Characteristics of the response of the microalga (Dunaliella viridis) to cerium compounds in culture

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Introduction

As is known, the anthropogenic impact on the environment has become a powerful environmental factor. The intensive development of nanotechnologies and nanobiotechnologies (Abbas et al., 2015), as an anthropogenic factor, has several important aspects:

1) new nanomaterials can have wide application and influence on various biological systems;
2) new materials can become a “tool” not only in medical technologies, but also in scientific research when studying biological objects;
3) development of a variety of test objects is needed to assess the safety of new nanomaterials.

Currently, there are various methods of screening new products for genotoxicity, cytotoxicity and specificity of new materials (Douk et al., 2012; Sahu et al., 2016). However, these methods are laborious, expensive, and do not always provide unambiguous biological answers. In this regard, the development and implementation of simple express methods of primary screening are relevant. Microalgae can be used as test objects during primary screening; and when creating new nanomaterials, not only knowledge of the physical and chemical characteristics of these materials, but also an assessment of their potential effect on biological systems of different levels of organization is necessary (from microorganisms to animals) (Hoet et al., 2004). It is important to note that the study of the response of biological objects to the presence of nanomaterials in the environment provides valuable information in the knowledge of the biology of the test object, in particular microalgae. Earlier, we developed a cellular bioindicator based on the Dunaliella viridis Teod. unicellular microalgae to assess the cytotoxic effect of biologically active substances, including autoimmune pathology (Klimova et al., 2016). The D. viridis culture can also be used in primary screening for the cytotoxic activity of cerium compounds. The D. viridis unicellular alga has several features and advantages compared to other microalgae, which supplements the arsenal of biotesting objects and the mechanisms of their effect. The absence of a cell wall in D. viridis ensures direct contact of xenobiotics with the plasmollemma, as in animal cells. This “allows” it to quickly change its shape and size depending on changes in cultivation conditions, which is an integral response to the presence of active compounds or changes in physical and chemical environmental factors (Bozhkov et al., 2010).

Currently, the mechanisms of the effect of nanoparticles, in particular cerium dioxide, are being actively investigated and it has been shown that cerium nanoparticles can change the structural organization of biomolecules and lead to changes in cell metabolism (Estevez & Erlichman, 2014; Cheng et al., 2013). However, many authors suggest a high degree of biological risk when using nanomaterials (Schubert et al., 2006; Mohs & Greig, 2017). Obviously, biosafety is decisive in the development of new drugs and medicinal substances. In this regard, the central tasks in the development of nanobiotechnology are the determination of the biological activity and possible cytotoxicity of nanomaterials and the long-term consequences of their interaction (Minchenko et al., 2013).

Recently, nanobiotechnology has been developing intensively; therefore, various properties of nanoparticles, which depend on their origin, concentration, and size, are of interest. It is known that CeO2 nanoparticles cause a positive biological effect. These particles are able to penetrate through biomembranes. At the same time, there are assumptions about a high degree of biological risks when using nanomaterials, and it is obvious that the biosafety of nanomaterials is decisive in the development of new products, including for medicine. The cytotoxicity of samples of cerium salts and cerium dioxide nanoparticles of different sizes was assessed at different concentrations using D. viridis. The cytotoxicity level by morphological and functional disorders of D. viridis was investigated, as determined by the change in cell shape, accumulation of inclusions, loss of flagellum, change in nature and movement, the formation of micro- and macroaggregates by D. viridis cells and exometabolite release. The cytotoxicity coefficient was calculated as a quotient of total detected changes divided by their number. It was shown that cerium salts (cerium (IV) ammonium nitrate and cerium (III) chloride) had pronounced cytotoxicity, which exceeded cytotoxicity values of the control by 7 and 6 times, respectively. Cerium dioxide nanoparticles with a size of 6 nm at a concentration of 0.01 M showed intermediate cytotoxicity, which exceeded control values by 3.5 times, and after the effect of nanoparticles with a size of 2 nm at a concentration of 0.1 M, the cytotoxicity coefficient corresponded to control values. The addition of inactivated blood serum to the incubation mixture resulted in a decreased cytotoxic effect of cerium dioxide. The use of D. viridis as a test system will supplement the arsenal of biotesting tools for nanomaterials and the study of the mechanisms of their effect.

