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ASSESSMENT OF THE DIFFERENT SIZE LEAD SULPHIDE NANOPARTICLES INFLUENCE ON ERYTHROCITES AND BLOOD PLASMA IN VITRO

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Disclosure of mechanisms for the implementation of toxic effects of lead compounds becomes necessary to assess the potential risks of their industrial use and to develop effective prevention measures. The aim of the study was evaluation *in vitro* of biochemical changes in rats erythrocytes and blood plasma under the action of lead sulfide nanoparticles depending on their size and their concentration. The obtained results showed a statistically significant increase in the antioxidant enzymes and the level of thiol groups activity in erythrocytes and blood plasma, as well as changes in the concentration of TBA(thiobarbituric acid)-active products and diene conjugates in erythrocytes under the action of lead nanoparticles. Biochemical changes in plasma and erythrocytes had the same tendencies, with erythrocytes parameters undergoing greater changes. The effect of $PbS_{26\text{-}34}$ showed a more active toxic effect, especially in dilutions of $10_{\text{-}3}$-$10_{\text{-}4}$ Mol/l.

Key words: lead, nanoparticles, erythrocytes, blood, antioxidant system, lipid peroxidation products, oxidative stress.

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ОЦІНКА ВПЛИВУ НАНОЧАСТИНОК СУЛЬФІДУ СВИНЦЮ РІЗНОГО РОЗМІРУ НА ЕРИТРОЦИТИ ТА ПЛАЗМУ КРОВІ IN VITRO

Розкриття механізмів реалізації токсичних ефектів наночастинок сполук свинцю стає необхідним для оцінки потенційних ризиків щодо їх промислового застосування та для розробки ефективних заходів профілактики. Мета дослідження - оцінка в умовах *in vitro* біохімічних змін плазми та еритроцитів крові щурів за дії наночастинок сульфіду свинцю в залежності від їх розмірів та концентрації. Отримані результати показали статистично достовірне зростання активності ферментів антиоксидантного захисту та рівня тіолових груп в еритроцитах і плазмі, а також зміни концентрації ТБК(тіобарбітурова кислота)-активних продуктів та дієнових кон'югатів в еритроцитах при дії сполук наночастинок свинцю. Біохімічні зміни плазми та еритроцитів мали однакові тенденції та залежали від концентрації дослідних речовин, при цьому показники еритроцитів зазнали більших змін. Вплив $PbS_{26\text{-}34}$ виявив більш активну токсичну дію, особливо у розведеннях 10^{-3}–10^{-4} Моль/л.

Ключові слова: свинець, наночастинки, еритроцити, кров, антиоксидантна система, продукти перикисного окислення ліпідів, оксидативний стрес.

The work is a fragment of the research projects "Investigation of cardiovasotoxic action mechanisms of heavy metal nanoparticles (on the problem of biosafety of nanomaterials)", state registration No. 0119U100182; "Investigation of the heavy metal nanoparticles toxic effects, search and substantiation of preventive measures", state registration No. 0116U000497; "Changes in internal organs and regulatory systems under the conditions of experimental damage and historical aspects of histology, cytology and embryology development in Ukraine", state registration No. 0116U000121 and "Study the of tissue reaction features and their modulation in lesions of various origins", state registration No. 0120U102691.

New physicochemical properties of engineered nanoparticles make them very attractive for industrial and biomedical use. Nowadays, the manufacture and application of nanotechnology products all over the world have reached industrial scale and have the potential for further growth and expansion.

It is known that the synthesized nanocrystals of lead compounds, the so-called "quantum dots", are successfully used in the manufacture of semiconductors, solar cells, biosensors, polymer composites, paints, electronic systems, including LEDs, flat light-emitting panels [4, 7, 12].

However, the social and commercial benefits of nanomaterials should not outweigh the potential negative health consequences for both nanoindustry workers and consumers of nanoproducts [6, 11, 14].

In our previous work on the effect of PbS NP 26–34 nm and 50–80 nm under subchronic intraperitoneal introduction and in acute intratracheal introduction it was indicated damage to heart myocardium and aorta [2, 3].

