The Use of Chemistry in Understanding the Pathogenic Mechanisms of Organophosphates Related Cardiac Toxicity

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Organophosphate substances, although known to exert their toxic effects mainly by inhibiting cholinesterase enzymes, associate a cardiac toxicity with pathogenic mechanisms that are not only limited to this anticholinergic effect. The aim is to discover the most reliable explanation for the late cardiac toxic effect of organophosphates in acute intoxication. The experimental research compres evaluation of oxidative stress system, serum electrolytes levels and evidences of myocardial injury (cardiac enzymes, histological examinations) while monitoring, through gas chromatography, the presence of the toxic at cardiac level, during a period of eight days evolution of an acute intoxication with trichlorfon at two different dosages (200 and 400 mg/kg body weight). Electrolyte disorders and the intervention of oxidative stress were not documented to be involved in this type of outcome. A pathogenic mechanism of myocarditis was documented in the evolution of the acute organophosphates intoxication due to a direct action of the toxic to the heart. The late effect is associated with a dysphasic accumulation of the organophosphate at myocardial level.

Keywords: gas chromatography, organophosphates, myocarditis, oxidative stress

Organophosphate substances continue to represent a threat to humans, through accidental or voluntary intoxications, and also through their potential usage as chemical weapons. [1-3] Although known to exert their toxic effects as inhibitors of cholinesterase, with acetylcholine accumulation [4-6] , clinical situations that must have had a different mechanism of toxicity were reported. In the evolution of an acute poisoning with organophosphate substances, sudden deaths were reported to appear between the fourth and the eighth day of evolution, when all the other commonly monitored tests (such as cholinesterase level of activity) have normalized. [7-9] In the literature, some experimental studies tried to explain the toxicological mechanisms of organophosphate with the hypothesis of myocarditis [10 -12] , electrolyte disorders [13-15] or oxidative stress injuries [16 -19].

The aim of the study was to investigate the hypotheses of organophosphate toxicity and to discovere the toxicological mechanisms that could lead to major interventions in the future management of this pathology. Our experimental research of an acute intoxication with organophosphate substances at two different dosages include the evaluation of oxidative stress system, serum electrolyte levels and evidence of myocardial injury (cardiac enzymes, histological examinations) [20-22] and monitors the presence of the toxic at cardiac level (through gas chromatography), over a period of eight days of evaluation in order to explain the late cardiac toxic effect of organophosphates after an acute intoxication.

Experimental part

Material an methods

Tested animals

Adult female (14 weeks of age) Wistar rats weighting 167.8 ± 16.1 g were used. They were previously acclimatized 5 days in the experiment room, housed four per cage, under controlled temperature conditions (22 ± 2°C), light (12-hr light–dark cycle) and humidity (50 ± 10%). The animals had *ad libitum* access to food (bread, milk, barley, fresh vegetal food, chow pellets) and tap water, except for the experimental period. The animals were randomly assigned to two experimental groups (8 per each group). The animal studies were approved by the Ethical Committee of the University of Medicine and Pharmacy "Gr. T. Popa", Iasi, Romania.

The chemical used was triclorfon – commercial substance Onefon 90: white powder mixable in water, consisting of 90% pure Triclorfon.

Method

The experiment was performed during two weeks using two different concentrations of Triclorfon 200 mg/kg bodyweight and respectively 400 mg/kg body weight. One day before the administration of Triclorfon, the experiment was prepared by adding 50 μL sodic heparin = 250 u.i in each tube of test, and by weighting the tested animals, labeling them and collecting blood samples for moment 0 (determination of cholinesterase level of activity, superoxiddismutase, creatinkinase, creatinkinase MB, lacticdehidrogenase - LDH, sodium, kalium, chlorium - reference values).

