JOURNAL OF BASIC PHARMACOLOGY AND TOXICOLOGY

Original Research Article

Prevention of toluene-induced brain neurodegeneration by atropine and neostigmine

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Abstract

We aimed to examine the neuroprotective effect of atropine or neostigmine in a rat model of acute toluene toxicity. Rats received intraperitoneal injections (i.p.) of toluene at a dose of 500 mg/kg. Neostigmine (30, 60 or 90 µg/kg, i.p.) or atropine (1 or 2 mg/kg, i.p.) were administered immediately after toluene injection. Lipid peroxidation (malondialdehyde; MDA), nitric oxide, reduced glutathione (GSH) and acetylcholinesterase (AChE) activity were measured in brain homogenates. Histopathological study of brain was also done. Results indicated that compared with the vehicle control, toluene caused: 1) increased brain lipid peroxidation; 2) increased nitric oxide; 3) decreased reduced glutathione; 4) inhibition of AChE activity; 5) cortical Lewy bodies, neuronal loss and degenerated Purkinje cells in cerebellum. Treatment with either atropine or neostigmine prevented the increase in brain MDA and nitric oxide and the decrease in reduced glutathione induced by toluene. AChE activity increased by atropine and neostigmine at 60 or 90 µg/kg. Either drug afforded neuronal protection. These effects were dose-dependent. These results suggest that single injection of toluene causes neuronal cell death which involves excessive cholinergic stimulation and oxidative stress and which is amenable to treatment with atropine or neostigmine.

Key words: toluene; neurotoxicity; cholinergic stimulation; acetylcholinesterase; atropine; neostigmine

1 Introduction

Volatile solvent abuse is a major health problem, especially among children and adolescents both in developed and developing countries [1, 2]. One of the most commonly abused solvents is toluene, which is widely used in industry, gasoline, and in household commercial products such as glues, adhesives, paint thinners and removers, making the solvent an easily accessible and cheap substance of abuse [3, 4]. Because of its highly lipophilic properties, toluene is rapidly absorbed from the lung and widely distributed in tissues, especially the brain [5]. Acute intoxication is associated with euphoria and excitation which is followed by prolonged relaxation, motor incoordination, dizziness, coma and even death from respiratory depression [4]. Chronic exposure to toluene is associated with serious neurotoxic effects including cognitive impairment, ataxia, and dementia [6-8]. Cerebral and cerebellar atrophy and white matter changes are major neuropathological findings in these subjects [6,7]. The mechanism (s) underlying these neurotoxic effects of toluene is largely undetermined but accumulating evidence implicate oxidative stress [9, 10], excitotoxic neuronal damage [3], and decreased neurotrophic factors [11].

Oxidative stress develops when there is an imbalance between the rate in which reactive oxygen and nitrogen species are produced and the capacity of the antioxidant defenses in the cell. The result is oxidative damage to cell membrane lipids, nucleic acids and proteins [12]. In this context, several factors make the brain particularly vulnerable to oxidative injury. The brain tissue is rich in highly polyunsaturated fatty acid side-chains, the preferred target for free radicals. The high basal O_2 consumption results in increased production of superoxide ($O_2^{\bullet-}$) by the mitochondria. The presence of excitotoxic amino acids, autoxidizable monoamine neurotransmitters and the high content of the redox metal iron in certain brain regions are other sources of reactive free radicals [12, 13]. The presence of oxidative stress has been demonstrated in the brain of experimental animals following toluene exposure. Thus, studies demonstrated increased lipid peroxidation products and protein carbonyls in different brain regions following exposure to toluene in rats [14-16]. Solvent inhalation was also found to increase oxidative DNA damage in the liver

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and kidney of rats [17]. Furthermore, studies demonstrated increased 3-nitrotyrosine in the caudate nucleus and nucleus accumbens [18] and increased generation of reactive oxygen species (ROS) in crude mitochondrial fractions from rat cerebellum, striatum, and hippocampus following i.p. injection of toluene [19].

In the treatment of poisoning with toluene, no specific antidotes are available and management is essentially supportive [20]. Exposure to toluene was found to inhibit acetylcholinesterase (AChE), the enzyme which hydrolyzes the neurotransmitter acetylcholine (ACh) at the neuronal synapse and thus terminates its action [14, 21]. The decrease in AChE activity could contribute to toluene-induced neurotoxicity, possibly via an increase in extracellular ACh levels. In this study, we therefore, aimed to investigate the effect of the muscarinic receptor antagonist atropine and the reversible AChE inhibitor neostigmine on brain oxidative stress and neurodegeneration following toluene injection in rats. These agents and also physostigmine have been used to treat bradycardia in acute toluene poisoning [22].

2 Materials and methods

2.1 Animals

Male Sprague-Dawley rats, weighing between 160-170 g obtained from Animal House of the National Research Centre, Cairo, were group-housed under temperature- and light-controlled conditions with standard laboratory rodent chow and water *ad libitum*. 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 (Publication No. 85-23, revised 1985).

2.2 Drugs and chemicals

Toluene (Sigma-Aldrich, St Louis, MO, USA), atropine (Nile Pharmaceutical Co., ARE) and neostigmine (Amriya Pharam. Ind, ARE) were used in the study. Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.3 Experimental design

Rats were randomly divided into seven groups, six rats each. Group 1 received the vehicle (paraffin oil) and served as negative control. Group 2 received toluene in paraffin oil (vol/vol) in a dose 500 mg/kg, intraperitoneally (i.p.) and served as positive control. Groups 3 and 4 received toluene (500 mg/kg, i.p) along with atropine (1 or 2 mg/kg, i.p.). Groups 5, 6 and 7 received toluene (500 mg/kg, i.p) along with neostigmine (30, 60 or 90 μg/kg, i.p.). Atropine or neostigmine or vehicle was administered immediately after the injection of toluene. Rats were euthanized 2 hr later by decapitation under light ether anesthesia for tissue collection. The brains were then quickly dissected out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, and stored at −80 °C. The brain was homogenized with 0.1 M phosphate buffer saline at pH 7.4 to give final concentration of 10% w/v for the biochemical assays.

