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Protective effects of thymoquinone on the neuronal injury in frontal cortex after chronic toluene exposure

Mehmet Kanter

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Abstract The aim of this study was designed to evaluate the possible protective effects of thymoquinone (TQ) on the neuronal injury in the frontal cortex after chronic toluene exposure in rats. The rats were randomly allotted into one of three experimental groups: A (control), B (toluene treated) and C (toluene treated with TQ); each group contain 10 animals. Control group received 1ml normal saline solution and toluene treatment was performed by inhalation of 3,000 ppm toluene, in a 8 h/day and 6 day/week order for 12 weeks. The rats in TQ treated group was given TQ (50 mg/kg body weight) once a day orally for 12 weeks starting just after toluene exposure. Tissue samples were obtained for histopathological investigation. To date, no histopathological changes of neurodegeneration in the frontal cortex after chronic toluene exposure in rats by TQ treatment have been reported. In this study, the morphology of neurons in the TQ treatment group was well protected. Chronic toluene exposure caused severe degenerative changes, shrunken cytoplasma, severely dilated cisternae of endoplasmic reticulum, markedly swollen mitochondria with degenerated cristae and nuclear membrane breakdown with chromatin disorganization in neurons of the frontal cortex. We conclude that TQ therapy causes morphologic improvement on neurodegeneration in frontal cortex after chronic toluene exposure in rats. We believe that further preclinical research into the utility of TQ may indicate its usefulness as a potential treatment on neurodegeneration after chronic toluene exposure in rats.

M. Kanter (✉)
Department of Histology and Embryology, Faculty of Medicine, Trakya University, Edirne 22030, Turkey
e-mail: mkanter65@yahoo.com

Keywords Toluene · Thymoquinone · Morphology · Frontal cortex · Rats

Introduction

Toluene is a volatile organic compound widely used as an industrial solvent, and it can also be found as an air pollutant in homes and buildings. Exposure to higher levels of toluene from inhalation is known to induce reproductive and developmental toxicity such as intrauterine growth retardation, premature delivery, and testicular atrophy (Donald et al. 1991). Several lines of evidence suggest that toluene affects the endocrine function of reproductive organs in both humans and animals.

In humans and animals, the primary effect associated with inhalation exposure to toluene is central nervous system (CNS) depression (Greenberg 1997; Pryor et al. 1987). Several studies, carried out with animals, reported an uptake of inhaled toluene in blood and brain (Benignus et al. 1981; Crofton et al. 1994). Toluene's affinity for the lipid-rich structures of nervous tissue results in CNS toxic effects within minutes (Benignus et al. 1981; Campagna et al. 2001).

The chronic abuse of solvents results in structural and functional impairment of a variety of organs. Thinner fume inhalation is an important cause of encephalopathy and may lead to irreversible brain damage. Particularly, toluene abuse has been shown to cause permanent changes in brain structures which correlate with neural dysfunction (Baelum 1991; Ladefoged et al. 1991; Hass et al. 1999). Furthermore, Mattia et al. (1993) demonstrated that intraperitoneal injection of toluene caused a significant elevation in the rate of reactive oxygen species generation and a reduction in levels of reduced glutathione (GSH) in the brain.

Reactive oxygen species, in turn, damage lipids, proteins and nucleic acid. This leads to neurodegenerative disorders, which mediate behavioral changes. Acute and chronic effects of toluene on neurons have been well documented (Burmistrov et al. 2001). Toluene can cause CNS depression, loss of memory and progressive brain and nerve damage (Benignus 1981).

Apoptosis, a type of programmed cell death, is a major event in normal development of the nervous system, playing an important role in the establishment of neuronal connections (Oppenheim 1991; Oppenheim 1996; Clarke et al. 1998). Apoptotic cell death is executed via molecular pathways that are mediated by the activation of caspases, a family of cysteine proteases (Zimmermann et al. 2001). Caspase-3, a main effector caspase, is strongly implicated in neuronal apoptosis (Cohen 1997), which occurs due to competition for, or limited supply of, neurotrophins that suppress the endogenous genetic death program. Evidence suggests that experimental axotomy or target elimination and withdrawal of neurotrophins result in an increase in apoptosis during development and in the adult (Henderson 1996; Hörtnagl and Hellweg 1997).

