Toxic doses of paraoxon alter the respiratory pattern without causing respiratory failure in rats

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Received 29 September 2006; received in revised form 1 December 2006; accepted 7 December 2006
Available online 15 December 2006

Abstract

Respiratory failure, through a combination of muscarinic, nicotinic, and central effects, is the primary cause of death in acute organophosphate poisoning. However, the mechanisms inducing respiratory failure remain unclear. In rats poisoned subcutaneously with paraoxon at doses near the LD\textsubscript{50}, we studied the pattern of respiration using whole body plethysmography and the occurrence of respiratory failure using arterial blood gases. Subsequently, we studied the effects of atropine on paraoxon-induced modification of ventilation and arterial blood gases. Fifty and 75%, but not 10% of the subcutaneous LD\textsubscript{50} of paraoxon induced marked and sustained signs and symptoms. At 30 min post-injection and throughout the study, there was a significant decrease in the respiratory frequency (34% (50\% versus solvent), and 29\% (75\% versus solvent)) and a significant increase in the expiratory time (72\% (50\% versus solvent) and 60\% (75\% versus solvent)) with no modifications of the inspiratory time. The tidal volume was significantly increased for the 75\% but not for the 50\% dose. Apnea was never detected. Even at the 75\% dose, paraoxon had no effects on PaO\textsubscript{2}, PaCO\textsubscript{2} or HCO\textsubscript{3}\textsuperscript{−}; however, a significant decrease in arterial pH was observed at 30 min $(7.34 \pm 0.07 \text{ versus } 7.51 \pm 0.01, p=0.03)$. Atropine completely reversed the paraoxon-induced respiratory alterations. We conclude that paraoxon, at doses equal to 50 and 75\% of the LD\textsubscript{50}, alters ventilation at rest without inducing respiratory failure during the study period.

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Keywords: Paraoxon; Atropine; Rats; Plethysmography; Arterial blood gases; Respiration

1. Introduction

Organophosphates are used daily throughout the world as pesticides. However, they remain a major health concern due to the large number of annual acute poisonings. Indeed, according to the World Health Organization data, there are more than 3 million organophosphate
intoxications and more than 220,000 deaths annually (Jeyaratnam, 1990; Segura et al., 1999).

The early and intermediate phases of toxicity of organophosphates are related to the inhibition of cholinesterase activity, resulting in the accumulation of acetylcholine within the synapses throughout the body which induces an overstimulation of the autonomic nervous system. Respiratory failure is considered the primary cause of death (Durham and Hayes, 1962; Lerman et al., 1988; Yamashita et al., 1997). However, its mechanism remains unclear. Organophosphate-induced acute respiratory failure is thought to result from a direct depressant effect on the respiratory center in the brainstem, constriction of and increased secretion by the airways, and paralysis of the respiratory musculature (Bartholomew et al., 1985). Data support the hypothesis that some organophosphates, including paraoxon, can also injure the air-blood membrane (Delaunois et al., 1992).

A number of experimental studies have demonstrated the detrimental effect of organophosphates on ventilation at rest in various species, ranging from rodents to primates. However, the clinical relevance of these findings remains debatable. Indeed, a large number of experiments were performed using chemical weapons (Aas et al., 1987; Anzuo et al., 1990; Edery and Berman, 1985; Gillis et al., 1988; Johnson and Wilcox, 1975; Lipp, 1976; Rickett et al., 1986; Worek et al., 1995) and the extension of these findings to pesticides remains questionable. A recent prospective study on human self-poisonings resulting from chlorpyrifos, fenthion, and dimethoate ingestion showed that the clinical findings, including the onset of respiratory failure requiring endotracheal intubation as well as the final outcome, were significantly different from each other (Eddleston et al., 2005). Finally, Segura and coworkers provided definitive evidence that paraoxon impaired the mechanics of breathing, although arterial blood gases were not measured (Segura et al., 1999). Surprisingly, in goats receiving intravenous dichlorvos followed by atropine, a significant alteration of the pattern of breathing was observed, without any significant modification of arterial blood gases (Bakima et al., 1989).

Thus, we conducted an experimental study to assess the effects of paraoxon on the pattern of respiration at rest using whole body plethysmography and the effects on arterial blood gases in awake rats. First, we confirmed the LD₅₀ of paraoxon administered subcutaneously. Secondly, we studied the effects of three doses, namely 10, 50, and 75% of the LD₅₀ of paraoxon, on ventilation at rest and on arterial blood gases in comparison with those induced by the solvent. Finally, we studied the antido-

tal effects of atropine on paraoxon-induced respiratory effects.

2. Materials and methods

All experiments were carried out within the ethical guidelines established by the National Institutes of Health and the French Minister of Agriculture.

2.1. Animals

Animals employed were Sprague–Dawley male rats (Iffa-Credo, France) weighing between 250 and 350 g at the time of experimentation. They were housed for 8 days before experimentation in a temperature- and light-controlled animal-care unit. They were allowed food and water ad libitum until one day prior to experimentation.

