High Dose of Pralidoxime Reverses Paraoxon-Induced Respiratory Toxicity in Mice

Pascal Houzé1, Thomas Berthin1, Jean-Herlé Raphalen2, Alice Hutin2, J. Frédéric Baud3

1Laboratoire de Biochimie, Hôpital Universitaire Necker-Enfants malades, Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France
2Département d’Anesthésie – Réanimation - SAMU de Paris, Hôpital Universitaire Necker-Enfants malades, Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France

Objective: The efficiency of pralidoxime in the treatment of human organophosphates poisoning is still unclear. In a rat model, we showed that pralidoxime induced a complete but concentration-dependent reversal of paraoxon-induced respiratory toxicity. The aim of this study was to assess the efficiency of pralidoxime in a species other than rats.

Methods: A dose of diethylparaoxon corresponding to 50% of the median lethal dose was administered subcutaneously to male F1B6D2 mice. Ascending single pralidoxime doses of 10, 50-100 and 150 mg kg−1 were administered intramuscularly 30 min after diethylparaoxon administration. Ventilation at rest was assessed using whole-body plethysmography and mice temperature was assessed using infrared telemetry. Results are expressed as mean±SE. Statistical analysis used non-parametric tests.

Results: From 30 to 150 min post-injection, diethylparaoxon induced clinical symptoms and a decrease in respiratory frequency, which resulted from an increase in expiratory and inspiratory times associated with an increase in the tidal volume. In the 10-, 50- and 100-mg kg−1 pralidoxime groups, there was a trend towards a non-significant improvement of paraoxon-induced respiratory toxicity. The 150 mg kg−1 dose of pralidoxime induced a significant reversal of all respiratory parameters.

Conclusion: In the present study, a toxic but non-lethal model of diethylparaoxon in awake, unrestrained mice was observed. By administering an equipotent dose of diethylparaoxon to rats, a 150 mg kg−1 dose of pralidoxime administered alone completely reversed diethylparaoxon-induced respiratory toxicity in mice. The dose dependency of reversal suggests that further studies are needed for assessing plasma concentrations of pralidoxime resulting in reversal of toxicity.

Keywords: Diethylparaoxon, pralidoxime, mice, plethymosgraphy, organophosphates

Introduction

Organophosphates are used daily worldwide as pesticides. However, they remain a major health concern because of the large number of annual acute poisonings. According to data by the World Health Organization, there are >3 million organophosphate intoxications annually and >220,000 deaths (1).

Respiratory failure is considered to be the primary cause of death in acute organophosphate poisonings (2). The underlying mechanism of organophosphate-induced respiratory failure remains unclear. Acute respiratory failure induced by organophosphates is thought to result from a direct depressant effect on the respiratory centre in the brainstem, constriction of and increased secretion by the airways and paralysis of the respiratory musculature (3). Data support the hypothesis that some organophosphates including diethylparaoxon can also injure the air–blood membrane (4).

Early toxicity caused by organophosphates is related to the inhibition of cholinesterase activity which results in the accumulation of acetylcholine within synaptic clefts throughout the body (5). This accumulation of acetylcholine induces overstim-
ulation of the autonomous nervous system resulting in the so-called cholinergic crisis.

Various oximes, including pralidoxime, obidoxime and Hi-6, are potent re-activators of organophosphate-induced cholinesterase inhibition in experimental conditions including in vitro as well as in vivo toxicity (6). In a cyclosarin model of poisoning in a cat, Sundwall (7) showed that the efficiency of oxime was related to its plasma concentration. Survival of animals was associated with plasma concentrations of pralidoxime ≥4 mg L⁻¹. In humans, the results are more controversial. Retrospective studies did not result in definitive conclusions. However, there were a number of biases, including the problem of ageing, numerous cholinesterase inhibitors involving carbamate insecticides that are known not to be reversed by oximes and dosage regimens of pralidoxime (1, 8, 9). Two randomised clinical trials resulted in opposite results (2); meanwhile, as expected, a meta-analysis concluded on the lack of efficiency of pralidoxime (8).

