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BLOOD ERYTHROCYTE INDICES IN RATS UNDER CONDITIONS OF ACETAMINOPHEN-INDUCED TOXIC INJURY AGAINST THE BACKGROUND OF ALIMENTARY PROTEIN DEFICIENCY

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Ключові слова: *еритроцити, гемоглобін, гематокрит, аліментарна депривація протеїну, токсичне ураження, ацетамінофен*
Ключевые слова: *эритроциты, гемоглобин, гематокрит, алиментарная недостаточность белка, токсическое поражение, ацетаминофен*

Abstract. Blood erythrocyte indices in rats under conditions of acetaminophen-induced toxic injury against the background of alimentary protein deficiency. Kopylchuk H.P., Nykolaichuk I.M. *Despite the available information on variations in the erythrocyte chain of homeostasis under the conditions of drug-induced toxic damage, the question of the biochemical characteristics of erythrocytes in the expose of toxic doses of paracetamol on the background of dietary imbalance in protein remains open. 96 white nonlinear rats were used for the study. During the experiment, the animals consumed a semi-synthetic diet AIN-93 in accordance with the recommendations of the American Institute of Nutrition on the principle of paired nutrition, taking into account the amount of dietary protein per kilogram of diet. Modelling of acute toxic lesions was performed by administration to experimental animals of acetaminophen per os at a rate of 1250 mg/kg body weight. We found that toxic damage by acetaminophen is a key factor in reducing the level of total hemoglobin in the blood of animals (40% compared to control) with a simultaneous decrease in its average concentration (18%) and content in a single erythrocyte (35%), indicating the development of hypochromia, which is an indicator of iron deficiency in the body (iron deficiency anaemia). The synergistic effect of nutritional protein deficiency and the exposition of toxic doses of acetaminophen is accompanied by a decrease in total erythrocytes (50% compared to control) due to their increased hemolysis (up to 32% in 0.9% NaCl) with a simultaneous decrease in hematocrit up to 16% and the average volume of erythrocytes (1.4 times compared with the control). At the same time, the established changes in oxyhemoglobin decrease against the background of increased fetal hemoglobin content under the conditions of toxic damage and alimentary protein deficiency may serve as an additional criterion for comprehensive assessment in the diagnosis of anaemic syndrome in inflammatory diseases of different genesis against nutrient-associated conditions. Thus, the study of changes in erythrocyte indices in the development of acetaminophen-induced lesions on the background of nutrients' alimentary deprivation can increase the effectiveness of monitoring the course of these pathologies and approaches to their correction.*

Реферат. Еритроцитарні індекси крові щурів за умов токсичного ураження ацетамінофеном на тлі аліментарної нестачі протеїну. Копильчук Г.П., Николайчук І.М. *Попри наявні відомості про варіації в еритроцитарній ланці гомеостазу за умов токсичного ураження медикаментозними ксенобіотиками, питання дослідження біохімічних характеристик еритроцитів при надходженні токсичних доз парацетамолу на тлі незбалансованості харчового раціону протеїном залишається відкритим. Для проведення досліджень нами використано 96 білих нелінійних щурів. Упродовж експерименту тварини споживали напівсинтетичний раціон AIN-93 відповідно до рекомендацій Американського інституту нутрієнтології за принципом парного харчування з урахуванням кількості харчового протеїну на кілограм дієти. Моделювання гострого токсичного ураження проводили шляхом введення per os дослідним тваринам ацетамінофену з розрахунку 1250 мг/кг маси тварини. Нами встановлено, що токсичне ураження ацетамінофеном виступає ключовим фактором зниження рівня загального гемоглобіну в крові тварин (на 40% порівняно з контролем) з одночасним зменшенням його середньої концентрації (на 18%) та вмісту в одному еритроциті (на 35%), що вказує на розвиток гіпохромії, яка є показником дефіциту заліза в організмі (залізодефіцитна анемія). При цьому синергічна дія аліментарної нестачі протеїну та надходження токсичних доз ацетамінофену супроводжується зниженням загальної кількості еритроцитів (на 50% порівняно з контролем) внаслідок їх посиленого гемолізу (до 32% у 0,9% розчині NaCl) з одночасним зменшенням рівня гематокриту до 16% та середнього об'єму еритроцитів (у 1,4 раза порівняно з контролем). Водночас установлені зміни зниження оксигемоглобіну на тлі підвищення вмісту фетального гемоглобіну за умов токсичного ураження при аліментарній нестачі протеїну можуть служити додатковим критерієм для комплексної оцінки в діагностиці анемічного синдрому за умов запальних захворювань різного генезу на тлі нутрієнтоасоційованих станів. Отже, дослідження змін еритроцитарних індексів при розвитку ацетамінофен-індукованих уражень на тлі аліментарної депривації нутрієнтів може дозволити підвищити ефективність спостереження за перебігом цих патологій та підходами щодо їх корекції.*

