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Research Article

Effect of *N*-acetylcysteine on time- dependent biochemical and oxidative changes after acute diisopropyl phosphorofluoridate poisoning in mice

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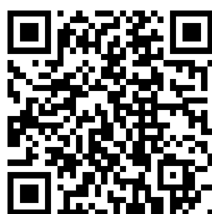

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Abstract

Objective: The aim of the study was to investigate the effect of an antioxidant, viz., *N*-acetylcysteine (NAC) against various biochemical and oxidative changes caused by acute diisopropyl phosphorofluoridate (DFP) poisoning in mice.
Materials and Methods: Effect of NAC (150 mg/kg) alone or in conjunction with atropine (10 mg/kg; 0 min), and/or 2-PAM (30 mg/kg; 0 min) on (i) LD_{50} of DFP, (ii) time for onset of signs and symptoms, and death after lethal doses of DFP, and (iii) time- dependent biochemical and oxidative damage after 0.50 LD_{50} DFP was investigated in Swiss albino mice.
Results: NAC (-60 min) did not confer any additional protection against DFP when measured by protection index (ratio of LD_{50} of DFP in protected and unprotected animals). However, NAC (-60 min, 0 min or +5 min) with atropine/ and or 2-PAM significantly delayed the onset of signs and symptoms, and time of death after 2.0 LD_{50} DFP. Further, NAC (-60 min) augmented the efficacy of atropine and 2-PAM in normalizing the butyrylcholinesterase levels in plasma, and acetylcholinesterase, reduced glutathione, malondialdehyde, glutathione peroxidase, catalase, and superoxide dismutase levels in brain.
Conclusion: The study indicates beneficial role of NAC in mitigating the toxicity of OP.
Keywords: Organophosphate, DFP, acute toxicity, Oxidative damage, N-acetylcysteine protection.

1. Introduction

Organophosphate (OP) compounds are extensively used in industries, agriculture, and public health. They are also employed as nerve agents (sarin, cyclosarin, soman, tabun and VX) in chemical warfare. OP compounds are responsible for widespread poisoning around the world, particularly in developing countries.[1,2] OP compounds act by irreversible inhibition of acetylcholinesterase (AChE) leading to accumulation of acetylcholine, a neurotransmitter at neural synapses and neuromuscular junctions. This leads to a wide variety of hypercholinergic effects that ultimately result in muscular dysfunction and nerve damage. Three types of effects like muscarinic, nicotinic, and central signs and symptoms are usually observed after OP poisoning.[3,4] There are evidences that OP toxicity is also mediated though generation of reactive oxygen species (ROS) leading to oxidative stress. Most of the studies relate to oxidative stress following OP pesticide exposure.[5-8] However, there is evidence on nerve agent-induced oxidative stress as well.[9]

Current management of OP poisoning includes administration of atropine, a muscarinic antagonist and pralidoxime (2-PAM), a cholinesterase reactivator (oxime).[10,11] However, the morbidity and mortality caused by OP poisoning remains high despite the use of specific antidotes.[8] Various reports indicate that OP-induced oxidative stress could be ameliorated by various antioxidants, usually in conjunction with the specific antidotes. Antioxidants are considered potentially safe antidotes for OP poisoning.[12] Since central nervous

system (CNS) is considered to be very vulnerable to oxidative damage, such molecules have been successfully used in alleviating several neuropathological conditions as well.[13] Therefore, antioxidants with minimal side effects are favored as pretreatment against a potential OP poisoning, and might be used in combination with the standard treatments. Out of several antioxidants tested against OP poisoning, low-molecular-weight thiols in particular have drawn special attention. One such compound is *N*-acetylcysteine (NAC), which was proposed as a novel treatment for acute OP poisoning.[14,15] NAC is a thiol compound with potent antioxidant and anti-inflammatory properties. Its antioxidant property is attributed to its ability to stimulate GSH synthesis and scavenge ROS.[14] Thereby, it minimizes OP-induced oxidative stress and enhances OP detoxification as well.[14,16] The aim of the present study was to investigate the protective efficacy of NAC alone or in adjunction with atropine and/ or 2-PAM against time- dependent biochemical and oxidative changes caused by acute diisopropyl phosphorofluoridate (DFP) exposure in mice. DFP is a structural analogue of Sarin and is more toxic compared to OP pesticides. The pathophysiological effects of DFP are very similar to nerve agents.[17]

2. Materials and methods

2.1 Chemicals and reagents

1) Ethylenediaminetetraacetic acid (EDTA), 2-PAM, atropine sulphate, sodium dodecyl sulphate (SDS), 2-thiobarbituric acid (TBA), o-phthaldialdehyde (OPT), Triton X-100, dimethyl sulfoxide (DMSO), metaphosphoric acid (MPA), acetylthiocholine iodide, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Trizma base and all other chemicals, reagents and buffers of highest purity- Sigma-Aldrich Inc. (St. Louis, USA).

2) Calbiochem make glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) diagnostic kits and DFP (> 99% purity)- Merck India Ltd. (Mumbai, India).

3) Bicinchoninic acid (BCA) protein assay kit- Pierce (Rockford, IL, USA).

2.2 Animals

Male Swiss albino mice (25-30 g) were procured from the animal facility of Defence Research and Development Establishment (DRDE), Gwalior. The animals were housed in polypropylene cages on dust free rice husk with free access to food (Ashirwad Brand, Chandigarh, India) and water *ad libitum*. Animals were maintained in controlled environmental conditions of ambient temperature (22 ± 2°C) and relative humidity of 40-60% in a 12:12 light: dark cycle.

The care and maintenance of the animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India, New Delhi. Animal experiments were carried out with the approval of Institutional Animal Ethical Committee of DRDE.

2.3 Solution preparation and treatments

DFP solution was freshly prepared in normal saline (0.9% sodium chloride solution) and propylene glycol in a ratio of 9:1 (v/v), respectively. Control animals received equivalent amount of normal saline-propylene glycol solvent. Atropine, 2-PAM and NAC were prepared fresh in normal saline. Volume of all the injections was kept between 0.10 to 0.20 ml. The dose, route and treatment time of atropine, 2-PAM and NAC were based on previous studies.[14,18] Route of administration of DFP was also based on previous study.[18] Sequence of treatments was: DFP- Atropine- 2-PAM.

