УДК 544.773.422:546.05-06 + 579.864.1:615.331

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ANTIBACTERIAL ACTIVITY OF CERIUM COLLOIDS AGAINST OPPORTUNISTIC MICROORGANISMS *IN VITRO*

The CeO_2 sol with the size of nanoparticles 2-4 nm has been synthesized. It has been determined that the synthesized nanocrystalline cerium has antibacterial activity in vitro against different groups of opportunistic microorganisms: clinical strains of Escherichia coli, Staphylococcus aureus and Candida albicans. The rate of viability depression of test-cultures depends on the concentration of cerium dioxide nanoparticles and time of incubation. It is shown that the sol interacts with the bacterial cell surface. It is suggested that the observed differences of antibacterial action of nanocrystalline cerium dioxide can be related to the structural characteristics of the cell surface.

K e y w o r d s: Escherichia coli, Staphylococcus aureus, Candida albicans, nanocrystalline cerium dioxide, antibacterial effect.

Nanopharmaceutics is an interdisciplinary area, that was formed to address the biological and medical problems with the use of new materials based on nanotechnology. Nanocrystalline cerium is one of these advanced materials. The possibilities of biomedical application of CeO_2 are presented in detail in reviews [1, 2]. In particular, we described for the first time the antiviral effects of nanocrystalline cerium [3, 4] and showed that the joint use of CeO_2 and *Lactobacillus bulgaricus* significantly increases probiotic properties of this microorganism [1]. On the other hand, literature data indicates the antibacterial activity as that typical of cerium dioxide nanoparticles [5, 6].

The aim of our work was to study antibacterial properties of CeO_2 *in vitro* against opportunistic microorganisms of different groups – clinical strains of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* by determining the rate of inhibition of the test cultures viability and visualization of the process of their interaction.

Materials and Methods. We have used cerium dioxide nanoparticles ~ 4.2 nm in size, that were synthesized by the method described earlier [7]. To 200 ml of a solution containing 3.26 g of $Ce(NO_3)_3$ (~0.01M) was added 1 g of citric acid (~ 0.005M), pH was adjusted to ~ 10.0 with aqueous ammonia (3M). The resulting light brown solution was refluxed for 8 h and then adjusted to pH= ~ 4.5 with 0.01 M nitric acid. Nanoparticles of cerium dioxide were separated by decantation and washed several times with a filter. The precipitate was transferred to a flask and dissolved in 200 ml of water; pH was adjusted to ~ 10 with aqueous ammonia. In the resulting clear sol was injected 10 ml of 50% solution of hydrogen peroxide and it was refluxed for 3 h. According to transmission electron microscopy (TEM) and electron diffraction (microscope Leo 912 AB Omega, magnification of 500000×) the obtained nanoparticles have high crystallinity and monodispersity (Fig. 1). In the UV-visible spectrum of the solution (spectrometer OceanOptics QE-65000) the absorption band was observed at 275-285 nm, which corresponds to the width of the band gap equal to 3.55-3.6 eV. According to dynamic light scattering (NanoSizer, Malvern Instruments) the hydrodynamic diameter of the obtained particles is about 7 nm, and zeta potential – about minus 20 mV (Fig. 2).

The study was performed using three cultures of opportunistic microorganisms, such as clinical strains of *S. aureus* 8325-4 (kindly provided to us by Professor V.S. Zuyeva, the N.F. Gamaleya Institute of Epidemiology and Microbiology, Russian Federation; the strain has plasmid-based resistance to gentamicin), *E. coli* UCM B-930 and *C. albicans* UCM Y-690 from the microorganism collection of IMV NAS of Ukraine. Cultures were previously grown on the elective solid nutrient media: *E. coli* on the ENDO medium (NSCAMB, Obolensk, Russia), *S. aureus* on the BAIRD-PARKER-Agar (Merck, Germany), *C. albicans* on the Sabouraud medium at 37 °C for 24 h.