2.2. Chemicals and drugs

Paraoxon (diethyl p-nitrophenyl phosphate) was obtained from Sigma–Aldrich Chemie Gmbh, Steinheim, Germany. Paraoxon was diluted in sterile distilled water to obtain a stock solution of 3.5 mg/ml. Several dilution of paraoxon were prepared (280, 140 and 28 µg/ml), to inject doses equal to 75, 50 and 10% of the LD₅₀. Solutions of paraoxon were preserved from light and stored at 4 °C during a maximum of 4 weeks.

The stability of these aqueous solutions of paraoxon was verified using high liquid performance chromatography with UV detection at 280 nm, on a Lichrosphere RP18 5 µm column (125 mm x 4 mm, Merck). The mobile phase was composed of water–methanol–acetonitrile (40/50/10%, v/v). Parathion was used as internal standard at concentration of 2 µg/mL. Using a flow gradient (0.4–0.9 ml/min.), the paraoxon was typically eluted at 6 min 36 s and parathion at 9 min 42 s.

Atropine sulfate was supplied by Sigma–Aldrich, Saint-Quentin Fallavier, France. Atropine sulfate was diluted in sterile distilled water in order to obtain a stock solution of 18 mg/ml. One dilution (9 mg/ml) was then prepared to inject doses corresponding to 10 mg/kg. This solution was freshly prepared the day of the experimentation.

Propionylthiocholine, 4,4′-dipyridyl disulfide (Aldrithiol®) dihydrated disodium phosphate, and monopotassium phosphate were obtained from Sigma–Aldrich (St Quentin Fallavier, France). Distilled water (Frésenius FrancePharma, Louviers, France) was used for preparation of the various reagents.

2.3. Study 1: median lethal dose (LD₅₀) of subcutaneous paraoxon in rats

Approximately 18 h prior to experimentation, the animals were fasted, but allowed free access to water. Following drug administration, animals were placed in individual cages, allowed to eat and drink, and maintained in the laboratory, which was temperature- and light-controlled. Every effort was
made to reduce the number of animals required for the study. Accordingly, the up-and-down method as proposed by Dixon (Dixon, 1991; Dixon and Mood, 1948) and refined by Bruce (Bruce, 1985, 1987), was employed.

Paraoxon was administered in awake, unrestrained animals, via subcutaneous injection in the neck. Animals were examined repeatedly during the first 4-h period after injection, then daily for 7 days, for evidence of toxicant-related side effects or other illness. At the end of the study period, animals were euthanized using a carbon dioxide chamber.

The animals were observed and the following signs were recorded: dyspnea, fasciculations, salivation and lacrimation, ataxia, paralysis, tonic-clonic seizures, coma, and death. Clinical observations were made before injection and thereafter at 5, 10, 15, 30, 60, 90, 120, 180, 240 min, then at 5, 6, 12, 24 h, and then daily up to 7 days post-injection. Each parameter was graded as absent, mild, moderate, or severe.

2.4. Study 2: effects of paraoxon on respiration at rest and arterial blood gases

2.4.1. Clinical examination

The animals were clinically observed while plethysmography measurements were performed. The following signs were noted: fasciculations, salivation and lacrimation, urination, defecation, ataxia, tonic–clonic seizures, coma, and death. The rectal temperature was measured at the same time.

2.4.2. Whole body plethysmography

Ventilatory parameters were recorded in a whole-body plethysmograph by the barometric method described and validated in the rat by Bartlett and Tenney (1970). The animals were placed in a rectangular Plexiglas chamber with a volume of 3 l connected to a reference chamber of the same size by a high-resistance leak to minimize the effect of pressure changes in the experimental room. The animal chamber was flushed continuously with humidified air at a rate of 5 l/min. During the recording periods, the inlet and outlet tubes were temporarily clamped and the pressures changes associated with each breath were recorded with a differential pressure transducer (Validyne MP, 45 ± 3 cm H2O, Northridge, CA), connected to the animal and reference chambers. During each measurement, calibration was performed by one injection of 1 ml of air into the chamber and the ambient temperature was noted. The spirogram was recorded and stored on a computer with an acquisition data card (PCI-DAS1000, Dipsi, Chatillon, France) using a respiratory acquisition software (Acquis1 Software, CNRS, Gif-sur-Yvette, France) for analysis off-line.

This technique was validated daily with a series of leak tests (a leak was signaled by a diminution of the signal amplitude exceeding 33% in 5 s). The quantification threshold corresponded to a minimum air volume injection of 30 µl. In the range of volumes tested (0.1 to 3 ml), the measurement was linear. The mean coefficient of intra-day variability (four series of 5 measurements carried out the same day) was 1.3 ± 0.2%. The mean coefficient of inter-day variability (25 measurements carried out on 3 different days) was 1.7 ± 0.1%. We verified that the mean CO2 measured using an Ohmeda 5250 RGM capnograph (rebreathing test) during clamping periods did not exceed 0.6% of the air contained in the chamber.