2.4 Details of Groups

2.4.1 Experiment 1 on determination of Protection Index (PI)

Groups	No of Mice	Treatments
1.	12	DFP [subcutaneous (s.c.)]
2.	12	DFP + Atropine [10 mg/kg; intraperitoneal (i.p.); 0 min]
3.	12	DFP + 2-PAM [30 mg/kg; intramuscular (i.m.); 0 min]
4.	12	DFP + NAC (150 mg/kg; i.p; -60 min)
5.	12	DFP + Atropine + NAC
6.	12	DFP+ 2-PAM + NAC
7.	12	DFP + Atropine + 2-PAM
8.	12	DFP + Atropine + 2-PAM + NAC

LD_{50} (median lethal dose) of DFP was determined in mice in the presence or absence of various combinations of atropine, 2-PAM and NAC by the method of Gad and Weil.[19] The PI was calculated as the ratio of LD_{50} of DFP in protected and unprotected animals.[18]

2.4.2 Experiment 2 on determination of time of onset of sign and symptoms, and death

Groups	No of Mice	Treatments
1.	6	2.0 LD_{50} DFP (s.c.)
2.	6	2.0 LD_{50} DFP + Atropine (10 mg/kg; i.p.; 0 min)
3.	6	2.0 LD_{50} DFP + 2-PAM (30 mg/kg; i.m.; 0 min)
4.	6	2.0 LD_{50} DFP + NAC (150 mg/kg; i.p; -60 min)
5.	6	2.0 LD_{50} DFP + NAC (0 min)
6.	6	2.0 LD_{50} DFP + NAC (+5 min)
7.	6	2.0 LD_{50} DFP + Atropine + NAC (-60 min)
8.	6	2.0 LD_{50} DFP + 2-PAM + NAC (-60 min)
9.	6	2.0 LD_{50} DFP + Atropine + 2-PAM
10.	6	2.0 LD_{50} DFP + Atropine + 2-PAM + NAC (-60 min)

After various treatments, time for onset of signs and symptoms, and death were recorded.

2.4.3 Experiment 3 on measurement of biochemical and oxidative stress markers

Groups	No of Mice	Treatments
1.	6	Control
2.	6	0.50 LD_{50} DFP (s.c.)
3.	6	0.50 LD_{50} DFP + Atropine (10 mg/kg; i.p.; 0 min)
4.	6	0.50 LD_{50} DFP + 2-PAM (30 mg/kg; i.m.; 0 min)
5.	6	0.50 LD_{50} DFP + NAC (150 mg/kg; i.p; -60 min)
6.	6	0.50 LD_{50} DFP + Atropine + NAC
7.	6	0.50 LD_{50} DFP+ 2-PAM + NAC
8.	6	0.50 LD_{50} DFP + Atropine + 2-PAM
9.	6	0.50 LD_{50} DFP + Atropine + 2-PAM + NAC

Identical studies with above groups were performed for 1 h, 24 h and 7 d post exposures. At specified time points, animals were anesthetized and blood was collected though retro-orbital plexus using heparinized capillary. Blood was used for measuring butyrylcholinesterase (BChE) activity in plasma. Thereafter, animals were killed by cervical dislocation and the brain was excised quickly, rinsed in 0.9% saline, blotted and weighed for the remaining assays. Various biochemical and oxidative stress markers were measured as follows:

2.4.3.1 BChE and AChE Assay [20]

AChE was measured in 10% brain homogenate prepared in enzymatic buffer. Thereafter, 2.840 ml phosphate buffer (pH 8.0), 10 µl of sample and 100 µl DTNB were mixed. Reaction was started by adding 50 µl of 0.075 M acetylthiocholine iodide and change in absorbance per minute was recorded for four minute duration at 410 nm using a micro plate reader (BioTek, USA). Plasma BChE was measured in 10 µl enzyme source. The plasma BChE and brain AChE activities were expressed as µmol acetylthiocholine hydrolyzed/ min/ ml blood and µmol acetylthiocholine hydrolyzed/ min/ g wet tissue, respectively.

2.4.3.2 GSH Assay [21]

Briefly, 1.5% homogenate was prepared in 1.92 ml phosphate EDTA buffer (pH 8.0), to this 0.5 ml MPA was added and centrifuged at 6000 rpm for 15 min. To 0.25 ml of the supernatant 2.25 ml of phosphate-EDTA buffer was added, from which 0.1 ml solution was taken and 1.8 ml of phosphate-EDTA buffer and 0.1 ml of OPT were added. The cocktail was incubated for 15 min (in dark) at room temperature. Fluorescence was measured at excitation 350 nm and emission 420 nm and values were expressed as µmol/g wet tissue.

2.4.3.3 Malondialdehyde (MDA) Assay [22]

A 10% tissue homogenate (in 1.15% KCl) was prepared and centrifuged for 10 min at 6000 rpm. To 0.1 ml of above supernatant, 0.2 ml of 8.1% SDS was added, followed by the addition of 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA, this was followed by boiling at 95°C for 1 h, cooled and centrifuged at 6000 rpm for 15 min. The supernatant was separated and its absorbance measured spectrophotometrically at 532 nm, and the values expressed as n moles MDA/ g wet tissue.

2.4.3.4 Antioxidant Enzymes Assay

GPx (µmol of NADPH oxidized/min/mg protein), GR (µmol of NADPH oxidized/min/mg protein), CAT (µmol of H_2O_2 degraded/min/mg protein) and SOD (Units/mg protein) were measured in tissue homogenate using commercial diagnostic kits. The protein content in the extractions was determined by BCA protein assay kit.

2.5 Data Analysis

The results are expressed as mean ± SEM (n=6). The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's test. Statistical significance was drawn at $*p<0.05$ and $**p<0.01$ levels using Sigma Stat software (Jandel Scientific Inc., CA, USA).

3. Results

3.1 Protection Index

Table 1 shows the effect of NAC in the presence or absence of atropine and/ or 2-PAM on LD_{50} of DFP in mice. The LD_{50} (s.c.) of DFP was found to be 3.86 (2.48-6.12) mg/kg, which was raised to 8.55 (5.20-14.29) by 2-PAM, resulting in a PI of 2.26 (1.37-3.81). However, atropine and NAC alone offered only marginal protection, whereas atropine and 2-PAM together produced maximum PI of 7.52 (4.54-13.64). Addition of NAC to atropine or 2-PAM alone and atropine + 2-PAM did not yield any additional protection.

3.2 Time of Onset of Sign and Symptoms, and Death

Table 2 summarizes the effect of NAC in the presence or absence of atropine and/ or 2-PAM on time of onset of sign and symptoms and time of death after acute DFP poisoning in mice. 2.0 LD_{50} DFP produced 100% mortality, and the time for onset of sign and symptoms and time of death were 3.23 ± 0.45 min and 12.3 ± 0.92 min, respectively. Atropine and 2-PAM alone reduced the mortality to 83% and 67%, respectively. In atropine alone the time for onset of sign and symptoms and time of death significantly extended to 8.83 ± 0.48 min and 23.3 ± 1.23 min, respectively. 2-PAM also extended the time for onset of sign and symptoms but not the time of death.

