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In vivo EFFECTS OF RARE-EARTH BASED NANOPARTICLES ON OXIDATIVE BALANCE IN RATS

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The purpose of the research was to find the influence of rare-earth based nanoparticles (CeO_2, $GdVO_2$: Eu^{3+}) on the oxidative balance in rats. We analyzed biochemical markers of oxidative stress (lipid peroxidation level, nitric oxide metabolites, sulfhydryl groups content) and enzyme activities (superoxide dismutase, catalase) in tissues of rats. It has been found that administration of both types of the nanoparticles increased nitric oxide metabolites and products of lipid peroxidation in liver and spleen within 5 days. At injections of $GdVO_2$: Eu^{3+} lipid peroxidation products, nitric oxide metabolites in serum at 5, 10 and 15 days of the experiment was also increased whereas the level of sulfhydryl groups decreased compared to the intact state and the control. In contrast, under the influence of nanoparticle CeO_2 level of diene conjugates were not significantly changed and the level of nitric oxide metabolites within 15 day even decreased. During this period, under the influence of both types of nanoparticles the activity of superoxide dismutase was increased, catalase activity was not changed. Oxidative stress coefficient showed the less pronounced CeO_2 prooxidant effect (2.04) in comparison to $GdVO_2$: Eu^{3+} (6.89). However, after-effect of both types of nanoparticles showed complete restoration of oxidative balance values.

Key words: nanoparticles CeO_2 and $GdVO_4$:Eu^{3+}, oxidative balance.

Reactive oxygen and nitrogen species (ROS, RNS) are constantly generated in the body from internal metabolism and external exposure [1, 2]. In normal cells, reactive oxidants are produced in a controlled manner in response to physiological cues and act as important signaling molecules to regulate such processes as cell division, inflammation, immune function, autophagy, and stress response [1]. Uncontrolled production of oxidants results in oxidative stress that impairs cellular functions and contributes to the development of cancer, chronic disease, and toxicity [2–4]. Considering the pathogenic role of the oxidative stress in the damage of vital functions, the use of nanoparticles as long-term antioxidants has great prospects [5].

The nonstoichiometry of cerium oxide in the nanocrystalline state enables its participation in various redox processes, in particular, in the inactivation of toxic reactive oxygen species such as hydrogen peroxide and nitric oxide radical. The study of nanoparticles (NPs) of cerium oxide as antioxidants [6] have shown an activity similar to the one of superoxide dismutase (SOD) [7–9] and catalase [10, 11] as well as neuroprotective [12] and anti-inflammatory [13] action. Cerium oxide exhibits also the radioprotective properties [14, 15]. Among the potential compounds for nanomedicines along with cerium oxide, the vanadium compounds attract much attention. The main insulin effects on carbohydrate and lipid metabolism are simulated by the vanadium

compounds *in vitro and in vivo*. The vanadium compounds, beyond the hypoglycemic effect, exhibit also antihypertensive and anticholesteric activity [16, 17]. In addition, these compounds exhibit antineoplastic action [18].

It is obvious that using of nanotechnology in medicine is possible only after the risk assessment. Some researchers have found negative — cytotoxic and proapoptotic effects of cerium oxide. It is shown that CeO_2-NPs can increase the production of reactive oxygen intermediates (ROI), reduce the level of glutathione as well as induce the oxidative stress (OS) [19]. It is shown that NPs of CeO_2 can cause the inflammation and damage of lungs in rats, and this effect is dose-dependent [20]. It is stated that the toxic effects of nanoparticles of cerium oxide can be realized through OS [21]. Also, high toxicity of vanadates impedes their use. The problem of the toxicity of these compounds can be solved by the creation of vanadates in nanocrystalline form. The orthovanadate nanoparticles doped with rare-earth elements (REE) allow profitable combining of biocompatibility, bioactivity, and optical properties caused by the presence of REE that allow using NPs as probes or lables in biomedical researches and diagnostics.

Data inconsistency on NPs effects can be determined by the peculiarities of the applied methods, by the use of different concentrations of nanoparticles, by different sensitivity of the cells and cell lines to the influences, and finally, by different duration of the experiment. It is indicated that not only the physical and chemical properties of the material, but also the size, shape and the presence of related compounds used in the synthesis of NP can determine the end result of the interaction of the particles with biological structures [22].

