



Onset Rate and Intensity of Signs of Organophosphate Poisoning Related to Paraoxon Dose and Survival in Rats

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Abstract

Introduction: Organophosphorus compounds (OP) bind to acetylcholinesterase (AChE) and inactivate it. In the synaptic cleft, undestroyed and accumulated acetylcholine produce the acute cholinergic effects. The aim of this study was to determine the frequency, speed of onset and intensity of certain signs of paraoxon poisoning depending on dose and outcome of poisoning.

Methods: The study was conducted in adult Wistar rats. The median lethal dose (LD₅₀) of paraoxon as well as protective ratio (PR) of atropine (10 mg/kg intramuscularly) was determined. Clinical signs of poisoning were observed: fasciculations, tremor, seizures, ataxia, piloerection, lacrimation, exophthalmos, bizarre/stereotypic behaviour and dyspnoea. The time from paraoxon injection to the first appearance of the sign of poisoning was recorded as well as the intensity of poisoning with evaluation at 10 time intervals throughout the 4 h observational period.

Results: The LD₅₀ of paraoxon was 0.33 mg/kg (subcutaneously) and PR of atropine was 2.73. Dose-dependent, piloerection occurred more often ($p = 0.009$) and at higher intensity ($p = 0.016$) at higher doses. Fasciculations, tremor, seizures and ataxia occurred significantly earlier at higher doses of paraoxon ($p = 0.015, 0.002, 0.021$ and 0.016 , respectively), as well as the intensity of seizure, tremor and fasciculation. Piloerection ($p = 0.002$) and seizures occurred more frequently ($p = 0.009$) in non-survivors. Fasciculations, tremor, seizures and ataxia occurred significantly earlier and at higher intensity in non-survivors ($p < 0.001$, for all parameters), as well as dyspnoea ($p = 0.009$ and $p = 0.048$). In atropine-protected rats, nicotinic effects persevered, so they were the prognostic parameter of the severity of the poisoning.

Conclusion: Seizures and fasciculations followed by tremor were strong prognostic parameters of the probability of lethal outcome of paraoxon poisoning. Also, the mentioned poisoning signs were with their intensity and speed of occurrence in a clear positive correlation with the administered dose of paraoxon. Even at high doses of paraoxon, atropine blocked the muscarinic (but not nicotinic) effects and somewhat mitigated the CNS toxic effects.

Keywords: Organophosphate; Acetylcholinesterase inhibitor; Paraoxon; Insecticide; Poisoning; Atropine.

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Introduction

In the body, organophosphorus compound (OPC) binds to the serine group of the enzyme

acetylcholinesterase (AChE, EC 3.1.1.7), phosphorylates it and creates a stable covalent bond

with it.¹ By binding to it, AChE becomes inactive and cannot perform its function, which is the breakdown of acetylcholine in the synaptic cleft.² Accumulated acetylcholine (ACh) stimulates various postsynaptic cholinergic receptors and exhibits its acute cholinergic effects: muscarinic (bronchorrhoea, bronchoconstriction, bradycardia, hypotension, miosis, hypersalivation, lacrimation, nausea, vomiting, increased motility of the bowels and of the bladder), nicotinic (mydriasis, tachycardia, hypertension, fibrillation, fasciculation and necrosis of skeletal muscles) and central (tremor, convulsions, motor incoordination, respiratory depression and coma).³ Besides acute cholinergic crisis, OP poisoning can manifest itself as intermediate syndrome, organophosphate-induced delayed neuropathy (OPIDN) and chronic organophosphate-induced neuropsychiatric disorder due to long-term low-level exposure.⁴

The OP-AChE bond does not spontaneously dissociate (hydrolyses) in the body, which is why OPs are called irreversible AChE inhibitors. The bond can be separated using AChE reactivators, so-called oximes, which are causal antidotes in OP poisonings.⁵ Unfortunately, the diversity of OPs makes oximes insufficiently universal and effective antidotes.⁶ An additional limitation of its efficacy is the process of dealkylation (so-called "ageing") through which the OP-AChE bond passes, after which it is not possible to separate OP and AChE.⁷ Ageing in some OPCs like the nerve agent soman is measured in minutes, while in organophosphorus insecticide (OPI) it is mostly measured in days.⁸

Although oximes are the only causal antidotes, their insufficient efficacy, especially in clinical practice, makes anticholinergic drugs still the main antidotes in OP poisoning. The most commonly used and most available anticholinergic drug is atropine, which blocks the muscarinic effects of OP poisoning, but not the nicotinic ones.⁹ The contribution to the protective effect of atropine is its lipophilicity, ie the ability to penetrate the CNS. There is an assumption whether more lipophilic anticholinergics would have better protection against OP poisoning than atropine. However, there are no clinical studies to corroborate this notion.

In addition to atropine and oximes (pralidoxime and obidoxime), anticonvulsants, primarily diazepam and midazolam, are also used in the treatment of OP poisoning. Anticonvulsants are

important, especially when the fact that the fatal outcome in OP poisoning most often occurs due to seizures and respiratory failure is taken into account.^{10,11}

Although most developed countries have banned the use of the most toxic pesticides, OPI poisonings, especially the intentional ones, are still common, especially in undeveloped and developing countries.^{12,13} Unfortunately, due to the high toxicity of OPCs, they continue to be abused for terrorist purposes and as nerve agents (tabun, sarin, soman and VX).¹⁴ Paraoxon (diethyl (4-nitrophenyl) phosphate) is the active metabolite of the OPI parathion.¹⁵ Due to its toxicity and chemical characteristics, it serves as a good experimental tool in the field of research of toxicity of nerve agents.