Due to the widespread prevalence of alimentary-dependent pathologies, the problem of balancing dietary intake of macro- and micronutrients requires an urgent solution [15]. Protein metabolism disorders – protein-energy deficiency, depletion of the intracellular amino acid pool [13], can often be caused by limited protein intake, their low nutritional value, deficiency of essential amino acids with simultaneous active use of textured soy proteins [11].

At the same time, quite often the course of alimentary-dependent diseases is intensified due to unsystematic and irrational use of a wide range of commonly available drugs for the correction of pathological conditions. The currently common antipyretic analgesics invariably include paracetamol (acetaminophen, N-acetyl-p-aminophenol), which is considered safe compared to non-steroidal anti-inflammatory drugs. The increased frequency of use of the drug without compliance with dosing and interval requirements in recent years has raised concerns about medication-associated impairment. In fact, acetaminophen-induced injuries have emerged as the leading cause of acute liver failure [9].

Among indices allowing to estimate the functional state of an organism, to carry out differential diagnostics of diseases, severity of their course, to predict a course of pathological process, there are hematological indexes

which, fluctuating within narrow limits, reflect physiological and pathological changes.

Erythrocytes are highly specialized and metabolically active blood cells that respond to functional changes in the body. Without studying the biochemical characteristics of red blood cells, it is impossible to fully assess the state of the organism as a whole and its ability to maintain homeostasis [2].

It is worth noting that administration of toxic doses of acetaminophen against the background of alimentary protein deprivation leads to increased levels of pathological forms of hemoglobin – methemoglobin and carboxyhemoglobin, which can be considered as markers of cytopathic hypoxia [4].

It is known that erythrocyte indices allow estimating both the size of erythrocytes and their hemoglobin content. They characterize the cells themselves rather than their number, so they are relatively stable parameters. Thus, the biochemical characteristics of erythrocytes can be considered a promising marker for studying the manifestations of pathological processes.

In view of the above, the aim of this work was to study erythrocyte blood indices in rats under conditions of acetaminophen toxicity against the background of alimentary protein deficiency.

MATERIALS AND METHODS OF RESEARCH

The studies were conducted on white mature outbred rats aged 2.5-3 months and weighing 110-130 g. During the experiment 96 animals were used. All manipulations with rats were performed in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes (Strasbourg, 1986) and the recommendations "Bioethical Expertise of Preclinical and Other Scientific Research Performed on Animals" (Kyiv, 2006).

The animals were divided into 4 groups: control and 3 experimental groups. The control group included intact rats kept on a complete semi-synthetic AIN-93 diet developed by the American Institute of Nutrition [14]. The animals of experimental group 1 consumed for 28 days a low-protein diet containing ⅓ of the conventional daily protein requirement. Rats of the experimental group 2 consuming a complete diet were modeled by *per os* injection of acetaminophen at the rate of 1250 mg/kg of animal weight in the form of suspension in a 2% solution of starch gel once a day for 2 days. Study group 3: animals were administered toxic doses of the drug against the background of alimentary protein deficiency. Rats were subjected to cervical dislocation on days 28 and 31 of the experiment, using diethyl ether to create medicated sleep [12, 13].

To determine the number of erythrocytes (RBC), 0.02 ml of whole blood was added to a test tube containing 4 ml of 3% NaCl solution and thoroughly mixed. The number of erythrocytes was counted by the conventional method in a Goryaev [7].

To determine hematocrit (HCT) we used hematocrit capillaries that were pre-treated with heparin solution (with activity of 5000 IU/ml) [7].

The content of total hemoglobin (Hb) was investigated using the reagent kit "Filisit-Diagnostics" (Dnipro, Ukraine).

Mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using the corresponding formulas [7].