Physical and chemical properties of nanoparticles that affect the expression of prooxidant or antioxidant effects of nanoparticles *in vitro* were identified earlier [23, 24]. It was discovered that microenvironment in biosystem strongly determines expression of NPs redox effects. So, the comprehensive assessment of the effects on biostructure and processes at different levels of living systems organization is required.

In the paper we have investigated the impact of $GdVO_4:Eu^{3+}$ and CeO_2 NPs on the oxidative balance *in vivo*. Based on evaluation of pro- and antioxidant parameters as well as on the level of biochemical markers of the OS in different tissues the response of organism was registered in the dynamics of NPs action. Luminescent properties of $GdVO_4:Eu^{3+}$ NPs have allowed evaluation of their distribution in body tissues. The correlation between the preferential accumulation of NPs in tissues and used parameters was found.

Materials and Methods

The aqueous colloidal solutions of cerium oxide [24] and europium doped orthovanadates ($GdVO_4:Eu^{3+}$) were synthesized by methods described previously [25, 26].

Synthesis of the $GdVO_4:Eu^{3+}$ and CeO_2 nanoparticles

The synthesis of $GdVO_4:Eu^{3+}$ and CeO_2 water colloidal solutions has been carried out according to the method reported earlier [24, 25]. $GdVO_4:Eu^{3+}$ nanoparticles with an average size of 25×8 nm, and CeO_2 with an average size of 8 nm have been obtained.

NPs were characterized using Transmission electron microscopy (TEM-125K electron microscope, Selmi, Ukraine). Standard deviation does not exceed ±10% from average size of the particle. $GdVO_4:Eu^{3+}$ and CeO_2 nanoparticles were stabilized by sodium EDTA and sodium citrate, respectively. The solutions were additionally dialyzed for 24 h against deionized water to remove the excess of ions and organics species. Dialysis membrane tubing with a molecular weight cutoff of "Cellu Sep H1" 6 KDa was used. All sols were transparent in transmitted light. The process solutions have physiological value pH = 7.2–7.8. The colloidal solutions were stored in sealed ampoules without changing their properties for more than 2 months at normal conditions.

Experimental procedure

Male Wistar rats (180–200 g body weight) and outbred mice (20±1 g body weight) were kept at 24 °C on a cycle of 12 h light/12 h darkness and had free access to a standard chow diet and drinking water *ad libitum*. Animals were killed by decapitation under anesthesia. The experiments were conducted according to the regulations of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and Law of Ukraine [27].

Animals were randomized into following groups:

1st group. Male rats (30 animals) injected intramuscularly with the aqueous solution of $GdVO_4$:Eu^{3+} NPs (0.5 ml, concentration — 0.2 g/l) once per day for 15 days.

2nd group. Male rats (30 animals) injected intramuscularly with the aqueous solution of CeO_2 NPs (0.5 ml, concentration — 0.2 g/l) once per day for 15 days.

Control group — male rats (30 animals) injected intramuscularly with 0.5 ml of sterile water for injection once per day for 15 days.

Intact control — the untreated rats which had free access to a standard chow diet and drinking water *ad libitum* were used for intact control (30 animals).

After 5 injections period from the every group it was taken out 15 animals for analysis of biochemical parameters in organs — brain, liver, lungs, kidneys, heart, spleen, pancreas, testes, skin (at the injection place). In the remaining part (15 rats) of the each group the experimental treatment lasted. Blood was taken from the tail vein, the biochemical parameters were evaluated at 5, 10 and 15 injections period and aftereffect.

The following parameters were determined in the blood plasma and tissues: the content of diene conjugates (DC), TBA-active products (TBA-AP), sulfhydryl groups (SH-groups), total metabolites of nitric oxide (mNO), nitrates, nitrites. In the erythrocyte hemolysates the activity of antioxidant enzymes — catalase and SOD was determined.

The DC level was measured spectrophotometrically according to the method of Stalna in the modification of Skornyakova et al. [28] at 233 nm.

The lipid peroxides were estimated according to the method of Uchiyma & Michara in the modification of Volchehorskiy and others using a thiobarbituric acid (TBA) test for malondialdegyde (MDA) absorbance was measured at 535 and 580 nm.

The catalase assay was carried out by the rate of hydrogen peroxide H_2O_2 utilization as described [29] at 410 nm.

The superoxide dismutase (SOD) activity was determined by quercetin oxidation in the modification of Kostiuk et al. [30] by monitoring at 406 nm.

The content of sulfhydryl (–SH)-groups in blood was measured by spectrophotometric method using Ellman's reagent at 412 nm.

