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Cardiac Effects of Magnesium Sulfate Pretreatment on Acute Dichlorvos-Induced Organophosphate Poisoning: An Experimental Study in Rats

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Abstract Although atropine and oximes are traditionally used in the management of organophosphate poisoning, investigations have been directed to finding additional therapeutic approaches. Thus, the aim of this study was to evaluate the cardiac effects of magnesium sulfate pretreatment on dichlorvos intoxication in rats. Rats were randomly divided into three groups as control, dichlorvos, and magnesium sulfate groups. After 6 h of dichlorvos or corn oil (as a vehicle) injection, venous blood samples were collected, and cardiac tissue samples were obtained. Biochemical analyses were performed to measure

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some parameters on serum and cardiac tissue. Immunohistochemical analyses of apoptosis and inducible nitric oxide (NO) synthase showed no change in cardiac tissue. Serum cholinesterase levels were markedly depressed with dichlorvos, and further suppressed markedly with magnesium sulfate pretreatment. Although we have demonstrated that serum NO levels in dichlorvos and magnesium sulfate groups were lower than the control group, cardiac tissue NO levels in magnesium sulfate group were higher than the other two groups. Mortality was not significantly affected with magnesium sulfate pretreatment. Uncertainty still persists on the right strategies for the treatment of organophosphate acute poisoning; however, it was concluded that our results do not suggest that magnesium sulfate therapy is beneficial in the management of acute dichlorvos-induced organophosphate poisoning, and also further studies are required.

Keywords Dichlorvos · Rat · Therapy · Magnesium sulfate · Nitric oxide

Introduction

Acute poisoning with organophosphate (OP) compounds produces characteristic symptoms arising from the inhibition of acetyl cholinesterase (ChE) in central and peripheral nervous systems. Acute symptoms are related to morbidity and mortality associated with the effect of the poisoning of central, respiratory, and cardiac systems [1]. Therapy of the poisoning is based on the administration of atropine and oximes as standard antidotes. When the atropine effectively antagonizes the muscarinic actions of acetylcholine excess (increased tracheobronchial secretions, bradycardia, salivation, and bronchoconstriction), oximes are well recognized as reactivates of inhibited ChE [2, 3]. Dysrhythmias can also occur in poisoning with OP compounds [4], and therapeutic management of these patients can be a trouble. Although the antidotes are used in the management of the poisoning, investigations have been directed to finding additional therapeutic approaches [5, 6]. One of these drugs is magnesium sulfate ($MgSO_4$).

Magnesium inhibits calcium entry into the cell via a noncompetitive blockade of the *N*-methyl-D-aspartate receptor [7], and it is also a physiological calcium antagonist at different voltage-gated channels [8]. It seems to be important and particularly effective in some dysrhythmias (i.e., torsade de pointes and ventricular tachycardia accompanying acquired long QT/QTU syndrome). Additionally, $MgSO_4$ is essential modulator of the electrical activity of cardiac cells [9], and effects on cardiac rate control have been also reported [10]. $MgSO_4$ has been taken into consideration in the management of the poisoning with OP compounds [11]. The reason why $MgSO_4$ may be beneficial in the poisoning may be expressed as follows: (1) $MgSO_4$ can suppress the acetylcholine release from motor nerve terminals and to antagonize the effects of poisoning with OP; (2) $MgSO_4$ can control premature ventricular contractions; and (3) it is considered to counteract the direct toxic inhibitory action of OP on sodium-potassium ATPase [10, 12].

$MgSO_4$ therapy in the poisoning usually reported on the effects of rhythm disturbances in clinical studies [13], and there are few studies evaluating the effects of $MgSO_4$ therapy on the poisoning in a long time period [14, 15]. However, $MgSO_4$ has not been administered routinely in poisoning with OP compounds, and also the effects of $MgSO_4$ on the poisoning is not clear enough and has not been sufficiently investigated by biochemical, histopathological, and immunohistochemical parameters in cardiac tissue. Thus, the aim of this study is to evaluate the possible effects of $MgSO_4$ pretreatment on dichlorvos poisoning in rats.

Materials and Methods

Animals

Male Wistar rats, weighing 200–400 g, were used in this study. Animals were kept in colony rooms with 12-h-light/dark cycles at a room temperature of 21±1°C and supplied with standard laboratory diet and tap water ad libitum. 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.