The aim of the study was to determine whether increasing doses of paraoxon affect the rate and intensity of signs of poisoning with an AChE inhibitor. The aim was also to determine whether the speed of onset and intensity of the appearance of certain signs of poisoning can be a prognostic sign of the fatal outcome of poisoning. In addition, the goal was to determine how clinical signs are manifested at high ($> LD_{100}$) doses of paraoxon in the presence of an antidote (atropine).

Methods

Animals

The study was conducted in adult male and female Wistar rats, weighing 200-240 g, purchased from Faculty of Natural Sciences and Mathematics, University of Banja Luka, the Republic of Srpska. The animals had access to food and water *ad libitum*. The room temperature was maintained at 20-22 °C, with a 12 h light and dark cycle. The study was approved by Ethics Committee for the Protection and Welfare of Experimental Animals in Biomedical Research, Faculty of Medicine, University of Banja Luka (Decision No 18/1/20). During the entire experiment, the "Guiding principles in the care of and use of laboratory animals" have been observed.

Chemicals

Paraoxon was purchased from Sigma Aldrich, St Louis, MO, USA. Application volume of the chemical was 1 mL/kg. Paraoxon was injected subcutaneously (sc) into the abdominal region, while atro-

pine was administered intramuscularly (im) into the right thigh. Stock solution of paraoxon (100 mg/mL) was dissolved in isopropyl alcohol. Final dilution for sc administration was made from saline (0.9 % NaCl) before injection. Atropine sulphate monohydrate was dissolved in saline up to the concentration of 10 mg/mL

Study design

In these experiments, lethality of paraoxon and an antidote potential of atropine was based on the 24 h survival.

a) Paraoxon and saline

Increasing doses of paraoxon were administered sc. Each group consisted of 6 rats and doses were determined by the “up and down” method (doses were: 0.2, 0.3, 0.35, 0.4 mg/kg). One minute after paraoxon injection, rats were injected with 1 mL/kg saline im. Based on the 24 h lethality, median lethal dose (LD_{50}) was calculated.

b) Paraoxon and atropine

Increasing doses of paraoxon were administered sc (doses were: 0.6, 0.9, 1.2 mg/kg). Each group consisted of 6 rats and doses were determined by the “up and down” method. One minute after the injection of paraoxon, the rats were injected with 10 mg/kg of atropine im. The outcome was protective ratio (PR) ie, ratio of LD_{50} in protected and in unprotected rats.

c) Clinical signs of poisoning

The following clinical signs of poisoning were observed: fasciculations, tremor, seizures, ataxia, piloerection, lacrimation, exophthalmos, bizarre/stereotypic behaviour, dyspnoea. The time from paraoxon injection to the appearance of the sign of poisoning was recorded. The intensity of poisoning was recorded as: 0 - absent, 1 - mild / moderate, 2 - severe. Evaluation was performed in the 5th, 10th, 15th, 30th, 60th, 90th, 120th, 150th, 180th, 210th, and 240th minute. To analyse the intensity of symptoms throughout the observed period (4 h), ToxScore was introduced, as a parameter that summed the intensity of each sign in all time intervals. TotToxScore was a measure that represented the sum of all ToxScore for one animal.

Statistics

The LD_{50} values were computed by means of the Pharm/PCS statistical software, according to the Litchfield and Wilcoxon (1949).¹⁶ IBM SPSS

21.0 software was used for other statistical procedures. After the normality of data distribution was analysed (Kolmogorov–Smirnov test), the appropriate parametric / nonparametric test was used (Student t-test / Man-Whitney U test, One Way ANOVA / Kruskal-Wallis test) as well as Chi-Squared test for categorical data.

Results

Paraoxon

a) Dose

The LD_{50} of paraoxon was 0.33 mg/kg sc (95 % CI: 0.31 - 0.36). In all rats, death occurred in the first hour of poisoning (mean \pm SD: 19.19 \pm 6.19, 95 % CI: 16.37 - 22.01).

Fasciculations occurred in 85.71 % of all rats, piloerection in 50.00 %, exophthalmos in 95.23 %, lacrimation in 54.76 %, tremor in 97.62 %, seizures in 83.33 %, ataxia in 66.67 %, stereotypic behaviour in 69.05 % and dyspnoea in 26.19 % rats.

The frequency of symptoms at different doses of paraoxon was not significant, except in the case of piloerection, where it occurred after doses of paraoxon 0.2, 0.3, 0.35 and 0.4 mg/kg in 0 %, 33.33 %, 66.67 % and 75.00 %, respectively ($\chi^2 = 11.667$, $p = 0.009$). The onset rate of poisoning clinical signs at different doses of paraoxon is shown in Table 1. Bizarre-stereotypic behaviour, although frequent (69.05 % of all animals), was not significantly different in either the intensity or the rate of sign on any parameter (data not shown).

Fasciculation, tremor, seizures, and ataxia occurred significantly earlier at higher doses of paraoxon. The intensity of signs measured 15 min and 4 h after paraoxon application relative to the dose applied is shown in Table 2. The time-point of 15th minute was taken as the time period when most of the signs of poisoning were manifested, yet most of the rats were still alive, and the time period of 4th hour was taken as the end of the observation period.