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It was of interest to investigate the biological response to nanoparticles depending on their origin, concentration and size (Celardo et al., 2011; Babu et al., 2014; Shcherbakov et al., 2015). Cerium is a chemical element that exists in two valence states +3 and +4. It is shown that in cases of transition to the nanocrystalline state, cerium dioxide changes physical and chemical properties and can show antioxidant activity in biological systems (Charhguo et al., 2017). At low concentrations, it can show anticancerogenic properties (Nourmohammadi et al., 2019). According to Schubert et al. (2006), cerium dioxide shows cytotoxic properties. Animal tests showed that the addition of cerium dioxide nanoparticles with a size of 6–7 nm at a dose of 175–250 mg/mL is accompanied by the death of 60% of experimental animals, while the dose of 100 mg/kg was not toxic.

The study of the effect of cerium salts and cerium dioxide nanoparticles with different characteristics on cell shape, mobility and the formation of cell aggregates in _D. viridis_ culture is of great interest, both to assess the potential ability of the microalgae to adapt to these xenobiotics and to develop a biotesting method.

As is known, the biological effect depends not only on the characteristics of the test xenobiotic, but also on the characteristics, or rather the functional state of the biological object (Bozhkov et al., 2010; Garcia-Saucedo et al., 2011). In this regard, it is of interest to determine the responses of _D. viridis_ to the addition of cerium compounds in combination with the components of human blood serum. Such an approach allows assessing both the possible interaction of nanoparticles with serum proteins and the change in their activity, and the effect of serum components on _D. viridis_ cells.

In this regard, in this paper, we used the _D. viridis_ test system to assess the possible cytotoxicity of cerium salts and cerium dioxide nanoparticles of various sizes and at various concentrations, the effect of serum components on cerium toxicity.

**Materials and methods**

The materials for the study were cerium salts and cerium dioxide nanoparticles (CDN), synthesized by Shcherbakov as described in Ivankov et al. (2010a, 2010b), Kuchibhatla et al. (2012) and Shcherbakov et al. (2017). Their characteristics are in the Table 1, taking into account the aspects of making, concentration and size.

| Options | Samples | Characteristics | Concentration, M | Nanoparticle size, nm |
|---------|---------|-----------------|------------------|-----------------------|
| Control | A       | Sodium citrate – stabilizer | 0.10            | –                     |
| Cerium salts | B Ce(IV) | Cerium (IV) ammonium nitrate | 0.01            | –                     |
|          | C Ce(III) | Cerium (III) chloride | 0.01            | –                     |
| Cerium dioxide nanoparticles | D CDN190 | Nanoceria aqueous sol obtained by microwave hydrothermal treatment | 0.10  | –6                   |
|          | E CDN190 | Nanoceria aqueous sol obtained by microwave hydrothermal treatment | 0.01  | –6                   |
|          | F CDN | Nanoceria obtained by hydrothermal treatment of a cerium(IV) ammonium nitrate aqueous solution | 0.20  | –3–4         |
|          | G CDN16 | Nanoceria stabilized with sodium citrate (ratio of nanoparticles and stabilizer 1:1) | 0.10  | –2                   |

Method for the determination of cytotoxic effects. The cytotoxicity of the test samples was assessed in a _D. viridis_ 20-day synchronized culture at a concentration of 15 million cells in 1 mL. Microalgae were cultivated on Artari medium in the Masyuk modification (Masyuk, 1973) at 25–27 °C under standard conditions (Bozhkov et al., 2010). To assess the cytotoxicity of the test substance, a suspension of _D. viridis_ unicellular algae and test samples were applied in equal volumes (50 μL). Also, inactivated serum was used as an adjuvant biological substrate in parallel studies. Inactivated serum (50 μL) was added to the test samples of cerium compounds. The serum was inactivated by incubating it at 56 °C for 20 minutes (Men’shikov, 1987). The altered cells were counted on a glass slide at 200 and 400 using light microscopy (Olympus BX53).