Rats were marked by cutting a portion of 1 – 2 cm^2 (or a strip) of hair from different regions of their fur, while they were anesthesized.. Each group was labeled by writing, on the specific cages, with a marker, the dose of Triclorfon used. At the same day, the established quantities of Onefon, equivalent to Triclorfon 200, respectively to 400 mg/kg body weight, were measured in a manner thought to simplify the phase of inoculation of the toxic, planned for the day after (to a dilution that will afford a simple rule for

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	Triclorfon 200 mg/kg.body-weight							
No. animal\Moment	0	%	20 min.	1 hour	3h	6h	12h	24h
(1)* 140 g	**22.6**	**100**	**144**	36.2	67.4	24,6	147	684
(2)* 170 g	**35.2**	**100**	**24. 5**	28.4	**31..5**	456	**24.8**	54.1
(3)** 180 g	**113**	**100**	**112**	118	286	306	486	259
(4)** 190 g	**120**	**100**	**24.1**	154	**546**	35,2	**28.3**	24.6
(5)* 150 g	**248**	**100**	645	**244**	938	**453**	91	**246**
(6)* 150 g	**186**	**100**	26	458	287	465	32	186
(7)** 150 g	**396**	**100**	134	**170**	254	**633**	784	**113**
(8)** 180 g	**544**	**100**	554	**144**	654	184	102	187
	Triclorfon 400 mg/kg.body-weight							
No. animal\Moment	0	%	20 min.	1 hour	3h	6h	12h	24h
(1)* 180 g	**54.2**	**100**	**24.6**	644	D			
(2)* 165 g	**241**	**100**	**684**	706	**250**	122	**96**	402
(3)** 160 g	**586**	**100**	**248**	D				
(4)** 185 g	**244**	**100**	**58.6**	44,2	**84.6**	40.2	**24.6**	148
(5)* 175 g	**154**	**100**	402	**154**	304	**180**	68.2	**168**
(6)* 190 g	**25.3**	**100**	D					
(7)** 170 g	**204**	**100**	486	**24.6**	208	**408**	670	**840**
(8)** 150 g	**64.8**	**100**	128	**102**	D			

Table 1
DYNAMIC OF CHOLINESTERASE LEVEL OF ACTIVITY AFTER ACUTE EXPERIMENTAL INTOXICATION WITH TRICLORFON AT TWO DIFFERENT DOSAGES: 200 MG/BW, RESPECTIVELY 400 MG/BW -* SEMIAUTOMATIC METHOD; ** AUTOMATIC METHOD; D: RAT HAVE DIED AFTER INTOXICATION

administration: 1 mL for 100 g animal). A dose of 200 mg/kgbw consisted of 20 mg pure Triclorfon per 100 g animal, which means 22.2 mg. Onefon 90 per 100 g animal.

For 8 rats weighting an approximate total amount of 2000 g, 440 mg powder of Onefon 90 stored in a graded chemistry flask with sealing stopper were measured at analytical balance. At the day of toxic administration to the rats, the powder was diluted with distilled water, which was added until it reached the sign of 20 mL and dissolved as solution by shaking the flask. By doing so, a solution of Onefon 90 administrable in the ratio of 1 mL to 100 g rat, for a dose of 200 mg/kg bw was obtained.

For the administrations of Triclorfon 400 mg/kg bw, preparations were similar, doubling the quantity of Triclorfon (880 mg instead of 440 mg) also dissolved, at the day of inoculation, in distilled water until it reached the sign for 20 mL.

The experiment started at 6 o'clock in the morning and ended at 22 o'clock the first day. For the collection of blood samples, 10 glass Pasteur pipettes were used. In order to avoid technical errors (possible haemolysis) pipette cleaning during the experiment was performed only with isotonic natrium chloride solution 9%$_0$. Blood was collected from the retroorbitar plexus without any previous anesthesia, except the anergy related to the intoxication. As an exception, for the sample collecting moments 12 and 24 h, the usage of small dosages of ethylic ether were necessary (by placing the rat in an oversaturated atmosphere of ethylic ether vapors).