Lack of thorough knowledge of the nanoparticles (including lead NP as one of the most common and highly toxic metals) effects on the body requires a wide range of medical and biological studies and necessitates the study of their mechanism of action both in vivo and at the level of cells and subcellular structures in in vitro.

In particular, the study of the interaction of erythrocytes and blood plasma antioxidant proteins with lead sulfide nanoparticles will allow to assess the direct effect of these NP on the state of the protein molecule, as well as on its functional (enzymatic) activity. The disclosure of lead compounds realization the toxic effects the mechanisms is necessary to assess the potential risks for their industrial application and to develop effective prevention measures.

The purpose of the study was evaluation *in vitro* biochemical changes in erythrocytes and blood plasma of rats under the action of lead sulfide nanoparticles depending on the size of the nanoparticles and their current concentration.

Materials and methods. Studies of the peculiarities of the cytotoxic effect of different sizes $PbS_{26\text{-}34nm}$ and $PbS_{50\text{-}80}$ lead sulfide NP, as well as lead nitrate Pb $(NO_3)_2$ were performed *in vitro* on erythrocyte suspension and blood plasma of adult male rats. Animals were kept in a vivarium for 2 weeks on a standard diet with free access to drinking water before the start of the experiment. Animals sacrificed in compliance with the requirements of humane treatment. Blood was collected with the addition of heparin, centrifuged for 5 minutes at 3000 V/min. At the end, plasma was taken for further research.

To obtain a suspension of erythrocytes, saline in the ratio of 1:3 was added to formed elements the precipitate in the test tube remaining after removal of plasma, mixed thoroughly and centrifuged for 5 minutes at 3000 V/min. Finally, the supernatant was removed. The procedure was repeated three times. Finally, a suspension of erythrocytes in saline was prepared.

Experimental procedure. Working solutions of lead compounds were added to the erythrocyte and plasma suspension in a ratio of 1:1. The final concentrations of the test compounds were 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} Mol/l. The incubation lasted 3 hours. At the end of the experiment, the parameters were determined in plasma, and to the erythrocyte suspension was added saline and centrifuged for 5 minutes at 3 at 3000 V/min, the supernatant was removed. Then 1 ml of distilled water, kept for 5 minutes and frozen at -20° C was added to the erythrocytes for their destruction.

Catalase and SOD activity were determined on an erythrocyte suspension *in vitro*; general level of thiol groups; the level of thiol groups in the composition of low molecular weight and high molecular weight compounds; concentrations of MDA (malonic dialdehyde) and diene conjugates. Plasma activity of catalase and SOD (superoxide dismutase), as well as the total level of thiol groups were determined [8].

Statistical processing of primary data was performed using Microsoft Excel 2003 and StatPlus LE, IBM SPSS Statistics Subscription (demoversion). Data were presented as medians with upper and lower quartiles M [Q1-Q3]. Significance of differences between indicators was assessed by the Mann-Whitney U-test with a significant difference at the level of $p<0.05$.

Results of the study and their discussion. Plasma studies indicate that low-sulfide lead and lead nitrate directly affect the activity of the enzyme catalase, reducing it (fig. 1–1). Namely: there was a statistically significant ($p<0.05$) decrease in catalase activity in blood plasma compared to intact cells in all dilutions of test substances (except for the minimum concentration of $PbS_{26\text{-}34}$ NP). It was found that the decrease in enzyme activity under the action of larger nanoparticles was more significant in comparison with $PbS_{26\text{-}34}$ NP in the whole range of concentrations (statistically significant changes ($p<0.05$) were determined at concentrations of 10^{-4} Mol/l). Statistically significant ($p<0.05$) changes of catalase activity were recorded at the maximum concentration of lead compounds in the incubation medium (the largest decrease in blood plasma was recorded under the action of lead nitrate, the smallest changes – under the influence of $PbS_{26\text{-}34}$ NP) when comparing the effects of both sizes PbS NP with $Pb(NO_3)_2$.