In a rat model of paraoxon poisoning in awake, unrestrained rats, we showed that a single high (50 mg kg⁻¹) dose of pralidoxime administered intramuscularly completely, albeit transiently, reversed paraoxon-induced toxicity (10). Thereafter, using this model, there was cumulative evidence that the ability of pralidoxime to reverse paraoxon-induced respiratory toxicity was strongly dependent on plasma pralidoxime concentrations (11).

The present study reports a model of paraoxon poisoning using a toxic but non-lethal dose (50% of the LD₅₀) in awake, unrestrained mice to test dose dependency in the efficiency of pralidoxime to reverse respiratory toxicity in mice.

Methods

All experiments were performed with the agreement of the animal ethical committee of the Faculty of Pharmacy of Paris (registered number P2.FB.073.09).

Animals

Animals employed were F1B6D2 male mice (Société Janvier, France) weighing 20 g at the time of the experiment. They were housed in a temperature- and light-controlled animal care unit; water and food was provided ad libitum.

Chemicals and drugs

Diethylparaaxon (diethyl p-nitrophenyl phosphate, CAS number 311-45-5, purity >90%) was obtained from Sigma-Aldrich (St Quentin Fallavier, France). Diethylparaaxon solutions were prepared as reported previously (12).

Pralidoxime (CAS number: 6735-59-7) methylsulfate (Contrathion©) was a gift by SERB Laboratory (Paris, France). Pralidoxime was diluted in isotonic saline solution to obtain 0.5, 2.5, 5 and 7.5 mg mL⁻¹ solutions, corresponding to 10, 50-100 and 150 mg kg⁻¹, respectively. All doses are expressed as pralidoxime cation, and a correction factor of 1.7 between pralidoxime and Contrathion* was used. The solutions were stored at +4°C during no more than 1 month.

Safety precaution

All solutions of diethylparaaxon were prepared under a fume hood using nitrile gloves, overalls and goggles, as reported previously (12).

Median lethal dose (MLD₅₀) determination

Every effort was made to reduce the number of animals required for the study. Accordingly, the up-and-down method, as proposed by Dixon (13) and refined by Bruce (14), was used. Diethylparaaxon was administered subcutaneously.

Clinical examination

The animals were clinically observed while plethysmography measurements were performed. The signs were noted and quantified according to the method described by De Candole et al. (15). Clinical examination was semi-quantitative and always realised by the same experimenter.

Core temperature

The temperature was measured using a rectal plummet (ref BIO-BIT-14, BIOSEB, Chaville, France) bound to an RSIC thermometer (BIOSEB, Chaville, France).

Whole-body plethysmography

Respiratory parameters were recorded in a whole-body plethysmograph by the barometric method described by Bartlett and Tenney (16) and adapted by Bonora et al. (17). The animals were placed in a rectangular Plexiglas chamber with a volume of 700 mL connected to a reference chamber of the same size by a high-resistance leak to minimise the effect of pressure changes in the experimental room. The animal chamber was flushed continuously with humidified air at a rate of 0.9 L min⁻¹. During recording periods, inlet and outlet tubes were temporarily clamped and pressure changes associated with each breath were recorded with a differential pressure transducer (Valdyne MP, 45±3 cm H₂O, Northridge, CA) connected to the animal and reference chambers. During each measurement, calibration was performed by one injection of 100 μL of air into the chamber, and the ambient temperature was noted. The spirogram was recorded and stored on a computer with a acquisition data card (PCI-DAS1000, Dipsi, Chatillon, France) using respiratory acquisition software (Elphy Software, CNRS-UNIC, Gif-sur-Yvette, France) for analysis offline.

Every morning the barometric pressure was measured and at each measure, ambient temperature in the chamber, temperature of the animal, tidal volume (Vₜ), inspiratory time (Tᵢ), expiratory time (Tₑ), total respiratory time (Tₜₒₜ = Tᵢ + Tₑ), respiratory frequency (f) and minute ventilation (Vₑ = Vₜ × f) were determined.
**Study designs**