Pre-treatment (-60 min), simultaneous treatment (0 min) and post-treatment (+5 min) of NAC alone did not exhibit any beneficial effects on any of the parameters. Now, for subsequent study, only pre-treatment of NAC was selected as in other studies. Adjunction of NAC with atropine or 2-PAM did not improve survival but marginally extended both time for onset of sign and symptoms and time of death. Atropine and 2-PAM together afforded 100% protection but the time for onset of sign and symptoms remained 10.8 ± 0.91 min. NAC treatment did not yield any additional protection.

3.3 Biochemical and Oxidative Stress Markers

Plasma BChE levels at different time intervals after various treatments are shown in Fig. 1. 0.50 LD_{50} DFP caused 97% inhibition of BChE at 1 h, which restored to 63% 24 h post exposure. NAC alone or with atropine and/ or 2-PAM did not improve the BChE activity. However, after 24 h a significant improvement in BChE activity was observed when NAC was given together with atropine and 2-PAM. Enzyme activity in all the treatment normalized by 7 d. Brain AChE activity at different time intervals after various treatments are shown in Fig. 2. 0.50 LD_{50} DFP caused 78% inhibition of AChE at 1 h, which improved to 65% and 29% by 24 h and 1 h post exposure, respectively. NAC alone or with atropine and/ or 2-PAM did not improve the enzyme activity. However, after 24 h marginal improvement in enzyme activity was noted after atropine + 2-PAM + NAC administration. Enzyme activity in after 2-PAM, 2-PAM + NAC, atropine + 2-PAM and atropine + 2-PAM + NAC treatments significantly improved after 7 d of treatment.

GSH content in brain homogenate after various treatments is depicted in Fig. 3. DFP significantly depleted the GSH levels 1 h and 24 h post exposure. Addition of NAC with atropine, 2-PAM and atropine + 2-PAM significantly restored the GSH levels at both the time intervals. Atropine and 2-PAM together also improved GSH levels after 24 h. GSH levels in all the treatments normalized after 7 d. Lipid peroxidation measured by brain MDA levels is illustrated in Fig. 4. Significantly elevated MDA levels were observed at 1 h and 24 h post DFP exposure, which favorably responded to all the treatments except atropine alone after 1 h. No change in MDA levels was observed in any of the treatments after 7 d.

GPx activity was not found to be affected by any of the treatments after 1 h and 7 d (Fig. 5). However, after 24 h the enzyme levels were significantly depleted by DFP, which was significantly restored by adjunction of NAC with atropine and 2-PAM. Also, NAC alone or with atropine or 2-PAM provided marginal protection compared to corresponding control. None of the treatments exerted any significant deleterious effect on GR levels at any time point (Fig. 6). CAT levels were significantly decreased by DFP 24 h post exposure. NAC with 2-PAM or with atropine + 2-PAM ameliorated the enzyme levels compared to corresponding control. 2-PAM, NAC, atropine + 2-PAM and atropine + NAC also provide marginal protection. However, the enzyme levels were not comparable to control (Fig. 7). Further, DFP significantly decreased the brain SOD levels 1 h post exposure, which was restored by atropine + 2-PAM or atropine + 2-PAM + NAC (Fig. 8). Marginal protection by atropine, NAC or atropine + NAC was also observed but the enzyme activity remained depleted compared to corresponding control. The enzyme levels were found to be unaffected by DFP 24 h and 7 d post exposure.

Table 1: Effect of NAC in the presence or absence of atropine and/ or 2-PAM on acute LD_{50} of DFP in mice

Sl. No.	Treatments	LD_{50} (mg/kg) (95% fiducial limits)	PI (95% fiducial limits)
1.	DFP	3.86 (2.48-6.12)	-
2.	DFP + Atropine	6.64 (4.89-9.74)	1.71 (1.26-2.52)
3.	DFP + 2-PAM	8.55 (5.20-14.29)	2.26 (1.37-3.81)
4.	DFP + NAC	4.49 (2.11-9.54)	1.16 (0.55-2.47)
5.	DFP + Atropine + NAC	7.13 (3.35-15.15)	1.85 (0.87-3.92)
6.	DFP + 2-PAM + NAC	5.66 (1.42-22.59)	1.47 (0.37-5.85)
7.	DFP + Atropine + 2-PAM	28.49 (16.93-52.13)	7.52 (4.54-13.64)
8.	DFP + Atropine + 2-PAM + NAC	28.28 (28.28-28.28)	7.32 (7.32-7.32)

Animals received various doses of DFP (s.c.) in the presence or absence of atropine (10 mg/kg; i.p.) and/ or 2-PAM (30 mg/kg; i.m.) and/or NAC (150 mg/kg; i.p.; -60 min). LD_{50} of DFP was determined by the method of Gad and Weil.[19] Protection Index (PI) was calculated as the ratio of LD_{50} of DFP in protected and unprotected animals.[18]

Table 2: Effect of NAC in the presence or absence of atropine and/ or 2-PAM on time of onset of sign and symptoms and time of death after acute DFP poisoning in mice

Sl. No.	Treatments	Mortality/ Exposed (% mortality)	Time for onset of sign and symptoms (min)	Time of death (min)
1.	2.0 LD_{50} DFP	6/6 (100)	3.23 ± 0.45	12.3 ± 0.92
2.	2.0 LD_{50} DFP + Atropine	5/6 (83.30)	8.83 ± 0.48**	23.3 ± 1.23**
3.	2.0 LD_{50} DFP + 2-PAM	4/6 (66.7)	7.50 ± 0.43**	15.8 ± 0.95
4.	2.0 LD_{50} DFP + NAC (-60 min)	6/6 (100)	3.10 ± 0.37	14.2 ± 0.40
5.	2 .0 LD_{50} DFP + NAC (-0 min)	6/6 (100)	3.17 ± 0.31	13.8 ± 0.95
6.	2.0 LD_{50} DFP + NAC (+5 min)	6/6 (100)	2.67 ± 0.42	12.8 ± 0.48
7.	2.0 LD_{50} DFP + Atropine + NAC + (-60 min)	5/6 (83.3)	9.10 ± 0.26**#	29.2 ± 2.2**
8.	2.0 LD_{50} DFP + 2-PAM + NAC (-60 min)	4/6 (66.67)	10.3 ± 1.0**@	27.0 ± 0.41**
9.	2 .0 LD_{50} DFP + Atropine + 2-PAM	0/6 (0.0)	10.8 ± 0.91**	>24 h**
10.	2.0 LD_{50} DFP + Atropine + 2-PAM + NAC (-60 min)	0/6 (0.0)	11.0 ± 1.2**	>24 h**

Animals received various doses of DFP (s.c.) in the presence or absence of atropine (10 mg/kg; i.p.; 0 min) and/ or 2-PAM (30 mg/kg; i.m.; 0 min) and/or N-acetylcysteine (NAC; 150 mg/kg; i.p.; -60 min, 0 min or +5 min). The time for onset of sign and symptoms and mortality was recorded. Values are mean ± SE (n = 6). **Significantly different from 2.0 LD_{50} DFP, and @significantly different from DFP + Atropine and DFP + 2-PAM at p<0.01.

