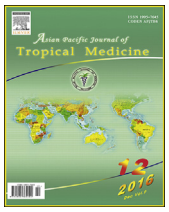
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm



Original research http://dx.doi.org/10.1016/j.apjtm.2016.11.005

Novel neuroprotective and hepatoprotective effects of citric acid in acute malathion intoxication

Omar M.E. Abdel-Salam[1✉], Eman R. Youness[2], Nadia A. Mohammed[2], Noha N. Yassen[3], Yasser A. Khadrawy[4], Safinaz Ebrahim El-Toukhy[2], Amany A. Sleem[5]

[1]*Department of Toxicology and Narcotics, National Research Centre, Tahrir St., Dokki, Cairo, Egypt*

[2]*Department of Medical Biochemistry, National Research Centre, Tahrir St., Dokki, Cairo, Egypt*

[3]*Department of Pathology, National Research Centre, Tahrir St., Dokki, Cairo, Egypt*

[4]*Department of Physiology, National Research Centre, Tahrir St., Dokki, Cairo, Egypt*

[5]*Department of Pharmacology, National Research Centre, Tahrir St., Dokki, Cairo, Egypt*

ARTICLE INFO

Article history:
Received 18 Jul 2016
Received in revised form 19 Aug 2016
Accepted 18 Sep 2016
Available online 10 Nov 2016

Keywords:
Citric acid
Malathion
Oxidative stress
Paraoxonase 1
Cholinesterase
Comet assay

ABSTRACT

Objective: To study the effect of citric acid given alone or combined with atropine on brain oxidative stress, neuronal injury, liver damage, and DNA damage of peripheral blood lymphocytes induced in the rat by acute malathion exposure.

Methods: Rats were received intraperitoneal (*i.p.*) injection of malathion 150 mg/kg along with citric acid (200 or 400 mg/kg, orally), atropine (1 mg/kg, *i.p.*) or citric acid 200 mg/kg + atropine 1 mg/kg and euthanized 4 h later.

Results: Malathion resulted in increased lipid peroxidation (malondialdehyde) and nitric oxide concentrations accompanied with a decrease in brain reduced glutathione, glutathione peroxidase (GPx) activity, total antioxidant capacity (TAC) and glucose concentrations. Paraoxonase-1, acetylcholinesterase (AChE) and butyrylcholinesterase activities decreased in brain as well. Liver aspartate aminotransferase and alanine aminotransferase activities were raised. The comet assay showed increased DNA damage of peripheral blood lymphocytes. Histological damage and increased expression of inducible nitric oxide synthase (iNOS) were observed in brain and liver. Citric acid resulted in decreased brain lipid peroxidation and nitric oxide. Meanwhile, glutathione, GPx activity, TAC capacity and brain glucose level increased. Brain AChE increased but PON1 and butyrylcholinesterase activities decreased by citric acid. Liver enzymes, the percentage of damaged blood lymphocytes, histopathological alterations and iNOS expression in brain and liver was decreased by citric acid. Meanwhile, rats treated with atropine showed decreased brain MDA, nitrite but increased GPx activity, TAC, AChE and glucose. The drug also decreased DNA damage of peripheral blood lymphocytes, histopathological alterations and iNOS expression in brain and liver.

Conclusions: The study demonstrates a beneficial effect for citric acid upon brain oxidative stress, neuronal injury, liver and DNA damage due to acute malathion exposure.

1. Introduction

Oxygen derived free radicals are produced in the cell from many sources. One important source is the mitochondrial electron transport chain where electrons that leaked from O_2 result in generation of superoxide anion radical. ($O_2^{\bullet-}$). The redox state of the cell is kept in balance due to a number of antioxidant mechanisms. These include both enzymatic (*eg.*, catalases, speroxide dismutases, and glutathione peroxidase) and non-enzymatic free radical scavengers (*eg.*, glutathione, α-tocopherol, ascorbic acid) [1,2]. Oxidative stress develops when there is an increase in oxidants and/or in adequate antioxidants [3]. Oxidative stress contributes to the development of several disease processes *eg.*, diabetes mellitus, cardiovascular disease, cancer, neurodegenerative and psychiatric disorders [4–7]. Owing to its high metabolic demand, the brain utilizes much O_2 with the consequent increased generation of reactive oxygen metabolites. Moreover, auto-oxidation of brain

✉First and corresponding author: Omar M.E. Abdel-Salam, Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt.
E-mail: omasalam@hotmail.com
Peer review under responsibility of Hainan Medical University.

neurotransmitters generating O_2 and quinones and the presence of redox-active metals capable of catalyzing free radical reactions increase the brain' oxidant burden. The brain is also rich in polyunsaturated fatty acids, which is the preferred substrate for free radical attack. These factors coupled with modest antioxidant mechanisms make the brain tissue particularly susceptible to oxidative stress [1,2,8].

Reactive oxygen metabolites are likely to contribute to the neurotoxic effects of organophosphate insecticides. In this context, exposure to malathion caused increased lipid peroxidation [9,10] increased nitric oxide, and decreased reduced glutathione (GSH) [10] in the rat brain. Lipid peroxidation increased in blood, liver [11,12] and in the rat erythrocytes as well [13]. Studies also indicated decreased activities of the antioxidant enzymes glutathione reductase and glutathione peroxidase in rat cerebral cortex [14] and activities of superoxide dismutase, catalase and glutathione peroxidase in human erythrocytes [15] also decreased after exposure to malathion. Moreover, the chain breaking antioxidants α-tocopherol and ascorbate were able to reduce lipid peroxidation and ameliorate the changes in antioxidant enzymes caused by malathion in rat and human erythrocytes [13,15].

Citric acid (2-hydroxy-1,2,3-propane-tricarboxylic acid) is a weak organic acid found in all animal tissues [16]. Cellular citrate is synthesized inside the mitochondria [17] while rich dietary sources include lemon, orange, tangerine and grapefruit [18]. Intracellular citric acid is important in the intermediary energy metabolism of the cell. Citrate is produced in the mitochondria from acetyl-CoA and oxaloacetate and enters the citric acid cycle (tricarboxylic acid cycle or Krebs cycle). The resulting high-energy intermediates; the reduced coenzymes nicotinamide adenine dinucleotide and flavin adenine dinucleotide are then utilized in the respiratory chain in the inner mitochondrial membrane to make ATP (adenosine 5′-triphosphate) for the cell's energy needs. Citric acid released into the cytoplasm *via* specific mitochondrial carriers is converted to acetyl CoA for the biosynthesis of fatty acids, lipids, and cholesterol [19].

Besides its role in the generation of energy, citrates have other important actions including down regulation of inflammation and reduction of lipid peroxidation [20–23]. Citrate reduces polymorphonuclear cell degranulation and attenuate the release of inflammatory mediators *eg.*, myeloperoxidase, platelet factor 4, interleukin 1β [18–20] and tumor necrosis factor-alpha [23]. Citric acid displayed hepatoprotective effects where it reduced hepatocellular damage evoked by carbon tetrachloride in rats [24,25]. It also decreased brain lipid peroxidation and inflammation and liver damage in mice treated with bacterial lipopolysaccharide endotoxin [23].

A defect in mitochondrial bioenergetics might be involved in the neurotoxic effects of malathion. This is because organophosphates can cause mitochondrial impairment [9,26–29]. Moreover, the administration of methylene blue, an antioxidant [30] and an enhancer of the electron transport chain [31] protected against the malathion-induced neurotoxicity [10]. Thus, in view of the bioenergetic, antioxidant and anti-inflammatory effects reported above for citrate, it looked pertinent to investigate the effect of citric acid administration on oxidative stress and brain damage in rats intoxicated with the organophosphate malathion. We also examined the possible modulation by citric acid of the effect of atropine, the muscarinic receptor antagonist and the antidote employed in the management of acute organophosphate poisoning [32,33]. Since, malathion has been shown to cause hepatocellular damage [34,35], the study was extended to include the liver tissue.

2. Materials and methods

2.1. Animals

Male rats of the Sprague–Dawley strain with body weight of (130–140) g were used. Rats were obtained from Animal House Colony of the National Research Centre. Rats allowed free access to standard laboratory food and water. Animal procedures were done in accordance to 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 purchased from El-Naser Chemical Co., Cairo. Citric acid and atropine were obtained from Sigma–Aldrich (St Louis, MO, USA). Other chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich.

2.3. Study design

Rats were randomly divided into different groups (6 rat/group). Group 1 was treated with *i.p.* saline (0.2 mL/rat) and served as negative control. Group 2–6 were *i.p.* treated with malathion at a dose of 150 mg/kg, along with saline (group 2), citric acid at 200 or 400 mg/kg (groups 3&4), atropine at 1 mg/kg (group 5) or citric acid at 200 mg/kg + atropine at 1 mg/kg (group 6). Rats were euthanized by decapitation 4 h after 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 later biochemical analyses. Homogenization of brain tissues were carried out using 0.1 M 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 product of lipid peroxidation was determined in tissue homogenates by the method of Nair and Turne [36]. In this assay thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid to form TBA-MDA adduct which can be measured colorimetrically at 532 nm.

2.4.2. Reduced glutathione

Reduced glutathione (GSH) was determined in tissue homogenates using the method of Ellman *et al.* [37]. The procedure is based on the reduction of Ellman's reagent [DTNB; 5, 5′-dithiobis (2-nitrobenzoic acid)] by the free sulfhydryl group on GSH to form yellow colored 5-thio-2-nitrobenzoic acid which can be determined using spectrophotometer at 412 nm.

2.4.3. Nitric oxide

Nitric oxide was determined using colorimetric assay where nitrate is converted to nitrite *via* nitrate reductase. Griess reagent

then act to convert nitrite to a deep purple azo compound that can be determined using spectrophotometer [38].

2.4.4. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined in supernatants using colorimetric glutathione peroxidase kit (Biodiagnostic, Egypt). The activity of GPx is expressed as mU/mL.