The level of catalase activity in erythrocytes was statistically significant ($p<0.05$) exceeded the control values of enzyme activity in intact erythrocytes (fig. 1–2) in both ionic and nanoform (at concentrations of 10^{-3}-10^{-7} Mol/l) after incubation with the studied lead compounds. It should be noted that lead sulfide nanoparticles with a size of 26–34 nm at concentrations of 10^{-3}-10^{-7} Mol/l caused a more pronounced increase in catalase activity compared to PbS nanoparticles with a size of 50-80 nm and lead nitrate in ionic form. Statistically significant difference ($p<0.05$) was observed in all dilutions of test substances except the minimum – 10^{-7} Mol/l. The increase in catalase activity under the action of lead compounds (especially lead sulfide sulfide of smaller size) in erythrocytes, probably due to activation of the antioxidant defense system in response on the development of oxidative stress.

Plasma SOD activity (fig. 2–1) was statistically significant ($p<0.05$) increased only in the study group with $PbS_{26\text{-}34}$ in higher concentrations (10^{-3}–10^{-5}), respectively statistically significant ($p<0.05$) differing from SOD in experiments with $PbS_{50\text{-}80}$ and $Pb(NO_3)_2$. The level of SOD activity in plasma did not change at lower concentrations of $PbS_{26\text{-}34}$, as well as after incubation with a larger PbS NP and lead nitrate.

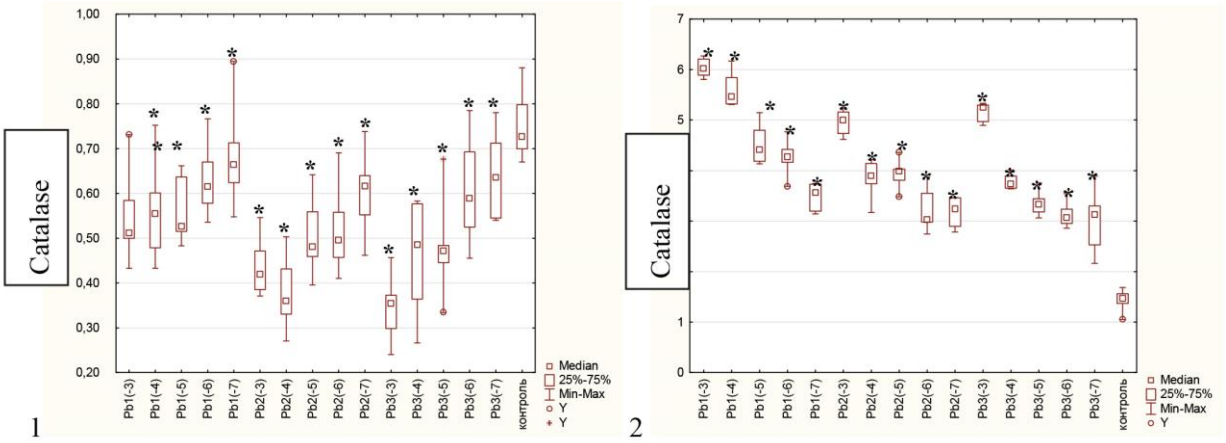

Fig. 1. Rats blood biochemical parameters dynamics after studied lead compounds incubation in ionic and nanoform at concentrations of 10^{-3}-10^{-7} Mol/l.

Notes: 1 – plasma catalase activity and 2 – erythrocyte catalase. * – statistically significant difference from control ($p<0.05$).

The activity of SOD in erythrocytes (fig. 2–2) was statistically significantly higher than intact erythrocytes after incubation with lead sulfide NP of both sizes and lead nitrate in concentrations (10^{-3}–10^{-5} Mol/l). The activity of SOD in erythrocytes gradually decreased with a further decrease in the concentration of lead compounds in the incubation medium. Indicator of SOD activity was observed only under the action of PbS NP s of both sizes at a concentration of 10^{-6} Mol/l, a statistically significant difference ($p<0.05$) compared with the control. The same indicator at a minimum concentration of 10^{-7} Mol/l was observed under the influence of $PbS_{50\text{-}80}$ and lead nitrate nanoparticles. Comparing the effects caused by the studied lead compounds, it should be noted that smaller PbS nanoparticles caused a more pronounced increase in SOD activity: the activity of SOD in erythrocytes was statistically significant ($p<0.05$) higher in comparison with the influence of PbS NP of larger size under the action of $PbS_{26\text{-}34}$ (at a concentration of 10^{-4} Mol/l); statistically significant ($p<0.05$) excess of SOD activity was recorded in dilutions of 10^{-4} and 10^{-6} Mol/l after incubation with the ionic form of lead.