The level of total nitric oxide metabolites was quantified according to L. C. Green et al. in the modification of Metelska and Humanova [31] by monitoring at 540 nm.

The nitrites content was measured according to Zvyagina [32] at 540 nm.

The nitrates content was estimated by the difference of total metabolites and nitrites. The nitrates content is expressed in μmol/ml of blood serum or μmol/g of tissue [33].

Identification of $GdVO_4$:Eu^{3+} NPs in the tissues of experimental animals

To study the dynamics of NP distribution in certain organs, $GdVO_4$:Eu^{3+} NPs were injected to male mice at the dose of 50 mg/kg intraperitoneally. In 1, 3, 7, 24 hours, 5, 10 and 30 days after injection in 5 animals were decapitated. The state of organs was examined by the autopsy. Brain, hypophysis, thymus, spleen, lungs, heart, kidney, adrenal gland, liver, testes, epididymis, and the ventral part of the prostate (VPP), suspension of epididymal sperms were taken. The blood was collected and separated to serum and pellet contained blood cells by centrifugation. Tissue samples were subjected to double freeze-thaw cycle, homogenized by glass homogenizer and the spot with diameter of 0.5 cm was applied to a glass and dried.

Based on the luminescence intensity of tissue samples, the semi-quantitative estimation of NPs content in different organs was carried out. Content of NPs was determined from the calibration curve "luminescence, rel. un — NPs content in a standard sample". Luminescence spectra were obtained using spectrofluorimeter based on the grating monochromator, luminescence was excited by He-Cd laser with $\lambda_{exc} = 325$ nm.

Results were expressed as the mean ± SEM. Differences between groups were determined using Student's t test. The values at $P < 0.05$ were regarded as reliable.

Used reagents

Heptane (Dow Chemical, Germany), isopropanol (BASF, Germany); TBA (ORGANICA, Germany), phosphoric acid (Chang Hui, China); DTNB (LOBA, Austria); H_2O_2 (Intersintez, Ukraine), ammonium molybdate (Hebei Hehua Energy Development Co., Ltd., China); EDTA (Akzo Nobel, China), TEMED (Himlaboreaktiv, Ukraine), quercetin (AppliChem, Germany), DMSO (Halychpharm, Ukraine); Griess reagent (LabMir, Ukraine), vanadium chloride (ALOPICH, USA); chloroform (CHEMICO GROUP, United Kingdom).

Results and Discussion

The study of metabolic changes under an influence of $GdVO_4:Eu^{3+}$ NPs and CeO_2 NPs was carried out using a set of parameters that characterize the free-radical processes, namely: the level of DC; MDA; the catalase; superoxide SOD activities; the content of -SH groups; the concentration of total stable metabolites of the nitric oxide cycle and nitrites in experimental animals tissues: brain, liver, lungs, kidneys, heart, spleen, pancreas, testes, skin (at the injection place).

Relations between the prooxidant and antioxidant reactions — the ability of pro- and antioxidants to protect the cell from the excess of free radicals were analyzed.

Doping of the vanadate NPs with europium ions supplies them luminescent properties and makes it possible to identify $GdVO_4:Eu^{3+}$ NPs accumulation in animal's tissues. The luminescence of $GdVO_4:Eu^{3+}$ nanocrystals is effectively excited in the visible range of spectrum and has the significant Stokes shift (more than 200 nm), allowing to get rid of the noise signal of the autofluorescence of biological objects. The features of NP accumulation in cells were investigated using the methods of luminescence spectroscopy and luminescence microscopy [34, 35].

Based on data of literature analysis of various NPs distribution in the body under the inhalation conditions, oral or intraperitoneal supplementation, the organs of excretory (kidney, lung, intestine), endocrine (hypophysis, adrenal and reproductive glands, ventral part of the prostate, epididymis), immune (thymus, spleen) system, metabolizing (liver) and such important organs as heart, brain, serum and blood clot containing erythrocytes and proteins were selected for the study [36–40].

The dynamics of NPs redistribution *in vivo* after the single injection of luminescent $GdVO_4:Eu^{3+}$ NPs is shown in Fig. 1. The semiquantitative analysis has revealed NPs accumulation mainly in the liver and spleen that is consistent with literature data *in vivo* distribution of NPs with similar characteristics [41], and reflects the significance of these organs in the body protection from xenobiotics, detoxification processes as well as the activation of immune system.

