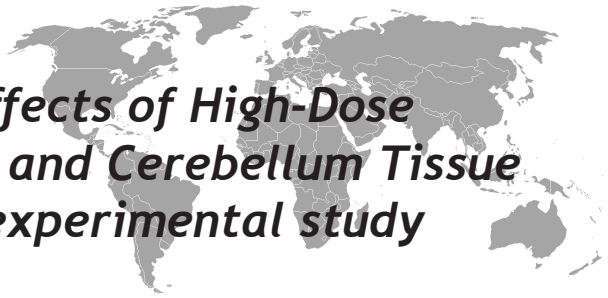

Examination of Apoptotic Effects of High-Dose Toluene on the Brain Cortex and Cerebellum Tissue During the Acute Phase: an experimental study

Ufuk Tas[1], Murat Ayan[2], Tuncay Kuloglu[3], Mustafa Suren[4], Duygu Cakıl[5], Birsen Ozyurt[1], Dürdane Aksoy Bekar[6], Mustafa Sarsılmaz[7]

ABSTRACT

The aim of this study is to examine the apoptotic effects of high-dose toluene on rat brain cortex and cerebellum tissues during the acute phase. Fourteen albino Wistar rats (180-200 g) were divided into two equal groups (n : 7). While Group I was used as a control group, Group II was applied to a high dose of toluene (5200 mg / kg / gavage). At the end of a three-hour experimental period brain tissue samples were taken from the killed animals and tissues were fixed in % 10 neutral formalin, then, embedded in paraffin and sectioned (thickness, 5 µm) to determine the immune reactivity of Bax, sections were stained immunohistochemically with avidin biotin-peroxidase method. At the same time the TUNEL method was used for detection of apoptosis in the brain cortex and cerebellum. As a result of the study, increased Bax immune reactivity was seen in the brain cortex (+++) and cerebellum (++++) of toluene treated rats compared to control. The numbers of TUNEL-positive cells were significantly higher in the cerebellum tissues of animals exposed to toluene than the control. Nevertheless there was not a significantly difference in terms of TUNEL positivity between the brain cortex of control and toluene exposed animals. As a result of this study it was shown that a high-dose of toluene can trigger apoptosis of the brain cortex and cerebellum in a very short period of time.

Key words: *Toluene, apoptosis, brain cortex and cerebellum, rat.*

Yüksek Doz Tolüenin Akut Dönemde Beyin Korteksi ve Serebellum Dokusu Üzerindeki Apoptotik Etkilerinin İncelenmesi; Deneysel Bir Çalışma

ÖZET

Bu çalışmada yüksek doz tolüenin rat beyin korteksi ve serebellum dokuları üzerinde akut dönemdeki apoptotik etkilerinin araştırılması amaçlandı. On dört adet Wistar Albino cinsi sıçan (180-200 g) iki eşit guruba (n:7) bölündü. Grup I kontrol grubu olarak kullanılırken Grup II'ye yüksek doz tolüen verildi (5200 mg/kg/gavaj). Üç saatlik deney periyodunun sonunda öldürülen hayvanlardan beyin doku örnekleri % 10'luk nötral formaline alınıp parafine gömülerek kesildi (5 µm). Bax immün reaktivitesinin belirlenebilmesi için kesitler avidin biotin peroksidaz yöntemi ile immünohistokimyasal olarak boyandı. Aynı zamanda beyin korteksi ve serebellumda apoptozun tespit edilmesi için TUNEL yöntemi uygulandı. Çalışma sonucunda tolüen verilen sıçanlarda beyin korteksi (+++) ve serebellumda (++++) artmış Bax immün reaktivitesine rastlanıldı. Tolüene maruz kalan hayvanların serebellum dokularında TUNEL pozitif hücre sayısı kontrol grubuna oranla anlamlı oranda yüksekti. Bunula birlikte beyin korteksi dokusnda tolüen ve kontrol grupları arasında TUNEL pozitifliği açısından anlamlı bir fark yoktu. Bu çalışma sonucunda yüksek doz tolüenin beyin korteksi ve serebellumda çok kısa sürede apoptozu tetikleyebileceği gösterilmiştir.

