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Nitric oxide synthase inhibitors protect against brain and liver damage caused by acute malathion intoxication

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ABSTRACT

Objective: To investigate the effect of N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor, and 7-nitroindazole (7-NI), a selective neuronal NOS inhibitor, on oxidative stress and tissue damage in brain and liver and on DNA damage of peripheral blood lymphocytes in malathion intoxicated rats.

Methods: Malathion (150 mg/kg) was given intraperitoneally (i.p.) along with L-NAME or 7-NI (10 or 20 mg/kg, i.p.) and rats were euthanized 4 h later. The lipid peroxidation product malondialdehyde (MDA), nitric oxide (nitrite), reduced glutathione (GSH) concentrations and paraoxonase-1 (PON-1) activity were measured in both brain and liver. Moreover, the activities of glutathione peroxidase (GPx) acetylcholinesterase (AChE), and butyrylcholinesterase (BChE), total antioxidant capacity (TAC), glucose concentrations were determined in brain. Liver enzyme determination, Comet assay, histopathological examination of brain and liver sections and inducible nitric oxide synthase (iNOS) immunohistochemistry were also performed.

Results: (i) Rats treated with only malathion exhibited increased nitric oxide and lipid peroxidation (malondialdehyde) accompanied with a decrease in GSH content, and PON-1 activity in brain and liver. Glutathione peroxidase activity, TAC, glucose concentrations, AChE and BChE activities were decreased in brain. There were also raised liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and increased DNA damage of peripheral blood lymphocytes (Comet assay). Malathion caused marked histopathological changes and increased the expression of iNOS in brain and liver tissues. (ii) In brain of malathion-intoxicated rats, L-NAME or 7-NI resulted in decreased nitrite and MDA contents while increasing TAC and PON1 activity. Reduced GSH and GPx activity showed an increase by L-NAME. AChE activity increased by 20 mg/kg L-NAME and 10 mg/kg 7-NI. AChE activity decreased by the higher dose of 7-NI while either dose of 7-NI resulted in decreased BChE activity. (iii) In liver of malathion-intoxicated rats, decreased MDA content was observed after L-NAME or 7-NI. Nitrite level was unchanged by L-NAME but increased after 7-NI which also resulted in decreased GSH concentration and PON1 activity. Either inhibitor resulted in decreased liver ALT activity. (iv) DNA damage of peripheral blood lymphocytes was markedly inhibited by L-NAME or 7-NI treatment. (v) iNOS expression in brain and liver decreased by L-NAME or 7-NI. (vi) More marked improvement of the histopathological alterations induced by malathion in brain and liver was observed after 7-NI compared with L-NAME.

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Conclusions: In malathion intoxicated rats, the neuronal NOS inhibitor 7-NI and to much less extent L-NAME were able to protect the brain and liver tissue integrity along with improvement in oxidative stress parameters. The decrease in DNA damage of peripheral blood lymphocytes by NOS inhibitors also suggests the involvement of nitric oxide in this process.

1. Introduction

Organophosphate insecticides are widely used compounds in agriculture, gardens, household and in veterinary [1]. These agents pose the risk of causing human neurotoxicity, both acute and chronic [2]. In acute intoxication, exposure to high concentrations of organophosphates, e.g., malathion result in headache, dizziness, ataxia, confusion, agitation, coma, muscle twitching, convulsions, muscle paralysis amounting to respiratory failure and death. These effects are mediated in large part by an inhibitory effect on AChE, the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses in the central and peripheral nervous system, autonomic ganglia, and motor end-plate [3,4]. Long-term complications of organophosphates have also been described including mood disorders, cognitive and memory impairments, psychiatric manifestations, and delayed polyneuropathy [5,6]. Moreover, exposure to these compounds has been implicated in the development of neurodegenerative disorders like Parkinson's disease [7,8]. Rats treated with malathion showed neuronal degeneration in cortex and hippocampus, reactive gliosis, increased glial fibrillary acidic protein immunostaining in hippocampus [9,10]. Besides their action on AChE, organophosphates are likely to exert their neurotoxic effects through other mechanisms such as impairment of mitochondrial dynamics and mitochondrial bioenergetics [11,12], oxidative and nitrosative stress [9,10,13–15]. In rats, exposure to malathion resulted in increased lipid peroxidation in brain, liver and blood [9,10,14,16,17]. The activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase and total antioxidant capacity (TAC) decrease in brain as well [9,10,14]. Human erythrocytes showed reduced activities of superoxide dismutase, catalase and GPx [13].

The role of nitric oxide (NO) in the development of neuronal damage in toxic and inflammatory conditions is a subject of paramount importance [18]. The gaseous molecule NO is produced from L-arginine via the action of the nitric oxide synthase enzyme (NOS)in the presence of O_2, nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin. Under physiological conditions NO is an important signaling molecule involved in neurotransmission, and in maintenance of vascular tone. The endothelial (eNOS) and neuronal (nNOS) isoforms of the enzyme account for constitutively formed low concentrations of NO under physiological conditions. A third isoform that is inducible NOS (iNOS) is not constitutively present but is expressed by glial cells (astrocytes and microglia) and other immune cells by the action of endotoxin lipopolysaccharide or cytokines such as interferon-γ, interleukin-1β or tumor necrosis factor-α(its expression increases) [19,20]. This generates excessive amounts of NO for longer lime with a resultant detrimental impact on tissue function and integrity [21]. This is largely due to the reaction of NO with O_2 to form reactive nitrogen oxide species, e.g., NO_2 and N_2O_3 or with superoxide anion ($O_2^{\bullet -}$) forming the highly reactive peroxynitrite ($ONOO^-$) (with these species being) capable of causing oxidation or nitrosylation of thiols, nitration of tyrosine residues, oxidation of lipids, protein and DNA [21–23]. The neurotoxic effects of acute malathion exposure in rats are associated with increased endogenous NO biosynthesis and iNOS expression in brain. In these studies, neuroprotection was associated with decreased level of NO and lower oxidative stress, thereby, suggesting a role for NO in the malathion neurotoxicity.

NOS antagonists offer the opportunity for evaluating the role of NO in the development of neuronal injury by organophosphates. In this study we investigated the effect of the non-selective NOS antagonist N^G-nitro-L-arginine methyl ester (L-NAME) and the selective neuronal NOS antagonist 7-nitroindazole (7-NI) on oxidative stress and neuronal injury in the rat brain following acute malathion exposure. Since the organophosphate has been shown to cause liver cell necrosis [9,24,25]and DNA damage of peripheral blood lymphocytes [26,27], the study was extended to investigate the role of NO in these effects of malathion.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 130–140 g from Animal House Colony of the National Research Centre were used in the study. They were allowed free access to standard laboratory food and water. All animal procedures followed the recommendations of the institutional Ethics Committee and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

Malathion (Commercial grade: 57%) was obtained from El-Naser Chemical Co. (Cairo, Egypt). L-NAME and 7-NI were purchased from Sigma–Aldrich (St Louis, MO, USA) and dissolved in normal saline. The other chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich.

2.3. Study design

Rats were randomly assigned into six different groups (6 rat/group). Group 1 received i.p. saline (0.2 mL/rat) and served as negative control. Groups 2–6 received i.p. malathion at a dose of 150 mg/kg and were treated i.p. with one of the following: saline (group 2), L-NAME at 10 or 20 mg/kg (groups 3 & 4), 7-NI at 10 or 20 mg/kg (groups 5 & 6). Rats were euthanized by decapitation 4 h post-drug administration; their brains were quickly removed on

ice-plate and washed with ice-cold phosphate-buffered saline at pH 7.4. Brains were weighed and stored at −80 °C for the biochemical assays. Homogenization of brain tissues was done using 0.1 mol/L phosphate buffer saline (pH 7.4) to give a final concentration of 20% w/v for the biochemical assays.

2.4. Biochemical analyses

2.4.1. Lipid peroxidation

Malondialdehyde (MDA), a lipid peroxidation product was determined in tissue homogenates using the method of Nair and Turne [28]. Thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid resulting in the formation of TBA-MDA adduct which can be measured using spectrophotometer at 532 nm.

