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Follow up studies on the respiratory pattern and total cholinesterase activities in dichlorvos-poisoned rats

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HIGHLIGHTS

- Study was performed in Sprague-Dawley rats.
- Dichlorvos effect on ventilation and cholinesterase activities from 5 min to 72 h.
- Respiratory effects returned to control values within 90 min.
- Cholinesterase activities were significantly decreased until 24 h.
- No correlation between respiratory effects and residual cholinesterase activities.

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ABSTRACT

A human prospective study confirmed that the severity and time-course of organophosphate poisonings depend on the compound. Our purpose was to assess the ventilation at rest and cholinesterase activities from 5 min to 72 h in rats poisoned with dichlorvos at 40% of the MLD (5.12 mg/kg). Ventilation at rest was recorded by whole body plethysmography and core temperature by infrared telemetry (DSI system). Results are expressed as mean ± SEM. Statistical analyses used two-way ANOVA. Dichlorvos induced the onset of respiratory effects within 5 min and hypothermia which peaked at 15 min, both reversed within 90 min post-injection. Dichlorvos significantly decreased respiratory frequency, resulting from an increase in expiratory time and associated with increased tidal volume. Tissues and whole blood cholinesterase activities were significantly decreased until the end of experiment. Our study showed that an inhibition of cholinesterase was correlated with an effect on respiratory functions at 15 min and 60 min. However, 24 h post-poisoning, the increase in cholinesterase activity was not completed while ventilatory parameters were within the normal range.

Respiratory effects were both qualitatively and quantitatively similar to those induced by diethylparaoxon. However the effects strongly differed between diethylparaoxon lasting hours while dichlorvos lasted tens of minutes.

1. Introduction

The early and intermediate phases of toxicity of OPs are related to the inhibition of cholinesterase activity, resulting in accumulation of acetylcholine within the synapses throughout the body which induces an overstimulation of the autonomic nervous system (Takahashi et al., 1991; Tsai et al., 2007). Respiratory failure is considered as the primary cause of death (Durham and Hayes, 1962; Lerman and Gutman, 1988; Yamashita et al., 1997). However, its mechanism remains unclear resulting from peripheral effects, including bronchorrhea, bronchoconstriction, and muscle weakness (Fryer et al., 2004; Gaspari and Paydarfar, 2009; Segura et al., 1999), and central apnea (Chang et al., 1990; Gaspari and Paydarfar, 2007).

According to the World Health Organization, OP toxicity is classified in four groups, (Ia, Ib, III, and IV) regarding the value of orally LD_{50} of each compound in rats (WHO, 2001). A recent prospective study on human poisonings resulting from chlorpyrifos, fenthion, and dimethoate ingestion showed that the clinical findings and severity of poisoning were significantly different from one

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compound to the other (Eddleston et al., 2005a) in terms of intubation, odds of death, the mode of death and response to treatment. Despite these data, human treatment of OP poisoning is similar for all pesticides (Tsai et al., 2007). In previous studies, we reported ventilatory effects in rats showing that a toxic but not lethal dose of diethylparaoxon, induced a decrease in respiratory frequency associated with an increase in the expiratory time and tidal volume (Houze et al., 2008; Villa et al., 2007). To test the hypothesis that the toxicity of OP is dependent on the nature of the compound, we studied the effects of dichlorvos on ventilation at rest in rats. In WHO classification, dichlorvos is ranged among extremely hazardous compounds (group Ib) whereas diethylparathion and its metabolite are in group Ia. Dichlorvos has been widely used as farming or domestic pesticide in South Asia and Mediterranean countries since 1955 (Brahmi et al., 2006; WHO, 2001; Yurumez et al., 2007). Regarding its high toxicity as well as its availability, dichlorvos is frequently involved in human poisonings (Eddleston et al., 2005b; Sungur and Guven, 2001).

