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Oxidative and histopathological alterations after sub-acute exposure of diisopropyl phosphorofluoridate in mice: Beneficial effect of *N*-acetylcysteine

Jebin Jacob John, D.P. Nagar, Niranjan L. Gujar, Rahul Bhattacharya*

Division of Pharmacology and Toxicology, Defence Research and Development Establishment, Jhansi Road, Gwalior, India

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ABSTRACT

Aims: Protective efficacy of *N*-acetylcysteine (NAC) was assessed against sub-acute diisopropyl phosphorofluoridate (DFP) poisoning in mice.

Main methods: Mice were allocated into nine groups of six each: vehicle control; DFP (0.125 LD_{50} ≈ 0.483 mg/kg bwt, s.c.); DFP + Atropine (ATR, 10 mg/kg bwt, i.p., 0 min); DFP + Pralidoxime (2-PAM, 30 mg/kg bwt, i.m., 0 min); DFP + NAC (150 mg/kg bwt, i.p., −60 min); DFP + ATR + NAC; DFP + 2-PAM + NAC; DFP + ATR + 2-PAM; and DFP + ATR + 2-PAM + NAC. Animals received various treatments for 21 d daily. Plasma butyrylcholinesterase (BChE) was measured after 7, 14 and 21 d of exposure. Brain acetylcholinesterase (AChE) and reduced glutathione (GSH), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) were measured (brain, liver and kidney) after 21 d of exposure. Histopathology, immunohistochemistry, and Western blot for inducible nitric oxide synthase (iNOS) and c-fos were also performed.

Key findings: DFP significantly reduced BChE and AChE levels. Diminished GSH, CAT, SOD (brain and liver), GPx, GR, and elevated MDA (Brain) levels were also observed. DFP caused notable histopathology (brain, liver and kidney) and over expression of iNOS, and c-fos proteins (brain). NAC enhanced the protective efficacy of ATR and 2-PAM in most parameters, without any appreciable protection in iNOS and c-fos expression.

Significance: NAC as an adjunct with ATR and 2-PAM, exhibited marked beneficial effects against sub-acute DFP poisoning, indicating its possible implications in the management of OP poisoning.

1. Introduction

Organophosphate (OP) compounds are extensively used in industries, agriculture and in human, veterinary, and public health management. Nerve agents like sarin, cyclosarin, soman, tabun, and VX are also highly toxic OP compounds, which pose threat as possible agents of chemical warfare and mass terrorism [1]. Indiscriminate use of OP compounds has a detrimental effect on the environment [2]. Toxicity of OP compounds is mainly attributed to inhibition of acetylcholinesterase (AChE) enzyme at neuronal and neuromuscular synapses that leads to excessive accumulation of acetylcholine (ACh), a neurotransmitter involved in nerve impulse transmission. This leads to overstimulation of cholinergic, nicotinic and muscarinic ACh receptors, causing various toxic manifestations like lacrimation, salivation, seizures, fasciculation, and tremors [3,4]. In addition to OP-induced cholinergic crisis, numerous non-cholinergic effects have also been recorded after OP poisoning [5]. Oxidative stress is one such non-cholinergic toxic phenomenon that has been shown to occur after acute and chronic OP pesticide poisoning [6–8]. Activation of *N*-methyl D-aspartate (NMDA) receptors accompanied by massive Ca^{2+} influx into the postsynaptic cells, excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) leading to neuronal degeneration are some major toxic manifestations reported after OP poisoning [9,10]. Elevated cytosolic free Ca^{2+} impairs many intracellular processes associated with Ca^{2+} mobilization and energy metabolism in the cell that leads to mitochondrial damage [11]. Mitochondrial dysfunction further leads to ROS generation, increased lipid peroxidation, compromised enzymatic and non-enzymatic antioxidant defence mechanism, impaired cellular metabolism of lipids, proteins, and carbohydrates, intranucleosomal DNA damage, etc. [6,12]. OP pesticide-

* Corresponding author at: Division of Pharmacology and Toxicology, Defence Research and Development Establishment, Jhansi Road, Gwalior 474 002, M.P., India.
E-mail address: rahul@drde.drdo.in (R. Bhattacharya).

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Nomenclature

AChE	Acetylcholinesterase
ATR	Atropine
BCA	Bicinchoninic acid
BChE	Butyrylcholinesterase
BSA	Bovine serum albumin
CAT	Catalase
ChE	Cholinesterase
CNS	Central nervous system
cAMP	Cyclic adenosine monophosphate
DFP	Diisopropyl phosphorofluoridate
DMSO	Dimethyl sulfoxide
DTNB	5, 5′-dithiobis-2-nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
HRP	Horseradish peroxidase
IEGs	Immediate early genes
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
ITFs	Inducible transcription factors
MDA	Malondialdehyde
MPA	Metaphosphoric acid
NAC	*N*-acetylcysteine
NMDA	*N*-methyl D-aspartate
NS	Normal saline
OBI	Organ-body weight index
OP	Organophosphate
OPT	o-phthaldialdehyde
PAGE	polyacrylamide gel electrophoresis
2-PAM	Pralidoxime
p-CREB	Phosphorylated cAMP response element binding protein
PBST	Phosphate buffered saline with Tween 20
PG	Propylene glycol
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBA	2-thiobarbituric acid

induced oxidative damage [6–8] is more commonly known compared to nerve agents [13,14]. We have recently reported dose and time-dependent oxidative stress after acute diisopropyl phosphorofluoridate (DFP) poisoning in mice [15]. DFP is a structural analogue of sarin and is more toxic compared to OP pesticides. It is known to mimic the toxicity of nerve agents [1,16].

Although, atropine (ATR; a muscarinic antagonist) and pralidoxime (2-PAM; a cholinesterase reactivator) are the drugs of choice for OP poisoning, prognosis of victims remains elusive due to several factors [3,17]. Central nervous system (CNS) is very sensitive to oxidative stress. Therefore, many antioxidants have been found to alleviate OP poisoning [14,17,18]. One such low-molecular-weight thiol compound is *N*-acetylcysteine (NAC), which has strong antioxidant and anti-inflammatory properties [19–21]. Even randomized clinical trial has been carried out with NAC for the management of OP pesticide poisoning [22]. However, in our recent study, appreciable protective effects of NAC could not be observed against acute DFP poisoning in mice [23]. Oxidative stress has often been implicated in delayed neuropathy caused by low dose long-term OP exposures [1,24]. Therefore, in the present study we investigated the role of NAC alone or in adjunction with ATR and/or 2-PAM against sub-acute toxicity of low-dose DFP exposure in mice. The study includes various oxidative stress markers, histopathology, immunohistochemistry and Western blot analysis for inducible nitric oxide synthase (iNOS) and c-fos protein expression.

2. Methods

2.1. Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA), 2-PAM chloride, ATR sulphate, NAC, 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), propylene glycol, bovine serum albumin (BSA), Trizma base, phosphate buffered saline with Tween 20 (PBST), and all other chemicals, reagents and buffers of highest purity were procured from Sigma-Aldrich Inc. (St. Louis, MO, USA). DFP (> 99% purity) was purchased from Merck India Ltd. (Mumbai, India).

2.2. Animals and housing conditions

Male Swiss Albino mice (25–30 g) bred in the Animal Facility of Defence Research and Development Establishment (DRDE), Gwalior were used in the present study. Mice were housed in polypropylene cages on dust free rice husk with free access to food (Ashirwad Brand, Chandigarh, India) and water ad libitum. Mice were kept 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 in compliance with the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Govt. of India, New Delhi. Animal experiments were carried out with the approval of Institutional Animal Ethical Committee of DRDE.

2.3. Treatment

DFP solution was freshly prepared in normal saline (NS; 0.9% sodium chloride in distilled water) and PG in a ratio of 9:1 (v/v), respectively. Control animals received equivalent amount of vehicle (NS-PG). ATR, 2-PAM and NAC were prepared fresh in NS. Volume of all the injections was kept between 0.10 and 0.20 ml. Mice were allocated into nine groups of six each: vehicle control; DFP (0.125 LD_{50} ≈ 0.483 mg/kg bwt, s.c.); DFP + ATR (10 mg/kg bwt, i.p., 0 min); DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); DFP + NAC (150 mg/kg bwt, i.p., −60 min); DFP + ATR + NAC; DFP + 2-PAM + NAC; DFP + ATR + 2-PAM; and DFP + ATR + 2-PAM + NAC. Animals received various treatments for 21 d daily. The dose, route, treatment time and sequence of treatments were based on previous studies [15,20,23]. NAC was administered one hour prior to DFP. ATR and 2-PAM were given within 10 s after DFP. Animals were anesthetized after 7, 14 and 21 d of exposure; blood was collected though retro-orbital plexus using heparinized capillary to measure plasma butyrylcholinesterase (BChE) activity. Animal body weight was recorded daily. Thereafter, Animals were killed by cervical dislocation after 21 d of exposure. Brain, heart, lung, liver, kidney, and spleen were quickly removed, rinsed in NS, blotted and weighed for determining the organ-body weight index (OBI). OBI was calculated as organ weight × 100 / animal body weight. Thereafter, brain, liver and kidney homogenate was prepared for measuring various biochemical and oxidative stress markers as discussed below.