2.4.5. Paraoxonase-1 activity

Paraoxonase-1 arylesterase activity was measured using phenylacetate as a substrate and the formation of phenol was measured spectrophotometrically by monitoring the increase in absorbance at 270 nm at 25 °C. One unit of arylesterase activity is defined as 1 μM of phenol formed per minute. Enzyme activity was calculated based on the extinction coefficient of phenol of 1 310 M^{-1} cm^{-1} at 270 nm, pH 8.0 and 25 °C and expressed kilo International Unit/Liter (kU/L) [39,40].

2.4.6. Acetylcholinesterase activity

Acetylcholinesterase activity was measured using the method of Ellman *et al.* [41,42]. 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 using spectrophotometric at 412 nm. AChE activity was expressed as μmol sulfhydryl (SH) groups/g tissue/min.

2.4.7. Butyrylcholinesterase activity

Butyrylcholinesterase (EC 3.1.1.8; BChE) activity was measured in brain supernatants using commercially available kit from Ben Biochemical Enterprise (Milan, Italy). The method is that of Ellman *et al.* [41]. Butyrylcholinesterase catalyzes the hydrolysis of butyrylthiocholine as substrate into butyrate and thiocholine. Thiocholine reacts with 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) forming a yellow chromophore which can be quantified using spectrophotometer.

2.4.8. Total antioxidant capacity

Total antioxidant capacity (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 benzensulfate to a colored product [29].

2.4.9. 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 quinoneimine which can be measured using spectrophotometer.

2.4.10. Liver enzymes

Reitman–Frankel procedure (Crowley 1967) was used for the colorimetric determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in liver. Alkaline phosphatase (ALP) activity was determined colorimetrically according to Belfield and Goldberg (1971). Commercially available kits from BioMérieux (France) were used for this purpose.

2.5. Comet assay

Isolation of peripheral blood leukocytes were done by centrifugation (30 min at 1 300 g) using Ficoll–Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After centrifugation, leukocytes were represented as a buffy coat, aspirated and washed twice with phosphate buffered saline (pH 7.4).

The comet assay was done at low temperature to minimize spontaneous DNA damage [44,45]. In brief, 100 μL of normal melting point agarose (at 0.7%) was applied onto a pre-cleaned microscope charged slide and a coverslip was gently applied. The coverslip was removed after the agarose solidified at 4 °C and 100 μL of low melting point agarose at 0.5% containing 1 500 peripheral blood lymphocytes were then added. The coverslip was replaced and the slide placed at 4 °C for solidification. The coverslip was then removed and a final layer of low melting agarose was added, coverslipped, and left to solidify for 10 min.

The coverslip was removed and the slide was immersed in 100 mL of fresh lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, 1% sodium hydroxide, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO (pH10)) at 4 °C for 1 h. The microgels slides were then removed from the solution and rinsed with DNA unwinding solution (300 mmol/L NaOH and 1 mmol/L EDTA, pH 13) for 30 min at 4 °C. Thereafter, the slides were placed on a horizontal gel electrophoresis chamber that is filled with DNA-unwinding solution. Gels were run for 30 min with constant current of 300 mA at 4 °C. Following electrophoresis, the microgels were neutralized with 0.4 M Trisma base at pH 7.5 for 10 min and finally, the slides were stained with 20 μL ethidium bromide (Sigma) at 10 μg/mL.

Examination of the slides was done at 400× magnification using a fluorescence microscope (IX70; Olympus, Tokyo, Japan). The apparatus was equipped with an excitation filter of 549 nm and a barrier filter of 590 nm and attached to an 'Olympus' video camera. The damaged cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side. The latter formed by the DNA containing strand breaks that were drawn away during electrophoresis. Samples were studied by counting the number of damaged cell per 100 cells as to calculate the percent of damaged cells.

2.6. Histological studies

The rats were killed by decapitation, and their brains and livers were quickly removed out. Slices were then were 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 (6 μm thick) were stained with hematoxylin and eosin (H&E), examined and photographed under light microscope using a digital camera (DP70, Tokyo). Adobe Photoshop version 8.0 was used for images processing.

2.7. Immunohistochemistry

Immunohistochemistry for iNOS was done 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. Sections were then rinsed with phosphate buffered saline, counterstained with H&E for (2–3) min, and dehydrated in ascending grades of ethanol. Slices were then soaked twice in xylene at room temperature for 5 min, mounted, and examined by light microscope.

2.8. Immunomorphometric analysis

Quantitative assessment of iNOS immunoreactivity was done with an Image Analyzer system (Leica Qwin 500IW, Cambridge, England).

2.9. Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (SPSS software; SAS Institute Inc., Cary, NC). A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Oxidative stress

3.1.1. Lipid peroxidation

In brain tissue, lipid peroxidation measured as malondialdehyde (MDA) showed significant increase by 45% after exposure to

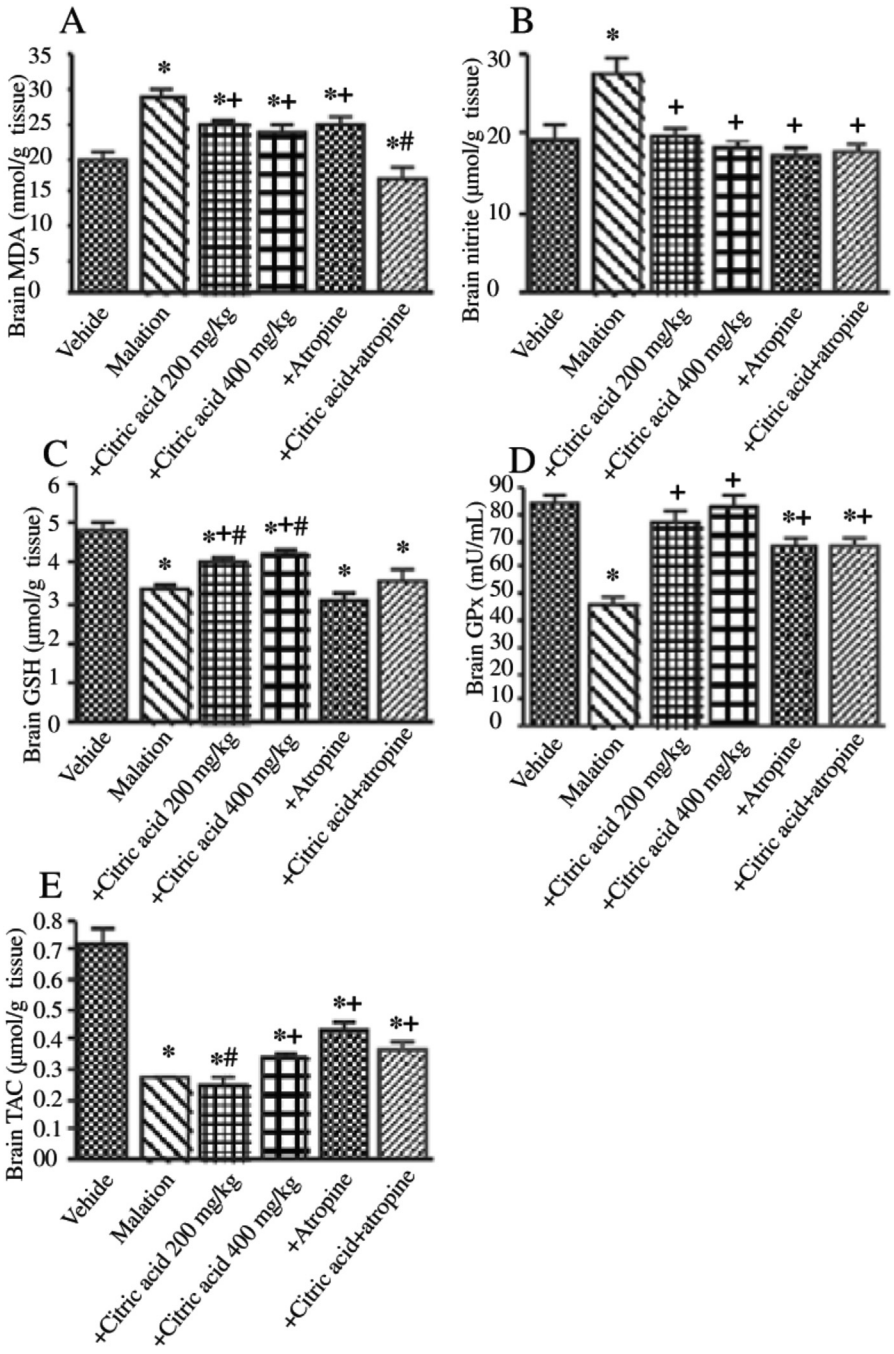


Figure 1. Oxidative stress markers in the brain of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine. *$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + atropine group.

malathion compared with the saline control group, (29.00 ± 1.20) *vs.* (20.00 ± 0.84) nmoL/g tissue. In rats treated with malathion, citric acid at 200 and 400 mg/kg resulted in significant decrease in the level of MDA by 14.8% and 18.6%, respectively (24.70 ± 0.92) and (23.60 ± 1.10) *vs.* (29.00 ± 1.20) nmoL/g tissue. No significant effect was observed for atropine but the combined treatment with atropine and citric acid reduced MDA by 41% compared with the malathion only group (17.10 ± 1.30) *vs.* (29.00 ± 1.20) nmoL/g tissue (Figure 1A).

In liver tissue, malathion induced a marked increase in membrane lipid peroxidation, as shown by the level of MDA (55.5% increase: saline control, (51.9 ± 2.1); malathion (80.7 ± 4.9) nmoL/g tissue). A significant decrease in MDA by 16.4% and 26.9% was observed after treatment with 200 mg/kg citric acid and after both atropine and citric acid (59.0 ± 3.7) and (67.4 ± 3.6) *vs.* (80.7 ± 4.9) nmoL/g tissue (Figure 2A).