Metabolic parameters in the tissues are consistent with the data of NPs distribution. Most significant increase of NOx was found in the liver and spleen tissues induced by $GdVO_4:Eu^{3+}$ NPs as well as CeO_2 NPs (Fig. 2).

Non-enzymatic LPO in the liver tissue induced by the orthovanadate NPs was more expressed than the process intensified by the CeO_2 NPs (Fig. 3). It should be noted there were no significant changes of the parameters in the other investigated tissues.

The data evidenced that $GdVO_4:Eu^{3+}$ NPs injections induced the LPO strengthening in serum (Fig. 4). In contrast to this, the stabilization of state at the certain level within two-week period of CeO_2 NPs treatment was observed. In control groups significant

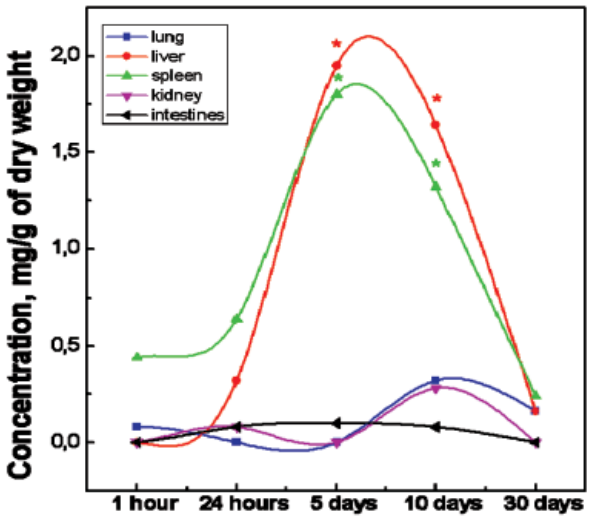

Fig. 1. **The semiquantitative analysis of luminescent $GdVO_4:Eu^{3+}$ NPs redistribution in tissues after the single injection:**
the luminescence intensity of other examined tissues was below the detection limit (not shown)
*$P < 0,05$ the differences of signal intensity significant versus background noise

fluctuations of the metabolic parameters were not observed.

Within the same period the changes in of NOx metabolism in blood serum, in response to the NPs $GdVO_4:Eu^{3+}$, may also be the evidence of OS deepening (Fig. 5, *A*). This increase, has a maximum on the 10^{th} injection, and was also significant for the level of nitrites (46%), and after 15 injections — 38% (Fig. 5, *B*, *C*). In contrast, NOx in serum was stable under the influence of CeO_2 NPs. Even the drop in NO metabolites was registered at the end of the experiment (Fig. 5, *B*, *C*).

The secondary regulatory mechanisms can prevent the negative effects of xenobiotics. Among inducible enzymes, SOD was remarkable increased under the action of CeO_2 NPs. Also that is possible that not only enzyme induction in response to the prooxidative effect of NPs takes place, but intrinsic SOD-mimetic activity of CeO_2 NPs define the final effect of NPs on oxidative metabolism as well. The greater increase in SOD activity under the action of CeO_2 (41%) as compared to the action of $GdVO_4:Eu^{3+}$ NPs (28%) (Fig. 6) can inhibit significantly the development of prooxidant

