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V. P. Rosalovsky

**HEMATOLOGICAL INDICES OF RATS
DURING THE FIRST HOUR AFTER CHLORPYRIFOS EXPOSURE***Institute of Animal Biology, National Academy Agrarian Sciences*

e-mail: ros.volodymyr@gmail.com

We studied the effects of acute chlorpyrifos intoxication on the erythrocytes resistance to acid hemolysis and certain hematological parameters (number of erythrocytes, leucocytes, monocytes, lymphocytes, granulocytes, and trombocytes, hemoglobin content, hematocrit, trombocrit, and platelet indices).

Acute exposure to 50 mg/kg CPF leads to a decrease in the resistance of main erythrocytes pool to acid hemolysis at 15 minutes after exposure. The number of leucocytes increased by 21,9%, of erythrocytes – by 9,0%, hemoglobin content – by 6,3%, compared to controls, at 15 minutes after exposure. The number of trombocytes decreased by 21,6% at 15 min, by 26,2% at 30 min, by 53,3% at 45 min, and by 56,0% at 60 min after exposure, compared to control means.

Key words: hematological parameters, chlorpyrifos, rats, intoxication.

В.П. Росаловський

**ГЕМАТОЛОГІЧНІ ПАРАМЕТРИ ЩУРІВ
ПРОТЯГОМ ПЕРШОЇ ГОДИНИ ПІСЛЯ ІНТОКСИКАЦІЇ ХЛОРПІРИФОСОМ***Інститут біології тварин НААН*

e-mail: ros.volodymyr@gmail.com

Фосфорорганічні сполуки (ФОС) у якості основних діючих агентів входять до складу великої кількості пестицидів, засобів побутової хімії, використовуються у хімічній промисловості, медицині (є компонентами препаратів що застосовуються для хіміотерапії туберкульозу і раку, міастенії, атонії кишок, лікування глаукоми). Висока токсичність та поширеність ФОС зумовлює значну небезпеку інтоксикацій цими речовинами. Хлорпірифос (ХПФ) є одним з найпоширеніших і найнебезпечніших для здоров'я людини представником цього класу сполук. ХПФ (О,О-Диетил-О-3,5,6-трихлор-2-піридилфосфотіоат ($C_9H_{11}Cl_3NO_3PS$)), відомий головним чином, як діюча речовина багатьох розповсюджених інсектицидів широкого спектру дії. Основний механізм токсичної дії ХПФ полягає в тому, що він інгібує холінестеразні ензими, спричиняючи, тим самим порушення синаптичної передачі.

Дослідження були проведені на 40 статевозрілих самцях білих лабораторних щурів лінії Вістар масою тіла 200–220 г. Було сформовано вісім груп тварин – чотири контрольних (K1, K2, K3, K4) і чотири дослідні (Д1, Д2, Д3, Д4) по 5 щурів у кожній. Декапітацію щурів за анестезії ефіром та відбір периферичної крові у тварин проводили через 15 (K1, Д1), 30 (K2, Д2), 45 (K3, Д3), 60 (K4, Д4) хв. після введення в їх організм хлорпірифосу. Відібрану кров розділяли на 2 частини. Для визначення гематологічних параметрів зразки крові вносили у пробірки (Terumo Europe N.V. (Бельгія)), які містили антикоагулянт ЕДТА-К2. Не пізніше ніж через 2 год. після взяття



кров аналізували на автоматичному гематологічному аналізаторі (Orphée Mythic 18, Швейцарія) за наступними показниками: кількість еритроцитів, лейкоцитів, лімфоцитів, моноцитів, гранулоцитів, тромбоцитів, вміст гемоглобіну, гематокрит, тромбокрит, середній тромбоцитарний об'єм і показник гетерогенності тромбоцитів за об'ємом. Дослідження резистентності еритроцитів до кислотного гемолізу проводили на еритроцитах відмитих загальноприйнятим методом. Стійкість еритроцитів до кислотного гемолізу визначали за методом Терскова і Гітельсона.