2.4.2. Reduced glutathione (GSH)

GSH was determined in tissue homogenates with the method of Ellman *et al* [29]. Reduction of Ellman's reagent (DTNB; 5,5′-dithiobis (2-nitrobenzoic acid)) by the free sulfhydryl group on GSH results in yellow colored 5-thio-2-nitrobenzoic acid which can be determined using spectrophotometer at 412 nm.

2.4.3. NO

NO measured as nitrite was determined in tissue homogenates using Griess reagent [30]. Nitrate is converted to nitrite via nitrate reductase. Griess reagent then act to convert nitrite to a deep purple azo compound which can be determined using spectrophotometer [30].

2.4.4. GPx activity

GPx activity in supernatants was determined by measuring NADPH oxidation at 340 nm using colorimetric GPx kit (Biodiagnostic, Egypt). One unit of GPx activity is equivalent to the amount of protein that oxidizes 1 mmol/L NADPH per minute. The activity of GPx is expressed as mU/mL.

2.4.5. Paraoxonase-1 (PON-1) activity

Arylesterase activity of PON-1 was spectrophotometrically measured using phenylacetate as a substrate. PON-1 catalyzes the cleavage of phenyl acetate resulting in phenol formation, the rate of which is measured by monitoring the increase in absorbance at 270 nm at 25 °C using Recording Spectrophotometer (Shimadzu Corporation). One unit of arylesterase activity is defined as 1 μmol/L of phenol formed per minute. Enzyme activity was calculated based on the extinction coefficient of phenol of 1310 mol/cm at 270 nm, pH 8.0 and 25 °C and expressed kilo International Unit/Liter (kU/L) [31,32].

2.5. AChE activity

AChE activity was measured according to the method of Ellman *et al* [33,34]. The method uses DTNB to measure the amount of thiocholine produced as acetylthiocholine is hydrolyzed by AChE. The color of DTNB adduct can be measured with spectrophotometer at 412 nm. AChE activity is expressed as μmol sulfhydryl (SH) groups/(g/min).

2.6. Butyrylcholinesterase(BChE)activity

BChE (EC 3.1.1.8) activity was measured in brain supernatants using commercially available kit from Ben Biochemical Enterprise (Milan, Italy). In this assay, BChE catalyzes the hydrolysis of butyrylthiocholine as substrate into butyrate and thiocholine. Thiocholine then reacts with DTNB forming a yellow chromophore which can be quantified with spectrophotometer [33].

2.7. TAC

TAC was measured in brain homogenates using a colorimetric kit obtained from Biodiagnostic (Egypt). In this method antioxidants in the sample react with a defined amount of exogenously provided hydrogen peroxide. The remaining hydrogen peroxide will be determined calorimetrically by an enzymatic reaction which involves the conversion of 3,5-dichloro-2-hydroxy benzenesulfate to a colored product [35].

2.8. Glucose

Glucose oxidase catalyzes the oxidation of glucose to gluconic acid with the formation of hydrogen peroxide. The latter reacts with phenol and 4-amino-antipyrine in the presence of peroxidase resulting in a colored quinonemine which can be measured using spectrophotometer [36].

2.9. Liver enzymes

Reitman–Frankel procedure was used for the colorimetric determination of aspartate aminotransferase (AST) activities and alanine aminotransferase (ALT) in liver homogenates [37]. Colorimetric determination of alkaline phosphatase (ALP) activity was done according to Belfield and Goldberg [38]. Commercially available kits from BioMérieux (France) were used.

2.10. Comet assay

Isolation of peripheral blood leukocytes was performed by centrifugation for 30 min at 1 300 *g* using Ficoll–Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). The comet assay was done at low temperature to minimize spontaneous DNA damage [39,40]. The details of the method were published elsewhere [9].

2.11. Histological studies

The rats were killed by decapitation and their brains and livers were quickly removed out. Slices were then fixed in 10% formalin (pH 7.4) for a minimum of 72 h, washed in tap water for 30 min, dehydrated using ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections (5 μm thick) were stained with hematoxylin and eosin (Hx & E), examined and photographed under light microscope using a digital camera (DP70, Tokyo). Adobe Photoshop version 8.0 was used for images processing.

2.12. Immunohistochemistry

Immunohistochemistry for iNOS was performed on paraffin-embedded sections that were deparaffinized and rehydrated. Sections were incubated in 0.3% H_2O_2 solution in methanol at room temperature for 30 min to block endogenous peroxidase activity. For antigen retrieval, sections were heated in a microwave oven at 720 W for 25 min and incubated with mouse

monoclonal iNOS antibodies (dilution 1: 50) at 4 °C overnight. Sections were then washed with phosphate buffered saline (pH 7.4) followed by incubation with biotinylated goat-anti-rabbit-immunoglobulin G secondary antibodies (dilution 1: 200) and streptavidin/alkaline phosphatase complex (dilution 1: 200) for 30 min at room temperature. The binding sites of antibody were visualized with 3,3′-diaminobenzidine (DAB). Thereafter, sections were rinsed with phosphate buffered saline, counterstained with H&E for 2–3 min, and dehydrated in ascending grades of ethanol. Slices were soaked twice in xylene at room temperature for 5 min, mounted, and examined by light microscope.

2.13. Immunomorphometric analysis

Quantitative assessment of iNOS immunoreactivity was performed at the Pathology Department, National Research Center using the Leica Qwin 500 Image Analyzer system (LEICA Imaging Systems Ltd, Cambridge, England) which consists of Leica DM-LB microscope with JVC color video camera attached to a computer system Leica Q 500IW.

2.14. Statistical analysis

Data are presented as mean ± SE. Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS software (SAS Institute Inc., Cary, NC). Differences were regarded as statistically significant at a probability value of less than 0.05.

3. Results

3.1. Oxidative stress

3.1.1. Lipid peroxidation

In brain tissue, malathion caused significant ($P < 0.05$) increase in malondialdehyde compared to saline control. Rats treated with malathion and L-NAME exhibited 19.7% and 24.1% ($P < 0.05$) decreases in brain malondialdehyde compared with the malathion control. Meanwhile, a significant decrease in malondialdehyde by 31.5% ($P < 0.05$) was observed after treatment with 7-NI at 20 mg/kg (Table 1).

A marked increase in the level of MDA by 59.5% ($P < 0.05$) was also seen in the liver tissue of malathion-treated rats compared with the saline control. A significant decrease in MDA by 18.0% ($P < 0.05$), 19.7% ($P < 0.05$) and 26.9% ($P < 0.05$) was observed after treatment with 2 mg/kg L-NAME, 10 mg/kg 7-NI and 20 mg/kg 7-NI, respectively compared with the malathion control (Table 2).

3.1.2. NO

Exposure to malathion resulted in a significant increase in NO concentration in brain tissue compared to vehicle control (46% increase; $P < 0.05$). In malathion-treated rats, the level of NO in brain was brought to near normal value by the administration of L-NAME or 7-NI (Table 1).

NO increased by 50.3% ($P < 0.05$) in the liver by malathion treatment. L-NAME did not significantly change the level of NO.

Table 3
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in the brain of rats given malathion alone or combined with either L-NAME or 7-NI.

Group	AChE activity (μmol SH/g/min)	BChE activity (U/L)
Vehicle	9.62 ± 0.51	179.30 ± 8.20
Malathion	6.77 ± 0.33*	115.20 ± 6.10*
Malathion + L-NAME 10 mg/kg	6.10 ± 0.41*	115.74 ± 4.90*
Malathion + L-NAME 20 mg/kg	8.37 ± 0.31^{+}	133.13 ± 7.00*
Malathion + 7-NI 10 mg/kg	8.63 ± 0.40^{+}	78.12 ± 3.10*$^{\#}$
Malathion + 7-NI 20 mg/kg	5.47 ± 0.38*$^{\#}$	69.12 ± 3.70*$^{\#}$

*$P < 0.05$ *vs.* saline group. $^{+}P < 0.05$ *vs.* malathion only group. $^{\#}P < 0.05$ *vs.* malathion + L-NAME (One way ANOVA and Duncan's test).