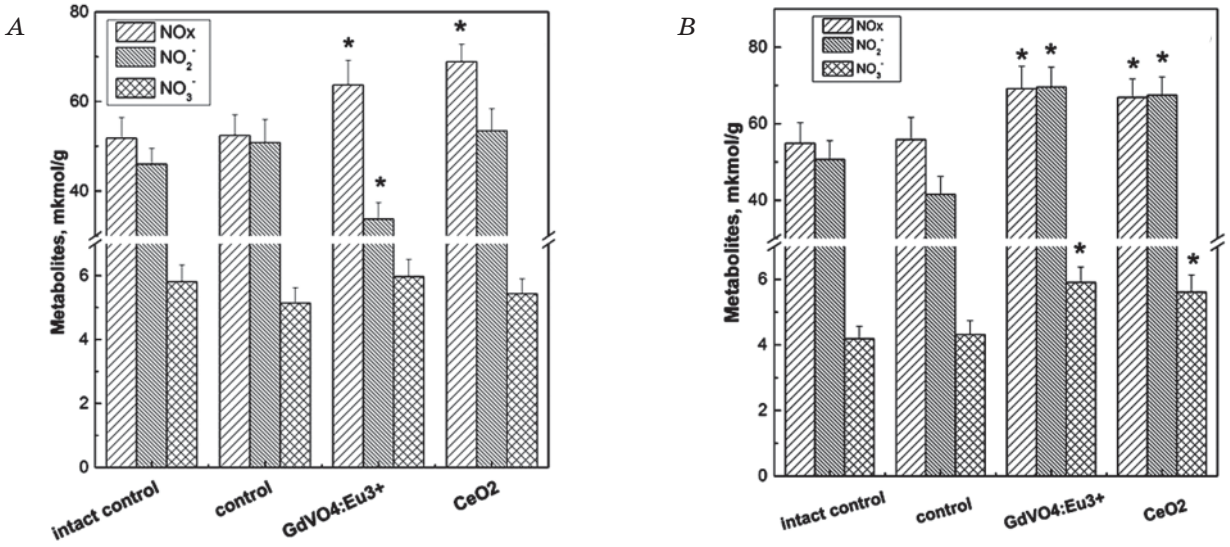

Fig. 2. **NPs influences on level of nitric oxide metabolites:**
A — liver; *B* — spleen; *hereafter* * $P < 0.05$, the differences significant versus control groups (differences between intact control and control groups are non significant)

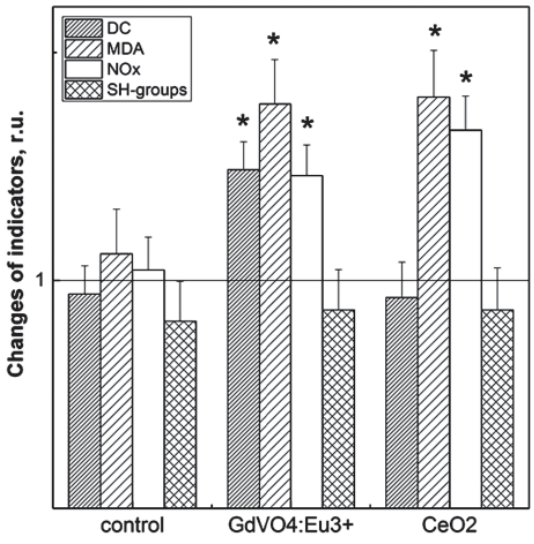

Fig. 3. **LPO parameters in liver:**
in relative units to the level of intact control group

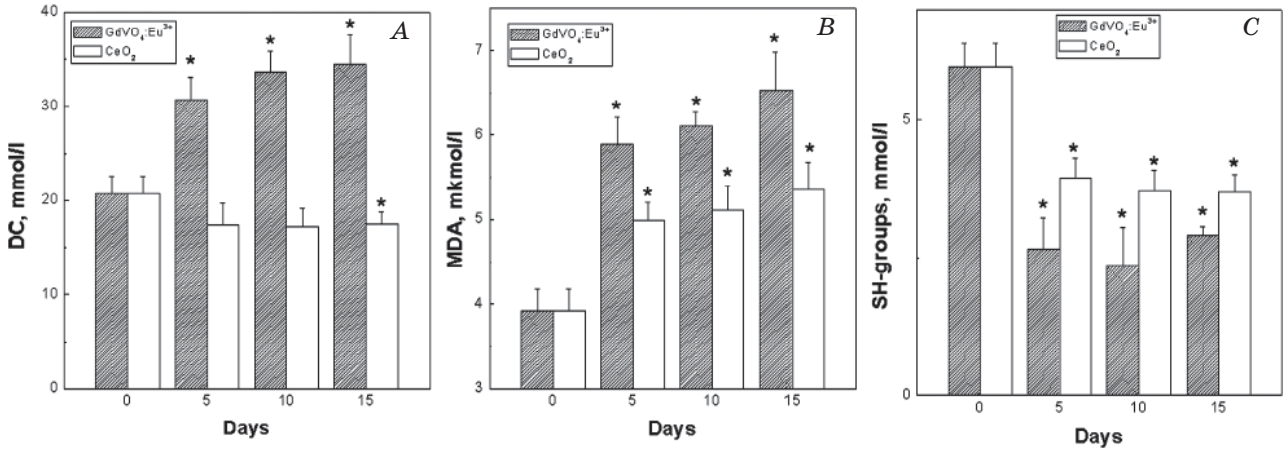

Fig. 4. **Biochemical parameters in blood serum:**
A — DC; *B* — MDA; *C* — SH-groups