Experimental Design and Serum/Tissue Sampling

Thirty-three rats were randomly divided into three groups: group 1 (control group, $n=8$) received corn oil (1 ml/kg) as a vehicle of dichlorvos; group 2 (dichlorvos group, $n=15$) received 30 mg/kg of dichlorvos [16]; and group 3 ($MgSO_4$ group, $n=10$) received 200 mg/kg of $MgSO_4$ immediately before 30 mg/kg dichlorvos administration [17]. All drugs and vehicle were administered intraperitoneally (i.p.), and after 6 h of injection, venous blood samples were collected by direct heart puncture under thiopental anesthesia. Then, cardiac tissue samples were obtained. The hearts were removed and divided into the two pieces for tissue samples and histopathological study. Specimens for tissue samples were weighed, washed two times with cold saline solution, placed into tubes, and stored in a deep freeze (−20°C) until processing. Specimens for immunohistochemical assays were fixed in 10% formaldehyde. If an animal died during experimental period (i.e., during 6 h), blood and tissue samples were not collected for experiment.

Chemicals and Drugs

Dichlorvos (30 mg/kg, prepared daily solution in corn oil, i.p., DDPV, Tarım-Veteriner Ilac Sanayi Ltd. Sti., Istanbul, Turkey), $MgSO_4$ (200 mg/kg, i.p., magnesium sulfate, Biopharma, Istanbul, Turkey), 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. $MgSO_4$ was administered as i.p. in calculated dose, immediately before the dichlorvos injection. Each animal received a total volume of 3 ml/kg of compounds tested or appropriate solvents at three different injection sites.

Biochemical Analysis

Biochemical analysis were performed to measure the serum levels of ChE, creatine kinase (CK), CK-MB, cardiac troponin I (cT-I), myoglobin (Mb), N-terminal probrain natriuretic peptide (Nt-proBNP), and nitric oxide (NO) and to determine the tissue levels of malondialdehyde (MDA), glutathione, and NO. Blood samples were collected at the end of the experiment and then promptly centrifuged at 2,500×*g*, 4°C, for 15 min. The plasma was removed and stored at −40°C until assayed. Serum ChE levels were measured by a colorimetric Ellman procedure (cholinesterase-ChE-Bi Kit, Spinreact, Spain) using Prestige 24i autoanalyzer (Prestige 24i, Tokyo Boeki Medical System, Japan) [18]. Serum CK and CK-MB level were measured by a colorimetric enzymatic procedure (Prestige 24i, Tokyo Boeki Medical System, Japan) using Prestige 24i autoanalyzer (Prestige 24 i LQ CK,

CK-MB Diagnostic Kit). Serum Mb and cT-I levels were analyzed with an automated two-site immunoassay using monoclonal antibodies by enzyme-linked immunosorbent assay (ELISA; DRG International, USA) specific for the Mb and cT-I. Absorbance was measured by a spectrophotometric method using Elecsys 2010 immunoanalyzer (EL_X 800 Universal Microplate Reader, Bio-Tek Instruments, Vermont, USA) at wavelength 450 nm. The concentration of Mb and cT-I in the samples was calculated by comparison with the standard curve. Serum Nt-proBNP level was determined with enzymatic immunoassay analytic technical method by ELISA (Diagnostic automation, Calabasas, USA). Absorbance was measured by a spectrophotometric method using Elecsys 2010 immunoanalyzer (EL_X 800 Universal Microplate Reader, Bio-Tek Instruments, Vermont, USA) at wavelength 450 nm. The concentration of NT-proBNP in the samples was calculated by comparison with the standard curve. Tissue MDA was measured by a spectrophotometric procedure published by Ohkawa et al. [19], and glutathione was evaluated using modified Ellman procedure [20].

NO Measurements

Plasma NO levels were measured by a NO/ozone chemiluminescence technique published by Alasehirli et al. [21], and cardiac NO levels were determined with the same chemiluminescence method as described previously [22]. Briefly, heart specimens were homogenized at 4°C with a Branson sonifier 150 (Danbury, CT, USA) in 5 vol. of phosphate-buffered saline (PBS; pH 7.4), then centrifuged at 7,000×*g* for 10 min and the pellets discarded. Supernatants were deproteinized with absolute ethanol at 0°C in a 1:2 *v*/*v* mix, incubated 30 min at 0°C followed by centrifugation at 7,000×*g* for 5 min. The pellets were discarded, and the supernatant was used to assess NO levels. NO was measured based on a gas-phase chemiluminescent reaction between NO and ozone with a Sievers NOA 280i (Boulder, CO, USA). All samples were analyzed in duplicates, with the person performing the testing blind to the origin of the specimen. A standard curve was established with a set of serial dilutions (0.1–100 μM) of sodium nitrate. Data collection and analysis was performed using the NOAnalysis™ software (version 3.21, Sievers, Boulder, CO, USA).

