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Methylene Blue as a Novel Neuroprotectant in Acute Malathion Intoxication

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ABSTRACT | The effect of methylene blue on oxidative stress and neuronal injury in rats following acute malathion intoxication was examined. Rats were intraperitoneally injected with malathion at 150 mg/kg body weight along with methylene blue at 5 or 10 mg/kg body weight, and euthanized 4 hr later. The levels of lipid peroxidation [malondialdehyde (MDA)], nitric oxide, and reduced glutathione (GSH), and the activities of paraoxonase 1 (PON1), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) were measured in the brain tissue. Histopathological examination and glial fibrillary acidic protein (GFAP) immunostaining were also carried out in this study. Malathion induced a significant elevation in brain lipid peroxidation (MDA) by 32.8% associated with an increased nitric oxide level by 51.4%. Following malathion exposure, brain GSH level fell by 67.7%, and brain AChE and BChE activities decreased by 25% and 60.4%, respectively. Malathion exposure also inhibited PON1 activity by 39.6% and decreased brain glucose level by 30%. Neuronal degeneration in the cortex and hippocampus and strong GFAP immunostaining in the hippocampus were observed in malathion-exposed animals. Methylene blue co-treatment at 10 mg/kg body weight decreased brain MDA by 17.8%, and at 5 and 10 mg/kg body weight, decreased nitric oxide level by 29.2% and 35.8%, respectively. There was no significant effect on the GSH level, but PON1 activity was increased by 22.9%–30.9% upon methylene blue co-treatment. BChE activity did not change but that of AChE increased after co-treatment with the lower dose of methylene blue. Rats receiving methylene blue co-treatment at 10 mg/kg body weight showed no degenerating neurons in the cortex, and occasional degenerating neurons and weak GFAP immunostaining in the hippocampus. Taken together, our results suggest that methylene blue is neuroprotective in acute malathion intoxication, and the neuroprotective effect is likely due to inhibition of oxidative stress and decreased glial cell activation.

KEYWORDS | Acetylcholinesterase; Butyrylcholinesterase; Glial cell activation; Glial fibrillary acidic protein Malathion; Methylene blue; Neuroprotection; Nitric oxide; Oxidative stress; Paraoxonase 1

ABBREVIATIONS | AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GFAP, glial acidic fibrillary protein; GSH, reduced glutathione; iNOS, inducible NOS; MDA, malondialdehyde; NOS, nitric oxide synthase; PON1, paraoxonase 1

CONTENTS

1. INTRODUCTION

Poisoning due to organophosphates, widely used as insecticides in the household, farm, and agriculture, constitutes an important health problem. One of the most widely used organophosphate insecticides is malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithionate). Malathion was introduced as a commercial chemical in 1950 and is used as a broad spectrum insecticide in agricultural and nonagricultural applications (e.g., the control of lice) [1]. Organophosphate insecticides target the enzyme acetylcholinesterase (AChE) which exists in the brain, endplate of skeletal muscle, and erythrocyte membrane. The most important function of AChE is to hydrolyze acetylcholine at the synaptic cleft, terminating its role in neurotransmission [2]. The symptoms and signs of acute organophosphate toxicity, including malathion poisoning, are principally due to inhibition of AChE both in the brain and in the peripheral tissue [3]. Long-term effects have also been described for organophosphate insecticides in the form of peripheral neuropathy and cognitive changes [4]. Moreover, epidemiological evidence implicated exposure to organophosphate insecticides in the increased risk for developing Parkinson's disease [5]. Besides their effect on AChE, malathion and other organophosphates have been shown to induce brain oxidative stress [6, 7]. The latter develops when the cell's antioxidant mechanisms are overwhelmed by the increased generation of reactive oxygen species and other radical and non-radical species with the resultant oxidation of membrane lipids, cellular proteins, and DNA [8]. Oxidative stress has been implicated in the development of neurodegenerative disorders, such as Parkinson's disease [9].

Methylthioninium chloride or methylene blue is a reduction-oxidation agent with potent antioxidant properties, which prevents the formation of mitochondrial oxygen free radicals [10]. It also acts as an enhancer of the electron transport chain, thereby promoting oxygen consumption [11]. The dye has a

number of clinical applications, and is used to treat methemoglobinemia, cyanide poisoning, as well as encephalopathy induced by the alkylating agent ifosfamide [12, 13]. Methylene blue readily crosses the blood-brain barrier, reaching high concentrations in the central nervous system [14]. The dye has recently been a focus of interest in view of a possible role in the therapy of neurodegenerative disorders. In this context, methylene blue has been shown to increase neuronal survival and decrease neurodegeneration in both in vitro and in vivo models of Huntington's disease [15]. In a genetic mouse model of Alzheimer disease, methylene blue is reported to reduce Aβ levels and improve learning and memory deficits [16]. This dye has also been demonstrated to prevent retinal neurodegeneration induced by intravitreal injection of rotenone in mice [17] and to decrease spinal cord injury due to ischemia-reperfusion [18]. The present study thus aimed to investigate the therapeutic potential of methylene blue in rats following acute malathion intoxication.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Male Sprague-Dawley rats, weighing 130–140 g of body weight, obtained from the Animal House of the National Research Centre (Cairo, Egypt), were used in the study. The rats were housed under temperature- and light-controlled conditions and provided with standard laboratory food and water ad libitum. The animal procedures were performed in accordance with the Ethics Committee of the National Research Centre (Cairo, Egypt) and followed the recommendations of the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and Chemicals

