WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 12, 309-356. Research Article ISSN 2277– 7105

NEUROBEHAVIORAL AND NEUROCHEMICAL CHANGES IN TOLUENE-TREATED RATS AND THE EFFECT OF ANTIOXIDANTS

Mariam M Atef[1], Asmaa F Galal[1*], Omar M.E. Abdel-Salam[1], Nermeen Shafee[3], Mariane G. Tadros [2], Amani E. Khalifa[2]

[1]Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt.

[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

[3]Department of Pathology, National Research Centre, Cairo, Egypt.

Article Received on
02 Oct 2015,

Revised on 27 Oct 2015,
Accepted on 20 Nov 2015

***Correspondence for Author**
Dr. Asmaa F Galal
Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt.

ABSTRACT

The present study aimed to investigate effects of repeated toluene administration on behavior, brain oxidative stress, dopamine, and neuronal damage in rats. The possibility of recovery after toluene abstinence was studied. Moreover, the role of antioxidant treatment with vitamin E and/or vitamin C was evaluated. Toluene was intraperitoneally (i.p.) administered once a day for 9 consecutive days at doses of 141.4, 282.8, 565.6 and 1131.2 mg/kg. Recovery was investigated using the highest dose (1131.2 mg/kg), two, four and eight weeks after cessation of toluene. To study the protective effects of antioxidants, vitamin E (α-tocopherol) (50 mg/kg, i.p.) and/or vitamin C (ascorbic acid) (50 mg/kg, i.p.) was co administered with toluene (565.6 mg/kg). Behavioral testing (grip strength, open field, and defensive aggression, water maze, forced swimming), was conducted in addition to measuring the lipid peroxidation marker malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO), acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and paroxonase-1 (PON-1) in brain homogenates. Striatal dopamine was also measured.Caspase-3, and tumor necrosis factor alpha (TNF-α) were evaluated using immunohistochemistry. Results indicated that toluene impaired motor function, and memory and produced depressant-like action in a dose-dependant manner, dose dependently. Significant increases occurred in brain levels of MDA, and NO levels accompanied by a marked decrease in GSH level compared to control group**.** Toluene also resulted in marked reduction in butyryl cholinesterase (BuChE),

acetylcholinesterase (AChE), and PON-1 activities. Toluene in addition resulted in markedly elevated dopamine level in the striatum. No significant recovery after 2 weeks while after 1, and 2 months, there was significant improvement in behavioral measures and biochemical changes compared to the control group. The concurrent administration of α-tocopherol and/or ascorbic acid conferred significant protection against deleterious effects of toluene, with protection being most effective with the combined administration of both antioxidants. These results indicate that: (1) short-term toluene administration results in motor and cognitive impairment accompanied by oxidative damage, neuroinflammation and apoptosis; (2) most of these changes are improved after abstinence from toluene; (3) Toluene's effects respond to antioxidant therapy with a combination of α-tocopherol and ascorbic acid, suggesting that oxidative stress is a major contributing event in this rat model of toluene neurotoxicity.

KEYWORDS: toluene; brain; behavioral; oxidative stress; α-tocopherol; ascorbic acid.

INTRODUCTION

The practice of glue inhalation is a growing social and health problem worldwide with increasing incidence in developing countries.[1][2] In Egypt, the problem is often underappreciated although it is associated with tremendous morbidity and mortality. According to ElKoussi and Bakheet (2011)[3], about 90.8 % of street children in unprivileged Upper Egypt admitted to inhale local household glue called 'Kolla' without any awareness of its detrimental health hazards. Toluene, a major component in glue and paint thinner, is known to exert severe impact on the central nervous system (CNS) resulting in numerous functional and structural impairments that could lead to devastating neurotoxicity and death in chronic abusers.[4] Toluene abusers are prone to neurobehavioral changes that start as anxiety, mood swings and irritability. With increasing the dose, abusers may develop coarse tremors, hearing impairment, nystagmus and ataxic gait.[5][6][7] However, mechanism underlying the neurotoxic effects of toluene is still not clear. In recent years, oxidative stress has emerged as a major causative factor in etiology of neurodegenerative diseases, including Alzheimer's, Parkinson's disease, and stroke.[8][9] Few reports have indicated that toluene can produce excess reactive oxygen species (ROS) in the CNS, both in vivo and in vitro.[10][11][12][13] There is a clear need to perform a comprehensive study addressing the effects of different doses of toluene, commonly encountered in toluene abuse, on neurobehavioral measures in relation to oxidative stress biomarkers. Besides, little is known about possible persistent structural damage to CNS that could be manifested as changes in

behavior and neurological function.[14] To extend knowledge in this area, it was imperative to evaluate a model of toluene-induced neurotoxicity and to develop an effective therapeutic approach. Therefore, it deemed necessary to establish dose-response curve of toluene effects. In the present study, a battery of behavioral tests was employed along with oxidative stress parameters that may mediate neurobehavioral effects of toluene. Previous studies demonstrated that antioxidant nutrients have important roles in cell function and have been implicated in processes associated with aging, including vascular, inflammatory and neurological damage.[15] The protective effect of vitamin E and C against cognitive decline and neurodegenerative diseases has been explored in several epidemiological and clinical studies.[16] Therefore, antioxidant therapy may be an important strategy for managing toluene-induced neurotoxicity. The present study was designed to further characterize changes in the effects of repeated exposure of toluene on a variety of behavioral tests. Moreover, we aimed to test whether oxidative stress plays a role in neurotoxicity caused by toluene. The study was further extended to evaluate the possibility of recovery after toluene withdrawal as well as the protection ability of treatment with vitamin E and/or vitamin C.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats, weighing 120-130g were used. Animals were obtained from the breeding colony maintained at the animal house of National Research Center (NRC, Cairo, Egypt). All animals were housed under conventional laboratory conditions throughout the period of experimentation at room temperature 25 ± 2°C, 60-70 % humidity and 12 h light/ dark cycle and fed standard laboratory pellets (20 % proteins, 5 % fats, 1 % multivitamins) and allowed free access to tap water. Animals were allowed at least one week of acclimatization before using them. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals. Equal group of 9 rats per each group were used in the study.

Chemicals

The following drugs were used: toluene (purity>99.5%) (Sigma, St. Louis, MO, USA), α-tocopherol (vitamin E) (Memphis co. Cairo, Egypt) and ascorbic acid (vitamin C) (Memphis co. Cairo, Egypt). Toluene was freshly prepared in paraffin oil to obtain the required doses.

Other chemicals were of analytical grade and were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Study design

The first set of experiments was designed to investigate the effect of intrapreitoneally injected toluene on behavior, brain oxidative stress, brain dopamine, and neuronal damage. Rats were treated with either vehicle (control) (group 1) or toluene in different doses of 141.4, 282.8, 565.6 and 1131.2 mg/kg (0.325 ml/kg, 0.65 ml/kg, 1.3 ml/kg, and 2.6 ml/kg) (groups 2-5) intrapreitoneally daily for 9 days. Rats were then subjected to behavioral testing (wire hanging test, forced swimming test, water maze test). Rats were sacrificed on the 10th day; brains were then dissected out and stored at -80^{0}C for further neurochemical measurements.

In the second set of experiments designed to evaluate the possibility of recovery after toluene withdrawal, rats were injected with toluene in 1131.2 mg/kg daily for 9 days, and then left for recovery. Thus, three different groups of rats received toluene intraperitoneally at 1131.2 mg/kg daily for 9 days and euthanized at 2, 4 and 8 weeks following the last dose of toluene. Brains were then dissected out and stored at -80^{0}C for further biochemical neurochemical measurements. Behavioral testing was performed on the 10th day of toluene administration (basal measurement) and 2, 4 and 8 weeks after stopping toluene (wire hanging test, forced swimming test, water maze test, open field test, defensive aggression test). The third set of experiments was designed to investigate the protective ability of treatment with the antioxidants ascorbic acid and α-tocopherol. Thus, different groups of rats received toluene intraperitoneally in 565.6 mg/kg daily for one week alone (control) or combined with either ascorbic acid (50 mg/kg, i.p.) (group 2), α-tocopherol (50 mg/kg, i.p.) (group 3) or both ascorbic acid and α-tocopherol (group 4). Treatments were continued for 9 days, rats were then euthanized on the 10th day, and their brains were then dissected out and stored at -80^{0}C for further biochemical and neurochemical measurements. Behavioral testing was performed on the 10th day of toluene administration (wire hanging test, forced swimming test, water maze test, open field test, defensive aggression test).

Behavioral testing

Grip strength test

To assess the muscular rigidity and grip strength, each rat was suspended by its forelimb on a metal rod about 30 cm into air which they immediately grasp with forepaws. The rat is

released to hang on with its forelimbs. Normal animals are able to catch the threat with the hind limbs, and to climb up with 5 seconds.[17]

Morris Water maze test

The water maze consists of a circular tank with 100 cm diameter and a wall 20 cm above the water level. A circular platform is hidden 2 cm below the water level. The water is made opaque using fuller earth, and is kept at about 23ºC during the experiment. Each session is consisted of three trials. The latency to find the platform is recorded as the time of placement of the rat in the water to the time it finds the platform.[18]

Prosolt's despair swim test

Rats were placed in the plastic cylinder in which there is no escape; for the first time are initially highly active, vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2-3 min activity begins to subside and to be interspersed with phases of immobility or floating of increasing length. After 5-6 min immobility reaches a plateau where the rats remain immobile for approximately 80% of the time. An animal is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position, its nose just above the surface.[19]

Open field test

The open field test was carried out in a square wooden arena (80cm x 80 cm x 40cm) with red walls, and white smooth polished floor divided by black lines into 16 equal squares (Pruus et al., 2002). The test was performed under white light in a quiet room. Each rat placed at the same corner Square, and observed during5 minute period. Each line crossed is scored as one unit of activity. Ambulation, rearing, and grooming were measured.[20]

Defensive aggression test

The rat lifted by its tail, and placed in a Plexiglas cage (60 x 31 x 41 cm high), and allowed to habituate for 30 seconds. The rat's reaction to five different stimuli was then assessed: A wooden rod moving slowly, Startle to an air puff, Poking with wooden rod at the flanks, Capturing with a gloved hand, urination, and defecation, and vocalization. The average score from each individual stimuli test was used in the statistical calculation.[21]

Biochemical assays

Lipid peroxidation: Brain malondialdehyde (MDA) content was determined according to the method of Uchiyama, and Mihara, (1978). In this assay, one molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) in acid medium (pH 2-3) at high temperature. The produced pink color is extracted by n-butanol, and then its optical density is determined at 532nm.

Reduced glutathione (GSH)

Brain GSH content was determined according to the method of Ellman (1959).The method is based on the reduction of Ellman's reagent (5, 5'- dithiobis (2-nitrobenzoic acid)) by SH group to form 1 mole of 2-nitro-5- mercaptobenzoic acid per mole of SH. The reaction product, 2-nitro-5- mercaptobenzoic acid, has an intense yellow color, and can be determined colorimetrically at 412 nm.

Nitric oxide (NO)

NO was determined as nitrite using Griess reagent, where the nitrite end product is used to determine nitric oxide production (Miranda et al., 2001).

Paraoxonase-1 (PON-1)

Arylesterase activity of paraoxonase was measured spectrophotometrically in brain supernatants using phenylacetate as a substrate (Higashino et al., 1972; Watson et al., 1995). In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol is measured by monitoring the increase in absorbance at 270 nm at 25ºC. The activity is expressed in kU/l.

Acetylcholinesterase activity

The procedure used for the determination of cholinesterase activity in the serum was a modification of the method of Ellman et al, (1961) as described by Gorun et al. (1978). The assay is based on measurement of thiocholine produced when acetylcholine is hydrolyzed; the color was read immediately at 412 nm.

Butrylcholinesterase activity

Cholinesterase activity in brain supernatant was determined by colorimetric method using butrylcholinesterase diagnostic kit (Biodiagnostic co., Egypt) according to the method of Knedel and Bottger, (1967).

Dopamine

Determination of striatal dopamine (DA) was carried out using HPLC. The separation of samples was performed on AQUA column C18 (150X4.6mm I.D., 5µm) Phenomenex, USA. Samples were then injected in the HPLC system under the following conditions: mobile phase 97/3 20 mM potassium phosphate buffer pH 3.0/methanol (v/v), at flow rate of 1.0 ml/min. The detection wave length was 270 nm and the injection volume was 40 µl (Pagel et al., 2000).

Histopathological examination

Brain sections were fixed in freshly prepared 10% neutral buffered formalin embedded in paraffin. Paraffin sections of five µm thick were prepared and stained with hematoxylin, and eosin (H&E) for histopathological examination. Sections were examined using a light microscope Olympus BH2 (Tokyo, Japan).

Immunohistochemistry

Immunohistochemical staining of anti-caspase 3, and TNF alpha (TNF-α) was performed with streptavidin-biotin. Sections of 4µm thickness were deparaffinized, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30min at room temperature. The specimens were then incubated with anti-caspase3, and TNFα antibody as the primer antibody at 1:100 dilutions. The specimens were counter stained with H&E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

Statistical analysis

Data were expressed as mean ± SEM values. Statistical analysis was conducted using one-way ANOVA test, followed by Tukey–HSD test for multiple comparisons.

RESULTS

Study I. Behavioral and biochemical alterations after repeated toluene exposure

Behavioral results

Wire hanging test

The repeated administration of toluene for 9 days decreased the latency to fall in the wire hanging test in a dose-dependent manner, indicating decreased grip strength by the toxicant. Thus the latency to fall decreased by 39.2, 68.3, 76.7 and 86.3% by toluene administered at doses of 141.4, 282.8, 565.6 and 1131.2 mg/kg, respectively (Fig. 1A).

Porsolt's forced-swimming test

The struggle time was significantly decreased in rats treated with toluene (141.4-1131.2 mg/kg i.p.) dose-dependently, suggesting a depressive-like effect for the toxicant. The percentages decrease in struggle time by the four doses of toluene were 24%, 41.4%, 69% and 78.5% compared with the vehicle control group (Fig. 1B).

Water maze test

The administration of toluene resulted in impaired cognitive performance leading to higher latencies to locate the platform. The effect was a dose-dependent one. Toluene given at 141.4-1131.2 mg/kg, i.p. increased escape latency by 118-649.3% (Fig. 1C).

