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Dose and Time-dependent Alterations in Various Cholinergic and Non-cholinergic Markers after Organophosphate Poisoning: Possible Role in Diagnosis

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ABSTRACT

Toxicity of organophosphates (OP) is mainly ascribed to inhibition of acetylcholinesterase (AChE) enzyme at the cholinergic synapses. This results in cholinergic crisis leading to various muscarinic, nicotinic and central effects. Additionally, there are several non-cholinergic effects of OP which are likely to exacerbate the toxicity and complicate diagnosis. The present study reports the dose (0.125 - 4.0 LD50) and time (1 h - 14 d)- dependent acute effect of diisopropyl phosphorofluoridate (DFP) on mice body weight, organ-body weight index (brain), butyrylcholinesterase (BChE) and β-glucoronidase (BG) activity in plasma, AChE activity, reduced glutathione (GSH) and malondialdehyde (MDA) levels in brain, and DNA damage in brain (agarose gel electrophoresis) and blood (comet assay). The study reveals a dose and time- dependent BChE inhibition up to 24 h and AChE inhibition up to 7 d. However, elevated BG levels were observed up to 1 h only after 1.0, 2.0, and 4.0 LD50 DFP. Diminished GSH levels up to 24 h and increased MDA levels at 4 h indicated oxidative stress. None of the treatments produced any DNA damage in soft tissues. In addition to cholinesterase, the study suggests possible relevance of measuring BG levels (non-cholinergic marker) in the diagnosis of OP poisoning.

Keywords: Organophosphate, DFP, acute toxicity, cholinergic and non-cholinergic markers, oxidative stress, DNA damage

1. INTRODUCTION

Since long organophosphate (OP) compounds have been extensively used as insecticides (malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, etc.) and herbicides (tribufos, merphos, etc.). OPs also find wide applications in the industry (plasticizers, antioxidants, softening agents, flame retardants, gasoline additives, etc.) and medical management of Myasthenia gravis and Glaucoma[1-3]. Extensive use of these compounds is responsible for various accidental and intentional exposures causing morbidity and mortality. Additionally, OP nerve agents (soman, sarin, tabun, VX, etc.) have been widely used in chemical warfare. Use of such agents in terrorism has escalated in the recent past, which is a matter of concern to both civil and military authorities[4-7].

Toxicity of OPs is primarily attributed to irreversible inhibition of acetylcholinesterase (AChE) enzyme at the cholinergic synapses. This results in the accumulation of acetylcholine (ACh), a neurotransmitter at the synaptic junction causing overstimulation and subsequent disruption of nerve impulse transmission in both central and peripheral nervous system. The cholinergic crisis involves various muscarinic, nicotinic and central effects[1,4,6]. The current treatments of OP poisoning like atropine and pralidoxime (2-PAM) are intended to alleviate such effects[8].

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Signs and symptoms of OP poisoning are predictable and intensely correlate with AChE activity[7]. However, OP toxicity is not mediated through cholinergic crisis alone. There are observed changes in non-cholinergic markers as well, which usually exacerbate the OP toxicity and complicate the diagnosis. These changes possibly could occur as secondary manifestations of cholinergic crisis[9]. Such effects include altered blood gas analysis, hypokalemia, hyperglycemia, acute pancreatitis, proteinuria, altered liver and kidney function test, etc. OP- induced generation of reactive oxygen species (ROS) leading to lipid peroxidation, oxidative stress and programmed cell death are also reported[10-14]. Usually the bed side tests to confirm OP poisoning include measurement of blood AChE and butyrylcholinesterase (BChE) activity[10]. Other techniques like detection of OP-adducts and antibodies have also been explored to confirm OP poisoning[11]. Measurement of plasma β-glucoronidase (BG) activity is also suggested as a biomarker of OP poisoning[15,16]. In the absence of a comprehensive study[17], we report here the acute effect of diisopropyl phosphorofluoridate (DFP) on AChE, BChE and BG activity in soft tissues of mice. Also, reduced glutathione (GSH) and malondialdehyde (MDA) levels were measured to assess oxidative stress, together with DNA damage in brain (agarose gel electrophoresis) and blood (comet assay). DFP is an anti-cholinesterase (OP) agent with structural similarity with Sarin, and is widely used as a simulant of nerve agent in experimental OP poisoning[18,19].

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Ethylenediaminetetraacetic acid (EDTA), Trizma base, o-phthaldialdehyde (OTP), Triton X-100, dimethyl sulfoxide (DMSO), acetylthiocholine iodide, 5, 5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), phenolphthalein mono-β-glucosiduronic acid, 2-thiobarbituric acid (TBA), ethidium bromide, sodium dodecyl sulphate (SDS), and normal melting point agarose (NMPA) were purchased from Sigma-Aldrich Inc. (St. Louis, USA). DFP (> 99 per cent purity), low melting point agarose (LMPA), and all other chemicals, reagents and buffers of highest purity were procured from Merck India Ltd. (Mumbai, India).

