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Original Contribution

Cardiac damage in acute organophosphate poisoning in rats: Effects of atropine and pralidoxime ☆

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Abstract Anticholinesterase poisoning is an important health problem in our country, and a complete understanding of its underlying mechanisms is essential for the emergency physician. Thus, we aimed to investigate the cardiac biochemical parameters and mortality in dichlorvos-induced poisoning in rats. Rats were randomly divided into 5 groups as control (corn oil), dichlorvos, atropine, pralidoxime, and atropine+pralidoxime groups. Immunohistochemical analyses of apoptosis and inducible nitric oxide synthase showed no change in cardiac tissue for all of the groups. Serum cholinesterase levels were suppressed with dichlorvos, and these reductions were inhibited with atropine and/or pralidoxime pretreatment. Serum levels of creatine kinase, creatine kinase-MB, cardiac troponin I, myoglobin, and N-terminal probrain natriuretic peptide were not affected with poisoning. Malondialdehyde and glutathione levels were not statistically significant between the groups. Although serum nitric oxide levels in the dichlorvos group were lower than those in the control group, cardiac nitric oxide levels in the atropine+pralidoxime group were markedly higher than those in the dichlorvos group. Atropine, pralidoxime, and atropine+pralidoxime pretreatments markedly reduced the mortality. In conclusion, our results implied that measured cardiac markers especially N-terminal probrain natriuretic peptide may not contribute to the early (first 6 hours) diagnosis of cardiotoxicity in dichlorvos-induced poisoning in rats. These results also showed that acute dichlorvos administration did not cause significant cardiac damage, and oxidative stress does not play a marked role in dichlorvos-induced poisoning. Besides, cardiac nitric oxide may produce protective effect on myocardium with atropine+pralidoxime therapy in rats.

1. Introduction

Because organophosphate (OP) compounds are the most widely used insecticides worldwide, poisoning with these compounds is especially an important environmental problem for developing countries [1]. Although accidental poisoning

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can occur after exposure to skin or inhalation, serious poisoning often follows suicidal ingestion [2]. According to the World Health Organization, 1 million serious accidental and 2 million suicidal poisonings with insecticides occur worldwide every year, and of these, approximately 200 000 die, mostly in developing countries [3]. Mortality rates were reported to be 9.0% for children and 9.6% for adults in a study performed in Turkey [4]. These OP compounds act as powerful inhibitors of acetylcholinesterase (AChE), resulting in accumulation of acetylcholine and overstimulation of cholinergic synapses in the central nervous system, somatic nerves, parasympathetic nerve endings, and sweat glands [5]. The continued stimulation and eventual paralysis of the acetylcholine receptors account for the clinical signs and symptoms of OP poisoning (OPP), including muscarinic, nicotinic, and central nervous system effects [6].

Cardiac complications and sudden death in OPP may take place after the poisoning [5,7]. Various arrhythmias and conduction disturbances due to sympathetic and parasympathetic overactivity have been reported with OPP [8,9]. In addition, hypertension-hypotension, noncardiogenic pulmonary edema, and myocardial damage in a few cases have also been described [2]. The mechanism by which OP induces cardiotoxicity has not been elucidated thus far, and it is difficult to pinpoint 1 mechanism as being the cause of cardiac toxicity related to OP. There are a lot of investigations about the cardiac toxicity of OPP, but its pathogenesis and underlying mechanisms are not known [10-12]. The current body of knowledge largely consists of limited studies and case reports. Therefore, many physicians may not be fully aware of the complications. There are no current available data concerning the cardiac biochemical markers, such as creatine kinase (CK), CK-MB, myoglobin (Mb), troponin I, and N-terminal probrain natriuretic peptide (NT-proBNP, a new marker) to determine cardiac damage in OPP.