Malathion was from Naser Chemical Company (Egypt). Methylene blue and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Study Design

Rats were randomly allocated into four groups with 6 rats in each group. Group 1 (normal control) received saline intraperitoneally (i.p.) (0.2 ml/rat). Group 2 received malathion (150 mg/kg body weight, i.p.). Groups 3 and 4 received malathion injection (150 mg/kg body weight, i.p.) along with methylene blue at 5 and 10 mg/kg body weight (i.p.), respectively. Rats were euthanized 4 hr later by decapitation under 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 tissue was homogenized in 0.1 M phosphate-buffered saline at pH 7.4 to give a final concentration of 20% (20 g per 100 ml) for the biochemical assays.

2.4. Biochemical Analyses

2.4.1. Determination of Lipid Peroxidation

The lipid peroxidation level in the brain tissue homogenates was determined by measuring the level of malondialdehyde (MDA) according to the method of Ruiz-Larrea et al. [19]. In this assay, MDA (along with its equivalents) reacts with thiobarbituric acid to produce a red colored complex that exhibits a peak absorbance at 532 nm.

2.4.2. Determination of Reduced Glutathione Level

The level of reduced glutathione (GSH) in the brain tissue homogenates was determined using the method of Ellman et al. [20]. The method is based on the reduction of Ellman's reagent by the sulfhydryl group of GSH to form 2-nitro-5-mercaptobenzoic acid, which is intense yellow in colour and determined spectrophotometrically at 412 nm.

2.4.3. Determination of Nitric Oxide Level

Nitrite, a relatively stable end product of nitric oxide, is mostly used as an indicator for the production of nitric oxide in biological systems. The nitric oxide level in the supernatants of the brain tissue homogenates was determined indirectly by measuring the level of nitrite using the Griess reagent, according to the method of Moshage et al. [21].

2.4.4. Determination of Paraoxonase 1 Activity

The arylesterase activity of paraoxonase 1 (PON1) in the supernatants of the brain tissue homogenates was

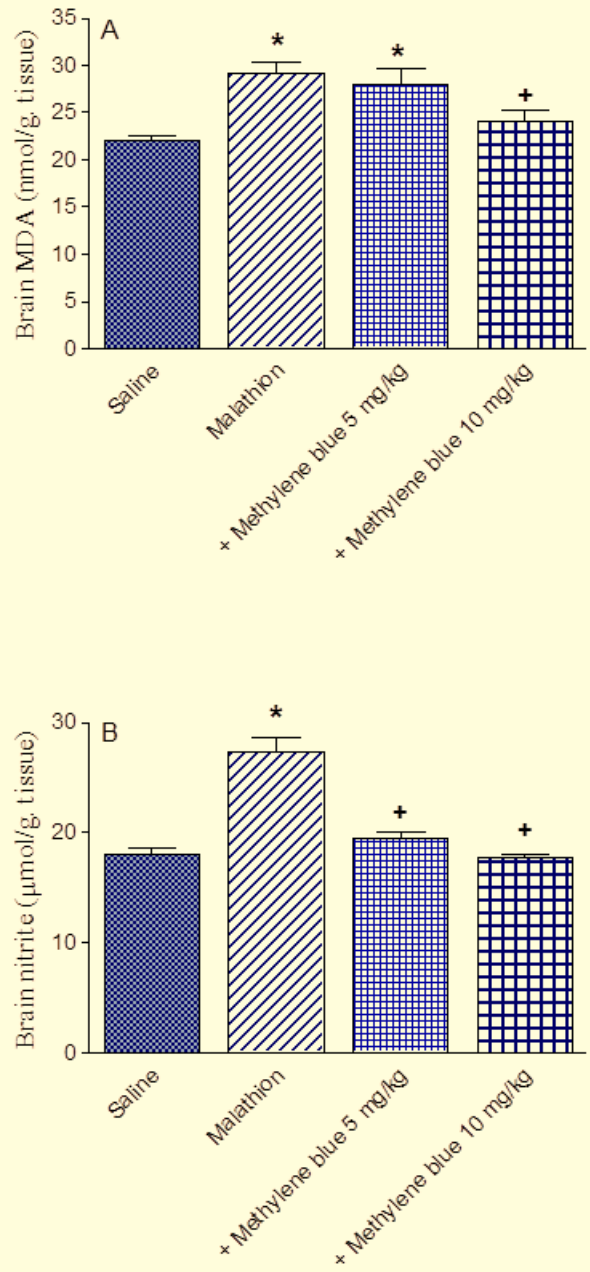

FIGURE 1. Effect of co-treatment with methylene blue (5 and 10 mg/kg body weight) on (A) malondialdehyde (MDA) and (B) nitrite (an indicator of nitric oxide) levels in the brain tissue of malathion (150 mg/kg body weight, i.p.)-exposed rats. *, $p < 0.05$ versus corresponding saline group; +, $p < 0.05$ versus malathion only group.

measured spectrophotometrically using phenyl acetate as a substrate, as described previously [22]. In this assay, PON1 catalyzes the cleavage of phenyl acetate, resulting in the formation of phenol. The rate of the formation of phenol is measured spectrophotometrically by monitoring the increase in absorbance at 270 nm.