Anahtar kelimeler: *Tolüen, apoptozis, beyin korteksi ve serebellum, sıçan*

Gazıosmanpaşa University, Faculty of Medicine, Departments of Anatomy[1], Emergency Medicine[2], Anesthesiology and Reanimation[4], Physiology[5] and Neurology[6] Tokat, [3]Firat University, Faculty of Medicine, Department of Histology and Embryology, Elazig, [7]Sifa University, Faculty of Medicine Department of Anatomy, Izmir, Turkey

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Correspondence: *Ufuk Taş, MD*
Gaziosmanpasa University, Faculty of Medicine, Department of Anatomy, 60000 Tokat, Turkey.
Tel: +90 356 2129500 (1232)
Fax: +90 356 2129417
E-mail: dr_ufuktas@hotmail.com

INTRODUCTION

Toluene is a liquid aromatic hydrocarbon that widely used in industry and addiction is observed commonly (1-3). The abuse of toluene like volatile substances is an important problem in the many countries, using of this substance with up to 10% to 15% of young people developed country (4). Due to the high percentage of toluene, addicts mostly prefer sniffing glue and paint thinner (2, 5, 6). Toluene can be taken through the respiratory, digestive system and the skin into the body and generally accumulates at highly vascularized tissues which are rich in terms of fat, a large part is destroyed in the liver and converted into hippuric acid and then is excreted through urine (1, 7, 8). Toluene may cause many clinical situations from a headache up to death depending on the dose and duration (1, 9). Exposure to high-dose toluene generally is observed at workers and dependents (10, 11). Researches on high-dose poisonings of toluene are mostly case studies based on the evaluation of cases of acute intoxication (12, 13). Since toluene mainly accumulates in nervous tissue, mostly chronic effects of toluene on the nervous system were investigated. Although, there are many studies examining the effects of chronic exposure to brain tissue (4, 14-16), only limited experimental studies examining the damage of acute and high doses of toluene (17, 18). Furthermore we didn't find any study about toluene induced apoptosis in brain and cerebellum tissue within a few hours. In this study we aimed to investigate the apoptotic effects of high-dose toluene on rat brain cortex and cerebellum tissues during the acute phase using immunohistochemical methods.

MATERIAL AND METHODS

Animals

Fourteen adult male Wistar-albino rats (250-300 g) obtained from Gaziosmanpasa University Biomedical Research Unit were randomly divided into two equal groups: Group I (n:7) as control and Group II (n:7) as treated with toluene. The experimental protocols were approved (2011 HADYEK-048) by the appropriate animal care committee of Gaziosmanpasa University.

Chemicals, route of exposure and dose selection

Toluene was administered by gavage without dilution described in previous study (19). The acute oral LD50 of toluene in adult rats ranged from 5.5 to 7.4 g/kg (20). We defined the benchmark dose as the maximum neurotoxic dose causing toxic damage without leading to death (19). Rats in Group I were administered serum through gavage, while Group II rats were given a single dose (5200 mg/kg/gavage) of 99.5% pure toluene (Sigma, St. Louis, Missouri, USA). Sample collection and preparations of brain cortex and cerebellum tissue. At the end of the three-hour experimental period all rats were killed by exsanguination under ketamine/xylazine (50/10 mg/kg) anesthesia. Brain tissues were removed directly and fixed in formalin solution for immunohistochemical evaluations. The paraffin-embedded brain specimens were cut into 4-5 µm sections and stained with Bax for immunohistochemical evaluation and TUNEL for apoptosis determination. Specimens were examined under a Novel N-800M light microscope.