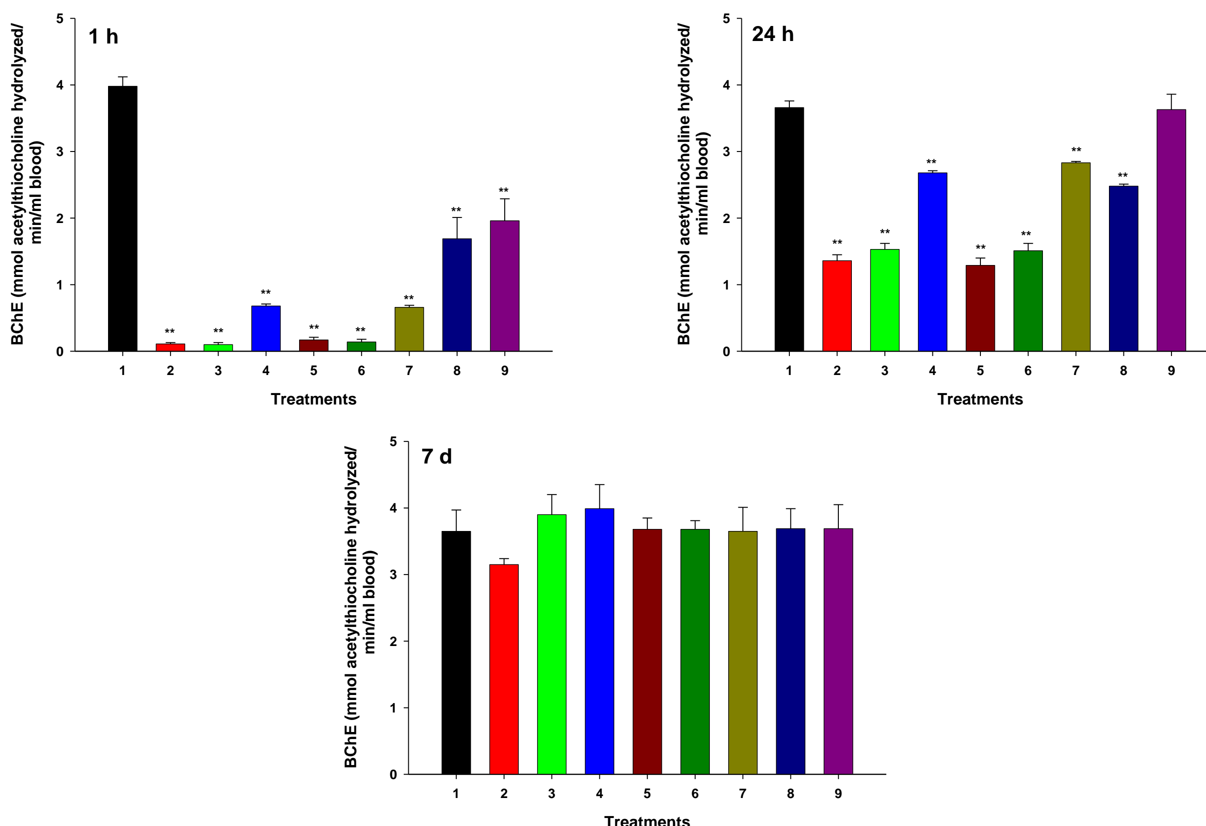

Fig. 1: Butyrylcholinesterase (BChE) activity in mice plasma at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). **Significantly different from corresponding control at $p<0.01$.

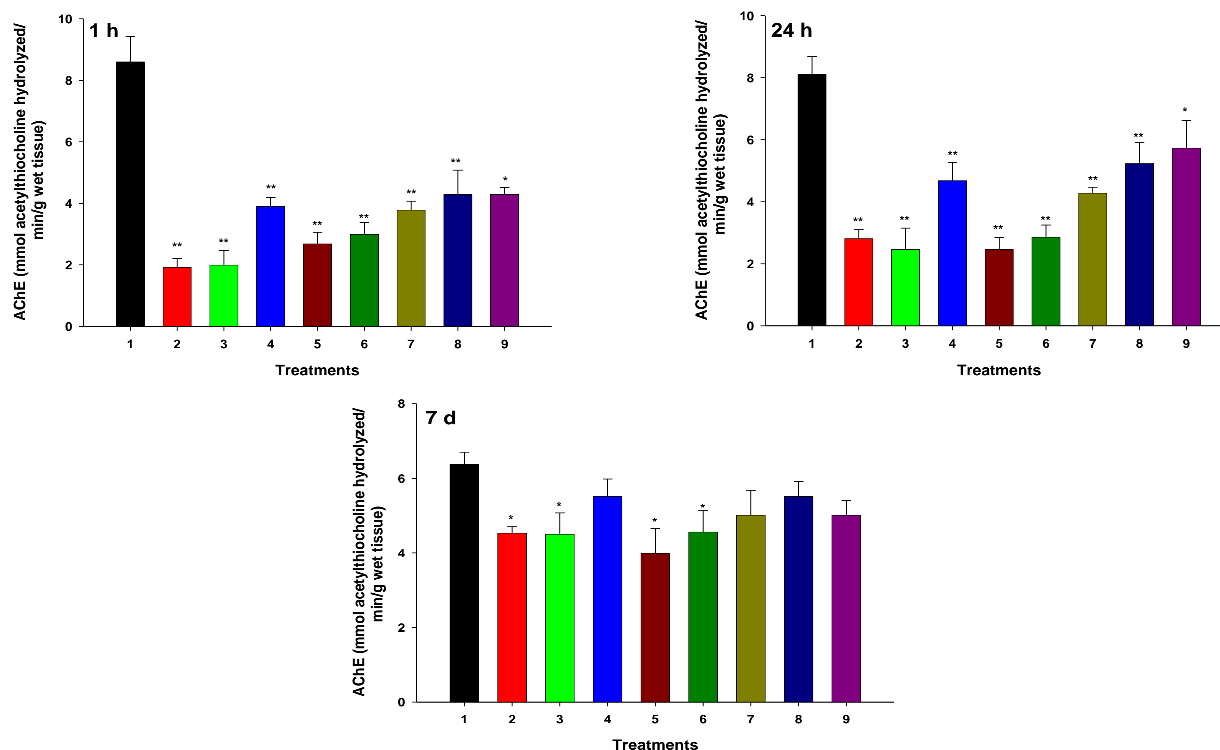

Fig. 2: Acetylcholinesterase (AChE) activity in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$. **Significantly different from corresponding control at $p<0.01$.