To our knowledge, no studies previously compared the respiratory toxicity in acute dichlorvos poisoning with cholinesterase activities in whole blood and tissues. Therefore, we determined, firstly, the median lethal dose (MLD) of dichlorvos administered subcutaneously; secondly, we studied the effects of 40% of the MLD of dichlorvos on ventilation at rest using whole body plethysmography and arterial blood gas analysis in awake rats between 5 min to 72 h post-injection. Finally, we studied the degree of inhibition of whole blood and tissue cholinesterase activities.

2. Materials and methods

All animal procedures used in this study were approved (P2.FB.071.09) by the Regional Ethical Committee of Animal Experimentation (University René Descartes, Paris Descartes).

2.1. Animals

Male Sprague-Dawley rats (200 and 250 g) were purchased from JANVIER (Route des chênes secs BP5, FR-53940 Le Genest-St Isle, France). Animals were housed in a light-and temperature- controlled setting with access to food and water *ad libitum*. After each experiment rats were euthanized using overdose of pentobarbital.

2.2. Chemicals and drugs

Dichlorvos (2,2-dichlorovinyldimethylphosphate, purity greater than 98.8%, CAS number 62-73-7), dimethylsulfoxyde, acetylcholinesterase of the electric eel *Electrophorus electricus* (AchE; 200 UI/mg), dihydrated monosodium phosphate, dihydrated disodium phosphate, chloroacetic acid, chloridric acid, sodium chloride, sodium hydroxide, triton X-100 and albumine bovine were obtained by Sigma–Aldrich (St Quentin Fallavier, France).

Dichlorvos was diluted in dimethylsulfoxide so as to obtain a mother solution of 60 mg/ml. A daughter solution was prepared extemporaneously in isotonic saline solution (3.84 mg/ml) to facilitate the injection of doses equal to 40% of the MLD. All solutions of dichlorvos were preserved at 4 °C in the dark for a maximum period of 4 weeks.

Anaesthtic drugs, ketamine (Ketalar®) and xylazine (Rompum®) were obtained from Parke Davis (Paris, France) and Bayer (Paris, France), respectively. Heparine solution (Choay®) at 25000 UI/5 ml was obtained from Sanofi Winthrop, France. [3H]Acetylcholine iodide (specific activity, 100 Ci/mmol) was obtained from PerkinElmer (Courtaboeuf, France) and was stored at −80 °C. Acetylcholine iodide (mw: 273g/mol, $C_7H_{16}INO_2$) was obtained from Fluka (Paris, France) and was stored at −80 °C. Precipath U (PPU) and Precinorm U (PNU) were obtained from Roche (France). Isoamyl alcohol was obtained from Merck (Fontenay Sous Bois, France). Distilled water (Frésenius FrancePharma, Louviers, France) was used for preparation of the various reagents.

2.3. Safety precaution

All solutions of dichlorvos were prepared under fume hood using nitrile gloves and goggles. Molar sodium carbonate solution was set under fume hood to neutralize immediately dichlorvos in the case of accidental spillage. Daily, after each experimental study, the same solution was used to decontaminate and clean up all materials and areas.

2.4. Methods

2.4.1. Clinical examination

The animals were clinically observed at each time of the study and were simultaneously assessed by two observers. The following signs were noted: fasciculation, salivation, lacrimation, urination, defecation, ataxia, tonic–clonic seizures, prostration, coma, and death (De Candole et al., 1953; Tsao et al., 1990). Qualitative assessment of each sign and symptom was made using a grading in severity, including, none (0), mild (1), moderate (2), and severe (3) according to that previously reported by De Candole. The central core temperature was simultaneously measured by infra red telemetry (Houze et al., 2008).

2.4.2. Determination of the average lethal doses (MLD)

The median lethal dose for 50% of rats (MLD) was determined using the up-and-down method previously reported by Bruce and refined by Dixon (Bruce, 1985; Dixon, 1991).

