

Methylene Blue Protects against Toluene-Induced Brain Damage: Involvement of Nitric Oxide, NF- κ B, and Caspase-3

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ABSTRACT | The effect of methylene blue on brain oxidative stress and neuronal damage after toluene poisoning in rats was investigated. Rats were treated intraperitoneally with toluene at 900 mg/kg either alone or in combination with methylene blue at 5, 10, or 20 mg/kg daily for 6 days. The levels of malondialdehyde, nitrite, and reduced glutathione (GSH), and the activity of paraoxonase-1 (PON1) were evaluated in the brain and serum. We also measured serum butyrylcholinesterase (BChE) activity, brain derived neurotrophic factor (BDNF), and nuclear factor kappa-B (NF- κ B). Histopathological examination and immunohistochemical staining for caspase-3 and glial fibrillary acidic protein (GFAP) in the brain were performed. Toluene-treated rats exhibited increased lipid peroxidation (malondialdehyde) and nitrite levels in the brain and serum and reduced brain GSH level. There were also decreased PON1 and BChE activities, decreased BDNF level, and increased NF- κ B level in the serum after toluene exposure. Neurodegeneration, cerebral edema, and marked degeneration of Purkinje cells were observed. There was increased caspase-3 immunostaining, but decreased immunostaining for GFAP in glial cells. Methylene blue induced a significant decrease in the levels of malondialdehyde and nitrite in the brain and serum of toluene-exposed rats. The GSH level did not change, but PON1 activity in the brain and serum increased. Methylene blue also decreased NF- κ B level, increased BChE activity, and increased BDNF level in a dose-dependent manner. Methylene blue protected against the neuronal damaging effects of toluene, decreased caspase-3 immunoreactivity, and restored GFAP positivity. These findings indicate a neuroprotective effect for methylene blue against the brain damage induced by toluene. This protection is likely to involve the inhibition of nitric oxide, NF- κ B, and caspase-3 by methylene blue treatment.

KEYWORDS | Apoptosis; Glial cells; Methylene blue; Nitric Oxide; Neuronal damage; Nuclear factor kappa-B; Oxidative stress; Toluene

ABBREVIATIONS | BChE, butyrylcholinesterase; BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GSH, reduced glutathione; H&E, hematoxylin and eosin; i.p., intraperitoneally; MDA, malondialdehyde; NF- κ B, nuclear factor kappa-B; PON1, paraoxonase-1; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; TNF- α , tumor necrosis factor- α

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1. INTRODUCTION

Solvent abuse is an important health problem worldwide, especially among children and adolescents [1]. One commonly abused solvent is methyl benzene or toluene found in glues/adhesives, varnishes, and paints [2]. Owing to its lipophylicity, toluene readily crosses the blood brain barrier with consequent effects on behavior, cognition, and neuronal integrity [3]. The acute effects include cheerfulness followed by relaxation and lethargy. Motor impairment in the form of ataxia and confusion, which might proceed to coma and death, can also develop [4, 5]. Chronic abuse also results in serious and long-lasting effects

on the central nervous system, including white matter abnormalities, brain atrophy, leukoencephalopathy, and dementia [6, 7]. Studies in rodents indicated a decreased motor activity, the development of depressive-like behavior, impaired memory [8, 9], hippocampal neuronal loss, and decreased neurogenesis [8, 10]. Oxidative stress has been implicated in the development of toluene neurotoxicity. Studies indicated that toluene (0.5–1.5 g/kg, via intraperitoneal injection) increased the generation of reactive oxygen species (ROS) in crude mitochondrial fractions from rat cerebellum, striatum, and hippocampus [11]. Lipid peroxidation products increased in the hippocampus, cortex, and cerebellum [12] and protein car-

bonyls increased in the frontal cortex and cerebellum of rats following weeks of toluene inhalation [13]. Oxidative DNA damage has also been detected in the liver and kidney of rats after toluene inhalation for 7 days [14].

Methylthioninium chloride or methylene blue dye is used in humans for treating methemoglobinaemia, reducing methemoglobin to hemoglobin [15] and also as an antidote for cyanide poisoning [16]. Other clinical applications for the dye include the treatment of refractory hypotension due to sepsis in adults [17] and infants [18], and treatment of encephalopathy associated with the chemotherapeutic agent ifosfamide [19]. Recently, methylene blue has received much attention in view of studies suggesting the usefulness of the dye in treating mitochondrial dysfunction [20]. The latter is involved in the pathogenesis of several neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease [21]. Methylene blue displayed neuroprotective effects against retinal neurodegeneration induced by intravitreal rotenone injection in mice [22], spinal cord injury due to ischemia-reperfusion in rabbits [23], neurodegeneration in mice model of Huntington's disease [24], and rotenone-induced nigrostriatal cell loss in rats [25]. This neuroprotective action of methylene blue is thought to result from its decreasing the production of superoxide anion and hence protection of the mitochondria, increased oxygen consumption, and decreased anaerobic glycolysis [20, 26]. Other suggested mechanisms include nitric acid blockade [27], as well as decreased tumor necrosis factor- α (TNF- α) and caspase-3 activity (decreased apoptosis) [25].

In the present study we investigated the effects of methylene blue on oxidative stress and neuronal damage in the brain of rats exposed to toluene.

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats, obtained from the animal house of the National Research Centre (Cairo, Egypt), weighing 130–140 g, were group-housed under temperature- and light-controlled conditions with standard laboratory rodent chow and water provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the Nation-

al Research Centre and followed the recommendations of the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and Chemicals

Methylene blue, toluene, and other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO, USA).

2.3. Study Design

Rats were allocated into different groups ($n = 7$ per group) and treated with toluene at a dose of 900 mg/kg intraperitoneally (i.p.) daily for 6 days. Starting at the time of toluene exposure, rats were also treated with methylene blue at 5, 10, or 20 mg/kg or saline (control group) given via the intraperitoneal route. A 5th group ($n = 7$) received the vehicle (paraffin oil), i.p., daily for 6 days, while a 6th group received methylene blue only at a dose of 20 mg/kg and was subjected to pathological examination only. Rats were euthanized on the 7th day of different treatments, and their brains were then dissected out on an ice-cold plate and stored at -80°C for further biochemical measurements.

2.4. Determination of Lipid Peroxidation

Lipid peroxidation was assessed by measuring the level of malondialdehyde (MDA) according to the method of Ruiz-Larrea et al. [28] in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex that exhibits a peak absorbance at 532 nm.

2.5. Determination of GSH

Brain GSH level was determined spectrophotometrically by the Ellman's method [29]. The procedure is based on the reduction of the Ellman's reagent by $-SH$ groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which is determined spectrophotometrically at 412 nm.

2.6. Determination of Nitric Oxide

Nitrite, the stable end product of nitric oxide, is mostly used as an indicator for the production of ni-

tric oxide. Nitric oxide measured as nitrite was determined using the Griess reagent, according to the method described by Moshage et al. [30].

