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# Evaluation of Selected Toxicity Endpoints in Ovary Cells Exposed to Nanoceria

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## 1. Introduction

Cerium dioxide nanoparticles (nanoceria, $CeO_2$NPs) due extensive commercial use are included on the priority list of nanomaterials requiring evaluation in term their toxicity by the Organization for Economic Cooperation and Development (OECD 2011). $CeO_2$NPs are used as: catalyst for automobile emission control; polishing agent; buffer layer for superconductors; ultraviolet absorbent; heat resistant alloy coatings, and many others (NIA 2010).

The consequence of widespread usage of nanomaterials could be their unintentional or accidental release into workplaces or ecosystems, thus increasing cerium concentration in these environments. A significant fraction of nanoceria released from consumer products and industrial processes can be expected to reach industrial and municipal wastewaters treatment plants and consequently biosolids, which can lead to adverse effects (NIA 2010). A large part of production of $CeO_2$NPs is used as a catalyst support in diesel engine to reduce fuel consumption, particulate matter and greenhouse gas emissions (NIA 2010). Such usage of nanoceria is extremely advantageous to the consumer and environment, but it can be expected, that a large number of $CeO_2$NPs will be emitted to the ambient air and finally to the watercourses and soil.

Although the production of $CeO_2$NPs is currently in high tonnage, the existing toxicological data were considered insufficient to assess the risks which it may pose to humans and the environment, especially that

the differences in the toxicity of nano and micro cerium dioxide are reported (NIA 2010, Arnold et al. 2013).

Several publications have demonstrated harmful effects of $CeO_2$NPs on aquatic or soil organisms, for instance algae (Rodea-Palomares et al. 2011, Rogers et al. 2010, Van Hoecke et al. 2009) or nematode (Zhang et al. 2011, Arnold et al. 2013). For human, the toxicological studies carried out in the last years give inconsistent picture of toxicity of nanoceria, especially regarding the oxidant/antioxidant effect. Some *in vitro* and *in vivo* studies demonstrated that nanoceria possess antioxidant activity and therefore may be useful in biomedical applications for protecting cells against radiation damage, oxidative stress, or inflammation (NIA 2010, Hirst et al. 2009, 2013). On the other hand, several publications have demonstrated harmful, cytotoxic and oxidative effects of $CeO_2$NPs on somatic cells (Park et al. 2008, Kim et al. 2010, Kroll et al. 2011).

In toxicity assessment of nanoceria it is also important to identify their potential long-term effects of exposure.The DeMarzi et al. (2013) demonstrated time-dependent differences in the cytotoxic activity of $CeO_2$-NPs. During 24h exposure of lung epithelial cells, intestinal epithelium and liver cells, they did not find any cytotoxic effect, but they observed the DNA damage in all cells. Until after 10 days exposure they observed a significant reduction in cell viability. The time-depended toxicity of $CeO_2$NPs was also observed in aquatic organisms (Van Hoecke et al. 2011). The above-mentioned results demonstrate that the evaluation of nanoceria toxicity is an open problem requiring basic researches.

The purpose of this study was to assess the cytotoxic effects of $CeO_2$NPs on reproductive system cells (CHO-9) in short, and long-time of exposure. It was also relevant to identify the ability of $CeO_2$NPs to induce DNA damage in the cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

$CeO_2$NPs with nominal particle size below 25 nm were purchased from Sigma-Aldrich (Cat. No.: 544841). The media for cell cultures were provided by Gibco BRL (Life Technologies Ltd. Paisley, UK). Other reagents were supplied by Sigma Chemical Co. (St Louis, MO, USA), if not indicated otherwise.

### 2.2. Preparation and characterization of cerium dioxide nanoparticles

The morphology of $CeO_2$NPs was characterized using scanning electron microscopy (SEM, Zeiss Ultra Plus). The particle size distribution of $CeO_2$NPs was carried out using the Nanoparticle Tracking Analysis (NTA) (NS500, Nanosight Ltd., UK). Specific surface area was measured by means of the Brunauer, Emmett and Teller (BET) technique (Gemini 2360, Micromeritics). All these measurements were carried out at the Institute of High Pressure Physics of the Polish Academy of Sciences (Warsaw).

Before each experiment, the "stock" solution of $CeO_2$NPs (1 mg/ml) was prepared by suspending the powder in cold phosphate-buffered saline (PBS), and sonicated on ice at high energy level of 420 J/cm$^3$ (30 sec. with amplitude 90%) (Sonica Q 700, Qsonica LLC, USA). Then, the particular concentrations of nanoceria were prepared immediately before the toxicity tests in a serum-free culture medium.

### 2.3. Cell culture and treatment

The Chinese hamster ovary cells (CHO-9 were cultured as a monolayer in a F-10 medium supplemented with 10% FBS and with 1% antibiotic-antimycotic, and incubated in a 5% $CO_2$ atmosphere at 37°C.

### 2.4. Cell cytotoxicity assays

CHO-9 cells were exposed to different concentrations of $CeO_2$NPs for 24 or 72 hrs. The viability of the cells was assessed with three cytotoxicity assays: MTT and WST-1 tetrazolium salts reduction assays and neutral red uptake test (NRU). The assays were conducted according to INVITTOX Protocols No. 17 (for MTT) and No. 64 (for NRU), and according to the ROCHE manufacturer's instructions (for WST-1) using a Synergy 2 microplate reader (BioTek, USA). Based on the absorbance measurement values obtained in the tests, the viability ratio of cells exposed to $CeO_2$NPs, i.e. the percentage of viable cells compared to control (cells without $CeO_2$NPs), was calculated.