TQ was isolated as the principal active ingredient from the volatile oil of the black seeds (*Nigella sativa*) (Mahfouz and El-Dakhakhny 1960). The seeds of the plant were shown to contain a fixed oil (>30% wt/wt) and a volatile oil (0.40–0.45%) (El-Alfy et al. 1975). The volatile oil has been shown to contain 18.4–24.0% TQ (2-isopropyl-5-methyl-1,4-benzoquinone) (Aboutabl et al. 1986). TQ has been shown to attenuate eicosanoid generation (Houghton et al. 1995), lipid peroxidation (Nagi et al. 1999), cisplatin nephrotoxicity (Badary 1999), ifosfamide Fanconi syndrome (Badary et al. 1997), tetrachloride hepatotoxicity (Gharably et al. 1997), doxorubicin cardiotoxicity (Al-Shabanah et al. 1998), histamine release (Chakravarty 1993) and diabetic nephropathy (Kanter 2009). In spite of these studies, there is no available information on the effect of TQ on neuronal injury. The present study aimed to investigate the possible protective effects of TQ on the neuronal injury in frontal cortex after chronic toluene exposure.

Materials and methods

Experimental Procedure

TQ was obtained from Sigma Chemical Co., St. Louis, MO, USA. It was dissolved by the initial addition of DMSO, followed by the addition of normal saline (the final concentration of DMSO was <0.5%). The solution was administered at a dose of 50 mg/kg body weight once daily by using intra-gastric intubation for up to 12 weeks (Fararh et al. 2005).

Animals

Forty healthy male Wistar albino rats, weighing 200–250 g and averaging 16 weeks old were utilized in this study. The animals were purchased from a local supplier (Ankara Laboratories), and housed in individual cages (360 × 200 × 190 mm) 1 month before the start of the experiments. Food and tap water were available ad libitum. In the windowless animal quarter automatic temperature (22 ± 2°C) and lighting controls (light on at 07 AM and off at 09 PM: 14 h light/10 h dark cycle) was performed. Humidity ranged from 50 to 55%. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Experimental design

The rats were randomly allotted into one of three experimental groups: A (control), B (toluene treated) and C (toluene treated with TQ); each group contain 10 animals.

Solvent exposures, drug preparation and sample collection

Control group received 1ml normal saline solution and in group B and C were exposed to 3,000 ppm toluene (Merck, 99.5%) in inhalation chambers equipped with a trap, and designed to sustain dynamic and adjustable airflow (11,250 mg/m^3) for 8 h/day (from 08 am to 04 pm) and 6 day/week order for 12 weeks. The rats in TQ treated group was given TQ (50 mg/kg body weight) once a day orally for 12 weeks starting just after toluene exposure. The solvent concentration in the chambers was continuously monitored using a gas chromatography (GC). Control group (A) animals were housed in identical chambers ventilated with fresh air. At the end of the solvent exposure, all animals were anesthetized with i.p. injection of sodium thiopenthal (100 mg/kg, Sigma, St. Louis, MO, USA). Twenty minutes later, the anesthetized rats were sacrificed and frontal cortex tissues were removed for histological investigation.

Histopathological procedures

Biopsies from the frontal cortex tissues of the rats were harvested and tissue fragments were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned at 5 μm thickness and then, stained with hematoxylin and eosin (H&E). The preparations were evaluated by means of a bright-field microscope and photographed (Optiphot 2; Nikon, Tokyo, Japan).

Immunohistochemical procedures

Harvested frontal cortex tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm thickness. Immunocytochemical reactions were performed according to the ABC technique described by Hsu et al. (1981). The procedure involved the following steps: (1) endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 30 min; (2) the sections were washed in distilled water for 10 min; (3) nonspecific binding of antibodies was blocked by incubation with normal goat serum (DAKO × 0907, Carpinteria, CA) with PBS, diluted 1:4; (4) the sections were incubated with specific a rabbit polyclonal anti-caspase-3 antibody (Cat. # RB-1197-P, Neomarkers, USA), diluted 1: 50 for 1 h, and then kept at room temperature; (5) the sections were washed in PBS for 3 × 3 min; (6) the sections were incubated with ABC complex (DAKO LSAB 2 Kit) for 20 min; (7) the sections were washed in PBS for 3×3 min; (8) peroxidase was detected with an aminoethylcarbazole substrate kit (AEC kit; Zymed Laboratories); (9) the sections were washed in tap water for 10 min and then dehydrated; (10) the nuclei were stained with hematoxylin; and (11) the sections were mounted in DAKO paramount. All dilutions and thorough washes between steps were performed using phosphate buffered saline unless otherwise specified. All steps were carried out at room temperature unless otherwise specified. As a negative control, primary antibody was replaced with PBS.