2.4 Biochemical analysis

2.4.1 Lipid peroxidation

Lipid peroxidation products in the brain homogenates were assayed by measuring the level of malondialdehyde (MDA) where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having a peak absorbance at 532 nm [23].

2.4.2 Nitric oxide

The level of nitric oxide, measured as nitrite, was determined using the Griess reagent. Nitrite, a stable end-product of nitric oxide, is used as an indicator of the production of nitric oxide. In this assay, nitrate is converted to nitrite by nitrate reductase. The Griess reagent then reacts with nitrite forming a deep purple azo compound. The absorbance is read at 540 nm using a spectrophotometer [24].

2.4.3 Reduced glutathione

Brain reduced glutathione (GSH) was determined in homogenates according to Ellman et al. [25]. The procedure is based on the reduction of Ellman´s reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid, which is intense yellow in color and determined spectrophotometrically at 412 nm.

2.4.4 Acetylcholinesterase activity

The procedure used was a modification of the method of Ellman et al. [26] as described by Gorun et al. [27]. The principle of the assay involves the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color is read immediately at 412 nm.

2.5 Histological study

The brain from different groups was removed and fixed in 10% formol saline. 5 μm thick paraffin sections were stained with haematoxylin and eosin. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo).

2.6 Statistical analysis

Results are presented as mean ± SEM. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for multiple group comparison. Statistical significance was considered at a probability value of less than 0.05. GraphPad

Prism 6 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA) was used.

3 Results

3.1 Biochemical results

3.1.1 Lipid peroxidation

In the toluene only treated group, the MDA content in brain tissue increased significantly by 51.7% compared with the vehicle group (29.3 ± 1.0 vs. 19.31 ± 0.98 nmol/g tissue). Either atropine or neostigmine inhibited lipid peroxidation (MDA) induced by toluene. The level of MDA decreased by 42.4% and 53.9% after treatment with 1 and 2 mg/kg atropine, respectively and decreased by 57.2% and 56.8% by 60 and 90 µg/kg neostigmine, respectively (Fig. 1).

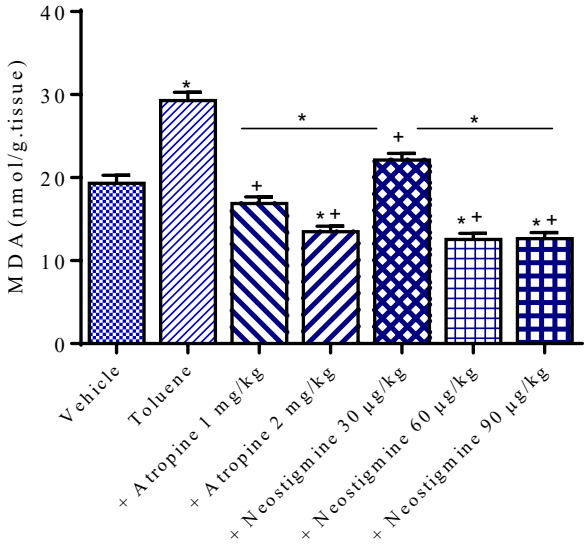

Figure 1. Effect of atropine or neostigmine on the level of malondialdehyde (MDA) in brain of toluene-treated rats. Results are mean ± SEM (n = 6). $^{*}p < 0.05$ versus vehicle and between different groups as shown on the graph; $^{+}p < 0.05$ versus toluene control group.

3.1.2 Nitric oxide

In the brain tissue of toluene only treated group, the level of nitric oxide significantly increased by 37.8% compared to the vehicle control value (29.5 ± 1.58 vs. 21.4 ± 0.85 µmol/g tissue). Atropine treatment given immediately after toluene injection reduced nitric oxide levels by 30% and 42.0%, respectively, compared with the toluene only group. Similarly, treatment with neostigmine at doses of 60 and 90 µg/kg decreased brain nitric oxide levels by 32.5% and 56.2%, respectively (Fig. 2).

3.1.3 Reduced glutathione

The GSH level in the brain of toluene only treatment group was significantly lower compared to that of the vehicle group (43.0% decrease; 2.05 ± 0.13 vs. 3.6 ± 0.11 µmol/g tissue). The administration of 1 and 2 mg/kg atropine increased GSH level by 28.3% and 21.5%, respectively, compared with the toluene only group. Meanwhile, treatment with neostigmine at doses of 30, 60 or 90 µg/kg resulted in 36.1%, 53.6% and 64.9% increments in brain GSH, respectively (Fig. 3).

3.1.4 Acetylcholinesterase activity

The activity of AChE in brain was significantly lower by 78.4% in rats treated with toluene compared with the vehicle control. The administration of atropine at 1 or 2 mg/kg significantly increased AChE activity in brain homogenates by 127.1% and 290%, respectively, compared with the toluene only group. Similarly, neostigmine given at 60 or 90 µg/kg increased brain AChE activity by 158.6% and 181.4%, respectively (Fig. 4).