2.2 Animals

Male Swiss Albino mice (25 g - 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 as the bedding material with free access to food (Ashirwad Brand, Chandigarh, India) and water ad libitum. They were maintained in controlled environmental conditions of ambient temperature (22 ± 2 °C) and relative humidity of 40-60 per cent 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 Treatment

The acute (24 h) median lethal dose (LD50) of DFP by subcutaneous (s.c.) route in mice was found to be 3.86 (2.48 - 6.12) mg/kg. Values in parenthesis are fiducial limits at 95 per cent confidence interval (our unpublished work). A total of one hundred and eighty two animals were randomised and used for the present study. Initially 170 animals were divided into four groups of thirty and one group of fifty animals each as follows: (1) Control; n= 30, (2) 0.125 LD50 DFP; n= 30, (3) 0.25 LD50 DFP; n= 30, (4) 0.50 LD50 DFP; n= 30, and (5) 1.0 LD50 DFP; n= 50. In group 5 only, the surviving animals (n = 6) were considered for the study. Additional two groups (2.0 and 4.0 LD50 DFP) of six animals each were included in BG assay studies alone. Control animals received equivalent amount of carrier (10 per cent PG-NS; normal saline- propylene glycol). Animal body weight was recorded daily and blood was collected from the retro-orbital plexus of ether anesthetised animal at 1 h, 4 h, 24 h, 7 d, and 14 d post-exposure, using heparinised capillary. Blood was used for comet assay or single cell gel electrophoresis (SCGE) and measurement of BChE and BG activity in plasma. Thereafter, animals were killed by cervical dislocation and the brain was excised quickly. The brain was rinsed in 0.9 per cent saline, blotted and weighed to determine the Organ-Body Weight Index (OBI). The OBI (Brain) was calculated as the ratio of brain weight X 100/ animal body weight.

2.4 BChE and AChE Estimation

Cholinesterase activity in blood plasma and brain tissue homogenate were determined by the method of Ellman[20] , *et al*. For the measurement of AChE, a 10 per cent brain homogenate was prepared in enzymatic buffer using tissue rupter for 3 min - 5 min at 4 °C. In this method, 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). For the measurement of plasma BChE a 10 µl enzyme source was taken. The plasma BChE and brain AChE activities were expressed as µmol acetylthiocholine hydrolysed/min/ml blood and µmol acetylthiocholine hydrolysed/min/g wet tissue, respectively.

2.5 BG Estimation

The amount of BG released in plasma was measured by the method of Fishman[21], *et al.* Measurement of BG activity was initiated by adding 100 µl of 6 mM substrate (phenolphthalein mono β-glucosiduronic acid) in 100 µl of 0.2 M sodium acetate buffer which was followed by addition of 50 µl of sample. Above mixture was kept at 37 °C in incubator for 4 h after which 0.75 ml H_2O and 0.5 ml glycine NaOH buffer (200 mM; pH 11.7) containing 0.2 per cent SDS was added. Centrifugation of the above samples was done at 1000 g for 15 min and absorbance of supernatant was measured at 540 nm on a micro plate reader.

2.6 GSH Estimation

GSH levels were assayed in tissue homogenate following the method of Hissin and Hilf[22]. Briefly, 1.5 per cent homogenate was prepared in 1.92 ml phosphate EDTA buffer (pH 8.0), to this 0.5 ml MPA (metaphosphoric acid) 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.

2.7 MDA Estimation

To assess the amount of lipid peroxidation, MDA levels in brain tissue was assayed following the method of Okhawa[23], *et al.* Briefly, a 10 per cent tissue homogenate (in 1.15 per cent KCl) was prepared and centrifuged for 10 min at 6000 rpm. To 0.1 ml of above supernatant, 0.2 ml of 8.1 per cent SDS was added, followed by the addition of 1.5 ml of 20 per cent acetic acid (pH 3.5) and 1.5 ml of 0.8 per cent 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.

2.8 Comet Assay

Comet assay was performed by the method of Singh[24], *et al.* with some minor modifications. Microscopic slides were washed using 10 per cent chromic acid. Dust free slides were then dipped in 1 per cent NMPA and allowed to solidify. 10 per

cent whole blood was mixed with 1 per cent LMPA and poured onto the slides. Slides were covered with cover slips and kept on ice for 10 min. Cover slips were removed and second layer of 1 per cent LMPA was added onto the slides which were again covered with new cover slips on ice. After 10 min, cover slips were removed and slides were dipped in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1 per cent Triton X-100 and 10 per cent DMSO) and kept at 4 °C for 2 h. The above procedure was performed in dark to minimize DNA damage. After 2 h, slides were removed from lysis buffer and excess buffer was removed from surface. Thereafter, the slides were processed for electrophoresis. Slides were incubated in running buffer (10 N NaOH, 200 mM EDTA) for 30 min to unwind DNA. Electrophoresis of slides was carried out at 24 V and 300 mA for 30 min. Slides were then placed on a dry surface and neutralisation buffer (0.4 M Tris, pH 7.5) were added on slides for 3 times at interval of 5 min. Slides were then stained with ethidium bromide for 5 min and washed with chilled distilled water. Cover slips were placed and slides were scored immediately at 40x under fluorescent microscope (Leica, Germany) for the visualisation of image.

2.9 DNA Isolation and Fragmentation

DNA isolation and fragmentation assay was performed by phenol:chloroform method of Sambrook and Russell[25]. DNA isolated from a 10 per cent brain homogenate was electrophoresed on 1.2 per cent agarose gel impregnated with ethidium bromide; 1 kb DNA served as standard. Gel was visualised under UV gel imaging system (FluorChem™ SP, Alpha-Innotech, USA).

