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NEW ORIGINAL *IN VITRO* METHOD TO ASSESS CHOLINESTERASE REACTIVITY IN ORGANOPHOSPHATE POISONING

L. Şorodoc[1,2], Cătălina Lionte[1,2], Ema Largu[3], O. Petriş[2,4]
"Gr.T. Popa" University of Medicine and Pharmacy Iaşi
School of Medicine
1. Internal Medicine Department
4. Nursing Department
"Sf. Ioan" Emergency Clinic Hospital Iaşi
2. Medical Clinic
3. Biochemistry and Toxicology Laboratory

NEW ORIGINAL *IN VITRO* METHOD TO ASSESS CHOLINESTERASE REACTIVITY IN ORGANOPHOSPHATE POISONING (Abstract) : Assessment of organophosphate poisoning could benefit from a safe, non-expensive, easy to perform, quick (< 1 hour) test, which evaluates the level of cholinesterase activity "in vitro" regarding to the capability of oximes to reactivate OF-blocked cholinesterase. In the proposed protocol, 0.5 mL of sample serum is incubated, prior to the evaluation of level of cholinesterase activity, with 5 μL of a Toxogonin® dilution (0.125 mg) for 30 minutes at 37°C. For the standardization of the newly proposed protocol, several important issues were documented in the present article. The new original method of assessing cholinesterase reactivability will consist in an advantage for the diagnosis, prognostic evaluation and therapeutic orientation in OF intoxication. **Key words** : ORGANOPHOSPHATE, CHOLINESTERASE, TOXOGONIN

Organophosphate (OF) poisoning continues to represent an important medical issue through its high prevalence and severity among toxic pathology. Despite the existence of efficient antidotes, the prognosis of this intoxication remains reserved.

Diagnosis in OF poisoning is based on the assessment of plasma cholinesterase (ChEs) activity, this being the most constantly affected enzyme in OF poisoning (1,2). A method limitation results from a very wide interval of normal values for ChEs activity. Due to this, in medical practice, a relatively low value may represent the normal, while a so-called normal value may be detected when inhibition of ChEs activity is present (3,4,5,6).

Reversibility of cholinesterase inhibition after Toxogonin® (Tox) administration characterizes, with few exceptions, OF poisoning. The reactivation through Tox consist in the release of ChEs from the complex with OF, with the consequent regain of its enzymatic capabilities on choline compounds, expressed by an increase in the ChEs level of activity towards normal values. Thus, in case of suspected OF poisoning, evaluation of reactivability of ChEs activity levels will add important new perspectives for the diagnosis, compared with only the simple detection of the plasma ChEs activity.

In a specific "in vitro" protocol of testing, adding Tox to the serum sample will generate a recovery of the ChEs activity only in circumstances of acute OF poisoning. ChEs activity will not be significantly influenced if there is no previous blockage

of ChEs by OF. Tox-sensibilization method is based on the reactivatory effect of Tox, which increases the level of ChEs activity when the enzyme is blocked by OF. This effect is possible only in the initial period after intoxication (generally the first 48-72 hours). Then, the enzyme becomes irreversibly blocked by OF, called "ageing process" (a dezalkylation of the complex ChEs–OF). During this interval, obidoximes are efficient in recovering ChEs from their complexes with OF. This period can be longer, if the absorption still continues (i.e. additional ignored sites where absorption of poison persist, such as head hear, fat tissue, enteroenteric circuits etc.), or shorter (depending on the type of OF involved, different ways the poisoning was produced etc.) (7,8,9).

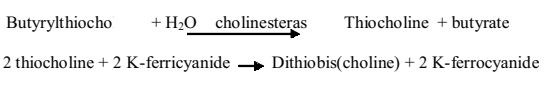
Another advantage of this test is the possibility to assess if the enzyme is still responsive to Tox, and sustains the continuation of Tox treatment, an expensive and even harmful treatment if the ChEs complex is „aged" (instead of unbinding the OF from the enzyme by linking with it, Tox will bind the enzyme, decreasing even more its level of activity) (10).

The aim of this study is to propose and standardize an original laboratory test, helpful for diagnosis with high sensitivity the OF poisoning, allowing a more orientated therapeutic approach.

MATERIAL AND METHOD

Serum samples from 23 consecutive patients, admitted in "Sf.Ioan" Emergency Clinic Hospital of Iasi, during 2009-2010, were used in the study. Inclusion criterion was the diagnosis of acute OF poisoning. Exclusion criteria were: age less than 18 years, mixed poisonings, refuse of patient or patient's family to sign the informed consent, associated chronic therapies with either: ibuprofen, procainamide, phenazopyridine, L-Dopa (which are known to interfere with the spectrophotometric method for detection ChEs levels of activity used in the study). Reference serum samples were analyzed from patients free from pesticide exposure, as follows: 23 patients with severe liver disease, and 23 patients without hepatic disorders.