Excessive reactive oxygen species (ROS) and lipid peroxidation (LPO) generation have been found to be involved in many diseases. Recent findings indicate that toxic manifestations induced by OP may be associated with an enhanced production of ROS. Some studies reported that OPP can cause LPO [13,14]. It is known that ROS can initiate apoptosis and organ lesions. Organophosphate insecticides are also capable of inducing programmed cell death (apoptosis) by multifunctional pathways. When cells are exposed to oxidative stress, they often die by apoptosis or necrosis. Under conditions of higher stress, the cellular impairment is so high that apoptosis is suppressed, leading to cell death by necrosis, which causes further tissue damage and an intense inflammatory response [15,16]. Because no experimental results have yet been reported about the effects of dichlorvos on LPO of rat heart, dichlorvos attracted our interest because of its effect on LPO and the activities of some antioxidant enzymes. Moreover, there is no published study about nitric oxide (NO), inducible NO synthase (iNOS), and apoptosis for cardiac tissue with acute dichlorvos-induced poisoning.

The standard therapy for coping with overt somatic effects comprises anticholinergic agents (mainly atropine), anticonvulsants (diazepam as the first choice), and reactivators of AChE (oximes). Anticholinergic effects of atropine are of lifesaving importance in the settings of OP intoxication [17]. However, the effects of atropine and/or pralidoxime (PAM) therapy on cardiac parameters in acute dichlorvos poisoning are not known. Thus, the aim of the present study was to investigate the cardiac biochemical markers, oxidative stress parameters, NO, immunohistochemical changes (iNOS and apoptosis) concerning cardiac damage and mortality in dichlorvos-induced poisoning in rats. In addition, the effects of atropine and/or PAM therapy on cardiac damage and mortality in acute dichlorvos poisoning were investigated.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 200 to 400 g, were used in the present study. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was approved by the local ethics committee.

2.2. Experimental design

Fifty-four rats were randomly divided into 5 groups. Group 1 (control group, n = 8) received 1 ml/kg of corn oil [18]. Group 2 (dichlorvos group, n = 15) received 30 mg/kg of dichlorvos [19]. Group 3 (n = 10) received 10 mg/kg of atropine 5 minutes before 30 mg/kg of dichlorvos [20]. Group 4 (n = 10) received 40 mg/kg of PAM 5 minutes before 30 mg/kg of dichlorvos [21]. Group 5 (n = 10) received 10 mg/kg of atropine and 40 mg/kg of PAM before 30 mg/kg of dichlorvos. All drugs and vehicle were administered intraperitoneally (i.p.).

2.3. Analysis of serum and tissue samples

All rats were killed by decapitation under thiopental sodium anesthesia. After 6 hours of dichlorvos or corn oil injection, venous blood samples were collected by direct heart puncture, and cardiac tissue samples were obtained.

2.4. Drugs and chemicals

Dichlorvos (30 mg/kg, prepared daily solution in corn oil, i.p., DDPV; Tarim-Veteriner Ilac Sanayi Ltd Sti, Istanbul, Turkey), atropine (10 mg/kg, stock solution was dissolved in ethanol, prepared daily solution in saline, i.p.; Sigma-Aldrich Chemical Co, St Louis, Mo), PAM methylsulfate (40 mg/kg,