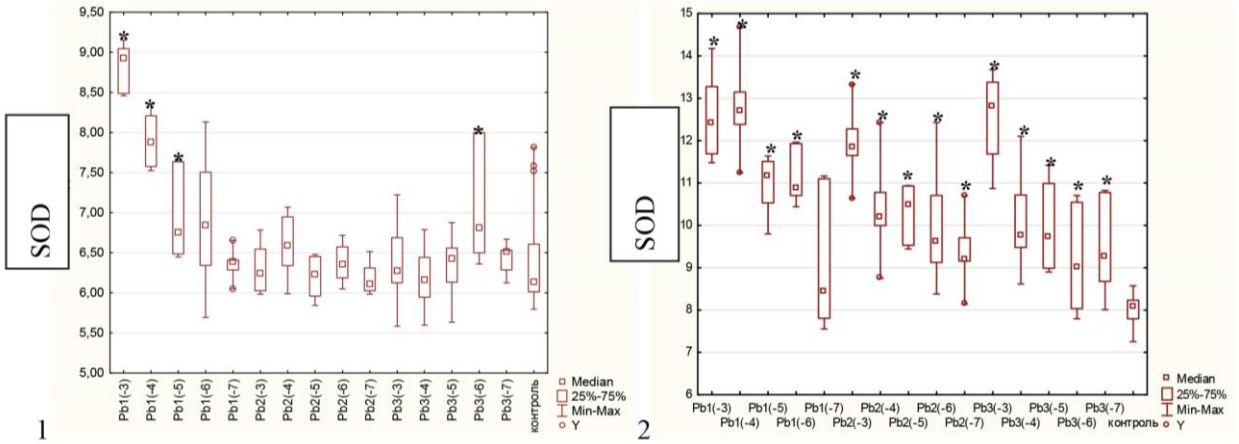

Fig. 2. Rats blood biochemical parameters dynamics after studied lead compounds incubation in ionic and nanoform at concentrations of 10^{-3}-10^{-7} Mol/l.

Notes: 1 – Activity of plasma SOD and 2 – erythrocytes. * – statistically significant difference from control ($p<0.05$).

The level of thiol groups in blood plasma (fig 3–1) was statistically significant ($p<0.05$) increased in the whole range of concentrations after incubation with the studied lead compounds. The most pronounced changes occurred under the action of PbS nanoparticles of both sizes at maximum concentrations (10^{-3}–10^{-4} Mol/l), and especially – $PbS_{26\text{-}34}$.

The increase in the total level of thiol groups in blood plasma may be due to changes in the conformation of protein molecules, which is a result of the destruction of their quaternary structure after incubation with lead compounds, which leads to the appearance of free SH groups that interact with Elman's reagent. Changes in the structure of the protein molecule can cause changes in the functional activity of enzymes as a component of antioxidant protection (SOD, catalase). These mechanisms play a leading role in the pathogenesis of the toxic effects of lead compounds.

The total level of thiol groups, thiol groups in the composition of low molecular weight compounds and in the composition of high molecular weight erythrocytes increase statistically significantly from the

control indicator in all experimental groups, and this indicator decreases with increasing dilution. The total level of thiol groups in the experiments with PbS_{50-80} and $Pb(NO_3)_2$ is restored at dilutions of 10^{-7} Mol/l and 10^{-6}–10^{-7} Mol / l, respectively (fig. 3–2).

There was a difference between the levels of thiol groups in the composition of low molecular weight erythrocytes (Fig 3–3) under the action of lead nitrate in ionic form and nanoparticles with a size of both 26–34 nm and 50–80 nm (statistically significant difference) ($p<0.05$) was observed in all dilutions of test substances (except for insignificant increase in PbS_{50-80} at a concentration of 10^{-5} Mol/l). of smaller lead size. Statistically significant difference ($p<0.05$) between the indicators in the groups under the influence of PbS nanoparticles of different sizes was recorded in dilutions of 10^{-3}–10^{-5} Mol/l. The increase in the thiol groups level in low molecular weight compounds may be due to the increase in glutathione levels in erythrocytes and may be a manifestation of compensatory processes in response to the growth of ROS production and the development of oxidative stress.