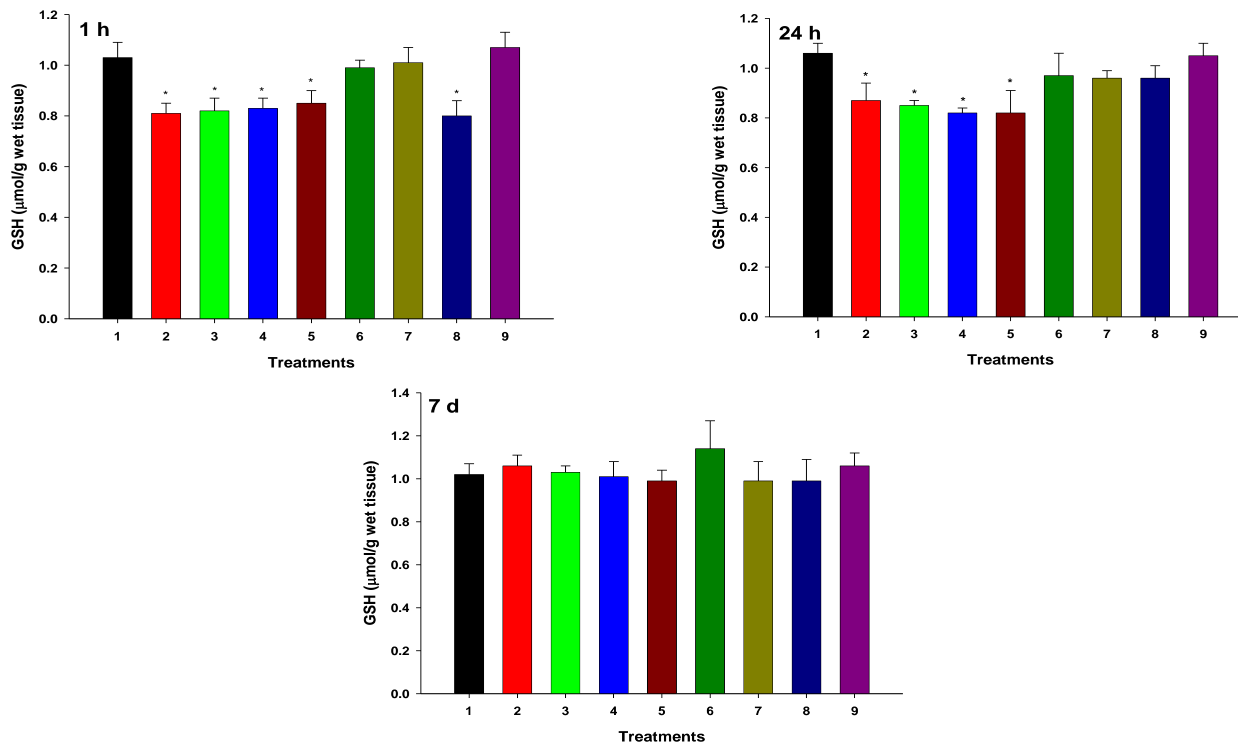

Fig. 3. Reduced glutathione (GSH) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$.

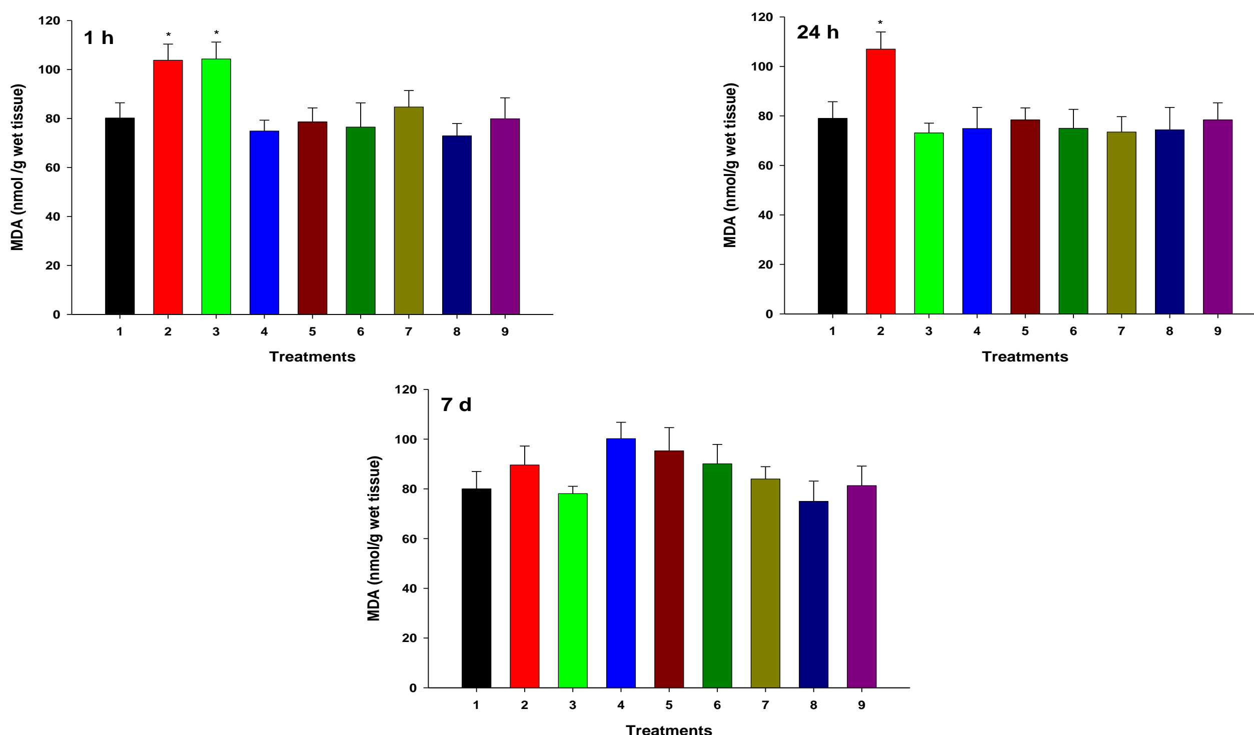

Fig. 4. Malondialdehyde (MDA) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$.

