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Perspective

NOVEL BIOLOGICAL MARKERS FOR DIAGNOSIS AND PROGNOSIS OF POISONING WITH ORGANOPHOSPHATES, WITH SPECIAL REFERENCE TO ACYLPEPTIDE HYDROLASE

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Finding specific markers for changes in the biological systems after taking drugs or the consequences of exposure to toxic chemicals in the environment are increasingly important. Herein, the available biomarkers for documenting the interaction of organophosphorus compounds (neuroparalytic agents, pesticides, and drug preparations) with the biological systems as inhibition of cholinesterase group enzymes (AChE and BuChE) are reviewed. The advantages in the study of the activity of acylpeptide hydrolase in the blood for prevention and diagnosis of the organism changes after interaction with an organophosphorus compound are highlighted. ***Biomed Rev*** **2022; 33: 43-48**

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Analysis of tissues and body fluids for chemicals, metabolites of chemicals, enzymes and other biochemical substances has been used to document the interaction of these chemicals with biological systems. Measurements of these substances, now referred to as "biomarkers", are recognized as providing data linking exposure to a chemical with internal dose and outcome and as relevant to the process of risk assessment. In other words, a physical sign or laboratory measurement that occurs in association with a pathological process and that has putative diagnostic and/or prognostic utility.

One important process is to establish the qualitative and quantitative relationship of the biomarker (a) to exposure to a chemical, and (b) to the selected end-point. Desirable characteristics of biomarkers include:

(1) the marker (measurement)
   (a) reflects the interaction (qualitative or quantitative) of the host biological system with the chemical of interest, (b) has known and appropriate specificity and sensitivity to the interaction, and (c) is reproducible qualitatively and quantitatively with respect to time (short- and long-term);
(2) the analytical measurement has defined and appropriate accuracy and precision;
(3) the marker is common to individuals within a popula-

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tion or subgroup and is of defined variability within the normal, non-exposed population or group of interest, and (4) the marker is common between species.

Methods for assessing exposure to a chemical fall into two categories (1): (i) measurement of levels of chemical agents and their metabolites and/or derivatives in cells, tissue, body fluids or excreta, and (ii) measurement of biological responses such as cytogenetic and reversible physiological changes in the exposed individuals.

Measurement of covalent adducts formed between chemical agents and cellular macromolecules (proteins, DNA), or their excretion products have characteristics of both categories listed above.

In short, the most important criteria of biomarkers include: (i) biological specificity, (ii) clarity of interpretation, (iii) time and permanence of response, (iv) reliability, (v) methodological considerations, (vi) relative sensitivity, (vii) validation in the field, and (viii) linkage to higher-level effects

How do you know if you have been exposed to something harmful?

One way is to have data from continuous monitoring of the individual's environment. (While this would be very useful, it is seldom, if ever, done). Other ways to determine exposure are: (i) measure residues on clothing or skin, (ii) measure residues in blood, urine, saliva (of note, all short lived), and (iii) analyze proteins modified by exposure.

Is it dangerous to be exposed to chemical compounds from the group of organophosphates (OPs)?

Herein, I would like to highlight the problem of tracing and the consequences of human exposure to chemical substances with the example of phosphoorganic compounds. I may list two reasons for choosing these compounds. One is "sentimental": these are the compounds I started dealing with many years ago as the first task on my scientific journey. The other is that even now, as an expert in the toxicology of chemicals, I am forced to explain whether the food we eat is protected from pesticide contamination, who and how provides this, *etc*.

Almost every person is or has been exposed to OP insecticides in their home or work environment or as trace dietary contaminants. Intoxication by OPs, occasionally leading to death, represents up to 80% of pesticide-related hospital admissions. Organophosphates are also major chemical warfare agents with extensive stockpiles as a continuing threat worldwide. Selected OPs are used in medicine as anthelmintics and ectoparasiticides and for the therapy of glaucoma and have been tested for myasthenia gravis and Alzheimer's disease. Thus, OPs are essential tools and probes both in agriculture and medicine.

Current issues of organophosphates toxicology

Toxicological problems with OPs can be listed as follows: (i) possible long-term effects of chronic low-level exposures, (ii) genetic susceptibility to OP toxicity, (iii) developmental toxicity and neurotoxicity, (iv) common mechanism of action, and (v) possible additional OP targets.

Organophosphates possible targets of action

- OP protein targets - *serine hydrolases*:
  - Serine proteases (≈125 members): trypsin, thrombin, activated factor Xa
  - Metabolic serine hydrolases (≈115 members): lipases, peptidases, esterases, thioesterases, and amidases
- OP protein targets - *non serine targets*:
  - tyrosine in purified tubulin, kinesin, albumin, transferrin, alpha-2-glycoprotein, apolipoprotein, and small synthetic peptides

The most popular and well known biomarkers of exposure to OPs including nerve agents and pesticides are biomarkers of ***effect*** (changes in cholinesterase's activity in plasma and erythrocytes) and biomarkers of ***exposure*** (monitoring OPs and their metabolites in biological samples – blood and urine).