Electron microscopy

For electron microscopy, frontal cortex tissue specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for three hours at 4°C, washed in the same buffer for one hour at 4°C, and post-fixed with 1% osmium tetraoxide in sodium phosphate buffer for one hour at 4°C. The tissues were then dehydrated in graded series of ethanol starting at 50% each step for 10 min and, after two changes in propylene oxide. The tissue specimens were embedded in araldite. Ultrathin sections were prepared with Mg-uranyl acetate and lead citrate for the electron microscopic (Jeol JEM 1010) evaluation.

Microscopic examination

Histological specimens of the frontal cortex were examined under light microscopy, with the examination carried out at a magnification of 400 and the counts of neurons determined per square millimeter with the use of a standardized ocular grid. Apoptotic cells (TUNEL positive neurons) were counted. The distribution of neurons was examined in the sections from the specimens were subjected by using terminal dUTP nick end-labeling (TUNEL). Tissue sections were examined under light microscopy (400×) and the number of the neurons counted within random high-power fields using a Nikon Optiphot 2 light microscope incorporating a square graticule in the eyepiece (eyepiece ×10, objective ×40, a total side length of 0.25 μm^2). Neuron density was assessed by counting the number of cells in 400 high power fields amongst the frontal cortex tissue preparations of each group. The neuron density in each site was calculated and recorded as the number of neurons/μm^2. The tissue compartments were used to record neuron distribution in the frontal cortex tissues.

Evaluation of apoptosis

Apoptosis was evaluated by the TUNEL assay. The TUNEL method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (TdT-Fragel™ DNA Fragmentation Detection Kit, Cat. No. QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer's instructions. Five-μm-thick frontal cortex tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series as described previously. They were then incubated with 20 mg/ml proteinase K for 20 min and rinsed in TBS. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10–30 min and then TdT-enzyme, in a humidified atmosphere at 37°C, for 90 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 min and incubated with blocking buffer for 30 min. Each step was separated by thorough washes in TBS. Labelling was revealed using DAB, counter staining was performed using Methyl green, and sections were dehydrated, cleared and mounted.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). All data were presented in mean (±) standard deviations (SD). Differences in measured parameters among the three groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann–Whitney *U*-test. These differences were considered significant when probability was less than 0.05.

Results

The initial study consisted of 13 rats in the toluene-treated group. Three animals died in the toluene-treated group during the experiment and the rest of rats had loss of righting reflex and psychomotor impairment.

Histopathological and ultrastructural findings

In the control group, the morphology of neurons in the frontal cortex tissues was normal (Fig. 1a). In the toluene treated group, the most consistent findings occurring in the histological tissue sections stained with H&E were those indicating severe