3.1.2. Nitrite

Malathion induced a significant rise in the level of brain nitrite by 42% compared with the saline group (27.4 ± 2.2) *vs.* (19.3 ± 1.6) μmol/g tissue. Treatment with citric acid significantly reduced brain nitrite by 28.8% and 33.9%, respectively, compared with the malathion only treatment group (19.5 ± 0.9) and (18.1 ± 1.3) *vs.* (27.4 ± 2.2) μmol/g tissue. A significant decrease in brain nitrite by 37.2% and 35% was also observed in rats treated with either atropine or atropine and citric acid (17.2 ± 1.0) and (17.80 ± 0.68) *vs.* (27.4 ± 2.2) μmoL/g tissue (Figure 1B).

In the liver tissue of malathion only treated rats, a significant increase in nitrite was also observed as 39.3% increase: saline control, (44.0 ± 2.6); malathion (61.3 ± 4.0) μmoL/g tissue. The administration of citric acid, atropine or their combination, however, had no significant effect on liver MDA (Figure 2B).

3.1.3. Reduced glutathione

Compared with the saline control group, the level of brain GSH decreased by 29.8% in the malathion only treated rats (3.40 ± 0.11) *vs.* (4.83 ± 0.24) μmol/g tissue. Brain GSH showed significant rise by 19.1% and 23.5% after treatment with citric acid at 200 and 400 mg/kg, respectively (4.05 ± 0.12) and (4.20 ± 0.18) *vs.* (3.40 ± 0.11) μmol/g tissue. The administration of atropine alone or combined with citric acid, however, had no significant effect on brain GSH (Figure 1C).

A significant decrease in GSH by 30.4% was also observed in the liver of malathion only treated rats (8.00 ± 0.25) *vs.* (11.50 ± 0.61) μmol/g tissue. Rats treated with 200 m/kg citric acid or both atropine and citric acid had 17% and 17.5% increments in liver GSH compared with the malathion only treated group (9.36 ± 0.21) and (9.42 ± 0.3) *vs.* (8.00 ± 0.25) μmol/g tissue (Figure 2C).

3.2. Glutathione peroxidase activity

Malathion resulted in a significant decrease in brain GPx activity by 44.9% saline control, (83.9 ± 3.5); malathion (46.2 ± 2.9) mU/mL. Rats treated with citric acid showed significant increments in GPx activity by 67.3% and 79%, respectively, compared with the respective malathion only control group (77.3 ± 4.0) and (82.7 ± 4.3) *vs.* (46.2 ± 2.9) mU/mL. A significant increase in brain GPx activity by 47.2% and 47.4% was also observed in rats treated with malathion along with atropine or both atropine and citric acid compared with the malathion only control group (68 ± 3.0) and (68.1 ± 3.6) *vs.* (46.2 ± 2.9) mU/mL (Figure 1D).

3.3. Total antioxidant capacity

Rats treated with only malathion had significantly decreased TAC in their brains by 62.5% compared with their saline control counterparts (0.268 ± 0.010) *vs.* (0.714 ± 0.060) μmol/g tissue. A significant increase in brain TAC by 27.2%, 58.9% and 37.3% was observed in rats treated with malathion along with 400 mg/kg citric acid, atropine or both atropine and citric acid compared

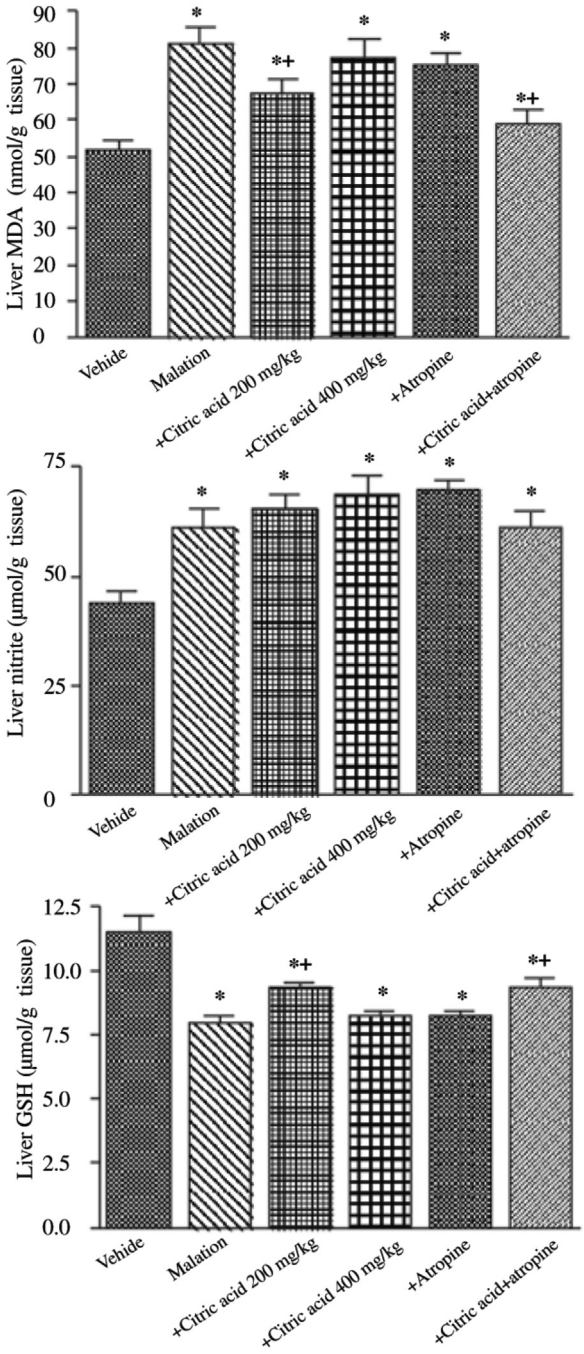


Figure 2. Oxidative stress markers in the liver tissue of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine. *$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + atropine group.

with the malathion only control group (0.341 ± 0.010), (0.426 ± 0.030) and (0.368 ± 0.020) *vs.* (0.268 ± 0.010) μmol/g tissue (Figure 1E).

3.4. Acetylcholinesterase activity

Rats treated with only malathion showed 30.7% inhibition in brain AChE activity compared with the saline control group (4.71 ± 0.23) *vs.* (6.80 ± 0.41) μmoL SH/g/min. A significant increase in brain AChE activity by 47.1%, 32.9%, and 25.3% was observed in rats treated with malathion along with 400 mg/kg citric acid, atropine or both atropine and citric acid compared with the malathion only control group (6.93 ± 0.47), (6.26 ± 0.22) and (5.90 ± 0.31) *vs.* (4.71 ± 0.23) μmol SH/g/min (Figure 3).

3.5. Butyrylcholinesterase activity

In malathion only-treated rats, brain BChE activity was inhibited by 38.2% compared with the saline control group (120.51 ± 7.60) *vs.* (195.14 ± 13.70) U/L. Further significant decrease in brain BChE activity by 24.4%, and 24.5% was observed in rats treated with malathion along with 400 mg/kg citric acid or both atropine and citric acid compared with the malathion only control group (91.1 ± 6.0) and (91.0 ± 4.8) *vs.* (120.51 ± 7.60) U/L (Figure 4).

3.6. Paraoxonase-1 activity

Compared with the saline control group, malathion only-treated rats showed a significant inhibition of brain PON1 activity by 36.9% (7.95 ± 0.54) *vs.* (12.6 ± 0.42) kU/L. No significant effect for atropine was observed on PON1 activity in malathion-treated rats. The enzyme activity in the brain, however, showed further inhibition by 19.1%, 23.3% and 24.9% following treatment with citric acid at 200 or 400 mg/kg and both atropine and citric acid compared with the malathion only control group (Figure 5A). In the liver tissue, malathion caused 34.8% inhibition in PON1 activity (21.7 ± 1.2) *vs.* (33.27 ± 1.60) kU/L. There was no significant effect for citric acid alone on PON1 activity in malathion-treated rats. Treatment with only atropine inhibited the activity of the enzyme by 20.6% (17.23 ± 0.83) *vs.* (21.7 ± 1.2) kU/L, whereas citric acid in combination with atropine increased PON1 activity by 26.7% compared with the malathion only control group (27.5 ± 1.5) *vs.* (21.7 ± 1.2) kU/L (Figure 5B).

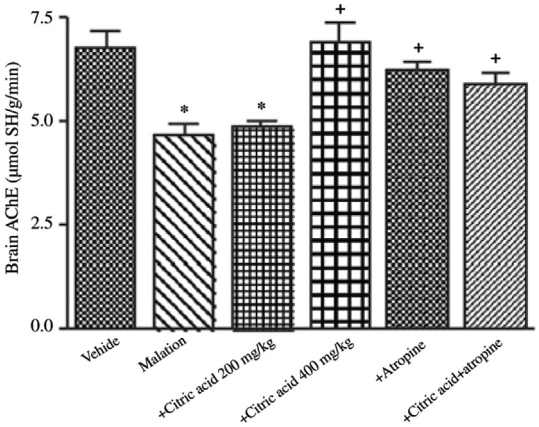


Figure 3. Acetylcholinesterase (AChE) activity in the brain of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine.
*$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group.

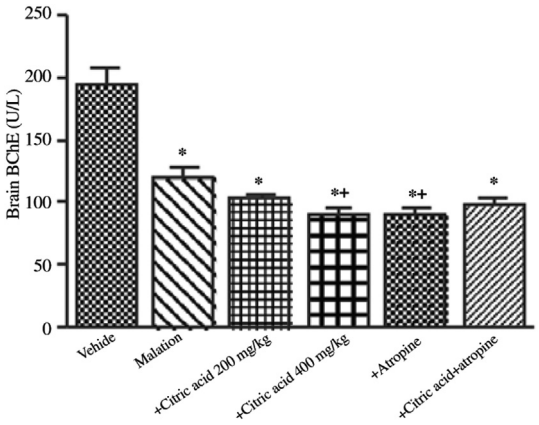


Figure 4. Butyrylcholinesterase (BChE) activity in the brain of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine.
*$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group.