Integral cytotoxicity indices were determined as the coefficient of spontaneous cytotoxicity CSP (for the control culture) and the coefficient of induced cytotoxicity CC (in the test samples) (Klimova et al., 2010). The coefficient of spontaneous cytotoxicity was calculated according to the formula:

\[
C_{SP} = \frac{a + b + c + d + e + f}{n},
\]

where the various types of the cytotoxic effect were assigned as a, b, c, d, e, f. It was decided that: a – change of cell shape (1 point), b – inclusion accumulation (2 points), c – loss of flagellum (3 points), d – change in the nature and direction (loss of mobility, 4 points), e – formation of aggregates (5 points), f – exometabolite release (6 points), n – amount of types of observed morphological and structural changes. In the absence of any disorders in the numerator, only the revealed disorders remain with corresponding points. In the absence of a certain type of disorder, its value was assigned as “0”.

The research scheme of sample cytotoxicity. We conducted five independent experiments; each had three repetitions, according to the scheme. The samples were composed as follows: Control of _D. viridis_ + saline; _D. viridis_ + serum; _D. viridis_ + cerium salt; _D. viridis_ + nanoceria; _D. viridis_ + nanoceria + serum.

The statistical processing of the obtained results was performed by Statistica 6.1 program (StatSoft Inc., USA). To compare average values ANOVA was used, with values _P_ < 0.05 considered statistically significant. Data are expressed as the x ± SE of at least five independent experiments.

**Results**

Characteristics of a _D. viridis_ test culture in the control sample. In all the control samples of _D. viridis_, the cells were oval, the mobility was 95.0 ± 1.1%, aggregates in the culture were not formed, and the cells maintained their usual straight movement (Fig. 1). The level of spontaneous cytotoxicity for the control culture was CSP = 2.0 ± 0.02 units.

The presence of such spontaneous cytotoxicity in the control culture is explained by the fact that such a culture is due to the presence of a natural cell death, the presence of cells in different phases of the cell cycle and different functional states. In this regard, this level of cytotoxicity is a spontaneous level.

**Fig. 1.** _D. viridis_ cells in the control, bar 10 μm

Characteristics of a _D. viridis_ test culture after incubation with cerium dioxide salts. After incubation of _D. viridis_ with cerium salt (IV) heptahydrate at a concentration of 0.01 M, the cell shape changed, and most of the cells were pear-shaped (Fig. 2). The cells lost flagella and the movement passed from straight to circular. Intracellular inclusions were observed in the cells (which were absent in the control), some of the cells were formed by micro- and macroaggregates surrounded by exometabolites. The cytotoxicity coefficient in this case was CC = 14.0 ± 0.08 units, i.e. 7 times greater than the control sample.
After incubating the *D. viridis* culture with cerium (III) nitrate at the same concentration (0.01 M), some of the cells had a pear shape, and other cells became rounded (Fig. 3a). Some cells lost mobility. The cells formed macroaggregates surrounded by exometabolites (Fig. 3b). The cytotoxicity coefficient was lower than for heptahydrate, but their number was 11.0 ± 0.05 units. Consequently, various cerium salts showed toxicity, while there were small quantitative differences.

The effect of cerium dioxide nanoparticles of different sizes and different concentrations on *D. viridis*. Incubation of the *D. viridis* control culture with the addition of CeO₂ nanoparticles of different sizes and different concentrations changed the nature of the cells in the culture.

![Fig. 2. *D. viridis* cells after incubation with cerium (IV) ammonium nitrate: arrows indicate pear-shaped *D. viridis* cells; bar – 10 µm](image1)

![Fig. 3. *D. viridis* cells after incubation with cerium (III) chloride: a – rounded cells, b – formation of a macroaggregate with exometabolite release; bar – 10 µm](image2)

![Fig. 4. Formation of microaggregates by *D. viridis* cells after incubation with cerium dioxide nanoparticles (CDNs190), bar 10 µm](image3)

![Fig. 5. The effect of cerium dioxide nanoparticles on structural and functional changes in the *D. viridis* cellular bioindicator (sample CDN190 – 0.01 M); bar – 10 µm](image4)

So, after incubating the *D. viridis* test system with cerium dioxide (CDNs190) at a concentration of 0.1 M, similar cytotoxic effects were observed, as after the effect of cerium salts, but less severe. Culture cells formed a large number of microaggregates with 10–15 cells in the aggregate (Fig. 4). The cytotoxicity coefficient was 8.5 ± 0.06 units, i.e. half as much as the cerium salt.