Determination of Cardiac Apoptosis

Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed using an apoptosis detection kit (Takara Bio, 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. In brief, at the end of experiment, the cardiac samples were collected in 10% paraformaldehyde in PBS (pH 7.4) and four longitudinal sections were cut and further fixed in 10% formaldehyde in PBS for 24 h at room temperature. Fixed tissues were embedded in a paraffin block and two slides at 4–5-mm thickness were cut from each tissue block. After rinsing with PBS, slides were coverslipped with mounting medium containing diaminobenzidine (DAB) to permit total nuclei counting. The sections were counterstained with hematoxylin and eosin. Myocytes in which the nucleus was obviously labeled with DAB were defined as TUNEL positive. For each slide, ten fields were randomly chosen, and using a defined rectangular field area (×40 objective), a total of 100 cells per field were counted. Assays were performed in a blinded manner.

Immunohistochemical Analyses

Immunohistochemical staining of cardiac tissues was carried out by deparaffinization, dehydration and incubation in citrate buffer. Immunohistochemical procedures for detecting inducible NOS (iNOS) were performed by using ImmunoCruz^{TM} Staining System (Santa Cruz Biotechnology, California, USA) according to the manufacturer's instructions. Semiquantitative method was used for immunohistochemical evaluation. Immunolabeling intensity was graded as no staining (0), mild (1), moderate (2), and strong (3). Scoring was performed in a blinded manner.

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 two groups was performed by a one-way analysis of variance followed by Student–Newman–Keuls multiple comparisons test. A Fisher's exact test was used to detect mortality difference between groups. Scores were analyzed using Kruskall–Wallis variance analysis. In all tests, p values less than 0.05 were considered to be statistically significant.

Results

Mortality observed in dichlorvos and control groups were 47% and 0%, respectively, and this incidence was 20% for $MgSO_4$ group ($p>0.05$, Fig. 1). Serum ChE levels were markedly depressed with dichlorvos (from 192.5±51 U/l, $n=15$ to 93.6±18.2 U/l, $n=8$, $p<0.05$). Additionally, serum ChE levels were further suppressed with $MgSO_4$ pretreatment (35.6±28.7 U/l, n=10, $p<0.05$). MDA (Fig. 2a) and glutathione (Fig. 2b) measured as oxidative stress parameters, and changes were found to be statistically insignificant ($p>0.05$) between the groups. Although we have demonstrated that serum NO levels in dichlorvos and $MgSO_4$ groups were lower than the control group (Fig. 3b), cardiac tissue NO levels in $MgSO_4$ group was higher than the other two groups ($p<0.05$; Fig. 2b). Serum CK levels of control, dichlorvos, and $MgSO_4$ groups were 439.8±265.2, 484.6±233.8, and 677±315 IU/L ($p>0.05$), respectively. Values for CK-MB in the same groups were 449.8±303.3, 475.0±255.1, and 640.8±349.8 IU/L ($p>0.05$), respectively. cT-I levels were