Table 1
Malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), total antioxidant capacity (TAC), glutathione peroxidase (GPx) activity and paraoxonase-1 (PON-1) activity in the brain of rats treated with malathion alone or combined with either L-NAME or 7-NI.

Group	MDA (nmol/g·tissue)	NO (μmol/g·tissue)	GSH (μmol/g·tissue)	TAC (μmol/g·tissue)	GPx activity (mU/mL)	PON-1 activity (kU/L)
Vehicle	21.70 ± 1.30	21.50 ± 0.97	4.54 ± 0.11	0.633 ± 0.028	85.20 ± 4.00	12.86 ± 0.67
Malathion	32.37 ± 1.62*	31.44 ± 2.00*	3.10 ± 0.20*	0.259 ± 0.012*	45.10 ± 1.60*	7.45 ± 0.21*
Malathion + L-NAME 10 mg/kg	26.00 ± 1.43*	20.70 ± 1.00*$^{+}$	3.03 ± 0.18*	0.268 ± 0.027*	72.27 ± 4.40*$^{+}$	8.00 ± 0.29*
Malathion + L-NAME 20 mg/kg	24.57 ± 0.83*$^{+}$	17.40 ± 0.58*$^{+}$	3.81 ± 0.15*$^{+}$	0.388 ± 0.022*$^{+}$	72.94 ± 3.00*$^{+}$	9.63 ± 0.37*$^{+}$
Malathion + 7-NI 10 mg/kg	29.51 ± 1.33	16.30 ± 0.85*$^{+}$	2.82 ± 0.14*$^{\#}$	0.308 ± 0.020*$^{\#}$	48.63 ± 2.10*$^{\#}$	6.72 ± 0.18*$^{+\#}$
Malathion + 7-NI 20 mg/kg	22.23 ± 0.92*$^{+}$	16.00 ± 0.73*$^{+}$	2.33 ± 0.10*$^{+\#}$	0.382 ± 0.016*$^{+}$	48.00 ± 3.30*$^{\#}$	9.15 ± 0.40*$^{+}$

*$P < 0.05$ *vs.* saline group. $^{+}P < 0.05$ *vs.* malathion only group. $^{\#}P < 0.05$ *vs.* malathion + L-NAME. (One way ANOVA and Duncan's test).

Table 2
Malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) and paraoxonase-1 (PON-1) activity in the liver of rats given malathion alone or combined with either L-NAME or 7-NI.

Group	MDA (nmol/g·tissue)	NO (μmol/g·tissue)	GSH (μmol/g·tissue)	PON-1 activity (kU/L)
Vehicle	42.00 ± 3.56	32.40 ± 1.10	12.23 ± 0.87	43.70 ± 2.10
Malathion	67.00 ± 2.80*	48.70 ± 1.54*	8.81 ± 0.34*	22.40 ± 0.89*
Malathion + L-NAME 10 mg/kg	63.70 ± 3.20*	48.00 ± 1.70*	8.56 ± 0.33*	21.40 ± 1.40*
Malathion + L-NAME 20 mg/kg	54.90 ± 3.00*$^{+}$	55.00 ± 3.10*	9.64 ± 0.25*	22.70 ± 0.93*
Malathion + 7-NI 10 mg/kg	53.80 ± 1.80*$^{+}$	79.95 ± 3.80*$^{+\#}$	6.93 ± 0.21*$^{+\#}$	13.50 ± 0.67*$^{+\#}$
Malathion + 7-NI 20 mg/kg	49.00 ± 2.60*$^{+\#}$	71.30 ± 2.50*$^{+\#}$	7.43 ± 0.41*$^{\#}$	14.90 ± 0.84*$^{+\#}$

*$P < 0.05$ *vs.* saline group and between different groups as indicated on the graph. $^{+}P < 0.05$ *vs.* malathion only group. $^{\#}P < 0.05$ *vs.* malathion + L-NAME.

Table 4

Brain glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the liver tissue, and percentage of comets from blood lymphocytes in rats after treatment with malathion alone or combined with L-NAME or 7-NI.

Group	Glucose (μg/g·tissue)	AST activity (U/g·tissue)	ALT activity (U/g·tissue)	Comets (%)
Vehicle	325.0 ± 11.1	29.8 ± 1.6	21.3 ± 1.0	5.0 ± 0.2
Malathion	215.2 ± 8.3*	71.2 ± 3.4*	77.0 ± 4.2*	81.0 ± 3.7*
Malathion + L-NAME 10 mg/kg	150.0 ± 7.1*+	63.0 ± 4.2*	65.4 ± 3.5*	30.0 ± 2.2*
Malathion + L-NAME 20 mg/kg	177.0 ± 10.3*+	59.8 ± 2.6*	20.3 ± 1.4*+	26.5 ± 1.5*
Malathion + 7-NI 10 mg/kg	183.0 ± 9.5*	68.1 ± 2.7*	68.0 ± 3.9*	44.0 ± 2.1*
Malathion + 7-NI 20 mg/kg	90.7 ± 4.8*+	50.3 ± 2.1*+#	43.5 ± 1.7*#	34.5 ± 1.8*+#

*$P < 0.05$ *vs*. saline group and between different groups. +$P < 0.05$ *vs*. malathion only group. #$P < 0.05$ *vs*. malathion + L-NAME (One way ANOVA and Duncan's test).

