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# Amelioration of fenitrothion induced oxidative DNA damage and inactivation of caspase-3 in the brain and spleen tissues of male rats by *N*-acetylcysteine

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ABSTRACT

*N*-acetylcysteine (NAC) has largely been used as an effective chemo- protective agent owing to their beneficial effect in restoring several physiological parameters and relieving oxidative stress. Interestingly, it has been suggested that NAC mechanisms of action extend beyond being a precursor to the antioxidant glutathione and that they may involve several neurotropic and inflammatory pathways. Exposure to fenitrothion, an organophosphorus insecticide, promotes oxidative stress and induces several deleterious changes in the immune response and various tissues including cerebrum and spleen. The main objective of our study was to investigate ameliorative efficacy of *N*-acetylcysteine for immunological and neurological alterations and oxidative DNA damage induced by fenitrothion toxicity in cerebrum and spleen tissues of male rats. Our results revealed that oral exposure to fenitrothion for 30 days caused a reduction in the erythrocyte count in addition to leukocytosis, lymphocytosis, and neutrophilia. Also, this route of administration increased the serum levels of LDH, TNF-α, and IL-2 with reduction in serum immunoglobulins (IgG & IgM) concentrations. Furthermore, a significant downregulation in the antioxidant markers (GSH & SOD) with an elevation of free radical (MDA) levels were noticed. Regarding the brain, fenitrothion administration inhibited AchE activity and increased brain GABA, serotonin and dopamine levels. Moreover, it induced an elevation in oxidative DNA damage indicated by 8-hydroxy 2-deoxyguanosine (8OH2dG) and mRNA expression of pro-apoptotic genes, including Bax, and p53, but Bcl-2 expression was reduced. *N*-acetylcysteine co-treatment restored the normal physiological tone in most of these parameters. Immunostaining for GFAP and Caspase-3 markers in the brain and spleen tissues were increased respectively. In conclusion, *N*-acetylcysteine supplementation has an ameliorative effect against immunotoxic, neurotoxic and oxidative DNA damage induced by fenitrothion exposure.

## 1. Introduction

Organophosphorus (OP) pesticides are widely used chemicals in agriculture for pest control [1]. Humans are exposed to these pesticides either directly as agricultural workers or indirectly through food consumption. Moreover, a significant amount of these pesticides and their metabolites, which reach estuaries and rivers through run-off from farmland, are evidently toxic to the wildlife [2]. The neurological effect is considered the primary target of OP via its inhibiting effect on acetylcholinesterase [3]. Fenitrothion [*O*, *O*-dimethyl O- (4-nitro-m-tolyl) phosphorothioate] (FNT) is a broad-spectrum OP insecticide used in agriculture to control the chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains, and cotton. [4]. In addition to the FNT-induced oxidative stress causing deleterious changes, the male reproductive system is considered as a secondary target for OP pesticides [5]. Neuronal necrosis has been observed in multiple cortical and subcortical regions in experimental rats exposed to OPs [6] as fenthion [7]. Furthermore, FNT induced atrophy of lymphoid tissues and suppressed different immune functions [8,9].

N-acetylcysteine, a thiol-containing agent, has several actions including anti-inflammatory properties, antioxidant activity, and intracellular glutathione level enhancement [10]. It can cross the blood-brain barrier in both rodents and human [11,12]. NAC has been used to relief paracetamol intoxication and as a mucolytic agent [13]. It can

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decrease the early death of spinal motoneurons and delay the degeneration of sensory neurons [14,15]. The present study was designed to evaluate the impact of fenitrothion oral administration for 30 days on some hematologic and immunologic parameters, induction of oxidative stress and its deleterious action on the brain and spleen in addition to the protective effect of NAC in male Sprague Dawley rats.

## 2. Materials and methods

### 2.1. Animals

Eighty (80) male adult Sprague Dawley rats of 6 weeks age, weighing between 160 ± 10 g were obtained from the Laboratory Animal Research Unit, Faculty of Veterinary Medicine, Zagazig University, and acclimatized for a 2 week before treatment. The rats were housed in metal cages and provided with basal diet and water ad libitum. All rats were maintained with standard environmental conditions throughout the experimental period (12 h light/dark cycles, 60% relative humidity, 24 °C room temperature). All protocols were approved by Institutional Animal Ethical Committee (IAEC) in accordance with the guiding principles of the National Institutes of Health (NIH) for the care and use of laboratory animals.

### 2.2. Chemicals and reagents

Fenitrothion (Sumithion KZ 50% EC) was purchased from the Kafr El-Zayat Company for Pesticides and Chemicals (Kafr El-Zayat, Egypt). N-acetylcysteine was obtained from Sigma Chemicals (St Louis, MO, USA).

### 2.3. Animal treatment

Rats were randomly divided into four equal groups (n = 20/group). Group 1 (control group) which orally received distilled water; Group 2 (NAC-treated group) which orally received NAC in a dose of 200 mg/kg BW [16] daily for 30 days; Group 3 (fenitrothion-treated group) received oral fenitrothion (dissolved in distilled water) at a dose of 20 mg/kg BW day per day for 30 days (1/30 $LD_{50}$) [17]; and Group 4 (NAC + fenitrothion-treated group) which was simultaneously treated with NAC (200 mg/kg BW) and fenitrothion (20 mg/kg BW) at the same mentioned route and duration. Body weight of each rat was recorded during the experiment and the dosing volume of stock was adjusted accordingly. Clinical signs and mortality rate were recorded daily.

### 2.4. Blood samples

At the end of the experimental period (on day 31), rats from different groups were fasted overnight. The blood samples were collected from retro-orbital plexus, a sample of 0.5 ml was collected into anticoagulant tubes (EDTA 10%) for estimation of hematological parameters, and another 3 ml was collected into test tubes and left to clot (30 min at room temperature); the serum was separated by centrifugation (4500 rpm, 20 min) and stored at −20 °C until analyzed.