2.4.5. Determination of AChE Activity

The AChE activity in the supernatants of the brain tissue homogenates was measured according to the method described by Ellman et al. [23]. The principle of the method is based on the spectrophotometric measurement of the thiocholine formation resulting from the hydrolysis of acetylthiocholine by AchE.

2.4.6. Determination of Butyrylcholinesterase Activity

The butyrylcholinesterase (BChE) activity in the supernatants of the brain tissue homogenates was measured spectrophotometrically using a commercially available kit (Ben Biochemical Enterprise, Milan, Italy). In this assay, BChE catalyzes the hydrolysis of butyrylthiocholine to give rise to butyrate and thiocholine. The resulting thiocholine reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), forming a colored compound that absorbs light maximally at 405 nm. The increase in absorbance per unit time at 405 nm is proportional to the activity of BChE in the sample.

2.4.7. Determination of Glucose Level

The level of glucose in the supernatants of the brain tissue homogenates was measured according to a standard glucose oxidase method [24]. In this assay, glucose, in the presence of glucose oxidase, is converted to hydrogen peroxide and gluconic acid. The produced hydrogen peroxide reacts with phenol and 4-amino-antipyrine in the presence of a peroxidase to yield a colored quinoneimine, which is measured spectrophotometrically.

2.5. Histopathological Examination

Brain tissues were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin using standard procedures. Sections of 5 µm

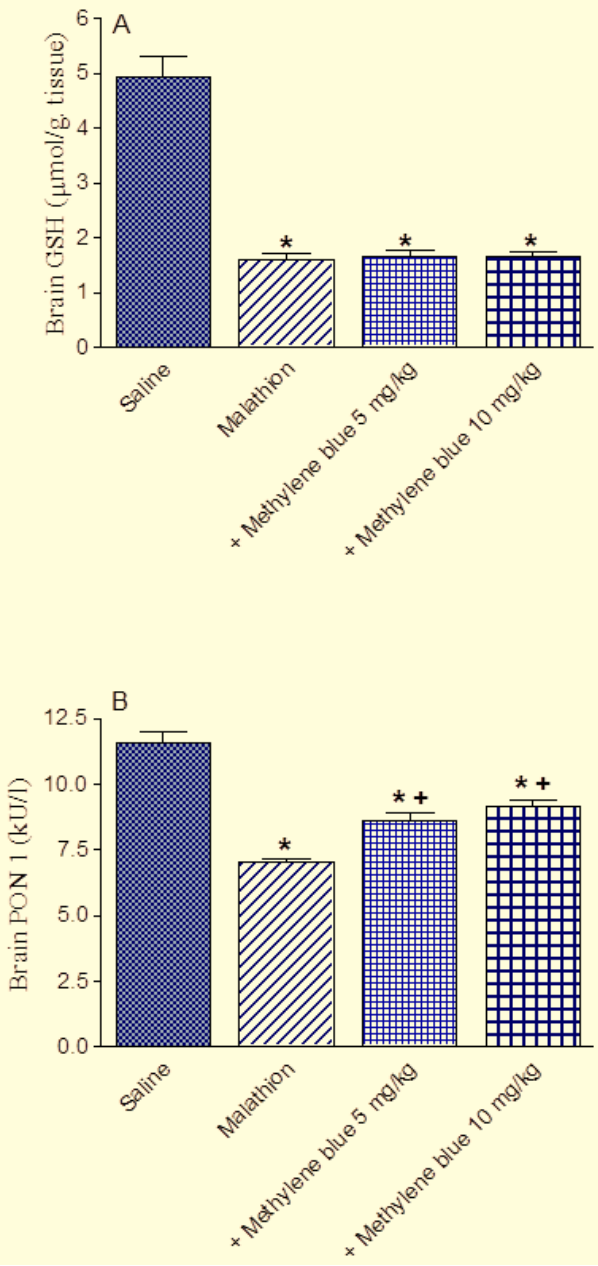

FIGURE 2. Effect of co-treatment with methylene blue (5 and 10 mg/kg body weight) on (A) reduced glutathione (GSH) level and (B) paraoxonase 1 activity (PON1) in the brain tissue of malathion (150 mg/kg body weight, i.p.)-exposed rats. *, $p < 0.05$ versus corresponding saline group; +, $p < 0.05$ versus malathion only group.

thickness were obtained and stained with hematoxylin and eosin (H&E) for histopathological examination under a light microscope.