2.10 Data analysis

The results are expressed as mean ± SEM (n=6). The data were analysed 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

The dose and time- dependent effect of DFP on mice body weight is depicted in Fig. 1. Animals received s.c. injection of various doses of DFP and were monitored for animal weight gain from 0 d to 14 d. Control animals received equivalent amount of carrier (PG-NS). Each group comprised of 6 animals, except DFP 1.0 LD50 group, where 10 animals were treated and only 6 surviving animals were included in the experiment. The data show that none of the treatments exhibited any adverse effect on animal body weight gain and all the treatment groups were comparable to control. Animal weight in control groups usually varied between 27.0 ± 0.83 g and 31.0 ± 2.3 g.

3.2 Organ-Body Weight Index (Brain)

Figure 2 illustrates the OBI (brain) in different treatment groups at various time intervals. None of the treatments exerted any deleterious effect on OBI (brain) when compared to control, which in case of control animals varied between 1.16 ± 0.04 and 1.25 ± 0.02.

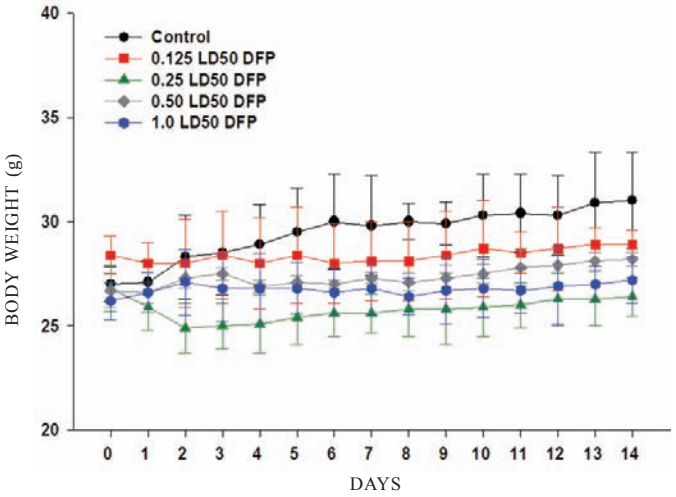

Figure 1. Effect of DFP on body weight of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6).

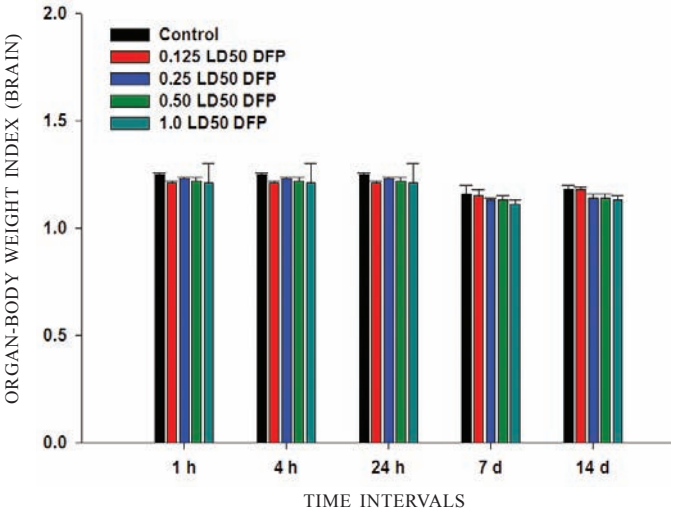

Figure 2. Effect of DFP on organ-body weight index (brain) of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6).

3.3 BChE and AChE Activity

DFP- induced dose and time- dependent plasma BChE inhibition is shown Fig. 3. All the doses of DFP significantly ($p< 0.01$) inhibited the enzyme activity up to 24 h post exposure compared to control. The mean enzyme activity in control plasma was 3.47±0.18 µmol acetylthiocholine hydrolysed/ min/ ml blood. Maximum BChE inhibition of 98 per cent was observed in 1.0 LD50 DFP at 1 h, which progressively decreased to 68 per cent by 24 h. Similarly, 0.125 LD50 DFP showed 91 per cent inhibition at 1 h, which progressively tapered to 37 per cent by 24 h. A conspicuous dose and time-dependent inhibition of the enzyme was observed. With the passage of time, a progressive reactivation of the enzyme was visible in all the treatment groups, and enzyme activity was found to normalize by 7 d post exposure. Figure 4 shows the dose and time- dependent effect of DFP on brain AChE activity. The data