### 2.5. Tissue samples

At necropsy, the whole brain and spleen were removed and rinsed with normal saline. Parts of the cerebral and splenic tissue specimens were snap-frozen by immersion in liquid nitrogen and kept at (−80 °C) for later gene expression analysis. Other parts of the cerebrum and spleen were then fixed at 10% buffered formalin for 72 h and subsequently transferred to 70% ethanol for use in immunohistochemistry and histopathology. The rest of the cerebrum or spleen tissue were then homogenized for 5 min in 0.115 M chilled phosphate buffer saline (1:5 w/v) using tissue homogenizer (Potter–Elvehjem), then centrifuged at 14,000 rpm at 4 °C for 15 min, and the supernatants were separated for biochemical analysis.

### 2.6. Hematological analysis

Blood samples collected with 10% EDTA were analyzed using a Hemascreen 18 automatic cell counter (Hospitex, Osmannoro-Sesto Fiorentino, Italy) to obtain measures of RBC, Hb, PCV, MCV, MCHC, and WBC counts. Blood films were fixed with absolute methyl alcohol, then stained with Giemsa and examined for differential leukocyte count [18].

### 2.7. Inflammatory cytokines assay

Serum samples were used for evaluation of inflammatory cytokines, interleukin (IL)-2 and tumor necrosis factor (TNF)-α concentrations using commercial ELISA kits purchased from ThermoFisher Scientific (Invitrogen, BMS634 & KRC3011) respectively.

### 2.8. Oxidative stress markers analysis

Serum samples were used to measure the reduced GSH concentration according to Jollow et al. [19], SOD activity as the method described by Nishikimi et al. [20], and MDA (lipid peroxidation marker) was determined according to Ohkhawa et al. [21].

### 2.9. Analysis of acetyl cholinesterase and 8-hydroxy-2′-deoxyguanosine

Concentrations of AChE and 8-hydroxy-2-deoxyguanosine in brain tissue homogenate were determined using commercial ELISA kits obtained from CUSABIO Cat. No CSB-E11304 and CSB-E10526 respectively.

### 2.10. Determination of brain neurotransmitters

Gamma-aminobutyric acid (GABA), serotonin and dopamine levels in brain homogenate were measured using commercial ELISA kits purchased from EIAab and MyBiosource (Cat. No. E0900r, MBS166089, and MBS296895 respectively).

### 2.11. Real time-quantitative PCR (RT-qPCR) analysis

Total RNA extraction from tissue samples was performed using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) following the manufacturer's instructions. A tissue sample weighting around 30 mg was added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. For homogenization of samples, tubes were placed into the adaptor sets, which are fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in a 2-min high-speed (30 Hz) shaking step. RNA concentration and purity from each sample were estimated using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, USA). The total RNA was then used as a template for one-step PCR using a 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH). The primers used were supplied from Metabion (Germany) and listed in Table 1 according to Banni et al. [22], Mehmeti et al. [23] Aliparasti et al. [24] and Deng et al. [25] respectively. The 25 µl RT-PCR reaction mixtures contained 12.5 µl of the 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µl) (Thermo Fisher), 0.5 µl of each forward and reverse primer of 10 pmol concentration, 10.25 µl of water, and 1 µl of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine. The thermal cycling conditions consisted of reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 15 min and amplification for 40 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Each sample was measured in triplicate. A melting curve analysis was performed following PCR amplification. The amplification curves and CT

**Table 1**
Primer sequences for the quantitative real-time PCR.

| Gene | | Primer sequence | Reaction conditions |
|---|---|---|---|
| β-actin | F | TCCTCCTGAGCGCAAGTACTCT | 50 °C-30 min/94 °C-15 min/94 °C-15 s/60 °C |
| | R | GCTCAGTAACAGTCCGCCTAGAA | 30s/72 °C-30s (40 cycle)/94 °C-1 min/60 °C-1 min/94 °C-1 min (1 cycles) |
| BAX | F | CCAGGACGCATCCACCAAGAAG | 62 °C-30 s (40 cycle)/62 °C-1 min |
| | R | CCCAGTTGAAGTTGCCGTCTGC | (1 cycles) |
| P53 | F | GTCGGCTCCGACTATACCACTATC | 60 °C-30 s (40 cycle)/60 °C-1 min |
| | R | CTCTCTTTGCACTCCCTGGGGG | (1 cycles) |
| Bcl2 | F | ATCGCTCTGTGGATGACTGAGTAC | 68 °C-30 s (40 cycle)/68 °C-1 min |
| | R | AGAGACAGCCAGGAGAAATCAAAC | (1 cycles) |

**Table 2**
Effects of NAC oral administration (200 mg/kg BW) and fenitrothion (20 mg/kg BW) and their combination on male rat hematological parameters. Data are expressed as mean ± SE, (n = 8–10).