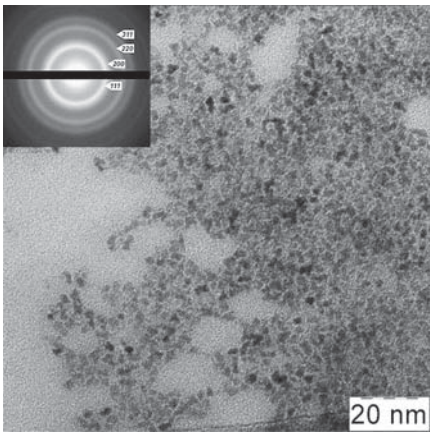

Fig. 1. Electron-microscopic image and diffraction of cerium dioxide nanoparticles

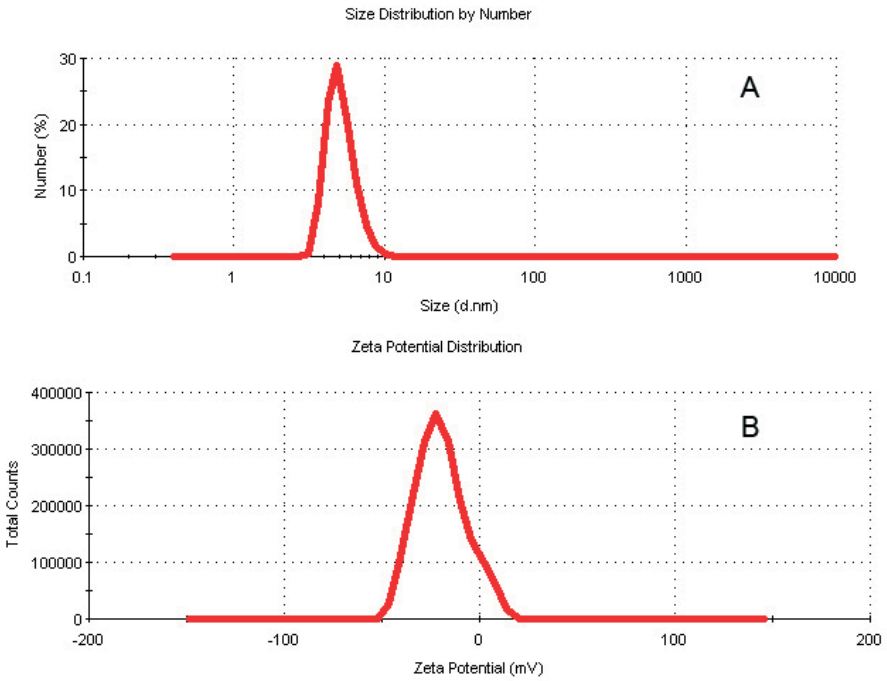

Fig.2. Dynamic light scattering data: A – distribution of the hydrodynamic diameter of cerium dioxide particles; B – distribution of particles zeta potential

To determine the antimicrobial action of cerium nanoparticles, daily culture of *E. coli*, *S. aureus* and *C. albicans* were washed from agar medium with sterile saline, twice washed by centrifugation at 3000 rpm for 10 minutes and then resuspended in saline [8]. To obtain the final concentration of cerium dioxide 1:10, 1:100, 1:1000 from the initial, the required volume of the nanocrystalline cerium solution was added to the tubes with the microbial suspension (the concentration of microorganisms was 1×10^5 cells / ml). Suspension of microorganisms without adding CeO_2 was used as a control. All tubes were kept in a thermostat at a temperature of 37 °C. After 1, 3, 6, 12 and 24 hours of incubation, 100 ml of the suspension was collected from each sample and plated onto a Petri dish with elective agar medium [9].

Electron-microscopic examination was carried out to determine the interaction between cerium dioxide nanoparticles and opportunistic microorganisms [11, 12]. The suspension of microbial cells was prepared by a similar scheme. The concentration of each culture was 1×10^6 cells / ml. To the tubes that contained 1 ml of microbial suspension was added an equal volume of initial cerium

dioxide sol to obtain the final concentration of nanoparticles. Saline in the amount of 1 ml was added to the control samples instead of cerium dioxide nanoparticles [10]. Test tubes were incubated in a thermostat at 37 ° C, samples were taken after 15, 60 and 180 minutes of incubation. Electron microscopy of samples was performed at 80 keV. Voltage was chosen to prevent any violation of the cells during the study. As it was noted above, during the preparation of samples for electron microscopy, saline was used instead of distilled water to maintain cell viability, due to the duration of the longest incubation period (3 hours).

Statistical data processing was carried out in accordance with the recommendations [13, 14]. In the case of processing the obtained results using the computer program Origin pro 8.5 by variation statistics, numerical data were presented as arithmetic means and standard errors (M ± m). Numerical data obtained using the software package BioStat 2009 Professional 5.8.1 were presented as median, first and third interquartile range.

