JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v10i1.287

# Regarding the mechanisms of the changes of acetyl cholinesterase (ACE) enzyme activity from erythrocyte membrane under the action of pesticides

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**Received:** 11 June 2019 **Accepted:** 01 March 2020 **Available Online**: 31 March 2020

ABSTRACT

The kinetics and mechanisms of the action of some pesticides, such as pentachloronitrobenzene, trichloracetic acid, rogor, sodium pentachlorphenol, chlorophos, heptachlor and photodynamic herbicides on acetylcholinesterase activity and mechanical stability of red blood cells under ultrasound action was studied. The degree of change in the resistance of erythrocytes treated with the pesticides to ultrasound action was determined. The data on the altering of structure-function characteristics correlate well with the results of toxic action of these compounds on different biological objects. These results may be used for selective search of the preparations with lower toxicity and higher economical efficiency.

Аннотация

Изучена кинетика и механизмы влияния некоторых пестицидов: пентахлорнитробензола-ПХНБ, трихлоруксусной кислоты (ТХУ), рогора, пентахлорфенолата натрия (ПХФ-Na), хлорофоса, гептахлора и фотодинамических гербицидов на мембранную активность ацетилхолинэстеразы и механическую стабильность эритроцитов под воздействием ультразвука. Определена степень изменения резистентности обработанных пестицидами эритроцитов к ультразвуку (УЗ). Данные о нарушении структурно-функциональных свойств эритроцитов коррелируют с результатами токсического воздействия этих препаратов на различные биологические объекты. Полученные данные могут быть использованы для селективного поиска препаратов с наименьшей токсичностью и экономической эффективностью

**Keywords:** Pesticides, ultrasound, acetylcholine, RBC hemolysis

## 1. Introduction

Pesticides are chemical compounds used for the struggle against pathogenic organisms. World assortment of these preparations is more than 10000 names based on more than 600 chemical compounds of different classes. However, despite of advantages of the chemical method of plant protection there are some disadvantages too. First of all, preparations accumulate in the environment; stable populations of harmful organisms are formed that turn into new resistant species, useful inhabitants of the biosphere suffer greatly, as well as the health of human population [1]. Finally, it leads to the violation of natural biosynthesis in the biosphere. Many authors confirm that high level of morbidity of different etiologies may be caused by the influence of technogenic pollution of the environment on the human health [4]. Humans are under the influence of a huge number of various chemical substances during their life. They enter the body in different ways. Due to the connection with the development of technogenic factors the population of large cities is strongly affected by heavy

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metals like lead, chromium, copper and cadmium and also by aromatic hydrocarbons and pesticides. Compounds of these substances have an adverse effect on the functional state of the digestive system, pancreatic cells, and also cause irritation of the mucous membrane of the small intestine It is confirmed that the increase of chromosomal aberration rate in the lymphocytes of peripheral blood of the persons with chronic lead intoxication is the result of industrial contact with pesticides [1,2]. Presence of induced chromosomal aberrations may be the evidence of the mutagenic action of industrial factors on human population. Thus, the increased rate of mutations in cells of persons and animals contacted with heavy metals has been proved . This process may be the reason of aborts, congenital malformations, hereditary diseases and other pathologies [2]. So, it is necessary to predict the mutagenic danger of substances with which persons may get in contact. Large work is being done in many countries to overcome these disadvantages. Toxic pesticides are replaced by substances with decreased toxicity that are not capable to accumulate in the environment and living organisms. Replacement of DDT (Dichlorodiphenyltrichloroethane) by methyl chloride, dichlor and other insecticides is a very actual point. Such chemical substances promote the changes of blood content and it results in abnormal protein levels in plasma [4,6]. It needs the constant medical check-up. The research of scientists of National Cancer Institute /USA/ on donors showed that pesticides action on organism twice increases the risk of multiple myeloma-malignant cancers of the bone marrow development in the age interval from 30 till 94 . Agricultural employers have been shown to have increased risk of hematological diseases development with a lethal outcome compared to the total human population [6].