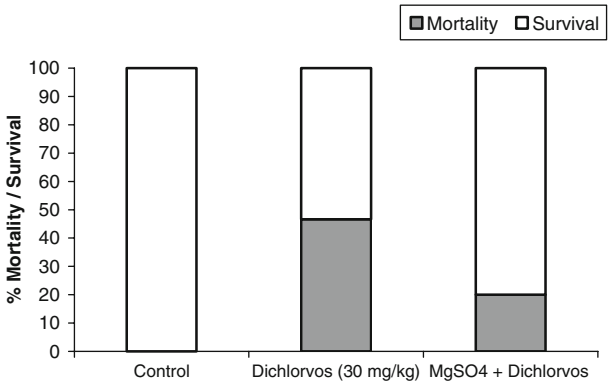

Fig. 1 Mortality rate for the groups

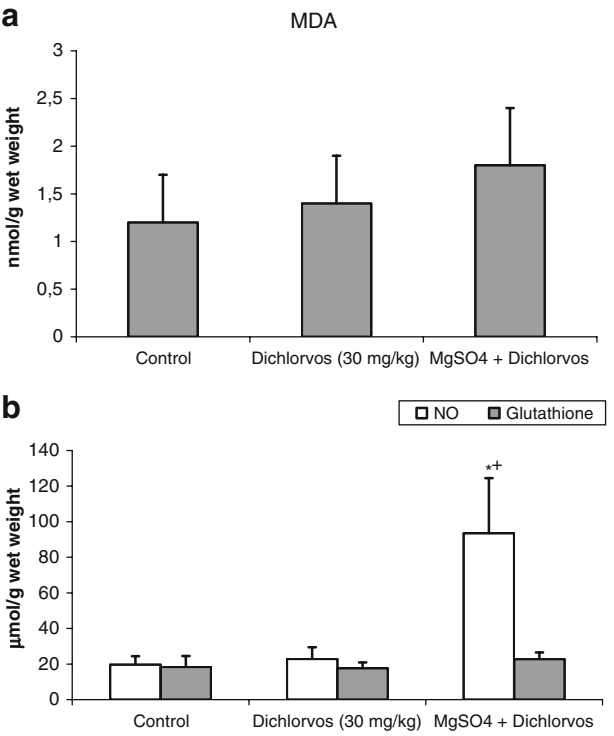

Fig. 2 Malondialdehyde levels (**a**) and nitric oxide and glutathione levels (**b**) in the cardiac tissue for the groups. *$p<0.05$ when compared to control group. $^{+}p<0.05$ when compared to dichlorvos group

0.5±0.3, 0.9±0.6, and 0.9±0.4 ng/ml ($p>0.05$) and Mb levels were 19.0±11.9, 18.0±9.2, and 15.8±11.5 ng/ml ($p>0.05$) for the control, dichlorvos, and $MgSO_4$ groups, respectively. Nt-proBNP levels between the groups (191.4±21.3, 226.0±28.2, and 217.7±59.7 fmol/ml for the control, dichlorvos, and $MgSO_4$ groups, respectively) were found to be statistically insignificant ($p>0.05$) (Fig. 3a). TUNEL assay for cardiac apoptosis was not observed, and immunohistochemical analyses performed for iNOS were not significantly changed between the groups ($p>0.05$). Inducible NOS scores of control, dichlorvos, and $MgSO_4$ groups were 1±1.1, 1.6±0.7, 0.8±0.5, respectively ($p>0.05$).

Discussion

The main four findings of this study are as follows: (1) serum ChE levels were further suppressed with $MgSO_4$ pretreatment; (2) mortality has not been markedly reduced by $MgSO_4$ pretreatment;(3) while tissue NO levels were elevated, serum NO levels were decreased with $MgSO_4$ pretreatment; and (4) cardiac apoptosis was not induced by 30 mg/kg dichlorvos-induced poisoning within first 6 h in rats.

The most striking result of the study was the observation that $MgSO_4$ pretreatment markedly reduced the serum ChE levels. The mechanism of ChE suppression with $MgSO_4$ in the present study is not known. It has been demonstrated that the mortality rate and hospitalization days of patients who received $MgSO_4$ treatment were significantly lower than those who had not received $MgSO_4$ and suggested that administration of $MgSO_4$ concurrent to conventional therapy in OP acute human poisoning is beneficial by reducing

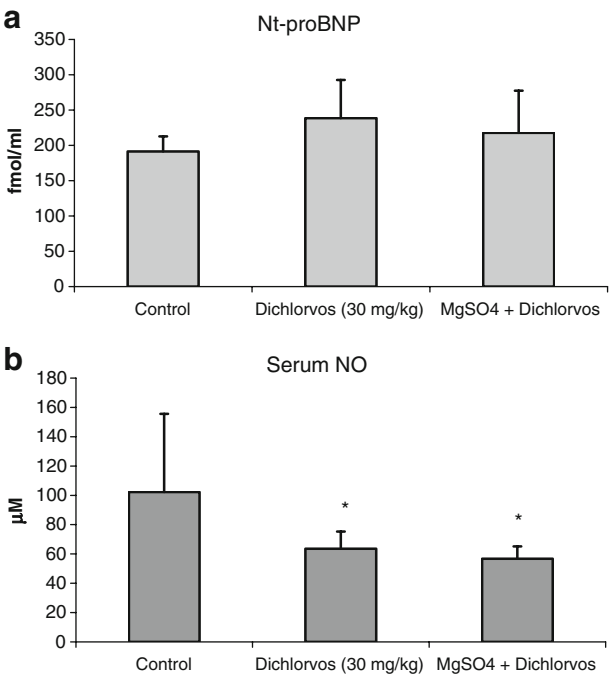

Fig. 3 Serum Nt-proBNP (**a**) and NO (**b**) levels in the groups. *$p<0.05$ when compared to control group

