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Mode of Cell Death in Mouse Brain Following Early Exposure to Low-Dose Trichloroethane: Apoptosis or Necrosis

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Abstract

The goal of this study was to investigate, *in-vivo*, the predominant mechanism of cell death, apoptosis versus necrosis, in the mature mouse brain exposed early to a ubiquitous environmental toxicant trichloroethane (TCE). A subset of male albino mice was injected intraperitoneally twice weekly for three weeks with TCE (100 and 400µg/kg). All animals were followed up for signs of toxicity and mortality. Changes in neural tissues were histpathologically evaluated. Biomarkers of brain cell number were also studied. The results showed that TCE insult triggered significant alterations in the microstructure of the brain tissues compared to controls. Mitotic figures and apoptotic changes such as chromatin condensation and nuclear fragments were also identified. Cell death analysis demonstrates that cell apoptosis with necrosis was evident in the TCE-treated groups. The percent of necrosis was quantified as 20.09 ± 2.57% at 100µg/kg TCE, 30.57 ± 5.18% at 400µg/kg TCE, and 12.67 ± 1.25% in controls. However, the percent of apoptosis was quantified as 29.18 ± 1.51% at 100µg/kg TCE, 20.14 ± 2.12% at 400µg/kg TCE, and 8 ± 1.25% in controls. There was also a significant reduction in the brain DNA content in the TCE-treated groups. Agarose gel electrophoresis is also provided further biochemical evidence of apoptosis by showing internucleosomal DNA fragmentation. These results correlated with neurobehavioral impairment. These findings indicate that TCE induces degeneration and apoptotic cell death in mouse brain, suggesting a crucial role played by apoptosis in TCE neurotoxicity.

Keywords

Trichloroethane, Neurotoxicity, Neurobehavior Abnormalities, Apoptosis, DNA Fragmentation, Mouse

1. Introduction

The nervous system has emerged as one of the major toxicity targets of environmental toxicants. There is a growing literature that suggests that early exposure to environmental chemicals have been shown to adversely affect brain histology, function, and consequently behavior later in life both in rodents and humans [1-5]. Evaluating the toxicity of chemicals, such as trichloroethane (TCE), is one of the most concerned issues in the modern society. Some of these toxicities show immediate impact; others can result in subtle alterations that are delayed in their expression [6]. TCE, a ubiquitous environmental toxicant, is a volatile organic solvent that has been used in large quantities as a dissolvent, metal degreaser, chemical intermediate, and component of consumer products [7-8]. Originally produced as a safer alternative to other chlorinated solvents, the acute and chronic toxicities of TCE are relatively low. However, there is a risk of toxic effects to those that encounter TCE in high concentrations in the workplace or recreationally abuse the solvent [8]. It has been reported that severe exposures of humans to TCE have resulted in sensitization of the heart to epinephrine-induced arrhythmias and mild hepatorenal effects

[9], but the central nervous system (CNS) is considered the principal target. Acute exposures to volunteers have produced impaired performance in tests of manual dexterity, eye–hand coordination, perceptual speed, and reaction time [2, 8, 10-11].

Cellular effects of environmentally foreign chemicals might implicate recruitment or de-repression of cell death mechanisms that ultimately may turn out to be either protective (as in carcinogenesis) or deleterious (as in neurodegeneration) to the organism. Whether a cell survives or dies in the presence of a chemical insult is often determined by proliferative status, repair enzyme capacity, and the ability to induce proteins that either enhance or prevent the cell death process. The discovery that apoptosis and certain other cell death modalities can also be utilized to eliminate genetically damaged cells, thereby protecting the organism from cancer, has suggested the possibility that low level chronic exposure to potentially dangerous chemicals may act not only by triggering oncogenic reactions but also by inhibiting tumor surveillance [12]. Conversely, the finding that components of the apoptotic program, such as caspases, can also be involved in nonapoptotic signaling pathways has pointed to the complexity of the cellular effects exerted by toxic chemicals [13].