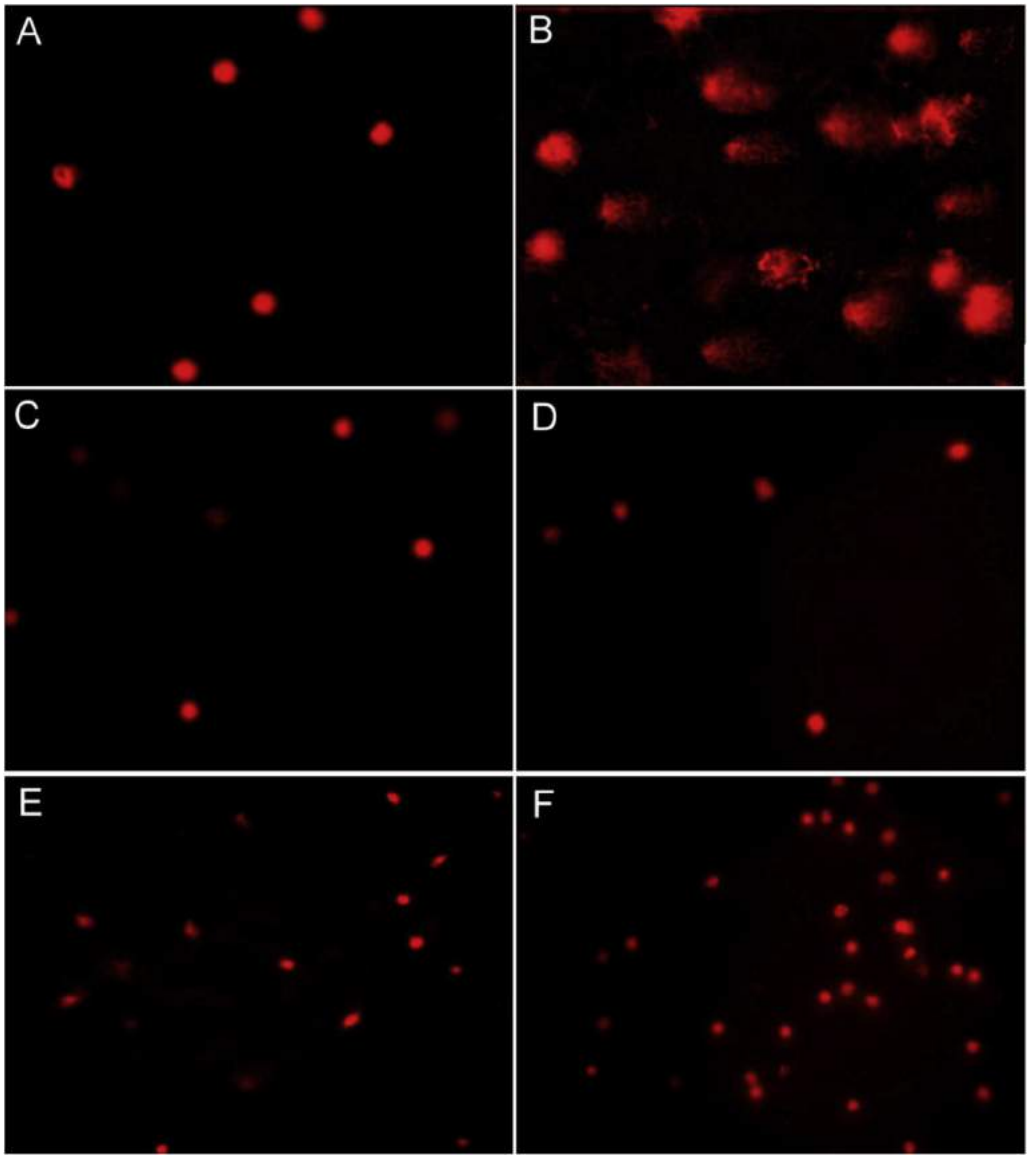

Figure 1. Representative fluorescence images of comets from blood lymphocytes of rats following malathion alone or combined L-NAME or 7-NI. (A) Saline control. (B) Malathion. (C & D) Malathion + L-NAME 10 or 20 mg/kg. (E & F) Malathion + 7-NI 10 or 20 mg/kg.

In contrast significant increase in NO by 64.0% ($P < 0.05$) and 6.4% ($P < 0.05$) in liver of malathion-treated rats was observed after the administration of 7-NI at 10 and 20 mg/kg, respectively compared with the malathion control group (Table 2).

3.1.3. GSH

In malathion alone-treated rats, brain GSH fell by 31.7% ($P < 0.05$) compared with the saline group. GSH increased by 22.9% ($P < 0.05$) in the malathion and 20 mg/kg L-NAME

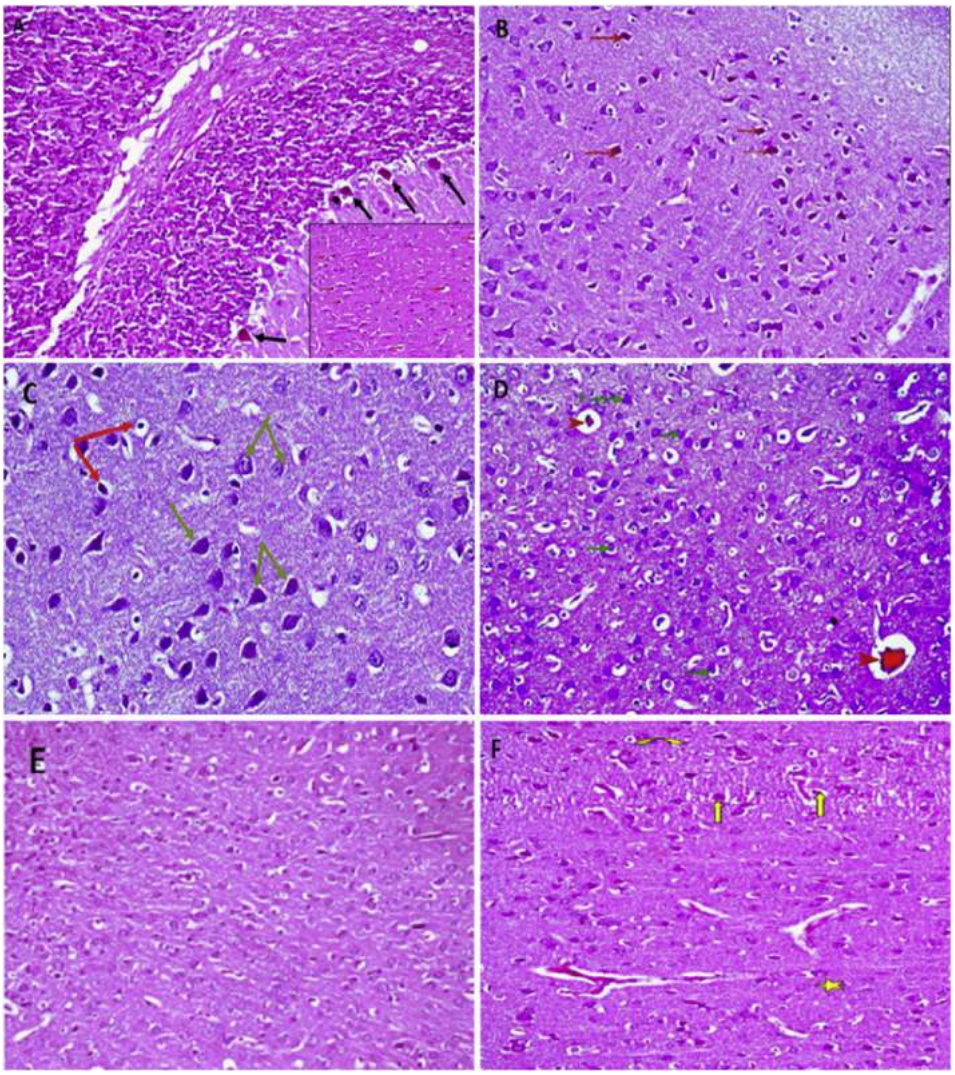

Figure 2. Hematoxylin and Eosin (Hx& E) stained brain sections from rats treated with (A) malathion; (B) malathion + L-NAME 10 mg/kg; (C) malathion + L-NAME 20 mg/kg; (D) malathion + 7-NI 10 mg/kg; (E) malathion + 7-NI 20 mg/kg; (F) saline.
Black arrows: degenerated Purkinje cells. Red arrows: pyknotic neuronal cells. Green arrows: normal neuronal cells. Red arrows heads: congested blood vessels. Curved yellow arrow: normal granular layer. Yellow arrow:healthy Purkinje cell layer. Yellow star: normal molecular layer (×200).

group compared with the malathion only group. In contrast, 24.8% ($P < 0.05$) decrease in brain GSH was observed in the malathion and 20 mg/kg 7-NI group compared with the malathion control (Table 1).

In the liver, GSH showed 28.0% ($P < 0.05$) decrease after malathion exposure compared with the saline group. There was no significant change in GSH content by the administration of L-NAME. In contrast, 7-NI given at 10 mg/kg to malathion-treated rats caused further and significant decline in GSH by 21.3% ($P < 0.05$) (Table 2).

3.1.4. TAC

Malathion resulted in significantly decreased brain TAC by 59.1% ($P < 0.05$) compared with the saline control group. Rats treated with malathion and 20 mg/kg L-NAME or 20 mg/kg 7-NI exhibited significant increase in brain TAC by 49.8% ($P < 0.05$) and 47.5% ($P < 0.05$), respectively compared with the malathion only treatment group (Table 1).

3.1.5. GPx activity

In the malathion only group, GPx activity in brain decreased by 47.1% ($P < 0.05$) compared with the saline control. In malathion intoxicated rats brain GPx activity increased by 60.3% ($P < 0.05$) and 61.6% ($P < 0.05$) following L-NAME at 10 and 20 mg/kg, respectively. However, the administration 7-NI had no significant effect on GPx activity in malathion-treated rats (Table 1).

3.1.6. PON-1 activity

In brain tissue of malathion only treated rats, PON-1 activity decreased by 42.1% ($P < 0.05$) compared with the saline group. In malathion-treated rats, 7-NI given at 10 mg/kg resulted in further decrease in PON-1 activity by 23.3% ($P < 0.05$). The administration of either 20 mg/kg L-NAME or 20 mg/kg 7-NI to malathion intoxicated rats was associated with 29.3% ($P < 0.05$) and 22.8% ($P < 0.05$) increments in brain PON-1 activity, respectively as compared to the malathion control group (Table 1).