Встановлено, що гостра інтоксикація щурів ХПФ у дозі 50 мг/кг впродовж першої години після введення спричиняє: зростання кількості лейкоцитів на 29,1%, еритроцитів – на 9%, вмісту гемоглобіну – на 6,3% , порівняно до контролю через 15 хв після інтоксикації. зменшення кількості тромбоцитів через 15 хв- на 21,6%, через 30 хв – на 26,2%, через 45 хв – на 53,3% і через 60 хв – на 56,0% порівняно з контрольними значеннями; зниження резистентності основного пулу еритроцитів до кислотного гемолізу у групі E1.

Ключові слова: гематологічні показники, хлорпірифос, щурі, інтоксикація.

В. П. Росаловский.

ГЕМАТОЛОГИЧЕСКИЕ ПАРАМЕТРЫ КРЫС В ТЕЧЕНИЕ ПЕРВОГО ЧАСА ПОСЛЕ ИНТОКСИКАЦИИ ХЛОРПИРИФОСОМ

Институт биологии животных НААН

e-mail: ros.volodymyr@gmail.com

Было проведено исследование влияния острой интоксикации хлорпирифосом на резистентность эритроцитов к кислотному гемолизу и некоторые гематологические показатели (количество эритроцитов, лейкоцитов, моноцитов, лимфоцитов, гранулоцитов, тромбоцитов, содержание гемоглобина, гематокрит, тромбокрит и тромбоцитарные индексы).

Острая интоксикация крыс ХПФ в дозе 50 мг/кг вызвала снижение резистентности основного пула эритроцитов к кислотному гемолизу через 15 минут после введения. Был зафиксирован рост количества лейкоцитов на 29,1%, эритроцитов - на 9,0%, содержания гемоглобина - на 6,3%, по сравнению с контролем, через 15 мин после интоксикации. Количество тромбоцитов снизилось на 21,6% через 15 мин, на 26,2% через 30 мин, на 53,3% через 45 мин и на 56,0% через 60 мин после введения, по сравнению с контрольными значениями.

Ключевые слова: гематологические показатели, хлорпирифос, крысы, интоксикация.

INTRODUCTION

Organophosphates (OPs) are the main active substances in many agriculture pesticides and household chemicals; they are also used in the chemical industry, medicine (tuberculosis and cancer chemotherapy preparations, drugs against myasthenia gravis, gastrointestinal atony, and glaucoma). Chlorpyrifos (CPF) is one of the most common and most dangerous of these compounds. CPF (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate ($C_9H_{11}Cl_3NO_3PS$)) is known as the active

ingredient of many common broad-spectrum insecticides used in agriculture to protect vineyards, vegetables, citrus fruits and cereals from pests, and, that is particularly dangerous, to control insects in homes (Needham, 2005, Salyha, 2010a).

The main mechanism of CPF toxicity is inhibition of cholinesterase enzymes, causing disruption of synaptic transmission. Till recently, this was considered as a key and almost only toxicity mechanism of CPF, and all OPs in general, but the latest studies have convincingly shown that the toxic effect of OPs is more complex and is not limited to anticholinergic action. Besides cholinesterases, other potential molecular targets of OPs were found (Eaton, 2008; Salyha, 2010a; Salyha, 2010b; Abmali, 2011; Salyha, 2013). The free radical oxidation, a universal pathophysiological phenomenon in many pathological conditions, also plays a significant role in reaction to the toxic effects of various xenobiotics, including OPs and CPF in particular (Salyha, 2010b; Elsharkawy, 2013). Undoubtedly, in case of intoxication, blood is affected as well as other tissues, as haematological parameters are important markers of physiologic conditions of the body. Blood is a key homeostatic system of the organism (Savithri, 2010; Khaybullina, 2012; Salyha, 2013). Study of the blood system in context of homeostatic adaptive reactions to the toxic exposures is essential for understanding the pathologic mechanisms at cellular and tissue levels.

Under the effect of different compounds, particularly poisons, the nonspecific signs of alterations in peripheral blood mainly include changes in the total white blood cells number (mild leukocytosis) and leukocyte formula (lymphopenia, neutrophilia, sometimes with a left shift, eosinopenia or eosinophilia). In their turn, alterations in red blood are less prominent and are manifested mainly by a slight decrease in hemoglobin and erythrocytes number. Chronic exposure may lead to erythrocytosis with normal hemoglobin, leucopenia with left shift of the leucocytes formula, toxic granularity of neutrophils, and reduction of erythrocyte sedimentation rate (Khaybullina, 2012). Nevertheless, there is a relatively high variability of quantitative hemato-cytological parameters, depending on physicochemical features of the toxic factor, duration of its action, dose, intake route and so forth (Eaton, 2008; Savithri, 2010).

