BIOCHEMICAL AND HAEMATOLOGICAL CHANGES IN PERIPHERAL BLOOD OF RATS EXPOSED TO CHLORPYRIFOS: PROTECTIVE EFFECT OF VITAMINS A AND E COMBINATION

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Chlorpyrifos is a highly toxic organophosphate compound. It is still among the most widely used insecticide, and the main mechanism of its toxicity is associated with inhibition of cholinesterases. Along with the anticholinesterase action, CPF may affect other biochemical mechanisms, particularly through disrupting pro- and antioxidant balance and inducing free-radical oxidative stress.

We studied the action of A and E vitamins on the basic haematological and biochemical parameters of rat peripheral blood after 12 hours of a single chlorpyrifos intoxication. Exposure to 70 mg/kg chlorpyrifos caused a decrease in the total number of red blood cells (RBCs), platelets, and total haemoglobin content. We also observed a decrease in the acid haemolysis resistance of RBCs in peripheral blood of CPF-poisoned rats. Combined exposure to chlorpyrifos and vitamins A and E caused changes in haemolysis resistance of RBCs, approaching the control values. In addition, it was found that chlorpyrifos intoxication disrupt prooxidant-antioxidant balance as evidenced by the increase of lipid peroxidation products: lipid hydroperoxides and thiobarbituric acid reactive substances. However, administration of vitamins A and E during intoxication provided levelling effect on the formation of lipid peroxidation products. CPF intoxication caused an increase of the catalase activity, while superoxide dismutase, glutathione peroxidase and glutathione reductase activities and the content of reduced glutathione decreased. It was revealed that combination of vitamins A and E cause corrective effect at the platelets quantity, and lipid hydroperoxides thiobarbituric acid reactive substances amount of rat peripheral blood.

Keywords: chlorpyrifos, vitamin E, vitamin A, intoxication, rats.

INTRODUCTION

Organophosphorous compounds (OPs) belong to a group of phosphonic or phosphoric acid derivatives, widely used in agriculture, primarily as components of insecticides, defoliants and means against animal ectoparasites. The most frequent causes of acute OP poisonings are violation of personnel safety regulations, accidental intake, and suicidal attempts [13].
The main mechanism of OP toxicity is cholinesterase (ChE) inhibition in plasma and erythrocytes, caused by phosphorylation of serine hydroxyl residue in the enzyme molecule. Resulting accumulation of non-hydrolyzed acetylcholine leads to violations in neuromuscular synaptic transfer and respiratory depression [2, 22].

A typical representative of OPs is Chlorpyrifos (CPF) \((\text{C}_9\text{H}_{11}\text{Cl}_3\text{NO}_3\text{PS})\), both in pure form and in combinations with other substances, is widely used in agriculture, industrial and home disinfection, to control harmful and synanthropic insects [9, 24, 28].

Numerous studies indicate neuro-, hepato- and cytotoxic effects of CPF [2, 8, 20-21, 23]. There is evidence that CPF intoxication leads to disruption of pro- and antioxidant balance and causes formation of reactive oxygen species (ROS). The latter have high reactivity and can modify the biological structure of blood cells, including erythrocytes, impairing their resistance to damage and membrane integrity [6, 22].

Red blood cells transport oxygen and carbon dioxide between lungs and tissues. They can be subjected to oxidative damage due to the high oxygen tension, high content of polyunsaturated fatty acids (PUFAs) in plasma membranes. Moreover, due to hemoglobin oxidation, ferrum valence changes, and the emitted electron can modify the oxygen molecule \((\text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + e^-)\). However, RBCs have an antioxidant defence system, which includes enzymatic components superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPO), glutathione reductase (GR) and non-enzymatic ROS-scavengers: reduced glutathione (GSH) and vitamins A and E [2].

Due to the double bonds, vitamin A is involved in the regulation of redox reactions. Another fat-soluble antioxidant is vitamin E. Its action is aimed to activate tissue respiration and keep the steady level of free radical peroxidation. Tocopherol reacts with ROS, producing inactive compounds and disrupting the ROS formation chain; it protects PUFAs of the cell membrane. It is known that vitamin E increases the biological activity of vitamin A, preventing oxidation of its unsaturated side chain [1,2].