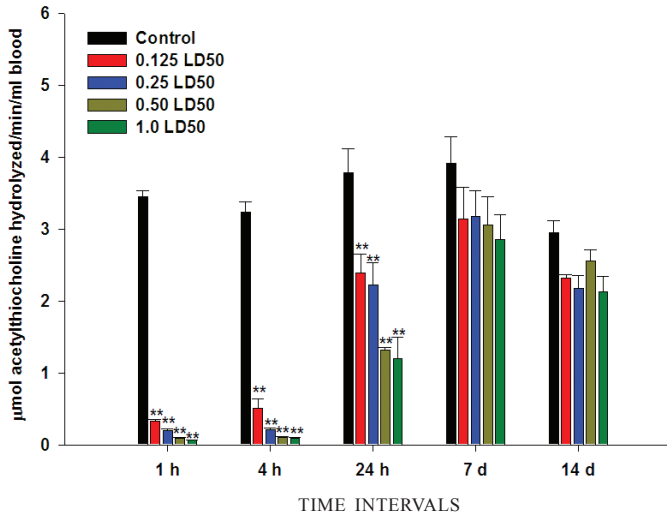

Figure 3. Effect of DFP on plasma butyrylcholinesterase activity of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6). Statistically significant at **p< 0.01 compared to corresponding control.

reveal a noticeable dose and time- dependent inhibition of the enzyme up to 7 d post exposure. The mean enzyme activity in control brain homogenate was estimated to be 7.32 ± 0.23 µmol acetylthiocholine hydrolysed/ min/ g wet tissue. Maximum AChE inhibition of 92 per cent was observed in 1.0 LD50 DFP at 1 h, which progressively decreased to 54 per cent by 7 d. Similarly, 0.125 LD50 DFP showed 36 per cent inhibition at 1 h, which progressively decreased to 8 per cent by 7 d. AChE inhibition in 0.25 LD50 and 0.125 LD50 DFP was not significant compared to control after 24 h, while the enzyme in 1.0 LD50 and 0.50 LD50 group remained significantly inhibited up to 7 d. The enzyme activity in all the treatment groups was found to normalize by 14 d post exposure.

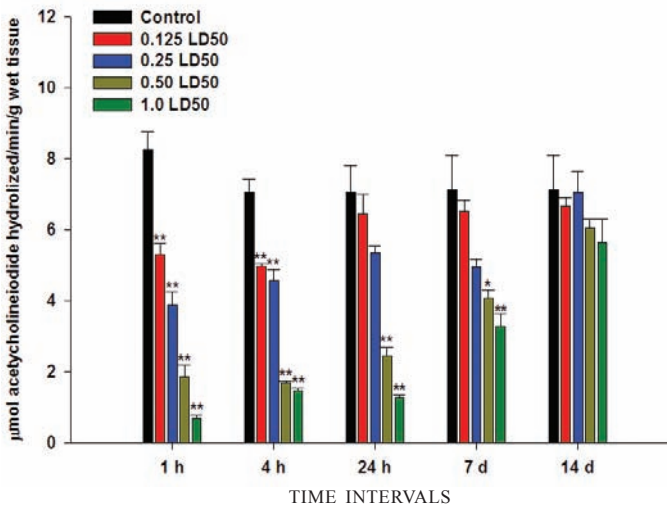

Figure 4. Effect of DFP on brain acetylcholinesterase activity of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6). Statistically significant at **p< 0.01 compared to corresponding control.

3.4 BG Activity

A dose and time- dependent effect of DFP on plasma BG activity in mice is illustrated in Fig. 5. None of the treatments at 0.125 LD50 - 0.50 LD50 DFP produced any change in plasma BG levels at any time point. However, in 1.0 LD50 group at 1 h alone, a significant (p<0.05) increase in BG activity was observed compared to control. When the dose of DFP was further raised to 2.0 LD50 and 4.0 LD50, the BG levels were found to be elevated further in a dose-dependent manner. In these groups all the animals were bled immediately prior to death (10 min - 15 min). Here, the increase in BG levels was visible at 1 h post exposure alone.

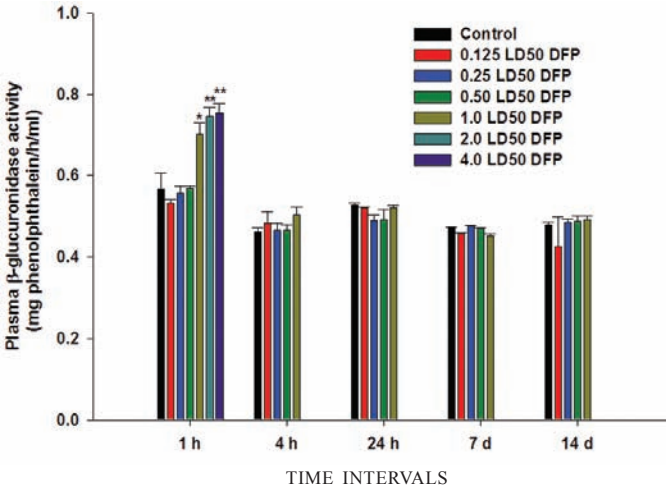

Figure 5. Effect of DFP on plasma β -glucuronidase activity of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Additional groups of 2.0 and 4.0 LD50 DFP were included here, where the animals were bled just prior to death (10 min - 15 min). Values are mean ± SEM (n= 6). Statistically significant at *p< 0.05 and **p< 0.01 compared to corresponding control.

3.5 GSH and MDA Levels

Figure 6 depicts the GSH levels in brain homogenate after various treatments. All the doses of DFP significantly diminished the GSH levels at 1 h. However, significant decrease compared to corresponding control was visible up to 24 h post exposure after 0.50 LD50 and 1.0 LD50 DFP alone. No appreciable dose or time-dependent effect was visible. GSH levels in DFP treated groups were found to be comparable to control after 7 d. MDA levels in brain homogenate after various treatments are shown in Fig. 7. The MDA levels were found to be significantly elevated (p<0.05) in 0.5 LD50 and 1.0 LD50 DFP at 4 h post exposure alone. No significant change in MDA levels was observed in any other dose or time point.