Results and Discussion. Investigations of the antimicrobial action of nanocrystalline cerium were performed using three cultures of opportunistic microorganisms: *S. aureus*, *C. albicans* and *E coli*. Significant differences were detected in sensitivity of various opportunistic microorganisms to the presence of CeO_2 in saline (Fig. 3). Thus, the presence of CeO_2 hardly oppressed viability of *E. coli*. A certain (relative to accurate values of control – 2.79 ± 0.09 lg CFU / ml) reduction in the number of microorganisms in the suspension in the presence of 1.0 and 0.1 mM CeO_2 was detected only after 24 h of incubation (1.11 ± 0.02 and 1.92 ± 0.07 lg CFU / ml respectively ($P < 0.01$)). The lack of the reliable effect of growth inhibition of microorganisms in the presence of nanocrystalline cerium in concentration of 10.0 mM CeO_2 should be noted, that may be associated with a high concentration of particles and their agglomeration that defined the ability of Ce^{3+} to interact with phosphates.

S. aureus was highly sensitive to the presence of cerium dioxide nanoparticles in saline. The highest concentrations of nanocrystalline cerium, taken in the study, was ineffective as in the case of *E. coli*, but the addition of 1.0 mM CeO_2 reduced the number of viable microorganisms (significant relative to the control values – 3.09 ± 0.23 lg CFU / ml) already at the 3rd hour: 2.55 ± 0.11 lg CFU / ml ($P < 0.01$). With the increase of cultivation time, a gradual reduction in the number of live *S. aureus* was observed. After 24-hour contact the number of microorganisms was 1.53 ± 0.07 lg CFU / ml. A tenfold decrease in the concentration of CeO_2 was accompanied by the loss of identified effect.

C. albicans was most sensitive to the presence of nanocrystalline cerium in saline. If the cells concentration in a control suspension was 3.05 ± 0.20 lg CFU / ml, a significant reduction in the number of viable cells was observed in the presence of 1.0 mM CeO_2 already after the first hour of contact: 2.49 ± 0.07 lg CFU / ml ($P < 0.01$). The effect of reducing the number of viable cells in the suspension was found for all the concentrations of nanocrystalline cerium at the 3rd hour of incubation. Further cultivation of *C.albicans* in the presence of 1.0 mM CeO_2 nanoparticles was accompanied by complete inhibition of their viability. Higher concentration of CeO_2 (10.0 mM) completely oppressed the ability of *C. albicans* to grow after 12 hours of contact. Unlike *E. coli* and *S. aureus*, the lowest concentration of nanocrystalline cerium (0.1 mM) effectively reduced the viability of *C. albicans*: after 24 hours of contact the number of living cells was an order of magnitude lower than in controls and was 1.96 ± 0.02 lg CFU / ml.

Thus, among the studied opportunistic bacteria *C. albicans* were most sensitive to the presence of synthesized nanocrystalline cerium. The presence of CeO_2 reduced less effectively the viability of *S. aureus* and practically did not change the viability of *E. coli*. When analyzing the results it can be taken into account that the investigated microorganisms were characterized by different composition of the cell wall. *E. coli*, which belong to the gram-negative bacteria, have a more complex structure of the shell: except for the outer layer of murein, peptidoglycan layer is located in periplasm and outer membrane containing lipopolysaccharide. Preservation of stability of lipopolysaccharide layer is provided by divalent calcium cations. No significant impact of nanocrystalline cerium on the viability of *E.coli* can be caused by many reasons: first, similar to calcium ions stabilizing influence of CeO_2 on the lipopolysaccharide membrane of bacteria. In addition, the presence of periplasm space, where the splitting of the majority of nutrients entering the bacterial cell take place, providing some autonomic metabolism and prevents direct contact between nanocrystalline cerium and cells membrane.