The two basic forms of cell death, necrosis and apoptosis, play crucial roles in development, homeostasis, and pathogenesis [14]. Necrosis has long been considered an uncontrolled form of cell death, with morphological features of loss of plasma membrane integrity, organelle swelling, and leakage of cell contents. In contrast, apoptosis is a tightly regulated form of cell death characterized by nuclear shrinkage and fragmentation, membrane blebbing, and apoptotic body formation. However, growing evidence has described an active and well-orchestrated form of necrosis, termed necroptosis [15]. Measurements of DNA and cell protein fractions are utilized to assess general impacts on cell CNS development [16]. Because each neural cell contains only a single nucleus [17], the DNA content (DNA per brain region) reflects the total cells number/ density and the DNA concentration (DNA per unit tissue weight) reflects the cell packing density. As cells enlarge, their protein content rises while DNA remains constant, so the total protein/DNA reflects relative cell size; similarly, the development of neuritic projections increases the membrane surface area, increasing the ratio of membrane protein/total protein. The rationale and mechanistic basis for each of these markers was presented in earlier investigations [18-19].

The CNS has emerged as one of the major toxicity targets of environmental toxicants. Although acute exposure of toxicants contributes to apoptosis and necrosis of brain cells, chronic and sub-lethal exposure is prevailing in the general public [3, 8]. Due to the unusual long half-lives of some of toxicants in mammalian body [20], chronic and low level exposure to humans could cause long-term unwanted health effects.

However, to the best knowledge we have, no study had designed to determine whether apoptosis is involved in TCE-induced cell death in mouse brain. The current study aimed to examine the mode of neural cell death following exposure to environmental toxicant TCE, with a range comparable to that in humans [7]. Groups of male mice were treated with TCE, and the markers of neurobehavior and their association with brain damage were evaluated. We also utilized biomarkers of brain cell number to further examine the impact of TCE on the type of neural cell death. The current data support a hypothesis that apoptosis is quantitatively relevant mechanism of neural cell death following early exposure to low-dose TCE in mouse brain.

2. Materials and Methods

2.1. Animals and Experimental Protocols

A total of twenty four male albino mice (19-21 g), aged between three and four weeks were used in this study. Animals were inbred in animal house of the Zoology Department. All experiments were carried out in the same centre. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were housed in polycarbonate cages in a room with controlled temperature (24 ± 1ºC), humidity (55 ± 5%), a 12 hour cycle of light and dark, and were fed laboratory pellet chows and water ad libitum. The experiment was performed after a stabilization period in the laboratory for several days.

The study comprised four groups of 6 animals: group A - untreated group (sham controls), group B - corn oil group (vehicle controls), group C - 100 µm/kg TCE, and group D - 400 µm/kg TCE. The experimental animals were injected intraperitoneally (i.p.) twice weekly for three weeks. The administration occurred at a defined time (10:00 am). TCE (99% purity) (obtained from the Baxter International) was diluted in corn oil to obtain the selected doses. The doses were calculated and delivered in 80 - 100 µl corn oil based on their body weights [7, 21-22]. TCE doses were selected because our preliminary investigations have shown that it causes significant brain degeneration and produces neurobehavioral impairment. The treatment window was selected because this is the critical development window in the mouse [23].

2.2. Clinical Assessment

Comprehensive teratological evaluations of all experimental animals were performed. These include animal survival, body and brain weight, and clinical signs.

2.2.1. Animal Survival

During the course of the exposure period, animals were assessed for morbidity and mortality that may result from toxicity twice daily, midmorning and late afternoon. Night deaths were recorded the next morning. Two independent observers confirmed the cause of death to exclude TCE-nonrelated mortality.

2.2.2. Body Weight Changes

Mouse body weight in controls and TCE-treated groups was

monitored and assessed on a weekly basis.