2.7. Determination of PON1 Activity

The arylesterase activity of PON1 was measured spectrophotometrically using phenyl acetate as a substrate [31, 32]. In this assay, PON1 catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C. The working mix consisted of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl₂ and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer were added to the above mix and the changes in absorbance were recorded following a 20 s lag time. One unit of arylesterase activity is equal to 1 µmole of phenol formed per minute. The PON1 activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 M⁻¹cm⁻¹. Blank samples containing water were used to correct for the spontaneous hydrolysis of phenyl acetate.

2.8. Determination of BChE Activity

Butyrylcholinesterase (BChE) activity in the brain tissue supernatants was determined by a colorimetric method using butyrylcholinesterase diagnostic kit (Chronolab, Barcelona, Spain). The principle of the method is that BChE hydrolyzes butyrylcholine to butyrate and thiocholine. The latter reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) forming 5-mercapto-2-nitrobenzoic acid (5-MNBA). The rate of 5-MNBA formation, measured spectrophotometrically, is proportional to the enzymatic activity of BChE in the sample.

2.9. Quantification of NF-κB

The level of NF-κB was measured in the serum using a commercially available ELISA kit (Glory Science, Del Rio, TX, USA). The kit uses a double antibody sandwich enzyme linked immunosorbent assay to assay the level of NF-κB.

2.10. Quantification of BDNF

The level of BDNF protein was detected in the serum using a sandwich-type immunoassay kit (Glory Sci-

ence, Del Rio, TX, USA) according to the manufacturer's instructions.

2.11. Histopathological Examination

The brains of different groups were removed and fixed in 10% formol saline, and the 5 µm thick paraffin sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope.

2.12. Immunohistochemical Staining

2.12.1. Caspase-3

Immunohistochemical staining of caspase-3 was performed with sections of 4 µm thick that were deparaffinized and incubated with a fresh solution of 0.3% hydrogen peroxide (H₂O₂) in methanol for 30 min at room temperature. The deparaffinized brain slides were then incubated with antibodies for caspase-3 (diluted 1:50). Positive cells were determined with streptavidin biotin-peroxidase secondary antibody (Dako, Carpinteria, CA, USA). The binding sites of antibody were visualized with the chromogen 3,3'-diaminobenzidine. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted on slides. The immunostaining intensity and cellular localization of caspase-3 were analyzed by light microscopy.

2.12.2. Glial Fibrillary Acidic Protein

Immunostaining was performed using the avidin-biotin peroxidase technique for localization of glial fibrillary acidic protein (GFAP). Paraffin sections (4 µm thick) mounted on coated slides were deparaffinized and incubated in 10 mM citrate buffer for 15–20 min to unmask antigens. Sections were incubated in H₂O₂ for 10 min to abolish endogenous peroxidase activity. Then, sections were hydrated, washed, and heated in buffered citrate, and incubated for 30 min followed by incubation with horse serum for 2 h at room temperature to inhibit nonspecific immunoreactions. Primary monoclonal anti-GFAP serum (AM020-5M, Bio-Genex, Fremont, CA, USA) was applied at 1:5000 dilutions. Sections were incubated with primary monoclonal antiserum for 36 h at 4°C, washed, and then incubated with biotinylated secondary antibodies (REF85-9043, Zymed, San Francisco, CA, USA) (1:200) for 5 h, followed by

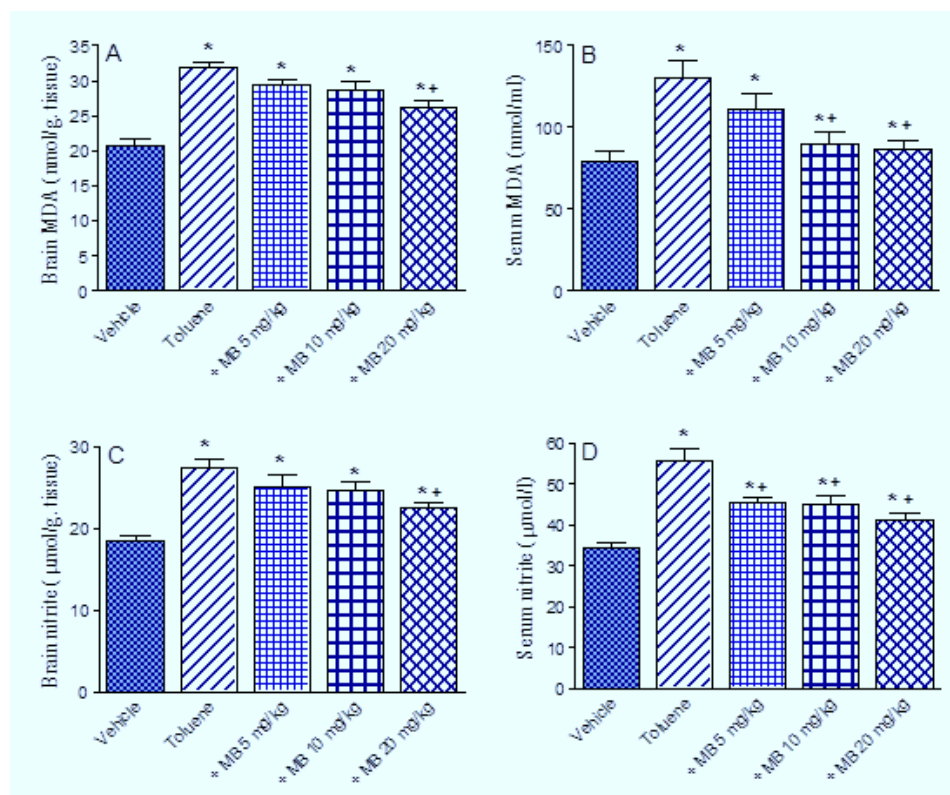


FIGURE 1. Effect of methylene blue (MB) on toluene-induced increases in the levels of MDA (panels A and B) and nitrite (panels C and D) in the brain and serum in rats. *: $p < 0.05$ vs. corresponding vehicle group; +: $p < 0.05$ vs. toluene alone group.

avidin-biotin peroxidase complex. Finally, the immune reaction was visualized with 0.05% 3,3'-diaminobenzidine. The slides were counter stained with Mayer's hematoxyline before mounting.

2.12.3. Image Analysis

Optical density measurements of immunoreactivity were performed with a computer-assisted image analysis system (Leica QWin 500 Image Analyzer, Leica Imaging Systems, Cambridge, England) at the Image Analyzer Unit, Pathology Department, National Research Centre (Cairo, Egypt). Images were composed with a high precision illuminator, a digital camera, and a computer with specific image analysis software. The mean optical density of each region was bilaterally measured on selected brain regions, using consecutive sections in each subject. The de-

gree of reaction was chosen by the color-detect menu. The areas of reactivity were masked by binary color, and the area was measured by using an objective lens of magnification 40× and eye lens 10× (total magnification: 400×). The morphometric analysis was carried out on caspase-3 and GFAP stained slides, which stain the tissue blue and the positive cells with brown color. A total of 10 measurements were taken per region by an investigator blinded to the experimental groups. The measurements were averaged to obtain a mean per region for each rat.