The level of thiol groups also increased statistically significantly in the composition of macromolecular compounds (fig. 3) in erythrocytes after incubation with the entire concentration range lead sulfide NP $Pb(NO_3)_2$ (fig. 3–4). The level of thiol groups in the composition of macromolecular compounds of erythrocytes is restored in dilutions of 10^{-5}–10^{-7} Mol/l in the experiment with $Pb(NO_3)_2$ and 10^{-7} in the experiment with PbS_{50-80}.

A difference was found between the levels of thiol groups in the composition of macromolecular compounds under the action of PbS NP of each size and the effect of $Pb(NO_3)_2$, namely: a statistically significant increase ($p<0.05$) was observed in all dilutions of test substances under the influence of PbS_{26-34} NP and only under the action of the maximum concentration of PbS_{50-80} NP in the incubation medium. It should be noted that a more significant increase in the level of thiol groups in the composition of macromolecular compounds caused a lower lead sulfide low*sulfide (statistically significant difference ($p<0.05$) between the indicators was recorded in dilutions of 10^{-3}, 10^{-4} and 10^{-7} Mol/l).

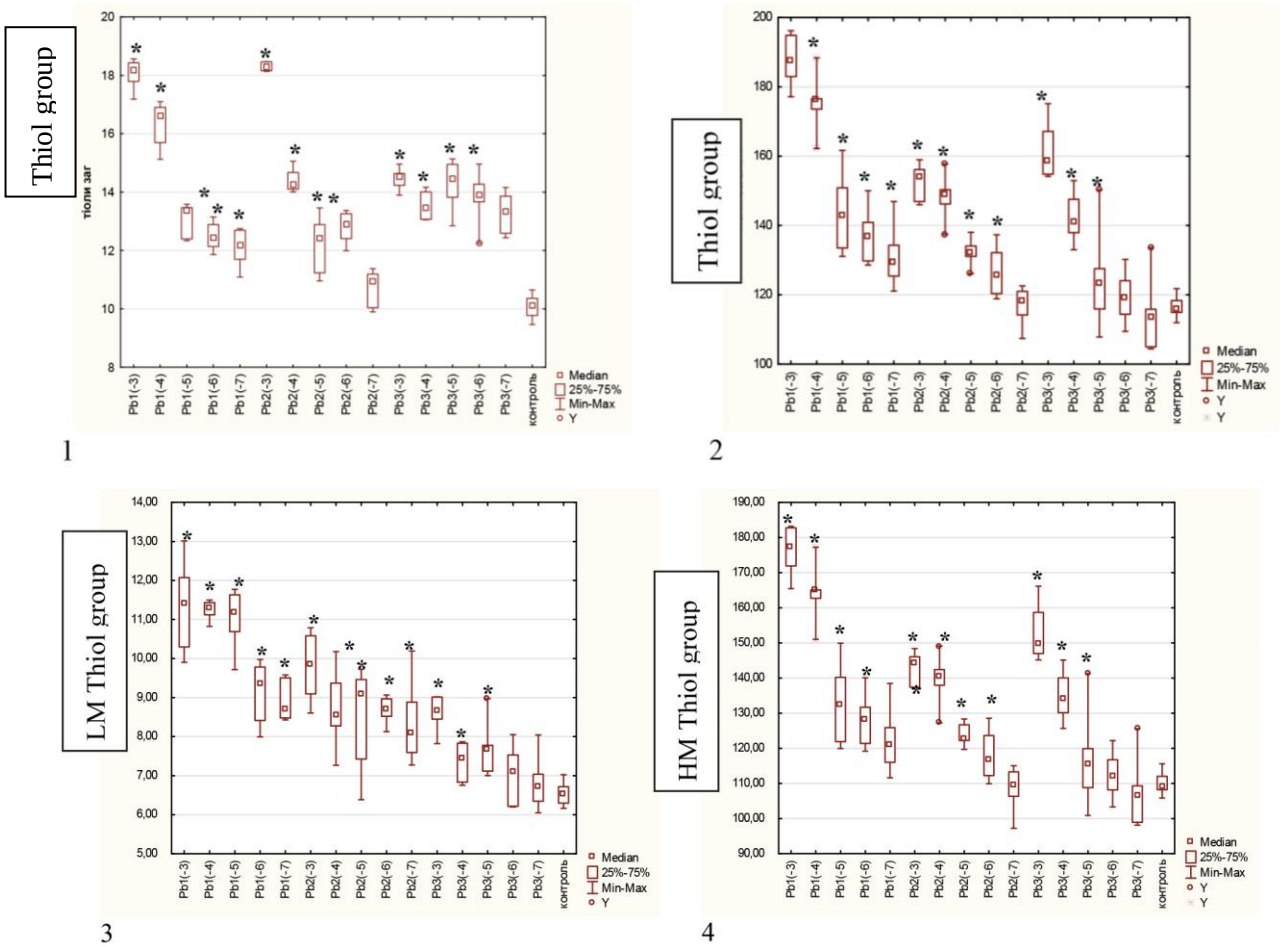

Fig. 3. Rats blood biochemical parameters dynamics after studied lead compounds incubation in ionic and nanoform at concentrations of 10^{-3}–10^{-7} Mol/ l.

Notes: 1 – total thiol groups of plasma; 2 – common thiol groups of erythrocytes; 3 – thiol groups as a part of low-molecular compounds of erythrocytes, 4 – thiol groups as a part of high-molecular compounds of erythrocytes. * – statistically significant difference from control ($p<0.05$).