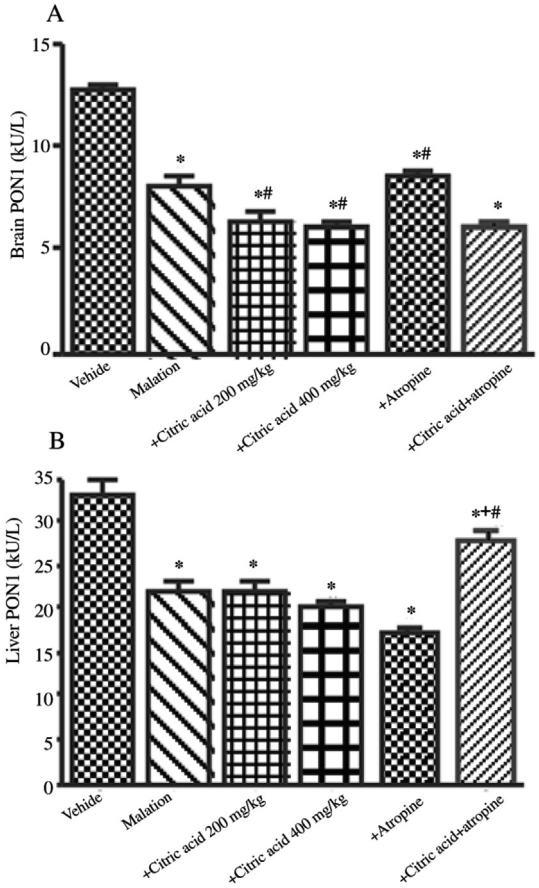


Figure 5. Paraoxonase-1 (PON-1) activity in the brain and liver of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine.
+$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + atropine group.

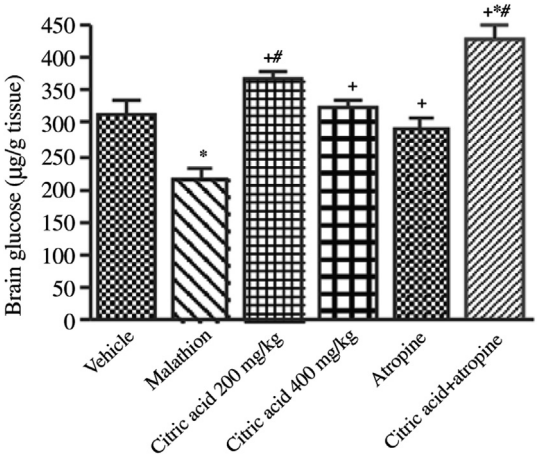


Figure 6. Brain glucose level in rats treated with malathion and the effect of citric acid, atropine or citric acid combined with atropine.
*$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + atropine group.

3.7. Brain glucose

Compared with the saline-treated rats, a significant decrease in brain glucose concentrations by 30.6% was observed in the malathion only treated group (218.0 ± 13.0) *vs*. (314.0 ± 9.6) μg/g tissue. A significant increase in glucose concentrations by 69.3%, 49.7%, 33.9%, and 97.2%, respectively, was observed in brain of rats treated with malathion along with (200–400) mg/kg citric acid, atropine or both atropine and citric acid compared with the malathion only control group (Figure 6).

3.8. Liver enzymes

Results are presented in Figure 7. Malathion caused significant elevation in liver ALT and AST by 83.9% and 130.8%, respectively compared with saline-treated rats (84.6 ± 4.1) *vs*. (46.0 ± 3.2) and (83.1 ± 3.7) *vs*. (36.0 ± 1.9) U/g tissue. Citric acid given at 200 and 400 mg/kg significantly decreased liver

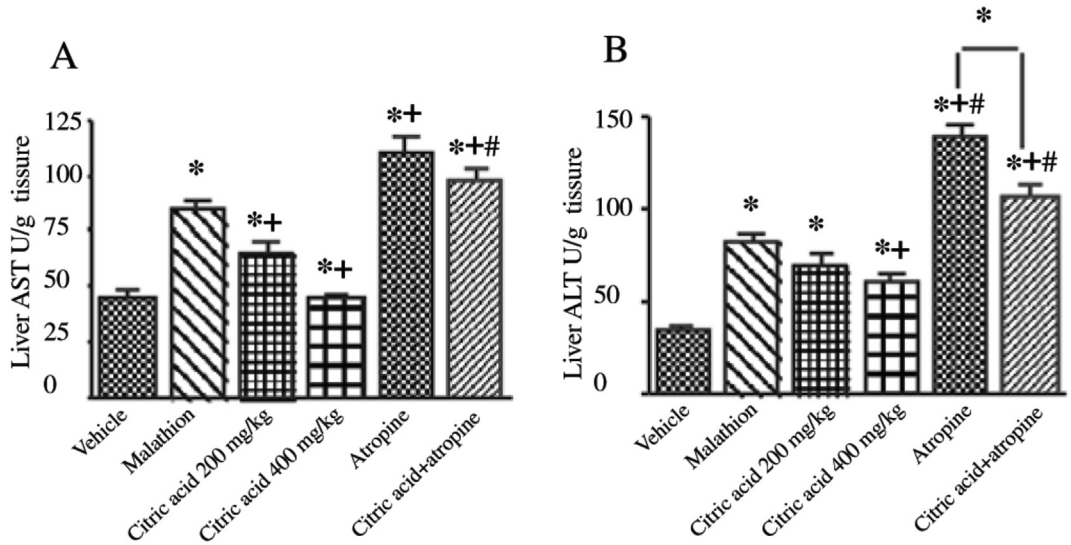


Figure 7. AST and alanine ALT activities in the liver tissue of malathion-treated rats and the effect of citric acid, atropine or citric acid combined with atropine.
*$P < 0.05$ compared with saline group and between different groups as indicated in the figure. +$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + citric acid.

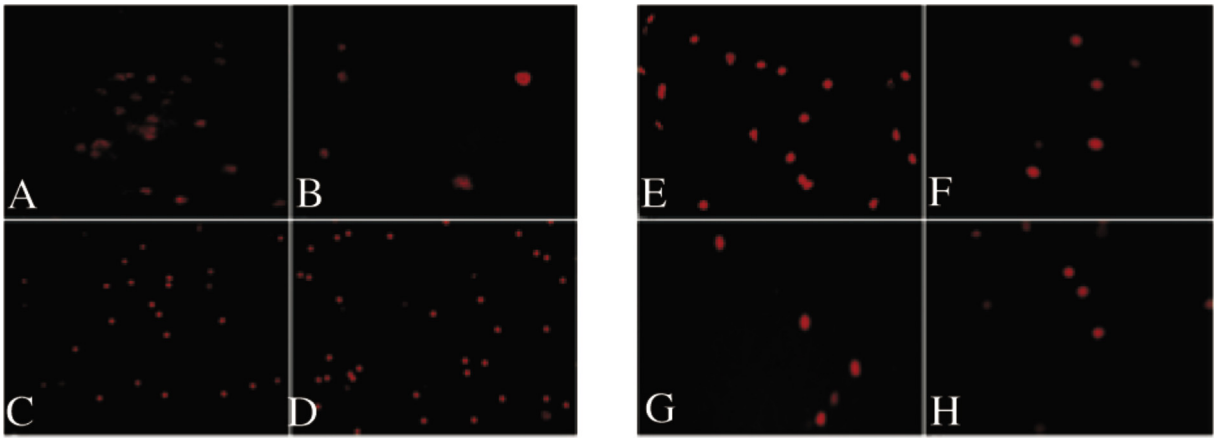


Figure 8. Representative fluorescence images of comets from blood lymphocytes of malathion and citrate/atropine-treated rats.
A and B: malathion (control); C: malathion + citric acid 200 mg/kg; D: malathion + citric acid 400 mg/kg; E and F: malathion + atropine 1 mg/kg; G and H: malathion + citric acid 200 mg/kg + atropine 1 mg/kg.

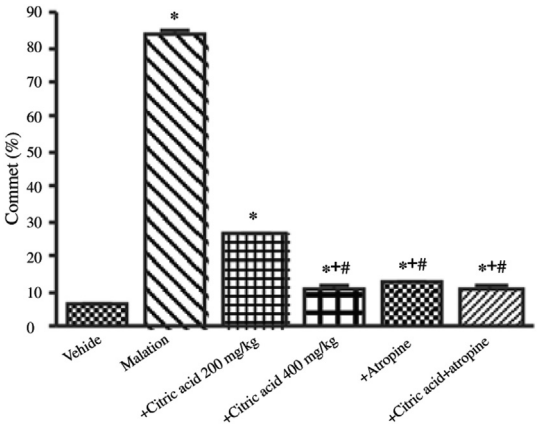


Figure 9. The percentage of comets from blood lymphocytes of rats treated with malathion alone or combined with citric acid and or atropine. Means ± SE. *$P < 0.05$ compared with saline group. +$P < 0.05$ compared with malathion only group. #$P < 0.05$ compared with malathion + citric acid 200 mg/kg group.

AST by 22.7% and 47.4%, respectively, compared with malathion only group (65.4 ± 5.2) and (44.5 ± 2.1) *vs*. (84.6 ± 4.1) U/g tissue. Meanwhile, there was 26% decrease in liver ALT by citric acid at 400 mg/kg (61.5 ± 4.2) *vs*. (83.1 ± 3.7) U/g tissue. In contrast, significant increase in AST and ALT by 30% and 67.3% was observed in malathion + atropine compared with malathion only group. On the other hand, rats treated with malathion + citric acid + atropine showed 14.3% and 29% increments in AST and ALT compared with malathion only group.

3.9. Comet assay

Malathion injection induced DNA fragmentation in blood lymphocytes. The comet percentage of lymphocytes in malathion only treated rats was 83.5% ± 1.34% compared with saline control value of 6.2% ± 0.47% ($P < 0.05$). Citric acid administered at 200 or 400 mg/kg to malathion-treated rats resulted in a dose-dependent decrease in the % of damaged cells by 68.9% and 87.2% (26.00% ± 0.81%) and (10.70% ± 0.98%) *vs.* (83.50% ± 1.34%). The comet percentage of peripheral blood lymphocytes after treatment with atropine and citric acid + atropine was significantly reduced by 85% and 86.8% compared with the malathion only group (12.50% ± 0.56%) and (11.00% ± 0.71%) *vs.* (83.5% ± 1.34%) (Figures 8 and 9).