3.6 DNA Damage

The comet assay or SCGE was performed in mice blood from all the treatment groups at all the time points. Representative images of lymphocyte comets from control and 1.0 LD50 DFP treated group after 14 d exposure are depicted in Fig. 8 (a) and 8 (b), respectively. On the basis of product of tail length and relative tail intensity, no significant difference was observed between control and DFP treated groups, thereby not

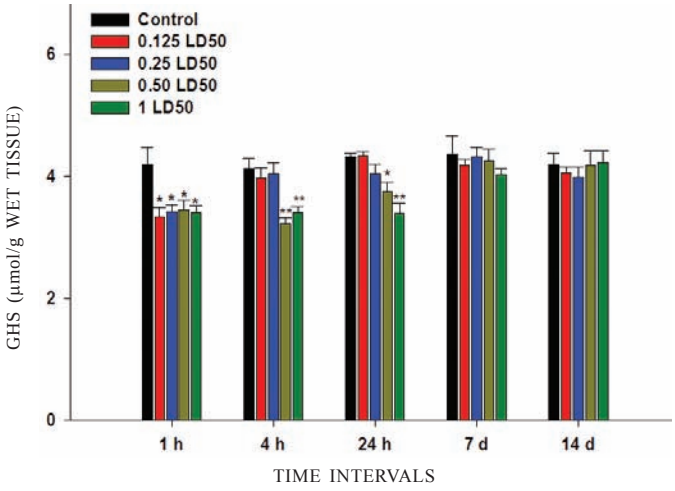

Figure 6. Effect of DFP on brain reduced glutathione (GSH) levels of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6). Statistically significant at *p< 0.05 and **p< 0.01 compared to corresponding control.

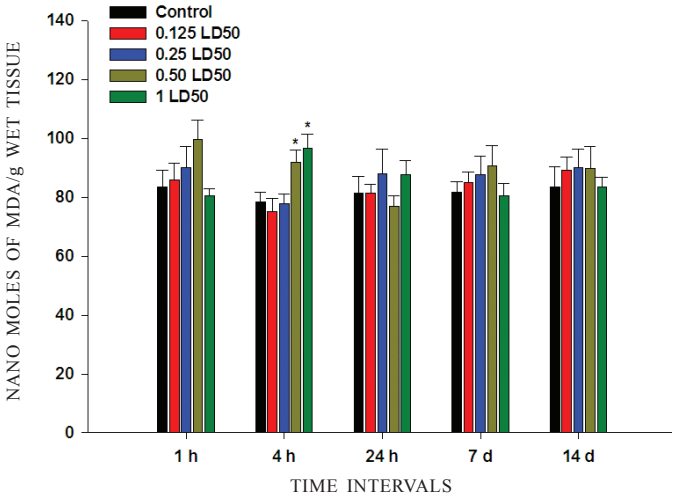

Figure 7. Effect of DFP on brain malondialdehyde (MDA) levels of mice. Animals received various doses of DFP (s.c.). Control animals received equivalent amount of carrier. Values are mean ± SEM (n= 6). Statistically significant at *p< 0.05 compared to corresponding control.

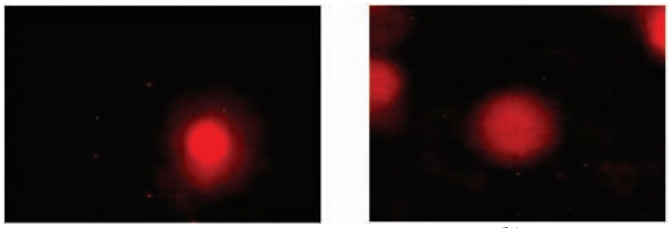

Figure 8. Representative photomicrograph of comet assay performed in mice blood. (a) Control and (b) 1.0 LD50 DFP 14 d post exposure. Magnification: 40x.

indicating any DNA damage. Figure 9 represents the agarose gel electrophoresis for qualitative analysis of DNA extracted from whole brain homogenate of mice receiving various treatments after 14 d of exposure. Comparison of control gel with 0.125 LD50 - 1.0 LD50 DFP did not show any change in gel pattern indicating that none of the treatments caused any necrotic or apoptotic kind of DNA damage.