In order to reinforce the assessment of cholinesterase level, the blood samples were investigated using two methods, that had different procedural principles, one using a semiautomatic analyzer, and the other an automatic one. We used the colorimetric method of kinetic determination of the level of activity of serum cholinesterase. Reference interval is 4.65–10.44 U/mL (females) and 5.90–12.22 U/mL (males). Hemolyzed serum was rejected from analyze. Serum was separated from the clot (after centrifugation) in an interval of maximum 4 h, and the storage time was of a maximum of 6 h, at room temperature. Serum cholinesterase (**nachEs**) was determined from blood samples collected at the moments: 0, 20 min, one hour, 3, 6 , 12, 24 h (the second day). Superoxid dismutase (**SOD),** for assessment of oxidative stress, was determined from blood samples collected at the moments: 0, 20 min, 1, 3, 6, 12, 24 h. Electrolytes (Na, K, Cl**)** were determined from blood samples collected at the moments: 0, 6, 12, 24 h (the second day), the third day, the fourth day, the fifth day, the sixth day, the seventh day, the eighth day. Creatinkinase (**CK),** creatinkinase isoenzime MB (**CK-MB), lactic** dehidrogenase (**LDH**) were determined at the same moments as those for electrolytes: 0, 6, 12, 24 h (the second day), the third day, the fourth day, the fifth day, the sixth day, the seventh day, the eighth day. After the first 12 h, and then daily, starting from the second day, one rat from each group was killed, in order to allow an 8 day monitoring of the proposed parameters: electrolytes, CK, CK-MB, LDH. The heart of all the sacrificed rats was collected in order to obtain a dynamic evaluation of cardiac lesions, after 12 h and then, daily. The heart was washed with physiologic serum, weighted, and a fragment from it was collected. This fragment was also weighted and, after that, used for the quantitative determination of organophosphate presence, through gas chromatography. The remaining part of the rat heart was processed for histological examination.

Results and discussions

After acute experimental intoxication with Triclorfon at two different dosages: 200 mg/bw, respectively 400 mg/bw, four rat have died after intoxication with high dose, in the first day (one in 20 min, one in the first hour, and others two in the third hours after inoculation).

In our study, no disorders in the serum level of sodium, kalium or chloride were documented, to correlate with organophosphate toxicity, in both concentrations that were tested.

No correlation between intoxication and the dynamic of superoxid dismutase values was documented, this behaving as an independent variable without any correlation with the intoxication.

The dynamic of Serum cholinesterase level after acute experimental intoxication with Triclorfon at two different dosages: 200 mg/bw, respectively 400 mg/bw was represented in table 1.

We did not observe a significant correlation between the high level of serum cholinesterase and the mortality of the rats, or with the level of cardiac lesions. CK, CK MB, LDH levels in serum were determined in order to monitor possible toxic myocarditis which may arise in the development of organophosphate intoxication. During the 8 days interval of monitoring, the dynamic of myocardial enzymes revealed a curved shape with a maximum in the 5^{th} – 6^{th} day of intoxication (table 2 and 3).

This dynamic is better illustrated in figure 1.

Experiment with Triclorfon 400 mg/kg.body weight – values determined for myocardial enzymes (U/l)

No. animal\Moment		0	6 h	12 h	day 2	day 3	day 4	day 5	day 6	day 7	day 8
(1) 140 g	LDH	284		262	*S						
	CK	82		92							
	CK-MB	16		24							
(2) 170 g	LDH	275		302		288	*S				
	CK	84		82		74					
	CK-MB	22		18		20					
(3) 180 g	LDH	198		ser hemolizat		ser hemolizat		822	*S		
	CK	76						340			
	CK-MB	19						180			
(4) 190 g	LDH	246		226		256		654		454	*S
	CK	58		82		76		420		98	
	CK-MB	14		18		16		112		32	
(5) 150 g	LDH	238	344		276	*S					
	CK	84	78		68						
	CK-MB	18	14		18						
(6) 150 g	LDH	ser hemolizat						*S			
	CK										
	CK-MB										
(7) 150 g	LDH	303	ser hemolizat		344		322		1124	*S	
	CK	68			68		84		312		
	CK-MB	16			20		18		142		
(8) 180 g	LDH	286	272		302		294		1584		462
	CK	64	88		66		80		264		82
	CK-MB	18	20		14		22		148		28