2.3. Evaluation of Motor Behaviour

Motor behaviour was assessed throughout the course of our study in all experimental groups according to the procedure described previously [22]. About two hours after each treatment, spontaneous turning behaviour was evaluated every fifteen minutes for two hours post-insult. After being placed in a round cage, the number of rotations was recorded manually. One rotation was computed when the mouse completely circled the cage. Five separate counting periods of three minutes each, separated by fifteen minutes intervals, were made. Values are reported as the mean rotation number during the five counting periods per hour. The same mice were also evaluated soon after awakening and twenty four hours later for failure to fully extending the left forepaw and for contralateral turning when pulled by the tail. Under this condition all mice showed a clear circling to the left side.

2.4. Neurological Tests

Neurological evaluation of motor sensory functions was carried out two hours prior and post each treatment. The examiners were blind as to the procedure that the mouse had undergone. Evaluations were always performed between 10:00 am and 11:00 am to exclude behavioural changes based on circadian rhythm. The neurological examination consisted of six tests [22, 24]: (i) spontaneous activity; (ii) symmetry in the movement of four limbs; (iii) forepaw outstretching; (iv) climbing; (v) body proprioception; and (vi) response to vibrissae touch. The score assigned to each mouse at completion of the evaluation equalled the sum of all six test scores.

2.5. Histopathological Studies

After dissection, 10%-formalin-fixed brain tissues were processed in a series of graded ethanol solutions and embedded in paraffin wax. Paraffin sections were cut at 6-8 µm thickness, deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E) and examined under a light microscope (Leica, Germany) for histopathology.

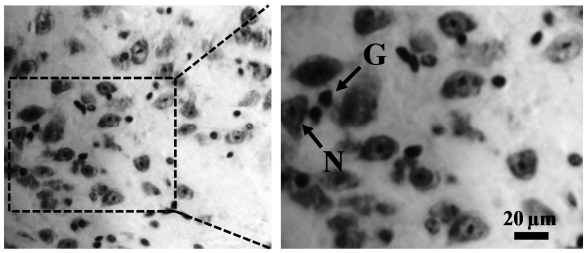

Fig. 1. *A photograph of H&E-stained brain section (X100) showing a neuron (N) and glia (G) (21).*

Neurons and glia were determined as described previously [1, 21, 25-27]. On H&E 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 nuclei and nucleoli were not clearly distinguishable (although in most cases they were) (Fig. 1).

Necrotic cells were distinguished from apoptotic cells by their morphological criteria [28]. Cells undergoing necrosis were identified using the following morphological criteria: increased eosinophilia, cell swelling and lysis, loss of architecture, karyolysis, and karyorrhexis. However, cells undergone apoptosis were identified by morphological criteria, such as pyknosis, cell shrinkage, DNA fragmentation, chromatin condensation and margination, and apoptotic bodies (Fig. 2).

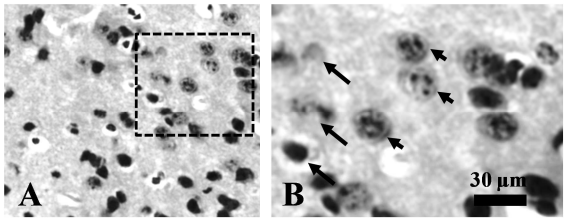

Fig. 2. *Light photomicrograph of hematoxylin and eosin–stained sections of brain tissues (X100). B, High-power view of the area indicated in panel A. Note: distinguishing characteristics of cell degeneration are: karyorrhexis, karyolysis, nuclear pyknosis, apoptotic nuclei, DNA fragmentation, and chromatin condensation.*

2.6. Microscopy and Cell Scoring

Brain sections were viewed and imaged using light microscopy (Leica, Germany). Dying/dead cells were counted in 10 high-power fields using ImageJ software (version 1.45). All histological assessments were made in a blinded fashion by two investigators.