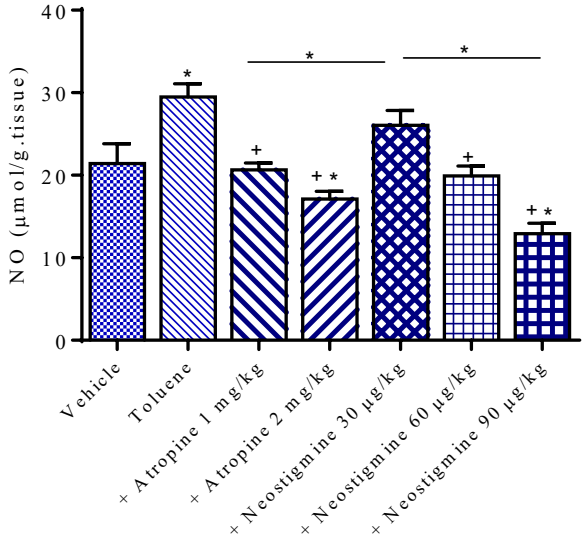

Figure 2. Effect of atropine or neostigmine on brain nitric oxide (NO) in toluene-treated rats. Results are mean ± SEM (n = 6). $^{*}p < 0.05$ versus vehicle and between different groups as shown on the graph; $^{+}p < 0.05$ versus toluene control group.

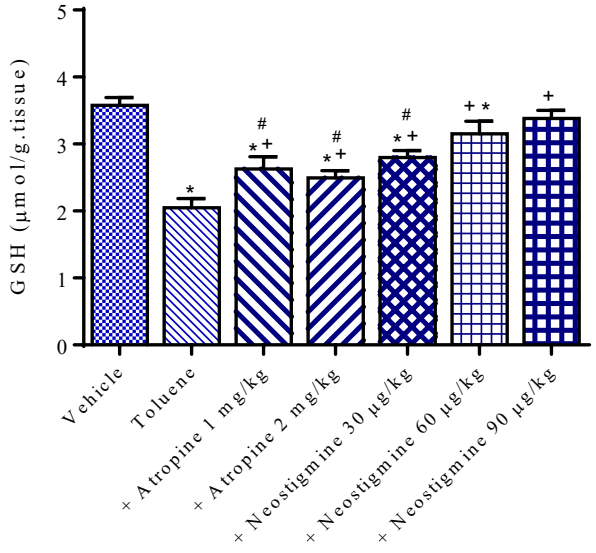

Figure 3. Brain reduced glutathione (GSH) in rats treated with toluene alone or with atropine or neostigmine. Results are mean ± SEM (n = 6). $^{*}p < 0.05$ versus vehicle; $^{+}p < 0.05$ versus toluene control group; $^{\#}p < 0.05$ versus neostigmine 90 µg/kg.

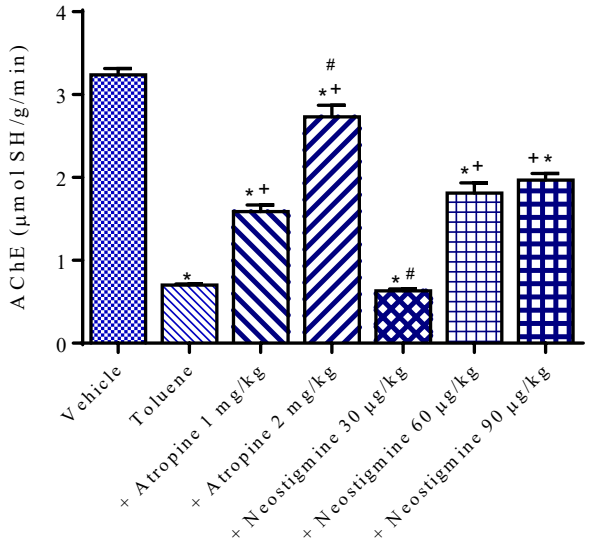

Figure 4. Brain acetylcholinesterase activity (AChE) in rats treated with toluene alone or with atropine or neostigmine. Results are mean ± SEM (n = 6). $^{*}p < 0.05$ versus vehicle; $^{+}p < 0.05$ versus toluene control group; $^{\#}p < 0.05$ versus neostigmine 90 µg/kg.

3.2 Histological results of brain

3.2.1 Cerebral cortex

H&E stained sections of the cerebral cortex from the vehicle control group showed normal neurons having either single or double nuclei with prominent nucleoli surrounded with basophilic cytoplasm. No reactive gliosis is present and few capillaries are also seen (Fig. 5A). Sections from rats given toluene only showed cortical Lewy bodies, neuronal loss and glial cells. Some neurons appeared necrotic; others had red coloration (hypereosinophilic) which is due to pyknosis or degradation of the nucleus and loss of Nissl bodies (Fig. 5B &C). Sections from rats treated with toluene along with atropine at 1 mg/kg showed some neurons appearing normal, others appearing eosinophilic and degenerated (Fig. 5D). With the higher dose of atropine, most neurons appeared normal, but some appeared necrotic and kayorrhatic (Fig. 5E). The cerebral cortex of rats treated with toluene along with neostigmine at 30 µg/kg showed that the brain tissue still suffered from pathological changes in the form of necrotic, hypereosinophilic neurons while some neurons exhibited chromatolysis (Fig. 5F). The higher dose of neostigmine (90 µg/kg) induced more improvement in pathological changes in the form of normal appearing neurons although congestion of cerebral blood vessel was still present (Fig. 5G).

3.2.2 Cerebellum

In sections from the vehicle group, the Purkinje cells in cerebellum appeared normal (Figs. 6A). Toluene caused degeneration of Purkinje cells in cerebellum (Figs. 6B). After treatment with atropine at 1 mg/kg, the Purkinje cells still suffered from degeneration, few appeared normal and there was white matter vacuolization (Fig. 6C). After treatment with the higher dose of atropine, most of Purkinje cells appeared normal, but white matter vacuolization and congestion in blood vessel were seen (Fig. 6D). With neostigmine being given at 30 µg/kg, Purkinje cells appeared normal while vacuolization in white matter and congestion in blood vessel were seen (Fig. 6E). The higher dose of 90 µg/kg was associated with normal Purkinje cells in cerebellum (Fig. 6F).