2.6. Immunostaining Analysis

Serial brain sections cut at 5 µm thickness were mounted on positively charged glass slides using a secondary ultravision detection system (Thermo Fisher Scientific, Waltham, MA, USA). Sections were deparaffinized, hydrated, and incubated (in humid chambers at room temperature) with hydrogen peroxide blocking solution for 15 min. Slides were then washed twice in phosphate buffer, incubated with pepsin digestive enzyme, and then washed 4 times in phosphate buffer. Ultra violet block was applied and incubated for 5 min. Primary antibodies against glial fibrillary acidic protein (GFAP, Thermo Fisher Scientific) were then applied on the serial sections. Sections were then washed and biotinylated with goat anti-polyvalent antibody. Secondary antibody was applied for 10 min, washed, then followed by incubation with streptavidin peroxidase for 10 min and washed again. To develop color reaction, one drop of diaminobenzidine (DAB) plus chromogen was added to 2 ml of DAB plus substrate, mixed, and applied on tissues for 5–15 min. Sections were then counterstained with Mayer's hematoxylin. Coverslips were applied using mounting media. Positive reaction appeared as brown color.

2.7. Quantitative Image Analysis

Quantitative analysis of the degenerating neurons in the cortex was performed to determine the labeling index, which denotes the percentage of degenerating cells. Briefly, labeling index = [(number of degenerating neurons in 5 high power fields) ÷ (number of all neurons in these fields)] × 100 [25]. The degenerating cells in 5 random high power fields were counted on the screen using Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,) in the Pathology Department at the National Research Centre (Cairo, Egypt).

2.8. Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA), followed by Duncan's multiple range test

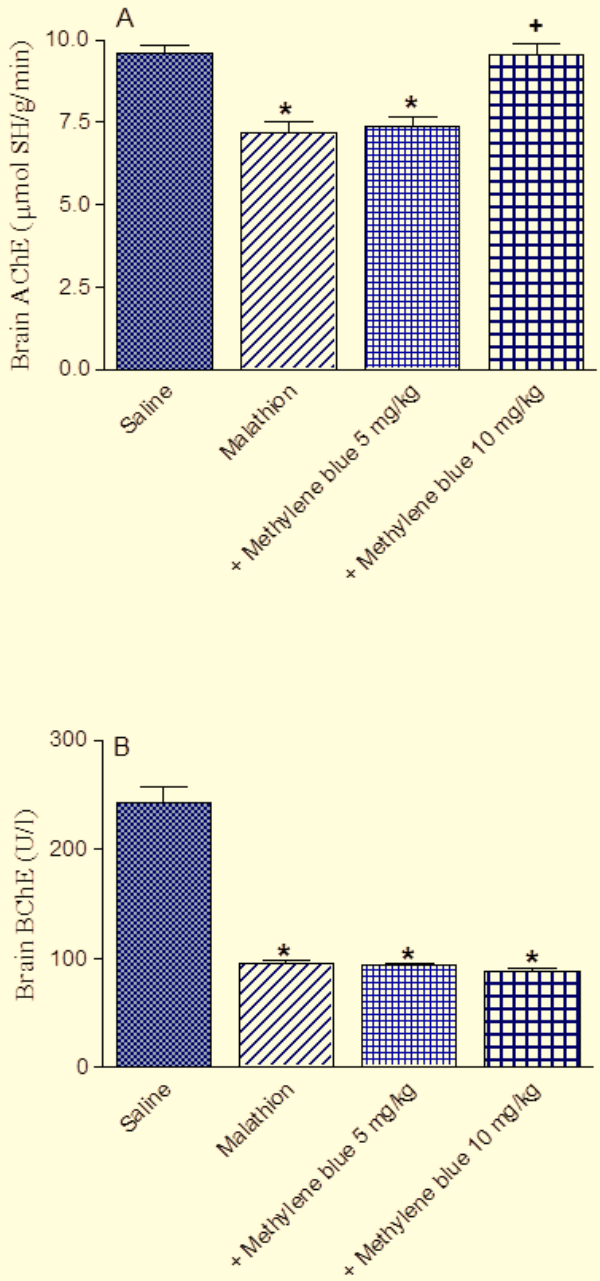

FIGURE 3. Effect of co-treatment with methylene blue (5 and 10 mg/kg body weight) on (A) acetylcholinesterase (AChE) activity and (B) butyrylcholinesterase (BChE) activity in the brain tissue of malathion (150 mg/kg body weight, i.p.)-exposed rats. *, $p < 0.05$ versus corresponding saline group; +, $p < 0.05$ versus malathion only group.

using SPSS software (SAS Institute Inc., Cary, NC, USA). A probability value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Biochemical Studies

3.1.1. Oxidative Stress

Rats treated with malathion alone exhibited elevated lipid peroxidation, as assessed by the MDA level that showed a significant increase by 32.8% compared with the saline-treated group (29.2 ± 1.2 versus 22.0 ± 0.62 nmol/g tissue, $p < 0.05$) (**Figure 1A**). The nitric oxide concentration in the brain, as assessed by measuring the nitrite level, was also increased by 51.4% in malathion-exposed animals compared with the control group (27.4 ± 1.3 versus 18.1 ± 0.65 μmol/g tissue, $p < 0.05$) (**Figure 1B**), whereas the brain GSH level was reduced by 67.7% after malathion treatment (1.59 ± 0.11 versus 4.92 ± 0.40 μmol/g tissue, $p < 0.05$) (**Figure 2A**). Meanwhile, the brain PON1 activity was significantly decreased by malathion exposure as compared with the control group ($p < 0.05$) (39.6% decrease: 7.0 ± 0.16 versus 11.6 ± 0.38 kU/L, $p < 0.05$) (**Figure 2B**).