The design of the study was to assess respiratory parameters in unrestrained animals using plethysmography. Whole body plethysmography requires the simultaneous measurements of pressure, as well as ambient and rectal temperatures. However, using unrestrained rats precluded the continuous measurement of the temperature using a rectal probe, while it is well established that toxic doses of organophosphates induce highly significantly effects on core temperature (Coudray-Lucas et al., 1981). Thus, the rectal temperature was measured in a separate study. The animals were placed in the plethysmography chamber under the same conditions as the others, but were temporarily restrained for measurement of rectal temperature using a Plexiglas cylinder (internal diameter: 6.5 cm, adjustable length up to 20 cm) (Harvard Apparatus, Inc., Holliston, MA).

2.4.3. Measurement of arterial blood gases

The day before the study, the animals were anesthetized with ketamine (Ketalar®) 70 mg/kg and xylazine (Rompun®) 10 mg/kg intraperitoneally and then placed on a warming blanket with a regulating thermostat. A rectal probe permitted feedback control of the temperature. The adequacy of anesthesia was judged by complete immobility, deep sleep, and lack of response to painful stimuli.

The femoral artery was catheterized with silastic tubing: external and internal diameters 0.94 and 0.51 mm, respectively; length 30 cm (Dow Corning Co., Midland, MI). The arterial catheters were then tunneled subcutaneously and fixed at the back of the neck. Heparinized saline was injected into the catheter to avoid thrombosis and catheter obstruction. Then, the rats returned to their individual cages for a minimum 24-h recovery period, to allow for complete washout of the anesthesia. On the day of the experiment, rats were placed in a restraining chamber. Before drug injection, the catheter was exteriorized, purged, and its permeability verified. No major problems were encountered during catheterization, drug administration, or collection of arterial blood samples.

The day of the experimentation, rats were placed individually in a Plexiglas cylinder (internal diameter: 6.5 cm, adjustable length up to 20 cm) (Harvard Apparatus, Inc, Massachusetts). The Plexiglas cylinders, as provided, had 4 openings on the cranial extremity and two longitudinal openings in the ventral and dorsal faces. Additionally, we placed several holes at the cranial end of the cylinders in the order to prevent CO2 rebreathing. One hundred microliters of arterial blood samples were collected in preheparinized syringes and immediately analyzed on a Rapidlab® 248, (Bayer Diagnostics). Paraoxon was injected subcutaneously in the neck. Blood samples were taken using the femoral arterial catheter. One hundred microliters of blood were collected before paraoxon or solvent injection, then every 15 min up to 210 min post-injection. The temperatures of the animals were systematically recorded at each sampling time.
2.4.4. Whole blood cholinesterase activity

From catheterized animals, one hundred and fifty micro- liters of heparinized blood were collected in a glass tube at the same time as arterial blood gases. Fifty microliters of blood were transferred to an Eppendorf tube. The sample was then diluted to 1/20 in water. This preparation was immediately frozen at −20 °C until the measurement. The whole blood cholinesterase activity (total cholinesterase activity) was determined by UV kinetic method (324 nm) according to Augustinsson et al. (1978). Briefly, 100 µl of hemolyzed whole blood (1:20 in water) were mixed to 2850 µl Aldrithiol® (0.1 mM in phosphate buffer 50 mM, pH 8). The mixture was incubated for 2 min at 30 °C, then 50 µl of the substrate (propionylthiocholine 0.3 M in distilled water) were added. After mixing, absorbance was determined at 30 °C after an interval of 30 s during 5 min. Sample activity (IU 1−1) was assessed using change in absorbance per min multiplied by a factor (60,000) determined according to various parameters (sample volume, total reaction volume, molecular absorbance coefficient of reaction product, ...). On each hemolyzed sample, hemoglobin was determined according to the method of Drabkin (Drabkin, 1949) in order to express activity in IU g −1 hemoglobin. The method was linear between 1 and 5 min, with a good between-runs precision (coefficient of variation <6%, n = 5). In our results, the total cholinesterase activity of treated groups is expressed as a percentage of the control group activity.

2.4.5. Study design

2.4.5.1. Effects of paraoxon on ventilation at rest and arterial blood gases. Ventilation at rest was studied in animals randomized to four groups of 8 animals each, namely the control, 10, 50, and 75% × LD50 groups. The first measurement was performed after a period of accommodation of 30–60 min while the animal was quiet and not in deep or rapid eye movement sleep which can be roughly estimated from their behavior, the response to noise, and the pattern of breathing. Measurements were made 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 every 15 min during 4 h, each record lasting about 60 s. The following parameters were measured: the tidal volume (VT), the inspiratory time (TI), the expiratory time (TE), and the total respiratory time (TOT = TI + TE). Additional parameters were calculated: the respiratory frequency (f), the minute ventilation (VE = VT × f), the TET/TOT ratio, and the mean inspiratory flow (=VT/TE). Due to the lack of impairment of respiratory parameters in the 10% group, we considered it unnecessary to measure the arterial blood gases and whole blood cholinesterase activities in this group. Arterial blood gases and cholinesterase activities were thus measured in catheterized animals having received the solvent, 50, and 75% × LD50 of paraoxon. Blood specimens were collected before any treatment and then 30, 60, 90, 150, and 210 min after paraoxon injection.

The rats were subsequently euthanized using a carbon dioxide chamber.