2.13. Statistical Analysis

Data are expressed as means \pm standard error. Statistical analysis of the measurements was performed using SPSS for Windows, v.13 statistical package (Chicago, IL, USA). Data were analyzed by one-way

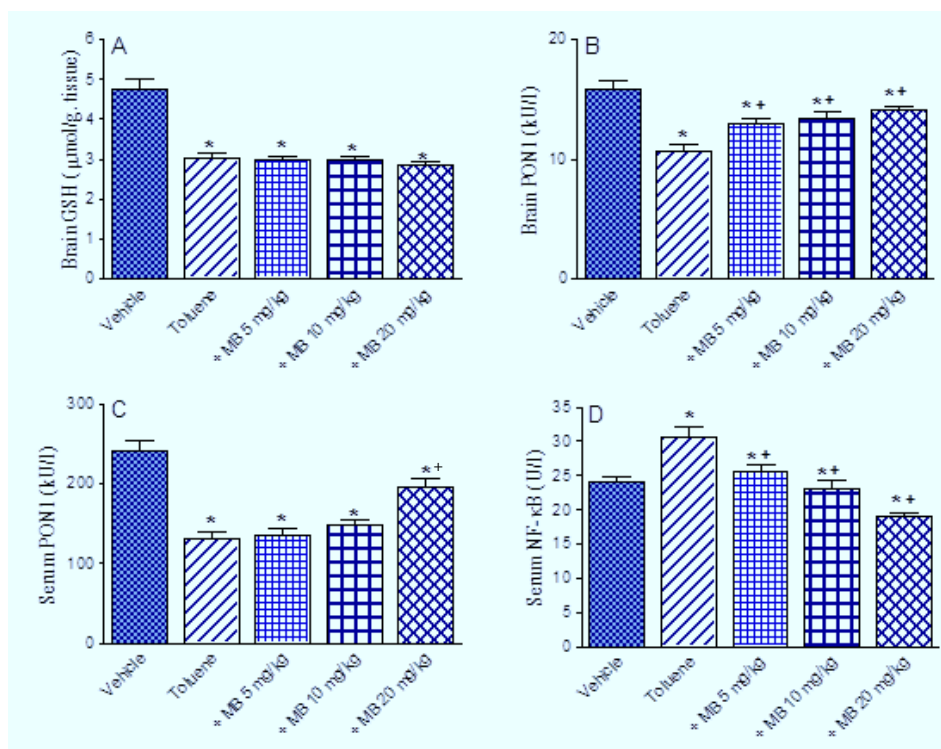


FIGURE 2. Effect of methylene blue (MB) on toluene-induced changes in GSH level and PON1 activity in the brain (panels A and B) and PON activity and NF-κB level in the serum in rats. *: $p < 0.05$ vs. corresponding vehicle group; +: $p < 0.05$ vs. toluene alone group.

analysis of variance, followed by Duncan's multiple range test for post hoc comparison of group means. Effects with a probability of $P < 0.05$ were considered to be statistically significant.

3. RESULTS

3.1. Biochemical Results

3.1.1. Oxidative Stress

The administration of toluene in the rats in this study produced markedly increased oxidative stress as indicated by increased lipid peroxidation (MDA) in the brain (54.2% increase: 31.93 ± 0.21 vs. 20.71 ± 1.0 nmol/g.tissue) and serum (65.2% increase: 130.51 ± 10.8 vs. 79.0 ± 6.1 nmol/L) (Figure 1A and 1B). The level of nitric oxide in the brain increased by 48.5% (27.32 ± 1.24 vs. 18.4 ± 0.7 μmol/g.tissue) and in

serum by 62.2% (55.63 ± 3.1 vs. 34.3 ± 1.6 μmol/L) (Figure 1C and 1D). Meanwhile, brain GSH content fell by 36.3% in toluene-treated rats compared with their saline-treated counterparts (3.0 ± 0.13 vs. 4.77 ± 0.26 μmol/g.tissue) (Figure 2A).

No significant change was observed in brain MDA level in rats treated with toluene and methylene blue at 5 or 10 mg/kg. The higher dose (20 mg/kg), however, resulted in a 71.6% decrease in brain MDA level compared with the toluene alone group (26.3 ± 0.96 vs. 31.93 ± 0.21 nmol/g.tissue). On the other hand, serum MDA level decreased by 31.5% and 33.6% after treatment with 10 and 20 mg/kg of methylene blue, respectively, as compared to the toluene alone group value (89.4 ± 8.1 and 86.7 ± 5.3 vs. 130.51 ± 10.8 nmol/L) (Figure 1A and 1B).

Methylene blue treatment (20 mg/kg) significantly decreased nitric oxide level in the brain by 16.9% compared to the toluene only group (22.7 ± 0.62 vs. 27.32 ± 1.24 μmol/g.tissue). Significant inhibition of

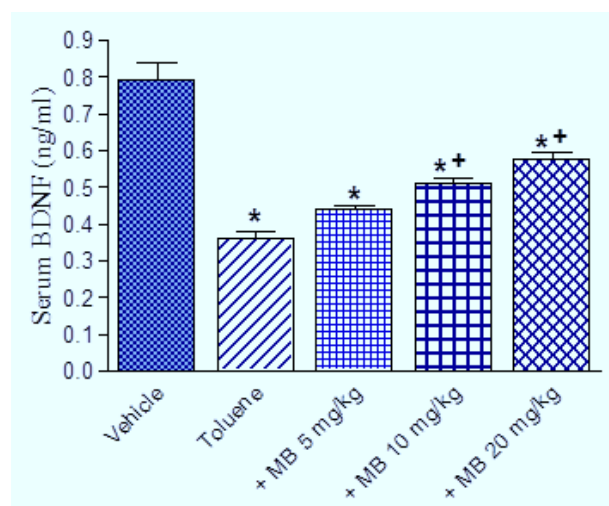


FIGURE 3. Effect of methylene blue (MB) on toluene-induced decreases in serum BDNF level in rats. *: $p < 0.05$ vs. vehicle group; +: $p < 0.05$ vs. toluene alone group.

serum nitric oxide level by 18.2%, 19.1%, and 26.1% was also observed after co-treatment with methylene blue at 5, 10, and 20 mg/kg, respectively, as compared to the toluene alone group (45.5 ± 1.3 , 45.0 ± 2.2 , and 41.1 ± 1.7 vs. 55.63 ± 3.1 $\mu\text{mol/L}$) (**Figure 1C and 1D**).