the hospitalization days and rate of mortality [10]. Although Pajoumand et al. [10] administered $MgSO_4$ to the patients via intravenous infusion at dose of 4 g/day (60 mg/kg for 70 kg), the $MgSO_4$ dose in our study was 200 mg/kg via i.p. as used by Mochizuki et al. [17] in rats. The results of our experiments suggest that single-dose $MgSO_4$ therapy may be a disadvantage of therapy in poisoning with OP compounds. Although there was a reduction in the mortality with $MgSO_4$ treatment (from 47% to 20%), this decrease in incidence did not reached statistical significance in the present study. Thus, $MgSO_4$ therapy may not positively affect the survival in dichlorvos poisoned rats.

High concentrations of magnesium enhance the synthesis of nitric oxide, in part through the upregulation of endothelial nitric oxide synthase in human umbilical vein endothelial cells [23]. Since endothelial NOS (eNOS) and neuronal NOS (nNOS) are generally referred to as constitutively expressed and calcium-dependent enzymes [24], $MgSO_4$ may affect the NO synthesis through its calcium channel blocking properties [25]. As shown in the present study, cardiac tissue NO was stimulated by $MgSO_4$ therapy. Further studies are required to explain the possible mechanism for the impression; while tissue NO levels were elevated, serum NO levels were decreased with $MgSO_4$ pretreatment. Since iNOS staining were not changed in the present study, it is likely that cardiac iNOS is not involved in this effect. However, there is evidence that OP pesticides may appear to affect intracellular ion balance, particularly calcium and specifically target constitutive nitric oxide synthase (eNOS and nNOS)-mediated processes [26].

Cardiac MDA and glutathione, oxidative stress parameters evaluated in this study, were not markedly affected by dichlorvos. There was a tendency toward an elevation in cardiac MDA levels with $MgSO_4$ treatment in the present study, but these changes did not reach statistical significance. This may be related to the fact that our experimental period was not long enough to detect marked changes. There is no published experimental study to show

the effect of dichlorvos on cardiac MDA levels. It has been documented that dichlorvos produced toxic effect on isolated hepatocytes by increased MDA production [27]. Cardiac glutathione levels were not different between the groups in the present study. However, it has been reported that glutathione levels decreased with dichlorvos exposure in the rat brain [28]. Cardiac markers (CK, CK-MB, cT-I, and Mb) and Nt-proBNP were not affected by dichlorvos or $MgSO_4$ therapy in the first 6 h. These data may suggest that these markers were not markedly modified and may not give an information about the cardiac toxicity in acutely dichlorvos poisoning.

Although we were not able to detect apoptosis in our experiments, apoptosis can be induced in the rat endometrium following chronic dichlorvos exposure [29]. It is known that reactive oxygen species can initiate apoptosis [30], but oxidative stress parameters (MDA and glutathione) did not change in the present experiments. This may explain the lack of evidence for apoptosis in our experiments.

Finally, it seems to us that, uncertainty still persist on the right strategies for the treatment of OP acute poisonings and that, at present, only small a study suggests a benefit from new treatments such as $MgSO_4$, and much larger trials are needed [31]. In fact, the only studies suggesting beneficial effects are very small; they are carry out on a local basis [10], no multicentric studies are available, and finally, results are conflicting [14]. In conclusion of in this light, our results do not suggest that $MgSO_4$ therapy may be beneficial in the management of acute dichlorvos-induced OP poisoning. Further studies related to the improvement of the management of poisoning with OP compounds are required.

Limitations to the Study Exist

The methodology used in this study contains some limitations. First, we used rat models of OP poisoning, which did not completely reflect the clinical situation. The second one is that $MgSO_4$ was administered before the poisoning. However, patients admit to the emergency department following the poisoning. The last reason for limitation is that there was absence of a group exposed to $MgSO_4$ only. It is almost impossible to discriminate the effects due to $MgSO_4$, those due to dichlorvos, and those due to the interaction between the two substances.

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