# Changes in erythron of experimental rats under influence of pyrite ore

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# Changes in erythron of experimental rats under influence of pyrite ore

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**Abstract.** On white non-breeding rats' males (n=60) were determined the experimental chronic intoxication of copper-zinc pyritic ore on rat erythron. The experimental model of chronic intoxication was created on rats by giving them water suspension of ore in a dose of 600 mg/kg of rats' body weight within two months. On the 30-th day of intoxication of copper-zinc pyrite ore there was a decrease in peripheral blood the number of erythrocytes and hemoglobin in 1,2 times compared with the control group ($p<0,05$). It is established that under the influence of ore the structure of cells of rats' marrow was changed, it was observed reduction of quantity of young cellular forms of erythroidal row. Observed shifts of erythropoiesis are caused by presence of heavy metals in content of ore, which accumulate in small doses in the body, cause the erythropoiesis suppression.

## 1. Introduction

The problem of pollution of the environment by heavy metal compounds is urgent and requires an in-depth study of the processes of the effects of these compounds on the human and animal bodies. The study of metall-induced anemia and possible ways of correcting it is extremely important for the Republic of Bashkortostan, which ranks second in the Ural region in terms of industrial potential and is the largest industrial center in Russia. More than 80 minerals represented by pyrite, chalcopyrite, chlorite, and others have been found in copper-zinc deposits. In addition to the main components (copper, zinc, sulfur, gold and silver), the ore contains lead, selenium, cadmium, nickel, cobalt, arsenic, thallium and barium. It is known that metals such as iron, copper, zinc, molybdenum, cobalt, manganese are essential for the life of the body. However, heavy metals and their compounds, settling on the skin, nails and hair, penetrating the body through air, water, food, accumulate in it and have a toxic effect on the stability of the membranes of blood cells, immune, endocrine and central nervous systems [1]. According to previous studies, cadmium and lead are one of the leading places among the most dangerous metals pollutants [2]. In the pathogenesis of lead-induced anemia there is a decrease in the formation of erythropoietin in the kidneys, damage to the stem cells of the red bone marrow, violation of the synthesis of hemoglobin, a decrease in the osmotic resistance of red blood cells [3]. Cadmium accumulates in the kidneys (30-60%) and liver (20-25%), which leads to the violation of cellular adhesion and activation of peroxidation of lipids, this element binds carboxyl, amino- and sulfhydryl groups, causing necrosis and cell apoptosis.

The study of the quantitative and qualitative composition of erythroblastic islets (EI) of bone marrow - cellular associations consisting of a centrally located macrophage with the 'crown' of erythroid cells of varying degrees of maturity, allows to study features of interaction between cells, characterize the effects of various toxic substances on erythropoiesis. Morphological analysis of EI makes it possible to determine in the unit the volume of bone marrow the number of colonizing units of erythrocytes (CUE) entered into differentiation, to assess the synchronicity of waves of amplification, as well as to study the nature of intercellular interactions in EI under various experimental pathologies. The purpose of this study was to determine the effects of heavy metal compounds in copper-zinc pyrite ore on the central and peripheral parts of erythron in vivo conditions.

## 2. Materials and methods

The work was carried out on 60 outbred albino male rats aged 3-4 months weighing 200.8±10.5 g. This study was carried out in accordance with the principles of the Basel Declaration and the Principles of Good Laboratory Practice (Order No. 544 of Federal Agency for Technical Regulation and Metrology, December 02, 2009, GOST R 53434-2009). The animals were kept in standard cages (n=6) with free access to water and food at the temperature of the air in the vivarium+24±2°C with the 2010/63/EU Directive on the Protection of Animals Used for Scientific Purposes. All painful manipulations with animals in the taking of peripheral blood and euthanasia by decapitation were carried out under ethereal anesthesia in a separate room from the vivarium and operating room. A sample of the ore was provided by Uchalinskyi Mining and Refining Plant (Uchaly, the Republic of Bashkortostan). The ore was crushed to a powdery state. To create an experimental model of chronic intoxication of copper-zinc pyrite ore to an experimental group of animals (n=40) daily an hour before the standard feeding orally for two months injected water suspension powder copper-zinc pyrite ore. Component analysis of the ore sample was determined by the method of atomic-absorption spectrometry. The injected dose of ore for the experimental group of rats was calculated at the minimum allowable concentration (MAC) of lead and cadmium in copper-zinc pyrite ore - 300 micrograms/kg and 600 mg/kg of animal's weight, respectively.