2.4.5.2. Reversal of the paraoxon-induced respiratory effects using atropine. Plethysmography revealed that the maximum effects of paraoxon on ventilation at rest were observed 30 min post-injection in the 75% group. Thus, atropine was administered 30 min after paraoxon. Atropine sulfate was administered subcutaneously at a dose of 10 mg/kg. The respiratory parameters were measured before paraoxon administration, then at 15 and 30 min after paraoxon injection. Thereafter, measurements were made at 15, 30, 60, 75, 120, 180 min after injection of atropine. Arterial blood gases were measured at the same times as plethysmography measurements.

The rats were subsequently euthanized using a carbon dioxide chamber.

2.4.5.3. Effects on the whole blood cholinesterase activities. The whole blood cholinesterase activities were measured in the solvent, 50%, and 75% groups as well as in the 75% groups treated with atropine. Activities were determined before any treatment and at 30, 60, 90, 150, 210 min post-injection.

2.5. Statistical analysis

The results are expressed as mean ± S.E.M. Baseline values were compared using the Kruskall–Wallis one-way analysis of variance followed by multiple Dunn’s comparison tests. For each animal, and for each parameter at each sampling time, we calculated the difference between the value at that time and its corresponding baseline value. The areas under the curve (AUC) after paraoxon, atropine or solvent injection until the completion of the study were calculated using the trapezoidal method (Tallarida and Murray, 1981). The AUCs were compared using the Kruskall–Wallis one-way analysis of variance followed by multiple Dunn’s comparison test. In each study we also compared the effect of treatment at each time using the Kruskall–Wallis one-way analysis of variance followed by multiple Dunn’s comparison test or the Mann-Whitney test. All tests were two-tailed and performed using Prism version 3.0 (GraphPad Software, Inc., San Diego, CA). A p-value of less than 0.05 was considered significant.

3. Results

3.1. Study 1: estimated lethal dose (LD50) of subcutaneous paraoxon in rats

The LD50 by the subcutaneous route was 0.430 mg/kg. The animals exhibited various signs including fasciculations, dyspnea, lacrimation, and ataxia.

3.2. Study 2: effects of paraoxon on respiration at rest and on arterial blood gases

3.2.1. Clinical findings

No animals died during the 240 min study period of the plethysmography study. Four (4/8) animals died
Values are presented as mean ± S.E.M.

in the 75% group catheterized for arterial blood gas measurement. Animals in the solvent and 10% group were free of symptoms. In animals receiving the 50 and 75% dose, the most frequent clinical abnormalities were fasciculations, dyspnea, defecation, urination, and ataxia. There were no significant differences in the mean baseline body temperatures. In contrast, there was a significant decrease in the mean body temperatures at the completion of the study in the 50 (p<0.05) and 75% (p<0.001) groups in comparison with the solvent. The mean body temperatures at the completion of the study in the solvent, 10, 50, and 75% groups were, 37.7 ± 0.3, and 35.3 ± 0.5 °C, respectively. During the arterial blood gas study, symptoms were more marked at the 50 and 75% doses than the rats in the plethysmography study. In the 75% group, two animals out of five died. Rats which eventually died presented intense dyspnea, bradypnea with prolonged expiratory phase, and stertorous breathing. Generalized clonus was frequently observed. Gasping respirations were followed by death.

3.2.2. Comparison of baseline values

There were no significant differences in ventilatory parameters or arterial blood gases among the groups (Tables 1 and 2).

3.2.3. Effects of toxic doses of paraoxon on ventilation at rest and arterial blood gases

3.2.3.1. Ventilation at rest. There were no significant differences in any parameters between the solvent and the 10% groups. In contrast, the 50 and the 75% doses induced a rapid onset of significant alterations of the ventilatory pattern that plateaued 30 min post-injection. The comparisons of the AUCs showed that there were significant differences between the solvent and the 50 and 75% groups with regard to the f (p<0.0001), the \( \frac{T_{T}}{T_{TOT}} \) (p<0.0001), the \( T_{E} \) (p<0.0001), the \( T_{I}/T_{TOT} \) (p<0.0001), and the \( V_{T} \) (p<0.0001) (Fig. 1a). In contrast, there were no significant effects on the \( T_{I} \) of paraoxon at any dose. There were no significant effects on the \( V_{T} \) in the 50% group. There were no significant effects on the \( V_{E} \) in the 75% group, or in the 10% group versus the 50% group.

In comparison with the solvent group, with respect to f, 34% (50% versus solvent), and 29% decreases (75% versus solvent) were observed; conversely, regarding the \( T_{TOT} \), there were 51% (50% versus solvent), and 41% increases (75% versus solvent). For the \( T_{E} \), there were 72% (50% versus solvent) and 60% increases (75% versus solvent). For the \( T_{I}/T_{TOT} \), there were 25% increases in both the (50% versus solvent) and (75% versus solvent) groups. Regarding the \( V_{T} \), there were 13% (50% versus solvent) and 32% increases (75% versus solvent). In the 50% group, but not in the 75% group, there was a significant decrease (25%) in the \( V_{E} \) in comparison with the solvent. This effect resulted from the significant decrease of the f (34%) with no significant effect on the \( V_{T} \). The effects of the 50% and the 75% doses were sustained and remained significant at the end of the study, 240 min post-injection (Fig. 1b).