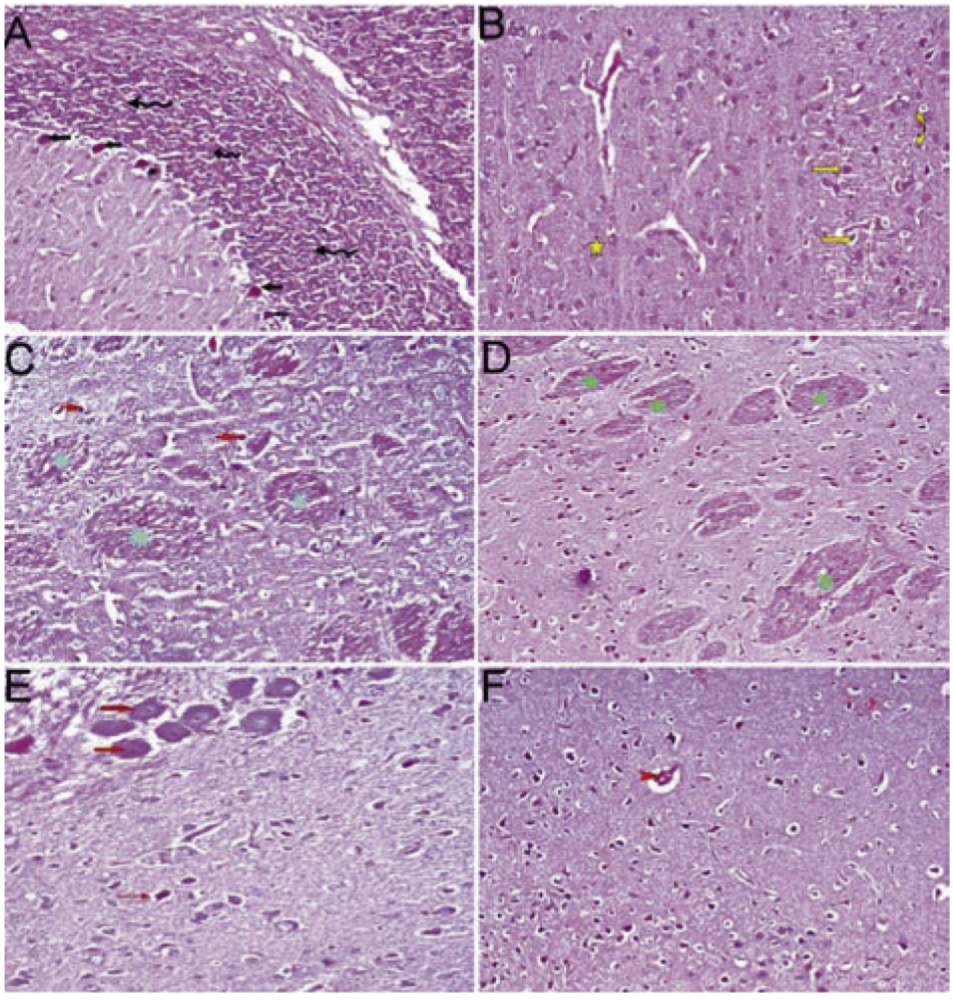


Figure 10. Hematoxylin and eosin (H&E) stained sections of cerebral cortex of rats.
Treated with (A) Saline: normal structure; the granular layer (curved yellow arrow), Purkinje cell layer (yellow arrow) and the molecular layer (yellow star). (B) Malathion 150 mg/kg: lymphocytic infiltration (wavy arrows), destruction of Purkinje cells layer (short arrows). (C) Malathion + citric acid 200 mg/kg: congested blood vessels (red arrow heads), and reactive gliosis (green stars). (D) Malathion + citric 400 mg/kg: reactive gliosis (green stars). (E) Malathion + atropine: generalized astrogliosis (thick red arrows). (F) Malathion + citric acid + atropine: normal glial tissue with minimal congested blood vessels (red arrow heads) (H&E × 200).

3.10. Histopathological results

3.10.1. Brain

Rats treated with saline (control group) showed normal histological picture of brain tissue (Figure 10A). Rats treated with only malathion showed marked changes in the form of meningitis and lymphocytic infiltration associated with destruction of most of the Purkinje cells layer (Figure 10B). On the other hand there was marked improvement after the administration of citric acid although congested blood vessels and reactive gliosis were seen (Figure 10C and D). Rats treated with malathion and only atropine exhibited near normal brain tissue except for generalized astrogliosis as demonstrated by cellular hypertrophy (Figure 10E). Sections from rats treated with malathion + citric acid + atropine showed only congested blood vessels (Figure 10F).

3.10.2. Liver

Saline-treated rats showed normal histology of hepatic tissue (Figure 11A). Rats treated with malathion showed liver structural damage along with disarrangement of hepatic lobules with formation of fibrotic strands. Necrosis and vacuole formation in hepatocytes, sinusoidal enlargement, and leucocytic infiltration were seen. Moreover, dilation and congested blood vessels with hemorrhage were noted (Figure 11B). Rats treated with both malathion and citric acid (200 mg/kg) exhibited improved hepatic tissue structure, with minimal vacuolated hepatic cells (Figure 11C). Treatment with the higher dose of citric acid (400 mg/kg) improved hepatic cells except for minimal lymphocytic infiltration around the hepatic vessels (Figure 11D). Meanwhile, rats given both malathion and atropine exhibited near normal liver tissue with normal architecture and distinct hepatic cells except for blood vessel dilatation (Figure 11E). Likewise, rats treated with malathion, atropine and citric acids showed only minimal congested hepatic vessels (Figure 11F).

3.11. Immunomorphometric analysis of iNOS immunoreactivity

3.11.1. Brain

Figure 12 shows the mean optical density measurements of iNOS immunoreactivity ± SE (%) in cerebral cortex of rats treated with saline, malathion and malathion along with citric acid and/or atropine. There was negligible iNOS expression in the control group. Rats treated with only malathion exhibited increased iNOS expression compared with the saline treated group (213.6% increase: (4.92 ± 0.35) *vs.* (0.22 ± 0.10)). In malathion-treated rats, citric acid caused a significant and dose-dependent decrease in iNOS immunoreactivity (63% and 95.3% decrements: (1.82 ± 0.16), (0.23 ± 0.05) *vs.* (4.92 ± 0.35)). A significant and marked decrease in iNOS expression by 89.2%

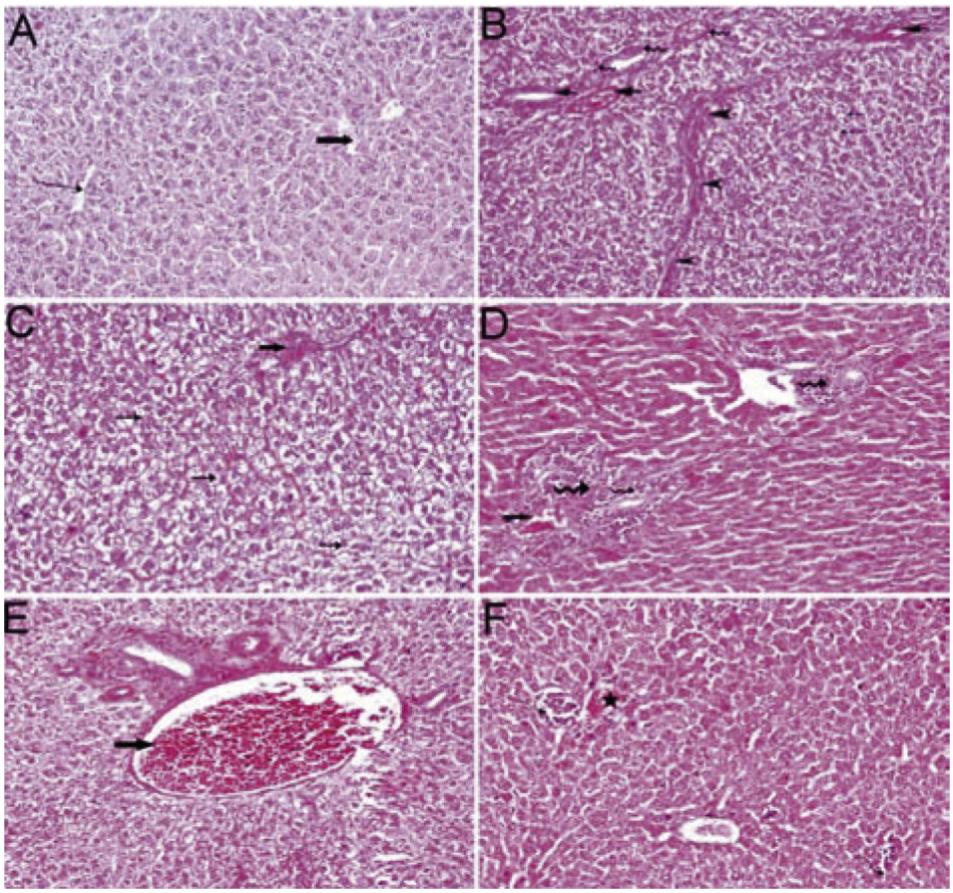


Figure 11. Hematoxylin and eosin (H&E) stained liver sections from rats.
Treated with (A) Saline: normal hepatic lobules; the central vein (long thin arrow), portal triad. (B) Malathion (150 mg/kg): congested blood vessels (thick arrows), fibrotic strands (arrow heads), leucocytic infiltration (wavy arrows), and vacuolar hepatocytes (thin arrows). (C) Malathion + citric acid 200 mg/kg: minimal congestion of the central vein (thick arrow), vacuolated hepatocytes (thin arrows). (D) Malathion + citric acid 400 mg/kg: leucocytic infiltration around hepatic vessels (wavy arrows), and minimally congested vessels (thick arrow). (E) Malathion + atropine: well formed hepatic tissue and dilated congested hepatic vessels (thick arrow). (F) Malathion + citric acid + atropine: foci of inflammatory cells (wavy arrow), foci of necrotic tissue (star), congested blood vessels (thick arrow) (H&E × 200).