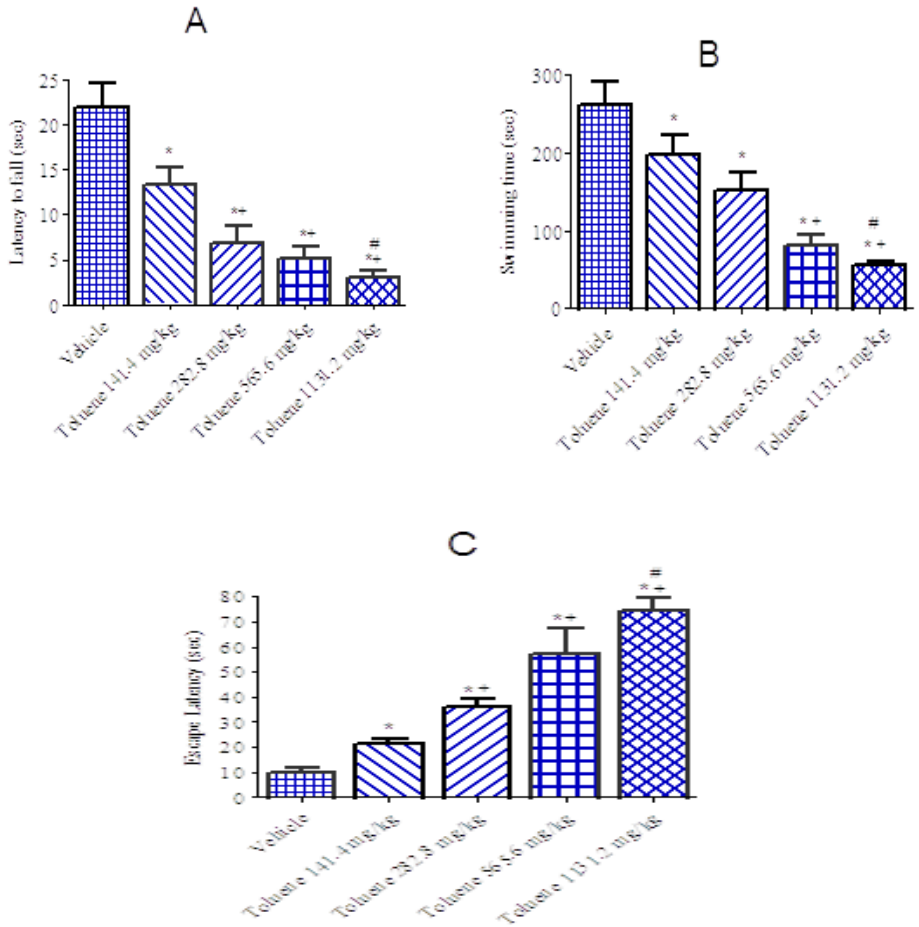


Fig. 1. Effect of 9 days treatment with different doses of toluene on (A) Grip strength test. Bars represent the latency to fall. (B) Forced swimming test. (c) Morris Water Maze test. Bars represent latency to reach the hidden platform. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene 141.4mg/kg. # p<0.05 vs. toluene 565.6mg/kg.

Biochemical results

Malondialdehyde

The administration of toluene (141.4-1131.2 mg/kg) resulted in marked and significant elevation in the level of MDA in the cortex (80.1-632.1%), striatum (80-721.8%) and rest of brain (79.9-608.3%) compared to the vehicle control group (Fig. 2A).

Reduced glutathione

GSH content was decreased, as a result of toluene administration, in cortex, striatum and rest of the brain by a comparable percent (~45-90.3%), as compared to the control group (Fig. 2B).

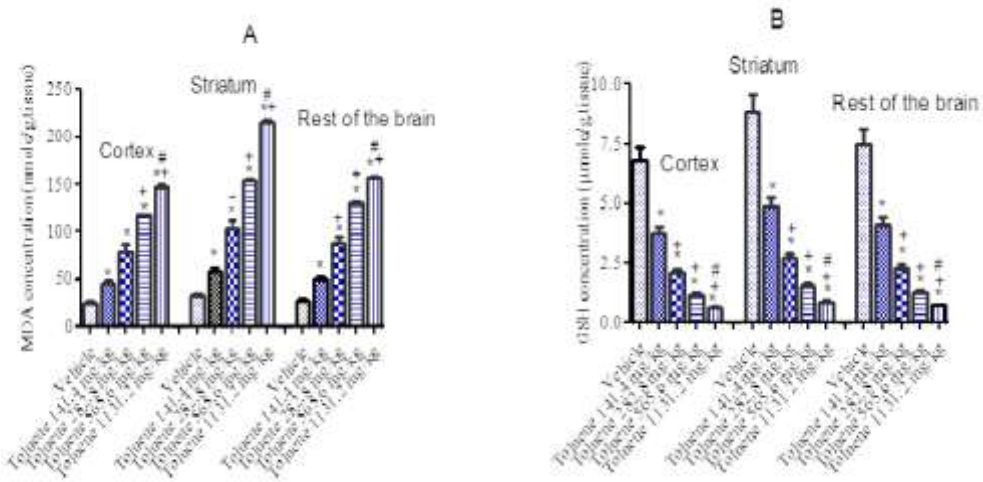


Fig. 2A-B. Brain MDA and GSH. Effect of 9 days treatment with different doses of toluene. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene 141.4 mg/kg. # p<0.05 vs. toluene 565.6mg/kg.

Nitric Oxide

The administration of toluene resulted in marked and significant elevation in the level of NO in the cortex (30.2-100.4%), striatum (-26.7-98.9%) and rest of brain (-33.9-105.5%) compared to the vehicle control group (Fig. 2C).

Paroxonase activity

Toluene injection resulted in significant decrease in PON-1 activity in cortex, striatum and rest of the brain by comparable percentages (-40-90 %) as compared to control group (Fig. 2D).

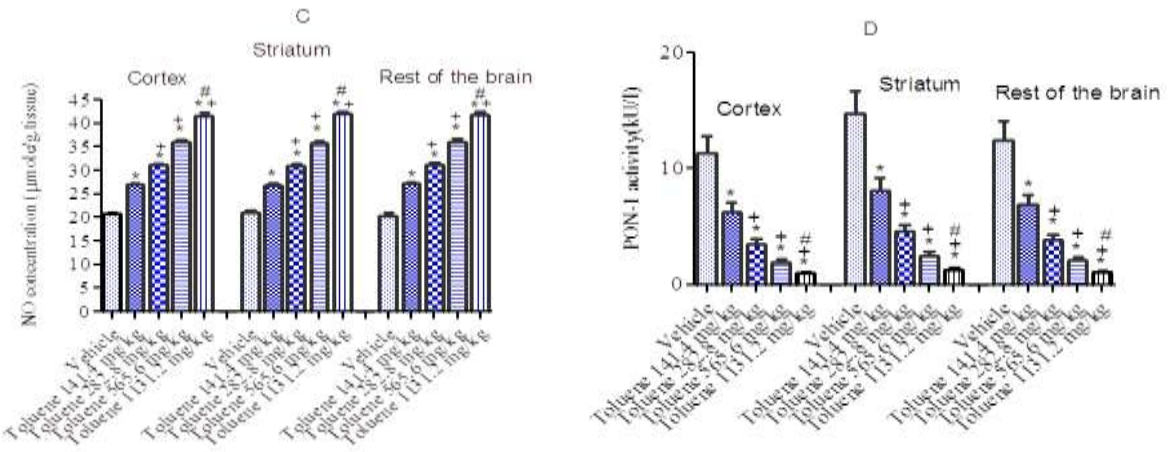


Fig. 2C-D. Brain nitric oxide and PON1 activity. Effect of 9 days treatment with different doses of toluene. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene 141.4 mg/kg. # p<0.05 vs. toluene 565.6mg/kg.

Acetylcholinesterase activity

Toluene injection resulted in significant decrease in AChE activity in cortex, striatum and rest of the brain by comparable percentages (-44.6-91 %) as compared to control group (Fig. 3A).

Butyrylcholinesterase activity

Toluene injection resulted in significant decrease in BuChE activity in cortex (-45.3-91%), striatum (-45-91%) and rest of the brain (-45.1-91%) as compared to corresponding control value (Fig. 3B).

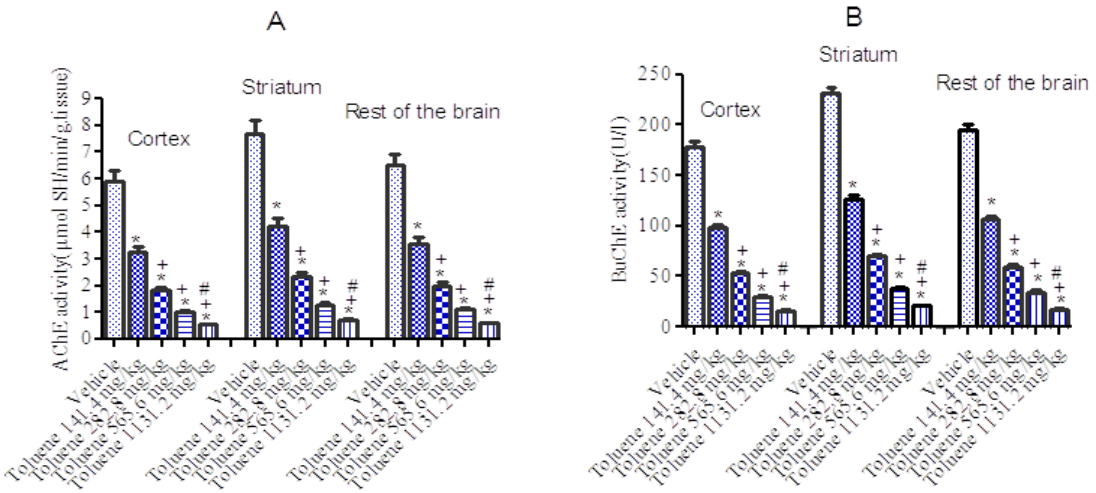


Fig. 3A-B. Brain AChE and BuChE activities. Effect of 9 days treatment with different doses of toluene. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene 141.4 mg/kg. # p<0.05 vs. toluene 565.6mg/kg.

Dopamine

The administration of toluene (565.6 mg/kg) resulted in markedly elevated dopamine level in the striatum by 483.1% (19.05 ± 0.3 vs. 3.27 ± 0.06 µg/g.tissue) compared to the control value (Fig. 4).

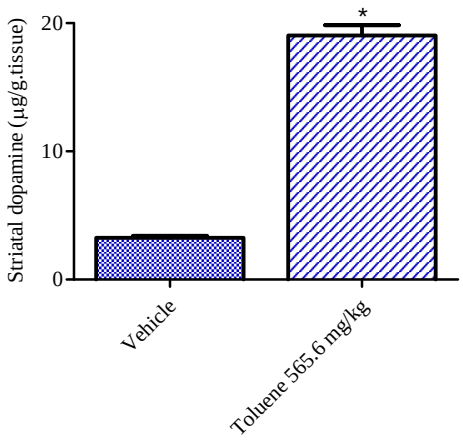
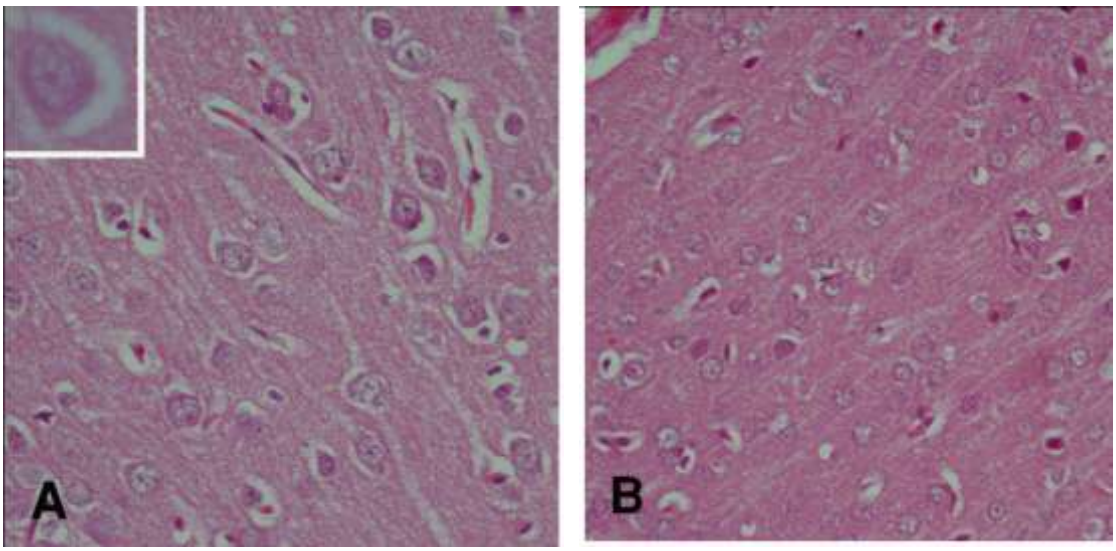


Fig. 4. Striatal dopamine content. Effect of 9 days treatment with toluene at 565.6 mg/kg. *p<0.05 vs. vehicle-treated group.

Histopathological and Immunochemistry results

Histopathological examination of H & E sections of cerebral cortex and hippocampus revealed that i.p. injection of toluene resulted in a dose-dependent damage in brain tissue. Undefined nucleoli, fragmented DNA, as well as and dark neurons were observed indicative of early signs of degeneration (Fig. 5 & 6).