2.7. Genomic DNA Isolation and Electrophoresis

Genomic DNA was isolated from brain mice of controls and TCE-treated groups using a QIAamp DNA Mini Kit (Qiagen). In brief, up to 25 mg of tissue samples were ground into small pieces and homogenized in DNA lysis buffer and proteinase K (2 mg/mL) and incubated in the same buffer overnight at 56ºC. Samples were treated with RNase A (20 mg/mL), purified on spin column and eluted with Tris/EDTA buffer. The quality of extracted DNA and its concentration were measured by UV-spectrophotometry (BioPhotometer, eppendorf), with absorbance A260/A280 nm ratios at pH 8.0 between 1.7 and 2 for all samples. To determine the integrity of the extracted DNA, three micrograms of each DNA extract were fractionated by electrophoresis on 1.5% agarose gel. The gel was stained with GelRed™ and the DNA was visualized using a UV light source. The results of DNA gel electrophoresis were photographed and stored.

2.8. Statistics

The statistical analysis was 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 the 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. Impact of TCE on Animal Survival

No mortality has been recorded among mice in all groups along the course of the experiment except for one death case out of the six in 400 µg/kg TCE-treated group recorded four weeks post-TCE exposure.

3.2. Impact of TCE on Body and Brain Weight

No significant alterations were observed in body weight and relative organ weight (brain and heart) between animals non and received TCE treatment, making TCE doses a NOAEL (no-observed-adverse-effect level) for animals in this model.

3.3. Impact of TCE on Motor Behavior

It has been reported that environmental toxicants induce neurobehavioral abnormalities [29-33]. Here we monitored changes in neurobehavioral performance in controls and TCE treatment groups. TCE-treated groups exhibited turning behavior starting two hours following treatment, but vehicle alone had no effect on motor behavior when applied under control conditions.

The results revealed a significant turning behavior in 100 and 400 µg/kg TCE treatment groups ($P < 0.043$, $P < 0.0098$ vs. controls, respectively) compared to controls (Table 1). However, controls groups did not show any turning behavior.

Table 1. *Effect of TCE insult on turning behavior.*

Treatment	Turning behavior
Sham-treated (n=6)	20 ± 10
Vehicle-treated (n=6)	25 ± 18
TCE-treated (100 µg/kg n=6)	39 ± 124#
TCE-treated (400 µg/kg n=6)	51 ± 124##

Turning behavior is expressed as the mean ± SEM of the number of rotations per hour. #P < 0.05 compared to controls.

3.4. Histological Examination

We next investigated the impact of TCE exposure on the brain cytoarchitecture and cell viability. Representative photographs of HE-stained sections are shown in Fig. 2. The brain was intact in control-operated animals with many large motoneurons (Fig. 3A), and no change was seen in neural cells including neurons and glia. Moreover the nuclei of neural cell appeared normal in vehicle group (data not shown). However, many neurons and glia disappeared in the brain from mice exposed to 100µg/kg TEC, and necrotic changes and neurophil vacuolation became evident (Fig. 3A). In addition, some neurons exhibited apoptotic characteristics such as cell shrinkage, chromatin condensation and nuclear budding (Fig. 3A, arrows). Moreover, there were also apoptotic nuclei with condensed chromatin and nuclear fragments were evident in TCE treatment groups (Fig. 3A, arrows). Apoptotic bodies were also verified (Fig. 3A, arrowheads). These results demonstrate that TCE is critical for the induction of DNA fragmentation and chromatin condensation

Next, we quantitatively examined the impact of TCE insult on the neural cells. Analysis of cell death revealed the percent of dead cells was significantly ($P < 0.001$) increased by up to 2.5-fold in TCE-treated groups compared to controls (20.78 ± 1.74% in controls; Fig. 3A), but vehicle alone had no significant effect when applied under control condition. However, 100 µg/kg TCE treatment group showed lower percent of neural cell death (49 ± 2.68%) compared to 400 µg/kg TCE treatment group (53.21 ± 5.13%; Fig. 3B). Moreover, there was no significant difference in the percent of neural cell death between 100 and 400 µg/kg TCE treatment groups (Fig. 3B).