Methylene blue co-treatment did not significantly affect the level of brain GSH in toluene-intoxicated rats (**Figure 2A**).

3.1.2. PON1 Activity

In rats treated with toluene, there was significant inhibition of PON1 activity in the brain (32.6% inhibition: 10.7 ± 0.52 vs. 15.87 ± 0.73 kU/L) and serum (45.2% inhibition: 132.18 ± 7.4 vs. 241.4 ± 12.5 kU/L) compared with the saline control group. The combined administration of toluene and methylene blue resulted in increased PON1 activity. In the brain, PON1 activity increased by 21.5%, 25.2%, and 31.8% by methylene blue at 5, 10, and 20 mg/kg, respectively. Meanwhile, serum PON1 activity increased by 48.1% after treatment with methylene blue at 20 mg/kg, compared with toluene alone group (195.8 ± 10.0 and 73.4 ± 4.1 vs. 132.18 ± 7.4 kU/L) (**Figure 2B and 2C**).

3.1.3. NF- κ B Level

In toluene-treated rats, serum NF- κ B level showed significant increase by 27% compared with that in the saline control group (30.64 ± 1.5 vs. 24.13 ± 0.55 U/L). Rats treated with both toluene and methylene blue at 5, 10, and 20 mg/kg exhibited 16.8%, 24%, and 38% decrements in NF- κ B level, respectively, as compared with the toluene alone group (25.51 ± 1.5 , 23.05 ± 1.2 , and 18.98 ± 0.56 vs. 30.64 ± 1.5 U/L) (**Figure 2D**).

3.1.4. BDNF Level

A significant decrease in BDNF level by 54.4% was observed in the serum of rats given toluene alone compared with the saline control value (0.36 ± 0.02 vs. 0.79 ± 0.05 ng/ml). In toluene-treated rats, the administration of methylene blue resulted in a dose-dependent increase in serum BDNF level by 22.2%, 41.7%, and 59.7%, respectively, as compared with the toluene only treatment group (0.44 ± 0.01 , 0.51 ± 0.015 , and 0.575 ± 0.017 vs. 0.36 ± 0.02 ng/ml) (**Figure 3**).

3.1.5. BchE Activity

Toluene administration induced significant inhibition of serum BChE activity by 44.7% compared with the saline control value (121.1 ± 5.7 vs. 219.13 ± 11.7 U/L). Rats treated with methylene blue along with toluene exhibited increased BChE activity in a dose-dependent manner. BChE activity increased by 45.9%, 50%, and 79.2% by methylene blue at 5, 10, and 20 mg/kg, respectively. Values were 176.69 ± 8.3 , 181.66 ± 6.2 , and 217 ± 10.0 U/L for toluene plus methylene blue at 5, 10, and 20 mg/kg, respectively, and 121.1 ± 5.7 U/L for toluene alone group (**Figure 4**).

3.2. Histopathological Results

3.2.1. Cerebral Cortex

Examination of H&E stained sections from saline-treated rats showed the normal structure of the cerebral cortex (**Figure 5A**). Rats treated with methylene blue at 20 mg/kg showed normal histological structure except for a congested blood vessel (**Figure 5B**). Rats treated with toluene exhibited neurodegenera-

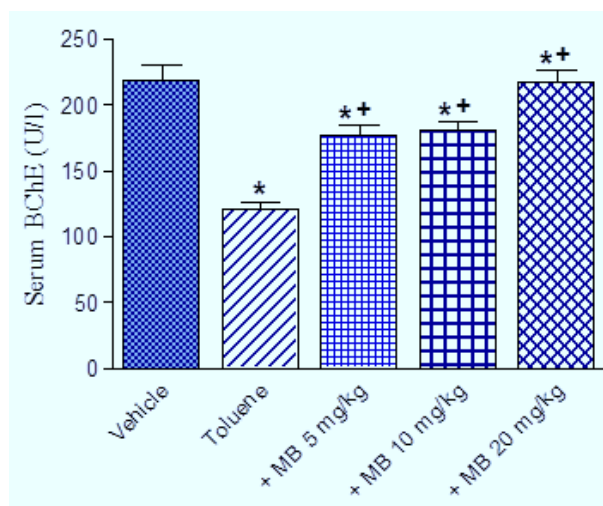


FIGURE 4. Effect of methylene blue (MB) on toluene-induced decreases in serum BChE activity in rats. *: $p < 0.05$ vs. vehicle group; +: $p < 0.05$ vs. toluene alone group.

tion with pyknotic and apoptotic neurons. Some neurons appeared with intense acidophilia and contrasting basophilia. Cerebral edema and marked degeneration of Purkinje cell were observed (Figure 5C and 5D). Rats treated with both toluene and methylene blue at 5 mg/kg showed degenerative changes, intense acidophilia, and contrasting basophilia of the pyknotic nuclei. Cerebral edema and degeneration of some Purkinje cells were seen (Figure 5E and 5F). Sections from rats treated with both toluene and methylene blue at 10 mg/kg showed some improvement in the pathological changes in the form of no edema although degenerated neurons, few apoptotic neurons, darkly stained neurons, as well as neuronal loss were seen. There was also degeneration of some Purkinje cells (Figure 5G and 6D). Sections from rats treated with both toluene and methylene blue at 20 mg/kg showed normally appearing neurons but also apoptosis in some neurons and degeneration in others. Degeneration of some Purkinje cells was observed (Figure 5H and 6E). Sections from rats treated with both toluene and methylene blue at 20 mg/kg showed normally appearing neurons but also apoptosis in some neurons and degeneration in others. Degeneration of some Purkinje cells was observed (Figure 5H).

3.2.2. Cerebellum

As shown in Figure 6A the cerebellum of control (saline)-treated rats exhibited the normal structure. Rats treated with toluene exhibited marked degeneration of Purkinje cells (Figure 6B). Rats treated with both toluene and methylene blue showed improvement in the pathological changes although degeneration of some Purkinje cells was still seen (Figure 6C, 6D, and 6E).

3.3. Immunohistochemical Results

3.3.1. Caspase-3 Immunoreactivity

No caspase-3 immunoreactivity was detectable in the brain of control (saline-treated) rats (Figure 7A). Rats treated with toluene exhibited strong caspase-3 immunostaining (Figure 7B). Caspase-3 positive cells were reduced in a dose-dependent manner in rats treated with toluene along with methylene blue at 5, 10, or 20 mg/kg (Figure 7C, 7D, and 7E). Rats treated with methylene blue alone showed mild caspase-3 immunoreactivity (Figure 7F). Figure 8 shows the binary image morphometric measurement for the percentage area of apoptotic cells of control group, group treated with toluene, group treated with toluene along with methylene blue at 20 mg/kg, and group treated with methylene blue alone. Quantitative analysis of caspase-3 immunostaining is shown in Figure 9.