| Groups<br>Parameters | Control | NAC | Fenitrothion | NAC+ fenitrothion |
|---|---|---|---|---|
| RBCs $\times 10^6$/μl | 7.62 ± 0.11$^{ab}$ | 7.81 ± 0.11$^{a}$ | 6.02 ± 0.15$^{c}$ | 7.26 ± 0.06$^{b}$ |
| Hb gm% | 14.93 ± 0.26$^{a}$ | 15.17 ± 0.11$^{a}$ | 13.96 ± 0.61$^{a}$ | 14.13 ± 0.09$^{a}$ |
| PCV % | 49.35 ± 0.96$^{ab}$ | 51.89 ± 0.62$^{a}$ | 46.88 ± 0.54$^{b}$ | 49.54 ± 0.63$^{ab}$ |
| MCV fl | 64.76 ± 0.65$^{b}$ | 66.45 ± 0.39$^{b}$ | 78.20 ± 1.21$^{a}$ | 67.33 ± 0.61$^{b}$ |
| MCHC % | 30.27 ± 0.21$^{a}$ | 29.25 ± 0.22$^{a}$ | 29.68 ± 0.97$^{a}$ | 28.48 ± 0.18$^{a}$ |
| TLC $\times 10^3$/μl | 7.17 ± 0.17$^{c}$ | 7.04 ± 0.07$^{c}$ | 15.54 ± 0.45$^{a}$ | 11.47 ± 0.27$^{b}$ |
| Lymphocyte $\times 10^3$/μl | 5.64 ± 0.25$^{c}$ | 5.72 ± 0.18$^{c}$ | 9.53 ± 0.30$^{a}$ | 7.10 ± 0.13$^{b}$ |
| Neutrophil $\times 10^3$/μl | 1.02 ± 0.07$^{c}$ | 0.95 ± 0.07$^{c}$ | 5.71 ± 0.22$^{a}$ | 3.90 ± 0.11$^{b}$ |
| Eosinophil $\times 10^3$/μl | 0.20 ± 0.03$^{a}$ | 0.23 ± 0.04$^{a}$ | 0.20 ± 0.03$^{a}$ | 0.18 ± 0.03$^{a}$ |
| Monocyte $\times 10^3$/μl | 0.24 ± 0.02$^{a}$ | 0.31 ± 0.05$^{a}$ | 0.34 ± 0.05$^{a}$ | 0.26 ± 0.06$^{a}$ |

Means within the same column carrying different superscript letters are significantly different (P ≤ 0.05).

values were determined by the Stratagene MX3005P software. All of the different RT-PCR processes and reporting comply with MIQE guidelines [26]. The expression level of the target genes was normalized to that of β-actin, and the relative fold changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ comparative method [27–29].

### 2.12. Histopathology and immunohistochemistry evaluation

The brain and spleen tissue samples from rats of the different groups previously collected and fixed in 10% buffered neutral formalin were processed to get 5 μm thick paraffin sections. Hematoxylin and Eosin (H &E) stain was done according to standard protocols for histopathological examination [30]. Immunohistochemical detection of Glial fibrillary acidic protein (GFAP) and caspase-3 in the cerebrum and spleen respectively were performed using primary rabbit anti-GFAP antibody, primary rabbit anti-caspase-3 antibody and secondary antibody from Abcam, Cambridge, MA. The avidin–biotin complex technique was applied. The technique protocol was standardized following the manufacturer's instructions. Negative controls were done using the same steps but with antibody free phosphate buffered saline applied instead of the primary antibodies [31]. Three slides per rat at least were used for evaluation in all groups. Brain GFAP and Splenic Caspase-3 immunostaining density were calculated by using FIJI/ImagJ® software and the positive area was expressed as a percent of control [32,33].

### 2.13. Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using a one-way analysis of variance (ANOVA) test followed by post hoc Tukey's test to determine any significant differences between the different experimental groups. An n = 8–10 animals per experimental group was applied. A p-value ≤0.05 was considered statistically significant.

## 3. Results

### 3.1. Clinical signs and mortality rate

Rats which received fenitrothion (Gp.3, 20 mg/kg BW for 30 days) exhibited salivation, depression, decrease food intake, and soft stool with mortality rate 45% during the course of exposure, while rats co-treated with NAC and fenitrothion (Gp.4) showed less severe clinical signs, with mortality rate reduced to 27.5% compared to fenitrothion intoxicated rats.

### 3.2. Erythrogram and leukogram

NAC-treated rats showed no significant changes in the erythrocyte count, Hb content, PCV, MCV and MCHC compared to control group, while rats intoxicated with fenitrothion showed a significant decrease in erythrocyte count and a significant increase in MCV with non-changes in Hb content, PCV and MCHC percentages (macrocytic normochromic anemia) in comparison to control group. However, rats treated with NAC and fenitrothion showed non-significant changes in the erythrogram (Table 2). NAC-treated rats showed also no significant changes in the total leukocyte, lymphocyte, neutrophil, eosinophil and monocyte counts; however, a significant increase of these parameters was noticed in fenitrothion intoxicated rats compared to the control group. The co-treated group showed a significant decrease in total leukocyte, lymphocyte, and neutrophil counts in addition to non-significant changes in eosinophil and monocyte counts when compared to fenitrothion intoxicated rats (Table 2).

### 3.3. Biochemical analysis and inflammatory cytokines

NAC administrated group showed no significant changes in serum LDH, but in the fenitrothion intoxicated group, a significant increase in serum LDH in comparison to control was observed. However, the co-treated group showed a significant decrease in serum LDH compared to fenitrothion intoxicated rats. Furthermore, no significant changes in

**Table 3**
Effects of NAC oral administration (200 mg/kg BW) and fenitrothion (20 mg/kg BW) and their combination on male rat serum LDH, IgG, IgM, SOD, MDA, TNF-α, IL-2 levels between different groups. Data are expressed as mean ± SE (n = 8–10).

