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Effect of inorganic nanoparticles and organic complexes on their basis on free-radical processes in some model systems

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Aim. Evaluation of free–radical activity of rare–earth based nanoparticles (NPs) (orthovanadates and CeO_2) with different geometrical parameters, and organic complexes formed on their base with methylene blue (MB) photodynamic dye in abiotic and biotic systems (homogenate of liver, isolated mitochondria and isolated hepatocytes). **Methods**. Effects of NPs were estimated using luminol-dependent chemiluminescence (ChL) and by measurement of the final product of lipid peroxidation – malondialdehyde (MDA). **Results**. According to the ChL data in abiotic systems all NPs demonstrated antiradical activity. In biotic systems spherical extra small (1–2 nm) NPs of both types showed prooxidant effects of different degree; CeO_2 of 8–10 nm have demonstrated a week antioxidant effect. The data of ChL correlated with the measurements of MDA-level. The effects of «NP-MB» complexes were the same as the corresponding «bare» NPs in different examined systems. The most prooxidant NPs in the presence of glutathione (GSH) did not aggravate free-radical processes. NPs demonstrated a more pronounced prooxidant effect in cells at pH 7.8 that may be a result of pH-dependent changes in protonated GSH. **Conclusions.** Differences in the effects of NPs in the biotic systems depend on their geometric parameters that determine their penetration and interaction with the cellular structures. This is also related to the processes on the NPs surface as well as in the near-surface layers.

Keywords: nanoparticles, luminol-dependent chemiluminescence, prooxidant, antiradical.

Introduction

The understanding of the mechanisms of implementation of nanoparticles (NPs) and creation of NP-based structures providing necessary effects *in situ* are of great importance for solving a wide range of tasks in the nanomedicine area. The NPs size, shape, chemical composition, methods of synthesis, the presence of functional chemical groups on the NPs surface, heterogeneity and porosity, hydrophilicity and hydrophobicity, agglomeration state – all these factors determine the NPs reactivity [1, 2].

Biological and medical application of the rare-earth based NPs attracts a lot of attention [3]. Their luminescent properties allow *in vitro* and *in vivo* imaging. There is also a lot of reports about CeO_2 antioxidant properties [4, 5]; of special interest are those, which stated that CeO_2 acted similarly to superoxide dismutase (SOD) [6] and catalase [7, 8]. However, some other experiments demonstrated that the CeO_2 NPs produced reactive oxygen species (ROS), provoked inflammatory, lipid peroxidation, etc., and exerted cytotoxicity by the apoptotic process [9–11].

It is widely accepted that the NPs toxic effects can be mediated mainly by generation of free-radicals (induction of oxidative stress) when the particles interact with biological objects. NPs-induced oxidative stress is determined by their influence on the

mitochondrial functional state. Mitochondria (mitochondrial electron transport chain) are the primary sources and target of ROS in the cell [12], and they are very sensitive to oxidative damage. NPs can alter the mitochondrion function by embedding in a respiratory chain and absorbing/giving electrons or mechanically injuring the mitochondrion membranes.

The data obtained *in vitro* and *in vivo* can differ significantly, because the final effect of NPs depends considerably on the environment of the particles. Surface properties are very important factors in the NPs-bioobjects interaction. Thus, the final effect of the «NP-organic compound» hybrid complexes on the free radicals generation may differ from the NPs direct influence. For example, methylene blue (MB) is widely used in photodynamic therapy [13]. The «NPs-MB» complex can have an enhanced effect of phototherapy, whereas the inorganic component (NPs, possessing pronounced antioxidant properties like CeO_2) can protect healthy cells against further damage and reduce drug side effects. On the other hand, glutathione (GSH) plays a key role in the thiol antioxidant buffer system and in adaptive processes of the cell. It takes part in neutralization of peroxides of lipids, preserves SH-groups of proteins against oxidation, reduces S-S-binding induced by oxidative stress, is involved in the xenobiotics detoxication [14]. Thus, the NPs modification by MB or the NPs-GSH interaction is able to change completely the NPs properties and therapeutic effect.