3.3.2. GFAP Immunoreactivity

Immunohistochemical staining of the cerebral cortex showed strong positive immunoreactivity for GFAP in star shaped glial cells and their processes in the saline-treated group (Figure 10A). The administration of toluene induced marked decrease in immunohistochemical staining for GFAP (Figure 10B). In toluene-intoxicated rats, methylene blue treatment increased the immunoreactivity for GFAP in a dose-dependent manner (Figure 10C, 10D, and 10E). Rats treated with methylene blue alone at 20 mg/kg showed strong brown positive for GFAP in star shaped glial cells and their processes nearly identical to the saline control group (Figure 10F). This suggested that methylene blue alone had no significant effect on GFAP expression in glial cells. Figure 11 shows the binary image morphometric measurement

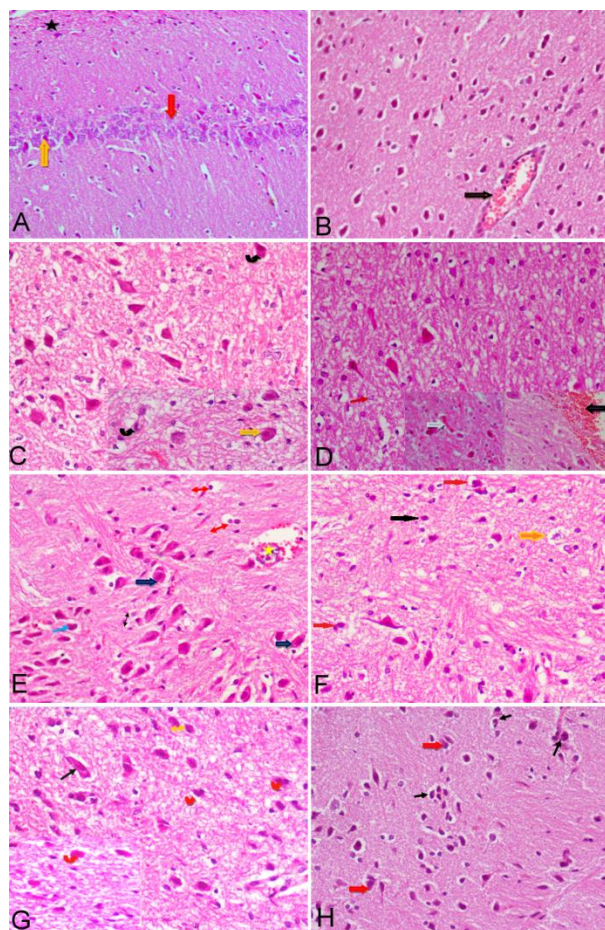


FIGURE 5. Photomicrographs of representative brain sections from the cerebral cortex of rats. In (A), saline-treated group showed normal structure in granular layer (red arrow), Purkinje cell layer (yellow arrow), and molecular layer (star). In (B), methylene blue alone group showed congested blood vessel (arrow). In (C), toluene alone group showed pyknotic (red arrow) and apoptotic (curved arrow) neurons, and intense acidophilia (yellow arrow). In (D), toluene alone group showed euchromatic nucleated neurons (red arrow) and hemorrhage in meninges above the surface (lower part: black arrow). In (E), toluene + methylene blue 5 mg/kg group showed intense acidophilia (black arrow), pyknotic nuclei (blue arrow), and congested blood vessel (star). In (F), toluene + methylene blue 10 mg/kg group showed infiltrative cells, degenerated neurons (yellow arrow), and apoptotic neurons (red arrow). In (G), toluene + methylene blue 10 mg/kg group showed degenerated (red arrow) and darkly stained neurons (black arrow), and apoptotic neurons (yellow arrow). In (H), toluene + methylene blue 20 mg/kg group showed normally appearing neurons, apoptosis in some neurons (black arrow), and degeneration in others (red arrow). All images are 400 \times and H&E staining.

for GFAP percentage areas of control group, group treated with toluene, group treated with toluene along with methylene blue at 20 mg/kg, and methylene blue alone group. Quantitative analysis of GFAP immunostaining is presented in **Figure 12**.

4. DISCUSSION

The present findings indicate for the first time the potential of methylene blue to protect against neuronal death caused by toluene. In this study, the administration of toluene resulted in an increase in oxidative stress in the rat brain and serum as indicated by the marked elevation of the lipid peroxidation product MDA [33]. The brain tissue, which is rich in polyunsaturated fatty acids (PUFAs), is most vulnerable to free radical attack. Oxidation of PUFAs in cell membranes can lead to lipid peroxidation, result-

ing in damage to cellular biomembranes and producing lipid hydroperoxides or lipid alkoxyl radicals [34]. The presence of oxidative stress in the brain of toluene-treated rats is also indicated by the observed decrease in the concentration of brain GSH. The tripeptide GSH (L- γ -glutamyl-L-cysteinyl-glycine)—the most abundant non-protein thiol intracellularly, is an important cellular antioxidant that acts as a substrate for glutathione peroxidase. It also directly scavenges ROS, such as hydroxyl radicals and peroxyl radicals [34, 35]. The decrease in brain GSH by toluene is thus likely to be due to the consumption of the antioxidant by the increased generation of ROS. We also observed marked increase in nitric acid concentration in the brain and serum of toluene-treated rats. Nitric oxide is a highly-diffusible molecule which acts as a cellular messenger in the brain and also mediates vasodilatation [36]. Nitric oxide also exerts neurotoxic effects, depending on its concentrations. This occurs in inflammatory and toxic states due to increased generation of nitric oxide from in

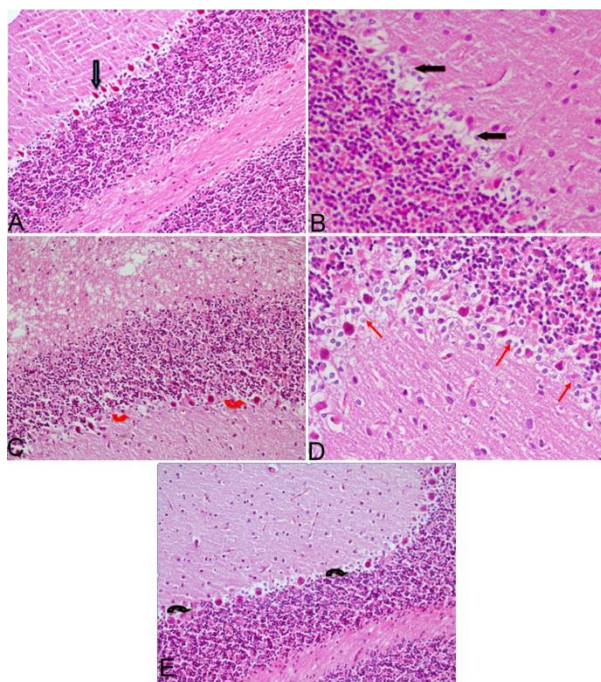


FIGURE 6. Photomicrographs of representative brain sections from the cerebellum of rats. In (A), saline-treated group showed normal Purkinje cells. In (B), toluene alone group showed marked degeneration of Purkinje cells (black arrow). In (C), toluene + methylene blue 5 mg/kg group showed degeneration of some Purkinje cells (red curved arrows). In (D) toluene + methylene blue 10 mg/kg group showed degeneration of some Purkinje cells (red curved arrow). In (E), toluene + methylene blue 20 mg/kg group showed degeneration of some Purkinje cells (black curved arrow). All images are 400 \times and H&E staining.