As a material of the study there were taken peripheral blood, bone marrow and kidneys of intact and experimental rats. Blood and bone marrow were determined on the 30$^{th}$ and 60$^{th}$ day of the experiment. Blood from the tail vein was collected into micro-tubes with EDTA, and then determined on the veterinary semi-automatic hematology analyzer Vet Exigo 19 (Sweden). There was determined the number of red blood cells (RBC) and the concentration of hemoglobin (HGB). Blood for the counting of reticulocytes was stained in vitro with a ready-made solution of brilliant cresol blue. The reticulocytes were counted using the MECOS-C2 Soft (Russia) automatic microscopy complex on the AXIO Lab.A1 microscope (ZEISS, Germany) with an increase in x900 using oil. The content of erythropoietin in serum (EPO) was determined by solid phase immunoassay analysis using a set of reagents "Erythropoietin-IFA-BEST" (Vector-BEST, Russia) on the micro plate assay analyzer Tecan INFINITY (Austria). For the morphological study on the 60$^{th}$ day of the experiment, there were taken the intact and experimental rats' pieces of kidneys measuring 0.5x0.5 sm. The tissues were fixed in a 10% buffer solution of formalin and after the standard histological wiring prepared slices of 7 microns thick, and then stained them with hematoxylin and eosin. The bone marrow for the study was obtained from the femurs. The total number of EIs was counted in Goryaev's chamber. The rest of EI were fixed on May-Grunewald stained eosin - methylene blue, and then stained by Gimza. The EIs were calculated by light microscopy with an increase of x900 using oil. EIs were classified into 5 classes of maturity according to Zakharov [4]. The "crown" of the first class EI (EI1) was represented by proerythroblasts and basophile erythroblasts with a number of cells from 2 to 8 (*de novo* erythropoiesis); the "crown" of second class EI (EI2) was represented by basophil and early polychromatophile erythroblasts (rubricytes) with number of cells from 9 to 16. The "crown" of third class EI (EI3) contained polychromatophilic erythroblasts, oxyphilic normoblasts and reticulocytes with a number of cells from 17 to 32. The crown of involutionary islands (EIinv) consisted of polychromatic and oxyphilic erythroblasts and reticulocytes (<16 nucleated cells). The crown of reconstructed (EIrec) islands contained mature cells (oxyphilic erythroblasts and reticulocytes) and young proerythroblasts and/or basophilic erythroblasts (*de repeto* erythropoiesis).In addition, there were counted the number of free separate macrophages per 100 EI and the number of EI, that "crown" was consisted only of reticulocytes [4]. To assess the level of development of erythropoiesis, there were calculated the following indicators:

- A1 – intensity of CUEs involvement into differentiation;
- A2 – intensity of CUEs involvement into erythropoiesis (EI1 +EIrec);
- A3 – maturation of erythroid cells ((EI3 + EIinv)/(EI1 + EI2 + EIrec));
- A4 – repeated involvement of macrophages into erythropoiesis (EIrec/EIinv).

The statistical processing of the data was carried out in the Russified license program Statistica 10 (StatSoft, USA). The results were analyzed by standard methods of descriptive statistics: the means and standard errors of means (M±m) were calculated. The groups were compared by nonparametric methods by Mann–Whitney tests. The differences were significant at $p<0.05$.

## 3. Results and discussion

In the study of peripheral blood indicators of rats of the experimental group, it was found that by the 30th day the RBCs and HGB in comparison with the control group decreased by 1.2 times (table 1). On the 60th day there were no reliable differences, although on average both the RBCs and HGB were significantly lower than the rates of intact animals. The bone marrow of the rats of the experimental group also began to respond to experimental toxic effects, on the 30th and 60th days of experiment the number of reticulocytes in the peripheral blood was increased by ~2 times the control value.

**Table 1.** Indicators of the peripheral part of erythron in chronic intake of copper-zinc pyrite ore.

| Parameter | RBC, $\times 10^{12}$/l | HGB, g/l | Reticulocytes, (x109/l) | EPO (mME/ml) |
|---|---|---|---|---|
| Control (n=20) | 7.7±0.1 | 142.5±1.5 | 1.4±0.1 | 2.3±0.2 |
| Day 30 (n=20) | 6.3±0.3* | 119.2±4.2* | 2.2±0.8* | 1.8±0.2* |
| Day 60 (n=20) | 6.7±0.5* | 133.8±9.1 | 2.7±0.1* | 2.3±0.3 |

Note. Here and in the table. 2, 3, 4: $p<0.05$ in comparison with *control group.

