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Acute dichlorvos poisoning induces hemorheological abnormalities in rabbits via oxidative stress

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Abstract. Dichlorvos is an important insecticide used largely. Some studies have demonstrated that organophosphate pesticide has effects on erythrocyte membrane structures, which is critical to erythrocyte function and hemorheology. The aim of the present study was to explore the effect of oxidative stress on hemorheological changes during dichlorvos poisoning in rabbits. Data indicated that after dichlorvos exposure the hematocrit adjusted viscosity at high shear rate increased and erythrocyte membrane fluidity decreased. Data obtained from plasma showed that lipid peroxidative substance-malonaldehyde was elevated and superoxide dismutase was reduced. In summary, oxidative stress does occur in dichlorvos poisoning and may lead to hemorheological alterations. The changes of hemorheology may be responsible for the pathophysiology of the dichlorvos poisoning.

Keywords: Dichlorvos poisoning, oxidative stress, hemorheology, hematocrit adjusted viscosity

1. Introduction

Organophosphate (OP) compounds are the most extensively used insecticides worldwide. The poisoning with these compounds is especially an important environmental problem for developing countries [43]. Among the OP pesticides, dichlorvos (dimethyl 2,2-dichlorovinyl phosphate, DDVP) is one of the most widely used in agriculture [24].

It is well known that the OP compounds act as powerful inhibitors of acetylcholinesterase (AChE), resulting in the accumulation of acetylcholine and overstimulation of cholinergic synapses in the central nervous system, somatic nerves, parasympathetic nerve endings, and sweat glands [29]. Recently there

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were evidences indicated that AChE would relate to the hemorheological parameters [4, 12]. Acute organophosphate poisoning (AOPP) can manifest three different phases of toxic effects, namely, acute cholinergic crisis, intermediate syndrome (IMS), and delayed neuropathy. But AChE inhibition does not explain all the symptoms of OP intoxication [38, 44]. For example, the renal dysfunction secondary to OPs exposure in humans was found not to be correlated with the degree of cholinesterase suppression [28, 36]. Hence, mechanisms other than AChE inhibition may be involved in the progression of AOPP.

Pesticides have been reported to induce the generation of reactive oxygen species (ROS) *in vitro* and *in vivo* [1, 7]. ROS play an important role in the toxicity of OP compounds [24]. Oxidative damage by free radicals or ROS could result in lipid peroxidation and protein modification, causing changes in membrane properties and cell dysfunction [41].

Although oxidative stress is not specific to organophosphate poisoning and can occur with other classes of pesticides, studies have shown the increased oxidative stress and damage in both acute and chronic OP poisoning in humans [8, 39, 45]. OP pesticides may induce oxidative stress, leading to the generation of free radicals and the alteration in antioxidants [e.g., glutathione (GSH)], oxygen free radicals, the scavenging enzyme system [e.g., superoxide dismutase (SOD)] and lipid peroxidation [evaluated by plasma malonaldehyde (MDA)] [1, 2].

Available evidences indicated that OP poisoning could induce alteration in erythrocytes, such as the increase of lipid peroxidation, and the reduction of plasma membrane fluidity [20]. It is known that normal erythrocyte membrane structure is very important for fluidity [31]. Some recent papers showed that there were correlations between oxidative stress and hemorheological properties [10, 11, 26]. Therefore, it would be valuable to assess the effects of OP on the hemorheological properties of blood. However, to our knowledge, hemorheology in AOPP has been not well investigated. In present study, we created a rabbit model for AOPP and examined the changes in biomechanical properties of both erythrocytes and whole blood. We found that oxidative stress occurred in dichlorvos poisoning and may be responsible for the hemorheological alterations.

2. Materials and methods

2.1. Animals

New Zealand rabbits weighing 2.0–2.5 kg (provided by Peking University Animal Breeding Unit) were housed under the standard laboratory conditions. Prior to dichlorvos injection, all rabbits were allowed to acclimatize for 1 week. The animal protocol of this work was approved by the ethical animal committee of Peking University.