4 Discussion

Toluene is one of the most commonly abused organic solvents worldwide. Chronic abuse has been reported to cause cognitive impairment [28]. This study provided the evidence that even brief exposure to high dose of the solvent can result in neuronal loss. We showed that a single injection of toluene caused cerebral neuronal necrosis. An earlier report demonstrated that the cerebral cortex from rats given toluene i.p., at a dose of 900 mg/kg for two consecutive days exhibited death and apoptotic neurons, perineuronal vacuolation, and glial cells [29]. Other studies reported the presence of darkly stained neurons, DNA fragmentation, neurons with acidified cytoplasm and undefined nucleoli and cerebral oedema in brain from rats treated with the solvent i.p., at 900 mg/kg for 6-9 consecutive days [14, 16]. Toluene (1.3 ml/kg) given i.p., for 5 consecutive days was also found to result in pyknotic

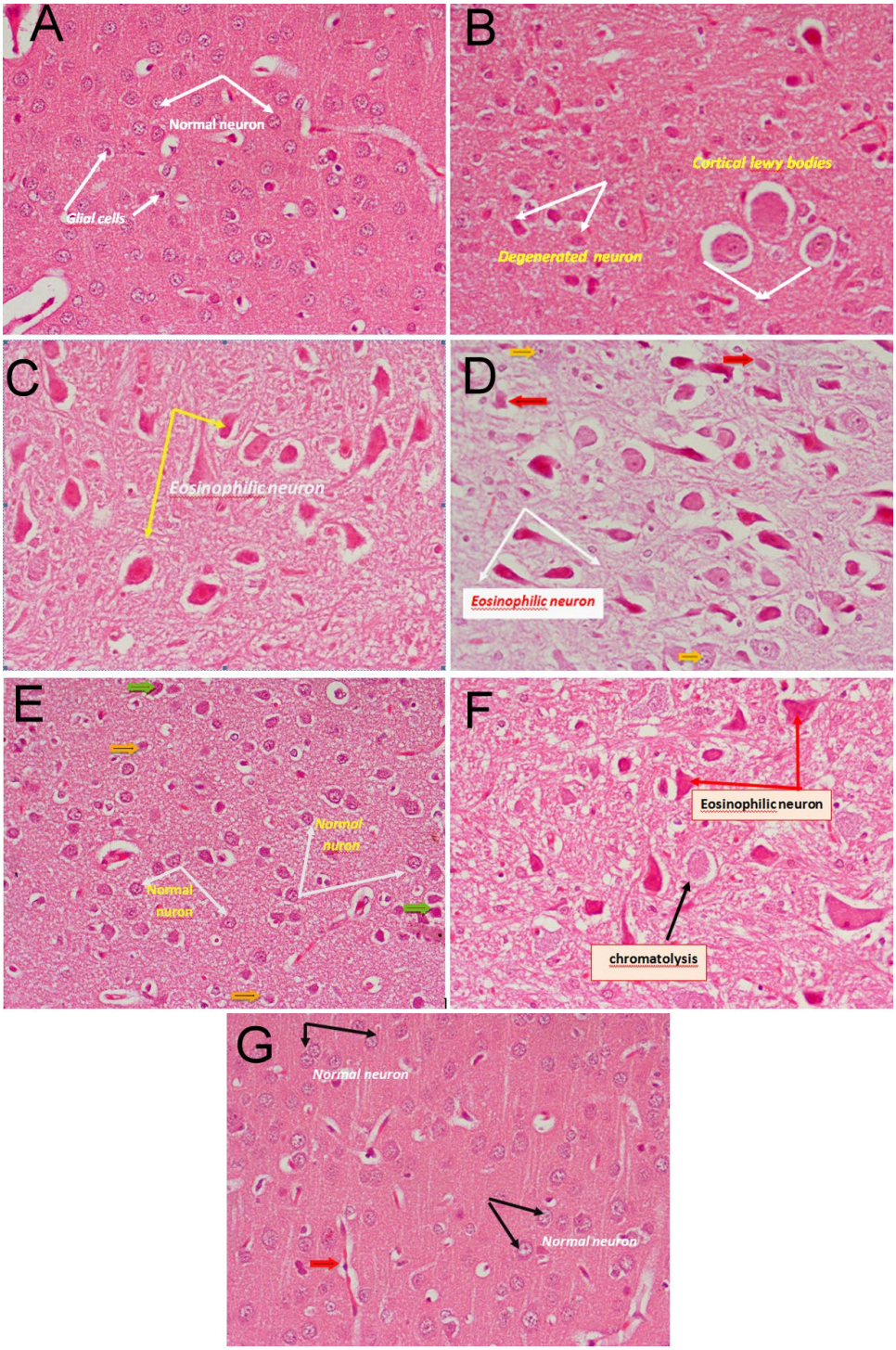

Figure 5. Representative photomicrographs of the cerebral cortex after treatment with (A) vehicle showing normal histological structure of the cerebral cortex; (B) toluene only showing Lewy bodies and degenerated neurons; (C) another filed toluene only showing eosinophilic neuron; (D) toluene along with atropine at 1 mg/kg showing some neurons appearing normal (orange arrow), others easinophilic and some degenerated (red arrow); (E) toluene along with atropine at 2 mg/kg showing most of neuron appearing normal, few appeared degenerated in the form of necrosis (orange arrow) and kayorrhatic (green arrow); (F) toluene along with neostigmine at 30 µg/kg showing hypereosinophilic neurons (red arrows) and some neurons with chromatolysis (black arrow); (G) toluene along with neostigmine at 90 µg/kg showing normal neurons and congestion of cerebral blood vessel (red arrow) (Hx&E x400).