The development of effective methods of physical and chemical characteristics of RBC in different pathologies and under the influence of negative factors of environment is one of the important biomedical problems. Fundamental similarity of structural and functional organization of all cell membranes and established total mechanisms of their responses to some pathological conditions make possible to use RBC as a model. That's why it is important to develop the method to study the changes in RBC membranes under various stresses that model certain pathological processes. Application of ultrasound that is used widely for direct and selective effect on cells may give information about mechanical resistance of RBC, since ultrasound can cause a hemolytic effect. Since it is known that pesticides have a damaging effect on cell membranes, one can study the regularities of effects of chlorine-containing pesticides in the model system of RBC suspension. Study of ultrasonic hemolytic resistance of RBC is relevant because pesticides are widely used. The changes of mechanic resistance of RBC treated with pesticides may be used a characteristic of their damaging action on a quantitative level [2].

Getting into the body, drugs may function as powerful blockers of ACE. Apparently, drugs dissolving in the hydrophobic region of erythrocyte membranes, may later form a complex with cytochrome, and leading to the change in membrane stability both directly and indirectly. So, it became necessary to analyze the mechanism of action of ultrasound (US) on RBC membranes, as it has been reflected in our published works [2,3].

There are several mechanisms of the effect of ultrasound on cells and cell suspension listed below:

- the heating (temperature factor);
- chemical damage caused by the action of free radicals;
- mechanical damage caused by shock waves and acoustic flows (mechanic factor).

The purpose of this work was to study kinetics of membrane-bound ACE in presence of chlorine-containing pesticides, as well as to obtain quantitative criteria for comparative evaluation of the action of pesticides.

The dependence of hemolytic activity of some pesticides and herbicides on the level of initial activity of cellular enzymes, on the ability of the drug to reduce membrane fluidity has been studied in a number of works [2,3,5]. However, the kinetic characteristics of the action of chlorinated pesticides on RBC hemolysis and the resistance of RBC to mechanical factors, as well as investigation of certain patterns of physiological activity of these compounds have not been studied yet.

Previously, it was shown that the damaging effect of insecticides in biological systems is directly related to their effect on the lipid phase of biological membranes [3]. However, quantitative criteria for their effect on RBC have not been investigated yet. Research on quantitative regularities of the damaging action of physiologically active compounds (PAC) would be actual.

The aim of this work is to study the kinetics of the action of chlorine-containing pesticides on the activity of membrane-bound ACE-ase, as well as the obtained quantitative criteria for comparative analysis.

## 2. Material and Methods

As the material we used the suspension of red blood cells isolated from donor blood. The specific activity of ACE was determined by the potentiometric method with automatic registration process. Kinetics of RBC hemolysis in the isotonic medium under the action of pesticides was studied by the photocalorimetric method in suspension (107-108 cells per 1 ml). We used sodium salt of trichloroacetic acid (herbicide), sodium pentachlorophenol (herbicide), trichlorfon (insecticide), pentachloronitrobenzene (fungicide) and rogor (pesticide).

RBC suspension extracted by precipitation from 3 ml of donor fresh blood treated by heparin or citrate ( 0,5 ml of citrate + 2,5 ml of whole blood ) and twice washed from plasma by 0,9% NaCl isotonic solution was used. Centrifugation was performed at 6000 rpm for 10 min (3 times). The washed cells were suspended in 8 ml of saline solution. The suspension of RBC diluted with saline solution

in a ratio of 0,5 ml over 23,5 ml of saline solution (50 times) has been prepared for the research of US hemolysis. Cell concentration of suspension is 30·106 cell/ml. We also carried out a spectrophotometric assessment of mechanical and physical factors (including US fields) kinetics of action on biological membranes. This method allows to investigate the hidden damages of RBC membranes. Resistance of RBC was studied on the basis of method of photometric automatic registration of process of hemolysis of RBC under the influence of continuous ultrasound at the frequency 0,88 MHz in frames of intensity 0,1-1,0 v/sm2 at the constant temperature.

Activity of membrane-associated ACE was determined by the potentiometric method. The substrate was acetylcholine chloride (AC) with initial concentration of 2.5 mM in a measuring cell. To study the kinetics of enzymatic hydrolysis, an incubation mixture was used consisting of a 0.9% NaCl solution containing 2.5 mM Tris-HCl, a suspension of RBC (5-8·10$^4$ cell / ml), ethanol (no more than 2 % by volume). The speed of reaction in the process of enzymatic hydrolysis of AC has been calculated from experimental kinetic curves at the change of pH of the incubated mixture in control and after the treatment by AC. Incubation time of RBC with preparations was equal to 2 min. Kinetic analysis of the results of the determination of ACE activity of RBC before and after treatment of preparations assessed by estimation of enzyme activity through the change of relative activity (A) (by the tangent of the slope of the kinetic curve) at the action of preparation.