The concentration of TBA-active (thiobarbituric acid) products in erythrocytes (malonic dialdehyde is one of the TBA-active products) increased statistically significantly after incubation with the studied lead compounds at concentrations from 10^{-3} Mol/l to 10^{-6} Mol/l (except for an insignificant increase in exposure to lead nitrate at a concentration of 10^{-5} Mol/l) (fig. 4–1). It should be noted that the most significant increase in the concentration of TBA-active products was observed after incubation of erythrocytes with $PbS_{26\text{-}34}$ at concentrations of 10^{-3} and 10^{-4} Mol/l. At a minimum concentration of 10^{-7} Mol/l lead nanoparticles of both sizes did not cause changes in this indicator compared with intact erythrocytes. At the same time, a significant increase in TBA-active products in erythrocytes was recorded, when studying $Pb(NO_3)_2$ in the lowest concentration. Since the concentration of MDA is maintained at a certain level with the participation of antioxidant enzymes, its increased levels are a marker of lipids peroxidation.

Diene conjugates are known to be primary products of lipids peroxidation. The concentration of diene conjugates in erythrocytes (fig. 4–2) was statistically significantly higher than the control value after incubation with test compounds of lead in both ionic form and nanoform at concentrations of 10^{-3}–10^{-7} Mol/l.