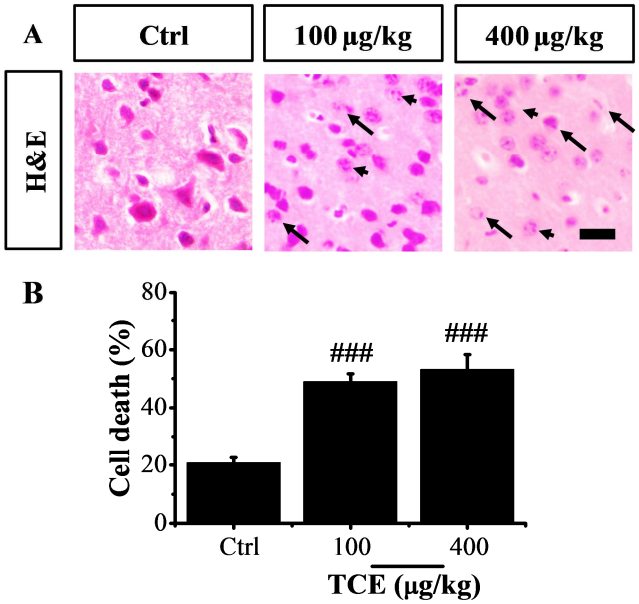

Fig. 3. *Cell death in mouse brain after TCE insult. (A) Photomicrographs of control and TCE-treated mouse brain, H&E X, 40X. The control group showed no or little histological changes (normal nuclear form). Neural cells were partly lost and some cells exhibited necrotic characteristics after TCE insult (arrows). In TCE treatment groups, the nuclei also exhibited typical apoptotic morphology as condensed and fragmented (arrows). The apoptotic bodies could also be detected (arrowheads). (B) Quantification of neural cell death. Data are expressed as the mean ± SEM (n = 6). #Significantly different from the controls. #P ≤ 0.05.*

Here we investigated the predominant mode of neural cell death in response to TCE insult (Fig. 4). Using the standard of morphological criteria of necrosis and apoptosis, we found that TCE exposure leads to significant increase in the percent of necrotic and apoptotic cells compared to controls (Fig. 4A & B), but vehicle alone had no significant effect on when applied under control condition (data not shown). Specifically, 100 µg/kg TCE-treated group showed higher apoptosis 1.5-fold ($P = 0.004$, 29.18 ± 1.51%) compared to 400 µg/kg TCE-treated group (20.14 ± 2.12%, Fig. 4A & B). However, 400 µg/kg TCE treatment group showed higher necrosis 1.5-fold ($P = 0.03$, 30.57 ± 5.18%) compared to 100 µg/kg TCE-treated group (20.09 ± 2.57%, Fig. 4A & B).

Taken together, these results suggest that apoptosis contributes to neural cell death post-insult in this *in-vivo* mouse model of a chemical-induced brain damage.

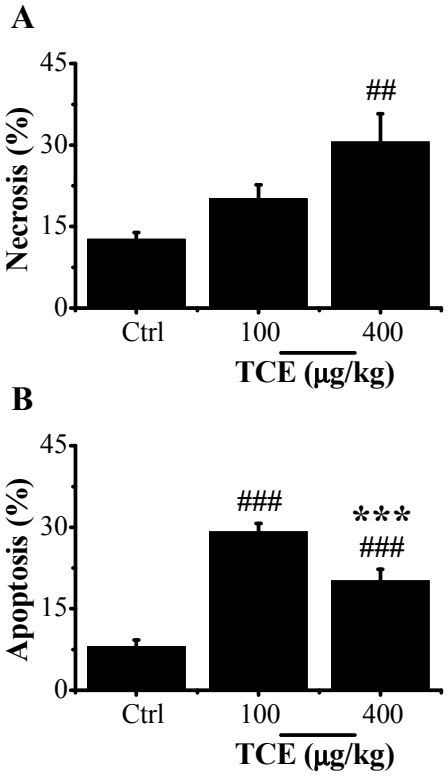

Fig. 4. *Effect of TCE insult on the type of neural cell death. The number of (A) necrotic and (B) apoptotic cells is expressed as a percent of the total number of neural cells that estimated by evaluating the number of microscopic fields. Data are expressed as the mean ± SEM (n = 6). #Significantly different from the controls. *Significantly different from the 100 µg/kg TCE-treated groups. #/*P ≤ 0.05.*