* S: rats were previously sacrified

Table 2
DYNAMIC OF MYOCARDIAL ENZYMES AFTER ACUTE EXPERIMENTAL INTOXICATION WITH TRICLORFONE AT DOSE OF 200 mg/BODY WEIGHT

Experiment with Triclorfon 200 mg/kg.body weight – values determined for myocardial enzymes (U/l)

No. animal\Moment		0	6 h	12 h	day 2	day 3	day 4	day 5	day 6	day 7	day 8
(1) 180 g	LDH	254	**D								
	CK	68									
	CK-MB	18									
(2) 165 g	LDH	198		204		186		796	*S		
	CK	83		84		88		512			
	CK-MB	14		16		14		368			
(3) 160 g	LDH	306	**D								
	CK	74									
	CK-MB	21									
(4) 185 g	LDH	341		286		344		684		864	*S
	CK	86		74		96		468		240	
	CK-MB	17		20		22		248		122	
(5) 175 g	LDH	282	264				344	*S			
	CK	48	44				60				
	CK-MB	14	18				16				
(6) 190 g	LDH	ser hemolizat	**D								
	CK										
	CK-MB										
(7) 170 g	LDH	384	ser hemolizat		354		486		1478		348
	CK	58			41		43		452		88
	CK-MB	10			21		20		214		76
(8) 150 g	LDH	202	**D								
	CK	88									
	CK-MB	18									

* S: rats were previously sacrified; **D: rat has died after intixication

Table 3
DYNAMIC OF MYOCARDIAL ENZYMES AFTER ACUTE EXPERIMENTAL INTOXICATION WITH TRICLORFONE AT DOSE OF 400 mg/BODY WEIGHT

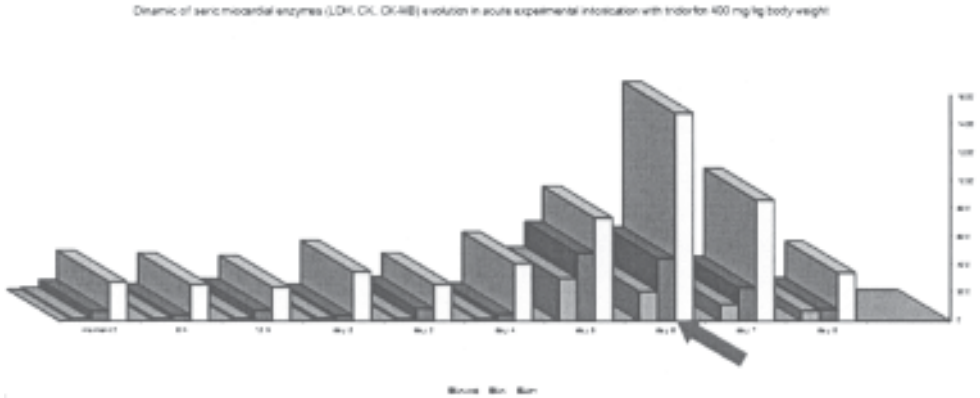

Fig. 1 Dynamic of seric myocardial enzymes (LDH, CK, CK-MB) evolution in acute experimental intoxication with triclorfon 400 mg/kg body weight

We evaluated the dynamic of Triclorfon concentrations in myocardial tissue after acute experimental intoxication with Triclorfon 200 mg/body weight.

Gas chromatography results demonstrated a biphasic accumulation of the insecticide at heart level.