***Table 1**. The scoring of the immune reactivity (Bax)*

Grade	*Symbol*
absent	*(-)*
very few	*(±)*
few	*(+)*
medium	*(++)*
high	*(+++)*
very high	*(++++)*

Immunohistochemistry

Paraffin embedded brain cortex and cerebellum tissues were dissected at 4-5 µm and deparaffinized in xylene, then dehydrated with alcohol series. The brain cortex and cerebellum tissue were then placed in distilled water and boiled in citrate buffer solution (pH:6.0) in a microwave oven (750W) for 7+5 minutes for antigen retrieval. To prevent endogenous peroxidase activity sections were treated with 3% hydrogen peroxide.

***Table 2**. Semi-quantitative evaluation of Bax staining in brain cortex and cerebellum tissues of control and toluene-treated rats (n: 7 for each group).*

Tissue	*Control*	*Toluene*
Brain cortex	*(+)*	*(+++)*
Cerebellum	*(+)*	*(++++)*

The number of the positive staining was recorded as very few (±), few (+), medium (++), high (+++) and very high (++++).

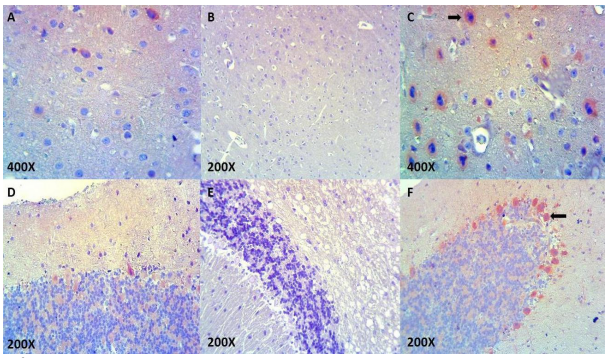

***Figure 1**. Bax staining in the brain cortex (400x) and cerebellum (200x) tissues of the animals in the toluene and control group. A small amount of BAX (+) positive cells in the brain cortex (A) and cerebellum (D) of the control group. Negative control of the brain cortex (B) and cerebellum (E) tissue of animals exposed to toluene. BAX positive cells in the brain cortex (C) (+ + +), and cerebellum (F) (+ + + +) tissues of animals exposed to toluene.*

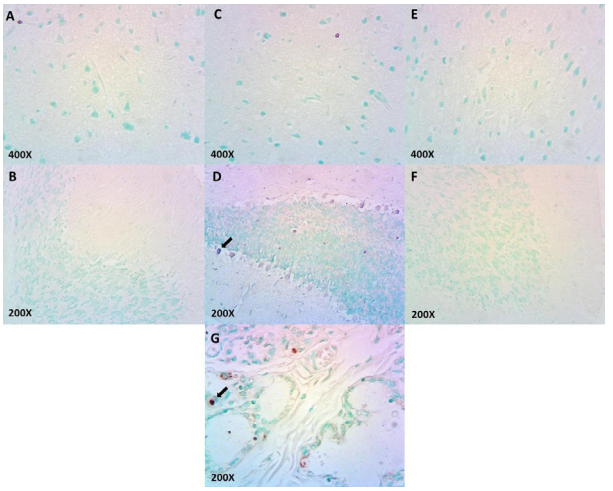

***Figure 2**. TUNEL staining in the brain cortex (400x) and cerebellum (200x) tissues of the animals in the toluene and control group. The brain cortex (A) and cerebellum (B) tissues of the control group. While in the brain cortex (C) there was no significant difference between toluene group and control group in terms of the number of apoptotic cells, a significantly number of apoptotic cells in the cerebellum tissue (D) are observed. Negative controls of the brain cortex (E) and cerebellum (F) tissues of animals exposed to toluene. Positive control staining in the gastric tissue (G).*

Tissues were treated with Ultra V Block (Ultra V Block, TA-125-UB, Thermo Fisher Scientific Inc, USA) solution to prevent background staining and then incubated with primer antibody Bax (mouse monoclonal IgG, Santa Cruz Biotechnology, sc–32239, California, USA) for 60 minutes. Secondary antibody application (biotinated anti-mouse IgG, Diagnostic BioSystems, KP 50A, Pleasanton, USA) was performed for 30 minutes. After streptavidin horseradish peroxidase treatment for 30 minutes and 3-amino-9-ethyl carbazole chromogen treatment, contrast staining was carried out using Mayer's hematoxylin. Phosphate buffered saline (PBS) was used instead of primary antibody for the negative control. Tissues treated with PBS and distilled water were covered with an appropriate covering solution.