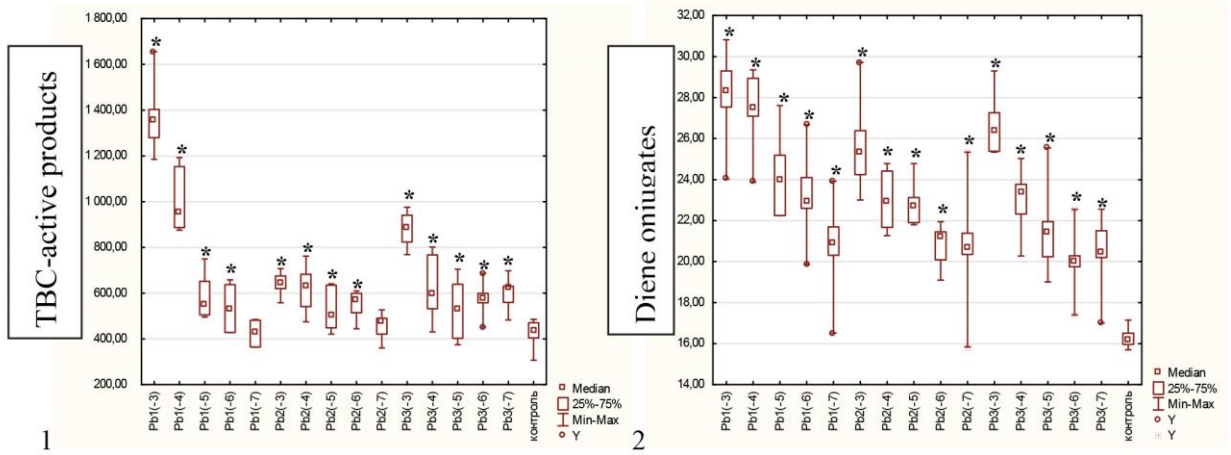

Fig. 4. Rats blood biochemical parameters dynamics after studied lead compounds incubation in ionic and nanoform at concentrations of 10^{-3}–10^{-7} Mol/l.

Notes: 1 –TBA-active products; 2 – Diene conjugates. * – statistically significant difference from control (P<0.05).

Throughout the concentration range, a greater increase in diene conjugates resulted in lower levels of lead sulfide. A statistically significant difference ($p<0.05$) was observed in comparison with larger PbS NP in dilutions of 10^{-4} and 10^{-6} Mol/l; and in comparison with $Pb(NO_3)_2$. Statistically significant excess ($p<0.05$) was determined at concentrations of 10^{-4}–10^{-6} Mol/l.

Thereby, decreased plasma catalase in blood plasma activity further confirms that lead sulfide NP have prooxidant effects in the cell, stimulating increased reactive oxygen species (ROS) formation and inhibiting the antioxidant defense systems activity, leading to the development of oxidative stress and lipids peroxidation.

Catalase decomposes hydrogen peroxide, oxidizes low molecular weight alcohols and nitrites in the presence of hydrogen peroxide and participates in cellular respiration processes. Its growth in erythrocytes in our study can be explained by the increase in the content of hydrogen peroxide against the background of the lack of influence on the lead ions enzyme active center [10]. This effect of lead ions can be explained by the fact that catalase contains heme in its active center [13].

A positive correlation was found in workers engaged in steel production between the content of lead in the blood and the activity of SOD or the content of malonic dialdehyde (MDA) [15]. The addition of lead at doses of 10 mg/kg or 20 mg/kg caused inhibition of SOD activity in peripheral erythrocytes *in vitro* and *in vivo*, while in our study this figure increases, indicating other mechanisms of oxidative stress [5, 9].

Changes in the activity of SOD as metalloenzymes in plasma and erythrocytes can have different causes, which is primarily due to oxidative stress caused by Pb^{2+} ions. It is possible that the increase in the activity of SOD is due to the mobilization of low molecular weight antioxidants that reduce the amount of peroxide radicals that can attack the enzyme molecule and inhibit its activity.

The increase in the level of thiol groups in the composition of macromolecular compounds may be due primarily to changes in the protein molecules conformational properties and the release of thiol groups, because erythrocytes are not able to synthesize proteins de novo.

Diene conjugates are formed mainly in the first stage of lipid peroxidation, malonic dialdehyde is formed in the body during the degradation of polyunsaturated lipids by reactive oxygen species. Both serve as a marker of lipids peroxidation and oxidative stress [1]. The increase in malonic dialdehyde is explained by the activation of antioxidant enzymes that are responsible for the neutralization of lipid peroxidation triggers [10].

Conclusion

There was a statistically significant increase in the activity of antioxidant enzymes (SOD, catalase) and the level of thiol groups in erythrocytes and blood plasma, as well as changes in the concentration of TBA-active products and diene conjugates in erythrocytes under the action of lead nanoparticles, which may indicate significant stress protective systems of the body against the background of oxidative stress activation.

The results of the effect lead nanoparticles compounds study on antioxidant plasma proteins and erythrocytes showed a dependence on the test substances concentration. Biochemical changes in plasma and erythrocytes had the same tendencies, with erythrocyte parameters undergoing greater changes. The effect of $PbS_{26\text{-}34}$ showed a more active toxic effect, especially in dilutions of $10_{-3} – 10_{-4}$ Mol/l.

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