2.4.3. Whole body plethysmography

Respiratory parameters were recorded in a whole-body plethysmography using the barometric method validated in the rat by Bartlett and Tenney (1970), and described in our previous studies with minor modifications (Houze et al., 2010, 2008; Villa et al., 2007). The following parameters were measured: barometric pressure, chamber temperature, core temperature, respiratory frequency (f), inspiratory time (T_I), expiratory time (T_E), total time ($T_{TOT} = T_I + T_E$), tidal volume (V_T), and minute ventilation ($V_E = V_T \times f$).

2.4.4. Measurement of arterial blood gases

The femoral catheterization and the measurement of arterial blood gases were described in a previous study (Villa et al., 2007). Arterial blood samples were collected in heparinized syringes and immediately analyzed on a Rapidlab® 248, (Bayer Diagnostics).

2.4.5. Measurement of whole blood and tissues cholinesterases (ChE) activity

2.4.5.1. Blood sampling. Rats were anaesthetized with pentobarbital and blood collection was made by cardiac puncture (in heparinized syringes). All blood sampling were collected in heparinized Eppendorf cups and stored at −80 °C until the assay.

2.4.5.2. Preparation of tissues homogenates. Before the collection of tissues rats were anesthetized and thoracotomised. The right auricle of the heart was excised for exsanguination and all tissues were perfused simultaneously *via* the left ventricle with 60 ml of heparinized saline solution at 37 °C while the heart was still beating (Olivier et al., 1990). The volume of perfusion used in our study was necessary to remove all circulating blood volume, plasma butyrylcholinesterase or residual hemoglobin. The circulating blood volume in rat was comprised between 50 and 70 ml/kg (Morton et al., 1993). To check if the perfusion was correctly made we observed the mucous membranes and the extremities. During the perfusion we observed pale mucous membranes of the conjunctiva or inside the mouth, pale tongue, and pale extremities and liver turned pale. After exsanguination and perfusion, tissues (forebrain, brainstem, lungs, thigh and diaphragm muscles) were collected and stored at −80 °C. Frozen tissues were diluted in 9 volumes of 0.1 M sodium phosphate buffer (1:10, w:v) (pH 7.4) with 1% Triton X-100, followed by homogenization of the tissues for 30 s (Ultraturax IKA, T10 model, IMLAB, Lille, France). The supernatant was collected after centrifugation at 4 °C for 10 min at 4000 rpm and the total protein concentration was measured by the method of Lowry (Lowry et al., 1951) on modular® (Roche, France) using bovine serum albumin as standard and samples were frozen at −80 °C until radiometric assay.

2.4.5.3. Radiometric assay. Total cholinesterase (butyrylcholinesterase and acetylcholinesterase) activity was measured radiometrically by the method of Johnson and Russell (1975) using [3H]acetylcholine iodide as substrate. This assay was based on the selective extraction of the hydrolysis product, [3H]acetic acid, into a scintillation fluid. The incubation mixture was 0.12 ml containing 5 mM [3H]acetylcholine iodide and 50 mM phosphate buffer, pH 7.0, with 10 μl samples or control samples (Precipath: PPU and Precinorm: PNU) or acetylcholinesterase at 105 UI/l (hydrolysis total) for 15 min at room temperature. The reaction was stopped by adding 0.1 ml of a solution with the composition 1.0 M monochloroacetic acid, 2 M sodium chloride, and 0.5 M sodium hydroxide. Then 0.6 ml of isoamyl alcohol was added to the mixture, shaken and centrifuged for 10 min at 4000 g. [3H]Acetic acid was selectively extracted from the acid solution into 3 ml of a scintillation fluid added to the reaction vial. Cholinesterase activities measured in whole blood were reported as cpm/min per/ml of erythrocytes and in homogenates tissues in cpm/min per/g protein. Cholinesterase activities were expressed as the percent of control activity and plotted as a function of time after treatment.

2.4.5.4. Validation of the assay. The method was validate over a linear range of 0–1.75 UI/ml. The limits of detection (LOD) and quantification (LOQ) were 0.03 UI/ml and 0.09 UI/ml. Concerning the precision of the method, coefficients of variation have been calculated on replicates of 10 samples at different enzymatic activity.