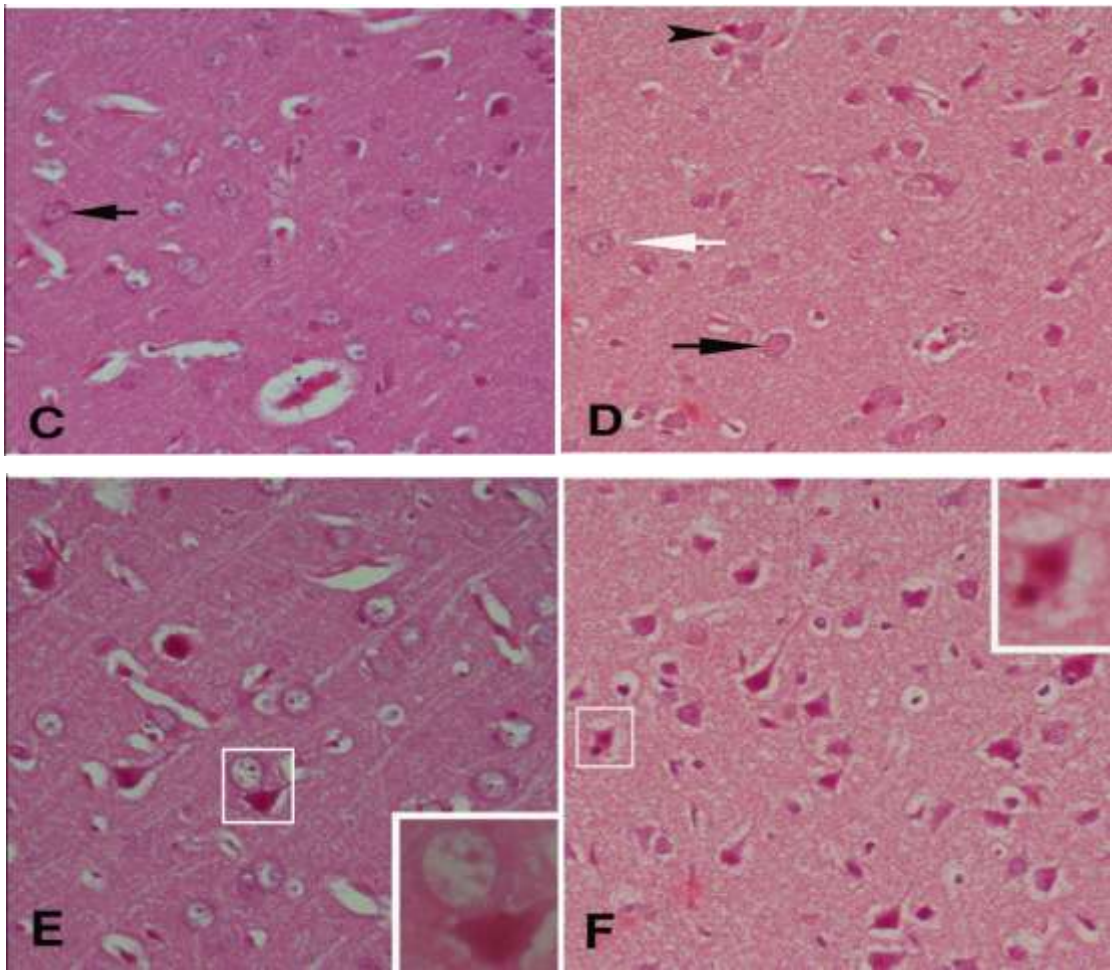


Fig. 5. Representative photomicrographs of sections of cerebral cortex of rats treated with (A): saline showing normal neurons with large vesicular nuclei and well defined nucleoli (as appear in the upper left corner of the figure). (B): vehicle showing the same result as the control group. (C): toluene at 141.4 mg/kg showing some neurons with nuclei darker than normal (arrow). (D): toluene at 282.8mg/kg showing 2 degrees of degeneration in neurons, the first one where neurons appear with nuclei darker than normal (black arrow) and the second one where neurons appear with acidified cytoplasm and small dark nucleus (arrowhead) if compared to normal neurons (black arrow). (E): toluene at 565.6 mg/kg showing neurons with acidified cytoplasm and dark small nuclei become more apparent (as shown in the right lower corner of the figure). (F): toluene at 1131.2 mg/kg showing a greater number of neurons with acidified cytoplasm and small dark nuclei (as shown in the upper left corner of the figure) than that seen in the previous group. (Hx. & E. X 200 & 500 for the highly magnified parts).

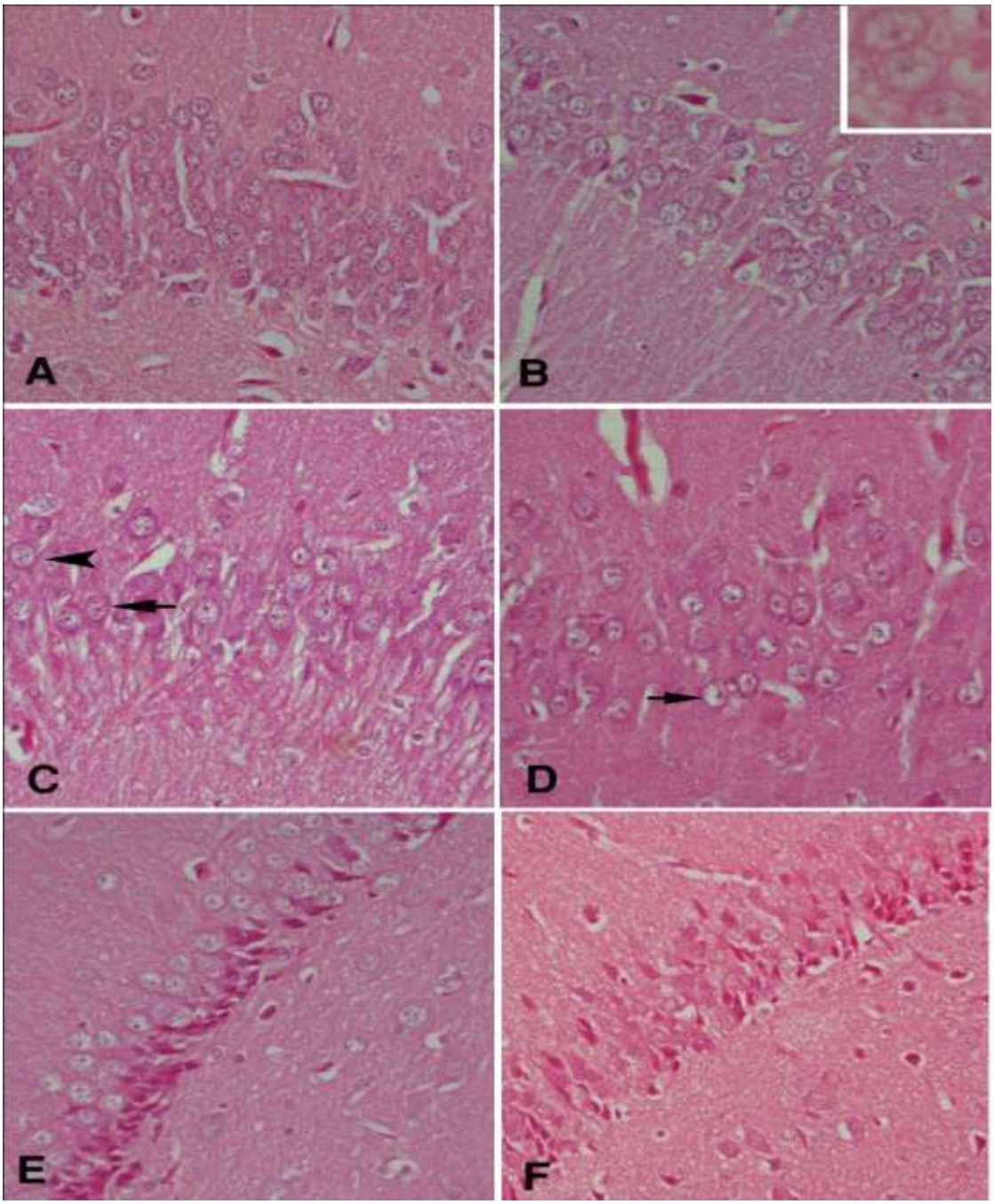


Fig. 6. Representative photomicrograph of sections of hippocampal area of rat treated with (A): saline showing many layers of neurons with normal large vesicular nuclei. (B): vehicle showing the same result as control group, the upper right corner shows normal neurons. (C): 141.4 mg/kg toluene shows a few cells with fragmented DNA and undefined nucleoli (arrowhead), while most of the cells appear normal (arrow). (D): 282.8 mg/kg toluene shows the same result as the previous group. (E): 565.6 mg/kg toluene shows noticeable reduction in thickness of this area with the appearance of some dark neurons. (F): 1131.2 mg/kg toluene shows many neurons with dark nuclei or with undefined nucleoli. (Hx. & E. X 200).

Immunohistochemistry

Caspase-3 expression

In the control group, the expression of the apoptotic marker, caspase-3, in brain was undetectable in the cytoplasm of cortical and hippocampal areas. Caspase-3 immunoreactivity was enhanced in the cytoplasm of rats treated with toluene (Fig. 7 & 8).

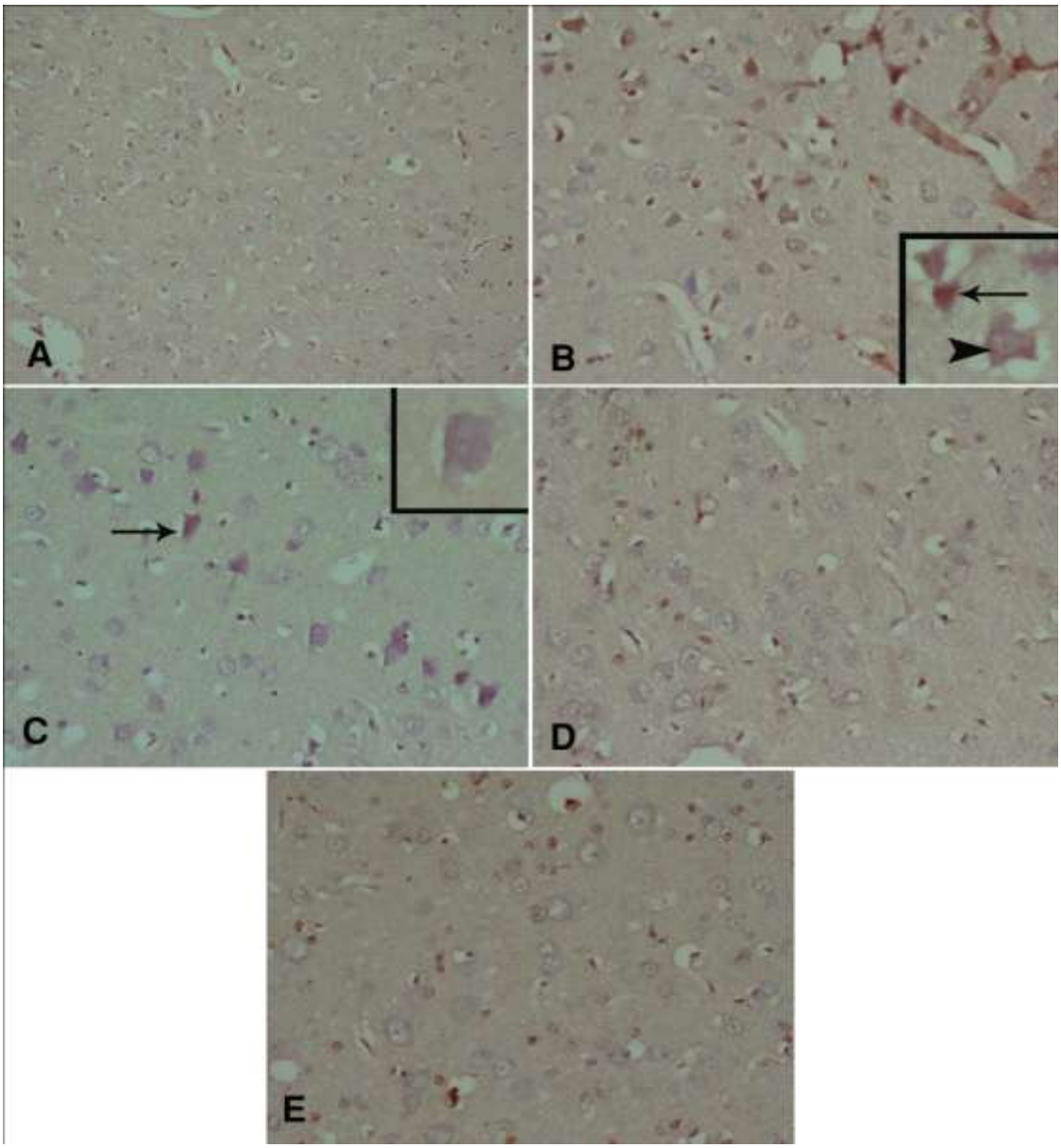


Fig. 7. Representative photomicrographs of scetions of cerebral cortex stained with antibodies against caspase 3 from (A): vehicle-treated rat showing negative result for caspase stain denoting no apoptotic cells are found. (B): 565.6 mg/kg toluene -treated rat showing many cells gave positive result for caspase stain. The lower right corner shows different degree of apoptosis either with darkening of nuclei (arrow) or with normal nuclei (arrow head). (C): 565.6 mg/kg toluene + vitamin C showing some cells that are still giving positive result for caspase stain. (D): 565.6 mg/kg toluene + vitamin

E showing negative result for caspase stain all over the field. (E): 565.6 mg/kg toluene + vitamin C & E showing the same result as the previous group. (caspase x 100 (A) & 200 for the rest).