2.2. Reagents

Dichlorvos 80EC was purchased from Tianjin pesticide Co. (Tianjin, China). Kits for plasma MDA and AChE assays were purchased from Nanjing Jiancheng Agent Co. (Nanjing, China). The SOD assay kit was bought from Dojindo Molecular Technologies (Dojindo, Gaithersburg, MD).

2.3. Treatment

The rabbits were randomly divided into two experimental groups: control group (N = 4), and dichlorvos group (N = 6). Dichlorvos 80EC diluted 1000 times with distilled water by sonication was given

intraperitoneally (i.p.) at a dose of 5 ml/kg body weight [13]. An equivalent amount of distilled water was given in the same way to the control rabbits. Since two out of 6 rabbits died during the experiment in the diclorvos group, plasma and blood samples were collected and analyzed in the remaining four rabbits. No deaths were recorded in the control group.

2.4. Plasma and blood samples

At 30 min and 24 h of the experiment, blood samples anticoagulated with trisodium citrate (2.5 mg/ml) or heparin (15 U/ml) were collected from auricular vein. Then samples were promptly centrifuged at 3000 rpm, for 10 min. The plasma was removed and stored at −20 °C until used. The protocols are in accordance with the guidelines for rheological measurements [9].

2.5. Measurement of plasma acetylcholinesterase (AChE)

Plasma acetylcholinesterase levels were measured by following a colorimetric Ellman procedure [18] using a true choline esterase TChE Kit (Nanjing Jiancheng Agent Co., China). The rate of hydrolysis of acetylthiocholine was measured at 412 nm wavelength (ANTAI 838 microplate reader, China) by the reaction of thiocholine with DTNB to generate the yellow 5-thio-2-nitrobenzoate anion.

2.6. Hematocrit adjusted viscosities of whole blood (HV)

Whole blood viscosities at 200 s^{-1} shear rates were measured with an automatic Viscometer (LG-R-80B, Steellex Co., Beijing, China). Whole blood was drawn in micropipette and centrifuged at 12000 rpm, for 10 min in a 3F-2 microdosis super-speed hydroextractor (Beijing, China). Hematocrits (Hct) were then obtained. The hematocrit adjusted viscosities (HV) were calculated from the equation: HV (200 s^{-1}) = (Whole blood viscosities−1)/Hct.

2.7. Plasma fibrinogen concentrations

Blood samples anticoagulated with trisodium citrate were centrifuged for 10 min at 3000 rpm and plasma was collected. Fibrinogen concentration was measured on a Coagulant Analyzer (RABRE, Steellex Co., Beijing, China).

2.8. Measurement of erythrocyte deformation index

Forty ul heparinized blood was suspended in 1 ml of 15% PVP buffer (w/v, pH 7.4, 290 mOsm/kg) with a viscosity of 15 mPa.s. The deformation indices (DI) at shear rates of 50–1000 s^{-1} were measured with a traditional ektacytometer (LG-B-190 Steellex Co., Beijing, China) [47].

2.9. Fluorescent probing of erythrocyte membrane fluidity

Erythrocyte membrane fluidity was measured using steady-state fluorescence anisotropy of the probe DPH incorporated into the red blood cell membranes with an F-4500 fluorescence spectrophotometer (HITACHI, Japan) [40]. A suspension of red blood cells was incubated with DPH at 37°C for 30 min. The fluorescence polarization parameter P was determined [6]. Wavelengths of 360 and 430 nm were used

for excitation and emission, respectively. Measurements were completed within 2–3 h after the harvests of samples.

2.10. Measurements of erythrocyte osmotic fragility

The osmotic resistance of red blood cells was measured [22]. The blood samples were centrifuged at 3000 rpm for 10 min and the packed cells were resuspended in buffers with osmotic pressures ranging from 0 to 295 mOsm/kg. The cell suspensions were left to equilibrate at room temperature for 1 h and then centrifuged at 3000 rpm for 10 min. Transmission rate (Tr) of the supernatant was determined at 540 nm with UNICO UV-2000 spectrophotometer. Osmotic fragility curves were obtained by plotting the percentage of haemolysis against osmotic pressures. Haemolysis rate (Hr) = Tr(295)−Tr/Tr(295)−Tr(0) × 100%.