It is important that CPF poisoning (especially acute), besides other effects, can cause hypoxia (Salyha, 2010a). The performance stability of erythrocytes is often used in experimental medicine as a way to assess their functional state (Sybirna, 2010; Ambali, 2010). Unfortunately, the few available literature data on haematological parameters of animals under CPF action are often incomplete and even contradictory (Savithri, 2010). Therefore, the aim of this study was to investigate changes in basic hematological parameters and *hemolysis resistance* of the *erythrocyte membrane* at 15, 30, 45, and 60 min after CPF exposure to rats.



MATERIALS AND METHODS

Forty adult male white Wistar rats weighing between 200 and 220g served as subjects for this study. Rats were housed under standardized laboratory conditions, with 12h dark/light cycle and free access to food and tap water ad libitum. All procedures were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and General Ethical Principles of Experiments using Animals (First National Congress of Bioethics, Kyiv, 2001).

The animals were randomly divided into eight groups: four control (C1, C2, C3, C4) and four experimental (E1, E2, E3, E4) groups, each comprising of five animals. All treatments were administered at a dose of 50 mg/kg. Administration of CPF was performed via oral gavage. Chlorpyrifos was diluted in sunflower oil. The control groups received the equivalent volume of pure oil.

At the end of the test period, the rats were sacrificed by decapitation after light ether anesthesia (at 15 min (groups C1 and E1), 30 min (C2 and E2), 45 min (C3 and E3), and 60 (C4 and E4) min after dosing, to obtain samples of peripheral blood.

The blood samples were collected into test tubes (*Terumo Europe N.V.*, Belgium) with anticoagulant, K2-EDTA. Not later than 2 hours after sampling, blood was analyzed with the automatic hematological analyzer (*Orphée Mythic 18*, Switzerland) to examine for total red blood cells (RBC), white blood cells (WBC), lymphocytes, monocytes, granulocytes, platelets, haemoglobin (Hb) concentration, hematocrit, plateletcrit, mean platelet volume and platelet volume heterogeneity index.

Erythrocytes resistance to acid hemolysis was assessed by Terskov-Hitelson (Gitel'zon, 1959): photometric measurement of the decrease in the red blood cells number caused by adding 0.002N HCL in saline. The red blood cells were counted at regular intervals (30 sec).

The experimental data were processed by using the *OriginPro 8* software. To compare between the data of the control and those of treatments, Student t-test was used. Values obtained were expressed as Mean \pm SD, Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Analyzing the hematological parameters of rat peripheral blood during the first hour after exposure to CPF, we found significant changes in the number of blood particles and in the acid hemolysis resistance of red blood cells. Below we present the CPF effects on parameters of blood particles number.

After the exposure, significant changes occurred in the total number of white blood cells. So, in the blood of group E1, i.e. at 15 min after CPF exposure, was observed a significant increase (by approximately 29,1%) in the number of white blood cells ($p < 0,05$), compared with controls (Table 1).