| Table 1 Baseline values of ventilation at rest in the solvent, 10, 50, and 75% LD50 paraoxon groups |
|-----------------|-----------------|-----------------|-----------------|
|                 | Solvent         | 10% DL50        | 50% LD50        | 75% LD50        |
| \( f \) (b/min) | 115.8 ± 5.1     | 107.9 ± 5.1     | 110.6 ± 4.4     | 116.1 ± 3.5     |
| \( T_{TOT} \) (s) | 0.52 ± 0.02 | 0.57 ± 0.03     | 0.56 ± 0.02     | 0.52 ± 0.02     |
| \( T_{I} \) (s)   | 0.18 ± 0.01     | 0.18 ± 0.01     | 0.20 ± 0.01     | 0.17 ± 0.01     |
| \( T_{E} \) (s)   | 0.35 ± 0.01     | 0.38 ± 0.02     | 0.35 ± 0.01     | 0.35 ± 0.01     |
| \( T_{I}/T_{TOT} \) | 0.34 ± 0.01 | 0.36 ± 0.01     | 0.32 ± 0.01     | 0.32 ± 0.01     |
| \( V_{T} \) (µl)  | 2069 ± 107.6    | 1891 ± 106.0    | 1704 ± 45.5     | 1863 ± 125.6    |
| \( V_{E} \) (µl/min) | 240711 ± 18528 | 204290 ± 16972  | 186122 ± 9325   | 222389 ± 16107  |

Values are presented as mean ± S.E.M.

| Table 2 Baseline values of arterial blood gases in the solvent, 50, and 75% LD50 paraoxon groups |
|-----------------|-----------------|-----------------|-----------------|
|                 | Solvent         | 50% LD50        | 75% LD50        |
| pH              | 7.48 ± 0.01     | 7.50 ± 0.01     | 7.51 ± 0.01     |
| \( PaO_{2} \) (kPa) | 12.77 ± 0.20 | 12.06 ± 0.21    | 12.19 ± 0.28    |
| \( PaCO_{2} \) (kPa) | 5.26 ± 0.22 | 4.84 ± 0.08     | 4.82 ± 0.06     |
| \( HCO_{3}^{-} \) (mmol/l) | 28.68 ± 0.98 | 27.84 ± 0.95    | 27.16 ± 0.86    |

Values are presented as mean ± S.E.M.
Fig. 1. (a) Effects of solvent (white), paraoxon 10% LD50 (vertical line), paraoxon 50% LD50 (slanting line), and paraoxon 75% LD50 (cross-ruling) on the ventilatory pattern. Each group consisted of eight rats. Data are represented by areas under the curve (AUC). Paraoxon induced a significant decrease in respiratory frequency, a significant increase in total time, and expiratory time for the 50 and 75% LD50 groups, a significant increase in the tidal volume for the 75% LD50 group only, and a significant decrease in the minute volume for the 50% LD50 group. Differences among groups were compared using the Kruskall–Wallis test followed by Dunn’s test. (b) Effects of solvent (square), paraoxon 10% LD50 (circle), 50% LD50 (rhomb) and 75% LD50 (triangle) on ventilatory pattern. Each group consisted of eight rats. Data are represented by mean ± S.E.M. at each time after injection. At each time, paraoxon induced a significant decrease in respiratory frequency, a significant increase in total time, expiratory time, and tidal volume for the 50% LD50 and 75% LD50 groups, until the end of the study. Differences among groups at each time were compared using the Kruskall–Wallis test followed by Dunn’s test. Paraoxon 50% LD50 vs. solvent: *p < 0.05, **p < 0.01 and ***p < 0.001. Paraoxon 75% LD50 vs. solvent: *p < 0.05, **p < 0.01 and ***p < 0.001.
3.2.3.2. Arterial blood gases. As there was no impairment of the ventilation at rest in the 10% group, the arterial blood gases were compared only in the solvent, 50%, and 75% groups. The AUCs regarding the arterial pH, the PaCO2, the PaO2, and the blood bicarbonate concentrations were not significantly different (Fig. 2a). Regarding the effect of time and treatment, the arterial pH was significantly lower at 30 min post-injection in the 75% group compared to the 50% and solvent groups (7.34 ± 0.07, 7.51 ± 0.01, and 7.51 ± 0.01, respectively; \(p = 0.03\)) (Fig. 2b).

In the 75% group, four animals died before the completion of the arterial blood gas study. The delays from paraoxon injection to death were 15, 30, 60, and 165 min, respectively. Fig. 3 shows individual values of arterial blood gases in the four rats. Values of arterial pH before death were 7.47, 7.00, 6.97, and 7.56, respectively. Values of arterial PaCO2 before death were 3.6, 9.1, 3.4, and 1.2 kPa, respectively. Values of arterial PaO2 before death were 10.8, 6.3, 12.8, and 15 kPa, respectively. Values of arterial blood bicarbonate before death were 19.5, 16.5, 5.7, and 8.1 mmol/l, respectively.
Fig. 3. In the 75% group, four animals died before the completion of the arterial blood gas study. This figure shows individual values of arterial blood gases in the four rats.