Biomarkers of effects

- Red blood cell AChE (Fig. 1)
- Muscarinic receptors in lymphocytes
- Lymphocyte neuropathy target esterase

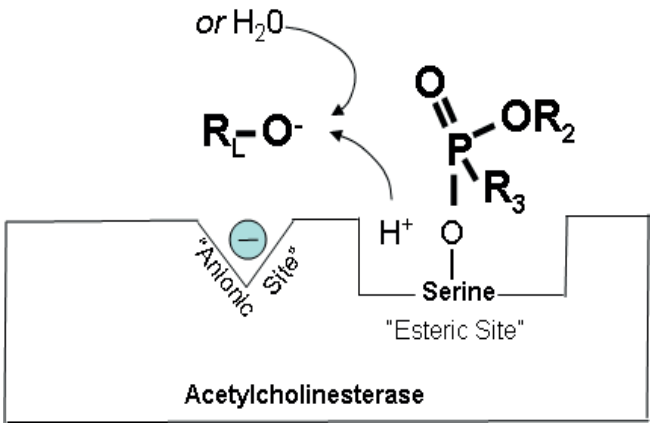

Figure 1. Organophosphates and AChE metabolic interactions.

Biomarkers of exposure (2)
An ideal biomarker of exposure should be chemical specific, available by minimally invasive techniques, detectable even at very low levels, easy to measure, and relatable quantitatively to certain prior exposures.

- Plasma butyrylcholinesterase (BuChE)
- Red blood cell acetylcholinesterase (AChE)
- OPs in blood
- OP metabolites in urine
- OP adducts to blood proteins (serum albumin on tyrosine 411)

Biomarkers of susceptibility
Genetic factors can modulate the response to neurotoxic chemicals.

- Paraoxonase 1 status
- BuChE polymorphisms
- Cytochromes P450s

Why are new biomarkers needed?

- Not all OPs inhibit AChE and BuChE
- OP doses too low to inhibit AChE cause toxicity
- Only some people have symptoms
- Toxic symptoms depend on the identity of the OP

In our old experiments when comparing the changes in cholinesterase activities in blood, liver and brain of poisoning with nerve gases animals, our data correspond to well known facts from the literature. Some new data concerned the changes in **acylpeptide hydrolase (APH)** activity. N-Acylpeptide hydrolase (EC 3.4.19.1) catalyzes the hydrolysis of N-acylated peptide substrates of various sizes and with different types of acyl groups (acetyl, chloroacetyl, formyl, and carbamyl) to generate an acylamino acid and a peptide with a free NH (3, 4). The enzyme help the post-translational acetylation of intracellular proteins and peptides. It is localized in hepatocytes, brain cells and erythrocytes. It is generally assumed to be an efficient means of protecting these substances from proteolytic degradation in eukaryotic cells, and thus of increasing their half-life (5). Thus, APH and the proteasome act in coordination to clear cytotoxic denatured proteins from cells. In principle, all serine hydrolases have the capacity to react with OP compounds, thus the characterization of members of this class of enzymes in biological systems would provide a useful resource for the identification potential OP targets. Acylpeptide hydrolase have been shown to be an essential target for reaction with organophosphates pesticides (6).

The main characteristics of the enzyme could be summarized like that:

Acylpeptide hydrolase

- *Enzyme reaction*: hydrolysis of N-acylated peptides to generate an acylamino acid and a peptide with a free N-terminus shortened by one amino acid.
- *Substrates*: peptides of various sizes with different types of N-terminal acyl groups (formyl, acetyl, carbamyl and chloroacetyl).
- *Tissue localization*: erythrocytes, brain, hepatocytes, enterocytes, lungs, heart.
- *Cellular localization*: cytosol, 732 aa, 75 kDa, tetrameric structure, catalytic triad: Ser^{587}, Asp^{675}, His^{707}

Acylpeptide hydrolase (known functions)

- *Clears* cytotoxic denatured proteins from cells.
- Cleaves *amyloid-b peptide* (Alzheimer's disease), proposed cognitive enhancement effect.
- APH gene is deleted in certain *cancers* (e.g., multiple myeloma).
- Also active on *small* acetylated bioactive peptides, such as **β-endorphin and α-melanocyte-stimulating hormone.**
- APH is *inhibited* by diisopropyl fluorophosphate (DFP), chlorpyrifos oxon, dichlorvos and naled.

The listed characteristics of the enzyme allow us to argue that **Erythrocyte-APH** is potential **BIOMARKER for OP exposure** (7) because is (i) easily *accessible* target, (ii) the enzyme activity could be determined *outdoor*. (iii) most OP have *higher affinity* to APH than to AChE, and (iv) the inhibitory potency of OP to APH does not depend from *thionate/ oxon swich*.