Table 1. Haemogram of control and chlorpyrifos treated rats

Parameters	C1	E1	C2	E2	C3	E3	C4	E4
RBC, $10^{12} L^{-1}$	7.5±0.20	8.1±0.30*	7.2±1.1	7.1±0.3	7.3±1.9	6.3±0.1	7.9±2.7	5.9±0.8
HB gL ⁻¹	138.5±3.5	154.1±4.9*	137.5±3.3	129.2±4.1	140.1±4.4	130.5±3.4	136.3±2.6	135.3±1.7
PCV (%)	41.5±1.0	44.1±0.9*	43.2±1.3	42.3±1.7	40.3±2.1	39.7±2.0	41.9±1.8	38.5±1.5
MCV fl	55.5±3.6	54.2±2.6	53.1±4.9	59.5±3.5	54.0±4.3	63.5±5.2	56.3±3.1	65.5±4.9
WBC $10^9 L^{-1}$	12.7±0.8	16.4±1.5*	13.5±0.9	12.1±1.6	12.1±1.4	9.5±0.6*	11.3±1.2	8.0±0.9*
Lymphocytes $10^9 L^{-1}$	9.6±1.7	11.3±2.4	9.7±1.8	9.1±1.1	8.8±1.4	6.9±1.5	8.1±1.3	6.0±1.4
Granulocytes $10^9 L^{-1}$	2.7±0.6	4.6±0.3*	3.5±1.0	2.6±0.2	3.1±0.6	2.3±0.5	3.0±0.6	1.6±0.7
Monocytes $10^9 L^{-1}$	0.4±0.1	0.6±0.1	0.3±0.1	0.4±0.2	0.2±0.1	0.2±0.1	0.2±0.4	0.3±0.1
Thrombocytes $10^9 L^{-1}$	572.3±32.9	448.9±28.5*	544.5±41.1	401.8±40.3*	597.2±58.9	278.7±31.4*	521.9±46.7	229.5±35.1*
Mean Platelet Volume, MPV fl	6.7±0.1	6.6±0.1	6.9±0.3	6.8±0.2	6.7±0.2	6.9±0.2	6.6±0.2	6.5±0.1
Platelet volume distribution PDW %	25.4±4.1	19.4±3.8	22.2±5.8	21.6±3.4	24.58±3.1	15.4±5.5	23.5±4.8	20.6±2.3

* - $p < 0.05$ significant differences from the control



On the other hand, at other studied time points, leukocyte count showed reversal dynamics: it tended to decrease by 10.37% in E2, and in the E3 and E4 groups it decreased significantly by 21,9% and 28,9% ($p < 0,05$), respectively, compared to control values. Moreover, E1 group showed a significant increase in the number of granulocytes by 67,4% ($p < 0,05$), compared with controls. No significant change in the number of monocytes, lymphocytes and granulocytes in groups E2, E3, E4 was observed. Increase of white blood cells in E1 may be related to their release from lymphoid-myeloid complex into blood to neutralize CPF-induced damage. The gradual decrease in E2, E3, and E4 may be related to the decrease in the oxidative stress-caused damage and completion of the disposal of damaged blood cellular components. These changes in characteristics of the white blood resonate with studies of other authors (Hissin, 1976), claiming that the number of white blood cells can change under the influence of organophosphorous pesticides. It is also reported that changes in the leukocyte number can be caused by CPF poisoning and depend on the severity of caused damage (Elsharkawy, 2013).

The number of platelets decreased significantly in all experimental groups, compared with intact animals. The intensity of these changes increased with time: the total platelet count decreased by 21.6% in group E1, by 26.2% in E2, by 53.3% in E3, and by 56.0% in E4, compared with control values. However, the rate of platelet volume distribution and average volume of platelets did not show any significant changes. The decrease in the number of thrombocytes can be caused by the formation of antigen-antibody complexes with platelet surface antigens, or by the changes in platelet membrane permeability, caused by oxidative stress (see, for instance, Sybirna, 2010; Ambali, 2011; Elsharkawy, 2013). It is known that changes in the platelet number can serve as an early sign of various intoxications.

Significant increase in number of red blood cells (by almost 9%, compared to the control group) was found only in group E1, i.e. 15 min after intoxication. We also observed here a significant increase in hemoglobin by 11% ($p < 0,05$), hematocrit by 6,3% ($p < 0,05$), compared to control. Such changes could be caused by hypoxia that occurs in the first few minutes after acute CPF poisoning, a powerful initiator of erythropoietin's synthesis and increased release of erythrocytes to the bloodstream from the depot, that initiate increasing hemoglobin content.. Within the same time intervals, erythrocyte count, total hemoglobin, the average volume of red blood cells showed no significant changes, compared to control.

Toxic substances affect the blood system and hematopoiesis in many ways: besides the bone marrow damage and abnormal hemoglobin transformation, they can also alter the degree of hemolysis of red blood cells. Violation of the erythrocyte membrane integrity, changes in surface properties of the lipid bilayer and protein conformation under the influence of toxic substances change the functional ability of erythrocytes to bind different compounds (Dudok, 2009; Ferents, 2014). Acid

erythrograms (Fig. 1) show the negative impact of CPF on the stability of erythrocyte membranes.