neurons, infiltrative cells, glial cells and cerebral oedema [30]. Shrinkage of neurons and vacuolation in neuropil in prefrontal cortex were reported by Meydan et al. [31] in rats injected i.p., with 0.5 ml/kg of toluene for 2 weeks. Moreover, Duncan et al. [32] exposed adolescent rats to toluene (3000 p.p.m.) for 8 weeks and observed white matter abnormalities. Chronic toluene abusers showed evidence of cerebellar degeneration in the form of intention tremors, unstable gait and nystagmus [33, 34]. In the present study and in previous reports [29], we demonstrated degeneration of cerebellar Purkinje cells following toluene injection in the rat, suggesting that inhalant abuse may lead

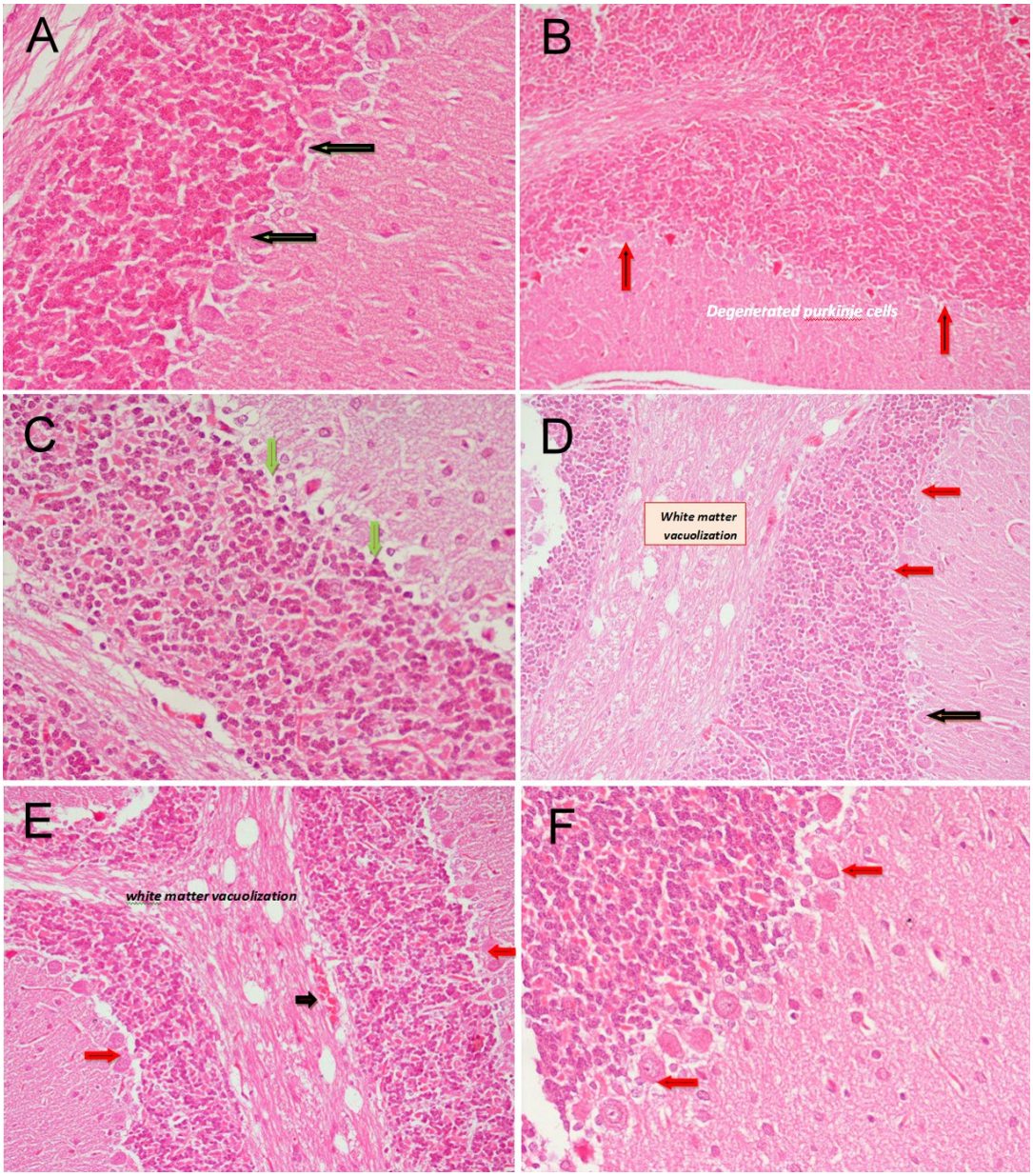

Figure 6. Representative photomicrographs of the cerebellum after treatment with (A) vehicle showing the normal Purkinje cells (black arrow); (B) toluene only showing degeneration in most of Purkinje cells; (C) toluene along with atropine at 1 mg/kg revealing degenerated Purkinje cells; (D) toluene along with atropine at 2 mg/kg showing Purkinje cells still suffer from degeneration (red arrow), few appeared normal (black arrow) with white matter vacuolization; (E) toluene along with neostigmine at 30 µg/kg showing that some Purkinje cells appeared normal (red arrow), vacuolation in white matter and congestion in blood vessel (black arrow); (F) toluene along with neostigmine at 90 µg/kg showing normal Purkinje cells (red arrow) (HX&Ex400).

to impaired cerebellar function as a result of Purkinje cell affection with prolonged exposure.