2.4. BChE and AChE estimation

Plasma BChE and brain AChE activity was measured by Ellman's method [25]. 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 minutes 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 ACh hydrolyzed/min/ml blood and µmol ACh hydrolyzed/min/g wet tissue, respectively.

2.5. Reduced glutathione (GSH) estimation

Tissue GSH levels were measured by the method discussed elsewhere [26]. 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.

2.6. Malondialdehyde (MDA) estimation

The lipid peroxidation in brain homogenate was quantified by MDA levels [27]. 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.

2.7. Estimation of antioxidant enzymes

Glutathione peroxidase (GPx; µmol of NADPH oxidized/min/mg protein), glutathione reductase (GR; µmol of NADPH oxidized/min/mg protein), catalase (CAT; µmol of H_2O_2 degraded/min/mg protein) and superoxide dismutase (SOD; Units/mg protein) were measured in tissue homogenate using Calbiochem make diagnostic kits from Merck India Ltd. (Mumbai, India). The protein content in the extractions was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

2.8. Histopathology

Brain, kidney and liver were pruned and fixed in 10% buffered formalin solution for 24 h at room temperature. After proper fixation organs were dehydrated using graded series of alcohol and toluene in tissue processor (Leica TP 1020, Germany), and subsequently embedded in paraffin wax using block shaped scaffoldings. After cooling, multiple sections of 4 µm thickness from each block of liver and kidney, and 8 µm thickness of brain were cut on rotator microtome (Microm, Germany). Sections were mounted on slide containing albumin coating, which were latter air dried for overnight. The sections were deparaffinized and stained with hematoxylin and eosin [28] in auto-stainer (Leica, Germany), and covered with cover slip by auto cover slipper (Leica, Germany). After drying, the sections were photographed under light microscope DMLB using DM 500 digital camera (Leica, Germany).

2.9. Immunohistochemistry for iNOS and c-fos

Sections of 8–10 µm thickness were collected from paraffin embedded brain tissues after neutralizing with 10% neutral formalin-fixed buffer. The tissue sections were deparaffinized in xylene and dehydrated using different grades of alcohol. After rehydration with water, retrieval of antigen was achieved by micro-waving the sections in citrate buffer (pH 6.0) at 100 °C for 3 cycles (each cycle for 5 min) for immunostaining. After blocking with 5% BSA for 2 h in room temperature, the sections were stained for iNOS/c-fos using polyclonal anti-rabbit cerb-B2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to instructions of the manufacturer. Staining was carried out at 4 °C overnight and the excess antibody was washed away with PBST (pH 7.5–7.6). The tissues were incubated with HRP conjugated mouse immunoglobulin for 45 min at room temperature. Peroxidase activity was demonstrated with 0.5% diamino-benzidine for 5 min, and the sections were counterstained with hematoxylin. Positive and negative controls were performed at the same time for each section. Positive iNOS/c-fos staining was identified in the form of membrane staining. Scoring for iNOS/c-fos was based on the percentage of cells stained, as well as intensity of membrane staining. Staining of iNOS/c-fos in brain section was scored from 0 to 3 according to ASCO guidelines [29]. The brain sections were scored as 0, 1+, 2+ and 3+. Score of 0 and 1+ was considered negative for iNOS/c-fos protein expression. While 2+ score, (equivocal/borderline) was defined as complete membrane staining that is non-uniform or weak in < 30% of cells. A 3+ score indicated protein expression.

Table 1
Levels of plasma butyrylcholinesterase (BChE) and brain acetylcholinesterase (AChE) in mice 21 d after various treatments (values are mean ± SEM; $n = 6$).

Sl. No.	Treatments	Plasma BChE (µmol acetylthiocholine hydrolized/min/ml blood)			Brain AChE (µmol acetylthiocholine hydrolized/min/g wet tissue)
		7 d	14 d	21 d	21 d
1.	Control	4.08 ± 0.04	3.38 ± 0.10	4.69 ± 0.34	7.28 ± 0.08
2.	DFP	0.88 ± 0.02**	1.06 ± 0.15**	1.35 ± 0.10**	4.08 ± 0.48**
3.	DFP + ATR	0.94 ± 0.13**	1.08 ± 0.10**	1.16 ± 0.11**	3.16 ± 0.32**
4.	DFP + 2-PAM	0.97 ± 0.06**	1.34 ± 0.16**	1.18 ± 0.26**	4.36 ± 0.36**
5.	DFP + NAC	1.06 ± 0.07**	1.74 ± 0.19**	1.31 ± 0.09**	3.56 ± 0.36**
6.	DFP + ATR + NAC	0.95 ± 0.01**	1.31 ± 0.10**	1.61 ± 0.29**	3.52 ± 0.28**
7.	DFP + 2-PAM + NAC	1.13 ± 0.12**	1.30 ± 0.18**	1.51 ± 0.12**	4.64 ± 0.48**
8.	DFP + ATR + 2-PAM	1.07 ± 0.07**	1.53 ± 0.20**	1.29 ± 0.11**	5.32 ± 0.56**
9.	DFP + ATR + 2-PAM + NAC	1.12 ± 0.14**	2.12 ± 0.32**	3.99 ± 0.12	5.96 ± 0.42*

Treatments include: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC.

* Significantly different from corresponding control at $p < 0.05$.

** Significantly different from corresponding control at $p < 0.01$.

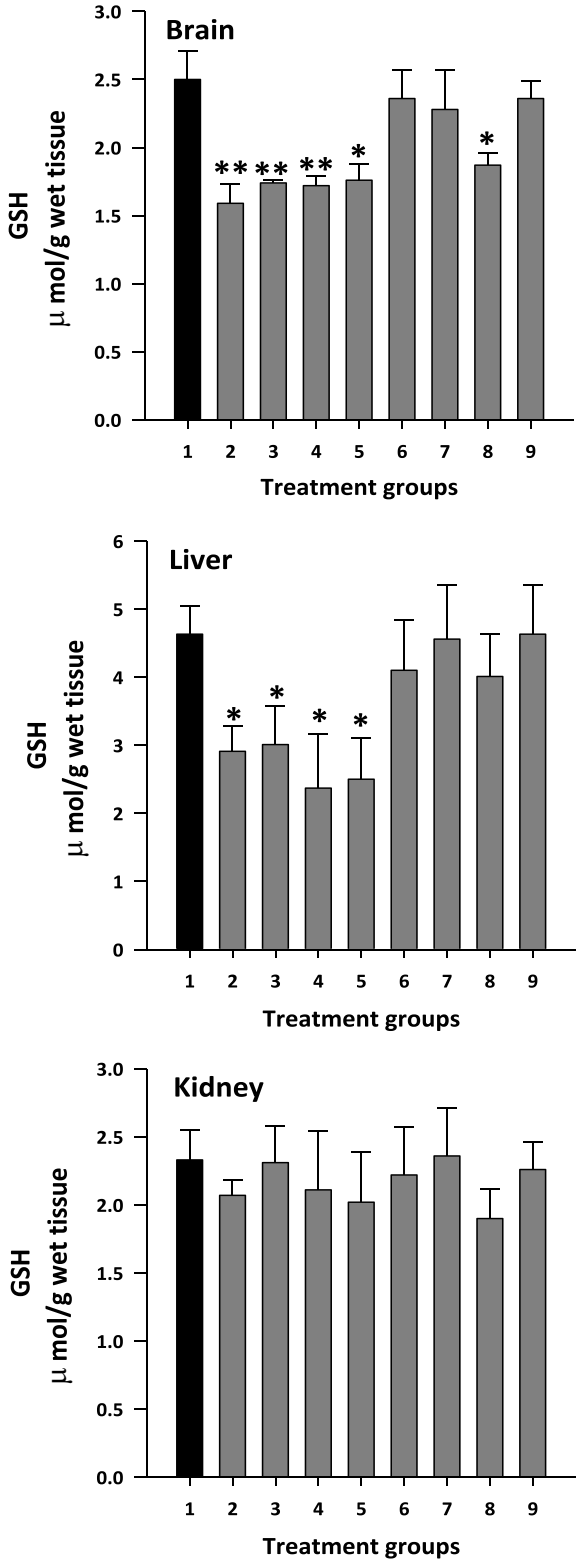


Fig. 1. Levels of reduced glutathione (GSH) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.

*Significantly different from corresponding control at $p < 0.05$.