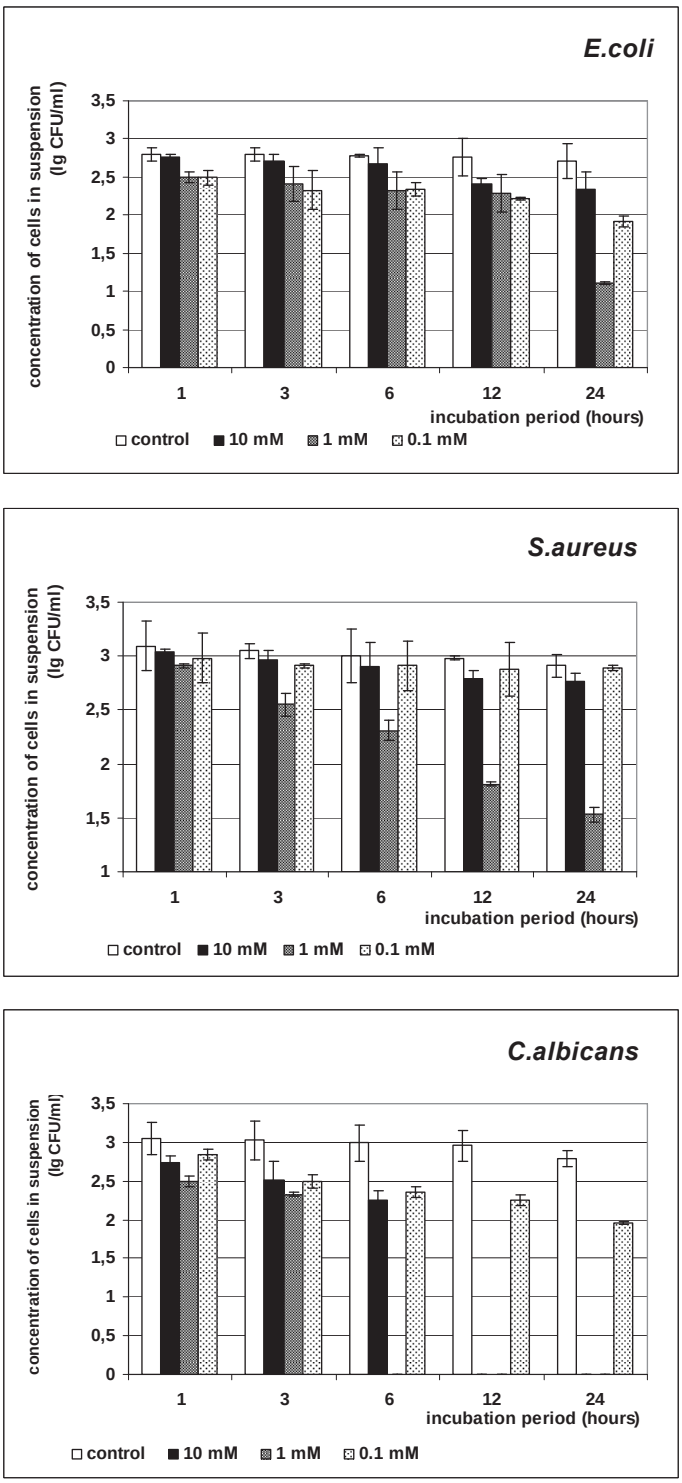

Fig. 3. Effect of different concentrations of CeO_2 on the growth of opportunistic microorganisms

In gram-positive bacteria, that include *S.aureus*, all the enzymes are allocated to the environment. Nutrients are coming and metabolic products are standing out through the cell wall that has pores 5-6 nm in size. A characteristic feature is the presence of teichoic acids: chains that consist of 8-50 residues of glycerol or rybitol, interconnected by phosphate bridges. Using phosphates, teichoic acids demonstrate amide-type binding to the murein [15]. In this regard, the presence of CeO_2 may

inhibit the metabolism of bacterial cells, resulting in a decrease in their viability we have found.

Unlike bacteria, the cell wall of *C. albicans* has not got a murein skeleton, the shell is represented by carbohydrate fibrillar matrix (glucan) and a marker polymer – chitin (polymer of N-acetylglucosamine). It is known that an important component of apical growth of mycelium is the balance between synthesis and lysis of the cell wall [16] chitinase and chitin synthetase activity [8]. The interaction between CeO_2 and components of the cell wall of *C. albicans* probably causes the irreversible changes in the latter, and as a result – blocking capabilities of the fungus own enzymatic activity and complete loss of its viability.

For further detailing the impact of nanoparticles of cerium dioxide on the opportunistic microorganisms their electron-microscopic study was carried out. The images of control samples of microorganisms that were not subjected to the influence of nanocrystalline cerium showed that the experiment conditions (centrifugation and the use of saline instead of distillate) make some changes to the appearance of cells without causing their destruction, and single crystals of sodium chloride are also present on the surface of cells.