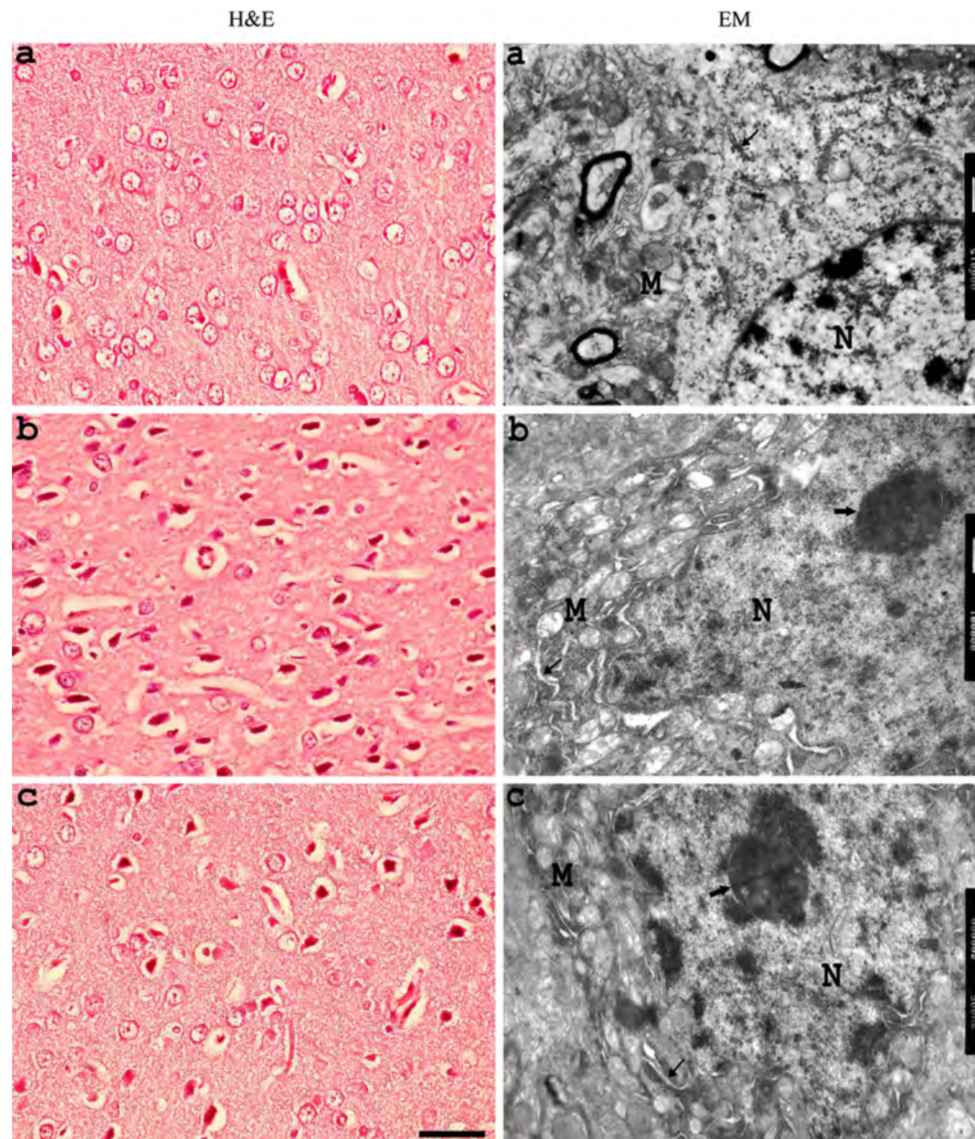

Fig. 1 Representative light microphotographs showing the morphology (**a**–**c**) of the frontal cortex tissue after chronic toluene exposure by hematoxylin-eosin. **a** Control rats: Normal frontal cortex tissue histology is seen. **b** Toluene treated rats: Severe degenerative changes, shrinkage cytoplasma and extensively dark picnotic nuclei in neurons of the frontal cortex tissue are seen. **c** Toluene treated rats with TQ: Effective preventing of degenerative changes and shrunken in cytoplasma and nuclei are prominent in neuronal cells (H&E, *scale bar* 50 μm). Representative transmission electron microphotographs showing the ultrastructure of neurons in the frontal cortex tissues after chronic toluene exposure (**a**–**c**). **a** Control rats: The ultrastructure of cytoplasma and cytoplasmic organelles showed a normal appearance. **b** Toluene treated rats: disintegrating neuron containing shrunken cytoplasma, severely dilated cisternae of endoplasmic reticulum and markedly swollen mitochondria resulting in loss of matrix density in varying stages of destruction were observed. Typical necrotic neurons had smaller clumps of irregularly shaped, condensed chromatin with nuclear membrane breakdown. **c** Toluene treated rats with TQ: Although TQ treatment was effective preventing the above mentioned findings, slightly dilated cisternae of endoplasmic reticulum was still observed. (*thick arrow*: nucleolus, *N*: nucleus, *thin arrow*: cisternae of endoplasmic reticulum, *M*: mitochondria, *Scale bars* 500 nm)

degenerative changes, shrunken cytoplasma and extensively dark picnotic nuclei in neurons of the frontal cortex tissues (Fig. 1b). The dark stained nucleus and the nerve cells showing the pathologic changes were reduced in the TQ -treated rats. In these groups, the severity of degenerative changes in the cytoplasma and especially in the nuclei of cells was less than that in the only toluene treated group (Fig. 1c).