3.5. Analysis of DNA Fragmentation

Nucleosomal fragmentation by endonuclease cleavage is a well-defined biochemical marker of cells undergoing apoptosis which results in DNA fragments with multiples of 180 bp, as compared to necrosis that causes nonspecific degradation of DNA into random-sized fragments [34]. To substantiate our histological evidence for apoptosis, nuclear DNA from controls and TCE-treated groups was analyzed by agarose gel electrophoresis. Gel electrophoresis of the total genomic DNA from brain of four groups was presented in Fig. 5B. DNA from the sham-operated brain was largely intact and exhibited little migration in the gel (Fig. 5A, lanes 3 and 4). No or little internucleosomal DNA fragmentation was observed in vehicle group. However, DNA from the TCE groups exhibited a characteristic nucleosome ladder which might result from DNA endonucleolytic digestion (Fig. 5A, lanes 5 and 6). A smear pattern resulting from random DNA degradation suggests that necrosis might have occurred concurrently with apoptosis. Results from DNA fragmentation analysis demonstrate that TCE treatment largely increased cell apoptosis and necrosis in mouse brain.

To further investigate the predominant mode of cell death, biomarkers of brain cell number were examined (Fig. 5B). TCE-treated groups showed a significant decrease in the DNA concentration compared to controls, but vehicle alone had no significant effect when administrated under control condition. Specifically, treatment with 100 µg/kg TCE significantly (P = 0.003) decreased DNA concentration 1.3-fold (41.31 ± 1.3) compared to controls (54.12 ± 0.67; Fig. 5B). However, treatment with 400 µg/kg TCE reduced DNA concentration ~ 1.5-fold (P = 0.00013; 37 ± 2.1) compared to controls. Moreover, there was no significant difference in the DNA concentration between 100 and 400 µg/kg TCE-treated groups (Fig. 5B).

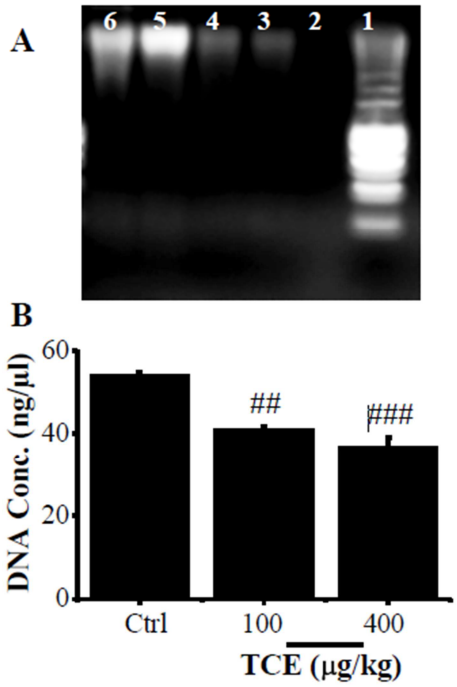

Fig. 5. *Effect of TCE insult on the DNA biomarkers (DNA quality and integrity). (B) Agarose gel electrophoresis of DNA isolated from brain of the controls and TCE-treated groups. Lane 1 indicates DNA marker; lane 2, negative control; lane 3, sham control; lane 4, vehicle control; lane 5, 100 µg/kg TCE-treated group; and lane 6, 400 µg/kg TCE-treated group. Almost no DNA degradation was detected in sham controls. DNA ladder with oligonucleosome fragments appeared with a smear pattern in TCE groups. (A) Quantification of DNA concentration. Data are expressed as the mean ± SEM (n = 6). #Significantly different from the controls. #P ≤ 0.05.*

4. Discussion

In the current study, we have demonstrated apoptosis, a form of genetically programmed cell death, as a potential mechanism mediating neural death in the brain. We sought to use a combination of techniques to obtain morphological and biochemical evidence for apoptosis, including histopathological examination with diverse staining techniques and DNA gel electrophoresis. Although other forms of cell death might have occurred, our data suggest that apoptosis contributes to the loss of neural cells in the brain in response to TCE exposure.

