Acute cardiotoxic effects of high dose toluene: an experimental study

Yüksek doz toluenin akut dönemdeki kardiyotoksik etkisi: Deneysel bir çalışma

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ABSTRACT

Objective: This study aimed to investigate the acute cardiotoxic effects of high dose toluene and its damage mechanisms on heart tissue in the acute period.

Methods: Twenty adult male Wistar Albino rats (200-220 g) were used in this controlled experimental animal study. Animals were divided into two equal groups: a control group (Group 1) and a high dose (6 mL/kg/gavage) toluene-administered group (Group 2). Arterial blood pressure (BP) and heart rate (HR) values were measured at 30th, 60th and 90th minutes after toluene was administered. At the end of the experimental period, blood samples and heart tissues were taken from the rats. Serum troponin T levels were assayed. Heart tissue sections were stained using routine histological methods and examined under a light microscope. In addition, the sections were immunohistochemically stained using the avidin-biotin-peroxidase method to determine caspase-3 immunoreactivity and TUNEL to detect apoptosis. To compare the apoptotic index, the Mann-Whitney U test was used. For comparisons between the two groups, the independent t-test was used. In addition, time-based changes of intra-group parameters were evaluated using paired t-tests.

Results: BP and HR values were low in toluene-treated rats compared to the control group. Troponin T levels were increased in toluene-administered animals as compared with controls [Toluene group: 0.140 (0.010-2.000) ng/mL vs control group: 0.010 (0.010-0.010) ng/mL, p=0.01]. Histopathologic examination of heart tissue sections showed congestion and edema in toluene administered rats. Higher TUNEL positivity and (+++) immunoreactivity for caspase-3 protein were observed in the toluene group compared to the control group.

Conclusion: The present study demonstrated that high doses of toluene cause apoptosis and may lead to impairment of cardiac function in the acute period. (Anadolu Kardiyol Derg 2013; 13: 3-8)

Key words: Toluene, cardiovascular toxicity, immunohistochemistry, apoptosis, troponin T

ÖZET

Amaç: Bu çalışma yüksek doz toluenin akut dönemde kalp dokusunun üzerindeki kardiyotoksik etkilerini ve hasar mekanizmanını araştırmak amacıyla yapıldı.

Yöntemler: Bu kontrollü deneysel hayvan çalışmasında 20 adet Wistar Albino cinsi sıçan (200-220 g) kullanıldı. Hayvanlar kontrol (Grup 1) ve yüksek doz toluen (6 mL/kg/gavaj) verilen grup (Grup 2) olmak üzere iki eşit gruba ayrıldı. Toluennin verildikten sonra 30, 60 ve 90. dakikalarda arteriyel kan basınçını (KB) ve kalp hızını (KH) ölçülü. Deney periyodunun sonunda hayvanlar ölüldürülecek ve doku örnekleri alınmalıdır. Serum troponin T seviyeleri değerlendirildi. Kalp doku örnekleri rutin histolojik yöntemlerle boylanarak ilk mikroskobu altında incelemeler. Ayrca kesetler kaspaz-3 immünreaktivitesinin değerlendirilmesi içinavidin-biotin-peroksidaz yöntemi ile immünohistokimyasal olarak ve apoptozun tespiti için TUNEL ile boyanan. Apoptotik indeksi ve karsılaştırmak için Mann-Whitney U testi kullanıldı. İki grubun sayısal değerleri karsılaştırmak için bağımsız t-testi kullanıldı.

Bulgular: Kontrol grubu ile karsılaştırdığında, toluen verilen hayvanlarla KB ve KH seviyeleri düştü. Toluennin verilen hayvanlarda troponin T seviyeleri artmıştır (Kontrol grubu: 0.010 (0.010-0.010) ng/mL, Toluennin verilen grubu: 0.140 (0.010-2.000) ng/mL, p=0.01). Toluennin verilen kesetlerin kalp doku örneklerinin histopatolojik incelenmesinde konjesyon ve ödем gözlandı. Kontrol grubu ile karsılaştırdığında toluen verilen grupta kaspaz-3 immünreaktivitesinin (+++) ve TUNEL pozitivitesinin arttığı gözlandı.