In the presented time intervals (15 min and 4 h after paraoxon), the intensity of seizures, tremor and fasciculations increased significantly with increasing doses. Piloerection was significantly

Table 1: Onset rate of clinical signs of poisoning at different doses of paraoxon (minutes after application of paraoxon)

Sign (ToO)	Paraoxon dose (mg/kg sc)				p value
	0.2	0.3	0.35	0.4	
Fasciculations	35.67 ± 17.95	34.25 ± 22.17	20.83 ± 1.84	12.70 ± 5.08	0.015*
Tremor	24.60 ± 7.92	19.33 ± 14.18	19.67 ± 31.29	7.67 ± 1.83	0.002*
Seizures	35.25 ± 20.81	24.78 ± 25.67	18.10 ± 18.73	10.42 ± 3.23	0.021*
Ataxia	47.60 ± 28.02	41.43 ± 51.82	33.89 ± 63.30	9.43 ± 3.10	0.016*
Piloerection	-	6.50 ± 3.32	7.75 ± 6.23	7.00 ± 2.83	0.972*
Exophthalmos	7.00 ± 3.46	5.82 ± 3.63	6.67 ± 3.75	3.92 ± 2.07	0.120*
Lacrimation	28.60 ± 24.34	39.50 ± 27.5	19.67 ± 9.58	17.00 ± 9.74	0.412*
Dyspnoea	29.50 ± 4.95	22.75 ± 12.53	27.33 ± 24.85	15.00 ± 7.07	0.795**

ToO – time of occurrence (minutes after application of paraoxon)

*Kruskal-Wallis test, **One-Way ANOVA, Red colour – statistically significant Values: mean ± standard deviation

Table 2: Intensity of clinical signs of poisoning 15 min and 4 h after administration of increasing doses of paraoxon

Sign (Intensity)	Paraoxon dose (mg/kg sc)				p value*
	0.2	0.3	0.35	0.4	
Fasciculations					
15 th min	0	0.45 ± 0.82	0.58 ± 0.67	1.11 ± 0.93	0.052
240 th min	0.33 ± 0.52	0.56 ± 0.52	0.56 ± 0.73	2.00 ± 0.00	0.061
Tremor					
15 th min	0.33 ± 0.52	0.82 ± 0.87	1.50 ± 0.67	1.78 ± 0.44	0.002
240 th min	0.17 ± 0.41	0.56 ± 0.53	1.00 ± 0.00	1.00 ± 0.00	0.044
Seizures					
15 th min	0	0.91 ± 0.94	0.92 ± 0.79	1.67 ± 0.50	0.004
240 th min	0	0.11 ± 0.33	0.40 ± 0.55	1.00 ± 0.00	0.069
Ataxia					
15 th min	0	0.45 ± 0.69	0.33 ± 0.49	0.44 ± 0.52	0.329
240 th min	0.33 ± 0.52	0.44 ± 0.88	1.00 ± 0.71	1.00 ± 0.00	0.271
Piloerection					
15 th min	0	0.27 ± 0.47	0.83 ± 0.72	0.67 ± 0.50	0.016
240 th min	0	0	0	0	-
Exophthalmos					
15 th min	1.00 ± 0.63	1.09 ± 0.53	1.50 ± 0.52	1.67 ± 0.50	0.055
240 th min	1.17 ± 0.98	1.22 ± 0.67	1.40 ± 0.55	2.00 ± 0.00	0.721

*Kruskal-Wallis test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)

higher at higher doses at the 15th min, however, it disappeared in all rats 45 min after poisoning.

To analyse the intensity of symptoms throughout the observed period (4 h), ToxScore was introduced, as a sum of the intensity of each sign in all time intervals. A significant difference was found in ToxScore values relative to dose regarding fasciculations and seizures (Figure 1). Other parameters were not significant.

TotToxScore is a measure that represents the sum of all ToxScores for one animal. TotToxScore values are shown in Figure 2. Although the difference was not statistically significant, an increase in the value of TotToxScore is noticeable, as a joint parameter of the intensity and duration of signs of

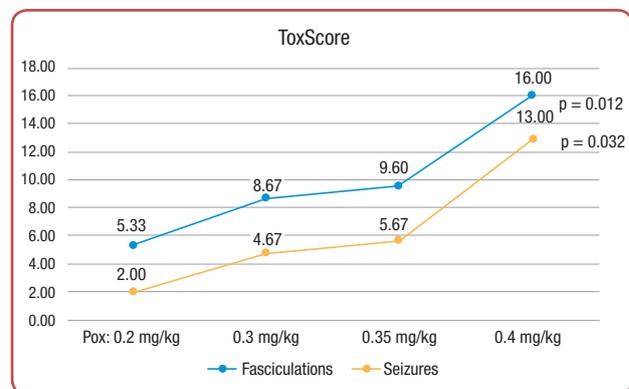


Figure 1: ToxScore for fasciculations and seizures relative to dose of paraoxon (Pox)

*Kruskal-Wallis test; ToxScore: the sum of the values of each sign in each point of time

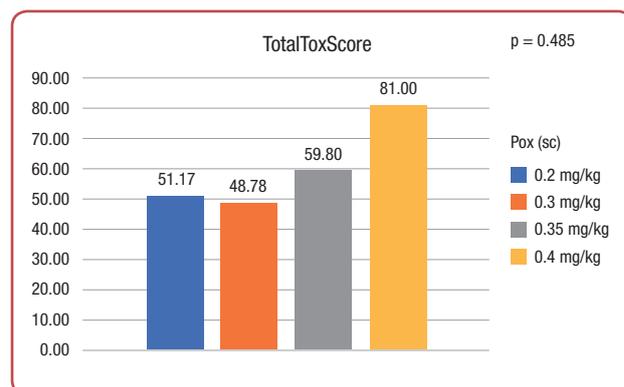


Figure 2: Mean TotToxScore relative to dose of paraoxon (Pox)

*TotToxScore: the sum of the values of all sign in each point of time; Kruskal-Wallis test

poisoning, especially at high doses.

b) Survival

Analysing the frequency of clinical signs in relation to whether the rat survived or not, a significant difference was found regarding the piloerection: 23.81 % of survivors and 76.19 % of non-survivors had piloerection ($\chi^2 = 11,524$, $p = 0.002$). The same goes for seizure frequency (66.67 % in survivors vs 100.00 % in non-survivors have had seizures, $\chi^2 = 8,400$, $p = 0.009$). The speed of onset of clinical signs of poisoning relative to whether or not the animals survived is shown in Table 3. All signs of poisoning occurred earlier in non-survivors compared to survivors.