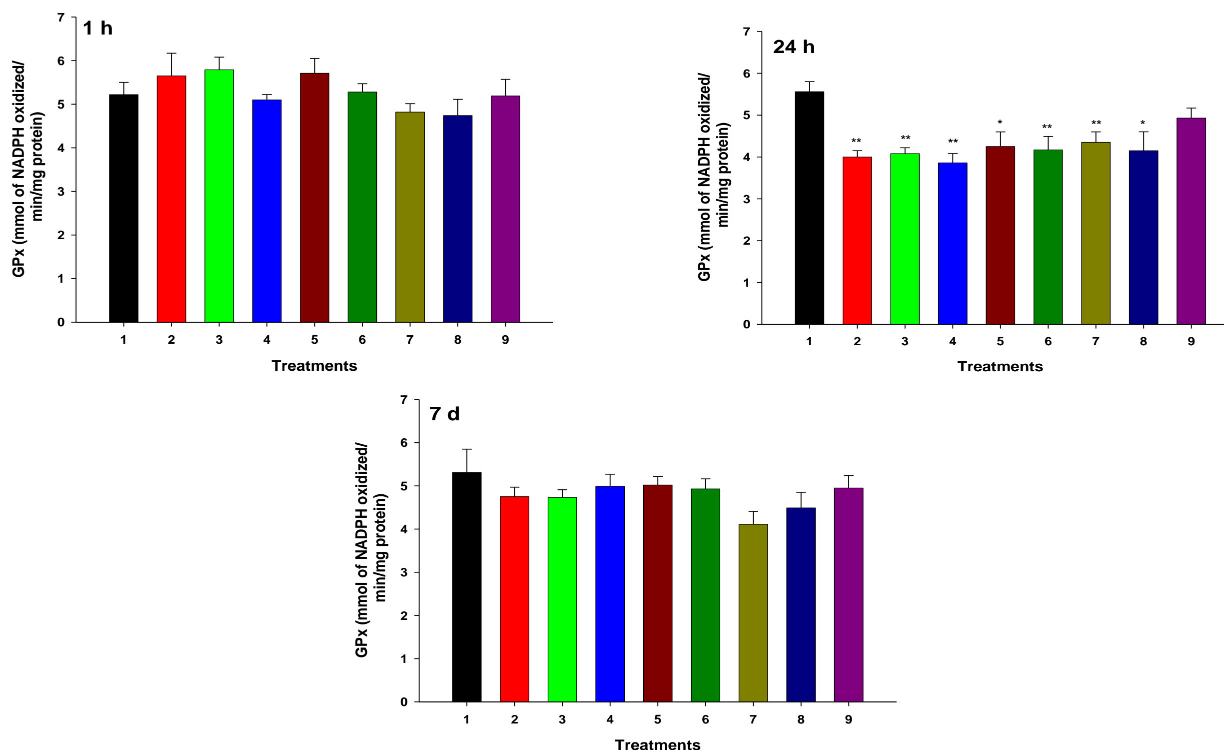

Fig. 5. Glutathione peroxidase (GPx) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$. **Significantly different from corresponding control at $p<0.01$.

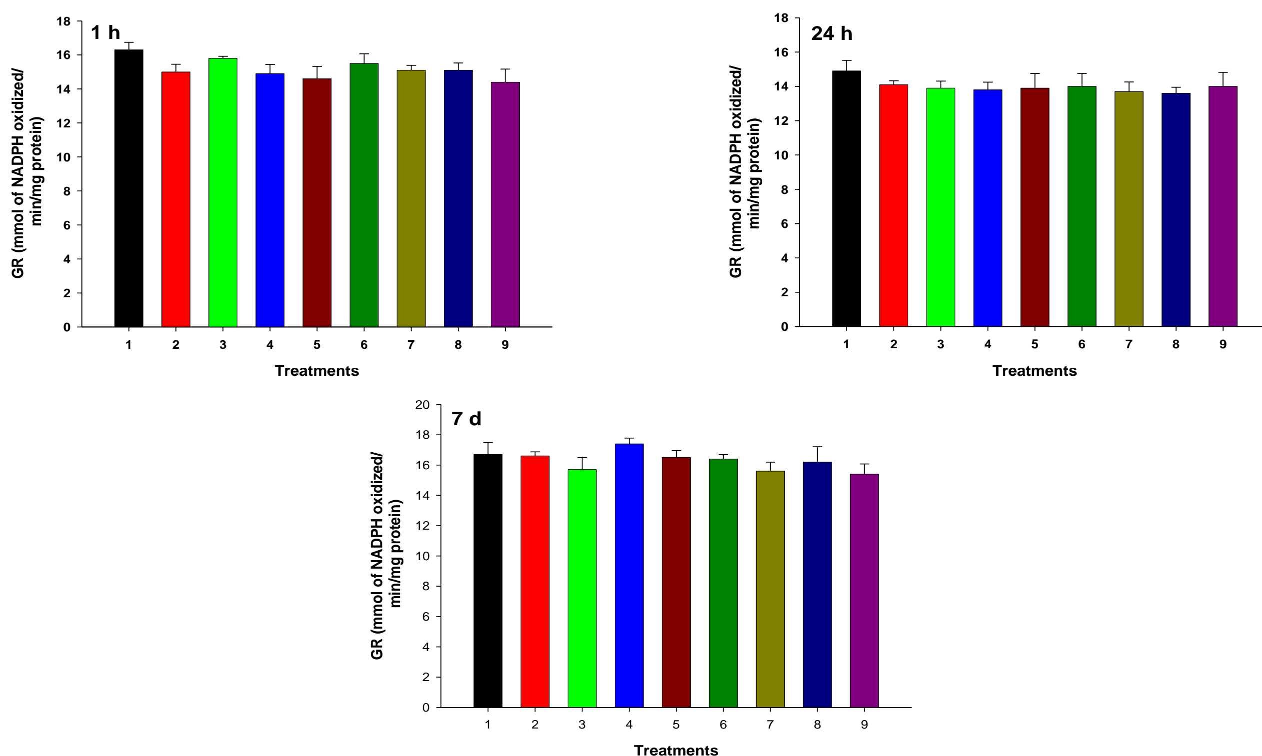

Fig. 6. Glutathione reductase (GR) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6).