According to existing data, the consequences of OP intoxication include changes in the activity of antioxidant system enzymes and initiation of lipid peroxidation (LPO) [22].

There are data on the use of vitamins C, E, A in the OP intoxications treatment [10, 24]. However, these works relate to antioxidant defence system, while the physiological parameters of blood components are studied rather poorly [2].

The aim of this work was to study the main haematological and biochemical parameters of rat peripheral blood after 12 hours of a single CPF exposure and in combination with vitamins A and E.

**MATERIALS AND METHODS**

The study was conducted on 20 adult male Wistar rats of 180-220 g body weight. The animals were kept in standard vivarium conditions, with 12-hour dark/light mode and unlimited access to water and lab food. All manipulations with animals were carried out according to the European Convention “On protection of vertebrate animals used for experimental and scientific purposes” (Strasbourg, 1986) and “General ethical principles of experiments on animals” (First National Congress on Bioethics, Kyiv, 2001).

Animals were divided into four groups: 1 control (C) and 3 experimental (E1, E2, E3) groups of 5 rats each. Animals in group E1 were exposed to 70 mg/kg chlorpyrifos in oil solution; E2 group animals received 0.055 g vitamin A (in the retinol palmitate form) and 0.1 g vitamin E (alpha tocopherol in the acetate form); animals from E3 group were exposed to both CPF solution (70 mg/kg) and vitamins A (0.055 g) and E (0.1 g).
animals of groups E2 and E4 were administered of vit. A and E solution 5 minutes later after CPF exposure. All exposures were conducted intragastrically via oral gavage. The control group received a corresponding amount of pure oil. After 12 hours of CPF, animals were euthanized with ether anaesthesia and decapitated and peripheral blood was collected.

For study of haematological parameters, blood samples were put in test tubes (Terumo Europe NV (Belgium)) containing as anticoagulant EDTA-K$_2$. No later than two hours after sampling, blood was studied in automatic haematological analyzer (Orphee Mythic 18, Switzerland). The following parameters were studied: the number of RBCs, white blood cells, lymphocytes, monocytes, granulocytes, platelets, haemoglobin concentration, haematocrit, and mean platelet volume and platelet volume heterogeneity index. RBC resistance to acid haemolysis was determined by Terskov and Gitel’zon [7].

For biochemical studies, heparinized blood samples were centrifuged for 15 minutes at 1,500 g. After plasma separation, erythrocytes were washed three times with 0.15 M NaCl solution. Haemolysates were obtained by three times freezing-thawing of aqueous suspensions of RBCs and their subsequent centrifugation at 10,700 g for 15 min.

Cholinesterase (ChE) (EC 3.1.1.8) activity in plasma was determined by Karpysh-tshenko [12], using the commercial kit by “Filisit-Diagnostics” (Ukraine). Optical absorbance was measured spectrophotometrically at 540 nm against distilled water.

Superoxide dismutase (SOD) (EC 1.1.15.1.) activity was determined by Dubinina et al. [5], based on the reduction of nitroblue tetrazolium to nitroformazan by superoxide anion, that are formed in the reaction between phenazine methosulphate and NADPH. SOD activity was expressed in arbitrary units per 1 mg protein.

Catalase (CAT) (EC 1.11.1.6) activity in the haemolysates was determined by Koroliuk [15] with modifications. The method is based on the ability of hydrogen peroxide to form a stable colored complex with molybden salts. Enzyme activity was expressed in mmol H$_2$O$_2$/min per 1 mg protein using a molar absorption coefficient 22200 M$^{-1}$cm$^{-1}$.

Glutathione peroxidase (GPO) (EC 1.11.1.9) activity was determined by the rate of GSH oxidation before and after incubation with tertiary butyl hydroperoxide. This colour reaction is based on the interaction between SH-groups with the 5,5’-dithiobis(2-nitrobenzoic acid) (DTNBA), resulting in a colored product, thionitrophenyl anion [18]. Quantity of the thionitrophenyl anion is directly proportional to the number of SH-groups reacted with DTNBA. Enzyme activity was expressed in μmol GSH/min per 1 mg protein.