**Significantly different from corresponding control at $p < 0.01$.

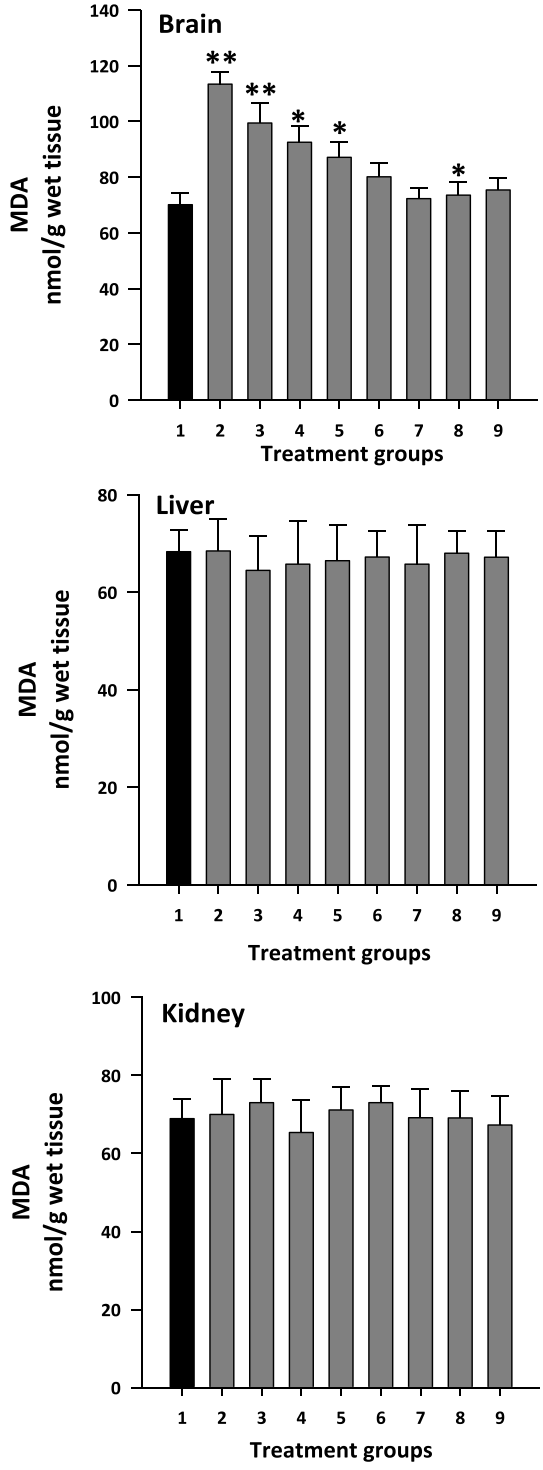


Fig. 2. Levels of malondialdehyde (MDA) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.

*Significantly different from corresponding control at $p < 0.05$.

**Significantly different from corresponding control at $p < 0.01$.

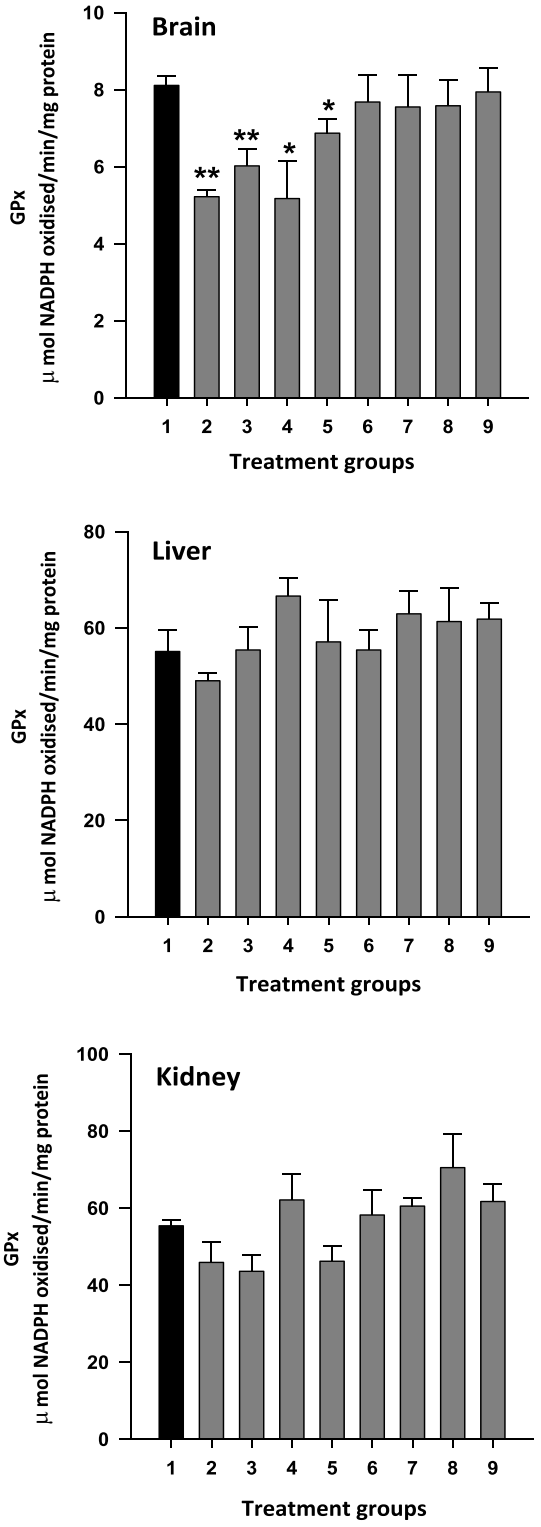


Fig. 3. Levels of glutathione peroxidise (GPx) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.
*Significantly different from corresponding control at $p < 0.05$.

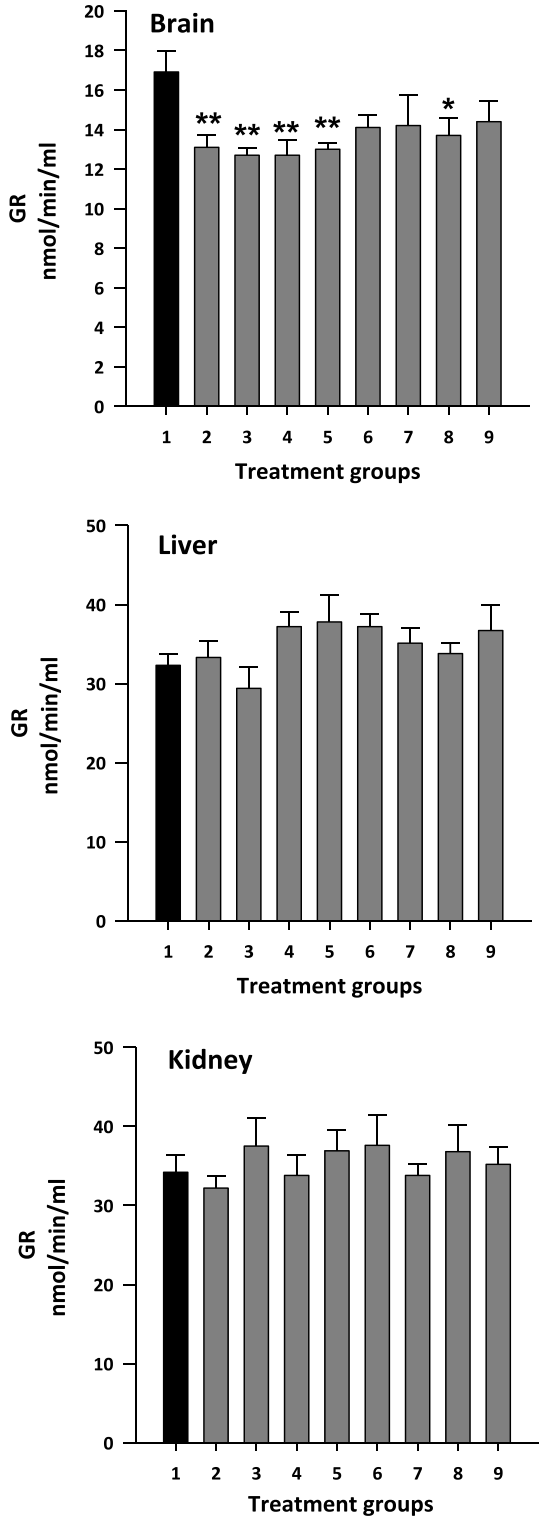
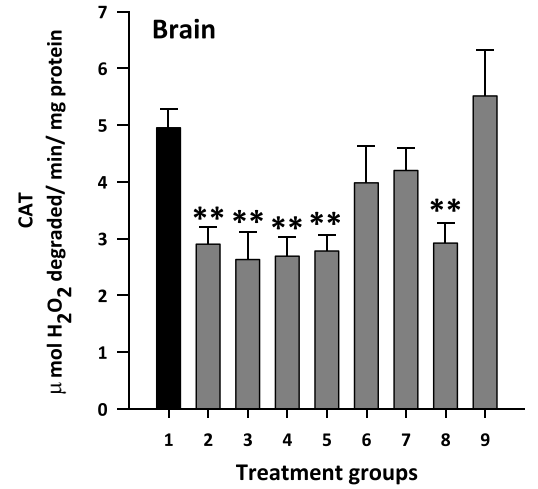
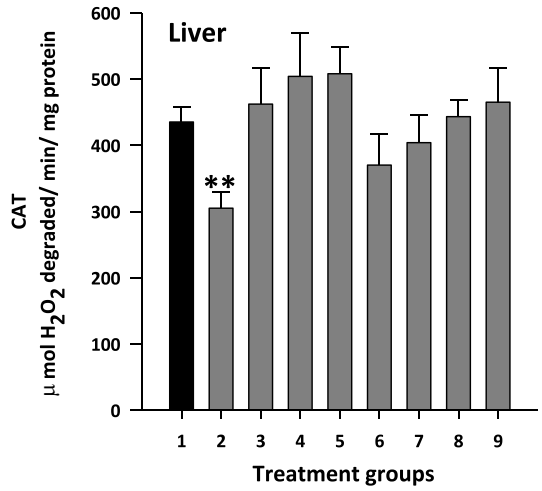


Fig. 4. Levels of glutathione reductase (GR) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.
*Significantly different from corresponding control at $p < 0.05$.
**Significantly different from corresponding control at $p < 0.01$.