Determination of ACE activity in the suspension of RBC was carried out also by the measurement of the kinetics of the damage of RBC in the process of ultrasound hemolysis (f=0.88 MHz and I=0.3 V/ cm$^2$). We used the method of US hemolysis that was developed before [2,9]. The speeds of cell mechanical disruption in the sonification was determined by kinetic curves of ultrasonic disintegration before and after treatment by preparations ($V_0$ and $V_1$). These indices are the characteristics of mechanic resistance of RBC membranes.

## 3. Results

In Table 1 one can see structural formulas of substances tested in the current study.

Detected inactivation of the external ACE enzyme indicates that the inhibitory effect of chlorine-containing pesticides is caused by the damage of the membranes of erythrocytes, with which ACE is associated.

As a criterion for assessing the effect of the studied pesticides on the activity of erythrocytes, the concentration of the drug causing enzyme inactivation by 50% ($CA_{50}$) was used. It was found that in the studied concentrations, these pesticides have certain antibacterial activity (Table 1). Table 1 shows that the studied compounds are not specific acetylcholinesterase inhibitors, as they reduce enzyme activity at significantly higher concentrations ($CA_{50}$ – 120 and 10$^{-1}$ – 10$^{-3}$ mM) than known anti-toxic agents such as phosphine, amiton, etc. ($CA_{50}$ ~ 106-108 mM) [6].

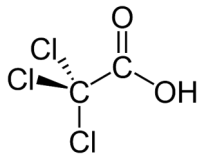
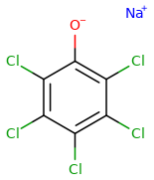
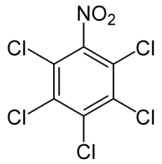
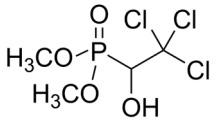
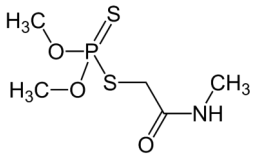
The detected inactivation of the external enzyme (i.e. acetylcholinesterase) suggests that the inhibitory effect of chlorine-containing pesticides is due to the damage of RBC membranes, which are associated with the enzymatic action of ACE [3].

These changes are caused either by the adsorption of the studied pesticides on RBC membranes, or by the inclusion of those substances into the lipoprotein structure of the RBC membrane [7].

One of the extreme manifestations of the modifying effect of chemical compounds on erythrocyte membranes, leading to disruption of cell integrity, is hemolysis. It was found that PCP-Na, chlorophos and rogor possess their own hemolytic activity in isotonic medium, and a treatment of red blood cells by THA-acid and PCNB does not lead to their hemolysis.

From fig 1 and table 2 it can be seen that the hemolytic effect naturally depends on the concentration of the pesticide: with an increase in concentration of the drug in the incubation medium, the hemolysis time t and half-life t50 decreases, and the rate of hemolysis (V) increases accordingly. The method of studying the quantitative

**Table 1 |** The effect of pesticides on the hemolytic activity of erythrocytes. ффективность пестицидов на гемолитическую активность эритроцитов.

| Chemical | Structural formula | $CA_{50}$ (mM) |
|---|---|---|
| TCl acid (trichloroacetic acid) |  | 120 |
| PCP-Na (sodium pentachlorophenolate) |  | 5·10$^{-1}$ |
| PCNB (pentachloronitrobenzene) |  | 2·10$^{-2}$ |
| Chlorophos (trichlorphon) |  | 5·10$^{-3}$ |
| Rogor |  | --- |

characteristics of hemolysis of red blood cells in the field of ultrasound allows to determine the lowest concentration of the drug that has hemolytic activity in each case.

It can be seen in figure 1 that the kinetic curves are S-shaped with a certain period of induction, and accordingly the rate of hemolysis depends on the concentration of the drug. With the increase in concentration of the pesticide in the incubation medium, the rate of hemolysis increases, and the induction period decreases. The obtained data indicate a strong modifying effect of PCP-Na and chlorophos on the membrane structure of red blood cells, leading to hemolysis of red blood cells.