The intensity of the signs at 15 min after paraoxon application in relation to whether the rat survived

Table 3: Onset rate of clinical signs of paraoxon poisoning in relation to whether rats survived or not

Sign (To0)	Survived		p-value
	Yes	No	
Fasciculations	35.44 ± 16.94	12.61 ± 4.25	<0.001*
Tremor	26.40 ± 24.02	7.38 ± 1.91	<0.001*
Seizures	33.14 ± 23.74	9.81 ± 3.12	<0.001*
Ataxia	52.33 ± 55.77	8.77 ± 2.05	<0.001*
Piloerection	8.40 ± 5.64	6.81 ± 3.97	0.646*
Exophthalmos	6.60 ± 3.35	4.70 ± 3.16	0.025*
Lacrimation	32.33 ± 22.28	14.37 ± 6.59	0.086*
Dyspnoea	33.67 ± 11.18	12.00 ± 5.83	0.004**

To0 – time of occurrence (minutes after application of paraoxon)

*Man-Whitney U-test, ** Student t-test, Red colour – statistically significant Values: mean ± standard deviation

Table 4: Signs intensity at 15 min after paraoxon administration in relation to whether the rat survived or not

Sign (To0)	Survived		p-value*
	Yes	No	
Fasciculations	0.09 ± 0.44	1.17 ± 0.73	<0.001
Tremor	0.71 ± 0.78	1.76 ± 0.44	<0.001
Seizures	0.43 ± 0.75	1.59 ± 0.51	<0.001
Ataxia	0.19 ± 0.40	0.53 ± 0.62	0.059
Piloerection	0.19 ± 0.40	0.88 ± 0.60	0.646
Exophthalmos	1.14 ± 0.48	1.59 ± 0.62	0.011
Lacrimation	0.33 ± 0.48	0.18 ± 0.39	0.416
Dyspnoea	0	0.24 ± 0.56	0.048

*Man-Whitney U-test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)

or not is shown in Table 4.

Apart from lacrimation and stereotypy, all other signs were of higher intensity in non-survivors. The most significant difference in intensity was recorded in fasciculations, tremor and seizures, where high significance was found ($p < 0.001$).

Paraoxon and atropine

a) Dose

The PR of atropine was 2.73 (LD_{50} of paraoxon when atropine was administered 1 min after was 0.91 mg/kg sc (95 % CI: 0.67 - 1.25)). In all rats, death occurred during the first hour of poisoning (mean ± SD: 14.00 ± 3.74, 95% CI: 10.87 - 17.13). Fasciculations occurred in 77.78 % of all rats, piloerection in 33.33 %, exophthalmos in 94.44 %, lacrimation in 22.22 %, tremor in 100.00 %, seizures in 94.44 %, ataxia in 44.44 %, stereotypic behaviour in 38.89 % and dyspnoea in 55.56 % of rats.

In atropine-protected rats, the dose had a significantly smaller effect on the speed and frequency of

symptoms. The frequency of any poisoning signs was not affected by the dose. As for the onset of clinical signs manifestation, a significant difference was found only in fasciculations ($p = 0.038$) and tremor ($p = 0.007$) (Table 5).

The intensity of the signs persisted throughout the observation period. The intensities of certain symptoms at 15th min and after 4 h relative to the dose are shown in Table 6. The cross-section at the 15th minute was taken as the time period when most of the signs of poisoning were present, yet most of the rats were still alive, while the 4th h was taken as the end of the observation period.

Table 5: Onset rate of clinical signs of poisoning at different doses of paraoxon with atropine protection (10 mg/kg im) (one minute after application of paraoxon)

Sign (To0)	Paraoxon dose (mg/kg sc)			p-value
	0.6	0.9	1.2	
Fasciculations	28.83 ± 30.75	7.60 ± 1.95	10.33 ± 4.73	0.038*
Tremor	12.83 ± 5.85	8.33 ± 1.03	5.00 ± 2.19	0.007*
Seizures	18.20 ± 15.21	9.50 ± 1.64	8.50 ± 4.64	0.170**
Ataxia	51.50 ± 79.20	8.00 ± 2.83	70.50 ± 85.56	0.444*
Piloerection	4.33 ± 1.15	6.00 ± 3.60	5.17 ± 2.56	0.507*
Exophthalmos	3.20 ± 1.64	4.00 ± 2.89	6.00 ± 0.89	0.134*
Lacrimation	111.00 ± 0.00	105.00 ± 140.00	6.00 ± 0.00	0.632*
Dyspnoea	63.40 ± 46.47	25.33 ± 31.77	52.50 ± 44.55	0.504**

To0 – time of occurrence (minutes after application of paraoxon)

*Kruskal-Wallis test, **One-Way ANOVA. Red colour – statistically significant Values: mean ± standard deviation

Table 6: Intensity of clinical signs of poisoning 15 min and 4 h after the application of paraoxon and atropine (10 mg/kg, im, one minute after) depending on the dose of paraoxon

Sign (Intensity)	Paraoxon dose (mg/kg sc)			p-value*
	0.6	0.9	1.2	
Fasciculations				
15 th min	0.89 ± 0.37	0.71 ± 0.32	0.55 ± 0.24	0.613
240 th min	1.00 ± 0.00	1.00 ± 0.00	2.00 ± 0.00	0.011
Tremor				
15 th min	1.17 ± 0.41	2.00 ± 0.00	1.8 ± 0.45	0.015
240 th min	0.67 ± 0.52	1.00 ± 0.00	1.00 ± 0.00	0.472
Seizures				
15 th min	0.83 ± 0.98	2.00 ± 0.00	2.00 ± 0.00	0.017
240 th min	0	0.50 ± 0.71	2.00 ± 0.00	0.022
Ataxia				
15 th min	0.33 ± 0.52	0.40 ± 0.55	0.20 ± 0.45	0.797
240 th min	0.33 ± 0.52	0.50 ± 0.71	2.00 ± 0.00	0.073
Dyspnoea				
15 th min	0.17 ± 0.41	0.20 ± 0.45	0.40 ± 0.89	0.961
240 th min	0.33 ± 0.52	0.50 ± 0.71	1.00 ± 0.00	0.301

*Kruskal-Wallis test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)

ToxScore, although without statistical significance, showed a clear increase in fasciculations, tremor, seizures, ataxia, and dyspnoea with increasing doses of paraoxon (Figure 3).