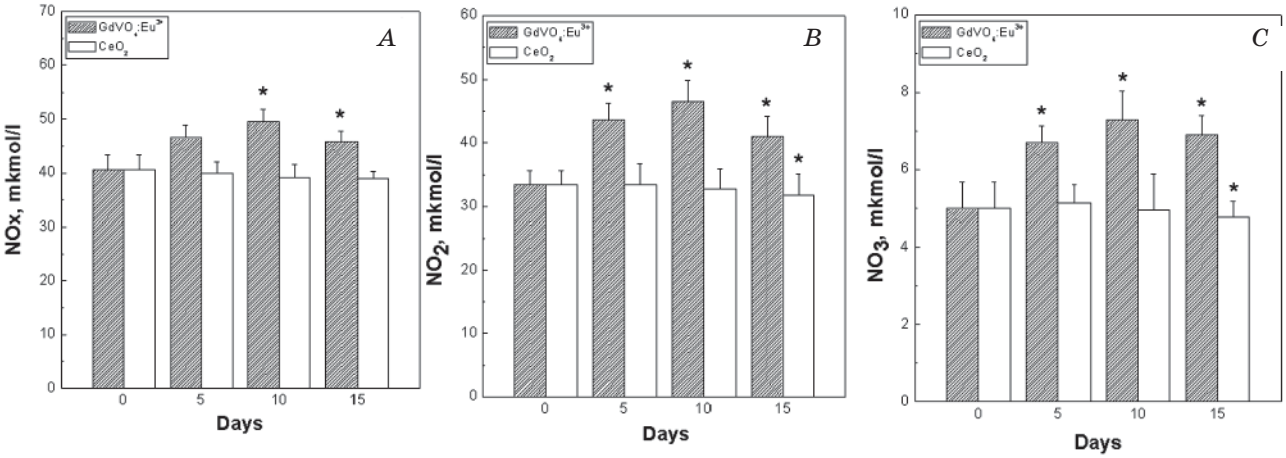

Fig. 5. **Nitric oxide level in blood serum:**
A — NOx; *B* — NO^{2-}; *C* — NO^{3-}