flammatory cells via the action of the inducible form of nitric oxide synthase [37]. It is largely thought that this neurotoxic action of excess nitric oxide is mediated by peroxynitrite anion (ONOO^-) generated by the reaction of nitric oxide with the superoxide anion. Moreover, the reaction of nitric oxide with molecular oxygen results in the formation of dinitrogen trioxide, and nitrogen dioxide, leading to nitrosylation and/or nitration of proteins and lipids [38]. The present observations are thus consistent with other studies that showed increased generation of

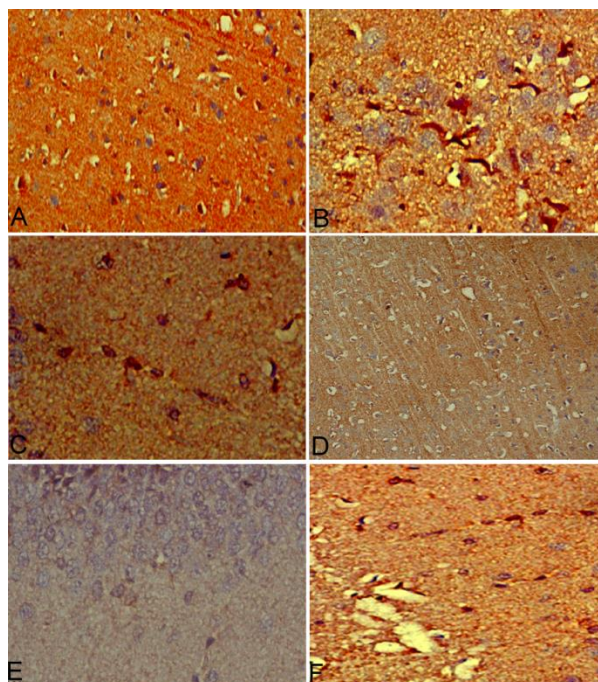


FIGURE 7. Caspase-3 expression in the cytoplasm of the brain cells in the cerebral cortex. In (A), saline control showed no caspase-3 immunoreactivity. In (B), toluene alone group showed strongly positive caspase-3 expression (dark brown). In (C), toluene + methylene blue at 5 mg/kg group showed moderate positive caspase-3 immunoreactivity. In (D), toluene + methylene blue at 10 mg/kg group showed decrease in caspase-3 immunoreactivity compared with toluene alone group. In (E), toluene + methylene blue at 20 mg/kg group showed decrease in caspase-3 immunoreactivity compared with toluene alone group. In (F), methylene blue alone group showed mildly positive caspase-3 immunoreactivity. All images have a magnification scale of 400.

ROS in crude mitochondrial fractions from rat cerebellum, striatum, and hippocampus following toluene exposure (0.5–1.5 g/kg, i.p.). This increase in ROS peaked within 2 h and was maintained throughout the next 24 h despite fading blood levels of toluene [11]. Oxidation of proteins in the cortex and cerebellum [13] and oxidative DNA damage in the liver and kidney [14] have also been reported in rats following repeated inhalation of toluene. The present study

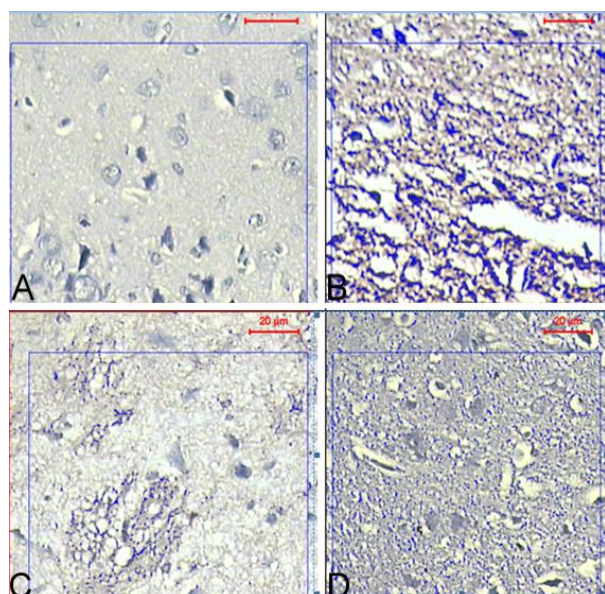


FIGURE 8. Binary image morphometric measurement for percentage area of apoptotic cells in the cerebral cortex stained by caspase-3 showing the positive brown cells (apoptotic cells) detected by blue color. (A): saline control group; (B): toluene alone group; (C): toluene + methylene blue at 20 mg/kg group; and (D): methylene blue alone group.

showed that the administration of methylene blue resulted in decreased lipid peroxidation in the brain and serum of toluene-treated rats. Methylene blue had no significant effect on brain GSH, but decreased the increment of nitric oxide concentration by toluene. Methylene blue is an inhibitor of nitric oxide synthase and guanylyl cyclase [39, 40] and has been shown to decrease brain nitrite/nitrate content and the number of cortical cells showing inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) activation in ischaemic/reperfusion brain injury [27]. This effect of methylene blue on nitric oxide might be involved, at least in part, in its neuroprotective action.