3.3. Study 3: effects of atropine on paraoxon-induced alteration of ventilation at rest and arterial blood gases

3.3.1. Comparison of baseline values

There were no significant differences in ventilatory parameters among the three groups (Table 3).

3.3.2. Effects of atropine on paraoxon-induced alteration of ventilation at rest

The 10 mg/kg dose of atropine induced a significant effect on all respiratory parameters with the exception of $V_T/T_I$ (Fig. 4a). All parameters returned to values not significantly different from those in the solvent group. Regarding the $T_I$, there was a significant decrease in the paraoxon + atropine group in comparison with the paraoxon + solvent group ($p<0.01$). The maximum effect occurred 30 min after injection of atropine and remained significant over the 180 min of the study period (Fig. 4b).

3.3.3. Arterial blood gases

There were no significant differences in arterial blood gases between the 75% and 75% + atropine groups (Tables 4 and 5).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Baseline values of ventilation at rest in the solvent, 75% paraoxon, and 75% + atropine groups</th>
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<td>Solvent + solvent</td>
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<td>$f$</td>
<td>124.2 ± 5.379</td>
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<td>$T_{TOT}$</td>
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<tr>
<td>$T_I$</td>
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<td>$T_E$</td>
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<td>$V_E$</td>
<td>241988 ± 23352</td>
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Values are presented as mean ± S.E.M.
Fig. 4. (a) Effects of the injections of both solvents (white), paraoxon 75% LD₅₀ followed by the solvent (slanting line), and paraoxon 75% LD₅₀ followed by atropine (cross ruling) on ventilatory pattern. Each group consisted of five rats. Data are represented by the areas under the curve (AUC). In animals poisoned with paraoxon, atropine induced a reversal of paraoxon-induced alteration in ventilatory pattern. Differences among groups were compared using the Kruskall–Wallis test followed by Dunn’s test. (b) Effects of the injection of solvent (circle), paraoxon 75% LD₅₀ followed by the solvent of atropine (triangle), paraoxon 75% LD₅₀ followed by atropine 10 mg/kg (square). Each group consisted of five rats. Data are represented by mean ± S.E.M. at each time after injection. Atropine induced a complete reversal of paraoxon-induced alteration in ventilatory pattern. Differences among groups at each time were compared using the Kruskall–Wallis test followed by Dunn’s test. Paraoxon + atropine vs. paraoxon + solvent: *p < 0.05, **p < 0.01 and ***p < 0.001. Paraoxon + atropine vs. solvent + solvent: + p < 0.05, ++p < 0.01 and +++p < 0.001. Paraoxon + solvent vs. solvent + solvent: ◦p < 0.05, ◦◦p < 0.01 and ◦◦◦p < 0.001.
Table 4
Baseline values of arterial blood gases in the 75 and 75% + atropine groups

<table>
<thead>
<tr>
<th></th>
<th>75% LD50</th>
<th>75% LD50 + atropine</th>
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</thead>
<tbody>
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<td>pH</td>
<td>7.47 ± 0.01</td>
<td>7.48 ± 0.02</td>
</tr>
<tr>
<td>PaO2</td>
<td>12.82 ± 0.35</td>
<td>12.92 ± 0.19</td>
</tr>
<tr>
<td>PaCO2</td>
<td>5.59 ± 0.08</td>
<td>5.42 ± 0.23</td>
</tr>
<tr>
<td>HCO3⁻</td>
<td>29.76 ± 0.73</td>
<td>29.24 ± 1.25</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M.

3.4. Study 4: effects of solvent, paraoxon alone, and paraoxon + atropine on whole blood cholinesterase activity

3.4.1. Comparison of baseline values

There were no significant differences in blood cholinesterase activities among the four groups (Table 6).

3.4.2. Effects of solvent, paraoxon alone, and paraoxon + atropine

The 50 and 75% LD50 dose induced significant decreases in whole blood cholinesterase activities in comparison with the solvent group (Fig. 5). The whole blood cholinesterase residual activities in the 50 and 75% groups were 43 and 45% of that of the solvent activity. The 10 mg/kg dose of atropine had no significant effects on the paraoxon-induced inhibition of the whole blood cholinesterase activity.

Table 5
Effects of atropine on arterial blood gases in the 75 and 75% + atropine groups

<table>
<thead>
<tr>
<th></th>
<th>75% DL50</th>
<th>75% DL50 + atropine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaO2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaCO2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO3⁻</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M.

4. Discussion

In this study, we demonstrated that paraoxon at doses approaching the LD50 (0.43 mg/kg, in accordance with the previously reported 0.426 mg/kg LD50 (RTECS®, 2005)) significantly impaired ventilation at rest but did not significantly alter arterial blood gases. A single high dose of atropine completely reversed the effects of paraoxon on the ventilation pattern during the 210 min following injection. One limitation of our study results from the fact that we did not use the Litchfield and Wilcoxon method to assess the LD50 of paraoxon but rather the up-and-down method. The Litchfield and Wilcoxon method is a complete method allowing the assessment of not only the LD50 but also the slope of toxicity. However, this method requires many animals. As the LD50 of paraoxon has been already reported, our
aim was only to confirm the LD₅₀ in the animal species we used for the study. Thus, we used a well-described method using a smaller number of animals than the classic method.