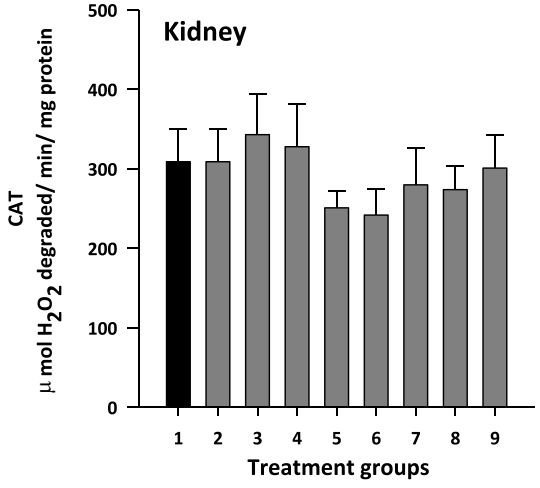
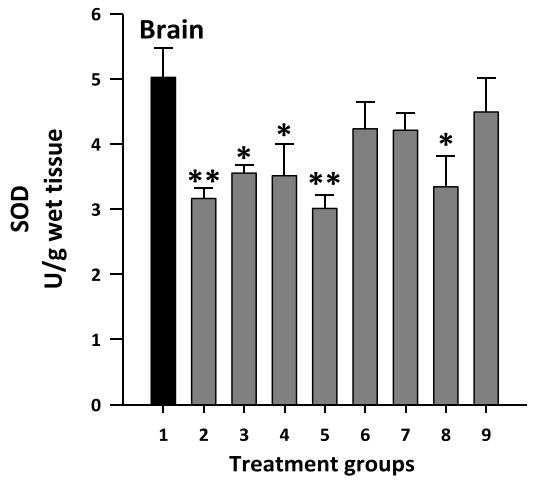
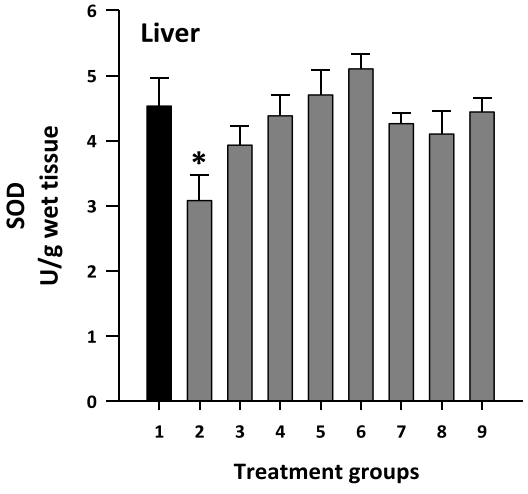


Fig. 5. Levels of catalase (CAT) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.

*Significantly different from corresponding control at $p < 0.05$.

**Significantly different from corresponding control at $p < 0.01$.





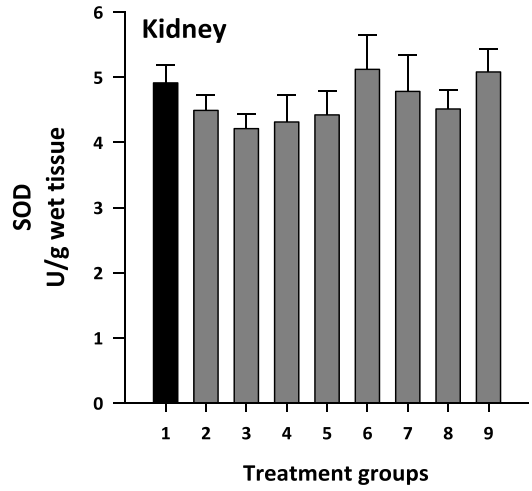


Fig. 6. Levels of superoxide dismutase (SOD) in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.

*Significantly different from corresponding control at $p < 0.05$.

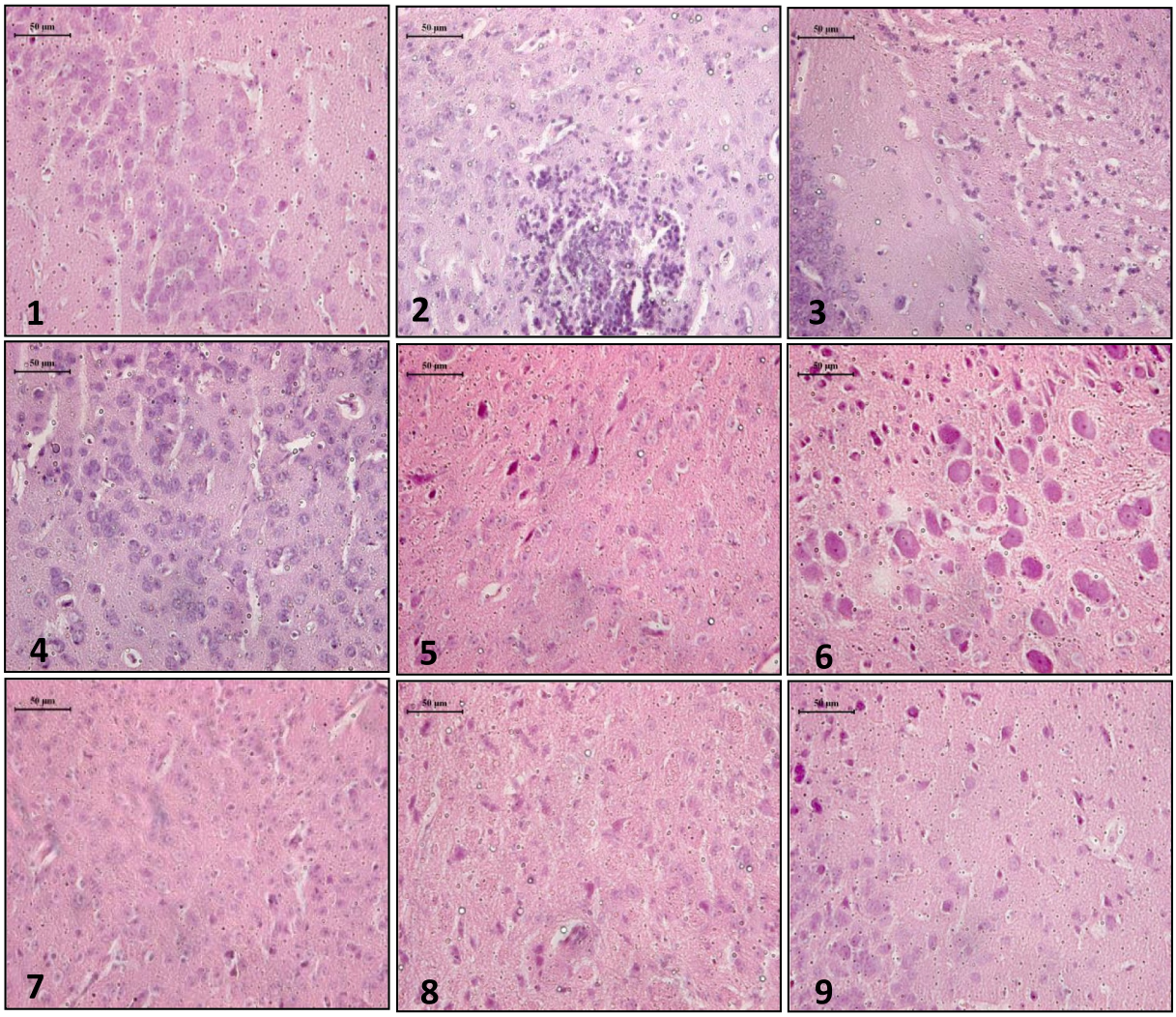


Fig. 7. Histopathology of mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. H&E.