Osmotic hemolysis of erythrocytes was examined in 10 mmol/L Tris-HCl medium (pH 7.4) with different concentrations (0.15-0.9%) of NaCl. Hemolysis of erythrocytes was estimated by measuring absorbance in the supernatant of samples at 543 nm: hemolysis degree (in %)=[A1/A2]×100%, where A1 is absorbance of supernatant of experimental sample; A2 is absorbance in complete hemolysis of control sample [2].

Quantitative determination of the ligand form of hemoglobin, oxyhemoglobin (HbO_2), was performed by spectrophotometric method, which is based on the ratio of molar extinction coefficients of analytical wavelength (D_{max}=560-580 nm; D'_{max}=535-560 nm) when the maximum level of oxygenation of the molecule is reached [5].

The level of fetal hemoglobin (HbF) was estimated by a method *Singer et al.* based on its resistance to the denaturing action of alkali, followed by precipitation of coagulated proteins $(NH_4)_2SO_4$ and spectrophotometrical determination of HbF concentration in the supernatant at 540 nm.

Statistical analysis of the data was performed using Microsoft Office Excel 2016. The values obtained in the groups of experimental animals were compared with the control using Student's t-test. For this purpose we calculated the arithmetic mean of 24 independent determinations and standard deviations. The level of significance or probability of error was $p \leq 0.05$ [1].

RESULTS AND DISCUSSION

The results of the studies showed a decrease in the total number of erythrocytes in all experimental groups of rats compared with the values of the control. The RBC values in the blood of the animals that consumed ⅓ of the daily protein requirement, and under the introduction of toxic doses of acetaminophen in conditions of balanced diet were lower by 30% and 42%, respectively, compared with the control, while under the conditions of combined

action of two adverse factors, the maximum decrease (by 50%) in the number of red blood cells was observed (Table).

The causes of erythrocytopenia under these experimental conditions can be both increased hemolysis of erythrocytes and erythropoiesis disorders. It is known that the main physiological regulator of this process is erythropoietin, 90% of which is synthesized by kidney cells, and in small amounts (10%) by hepatocytes.

Blood erythrocyte indices in rats under conditions of acetaminophen toxicity and a concurrent alimentary protein deficiency (M±m)

Index	Groups of animals			
	C	1	2	3
Erythrocytes (RBC), $\times 10^{12}$/L	5.1 ± 0.34	3.5 ± 0.26*	2.9 ± 0.13*	2.5 ± 0.18*
Hemoglobin (Hb), g/L	146 ± 12.22	114 ± 8.78*	87 ± 4.85*	83 ± 5.03*
Hematocrit (HCT), %	49 ± 3.14	37 ± 2.66*	24 ± 1.27*	16 ± 0.98*
Mean corpuscular volume (MCV), fL	114.2 ± 10.94	102.7 ± 9.86	87.4 ± 6.89*	84.2 ± 6.74*
Mean cell hemoglobin (MCH), pg	25.2 ± 2.08	22.8 ± 1.92	12.5 ± 0.65*	12.6 ± 0.78*
Mean corpuscular hemoglobin concentration (MCHC), g/L	302.1 ± 18.75	244.7 ± 12.58*	254.1 ± 11.63*	248.8 ± 12.04*

Notes: C – control group; 1 – rats on a low-protein diet; 2 – rats with acetaminophen toxic injury; 3 – rats with acetaminophen-induced injury and a concurrent alimentary protein deficiency; * – statistically significant difference as compared with the control ($p \leq 0.05$).

Given that under conditions of acetaminophen-induced toxic injury combined with previous keeping the animals on a low-protein diet, there are pathological changes in renal morphology [6], the formation of erythropoietin by renal cells is presumably suppressed, which can be considered a prerequisite for the reduction of erythrocytes.

On the other hand, the results of the study of hemolysis as another cause of the decrease in the count of erythrocytes under these experimental conditions are shown in Figure 1. When incubating erythrocytes in 0.15% and 0.3% NaCl solutions (the most hypotonic ones) enhanced cell hemolysis is observed in all studied animal groups. At the same time, in the range of 0.6% -0.9% solutions of sodium chloride in the blood of intact animals, only 2% of erythrocytes are subject to hemolysis. An increased level of erythrocyte hemolysis is observed at 0.6% NaCl in animals affected by acetaminophen regardless of the amount of protein in the diet. The maximum percentage of hemolysis (32%) was observed when the erythrocytes in protein-deficient animals with toxic injuries were incubated in 0.9% NaCl isotonic solution (Fig. 1).