The co-administration of methylene blue at 5 mg/kg body weight had no significant effect on the brain MDA level of malathion-exposed animals (27.9 ± 1.8 versus 29.2 ± 1.2 nmol/g tissue, $p > 0.05$). Co-treatment with methylene blue at 10 mg/kg body weight, however, decreased the brain MDA level by 17.8% in malathion-exposed animals (24.0 ± 1.3 versus 29.2 ± 1.2 nmol/g tissue, $p < 0.05$) (**Figure 1A**). The brain nitric oxide level in malathion-exposed animals was also significantly decreased by 29.2% and 35.8% after co-administration of methylene blue at 5 and 10 mg/kg body weight, respectively (19.4 ± 0.72 and 17.6 ± 0.43 versus 27.4 ± 1.3 μmol/g tissue, $p < 0.05$) (**Figure 1B**). Methylene blue co-administration had no significant effect on the brain GSH level in malathion-exposed animals (**Figure 2A**). In contrast, the brain PON1 activity in malathion-exposed animals was significantly increased by 22.9% and 30.9% after co-administration of methylene blue at 5 and 10 mg/kg body weight, respectively (8.6 ± 0.31 and 9.16 ± 0.22 versus 7.0 ± 0.16 kU/L, $p < 0.05$) (**Figure 2B**).

3.1.2. AChE and BChE Activity

The brain AChE activity was significantly decreased by 25% (7.2 ± 0.31 versus 9.6 ± 0.22 µmol SH/g/min) after malathion injection. The brain AChE activity in malathion-exposed rats was increased by 32.5% after co-administration of methylene blue at 10 mg/kg body weight (**Figure 3A**). This dose of methylene blue completely reversed malathion-induced inhibition of the brain AChE activity. In contrast, methylene blue at 5 mg/kg body weight showed no effect on the brain AChE activity in malathion-exposed animals (**Figure 3A**). Malathion exposure caused a 60.4% decrease in the brain BChE activity. Co-administration of methylene blue to malathion-exposed rats, however, showed no significant effect on the brain BChE activity (**Figure 3B**).

3.1.3. Glucose Level

A significant 30% reduction in the brain glucose concentration was observed in malathion-exposed rats compared with the control group. Methylene blue at 5 and 10 mg/kg body weight given to malathion-exposed rats resulted in 62.1% and 50.4% increments in the brain glucose concentration, respectively (**Figure 4**).

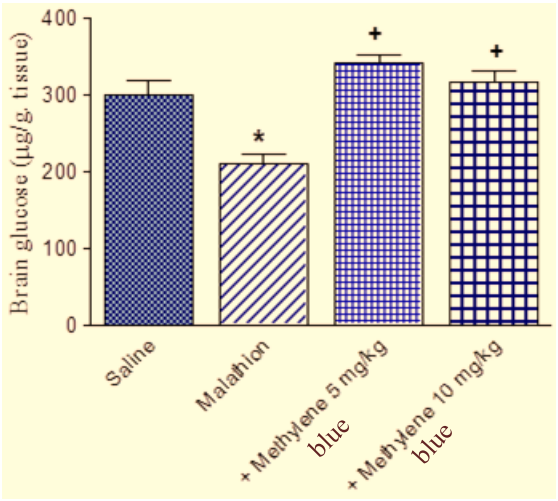

FIGURE 4. Effect of co-treatment with methylene blue (5 and 10 mg/kg body weight) on brain glucose in malathion (150 mg/kg body weight, i.p.)-exposed rats. *, $p < 0.05$ versus corresponding saline group; +, $p < 0.05$ versus malathion only group.

3.2. Histopathological Studies

The normal histology of the cortex from rats in the control group is shown in **Figure 5A**. The cortex of rats treated with malathion showed numerous degenerating neurons which appeared shrunken and dark (**Figure 5B**). Rats that received malathion plus methylene blue at 5 mg/kg body weight still showed many degenerating neurons, clumped neuropil, and some dilated blood vessels (**Figure 5C**). With the higher dose of methylene blue co-administration (10 mg/kg body weight), sections from the cortex of malathion-exposed rats showed no degenerating neurons. Neurons appeared normal with their basophilic cytoplasm, vesicular nuclei, and prominent nucleoli in a fine fibrillary background. However, some dilated blood vessels were still seen (**Figure 5D**).

Quantitative image analysis of sections from the cerebral cortex showed that there were no degenerating neurons observed in the cortex of the saline-treated group. Malathion-exposed rats had a labeling index of 89% compared with labeling indices of 73% and 1.2% in malathion-exposed rats co-treated with 5 and 10 mg/kg body weight of methylene blue, respectively (**Figure 5E**).