For evaluation of NPs effects on the biosystem oxidative balance several methods are required. In the present research the NPs impact on the redox processes was investigated by the method of luminol-dependent chemiluminescence (ChL) with Fenton's reagent. Additionally, the end-products of lipid peroxidation – malondialdehyde (MDA) was measured.

Materials and Methods

Synthesis of NPs. In present research nanoparticles with different form-factors and sizes were used: spherical – $GdYVO_4:Eu^{3+}$ (1–2 nm), CeO_2 (1–2nm and 8–10 nm); spindle – $GdVO_4:Eu^{3+}$ (25 × 8 nm); rod-like – $LaVO_4:Eu^{3+}$ (57 × 6–8 nm). NPs were characterized using the transmission electron microscopy (TEM-125K electron microscope, Selmi, Ukraine) [15].

The synthesis of $nReVO_4:Eu^{3+}$ (Re = Gd, Y, La) and CeO_2 water colloidal solutions was carried out according to the method reported earlier [16, 17, 6]. The final concentration of NPs in each sample was 0.05 g/l.

Preparation of complexes NPs-MB. Preparation of «NPs-MB» complexes was performed in accordance to [18]. Briefly, water colloidal solution of NPs with concentration of 1 g/l was mixed with water solution of MB. After addition of NPs to MB solution the part of dye monomers (λ_{max} = 665 nm) decreased and part of H-type dimers (λ_{max} = 568 nm) increased that provides an evidence about the formation of «NPs-MB» hybrid particles [19]. Concentration of NPs in the solution was 0.5 g/l, MB – 10^{-4} M.

An ability of the complexes to influence the free-radical processes in biotic system was investigated using luminol-dependent ChL (described below).

Biological material preparation. Hepatocytes were isolated from male Wistar normal rats with body weight of 180–200 g by the method described earlier [20] in accordance with International Rules of «The European Convention for the protection of vertebrate animals used for experimental and other scientific purposes» (Strasbourg, 1986). The number of cells was counted and their viability was evaluated by trypan blue exclusion. In the experiments the preparations with viability higher than 90 %, homogenate of liver (cell-free system: after decapitation liver was elicited and placed in ice medium of homogenization – 0.05 M tris-buffer pH = 7.4; homogenate was filtered through the double layer of nylon), and isolated mitochondrion were used. Isolation of mitochondrion was performed using the differential centrifugation method ascribed elsewhere [21].

Luminol–dependent chemiluminescence. ChL was stimulated by Fenton's reagent. Abiotic system for estimation of the NPs ability to generate free radicals contained 0.05 M tris-buffer, pH = 7.4, 50 μM luminol, 10 μM Fe^{2+}; the investigated NPs with final concentration of 0.05 g/l. H_2O_2 (1.35 mM) were added to activate the reaction. The ChL spectra were measured using chemiluminometer Lum-5773 (Russia). The light sum and intensity of ChL spectra were measured during 5 min.

The biotic cell-free system for estimation of pro- or antioxidant properties of NPs, their complexes