In the control group, the ultrastructure of neurons in the frontal cortex tissues was normal (Fig. 1a). In the toluene treated group, the most consistent findings occurring in the ultrathin sections stained with Mg–uranyl acetate and lead citrate were those indicating severe degenerative changes, shrunken cytoplasma, severely dilated cisternae of endoplasmic reticulum and markedly swollen mitochondria resulting in loss of matrix density with degenerated cristae were observed in neurons of the frontal cortex. The nucleus of these cells seem to have increased evidence of nuclear membrane breakdown with chromatin disorganization and severely shrunken appearance, all signs of advanced cell death (Fig. 1b). Although TQ treatment was effective in preventing the dilatation of endoplasmic reticulum, mitochondrial degeneration and irregularly shaped nuclei, the irregularly shaped chromatin clumps and moderately mitochondrial swollen was still observed in neurons (Fig. 1c).

Immunohistochemical findings

Mild caspase-3 immunoreactivity was observed in the cytoplasm of neurons in control rats (Fig. 2a). The micrographs showed apoptotic neurons by caspase-3 immunohistochemistry after chronic toluene exposure in rats. The caspase 3 immunopositivity was increased in degenerating neurons of the frontal cortex tissues following toluene exposure (Fig. 2b). Treatment of TQ markedly reduced the immunoreactivity of degenerating neurons after chronic toluene exposure (Fig. 2c).

TUNEL findings

The amount of neuronal cells apoptosis was quantitatively higher in the toluene treated group (Fig. 2b) than the control group (Fig. 2a). Treatment of TQ markedly reduced the reactivity and the number of apoptotic cells (Fig. 2c).

On the other hand, the number of apoptotic neurons were increased significantly in toluene treated rats compared to control ($P < 0.0001$) and toluene treated with TQ ($P < 0.01$) rats' frontal cortex tissues (Table 1).

Discussion

Despite the widespread abuse of toluene and other solvents, the molecular sites of action of these compounds are largely unknown (Balster 1998). More is known about the behavioral effects of these substances: toluene and other abused solvents produce effects similar to that of CNS depressants. These effects include psychomotor impairment (Moser and Balster 1986), excitation and then inhibition of locomotor activity (Warren et al. 2000), and loss of righting reflex and sedation (Tegeris and Balster 1994). In addition, peripheral nerve dysfunction has been reported (Echeverria et al. 1991). Mattia et al. (1993) demonstrated that intraperitoneal injection of toluene caused a significant elevation in the rate of reactive oxygen species (ROS) generation and a reduction in glutathione (GSH) levels in the brain. Long-term toluene abuse has led to neuropsychiatric and neurobehavioral disorders, which in many cases, but not all, were reversible. Some chronic toluene abusers have developed structural CNS damage (Zabedah et al. 2001; Greenberg 1997). Volatile substances containing toluene are frequently abused by young people, thus leading to neurological damage. In fact, glue sniffing is frequent among secondary school children and young adults because it is more easily available and cheaper than other drugs (Zabedah et al. 2001).

Acute and chronic effects of toluene on neurons have been well documented (Burmistrov et al. 2001). In addition, toluene inhalation was found to enhance astrocyte activation (Gotohda et al. 2000). Astrocytes are known to play an important role in survival of neurons in the brain and they have been implicated in the regulation of ionic environments which are required for proper physiological function of neurons (Moonen et al. 1990). Glial cells exhibit early cellular responses following a variety of insults to the CNS (Papadopoulos et al. 1999). Reactive gliosis is a reaction of astrocytes to neuronal damage resulting from physical or chemical insults.

Pathological studies of the effects of toluene inhalation based on biopsies of the sural nerve have shown swelling of the axons and an extremely thin lamella of the myelin sheath (Altenkirch et al. 1977). Coskun et al. (2006) suggested that increased lipid peroxidation, reduced antioxidant enzyme activities, and ultrastructural changes found in their study indicates that chronic toluene inhalation might be involved free radical processes. In the present study, chronic toluene exposure caused severe degenerative changes, shrunken cytoplasma, slightly dilated cisternae of endoplasmic reticulum, markedly swollen mitochondria with degenerated cristae and nuclear membrane breakdown with chromatin disorganization in neurons of the frontal cortex. These findings are in agreement with the results of other investigators on toluene -induced neurotoxicity in different animals (Altenkirch et al. 1977; Coskun et al. 2006; Saavedra et al. 1996; Korbo et al. 1996).