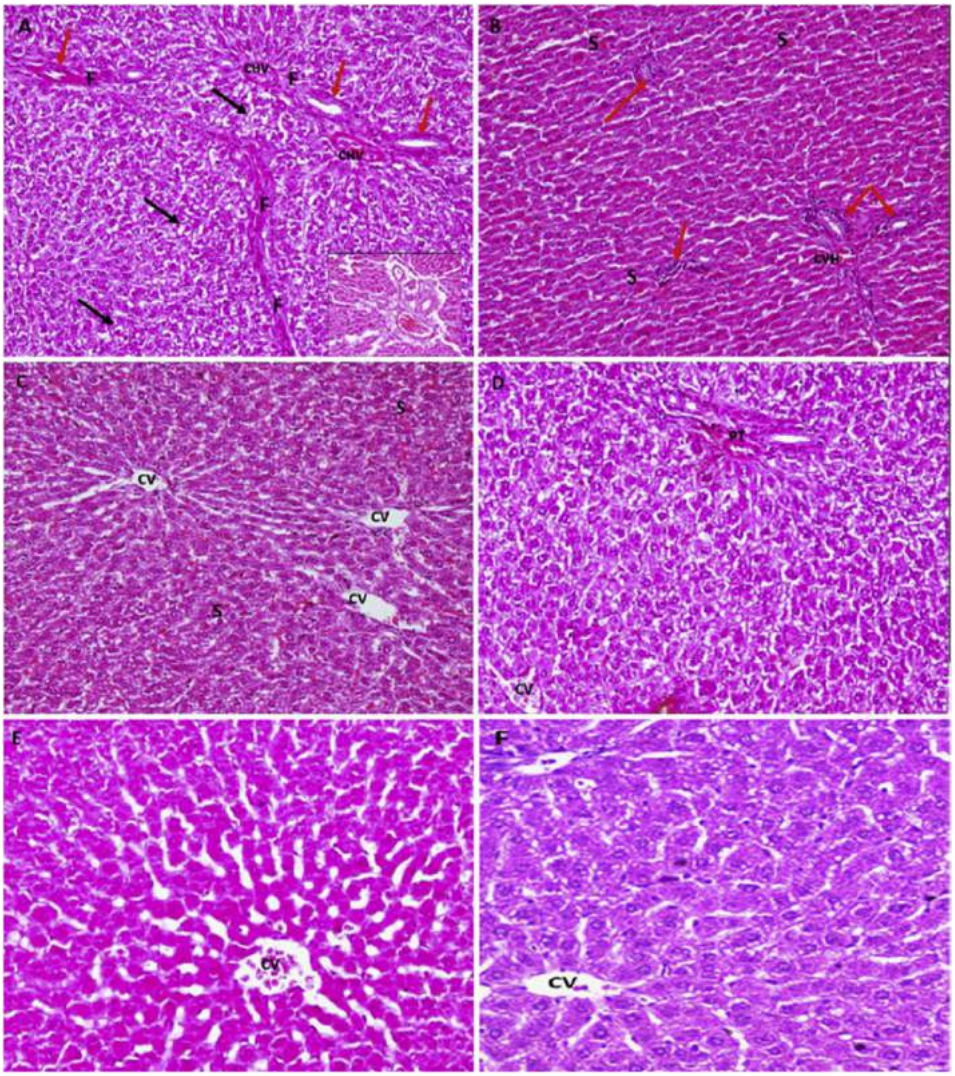

Figure 3. Hematoxylin and Eosin (Hx& E) stained liver sections from rats treated with (A) malathion; (B) malathion + L-NAME 10 mg/kg; (C) malathion + L-NAME 20 mg/kg; (D) malathion + 7-NI 10 mg/kg; (E) malathion + 7-NI 20 mg/kg; (F) saline.
Black arrows: vacuolar degenerated hepatocytes. Red arrows: inflammatory cells. F: Fibrosis. CHV: congested hepatic veins. S: dilated sinusoids. CV: central veins. PT: portal tract (×200).

In the liver, PON-1 activity decreased by 35.4% ($P < 0.05$) in the malathion only group compared with the saline group. PON-1 activity did not change after L-NAME but showed further decrease by 39.5%–33.5% after 7-NI compared with the malathion control (Table 2).

3.2. AChE and BChE activities

In the malathion only group, AChE and BChE activities in brain decreased by 29.6% ($P < 0.05$) and 35.7% ($P < 0.05$), respectively compared with the saline group. In malathion-treated rats, the administration of 20 mg/kg L-NAME was associated with a significant increase in AChE activity by 23.6% ($P < 0.05$) compared with the malathion only group. A significant increase in AChE activity by 27.5% ($P < 0.05$) was also observed after treating rats with 10 mg/kg of 7-NI. The higher dose, however caused 19.2% ($P < 0.05$) decrease in AChE activity in rats given malathion. On the other hand, a significant decrease in BChE activity by 32.2% ($P < 0.05$) and 40.0% ($P < 0.05$) occurred after 7-NI at 10 mg/kg and 20 mg/kg, respectively compared to the malathion only group (Table 3).

3.3. Brain glucose

Malathion resulted in a significant decrease in brain glucose concentrations by 33.8% ($P < 0.05$) compared with the saline group. In malathion treated rats, brain glucose level showed further decrease by 30.3%–17.7% ($P < 0.05$) following L-NAME and by 15.0%–57.8% ($P < 0.05$) after 7-NI administration compared with the malathion control (Table 4).

3.4. Liver enzymes

In malathion only treated rats, significant elevations in liver ALT and AST were observed compared with the saline group [138.9% and 261.5% increments: ($P < 0.05$)]. In rats treated with malathion, 7-NI caused a significant decrease in liver AST by 29.5%. Meanwhile, administration of either 20 mg/kg L-NAME or 20 mg/kg 7-NI caused a significant decrease in liver