TotToxScore values for paraoxon poisoning followed by atropine administration are shown in Figure 4. Total Tox Score, although without statistical significance, showed a clear increase with higher dose.

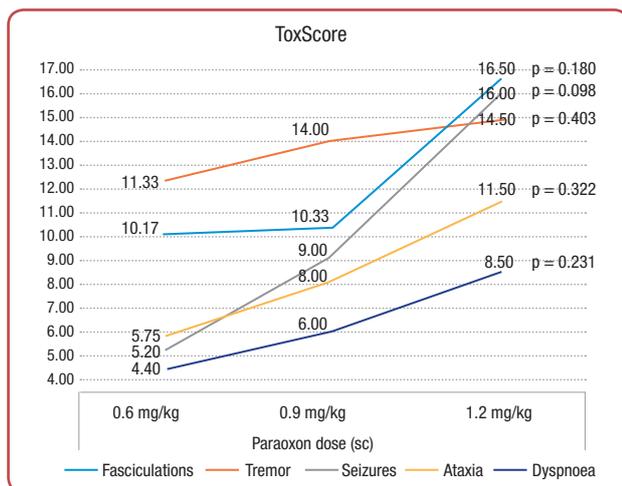


Figure 3: Dose-dependent ToxScore (paraoxon with atropine protection)

* Kruskal-Wallis test; ToxScore: the sum of the values of each sign in each point of time

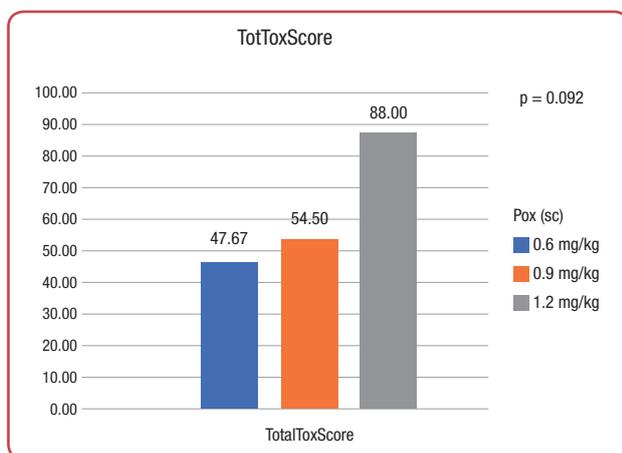


Figure 4: Mean TotToxScore relative to dose of paraoxon with atropine protection

*TotToxScore: the sum of the values of all sign in each point of time; Kruskal-Wallis test

b) Survival

There was no significant difference in the frequency of symptoms in relation to whether the rat survived or not. It was only observed that ataxia was more common in survivors than in non-survivors (70.00 % vs 12.50 %, respectively; $\chi^2 = 5,591$, $p = 0.025$) as well as fasciculations (100.00 % vs 50.00 %, respectively; $\chi^2 = 6,429$, $p = 0.023$).

Table 7: Onset rate of clinical signs of paraoxon poisoning with atropine administration in relation to whether rats survived or not

Sign (To0)	Survived		p-value
	Yes	No	
Fasciculations	21.70 ± 24.76	6.25 ± 0.96	0.004*
Tremor	10.50 ± 5.58	6.50 ± 2.50	0.083*
Seizures	14.67 ± 11.72	8.38 ± 3.42	0.117**
Ataxia	50.43 ± 69.43	10.00 ± 0.00	-
Piloerection	5.00 ± 1.63	5.50 ± 4.95	-
Exophthalmos	4.22 ± 1.86	4.75 ± 2.71	0.673*
Lacrimation	157.50 ± 65.76	6.00 ± 0.00	0.333*
Dyspnoea	60.50 ± 39.27	7.00 ± 1.41	0.098**

To0 – time of occurrence (minutes after application of paraoxon). Red colour – statistically significant; *Man-Whitney U-test, ** Student t-test; Values: mean ± standard deviation

Table 8: Signs intensity at 15 min after paraoxon administration with atropine protection in relation to whether the rat survived or not

Sign (To0)	Survived		p-value*
	Yes	No	
Fasciculations	1.10 ± 0.74	0.50 ± 0.55	0.147
Tremor	1.50 ± 0.53	1.83 ± 0.41	0.313
Seizures	1.30 ± 0.95	2.00 ± 0.00	0.220
Ataxia	0.40 ± 0.52	0.17 ± 0.41	0.492
Piloerection	0.10 ± 0.32	0.17 ± 0.41	0.875
Exophthalmos	0.80 ± 0.42	1.00 ± 0.63	0.635
Dyspnoea	0.30 ± 0.67	0.17 ± 0.41	0.875

* Man-Whitney U-test; Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)

A significant difference in the onset of symptoms relative to whether or not a rat survived was observed only regarding fasciculations. The onset rate of poisoning clinical signs relative to whether or not the animals survived is shown in Table 7. Most signs of poisoning occurred earlier in non-survivors, but statistical significance was found only regarding fasciculations ($p = 0.004$). The intensity of any signs of poisoning did not differ significantly in survivors and non-survivors (Table 8).