To date, no histopathological changes neurodegeneration in the frontal cortex after chronic toluene exposure in

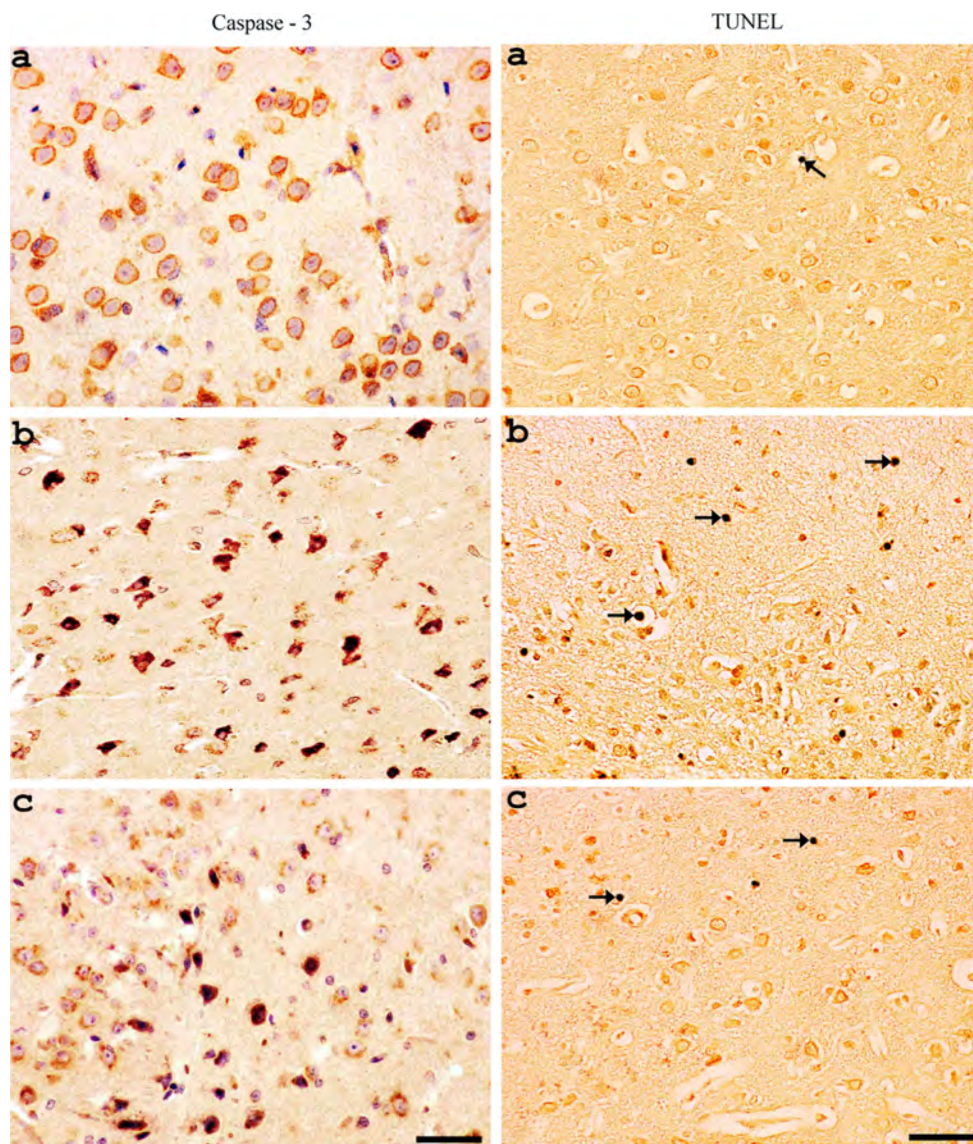

Fig. 2 Representative light microphotographs showing the apoptosis of the frontal cortex tissue after chronic toluene exposure by caspase-3 and TUNEL methods. Caspase-3. **a** Control rats: neuronal cells are mildly stain with the anti-caspase-3 antibody. **b** Toluene treated rats: The caspase-3 immunopositivity was strongly increased in degenerating neurons of the frontal cortex tissue after chronic toluene exposure. **c** Toluene treated rats with TQ: Treatment of TQ markedly reduced the immunoreactivity of degenerating neurons after toluene exposure. TUNEL. **a** Control rats: a few TUNEL-positive cells are observed in the frontal cortex tissue. **b** After toluene inhalation, positivity is mainly observed in neuronal cells of the frontal cortex tissue. **c** Toluene treated rats with TQ: TQ treatment labelled neuronal cells are rarely observed in the frontal cortex tissue. (*Arrow*: TUNEL positive neuronal cells) (Immunoperoxidase, haematoxylin counter-stain, TUNEL *scale bar* 50 μm)