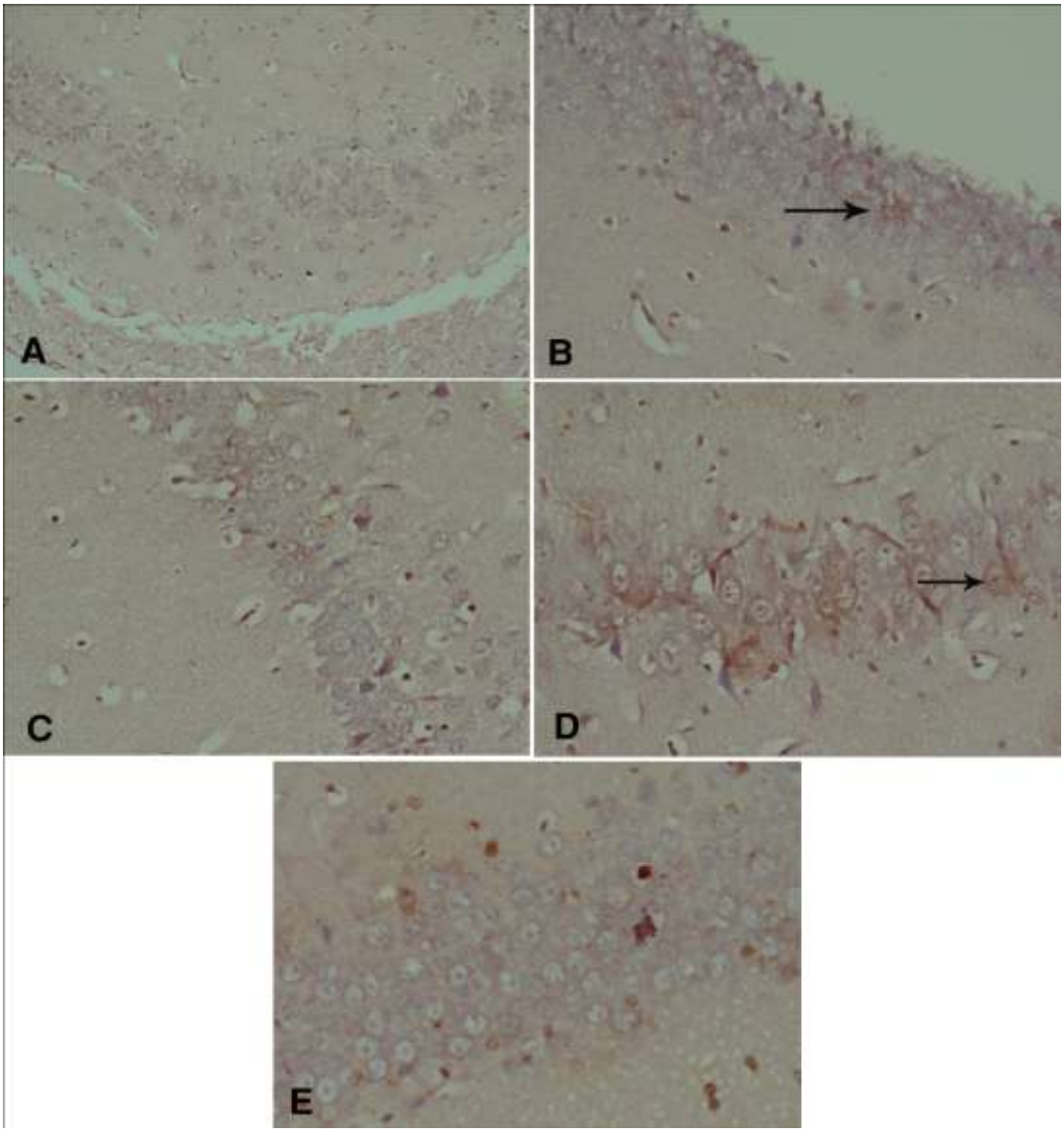


Fig. 8. Representative photomicrographs of scetions of hippocampal area stained with antibodies against caspase 3 from (A): vehicle-treated rat showing no positive result for caspase stain denoting no apoptotic cells are marked. (B): 565.6 mg/kg toluene -treated rat showing some cells giving a positive result for the stain (arrow & at the upper layers). (C): 565.6 mg/kg toluene + vitamin C showing few cells that give a faint positive result for the stain (arrow). (D): 565.6 mg/kg toluene + vitamin E showing negative result for the stain. (E): 565.6 mg/kg toluene + vitamin C & E showing negative result for the stain. (caspase x 100 (A) & 200 for the rest).

TNF-α expression

TNF-α immunoreactivity was rarely observed in the cytoplasm of the control group. An obvious increase in TNF-α immunoreactivity was noted in toluene-treated rats (Fig. 9 & 10).

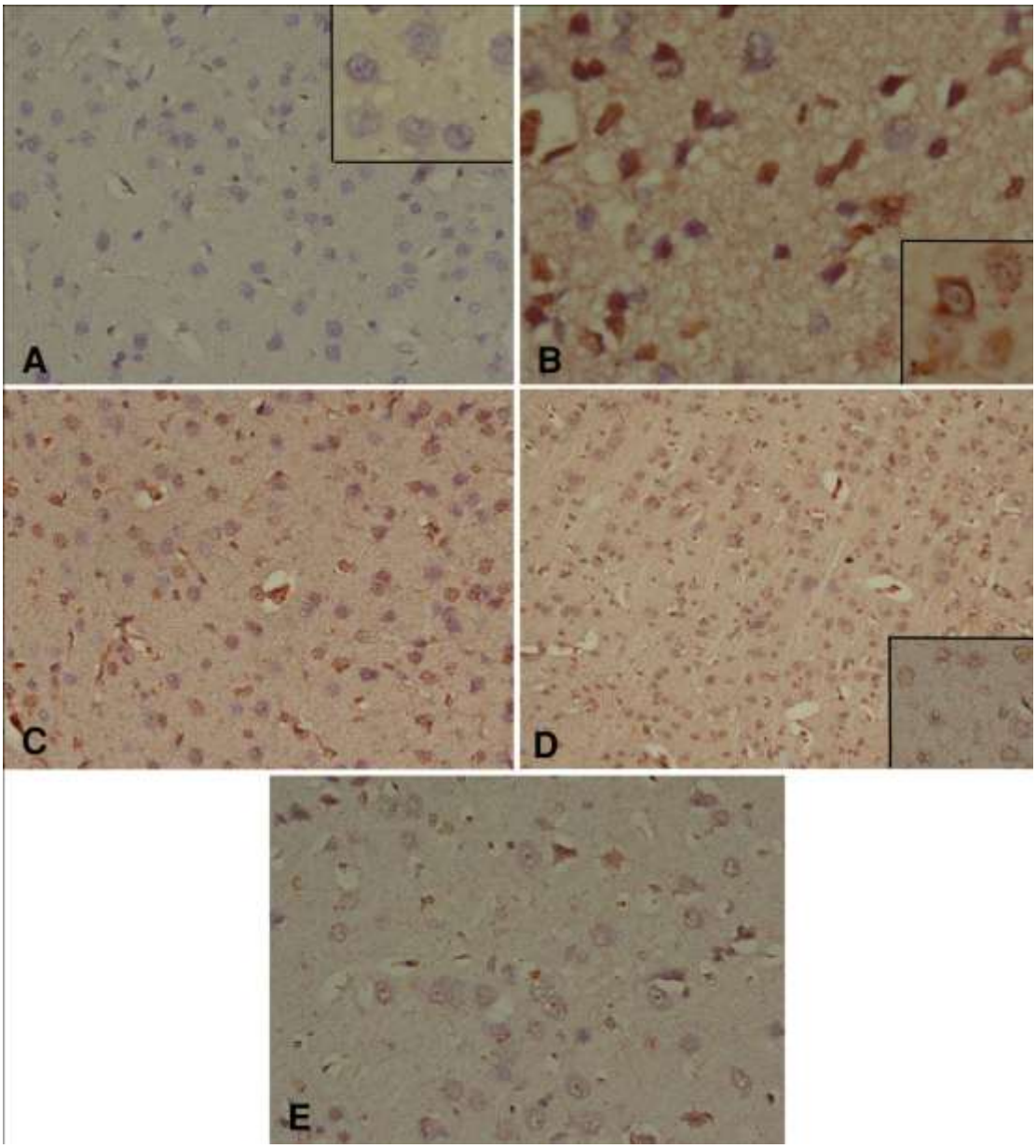


Fig. 9. Representative photomicrographs of scetions of cerebral cortex stained with antibodies against TNF –α (A): of a control rat showing negative result for TNF –α stain. (B): 565.6 mg/kg toluene -treated rat showing many cells gave positive result for the stain. The lower right corner shows positive cells. (C): 565.6 mg/kg toluene + vitamin C showing some cells that are still giving positive result. (D): 565.6 mg/kg toluene + vitamin E showing negative result in almost all the cells. (E): 565.6 mg/kg toluene + vitamin C & E showing negative result all over the field. (TN–α F x 100 (A, C & D), 200 (B & E) & 500 (the highly magnified parts).

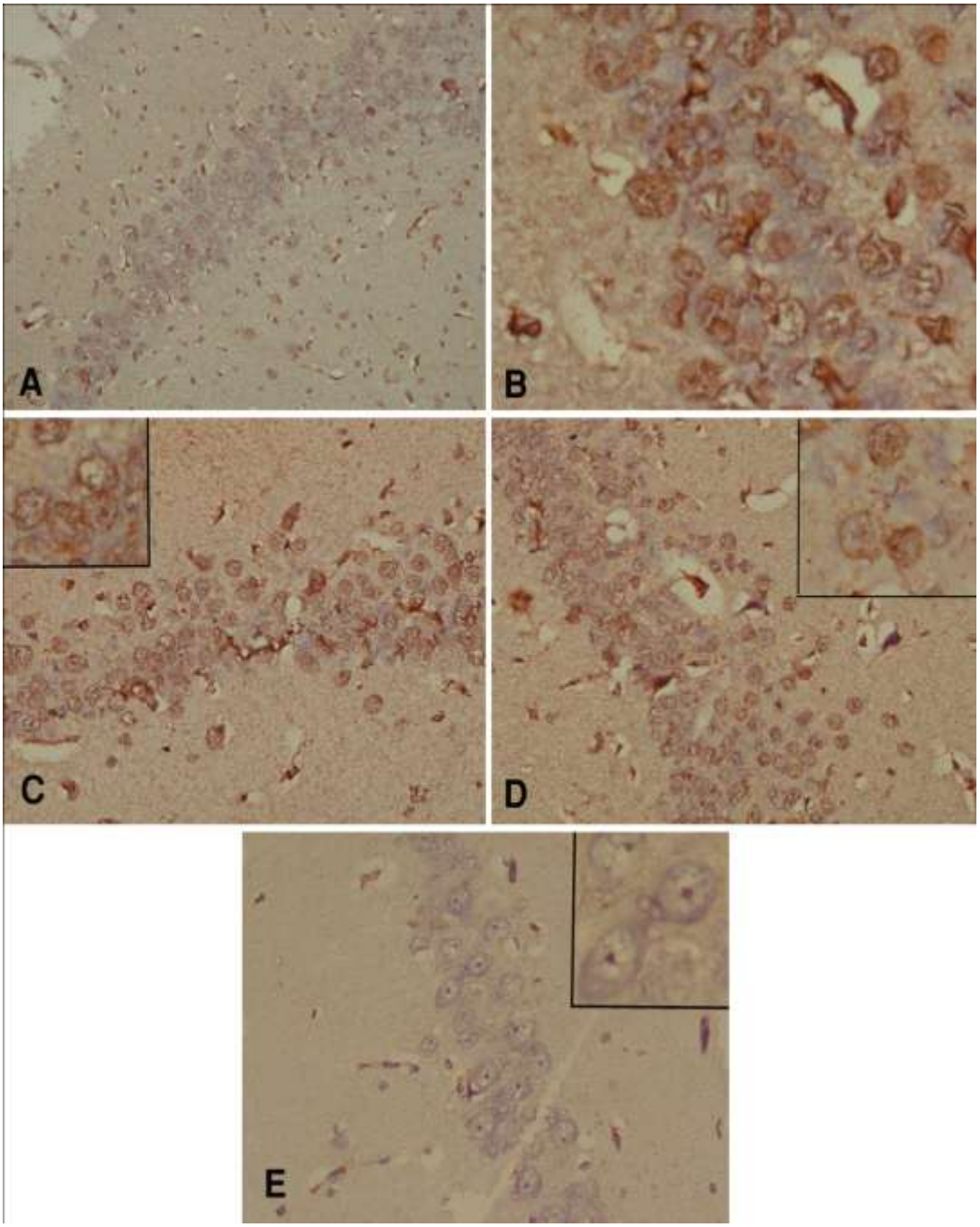


Fig. 10. Representative photomicrographs of scetions of hippocampus stained with antibodies against TNF –α (A): vehicle-treated rat showing no positive result for TNF –α. (B): 565.6 mg/kg toluene -treated rat showing some cells giving a positive result for the stain. (C): 565.6 mg/kg toluene + vitamin C showing a few cells that give a positive result for the stain. (D): 565.6 mg/kg toluene + vitamin E showing very faint positive result for the stain in some cells. (E): 565.6 mg/kg toluene + vitamin C & E showing negative result for the stain. (TNF x 100 (A), 200 (C, D & E) & 500 (B & the highly magnified parts).

Study II. Effect of abstinence from toluene on behavior and biochemistry

Behavioral results

Wire hanging test

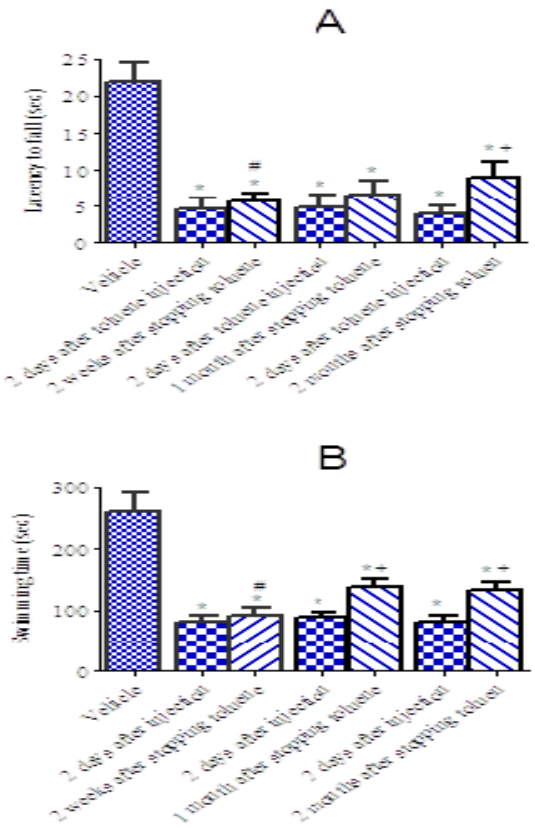
After toluene injection at 1131.2 mg/kg, the latency to fall remained high at 2 and 4 weeks after toluene cessation. Grip strength increased by only 24% and 35.2% compared with the corresponding basal value. Only after 2 months of cessation of toluene, we observed improvement of grip strength reflected in increased latency to fall by 125% *vs.* corresponding basal value (Fig. 11A).

Porsolt's forced-swimming test

Cessation of toluene administration resulted in increased struggle time in a time-dependently. The swimming time increased by 12.4%, 55.1% and 66.4% at 2 weeks, 4 weeks and 8 weeks following toluene cessation, respectively (Fig. 11B).

Water maze test

Cessation of toluene results in improved ability to find the hidden platform in a time-dependent manner. Thus, the escape latency decreased by 73.6, 84.3, and 83.5% at 2, 4 and 8 weeks after stopping toluene compared with the corresponding basal values (Fig. 11C).