TUNEL assay

ApopTag plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Cat no: S7101, USA) were used for detection of apoptotic cells. According to the instructions of the manufacturer sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in PBS. Tissues were incubated in a 0.05% proteinase K solution and were incubated with 3% hydrogen peroxide for five minutes to prevent endogenous peroxidase activity. Later washing with PBS, the tissues were placed in equilibration buffer for six minutes and in working solution (70% reaction buffer plus 30% TdT enzyme) at 37°C under moist conditions for one hour. Stop/wash buffer were applied during for 10 minutes and then anti-digoxigenin-peroxidase for 30 minutes. Diaminobenzidine (DAB) substrate was used to showing apoptotic cells. Sections were counterstained with methyl green. Stomach tissue was used for a positive control. PBS was used instead of the Tdt enzyme on the negative control. Cells with green nuclei after TUNEL staining using methyl green were considered normal, whereas cells with brown nuclei were considered apoptotic.

Evaluations of tissue sections

Preparations were observed and photographed (Figure 1 and Figure 2) using a research microscope (Novel N-800M). Apoptotic (TUNEL positive) cells were counted in at least eight areas per tissue section, in two sections from each animal, at 400X magnification. Bax staining was evaluated according to the method described previously (21) (Table 1).

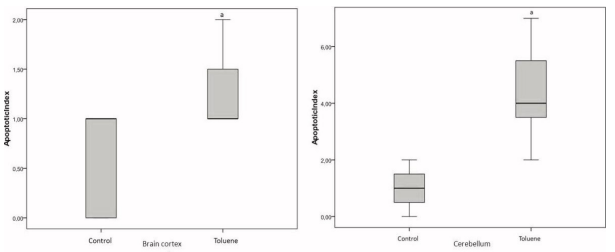

***Figure 3**. Apoptotic index of TUNEL positive cell in the brain cortex and cerebellum tissues of the animals in the toluene and control group a, p<0.01 compared to control group in cerebellum a, p>0.05 compared to control group in brain cortex*

Statistical analyses

Results are expressed as mean ± standard deviation (SD). Mann-Whitney U test were used for statistical analysis. All analyses were performed using SPSS version 15.0 software. P values of <0.05 were considered to be statistically significant.

RESULTS

Immunohistochemical findings

Bax protein was visualized by immunohistochemical staining of brain cortex and cerebellum tissues cross-sections of rats in the toluene and control groups (Figure 1). The results were evaluated semi-quantitatively. There were significant increases in Bax immune reactivity in the brain cortex (+++) and cerebellum (++++) tissue of rats in the toluene group, whereas there were little staining (+) in the control group (Table 2).

TUNEL findings

TUNEL staining was shown in the brain cortex and cerebellum tissue sections of rats in the toluene and the control group (Figure 2). The numbers of TUNEL-positive cells in the cerebellum tissues of animals exposed to toluene were significantly higher than the control group (p <0.05). However there was not a significantly difference in terms of TUNEL positivity between the brain cortex of control and toluene exposed animals (Figure 3).