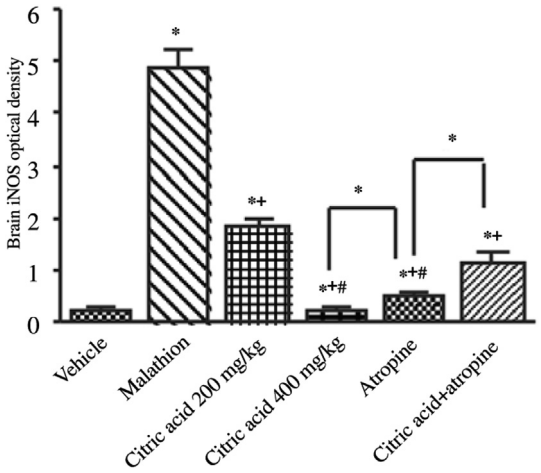


Figure 12. Optical density measurements of iNOS immunoreactivity ± SE (%) in the cerebral cortex of rats treated with malathion and the effect of citric acid, atropine or citric acid combined with atropine.
*P < 0.05 compared with saline group and between different groups as shown on the graph. +P < 0.05 compared with malathion only group. #P < 0.05 compared with malathion + citric acid 200 mg/kg group.

and 76.4% was also observed following atropine alone or combined with citric acid (0.53 ± 0.02), (1.162 ± 0.17) *vs.* (4.92 ± 0.35).

3.11.2. Liver

Quantitative measurements of iNOS immunoreactivity in the liver from rats exposed to malathion revealed marked and significant increase in iNOS immunoreactivity compared to the saline-treated group 741.7% increase: (7.55 ± 0.17) *vs.* (0.897 ± 0.120). Rats treated with malathion and citric acid exhibited 92.4% and 89.8% decrements in iNOS immunoreactivity, respectively, as compared with the malathion only group (0.57 ± 0.10) and (0.773 ± 0.130) *vs.* (7.55 ± 0.17). Treatment with atropine resulted in 98.4% decrease in iNOS immunoreactivity. Meanwhile, atropine combined with citric acid resulted in 79.9% decrease in iNOS immunoreactivity as compared with the malathion only group (1.516 ± 0.270) *vs.* (7.55 ± 0.17) (Figure 13).

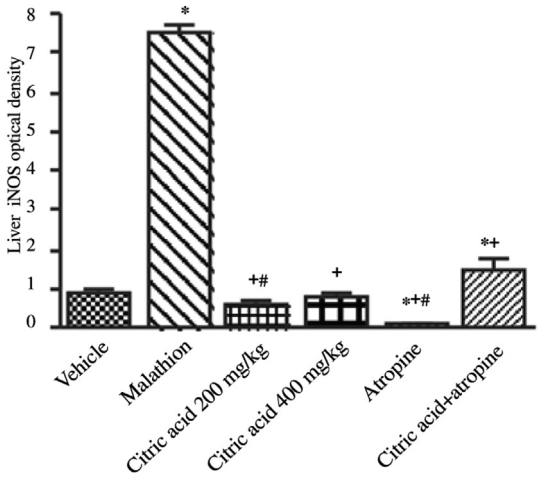


Figure 13. Optical density measurements of iNOS immunoreactivity ± SE (%) in the liver of rats exposed to malathion and the effect of citric acid, atropine or citric acid combined with atropine.
*P < 0.05 compared with saline group and between different groups as shown on the graph. +P < 0.05 compared with malathion only group. #P < 0.05 compared with malathion + citric acid 200 mg/kg group.

4. Discussion

Several recent studies indicated a role for reactive oxygen metabolites and increased oxidative stress in the neurotoxic effects of organophosphate insecticides [9,10,15]. In this study, the decrease in reduced glutathione, GPx activity, TAC and the increase in the lipid peroxidation product malondialdehyde is evidence of increased generation of reactive oxygen metabolites and other free radicals capable of inducing lipid peroxidation of the cell membrane polyunsaturated fatty acids [4]. Organophosphates result in increased free radicals either as a part of their biotransformation or in the detoxification pathway [46]. These agents can also cause mitochondrial impairment [9,29], which could lead to increased reactive oxygen species. The latter in turn could result in further damage to the mitochondria, thereby, generating a vicious circle [47,48]. In this context, malathion caused the increased formation of superoxide anion in sub-mitochondrial particles in hippocampus and inhibition of mitochondrial complex IV [9]. There was also a decrease in complex I activity in the hippocampus of malathion-exposed animals [28]. Other organophosphates induced alterations in mitochondrial dynamics such as increased mitochondrial length and reduced number of mitochondria as well as impaired axonal transport. These changes occurred at insecticide concentrations that failed to affect acetylcholinesterase activity [27]. In this study we hypothesized that citric acid by virtue of its antioxidant and bioenergetic actions would be able to decrease the malathion-induced neurotoxicity. Our findings validate this assumption. We have shown that the concurrent administration of malathion and citric acid resulted in markedly decreased neuronal damage due to the insecticide. Brain MDA decreased after citric acid administration and the decline in GSH, GPx activity and TAC were all reduced by citric acid. DNA damage of peripheral blood lymphocytes was also markedly decreased. Moreover, the histopathological alterations in the brain caused by malathion were ameliorated by treatment with citric acid. The present study thus suggests a novel action for citric acid in the protection against acute malathion intoxication.

Citrate occupies a central role in cellular intermediary metabolism. It occurs as an intermediate in the tricarboxylic acid or Krebs cycle, which generates energy through the oxidation of acetate. Energy in the form of reduced pyridine nucleotides are then used in the mitochondrial respiratory chain for synthesis of adenosine 5′-triphosphate [17]. Energy derived through glycolysis and tricarboxylic acid cycle is indispensable for maintaining neuronal and astrocytic functions and integrity [49,50]. In their study, Ying *et al.* [51] have shown that, excessive activation of PRAP1. Excessive activation of PRAP1 induces neuronal and astrocytes death by decreasing the levels of cytosolic NAD+ and impairing glycolysis. Provision of the tricarboxylic acid cycle substrates *eg.*, α-ketoglutarate or pyruvate (though not glucose) reduced this cell death [51]. In the same way, it is possible that a reduction in brain metabolism is related to the neurotoxic actions of malathion and this was amenable to treatment with citrate.

Besides its bioenergetic role, intracellular citrate is also important for the synthesis of fatty acids, isoprenoids and cholesterol [52]. Citrate exerted anti-inflammatory actions *eg.*, decreased release of myeloperoxidase, interleukin 1β, platelet factor 4 and elastase [20–22,53]. Citrate also displayed antioxidative effects reducing lipid peroxidation [21,23]. These actions of citrate are likely to be involved in protecting brain

neurons from deleterious effects of malathion. It has also been suggested that citrate released by astrocytes may modulate neuronal excitability by chelating Ca^{2+} and Mg^{2+} and thus regulating their extracellular concentrations [54]. This might provide another mechanism for the ability of citrate to protect neurons from malathion. There is also an evidence for the importance of cytoplasmic citrate availability in maintaining genome stability. In their study, defects in the mitochondrial citrate carrier SLC25A1 that releases citrate into the cytoplasm resulted in chromosomal breaks [55].

In this study, malathion caused increased brain nitric oxide and iNOS expression which decreased following treatment with citric acid. These observations suggest a pivotal role for nitric oxide in the development of malathion neurotoxicity. The gaseous molecule nitric oxide has an important role in the brain as intracellular messenger and in maintaining vascular tone owing to its vasodilator action [56]. In brain tissue, the excessive formation of nitric oxide in inflammatory and toxic states from astrocytes and microglia by the inducible nitric oxide synthase can lead to neuronal death. Increased activation of neuronal nitric oxide synthase during certain pathological conditions can also result in the release of excessive amounts of nitric oxide and ensuing neurotoxicity. Nitric oxide itself is relatively non-toxic, but can react with superoxide resulting in the formation of the peroxynitrite radical ($ONOO^-$), and also with oxygen to yield NO_2 and N_2O_3 capable of inducing lipid peroxidation, oxidation or nitrosylation of thiols *e.g.* glutathione or protein thiols and DNA damage [57,58]. Energy depletion occurs because of an inhibitory action on cytochrome oxidase, mitochondrial respiration, glycolysis, and the induction of mitochondrial permeability transition [59].

In this study, we observed decreased arylesterase activity of PON1 in the brain and liver of malathion exposed rats. The enzyme PON1 hydrolyzes several organophosphorus compounds, carbamates, nerve agents and many other xenobiotics [60,61]. The catalytic efficiency of PON1 in hydrolyzing specific organophosphate insecticides determines the individual's susceptibility to these agents [62–65]. The activity of the enzyme in plasma is also affected by exposure to organophosphate insecticides and is associated with marked inhibition of plasma BChE [66–68]. The PON1 enzyme also has an antioxidant action [40] and a peroxidase-like activity [69]. Paraoxonase-1 is inactivated by oxidants [70,71]. The decrease in enzyme activity by malathion might thus involve oxidative modification and/or direct inhibition by the organophosphate. In brain tissue of malathion-treated rats, the enzyme activity, however, showed further inhibition by citric acid and by both atropine and citric acid. Similar changes were not observed in liver tissue where citric acid alone had on PON1 activity in malathion-treated rats. These findings might suggest that changes in enzyme activity are not related to the protective action of citric acid in this study.