Sonuç: Bu çalışma sonucunda yüksek doz toluenin akut dönemde apoptoza ve kardiyak fonksiyonlarda bozulmaya yol açabileceği sonucu çıkmaktadır. (Anadolu Kardiyol Derg 2013; 13: 3-8)

Anahtar kelimeler: Toluen, kardiyovasküler toksite, immünohistokimya, apoptoza, troponin T
**Introduction**

Toluene, a clear aromatic hydrocarbon with a sharp odor (C\(_{6}\)H\(_{5}\)CH\(_{3}\)-CAS No: 108-88-3) is commonly used in industry to manufacture products ranging from gasoline to cleaning agents. As a result, people can be exposed to toluene through drinking water, food, air, and various consumer items (1, 2). Addiction to toluene and similar volatiles is a major problem in developing countries, as well as, in developed countries such as the United Stated (3, 4). Because of easy access, toluene addicts frequently prefer glue and thinner (5). Addicts, suicidal individuals, and others exposed to high levels of toluene may inhale, ingest, or absorb the toluene via the skin. After consumption, the toluene builds up in many tissues, especially those rich in fat (1, 6). A major part of the toluene is metabolized in the liver, converted into hippuric acid and excreted in urine (7).

Toluene may cause many clinical problems ranging from headache to death depending upon the dose taken and duration of exposure (8, 9). Although it is reported that damage could occur in the nervous system, liver, kidney, and heart as a result of chronic exposure (10-12), no explanation has been found for sudden deaths as a result of acute exposure. Some researchers have surmised that deaths could be due to ventricular arrhythmia (13). A significant portion of studies investigating the effects of toluene on the heart examines clinical cases of addicted individuals or people exposed to high levels of toluene due to accidents or attempted suicides. Experimental studies on this issue are quite limited (5, 14) and, although the majority of existing experimental studies evaluate chronic exposure.

We have not come across an extensive study, which evaluates heart tissue damage as a result of high levels of toluene exposure using electrocardiographic, histological, immunohistochemical, and biochemical methods in the literature.

The aim of the present study was to investigate the toxic effects of high levels of toluene on heart tissue in rats using electrocardiographic, histological, immunohistochemical and biochemical methods.

**Methods**

**Study design**

This is an experimental controlled animal study.

**Animals**

Twenty adult male Wistar-albino rats (200-250 g) were randomly divided into two equal groups: a control group (Group 1; n=10) and a group treated with toluene (Group 2; n=10). The experimental protocols were approved by the appropriate Animal Care Committee (HADYEK-045).

**Chemicals, dose level, dose selection, and route of exposure**

The most frequent route of toluene exposure is inhalation. However, oral administration of toluene has been reported to be more reliable in experimental studies dealing with heart rate (HR) and blood pressure (BP) (14). Therefore, toluene was administered orally in the present study. The acute oral LD50 of toluene in adult rats ranged from 5.5 to 7.4 g/kg (1). We defined the benchmark dose as the maximum dose causing toxic damage without leading to death (15). Rats in Group 1 were administered serum through gavage, while Group 2 rats were given a single dose (6 mL/kg/gavage) of 99.5% pure toluene (Sigma, St. Louis, Missouri, USA). Since toluene is not corrosive, it was administered through gavage without dilution (15). The experiment was completed 150 minutes after toluene was administered.

**Measurements of arterial blood pressure (BP) and electrocardiogram (ECG) recordings**

Rats were anaesthetized with 10 mg/kg xylazine hydrochloride (Rompun®, Bayer, Turkey) and 50 mg/kg ketamine hydrochloride (Alfamine®, Egevet, Turkey). Polyethylene (PE) catheters were inserted into the lower abdominal aorta via the left femoral artery. Arterial blood pressure and the ECG were recorded (KMA-800, Petas, Turkey and IRMA TRUPOINT™) in 30 minutes intervals starting from the administration of toluene.

**Sample collection**

At the end of the 150-minute experimental period, blood samples were taken and all rats were killed by exsanguination. Blood samples were collected into routine biochemical test tubes for determination of troponin T (TnT) levels. Heart tissues were removed directly and fixed in formalin solution for histopathological and immunohistochemical evaluations.

**Biochemical analysis of serum**

For the biochemical analysis of the serum, blood samples were collected into vacutainer tubes with K-EDTA as an anticoagulant. Plasma samples were separated by centrifugation (at 1,000 g for 10 min. at 4°C) and TnT levels (upper limit of normal <0.01 ng/mL) were measured using a Cobas C 501 auto analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with commercial kits (Roche Diagnostics GmbH, Mannheim, Germany).

**Microscopic examination of heart tissue**

Heart tissues were removed directly and fixed in 10% neutral formalin solution. The paraffin-embedded heart specimens were cut into 5 μm sections and stained with hematoxylin-eosin (H&E) for histopathological evaluation. Specimens were examined under a Novel N-800M light microscope (Nanjing Jiangnan Novel Optics Co. Ltd, Nanjing China).