In the present study, the administration of toluene was associated with increased brain malondialdehyde, an end product of lipid peroxidation [35], coupled with depletion of reduced glutathione. Glutathione (γ-L-glutamyl-L-cysteinylglycine) is the most important brain antioxidant and free radical scavenger which maintains the redox balance in the cell by shuttling between its reduced (GSH) and oxidized form (glutathione disulfide or GSSG). Glutathione protects cells by directly scavenging superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($ONOO^-$). Glutathione also detoxifies hydroperoxides, and lipid peroxides via enzymatic reactions involving glutathione peroxidase (GPX) and peroxiredoxins [36, 37]. Our findings suggest that increased free radical generation in brain of toluene intoxicated rats. Oxidative stress is a major contributor to brain damage after exposure to toluene. Reactive oxygen species (ROS) generated during toluene catabolism involves cytochrome P450 oxidation and aldehyde dehydrogenase oxidation resulting in superoxide anion and subsequently formation of hydroxyl radical [19]. Gerasimov et al. [38] showed that exposure to toluene (3000 ppm) increases extracellular dopamine levels in prefrontal cortex of rats. The increase in ROS formation after toluene, therefore, may also be due to autoxidation of monoamine neurotransmitters. Other studies showed increased brain

peroxidation associated with a reduction in the antioxidant and free radical scavenger reduced glutathione and in antioxidant enzymatic activities eg., superoxide dismutase and glutathione peroxidase after toluene exposure in rats [14, 16, 31]. Tokunaga et al. [17] exposed rats to toluene for one week and observed increased 8-hydroxy-2′-deoxyguanosine in liver and kidney indicative of oxidative damage to DNA. The findings that treatment with antioxidants such as vitamin C, vitamin E and omega-3 can reduce oxidative stress and neurodegeneration in the brain of toluene-exposed rats lends further support to the pivotal role of free radical mediated damage in toluene neurotoxicity [14, 31].

Our results also show that nitric oxide levels were increased in brain of toluene intoxicated rats which is in accordance with previous studies [16, 29]. Riegel et al. [18] found increased 3-nitrotyrosine in nucleus accumbens 2 hr after a single i.p., injection of 600 mg/kg toluene and in the caudate nucleus and nucleus accumbens 24 hr after repeated administration of toluene for one week in rats, thereby, suggesting the involvement of nitric oxide generation in inducing the toluene neurotoxicity. In addition to the physiological roles of brain nitric oxide as a signaling molecule and a vasodilator, excessive amounts of nitric oxide generated during pathological states mediates neurotoxicity. Nitric oxide reacts with several free radicals including the superoxide radical ($O_2^{\bullet-}$) resulting in the formation of the strong oxidant peroxynitrite (ONOO). It also reacts with oxygen resulting in more reactive nitrogen species such as dinitrogen trioxide (N_2O_3), and nitrogen dioxide (NO_2) capable of causing nitrosylation and/or nitration of proteins and lipids [39, 40].

There is evidence that toluene inhibits acetylcholinesterase (AChE), the enzyme which terminates the action of the neurotransmitter acetylcholine (ACh) at the neuronal synapse [21] which will result in increased extracellular ACh levels. In this study, we demonstrated significant and marked inhibition of AChE activity in brain from toluene-treated rats. Atef et al. [14] found that toluene induced significant decrease in AChE and butyrylcholinesterase (BChE) activities in cerebral cortex, striatum and rest of the brain tissue. We have also shown that toluene (i.p.) caused significant inhibition of brain and serum BChE activity by 34.6% and 44.7%, respectively [16, 29]. BChE also hydrolyzes ACh and many other substances as well [41]. Wang et al. [42] reported decreased AChE, choline acetyltransferase (ChAT) and ACh levels in mice exposed to volatile organic compounds including toluene. Moreover, Bale et al. [43] showed that nicotinic acetylcholine receptors (nAChRs) are inhibited by toluene. ACh is important in cognitive functioning including attention and memory [44]. The action of toluene on the cholinergic system might thus be involved in the cognitive changes in exposed individuals.

In this study, we investigated the effect of the muscarinic receptor antagonist atropine and the reversible AChE inhibitor neostigmine on brain oxidative stress and neurodegeneration following toluene injection in rats. The muscarinic anticholinergic drug atropine is the antidote used in the treatment of acute organophosphorus toxicity by blocking the effect of increased ACh the muscarinic cholinergic synapses [45]. Neostigmine is a short acting reversible anticholinesterae used in the treatment of myasthenia gravis and to reverse nondepolarizing neuromuscular blocking agents [46]. Neostigmine binds to the anionic site and reversibly inhibits AChE, thereby, acting as a competitive inhibitor of endogenous ACh [47]. Given as a pretreatment, neostigmine or physostigmine antagonized the inhibition of cholinesterase by the organophosphorus compound parathion; the effect being due to carbamylation of AChE making it unavailable to phosphorylation by organophosphates [48]. Our results show that either atropine or neostigmine was able to afford neuroprotection which involves decreased free radicals evidenced by the inhibition of lipid peroxidation and nitric oxide release along with sparing of the antioxidant reduced glutathione in brain tissues. These effects of atropine or neostigmine could be due to their effects in preventing the action of increased ACh on cholinergic receptors. Other mechanisms might also be involved in the effects of atropine or neostigmine observed in the present study. Bitzinger et al. [49] reported that treatment with neostigmne (75 μg/kg) or physostigmine (100 μg/kg) significantly reduced the production of ROS from rat polymorphonuclear neutrophils during sepsis. Pollak et al. [50] showed that the anticholinesterases rivastigmine, tacrine and neostigmine inhibited the release of the inflammatory procytokine interleukin-1β in hippocampus and blood. Moreover, neostigmine was shown to reduce the inflammatory procytokine tumour necrosis factor-alpha and to increase interleukin-10 levels in heart tissues after pressure overload [51].