**Median lethal dose of subcutaneous diethylparaoxon in mice**
Lethal dose 50 (LD$_{50}$) of diethylparaoxon reported in the literature was 0.27 mg kg$^{-1}$ for rodents. Diethylparaoxon was subcutaneously injected in the neck of mice at different doses equal to 0.5, 1 and 1.5 of this LD$_{50}$. For each dose, if the mouse died in the 24 h after the injection, the second mouse received the same dose divided by a factor of 1.3. If the mouse survived, the next dose was multiplied by the same factor 1.3 and so on. If the injection of diethylparaoxon to two consecutive mice produced two different results (one dead and one alive), the injection of doses multiplied or divided by 1.3 continued for four successive mice. For each dose, LD$_{50}$ calculation was done according to the Bruce and Dixon method (13, 14). MLD$_{50}$ is the median value obtained from three tested doses. At the end of the experiments, the mice were euthanized with an injection of 1 mL of sodium pentobarbital solution to 10 mg L$^{-1}$.

**Mice core temperature measurement**
Measurements were performed using a rectal plummet at the recording times of plethysmography ($T_{5}$, $T_{10}$, $T_{15}$, $T_{20}$, $T_{30}$, $T_{45}$, $T_{60}$, $T_{90}$, $T_{120}$, $T_{150}$ and $T_{180}$ min) to establish temperature profiles in the control (n=5) and diethylparaoxon groups (n=5). The control group received the solvent of diethylparaoxon (isotonic saline solution, SC). The treated group received diethylparaoxon (50% of MLD$_{50}$, SC). Temperature values were used, at each time, for calculating respiratory parameters in the plethysmography study.

**Plethysmography study of the effects of diethylparaoxon on ventilation at rest in mice**
Ventilation at rest was studied in two groups of five animals. The control group received the solvent of diethylparaoxon (isotonic saline solution, SC). The treated group received diethylparaoxon (50% of MLD$_{50}$, SC). The first plethysmography measurement was performed after accommodation for 30 min. Measurements were done three times to obtain baseline values. Then, the animal was gently removed from the chamber for the subcutaneous injection and replaced in the chamber for another session of respiratory recording. Ventilation was recorded during 90 s at the different recording times of plethysmography ($T_{5}$, $T_{10}$, $T_{15}$, $T_{20}$, $T_{30}$, $T_{45}$, $T_{60}$, $T_{90}$, $T_{120}$, $T_{150}$ and $T_{180}$ min). At the end of the experiments, mice were euthanized with an overdose injection of sodium pentobarbital solution.

**Reversal of diethylparaoxon-induced respiratory toxicity by increasing doses of pralidoxime cation**
The pralidoxime cation (10, 50, 100 and 150 mg kg$^{-1}$) was administered intramuscularly in the upper thigh when maximum effects of diethylparaoxon were observed. Ventilation was studied with the same design as that used in previous study 3. At the end of the experiments, the mice were euthanized with an overdose injection of sodium pentobarbital solution.

**Statistical analysis**
Results are expressed as mean±SEM. Graphs, statistical analysis and areas under curves (AUC) determinations were performed using Prism version 5.0, GraphPad Software (San Diego, CA, USA).

**Mice central temperature measurement**
Two groups of five animals were used. Groups were compared using a two-way analysis of variance for repeated measurements. For each parameter, a significant treatment×time interaction was found (p<0.001); this analysis was followed by multiple pairwise comparisons, the global risk being fixed at p<0.05. All tests were two-tailed.

**Plethysmography study of effects of diethylparaoxon on ventilation at rest in mice**
Two groups of five animals were used. The same analysis was performed as reported previously.

**Reversal of diethylparaoxon-induced respiratory effects by increasing doses of pralidoxime cation**
Six groups of five animals were used. The same analysis was performed. The question was to know whether pralidoxime treatment had any effects on the respiratory toxicity induced by diethylparaoxon. Accordingly, effects of the treatments in diethylparaoxon-poisoned rats were compared with the effects of the solvent of pralidoxime administered to diethylparaoxon-poisoned animals. Therefore, comparisons were performed with the Bonferroni adjustment, the global risk being fixed at p<0.05. All tests were two-tailed.

The AUC from 30 to 180 min (AUC$_{30–180}$) after diethylparaoxon, pralidoxime, or solvent injection until the completion of the study were compared by one-way analysis of variance followed by comparison test performed with the Bonferroni adjustment, the global risk being fixed at p<0.05. All tests were two-tailed.