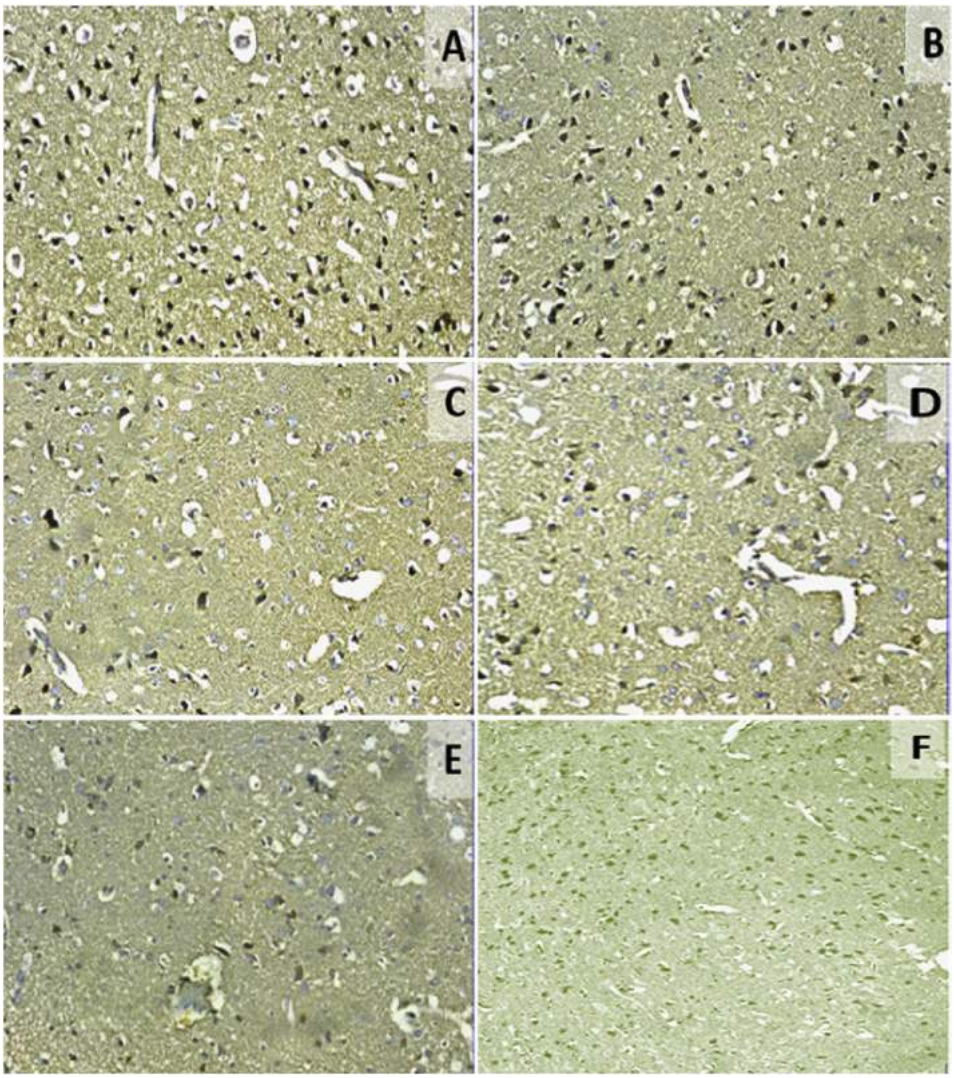

Figure 4. Representative light microphotographs showing immunohistochemical reaction of iNOS of the brain after.
(A) Malathion. (B) Malathion + L-NAME 10 mg/kg. (C) Malathion + L-NAME 20 mg/kg. (D) Malathion + 7-NI 10 mg/kg. (E) Malathion + 7-NI 20 mg/kg. (F) Saline (control rats) (×200).

ALT by 73.7% ($P < 0.05$) and 43.5% ($P < 0.05$), respectively as compared with the malathion control group (Table 4).

3.5. Comet assay

Malathion caused marked and significant ($P < 0.05$) DNA fragmentation in blood lymphocytes with the comet percentage of lymphocytes being 81.0 ± 3.7% compared with the saline control value of (5.0 ± 0.3)%. The administration of either L-NAME or 7-NI to malathion intoxicated rats caused significant and a dose-dependent decrease in the % of damaged cells by 63.0%–67.3% and 45.7%–57.4%, respectively compared with the malathion control (Figure 1 & Table 4).

3.6. Histopathological results

3.6.1. Brain tissue

Rats treated with only malathion showed severe histopathological alterations in the form of destruction of most of Purkinje cells layer which exhibited nuclear pyknotic degeneration, intense basophilia and apoptotic nuclei of neuronal cells (Figure 2A). The administration of L-NAME along with malathion resulted in a dose-dependent amelioration of malathion-induced degenerative changes and healthy neuronal cells with fewer group of degenerative features were seen after L-NAME at 20 mg/kg (Figure 2B and 2C). On the other hand, rats treated with 7-NI at 10 & 20 mg/kg, respectively showed normally looking neuronal tissue except for congested neuronal vessels (Figure 2D and 2E). The higher dose of 7-NI resulted in normal looking cells in form of euchromatic nucleated neurons (Figure 2E). Saline treated rats exhibited the normal histological picture of brain tissue (Figure 2F).

3.6.2. Liver tissue

Malathion resulted in severe damage of liver structure along with disarrangement of hepatic lobules with formation of fibrotic strands. The hepatic cells showed degenerative features in form of vacuolar ballooning cytoplasm with pyknotic nuclei, inflammatory infiltrations especially around hepatic vessels which appeared mostly dilated and congested with bile ductal

epithelium hyperplasia (Figure 3A). Rats treated with malathion along with L-NAME at 10 & 20 mg/kg had the normal hepatic architecture with some of the hepatocytes losing their cellular membrane, with dilated and congested hepatic vessels and sinusoids (Figure 3B and C). On the other hand, rats treated with malathion along with 7-NI (10 & 20 mg/kg) showed hepatic tissue improvement with normal hepatic architecture as most of the hepatocytes retained their cellular membrane with very minimal congested hepatic vessels and sinusoids (Figure 3D and E). In particular, the highest dose of 7-NI resulted in much better improvement of hepatic tissue with totally normally hepatocytes with normal thickness of hepatic vessels (Figure 3E). Saline-treated rats showed the normal histological picture of hepatic tissue (Figure 3F).

3.7. Immunohistochemical results

3.7.1. Brain tissue

Sections prepared from the brain tissue revealed immune-reaction of iNOS in form of fine brown granules within the cytoplasm of neuronal cells. Malathion resulted in wildly distributed strong immune-reaction, while rats given malathion and L-NAME showed moderate immune–reaction of iNOS. In contrast, rats treated with malathion and 7-NI had mild immune-reaction of iNOS. The saline-treated group had faint negligible immune-reaction of iNOS (Figure 4).

3.7.2. Liver tissue

Sections prepared from the liver tissue showed immune-reaction of iNOS in form of fine brown granules within the cytoplasm of hepatocytes. Rats given only malathion exhibited strong immune-reaction, while the groups treated with malathion and L-NAME showed moderate immune–reaction of iNOS which was widely distributed. Meanwhile, 7-NI resulted in mild to faint immune-reaction of iNOS. Saline-treated rats had negative immune-reaction of iNOS (Figure 5).

3.8. Immunomorphometric analysis of iNOS immunoreactivity

3.8.1. Brain

Activated iNOS expression was localized in the cytoplasm and cell wall of the neuronal cells and which was marked by the blue color in image analyzer system to be measured as an area %

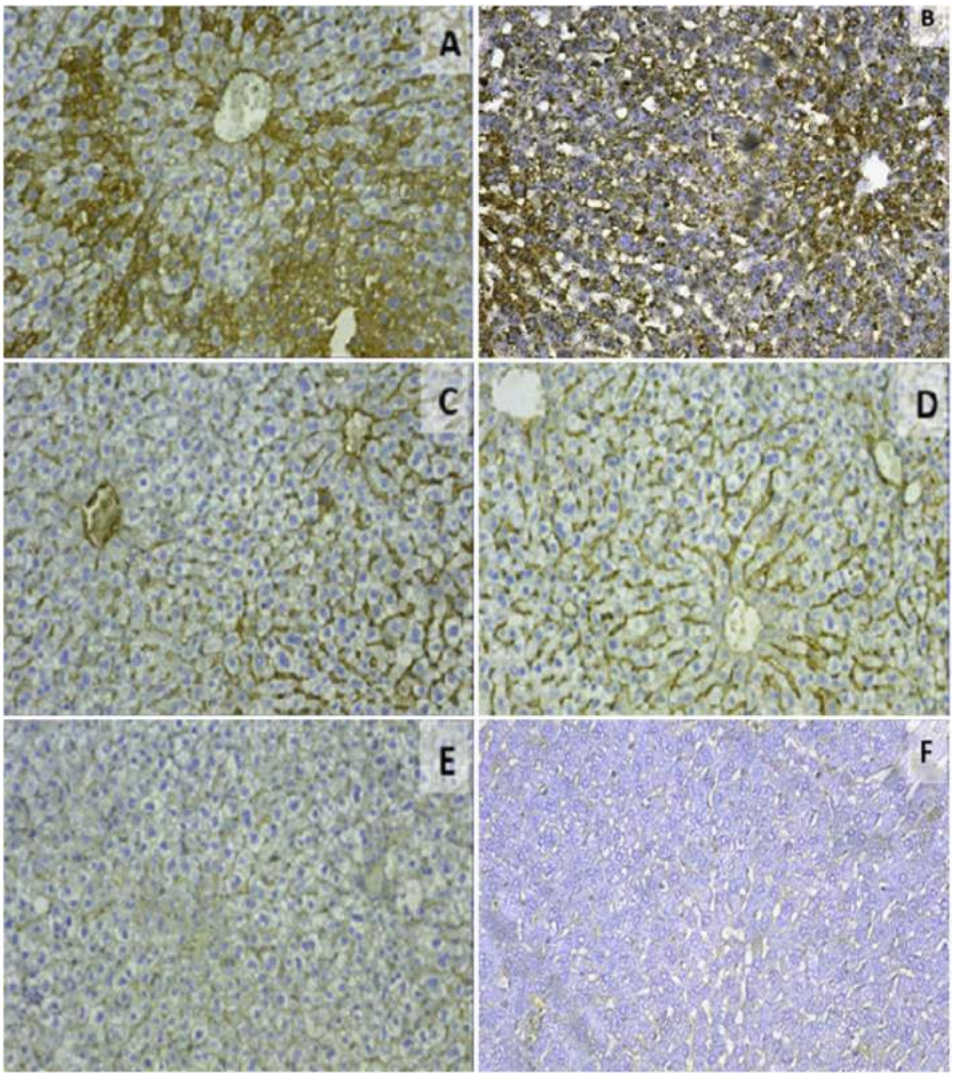

Figure 5. Representative light microphotographs showing immunohistochemical reaction of iNOS of the liver after.
(A) Malathion. (B) Malathion + L-NAME 10 mg/kg. (C) Malathion + L-NAME 20 mg/kg. (D) Malathion + 7-NI 10 mg/kg. (E) Malathion + 7-NI 20 mg/kg. (F) Saline (control rats) (×200).