Our results demonstrated increased NF- κ B level in the serum after toluene exposure. NF- κ B is a transcription factor involved in the regulation of several genes encoding immune and inflammatory mediators. NF- κ B is kept in the cytoplasm in an inactive state by binding to the inhibitory molecule I κ B. NF- κ B is activated by cytokines, such as tumor necrosis

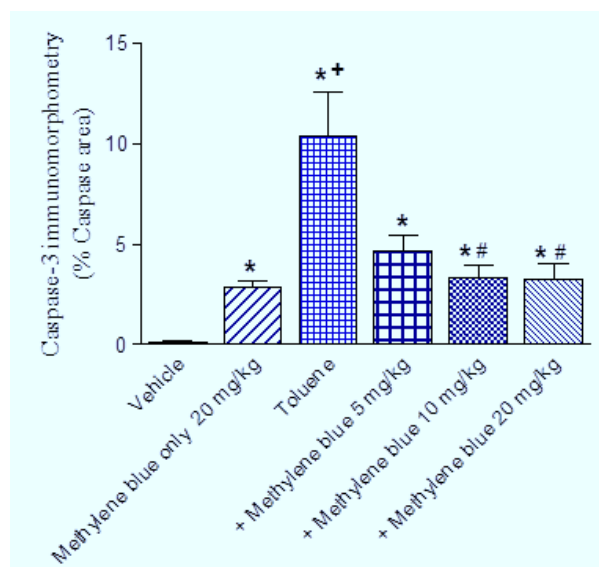


FIGURE 9. Optical density measurement of caspase-3 immunoreactivity in the brain of rats treated with toluene and toluene along with methylene blue. Data represent % area of apoptotic cells. *: $p < 0.05$ vs. vehicle; +: $p < 0.05$ vs. methylene blue alone; #: $p < 0.05$ vs. toluene alone.

factor (TNF)- α , interleukin- 1β , growth factors, bacterial lipopolysaccharide, and oxidants. Once activated, NF- κ B is released from the I κ B-NF- κ B complex and translocates to the nucleus and induces the expression of genes encoding cytokines, enzymes such as iNOS and cyclooxygenase-2, cell adhesion molecules, monocytes chemoattractant protein-1 (MCP-1), and acute-phase proteins [41, 42]. NF- κ B is a redox-sensitive factor and its activation is controlled by intracellular ROS as shown in cells exposed to H_2O_2 [43]. Moreover, the nuclear translocation of NF- κ B and the degradation of I κ B- α subunit were inhibited in cells overexpressing glutathione peroxidase following exposure to TNF- α or H_2O_2 [44]. The decrease in serum NF- κ B level in toluene-treated rats by methylene blue seen in the present study is thus likely to be the result of decreased oxidative stress and hence reduced activation of NF- κ B.

PON1 is an esterase and lactonase that is synthesized in the liver and released into the circulation where it binds to high-density lipoproteins (HDL) [45, 46]. PON1 plays an important role in the metab-

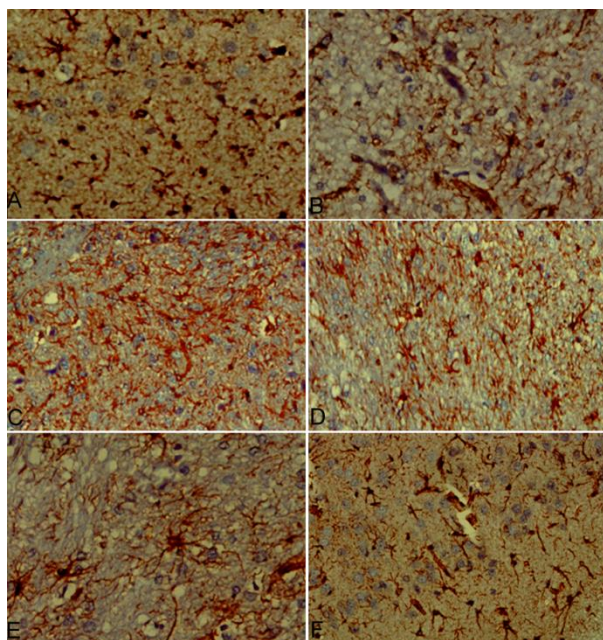


FIGURE 10. GFAP expression in the cerebral cortex of rats. In (A), saline control group showed positive immunoreactivity for GFAP in star shaped glial cells. In (B) toluene alone group showed decrease in GFAP immunoreactions. In (C, D, and E), the groups treated with toluene along with methylene blue at 5, 10, and 20 mg/kg, respectively, showed increase in immunoreactivity for GFAP in a methylene blue dose-dependent manner. In (F), methylene blue alone group showed increase in immunoreactivity for GFAP nearly identical to saline control group and some neuroglial cell processes still retained more branching. All images have a magnification scale of 400.

olism of organophosphorus insecticides, nerve agents (e.g., sarin and soman), as well as many xenobiotic compounds [47, 48]. The enzyme exerts peroxidase activities and this action is likely to be of value in neurodegenerative disorders in which there is inappropriately increased levels of oxidative stress [49]. In this context, reduced serum PON1 activity has been found in patients suffering from Alzheimer's disease or other dementias [50], multiple sclerosis [51], and autism [52]. The enzyme is redox sensitive and has been shown to be inactivated by oxidative stress [53]. In the present study and consistent with

previous observations [9], the activity of PON1 in the brain and serum decreased in rats treated with toluene, which was bunted by methylene blue co-treatment. The inhibition of PON1 activity by toluene is thus likely to result from inactivation of the enzyme through increased oxidative stress.

In this study, we also demonstrated that the administration of toluene was associated with decreased serum level of BDNF. The latter belongs to the neurotrophin family of growth factors which also include nerve growth factor, neurotrophin-3, neurotrophin-4/5, and neurotrophin-6. These growth factors are involved in neuronal maturation, survival, synaptic plasticity, and neurogenesis [54, 55]. BDNF is widely distributed in the central nervous system [56] and has been shown to exert neuroprotective effects in models of cerebral ischaemia [57] and experimental autoimmune encephalomyelitis [58]. Recent evidence linked BDNF to a number of neuropsychiatric disorders where low serum levels were found in non-treated patients with depression [59] or schizophrenia [60], and the levels were normalized upon depression remission and following cognitive training of the schizophrenic patients. BDNF has also been suggested as a reliable marker of neurotoxicity. Release of BDNF from the neurons and astrocytes in culture increased following exposure to cytotoxic levels of neurotoxic agents [61]. Studies showed increased expression of nerve growth factor and BDNF in the hippocampus in mice exposed to toluene at 9 ppm [62] or 500 ppm [63]. Other researchers reported decreased BDNF and glial cell line-derived neurotrophic factor (GDNF) in the spinal cord of rats exposed to toluene (1500 ppm for 4 h per day) for 7 days [64]. Our findings, however, indicate inhibition of serum BDNF following higher doses of toluene exposure. BDNF in serum of toluene-treated rats increased after treatment with methylene blue. This finding is likely to reflect the neuroprotective action of the dye. Other studies reported increased levels of BDNF in the brain of transgenic mouse model of Huntington's disease by feeding mice with methylene blue in chow [24].