**Results**

**MLD$_{50}$ of subcutaneous diethylparaoxon in mice**
MLD$_{50}$ of diethylparaoxon by subcutaneous administration was 1 mg kg$^{-1}$. Therefore, in the following experiments, a 0.5 mg kg$^{-1}$ dose equal to 50% of MLD$_{50}$ was used.

**Plethysmography study of effects of diethylparaoxon on ventilation at rest in mice**
There were no significant differences in baseline temperature ($T_{5}$) between control and diethylparaoxon groups.

Following the injection of 0.5 mg kg$^{-1}$ of diethylparaoxon, a significant decrease in the temperature was observed 10 min post-injection. Minimal values of 27.8°C±0.11°C in the diethylparaoxon group versus 37.3°C±0.15°C in the control group were observed 45 min post-injection, and the temperature remained below the normal range ranged from 37.0 to 37.5°C during the 180 min of the experiment (Figure 1).
Baseline values
There were no significant differences in respiratory parameters ($f$, $T_E$, $T_I$, $T_{TOT}$, $V_T$, and $V_E$) between control and diethylparaoxon groups.

Clinical findings
Compared with the control group, in the group in which mice were poisoned with diethylparaoxon, there was an onset of prostration, tremulation, salivation and urination within 10 min post-injection. These clinical signs progressively disappeared 60 min post-injection, except prostration which persisted for 150 min.

Plethysmography
At 30 min after diethylparaoxon injection, in comparison with the control group, the $T_E$, $T_I$, $T_{TOT}$ and $V_T$ values were significantly increased in the diethylparaoxon group (0.63±0.13 vs. 0.27±0.03 s (p<0.001), 0.47±0.11 vs. 0.17±0.02 s (p<0.001), 0.16±0.02 vs. 0.11±0.01 s (p<0.01) and 420±32 vs. 200±15 µL (p<0.001), respectively; Figures 2 and 3). Conversely, $f$ was significantly lower in the diethylparaoxon group than in the control group (113±30 vs. 235±32; Figure 4). There was no significant difference in the $V_E$ value at any time in the groups (Figure 3). All respiratory parameters of the diethylparaoxon group became non-significantly different from the control group values after 120 min, except $V_T$ which remained significantly different from that of the control group until the completion of the study.

Reversal of diethylparaoxon-induced respiratory effects by increasing doses of the pralidoxime cation

Baseline values
There were no significant differences in respiratory parameters ($f$, $T_E$, $T_I$, $T_{TOT}$, $V_T$, and $V_E$) between control, diethylparaoxon and pralidoxime cation groups.

Clinical findings
In comparison with the control group, mice poisoned with diethylparaoxon exhibited prostration, fasciculation, salivation and urination occurring 10 min post-injection. In mice treated with 10-mg kg$^{-1}$ pralidoxime cation, the symptoms...
persisted. In mice treated with 50- and 100- mg kg\(^{-1}\) pralidoxime cation, the symptoms, except prostration, disappeared at 45 min (15 min after pralidoxime cation injection). In mice treated with 150 mg kg\(^{-1}\) pralidoxime cation, the symptoms completely disappeared at 15 min post-pralidoxime injection.

**Plethysmography**

In the 10-, 50- and 100-mg kg\(^{-1}\) pralidoxime groups, \(f, T_e, T_i\), and \(T_{TOT}\) were non-significantly corrected by the pralidoxime cation. In the 150 mg kg\(^{-1}\) group, the pralidoxime cation induced a significant reversal of \(f, T_e, T_i\) and \(T_{TOT}\) values at 45 min post-paraoxon injection and the parameters returned to normal values at 90–180 min post-paraoxon injection. There were no significant effects of pralidoxime at any dose and at any time on \(V_T\) and \(V_E\).

The comparison of AUC\(_{30–180}\) showed that there were significant differences between the diethylparaoxon-saline versus the 150 mg kg\(^{-1}\) diethylparaoxon-pralidoxime groups with regard to \(f, T_e, T_i\) and \(T_{TOT}\) values (Figure 5).