The reduced osmotic resistance of erythrocytes obtained from blood of acetaminophen-affected animals can be explained by the destructive effect of a highly reactive metabolite, N-acetyl-p-benzoquinonimine, on red blood cell membranes due to the intensification of lipid peroxidation, reduction of antioxidant potential, as well as Na^+/K^+- and Ca^{2+}-ATPase malfunction [3].

Reduced count of erythrocytes and their increased hemolysis in the groups of experimental animals is accompanied by a decrease in hematocrit level and decreased average erythrocyte volume compared with the control values (Table). Thus, the combined effect of two adverse factors, acetaminophen toxic injury and protein deficiency, is accompanied by a maximum decrease in the HCT level up to 16%.

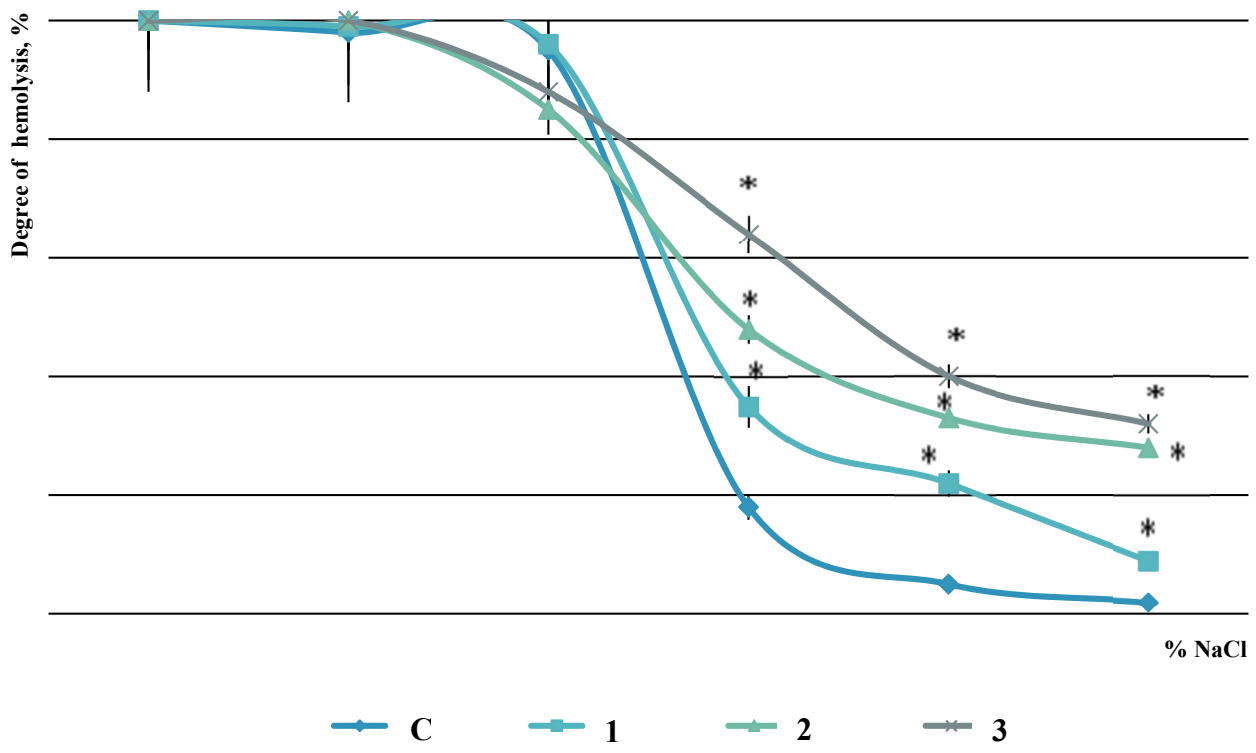
In fact, HCT is the ratio of the volume of formed elements, where 99% are erythrocytes, of the volume of blood plasma. Therefore, a decrease in this index correlates with a decrease in RBC (Table 1), and an increase in plasma content. The HCT level is regarded a control criterion for differential diagnosis of hemodilution [7].

The index of the average erythrocyte volume in the experimental groups of animals is reduced only if toxic doses of acetaminophen are administered, regardless of the protein content in the diet, as indicated in Table 1. The index of the average erythrocyte volume may be attributed to the disruption of the process of erythropoiesis, resulting in the formation of erythrocytes of reduced size and distorted shape [7].