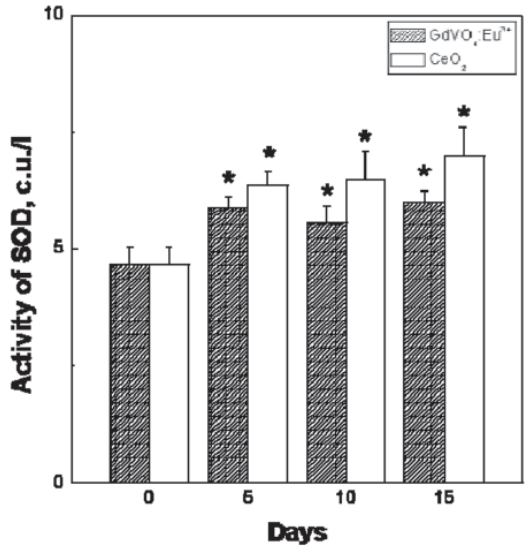

Fig. 6. **Dynamics of SOD activity in blood serum induced by the NPs**

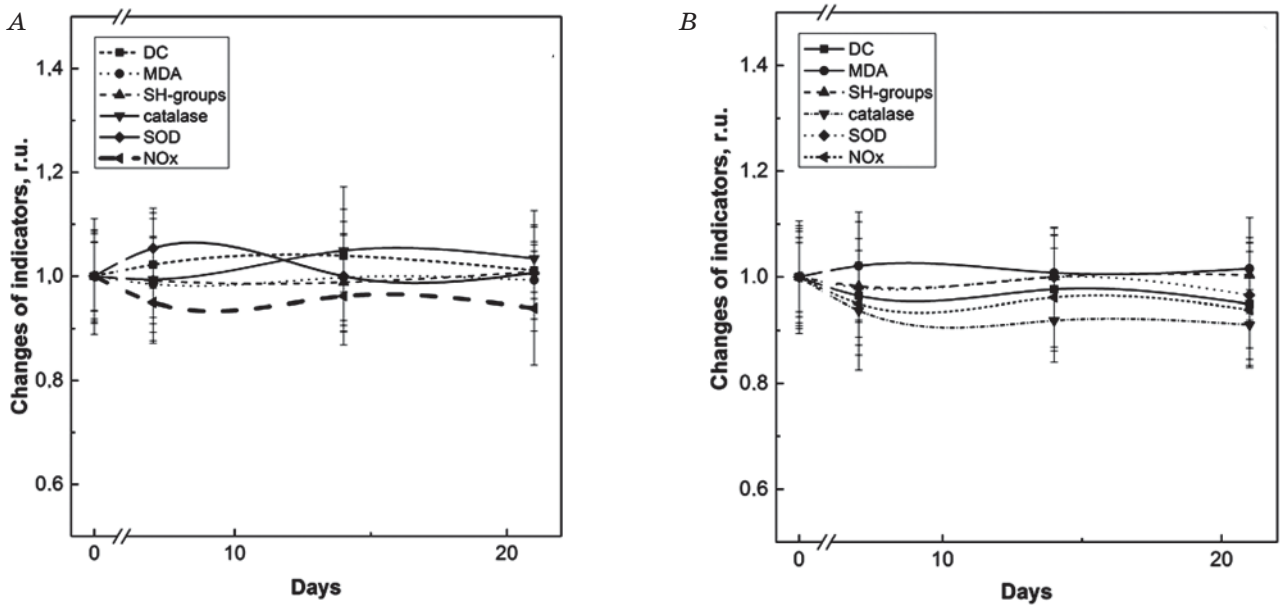

Fig. 7. **Aftereffect of NPs action on the parameters of oxidative balance:**
A — the orthovanadates NPs effect; *B* — the cerium oxide NPs effect (days after the last injections, at zero point- intact control as unit)

reactions, the accumulation of LPO derivatives and reduces the coefficient of oxidative stress (COS). COS reflects changes in the level of LPO derivatives and indexes of the antioxidants reactions [42]. That parameter was 6.89 for $GdVO_4:Eu^{3+}$ NPs at the end of experiment and only 2.04 for CeO_2 NPs.

Despite greater metabolic changes under the influence of $GdVO_4:Eu^{3+}$ NPs within two weeks of injection, in 7 days after the last injections of the both types of NPs any differences with intact organism was not registered (Fig. 7).

Temporary nature of changes in metabolism under the influence of NPs indicates rapid compensation of the oxidative imbalance induced by the NPs.

Thus we can summarize, that orthovanadate ($GdVO_4:Eu^{3+}$) and cerium oxide (CeO_2) NPs exerted free radical processes *in vivo* — LPO activation as well as antioxidant reactions. Starting from the early stage of NPs influence the output of peroxide products (DC and MDA) was registered. Such activation of free radical processes registered in tissues and serum causes significant changes in antioxidant reactions. In consistence with COS estimations, the prooxidant effect of $GdVO_4:Eu^{3+}$ NPs exceeded significantly the effect of CeO_2 NPs and the deepening of imbalance occurred over time. Unlike $GdVO_4:Eu^{3+}$ NPs, metabolic stabilization in response to the CeO_2 NPs lasted for two weeks. Furthermore, at the end of the experiment, the fall of DC level and significant increase in SOD activity was marked.

The injections of NPs of both types caused fast temporal changes in NO metabolism in liver and spleen, reflected by the increase in NOx content, nitrates and nitrites. But the opposite changes in NOx cycle in blood serum were observed in response to the action of $GdVO_4:Eu^{3+}$ NPs and CeO_2 NPs, which may reflect the peculiarities of influences. Thus, the increase in NOx level in blood plasma was observed at the influence of $GdVO_4:Eu^{3+}$ NPs only. Unlike that, the significant drop in the concentration of nitrates and nitrites in blood plasma was observed at the influence of CeO_2 NPs. The direct interaction of $NO^{\cdot}$ radicals with CeO_2 NPs and their neutralization, inhibition of gene expression or the activity of eNOS enzyme as well as the increase of endogenous inhibitors of NO synthase can be involved in observed effects. That requires detailed study of the mechanisms and regulatory ways involved in the process.

In addition the significant increase in SOD activity within two weeks at the influence of NPs of both types has antioxidant actions. The progression of the process under the influence of $GdVO_4:Eu^{3+}$ NPs is confirmed also by accumulation of LPO derivatives and by the calculations of COS.

In addition, luminescent properties allowed tracing dynamics of $GdVO_4:Eu^{3+}$ NPs in tissues.

The maximum of the NPs accumulation was observed in liver and spleen that correlated with fast changes of the analyzed indexes in these tissues. NPs in trace quantities were found in other examined tissues. NPs removal through the kidney and lungs was accompanied by a slight rise of NPs luminescence in these organs and occurred against a background of luminescence decrease in liver and spleen to 30th day after the injection of NPs.