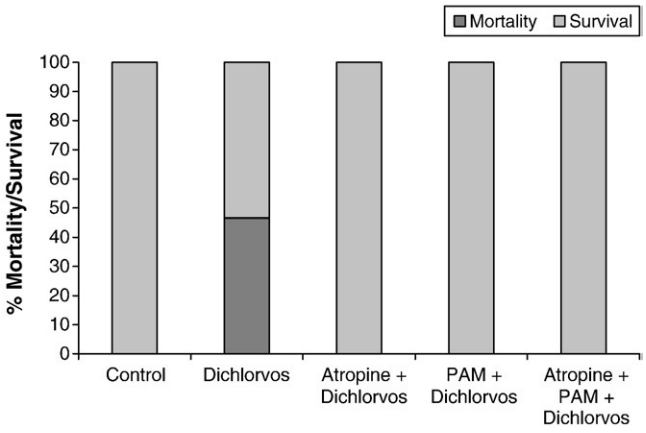

Fig. 1 Mortality rates for the groups. For the control and dichlorvos groups: $P = .0223$; odds ratio, 0.05965; 95% confidence interval, 0.002942-1.209. For the atropine and dichlorvos groups: $P = .0202$; odds ratio, 18.529; 95% confidence interval, 0.9199-373.25. For the PAM and dichlorvos groups: $P = .0202$; odds ratio, 18.529; 95% confidence interval, 0.9199-373.25. For the atropine +PAM and dichlorvos groups: $P = .0202$; odds ratio, 18.529; 95% confidence interval, 0.9199-373.25.

i.p., Contrathion 2%; Laboratories SERB, Paris, France), and thiopental sodium (120 mg/kg, dissolved in saline, i.p., Pental Sodyum; I.E. Ulagay, Istanbul, Turkey) were used in the present study. Control injections were made with corn oil as appropriate volume. Each animal received a total volume of 3 mL/kg of compounds tested or appropriate solvents at 3 different injection sites. Trichloroacetic acid, 2-thiobarbituric acid, butylated hydroxytoluene crystalline, sodium hydroxide anhydrous, 5,5-dithio-bis (2-nitrobenzoic acid), sodium citrate solution, Trizma hydrochloride reagent, potassium phosphate monobasic, disodium EDTA molecular biology reagent, and vanadium (III)–hydrochloride were purchased from the Sigma-Aldrich Chemical Co. Disodium hydrogen phosphate and absolute ethanol were purchased from the AppliChem (Biochemica, Darmstadt, Germany). All other chemical used were analytical grade.

2.5. Biochemical analysis

Biochemical analysis was performed to measure serum levels of cholinesterase (ChE), CK, CK-MB, cardiac troponin I (Tn-I), Mb, NT-proBNP, and NO and to determine the tissue levels of malondialdehyde (MDA), glutathione, and NO.

2.5.1. Measurements of blood samples

Serum ChE levels were analyzed using the colorimetric Ellman procedure [22], the serum CK and CK-MB levels were analyzed by a colorimetric enzymatic procedure, and all were measured using a Prestige 24i analyzer (Tokyo Boeki Medical System, Tokyo, Japan). Serum Mb, Tn-I, and NT-proBNP levels were analyzed with enzyme-linked immunosorbent assay methods. Absorbance was measured by a spectrophotometric method using Elecsys 2010 immunoanalyzer (ELx 800 Universal Microplate Reader; Bio-Tek Instruments Inc, Winooski, Vt) at wavelength of 450 nm.

2.5.2. Assessment of tissue samples

Tissue specimens were homogenized and centrifuged, and the supernatant was analyzed for NO, LPO, and glutathione levels. Both serum and tissue NO levels were measured by a NO/ozone chemiluminescence technique published by Alasehirli et al [23]. Lipid peroxidation was estimated using the thiobarbituric acid reaction according to Ohkawa et al [24]. Modified Ellman method [25] was used for cardiac tissue glutathione analysis, and the levels were measured using a spectrophotometer.

2.6. Determination of cardiac apoptosis

Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed using an apoptosis detection kit (Takara Bio Inc, Otsu, Shiga, Japan) according to the manufacturer's instructions. Cardiac apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick-end labeling (TUNEL) staining assay. Assays were performed in a blinded manner.

2.7. Immunohistochemical analysis

Immunohistochemical staining of cardiac tissues was carried out by deparaffinization, dehydration, and incubation in citrate buffer. Immunohistochemical procedures for detecting iNOS were performed using ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) according to the manufacturer's instructions. Semiquantitative method was used for immunohistochemical evaluation.