If in the control samples of *E. coli* cells (Fig. 4, a) the contamination with salt crystals without violation the integrity of the cells is visible, after fifteen minutes of culture incubation with CeO_2 the redistribution of nanoparticles in the suspension can be noted: their concentration in the environment reduced, and the cells formed around the electron-dense layer 70-120 nm thick (Fig. 4, b). It should be noted that this layer is more dense and thick at the cellular pole. A similar pattern is observed after one hour incubation of bacteria: CeO_2 are unevenly located along the cell membrane and concentrated at the cellular pole (Fig. 4, b). Perhaps this effect is associated with the redistribution of cell components that are also concentrated at the cellular pole [17, 18].

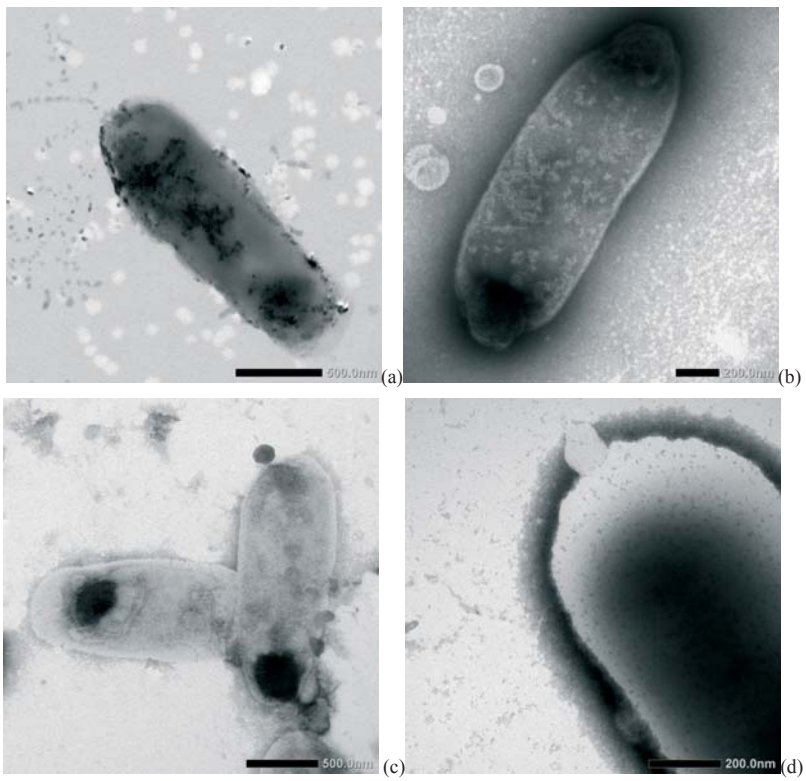

Fig. 4. Electron-microscopic images of interactions between *E. coli* UCM B-930 cells and CeO_2

(a) – culture control in saline;

(b) – incubation with cerium dioxide nanoparticles for 15 min.;

(c) – incubation with cerium dioxide nanoparticles for 60 min.;

(d) – incubation with cerium dioxide nanoparticles for 180 min.

After three hours of incubation, the layer of CeO_2 around the cells is more dense and uniform, with

no space between the cell membrane, its average thickness is 40-70 nm. Remarkable accumulation of CeO_2 in the central part of cells may be associated with nanoparticles ability to penetrate inside. Unfortunately, the technique used for the samples preparation for electron microscopy does not allow to state that the CeO_2 are located inside the cells rather than on its surface. Clarification of this issue is a task of our future studies.

S. aureus cells (Fig. 5, a) in the control samples have a rounded shape that is typical of the electronic images of the culture without any contrasting agents. Fifteen (15) minutes of cells suspension incubation with CeO_2 is accompanied by the formation of loose uniform 20-30 nm thick electron-dense layer around the cells (Fig. 5, b). After an hour of incubation, CeO_2 layer around the cell membrane has a greater density. It should be noted the presence of CeO_2-free space between cell membrane and a electron-dense layer of nanoparticles around the cell, it can be assumed that nanoparticles are concentrated at the surface of membrane carbohydrate complexes (polysaccharide capsule of *S. aureus* (Fig. 5, c)). After three hours of incubation the uniform distribution of nanocrystalline cerium throughout the cell surface (Fig. 5, d) was observed, and density of the nanoparticles layer was also uniform in the space between the bacteria that were in direct contact. The results have something in common with the described facts of free penetration of nanoparticles into clusters of cells that are part of biofilm [19].