The method used to determine the level of ChEs activity is a colorimetric technique, and relay on a Vitros System Chemistry 5,1/FS in which 11 ěL drop of sample is deposited on a dry, multilayered, analytical element coated on a polyester support type of slide and evenly distributed by the spreading layer to the underlying layers. ChEs brought in the reaction from the sample will hydrolyze butyrylcholine to thiocholine. The liberated thiocholine will reduce potassium hexacyanoferrate II, as it is shown below:



The rate of color loss is monitored by reflectance spectrophotometry and is proportional to the amount of ChEs activity present in the sample. Reference interval is: 4.65 – 10.44 U/mL (females), and 5.90 – 12.22 U/mL (males).

The idea of the research was to determine the level of ChEs activity using two samples of 0.5 ml serum obtained from the same specimen of blood, one analyzed without any preparation, using the conventional protocol, and the second one, after a preliminary incubation (half an hour at 37°C on an water thermostat – to reproduce, at least at minimum, the conditions within the human body) with Toxogonin® (active ingredient - obidoxime chloride, 1 mL ampoule, 250 mg/mL, manufactured by Merck Pharma GmbH). The blood samples were centrifuged immediately after collection. Each determination used 0.5 mL of serum, which was separated from the clot right after centrifugation (technical specifications of the kit producer being an interval of

maximum 4 hours), and processed after a maximal period of 24 hours (the kit producer recommendations are less than 7 days) while stored in refrigerator (at 2-8°C, never frozen), (11-13).

Several phases of the research were considered necessary, due to the need of obtaining answers on several issues regarding the new test.

I. Adding Tox, "in vitro", to a serum sample collected from a patient with acute OF poisoning, will generate different results for the levels of ChEs activity than by testing only the simple serum of the same patient ?

For this, blood samples were collected from patients admitted with acute OF intoxication and centrifuged as described. 0.5 mL from that serum was directly analyzed to determine the level of ChEs activity, and another 0.5 mL from the same sample was first incubated with Tox, being analyzed for the same parameter after the incubation. In the event of increasing values for the level of ChEs activity, we can consider that we have an "in vitro" effect of reactivation of the blocked enzyme through contact with Tox.

II. Are these results (increased values for the level of ChEs activity when is performed an initial incubation with Tox), the simple consequence of a colored substance insertion (Tox) in a colorimetric spectrophotometric working analyzer ?

For this, the new method was tested in patients without OF exposure : some associating liver diseases and some without any hepatopathy. Those samples were analyzed as simple serums and also, after an initial incubation period with Tox – with the new protocol. A lack of increase in ChEs activity for the samples associating incubation with Tox, both in patients with initially normal values of ChEs (subgroup non-intoxicated, without hepatopathy), or with initially decreased values (due to an impaired synthesis of ChEs secondary to liver disorders), can support the idea that the newly proposed test is characteristic for OF poisoning.

III. Which dilution of Tox has to be used for the serum incubation ?

Despite the temptation for using high concentration of Tox in order to obtain a maximal effect on ChEs reactivation, the risks for bias will increase with the use of large volumes or quantities of Tox. For example, the case of ibuprofen (mentioned in the technical references from the cholinesterase kit producer) is very suggestive : therapeutic serum concentration of 4 mg/dL associate no bias, at values of less than 20 mg/dL the interference is negligible (bias < 0.3 U/mL), but at concentrations over 40 mg/dL the error exceed 2.3 U/mL (14).

Thus, the dilution better to be used was estimated in relationship with the therapeutic level for Tox used in medical practice. The treatment is generally started with 2 ampoules of 250 mg Tox, for a total volume of blood of 5000 – 5500 mL, resulting in 0.1 mg / ml of blood. For a volume of 6 ml of blood collected in a vaccuette, results a need of 0.6 mg of Tox / vaccuette correspondent to an average of 2 ml of serum obtained by centrifugation from one vaccuette. A draft calculation associates 0.3 mg Tox for 1 mL serum, which signifies 0.15 mg Tox for the volume of 0.5 mL serum requested by the method. If we dilute the content of one ampoule of Tox (1 mL, containing 250 mg obidoxime) in proportion of 1/10 (0.5 mL Tox + 4, 5 mL NaCl 0.9%), 5 μL of this dilution will contain 0.125 mg.