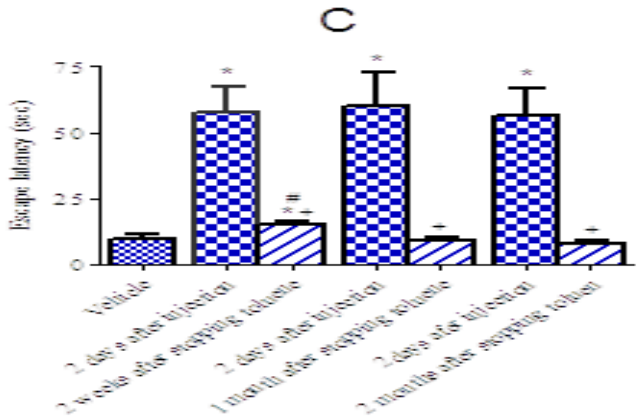


Fig. 11. Effect of recovery after toluene withdrawal (2 weeks, 1 month, and 2 months after stopping toluene (1131.2 mg/kg daily for 9 days) on (A) Grip strength test. Bars represent the latency to fall (sec). *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene injection. # p<0.05 vs. 2 months after stopping toluene injection. (B) Forced swimming test & (C) Morris Water Maze test. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene injection. # p<0.05 vs. 1 or 2 months after stopping toluene injection.

Open field test

Cessation of toluene was associated with improvement in the ambulation frequency. The effect was time-dependent in that the ambulation frequency increased by 93.6, 113.5, and 151.5% compared to the corresponding basal level at 2, 4 and 8 weeks after stopping toluene administration, respectively (Fig. 11A). Increments in frequency of rearing by 260, 231.8, and 624.8% compared to the corresponding basal level was observed 2, 4 and 8 weeks after stopping toluene administration, respectively(Fig. 12B). Cessation of toluene was also associated with improvement in the grooming frequency. The effect was time-dependent in that the number of ambulation increased by 176.5, 276.3, and 489.5% compared to the corresponding basal level at 2, 4 and 8 weeks after stopping toluene administration, respectively (Fig. 12C).

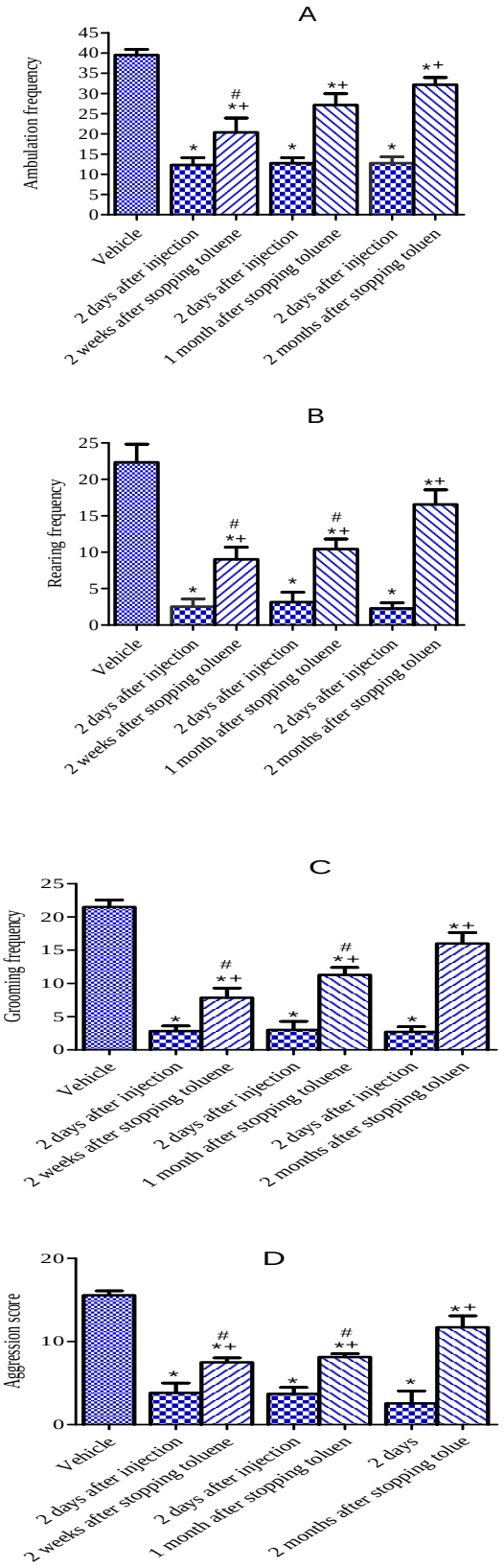


Fig. 12. Effect of recovery after toluene withdrawal (2 weeks, 1 month, and 2 months after stopping toluene (1131.2 mg/kg daily for 9 days) on (A) Ambulation frequency. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene

injection. # p<0.05 vs. 1 or 2 months after stopping toluene injection. (B) Rearing frequency. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene injection. # p<0.05 vs. 1 or 2 months after stopping toluene injection. (C) Grooming frequency. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene injection. # p<0.05 vs. 1 or 2 months after stopping toluene injection. (D) Aggression. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. corresponding 2 days after toluene injection. # p<0.05 vs. 2 months after stopping toluene injection.

Defensive aggression test

Cessation of toluene was associated with improvement in the defensive aggression score in a time-dependent manner. The defensive score increased by 80%, 119.2%, 355.5% compared to the corresponding basal level at 2, 4 and 8 weeks after stopping toluene administration, respectively (Fig. 12D).

Biochemical results

Malondialdehyde

Cessation of toluene was associated with decreased MDA in a time-dependent manner in that the MDA decreased in cortex, striatum and rest of brain by -20.5-37.4%, -61.5-87.5%, and 15-67.3% compared to the corresponding basal level at 2 and 8 weeks after stopping toluene administration, respectively (Fig. 13A).

Reduced glutathione

Cessation of toluene was associated with increased GSH in a time-dependent manner in that the GSH increased in cortex, striatum and rest of brain by 30.3%, 52.9%, and 90.3% and by 96.9%, 61.2% and 263.9%, and by 100%, 127.1% and 273.6% compared to the corresponding basal level at 2 and 8 weeks after stopping toluene administration, respectively. The level of GSH, however, did not reach the saline-control values after 2 months of stopping toluene (Fig. 13B).

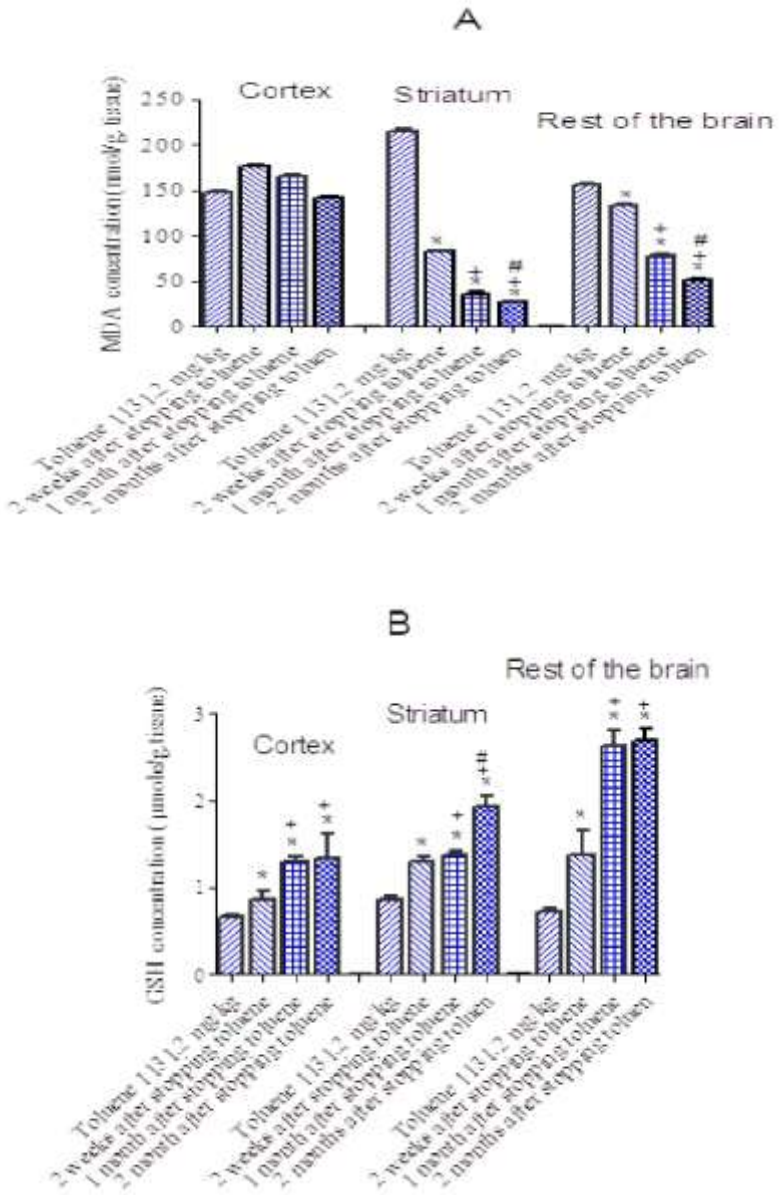


Fig. 13A-B. Effect of cessation of toluene exposure on (A) brain MDA. (B) brain GSH. *p<0.05 vs. toluene-treated group. + p<0.05 vs. corresponding 2 weeks after stopping toluene injection. # p<0.05 vs. 1 month after stopping toluene injection.

Nitric Oxide

Significant decrease in NO in the striatum and rest of brain by 25.8% and 24.7% was observed 1 month after stopping toluene. Nitric oxide showed significant decrease by 20.4%, -34.7% and -35.2% compared to the corresponding basal level at 2 months after stopping toluene administration (Fig. 13C).

Paroxonase activity

Stopping toluene was associated with increased PON-1 activity in a time-dependent manner in that the PON-1 increased in cortex, striatum and rest of brain by -9.8%, -534.6%, and -87.3% and 28, 549.2 and 250.9% and by 80%, 580.8% and 244.5% compared to the corresponding basal level at 2, 4 and 8 weeks after stopping toluene administration, respectively (Fig. 13D).

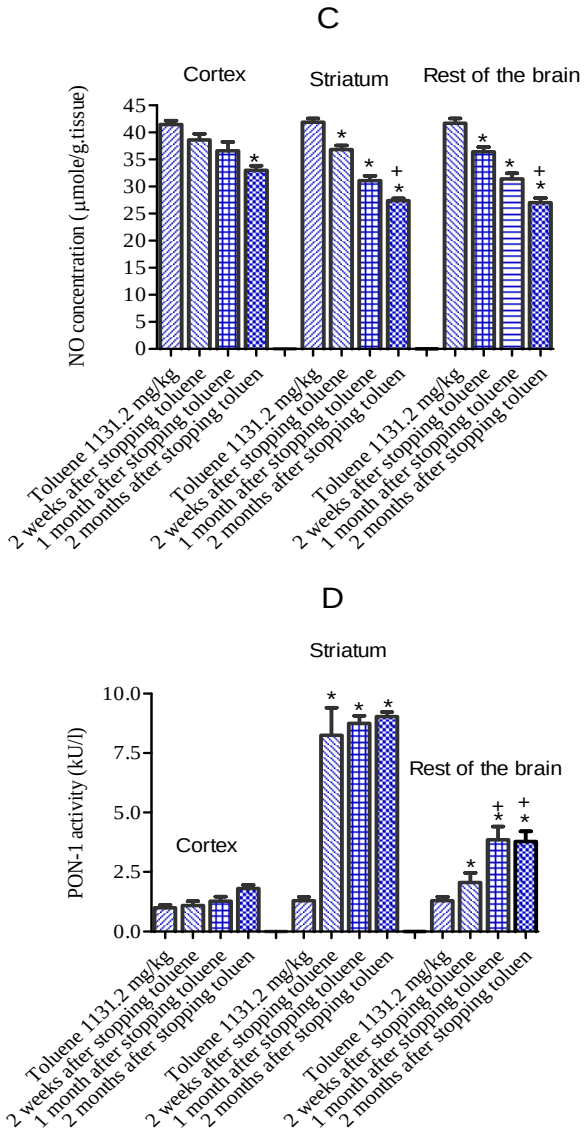


Fig. 13C-D. Effect of cessation of toluene exposure on (C) brain NO. (D) brain PON-1. *p<0.05 vs. toluene-treated group. *p<0.05 vs. toluene-treated group. + p<0.05 vs. 2 weeks after stopping toluene injection.

Acetylcholinesterase activity

Cessation of toluene was associated with increased AChE activity in a time-dependent manner. Significant increase by 17% was seen in the cortex 2 months after stopping the solvent. In contrast significant increases by 311.6-694.2% and by 122-284.7% were observed in the straitum and rest of brain at 2-8 weeks after stopping toluene (Fig. 14A).

Butyrylcholinesterase activity

Cessation of toluene was associated with increased BuChE activity in a time-dependent manner in that the BuChE increased in cortex, striatum and rest of brain by 16%, 269%, and 108.8% and by 10.7, 645.6 and 188.2% and by 17%, 693.4% and 233.9% compared to the corresponding basal level at 2, 4 and 8 weeks after stopping toluene administration, respectively (Fig. 14B).

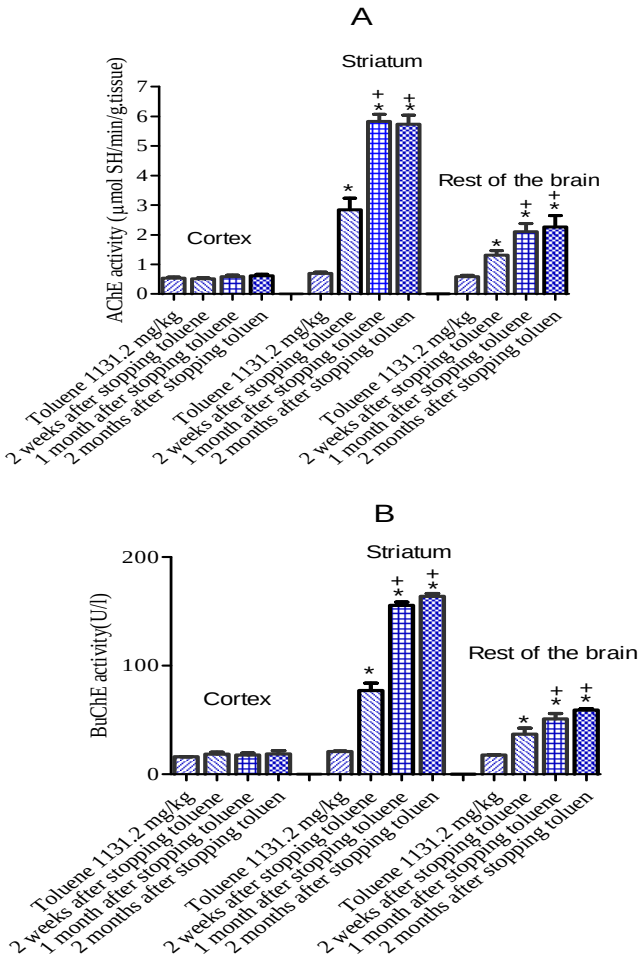


Fig. 14A-B. Effect of cessation of toluene exposure on (A) brain AChE activity. (B) brain BuChE activity.). *p<0.05 vs. toluene-treated group. + p<0.05 vs. 2 weeks after stopping toluene injection.