After incubation of the *D. viridis* test system with cerium dioxide (CDN190) at a concentration less than 10 times – 0.01 M, all the cells acquired a deformed rounded shape after 30 minutes. Cerium dioxide nanoparticles were present in the cells, while they lost flagella and formed large clusters around nanoparticles, aggregations on “islands” with nanoparticles. The cytotoxicity coefficient (CC) in the CDN190 sample was 7.2 ± 0.31 units (Fig. 5), i.e. slightly less than with a higher concentration of CDN190. Consequently, there is no direct correlation between the concentration of cerium nanoparticles and the biological response, at least in the concentration range of 0.01–0.10 M, which suggests that they act according to all or nothing.
Pear-shaped. Most of the cells (83.0 ± 3.3%) lost mobility. It was revealed that 45% of the cells formed macroaggregates (up to 100 cells in the aggregate, Fig. 6). Cells in such clusters contained a large number of inclusions, the size of the CDN nanoparticles was 3–4 nm and they penetrated into the cells. The effect of cerium dioxide nanoparticles (CDN) at a given concentration resulted in cells partially losing flagella. The cytotoxicity coefficient (CC) for this sample – CD 0.2 M was 3.6 ± 0.29 units (Fig. 6) was significantly less compared with CDN190 and slightly differed from the control sample.

![Fig. 6. The effect of cerium dioxide nanoparticles (CDN – 0.2 M sample) on the structural and functional changes in the D. viridis cellular bioindicator; bar – 10 µm](image)

After adding cerium dioxide nanoparticles (CDN16) to the D. viridis test system with a size of 2 nm, at a concentration of 0.1 M, changes in the slow rate of cell movement were observed. No significant change in the number of cells with altered morphology was found. The cells contained a large number of inclusions. The number of mobile cells was 52.0 ± 5.6%, the number of cells with lost flagella was only 9%, D. viridis culture did not form aggregates. After the co-incubation of D. viridis and cerium dioxide nanoparticles (CDN16) at this stage, the appearance of dividing cells (about 10%) was noted (Fig. 7). The cytotoxicity coefficient (CC) in the CDN16 sample was only 2.8 ± 0.09 units, i.e. did not differ from the control variant.

![Fig. 7. Co-incubation of D. viridis and cerium dioxide nanoparticles (CDN16): arrows indicate complexes of nanoparticles with proteins; bar – 10 µm](image)

Consequently, cerium salts and cerium dioxide nanoparticles had different cytotoxic effects on D. viridis microalgae. At the same time, D. viridis showed various responses in the presence of salts or nanoceria in the medium. Cerium dioxide nanoparticles CDN16 at a concentration of 0.1 M did not show toxicity and could even accelerate proliferation.

![Fig. 8. Morphological changes of the D. viridis bioindicator after the contact with serum (a) or serum and cerium dioxide nanoparticles (CDN) (b); bar – 10 µm](image)

When D. viridis was co-incubated with cerium salts (IV) ammonium nitrate or cerium (III) chloride) and inactivated serum, the cells changed their morphology: about 60% of the cells were round, exome-tabolites were released, micro- and macroaggregates were formed (Fig. 8b). The cytotoxicity coefficient was CC = 12.2 ± 2.9 units for cerium (IV) ammonium nitrate salt and CC = 10.3 ± 1.5 units for cerium (III) chloride salt, respectively, which was lower than in cerium salts only.

After adding an aqueous sol of nanoceria at a concentration of 0.1 M, which did not contain a stabilizer (CDNs190), and inactivated serum to the D. viridis test system, the cytotoxicity coefficient was reduced to 8.5 ± 0.06 (Fig. 9d). D. viridis cells in the presence of serum and cerium dioxide nanoparticles (CDN190) acquired an irregular shape of 76.0 ± 0.8%, the movement of the cells was disrupted due to the loss of flagella. Most of the cells were immobile (72.3 ± 1.8%), the cells formed clusters. A small number of cells (13.0%) rotated around their axis. Co-incubation of the D. viridis test system with serum and cerium dioxide nanoparticles (CDN) resulted in a change in the cells (Fig. 9c). When nanoparticles (CDN16) and serum were added to the incubation mixture, the oval cell forms was detected, the rate of movement was at the control level, and the cytotoxicity coefficient was 2.1 ± 0.1 units (Fig. 9b). Thus, the effect of serum on the cells of the D. viridis tests system effects, leading to a decrease in the cytotoxicity coefficient to the control level (Table 2).