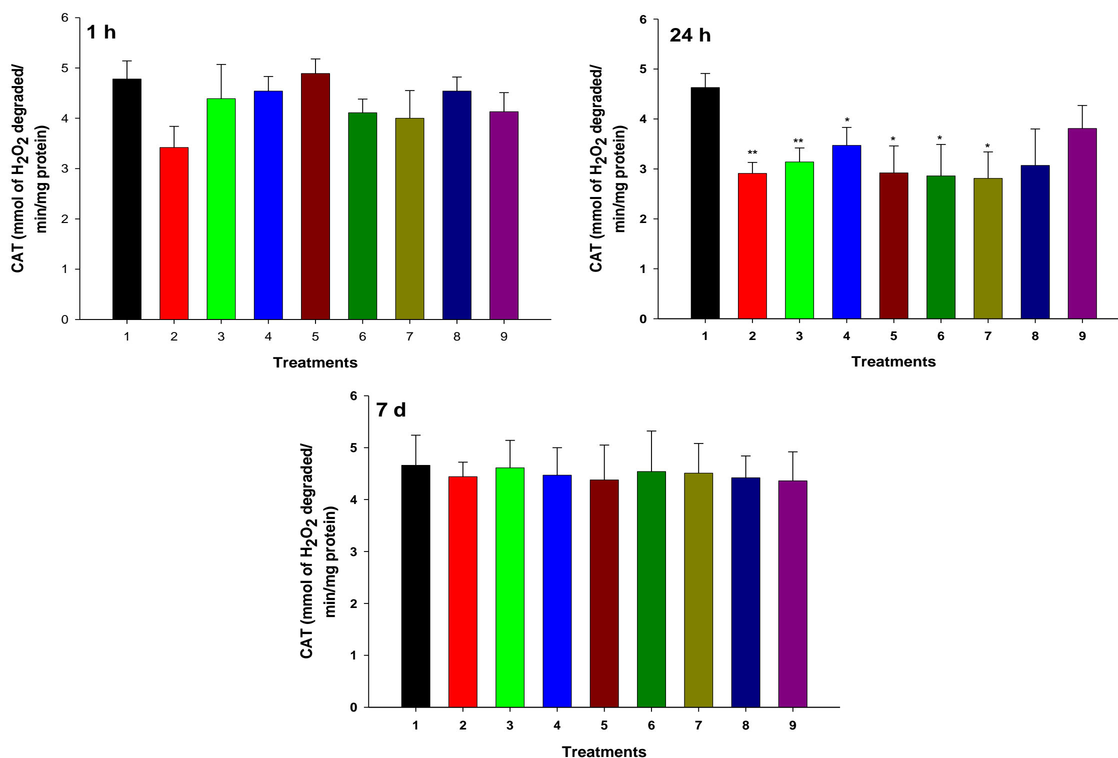

Fig. 7. Catalase (CAT) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$. **Significantly different from corresponding control at $p<0.01$.

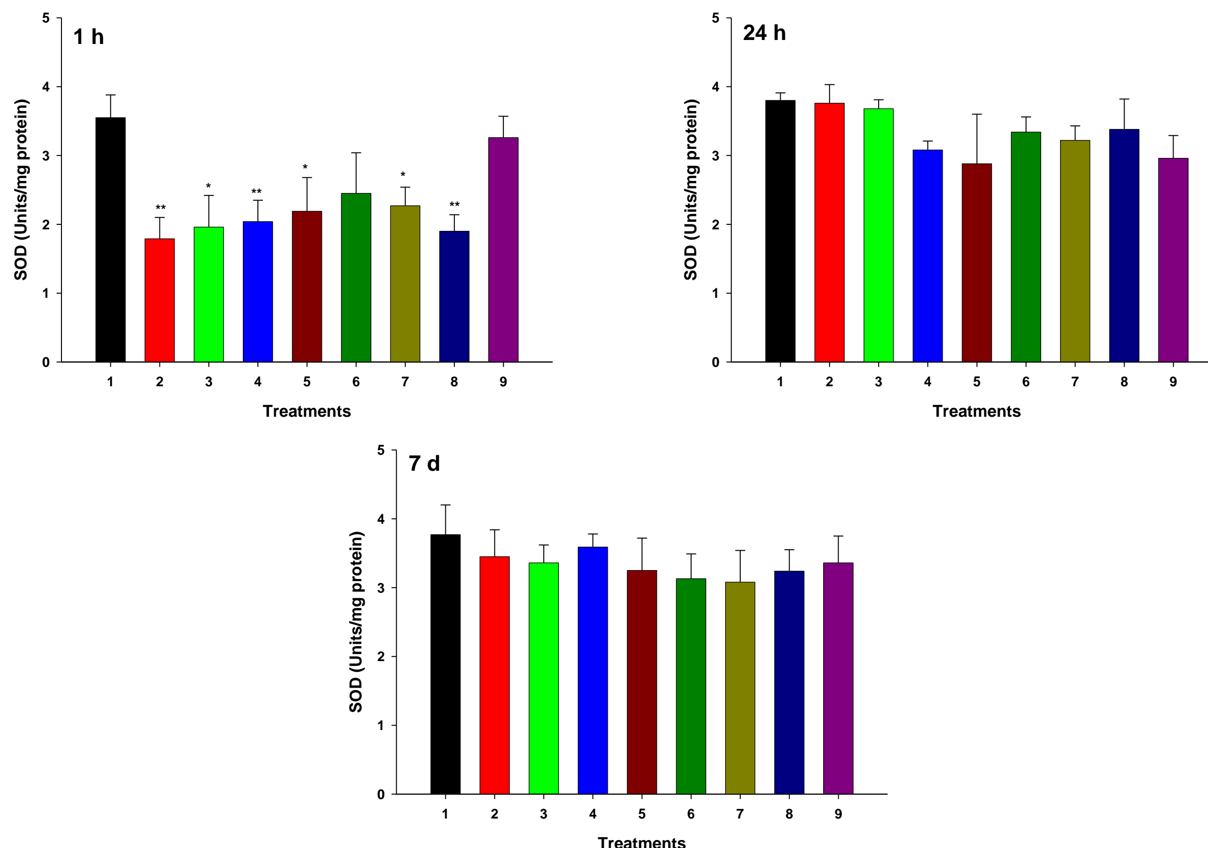

Fig. 8. Superoxide dismutase (SOD) levels in mice brain homogenate at different time intervals after various treatments: (1) Control; (2) 0.50 LD_{50} DFP; (3) 0.50 LD_{50} DFP + Atropine; (4) 0.50 LD_{50} DFP + 2-PAM; (5) 0.50 LD_{50} DFP + NAC; (6) 0.50 LD_{50} DFP + Atropine + NAC; (7) 0.50 LD_{50} DFP+ 2-PAM + NAC; (8) 0.50 LD_{50} DFP + Atropine + 2-PAM and (9) 0.50 LD_{50} DFP + Atropine + 2-PAM + NAC. Values are mean ± SE (n= 6). *Significantly different from corresponding control at $p<0.05$. **Significantly different from corresponding control at $p<0.01$.