2.11. Estimation of lipid peroxidation

Malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress [16] can be used in biomaterials as an indicator of cell membrane injury [19, 35]. MDA determination is based on spectrophotometric or spectrofluorimetric measurement of the condensation product formed from MDA and 2-thiobarbituric acid (TBA). By measuring the absorbance at 532 nm after reaction, the result could be obtained, and were expressed as μmol/L.

2.12. Determination of plasma SOD levels

The superoxide dismutase (SOD) activities were measured by using a water-soluble formazan dye kit (Dojindo Molecular Technologies, Gaithersburg, MD).

2.13. Statistics

Each measurement was performed at least in triplicate under the same condition. All data were analyzed by the ANOVA analysis of variance of the SPSS 16.0 statistical software. Differences were considered significant when $p < 0.05$. Control group (N = 4) at 30 min and 24 h after injection of distilled water were performed in parallel. Mean values of all the dates in different points were used as the overall control value since there were no significant differences among them.

3. Results

Mortality and survival rates for both groups were determined within the first 6 h. All the rabbits from dichlorvos group appeared signs of poisoning signs (fatigue, ataxia, excess of secretions, miosis, and respiratory distress) within 3–15 min. These signs disappeared fairly rapidly, within 30 min. Two out of six rabbits were dead in the dichlovos group. The mortality rate was 33%. There were no cholinergic findings and deaths in the control group during the first 6 hours.

Table 1
The concentrations of plasma acetylcholinesterase (AChE) in control and dichlorvos groups

	Control (N = 8)	Dichlorvos 30 min (N = 4)	Dichlorvos 24 h (N = 4)
AChE (U/ml)	12.6 ± 1.95	9.87 ± 2.01**	10.64 ± 1.14*

Data are presented as mean ± S.D.
*$p < 0.05$ vs control; **$p < 0.01$ vs control.

3.1. Concentration of plasma AChE

To evaluate the effectiveness of dichlorvos poisoning, we monitored the changes of plasma AChE levels. As Table 1 shows, mean plasma AChE activities had significant reduction at 30 min (9.87 ± 2.01 U/ml) and 24 h (10.64 ± 1.14 U/ml) in the dichlorvos group compared with those in the control group (12.6 ± 1.95 U/ml).

3.2. Hematocrit adjusted viscosity (Fig. 1A)

As described in methods, the hematocrit adjusted viscosities (HV) of whole blood at high shear rate (200 s^{-1}) were calculated from whole blood viscosities and hematocrits (data not shown). HV had no changes at 30 min (5.64 ± 0.32 mPa.s), but was elevated obviously at 24 h (7.00 ± 0.98 mPa.s) ($p < 0.05$) after dichlorvos exposure as compared with control group (5.69 ± 0.34 mPa.s).

3.3. Plasma fibrinogen concentration (Fig. 1B)

The plasma fibrinogen concentrations had no obvious alteration 30 min (2.51 ± 0.36 g/L), but increased significantly at 24 h (3.84 ± 0.94 g/L) after dichlorvos exposure compared with control group (2.53 ± 0.45 g/L) ($p < 0.05$), which were parallel to the changes of blood hematocrit adjusted viscosity.