Discussion

Paraoxon is one of the most toxic OPIs and is therefore banned from use as an insecticide in most countries. The obtained LD_{50} of paraoxon was 0.33 mg/kg sc, which roughly corresponds to the data from the literature.¹⁷ Death in almost all animals occurs in the first two hours of OP

poisoning, which is consistent with the data obtained in this study.¹⁸

The LD₅₀ of paraoxon, when 10 mg/kg of atropine is administered after 1 minute, was 0.91, so the PR of atropine was 2.73, similar to the data in the literature.¹⁹ Atropine is used as an antidote for poisoning with AChE inhibitors. It is much more effective in carbamate poisoning than in those caused by OPCs.¹⁹ It is logical that PR will be significantly higher in carbamates than OP poisoning given that carbamates are reversible AChE inhibitors, so the spontaneous reactivation of AChE makes the task of atropine as an antidote less demanding.²¹⁻²³ Considering the dose of atropine, 10 mg/kg im was selected because it is the most frequently used dose; therefore the results obtained in this experiments are comparable to the results of other researchers.²⁴ Krutak-Kol and Domino tried different doses of atropine against paraoxon poisoning: 10, 33 and 100 mg/kg and found that higher doses did not increase survival. Moreover, a dose of 100 mg/kg was significantly less effective than the dose of 10 mg/kg.²⁵ Repeated lower doses of atropine over time might potentially give better results.²⁶

The presence, time of occurrence and intensity of paraoxon poisoning signs were examined. The same parameters of poisoning were also observed at high lethal doses of paraoxon, in animals given atropine. Those signs that can be noticed only by observation without manipulation of the animal were analysed.

Lacrimation occurs by excessive stimulation of muscarinic receptors. Although not life-threatening, it can serve as a good indicator of muscarinic excitation, as well as sign of adequate atropinisation during treatment.³ Neither the intensity nor the rate of lacrimation occurred statistically significantly at different doses of paraoxon and in relation to the survival rate. However, when atropine was administered after paraoxon, lacrimation was significantly less common and occurred later, although significantly higher doses of paraoxon were administered (only 22 % vs 55 % when atropine was not administered). Regardless of the dose, the results obtained were expected since it is well known that atropine is an anti-muscarinic drug. The existence of lacrimation can indirectly indicate that there are other effects of muscarinic excitation (hypotension, bradycardia).

Muscarinic effects that are directly life-threatening are bronchoconstriction and bronchorrhoea. Dyspnoea was monitored as a parameter indicating respiratory failure, which in studies by other researchers proved to be the main cause of lethality in OPI poisoning.²⁷⁻²⁹ The disadvantage of this study is that dyspnoea was only subjectively observed, without objective measuring of respiratory failure degree. Sometimes, in severe seizures dyspnoea could be noticed only when the intensity of seizures subsided. Nevertheless, a clear link was found in the intensity and speed of occurrence of dyspnoea in relation to whether the animal survived or not. In animals treated with atropine after paraoxon administration, atropine delayed and reduced the intensity of dyspnoea, although these were significantly higher doses of paraoxon. Houze et al analysed whether respiratory failure in OP poisoning was due to central or peripheral muscarinic effects.³⁰ He showed that respiratory failure was almost completely corrected by atropine and not corrected at all by 100 times the equimolar dose of N-methylatropine and concluded that respiratory failure is due to central muscarinic effects. On the other hand, Villa et al found that 50 % and 75 % of LD₅₀ paraoxon caused alterations in ventilation, but did not cause respiratory failure.³¹

Fasciculations were a sign that, along with seizures, proved to be the most consistent parameter of the poisoning severity. Fasciculations occurred faster and were of higher intensity when the dose of paraoxon as well as survival of rats was observed. This can be explained by the fact that among the examined signs, fasciculations are those that follow overstimulation of nicotinic receptors on the neuromuscular junction. If it is assumed that according to the Poison Severity Score (PSS) in humans, any occurrence of nicotine signs of poisoning is considered at minimum as moderate poisoning, then it can be concluded that fasciculations, as a parameter of nicotine effects of poisoning, directly indicates the severity of poisoning.³² An additional problem is lack of effective antidote that would function as a nicotine antagonist, without serious side effects.^{33, 34} As expected, atropine administration did not affect the rate and intensity of fasciculations - they were of higher intensity and occurred earlier, because the dose of paraoxon was higher.

Muscle tremor was also a sign of inappropriate stimulation of nicotinic receptors on the neuro-

muscular joint, ie weakness of skeletal muscles. Tremor has been more often described in the literature as part of the delayed onset of extrapyramidal syndrome, rather than acute OP poisoning sign.³⁵ Since the monitoring of poisoning signs was based on observation, tremor served as a good indicator of muscle weakness. A strong relationship was found between both the speed of onset and the intensity of tremor, and the dose of paraoxon or the lethal outcome in rats. As expected, the results did not change when atropine was given after paraoxon, but the tremor occurred earlier and was of stronger intensity at higher doses and in non-survivors.

ACh is also found in the preganglionic fibres of the sympathetic nervous system. Piloerection is a sign that occurs as a consequence of excessive stimulation of preganglionic nicotinic receptors of the sympathetic nervous system.³⁶ Therefore, piloerection can be considered as potential predictor of sympathetic stimulation. The results of this study showed a strong relationship between the dose of paraoxon and the frequency of piloerection. Studies from other researchers show that the incidence of tachycardia in OP poisoning is 35-60 %, making it more common than bradycardia.³⁷ The alpha-1-adrenergic receptor, in addition to piloerection, in rats is also involved in retraction of the eyelids, leading to exophthalmos.³⁸ Unlike piloerection, exophthalmos remained most persistent throughout the observed period and had no statistical significance in the intensity and rate of exophthalmos in relation to paraoxon dose and rat mortality. The same results were obtained when atropine was administered after high doses of paraoxon.