2.10. Western blot analysis of iNOS and c-fos protein

Brain tissues were processed for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot. The protein content in the extractions was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were subjected to electrophoresis in 12% SDS-polyacrylamide gel, followed by transfer to polyvinylidene difluoride (PVDF) transfer membrane (Pall Pharmalab, Mumbai, India). After blocking with phosphate buffered saline containing 5% nonfat dry milk, the PVDF membrane was exposed to polyclonal iNOS antibody and monoclonal c-fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for whole night at 4 °C on a shaker. The membranes were incubated for 2 h in horseradish peroxidase (HRP) conjugated goat anti-goat secondary antibody (1:10,000), followed by signal amplification using chemiluminiscent peroxidise substrate. The signals were detected using an enhanced chemiluminiscent detection system (Fusion SL Vilber Loumart, Cedex, France). Further, for densitometric analysis, data were normalized with internal control and expressed as relative density (% control) for each band using ImageJ 1.48 V Wayne Rasband (NIH, MD, USA). The Western blot analysis was performed thrice and only representative blots with similar results are shown here.

2.11. 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. Animal body weight and OBI

None of the treatments was found to influence the animal body weight recorded daily and OBI of brain, heart, lung, liver, kidney and spleen measured after 21 d of exposure. Values in all the treatment groups were not significantly different from corresponding control (data not shown).

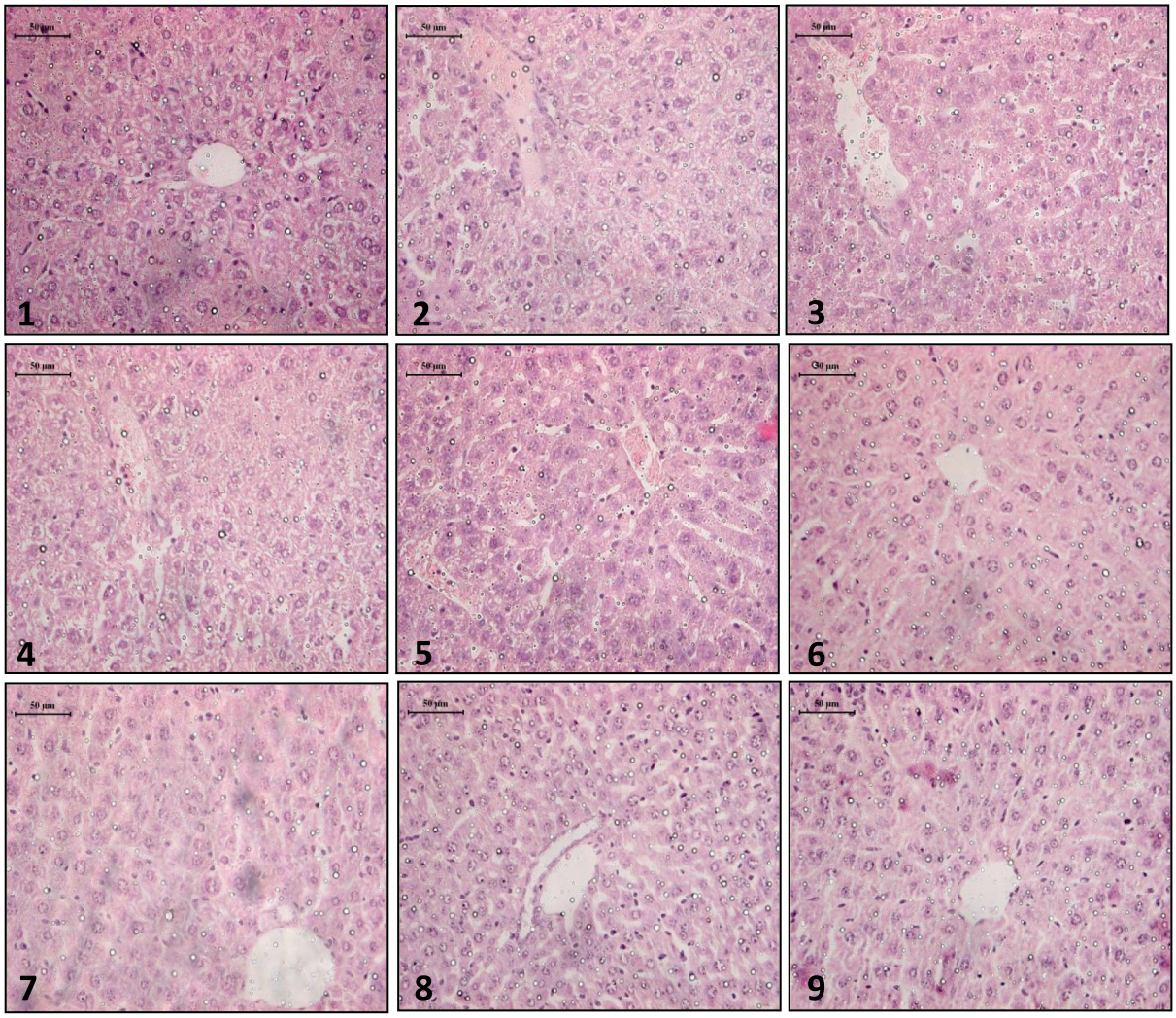


Fig. 8. Histopathology of mice liver 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}; s.c.); (3) DFP + ATR (10 mg/kg bwt; i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. H&E.

3.2. BChE and AChE activity

Effect of 0.125 LD_{50} DFP in the presence or absence of various treatments on plasma BChE levels (7, 14 and 21 d) and brain AChE levels (21 d) is shown in Table 1. DFP caused 78% BChE inhibition on 7 d, which persisted at 71% till 21 d after exposure. None of the treatments showed any beneficial effects. However, NAC in the presence of ATR and 2-PAM restored the BChE levels to normalcy by 21 d (15% inhibition). Brain AChE levels observed after 21 d of exposure were significantly ($p < 0.01$) reduced in all the treatment groups. Although, marginal improvement was observed in ATR + 2-PAM + NAC treated group, inhibition remained significant ($p < 0.05$).

3.3. GSH and MDA levels

GSH content in brain, liver and kidney after various treatments is depicted in Fig. 1. DFP significantly ($p < 0.01$) depleted the GSH levels in both brain and liver after 21 d of exposure. Except ATR + 2-PAM (brain), NAC in the presence or absence of ATR and/or 2-PAM significantly restored the GSH levels in both brain and liver. The GSH levels in kidney remained unchanged. MDA levels in brain, liver and kidney after various treatments is illustrated in Fig. 2. In both liver and kidney, the MDA levels were not found to be altered in any of the treatment groups. However, brain MDA levels were significantly elevated in DFP, DFP + ATR, DFP + 2-PAM, DFP + NAC, and DFP + ATR + 2-PAM treated groups. NAC together with ATR and/or 2-PAM significantly improved the MDA levels compared to control.

3.4. Antioxidant enzymes levels

Effect of NAC in the presence or absence of ATR and/or 2-PAM on brain, liver and kidney GPx levels after sub-acute DFP poisoning in mice is shown in Fig. 3. None of the treatments could alter the GPx levels in liver and kidney tissues. However, brain GPx levels was significantly depleted in DFP, DFP + ATR, DFP + 2-PAM, and DFP + NAC treated animals. NAC alone or in the presence of ATR and/or 2-PAM significantly improved the GPx levels compared to control. Fig. 4 shows the effect of various treatments on brain, liver and kidney GR levels. Brain GR levels were found to be significantly ($p < 0.01$) diminished by sub-acute treatment of DFP. Treatments of ATR, 2-PAM, NAC, and ATR + 2-PAM did not confer any protection, but NAC together with ATR and/or 2-PAM significantly alleviated the brain GR levels. Liver and kidney GR levels were not found to be changed compared to control. DFP was found to significantly ($p < 0.01$) deplete brain and liver CAT levels. NAC together with ATR and/or 2-PAM was found to significantly restore the brain GR levels. In case of liver, all the treatment regimens provided protection. The CAT levels in kidney remained unchanged (Fig. 5). The brain, liver and kidney SOD levels after various