Table 1 Some measured ChE and cardiac markers in serum for the groups

Groups	n	ChE (U/L)	CK (U/L)	CK-MB (U/L)	Tn-I (ng/mL)	Mb (ng/mL)	NT-proBNP (fmol/mL)
Control	8	191.7 ± 47.7	473 ± 267.4	492.8 ± 311.3	0.5 ± 0.4	19.8 ± 11.4	193 ± 20.5
Dichlorvos	15	77.4 ± 31.2 *	522.1 ± 325.9	487.5 ± 282.5	1.1 ± 0.8	16.1 ± 10.1	238.5 ± 54.2
Atropine	10	143.2 ± 79.9	396.4 ± 211.3	391.9 ± 196.1	0.8 ± 0.3	13 ± 7.8	200 ± 62.3
PAM	10	146.8 ± 90.4	708.8 ± 289.8	729.7 ± 297.3	0.9 ± 0.4	16.6 ± 10.6	197.4 ± 59.6
Atropine+PAM	10	161.4 ± 84.1	597.6 ± 268.3	587.3 ± 247.4	1 ± 0.4	8.5 ± 5.7	146.8 ± 90.4

* $P < .05$, significant decrease in ChE activities when compared with the control group.

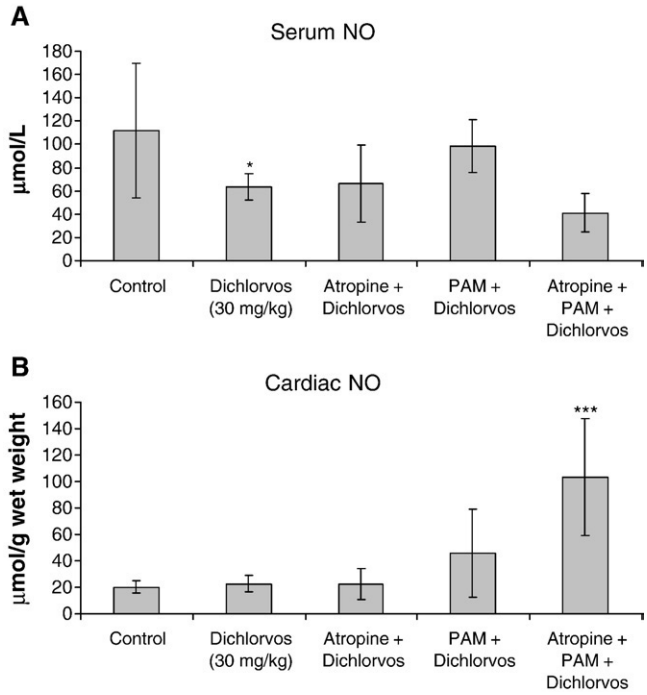

Fig. 2 Serum (A) and cardiac tissue NO levels (B) for the groups. *$P < .05$ when compared with the control group. ***$P < .001$ when compared with the dichlorvos group.

Immunolabeling intensity was graded as no staining (0), mild (1), moderate (2), and strong (3). Scoring was performed in a blinded manner.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Instat (version 3.05). All data are expressed as mean ± SD or the percentage incidence. Statistical comparison of more than 2 groups was performed by a 1-way analysis of variance followed by Student-Newman-Keuls multiple comparisons test. A Fisher exact test was used to detect mortality difference between groups. Scores were analyzed using Kruskal-Wallis variance analysis. In all tests, P values of less than .05 were considered to be statistically significant.

3. Results

Mortality and survival rates for all groups were determined within the first 6 hours. All the rats receiving acute 30 mg/kg doses of dichlorvos developed cholinergic signs (fatigue, tremor, cyanosis, excess of secretions, fasciculations, convulsion, and respiratory distress and arrest) within 3 to 5 minutes. These findings (fatigue, tremor, cyanosis, excess of secretions, and fasciculations) continued approximately one half to 1 hour after dichlorvos administration in rats. Seven of 15 rats (with prolonged convulsion and respiratory distress and arrest) were dead at the end of the experiment (the mortality rate was 46.6%). There were no cholinergic findings and deaths in the control, atropine, PAM, and atropine+PAM groups during the first 6 hours. Mortality and survival rates were found to be statistically significant between the groups ($P < .05$; Fig. 1).