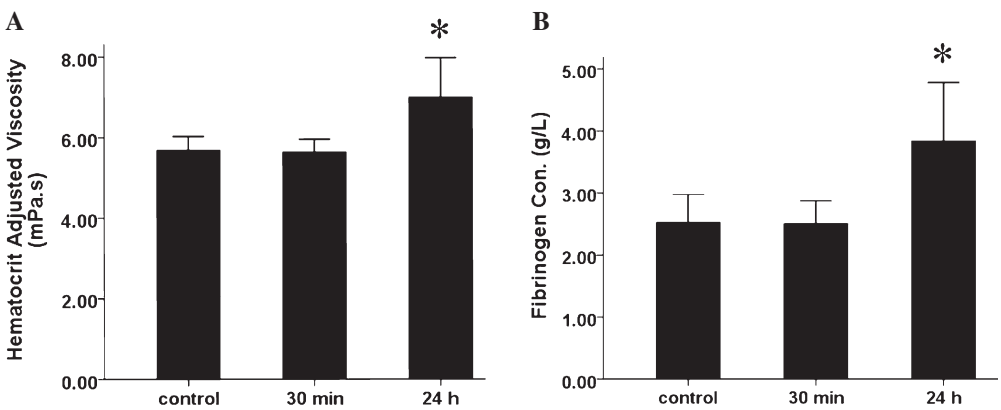

Fig. 1. The blood hematocrit adjusted viscosities (A) at high shear rate of 200 s^{-1} and plasma fibrinogen concentrations (B) in control and at 30 min and 24 h in dichorvos group. Data are presented as mean ± S.D. *$p < 0.05$ vs control.

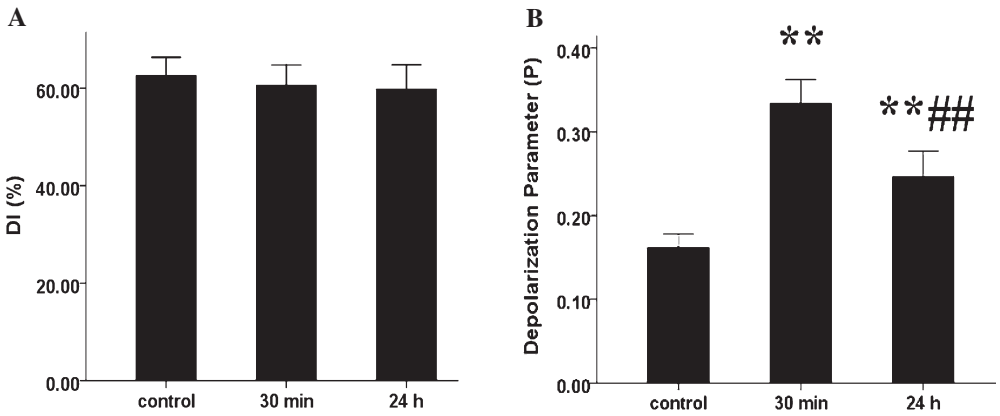

Fig. 2. The hemorheological properties of erythrocytes in control and at 30 min and 24 h in dichorvos group. (A) Erythrocyte deformation index (DI) (%) at shear rate of $1000\,s^{-1}$. (B) The fluorescence polarization parameter *P*. Data are presented as mean ± S.D. $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs 30 min after dichlorvos exposure.

3.4. Erythrocyte deformation index (Fig. 2A)

Erythrocyte deformation index (DI) can represent the ability of erythrocyte deformation. The lower the deformation index, the lower the deformability. In our study, there was a downward trend at 30 min and 24 h after dichlorvos treatment compared with control group. However there were no statistic differences among groups ($p > 0.05$).

3.5. Erythrocyte membrane fluidity (Fig. 2B)

In order to evaluate dichlorvos toxicity on the erythrocyte membrane, fluorescence probe (DPH) was used to analyze the structure and dynamics of erythrocyte membranes following dichlorvos exposure. The fluorescence polarization parameter *P* was 0.33 ± 0.028 in dichlorvos group at 30 min and 0.25 ± 0.031 at 24 h while it was 0.16 ± 0.016 in control group (Fig. 2B). Since *P* is inversely correlated with membrane fluidity, the data shows that treatment with dichlorvos induced a significant decrease in membrane fluidity.

3.6. Osmotic fragility measurements (Fig. 3)

Figure 3A shows the typical osmotic fragility curve of red blood cells in different groups. The haemolysis rates (Hr) at 145 mOsm/kg were compared among groups (Fig. 3B). Those were 57.36 ± 12.02% and 69.82 ± 4.72% respectively in the dichlorvos group at 30 min and 24 h, while that in control group was 51.51 ± 11.37%. Although there was an upward trend, but they did not show statistic differences (Fig. 3B).