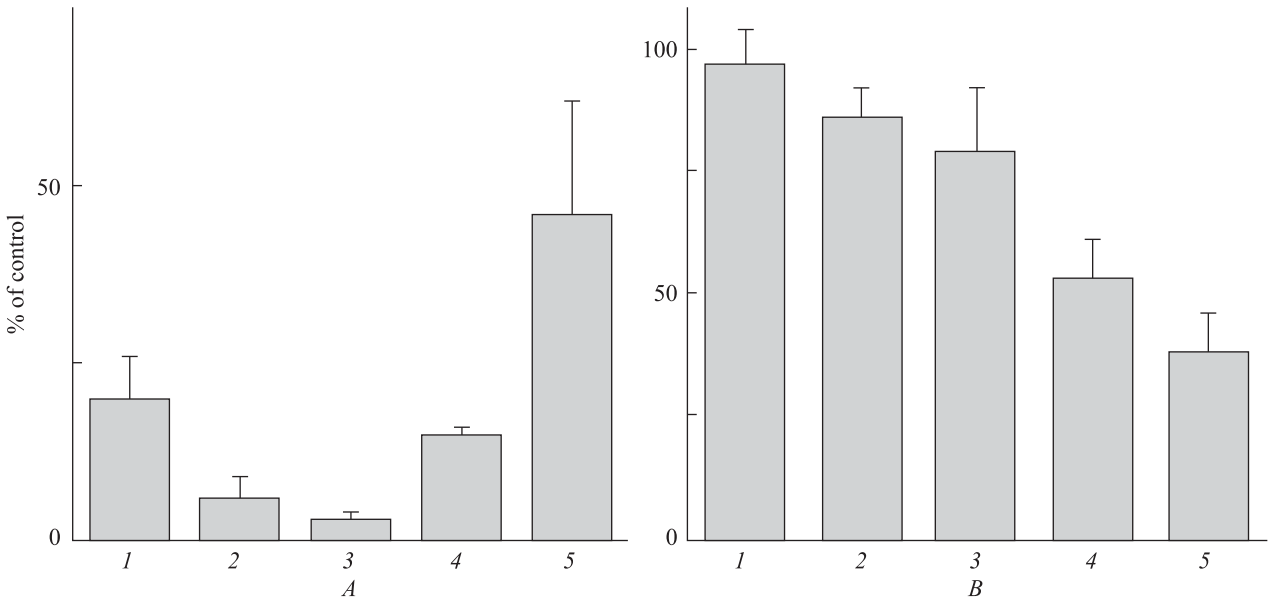

Fig. 1. Model systems: (*A*) – Chemiluminescence of Fenton's solution in the presence of nanoparticles. (*B*) – General antioxidant activity of NPs in the system Fe^{2+} – induced LP (level of MDA). *1* – spherical; *2* – spindle; *3* – rod-like; *4* – CeO_2 (1–2 nm); *5* – CeO_2 (8–10 nm)

«NPs-MB» (with final concentration of NPs 0.025 g/l) and effects of NPs in the presence of GSH (with final concentration of NPs 0.05 g/l and final concentration of GSH 0.5 mM) contained additionally homogenate of liver cells with final protein concentration of 70–80 μg/ml. Samples were incubated with NPs for 30 min at 37 °C and then placed into temperature-controlled cavity of chemiluminometer. ChL was measured as described above.

To estimate NPs influence on the oxidative balance of isolated hepatocytes, cells ($5x10^5$ cell/ml) were stained with the particles (final concentration was 0.05 g/l) in Krebs–Henseleit solution pH = 6.9, pH = = 7.4 and pH = 7.8 within 1 h. Then the mixture was centrifuged, the supernatant was removed and cells were replaced with 0.05 M tris-buffer, pH 7.4. Measurements of ChL were performed as described above.

Isolated mitochondrion (concentration of protein 42 μg/ml) after separation was resuspended in 0.1 M tris-buffer pH = 7.4. NPs (with final concentration of 0.05 g/l) were added, samples were incubated at 25 °C within 20 min and then placed in ice. Incubation of mitochondrion with NPs at 25 °C within 20 min is necessary for the development of effects of particles. It should be noted that no difference was observed during the experiment between the control samples (without NPs) which were kept at 25 °C within 20 min and the control (without NPs) samples stained in ice. Measurement of ChL was performed like for all systems described above.

Measurement of lipid peroxidation. The level of lipid peroxides, namely, MDA – a major end-product and an indicator of lipid peroxidation, was measured using the method described by Kumari et al. [22] with some modifications [23].

Measurement of total antioxidant activity of NPs. Each pattern contained: phosphate buffer pH = 7.45, 25 % suspension of lipoprotein of yolk, 25 mM Fe^{2+}, 0.3 % sodium dodecyl sulphate. NPs (with final concentration 0.05 g/l) were added to each sample (except control). The samples were heated in a water bath (37 °C) within 15 min and 20 % trichloroacetic acid and 0.01 M ionol were added to stop the reaction. The mixture was centrifuged, the supernatant fluid was removed and 1.8 ml of 0.5 % 2-thiobarbituric acid were added. The mixture was heated for 15 min at 100 °C in a water bath and cooled for 10 min to stop the reaction. The absorbance was measured

at 532 nm and 580 nm using SPECORD 200 («Analytik Jena») spectrometer. The results were presented as ΔD (percentage over the untreated control).