Consistent with previous observations [9], the administration of toluene resulted in significant inhibition of BChE in the serum. The cholinesterases—acetylcholinesterase (AChE) and BChE—both hydrolyze acetylcholine, but BChE differs in that it hydrolyzes many other substances as well. BChE is more abundant in the serum and is also found in the

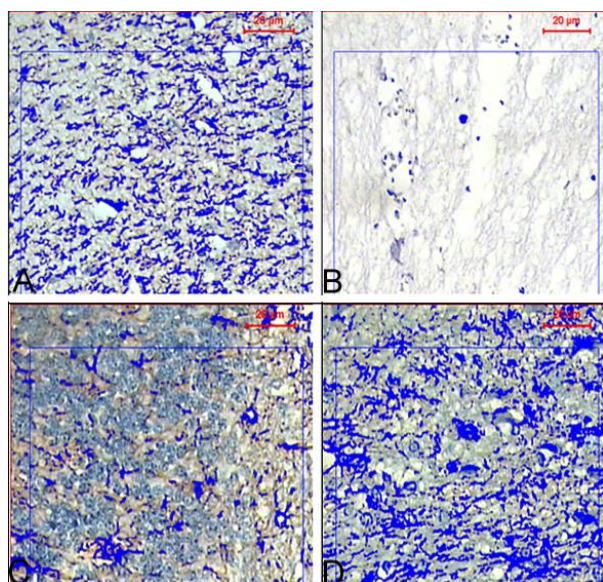


FIGURE 11. Binary image morphometric measurement for percentage GFAP areas in the cerebral cortex, showing the positive brown cells (glial cells) detected by blue color. (A): saline control group; (B): toluene alone group; (C): toluene + methylene blue at 20 mg/kg group; and (D): methylene blue alone group.

liver, heart, and brain [65]. In vitro, treatment with toluene resulted in an increase in synaptosomal membrane fluidity and decreased AChE activity [66]. Other researchers reported inhibition of AChE, choline acetyltransferase and acetylcholine levels after exposure to a mixture of benzene, toluene, xylene, and formaldehyde [67]. This action of toluene on cholinergic system could be of relevance to the cognitive changes seen in abusers of this solvent. It has also been suggested that inhibition of cholinesterase activity might be a useful marker for exposure to toluene.

The ability of methylene blue to protect against toluene neurotoxicity was also demonstrated on histopathological examination as well as by measurement of caspase-3 and GFAP immunoreactivity. Methylene blue reduced brain edema, pyknotic and apoptotic neurons, and the degeneration of Purkinje cells in a dose-dependent manner. Caspases are aspartate-specific cysteine proteases and members of the interleukin-1 β -converting enzyme family. These

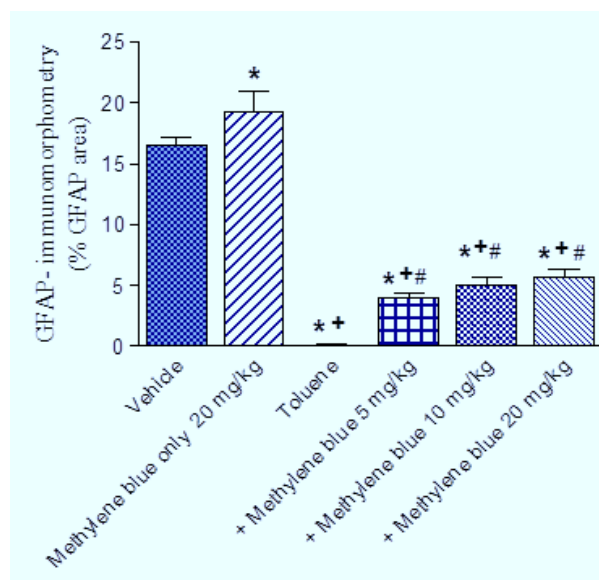


FIGURE 12. Optical density measurement of GFAP immunostaining in the brain of rats treated with toluene and toluene along with methylene blue. Data represent %area of viable glial cells. *: $p < 0.05$ vs. vehicle; +: $p < 0.05$ vs. methylene blue alone; #: $p < 0.05$ vs. toluene alone.

enzymes are produced in inactive forms and are activated in a protease cascade. Caspases are often classified into initiator (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and effector caspases (caspase-3, -6, -7). The latter ones with caspase-3 being considered the most important member, are responsible for the execution of apoptosis or programmed cell death, a morphologically distinct form of cell death characterized by DNA fragmentation and nuclear chromatin condensation, membrane blebbing, and shrinkage of the cell [68, 69]. Apoptosis is important during development, for the normal tissue homeostasis and also as a protective mechanism to get rid of damaged cells due to diseases or toxins [70]. On the other hand, inappropriate apoptosis has been implicated in the development of cancer, autoimmune diseases, and neurodegenerative disorders [69]. In this study, administration of toluene was found to induce strong caspase-3 immunostaining suggesting the involvement of caspase-3 activation in the neurotoxic action of this solvent. Our findings also indicated the ability of methylene blue to inhibit the activation of

caspase-3 in the brain of toluene-treated rats. Inhibition of apoptosis might thus be an important mechanism by which methylene blue protects against neurotoxicity caused by toluene and possibly other neurotoxicants. In this context, methylene blue was shown to decrease the expression of caspase-3 in the striatum and the degree of neuronal degeneration and gliosis in the striatum, substantia nigra, cortex, and hippocampus in rats treated with the nigrostriatal toxin rotenone [25]. Moreover, an inhibitory effect for methylene blue and its derivatives azure A, and azure B on caspase-1, -3, -6 activities has been demonstrated in vitro, in human colon carcinoma cells and human primary neurons, as well as in the liver of mice treated with lipopolysaccharide/galactosamine. This action involves the oxidation of a catalytic cysteine residue (Cys163) into sulfenic acid by the dye [71].

GFAP is a frequently used astrocytic marker. This protein forms a major protein constituent of glial filaments that is important in astrocyte cytoskeleton. Increased expression of GFAP is indicative of astrogliosis, a process in which astrocytes become activated in response to a variety of central nervous system insults [72]. Reactive astrocytes produce various cytokines such as interleukin-1 (IL-1), IL-6, IL-10, TNF- α and - β , interferon- α and - β , and neurotrophic factors (e.g., fibroblast growth factor, platelet-derived growth factor, and nerve growth factor) [73]. Reactive astrocytes form the glial scar and with cytokines and growth factors released from these cells, play an important role in limiting brain damage, but might also contribute to neuropathology [74]. We found that toluene exposure decreased immunostaining for GFAP in star shaped glial cells and their processes. Other researchers have shown that repeated inhalation of 1000 ppm toluene for up to 7 days in rats resulted in decreased GFAP in the thalamus [75]. These data suggested inhibition of GFAP in glial cells by toluene. Rats treated with methylene blue alone at 20 mg/kg showed GFAP expression in glial cells and their processes similar to the saline-treated rats. Thus, treatment with methylene blue alone had no significant effect on GFAP expression in glial cells. GFAP immunostaining increased in rats treated with toluene along with methylene blue (though not to the normal value). It is likely, therefore, that the increase in GFAP expression by methylene blue is a consequence of neuroprotection by the dye.

In summary, the findings in the present study indicate a neuroprotective action for methylene blue against the neurotoxic effects of toluene. This action of methylene blue is likely to involve inhibition of nitric oxide, NF- κ B, and caspase-3.

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