3.7. Evaluation of lipid peroxidation of plasma (Fig. 4A)

We estimated the level of lipid peroxidation of plasma in rabbits induced by dichlorvos injection by measuring MDA concentrations. As shown in Fig. 4A, MDA concentrations increased significantly ($p < 0.01$) at 30 min and 24 h after the exposure, as compared with the control.

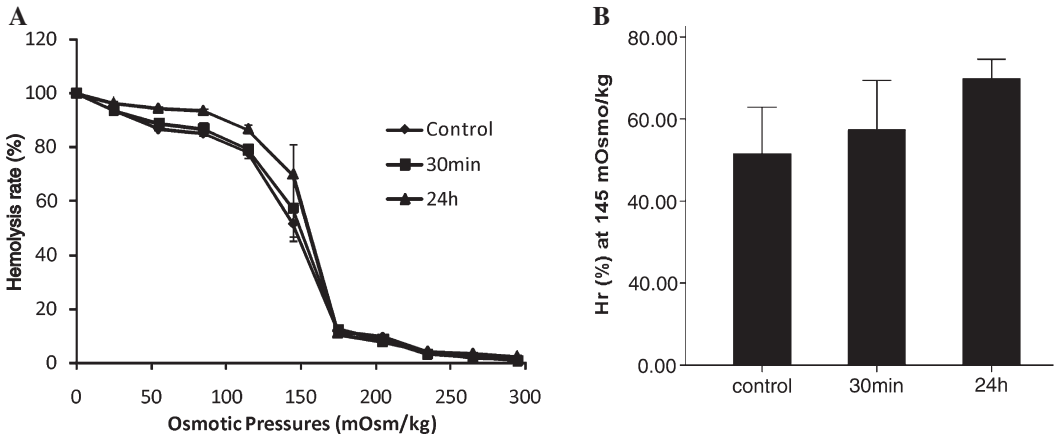

Fig. 3. The osmotic fragility of erythrocytes in control and at 30 min and 24 h in dichorvos group. (A) Typical osmotic fragility curves obtained by plotting the percentages of hemolysis rate against osmotic pressures. (B) The hemolysis rate at osmotic pressure of 145 mOsm/kg. Data are presented as mean ± S.D.

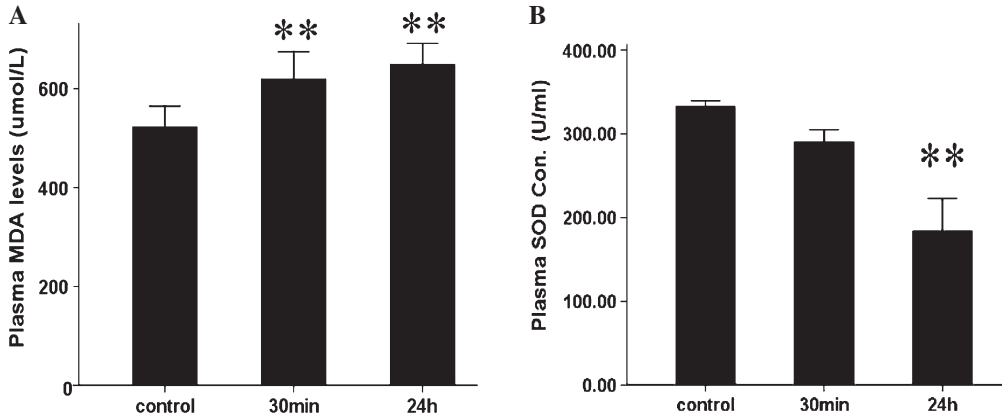

Fig. 4. Plasma MDA levels (A) and plasma SOD (B) in control and at 30 min and 24 h in dichorvos group. Data are presented as mean ± S.D. $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control.

3.8. Plasma SOD level (Fig. 4B)

Plasma SOD levels are shown in Fig. 4B. It was found that SOD levels decreased dramatically at 24 h ($p < 0.01$) after dichlorvos injection compared with the control concentration. The SOD concentration was 184.28 ± 38.22 U/mL at 24 h after the exposure, which is almost half of the normal concentration (332.67 ± 7.39 U/mL).