In summary, the present study showed that a single systemic injection of toluene in rats produced cerebral neuronal necrosis and degeneration of cerebellar Purkinje cells which was associated with increased brain oxidative stress and inhibition of AChE activity. The toluene-induced histopathological and biochemical alterations were prevented by treatment with atropine or neostigmine, suggesting that these agents might have a value in preventing neuronal injury in acute toluene toxicity and possibly the delayed neurotoxic consequences.

Conflicts of interest

The authors declare that there are no potential conflicts of interest.

Financial support

This work was not supported by research grants.

References

1. Filley CM. Toluene abuse and white matter a model of toxic leukoencephalopathy. Psychiatr Clin N Am. 2013; 36: 293–302. http://dx.doi.org/10.1016/j.psc.2013.02.008
2. Elkoussi A, Bakheet S. Volatile substance misuse among street children in Upper Egypt. Subst Use Misuse. 2011; 46 (Suppl 1): 35-39.
3. Eisenberg DP. Neurotoxicity and mechanism of toluene abuse. Einstein Quart J Biol Med. 2003; 19:150–159.
4. Lubman DI, Yucel M, Lawrence AJ. Inhalant abuse among adolescents: neurobiological considerations. Br J Pharmacol. 2008; 154: 316–326
5. Gerasimov MR. Brain uptake and biodistribution of [11C]toluene in nonhuman primates and mice. Methods Enzymol. 2004; 385: 334–349.
6. Rosenberg NL, Kleinschmidt BK, Davis KA, Dreisbach JN, Hormes JT, Filley CM. Toluene abuse causes diffuse central nervous system white matter changes. Ann Neurol. 1988;23:611–614.
7. Aydin K, Sencer S, Demir T, Ogel K, Tunaci A, Minareci O. Cranial MR findings in chronic toluene abuse by inhalation. AJNR Am J Neuroradiol. 2002; 23:1173–1179.
8. Fornazzari L, Pollanen MS, Myers V, Wolf A. Solvent abuse-related toluene leukoencephalopathy. J Clin Forensic Med. 2003; 10(2), 93–95. doi: 10.1016/S1353-1131(03)00035-X
9. Mattia CJ, LeBel CP, Bondy SC. Effects of toluene and its metabolites on cerebral reactive oxygen species generation. Biochem Pharmacol 1991; 42: 879-882.
10. Burmistrov SO, Arutyunyan AV, Stepanov MG, Oparina TI, Prokopenko VM. Effect of chronic inhalation of toluene and dioxane on activity of free radical processes in ratovaries and brain. Bull Exp Biol Med. 2001; 132(3): 832-6.
11. Gotohda T, Tokunaga I, Kitamura O, Kubo S. Toluene inhalation induced neuronal damage in the spinal cord and changes of neurotrophic factors in rat. Leg Med (Tokyo). 2007; 9(3):123–127. doi: 10.1016/j.legalmed.2006.11.014.
12. Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem. 1992; 59(5):1609–23. doi: 10.1111/j.1471-4159.1992.tb10990.x.
13. Floyd RA, Hensley K. Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging. 2002; 23: 795-807.
14. Atef MM, Galal AF, Abdel-Salam OME, Shafee N, Tadros MG, Khalifa AE. Neurobehavioral and neurochemical changes in toluene-treated rats and the effect of antioxidants. World J Pharmaceutical Res. 2015; 4(12):309–356.
15. Kodavanti PR, Royland JE, Moore-Smith DA, Besas J, Richards JE, Beasley TE, et al. Acute and subchronic toxicity of inhaled toluene in male Long-Evans rats: oxidative stress markers in brain. Neurotoxicology. 2015; 51:10–19. doi: 10.1016/j.neuro.2015.09.001.
16. Abdel-Salam OME, Youness ER, Morsy FA, Yassen NN, Mohammed NA, Sleem AA. Methylene blue protects against toluene-induced brain damage: involvement of nitric oxide, NF-κB, and caspase-3. Reactive Oxygen Species. 2016; 2(5): 371–87. doi: 10.20455/ros.2016.855.
17. Tokunaga I, Gotohda T, Ishigami A, Kitamura O, Kubo S. Toluene inhalation induced 8-hydroxy-2'-deoxyguanosine formation as the peroxidative degeneration in rat organs. Leg Med (Tokyo). 2003; 5(1):34–41.
18. Riegel AC,Ali SF,Torinese S, French ED. Repeated exposure to the abused inhalant toluene alters levels of neurotransmitters and generates peroxynitrite in nigrostriatal and mesolimbic nuclei in rat. Ann NY Acad Sci. 2004; 1025: 543–551. doi: 10.1196/annals.1316.079
19. Mattia CJ, Adams JD, Jr., Bondy SC. Free radical induction in the brain and liver by products of toluene catabolism. Biochem Pharmacol. 1993; 46(1):103–10. doi: 10.1016/0006-2952(93)90353-x.
20. Anderson, CE, Loomis, GA. Recognition and prevention of inhalant abuse. Am Fam Physician. 2003; 68: 869-74,876.
21. Engelke M, Diehl H, Tahti H. Effects of toluene and n-hexane on rat synaptosomal membrane fluidity and integral enzyme activities. Pharmacol Toxicol. 1992; 71(5):343–347.
22. Clinical Adult Neurology. Corey-Bloom J, David RB (Eds). Demos Medical; 3rd edition. 2009.
23. Nair V, Turner GA. The thiobarbituric acid test for lipid peroxidation: structure of the adduct with malondialdehyde. Lipids. 1984; 19: 804-805.
24. Archer S. Measurement of nitric oxide in biological models. FASEB J. 1993; 7(2): 340-360.
25. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959; 82(1):70–77. doi: 10.1016/0003-9861(59)90090-6.
26. Ellman GL, Courtney KD, Andres V, Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7:88–95. doi: 10.1016/0006-2952(61)90145-9.
27. Gorun V, Proinov I, Baltescu V, Balaban G, Barzu O. Modified Ellman procedure for assay of cholinesterases in crude enzymatic preparations. Anal Biochem. 1978; 86(1): 324-326.
28. Yucel M, Takagi M, Walterfang M, Lubman DI. Toluene misuse and long-term harms: A systematic review of the neuropsychological and neuroimaging literature. Neurosci Biobehav Rev. 2008;32(5):910-26. doi: 10.1016/j.neubiorev.2008.01.006.
29. Abdel-Salam OME, Sleem AA, Youness ER, Morsy FA. Exacerbation of toluene's neuro- and hepato-toxicity by amiodarone or chlorpropamide: Involvement of oxidative stress. Reactive Oxygen Species. 2019; 8(24):358–371.
30. Tumer AR, Atilla P, Onbasilar I, Biberoglu G, Muftuoglu S. Protective role of vitamin E on the harmful effects of toluene on brain tissue. Neurosciences. 2007; 12 (3): 191-197.
31. Meydan S, Altas M, Nacar A, Ozturk OH, Tas U, Zararsiz I, et al. The protective effects of omega-3 fatty acid against toluene-induced neurotoxicity in prefrontal cortex of rats. Hum Exp Toxicol. 2012; 31(11):1179–85. doi: 10.1177/0960327112457187.
32. Duncan JR, Dick ALW, Egan G, Kolbe S, Gavrilescu M, Wright D, et al. Adolescent Toluene Inhalation in Rats Affects White Matter Maturation with thePotential for Recovery Following Abstinence. PLoS ONE. 2012; 7(9): e44790. doi:10.1371/journal.pone.0044790
33. Isa MFM, Zain NRM, Gaillard F, Chee FKY. Toluene dependency, psychosis, and cerebellar syndrome. J Neuropsychiatry Clin Neurosci. 2013; 25:2, E42-43.
34. Gmaz JM, McKay BE. Toluene decreases Purkinje cell output by enhancing inhibitory synaptic transmission in the cerebellar cortex. Neurosci Lett. 2014; 560: 1– 6
35. Gutteridge JMC: Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem. 1995;41:1819–1828.
36. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004; 134: 489–492.
37. Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. Arch Physiol Biochem. 2007;113(4-5):234-58.DOI: 10.1080/13813450701661198
38. Gerasimov MR, Schiffer WK, Marstellar D, Ferrieri R, Alexoff D, Dewey SL. Toluene inhalation produces regionally specific changes in extracellular dopamine. Drug Alcohol Depend. 2002; 65(3):243-51.
39. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007; 87(1):315–424. doi: 10.1152/physrev.00029.2006.
40. Brown GC. Nitric oxide and neuronal death. Nitric Oxide. 2010; 23(3):153–65. doi: 10.1016/j.niox.2010.06.001.
41. Massoulie J, Sussman J, Bon S, Silman I. Structure and functions of acetylcholinesterase and butyrylcholinesterase.