| Groups<br>Parameters | Control | NAC | Fenitrothion | NAC+ fenitrothion |
|---|---|---|---|---|
| LDH (U/L) | 337.12 ± 6.19$^{c}$ | 350.00 ± 4.26$^{c}$ | 559.62 ± 5.39$^{a}$ | 488.87 ± 5.15$^{b}$ |
| IgG (ng/ml) | 8.64 ± 0.08$^{a}$ | 8.53 ± 0.09$^{a}$ | 4.71 ± 0.06$^{c}$ | 6.53 ± 0.07$^{b}$ |
| IgM (ng/ml) | 1.22 ± 0.01$^{a}$ | 1.21 ± 0.01$^{a}$ | 0.72 ± 0.01$^{c}$ | 0.89 ± 0.01$^{b}$ |
| GSH (mmol/L) | 2.23 ± 0.06$^{a}$ | 2.25 ± 0.05$^{a}$ | 0.71 ± 0.02$^{c}$ | 1.45 ± 0.04$^{b}$ |
| SOD (U/ml) | 3.95 ± 0.13$^{a}$ | 3.99 ± 0.08$^{a}$ | 1.18 ± 0.05$^{c}$ | 2.17 ± 0.06$^{b}$ |
| MDA (nmol/ml) | 14.76 ± 0.27$^{c}$ | 14.70 ± 0.49$^{c}$ | 46.21 ± 1.36$^{a}$ | 22.95 ± 0.52$^{b}$ |
| TNF-α (pg/ml) | 51.98 ± 1.93$^{c}$ | 53.75 ± 1.71$^{c}$ | 92.47 ± 1.74$^{a}$ | 73.22 ± 1. 20$^{b}$ |
| IL-2 (pg/ml) | 279.63 ± 1.69$^{c}$ | 280.35 ± 2.91$^{c}$ | 448.27 ± 6.22$^{a}$ | 337.53 ± 7.09$^{b}$ |

Means within the same column carrying different superscripts letters are significantly different (P ≤ 0.05).

serum immunoglobulin (IgG and IgM) were shown in NAC administrated group, but a significant decrease in their concentration was observed in fenitrothion treated group in comparison to the control. Compared to fenitrothion intoxicated group, the co-treated group revealed a significant increase in serum immunoglobulins (Table 3). Moreover, there are no significant changes in serum TNF-α and IL-2 in NAC-treated rats but a significant increase in the concentration of both was noted in rats treated with fenitrothion when compared to the control group. However, the combination group showed a significant decrease in serum TNF-α and IL-2 when compared to fenitrothion intoxicated rats (Table 3).

### 3.4. Antioxidant and oxidative stress markers

No significant changes in serum GSH, SOD, and MDA noticed in NAC-treated group. However, the fenitrothion treated group showed a significant decrease in serum GSH and SOD and a significant increase in serum MDA when compared to the control group. While the simultaneous administration of NAC and fenitrothion showed a significant increase in serum GSH and SOD with a significant decrease in serum MDA in comparison to fenitrothion intoxicated group (Table 3).

### 3.5. Acetyl cholinesterase, neurotransmitters and 8-hydroxy 2-deoxyguanosine (8OH2dG) in the cerebral tissue

Table 4 showed non-significant changes in AchE level in NAC treated rats; but it showed a significant decrease in its activity in the fenitrothion treated rats compared to the control group. Meanwhile, the NAC and fenitrothion combined group revealed a significant increase in AchE activity and a significant decrease in 8oH-2dG level in the brain tissue when compared to fenitrothion intoxicated rats. Moreover, rats treated with NAC exhibited non-significant changes in GABA, serotonin, dopamine, and 8oH-2dG levels in the brain tissue, while rats treated with fenitrothion showed a significant increase in these previous neurotransmitters and 8oH-2dG compared to the control group. Furthermore, our results confirmed that there was a significant decrease in GABA, serotonin, dopamine, and 8oH-2dG levels in the co-treated group when compared to the fenitrothion intoxicated rats.

### 3.6. Effects on Bax, p-53, and Bcl-2 mRNA expression

The transcriptional levels of Bax, p-53 and Bcl-2 mRNA expression in brain tissue of NAC-treated group revealed non-significant changes in the expression levels despite the decrease in Bax/Bcl2 ratio. Fenitrothion intoxicated group revealed significantly up-regulated expression levels in Bax and P-53, significantly down-regulated Bcl-2 mRNA expression and increased Bax/Bcl2 ratio compared to the control group. However, in the NAC and fenitrothion co-treated group, Bax and P-53 mRNA expression levels were down-regulated, the expression level of Bcl-2 mRNA was up-regulated and the Bax/Bcl2 ratio was also decreased compared to the fenitrothion intoxicated group (Fig. 1).

### 3.7. Histopathological and Immunohistochemical findings

The cerebral cortex of both the control and NAC-treated groups showed normal cerebral architecture. Normal histological structures of the hippocampus including intact granular layer, molecular layer, and purkinje cells were shown. On the other hand, in the brain sections of FNT-treated group, focal gliosis with shrunken neuron surrounded by mild sponigiosis as well as focal poliomalacia replaced by hemorrhages with numerous pyknotic neurons and glial clustering were seen in the cerebral tissue. Moreover, numerous shrunken hyperchromatic neurons and neurophagia were detected (Fig. 2). Marked apoptotic and necrotic changes in CA-1 region of hippocampus represented by shrunken neurons with dense pyknotic nuclei, scanty cytoplasm and marked spongiosis were noticed (Fig. 3). This corresponds to higher intensities in GFAP immunoreactivity in both the cerebral cortex and hippocampus of the fenitrothion exposed group compared to the control or NAC groups (Figs. 2 & 3). The cerebral cortex regions of the NAC + fenitrothion combination group restored a normal histological structure although mild degeneration of purkinje cells or focal apoptotic neurons in the dentate gyrus regions and mild edema of the hippocampal structures were shown (Figs. 2 & 3). A reduction in GFAP staining intensities of both regions was also evident when compared to the fenitrothion treated group (Figs. 2 & 3). The spleen in the control and NAC-treated rats showed a normal histological structure of both the white and red pulps (Fig. 4). In contrast, exposure to fenitrothion induced moderate to

**Table 4**
Effects of NAC oral administration (200 mg/kg BW) and fenitrothion (20 mg/kg BW) and their combination of male rat brain AchE, GABA, Serotonin, Dopamine, and 8oH2dG concentration. Data are expressed as mean ± SE, (n = 8–10).