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined by Carlberg [3]. This method is based on the catalytic NADPH-dependent reduction of oxidized glutathione, the intensity of which can be measured by the rate of extinction decline on the wavelength of NADPH maximum absorption 340 nm. Calculation of GR activity was carried out using molar absorption coefficient for NADPH 6200 M$^{-1}$ cm$^{-1}$. The activity of the enzyme was expressed in μmol NADPH/min per 1 mg of protein.

The concentration of reduced glutathione (GSH) was measured spectrophotometrically before and after the reaction, by Hissin [11]. The colour reaction is based on the interaction between SH-groups of GSH and DTNBA. The GSH content was calculated with the calibration curve.

The content of lipid hydroperoxides (LP) in erythrocyte mass was determined by [27], based on spectrophotometric measurement of optical density of products formed in the reaction between ammonium thiocyanate, Mohr’s salt and hydrochloric acid.
The concentration of thiobarbituric acid reactive substances (TBARS), characterizing the LPO rate, was determined by Korobeinikova, based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), in conditions of high temperature and acidic environment, with formation of a colored trimethyl complex consisting of one MDA and two TBA molecules [14].

Protein concentration was determined by Lowry method [16]. All reagents used were obtained from Sigma-Aldrich and Fluka (USA). Obtained data were analyzed statistically with Student’s t-test, using the program OriginPro 8. Data considered statistically significant at p <0.05.

RESULTS AND DISCUSSION

The basic integral marker of OP intoxication is a decrease in cholinesterase activity. The phosphorylation of cholinesterase leads to loss of its ability to hydrolyze acetylcholine. Because of this, we studied cholinesterase activity in blood plasma of all groups of animals (Fig. 1).

![Fig. 1. A blood plasma ChE activity of rats groups C, E1, E2, E3](image)

Here and later: * – p<0.05 significant difference compared to control group; # – p<0.05 significant difference compared to E1 group

The results indicate that CPF intoxication caused a decrease of ChE activity by 24.5% in the blood of group E1. Changes in the ChE activity are the primary characteristics of OP intoxication, as confirmed by the literature [17]. Notably, in the blood plasma of E3 animals, ChE activity decreased only by 14 %, comparing with the intact animals. No significant differences in ChE activity were found between E1 and E3 groups.

On the next research stage, we studied the haematological parameters of peripheral blood. The results are presented in Table 1.