The results were averaged from more than three measurements and statistically processed by means of the software Statistika v. 5.0 (StatSoft, USA) using the Student's t-criterion. The results differed statistically and significantly at $p< 0.05$.

Results and Discussion

For all types of NPs the antiradical activity has been observed using luminol-dependent ChL in the abiotic model system of Fenton stimulated reaction (Fig. 1, *A*). Measurement of the total antioxidant activity of NPs in the system of Fe^{2+}-induced lipid peroxidation (LP) revealed that CeO_2 NPs of both sizes reduced MDA level most significantly (Fig. 1, *B*). In spite of the antiradical activity detected in the abiotic system, investigation of free radical processes in liver homogenates preincubated with NPs showed a considerable increase of light sum for spherical orthovanadate particles. Extra small (1–2 nm) CeO_2 NPs also demonstrated prooxidant effects (Fig. 2, *A*). In isolated hepatocytes only the CeO_2 NPs (8–10 nm) keep the antiradical effect as compared to other NPs (Fig. 2, *B*), whereas spherical orthovanadate NPs have the most pronounced prooxidant effect. These data correlated with the results of MDA determination in isolated hepatocytes, for both types of extra small NPs (spherical orthovanadates and CeO_2) the prooxidant effect was also revealed (Fig. 2, *C*). At the same time, the antioxidant effect of CeO_2 (8–10 nm) was reproduced.

It is known from the literature that the mitochondrion is a general target for NPs in cells [24]. Their abnormal functioning under influence of NPs led to an increase of ROS and dysfunction of cells in general. In the case of extra small (1–2 nm) NPs we can expect that particles interact directly with membranes of mitochondria and injure them, provoke dysfunction of membrane pores and synthesis of ATP, etc. As can be seen from the Fig. 3, in the case of investigation of isolated mitochondrion by means of ChL, strong prooxidant effect of spherical orthovanadate NPs is reproduced.

Effects of NPs and their complexes with MB in both abiotic (Fig 4, *A*) and biotic (Fig. 4, *B*) systems, like in the case of unmodified NPs, differ significantly. It was shown that antiradical effects of all types of NPs in the abiotic system practically did not depend on the presence of organic constituent in the complexes. So, the microenvironment itself plays a key role in reactivity of NPs though any enhancement of prooxidant effect of spherical orthovanadate NPs at including MB into the nanocomplex was not achieved. In photodynamic therapy the therapeutic effect was achieved by irradiation at certain wavelengths and intensities. The absence of required conditions in the presented case may be a reason that complexes «NPs-MB» have no effects in the biotic system. This question is under investigation now.