| Groups<br>Parameters | Control | NAC | Fenitrothion | NAC+ fenitrothion |
|---|---|---|---|---|
| AchE (pg/mg) | 0.23 ± 0.008$^{a}$ | 0.20 ± 0.01$^{a}$ | 0.13 ± 0.005$^{b}$ | 0.21 ± 0.007$^{a}$ |
| GABA (ng/mg) | 0.164 ± 0.01$^{b}$ | 0.150 ± 0.01$^{b}$ | 0.270 ± 0.007$^{a}$ | 0.19 ± 0.005$^{b}$ |
| Serotonin (ng/mg) | 0.18 ± 0.005$^{b}$ | 0.16 ± 0.007$^{b}$ | 0.24 ± 0.006$^{a}$ | 0.185 ± 0.006$^{b}$ |
| Dopa amine (ng/mg) | 0.15 ± 0.006$^{b}$ | 0.15 ± 0.01$^{b}$ | 0.27 ± 0.004$^{a}$ | 0.17 ± 0.009$^{b}$ |
| 8oH2dG (ng/mg) | 0.17 ± 0.009$^{b}$ | 0.15 ± 0.01$^{b}$ | 0.24 ± 0.005$^{a}$ | 0.18 ± 0.008$^{b}$ |

Means within the same column carrying different superscripts letters are significantly different (P ≤ 0.05).

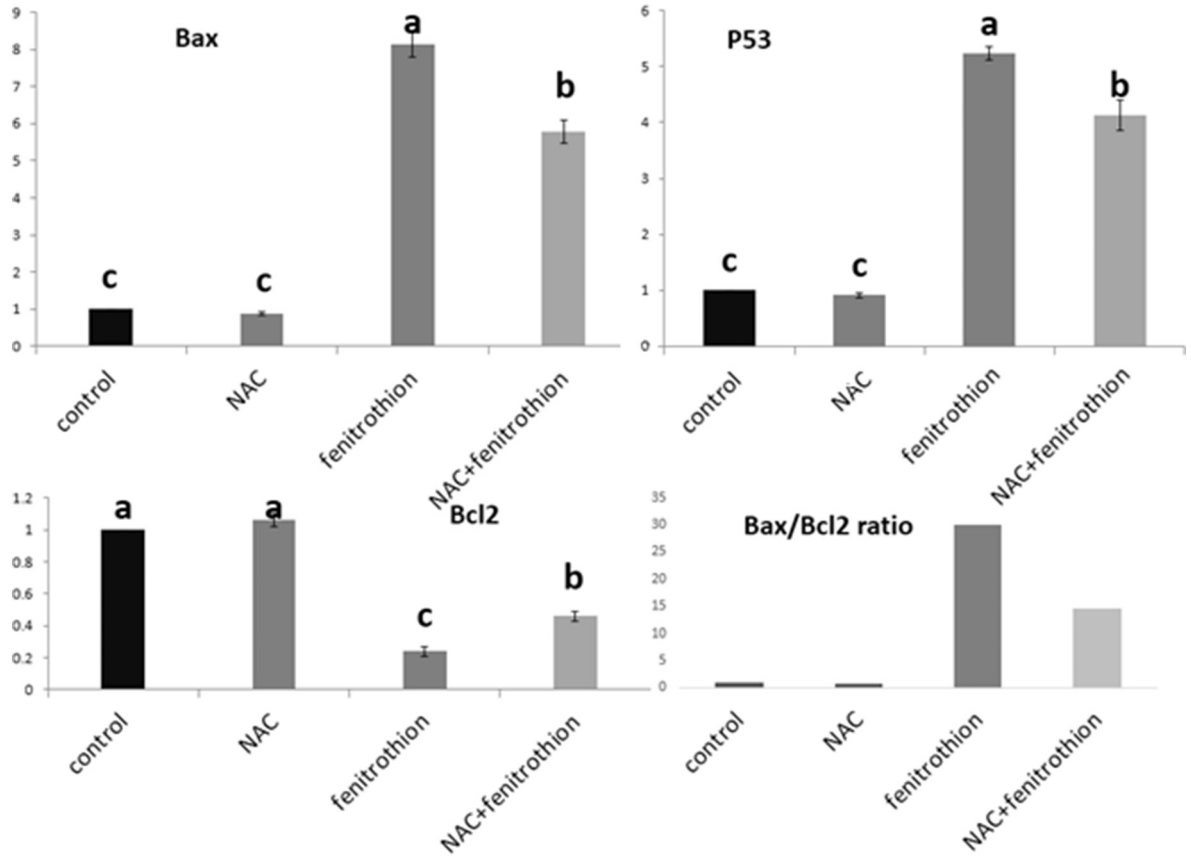


**Fig. 1.** Effects of NAC oral administration (200 mg/kg BW) and fenitrothion (20 mg/kg BW) and their combination on male rat brain Bax, p53, Bcl2 mRNA expression patterns and Bax/Bcl2 ratio. Data are expressed as mean ± SE.

severe white pulp depletion that was characterized by necrotic and apoptotic bodies. This was reflected as an elevation in the intensity of Caspase 3 immunoreactivity in both white and red pulps when compared to either the control or NAC groups (Fig. 4). NAC and fenitrothion co-treated group restored normal white and red pulps with mild germinal center formation (Fig. 4). Moreover, reduction in the