Histopathological and immunochemical results

Allowing recovery after toluene injection led to mild amelioration of the damaged cells after one month and the effect became more noticeable after 2 months in both the cortex, and hippocampal areas. In rats receiving 1131.2 mg/kg toluene and allowed to recover for 2 weeks, the cortex, and hippocampal area showed many neurons with dark nucleoli, and undefined nucleoli. While after 1 month of recovery, there was mild amelioration in the form of reduction of neurons number with undefined nucleoli. Rats receiving 1131.2 mg/kg toluene, and allowed to recover for 2 months, showed that most neurons appear normal, and there was a reduction in the degenerative effects of toluene on neurons (Fig. 15 & 16).

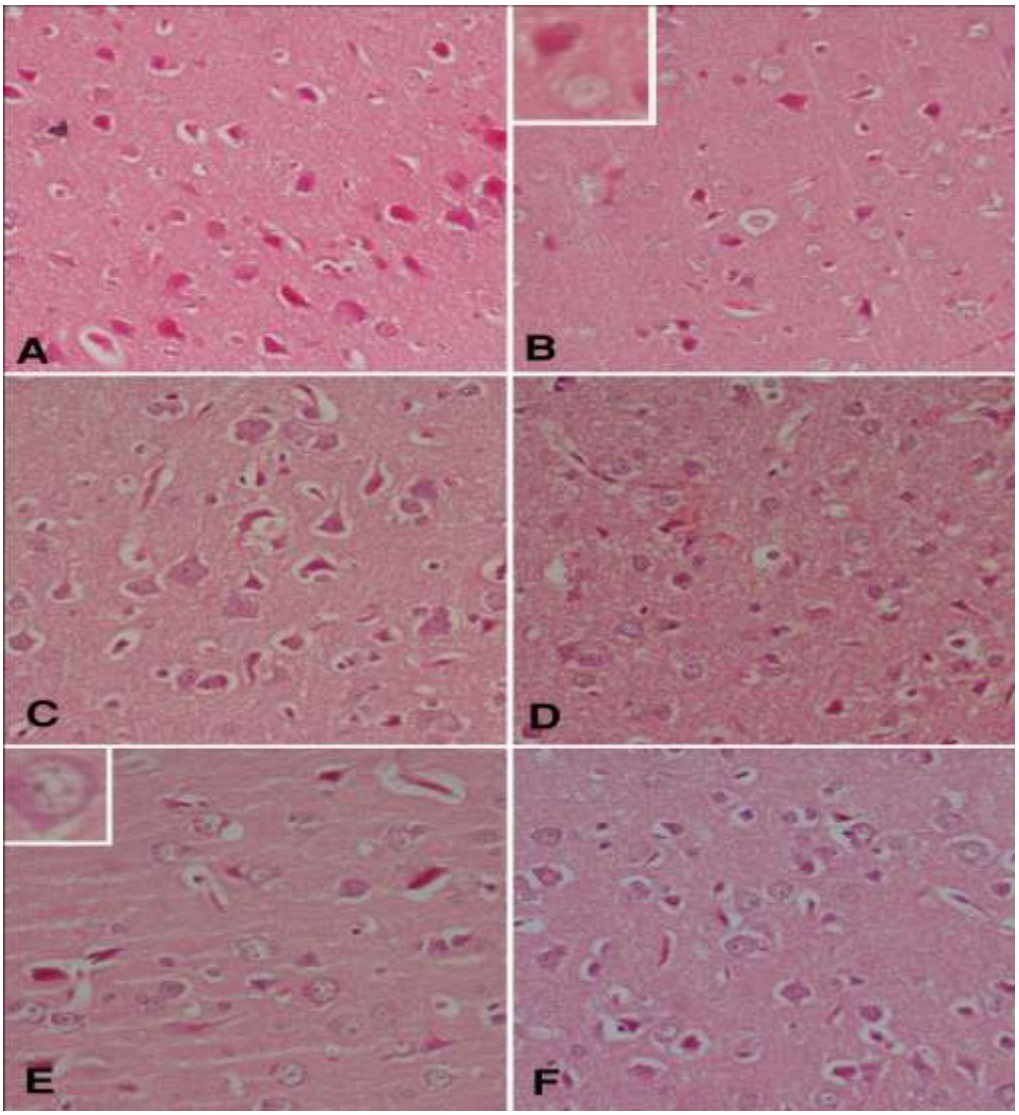


Fig. 15. Representative photomicrographs of sections of cerebral cortex of rats treated with (A): toluene at 1131.2 mg/kg and then allowed to recover for 2 weeks showing the same result as the group received 1131.2 toluene only without recovery. (B): toluene at 1131.2 mg/kg and then allowed to recover for a month showing noticeable reduction of

number of neurons with signs of degeneration. (C): toluene at 1131.2 mg/kg and then allowed to recover for 2 months showing marked amelioration of the degenerative effect of toluene on brain tissue. (D): toluene at 565.6 mg/kg and vitamin C showing that some neurons still have nuclei darker than normal. (E): toluene at 565.6 mg/kg and vitamin E showing marked amelioration of the degenerative effect of toluene on brain tissue. The upper left corner of the figure shows a neuron close to normal. (F): toluene 565.6 mg/kg and vitamin C & E showing normalization of brain tissue. (Hx. & E. X 200 & 500 for the highly magnified parts.

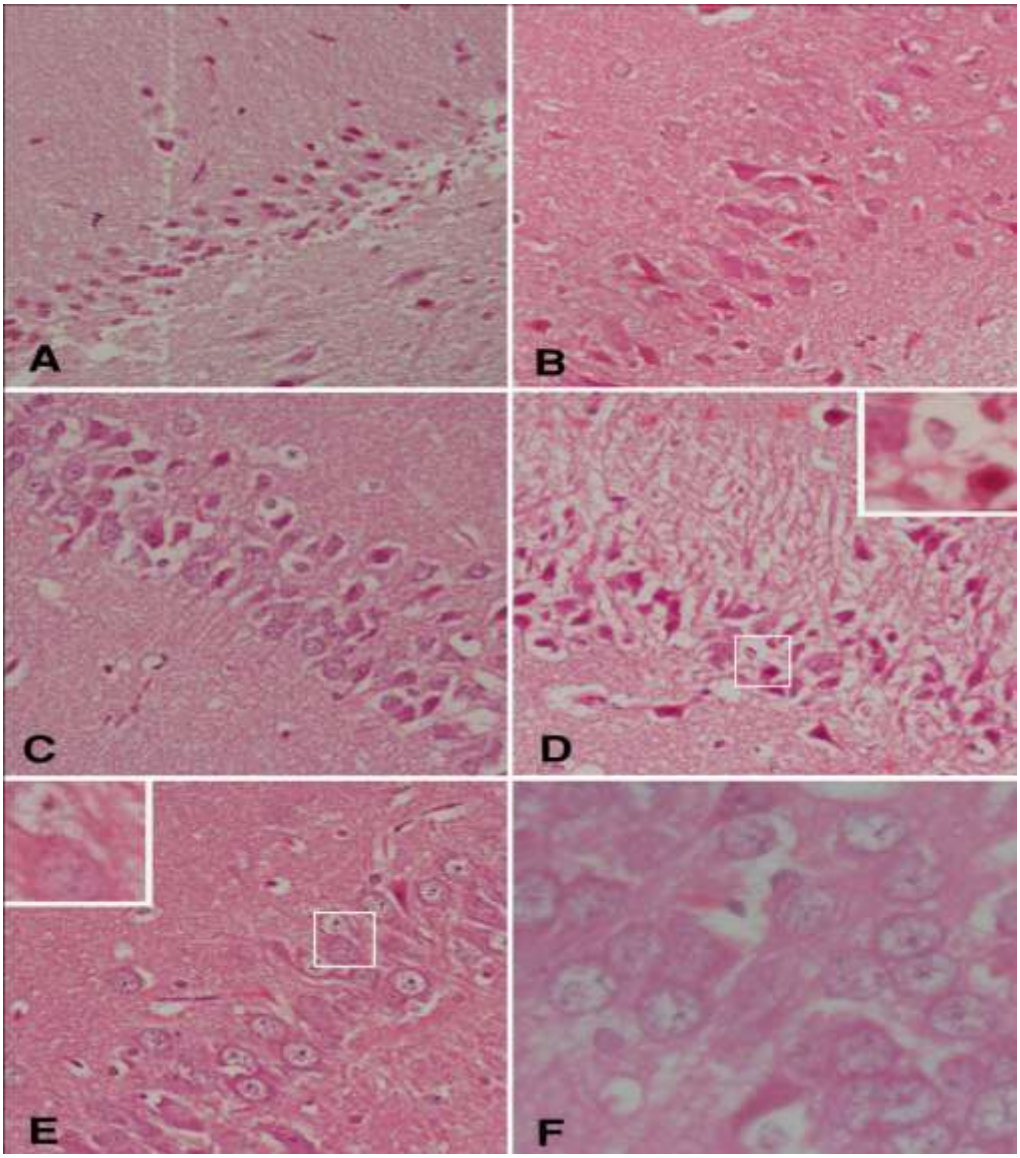


Fig. 16. Representative photomicrographs of a section of hippocampal area of rats treated with (A): 1131.2 mg/kg toluene that allowed to be recovered for 2 weeks showing the same result as the group that received 1131.2 mg/kg toluene without recovery. (B): 1131.2 mg/kg toluene and then allowed to recover for a month showing mild

amelioration in the form of reduction of neurons' number with undefined nucleoli, although there is mild deformation of tissue structure. (C): 1131.2 mg/kg toluene and allowed to recover for 2 months showing noticeable amelioration as most of neurons appear normal with restoration of the hippocampal area structure. (D): 565.6 mg/kg toluene and vitamin C showing little number of cells that appear normal, while many cells appear with acidified cytoplasm and dark small nucleus (as shown in the upper right corner of figure). (E): 565.6 mg/kg toluene and vitamin E showing many normal cells with large vesicular nuclei and some neurons with undefined nucleoli (notice the upper left corner of the figure). (F): 565.6 mg/kg toluene and vitamin C & E showing that most of neurons appear normal. (Hx. & E. X 200 & 500 for the highly magnified parts)

Study III. Effect of antioxidants on residual effects of toluene

Behavioral results

Wire hanging test

Compared with the vehicle-treated controls, rats treated with toluene at 565.6 mg/kg showed marked decrease in the latency to fall by 85.5%. Treatment with either ascorbic acid or α-tocopherol along with toluene resulted in prolongation of the time taken to fall. Rats treated with ascorbic acid or α-tocopherol showed 265% and 354.6% increments in the latency to fall compared with the toluene control group, respectively. The combination of ascorbic acid and α-tocopherol showed better effect than individual treatment (469.7% increase in the latency to fall compared with the toluene control group) (Fig. 17A).

Porsolt's forced-swimming test

Injecting toluene at 565.6 mg/kg resulted in decreased swimming time by 60% compared with the vehicle control. Swimming time increased following treatment with ascorbic acid or α-tocopherol by 23.4% and 65.4%, respectively as compared with the toluene-treated rats. The combined administration of ascorbic acid and α-tocopherol increased the swimming time by 98.7%, suggesting an additive effect for the antioxidants (Fig. 17B).

Water maze test

Toluene given at 565.6 mg/kg resulted in 537% increase in the latency to find the hidden platform compared with the vehicle control. In toluene treated rats, the administration of ascorbic acid or α-tocopherol resulted in significant decrease in escape latency by -50.1% and 61.9%, respectively as compared with the toluene-treated group. The escape latency

decreased by -72.6% after the combined administration of ascorbic acid and α-tocopherol as compared with the toluene-treated rats (Fig. 17C).

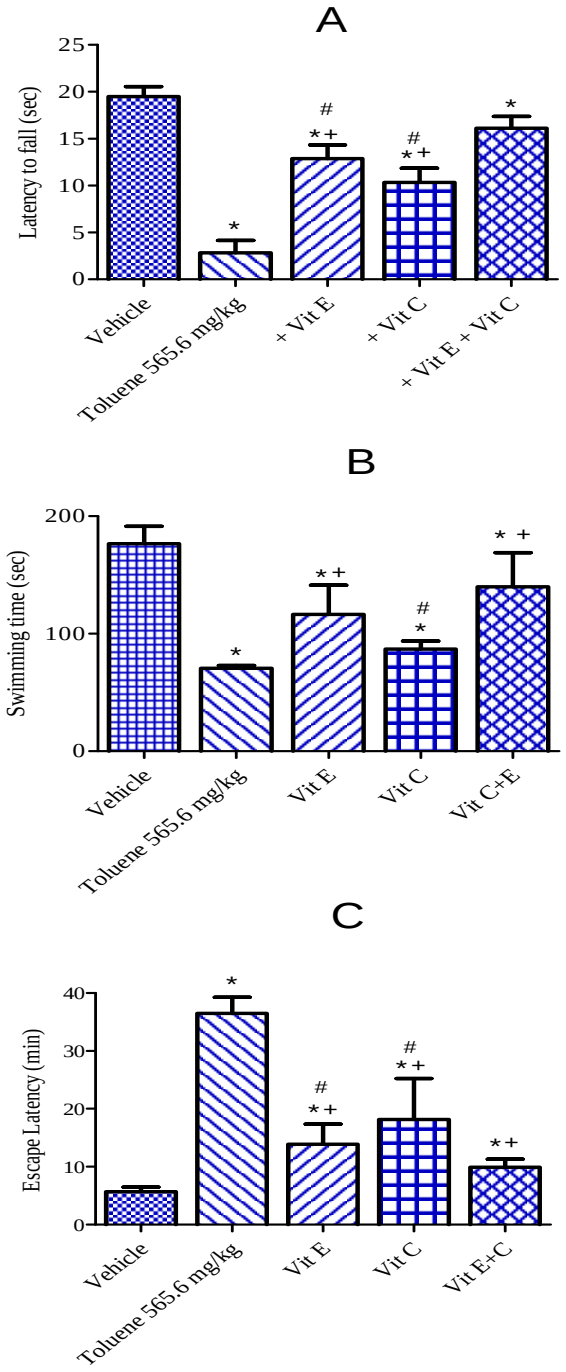


Fig. 17. Effect of treatment with vitamin E and/or vitamin C on toluene (565.6 mg/kg)-induced changes in: (A) Grip strength test. (B) Forced swimming test. (C) Morris Water Maze test. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Open field test

Rats administered toluene at 565.6 mg/kg for one week showed marked decrease in their ambulation by 68.3%, in rearing frequency by 81.9%, and in grooming frequency by 88.9%. The administration of ascorbic acid, α-tocopherol or both resulted in significant increase in ambulation frequency by 136, 160, 192.6%, respectively as compared with the toluene only treatment group. Rats treated with both ascorbic acid and α-tocopherol had ambulation frequency near to that of the vehicle treated group (Fig. 18A).