DISCUSSION

The majority of toluene is broken down via the cytochrome P-450 enzyme found in the liver and converted to hippuric acid, the major and last metabolite of toluene, which is excreted in the urine (22). Toluene and its metabolites affect the defense system in the body by means of oxidative stress and may cause damage in many tissues primarily including the brain (16, 23-25). While the mechanism of action of toluene is not exactly clear, authors mention several different mechanisms. Toluene modifies lipid composition of cell membrane and effects the Na / K-ATPase activity and thus increases the membrane fluidity (26). Some researchers have reported that toluene may affect Gabaergic, glutamatergic, serotonergic, dopaminergic pathways (2, 27). In addition, it is said that toluene may cause mitochondrial damage, and increase of pro-apoptotic proteins (Bax) in the cytoplasm and thus increases apoptotic activity (28, 29). Apoptosis known as controlled cell death without causing inflammation, it is characterized by cytoplasm shrinkage, membrane changes, and DNA fragmentation. Recently it is mentioned that apoptosis may be triggered with various factors and follow different pathways (30). In mitochondria-mediated apoptosis, which is the most popular of these, apoptosis is triggered by releasing of apoptogenic factors like, cytochrome c, apoptosis-inducing factor and smac / DIABLO from intermembranal space of mitochondria to cytosol. Cytochrome C released into the cytosol, the apoptosis complex consisting of C/Apaf-1/ATP/proxaptase-9 first activates caspase-9 and then caspase-3 (31, 32). Family member of Bcl-2 are effective in the increase or decrease of apoptotic activity. During the apoptosis period pro-apoptotic mediators which passing from the mitochondria into the cytosole (Bax, Bak, Bad), accelerate the apoptotic activity by increasing the release of cytochrome c (33, 34). Chromatin condensation, DNA fragmentation and catabolism of membrane proteins like morphological changes occur with Caspase-3 activation. (35). During apoptosis, these protein changes can be determined by immunohistochemical staining method and DNA damage can be determined with TUNEL staining method. In our study significantly increased Bax-positive cells was seen in the brain cortex and cerebellum tissues of rats exposed to toluene. This increase was evaluated in favor of the increase in apoptotic activity. The number of TUNEL-positive cells in the cerebellum tissues of animals exposed to toluene was significantly higher than

in the control group. However no significant difference was seen between the toluene group and control group in terms of TUNEL positivity in the brain cortex. El-Nabi Kamel and Shehata (2008) reported that the most affected tissue by toluene is the brain tissue (23). It was shown that toluene causes damage to the frontal cortex and brain stem and leads to an increase in apoptotic activity (36). Baydas et al have shown morphological changes (GFAP) in the cerebellum, cortex and hippocampus tissues of 60-70% toluene containing thinner exposed animals (37). Gotohda et al were demonstrated shrunken granule cells in the dentate gyrus of the hippocampus and shrunken Purkinje cells in cerebellum after toluene inhalation in rat brain (15). Hester et al demonstrated global gene expression changes in rat brain after toluene inhalation during an acute exposure. They found toluene exposure was associated with changing of genes in pathways associated with GABA receptor signaling and mitochondrial function (18). Heung-Sik Seo et al claim that acute toluene exposure (0-1000 mg/kg) reduces the rate of adult hippocampal neurogenesis but does not induce neural apoptosis in the hippocampus of adult mice (17). Kanter was demonstrated the significantly increased number of apoptotic neurons in chronic toluene treated rats compared to control animals in frontal cortex tissues using by TUNEL assay (14). Considering previous studies, it can be said that as a result of chronic toluene exposure especially may cause apoptotic changes and structural damage in the brain cortex and cerebellum tissue. Although the number of experimental studies in relation to acute exposure is very limited, it is mentioned some of these studies that toluene stimulates apoptosis lightly or does not induce apoptosis. However in our study, significantly increased Bax ımmunoreactivity was shown in the brain cortex and cerebellum tissues of animals given high doses of toluene, and that toluene is able to trigger apoptosis in a very short time like 3 hours. The numbers of apoptotic cells (TUNEL positive cell) in the cerebellum tissues of toluene treated animals were significantly higher but not in cortex compared to control. It may be concluded from this study, cortex of the brain tissue is affected more quickly than cerebellum in toluene poisoning. For better understanding of the pathogenesis of acute intoxication cases, it is necessary to make further studies in relation to acute high-dose exposure to toluene on the molecular level in the future.

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