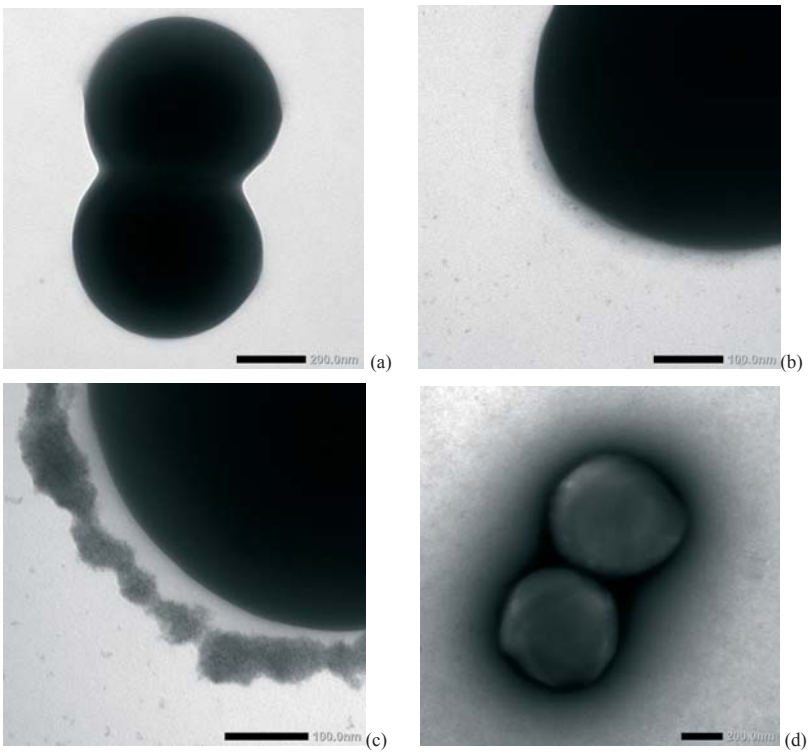

Fig. 5. Electron-microscopic images of interactions between *S. aureus* 8325-4 cells and CeO_2

(a) – culture control in saline;

(b) – incubation with cerium dioxide nanoparticles for 15 min.;

(c) – incubation with cerium dioxide nanoparticles for 60 min.;

(d) – incubation with cerium dioxide nanoparticles for 180 min.

For the *C. albicans* culture (Fig. 6) a similar, as for the previous two microorganisms, trend of gradual increase of CeO_2 concentration around the cell membrane during the experiment was observed. It should be noted that 15 and 60 minutes of CeO_2 contact with *C. albicans* cells resulted in the uneven accumulation of nanoparticles on the cells surface (Fig. 6, b, c). As in the case of *S. aureus*, a layer of CeO_2 is closely adjacent to the cell membrane that is probably due to the structural characteristics of cell walls of bacteria and fungi. Only after three hours of incubation the layer of CeO_2 around the cells was sufficiently uniform and reached a thickness of 150-200 nm

(Fig. 6, d). The chaotic arrangement of clusters of the nanoparticles around the cells makes it possible to assume that their influence on the cell membrane of *C. albicans* is destructive, so the membrane fragments separated from the cells under the influence of cerium dioxide can be seen around the microorganism (Fig. 6, c). This assumption is confirmed by the data of the number of live cells of *C. albicans* in the medium containing CeO_2 and can be an explanation for its bactericidal activity against this group of microorganisms.