These high values obtained in the fifth day of experiment are consistent with a process of myocarditis demonstrated at histological exam.

Normal aspect before acute intoxiccation (fig. 3).

In the first four days, the microscopic morphological aspects of myocardial tissue after acute experimental intoxication with Triclorfon 200 mg/body weight revealed: intense vascular congestion; moderate edema with dissociation of myocardial fibers (fig. 4 - 6); areas of fragmented myocardial fibers. After the fifth day of intoxication it was identified an intensification of edema

Triclorfon mg / kg . bw Sample:	200 Weight of fragmernt, (g)	Mass of metabolite (mg) detected gascromatographiic at the sample level	Triclorfon concentration in myocardial tissue (μg / g tissue)
1. (12h)	0.222	0.010	**45.05**
2. (II-th day)	0.285	0.013	**45.62**
3. (III-th day)	0.200	0.002	10
4. (IVa day)	0.260	0.006	23.08
5. (V-th day)	0.246	0.011	**44.72**
6. (VI-th day)	0.300	0.006	20
7. (VII-th day)	0.200	0.002	10
8. (VIII-th day)	0.340	0.0015	4.41

Table 4
DYNAMIC OF ORGANOPHOSPHORIC CONCENTRATION IN MYOCARDIAL TISSUE EXPRESSED AS A RATE FROM ABSOLUTE QUANTITIES INITIALLY ADMINISTERED, DURING ACUTE EXPERIMENTAL INTOXICATION WITH TRICLORFON 200 MG/ kg-body weight

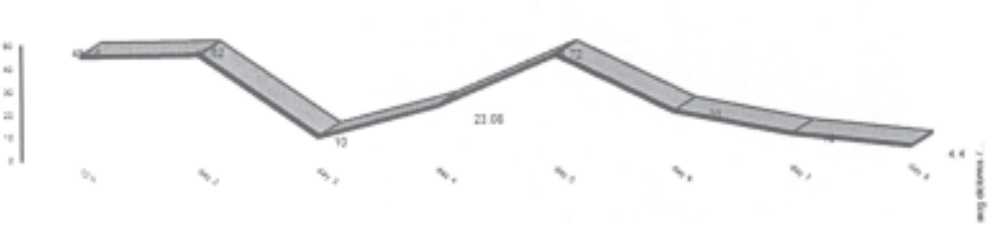

Fig. 2. Dynamic of organophosphate concentration in myocardial tissue expressed as a rate from absolute quantities initially administered, during acute experimental intoxication with Triclorfon 200 mg / kg body weight

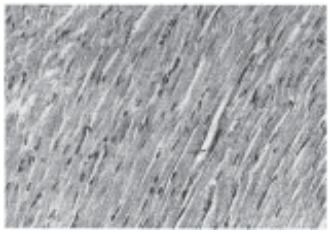

Fig. 3. Heart; normal aspects: observable transversal striations (ob.x40; col. H.E.)

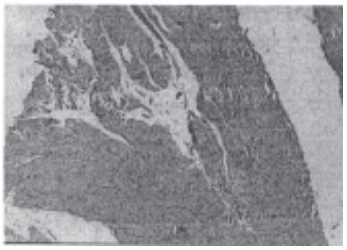

Fig. 4. Heart 400 mg Triclorfon at 120 hours: vascular congestion (ob.x10; col.H.E:)

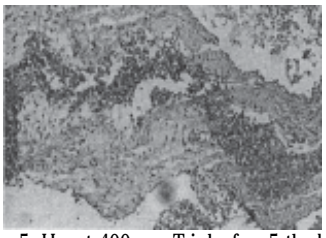

Fig. 5. Heart 400 mg Triclorfon 5-th day: Vascular wall with subendotelial hemoragy (ob.x20; col.H.E)

Fig. 6. Heart 400 mg Triclorfon 5-th day: vascular congestion (dilated blood vessels, filed with hematies)Edema with disociation of fascicules of miocardial fibres (longitudinal spaces - opticaly empty) (ob. x 20; col. H.E.)