Seizures initially occur due to stimulation of cholinergic receptors in the CNS and at this stage can be reversed by atropine. Prolonged seizure activity is due to excessive release of glutamate, when atropine is not effective.³⁹ For now, seizures are treated with combinations of anticholinergics and benzodiazepines but, given the role of glutamate in seizures, future therapies should include N-methyl-D-aspartate receptor (NMDA) antagonists.⁴⁰ The rate and intensity of seizures were clearly correlated to dose and especially to death in paraoxon poisoning. Seizures occur significantly less frequently in OPI than in nerve agents.⁴¹ In this study, seizures occurred in 83 % of rats and 100 % of non-survivors, which additionally

contribute to the characteristics of paraoxon as a nerve agents. Administration of atropine somewhat postponed and decreased the intensity of seizures. There was still a clear relationship between these parameters and paraoxon dose as well as fatal outcome, but seizures occurred later and were less intense than would be expected, given the paraoxon doses administered.

Ataxia is caused by stimulation of muscarinic receptors in the CNS. In addition, in acute OP poisoning, ataxia occurs as a consequence of muscle fatigue, but ataxia is more intense than would be expected from muscle fatigue alone.⁴² The results of this study show that ataxia occurred earlier at higher doses of paraoxon and in non-surviving rats.

ToxScore and TotToxScore are parameters that were introduced in order to see the persistence of symptoms related to the dose. Thus, the values of each sign in each point of time were summed, which made up the ToxScore, and the values of all ToxScores were added to calculate the TotToxScore for each animal. The pre-condition is that the animal survived the observation period. These parameters can also be used in future studies, when the protective effect of antidotes (either individually or in combination) can be compared, not only as a measure of survival, but also their ability to alleviate the signs of poisoning. ToxScore showed that the same parameters (seizure, fasciculation, tremor) that were significant indicators of the severity of poisoning lasted longer at higher doses. The TotToxScore had higher value at higher dose and, although the difference was not significant, showed a clear trend.

Conclusion

Seizures and fasciculations followed by tremor were strong prognostic parameters of the probability of lethal outcome of paraoxon poisoning. Also, the mentioned signs of poisoning were with their intensity and speed of occurrence in a clear correlation with the dose of paraoxon. Even at high doses of paraoxon, atropine blocked the muscarinic (but not nicotinic) effects, and somewhat mitigated the CNS effects.

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Conflict of interest

None.

References

- Adeyinka A, Muco E, Pierre L. Organophosphates. [Updated 2020 Aug 16]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK499860/>.
- Pope CN, Brimijoin S. Cholinesterases and the fine line between poison and remedy. *Biochem Pharmacol* 2018 Jul;153:205-16.
- Eddleston M. Novel clinical toxicology and pharmacology of organophosphorus insecticide self-poisoning. *Annu Rev Pharmacol Toxicol* 2019 Jan 6;59:341-60.
- Reddy BS, Skaria TG, Polepalli S, Vidyasagar S, Rao M, Kunhikatta V, et al. Factors associated with outcomes in organophosphate and carbamate poisoning: a retrospective study. *Toxicol Res* 2020 Feb 7;36(3):257-66.
- Jokanović M, Stojiljković MP, Kovač B, Ristić D. Pyridinium oximes in the treatment of poisoning with organophosphorus compounds. In: Gupta RC. Handbook of toxicology of chemical warfare agents. 3rd ed. Cambridge, MA, USA: Academic Press; 2020. p. 1145-1159.
- Worek F, Thiermann H, Wille T. Organophosphorus compounds and oximes: a critical review. *Arch Toxicol* 2020 Jul;94(7):2275-92.
- Antonijević B, Stojiljković MP. Unequal efficacy of pyridinium oximes in acute organophosphate poisoning. *Clin Med Res* 2007 Mar;5(1):71-82.
- Vanova N, Pejchal J, Herman D, Dlabkova A, Jun D. Oxidative stress in organophosphate poisoning: role of standard antidotal therapy. *J Appl Toxicol* 2018 Aug;38(8):1058-70.
- Henretig FM, Kirk MA, McKay CA Jr. Hazardous chemical emergencies and poisonings. *N Engl J Med* 2019 Apr 25;380(17):1638-55.
- Amir A, Raza A, Qureshi T, Mahesar GB, Jafferi S, Haleem F, Ali Khan M. Organophosphate poisoning: demographics, severity scores and outcomes from National Poisoning Control Centre, Karachi. *Cureus* 2020 May 31;12(5):e8371. doi: 10.7759/cureus.8371.
- Reddy BS, Skaria TG, Polepalli S, Vidyasagar S, Rao M, Kunhikatta V, Nair S, Thunga G. Factors associated with outcomes in organophosphate and carbamate poisoning: a retrospective study. *Toxicol Res* 2020 Feb 7;36(3):257-66.
- Kaushal J, Khatri M, Arya SK. A treatise on Organophosphate pesticide pollution: Current strategies and advancements in their environmental degradation and elimination. *Ecotoxicol Environ Saf* 2020 Oct 22;207:111483. doi: 10.1016/j.ecoenv.2020.111483.
- WHO. Health topics: mental health [Internet]. Geneva. [Cited: 2020-Dec-30]. Available from https://www.who.int/mental_health/prevention/suicide/en/PesticidesHealth2.pdf.
- Stojiljković MP. Nerve agents – a clear and present danger to mankind. *Scr Med* 2019;50(3):109-11.
- PubChem [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. PubChem Compound Summary for CID 9395, Paraoxon. [Cited 2021-Mar-02]. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/Paraoxon>.
- Litchfield JT Jr, Wilcoxon F. A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 1949 Jun;96(2):99-113.
- Misik J, Pavlikova R, Cabal J, Kuca K. Acute toxicity of some nerve agents and pesticides in rats. *Drug Chem Toxicol* 2015 Jan;38(1):32-6.
- Kassa J, Misik J, Hatlapatkova J, Zdarova Karasova J, Sepsova V, Caisberger F, Pejchal J. The Evaluation of the reactivating and neuroprotective efficacy of two newly prepared bispyridinium oximes (K305, K307) in tabun-poisoned rats - a comparison with trimesoxime and the oxime K203. *Molecules* 2017 Jul 11;22(7):1152. doi: 10.3390/molecules22071152.
- Holmstedt B. Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacol Rev* 1959 Sep;11:567-688.
- Stojiljković MP, Škrbić R, Jokanović M, Kilibarda V, Bokonjić D, Vulović M. Efficacy of antidotes and their combinations in the treatment of acute carbamate poisoning in rats. *Toxicology* 2018 Sep 1;408:113-124.
- Natoff IL, Reiff B. Effect of oximes on the acute toxicity of anticholinesterase carbamates. *Toxicol Appl Pharmacol* 1973 Aug;25(4):569-75.
- Kords H, Lüllmann H, Ohnesorge FK, Wassermann O. Action of atropine and some hexane-1.6-bis-ammonium derivatives upon the toxicity of DFP in mice. *Eur J Pharmacol* 1968 Jul;3(4):341-6.
- Albuquerque EX, Pereira EF, Aracava Y, Fawcett WP, Oliveira M, Randall WR, et al. Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proc Natl Acad Sci U S A* 2006 Aug 29;103(35):13220-5.
- Parkes MW, Sacra P. Protection against the toxicity of cholinesterase inhibitors by acetylcholine antagonists. *Br J Pharmacol Chemother* 1954 Sep;9(3):299-305.
- Krutak-Krol H, Domino EF. Comparative effects of diazepam and midazolam on paraoxon toxicity in rats. *Toxicol Appl Pharmacol* 1985 Dec;81(3 Pt 1):545-50.
- Rump S, Faff J, Szymańska T, Bak W, Borkowska G. Efficacy of repeated pharmacotherapy in experimental acute poisonings with fluostigmine. *Arch Toxicol* 1976 Aug 18;35(4):275-80.
- Eddleston M, Eyer P, Worek F, Mohamed F, Senarathna L, von Meyer L, et al. Differences between