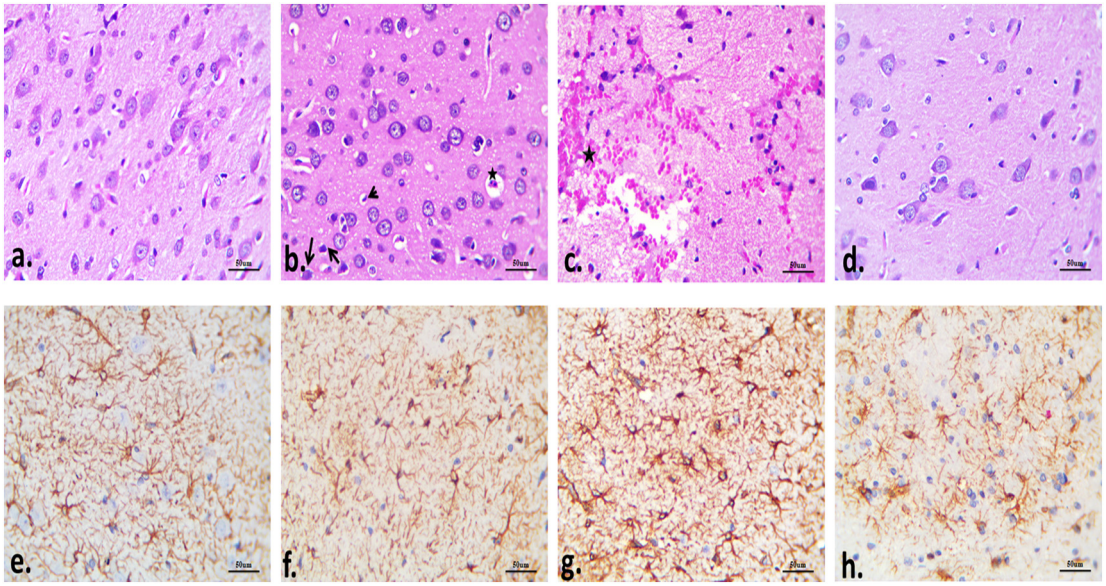


**Fig. 2.** Brain sections from (a) normal control rats, (b) NAC administered group showing normal cerebral architecture, (c) fenitrothion-treated rats, exhibits focal gliosis with shrunken neuron surrounded by mild spongiosis as well as focal poliomalacia replaced by hemorrhages with numerous pyknotic neurons and glial clustering, (d) rats administered combination of NAC and fenitrothion restored a normal histological structure although mild degeneration of purkinje cells or focal apoptotic neurons in the dentate gyrus regions. Hematoxylin and eosin stain; scale bare 50 μm. GFAP expression in the brain, (e & f) control and NAC treated groups revealed the normal limit immunopositive stainable of GFAP in cerebral cortex, (g) fenitrothion treated rats showed higher intensities in GFAP immunoreactivity in the cerebral cortex. A reduction in GFAP staining intensities of the cerebral region in the co-treated group (h). Bar = 50 μm.

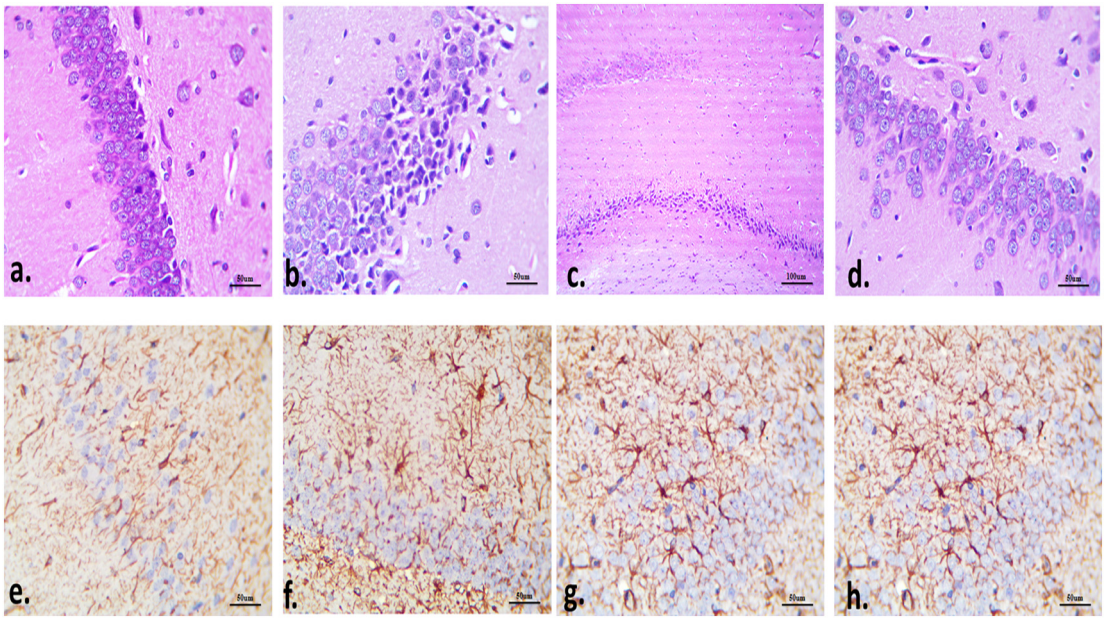


**Fig. 3.** Brain sections from (a) normal control rats, (b) NAC administered group showing normal histological structures of the hippocampus including intact granular layer, molecular layer, and purkinje cells, (c) fenitrothion-treated rats, exhibits marked apoptotic and necrotic changes in CA-1 region of hippocampus represented by shrunken neurons with dense pyknotic nuclei, scanty cytoplasm and marked spongiosis, while the co-treated group restored a normal histological structure with mild edema of the hippocampal structures (d). Hematoxylin and eosin stain. GFAP expression in the hippocampus, (e & f) control and NAC treated groups revealed the normal limit immunopositive stainable of GFAP in hippocampus region, (g) fenitrothion treated rats showed higher intensities in GFAP immunoreactivity in the hippocampus, and (h) co-treated group showed reduction in GFAP staining intensities of the hippocampus. Bar = 50 μm.

intensity of Caspase 3 immunoreactivity was clear in such group in comparison to the FNT-exposed group (Fig. 4).