The resistance of pesticide-treated erythrocytes to mechanical hemolysis under ultrasound was also studied using automatic registration of erythrocyte destruction kinetics in a spectrophotometer cell [2]. In control experiments, it was found that ethanol, used as a solvent for pesticides at a concentration of <0.1% by volume, does not have a noticeable hemolytic effect on erythrocyte cells.

For some pesticides and bactericides that cause hemolysis of erythrocytes in isotonic medium, it has also been shown that along with this, they stabilize erythrocyte membranes to hemolysis in hypotonic medium in certain concentrations (9 μm - 150 μm) [2,5]. It can be assumed that the modifying effect of pesticides on erythrocyte membranes also leads to change in their mechanical resistance to ultrasound. This is consistent with the data on the effect of surfactants on the mechanical stability of erythrocytes [3]. Table 2 presents quantitative indicators characterizing changes in the resistance of erythrocytes to ultrasound exposure under the influence of different concentrations of pesticides, indicating a change in the mechanical stability of cells in the presence of these chemical compounds.

It is also known that pesticides can affect the structure and biological activity of red blood cells. Erythrocytes are convenient model for studies on the damaging effect of various factors, including pesticides, on cell membranes, as

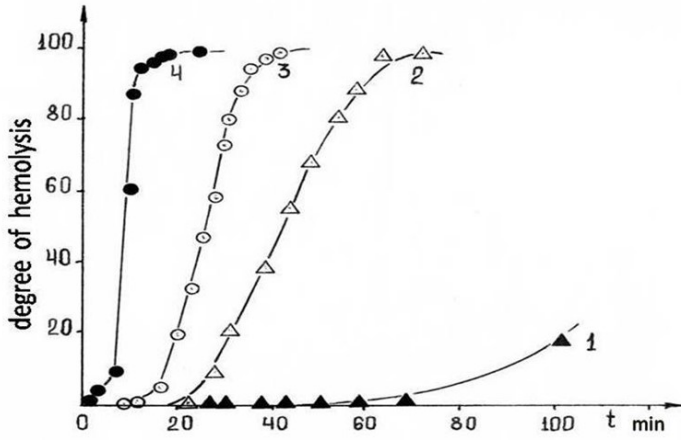



**Figure 1 |** The Hemolysis of red blood cells under the action of chlorine-containing pesticides, where curve 1is for TCl acid , curve 2 is for chlorophos, curve 3 is for PCP-Na, curve 4 is for rogor. On the abscissa axis there is hemolysis time (resistance) in minutes, on the ordinate axis there is light transmission in %.

**Table 2 |** The effect of pesticides of different concentration on the parameters of the ultrasound hemolysis and acetylcholinesterase activity in the estimated suspension of erythrocytes (the volume mode continuous, I = 0.4W / cm2, V=0.88 Mgs). Изучение влияния пестицидов различной концентрации на параметры УЗ гемолиза и активность АХЭ-азы в исследуемых суспензиях эритроцитов (режим воздействия УЗ непрерывный, I = 0,4 Вт / см2, V=0,88 МГц).

| Samples | Contentration [M] | t hem, c | V hem $c^{-1}$ | $C_{A\,50}$ (mM) |
|---|---|---|---|---|
| Testing | 0 | 500 ± 10 | 1,09 ± 0,03 | |
| PCNB | $10^{-4}$<br>$10^{-3}$ | 700 ± 22<br>1200 ± 150 | 0,60 ± 0,02<br>0,3 ± 0,1 | $2{\cdot}10^{-2}$ |
| TClA | (3-6)·10-3<br>(2-3)·10-2 | 420 ± 17<br>360 ± 19 | 1,12 ± 0,04<br>1,3 ± 0,1 | 120 |
| PCP Na | $10^{-6}$ -$10^{-5}$<br>$10^{-4}$-$10^{-3}$<br>$10^{-2}$ | 450 ± 27<br>415 ± 46<br>200 ± 3 | 1,2 ± 0,1<br>1,3± 0,1<br>4,0 ± 0,1 | $5{\cdot}10^{-1}$ |
| Heptachlor | $10^{-5}$<br>$10^{-4}$ | 565 ± 47<br>830 ± 43 | 0,97 ± 0,04<br>0,4± 0,1 | |
| Chlorophos | $10^{-3}$<br>$5{\cdot}10^{-3}$<br>$10^{-2}$ | 1550 ± 120<br>700 ± 80<br>250 ± 27 | 0,2 ± 0,2<br>0,6 ± 0,1<br>1,3 ± 0,1 | $5{\cdot}10^{-3}$ |