The lack of increasing of EPO was most likely due to the negative effect of the maximum allowable concentrations of heavy metal salts on the kidneys. Their peritubular cells could not produce the amount of erythropoietin, which could provide a full compensatory erythropoiesis, during which the content of erythropoietin in the serum increases 2 times or more [4]. Morphological examination of kidney tissue, conducted on the 60th day of the experiment, confirmed our assumption about the toxic damage of the tubular apparatus. On histological slices were revealed changes in the microcirculatory channel: venous hyperemia followed by an increase in permeability of capillary walls, increased vascular excretion of blood plasma, migration of white blood cells to the perivascular zone. The focal infiltration of kidney cannula tissue indicated tubule-interstitial jade and tubulopathy with signs of chronic pyelonephritis [5].

Previously, in our researches work was shown [1, 3] that chronic intoxication with the maximum allowable concentration of elements contained in copper-zinc pyrite ore is accompanied by pronounced changes in osmotic and acid resistance of RBC in experimental animals, which is undoubtedly associated with disturbances in the structure of cell membranes. However, in this study we have obtained evidence that this membrane dysfunction was caused not only by the direct toxic effects of heavy metal salts, but also by changes in the process of red blood cells formation during their differentiation and maturation in the bone marrow.

In the study of the state of bone marrow erythropoiesis (table 2) was found a slight increase of the absolute quantity of EI in the bone marrow of experimental animals. At the same time, in the bone marrow of rats of experimental groups began to increase the number of EI, which “crown” was consisted only reticulocytes. On the 30th day of the experiment, the number of such “reticulocyte” islets exceeded the control values by 13.5 times, by the 60th by 10.4 times.

**Table 2**. General characteristic of erythropoiesis in bone marrow with chronic intake of copper-zinc pyrite ore.

| Parameter | Absolute number of EI (x103/femur) | Reticulocyte EI (x103/femur) | Free separate macrophages (x103/femur) |
|---|---|---|---|
| Control (n=20) | 327.0±20.4 | 2.5±1.1 | 10.0±1.5 |
| Day 30 (n=20) | 362.5±22.8 | 33.8±3.5* | 44.7±3.4* |
| Day 60 (n=20) | 266.5±18.9 | 26.1±2.3* | 31.7±2.7* |

In physiological erythropoiesis, such an increase in the bone marrow number of EI with "reticulocyte crown" would be accompanied by a pronounced reticulocyte response of peripheral blood, but in our case we have registered very little increase in the number of reticulocytes in the peripheral blood. One of the reasons for this imbalance between the content of reticulocytes in the bone marrow and in the peripheral blood could be the previously discovered weak osmotic and acid resistance of the membranes of young erythroid cells, as a result of which most of the reticulocytes, due to the lack of their membrane resistance, were quickly subjected to hemolysis. In addition, this phenomenon could be caused by changes in the functional properties of both erythroid cells themselves at the stage of "oxyphilic erythroblasts - reticulocytes" and hemopoetic microenvironment of cells. It is possible that due to insufficient deformation ability, many reticulocytes could not pass through the walls of sinusoid bone capillaries into the bloodstream. At the same time, the change in the elastic properties of reticulocyte membranes may also affect their ability to form exosomes containing integrins α4β1 (the main molecules of the adhesion of erythroid cells, which contribute to the formation of intercellular contacts). It is known that before entering the vascular channel reticulocytes through exosomal transport are released from molecules that bind them to macrophages and components of extracellular matrix [6].

A detailed analysis of the distribution of different classes of maturity of EI (table 3) was found that on the 30th and 60th days of ore intoxication, the process of islet formation was stopped by primary accession of CUE to free bone macrophages (de novo erythropoiesis): EI1 in the bone marrow of experimental animals were not found throughout the experiment. EI2 were found very rarely. The oppression of de novo erythropoiesis was also evidenced by a significant increase of quantity of free macrophages (table 2): on the 30th and 60th day this parameter was exceeded the control value by 4.5 and 3.2 times respectively ($p<0.05$).

**Table 3**. Dynamics of the number of different classes of EI ($x10^3$/femur) with chronic intake of copper-zinc pyrite ore.

| Parameter | EI1 | EI2 | EI3 | EIinv | EIrec |
|---|---|---|---|---|---|
| Control (n=20) | 18.1±2.9 | 29.1±3.1 | 93.8±4.3 | 127.8±7.6 | 58.4±5.6 |
| Day 30 (n=20) | 0 | 2.1±0.9* | 70.4±6.1* | 250.6±15.7* | 39.4±2.7* |
| Day 60 (n=20) | 0 | 0 | 39.2±5.1* | 190.98±12.9* | 36.3±3.7* |

Erythropoiesis in the bone marrow of animals, receiving natural heavy metal salts, was supported by reconstruction (de repeto erythropoiesis), i.e. new EIs were formed only on the basis of bone macrophages that already had erythroid "crown" (EIrec), although this process was periodically slowed down. On the 30th and 60th day of the experiment, the number of EIrec in the bone marrow of experimental rats was reduced by 1.5 and 1.6 times, respectively, compared to the control group ($p<0.05$). With the increase in the duration of intoxication, the number of EI3 was steadily decreased, and this is understandable, as the EI3 of experimental animals created after Ei1 and EI2, as it happens in the case of physiological erythropoiesis. By the 60th day, the number of EIs3 were decreased by 2.4 times compared to the control group ($p<0.05$).