**TUNEL assay**

Apoptotic cells were detected using ApopTag plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Cat no: S7101, USA) based on the instructions of the manufacturer. Paraffin embed-
ded heart tissue was dissected into 5 µm sections. Sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in PBS. Tissues were incubated in a 0.05% proteinase K solution. Then tissues were incubated with 3% hydrogen peroxide for five minutes to prevent endogenous peroxidase activity. After 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 60 minutes. Tissues were then incubated in stop/wash buffer for 10 minutes and in anti-digoxigenin-peroxidase for 30 minutes. Apoptotic cells were observed using diaminobenzidine (DAB) substrate. Sections were counterstained with methyl green and sealed using proper covering solution. Stomach tissue was used as a positive control. PBS was used instead of the Tdt enzyme on the negative control. Preparations were observed and photographed using a research microscope (Novel N-800M). Cells with green nuclei after TUNEL staining using methyl green were considered normal, whereas cells with brown nuclei were considered apoptotic. Apoptotic (TUNEL positive) cells were counted in at least eight areas per heart section, in two sections from each animal, at 400X magnification.

**Immunohistochemistry**

For immunohistochemical caspase-3 staining, paraffin embedded heart tissue was dissected at 5 µm and deparaffinized in xylene, then dehydrated with alcohol series. The heart tissue was 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. Sections were treated with 3% hydrogen peroxide to prevent endogenous peroxidase activity. To prevent background staining, tissues were treated with Ultra V Block (Ultra V Block, TA-125-UB, Thermo Fisher Scientific Inc., USA) solution and then incubated with primer antibody caspase-3 (mouse monoclonal IgG, Santa Cruz Biotechnology, sc-7272, 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. For the negative control, phosphate buffered saline (PBS) was used instead of primary antibody. All other steps were the same. Tissues treated with PBS and distilled water was covered with an appropriate covering solution. Stained tissues were photographed using a research microscope (Novel N-800M). Caspase-3 staining was evaluated according to the method described previously (16) (Table 1). This analysis was performed in at least eight areas in each heart section, in two sections from each animal, at 400X magnification.

**Statistical analysis**

All statistical analyses were performed using SPSS for Windows version 15 (SPSS, Chicago, IL, USA). To compare the apoptotic index, the Mann-Whitney U test was used. The normality of TnT data was tested using the Kolmogorov-Smirnov test. For comparisons between the two groups, the independent t test was used. In addition, time-based changes of intra-group parameters were evaluated using paired t tests.

**Results**

**ECG and blood pressure**

HR and BP results for the toluene and control groups are given in Table 2. Average BP levels of toluene-administered rats were statistically lower than those of the control group (66±85 and 82±10 mmHg, respectively; p=0.003). In the toluene group, there were significant differences between the BP levels measured after 30 minutes and after 60 minutes (76±13 and 72±11 mmHg, respectively; p=0.03) and between those measured after 60 minutes and after 90 minutes (72±11 and 66±11 mmHg, respectively; p=0.006). In addition, pulse values taken after 30 and 60 minutes were significantly different (195±32 and 179±31 bpm, respectively; p=0.001).

**Biochemical findings**

The troponin T levels for the control and experimental groups are shown in Table 2. There was no change in troponin values in none of animals of the control group at the 150 minute. In toluene group, TnT values increased in five of ten animals and the in other 5 animals it remained unchanged. Minimum value of TnT at 150th minute was 0.01 ng/mL while maximum value was 5.15 ng/mL in toluene group. The median value of plasma TnT were significantly higher in toluene than in the control group at 150th minute (0.140 ng/mL (0.010-2.000) vs 0.010 ng/mL (0.010-0.010), p=0.01, respectively).

**Histopathological findings**

When evaluated under a light microscope, the control group appeared normal. On the other hand, histological examination of heart tissue sections obtained from toluene-administered rats showed edema and congestion (Fig. 1).

**TUNEL findings**

TUNEL positive cells from the hearts of control and toluene-treated rats are shown in Figure 2. In toluene-exposed rat hearts, the number of apoptotic cells was statistically higher compared to the control group (Fig. 3; p<0.01).
**Immunohistochemical findings**

Heart tissue sections of toluene and control group rats were immunohistochemically stained with caspase-3, and the results were semi-quantitatively evaluated. Very few caspase-3 stained cells were observed in the control group (±). However, there was high (+++) caspase-3 protein immunoreactivity in the hearts of toluene-treated rats (Fig. 1).

**Discussion**

We demonstrated that BP and HR values were low in toluene-treated rats compared to the control group. TnT levels increased in toluene-administered animals. Histopathologic examination of heart tissue sections showed congestion and edema in toluene administrated rats.