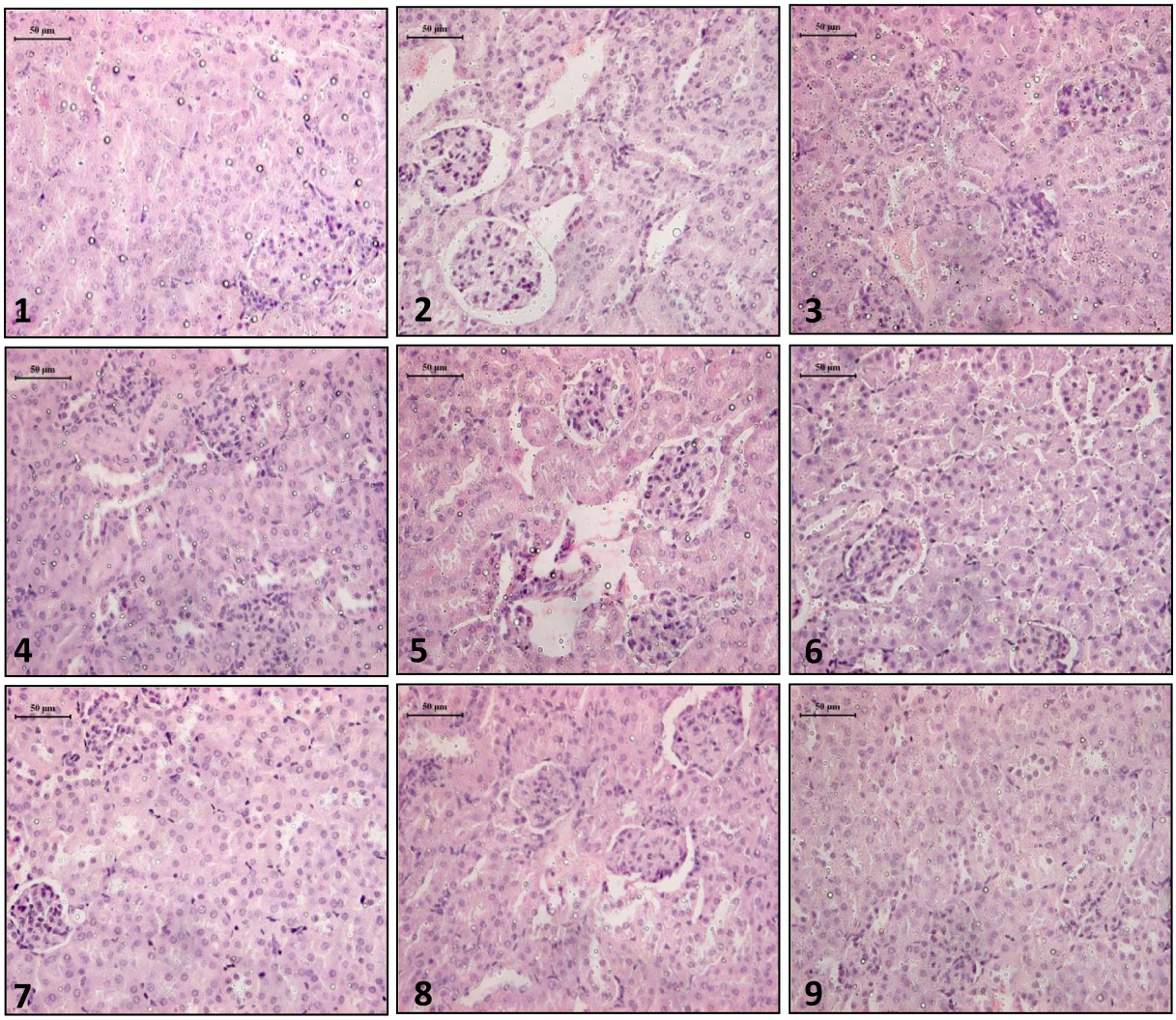


Fig. 9. Histopathology of mice kidney 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. H&E.

treatments are shown in Fig. 6. Sub-acute treatment of DFP significantly reduced the brain ($p < 0.01$) and liver ($p < 0.05$) SOD levels, which in case of brain did not favorably respond to ATR, 2-PAM, NAC and ATR + 2-PAM treatments. However, NAC in the presence or absence of ATR and/or 2-PAM exhibited significant protection. Protective efficacy of all the treatment regimens was obvious in case of liver. The kidney SOD levels were not influenced by any of the treatments.

3.5. Histopathology

Fig. 7(7.1–7.9) represents the brain photomicrographs of mice exposed to various treatments. Compared to control, DFP treated brain section revealed neuronal and neuroglial degeneration, particularly in peripheral region of cortex and cerebral cortex. This was characterized by the formation of perineuronal and perivascular spaces, mild necrosis, vacuolation, and dilation and congestion of blood vessels. Sub-acute treatment of DFP also induced cell shrinkage, pyknosis of nucleus and disappearance of nucleolus. Animals receiving ATR, 2-PAM or NAC alone displayed reduced neuronal lesions. However, mild necrosis in hippocampus region, dilation of blood vessels and vacuolation were still evident. Animals treated with ATR and/or 2-PAM in the presence of NAC exhibited normal histoarchitecture of brain with heterogeneous neuronal cells arranged in different layers. Mild apoptotic degeneration of neurons and dilated blood vessels especially in cortex indicated hypocellularity. Histopathology of liver is shown Fig. 8(8.1–8.9). Control liver showed normal hepatocytes, lobular architecture and cord pattern. DFP treated animals showed hyper-activation of Kuffer cells, vascular and perivascular infiltration of inflammatory cells, accumulation of edematous fluid, and dilation of sinusoids. Animals receiving ATR, 2-PAM or NAC alone displayed mild to moderate vasodilatation, congestion in blood vessels, and dilatation of sinusoids, accompanied by disturbed cord pattern and hepatic lesions. Animals treated with ATR and/or 2-PAM in the presence of NAC displayed normal hepatic cord arrangement, hepatic lobes and hepatocytes with normal hepatic parenchyma. Fig. 9(9.1–9.9) shows the effect of various treatments on histoarchitecture of kidney. Control animals showed normal structure of renal glomeruli, Bowman's capsule lined by squamous epithelium, distinct urinary space, the proximal tubule and distal convoluted tubules. DFP exposed animals showed increased cellularity in glomerulus, obliterations of urinary space between Bowman's capsule and glomerulus, hypertrophy of tubular epithelium, vascular congestion, and infiltration of inflammatory cells, in and around glomeruli. ATR, 2-PAM or NAC alone displayed mild congestion of blood vessels and dilation of suinocide while ATR + 2-PAM, ATR + NAC, 2-PAM + NAC, and ATR + 2-PAM + NAC showed normal glomeruli, Bowman's capsule and renal tubules.

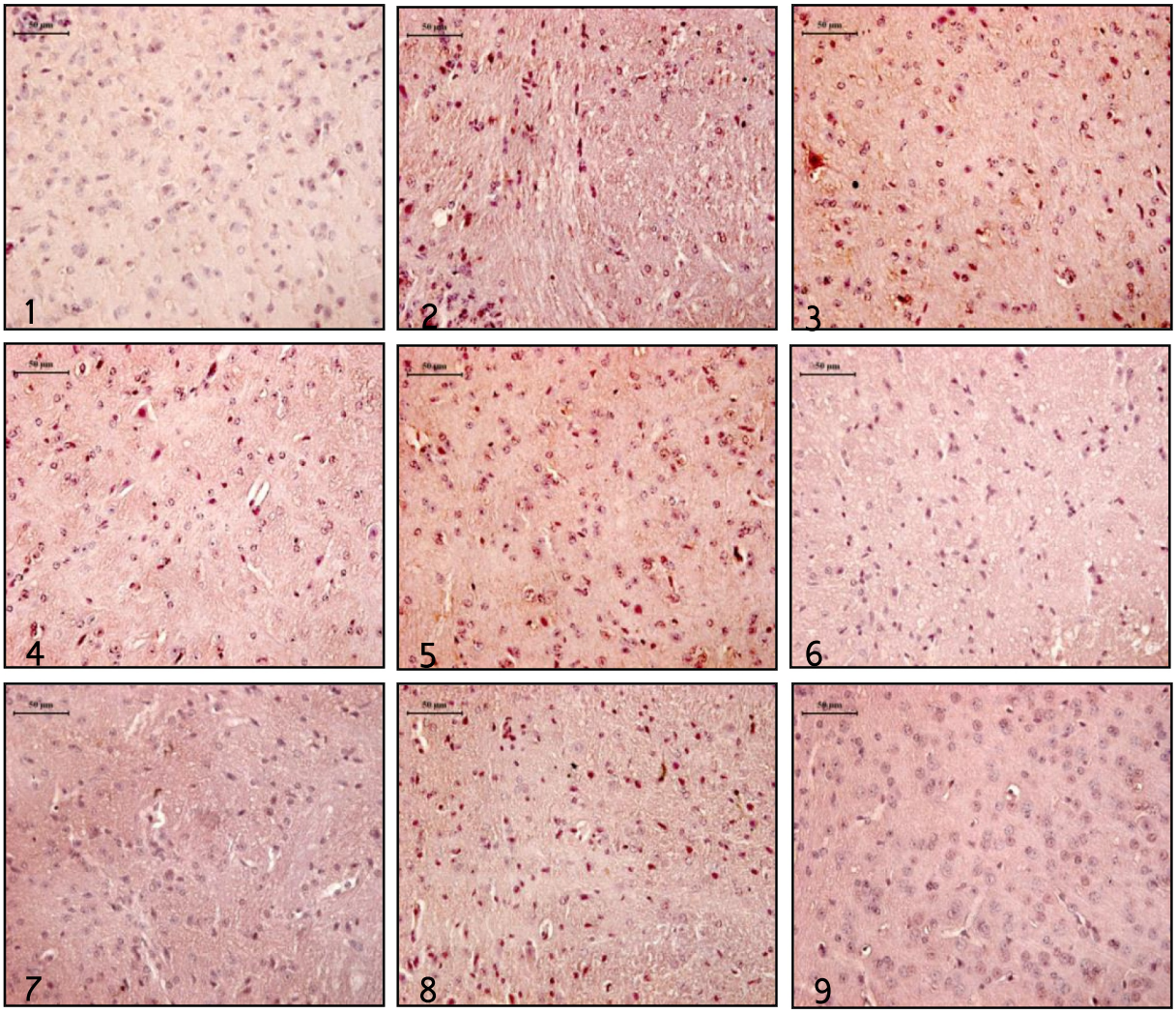


Fig. 10. Immunohistochemical localization of iNOS in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC.