In order to determine which dilution is the best to be used, 4 concentrations of Tox were tested for the smallest volume (5 μL) that was possible to be manipulated in the hospital laboratory : undiluted - for the amount of 1.25 mg/5 μL ; 1/10 - for the amount of 0.125 mg/5 μL ; 1/20 - for the amount of 0.0625 mg/5 μL ; 1/5 - for the

amount of 0.25 mg/5 μL.

IV. Is there any influence from the yellow color of the Tox used in the test, on the results offered by the spectrophotometer ?

For this, instead of serum, samples of NaCl 0.9% (without any ChEs) were investigated without and with a preliminary incubation with Tox, for spectrophotometric results on the level of ChEs activity. Ten samples were analyzed and the average difference between obidoxime incubated samples and those with only NaCl was considered as colorimetric influence of Tox, value that had to be subtracted from every result obtained for the reactivability of ChEs.

V. Is it necessary to constitute every time a fresh dilution for Tox, or is it possible to use a previously constituted dilution for many days (the whole surveillance period of an intoxicated patient), without interfering the rigourosity of the method ?

For this, in three of the patients included in the study, at different moments (a total of 19 comparative tests), the blood samples were comparatively assessed by incubating 0.5 mL of serum with 5 μL of Tox from a dilution instantly prepared on the moment of determination, and another 0.5 mL from the same sample of serum with Tox from a dilution prepared in the same week. Due to the known influence of sunlight on Tox, the prepared dilution was stored in a brown glass bottle.

All aspects of the clinical study were made with regard to the bioethics rules endorsed by European Union legislation, as well as the Romanian regulations for good clinical practice and clinical trials. The study protocols have the approval of the Bioethics Commission of the "Gr.T. Popa" University of Medicine and Pharmacy Iaşi.

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) 17 software for Windows 7.0. Comparisons of ChEs levels of activity at each time between the two methods used were assessed with unpaired *t*-test. For all tests, *p*-value less than 0.05 was considered significant.

RESULTS

Issue I. Values obtained for the level of ChEs activity determined using Vitros System Chemistry 5,1/FS on different samples of serum from 4 organophosphates poisoned patients, with the conventional method and a new method that involve a preliminary incubation of the serum (0.5 mL) with 5 μL of Tox (1,25 mg) for 30 minutes at 37°C are presented in Table I.

Increased values for the level of ChEs activity were considered as indicative for "in vitro" effect of reactivation of the blocked enzyme through contact with Tox.

Issue II. Values obtained for the level of ChEs activity on different serum samples from : A - patients without OF intoxication but associating liver disease (23 cases), and B - patients without any hepatopathy or insecticide exposure (23 cases), using the conventional method and a new method are presented in Table II.

Issue III. Comparative results for the level of ChEs activity determined with the conventional method and variations of the new methodology involving the preliminary incubation (30 minutes at 37°C) of

TABLE I
ChEs activity on different serum samples from OF poisoned patients.

Test: level of ChEs activity (U/mL)	Sample I	Sample II	Sample III	Sample IV
Conventional method	0.4	0.3	0.8	0.5
Tox – serum incubation method*	4	6.2	4.5	3.9

(*p value 0.0058)

TABLE II
Level of ChEs activity in patients without organophosphate poisoning

Test: level of activity of ChEs (U/mL)*		Conventional method	Tox – serum incubation method**
A.	Patients associating liver diseases	1.96±0.40	2.06±0.20
B.	Patients without hepatopathy or OF exposure	5.36±0.76	5.40±0.62

(*values expressed as mean ± standard deviation ; ** p value 0.47)

TABLE III
Level of ChEs activity on serum samples from patients with and without OF poisoning using different Tox dilutions

Level of ChEs activity (U/mL) Dilution of Toxogonin:	group	Conventional method*	Tox – serum incubation method
Undiluted for the amount of 1.25 mg/5 µL	1**	0.5±0.21	5.9
1/10 for the amount of 0.125 mg/5 µL			4.9
1/20 for the amount of 0.0625 mg/5 µL			4.7
1/5 for the amount of 0.25 mg/5 µL			5.2
Undiluted for the amount of 1.25 mg/5 µL	2***	5.31±0.72	5.1
1/10 for the amount of 0.125 mg/5 µL			4.3
1/20 for the amount of 0.0625 mg/5 µL			4.5
1/5 for the amount of 0.25 mg/5 µL			4.9
Undiluted for the amount of 1.25 mg/5 µL	3#	1.92±0.41	2.1
1/10 for the amount of 0.125 mg/5 µL			1.6
1/20 for the amount of 0.0625 mg/5 µL			1.7
1/5 for the amount of 0.25 mg/5 µL			1.9