4. Discussion

The overall results of this study indicated that exposure to dichlorvos could induce oxidative stress in plasma and changes in certain hemorheological parameters. All the rabbits receiving dichlorvos developed cholinergic signs and had 33.3% mortality rate. The poisoning signs disappeared fairly rapidly within 30 min, which is consistent with Giray et al.'s observation [21]. However, the activity of plasma acetyl-

cholinesterase was still low at 24 h after dichlorvos exposure. Acetylcholinesterase recovered more slowly than poisoning signs.

Whole blood viscosity is an important parameter in hemorheology, which studies the rheological properties of blood and blood cells. The major determinants of whole-blood viscosity are plasma viscosity, hematocrit and the properties of blood red cells including erythrocyte deformability and erythrocyte aggregation [17, 23, 30]. In order to eliminate the influence of hematocrit on whole-blood viscosity, hematocrit adjusted viscosity was calculated. Our data showed that the hematocrit adjusted viscosity at $200\,s^{-1}$ was elevated significantly at 24 h after dichlorvos exposure compared with control group. The increase of hematocrit adjusted viscosity would enhance the resistance of blood flow, influence the perfusion of microcirculation and then result in ischemic of important organs [46]. The plasma fibrinogen is the most important factor for plasma viscosity [25]. The changes of plasma fibrinogen were parallel to those of blood hematocrit adjusted viscosity. Hence the changes of plasma fibrinogen may play a role in the increase of hematocrit adjusted viscosity after intoxication.

Additionally erythrocyte deformability and membrane fluidity affect the hematocrit adjusted viscosity [32, 33]. The membrane is an important discriminating filter for cell functions, so it is a selective barrier for the presence of specific channels and pumps. These transport systems influence the ionic and molecular composition of the intracellular space [34]. The plasma membrane controls the activity of membrane proteins since the modifications of the physicochemical state of phospholipids can modulate the activity of these proteins, characterized by a dynamic structure [14]. All of these membrane functions dependence on membrane fluidity. Changes in phospholipids, fatty acids and in the cholesterol content modulate the membrane fluidity, which influences the enzymatic activity, and the functionality of receptors and channels. In this work, erythrocyte membrane fluidity was decreased immediately at 30 min and still in a low level at 24 h after dichlorvos injection. The decrease of membrane fluidity would reduce the erythrocyte deformability, and raise the hematocrit adjusted viscosity of blood. It could also influence the membrane functions described above and then result in pathological changes of tissue.

The erythrocyte membrane may be susceptible to oxidative damage due to the presence of polyunsaturated fatty acid, which may produce oxidative changes in cellular membrane. Recent studies pointed out that oxidative stress could be an important part of the mechanism of OP. In these studies, lipid peroxidation (LPO) has been suggested as one of the molecular mechanisms involved in OP-induced toxicity [3]. Our study found that the production of lipid peroxidation, MDA, were enhanced, and plasma SOD level decreased significantly after intoxication. These data imply that the decrease of erythrocyte membrane fluidity after dichlorvos exposure may be induced by oxidative damage.

We noticed that the MDA enhanced obviously 30 min later after exposure while the level of SOD began to decrease at 24 h. This suggests that the antioxidant systems did not reduce significantly in the early period of exposure, which indicates that the body may have a compensatory reaction to counteract the increased lipid peroxidation.

In this study, the level of plasma MDA is not parallel to the change of acetylcholinesterase. It suggests that the oxidative stress is independent of AChE inhibition [28, 36]. The primary toxicity associated with acute exposure to OP insecticides is cholinergic crisis resulted from inhibition of acetylcholinesterase activity [37]. Additional potential effects of OPs include delayed polyneuropathy, immunotoxicity, carcinogenesis, and endocrine, developmental, and reproductive toxicities [5, 42]. The lipid peroxidation may be responsible for additional potential effects of OPs [15, 27].

Overall, the results obtained in this study suggest that oxidative stress does occur in AOPP and may lead to hemorheological alterations. The changes of hemorheology may be responsible for the pathophysiology of the dichlorvos poisoning. Oxidative stress and hemorheological alterations may be mechanisms involved in the progression of dichlorvos poisoning.

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