## 4. Discussion

Our present study extends the previous research data on hematologic, immunotoxic, neurotoxic and apoptotic effects of fenitrothion oral administration in addition to expanding the potential effects of oral NAC treatment. The high mortality rate in fenitrothion intoxicated rats (50%) throughout the experimental period that may be due to respiratory failure [34]. In this study, the results of marked reduction in the erythrocyte count, elevation in MCV, and normal MCHC

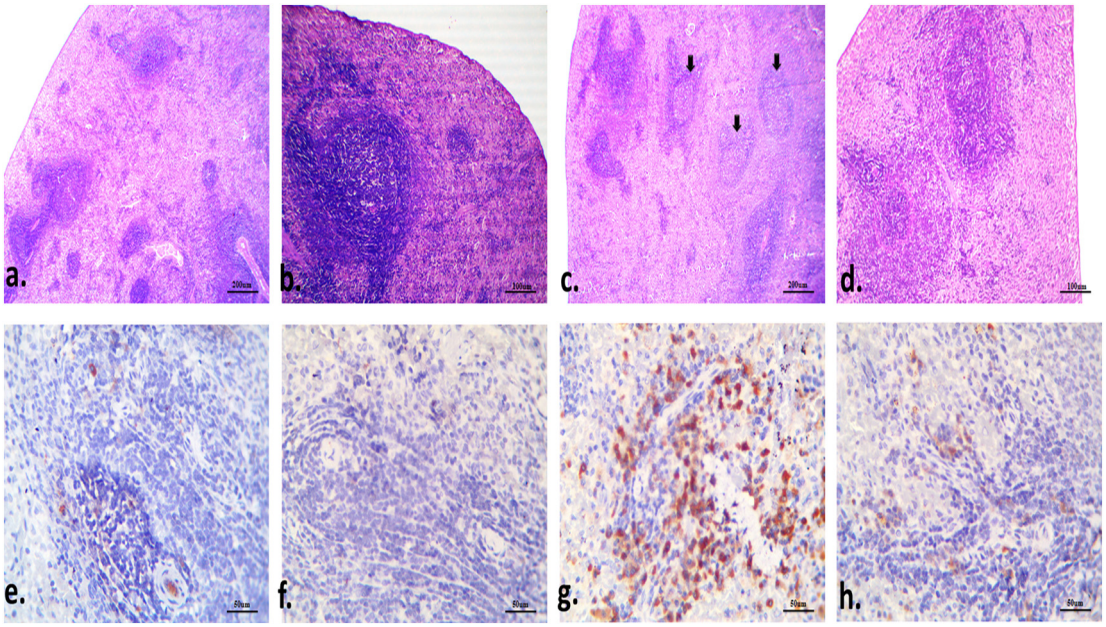


**Fig. 4.** Spleen sections from (a) normal control rats, (b) NAC administered group showing normal both the white and red pulps, (c) fenitrothion-treated rats, exhibits moderate to severe white pulp depletion that was characterized by necrotic and apoptotic bodies, (d) rats administered combination of NAC and fenitrothion revealed restored normal white and red pulps of with the mild germinal center formation. Hematoxylin and eosin stain. Caspase-3 expression in spleen, (e & f) control and NAC treated groups revealed very weak immunopositive of Caspase 3, (g) fenitrothion treated group, the splenic sections declared an elevation on the intensity of Caspase 3 immunoreactivity in both white and red pulps, and (h) co-treated group, showed reduction in the intensity of Caspase 3 immunoreactivity. Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

percentage in the fenitrothion intoxicated rats, exhibiting megaloblastic anemia, which may be due to the toxic effect of fenitrothion and/or its metabolites on the mitotic division of erythrocytes or metabolism of Vit B12 and/or folic acid in the body. These results are in accordance with Afshar et al. [35] who reported a reduction in the RBCs count in rats intoxicated with fenitrothion. Additionally, marked leukocytosis, lymphocytosis, and neutrophilia were observed in fenitrothion administrated group that clear the good defense mechanism of the body against chemical toxicity and inflammation [36], and the activation of the immune system and defense mechanism due to toxicity, are positive response for survival [37]. Our results are in harmony with other reports as Hundekari et al. [34]. Moreover, NAC daily administration in the co-treated group markedly improved the hematological parameters, elevated the erythrocyte, and lowered leukocyte counts, that attributed to the anti-inflammatory effect of NAC [38], which in turn, reduces the stimulation of bone marrow to release more leukocytes resulted from fenitrothion toxicity. Furthermore, fenitrothion intoxication induced marked elevation in the serum LDH activity due to pesticide stress that cause inflammation of the tissue [39,40] and reduction in the circulating immunoglobulin levels (IgG and IgM); this may be due to immunosuppressive effects of fenitrothion [41], which had a potent systemic cholinergic effect which induced corticosterone secretion, which decrease the production of antibodies, and produce atrophy of lymphoid organ [8,9]. These results were largely documented by histopathological alterations in spleen tissue due to fenitrothion toxicity, including moderate to severe white pulp depletion that was characterized by necrotic and apoptotic bodies. This was reflected as an elevation in the intensity of Caspase 3 immunoreactivity in both white and red pulps, which indicate apoptosis of the cells [42] resulted from fenitrothion toxicity. On the other hand, the reduction in the level of serum LDH and elevation of immunoglobulins concentrations in the co-treated group due to the anti-inflammatory and immunostimulant properties of NAC. It stimulated and strengthened the immune system [43] and improved T lymphocyte function [44].