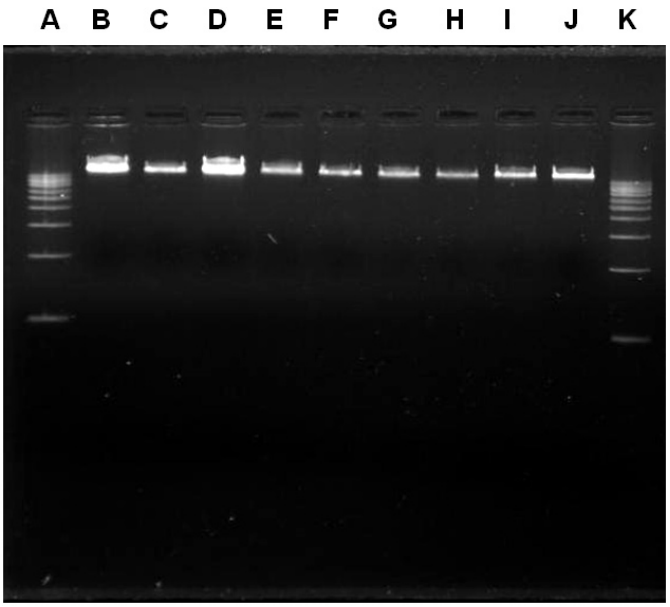

Figure 9. Representative photomicrograph of agarose gel electrophoresis of DNA extracted from whole brain of mice. Analysis was performed 14 d after exposure to various doses of DFP (s.c.). Control animals received equivalent amount of carrier. The lanes represent: (A) 1kb DNA ladder; (B) vehicle control; (C) 0.125 LD50 DFP-R1; (D) 0.125 LD50 DFP-R2; (E) 0.25 LD50 DFP-R1; (F) 0.25 LD50 DFP-R2; (G) 0.50 LD50 DFP-R1; (H) 0.50 LD50 DFP-R2; (I) 1.0 LD50 DFP-R1; (J) 1.0 LD50 DFP-R2 and (K) 1kb DNA ladder.

4. DISCUSSION

Identification of different biomarkers is crucial for correctly establishing the exposure of poison. At the same time, it is equally important to characterise such biomarker for designing appropriate therapeutic measures. Blood cholinesterases (AChE and BChE) have been widely used for monitoring OP poisoning for the following reasons:

(i) They react rapidly with OP at even low concentrations,
(ii) Good correlation exists between inhibition of esterases and acute sign and symptoms,
(iii) Besides BChE being present in plasma, AChE is also present in human red cells, and
(iv) The assay methods for both the esterases are simple and inexpensive[10,11,26].

However, susceptibility of cholinesterase is known to vary for different OPs due to existence of various isoenzymes with different turnover rates and region specificity[27,28].

Measurement of plasma BG levels has also been employed as a sensitive non-cholinergic biomarker of OP pesticide poisoning. BG and egasyn (carboxylesterase isozyme and

accessory protein of BG) are known to exist as a complex in liver microsomal membrane. The complex can easily dissociate in the presence of OP to release high levels of BG into the plasma[15,16]. Measurement of antioxidants enzyme, certain cytogenetic biomarkers such as chromosomal aberration, sister chromatid exchange, DNA fragmentation and SCGE have also been used for the detection of exposure to commonly used OP pesticides[11,29-31]. SCGE is a micro gel electrophoresis technique which detects DNA damage in individual cells[30]. However, most of these studies have been limited to OP pesticide exposures, and not applied to more toxic OP compounds like DFP and nerve agents. Therefore, the present study was designed to evaluate dose and time- dependent effect of DFP on various cholinergic and non-cholinergic markers by integrating some of the important biomarkers discussed above. Also, it was of importance to see that how long the changes were appreciably visible, and could be employed for confirming OP exposure even after a lapse of time. Some of the markers could be used as bed side tests, while others can be measured in tissues only in post mortem cases for retrospective confirmation of OP exposure.

OP pesticide- induced changes in animal body weight and relative organ-body weight ratio are known[32]. Also, brain AChE inhibition- induced down regulation of muscarinic receptors, decreased brain DNA synthesis, and reduced brain weight in offsprings are reported after chronic OP pesticide exposure[33]. However, in the present study no such phenomenon was observed. A significant inhibition of plasma BChE activity up to 24 h and brain AChE activity up to 7 d post exposure confirms the reliability of both the esterases in the diagnosis of OP poisoning. Also both the esterases exhibited a dose and time- dependent response to DFP. Although, percent inhibition of BChE was more intense compared to AChE, the effect on later was more lasting, indicating slower reactivation of AChE compared to BChE. Susceptibility of esterases to OP depends on their isoenzyme variants and turnover rates[27,28]. Usually, the effect of OP exposure is predominantly found in plasma cholinesterase[34]. Also, the trend of BChE and AChE inhibition in the present study was almost similar to another report[19]. The half- life of BChE is approximately 8 h to 16 h, and usually inhibition greater than 75 per cent is associated with neuromuscular blockade and pathophysiology[10]. If this is to be believed, then the time window in the present study was sufficient for the part reactivation of the inhibited enzyme and synthesis of the new enzyme as well. Hydrolysis is a rate-limiting reaction by which the phosphorylated cholinesterases are partly reactivated[1,8].

In the present study plasma BG levels were significantly elevated only after lethal doses of DFP, that too only up to 1 h post exposure. Whereas even low doses of OP pesticides have caused many fold increase in plasma BG levels[15,16]. This could be possibly due to difference in dissociation coefficient of BG-egasyn complex in case of DFP. Nevertheless, the role of BG as a non-cholinergic indicator of OP poisoning cannot be undermined. Enzymatic and non-enzymatic markers of oxidative stress have been shown to play a crucial role in the diagnosis of OP poisoning[1,8,9,29]. In present study, diminished GSH levels corroborate such findings. Although, increase in brain MDA levels appeared to be little delayed. Measuring the levels of various antioxidant enzymes would possibly ascertain their role in the diagnosis of OP poisoning. Our present study did not reveal any DNA damage, indicating that acute OP exposure is not likely to produce any DNA damage unlike chronic OP pesticide exposures[30,31].