The administration of ascorbic acid or α-tocopherol also resulted in significant increase in the ambulation by 132.2 and 161.3%, respectively as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in significantly higher increase in the rearing (by 265%) than either vitamin alone (Fig. 18B). Moreover, significant increase in grooming by 293.2, and 455.2%, respectively, was observed after ascorbic acid or α-tocopherol as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in significantly higher increase in grooming (by 637.2%) than either vitamin alone (Fig. 18C).

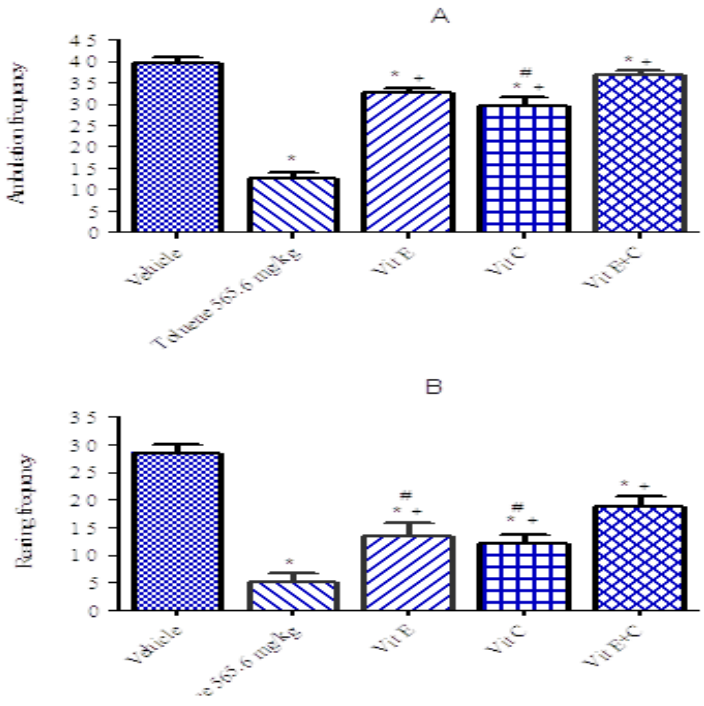


Fig. 18A-B. Effect of treatment with vitamin E and/or vitamin C on toluene (565.6 mg/kg)-induced changes in: (A) Ambulation frequency. (B) Rearing frequency. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Defensive aggression test

Rats administered toluene at 565.6 mg/kg for one week showed marked decrease in their defensive behavior by -80.8%. The defensive score decreased from 15.67±0.49 to 3 ± 0.26 ($p<0.001$). In toluene treated rats, the administration of ascorbic acid, α-tocopherol or both resulted in significant increase in the defensive behavior by 94.4, 166.7, and 338%, respectively as compared with the toluene only treatment group (Fig. 18D).

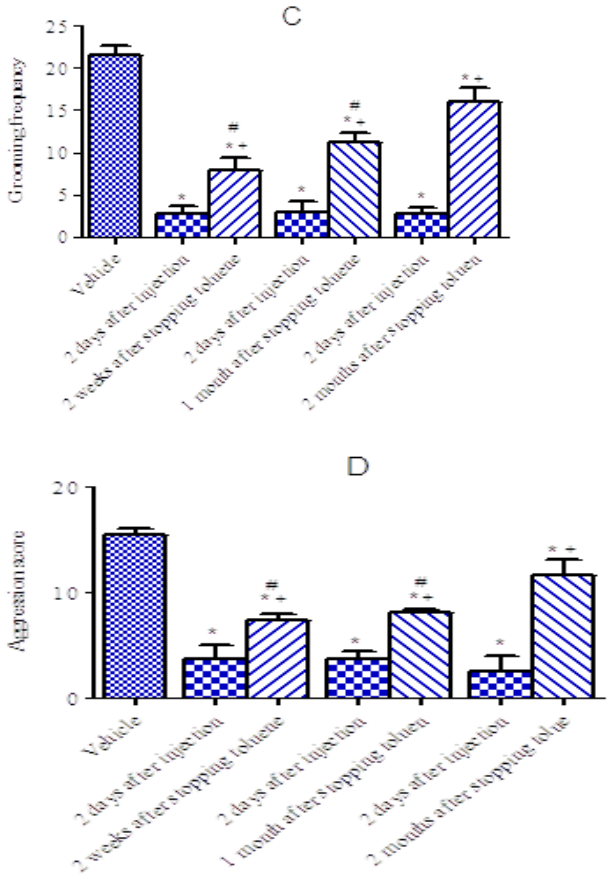


Fig. 18C-D. Effect of treatment with vitamin E and/or vitamin C on toluene (565.6 mg/kg)-induced changes in: (C) Grooming frequency. (D) Aggression. *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Biochemical results

Malondialdehyde

The administration of ascorbic acid or α-tocopherol resulted in significant decrease in lipid peroxidation as compared with the toluene only treatment group. The combined

administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 19A).

Reduced glutathione

The administration of ascorbic acid or α-tocopherol resulted in significant increase in GSH as compared with the toluene only treatment group (though not to saline control values). The combined administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 19B).

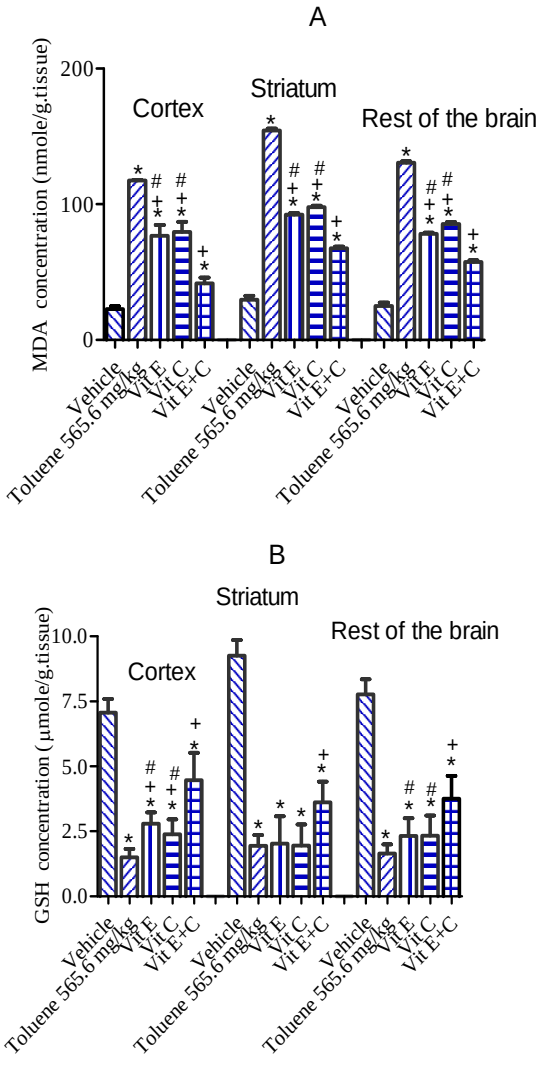


Fig. 19A-B. Brain MDA and GSH of toluene-treated rats and the effect of treatment with vitamin E and/or vitamin C *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Nitric Oxide

The administration of α-tocopherol but not ascorbic acid resulted in significant decrease in NO as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 19C).

Paroxonase activity

The administration of ascorbic acid or α-tocopherol resulted in significant increase in PON-1 as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 19D).

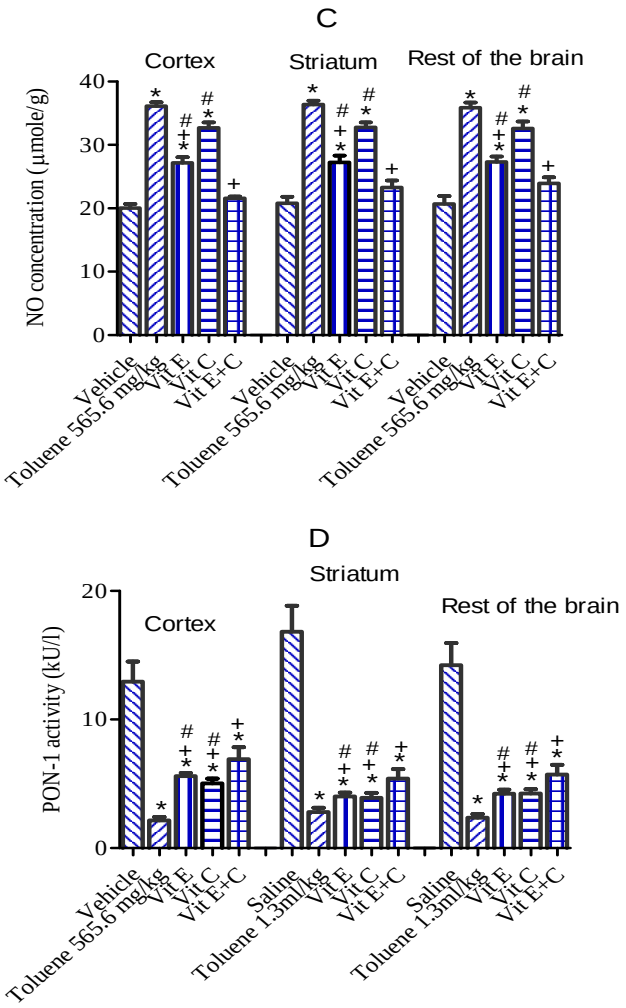


Fig. 19C-D. Brain nitric oxide and PON1 activity of toluene-treated rats and the effect of treatment with vitamin E and/or vitamin C *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Acetylcholinesterase activity

The administration of ascorbic acid or α-tocopherol resulted in significant increase in AChE as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 20A).

Butyrylcholinesterase activity

The administration of ascorbic acid or α-tocopherol resulted in significant increase in BuChE as compared with the toluene only treatment group. The combined administration of ascorbic acid and α-tocopherol resulted in better protective effect than either vitamin alone (Fig. 20B).

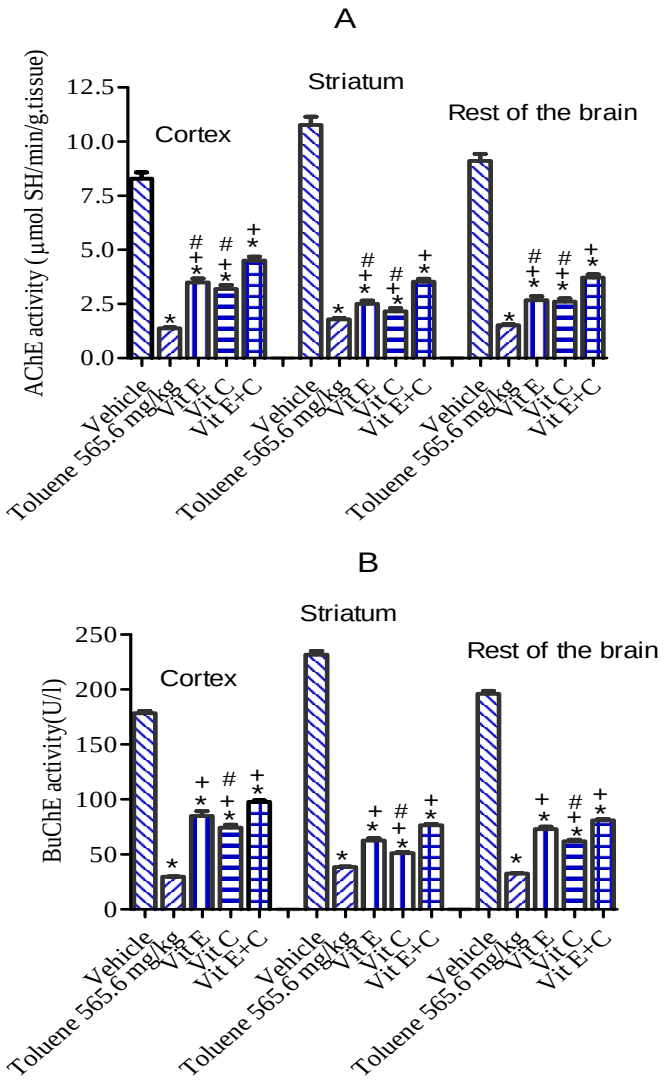


Fig. 20A-B. Brain ACHE and BuChE activities of toluene-treated rats and the effect of treatment with vitamin E and/or vitamin C *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Dopamine

The administration of either α-tocopherol or ascorbic acid decreased dopamine level by 45.3 and 40%, respectively compared to toluene control value. The combination of both ascorbic acid and α-tocopherol decreased dopamine by 69.5% compared to toluene- treated rats.