Thus, it was found that the uptake of NPs of both types causes the oxidative imbalance. The more intense character of peroxidation processes is observed in the case of $GdVO_4$:Eu^{3+} NPs. *In vivo* response to the CeO_2 NPs can be additionally modulated by its own antioxidant activity (SOD-mimetic). A week after injection of NPs of both types, the complete recovery of the oxidative balance of the organism was noted, which maintained to the end of experiment (3 weeks after the last injection).

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In vivo ЕФЕКТ НАНОЧАСТИНОК НА ОСНОВІ РІДКІСНОЗЕМЕЛЬНИХ ЕЛЕМЕНТІВ ЩОДО ОКСИДАТИВНОГО БАЛАНСУ У ЩУРІВ

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Метою дослідження було з'ясування впливу наночастинок на основі рідкісноземельних елементів (CeO_2, $GdVO_4$:Eu^{3+}) на прооксидантно-антиоксидантний баланс організму щурів. Аналізували біохімічні маркери окисного стресу (рівень пероксидації ліпідів, метаболіти циклу оксиду азоту, вміст сульфгідрильних груп), а також активність ензимів (супероксиддисмутази, каталази) у тканинах щурів. Виявлено, що введення наночастинок обох видів спричинює збільшення вмісту метаболітів циклу оксиду азоту та продуктів пероксидного окиснення ліпідів у печінці й селезінці до 5-го дня експерименту. У сироватці крові вміст продуктів пероксидного окиснення, метаболітів оксиду азоту в разі введення наночастинок $GdVO_4$:Eu^{3+} на 5-, 10- і 15-й день досліду також був підвищеним, порівняно з вихідним станом і відповідним контролем, тоді як рівень сульфгідрильних груп знижувався. На відміну від цього, під впливом наночастинок CeO_2 рівень дієнових кон'югатів достовірно не змінювався, а рівень метаболітів оксиду азоту до 15-го дня експерименту — знижувався. У цей же період під впливом наночастинок обох типів активність супероксиддисмутази збільшувалась, а каталази — не змінювалась. Розрахунок коефіцієнта оксидативного стресу показав, що прооксидантний вплив наночастинок CeO_2 виражений слабше (2,04), ніж у $GdVO_4$:Eu^{3+} (6,89). У період післядії незалежно від типу наночастинок спостерігалося повне відновлення показників окисного балансу у тварин.

Ключові слова: наночастинки $GdVO_4$:Eu^{3+} та CeO_2, оксидативний баланс.

In vivo ЭФФЕКТ НАНОЧАСТИЦ НА ОСНОВЕ РЕДКОЗЕМЕЛЬНЫХ ЭЛЕМЕНТОВ НА ОКСИДАТИВНЫЙ БАЛАНС У КРЫС

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Целью исследования было выяснить влияние наночастиц на основе редкоземельных элементов (CeO_2, $GdVO_4$:Eu^{3+}) на прооксидантно-антиоксидантный баланс организма крыс. Анализировали биохимические маркеры окислительного стресса (уровень пероксидации липидов, метаболиты оксида азота, содержание сульфгидрильных групп), а также измеряли активность энзимов (супероксиддисмутазы, каталазы) в тканях крыс. Установлено, что введение наночастиц обоих видов вызывает увеличение содержания метаболитов цикла оксида азота и продуктов пероксидного окисления липидов в печени и селезенке к 5-му дню эксперимента. В сыворотке крови содержание продуктов пероксидного окисления, метаболитов оксида азота при введении наночастиц $GdVO_4$:Eu^{3+} на 5-, 10- и 15-й день опыта также было повышенным, по сравнению с исходным состоянием и контролем, в то время как уровень сульфгидрильных групп снижался. В отличие от этого, под влиянием наночастиц CeO_2 уровень диеновых конъюгатов достоверно не изменялся, а метаболитов оксида азота к 15-му дню эксперимента — снижался. В этот период под влиянием наночастиц обоих типов активность супероксиддисмутазы увеличивалась, а каталазы — не изменялась. Расчет коэффициента окислительного стресса показал, что прооксидантное влияние наночастиц CeO_2 выражено слабее (2,04), чем у $GdVO_4$:Eu^{3+} (6,89). В период последействия независимо от типа наночастиц наблюдалось полное восстановление показателей прооксидантно-антиоксидантного баланса у животных.

Ключевые слова: наночастицы $GdVO_4$:Eu^{3+} и CeO_2, оксидативный баланс.