**Discussion**

Alterations of respiration have been consistently reported (1, 2, 9, 18, 19), and respiratory failure remains a major cause of mortality in organophosphate poisonings (1, 2, 9, 20). The underlying mechanisms of organophosphate-induced respiratory failure still remain unclear. A number of experimental studies have been conducted using chemical weapons (21-23). However, majority acute human organophosphate poisonings result from exposure to insecticides (2, 5). Dieth-

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**Figure 4.** Effects of solvent (white circle) and diethylparaoxon MLD50 (black circle) on the respiratory frequency. Each group comprised five mice. Data are represented by means±S.E.M. at each time point post-injection. Diethylparaoxon versus control: *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\)

**Figure 5.** Effects of solvent (white), diethylparaoxon MLD50 (black), 10 mg kg\(^{-1}\) pralidoxime (dotted), 50 mg kg\(^{-1}\) pralidoxime (horizontal line), 100 mg kg\(^{-1}\) pralidoxime (cross-ruling) and 150 mg kg\(^{-1}\) pralidoxime (vertical line) on the respiratory pattern. Each group comprised five mice. Data are represented by areas under the curve from 30 to 180 min (AUC\(_{30–180}\)). Diethylparaoxon versus control: *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\); 150 mg kg\(^{-1}\) pralidoxime versus diethylparaoxon: +\(p<0.05\), ++\(p<0.01\) and +++\(p<0.001\)
yparaoxon induces alterations of ventilation at rest in rats (10-12). Diethylparaoxon-induced respiratory effects are completely reversed by atropine but not by methylatropine (24); a finding that suggests a toxic effect on the central nervous system rather than on the peripheral and neuromuscular sites. In contrast, a high 50 mg kg\(^{-1}\) dose of pralidoxime administered intramuscularly induced a partial, albeit complete, reversal of respiratory toxicity after diethylparaoxon administration. This dose was selected to result in the greatest area under the plasma pralidoxime concentrations above 4 mg L\(^{-1}\), as reported by Sundwall in cats (7) and suggested by Willems in clinical studies (25).

The aim of this study was to validate the model of intoxication by diethylparaoxon in mice to assess the efficiency of pralidoxime in another species than rat, i.e. in mice. Therefore, we first determined the experimental MLD\(_{50}\) value of subcutaneous diethylparaoxon in mice using conditions described previously (10, 12). The MLD\(_{50}\) value for diethylparaoxon administered subcutaneously in mice reported in the literature was 0.27 mg kg\(^{-1}\) (26). We found that this MLD\(_{50}\) had no effect on mice. In contrast, Leposava and Radmanovic (26) reported a variable MLD\(_{50}\) ranging from 0.18 to 0.43 mg kg\(^{-1}\). Consequently, they used the method of Wilcox which requires the use of 40 animals for each series of dose; however, we used the method used by Bruce and Dixon (13, 14) and evaluated the MLD\(_{50}\) value of diethylparaoxon in mice at 1 mg kg\(^{-1}\). In the present study, we poisoned mice with a dose corresponding to 50\% of MLD\(_{50}\), i.e. 0.5 mg kg\(^{-1}\), to build a toxic but non-lethal model. We noted a difference regarding values of the subcutaneous MLD\(_{50}\) between mouse and rat. In rat, the MLD\(_{50}\) value of subcutaneous diethylparaoxon is 0.4 mg kg\(^{-1}\) (12) compared with 1 mg kg\(^{-1}\) in mice who were administered subcutaneously. Therefore, it was necessary to inject a larger dose in mice to observe the same magnitude of respiratory toxicity that was observed in rats.

Hypothermia is the main clinical feature of organophosphate poisoning (1, 6, 10-12). We observed that this dose of diethylparaoxon induced hypothermia in mice. The onset of significant and long-lasting hypothermia supports the assumption of validity of our model of paraaxon poisoning in mice. In addition to hypothermia, the main clinical signs observed during the phase of intoxication were fasciculations and prostration in animals. At the onset of maximal effects of diethylparaoxon, lactation and urination were also observed. Therefore, we found the same signs and symptoms in mice that were previously observed in rats who were poisoned with an equipotent dose (10-12).