At 12th hour after CPF exposure, a significant decrease in platelet count was observed in the peripheral blood of E1 group rats, which are consistent with results of our previous studies [19]. In addition, in this group was found a significant decrease in the number of RBCs (by 14 %) and haemoglobin content (by 8 %), compared with control values. According to S. F. Ambali, this decrease may be caused by the formation of cross-links between proteins and lipids of the cell membrane and inactivation of enzymes located in the cell. By-products of LPO can cause violations in composition and structure of membranes, extrude essential fatty acids, and inactivate membrane-bound enzymes. [2].
Table 1. Peripheral blood parameters in C, E1, E2, E3 groups of rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells, $10^{12}$/l</td>
<td>8.18±0.27</td>
<td>7.08±0.32</td>
<td>7.58±0.25</td>
<td>7.25±0.31</td>
</tr>
<tr>
<td>Total haemoglobin, g/l</td>
<td>153.8±3.37</td>
<td>141.5±4.05</td>
<td>149.3±5.32</td>
<td>142.8±5.8</td>
</tr>
<tr>
<td>Haematocrit, (HCT) l/l</td>
<td>0.44±0.03</td>
<td>0.39±0.04</td>
<td>0.4±0.03</td>
<td>0.39±0.13</td>
</tr>
<tr>
<td>Mean cell volume, (MCV) fl</td>
<td>54.54±1.66</td>
<td>55.07±2.65</td>
<td>53.36±2.18</td>
<td>56.08±2.48</td>
</tr>
<tr>
<td>Leucocytes, $10^9$/l</td>
<td>11.06±2.2</td>
<td>7.7±1.16</td>
<td>5.42±2.02</td>
<td>5.56±1.01</td>
</tr>
<tr>
<td>Lymphocytes, $10^9$/l</td>
<td>8.06±1.41</td>
<td>5.92±2.43</td>
<td>5.38±2.53</td>
<td>8.48±2.08</td>
</tr>
<tr>
<td>Granulocytes, $10^9$/l</td>
<td>1.12±0.7</td>
<td>0.62±0.29</td>
<td>0.2±0.07</td>
<td>0.44±0.21</td>
</tr>
<tr>
<td>Monocytes, $10^9$/l</td>
<td>1.9±0.25</td>
<td>1.15±0.56</td>
<td>0.92±0.44</td>
<td>1.44±0.32</td>
</tr>
<tr>
<td>Platelets, $10^9$/l</td>
<td>425.8±23.7</td>
<td>314.2±37.6</td>
<td>307.8±45.5</td>
<td>468.8±29.5</td>
</tr>
<tr>
<td>Mean platelet volume, (MPV) fl</td>
<td>6.6±0.33</td>
<td>7.5±0.85</td>
<td>7.4±1.02</td>
<td>7.3±0.99</td>
</tr>
<tr>
<td>Platelet distribution width, (PDW) %</td>
<td>20.02±3.47</td>
<td>17.72±4.4</td>
<td>22.98±8.27</td>
<td>16.22±3.36</td>
</tr>
</tbody>
</table>

Comments: Here and later: * – p<0.05 significant difference compared to control group; # – p<0.05 significant difference compared to E1 group.

We did not find any significant changes in the total amount of leukocytes, lymphocytes, granulocytes, monocytes in E1, E2, E3 groups, compared to control. In the E2 group, the platelet number slightly decreased, but this decrease was not statistically significant. In the E3 group was observed a significant increase in the total platelet number, compared to E1 group. It is possible, that increase total quantity of platelets may be associated with antioxidant properties of both vitamins. Being well known antioxidants, vitamin E and A prevent the free-radical mediated damage of the platelets [1, 2].

Moreover, vitamin E influences the cellular response to oxidative stress through modulation of signal-transduction pathways. According to S. F. Ambali [2], these pathways may provide the cellular regulation level. Lipid peroxidation can cause changes in rheological properties of erythrocyte membranes and membrane potential, increase their permeability for different ions that can initiate the process of haemolysis, and thus reduce the lifespan of erythrocytes [1].

To assess the stability of erythrocyte membranes, we studied their resistance to acid haemolysis (Fig. 2). The results are presented in Tab. 2.

Studying the acid haemolysis resistance of erythrocytes, we found significant changes in the stability of erythrocyte membranes (Table 2).

We observed a significant increase in the percentage of maximum haemolysis in all experimental groups. Therefore, it increased significantly to 33.8 % in the E1 group, to 39% in the group E2, and to 33 % in E3, compared to control. We found no significant differences between E1 and E3 groups in the percentage of maximum haemolysis.
It is known that CPF is able to generate ROS [2, 22]. The newly formed ROS can increase, directly or indirectly, the passive permeability of membranes to potassium and sodium ions and cause violations in the erythrocyte osmotic balance, thus reducing the lifespan of these cells [6, 25].

The level of LPO, both primary LP (lipid hydroperoxides) and secondary (TBARS), is a feature of ROS generation. The results of our study show that in group E1 the amount of LP in erythrocyte haemolysate significantly increased by 40 %, compared to control (Fig. 3, I).

At the same time, the number of primary lipid peroxidation products significantly (p<0.05) decreased (32 %) in the erythrocyte haemolysates of the E2 group, compared with the ones of intact animals. In the group E3, exposed to the combination of vitamin mixture and CPF, we observed more slight decrease in the number of LP than in E2: by 15%, compared with control. At the E3 group amount of primary lipid peroxidation products significantly decreased by 39% compared with E1 group.