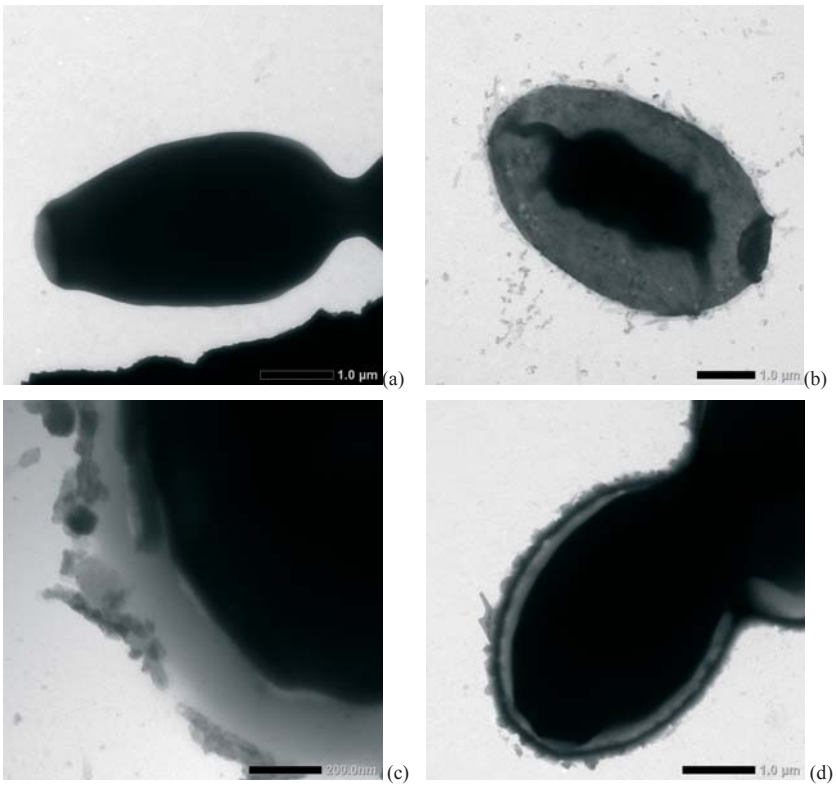

Fig. 6. Electron-microscopic images of interactions between *C. albicans* UCM Y-690 cells and CeO_2

(a) – culture control in saline;
(b) – incubation with cerium dioxide nanoparticles for 15 min.;
(c) – incubation with cerium dioxide nanoparticles for 60 min.;
(d) – incubation with cerium dioxide nanoparticles for 180 min.

So, for the synthesized CeO_2 sol the presence of antibacterial activity for gram-positive microorganisms and expressive antifungal action was shown. In general it can be argued that the antibacterial activity detected for nanocrystalline cerium *in vitro* offers the prospect of further investigation of synthesized CeO_2 nanoparticles to develop new approaches to the treatment of bacterial and/or fungal infections and development of new materials with antibacterial and/or antifungal properties that include CeO_2.

The work is performed in the framework of the research project “Development, creation and testing of nanobiomaterial composition based on cerium dioxide nanoparticles” DZ/492-2011.

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АНТИБАКТЕРІАЛЬНА АКТИВНІСТЬ КОЛОЇДІВ ЦЕРІЮ ПРОТИ УМОВНО-ПАТОГЕННИХ МІКРООРГАНІЗМІВ *IN VITRO*

Р е з ю м е

Був синтезований золь діоксиду церію з розміром наночастинок 2-4 нм. Визначено, що синтезовані наночастинки діоксиду церію виявляють антибактеріальну дію *in vitro* стосовно умовно-патогенних мікроорганізмів різних груп, а саме клінічних штамів *Escherichia coli, Staphylococcus aureus* та *Candida albicans.* Швидкість пригнічення життєздатності тест-культур залежить від концентрації наночасток та тривалості їх інкубації. Показано, що діоксид церію взаємодіє з поверхнею бактеріальної клітини. Висунуто припущення, що виявлені відмінності антибактеріальної дії наночасток можуть залежати від особливостей структурних характеристик поверхні клітин.

К л ю ч о в і с л о в а: *Escherichia coli, Staphylococcus aureus*, *Candida albicans,* нанокристалічний діоксид церію, антибактеріальний ефект.

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АНТИБАКТЕРИАЛЬНАЯ АКТИВНОСТЬ КОЛЛОИДОВ ЦЕРИЯ ПРОТИВ УСЛОВНО-ПАТОГЕННЫХ МИКРООРГАНИЗМОВ *IN VITRO*

Р е з ю м е

Был синтезирован золь диоксида церия с размером наночастиц 2-4 нм. Определено, что синтезированные наночастицы диоксида церия оказывают антибактериальное действие *in vitro* относительно условно-патогенных микроорганизмов различных групп: клинических штаммов *Escherichia coli, Staphylococcus aureus* и *Candida albicans.* Скорость угнетения жизнеспособности тест-культур зависит от концентрации наночастиц и длительности инкубации. Показано, что диоксид церия взаимодействует с поверхностью бактериальной клетки. Выдвинуто предположение, что обнаруженные отличия антибактериального действия наночастиц диоксида церия могут быть обусловлены особенностями структурных характеристик поверхности клетки.

К л ю ч е в ы е с л о в а: *Escherichia coli, Staphylococcus aureus*, *Candida albicans,* нанокристаллический диоксид церия, антибактериальный эффект.

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Отримано 16.05.2011