It is obvious that in the bone marrow of animals that have been chronically exposed to the maximum allowable concentrations of heavy elements of natural ore, the largest share of all EI were EIinv: their number exceeded the control 1.5 to 2.1 times (p 0.05). The quantity of EIinv in physiological erythropoiesis usually does not exceed 40% of the total number of EI of all maturity classes, and in our experiment in experimental animal groups the share of EIinv reached 72% [7]. As the “crown” of EIinv consists mainly of oxyphilic erythroblasts and reticulocytes, it became apparent that the chronic intake of mineral components of copper-zinc pyrite ore clearly slowed down the process maturation of erythroid cells at the stage of “oxyphilic erythroblasts – reticulocytes”.

Our supposition about the negative effects of the substances in pyrite ore on the erythroid tissue were confirmed in the analysis of estimated activity indicators of erythropoiesis (table 4). By the 60th day of chronic intoxication, the total number of EIs entering differentiation (A1) had decreased by 1.3 times. The rate of involvement of EI in in erythropoiesis (A2) on the 30th day decreased by 1.9 times, and by day 60 was by 2.1 times, which indicates a significant oppression of both erythropoiesis de novo and erythropoiesis de repeto.

**Table 4.** Indicators that characterize the activity of erythropoiesis in the bone marrow, with chronic intake of copper-zinc pyrite ore.

| Parameter | A1 (x103/femur) | A2 (x103/femur) | A3 (%) | A4 (%) |
|---|---|---|---|---|
| Control (n=20) | 385.4±24.7 | 76.4±8.0 | 2.2±0.2 | 0.5±0.1 |
| Day 30 (n=20) | 401.9±25.1 | 39.4±2.7* | 8.0±0.4* | 0.2±0.1* |
| Day 60 (n=20) | 302.8±22.2 | 36.3±3.7* | 6.5±0.3* | 0.2±0.1* |

The rate of maturation of erythroblasts (A3), on the contrary, in experimental animals compared to control rats significantly increased: on the 30th day by 3.6 times and on the 60th day by 3 times. The growth of this indicator proves that indeed one of the features of the toxic action of copper-zinc pyrite ore is the slowing down of the maturation of erythroid cells in the bone marrow of animals. The rate of repeated involvement of macrophages into erythropoiesis (A4) under the influence of pyrite ore components decreased by 2.5 times, which further indicates a weak activity of erythropoiesis de repeto, and most importantly - the absence of a pronounced compensatory reaction of erythron to chronic intoxication.

It is known that the most of the mineral components of copper-zinc pyrite ore are vital for the normal development of erythroid tissue: copper contributes to the absorption of iron ions in the intestine and the mobilization of its reserve from the liver and macrophages; nickel and cobalt support the synthesis of hemoglobin in erythroid cells, zinc prevents the development of iron deficiency anemia, improves the absorption of folic acid, and it is a part of the transcription factors that regulate the activity of the genome hemopoetic cells. However, when the maximum allowable concentrations of these and other trace elements in water or food are reached, the course of physiological erythropoiesis is damaged.

In our research, for the first time we described the features of the response of erythroid tissue to the introduction into the body of natural heavy metal salts, associated with the violation of intercellular interactions in morphological functional units of erythropoiesis - erythroblastic islands. Similar changes in experiments in vivo and in vitro were been reported previously in the study of the effects of carbon and silver nanoparticles, benzene, acute gamma radiation on erythropoiesis [8].

## 4. Conclusion

The maximum allowable concentrations of heavy metals, which are part of copper-zinc pyrite ore, completely inhibit the process of primary accession of CUE to free bone macrophages (de novo erythropoiesis). In chronic intake of components of copper-zinc pyrite ore, erythropoiesis is supported only by the reconstruction of EI, i.e. by forming islets based on the complexion of CUE with bone macrophages of EIinv. Weak reticulocyte response of the bone marrow to the toxic effects of heavy metal salts is caused by slowing the maturation of erythroid cells at the stage of “oxyphilic erythroblasts – reticulocytes”, as well as delay in the release of reticulocytes in the vascular bloodstream. Chronic intoxication with natural heavy metal salts is not accompanied by a full-fledged compensatory reaction of erythron, which is associated with the disruption of intercellular interactions in erythroid sprouts of blood and reduction of renal products of erythropoietin.

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