Brief information of our previous results

It concerns the problems with OP biomarkers in the context of our unpublished data from experiments with neuroparalytic agents (tabun and soman) on rats under an international contract with Dutch colleagues in 2009. In these experiments for the first time was shown a selective, significant and long lasting (up to 20% from the control after 1xLD50 at day 7^{th}) decrease of APH activity in blood of rats. The inhibition is selective for the tabun poisoning and not demonstrated after soman. It was also found an increased nitric oxide production in lung macrophages in rats treated with soman and most significantly with tabun. Significant signs of *oxidative stress* were observed in the early phases of soman and tabun intoxication (increased

corticosterone levels in plasma, decreased glutathione levels in liver and brain, decreased catalase, superoxide dismutase and glutathione peroxidase activities in erythrocytes, brain and liver). The signs of oxidative stress were more pronounced in tabun poisoned animals than in soman. That could be explained by the fact that soman poisoning developed very quickly with symptoms mainly of neurotoxicity. After soman treatment, unchanged blood and liver GSH-S-transferase and brain hydrolase activities were observed. Liver and brain carboxylesterase and blood GSH-S-transferase activities were also unchanged after tabun poisoning; however, some more changes in the peripheral organs were observed for longer period of time (increased inhibition of hydrolase, AChE and BuChE activity).

Accordingly, these experiments have demonstrated something very important: chronic exposure with low concentrations of OPs (for example, pesticides) is often not felt immediately, leading to a number of postponed in time pathological changes in internal organs and intoxicated behavior. Naturally, there is a strictly regulated state control of contamination, for example, of food products with OPs to prevent such pathological consequences. The speculations with the regulation of this control go beyond the ethical standards.

Regardless of the control of pollution by chemical substances (in the case of pesticides) of the environment and the environment in which millions of people work and live (farmers), it is worth to highlight the problem with a few examples.

Pesticides and workers

Approximately 3 million people suffer from poisoning and 200,000 die from pesticide poisoning annually in the world. Millions are exposed to hazardous work practices and insecure storage of pesticides; however, it is deliberate self-poisoning that causes most deaths, particularly in Asia. According to what it was found, it is recommended that researchers who carry out studies with cholinesterase enzymes do not lose sight of the delicacy of these enzymes in order to have the best possible results, not necessarily seek that the results are below a range established by the laboratory and take into account notes that the appearance of symptoms of intoxication depends more on the **rate** of *cholinesterase inhibition* than on the absolute level of activity found (8).

A retrospective cohort study conducted in Sukoharjo, Central Java, Indonesia examined a sample of 200 rice farmers was selected by fixed exposure sampling to analyze the effects of pesticide exposure and psychosocial determinants on neuropsychiatric disorders (depression and anxiety). Depression and anxiety are directly increased by high work stress, depression history of family members, grief and loss, and past trauma. Depression and anxiety are indirectly affected by the OP pesticide exposure, age through the use of personal protective equipment (PPE), and latest education through the use of PPE (9).

Long-term occupational or environmental exposure to OP/CB were associated with a reduced neurobehavioral functioning in a sample of agricultural workers (OE) and rural inhabitants (EE). Seasonal exposure to OP/CB consistenly inhibited BuChE activity in the EE and OE groups, and in the EE group this biomarker was the best predictor for reduced performance in logical, auditory and visual memory, inhibitory control of cognitive interference, constructional and planning abilities, executive functions, and motor speed and coordination.

The evidence presented supports the notion that improvements are needed in the regulation and control of the use of pesticides, especially when they are used near residential areas. This should be supported by stricter regulations for the sale and use of pesticides in order to contribute to achieving a higher level of sustainability for health and the environment (10).

The study showed that inhibition of AChE and BuChE enzyme activities exceeded workplace standards thereby confirming recent exposure to pesticides on exposed individuals belonging to both agricultural workers and the general population. These indicators of enhanced exposure were associated with cognitive impairment in the environmentally exposed subgroup, mainly in cognitive areas of attention, praxis, language/vocabulary and executive function (11).

It is worth noting that in the assessment of the health status of these farmers we have, in addition to direct testing of the effect of exposure to OPs, a number of *other* biochemical and cytological parameters, such as:

- Oxidative stress
  - primary *targets*: liver, brain, erythrocyte haemolysate
  - primary *parameters*: SOD, catalase, glutathione, GSSG-reductase, GSH-peroxidase, glucose-6-phosphate dehydrogenase, GSH-transferase
- Among all the psychological disorders, only sleep quality was affected by the inhibition of cholinesterase disorder. And, inhibition of the cholinesterase activity might be a key factor contributing to sleep disturbances in the workers. Evaluation of mental disorders is also crucial to improve wellbeing among OPs factory workers through workplace wellness programs and self-assessment tools for mental disorders (12).

These findings confirm the few data available in the literature that the poisoning with OPs is accompanying with more or less exert decreased antioxidant body capacity mainly with decreased enzyme activities of catalase, SOD and glutathione peroxidase – after some pesticides in human (13) in the development of type II paralysis in patients suffering from acute OP poisoning (14).

CONCLUSION

Achieving a better healthy environment for living a person in the conditions of the so-called European green deal will have a real effect only when it is convinced and calm about the results achieved. Unfortunately, conviction is very difficult to achieve in modern living conditions. No biomarkers are needed for this.

The easiest way to do not need a biomarker is illustrated in the picture below:

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