Figure 6A shows the normal histology of the hippocampus from the saline-treated rats. The hippocampal region of malathion-exposed rats showed numerous degenerating neurons and clumped neuropil in the molecular layer (**Figure 6B**). The hippocampus of rats treated with malathion plus 5 mg/kg body weight of methylene blue showed compact arrangement with some degenerating shrunken neurons. The molecular layer showed dilated congested vessels, indicating increased vascularity and clumped neuronal processes forming clumped neuropil (**Figure 6C**). Sections from rats treated with malathion plus 10 mg/kg body weight of methylene blue showed occasional degenerating neurons, which appeared shrunken and dark (**Figure 6D**).

3.3. GFAP Immunostaining

Malathion treatment of rats resulted in a marked increase of the GFAP staining in the molecular layer of the hippocampus compared with faint staining in the saline-treated group (**Figure 7A** and 7**B**). Strong

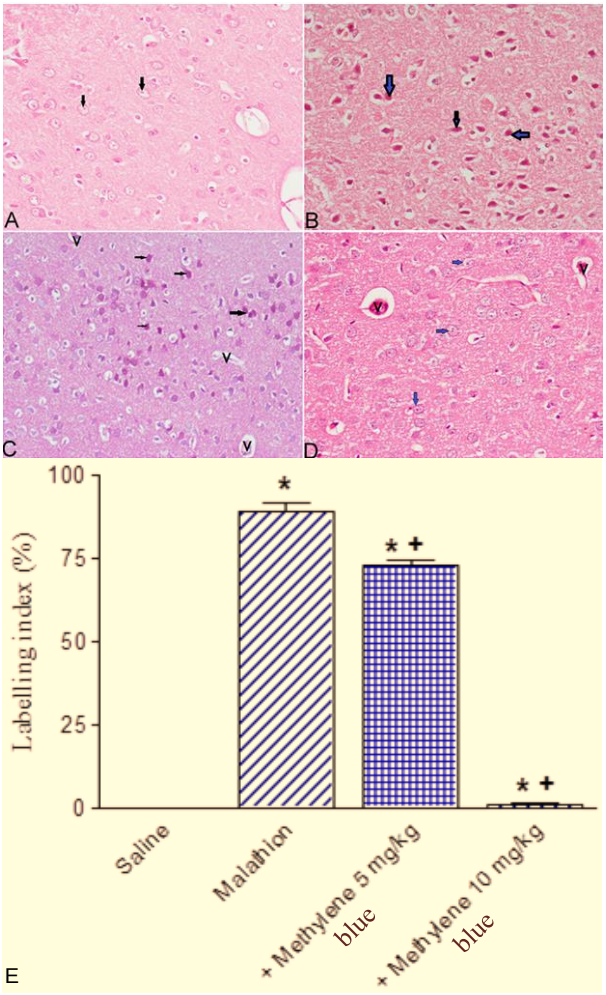

FIGURE 5. Photomicrographs of representative brain sections from the cortex (A-D) and quantitative analysis (E). In (A), saline-treated group showed normal neurons with prominent nuclei (arrows) in a fibrillary background. In (B), malathion-exposed group showed numerous degenerating neurons, which appeared shrunken and dark (arrows). In (C), malathion plus methylene blue (5 mg/kg body weight) group showed many degenerating neurons, which appeared shrunken and dark (arrows). Some dilated blood vessels were also seen (V letters). In (D), malathion plus methylene blue (10 mg/kg body weight) group showed normal neurons (arrows) in a fine fibrillary background and dilated blood vessels (V letters). All images are 400× and H&E staining. In (E), quantitative estimation of the degenerating neurons in the cortex.*, $p < 0.05$ versus corresponding saline group; +, $p < 0.05$ versus malathion group.

GFAP expression was still observed in animals treated with malathion plus 5 mg/kg body weight of methylene blue (**Figure 7C**). In contrast, there was weak GFAP expression after co-treatment of the malathion-exposed rats with 10 mg/kg body weight of methylene blue (**Figure 7D**).

4. DISCUSSION

In this study, acute administration of malathion to rats resulted in increased oxidative stress in the brain tissue as indicated by an increase in the lipid peroxidation product MDA and by the markedly reduced GSH level. Oxidative stress ensues when the brain antioxidant defenses are unable to cope with exceedingly generated free radicals. Oxidative stress results in damage to cell membranes, enzymes, and DNA as well as disruption of redox signaling and other cellular physiological processes. A number of non-protein antioxidant molecules (e.g., ascorbate, tocopherols, carotenoids) and enzymes constitute the cell's antioxidant machinery [8]. One important free radical scavenger in the brain is GSH, a tripeptide of glutamate, cysteine, and glycine. In addition to a direct scavenging action, GSH participates in reduction of free radicals and reactive oxygen species and oxidized sulfhydryl groups via acting as a co-substrate for antioxidant enzymes (e.g., GSH peroxidase, glutaredoxin), and the ratio between its reduced and oxidized forms determines the redox state of the cell [26]. The observed decrease in the brain GSH level is thus likely to be the result of increased free radical generation by malathion with the consequent consumption of the antioxidant. This notion is supported by other studies in which intraperitoneal injection of malathion into rats at doses of 50–150 mg/kg body weight resulted in increased brain lipid peroxidation [6] and decreased GSH peroxidase and glutathione reductase activities in the cerebral cortex [7]. Rats administered malathion orally also showed increased lipid peroxidation in erythrocytes [27]. Malathion increased lipid peroxidation (MDA) and decreased the activities of the antioxidant enzymes, including superoxide dismutase, catalase, and GSH peroxidase, in human erythrocytes in vitro [28]. Moreover, antioxidant treatment with vitamin C or vitamin E conferred partial protection against the changes in antioxidant enzymes and lipid peroxidation induced by malathion in erythrocytes [27, 28]. The results of