Organophosphate insecticides irreversibly bind to and inactivate the enzyme cholinesterase [72]. In mammals, both AChE and BChE hydrolyze the neurotransmitter acetylcholine but with differing specificity. This neurotransmitter is present in the post-synaptic neuronal membrane, at the myoneural junction, autonomic ganglia, and at the terminal endings of the post-ganglionic parasympathetic nerves [73]. Organophosphates thus results in acetylcholine accumulation at the neuronal synapse and excessive central and peripheral cholinergic activity [72,73]. Whereas the role for AChE in hydrolyzing acetylcholine is clear, the physiological role of BChE is less obvious [74]. Butyrylcholinesterase also detoxifies or catabolize ester-containing drugs. Humans with BChE deficiency are asymptomatic but exhibit increased sensitivity to the muscle relaxants suxamethonium and mivacurium, two BChE substrates, used in anesthesia [75]. In managing poisoning due to organophosphate, the antidote atropine, a cholinergic receptor antagonist, is frequently used to prevent the effects of excess acetylcholine at the muscarinic cholinergic synapses [32,33]. In this study, we demonstrate that atropine administered at time of exposure to malathion was capable of increasing brain cholinesterase activity and ameliorating the neuronal damage due to the insecticide. The agent also effectively inhibited DNA damage of peripheral blood lymphocytes. In the brain of malathion-exposed rats, atropine inhibited iNOS expression and the release of nitric oxide. Glutathione peroxidase activity and total antioxidant capacity also increased following treatment with atropine. These findings suggest a link between excessive cholinergic stimulation and the development of oxidative stress due to the organophosphate and that oxidative stress is a consequence to increased cholinergic stimulation.

Glucose is the main energetic fuel for energy-dependent brain functions and brain glucose consumption accounts for 45–60% of glucose used by the body [76]. In this study, a decrease in brain glucose in malathion-exposed rats was observed. It is not clear whether this represents an increase in energy production by neurons and astrocytes in face of the toxic challenge or is the result of impaired glucose brain transport. Nevertheless, recovery of brain glucose in malathion-exposed rat occurred following citrate, atropine or their combined administration, suggesting a link between the restoration of brain glucose level and neuroprotection by these agents.

In this study, the ability of malathion to cause DNA damage of peripheral blood lymphocytes was determined using the 'Comet assay'. This assay is useful for the detection of DNA strand breaks in mammalian cells [44]. Our results confirm previous studies indicating increased the DNA damage in peripheral blood of malathion-treated rats [77,78]. The number of comets produced by malathion in peripheral blood lymphocytes showed marked decrease in citric acid and/or atropine-treated rats.

Studies have shown that exposure to malathion is able to cause liver tissue damage which is mediated by free radicals [34,35]. We thus extended our observations in order to delineate an effect for citric acid on liver injury in malathion intoxicated rats. Our results showed that malathion increased liver lipid peroxidation and nitrite along with decreased GSH indicative of increased oxidative stress. There were also increased liver transaminases, a marker of liver cell damage. Only with citric acid at 200 mg/kg or citric acid-atropine combined treatment, there was decreased lipid peroxidation and an increase in hepatic GSH while transaminases decreased after treatment with citric acid at 400 mg/kg. These changes were not observed after atropine. Nevertheless, histopathological studies indicated a clear protective effect for citric acid, atropine, or their combination along with inhibition of iNOS expression in hepatocytes. The liver lobule and hepatocytes thus regained their morphological integrity after treatment with citric and/or atropine. It is likely; however, that functional recovery is not yet complete in view of the still increased lipid peroxidation and tissue transaminases.

Our results thus indicate that cholinergic receptor blockade with atropine was able to protect against the histopathological alterations in the brain and liver and markedly inhibited DNA damage in blood lymphocytes following acute malathion exposure in rats. The mechanism probably involves decreased oxidative stress that accompanied cholinesterase inhibition and the excessive central and peripheral cholinergic stimulation. The study also demonstrates for the first time that the administration of citrate was able to ameliorate the neurotoxicity and hepatotoxicity as well as DNA damage caused by malathion. These effects are likely to involve an antioxidant as well as a bioenergetic action of citric acid. Citrate might thus find a role in treatment of nervous system consequences following exposure to malathion and possibly other organophosphates.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans* 2007; **35**(Pt 5): 1147-1150.

[2] Weidinger A, Kozlov AV. Biological activities of reactive oxygen and nitrogen species: oxidative stress versus signal transduction. *Biomolecules* 2015; **5**(2): 472-484.

[3] Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997; **82**(2): 291-295.

[4] Solsona C, Kahn TB, Badilla CL, Álvarez-Zaldiernas C, Blasi J, Fernandez JM, et al. Altered thiol chemistry in human amyotrophic lateral sclerosis-linked mutants of superoxide dismutase 1. *J Biol Chem* 2014; **289**(39): 26722-26732.

[5] Markkanen E, Meyer U, Dianov GL. DNA damage and repair in schizophrenia and autism: implications for cancer comorbidity and beyond. *Int J Mol Sci* 2016; **17**(6); http://dx.doi.org/10.3390/ijms17060856.

[6] Peiró C, Romacho T, Azcutia V, Villalobos L, Fernández E, Bolaños JP, et al. Inflammation, glucose, and vascular cell damage: the role of the pentose phosphate pathway. *Cardiovasc Diabetol* 2016; **15**(1): 82.

[7] Huang WJ, Zhang X, Chen WW. Role of oxidative stress in Alzheimer's disease. *Biomed Rep* 2016; **4**(5): 519-522.

[8] Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* 2014; **24**(10): R453-R462.

[9] Delgado EH, Streck EL, Quevedo JL, Dal-Pizzol F. Mitochondrial respiratory dysfunction and oxidative stress after chronic malathion exposure. *Neurochem Res* 2006; **31**(8): 1021-1025.

[10] Abdel-Salam OM, Youness ER, El-N Esmail RS, Mohammed NA, Khadrawy YA, Sleem AA, et al. Methylene blue as a novel neuroprotectant in acute malathion intoxication. *React Oxyg Species* 2016; **1**: 165-177.

[11] El-Bini Dhouib I, Lasram MM, Annabi A, Gharbi N, El-Fazaa S. A comparative study on toxicity induced by carbosulfan and malathion in Wistar rat liver and spleen. *Pestic Biochem Physiol* 2015; **124**: 21-28.

[12] Karabag-Cobana F, Buldukb I, Limana R, Incec S, Cigercid I, Hazmane O. Oleuropein alleviates malathion-induced oxidative stress and DNA damage in rats. *Toxicol Environ Chem* 2016; **98**(1): 101-108.

[13] John S, Kale M, Rathore N, Bhatnagar D. Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes. *J Nutr Biochem* 2001; **12**(9): 500-504.

[14] Trevisan R, Uliano-Silva M, Pandolfo P, Franco JL, Brocardo PS, Santos AR, et al. Antioxidant and acetylcholinesterase response to repeated malathion exposure in rat cerebral cortex and hippocampus. *Basic Clin Pharmacol Toxicol* 2008; **102**(4): 365-369.

[15] Durak D, Uzun FG, Kalender S, Ogutcu A, Uzunhisarcikli M, Kalender Y. Malathion-induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E in vitro. *Environ Toxicol* 2009; **24**(3): 235-242.

[16] German JB. Antioxidants. In: Branen AL, Davidson PM, Salminen S, Thorngate JH III, editors. *Food additives*. 2nd ed. New York, Basel: Marcel Dekker, Inc.; 2002, p. 538.

[17] Fromm HJ, Hargrove MS. The tricarboxylic acid cycle. In: *Essentials of biochemistry*. Berlin Heidelberg: Springer-Verlag; 2012, p. 205-221.

[18] Penniston KL, Nakada SY, Holmes RP, Assimos DG. Quantitative assessment of citric acid in lemon juice, lime juice, and commercially-available fruit juice products. *J Endourol* 2008; **22**(3): 567-570.

[19] Franklin RB, Costello LC. Citrate metabolism in prostate and other cancers. In: Singh KK, Costello LC, editors. *Mitochondria and cancer*. Chicago: Springer; 2009, p. 61-78.

[20] Gabutti L, Ferrari N, Mombelli G, Keller F, Marone C. The favorable effect of regional citrate anticoagulation on interleukin-1beta release is dissociated from both coagulation and complement activation. *J Nephrol* 2004; **17**(6): 819-825.

[21] Gritters M, Grooteman MP, Schoorl M, Schoorl M, Bartels PC, Scheffer PG, et al. Citrate anticoagulation abolishes degranulation of polymorphonuclear cells and platelets and reduces oxidative stress during haemodialysis. *Nephrol Dial Transpl* 2006; **21**(1): 153-159.

[22] Tiranathanagul K, Jearnsujitwimol O, Susantitaphong P, Kijkriengkraikul N, Leelahavanichkul A, Srisawat N, et al. Regional citrate anticoagulation reduces polymorphonuclear cell degranulation in critically ill patients treated with continuous venovenous hemofiltration. *Ther Apher Dial* 2011; **15**(6): 556-564.

[23] Abdel-Salam OME, Youness ER, Mohammed NA, Morsy SMY, Omara EA, Sleem AA. Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. *J Med Food* 2014; **17**(5): 588-598.

[24] Abdel Salam OME, Sleem AA, Shaffie NM. Hepatoprotective effects of citric acid and aspartame on carbon tetrachloride-induced hepatic damage in rats. *EXCLI J* 2009; **8**: 41-49.

[25] Abdel Salam OME, Sleem AA, Shaffie NM. Protection against carbon tetrachloride-induced liver damage by citric acid. *Cell Biol Res Ther* 2015; **4**: 1.

[26] Kaur P, Radotra B, Minz RW, Gill KD. Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. *Neurotoxicology* 2007; **28**(6): 1208-1219.

[27] Middlemore-Risher ML, Adam BL, Lambert NA, Terry AV Jr. Effects of chlorpyrifos and chlorpyrifos-oxon on the dynamics and movement of mitochondria in rat cortical neurons. *J Pharmacol Exp Ther* 2011; **339**(2): 341-349.