Mean serum ChE activities were 77.4 ± 31.2 U/L in the dichlorvos group compared with 191.7 ± 47.7 U/L in the control group ($P < .05$; Table 1). There were no significant differences in serum ChE activities in the atropine, PAM, and atropine+PAM groups when compared with the control group. Creatine kinase, CK-MB, Tn-I, and Mb levels between the groups were found to be statistically insignificant ($P > .05$; Table 1). In addition, NT-proBNP levels showed no marked changes between the groups ($P > .05$; Table 1).

Mean serum NO levels were 63.6 ± 11.7 µmol/L in the dichlorvos group compared with 111.9 ± 57.7 µmol/L in the control group ($P < .05$; Fig. 2A). No significant differences in NO levels were recorded in the atropine, PAM, and atropine +PAM groups when compared with the dichlorvos group ($P > .05$). Cardiac tissue NO levels were 103.3 ± 44.3 µmol/g wet weight in the atropine+PAM group compared with 22.8 ± 6.6 µmol/g wet weight in the dichlorvos group ($P < .001$; Fig. 2B), and no significant differences were found between the other groups. No significant changes were also observed with cardiac MDA and glutathione levels ($P > .05$; Fig. 3).

Terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick-end labeling assay for cardiac apoptosis

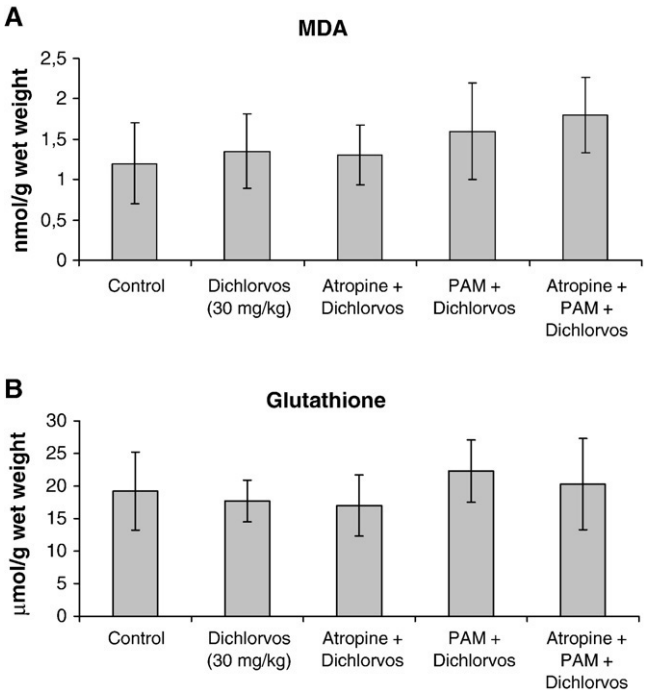

Fig. 3 Cardiac tissue glutathione and MDA levels for the groups.

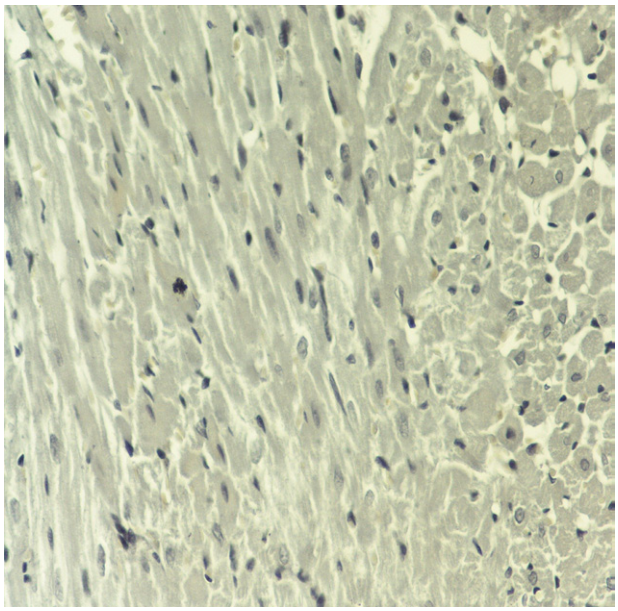

Fig. 4 Determination staining for cardiac apoptosis with TUNEL method in rat heart (apoptosis score, 0) (hematoxylin-eosin, original magnification ×40).