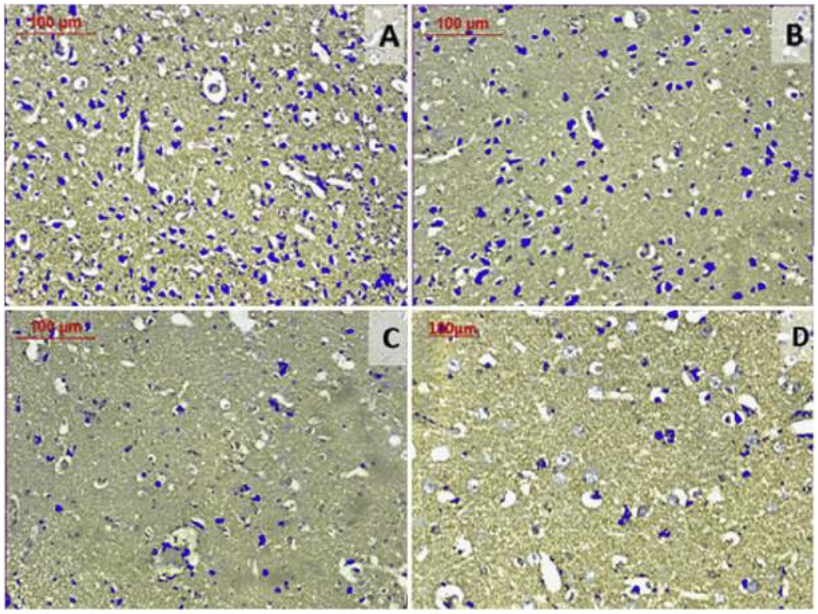

Figure 6. Binary images from image analysis system screen of brain tissue from different treated groups stained for iNOS showing the positive brown cells detected by blue color.
(A) Malathion. (B) Malathion + L-NAME 20 mg/kg. (C) Malathion + 7-NI 20 mg/kg. (D) Saline.

Table 5
Optical density measurements of iNOS immunoreactivity in cerebral cortex and liver of rats treated with malathion along with L-NAME or 7-NI.

Group	Brain iNOS optical density	Liver iNOS optical density
Vehicle	0.18 ± 0.07	0.38 ± 0.14
Malathion	5.30 ± 0.26*	13.00 ± 0.65*
Malathion + L-NAME 10 mg/kg	4.24 ± 0.13*$^{+}$	7.55 ± 0.42*$^{+}$
Malathion + L-NAME 20 mg/kg	3.71 ± 0.19*$^{+}$	6.21 ± 0.23*$^{+}$
Malathion + 7-NI 10 mg/kg	2.32 ± 0.12*$^{+\#}$	2.06 ± 0.05*$^{+\#}$
Malathion + 7-NI 20 mg/kg	2.05 ± 0.07*$^{+\#}$	1.72 ± 0.08*$^{+\#}$

Data presented are means ± SE (%). *$P < 0.05$ *vs.* saline group. $^{+}P < 0.05$ *vs.* malathion only group. $^{\#}P < 0.05$ *vs.* malathion + L-NAME (ANOVA and Duncan's test).

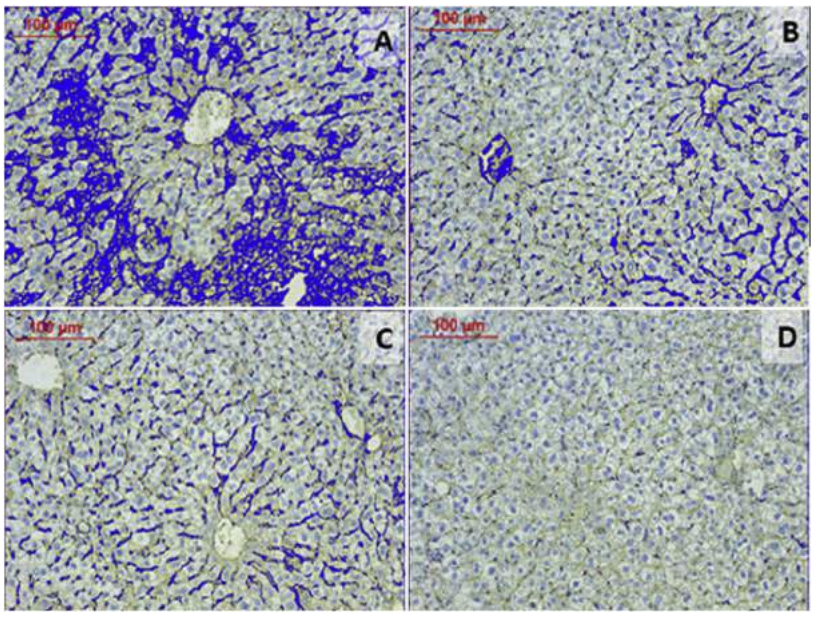

Figure 7. Binary images from image analysis system screen of liver tissue from different treated groups stained for iNOS showing the positive brown cells detected by blue color.
(A) Malathion. (B) Malathion + L-NAME 20 mg/kg. (C) Malathion + 7-NI 20 mg/kg. (D) Saline.

(mean ± S.E). There was negligible iNOS expression in the saline-treated control group. Highly expressed iNOS was seen in the group treated with only malathion followed by malathion + L-NAME 10 mg/kg group. Significantly less iNOS expression was observed in rats treated with malathion + L-NAME 20 mg/kg compared with the malathion only group (30.0% decrease). On the other hand, 56.2% and 61.3% decrements in iNOS immunoreactivity were observed after treatment with 10 mg/kg and 20 mg/kg 7-NI, respectively compared with the malathion control group (Figure 6 & Table 5).

3.8.2. Liver

Quantitative measurements of iNOS immunoreactivity in the liver revealed negligible iNOS expression in rats treated with saline. This contrasted with strong reactivity for iNOS in rats treated with only malathion. iNOS appeared in form of fine brown granules within the cytoplasm and cell wall of hepatocytes. A dose-dependent decrease in iNOS expression by 41.9% and 52.2% was seen after treatment with 10 and 20 mg/kg L-NAME, respectively compared with the malathion control group. On the other hand, much lower expression of iNOS by 84.1% and 86.8% was observed in the groups treated with 10 and 20 mg/kg 7-NI, respectively compared with the malathion only group (Figure 7 & Table 5).

4. Discussion

In this work malathion treatment led to a significant increase in lipid peroxidation in the brain (and liver) of rats. There were also significant decreases in brain GSH, GPx activity, and TAC, thereby, suggesting that the mechanism of neuronal cell damage induced by malathion involves the increased generation of free radicals. Other studies have also demonstrated increased lipid peroxidation in brain and erythrocytes [9,11,16] and decreased GPx and glutathione reductase activities in the cerebral cortex of malathion-treated rats [14]. The toxicant also resulted in increased MDA and decreased activities of the antioxidant enzymes GPx, superoxide dismutase, and catalase in human erythrocytes *in vitro* [13]. Other organophosphates like chlorpyrifos and diazinon resulted in an increase in reactive oxygen species, lipid peroxidation and oxidized glutathione in cerebellar granule neurons of mice [41]. Mitochondria are the major source of reactive oxygen metabolites and also the site for being attacked by free radicals [42,43]. Malathion has been shown to increase $O_2^{\bullet -}$ formation in sub-mitochondrial particles in hippocampus and to decrease the activities of mitochondrial complex I and IV and which would have profound effects on cellular energy production [11,15].