[28] Karami-Mohajeri S, Hadian MR, Fouladdel S, Azizi E, Ghahramani MH, Hosseini R, et al. Mechanisms of muscular electrophysiological and mitochondrial dysfunction following exposure to malathion, an organophosphorus pesticide. *Hum Exp Toxicol* 2014; **33**(3): 251-263.

[29] dos Santos AA, Naime AA, de Oliveira J, Colle D, dos Santos DB, Hort MA, et al. Long-term and low-dose malathion exposure causes cognitive impairment in adult mice: evidence of hippocampal mitochondrial dysfunction, astrogliosis and apoptotic events. *Arch Toxicol* 2016; **90**(3): 647-660.

[30] Bozkurt B, Dumlu EG, Tokac M, Ozkardes AB, Ergin M, Orhun S, et al. Methylene blue as an antioxidant agent in experimentally-induced injury in rat liver. *Bratisl Lek Listy* 2015; **116**(3): 157-161.

[31] Roy Choudhury G, Winters A, Rich RM, Ryou MG, Gryczynski Z, Yuan F, et al. Methylene blue protects astrocytes against glucose oxygen deprivation by improving cellular respiration. *PLoS One* 2015; **10**(4): e0123096.

[32] Jokanović M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. *Toxicol Lett* 2009; **190**(2): 107-115.

[33] Eddleston M, Chowdhury FR. Pharmacological treatment of organophosphorus insecticide poisoning: the old and the (possible) new. *Br J Clin Pharmacol* 2016; **81**(3): 462-470.
[34] Possamai FP, Fortunato JJ, Feier G, Agostinho FR, Quevedo J, Wilhelm Filho D, et al. Oxidative stress after acute and sub-chronic malathion intoxication in Wistar rats. *Environ Toxicol Pharmacol* 2007; **23**(2): 198-204.
[35] Nel-H Zidan. Hepato- and nephrotoxicity in male albino rats exposed to malathion and spinosad in stored wheat grains. *Acta Biol Hung* 2015; **66**(2): 133-148.
[36] Nair V, Turner GA. The thiobarbituric acid test for lipid peroxidation: structure of the adduct with malondialdehyde. *Lipids* 1984; **19**: 804-805.
[37] Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82**(1): 70-77.
[38] Archer S. Measurement of nitric oxide in biological models. *FASEB J* 1993; **7**(2): 340-360.
[39] Eckerson HW, Wyte CM, La Du BN. The human serum paraoxonase/arylesterase polymorphism. *Am J Hum Genet* 1983; **35**(6): 1126-1138.
[40] Haagen L, Brock A. A new automated method for phenotyping arylesterase (EC 3.1.1.2) based upon inhibition of enzymatic hydrolysis of 4-nitrophenyl acetate by phenyl acetate. *Eur J Clin Chem Clin Biochem* 1992; **30**(7): 391-395.
[41] Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; **7**: 88-95.
[42] Gorun V, Proinov I, Baltescu V, Balaban G, Barzu O. Modified Ellman procedure for assay of cholinesterases in crude enzymatic preparations. *Anal Biochem* 1978; **86**(1): 324-326.
[44] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; **175**(1): 184-191.
[45] Blasiak J, Gloc E, Drzewoski J, Wozniak K, Zadrozny M, Skórski T, et al. Free radical scavengers can differentially modulate the genotoxicity of amsacrine in normal and cancer cells. *Mutat Res* 2003; **535**(1): 25-34.
[46] Elersek T, Filipic M. Organophosphorous pesticides-mechanisms of their toxicity. In: Stoytcheva Margarita, editor. *Pesticides-the impacts of pesticides exposure*; 2011; http://dx.doi.org/10.5772/14020. Available from: http://www.intechopen.com/books/pesticides-the-impacts-of-pesticidesexposure/organophosphorous-pesticides-mechanisms-of-their-toxicity.
[47] Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer. *Cancer Metab* 2014; **2**: 17.
[48] Holmström KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Bio* 2014; **15**(6): 411-421.
[49] Attwell D, Laughlin SB. An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab* 2001; **21**(10): 1133-1145.
[50] Hertz L, Peng L, Dienel GA. Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J Cereb Blood Flow Metab* 2007; **27**(2): 219-249.
[51] Ying W, Chen Y, Alano CC, Swanson RA. Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *J Cereb Blood Flow Metab* 2002; **22**(7): 774-779.
[52] Inoue K, Zhuang L, Ganapathy V. Human Na+ -coupled citrate transporter: primary structure, genomic organization, and transport function. *Biochem Biophys Res Commun* 2002; **299**(3): 465-471.
[53] Bryland A, Wieslander A, Carlsson O, Hellmark T, Godaly G, Jones LM, et al. Gustatory processing: a dynamic systems approach. *Curr Opin Neurobiol* 2006; **16**(4): 420-428.
[54] Brekke E, Morken TS, Sonnewald U. Glucose metabolism and astrocyte-neuron interactions in the neonatal brain. *Neurochem Int* 2015; **82**: 33-41.
[55] Morciano P, Carrisi C, Capobianco L, Mannini L, Burgio G, Cestra G, et al. A conserved role for the mitochondrial citrate transporter Sea/SLC25A1 in the maintenance of chromosome integrity. *Hum Mol Genet* 2009; **18**(21): 4180-4188.
[56] Sobrevia L, Ooi L, Ryan S, Steinert JR. Nitric oxide: a regulator of cellular function in health and disease. *Oxid Med Cell Longev* 2016; **2016**. 9782346.
[57] Wink DA, Feelisch M, Vodovotz Y, Fukuto J, Grisham MB. The chemical biology of nitric oxide. In: Gilbert, Colton, editors. *Reactive oxygen species in biological systems*. New York: Kluwer Academic/Plenum Publishers; 1999, p. 245-291.
[58] Yuste JE, Tarragon E, Campuzano CM, Ros-Bernal F. Implications of glial nitric oxide in neurodegenerative diseases. *Front Cell Neurosci* 2015; **9**: 322.
[59] Brown GC. Nitric oxide and neuronal death. *Nitric Oxide* 2010; **23**(3): 153-165.
[60] La Du BN. Human serum paraoxonase/arylesterase. In: Kalow W, editor. *Pharmacogenetics of drug metabolism*. New York: Pergamon Press, Inc.; 1992, p. 51-91.
[61] Kulka M. A review of paraoxonase 1 properties and diagnostic applications. *Pol J Vet Sci* 2016; **19**(1): 225-232.
[62] Jørgensen A, Nellemann C, Wohlfahrt-Veje C, Jensen TK, Main KM, Andersen HR. Interaction between paraoxonase 1 polymorphism and prenatal pesticide exposure on metabolic markers in children using a multiplex approach. *Reprod Toxicol* 2015; **51**: 22-30.
[63] Carr RL, Dail MB, Chambers HW, Chambers JE. Species differences in paraoxonase mediated hydrolysis of several organophosphorus insecticide metabolites. *J Toxicol* 2015; **2015**. 470189.
[64] Holland N, Lizarraga D, Huen K. Recent progress in the genetics and epigenetics of paraoxonase: why it is relevant to children's environmental health. *Curr Opin Pediatr* 2015; **27**(2): 240-247.
[65] Marsillach J, Costa LG, Furlong CE. Paraoxonase-1 and early-life environmental exposures. *Ann Glob Health* 2016; **82**(1): 100-110.
[66] McDaniel CY, Dail MB, Wills RW, Chambers HW, Chambers JE. Paraoxonase 1 polymorphisms within a Mississippi USA population as possible biomarkers of enzyme activities associated with disease susceptibility. *Biochem Genet* 2014; **52**(11–12): 509-523.
[67] Bernal-Hernández YY, Medina-Díaz IM, Barrón-Vivanco BS, Robledo-Marenco Mde L, Girón-Pérez MI, Pérez-Herrera NE, et al. Paraoxonase 1 and its relationship with pesticide biomarkers in indigenous Mexican farmworkers. *J Occup Environ Med* 2014; **56**(3): 281-290.
[68] Medina-Díaz IM, Ponce-Ruiz N, Ramírez-Chávez B, Rojas-García AE, Barrón-Vivanco BS, Elizondo G, et al. Downregulation of human paraoxonase 1 (PON1) by organophosphate pesticides in HepG2 cells. *Environ Toxicol* 2016; http://dx.doi.org/10.1002/tox.22253.
[69] Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, et al. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 2000; **101**(21): 2510-2517.
[70] Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, et al. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic Biol Med* 1999; **26**(7–8): 892-904.
[71] Nguyen SD, Sok DE. Oxidative inactivation of paraoxonase1, an antioxidant protein and its effect on antioxidant action. *Free Radic Res* 2003; **37**(12): 1319-1330.
[72] Pohanka M. Inhibitors of acetylcholinesterase and butyrylcholinesterase meet immunity. *Int J Mol Sci* 2014; **15**: 9809-9825.
[73] Silman I, Sussman JL. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr Opin Pharmacol* 2005; **5**(3): 293-302.
[74] Çokuğraş AN. Butyrylcholinesterase: structure and physiological importance. *Turk J Biochem* 2003; **28**(2): 54-61.

[75] Delacour H, Dedome E, Courcelle S, Hary B, Ceppa F. Butyrylcholinesterase deficiency. *Ann Biol Clin (Paris)* 2016; **74**(3): 279-285.

[76] Shrayyef MZ, Gerich JE. Normal glucose homeostasis. In: Poretsky L, editor. *Principles of diabetes mellitus*. Epub: Springer; 2010, p.19-34 [Epub].

[77] Réus GZ, Valvassori SS, Nuernberg H, Comim CM, Stringari RB, Padilha PT, et al. DNA damage after acute and chronic treatment with malathion in rats. *J Agric Food Chem* 2008; **56**: 7560-7565.

[78] Ojha A, Srivastava N. *In vitro* studies on organophosphate pesticides induced oxidative DNA damage in rat lymphocytes. *Mutat Res Genet Toxicol Environ Mutagen* 2014; **761**: 10-17.