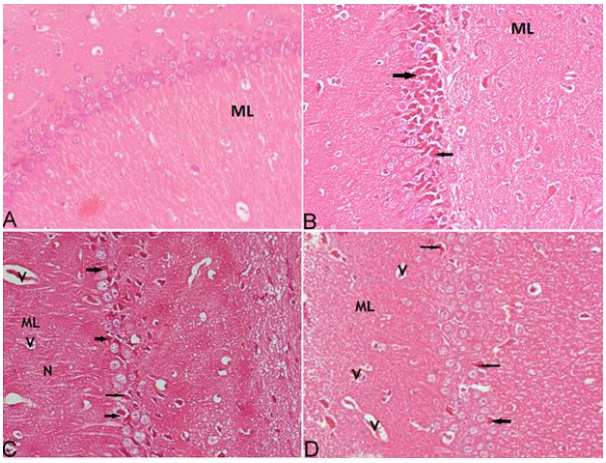

FIGURE 6. Photomicrographs of representative brain sections from the hippocampus of the rats. In (A), saline-treated group showed normal neurons and fine fibrillary molecular layer (ML). In (B), malathion-exposed group showed numerous degenerating neurons (dark neurons) (arrows). The molecular layer showed clumped neuropil. In (C), malathion plus methylene blue (5 mg/kg body weight) group showed some degenerating neurons (arrows). The molecular layer showed dilated blood vessels (V letters), and clumped neuronal axons (N letter). In (D), malathion plus methylene blue (10 mg/kg body weight) showed some degenerating neurons, which appeared dark and shrunken (arrows). The molecular layer showed some dilated blood vessels (V letters). All images are 400x and H&E staining.

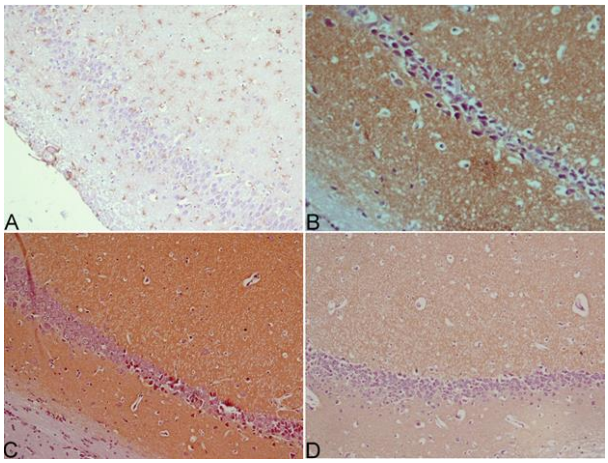

FIGURE 7. Glial fibrillary acidic protein (GFAP) immunostaining of the hippocampus of the rats. In (A), saline control group showed faint staining for GFAP in the molecular layer. In (B), malathion-exposed group showed strong diffuse positive staining of GFAP. In (C), malathion plus methylene blue (5 mg/kg body weight) group showed strong staining of GFAP in the molecular layer. In (D), malathion plus methylene blue (10 mg/kg body weight) showed weak staining of GFAP in the molecular layer. All images have a magnification scale of 200.

the present study showed that co-treatment with methylene blue at 10 mg/kg body weight was capable of reducing brain MDA formation in malathion-injected rats, indicating decreased lipid peroxidation by the dye. Methylene blue, however, showed no effect on brain GSH level in malathion-injected rats, suggesting that the decrease in lipid peroxidation by this dye was not due to changes in GSH level.

The current study also showed that malathion exposure resulted in a marked increase in brain nitric oxide level. Nitric oxide is derived from L-arginine by the action of nitric oxide synthase (NOS), which exists in three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible form (iNOS). Nitric oxide is an important intercellular messenger, involved in neuronal signaling, neural activity, and blood flow modulation [29]. On the other hand, iNOS expressed in microglial cells and astrocytes after proinflammatory cytokine stimulation can generate high fluxes of nitric oxide for periods of hours to days and has been implicated in neuronal energy failure and the development of neuronal injury [30, 31]. The toxic effects of nitric oxide are mediated by reactive nitrogen species derived from the interaction of nitric oxide with superoxide or oxygen. Of particular importance is the reaction between superoxide and nitric oxide to form the powerful oxidant peroxynitrite ($ONOO^-$), resulting in nitrosation of amines and thiols, nitration of tyrosine residues, lipid peroxidation, protein oxidation, and DNA base oxidation [29–31]. The results of the present study showed that after methylene blue co-treatment at 5 and 10 mg/kg body weight, the brain nitric oxide level in malathion-injected rats was decreased by 29.2% and 35.8%, respectively. Studies showed that methylene blue acted as a direct inhibitor of cerebellar NOS in vitro [32], and also inhibited brain (hippocampal) NOS activity in vivo [33].