Our present findings also show that malathion is capable of increasing endogenous NO biosynthesis and iNOS expression in the rat brain which is in agreement with previous reports [9]. Other researchers reported higher levels of citrulline, the by-product of NO synthesis in different brain regions following the administration of the organophosphates diisopropylphosphorofluoridate or parathion to rats [44,45]. These data thus suggests a role for NO in the neurotoxicity induced by malathion. NO itself might not be toxic to neurons but it is the formation of more reactive nitrogen oxides NO_2 and N_2O_3 or $ONOO^-$, which eventually results in nitrosylation and/or oxidation of proteins and sulfhydryls. This occurs when there is excessive generation of NO for long duration, e.g., from iNOS resulting in high local concentrations in the micromolar range. This contrasts with the transient and low levels of NO in the nanomolar range formed constitutively by nNOS and eNOS [21–23]. Neuronal NOS is the isoform found mainly in brain in neurons and in rat astrocytes and also is constitutively expressed in other tissues, e.g., in peripheral nitrergic nerves, heart, skeletal muscle and liver as well [19,20]. Neuronal NOS, however, could be also activated to generate NO following NMDA receptor stimulation during pathologic states of brain inflammation or ischemia. NO then binds to cytochrome *c* oxidase to inhibit cell respiration or interact with O_2 or $O_2^{\bullet -}$ to form more reactive nitrogen species [21]. This nNOS-derived NO has been implicated in the development of brain infarction after reversible focal brain ischemia in mice [46,47] and in brain demyelination following cuprizone treatment in mice [48]. Thus, besides NO released by mitochondria, microglia, macrophages and astrocytes, another source of increasingly formed NO in malathion-treated rats might be neuronal.

In this study we aimed to delineate the role of endogenously released NO in the development of oxidative stress and neuronal injury induced by malathion. For this purpose, the non-selective NOS antagonist L-NAME and the selective nNOS antagonist 7-NI were administered to malathion-intoxicated rats. Our findings indicate that 7-NI and also L-NAME inhibited the malathion-induced increase of lipid peroxidation and nitrite and increased TAC in rat brain. GSH and GPx activity showed an increase after L-NAME. The selective nNOS inhibitor 7-NI was able to confer marked protection against neuronal cell death caused by treating rats acutely with malathion. Interestingly, brain iNOS expression was markedly inhibited by 7-NI and to much less degree by L-NAME. These results, therefore, suggest that NO released by iNOS is the likely mediator of malathion-induced neuronal damage. Meanwhile, the much less degree of protection was observed after treatment with L-NAME is likely to be caused by non-selective inhibition of NO synthases would also result in vasoconstriction and consequent increase in tissue damage due to eNOS inhibition.

In the liver, NO is endued with protective effects since blockade of endogenous synthesis with non-selective NOS inhibitors resulted in aggravation of liver injury during ischaemia/reperfusion [49], liver transplanted graft [50], endotoxin injection [51] or obesity [52] in the rat. This cytoprotective effect of NO has been ascribed to a decrease in neutrophil accumulation in liver [49], loss of the protective function of eNOS [53], inhibition of platelet adhesion and consequent intravascular thrombosis, neutralizing oxygen free radicals [54]. NO also exerts injurious effects by forming reactive nitrogen species with O_2 or $O_2^{\bullet -}$ or with resultant oxidative/nitrosative stress [21–23]. In mice, acetaminophen toxicity was inhibited by nNOS inhibitors, while nNOS knockout mice displayed delayed toxicity as compared with wild type mice [55]. There have been several studies showing liver tissue damage following exposure to malathion in rats. The data reported highlighted the role of free radicals in mediating such injury [9,24,25]. In our studies, the liver of malathion-treated rats showed histological features of hepatocyte necrosis, leucocytic infiltrations, hemorrhage, and distorted architecture. Increased hepatic NO, iNOS expression, and malondialdehyde along with decreased GSH content were observed. There was also raised liver aminotransferases indicative of hepatocyte injury. The administration of 7-NI resulted in marked amelioration of the extent of histologic tissue damage and the degree of apoptosis inflicted by malathion. Notably, iNOS expression in liver tissue was markedly suppressed by 7-NI. These parameters also decreased by L-NAME but to a much lesser degree

compared with 7-NI. These observations strongly suggest an important role for excessive NO formed by iNOS during malathion intoxication.

In this study we have shown using the Comet assay that malathion is capable of causing DNA damage of peripheral blood lymphocytes which is in agreement with previous reports. The comet assay is a sensitive technique for detecting DNA strand breaks in mammalian cells where damaged cells have the shape of a comet having a brightly fluorescent head and a tail that is formed by the DNA containing strand breaks which migrated away from the nucleus during electrophoresis [39]. This technique is widely used for biomonitoring and genotoxicity testing and also to evaluate DNA damage/repair [56]. Here, the administration of either 7-NI or L-NAME to rats acutely treated with malathion resulted in marked decrease in the number of comets produced by malathion in lymphocytes. This finding suggests the involvement of NO or its reactive metabolites, e.g., $ONOO^-$ in inducing the DNA damage due to malathion, most likely via increased oxidative stress. In support of this notion, the ability of antioxidants to ameliorate the toxicant-induced DNA damage [57,58].

PON-1 is an enzyme of marked interest in view of its ability to modulate organophosphate toxicity [59]. The enzyme which possesses arylesterase and lactonase activities hydrolyzes the active metabolites (oxons) of a number of organophosphate insecticides and nerve agents [60]. Mice deficient in PON-1 showed marked sensitivity to chlorpyrifosoxon and diazoxon [61] while the administration of rabbit PON-1 to rats protected against toxicity due to paraxon [62] and dichlorvos [63]. PON-1 showed anti-oxidative and anti-inflammatory activities as well. Our present results indicate decreased PON-1 activity in brain and liver following exposure to malathion in rats. This finding is therefore in agreement with previously reported data [9,10]. Organophosphates have also been shown to downregulate PON-1 gene expression *in vitro*, human hepatocellular carcinoma cells exposed to methyl parathion and chlorpyrifos. This occurred together with increased levels of proinflammatory cytokines [64]. It has also been shown that farmers exposed to organophosphates in whom PON-1 192RR polymorphism (with enhanced catalytic activity) is present exhibited lower levels of oxidative stress markers in their sera [65]. In toxicity caused by organophosphates, the decrease in PON-1 activity is thus likely to result in increased oxidative stress and consequent cell injury. Paradoxically, the enzyme is also susceptible to inactivation by oxidative stress [66]. Meanwhile, the changes in PON-1 activity seen in malathion exposed rats following treatment with NOS synthase inhibitors might reflect the redox status of the cell.

Neurotoxicity caused by organophosphate insecticides is due to the ability of these compounds to inhibit the AChE enzyme with the consequent rise in acetylcholine concentration in neuronal synapses and motor end plate [3,4]. Whereas the role of AChE in hydrolyzing acetylcholine is clear, the physiological actions of BChE are still to be delineated [67]. Evidence, however, suggest a role for BChE in hydrolyzing excess acetylcholine [68] and protecting nerve terminals from excessive acetylcholine at the neuromuscular junction [69]. In this study, there was a significant decrease in both AChE and BChE in brain of rats treated with malathion. The administration of either L-NAME or 7-NI was shown to modulate the activities of these enzymes. This effect appears not to be involved in the neuroprotective action of the two NOS inhibitors in view of the decrease in AChE activity by the higher dose of 7-NI and the decrease in BChE activity by either dose of 7-NI. Rather, neuroprotection by L-NAME and 7-NI is likely at least in part to be result from the decrease in the raised levels of endogenous NO during malathion exposure and the decrease in oxidative stress.

Conflicts of interest statement

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

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