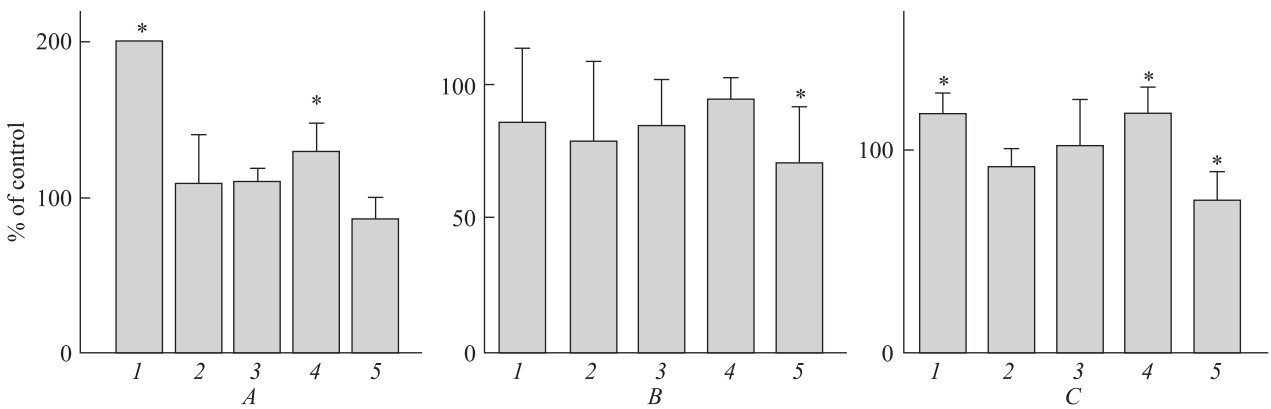

Fig. 2. (*A*) – Chemiluminescence of liver homogenate after 30 min and (*B*) hepatocytes after 1h exposure to NPs; (*C*) – MDA formation in isolated hepatocytes treated with NPs within 1 h (*$p < 0.05$ compared to control). *1* – spherical; *2* – spindle; *3* – rod-like; *4* – CeO_2 (1–2 nm); *5* – CeO_2 (8–10 nm)

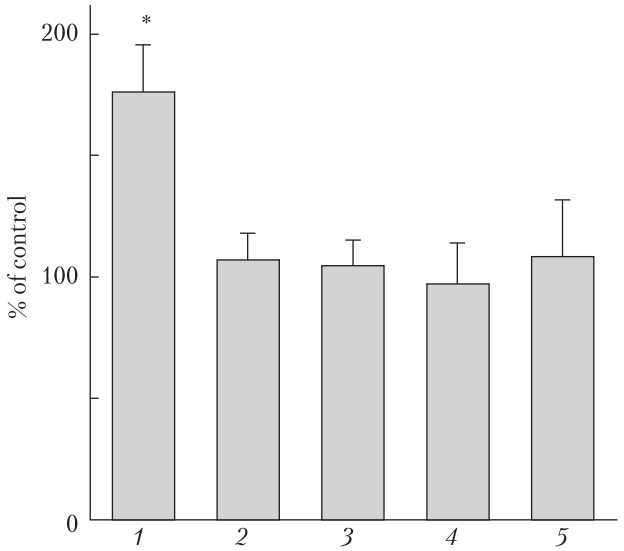

Fig. 3. Chemiluminescence of isolated mitochondrion after 20 min exposure to NPs at 25 °C (* $p < 0.05$ compared to control). *1* – spherical; *2* – spindle; *3* – rod-like; *4* – CeO_2 (1–2 nm); *5* – CeO_2 (8–10 nm)

Extra small NPs able to activate free-radical processes (spherical orthovanadate and CeO_2 1–2 nm particles) (Fig. 2, 3), in the presence of exogenous GSH did not show any prooxidant effect (Fig. 5). It is possible that the prooxidant effects of NPs in biosystems may be related first of all to the changes in GSH/GSSG (GSSG – oxidized form of glutathione) ratio due to direct interaction of NPs with reduced or/and oxidized form of compound or as a result of detoxication processes which are catalyzed by glutathione S-transferase. This process occurrs via transport of sulfur atom into compounds and formation of mercaptides, mercapturic acid, derivatives of GSH with the substances. This process is especially active in liver and, probably, provides the NPs neutralization.

It is known that high levels of reduced GSH and acidic conditions are associated with diminished chemical lethality, the influence of these parameters on the cellular response to oxidative stress was evaluated earlier [25]. It was shown that the oxidation of DCFH and 2-deoxyribose was inhibited by GSH, with about 4 times stronger inhibition efficacy at pH 6.8 than at pH 7.4. Thus, the authors concluded that the protonated form of GSH was more likely the inhibitory species. We have investigated an NPs influence on free-radical processes in isolated hepatocytes at physiological range of pH from 6.9 to 7.8.