In-vivo experimental studies are essential to assess toxicants with adverse risk to health at birth or later in life. The

in-vivo study of the toxicity of chemicals is of great importance because animal systems are extremely complicated, and the interaction of chemical compounds with biological components could lead to unique biodistribution, clearance, immune responses and metabolism. In recent study, we found that early exposure to low-dose TCE had detrimental impact on the integrity of mouse brain [21]. A large number of degenerative cells (pyknosis of nuclei, disruption of eosinophilic cytoplasm) were observed in response to TCE insult. However, little or no signs of degeneration were seen in the brains of controls, indicating that TCE induces degeneration in mouse brain.

It has been reported that environmental exposure to TCE is associated with several types of neurological deficit [2], whereas low occupational exposure is linked to the changes in neurobehavioral performance [11, 29]. In the current study, we used mouse model to investigate the impact of early exposure to TCE on the motor behaviour at later in lifespan. We found that TCE insult lead to significant neurobehavioral abnormalities. It should be noted that at the doses used in our experiments, TCE increases motor activity in the mice and induces turning behavior, but vehicle does not modify locomotion in control mice when applied alone. Although our dosing window and dose levels were not the same as previous studies [31-32], our results on TCE-related turning behavior are consistent with previous studies that used higher doses of TCE than those used in this study [32-33], suggesting the neurotoxicity of TCE.

During nervous system development, apoptosis is a physiological process of cell elimination [35]. However, in neurophathological conditions, apoptosis might contribute to neural cell damage by many stimuli such as ischemia, hypoxia, radiation, oxidative stress, excitatory neurotoxicity, and chemicals [36-38]. It is well known that histological assays are reliable tools to detect morphological changes due to environmental toxicants. In the present study, we found that TCE insult lead to significant increase in the percent of neural cells that met the definition of necrosis, i.e. cell swelling and lysis, vacuolation, karyorrhexis, and karyolysis. However, on a quantitative basis, the percent of apoptotic neural cells was approximately two orders of magnitude higher. This suggests that 55-60% of all dying/ dead cells were undergoing an apoptotic process characterized by cell shrinkage, chromatin condensation and margination, and apoptotic body formation. The similar morphological changes of some neural cells were observed by cresyl fast violet staining [21]. Abundant cells with apoptotic morphology based on the rounded, shrunken nature of nucleus and on the intense staining of the nucleus were seen in TCE-treated groups, suggesting that apoptosis is implicated in TCE-induced brain degeneration.

It has been reported that biomarkers of brain cells (DNA and protein concentrations) can be used as a measure of cell density [39]. In this study, we found that TCE insult induced a significant reduction in the brain DNA content compared to controls, an effect that was associated with neurobehavior abnormalities. This pattern is consistent with greater neuronal cell loss and reactive gliosis [21]. Related studies were conducted in which groups of rodents were continuously exposed to TCE for three months, followed by a four-month exposure-free period, ending with sacrifice [39-41]. Study endpoints included body weight, total and regional brain weight, brain protein, DNA. However, brain histology and neurobehavior were not evaluated. It was found that exposure to TCE significantly reduced DNA content in three investigated brain areas [39]. Because of the toxicological significance of reduced DNA content and reliability as a biomarker are unclear, the doses level used in this study is not characterized as a NOAEL or LOAEL (lowest-observed-adverse-effect level).