4. Discussion

Toxic effect of OP is not limited to cholinergic crisis alone, following inhibition of cholinesterases. It has also been opined that oxidative stress plays a crucial role in OP toxicity following acute and chronic exposures.[5-7] Increased lipid peroxidation and compromised enzymatic and non enzymatic antioxidant defence system are key components of OP toxicity. Generation of free radicals is known to initiate from hyperactivation of various enzymes resulting from intracellular calcium overload during OP poisoning[8]. The macromolecular targets of oxidative damage include lipids, proteins, both nuclear and mitochondrial DNA, and numerous genes, including those involved in modulating the production of free radicals.[23]

Role of antioxidants in mitigating OP toxicity is well known.[12,16] Antioxidants including the free radical scavengers are being extensively pursued by the Czech Armed Forces as antidotes against chemical warfare agents.[25] The most important agents for mitochondrial antioxidant protection are the GSH and multiple GSH-linked antioxidant enzymes. In light of this, NAC, a cell permeable GSH prodrug that acts by directly scavenging the free radicals and restoring glutathione and cysteine was considered as a promising candidate against fenthion [14] and malathion [24]-induced oxidative stress. NAC, as a precursor of cysteine (sulfur amino acid) confers oxidative protection by interacting directly with electrophiles or by facilitating generation of GSH. NAC is a small molecule and after internalization into the cell, it is deacylated to cysteine. As an antioxidant, NAC directly scavenges free radicals.[24] Additionally, it has mucolytic property due to which it has been recommended for the treatment of chronic bronchitis and chronic obstructive pulmonary disorder.[26] Therefore, it is expected to alleviate the OP-induced pulmonary toxicity as well.[14]

Role of oxidative stress in OP pesticide poisoning has been adequately discussed.[5-7] However, occurrence of similar phenomenon after OP nerve agent poisoning has not been sufficiently addressed.[28] In a recent study, we reported alterations in various cholinergic and non-

cholinergic markers after DFP poisoning.[27] Among non-cholinergic markers, diminished GSH and elevated MDA levels were observed in the brain, indicating possible oxidative damage. Lipid peroxidation plays a crucial role during oxidative stress, and elevated MDA levels are usually measured as a marker of lipid peroxidation.[22] Enhanced lipid peroxidation occurs due to direct interaction of OP with the cellular membrane and ROS. In the present study, oxidative stress was further characterized by various antioxidant enzymes after DFP poisoning, in the presence or absence of NAC alone or with atropine and/or 2-PAM. In the absence of data on PI of NAC against DFP, most of the comparisons were made on biochemical alterations caused by OP and their amelioration by NAC. The present study did not reveal any beneficial effect of NAC on the survival of animals. However, adjunction of NAC (-60 min) with atropine or 2-PAM was found to significantly extend the time for onset of sign and symptoms and time of death after 2.0 LD_{50} DFP. There was no difference in the efficacy of NAC if given as prophylactic, simultaneous or therapeutic treatment. Similar observations were also made by authors who reported that NAC alone could improve the survival of mice receiving acute high dose of fenthion.[14] In the present study, time-dependent inhibition of cholinesterases after 0.50 LD_{50} DFP almost followed the same pattern as reported earlier.[18,27] Although, AChE inhibition was more lasting, percent inhibition of plasma BChE was more compared to brain AChE. Usually, effect of OP is more conspicuous on plasma cholinesterase activity.[29] In the present study, beneficial effect of NAC alone on DFP- induced alterations in plasma BChE and brain AChE, GSH, MDA, GPx, GR, CAT, and SOD levels was not appreciable. However, NAC together with atropine and 2-PAM certainly exerted its protective effects. There are many studies where protective effect of NAC alone against OP pesticide- induced oxidative stress and cholinesterase inhibition has been documented.[14,24,30,31] The sensitivity of cholinesterases to OP pesticides and DFP may greatly vary for the reason that cholinesterase may exist as region-specific isozymes with different turnover rates and sensitivity to different OPs.[32] Therefore, the resultant excitotoxicity, intracellular calcium overload and oxidative stress, and their response to antioxidants may largely vary with different OPs. Antioxidant enzymes like GPx, GR, CAT, and SOD play crucial roles in the metabolism of ROS and protecting the cells from oxidative injury. Any insult to antioxidant enzymes exposes the cells to oxidative stress. Inhibition of GPx, SOD and CAT may possibly be due to decrease in cellular GSH content and increase in production of oxygen free radicals. Usually, glutathione redox cycle can abrogate low levels of oxidative injury, while CAT is implicated in alleviating severe oxidative stress. Decrease in CAT activity could be due to elevated levels of superoxide anion radical as a result of a reduction in SOD activity.[33]

In the present study, oxidative stress was observed at 24 h post exposure. However, there is a possibility that it could have initiated earlier because in our previous study DFP (0.50 and 1.0 LD_{50}) caused depletion in GSH levels and elevation in MDA levels as early as 4 h post exposure.[27]

Usually, combination of atropine and 2-PAM is widely employed for the management of OP poisoning. However, there have been extensive studies to use diverse pharmacological agents as adjuncts to augment the efficacy of atropine-oxime therapy [10,11] and antioxidants have exhibited immense promise in this regard.[14,24,30,31] In the present study, NAC as such was not anticipated to increase the survival of the animals. However, its ability to resolve the oxidative stress may be of enormous importance because antioxidants are known to abrogate neurodegenerative diseases, and survival of the victims due to delayed effects of OP becomes very crucial.[13,34] Possibly, beneficial effects of NAC may be better expressed against repeated OP exposure.[30]

5. Conclusion

The present study demonstrates the benefit of incorporating NAC in the atropine-oxime treatment regimen in mitigating the oxidative stress mediated OP toxicity. Such treatment is likely to enhance the prognosis of the victims exposed to OP pesticides and nerve agents. The study has relevance in the treatment of OP poisoning, possibly occurring through pesticide or nerve agent exposures. However, adequate pre-clinical animal studies need to conducted to unequivocally establish the efficacy of NAC.

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Declaration of Conflicting Interests

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