5. CONCLUSION

The present study indicates the relevance of measuring plasma BG levels together with cholinesterases to confirm acute OP poisoning. Also, non-specific, oxidative stress markers could supplement the standard biomarkers. More sensitive and reliable non-cholinergic biomarkers of OP poisoning remain to be identified in near future. This study could be of enormous importance to the clinicians involved in the diagnosis of OP poisoning.

REFERENCES

1. Watson, A.; Opresko, D.; Young, R.; Hauschild, V.; King, J. & Bakshi, K. Organophosphate nerve agents. *In* Hand book of toxicology of chemical warfare agents, edited by R.C. Gupta. Academic Press, CA, USA, 2009, pp. 43-67. doi: 10.1016/B978-0-12-374484-5.00006-7
2. Satoh, T. & Hosokawa, M. Organophosphate and their impact on the global environment. *Neurotoxicol.,* 2000, **21**(1-2), 223-7.
3. Paudyal, B.P. Organophosphorus poisoning. *J. Nepal Med. Assoc.*, 2008, **47**(172), 251-8.
4. Afshari, R.; Majdzadeh, R. & Balali-Mood, M. Pattern of acute poisonings in Mashhad, Iran 1993-2000. *J. Toxicol. Clin. Toxicol.,* 2004, **42**(7), 965-75. doi: 10.1081/CLT-200042550
5. Seto, Y. The sarin gas attack in Japan and the related forensic investigation. Organization for the Prohibition of Chemical Weapons. *In* Hand book of toxicology of chemical warfare agents, edited by R.C. Gupta, Academic Press, CA, USA, 2001, pp. 847-76.
6. Golime, R.; Afley, P.; Acharya, J. & Bhattacharya, B.K. Efficacy of antidotes (midazolam, atropine and HI-6) on nerve agent induced molecular and neuropathological changes. *BMC Neurosci.,* 2014, **15**(47), 1-11.
7. Okumura, T.; Taki, K.; Suzuki, K. & Satoh, T. The Tokyo subway sarin attack: A toxicological whole truth. *In* Hand book of toxicology of chemical warfare agents, edited by R.C. Gupta, Academic Press, CA, USA, 2009, pp. 25-32. doi: 10.1016/B978-0-12-374484-5.00004-3
8. Balali-Mood, M. & Saber, H. Recent advances in the treatment of organophosphorous poisonings. *Iran J. Med. Sci.,* 2012, **37**(2), 74-91.
9. Qian, Y.; Venkatraj, V.; Barhoumi, R.; Pal, R.; Datta, A.; Wild, J.R. & Tiffany-Castiglioni, E. The non-cholinergic mechanisms of organophosphorus toxicity in neural cell cultures. *Toxicol. Appl. Pharmacol.*, 2006, **219**(2-3), 162-71. doi: 10.1016/j.taap.2006.11.030
10. Jun, D.; Bajgar, J.; Kuca, K. & Kassa, J. Monitoring of blood cholinesterase activity in workers exposed to nerve agents. *In* Hand book of toxicology of chemical warfare agents, edited by R.C. Gupta, Academic Press, CA, USA,

