of neural cell count. Data represent mean ± SEM of n = 6 animals per groups. #$P \leq 0.05$. #Significantly different from the controls.

3.2 Neuron Count

Quantitative analysis of cell death revealed that the percent of degenerative neurons was significantly (P = 0.015) increased by up to 2.5-fold (36.67 ± 6.38%) in mice that received 100 µg/kg TCE compared to controls (14.67 ± 1.28%) (Figure 3A). However, the percent of degenerative neurons was significantly (P = 0.001) increased by up to 2.9-fold (43.33 ± 6.87%) in mice that received 400 µg/kg TCE compared to controls (Figure 3A). Moreover, there was no statistical difference (P = 0.743) in the percent of degenerative neurons between mice received 100 µg/kg TCE (36.67 ± 6.38%) and those received 400 µg/kg TCE (43.33 ± 6.87%) (Figure 3A).

The continued analysis also showed that the percent of neurons was significantly ($P < 0.05$) reduced by up to 1.3-fold in 100 µg/kg TCE-treated groups compared to controls (Figure 3B). Moreover, treatment with 400 µg/kg TCE reduced the percent of neurons 1.2-fold (47.83 ± 4.54%) compared to 100 µg/kg TCE treatment group (41.67 ± 2.23%; Figure 3B).

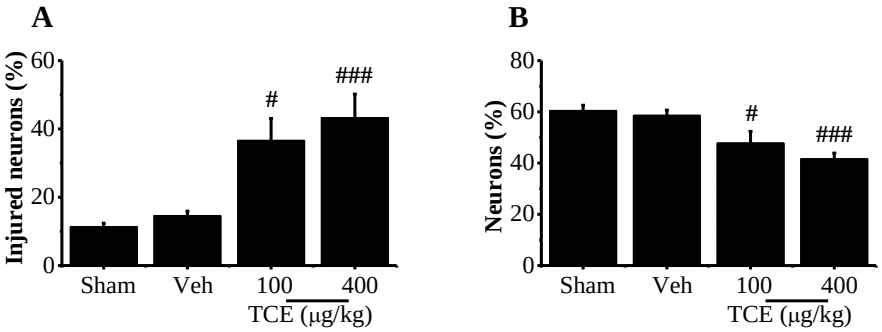

Figure 3. Injury responses of neurons. Evaluation was performed in high-power fields (X100) in control and TCE-treated mice. (A) Quantification of degenerative neurons. (B) Quantification of the percent of neurons. Data represent mean ± SEM of n = 6 animals per groups. #$P \leq 0.05$. #Significantly different from the controls.

3.3 Glia count

Cell death analysis revealed that the percent of degenerative glia was significantly (P = 0.002) increased by up to 2.2-fold (25.83 ± 2.86%) in mice that received 100 µg/kg TCE compared to controls (11.67 ± 1.89%) (Figure 4). However, the percent of degenerative glia was significantly (P = 0.00031) increased by up to 2.4-fold (28.5 ± 2.99%) in mice that received 400 µg/kg TCE compared to controls (Figure 4). There was no statistical difference (P = 0.856) in the percent of degenerative glia between mice received 100 µg/kg TCE (25.83 ± 2.86%) and those received 400 µg/kg TCE (28.5 ± 2.99%; Figure 4). This study also showed that the percent of glia was significantly ($P < 0.05$) increased by up to 1.3-fold in TCE treatment group compared to controls (Figure 4B). Specifically, treatment with 400 µg/kg TCE reduced the percent of glia 1.4-fold (55.61 ± 3.64%) compared to 100 µg/kg TCE treatment group (59.82 ± 2.06%; Figure 4B). Taken together with the data presented in Figure 3, these results indicate that although both neurons and glia were vulnerable to TCE, neurons were more susceptible to TCE than glia in this *in-vivo* model of brain injury.

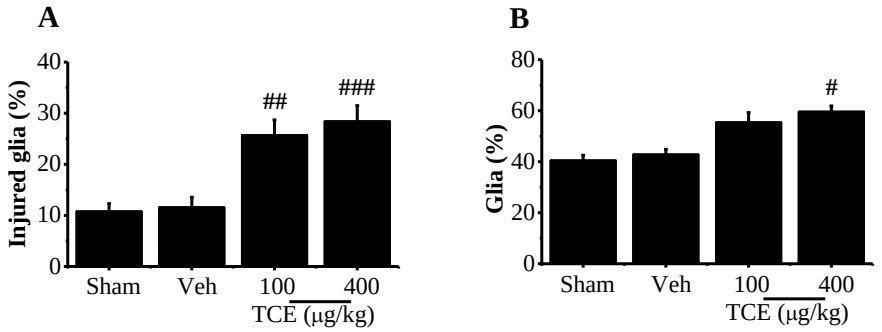

Figure 4. Injury response of glia. Evaluation was performed in high-power fields (X100) in control and TCE treated mice. (A) Quantification of degenerative glia. (B) Quantification of the percent of glia. Data represent mean ± SEM of n = 6 animals per groups. #$P \leq 0.05$. #Significantly different from the controls.