**Discussion**

Of great interest is the assessment of the possible modification of the toxic effect of cerium dioxide nanoparticles by their possible interaction with proteins, in particular, blood serum. This is important to know because in the bloodstream nanoparticles are primarily bound to proteins.

The effect of inactivated serum and cerium nanoparticles on D. viridis cells. In the first series of experiments, the possible effect of serum components on D. viridis cells was determined. The addition of serum into the D. viridis culture did not have a cytotoxic effect. When assessing the morphological changes of D. viridis cells and serum an oval cell shape was noted (Fig. 8a). The cell movement was slightly slowed down. The D. viridis immobile cells, on average, were not more than 9%, the number of cells that lost the flagellum – on average, 4%, the number of rounded cells was not more than 10.0 ± 1.1%. The cytotoxicity coefficient was CC = 2.5 ± 0.6 units, i.e. no significant differences compared with the control group were found.

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The results of this paper allow us to conclude that the addition of cerium (IV) ammonium nitrate, cerium (III) chloride or cerium dioxide nanoparticles at low concentrations (0.01–0.10 M), with different sizes, into the D. viridis culture was accompanied by severe morphological and functional changes in microalgae. Cerium dioxide nanoparticles showed less “activity” in relation to cell culture compared to cerium salts. The addition of cerium dioxide nanoparticles with blood serum into culture reduced the manifestation of morphological and functional changes in the test object. D. viridis culture can be used as a test object in the study of the biological effects of new substances and their compositions.

Fig. 9. Co-incubation of the D. viridis bioindicator, serum and studied samples: a – sodium citrate 0.10 M, b – CDN16 0.10 M, c – CDN 0.20 M, d – CDN190 0.01 M; bar – 10 μm

Due to the development of nanobiotechnology, biological systems that can be used as test objects or cell biosensors are being actively searched for (Jurado-Sanchez, 2018). In addition, bioethics and the principles of humane treatment of laboratory animals that have emerged in recent decades have become a new factor in the refusal to test animals for the purposes of toxico-biological evaluations. Perhaps the most important thing is that the formation of a hierarchical biological response to the presence of xenobiotics can be observed. The biological response hierarchy in the D. viridis culture is represented by several levels: 1 – change in the rate of cell movement and its nature (from straight to circular); 2 – change in the cell shape; 3 – formation of different types of cell aggregates – microaggregates (up to 15–15 cells in an aggregate) and macroaggregates (from 50 to 100 and more cells); 4 – the presence or absence of polysaccharide exometabolites around cellular aggregates.

The first level in the response hierarchy to the presence of xenobiotics in the medium is the change in the rate and nature of the movement. As is known, the movement of Dunaliella cells is performed by two flagella, the rotational movements of which are a result of the hydrolysis of macroergic bonds (Prevo et al., 2017). Consequently, the rate of Dunaliella cells movement depends on the state of the energy metabolism of the cell. A change in the form of movement indicates a change in the structural and functional characteristics of the movement apparatus itself (Hexin et al., 2016). Movement impairment (accompanied by loss of flagella) and D. viridis bioindicator cell shape is obviously associated with changes in the cytoskeleton components, which is also involved in intracellular transport, secretion regulation, receptor protein metabolism, signal transmission from plasma membrane receptors inside the cell (Radoservic et al., 1994; Shi et al., 2013).

Table 2

| Samples                      | C<sub>c</sub> units (x ± SE) without serum | C<sub>c</sub> units (x ± SE) after serum adding |
|------------------------------|------------------------------------------|-----------------------------------------------|
| D. viridis + saline (control)| 2.0 ± 0.02                               | 2.5 ± 0.61                                   |
| D. viridis + sodium citrate  | 3.2 ± 0.12*                              | 2.8 ± 0.40                                   |
| D. viridis + cerium (IV)     | 14.0 ± 0.08**                            | 12.2 ± 2.90*                                |
| D. viridis + cerium (III)    | 11.0 ± 0.05***                           | 10.3 ± 1.50*                                |
| D. viridis + CDN100          | 8.5 ± 0.05***                            | 7.1 ± 0.12*                                 |
| D. viridis + CDN0.01         | 7.2 ± 0.31*                              | 5.9 ± 0.21*                                 |
| D. viridis + CDN – 0.20M     | 3.6 ± 0.29*                              | 2.9 ± 0.30*                                 |
| D. viridis + CDN16 – 0.10M   | 2.8 ± 0.30                               | 2.1 ± 0.11                                  |

Note: * – the differences are significant relative to the control, P < 0.05, **– P < 0.01, ***– P < 0.001.