The observation that TCE insult had a significant effect on the DNA content raises the question of whether it also enhances internucleosomal DNA fragmentation. Degradation of the DNA during necrosis is very rapid and is not believed to contribute to the labeling reaction at the delayed time points [37]. Moreover, the necrotic cells do not show/uneven staining in the cytoplasm, and a lack of shrinking cytoplasm [42]. It is well known that the DNA of apoptotic cells is cleaved into multiples of 180 ± 20 bp by activated endonucleases, leading to `DNA ladders'. However, these ladders are not the sole criterion for identifying apoptosis [43]. The DNA ladders do not appear in some circumstances because the apoptotic cells are easily and rapidly cleared by neighboring cells; on the other hand, as little as 2% of apoptotic cells amongst necrotic cells can be detected as a ladder [37]. Therefore, caution must be used when interpreting DNA laddering results. In the current study, the DNA extracted from the brains of controls and TCE-treated groups was separated in agarose gels. Little or no sign of DNA fragmentation were seen in the controls. However, there was a significant internucleosomal DNA fragmentation in response to TCE insult. Furthermore, the time course of DNA fragmentation following TCE exposure was similar to that of the occurrence of dead cells determined by H&E staining, suggesting that degeneration induced by TCE might be correlated with the synthase of certain proteins, especially those apoptosis related ones.

The biochemical indices used in the current study have some limitations. Firstly, brain regions contain separate neuronal groupings so that drastic effects on a particular set of neurons are likely to be diluted with unaffected tissue. Accordingly, the alterations seen here for impacts on DNA suggest that there are actually much larger alterations in more restricted areas. Second, measurements of DNA are common to neural cells including neurons and glia, so that it is not clear from the current measurements alone which types of neural cells are targeted. Nevertheless, by examining impacts on a specific set of neural cell markers, we were able to verify adverse effects on specific cell population (data not shown). At the same time, more specific markers for neurons and glia, along with quantitative morphological studies, will be likely to give greater insight into the types of cells that are affected. Our interpretation that TCE insult sensitizes the developing brain to neuronal cell damage and reactive gliosis evoked by subsequent TCE insult is conjectural based on the biochemistry presented here. Finally, the relationship between

TCE's biochemical effects and adverse behavioral outcomes has already been specified [21, 44].

A further limitation is imposed by temporal alterations that may take place after toxicant-induced developmental damage. In the current study, we found much larger impacts on DNA marker in the cerebral cortex, whereas many of the impacts were reduced or absent later in lifespan (data not shown). Neuronal loss and reactive gliosis can be expected to produce this type of pattern. For example, prenatal exposure to nicotine, a prototypic cholinergic developmental neurotoxicant, evokes loss of neuronal cells and a consequent decrease in DNA, and although values subsequently return to normal, this occurs because of replacement of neurons with glia [16, 45]. Accordingly, determination of the targets for effects of TCE will likely require examining biomarkers and morphological changes over the course of development rather than just in adulthood.

The characterization of neural cell subpopulations that are especially susceptible to environmental insult represents a crucial new concept in toxicology. Although we did not identified specific targets, the fact that adverse impacts also extend to general biomarkers of brain cell number, size, and neuritic projections suggests that the impact is likely to involve multiple pathways and their attendant behaviors. Accordingly, sensitization to environmental exposures later in life may contribute to the increase in neuropsychiatric and learning disorders noted for offspring of women exposed to chemicals [16]. These findings have crucial implications in developmental toxicology, both in terms of common human exposure, but also in generating novel models that explore the concept of susceptible subpopulations from the viewpoint of non-genetic factors that may evoke sensitization to environmental toxicants.

In the current, we also demonstrate that increased turning behavior in TCE-treated mice associates with histological damage and reduction in the brain DNA content. Therefore, this parameter might be a useful predictor of damage and could be used to screen injured brain, which do not incur extensive brain damage, from further study where histological verification of the lesion is not possible. Further support for this conclusion comes from the observation that the TCE insult led to a significant reduction in the DNA content in comparison with DNA laddering could reflect a decrease in brain cell number / density, possibly as a result of cell death and/or inhibition of non-neuronal cell acquisition, although other interpretations are possible [40].

5. Conclusion

In conclusion, our findings demonstrate that early exposure to TCE induces degeneration and apoptotic cell death in mouse brain at later in life, suggesting that apoptosis might play a crucial role in neurotoxicity of TCE. These results also provide important insights into mechanisms of TCE-elicited neurotoxicity and call for further investigations about neuroprotective measures against neural cell damage by TCE.

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