In rats, we consistently showed that diethylparaoxon induced a highly reproducible respiratory toxicity with a decrease in \(T_E\) and an increase in \(V_T\) (10-12). Therefore, we studied the effects of diethylparaoxon on ventilation at rest in mice using the method of plethysmography, as described previously (12) which was adapted for mice as recommended by Bonora et al. (17). Regarding respiratory toxicity, diethylparaoxon induced a decrease in \(f\) and an increase in \(T_E\) and \(V_T\) in mice as well. In mice but not in rats, we also observed an increase in \(T_R\). Therefore, in mice, \(T_{TOT}\) which is the sum of \(T_E\) and \(T_R\) was induced by diethylparaoxon. \(V_T\) is the product of \(f\) by \(V_T\). As diethylparaoxon induced a decrease in \(f\) and an increase in \(V_T\), \(V_T\) remained unchanged. These effects were shown to be the maximum 30 min post-paraoxon injection and remained significantly increased up to 90 min post-injection with a progressive return to normal values within 180 min. This return to normal was different from what was observed in rats, in which diethylparaoxon-induced respiratory toxicity remained significant even 4 h post-injection (12).

In rats, intramuscular administration of 50 mg kg\(^{-1}\) pralidoxime induced complete reversal of respiratory toxic effects induced by diethylparaoxon, but this reversal was time-limited (11). Therefore, in mice, we tested the effects of ascending doses of pralidoxime at 10, 50, 100 and 150 mg kg\(^{-1}\). The antidotal effects were assessed using plethysmography in awake mice with the same time intervals for measurements as those used in diethylparaoxon poisoning. Regarding the clinical status, after pralidoxime administration, we observed a rapid disappearance of fasciculation, urination and lacrimation from 45 min post-paraoxon injection, i.e. 15 min post-pralidoxime injection. Prostration was weaned by 150 mg kg\(^{-1}\) pralidoxime and to a lesser degree by doses of 50 and 100 mg kg\(^{-1}\) of the pralidoxime cation. However, we observed that mice never completely physiologically recovered with doses below 150 mg kg\(^{-1}\).

Regarding respiratory effects, 10 mg kg\(^{-1}\) pralidoxime resulted in no detectable antidotal effects on diethylparaoxon intoxication as determined by whole-body plethysmography. Doses of 50 and 100 mg kg\(^{-1}\) of pralidoxime induced non-significant antidotal effects on diethylparaoxon. Only the 150 mg kg\(^{-1}\) dose of pralidoxime induced a significant complete reversal of paraaxon-induced respiratory toxicity. We observed a return to normal values of \(f\), \(T_E\) and \(T_R\). Antidotal effects appeared from 15 min post-pralidoxime injection and persisted until the end of the experiment. However, in mice but not in rats, pralidoxime did not improve alteration in \(V_T\). As all signs of paraaxon poisoning cleared with the 150 mg kg\(^{-1}\) dose of pralidoxime, we cannot exclude that the increase in \(V_T\) resulted from intrinsic effects of pralidoxime. There are a number of limitations in the present study. We tested only one toxic but non-lethal dose of diethylparaoxon. Therefore, we cannot assume that, at a greater dose, the mechanisms of paraaxon-induced respiratory toxicity would be the same. We did not measure arterial blood gas concentrations; therefore, we cannot assume that our model resulted in respiratory failure. In rats, an equipotent dose of diethylparaoxon did not result in alteration of arterial blood gases (12). We did not measure blood cholinesterase activities to assess the extent of inhibition induced by an injection of the study dose and the effect of pralidoxime on cholinesterase activity. However, in a
previous study in rats, a pharmacokinetic/pharmacodynamic relationship showed a quite complex correlation between plasma pralidoxime concentrations and blood cholinesterase activity (11).

Conclusion
The present study showed a toxic but non-lethal model of diethylparoxon poisoning in awake, unrestrained mice. Administering an equipotent dose of diethylparoxon to rats and using the same non-invasive method of plethysmography, we showed that a 150 mg kg−1 dose of pralidoxime administered alone, without atropine, completely reversed paroxon-induced respiratory toxicity in mice. The dose dependency of reversal suggests that further studies assessing the range of plasma concentrations of pralidoxime resulting in reversal of toxicity induced by diethylparoxon are required.

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