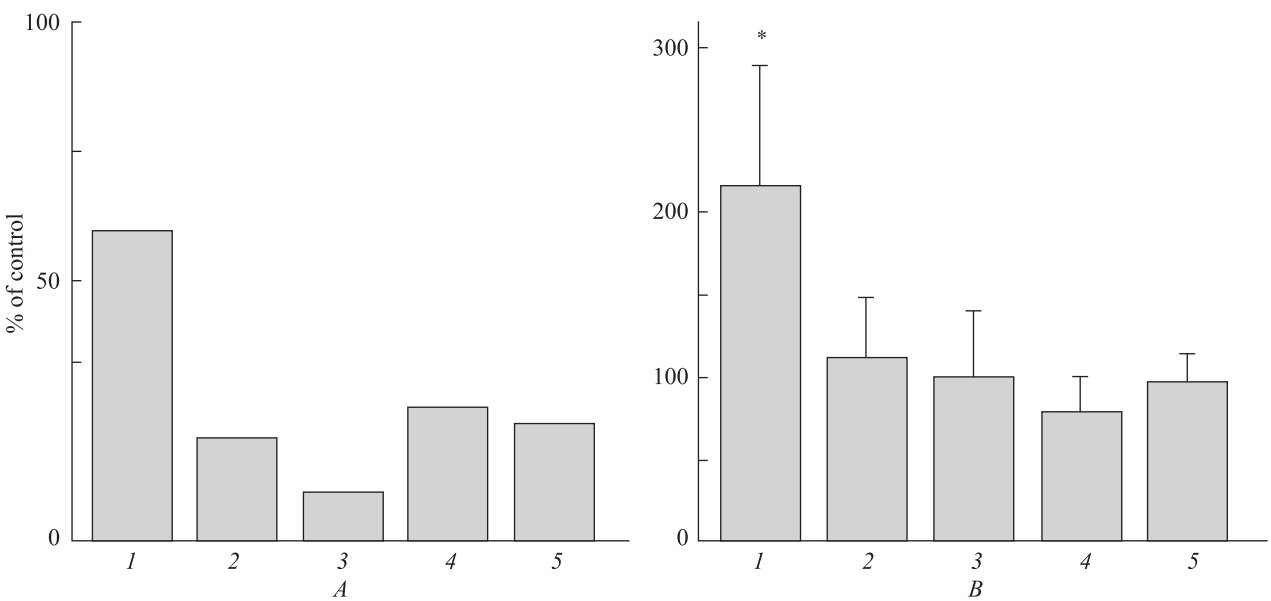

Fig. 4. (*A*) – Chemiluminescence with Fenton's reagent in abiotic system in the presence of complexes «NPs-MB»; (*B*) – Chemiluminescence of homogenate of hepatocytes after exposure to «NPs-MB» complexes. (*$p < 0.05$ compared to control). *1* – spherical + + MB; *2* – spindle + MB; *3* – rod-like + MB; *4* – CeO_2 (1–2 nm) + MB; *5* – CeO_2 (8–10 nm) + MB

The data have demonstrated a pH-dependent increase of MDA-level in cells after treatment with NPs (Fig. 6, *A*). This tendency was preserved when we analysed Chl in cells in the same conditions (Fig. 6, *B*). So, pH-dependent changes in the GSH redox balance is the base of higher level of oxidative disturbance induced by NPs at pH 7.8.

On the other hand, an inclination of extra small NPs to aggregation is probably a more important factor of damaging influence of NPs in biological systems. In this case we assume that such effect of NPs ($GdYVO_4:Eu^{3+}$ and CeO_2) can be explained by changing of the NPs agglomeration state [2] and mechanical injury of cellular structures. The virtual absence of NPs effect on isolated hepatocytes at physiological pH = 7.4 in the present experimental condition can be associated with a stronger integrity of antioxidant defense systems in cells as compared to other model biotic systems.

So, the final effect of NPs in living systems cannot be explained only by the structure of material, shape, covering, exposure time, or dose. At different structural levels of living systems the final effect of NPs depends on the microenvironment properties, adaptive and protective processes in response to the presence of NPs. The features of NPs influence must be considered since it is one of the most important characteristics of the toxicity of nanomaterials.