We chose to study the effects of paraoxon at doses near the LD₅₀ in awake rats. Similarly, Foutz and coworkers studied the effects of paraoxon administered by intracerebroventricular injection to awake and anesthetized cats on the ventilatory pattern, using whole body plethysmography (Fourtz et al., 1987). Intracerebroventricular injection was performed to avoid peripheral effects of paraoxon such as paralysis of respiratory muscles. In awake animals, paraoxon did not depress respiration but conversely stimulated respiration inducing a 4 to 5-fold increase in the respiratory frequency and minute volume. In contrast, pentobarbital or halothane anaesthesia induced opposite effects of paraoxon, with respiratory depression and even respiratory arrest.

We showed that paraoxon at 50 and 75% of the LD₅₀ induced the rapid onset of alterations in the ventilatory pattern with maximum effects occurring between 30 and 45 min after paraoxon injection, followed by a plateau until the study completion. There was a significant increase in the \( T_{TOT} \) and conversely a significant decrease in the \( f \). The increase in the \( T_{TOT} \) resulted from an increase in the \( T_E \), without any effects on the \( T_I \). Segura and coworkers studied the effects of parathion on the respiratory mechanics in unrestrained guinea pigs, using whole body plethysmography (Segura et al., 1999). There was a significant dose-dependent increase in the total lung resistance index in females, known to be more sensitive to acute toxicity of parathion. This effect was assessed using the determination of the enhanced pause, a calculated index dependent on several parameters including the \( T_E \) (Segura et al., 1999). Accordingly, prolongation of the \( T_E \) in our study strongly suggested peripheral airway obstruction and flow restriction increasing expiratory effort. This is even more underscored by resolution of the changes in the \( T_E \) with atropine. Paraoxon caused peripheral airway obstruction, preferentially expressed during exhalation. The lack of change in PaCO₂ suggested that alveolar ventilation was maintained. Thus, since the \( f \) was decreased, then the \( V_T \) had to increase, maintaining the minute alveolar ventilation and the PaCO₂ with fewer breaths.

A significant increase in the \( V_T \) was only observed in the 75% group. Consistent with this finding, there was a significant decrease in the \( V_E \) in the 50% group while in the 75% group, there was a significant increase in the \( V_E \) in comparison with the solvent. While our data did not show any significant effects of paraoxon on the \( V_T/T_I \) ratio, the simultaneous increase in \( V_T \) with no significant effects on \( T_I \) failed to support the hypothesis of a major depressant effect of paraoxon on the control of breathing within this range of doses. However, the reliability of plethysmography to measure the ventilation in awake animals can be questioned for the measurement of \( V_T \) which can be underestimated, but this technique has been validated against pneumotachography and direct plethysmography with a systemic error on \( V_T \) of <7% (Bonora et al., 2004).

Respiratory failure is defined by alteration of arterial blood gases while breathing room air. Arterial blood gases have been performed only in a very limited number of studies (Bakima et al., 1989; Bardin et al., 1987; Stefanovic et al., 2006). Our study did not show any decrease in PaO₂ in the 50 and 75% groups. There was even a trend towards an initial increase in PaO₂. There was no significant effect of the 50% dose on the arterial pH at any time. In contrast, there was a significant but limited effect of the 75% dose on the arterial pH at 30 min post-injection in comparison with the solvent group. There were no significant effects of the 50 or 75% doses on the PaCO₂. There were no significant effects of the 50 or 75% doses on the blood bicarbonate concentrations using the comparison of AUCs. However, the comparison of values at each sampling time showed that, at 30 min post-injection the blood bicarbonate concentration was lower, although not significantly, in the 75% group versus the solvent group. Our results are in agreement with those previously reported by Bakima and colleagues (Bakima et al., 1989) who administered intravenous dichlorvos to French alpine goats followed by atropine 5–10 min later. Within 2 min of dichlorvos administration, all the goats showed acute respiratory distress. Dynamic compliance decreased while pulmonary resistance significantly increased. However, neither minute ventilation, nor arterial oxygen or PaCO₂ were significantly altered. Atropine treatment reversed all the clinical and functional parameters. Similarly, Rickett and coworkers (Rickett et al., 1986) examined the effects of soman, sarin, tabun, and VX in cats. At the time of respiratory arrest, PaCO₂ and PaO₂ varied little from controls for each of the four agents. However, our results contrasted with those of Stefanovic and coworkers showing the presence of respiratory distress in the dichlorvos-treated rats (1.3 LD₅₀), in comparison to controls (Stefanovic et al., 2006). These authors reported a significant decrease in the pH, a significant decrease in the PaO₂, and a significant increase in the PaCO₂. Differences with our results could be related to differences.
in the organophosphate, the dose, and administration protocol.