Erythrocytes have no protein synthesis apparatus, so the adaptive properties of these cells and their role in the resistance of the organism largely depend on the ratio of prooxidants and antioxidants. Despite a well-developed

antioxidant system, erythrocytes are still vulnerable to oxidative stress, possibly due to the absence of mitochondria and, as a consequence, low energy supply. Extensive degradation of erythrocyte membrane lipids and proteins under oxidative stress leads to hemolysis [8], as confirmed by the results we obtained (Fig. 1) for acetaminophen-induced injury.

Thus, the synergistic effect of alimentary protein deprivation and the intake of toxic doses of acetaminophen is accompanied by a decrease in the total number of red blood cells due to their increased hemolysis with a simultaneous decrease in hematocrit and average red blood cell volume.



C – control; 1 – rats kept on a low-protein diet; 2 – rats with toxic lesions of acetaminophen; 3 – rats with acetaminophen-induced lesions on the background of alimentary protein deficiency; * – statistically significant difference compared to control ($p \leq 0.05$).

Fig. 1. The dependence of hemolysis on NaCl concentration for blood erythrocytes in rats under conditions of acetaminophen toxicity and a concurrent alimentary protein deficiency

Along with determining the count of erythrocytes, their average volume and hematocrit level, hemoglobin concentration analysis is important in determining the state of the erythrocyte system. Thus, we recorded a decrease in the total hemoglobin content in all experimental groups of animals as compared with the control (Table). Analysis of the obtained results indicates that the introduction of the toxin has a significant impact on changes in this index, regardless of the consumption of protein by the studied animals. The intake of an excessive amount of xenobiotic is accompanied by a disturbance of its effective detoxification with the formation of a highly reactive metabolite, N-acetylbenzoquinonimine, which conjugates with glutathione (GSH). Erythrocytes contain approximately 99.5% of blood GSH in the amount of 8.77 μmol/g hemoglobin [8]. Red blood cells with low GSH content are easily oxidized, which leads to the precipitation of hemoglobin and its deposition in the form of Heinz bodies. It has been reported [3, 8], that oxidative stress can lead to a significant accumulation of the oxidized form of glutathione (GSSG) in the liver and its release into the blood, followed by distribution between erythrocytes and plasma. For example, an increased plasma GSSG content can, in turn, cause oxidation of thiol groups of plasma proteins and/or proteins of erythrocyte basolateral membranes and their inactivation. In this case, the degree of iron oxidation of hemoglobin can change, which is accompanied by the formation of methemoglobin, as described in detail in our previous studies [4].

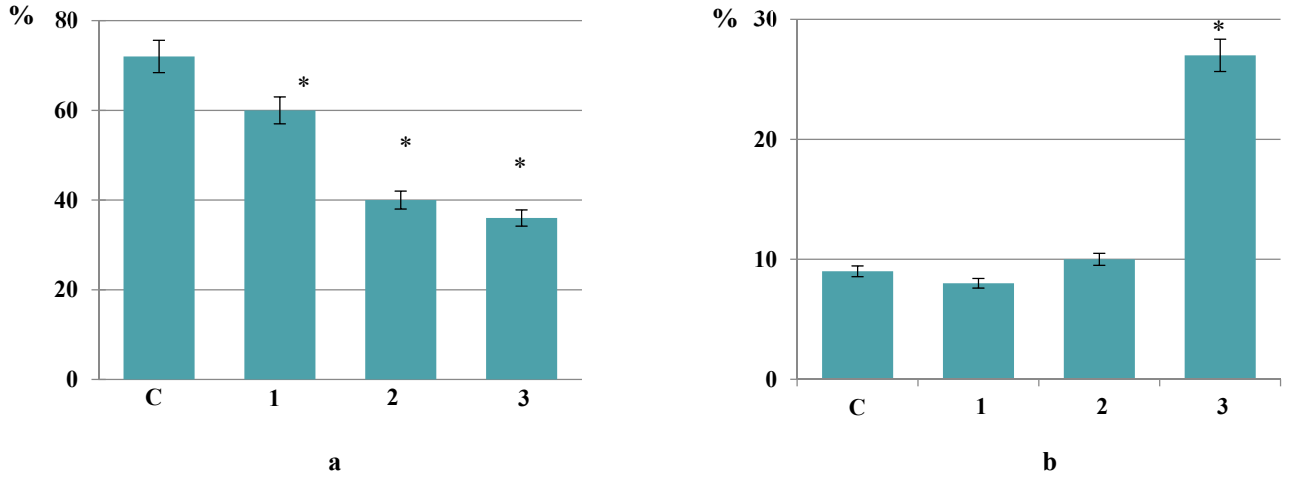
Regarding erythrocyte indices, a decrease in the average hemoglobin concentration in erythrocytes

(MCHC) was observed in the blood of all experimental groups of animals (Table), whereas a decrease in hemoglobin per erythrocyte (MCH) was registered only in animals with acetaminophen-induced injuries regardless of the diet (Table).