Changes in the content of TBARS were also found in the experimental groups (Fig. 3, II). So, the content of TBARS significantly increased in E1 (by 80 %) and E3 (by 49 %) groups, compared with control values. According to I. Amara and S. F. Ambali, the increase of lipid peroxidation products content shows the accumulation of hydrogen peroxide, nitrites, nitrates and other compounds, the amount of which exceeds the one that can be disposed in detoxification processes. These products can cause oxidative
damage to membranes through interaction with polyunsaturated fatty acids (PUFAs) and haeme iron in the erythrocytes [1, 2].

The catalase (CAT) and superoxide dismutase (SOD) activities are important indicators of the functional state of the erythrocyte antioxidant system. It is known that CAT is involved in utilization of hydrogen peroxide produced in the process of biological oxidation, decomposing it into water and molecular oxygen. In the presence of H₂O₂, it oxidizes low molecular alcohols and nitrites. SOD performs dismutation of superoxide radical into molecular oxygen or hydrogen peroxide [4].

We found a significant increase in CAT activity (by 34 %) in haemolysates of RBCs of E1 group, compared with the control (Fig. 4, I). Instead, SOD activity decreased significantly in erythrocytes haemolysate in groups E2 and E3, by 66 and 32 %, respectively (Fig. 4, II).

The glutathione system which includes glutathione and glutathione-dependent enzymes, has one of the leading roles in the utilization of ROS and the redox potential stability. This is due to synergism of glutathione-dependent enzymes, their participation...
in regeneration of some low-molecular antioxidants and the ability of glutathione to neutralize active oxygen intermediates through direct interaction [22]. We studied the content of GSH, GPO, and GR after 12 hours of CPF intoxication (Fig. 5).

![Graphs showing GSH, GPO, and GR activities](image)

**Fig. 5.** GSH (I) content, GPO (II) and GR (III) activities in the red blood cells of rats groups С, E1, E2, E3

The content of GSH decreased by 15 % in the E1 group, while in the group E3 it increased by 9 %, compared to the control. Data in this study also show that GSH content in the group E3 increased by 27 % if to compare with E1 group.

Activity of GPO in RBC haemolysates of E1 and E3 groups significantly decreased (by 70 % and 60 %, respectively), compared to the control values. GR activity decreased by 40 % in E1 and by 46 % in E2, compared with the control. Treatment of CPF-intoxicated rats with vit A and E significantly increased GR activity by 33 % in group E3 if to compare with E1 group.

The reduction-oxidation cycle of glutathione is involved in regulation of oxidative stress, when the stress level is low [1]. The decrease of GSH content in erythrocyte haemolysates of E1 group is obviously connected with its consumption to maintain redox balance, particularly, when CPF affects mitochondrial metabolism [26]. Instead, higher glutathione levels under conditions of the correction of the toxic CPF effects by the vitamin mixture may indicates a decrease in the intensity of LPO, and perhaps even normalization of oxidative processes in the structures of rat erythrocytes.
CONCLUSIONS

We found that acute exposure to 70 mg/kg CPF led to changes in haematological parameters: a decrease in the number of red blood cells, platelets, and total haemoglobin. After CPF exposure, a significant decrease in platelet count was observed in the peripheral blood of E1 group rats. In the E3 group we observed a significant increase in the total platelet number, compared to E1 group. We found slight corrective effect of vitamins A and E at the total number of platelets.

Besides, a decreased acidic haemolysis resistance in erythrocytes of CPF-intoxicated rats was found. The studied mixture of vitamins A and E showed a slight protective effect on these haematological parameters. The toxicant caused some biochemical changes, e.g. increase in the number of TBARS and lipid hydroperoxides content in the RBCs of rats after 12 hours of exposure. We found activation of LPO processes due to CPF intoxication and decrease in their intensity under the effect of vitamins A and E. We also found an increase in CAT activity, and decreased SOD, GPO, GR activities and GSH content.

In conclusion, we consider that the correction of oxidative stress by vitamins had a positive effect on the biochemical parameters of rats.