4. DISCUSSION

The purpose of the current study was to investigate the cytotoxicity effects of TCE on neurons and glia. This study provides the first evidence that early exposure to TCE results in a higher number of degenerative neural cells in the albino mice. This effect was seen at both the lowest and highest doses used, 100 and 400 µg/kg TCE, respectively. These doses are relevant to prenatal exposure that occurs in humans [15]. The results also showed that the counts of neurons and glia quantitatively changed in response to TCE.

Prior studies showed that perinatal TCE exposure affects the development of the CNS and adversely affects its functions [2, 4, 15, 24-26]. It has been reported that even relatively low occupational exposure levels of TCE may affect neuromotor function [27]. Recently, we found that exposure to TCE can impair motor performance (unpublished data). This is an acute behavioural response since mice did not show spontaneous turning behavior in control. Although our dosing window and dose levels were not the same as previous studies [17, 28], our results are consistent with results of previous studies [28-29] that used higher doses of TCE than those used in our study exposure. Collectively, current data suggest that brains of mature mice are sensitive to TCE, even at environmentally relevant levels, and that developmental TCE exposure has long lasting effects on the CNS, confirming that TCE is a neurotoxicant.

It is well known that histological assays are reliable tools to detect morphological alterations due to enviromental toxicants; hence, the histopathology of various treated tissues was examined. The results of cresyl fast violet staining indicated that a large number of degenerative neural cells appeared in response to TCE exposure. However, few number of degenerative cells were seen in controls, indicating that TCE induces neurotoxicity. In this study we also found that increased turning behavior in TCE-treated groups correlates with histological damage to brain cellular elements (unpublished data).

There is a growing literature that suggests that early exposure to environmental toxicants may be associated with an increased risk of neurological disorders, such as autism. This suggests that increases in neural cell counts are not necessarily advantageous [14]. It is speculative to suggest that the effects found here could be an indication of a predisposition toward autism. Using quantitative morphometric techniques, we found that TCE treatment induced a significant increase in the percent of degenerative neurons and glia compared to control. Many studies have reported that glia respond rapidly to injury and neurodegenerative disease [30]. Consistent with results of others [31] on neurotoxicity, the results of this study showed that the counts of glia increases in parallel with neuronal cell degeneration, suggesting that neurons are more sensitive to TCE mediated injury than are glia [8, 32].

Understanding how reactive gliosis, a common characteristic of many CNS pathologies, contribute to oligodendrocyte death with a subsequent loss of appropriate myelination following injury would be relevant not only for the pathogenesis of the injury but also for the CNS inflammation and is likely to suggest therapeutic approaches to CNS disorders. Activation of astrocytes and microglia is accompanied by the production of different toxic molecules, such as cytokines and reactive nitrogen and oxygen radicals [33], finally leading to the death of neurons and oligodendrocytes that where initially spread during the phase of primary damage. It is speculative to suggest that the increases in the percent of the degenerative glia in the TCE treated groups could be due to alterations in astrocytes, oligodendrocytes, or microglia because the Nissl stain employed in the current study does not distinguish between different types of glia. In the present study, we quantified all glial cell types together, consequently any specific type may be responsible for the changes reported here. No studies to date have investigated whether TCE directly impacts oligodendrocytes, but an *in-vitro* study did report concentration-dependent alterations in neural progenitor cells and in NG2-positive precursor cells. However, it is difficult to ascertain how the TCE concentrations used in culture studies correspond to doses of TCE used in *in-vivo* studies or to exposure that occurs in humans. Therefore, more studies with *in-vivo* administration of TCE are needed to determine what types of glia are contributing to the increases seen in this current study.

In conclusion, our findings demonstrate that exposure to TCE resulted in more glia, with significant neuronal cell loss, in mice. Future studies are needed to determine the mechanism for these alterations and the specific type of cells that are implicated.

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