3.6. Immunohistochemistry

Effect of various treatments on iNOS and c-fos protein expression in mice brain regions after 21 of exposure, following immunohistochemical localization are shown in Figs. 10 and 11, respectively. Both proteins were highly expressed in DFP treated animals, and NAC alone or with ATR and/or 2-PAM did not show any regression.

3.7. Western blot

Fig. 12 represents the effect of various treatments on iNOS and c-fos protein expression in mice brain regions after 21 d of exposure, following Western blot (A) and densitometric analysis (B). The iNOS expression was found to be significantly increased in DFP, DFP + NAC and DFP + ATR + 2-PAM + NAC treated groups, while a decrease was observed in DFP + 2-PAM, DFP + 2-PAM + NAC and DFP + ATR + 2-PAM treated groups. A significant increase of c-fos was observed in all the treatment groups compared with control. Maximum expression of c-fos protein was observed in DFP treatment. The expression level of iNOS and c-fos were complimentary to immunohistochemical results.

4. Discussion

The main treatment of acute OP poisoning includes ATR-oxime therapy followed by antiseizure drugs. Various neuroprotective agents have been advocated for minimizing the delayed neurological complications [30]. Oxidative stress, a non-cholinergic event plays a crucial role in OP-induced delayed neuropathy [6,8,13,14]. Oxidative stress is usually characterized by increased lipid peroxidation and impaired enzymatic and non-enzymatic antioxidant defence system. These events are preceded by intracellular calcium overload leading to free radical generation [14,31]. Usually lipids, proteins, nuclear and mitochondrial DNA, and several other genes involved in modulating the production of free radicals, are the major targets of oxidative insult [12,32]. Levels of various antioxidant enzymes like GPx, GR, GSH, SOD, and CAT are known to be depleted after acute or sub-acute OP exposures [33,34]. However, due to high turnover rate of many antioxidant enzymes in the tissue, their role in oxidative stress remains obscure [14]. Other than oxidative stress, many other non-cholinergic aberrations are also known to occur during OP poisoning [5,8,12]. However, most of the studies were limited to OP pesticide exposures [7,8,33,34]. Many genomic and proteomic changes have also been reported after more

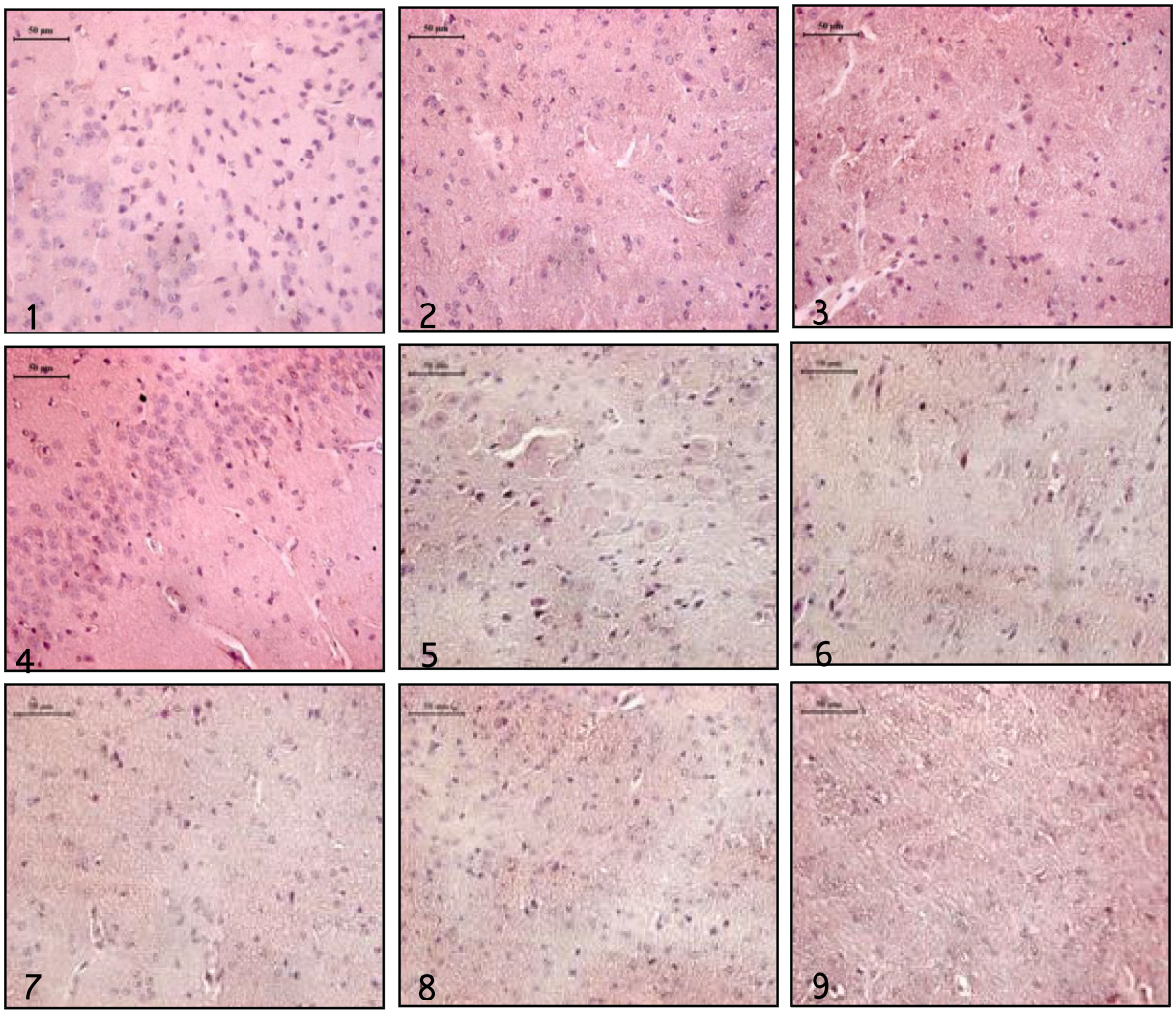


Fig. 11. Immunohistochemical localization of c-fos in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC.

toxic OP nerve agent poisoning [13,32,35]. However, antidotal efficacy of diverse pharmacological agents against such non-cholinergic changes has not been adequately addressed [14]. DFP is a nerve agent simulant, and in a recent study we elucidated several dose and time-dependent cholinergic and non-cholinergic changes after acute DFP poisoning in mice [15]. This study was immediately followed by our report on the effect of NAC in the presence or absence of ATR and/or 2-PAM on various time-dependent biochemical and oxidative changes caused by acute DFP poisoning in mice [23]. Oxidative stress is known to initiate delayed neuropathy caused by low dose long-term OP exposures [24]. Therefore, in the present study we anticipated a notable protection by NAC against a low-dose repeated exposure of DFP.

Oxidative stress is considered as a therapeutic target during OP poisoning. Antioxidants, particularly the free radical scavengers, and various endogenous antioxidant mimetics have been extensively explored against OP nerve agent poisoning [3,14,17]. NAC is a cell permeable GSH prodrug that acts by directly scavenging the free radicals and restoring GSH and cysteine levels in the tissue. NAC is a small molecule that after internalization into the cell, it is deacylated to cysteine. GSH and multiple GSH-linked antioxidant enzymes are essential for protecting mitochondria from oxidative damage [19,36]. NAC is also known to confer protection against chronic bronchitis, chronic obstructive pulmonary disorder, and neurodegenerative diseases [37,38]. In addition to its strong antioxidant potential, it has mucolytic properties and is likely to circumvent the pulmonary function deficits after OP toxicity [37].