In clinical practice, a decrease in MCH indicates the development of hypochromia, an indicator of iron deficiency in the body (iron deficiency anemia) or non-absorption of iron by bone marrow normoblasts, which leads to impaired heme synthesis [10].

Along with this, MCHC was investigated, which determines the degree of saturation of erythrocytes with hemoglobin regardless of the volume of the red cells, that is, it reflects the ratio of hemoglobin content to cell volume. Therefore, a decrease in the average concentration of hemoglobin (Table) may be caused by a minor disorder of its formation, which agrees with the results obtained regarding the drop in total blood hemoglobin and decrease in the total count of blood erythrocytes (Table).

In addition, a decrease in the values of erythrocyte parameters is accompanied by a decrease in the percentage content of oxyhemoglobin in the blood of all groups of rats with the lowest indices under acute toxic injury against alimentary protein deprivation (Fig. 2, a) and a simultaneous increase in fetal Hb level under these conditions (Fig. 2, b).



C – control; 1 – rats kept on a low-protein diet; 2 – rats with toxic lesions of acetaminophen; 3 – rats with acetaminophen-induced lesions on the background of alimentary protein deficiency; * – statistically significant difference compared to control ($p \leq 0.05$).

Fig. 2. Contents of oxyhemoglobin (a) and fetal hemoglobin (b) in the blood of rats with acetaminophen toxic injury and concurrent alimentary protein deficiency

Figure 2a shows that the critical factor for reducing O_2 saturation (SaO_2) by two times is the intake of toxic doses of acetaminophen as compared with the control group. In clinical practice, certain pathological conditions are accompanied by significant hypoxemia (SaO_2<90%), with reduced oxygen delivery and utilization leading to organ dysfunction. Thus, under conditions of hypoxia, anaerobic glycolysis is activated in erythrocytes, which leads to the accumulation of lactate, a decrease in the alkaline blood reserves with the development of intracellular acidosis.

The references [8] indicate that the course of pathological processes under hypoxia is characterized by increasing heterogeneity of hemoglobin. Among all types of hemoglobin, fetal hemoglobin is of particular interest, which, at the same partial pressure, more actively absorbs oxygen and discharges carbon dioxide. In humans, the amount of HbF greater than 1.5% is considered pathological.

We explain the growth of the HbF level under acute toxic injury with concurrent alimentary protein deficiency by the fact that it, being a chromoprotein evolutionarily adapted to stabilize tissue gas exchange in conditions of chronic hypoxia by higher affinity to oxygen than HbA1, responds specifically to tissue hypoxia in conditions of drug-induced liver injury. The increased concentration of HbF in erythrocytes is related to the development of compensatory reactions of the erythron in conditions of hypoxia and may be associated with partial derepression of gamma globin chain in the course of erythropoiesis disorders.

CONCLUSIONS

1. In this way, acetaminophen-induced injury is a key factor in the reduction of total blood hemoglobin in the animals with a simultaneous decrease in its average concentration and content in an individual

erythrocyte. The changes we identified in the decrease of oxyhemoglobin against the increased fetal hemoglobin content can serve as an additional criterion for a comprehensive assessment in the diagnosis of anemic syndrome in inflammatory diseases of different genesis under nutrient-associated conditions.

2. Thus, among the promising markers, there are biochemical indices of red blood cells, which are quite representative and can be determined in most clinical biochemical laboratories. Studies of changes in erythrocyte indices during the development of acetaminophen-induced injuries against the nutrient deprivation could enhance the effectiveness of monitoring the course of these pathologies and approaches to their correction.

Contributors:

Kopylchuk H.P. – conceptualization, writing – review & editing, supervision, project administration, funding acquisition;

Nykolaichuk I.M. – methodology, validation, formal analysis, investigation, writing – original draft, visualization.

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