2009, pp. 877-86.
doi: 10.1016/B978-012374484-5.00058-4

11. Lockridge, O.; Schopper, L.M. & Masson, P. Biomarkers of exposure to organophosphorus poisons: A new motif for covalent binding to tyrosine in proteins that have no active site serine. *In* Hand book of toxicology of chemical warfare agents, edited by R.C. Gupta, Academic Press, CA, USA, 2009, pp. 953-66.
doi: 10.1016/B978-012374484-5.00056-0
12. Pazdernik, T.L.; Emerson, M.R.; Cross, R.; Nelson, S.R. & Samson, F.E. Soman induced seizures: Limbic activity, oxidative stress and neuroprotective proteins, *J. Appl. Toxicol.,* 2001, **21**(1), S87–S94. doi: 10.1002/jat.818
13. Ranjbar, A.; Solhi, H.; Mashayekhi, F.J.; Susanabdi, A.; Rezaie, A. & Abdollahi, M. Oxidative stress in acute human poisoning with organophosphorus insecticides; a case control study. *Environ. Toxicol. Pharmacol*., 2005, **20**(1), 88-91. doi: 10.1016/j.etap.2004.10.007
14. Spradling, K.D. & Dillman III, J.F. The molecular toxicology of chemical warfare nerve agents. *In* Advances in molecular toxicology, edited by J.C. Fishbein, Elsevier Science Publisher B.V., Amsterdam, The Netherlands, 2011, pp. 111-44.
doi: 10.1016/B978-0-444-53864-2.00003-7
15. Soltaninejad, K.; Shadnia, S.; Afkhami-Taghipour, M.; Saljooghi, R.; Mohammadirad, A. & Abdollahi, M. Blood β-glucuronidase as a suitable biomarker at acute exposure of severe organophosphorus poisoning in human. *Hum. Exp. Toxicol*., 2007, **26**(12), 963-6.
doi: 10.1177/0960327107085349
16. Ueyama, J.; Satoh, T.; Kondo, T.; Takagi, K.; Shibata, E.; Goto, M.; Kimata, A.; Saito, I.; Hasegawa, T.; Wakusawa, S. & Kamijima, M. Glucuronidase activity is a sensitive biomarker to assess low-level organophosphorus insecticide exposure. *Toxicol. Lett.*, 2010, **193**(1), 115-19. doi: 10.1016/j.toxlet.2009.12.009
17. Mahle, D.A. Omic' evaluation of the region specific changes induced by non-cholinergic diisopropylfluorophosphate (DFP) exposure in Fischer 344 rat brain. Wright State University, OH, United States, 2012, PhD Thesis.
18. Bhattacharya, R.; Kumar, P.; Jeevaratnam, K.; Pandey, K.S. & Das Gupta, S. Therapeutic evaluation of bronchodilators as an adjunct against organophosphorous intoxication in mice. *Asia Pacific J. Pharmacol*., 1991, **6**(1), 75-80.
19. Kumar, P.; Swami, D.; Karade, H.N.; Acharya, J.; Jatav, P.C.; Kumar, A. & Meena, M.K. In vivo protection of diisopropylphosphorofluoridate (DFP) poisoning by three bis-quaternary 2 -(hydroxyimino) -N -(pyridin-3-yl) acetamide derivatives in Swiss mice. *Cell. Mol. Biol*., 2014, **60**(3), 53-9.
20. Ellman, G.E.; Courtney, K.D.; Andres jr., V. & Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, 1961, **7**(2), 88-95. doi: 10.1016/0006-2952(61)90145-9
21. Fishman, W.H.; Springer, B. & Brunetti, R. Application of an improved glucuronidase assay method to the study of human blood. *J. Biol. Chem.,* 1948, **173**(2), 449-56
22. Hissin, P.J. & Hilf, R. A fluorimetric method for determination of oxidised and reduced glutathione in tissue. *Anal. Biochem*., 1976, **74**(1), 214-26.
doi: 10.1016/0003-2697(76)90326-2
23. Okhawa, H.; Ohishi, W. & Yogi, K. Assay formulation lipid peroxidase in animal tissue by thiobarbituric acid reaction. *Anal. Biochem*., 1979, **95**(2), 351-8.
doi: 10.1016/0003-2697(79)90738-3
24. Singh, N.P.; McCoy, M.T.; Tice, R.R.& Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.,* 1988, **175**(1), 184-91. doi: 10.1016/0014-4827(88)90265-0
25. Sambrook, J. & Russell, W.D. Protocol: DNA isolation from mammalian tissue. *In* Molecular Cloning: A Laboratory Manual, edited by J. Sambrook & W.D. Russel, Cold Spring Harbor Laboratory Press, NY, USA, 2000, pp. 623-27.
26. Remor, A.P.; Totti, C.C.; Moreira, D.A.; Dutra, G.P.; Heuser, V.D. & Boeira, J.M. Occupational exposure of farm workers to pesticides: Biochemical parameters and evaluation of genotoxicity. *Environ. Intern*., 2009, **35**(2), 273-8. doi: 10.1016/j.envint.2008.06.011
27. Costa, L.G.; Cole, T.B.; Vitalone, A. & Furlong, C.E. Measurement of paraoxonase status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin. Chim. Acta*., 2005, **352**(1-2), 37-47.
doi: 10.1016/j.cccn.2004.09.019
28. Booth, G.M.; Connor, J.; Metcalf, R.A. & Larsen, J.R. A comparative study of the effects of selective inhibition of esterase isozymes for the mosquito Anopheles punctipennis. *Comp. Biochem. Physiol*., 1973, **44**(4), 1185-95.
29. Singh, M.; Sandhir, R. & Kiran, R. Erythrocyte antioxidants enzyme in toxicological evaluation of commonly used organophosphate pesticides. *Indian J. Exp. Biol.,* 2006, **44**(7), 580-3.
30. Garaj-Vrhovac, V. & Zeljezic, D. Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay: Pesticide genotoxicity revealed by comet assay. *Mutat. Res*., 2000, **469**(2), 279-85.
doi: 10.1016/S1383-5718(00)00092-9
31. Ündeger, U. & Basaran, N. Effects of pesticides on human peripheral lymphocytes in vitro: induction of DNA damage. *Arch. Toxicol*., 2005, **79**(3), 169-76.
doi: 10.1007/s00204-004-0616-6
32. Pawar, S. Toxicity of organophosphate insecticide on relative body and liver weights of mice. *Intern. J. Sci. Res*., 2015, **4**(1), 1694-6.
33. Eskenazi, B.; Bradman, A. & Castorina, R. Exposures of children to organophosphate pesticides and their potential adverse health effects. *J. Environ. Health Pers.,* 1999, **107**(3), 409-19. doi: 10.1289/ehp.99107s3409
34. Jintana, S.; Sming, K.; Krongtong, Y. & Thanyachai, S. Cholinesterase activity, pesticide exposure and health impact in a population exposed to organophosphates. *Intern. Arch. Occup. Environ. Health,* 2009, **82**(7), 833-42. doi: 10.1007/s00420-009-0422-9

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