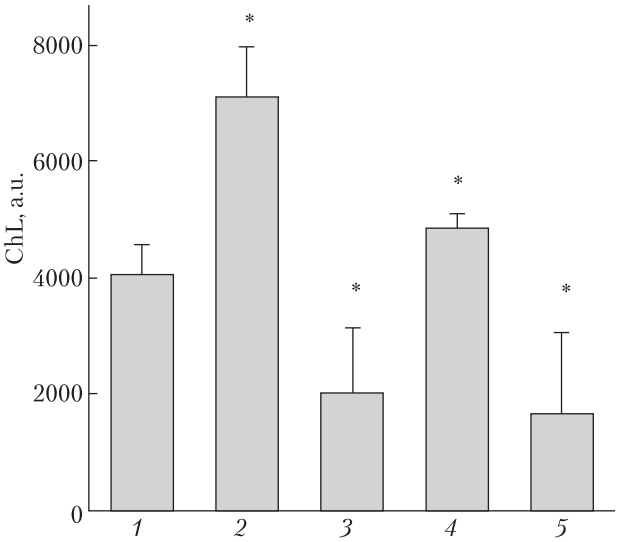

Fig. 5. Chemiluminescence of liver homogenate after exposure to NPs in the presence of exogenous GSH. (*p < 0.05 compared to control). *1* – control; *2* – spherical; *3* – spher + GSH; *4* – CeO_2 (1–2 nm); *5* – CeO_2 (1–2 nm) + GSH

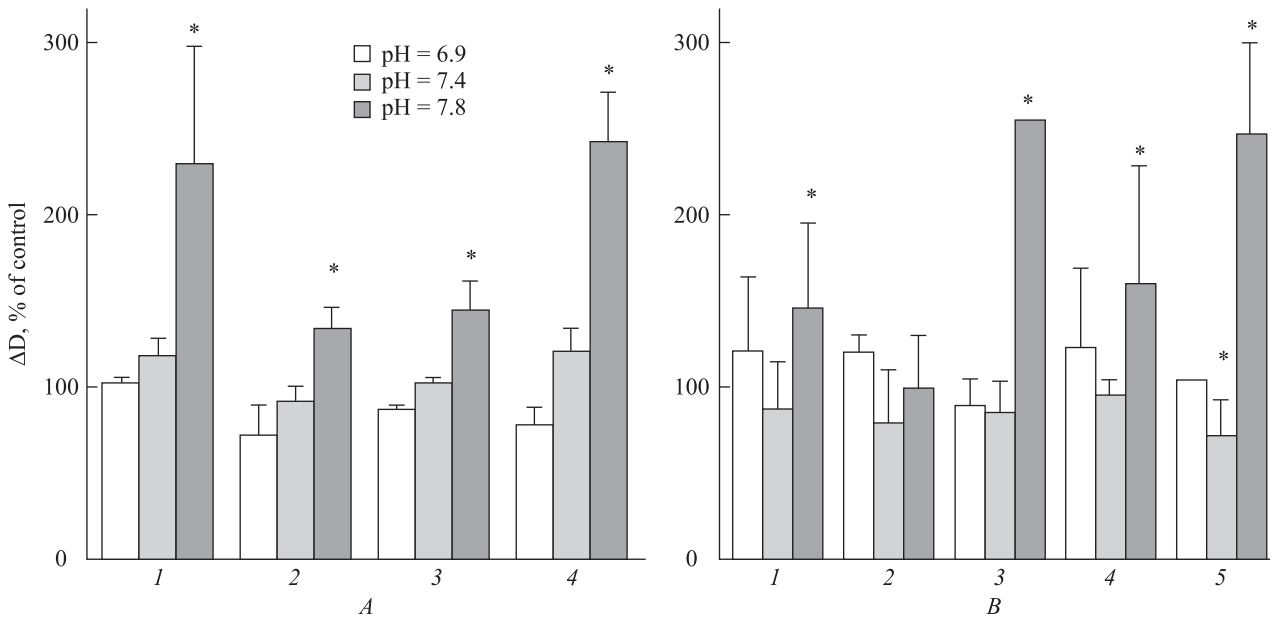

Fig. 6. (*A*) – Effect of NPs on MDA-level in isolated hepatocytes at different pH; (*B*) – Effect of NPs on the light sum of chemiluminescence in isolated hepatocytes at different pH. (*p < 0.05 compared to control). *1* – spherical; *2* – spindle; *3* – rod-like; *4* – CeO_2 (1–2 nm); *5* – CeO_2 (8–10 nm)

Conclusions

During interaction of nanoparticles with the cellular structures the final prooxidant or antioxidant effect may be determined not only by the properties of the particles, but also by their microenvironment in a biosystem. The expressed prooxidant activity of extra small NPs can also be associated with the changes of aggregative properties and high reactivity of these particles, the way they interact with nanoscale cellular structures or ability of NPs to change the balance of antioxidant defence system.