showed no staining in all groups (observed as apoptosis score of 0) (Fig. 4). Inducible NO synthase scores in the control, dichlorvos, atropine, PAM, and atropine+PAM groups were 9.0 ± 1.0, 11.0 ± 0.8, 16 ± 1.2, 8 ± 0.6, and 11 ± 1.1, respectively. Values are given as sum ± SD. However, there were no marked modifications in iNOS immunohistochemical analyses between the groups ($P > .05$).

4. Discussion

The results of the present study showed that serum ChE levels were decreased with dichlorvos, and these reductions were inhibited with atropine, PAM, or atropine+PAM pretreatments. All the rats receiving dichlorvos developed cholinergic signs and showed 46.6% mortality rate. There were no cholinergic findings and deaths in the control, atropine, PAM, and atropine+PAM groups during experimental period. Acute OP can lead to a potentially lethal state characterized by muscular paralysis, autonomic overstimulation, and cardiorespiratory failure. Besides, cardiac complications accompany poisoning with these compounds, which may be serious and often fatal [8,12]. Worldwide OP report of mortality rates are from 3% to 30% [26]. The mortality rate for poisoned patients who require ventilation is as high as 50% [1]. Up to 70% of patients with OPP have a high incidence of respiratory failure and a prolonged QT interval corrected for heart rate [1,12]. Our data showed that atropine, PAM, or atropine+PAM pretreatments markedly reduced mortality. Our results suggest that the cause of mortality in the dichlorvos-treated rats was not from myocardial damage but rather cholinergic poisoning and subsequent neurotoxicity. It should be mentioned that the results of animal experiments are limited with regard to extrapolating the data to human beings.

Although rhythm disturbances as cardiac effects of OP have been documented, it is not known whether the acute toxicity of OPs causes damage in heart tissue. Povoa et al [27] have reported that OPs induced acute poisoning with myocardial necrosis. Although this is the first case in the literature with histological confirmation of myocardial necrosis from OP intoxication, biochemical parameters of cardiac damage have not been examined. According to Saadeh et al [12], increased CK and lactate dehydrogenase levels with ST-segment elevation were found in 5 of 11 patients, but other cardiac parameters have not been studied. Changes of serum CK levels were reported in some toxicity studies [28,29], but serum CK-MB levels in OP have not been evaluated in an experimental study. Elevated CK in this study may be related to cholinergic crisis or rhabdomyolysis [30]. Serum levels of CK-MB were not changed with dichlorvos-induced OP in the present study. Traditionally used markers, CK and CK-MB, were not affected by atropine, and PAM pretreatment suggests that both CK and CK-MB were not associated with dichlorvos-induced cardiac damage in the first 6 hours. It is known that measurement of serum cardiac Tn-I levels is a sensitive and specific means for detecting myocardial damage in many mammalian species. Cardiac Tn-I and Mb have an important place to diagnose or eliminate the myocardial damage in the early hours of acute coronary syndrome [31], but these markers were not affected by dichlorvos intoxication in our study. Thus, the results of the present study imply that CK, CK-MB, Tn-I, and Mb may not be taken into consideration in the evaluation of cardiotoxicity related to OP.