(*mean ± standard deviation ; **p value 0.0007 between two methods used ; *** p value 0.83 ; #p value 0.059 between methods used)

TABLE IV
Level of ChEs activity with NaCl 0.9 % to replace the serum

Test: level of ChEs activity (U/mL)	No. samples	Conventional method	No. samples	Tox - serum incubation method*
NaCl 0.9% 0.5 mL	10	< 0.2	10	0.56±0.10

* p value 0.057

the serum (0.5 mL) with 5 µL of Tox from different dilutions (undiluted, diluted 1/5, 1/10 and 1/20) on samples of serum from : 23 patients intoxicated with organophosphates (1), 23 patients without any hepatopathy or insecticide exposure (2), and 23 patients without OF intoxication but associating liver disease (3) are presented in Table III.

Issue IV. Values obtained for the level of ChEs activity using NaCl 0.9 % to replace serum in the test methodology, determined with conventional method and with the new proposed method using Tox (0.125 mg – dilution 1/10), in order to test the influence of the Toxogonin® yellow color on the results offered by the spectrophotometer are presented in Table IV.

Issue V. Values obtained for the level of ChEs activity determined on serum samples from OF poisoned patients, using the new method that involves a preliminary incu-

TABLE V
ChEs activity using 1/10 Tox dilution fresh and previously constituted

Level of ChEs activity (U/mL) Tox – serum incubation method	Time elapsed from the moment of constitution of the dilution																		
	Sample 3 (16 h)		Sample 4 (24 h)		Sample 7 (48 h)		Sample 10 (72 h)		Sample 11 (96 h)		Sample 12 (120 h)			Sample 13 (144 h)			Sample 14 (168 h)		
	Subject		Subject		Subject		Subject		Subject		Subject			Subject			Subject		
	D*	G	D	F	F	G	F	G	F	G	D	F	G	D	F	G	D	F	G
Fresh Tox dilution	0.4	4.1	0.5	3.9	2.6	0.9	2.8	0.9	2.9	1.2	3	3.2	1	3.4	3.1	1	3.8	3.2	1.2
Previously-constituted Tox dilution**	0.4	4	0.6	3.9	2.6	0.9	2.6	0.8	2.8	1.2	3.1	3.2	1	3.5	3	1	3.7	3.2	1.3

* Letters D, G, F - were designated to poisoned patients, as they were included in the study;

** p value 0.42.

bation of the serum (0.5 mL for 30 minutes at 37°C with 5 μL of Tox (0.125 mg) 1/10 dilution, prepared in two manners: fresh, just before incubation with serum, and constituted previously with the occasion of the first determination are included in Table V.

DISCUSSION

The results obtained from analyzing issues I and II have supported our hypothesis that, in acute OF poisoning, additional information can be obtained using a laboratory method that test the effect of an "in vitro" incubation with Tox to generate a possible recovery in the level of ChEs activity. Results from Table II showed that in group A, level of ChEs activity is almost equal with both methods, because there is a decreased amount of enzyme synthesized by affected liver, while in group B, where ChEs activity is not influenced by anything, the differences between values obtained with both methods are inconclusive, with no statistical significance.

Technical aspects regarding the methodology of the test were assessed in issues III, IV and V revealing the best dilution of Tox to be used, which is 1/10, containing 0.125 mg / 5 μL (conclusion from issue III).

The presence of Tox in the sample (0.125 mg added to the 0.5 mL of serum) result in an artifact of 0.5 U/mL false increase in the level of ChEs activity which has to be subtracted from the values obtained with the newly proposed protocol (from those of samples incubated with obidoxime) - conclusion from issue IV, but this value was not statistically significant. Once constituted, the dilution of Tox can be safely used for at least one week, if stored in brown glass bottles (conclusion from issue V), because differences in the two dilutions used (fresh and previously prepared) is not statistically significant in patients analyzed.

CONCLUSIONS

Analyzing "in vitro" the Tox effect on restoring the level of ChEs activity resulted in a safe, non-expensive, easy to perform, quick (< 1 hour) test capable to offer new, interesting perspectives on OF intoxications. It clearly consists in an advantage for the diagnosis, prognostic evaluation and therapeutic orientation in organophosphate poisoning. These aspects are now evaluated in a prospective study, which will be finalized in May 2012. Finally, the desire of the authors is that professionals involved in the management of organophosphate poisonings will start to use the new proposed method, and communicate their personal considerations on this topic.

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