Although there are many studies about the health consequences of toluene in the literature, the mechanisms underlying the deleterious effects are unknown. Toluene, a highly toxic material, has been reported to cause decreases in antioxidant levels in many tissues and increases in peroxidation derivatives. In addition, it can affect the concentrations of electrolytes, such as sodium, potassium and calcium, as well as, the levels of many different neurotransmitters. Some investigators have maintained that it blocks sodium channels and thus could lead to arrhythmia (17). In studies dealing with the effects of toluene on the heart, very different findings have been reported for heart rate (tachycardia, bradycardia), blood pressure (hypotension, hypertension), and ECG (QRS enlargement, branch block, PR extension, QT changes) depending upon the dose and duration of exposure (18-20). In the present study, the average blood pressure values of rats administrated toluene were quite low compared to the control group. In addition, blood pressure values of rats 60 minutes after toluene administration were significantly lower than blood pressures after 30 minutes (p=0.001). Morvai et al. (21) found that intravenous injection of toluene induced a rapid and permanent decrease in blood pressure. Gordon et al. (14) reported that in rats administered 0.8-1.2 g of toluene orally via gavage,

**Table 2. Arterial blood pressure, heart rate and troponin T levels of toluene and control groups**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group (n=10)</th>
<th>Toluene group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, min</td>
<td>30 60 90 150</td>
<td>30 60 90 150</td>
</tr>
<tr>
<td>BP, mmHg</td>
<td>84±10 81±7 79±13</td>
<td>77±13^a 72±11^b 66±11</td>
</tr>
<tr>
<td>HR, per min*</td>
<td>198±21 196±35 193±38</td>
<td>195±32^c 179±31 177±37</td>
</tr>
<tr>
<td>TnT, ng/mL†</td>
<td>- - - 0.010 [0.010-0.010]</td>
<td>- - - 0.140 [0.010-2.000]^d</td>
</tr>
</tbody>
</table>

*Values are presented as mean ± standard deviation.
†Values are presented as median and interquartile range (Q1 to Q3).
Independent t and paired t tests were used.

a, p=0.003 comparison between 30th and 60th minute of toluene group
b, p=0.03 comparison between 60th and 90th minute of toluene group
c, p=0.01 comparison between 30th and 60th minute of toluene group
d, p=0.01 comparison between 150th minute of toluene and control group
BP - blood pressure, HR - heart rate, TnT - troponin T

**Legend**

- **Figure 1. Hematoxylin and eosin staining (A, B) and caspase-3 immunoreactivity (C, D, E) in heart tissue of control and toluene-treated rats.**
  - Congestion and edema are observed upon histological examination of heart tissue sections obtained from toluene-administered rats (B).
  - Very few caspase-3 stained cells were observed in the control group (C).
  - There was high (+++) immunoreactivity for caspase-3 protein in heart tissues of toluene-treated rats (D).
  - Negative control tissue for caspase-3 staining (E).

- **Figure 2. TUNEL staining for the control (A), toluene-treated (B), negative control (C), and positive control (D) groups.**
  - Stomach tissue was used as a positive control (D).
  - Increased TUNEL positive cells were seen in the heart tissue sections obtained from toluene-administered rats.
In the present study, histological examination of the heart tissue sections obtained from toluene-administered rats revealed edema and congestion. Immunohistochemical examination demonstrated high (+++) immunoreactivity for caspase-3 proteins and increased TUNEL positive (apoptotic) cell numbers were found in the heart tissues of toluene-treated rats.

Troponins are preferred biomarkers for showing ischemic heart damage in diagnosis of myocardial infarction (31). It was reported that sensitivity to troponin T and I in showing the heart muscle damage were virtually 100% and specificities 95% in hours from two to six (32, 33). Because of their high tissue sensitivity and specificity, troponin T and I are better biomarkers than others, such as creatine kinase-MB fraction (34, 35). In the present study, troponin T levels 150 minutes after toluene exposure were significantly higher compared to the control group. This increase was interpreted as heart muscle damage.

Study limitations
Firstly, the experiment was completed 150 minutes after toluene was administered because of the fact that the concern of animal may die from high dose toluene. Increases of apoptosis and troponin levels can be seen more clearly with longer exposure to toluene. Secondly, in our study TnT level was measured in serum. However, it is ideal to measure TnT levels both in serum and directly from myocardium tissue using by immunohistochemistry. Finally, we used TUNEL assay to detect apoptotic cell. In the future study, annexin V-fluorescein isothiocyanate (FITC) propidium iodide method can be used to detect the early apoptotic cells.

Conclusion
These data show that acute toluene exposure leads to apoptosis by increasing the caspase-3 activity and so it causes serious heart tissue damage within a very short period of time.

Conflict of interest: None declared

Peer-review: Externally peer-reviewed.


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