- organophosphorus insecticides in human self-poisoning: a prospective cohort study. *Lancet* 2005 Oct 22-28;366(9495):1452-9.
28. Namba T, Nolte CT, Jackrel J, Grob D. Poisoning due to organophosphate insecticides. Acute and chronic manifestations. *Am J Med* 1971;50(4):475-92.
 29. Ballantyne B, Marrs TC. Overview of the biological and clinical aspects of organophosphates and carbamates. In: Ballantyne B, Marrs TC, eds. *Clinical and experimental toxicology of organophosphates and carbamates*. Oxford: Butterworth-Heinemann; 1992. p. 3-14.
 30. Houze P, Pronzola L, Kayouka M, Villa A, Debray M, Baud FJ. Ventilatory effects of low-dose paraoxon result from central muscarinic effects. *Toxicol Appl Pharmacol* 2008 Dec 1;233(2):186-92.
 31. Villa AF, Houze P, Monier C, Risède P, Sarhan H, Borron SW, et al. Toxic doses of paraoxon alter the respiratory pattern without causing respiratory failure in rats. *Toxicology* 2007 Mar 22;232(1-2):37-49.
 32. Persson HE, Sjöberg GK, Haines JA, Pronczuk de Garbino J. Poisoning severity score. Grading of acute poisoning. *J Toxicol Clin Toxicol* 1998;36(3):205-13.
 33. Sheridan RD, Smith AP, Turner SR, Tattersall JE. Nicotinic antagonists in the treatment of nerve agent intoxication. *J R Soc Med* 2005 Mar;98(3):114-5.
 34. Turner SR, Chad JE, Price M, Timperley CM, Bird M, Green AC, Tattersall JE. Protection against nerve agent poisoning by a noncompetitive nicotinic antagonist. *Toxicol Lett* 2011 Sep 25;206(1):105-11.
 35. Reji KK, Mathew V, Zachariah A, Patil AK, Hansdak SG, Ralph R, Peter JV. Extrapyramidal effects of acute organophosphate poisoning. *Clin Toxicol (Phila)* 2016 Mar;54(3):259-65.
 36. Dhanarisi J, Shihana F, Harju K, Mohamed F, Verma V, Shahmy S, et al. A pilot clinical study of the neuromuscular blocker rocuronium to reduce the duration of ventilation after organophosphorus insecticide poisoning. *Clin Toxicol (Phila)* 2020 Apr;58(4):254-61.
 37. Saadeh AM, Farsakh NA, al-Ali MK. Cardiac manifestations of acute carbamate and organophosphate poisoning. *Heart* 1997;77(5):461-4.
 38. Kikuchi-Utsumi K, Ishizaka M, Matsumura N, Nakaki T. Alpha(1A)-adrenergic control of piloerection and palpebral fissure width in rats. *Auton Neurosci* 2013 Dec;179(1-2):148-50.
 39. Rusyniak DE, Nañagas KA. Organophosphate poisoning. *Semin Neurol* 2004 Jun;24(2):197-204.
 40. Stojiljković MP, Škrbić R, Jokanović M, Bokonjić D, Kilibarda V, Vulović M. Prophylactic potential of memantine against soman poisoning in rats. *Toxicology* 2019 Mar 15;416:62-74.
 41. Wadia RS, Sadagopan C, Amin RB, Sardesai HV. Neurological manifestations of organophosphate insecticide poisoning. *J Neurol Neurosurg Psychiatry* 1974 Jul;37(7):841-7.
 42. Peter JV, Sudarsan TI, Moran JL. Clinical features of organophosphate poisoning: a review of different classification systems and approaches. *Indian J Crit Care Med* 2014 Nov;18(11):735-45.