Endogenous cardiac hormone NT-proBNP may be secreted upon myocardial stress. Many publications describe this peptide as an excellent marker of left ventricle function and a simple and effective tool to detect heart failure or left ventricle dysfunctions [32]. In addition, it was reported that NT-proBNP level may contribute to the early diagnosis of cardiotoxicity in some poisoning [33,34]. To our knowledge, this is the first experimental study investigating the relationship between the serum NT-proBNP level and the toxicity of OP, and our data suggest that measuring the serum NT-proBNP levels may not contribute to the early diagnosis of cardiotoxicity with dichlorvos-induced OP in rats.

Data in the literature confirm that there is an intimate cross talk between NO overproduction and free radical overformation in the injured heart [35]. Whereas serum NO levels are suppressed with dichlorvos, cardiac NO levels are elevated with atropine+PAM pretreatment. The reason for these differences is not known. Nitric oxide takes part in termination of LPO reactions. Nitric oxide is an effective chain-breaking antioxidant in free radical–mediated LPO. A protective effect of NO on LPO has been shown by some

investigators [36]. Because it has been demonstrated that peroxynitrite formed from NO and superoxide has antioxidant and protective effects on myocardium [37], NO or peroxynitrite may produce a protective effect on myocardium in dichlorvos-induced OP in rats. However, it is clear that there was no change in iNOS expression; therefore, iNOS was not responsible for elevated NO levels in cardiac tissue. Thus, this alteration in NO levels may be related to endothelial or neuronal NO synthases.

The heart tissue may be susceptible to oxidative damage due to the presence of polyunsaturated fatty acids and oxygen, which may produce oxidative changes in myocytes. Recent studies point that oxidative stress could be an important part of the mechanism of OP. In these studies, LPO has been suggested as one of the molecular mechanisms involved in OP-induced toxicity [38]. Glutathione scavenges ROS directly or in a reaction catalyzed by glutathione peroxidase through the oxidation of 2 molecules of glutathione to a molecule of glutathione disulphide (GSSG) [16,39]. However, our results showed that cardiac MDA and glutathione levels were not affected by dichlorvos. Although the reason for this was not clear, it might be related to exposure period of the OP compound. The rats in the present experiment were acutely poisoned with dichlorvos. In addition, it can be expressed that glutathione did not play a role in the atropine, PAM, or atropine+PAM therapy in dichlorvos-induced poisoning.

It has been suggested that oxidative stress plays a role as a common mediator of apoptosis. The ability of oxidative stress to provoke apoptosis as a result of massive cellular damage has been associated with LPO and alterations of protein and nuclei [40]. It is known that the subchronic and chronic toxicity of OP may cause biochemical and histopathologic changes in different tissues such as liver, kidney, pancreas, vascular wall, and endometrium [14,15]. Subchronic methidathion and diazinon exposure caused histopathologic changes in heart tissue [14,38]. However, changes in these studies were associated with enhanced serum MDA production in rats. Domenicotti et al [41] found an apoptotic pathway dependent on glutathione depletion. Schafer and Buettner [42] found that the glutathione redox status can be indicative of the biological status of the cell. A decrease in the ratio of glutathione to GSSG may shift cells through different biological stages, such as proliferation, differentiation, apoptosis, and at very low values, necrosis. An impairment of the glutathione redox status could lead to excessive oxidative stress, necrosis, and eventually, the death of the organism [16]. Altogether, these results suggest that ROS can initiate apoptosis, but oxidative stress parameters (MDA and glutathione) did not change in the present study. This may explain the lack of evidence for apoptosis in our experiments.

There is a limitation of the study. Our experimental period was up to 6 hours after dichlorvos injection. Longer experimental periods for 2 to 3 days rather than 6 hours could have affected the results because this time course could have been too short to demonstrate the cardiac tissue injury markers or histopathologic changes.

In conclusion, our results imply that measured cardiac markers including CK, CK-MB, Tn-I, Mb, and NT-proBNP may not contribute to the diagnosis of cardiotoxicity in acute dichlorvos-induced poisoning. In addition, iNOS may not be responsible for elevated NO levels in cardiac tissue, although NO may have a protective effect on myocardium with atropine+PAM therapy in rats.

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