Four of eight rats studied using arterial blood gases after a 75% LD$_{50}$ dose died during the procedure. We believe there are two possible explanations for this observation. First, the effects of anaesthesia and surgical placement of arterial lines may have effectively lowered the lethal dose. On the other hand, the results of arterial blood gases collected in these animals suggest the onset of incompletely compensated metabolic acidosis (Fig. 3). It should be noted that only one animal had an increase in PaCO$_2$ before death while there was a progressive decrease in PaCO$_2$ in the three others. Furthermore, in spite of lower pH and PaCO$_2$, the PaO$_2$ remained within the upper limit of the normal range while breathing room air. Thus, the deaths cannot be attributed to hypoxia. Further insight on the mechanisms of death in these animals would require the assessment of hemodynamic parameters and plasma lactate concentrations.

We studied the effects of atropine in the 75% group. A 10 mg/kg dose of atropine sulfate administered subcutaneously 30 min after paraoxon completely and definitively corrected the paraoxon-induced impairment of the ventilatory pattern. Regarding the arterial blood gases, atropine did not induce any significant effects. Animals in the 50 and 75% LD$_{50}$ dose exhibited clinical findings suggestive of both muscarinic syndrome and nicotinic syndrome. In contrast, the alteration of ventilation at rest without impairment of arterial blood gases, the lack of apnea, as well as the complete and sustained effect of atropine supported the hypothesis of primarily muscarinic effects on the respiratory system. Thus, these data suggested that clinical examination does not allow any assumption about the mechanisms, muscarinic and/or nicotinic, of impairment of ventilation. The present results obtained in rats supported the assumption that male Sprague–Dawley rats may be more sensitive to paraoxon than humans as both muscarinic and nicotinic signs were observed with a moderate decrease in whole blood cholinesterase activity in comparison with that observed in humans.

Our study suffers from a number of limitations. We studied the effects of paraoxon only. Thus, we cannot assume that these results can be extended without caution to other compounds. A recent prospective study showed significant differences of frequency as well as severity of respiratory failure in humans self-poisoned with chlorpyrifos, fenthion or dimethoate (Eddleston et al., 2005). Rats are not the species having ventilation at rest the most similar to that of humans. Thus, extrapolation of our data to paraoxon effects in humans may not be justified. Furthermore, we used male rats, while sex-dependency of paraoxon respiratory effects were reported in guinea pigs (Segura et al., 1999). Thus, paraoxon effects on ventilation at rest in females remain to be determined. Finally, in our study, the duration of observation was limited to 210 min after injection. Thus, the late and even delayed effects of toxic doses of paraoxon on ventilation at rest remain unknown. We observed only a moderate decrease in whole blood acetylcholinesterase activity. However in rat diaphragm muscles, neuromuscular dysfunction was not observed until 70% of acetylcholinesterase activity was inhibited (Eyer, 2003). Similarly, in organophosphate poisoned humans, Thiermann and coworkers showed that cholinergic signs subsided when acetylcholinesterase activity was above 20% of the normal (Thiermann et al., 1999). We use atropine sulfate and one important limitation is that atropine may cross the brain blood barrier. Thus, disruption of ventilatory changes by atropine dose not necessarily indicate that it is peripherally mediated, sparing the respiratory control centres. Methyl atropine, an antimuscarinic that does not enter the central nervous system would have been useful here. In conclusion, the injection of 50 and 75% of the subcutaneous LD$_{50}$ of paraoxon induced overt poisoning in male Sprague–Dawley rats and significantly impaired ventilation at rest without inducing respiratory failure. The impairment of the ventilatory pattern induced by the 75% LD$_{50}$ was completely reversed with a single dose of atropine suggesting that muscarinic effects were the primary mechanism of alteration of ventilation at rest.

**Conflict of interest**

None.

**Acknowledgements**

We are indebted to Drs M. Bonora (Inserm E213, Croissance et Réparation du Poumon, Hôpital Saint Antoine, Bât Raoul Kourilsky, 184 rue du Faubourg Saint Antoine 75571 Paris cedex 12) and A. Foutz (Neurobiologie Génétique et Integrative, Institut de Neurobiologie Alfred Fessard, C.N.R.S., 91198 Gissur-Yvette cedex France) for teaching us whole body plethysmography. Dr Bonora kindly accepted to review the part of the manuscript devoted to whole body plethysmography. This study was supported in part by SERB Laboratories (75020 Paris, France).
Appendix A. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>CNRS</td>
<td>Centre National de la Recherche Scientifique</td>
</tr>
<tr>
<td>f</td>
<td>Respiratory frequency</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Blood bicarbonate concentration</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose 50</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Partial pressure of carbon dioxide in arterial blood</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of oxygen in arterial blood</td>
</tr>
<tr>
<td>pH</td>
<td>Arterial pH</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Tₑ</td>
<td>Expiratory time</td>
</tr>
<tr>
<td>Tᵢ</td>
<td>Inspiratory time</td>
</tr>
<tr>
<td>Tᵢ/TOT</td>
<td>Ratio of inspiratory time to total time</td>
</tr>
<tr>
<td>TOT</td>
<td>Total time</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vₑ</td>
<td>Minute ventilation</td>
</tr>
<tr>
<td>Vᵡ</td>
<td>Tidal volume</td>
</tr>
<tr>
<td>Vᵥ/Tᵢ</td>
<td>Ratio of tidal volume to inspiratory time</td>
</tr>
</tbody>
</table>

References