well as changes in other membranes [5]. ACE is an enzyme of the outer surface of erythrocyte membranes, brain cells, nerve tissue, etc. and it plays significant role in signal transmission through synapses since it catalyzes the hydrolysis of acetylcholine. ACE activity in blood and its components can serve as an additional diagnostic criterion for the analysis of some pathological conditions caused by toxic compounds. A number of studies have shown the dependence of hemolytic activity of some pesticides on the level of initial activity of cellular enzymes and the ability of the drug to reduce membrane fluidity [6, 7]. However, the kinetic characteristics of the action of a number of pesticides on erythrocyte hemolysis and their resistance to mechanical factors that characterize certain patterns of physiological activity of these compounds, have been previously studied, but insufficiently [8,9]. We have studied the kinetics and mechanisms of the influence of pesticides on ACE activity of red blood cells, namely, the change of erythrocyte resistance to ultrasound exposure in the presence of different concentrations of pesticides.

## 4. Concluding Remarks

Quantitative characteristics of the effect of chlorine-containing pesticides on the structural and functional activity of red blood cells were obtained. From the results presented in the table, it can be seen that the studied chlorine-containing drugs reduce the functional

(membrane) activity of red blood cells and change their mechanical hemolytic resistance to drugs and ultrasound, both individually and in combined action(see table 2.

The data obtained indicate that PCP Na, PCNB, heptachlor and Rohor are relatively weak inhibitors of the enzymatic activity of ACE in erythrocyte membranes, but have a pronounced structural-determinant effect, namely, they can cause hemolysis in an isotonic medium (Na PCP, Rohor, trichlorophon) and accelerate (Na PCP, THC, trichlorophon) or slow down (PCNB, heptachlor) the rate of hemolysis (see table 2). Therefore, quantitative indicators that characterize the ULTRASONIC hemolysis of red blood cells can be used as criteria for evaluating the membranotropic action of pesticides. At the same time, when pesticides and ultrasound are combined, their adsorption on the surface of the erythrocyte membrane is observed, which in turn leads to a slowdown in the hemolytic effect and, in turn, to a change in the qualitative and quantitative composition of membrane lipids [10]. The kinetic analysis method is the most effective for determining the mechanism of action of drugs on red blood cell membranes. This creates prerequisites for the search for new medicines for the human body and animals and the offer of new, more effective drugs for their use in agriculture and medicine.

## Заключение

Степень гемолиза и защитное действие некоторых поверхностно-активных веществ коррелируют с составом фосфолипидов в мембране эритроцитов; эти изменения могут также отражать нарушения ультраструктуры компонентов эритроцитов, содержащих гидролитические ферменты.

Получены количественные характеристики влияния хлорсодержащих пестицидов на структурно-функциональную активность эритроцитов. Из представленных результатов видно, что исследуемые хлорсодержащие препараты снижают функциональную (мембранную) активность эритроцитов и изменяют их механическую гемолитическую резистентность к лекарственным препаратам и ультразву, как индивидуально, так и в комбинации.

Полученные данные свидетельствуют о том, что ПХФ Na, ПХНБ, гептахлор и Рогор являются относительно слабыми ингибиторами ферментативной активности АПФ мембран эритроцитов, но обладают выраженным структурно-детерминантным действием, а именно могут вызывать гемолиз в изотонической среде (Na ПХФ, рогор, трихлорофон) и ускорять (NaCl ПХФNa, ТХУ-кислота, трихлорофон) или замедлять (ПХНБ, гептахлор) скорость гемолиза(см. таблицу 2). Поэтому количественные показатели, характеризующие УЗ гемолиз эритроцитов, могут быть использованы в качестве критериев оценки мембранотропного действия пестицидов.В то же время при совместном действии пестицидов и ультразвука наблюдается их адсорбция на поверхности мембраны эритроцитов, что в свою очередь приводит к замедлению гемолитического эффекта и к изменению качественного и количественного состава мембранных липидов[10]. Метод кинетического анализа является наиболее эффективным для определения механизма действия лекарственных средств на мембраны эритроцитов. Это создает предпосылки для поиска новых лекарственных средств для организма человека и животных и предложения новых, более эффективных препаратов для их применения в сельском хозяйстве и медицине.

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