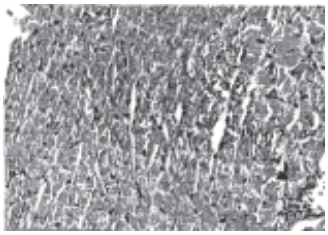

Fig. 7. Heart 400 mg. Triclorfon at 120 h: Fragmentations of myocardial fibers (ob x 40; col.H.E.)

with dissociation of myocardial fibers from their fascicules; aspects suggestive for toxic myocarditis.

The microscopic morphological aspects in myocardial tissue after acute experimental intoxication with Triclorfon 400 mg/body weight showed similar aspects to those observed for the dose of 200 mg/kg body weight (fig. 7).

The obtained values revealed similar patterns for organophosphoric dynamic in the myocardial tissue confirming our initial conclusion of a biphasic myocardial accumulation of toxic in Triclorfon poisoning. We have documented a mechanism that explains the pathogenesis of sudden deaths, reported in the literature to appear despite the apparent evolution with clinical and laboratory improvements. This possible outcome appears through reacumulation of organophosphoric substance at myocardic level, in the V-th - VI-th day of intoxication. In the myocardial tissue, a certain threat hold concentration of organophosphoric generates a process of myocarditis. The revealed mechanism explains the late appearance of these toxicological effects. Our study failed to confirm the involvement of oxidative stress in the pathogenesis of this intoxication [23 - 27]. Superoxid dismutase was monitored in subsets of animals, at heterogenous moments of time and no correlation between intoxication and the dynamic of superoxid dismutase values was documented, this behaving as an independent variable without any correlation with the intoxication. These findings were consistent with other studies, but did not assess the electrocardiographic changes that could have appeared in this type of intoxication - effect on QT interval, arrhythmias, torsades des points or asystole [28 - 36].

In our study, no correlation between disorders in the serum level of sodium, kalium or chloride and organophosphate toxicity was found, in both the concentrations that were tested. We can therefore conclude that, under the experimental conditions described, electrolyte disturbances were not proven to be involved in the pathophysiology of acute intoxication mechanisms of toxicity of organophosphate substances. This finding is in discordance with others studies [13 - 15].

Due to the fact that, in absolute values, the quantities of Triclorfon initially administered to the rats were not the same in one animal compared to another (a fixed dose of

200 mg/kg body weight results, in fact, in different quantities of Triclorfon administered according to the weight of the rat) the obtained values were expressed as a rate from the initial quantity of organophosphate administered to the animal. Similar pattern of organophosphate dynamic in the myocardial tissue was documented, straightening the initial conclusion of a biphasic myocardial accumulation of toxic in Triclorfon poisoning. In the myocardial tissue, the concentration of organophosphate in the 5^{th} - 6^{th} day of intoxication that generates a process of myocarditis poses a certain threat. The revealed mechanism explains the late appearance of these toxicological effects. We consider the diphasic evolution and the process of toxic myocarditis, to be the conditions that properly explain cardiac toxicity of Triclorfon.

Conclusions

Sudden deaths mentioned to appear in the evolution of the acute intoxication with organophosphate substances were documented to have myocarditis as a pathogenic mechanism, due to direct action of the toxic to the heart. The late effect is due to a biphasic accumulation of the organophosphate at myocardial level, as proved by the dynamic of Triclorfon concentrations into the heart, correlated with the monitored blood levels of cardiac enzymes and hystologic myocardial findings. Electrolyte disorders and the intervention of oxidative stress were not documented to be involved in this complication. As a result of the study we recommend that the possibility for an outcome associated with toxic myocarditis should always be taken into account and the patient should be monitored at least eight days from the moment of intoxication, also from this point of view.

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