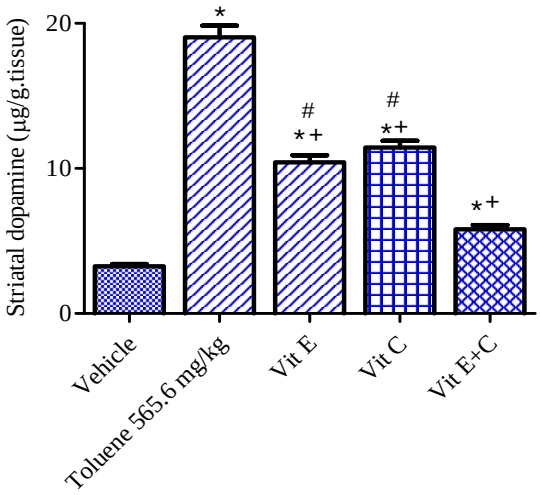


Fig. 21. Striatal dopamine content of toluene-treated rats and the effect of treatment with vitamin E and/or vitamin C *p<0.05 vs. vehicle-treated group. + p<0.05 vs. toluene group. # p<0.05 vs. toluene + Vitamin E + Vitamin C.

Histopathological and Immunochemistry results

Using ascorbic acid as a protecting agent caused a mild reduction of the damaging effect of toluene on brain tissue. On the other hand, α-tocopherol caused even a higher degree of protection against toluene . Meanwhile, there was an additive protective effect of combining both ascorbic acid and α-tocopherol. Few normal cells were observed in rats treated with ascorbic acid, while many normal cells with large vesicular nucleoli were seen after treatment with α-tocopherol. The combined treatement with α-tocopherol and ascorbic acid C resulted in most of neurons being normal in appearance (Fig. 15 & 16).

Immunohistochemistry

Caspase-3 expression

Mild decrease in caspase-3 expression was noted after administration of ascorbic acid, while administration of α-tocopherol resulted in marked reduction in caspase-3 immunopositivity. The combined administration of ascorbic acid and α-tocopherol resulted in negative

appearance in the number of cells containing caspase-3 compared to toluene (565.6 mg/kg)-treated rats (Fig. 17).

TNF-α

The co-administration of ascorbic acid resulted in less reaction compared with toluene (565.6 mg/kg)-only treated rats, while the administration of α-tocopherol resulted in amelioration of TNF-α immunereactive cells. TNF-α immunoreactivity was near to the saline control group in those treated with combination of ascorbic acid and E (Fig. 18).

DISCUSSION

The current study demonstrated that repeated toluene exposure for 9 days led to significant alterations at both neurochemical and behavioral levels. At the behavioral level, toluene impaired memory, motor coordination, decreased locomotion and rearing frequencies, decreased defensive aggression and induced depression in the rat dose-dependently. These behavioral alterations were accompanied by increased oxidative stress, proinflammatory cytokine (TNF-α), and apoptotic marker (caspase-3). Toluene also inhibited AChE, BuChE activities and increased striatal dopamine level. Histopathologic studies provided evidence of neurotoxicity such as fragmented DNA and undefined nucleoli in addition to reduction in thickness and the presence of dark neurons in brain of toluene-treated rats, reflecting early signs of degeneration. Treatment with vitamin E, or vitamin C or their combination conferred significant protection against these detrimental effects of toluene.

According to several investigators, repeated administration of toluene has been demonstrated to produce a wide array of CNS effects similar to CNS depressants.[22][23] Muscle coordination and gait impairment have been demonstrated even at very low doses in animal models and toluene abusers.[24][25] These findings are consistent with reports of ataxic gait, nystagmus, slurred speech and intention tremors in chronic toluene abusers. One likely explanation for the decreased locomotion and rearing could be the ataxic behavior commonly associated with toluene administration.[26] In the present study, the acute effects of toluene reflected mainly these CNS depressant effects including decreased locomotion and rearing, decreased CNS excitability, reduction of muscle tone (grip strength), reduced response to sensory stimuli, and increased ease of removal. Moreover, the finding that toluene administration affect both locomotion and rearing implicate that toluene affect both motor and cognitive functions, since rearing is related to cognitive processes related to spatial learning.[27] Similar to other CNS depressant drugs, toluene exerts biphasic locomotor

response, i.e. increased and decreased activity at low and high concentrations, respectively.[28][29][26][30] We only observed decreased activity at the doses used in the present study, suggesting that toluene at these doses exerted CNS depressant effects.

Toluene has been shown to interfere with cognitive, perceptional, and mental capabilities, hence; toluene abusers are prone to perceptual distortion, altered level of consciousness, psychosis, dementia, and visual hallucinations can emerge as a residual sequel of high dose exposure.[31] Our results showed that the escape latency in the water maze test was significantly increased in a dose dependent manner. The solvent led to higher latencies to locate the hidden platform, indicating learning and memory deficits. Another important finding in the current study is the depressant-like action induced by toluene. Toluene administration resulted in a decrease in the struggle time and increased immobility time in the Prosolt's despair test. Seo et al. (2010)[32] reported depressant effect that was evident up to 4 days following toluene exposure. In contrast, Cruz et al. (2009)[33] reported on the antidepressant actions of toluene 30 min after inhalation. The present data confirmed that toluene exposure induces depression in short term exposure. Defensive aggression test further characterize behavioral effects and allows quantitative evaluation of CNS activity.[34] In defensive aggression test, toluene delayed significantly the responses to different stimuli, thereby, suggesting decreased CNS excitability. Toluene interferes with the ability of animals to avoid situations that may provoke fear or frustrations under normal conditions. This result lends further support to the depressant-like behavior of toluene.

In the present study, the repeated injection of toluene was found to increase lipid peroxidation and NO paralleling with decreased GSH content, suggesting depletion of GSH by increased free radicals. These findings are in agreement with the results of other investigators who showed that toluene exposure stimulates the release of reactive oxygen species, which induces oxidative damage to lipids, proteins and nucleic acids.[35][36][37][12] Halifeoglu et al. (2000)[37] reported that toluene-containing thinner elevates the levels of malondialdehyde in serum of people working with paint thinner. The generation of reactive oxygen species, which cause neurodegeneration, may mediate the neurobehavioral impairments found in toluene-treated rats. Several investigators reported that memory is impaired by oxidative stress.[38][39]

Converging evidence shows that oxidative stress is a critical mediator of cognitive impairments such as those found in Alzheimer's disease, and that treatment with dietary

antioxidants can reverse these deficits. In Long-Evans rats trained on a spatial reference memory water-maze task, greater oxidative stress-related changes were observed in the hippocampus of rats with a spatial-learning impairment, compared to unimpaired animals of the same age.[40] Oxidative stress induced by hyperoxia significantly impaired water maze and radial maze performance of 3-month-old rats, effects that were partially attenuated by dietary vitamin E supplementation.[41][42] Oxidative stress occurs when there is an imbalance in cell's production of oxidants or reactive oxygen species and a reduction in its ability to eliminate them by endogenous antioxidant scavengers. The resulting accumulation of oxidative free radicals can directly damage proteins, lipids and DNA or cause the release of cytokines that lead to inflammation and further release of reactive oxidants. Oxidative stress could cause serious cellular damage, activate caspases, and ultimately lead to apoptosis of neuronal cells.[43]

Oxidative damage also triggers mitochondrial dysfunction that results in reduction of ATP. This drop down in energy leads to either apoptotic or necrotic cell damage. Our results showed apoptotic cell damage rather than necrotic, since we found up regulation in caspase-3 immunoreactivity in the cytoplasm of cortex and hippocampus following toluene administration. 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.[44][45, 46] Apoptotic cell death is executed via molecular pathways that are mediated by the activation of caspases, a family of cysteine proteases.[47] Caspase-3, a main effector caspase, is strongly implicated in neuronal apoptosis[48], which occurs due to competition for, or limited supply of, neurotrophins that suppress the endogenous genetic death program.[49][50]

In the present study, the expression of TNFα was increased in response to toluene administration in both cerebral cortex and hippocampus. TNFα is a pleiotropic cytokine that promotes inflammation and signals of death. Within the CNS, it is best characterized for its ability to initiate cascades associated with neuronal apoptosis and neurological impairment. TNFα clearly contributes to neuronal death in brain ischemia[51] and several neurodegenerative diseases, e.g. Parkinson's[52] Release of proinflammatory cytokines which might act by stimulating NO production or directly damaging the neurons by activating receptors that contain intracytoplasmic death domains involved in apoptosis.[53]

Moreover, our results showed that the activity of PON-1 was found to be substantially decreased in toluene-treated rats. PON-1 acts by protecting cells against lipid peroxides and decreased enzyme activity was found in neurodegenerative conditions of an inflammatory etiology and/or associated with increased oxidative stress.[54][55] Impaired enzyme activity would therefore result in more susceptibility of the cells to high levels of reactive oxygen species.

Being an abused substance, toluene has been shown to elevate dopamine levels in frontal cortex[56] and in nucleus accumbens[57][58] which is consistent with toluene‘s reported rewarding effects. The observed increase in striatal dopamine levels in the present work could be explained by the increase in the release of the neurotransmitter.[58][59][26][57] Another explanation is the reduction of dopamine turnover.[60] Riegel and collaborators (2004)[61] found that repeated administration of toluene could inhibit monoamine oxidase-A activity in certain brain regions leading to inhibition of catecholamine metabolism.

Cholinergic neurons are involved in the learning and memory functions.[62] Functional disturbances in cholinergic activity play a role in memory loss and related cognitive problems. Thus, restoration of cholinergic function may reduce the severity of the cognitive loss.[63][64] The neurotransmitter ACh is degraded at the synapse into choline and acetic acid by the enzyme cholinesterase. The reaction is necessary to allow a cholinergic neuron to return to its resting state after activation. Two cholinesterases exist in vertebrates, acetylcholinesterase (AChE) which is found mainly in blood and neural synapses and butyrylcholinesterase(BuChE) found mainly in the liver.[65] In the present study, AChE and BuChE activities decreased in brains of toluene-treated rats in a dose-dependent manner. Inhibition of AChE and BuChE leads to accumulation of intracellular Ach.[66] In rats, inhalation of toluene decreases striatal ACh release during and after toluene exposure. The mechanism is unlikely to be related to increased extracellular dopamine levels.[58] On the other hand, Honma and Suda (2004)[67] found that the i.p. injection of high doses of toluene (200-2000 mg/kg) increased ACh content in brain homogenate. Toluene reduced extracellular concentration of ACh in the striatum and hippocampus, possibly due to decreased release of ACh from cholinergic nerve endings. Thus, the increase in brain ACh could be a good marker of lowered activity of cholinergic neurons by the solvent (Honma and Suda, 2004).[67]

The present studies also tested whether the neurobehavioral, neurochemical and neuronal damage due to toluene is reversible following abstinence from the solvent. We also found that if rats were allowed to recover for 2 months after toluene administration, the changes caused by the solvent gradually returned to normal. Cessation of toluene injection led to improvements in grip strength, memory, and depressive-like behavior. These improvements were most evident in the group left 1-2 months to recover after stopping exposure to the solvent. Recovery of motor activity, exploratory activity and improvement in the defensive aggression score was evident as early as 2 weeks from stopping toluene injection. Cessation of toluene also led to gradual recovery in the biochemical alterations caused by the solvent. On the histopathological level, mild amelioration of the neuronal damage was seen after one month and the effect becomes more noticeable after 2 months in both the cortex, and hippocampal areas. These results collectively suggest reversibility from toluene induced neurotoxicity. Thus, the effects of toluene on cognitive functions (neurobehavioral effects) appear to be reversible, which in turn suggests that the impaired cognitive function of rats exposed to toluene depends on neurochemical changes, such as increased reactive oxygen species and impaired antioxidant defense systems, rather than on persistent structural changes. It should be noted, however, that, the observations relate to short time of exposure and not to long-term toxicity.

In this study, the role of antioxidant therapy with vitamin C and E on the toluene induced behavioral neurochemical and structural brain alterations were investigated. Studies linked an inadequate dietary supply of Vitamin C to cognitive impairment.[68][69] The brain has comparatively high concentrations of Vitamin C when most other organs are depleted, indicating the importance of this vitamin for the brain.[70][71] Vitamin C (ascorbate) has both antioxidant and non-antioxidant functions in the CNS. As an antioxidant, ascorbate acts directly to scavenge oxygen- or nitrogen-based radical species, and especially important is the scavenge of the superoxide radical, a major oxidizing free radical, capable of causing damage to the cell components.[72][73][74] Another important action of ascorbate also is to recycle α-tocopheroxyl radical back to α-tocopherol, thereby, combating oxidative stress.[75] In the cell, vitamin E has a crucial role as an antioxidant protecting the cell from oxidative damage by reactive free radicals.[76][77][78][79] It is also an important modulator of cell function and regulates specific enzymatic activities, membrane properties and signaling elements.[80][81][82][83] Studies have indicated neuroprotective effects of vitamin E, inhibiting lipid peroxidation[84], improving degeneration of hippocampal cells following ischemia[85]

and enhancing the recovery of neuronal damage of motor function after spinal cord injury.[86] Vitamin E can repair cellular disturbances by abrogating apoptotic signals, suppressing lipid peroxidation, and inflammation.[87]

In this study, we found that both agents were able to protect against the deleterious effects of the organic solvent, with the combination being more effective than either agent alone. The combination of antioxidant vitamin E and C treatment could obviously reduce the neuronal loss, apoptosis and efficiently rescue cognitive and behavioral impairment induced by toluene. Co administration of vitamin C and/or E reverted these changes, which could be explained by the improvement in the oxidative status of the cells. The administration of either vitamin E or C or their combination restored AChE and BuChE enzyme activities, possibly reflecting a neuroprotective effect of vitamin E and C on cholinergic neurons. These finding are in line with Ben Amara et al. (2011)[88] who showed that AChE and BuChE activities are decreased by free radicals and restored by antioxidant treatment.

In summary, it was demonstrated that short-term exposure to toluene induced a state of oxidative stress in the brain as well as neuroinflammation. The solvent inhibited motor activity caused cognitive deficits and exerted depressant-like action. Histopathologic changes indicative of early neurodegeneration was seen in the cortex and hippocampus. These effects of toluene were reversible upon discontinuing toluene and were significantly improved by combination of vitamin E and C supplementation which considerably reduced oxidative stress, augmented antioxidant mechanism and exerted an antiapoptotic effect. The combination of vitamin E and C ameliorated the pathological and functional alterations resulting from toluene administration. Our results thus suggest that vitamin C and E by virtue of their antioxidant and antiapoptotic actions could be considered as an important neurotherapeutic agent in those who abuse toluene.

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