In our previous study, mice were administered 0.50 LD_{50} DFP once and various biochemical and oxidative changes were measured 1 h, 24 h and 7 d post-exposure. Here, plasma BChE and brain AChE levels were diminished only up to 24 h and 7 d post-exposure, respectively [23]. In a separate study, 2-PAM (30 mg/kg, i.m.), ATR (10 mg/kg, i.p.), and NAC (150 mg/kg, i.p.) were given alone in mice. These treatments were not found to produce any sign or symptoms of toxicological significance (data not shown), and for brevity, these groups have not been included here. In our present study, 0.125 LD_{50} DFP was administered daily for 21 d and plasma BChE was measured after 7, 14 and 21 d of exposure, while other parameters in tissue homogenate were measured after 21 d of exposure. The present study shows diminished BChE and AChE levels even 21 d post-exposure. Possibly, due to low-dose sub-acute exposure, the normalization of crucial enzymes like BChE and AChE was very minimal. Decreased brain GSH, GPx, CAT (24 h), SOD (1 h), and elevated MDA (24 h) levels were observed in earlier study [23]. In the present study, decreased GSH, CAT and SOD (brain and liver), GPx and GR (brain), and increased MDA (brain) levels were observed 21 d post-exposure. Lipid peroxidation is known to initiate oxidative stress, and elevated MDA levels are indicator of lipid

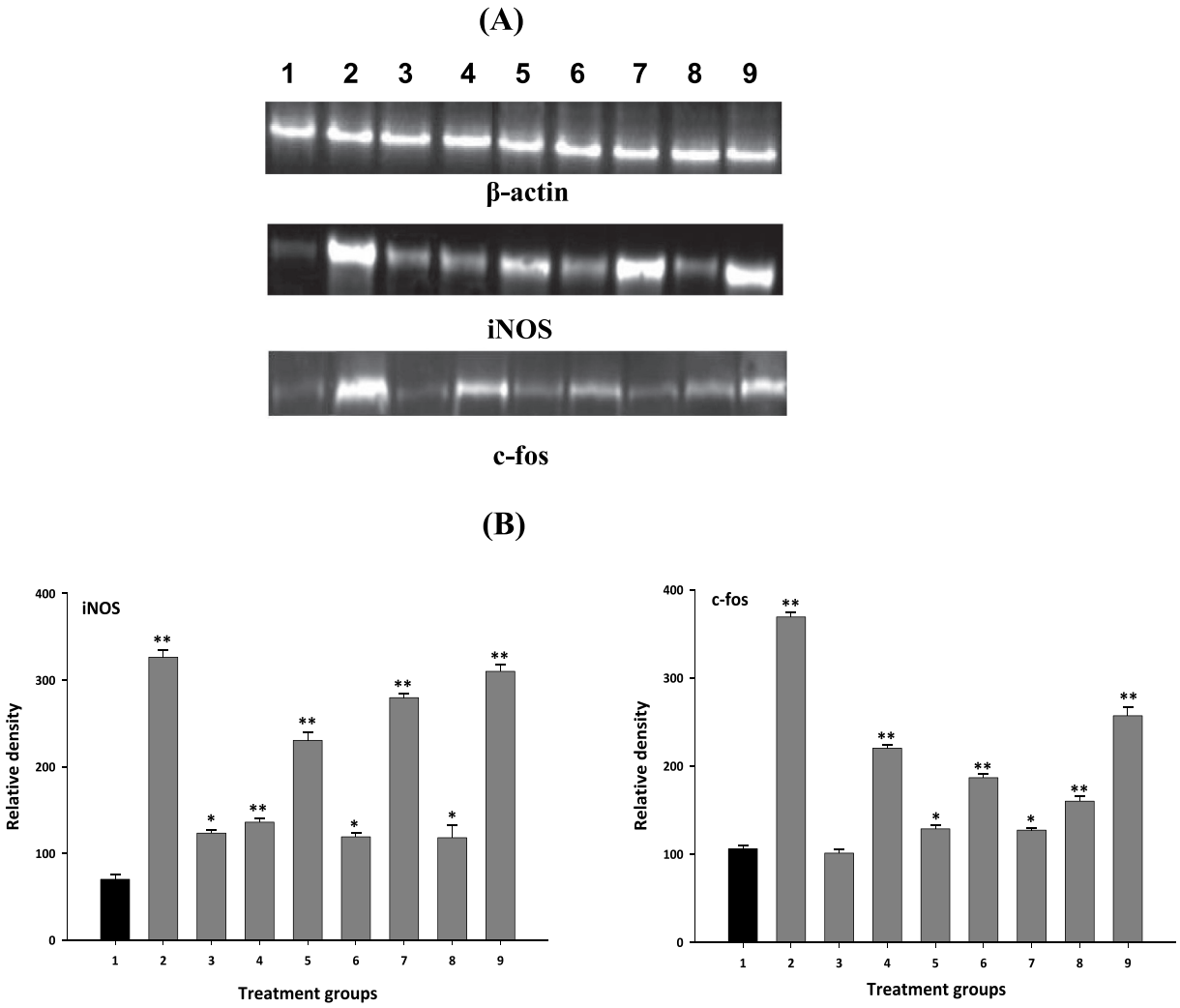


Fig. 12. Western blot (A) and densitometric analysis (B) of iNOS and c-fos protein expression in mice brain 21 d after various treatments: (1) Vehicle control; (2) DFP (0.125 LD_{50}, s.c.); (3) DFP + ATR (10 mg/kg bwt, i.p., 0 min); (4) DFP + 2-PAM (30 mg/kg bwt, i.m., 0 min); (5) DFP + NAC (150 mg/kg bwt, i.p., −60 min); (6) DFP + ATR + NAC; (7) DFP + 2-PAM + NAC; (8) DFP + ATR + 2-PAM; and (9) DFP + ATR + 2-PAM + NAC. Values are mean ± SEM; $n = 6$.
*Significantly different from corresponding control at $p < 0.05$.
**Significantly different from corresponding control at $p < 0.01$.

peroxidation [27]. Enhanced lipid peroxidation may occur due to direct interaction of OP with the cellular membrane and ROS [7]. Oxidative stress can also be evidenced by altered levels of antioxidant enzymes [33,34]. Antioxidant enzymes viz., GPx, GR, CAT, and SOD play crucial role in the metabolism of ROS, thereby preventing oxidative injury to the cells. Inhibition of antioxidant enzymes can be correlated with compromised cellular GSH contents and increased ROS [31]. Our present observations can be correlated with a previous work, where authors opined that exposure to acute dose of OP pesticide could lead to accumulation of lipid peroxides, but repetitive dose could increase lipid peroxidation and decreased antioxidants enzymes in various tissues [39]. Compared to our previous study [23], the present study shows delayed but more conspicuous protection by NAC, possibly due to sufficient time allowed to create an adequate pool of cysteine (sulfur amino acid) in challenging the OP toxicity [6]. Since oxidative stress is an important phenomenon underlying seizures and delayed neuropathy caused by OPs, the protective efficacy of NAC becomes obvious [14]. The present study also revealed various histopathological changes in various organs, particularly the brain, and all the changes favorably responded to various treatments involving NAC. There are several reports on OP pesticides-induced histopathological changes in rats after repeated exposures [40,41], and their attenuation by antioxidants [39,40].

Nitric oxide (NO) is a chemical messenger and a free radical produced during various biochemical processes catalyzed by nitric oxide synthase (NOS). NOS can cause oxidative damage to many cell types and neurons [10,11,42]. In the present study, alterations in iNOS expression after DFP were similar to that observed with malathion and mevinphos intoxication in rats [43,44]. Modulation of iNOS expression leads to excessive NO generation; the nitrosative stress may compound the oxidative stress. In the present study, identical results were obtained in immunohistochemistry of brain tissues and Western blot, while using the same protein. It is surprising to note that adjunction of NAC abrogated various oxidative and histopathological changes but failed to normalize the iNOS expression. Activation of any receptor in cholinergic or non-cholinergic pathway by neurotransmitters, oxidative stress or any other stimuli leads to a sequence of post transcriptional modifications, most likely phosphorylation. OP exposure is known to cause excessive phosphorylation, which may lead to modifications in phosphorylated cyclic adenosine monophosphate (cAMP) response element binding protein (p-CREB) [45,46]. This leads to activation of inducible transcription factors (ITFs) such as c-fos and c-jun within a short time [47]. Alterations in c-fos expression after OP pesticide poisoning have been associated with change in peripheral cytokine levels

and long-term neurobehavioral deficits [48]. In the present study, we assume that DFP-induced oxidative stress possibly evoked immediate early genes (IEGs), which resulted in significant increase in basal levels of c-fos. This could further result in activation of a cascade of target gene proteins involved in the development OP-induced neurotoxicity [49]. Although, amelioration of c-fos expression by NAC is known [47], we could not observe similar phenomenon in the present study. IEGs are known to be unstable and most rapidly degrading proteins [50]. Possibly, this could be the cause of inconsistency in c-fos levels in different treatments. The immunohistochemical localization of iNOS and c-fos activity was similar to the Western blot assay.

ATR and 2-PAM combination is the most popular treatment for OP poisoning. However, adjunction of diverse pharmacological agents has been sought to increase their efficacy, particularly to resolve the OP-induced delayed neuropathy [9,14]. Although, most of the drugs are intended to be used therapeutically, efficacy of many drugs has been experimentally established as a prophylaxis as well [51]. On the basis of our previous study [23], NAC was anticipated to confer protection against sub-acute toxicity of OP, which has been testified in the present study. Its protective efficacy was evidenced by amelioration of oxidative stress and histopathological changes. However, its effects on i-NOS and c-fos gene expression were not very appreciable.

5. Conclusion

The present study shows the beneficial effects of NAC as an adjunct with ATR and 2-PAM in alleviating the sub-acute toxicity of DFP in mice. The study indicates possible role of NAC in the management of OP poisoning.

Competing interests

The authors declare that there are no conflicts of interest.

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