rats by TQ treatment have been reported. Recent studies (Kanter 2008a) have attempted to identify the *Nigella sativa* has some neuroprotective and restorative effects on secondary pathochemical events after frontal cortex and brain stem injury in rats. These restorative effects mainly observed on oxidative stress and neuronal numbers and neuronal morphology.

In our previous report (Kanter 2008b), we concluded that Thymoquinone and *Nigella sativa* therapy causes morphologic improvement on neurodegeneration in hippocampus after chronic toluene exposure in rats. In the present study, in the TQ-treated rats' frontal cortex, the severity of degenerative changes in the cytoplasma and especially in the nuclei of cells was less than that in the only toluene treated group. The dark stained nucleus and the nerve cells showing the pathologic changes were reduced in the TQ -treated rats.

Apoptosis involves interactions among several protein families that regulate activation of proteolytic caspases. Although multiple pathways can induce apoptosis, the

Table 1 The numbers (number/μm^2) of apoptotic neurons (TUNEL positive neurons) in the frontal cortex tissues of A (control), B (toluene treated) and C (toluene treated with TQ) groups

Groups	Frontal cortex tissue
A	4.6 ± 0.3
B	56.7 ± 5.7[a]
C	32.3 ± 2.8[b]

Kruskal-Wallis test was used for statistical analysis. Values are expressed as means ± SD, $n = 10$ for each group

[a] $P < 0.0001$ compared to A group

[b] $P < 0.001$ compared to A group

[b] $P < 0.01$ compared to B group

mitochondrial pathway has most frequently been implicated in CNS apoptosis (Yuan and Yankner 2000). In our previous study, Kanter et al. (2010) suggest that L-carnitine may be beneficial to spermatogenesis and infertility following testicular irradiation by decreasing germ cell apoptosis. In our another study, there was a clear increase in the number of TUNEL positive apoptotic cells in the bile duct ligation treated rats in the liver parenchyma. Treatment of quercetine markedly reduced the reactivity and the number of TUNEL positive apoptotic cells (Kanter 2010).

Studies have shown that in rats toluene just like ethanol, has toxic effects on the brain development as well as disturbances in the cognitive functions (Hass et al. 1999). Previously it has been shown that toluene affects the apoptotic neurodegeneration in the cerebellar granular cell layer after prenatal exposure (Dalgaard et al. 2001). For hippocampus on postnatal day 6, the caspase-3 activity was higher in the toluene group compared to the control group (Ladefoged et al. 2004). In this study, likewise, in our previous study (Kanter 2008a), mild caspase-3 immunoreactivity was observed in the cytoplasm of neurons in control rats, whereas the caspase 3 immunopositivity was increased especially in neurons of the frontal cortex and brain stem following toluene exposure. Treatment of TQ markedly reduced the immunoreactivity of degenerating neurons after chronic toluene exposure.

A marked increase in number of apoptotic cells was observed in cerebellar granule cells at postnatal day (PND) 21 compared with the other age groups. Toluene induced a statistically significant increase in the number of apoptotic cells in the cerebellar granule layer at PND 21. The mean was increased from 37 in the control group to 71 in the toluene-exposed group. Thus, the granular cell layer in cerebellum is a highly relevant tissue with which to study toluene-induced apoptosis, because of the continuous migration of neurons and high frequency of neuronal apoptosis during the weaning period (Dalgaard et al. 2001). In the present study, the number of the apoptotic (TUNEL positive neurons) neurons was increased significantly in toluene treated rats compared to control and toluene treated with TQ rats' frontal cortex tissues.

We conclude that TQ therapy causes morphologic improvement on neurodegeneration in the frontal cortex tissues after chronic toluene exposure in rats. We believe that further preclinical research into the utility of TQ may indicate its usefulness as a potential treatment on neurodegeneration after chronic toluene exposure in rats.

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