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Вплив неорганічних наночастинок та органічних комплексів на їх основі на вільнорадикальні процеси в деяких модельних системах

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Мета. Оцінити вільнорадикальну активність наночастинок (НЧ) на основі рідкісноземельних елементів – ортованадатів та CeO_2 з різними геометричними параметрами і органічних комплексів на їх основі з фотодинамічним барвником метиленовим блакитним (МБ) в абіотичних і біотичних системах (гомогенат печінки, ізольовані мітохондрії, ізольовані гепатоцити). **Методи.** Ефекти НЧ оцінювали за допомогою люмінол-залежної хемілюмінесценції (ХЛ), а також вимірюючи рівень малонового диальдегыду (МДА) – кінцевого продукту пероксидації ліпідів. **Результати.** ХЛ показано, що в абіотичній системі всі НЧ демонструють антирадикальну активність. В біотичній системі сферичні екстрамалі (1–2 нм) НЧ обох типів різною мірою демонструють прооксидантну активність; CeO_2 розміром 8–10 нм демонстрував слабкій антиоксидантній ефект. Дані ХЛ корелюють з результатами, отриманими при вимірюванні рівня МДА. Ефект комплексів «НЧ-МБ» був таким же, як і у випадку «голих» НЧ. Найбільш прооксидантні НЧу присутності глутатіону (GSH) не посилювали вільно-радикальні процеси. В клітинах при pH = 7.8 НЧ демонстрували очікувані прооксидантні ефекти, що може бути пов'язано з pH-залежними змінами протонованого GSH. **Висновки.** Відмінності ефектів НЧ пояснюються їх геометричними параметрами, які впливають на проникнення і взаємодію частинок з клітинними структурами. Також це пов'язано з процесами, що проходять як на поверхні НЧ, так і в приповерхневому шарі.

Ключові слова: наночастинки, люмінол-залежна хемілюмінесценція, прооксидантний, антирадикальний.

Влияние неорганических наночастиц и органических комплексов на их основе на свободнорадикальные процессы в некоторых модельных системах

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Цель. Оценить свободнорадикальную активность наночастиц (НЧ) на основе редкоземельных элементов – ортованадатов и CeO_2 с различным геометрическими параметрами и органических комплексов на их основе с фотодинамическим красителем метиленовым голубым (МГ) в абиотических и биотических системах (гомогенат печени, изолированные митохондрии, изолированные гепатоциты). **Методы.** Эффекты НЧ оценивали с помощью люминол-зависимой хемилюминесценции (ХЛ), а также измеряя уровень малонового диальдегида (МДА) – конечного продукта пероксидации липидов. **Результаты.** ХЛ было показано, что в абиотической системе все НЧ демонстрируют антирадикальную активность. В биотической системе сферические экстрамалые (1–2 нм) НЧ обоих типов в разной степени демонстрируют прооксидантную активность; CeO_2 размером 8–10 нм демонстрирует слабый антиоксидантный эффект. Данные ХЛ коррелируют с данными, полученными при измерении уровня МДА. Эффект комплексов «НЧ-МГ» был таким же, как и в случае «голых» НЧ. Наиболее прооксидантные НЧ в присутствии глутатиона (GSH) не усугубляли свободно-радикальные процессы. В клетках при pH = 7.8 НЧ демонстрировали ожидаемые прооксидантные эффекты, что может быть связано с pH-зависимыми изменениями протнированного GSH. **Выводы.** Различия эффектов НЧ объясняются их геометрическими параметрами, которые влияют на проникновение и взаимодействие частиц с клеточными структурами. Также это связано с процессами, проходящими как на поверхности НЧ, так и в приповерхностном слое.

Ключевые слова: наночастицы, люминол-зависимая хемилюминесценция, прооксидантый, антирадикальный.

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