Moreover, the neuroprotective and cardioprotective effects of methylene blue in experimental cardiac arrest were attributed to the effect of the dye on NOS and guanylyl cyclase [34, 35]. Methylene blue reduced cerebral tissue nitrite/nitrate content, blood-brain barrier permeability, and the number of cortical cells showing iNOS and eNOS activation [35]. Inhibition of NOS and decreased brain nitric oxide levels might be an important mechanism through which methylene blue provides neuroprotection against malathion-induced toxicity.

We also demonstrated a significant inhibition of the brain PON1 activity in malathion-exposed rats. The paraoxonase family of enzymes, which comprise three members, is involved in the hydrolysis of the active metabolites of a number of organophosphate insecticides, nerve agents, and many other xenobiotics [36]. Studies showed that PON1 is important in the detoxication of diazoxon, paraoxon, dichlorvos, and chlorpyrifos oxon [36–38] and a low PON1 status appeared to increase the susceptibility to organophosphate toxicity in humans [39]. The decrease in brain PON1 enzyme activity by malathion in the current study is in line with the finding of decreased PON1 activity following acute intoxication with organophosphates and in farm workers exposed to anticholinesterase pesticides [40, 41]. Moreover, in subjects handling organophosphate agricultural pesticides, low plasma PON1 activity and low PON1catalytic efficiency were both significantly associated with greater BChE inhibition [42]. The low PON1 activity in the brains of rats exposed to malathion in the present work might be due to inactivation of the enzyme by malathion itself or secondary to increased free radicals and nitric oxide resulting in oxidative stress. Oxidative stress has been shown to cause inactivation of PON1 [43]. Thus, it is possible that the methylene blue-induced increase of PON1 activity in the brains of the rats receiving malathion is due to a decrease in oxidative stress.

Organophosphate insecticides are irreversible inhibitors of AChE [2]. This action leads to an increased concentration of the neurotransmitter acetylcholine in neuronal synapse and neuromuscular junction, resulting in excessive cholinergic stimulation both centrally and peripherally [3]. There is evidence that methylene blue inhibits cholinesterase activity [44–46]. Methylene blue inhibited the esterase activity of acetylcholinesterase of human plasma (also known as pseudocholinesterase or BChE) and bovine acetylcholinesterase [44]. Küçükkilinç and Ozer [45] found that methylene blue acted as a complex inhibitor of human plasma BChE. Recently, Petzer et al. [46] reported that methylene blue and also its metabolite azure B, acted as a reversible and competitive inhibitor of AChE and BChE. In the present study, methylene blue co-treatment did not alter the brain BChE activity in malathion-exposed rats. The brain AChE activity of malathion-exposed rats, however, was increased by the dye. Similar observations were reported in rotenone-exposed rats where methylene blue prevented the decline in brain AChE activity induced by the neurotoxicant [47].

In this study, the histopathological examination of the brain tissue indicated a clear neuroprotective effect of methylene blue in malathion intoxication. Administration of the dye at 10 mg/kg body weight to malathion-exposed rats was associated with normally appearing cortex while degenerating neurons were occasionally observed in the hippocampus compared with marked neuronal degeneration in the malathion-exposed rats not receiving methylene blue. These observations were confirmed by quantitative analysis of the number of the degenerating neurons in the cortex. The results of the present study demonstrated increased expression of GFAP in the hippocampus following malathion injection, which was in contrast to weak GFAP expression after co-treatment of the malathion-exposed rats with methylene blue at 10 mg/kg body weight. GFAP is a major protein constituent of glial filaments in astrocytes, which is important in cytoskeleton support. GFAP is a marker for glia cell activation and reactive gliosis, where the central nervous system insults (e.g., ischemia, trauma, or toxins) result in the activation of microglia and astrocytes into their activated phenotypes with increased GFAP expression. GFAP might be a useful marker for various types of brain damage, and measurement of GFAP could be used to monitor glial pathology [48] Astroglial cells have a role in tissue repair via the formation of the glial scar. These cells also secrete a number of growth factors (e.g., nerve growth factor, brain-derived neurotrophic factor) and inflammatory cytokines (e.g., interleukin-1, interleukin-6) and are thus involved in the local inflammatory response. Astroglial cells express iNOS, and the high amounts of nitric oxide produced by iNOS could be neurotoxic. Under pathological conditions, astroglial activation thus could result in neuronal death [49]. Inhibition of glia

cell activation by methylene blue might therefore account for its neuroprotective effect observed in the present study.

In summary, the results of the present study demonstrated that co-administration of the redox active dye methylene blue protected the brain against malathion-induced neurotoxicity in rats. The neuroprotective effect of methylene blue likely resulted from the decreased formation of nitric oxide, reduced oxidative stress, and inhibition of glial cell activation in the brain of the malathion-exposed animals.

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