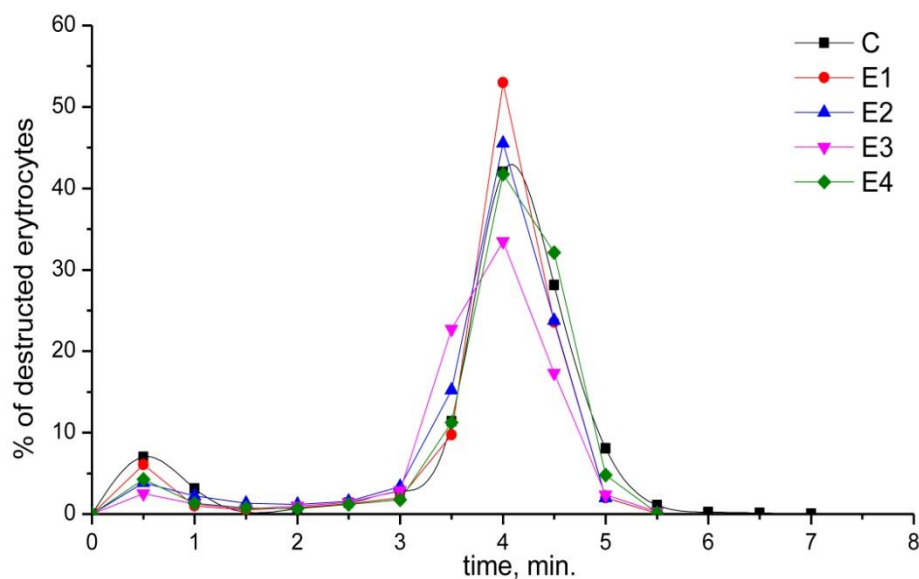


Fig. 1. Erythrograms of acid hemolysis and parameters of erythrograms of intact and exposed animals (1h after chlorpyrifos dosing)

In group E1, 15 min after CPF exposure, we observed decreased resistance to acid hemolytic of the main pool of red blood cells, showed by a significant increase in the ratio of hemolyzed red blood cells. In this group, total erythrocyte hemolysis occurred faster than in controls. At 30 min after intoxication (group E2), maximum red blood cells hemolysis percentage slightly decreased, compared with group E1, and a total destruction of red blood cells left unaltered. In Group E3 (45 minutes after experiment beginning) the percentage of damaged red blood cells significantly decreased up to 33.5%, compared to control ($p < 0.05$). However, the increased area under the hemolysis curve of both right and left inflexions indicates the emergence of erythrocytes fraction with increased resistance to acid hemolytic (Fig. 1). After 60 min after CPF exposure, in group E4 the number of red blood cells destroyed by hemolytic approached the control values. In the peripheral blood of rats, the appearance of erythrocytes that are more resistant to acid hemolytic can be explained by substitution of the pool of destroyed erythrocytes with cells that are more resistant to hemolysis.



Acid erythrogram method helps to assess the condition of hydrophobic and protein components of erythrocyte membranes and to form morphologically homogeneous groups of erythrocytes by their age. The most resistant to hemolytic are the young red blood cells that are situated on the right side of the erythrogram. Aging of the erythrocytes is accompanied by a gradual decrease in their resistance, manifested in shifting the curve to the left.

CONCLUSIONS

An *acute exposure* to chlorpyrifos (50 mg/kg), 15 min *after dosing*) caused the increase in the white blood cells number by 29.1 percent, increase in red blood cells number by 9 percent, and increase in haemoglobin by 6.3 percent compared with control.

We also registered a decrease in the platelet number by 21.6 percent (15 min after dosing, group E1), by 26.2 percent (after 30 min, E2), by 53.3 percent (after 45 min, E3), and by 56.0 percent (after 60 minutes, E4), compared with control values. We registered decreased acid hemolysis resistance of the main pool of red blood cells in group E1 15 min after intoxication);

In conclusion, significant adverse changes in hematological parameters are reported to be associated with exposure to CPF, in this present study. This therefore suggest that exposure to CPF may be considered to be among the risk factors for the development of anaemic condition. Hence, exposure to this drug should be minimized.

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