For example, according to Reilein et al. (2001), loss of mobility and change in the cell shape due to dysfunction of individual organelles is the result of changes in the effect of several motor proteins, disruption of proper transport due to dysregulation of their activity. As shown, change in the nature of cell movement from straight to rotational or loss of mobility directly depends on the functioning of the cytoskeleton microfilament contractile systems: actin-myosin in microfilaments (Vale & Fletterick, 1997) and tubulin-dinne interactions in microtubules of sliding systems (Schroer, 1994).

Stabilizing and structural dysfunction of the cytoskeleton can disrupt cytoskeleton contractile filaments, induce exometabolite release by the algal cells as a protective factor that envelops the cells and reduces the concentration of the toxic agent surrounding the cell.

According to Sharifi (2012), nanoparticles can more easily penetrate the protective barriers of living organisms and penetrate directly into organs and tissues. Cerium compounds cause the formation of micro- and macroaggregates, which can be inducers of the adhesive properties of the bioindicator cell membranes.

As known, D. viridis rapidly changes the cell shape due to the loss of the cell wall and adaptation to reservoirs with very different concentrations of sodium chloride (from 0.5 to 3.0 M). As shown by Chen & Jiang (2009), the unicellular Dunaliella algae can modify the osmotic response and the concentration of glycerol in response to changes in salinity in the medium. Due to this, changes in the medium osmosis or the effect of the test xenobiotics on the activity of ions channels or the cell cytoskeleton leads to a rapid change in the cell shape.

Finally, the third level of a culture biological response to the presence of toxic xenobiotics is the formation of cell aggregates. The aggregation was described more than 100 years ago; however, the mechanisms of these have not been finally established. Today, it can be argued that aggregation in the D. viridis culture is the reaction of cells to stress fac-
tors of various nature (Bozhkov, 2010; Rostama et al., 2012). The formation of aggregates depends on cell mobility, secretory activity and characteristics of the cell surface apparatus. It is considered that substances can have an adjuvant effect, for example, cytokines (IL-1β, IL-2, TNF-α, IFN-α, IFN-γ) affect high-affinity receptors on target cells and can cause biological effects at small concentrations (Fontes et al., 2017; Tomlénovich & Show, 2011).

Currently, cerium dioxide and its materials are widely used as sensors or catalysts (Ezu et al., 2013). A number of studies have shown that cerium dioxide nanoparticles have antioxidant properties and can be used in biology and medicine as inorganic antioxidants. Vibrio fischeri and Escherichia coli microorganisms have shown that cerium dioxide nanoparticles are not toxic (Kurvet et al., 2017).

When D. viridis was used as a test object, cerium salts changed the rate and cell movement, and induced the formation of cell aggregates, i.e. they influenced all hierarchical stages of formation of a stress response, i.e. showed toxicity. Cerium dioxide nanoparticles had half as much severe toxicity compared to cerium salts or were not toxic to cerium dioxide at a concentration of 0.20 M and CDN16 at concentration of 0.10 M. Serum components reduced toxicity for both cerium salts and nanoparticles. This indicates that cerium can form complexes with organic substances that are not toxic.

Conclusion

The D. viridis unicellular algae culture is a promising bioindicator in a comparative study of the cytotoxicity of nanomaterials. Cerium salts (cerium(IV) ammonium nitrate, and cerium (III) chloride) at concentration of 0.01 M showed high cytotoxicity with respect to the D. viridis cell test system and caused all kinds of identified structural and functional cell disorders, including the formation of macroaggregates and release of exometabolites. Cerium dioxide nanoparticles CDN5190 (0.10 M) showed significantly less cytotoxicity than cerium salts, and CDN16 nanoparticles with a size of 2 nm at a concentration of 0.10 M did not have toxicity. The addition of inactivated serum with nanoparticles reduced the manifestation of cytotoxicity caused by cerium dioxide nanoparticles.

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