ЗМІНИ БІОХІМІЧНИХ І ГЕМАТОЛОГІЧНИХ ПОКАЗНИКІВ ПЕРИФЕРИЧНОЇ КРОВІ ЩУРІВ ЗА ДІЇ ХЛОРПІРИФОСУ: ЗАХИСНИЙ ВПЛИВ ВІТАМІНІВ А ТА Е

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Хлорпірифос є високотоксичною фосфорорганічною сполукою. Він залишається одним з найбільш поширенних інсектицидів. Основний механізм його токсичності пов’язаний з інгібуванням холінестерази. Поряд із антихолінестеразними механізмами хлорпірифос може чинити вплив через інші біохімічні механізми, зокрема, через порушення про- і антиоксидантний баланс викликаючи генерацію вільних радикалів та спричиняти оксидативний стрес.

Ми вивчили дію вітамінів А і Е на основні гематологічні та біохімічні параметри периферичної крові щурів через годин 12 після одноразової інтоксикації хлорпірифосом. Введення хлорпірифосу у дозі 70 мг/кг спричиняло зниження загальної кількості еритроцитів, тромбоцитів і загального вмісту гемоглобіну. Ми виявили зниження стійкості еритроцитів периферичної крові до кислотного гемолізу у щурів інтоксикованих хлорпірифосом. Отруєння хлорпірифосом спричиняло зростання кількості продуктів перекисного окислення ліпідів: гідроперекисів ліпідів і ТБК-активних продуктів.

Введення вітамінів А і Е при інтоксикації спричиняє нівелюючий ефект на формування продуктів перекисного окислення ліпідів. У разі інтоксикації хлорпірифосом активність каталази зростала, тоді як активність супероксиддисмутази, глутатіонпероксидази, глутатіонредуктази і вміст відновленого глутатіону знижувалась.

Ключові слова: хлорпірифос, вітамін Е, вітамін А, інтоксикація, щурі.

ИЗМЕНЕНИЯ БИОХИМИЧЕСКИХ И ГЕМАТОЛОГИЧЕСКИХ ПОКАЗАТЕЛЕЙ ПЕРИФЕРИЧЕСКОЙ КРОВИ КРЫС ПОД ВОЗДЕЙСТВИЕМ ХЛОРПИРИФОСА: ЗАЩИТНОЕ ВЛИЯНИЕ ВИТАМИНОВ А И Е

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Хлорпирофос является высокотоксичным фосфорорганическим соединением. До сих пор он остается одним из самых распространенных инсектицидов. Основной механизм его токсичности связан с ингибиторием холинэстеразы. Наряду с антихолинэстеразными механизмами хлорпирофос может оказывать влияние через биохимические механизмы, в частности, посредством нарушения про- и антиоксидантного баланса вызывая генерацию свободных радикалов и обусловить возникновение окислительного стресса.

Мы изучили воздействие витаминов А и Е на основные гематологические и биохимические параметры периферической крови крыс через часов 12 после однократ-
ной интоксикации хлорпирифосом. Введение хлорпирифоса в дозе 70 мг/кг вызыва-
ло снижение общего количества эритроцитов, тромбоцитов и общего содержания
гемоглобина. Мы обнаружили снижение устойчивости эритроцитов перифериче-
ской крови к кислотному гемолизу у крыс, интоксичрованных хлорпирифосом. От-
равление хлорпирифосом приводит к повышению количества продуктов перекисно-
го окисления липидов: гидроперекисей и ТБК-активных продуктов.

Вместе с тем, введение витаминов A и E при интоксикации вызывает нивели-
рующий эффект на формирование продуктов перекисного окисления липидов. При интоксикации хлорпирифосом активность катализы повышалась, тогда как ак-
тивность супероксиддисмутазы, глутатионпероксидазы, глутатионредуктазы и со-
держание восстановленного глутатиона снижались. Обнаружено, что сочетание
витаминов A и E вызывает корректирующее воздействие на количество тромбоци-
тов, содержание гидроперекисей и ТБК-активных продуктов в перифери-
ческой крови крыс.

**Ключевые слова:** хлорпирифос, витамин Е, витамин A, интоксикация, крысы.

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