Oxidative stress is produced when pro-oxidant is not balanced with antioxidants, resulting in damage of cells. Lipid peroxidation products as MDA and endogenous oxygen free radical scavengers such as reduced glutathione and SOD are effective markers to evaluate oxidative stress caused by fenitrothion toxicity. This study showed a severe reduction in serum GSH and SOD demonstrating the depletion of antioxidant enzymes of the body; in addition, a marked elevation in free radical generation (MDA) levels was shown because of the tissues poisoned by fenitrothion pesticide [34]. *N*-acetylcysteine is a free-radical scavenger which protects cells against the toxicity of chemicals through decreasing the oxidative stress caused by fenitrothion. In this study, the decrease of oxidative stress was indicated by improving the antioxidant enzymes (GSH & SOD) and reducing the lipid peroxidation formation (MDA) in the co-treated group. The protective effect of *N*-acetylcysteine against oxidative stress attributed to its ability to increase the synthesis of glutathione [44,45], reduce the metabolites resulted from metabolism of fenitrothion, and increase the secretion of it outside the body. Also, *N*-acetylcysteine is a precursor of GSH as it is a thiol compound, so it increases the reduced glutathione concentration that detoxifies fenitrothion toxicity and reduces MDA formation [46,47]. Our results agree with Kaya et al. [48], Sathish et al. [49], Uraz et al. [38] and Vahdati-Mashhadian et al. [50].

We also observed the marked elevation in inflammatory cytokines (TNF-alpha and lL-2) may be resulted from increasing the production of T cell-derived cytokines to initiate the inflammation in the tissue due to fenitrothion toxicity. Acute toxicity with organophosphorus pesticide is usually associated with inflammatory conditions which are accompanied by the increased expression of inflammatory cytokines as TNF-α [51,52]. Administration of NAC in the co-treated group significantly reduces the release of inflammatory markers (TNF-alpha and lL-2) due to the ability of NAC as an anti-inflammatory agent to prevent the secretion of pro-inflammatory cytokines in addition to reducing their expression and also reducing the activation of NF-kB induction [53]. Moreover, NAC effectively lowers the level of TNF-a [54] and its thiol content inhibits the synthesis of pro-inflammation mediators [44].

AChE is an enzyme that degrades the neurotransmitter acetylcholine (ACh) into choline and acetic acid. Oral toxicity of fenitrothion produced a marked reduction in AChE activity as it is capable of penetrating the brain tissue and inhibiting brain acetyl cholinesterase activity [9,55,56]. In addition, it inactivates AChE by phosphorylating the serine hydroxyl group located at the active site of AChE. The phosphorylation occurs with loss of an organophosphate leaving group and establishment of a covalent bond with AChE [57]. The primary mechanism of action of organophosphate pesticides is inhibition of carboxyl ester hydrolases, particularly acetyl cholinesterase. Our results are linear with Trottier et al. [58] and Hundekari et al. [34]. Moreover, fenitrothion induced neurotoxicity caused a marked elevation in brain GABA, serotonin (5-HT) content, and dopamine with induction of oxidative DNA damage represented by elevation of brain 8OH2dG concentration. Ahmed et al. [59] have previously reported similar changes in brain neurotransmitters after OPs exposure. Additionally, pesticide exposure has been reported to cause alterations in the neurotransmitter system [60,61]. This might be due to modulation of peripheral and central benzodiazepine receptors [62], and disturbances in the synthesis and degradation of amino acids by the liver [63]. Additionally, marked histopathological changes in the brain tissue were seen following fenitrothion toxicity, including focal gliosis with shrunken neuron surrounded by mild spongiosis as well as focal poliomalacia replaced by hemorrhages with numerous pyknotic neurons and glial clustering in the cerebral tissue. Moreover, marked apoptotic and necrotic changes in CA-1 region of hippocampus represented by shrunken neurons with dense pyknotic nuclei, scanty cytoplasm and marked spongiosis were observed. Immunohistochemically higher intensities of GFAP immunoreactivity were exhibited in both the cerebral cortex and hippocampus, suggesting that there was an inflammatory response that caused neuronal injury and astrogliosis induced by organophosphorus toxicity [64–66] and indicating that fenitrothion may have elevated the neuronal damage and induced changes in the cytoskeleton that exposed the GFAP antigenic sites [67]. Furthermore, Bcl-2 members regulate apoptosis through controlling the membrane potential of mitochondria and preventing the cytochrome-c release from mitochondria into the cytoplasm, leading to the increase in the longevity of the cell which, consequently increases the possibility of precipitation for malignity [68,69]. In addition, p53 gene arrests the cell cycle at the late G1 phase in DNA damage and spares time for DNA repair. Meanwhile, if DNA is repaired p53 diminishes and then the cycle will be completed. If the repair attempt is unsuccessful, p53 leads the cell to apoptosis [70,71]. Therefore, if p53 gene is damaged, the risk of tumorigenesis will increase due to the increased amount of DNA damage [70]. In the present work, the effect of fenitrothion toxicity on brain cell apoptosis has been shown, the results showed a marked up-regulation in the mRNA expression of Bax and p53, genes promoting cell apoptosis. Also, there was down-regulation in the mRNA expression of bcl2, which regulates and reduce the death cycle. Therefore, the role of fenitrothion intoxication in brain cell death was demonstrated through the increased amount of DNA damage. Meanwhile, NAC supplementation was able to reduce the brain neurotransmitters (GABA, serotonin, and dopamine) in the combination group. Moreover, it has been hypothesized to induce modulation of glutamate and dopamine neurotransmission, due to its antioxidant activity [72]. Also, it able to reduce the expression of many important neuro-inflammatory markers including TNF-alpha, matrix metalloproteinase, interleukin 1beta, and nitric oxide synthase [73,74] leading to the reduction of the inflammation in the brain tissue.

In conclusion, these results of this study have demonstrated that intoxication with fenitrothion induced hematological changes, immunotoxic properties and oxidative stress in the brain and spleen tissues that could be ameliorated by NAC co-treatment.

## Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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