Prog Brain Res. 1993; 98:139–146. doi: 10.1016/s0079-6123(08)62391-2.
42. Wang F, Li C, Liu W, Jin Y. Potential mechanisms of neurobehavioral disturbances in mice caused by sub-chronic exposure to low-dose VOCs. Inhal Toxicol. 2014;26(4):250-258. doi: 10.3109/08958378.2014.882447.
43. Bale AS, Meacham CA, Shafer TJ, Bushnell PJ. Inhibitory effects of volatile organic compounds on neuronal nicotinic acetylcholine receptors. Presented at Society for Neuroscience, New Orleans, LA, 11/8-12/2003.
44. Wilkinson DG, Francis PT, Schwam E, Payne-Parrish J. Cholinesterase inhibitors used in the treatment of Alzheimer's disease: the relationship between pharmacological effects and clinical efficacy. Drugs Aging. 2004; 21:453–478.
45. Jokanovi M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. Toxicol Lett. 2009; 190(2):107-115.
46. Colovic MB, Krstic DZ, Lazarevic- Pasti TD, Bondzic AM, Vasic VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol. 2013; 11(3): 315–335. doi: 10.2174/1570159X11311030006
47. Watchtel RE. Comparison of anticholinesterases and their effects on acetylcholine-activated ion channels. Anesthesiology. 1990;72:496-503.
48. Jun JW, Kim YC. Protective effect of physostigmine and neostigmine against acute toxicity of parathion in rats. Arch Pharm Res. 1991; 14(4): 330-335.
49. Bitzinger DI, Gruber M, Tümmler S, Malsy M, Seyfried T, Weber F et al. In vivo effects of neostigmine and physostigmine on neutrophil functions and evaluation of acetylcholinesterase and butyrylcholinesterase as inflammatory markers during experimental sepsis in rats. Mediators of Inflammation 2019, Article ID 8274903. https://doi.org/10.1155/2019/8274903
50. Pollak Y, Gilboa A, Ben-Menachem O, Ben-Hur T, Soreq H, Yirmiya R. Acetylcholinesterase inhibitors reduce brain and blood interleukin-1beta production. Ann Neurol. 2005;57:741–745.
51. Freeling J, Wattier K, LaCroix C, Li YF. Neostigmine and pilocarpine attenuated tumour necrosis factor α expression and cardiac hypertrophy in the heart with pressure overload. Exp Physiol. 2007; 93(1): 75–8.DOI: 10.1113/expphysiol.2007.039784