### 2.5. Clonogenic assay (Colony Forming Efficiency Assay, CFEA)

The clonogenic assay was conducted according the procedure described by Franken et al. (2006). The cells were seeded in Petri dish 60 x 15 mm (21 cm$^2$) at a density 500 cells/dish, together with different

concentrations of $CeO_2$NPs for 7 days (the time required to form colonies). After this time the colonies were counted and plating efficiency ratio (PE) and surviving fraction ratio (SF) were calculated in the following way:
PE = no. of colonies formed / no. of cells seeded,
SF = no. of colonies formed after treatment / no. of cells seeded x PE.

### 2.6. Alkaline comet assay

The alkaline comet assay (Single Cell Gel Electrophoresis, SCGE) was performed as described in Wojewodzka et al. (1998). The measurements were carried out at Institute of Nuclear Chemistry and Technology in Warsaw. The cells were exposed to different concentrations of $CeO_2$NPs for 24 h. After electrophoresis the preparations were subjected to computerized image analysis using the software Comet Assay IV Image Analysis System from Perceptive Instruments Ltd. For the assessment of the level of DNA damage, the percentage of DNA in the comet's tail was applied.

### 2.7. Data analysis

Cytotoxicity tests were performed in at least three independent experiments with at least three replications for each treatment. Based on the analysis of a series of dose–response curves, the $IC_{50}$ values (the concentrations of nanoceria that reduced cell viability by 50% compared to control) were evaluated using non-linear regression analysis at 95% confidence interval. The CFEA test was carried out in the three separated experiments with three replications for each concentration of nanoceria. The results were presented as surviving fraction ratio (SF) ± standard deviation (SD). SF=1 was set for the control. CFEA data were analyzed by Student's test for comparison between two groups. Results of comet assay are presented as mean values obtained from five experiments, ± (SD). The significance of differences between the mean values was assessed using the paired Student's *t*-test. The software employed for statistical analysis was Statistica, version 7.1.

# 3. Results

## 3.1. Characterization of cerium dioxide nanoparticles

The hydrodynamic diameters of $CeO_2$NPs assessed by NTA method were slightly larger than the nominal particle sizes (< 25 nm). A major part of particles were 53 nm (Mode = 53; Mean 108 nm ± 43) (data not shown). This suggests that $CeO_2$NPs did not exist as single particles, but tended to form aggregates/agglomerates. The results obtained with NTA analysis were confirmed by SEM pictures, where aggregates of particles were found (Fig. 1). The specific surface area in BET analysis measured as BET = 33,3199 $m^2/g$, indicate the presence of small particles.

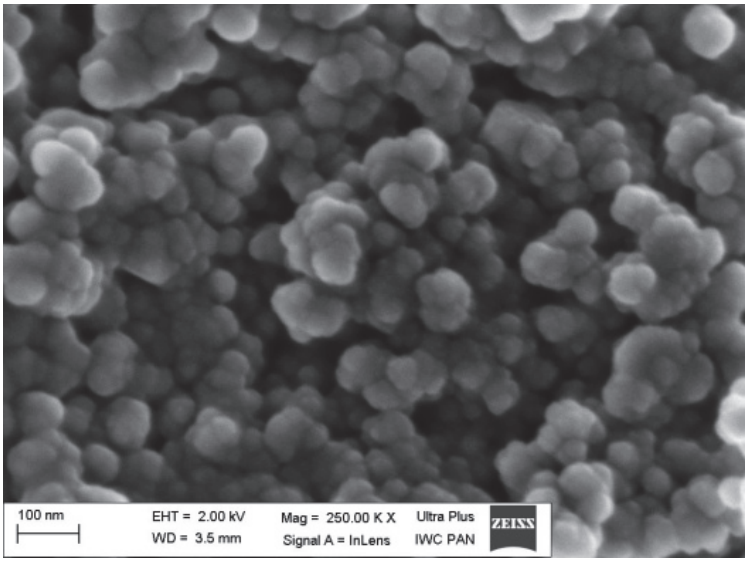


**Fig. 1.** SEM image of $CeO_2$NPs (< 25 nm)
**Rys. 1.** Obraz $CeO_2$NPs (< 25 nm) ze skaningowego mikroskopu elektronowego (SEM)

## 3.2. Cell cytotoxicity assays

In order to assess the cytotoxicity of $CeO_2$NPs, three assays were conducted: MTT and WST-1 tetrasolium salt reduction assays, which assess the metabolic activities of cells, and NRU assay which measures cells membrane permeability. The results of the MTT and NRU viability assays showed that $CeO_2$NPs were cytotoxic toward CHO-9 cells ex-

posed for both 24 h and 72 h. Cytotoxicity doses were at 100-400 µg/ml depending on cytotoxicity endpoint. An increase of the toxicity of $CeO_2$NPs along with the time of exposure of the cells was observed. In contrast, when WST-1 test was applied, the reduction in metabolic activation was observed only after 72 h of CHO-9 cells exposure. After 24 h of exposure of the cells to $CeO_2$NPs only a slight decrease in cell viability was observed, with the highest concentrations used, so it was impossible to achieve a 50% decrease in viability of the culture. Therefore, in this case the $IC_{50}$ values are theoretical values, determined by extrapolation (Fig. 2).

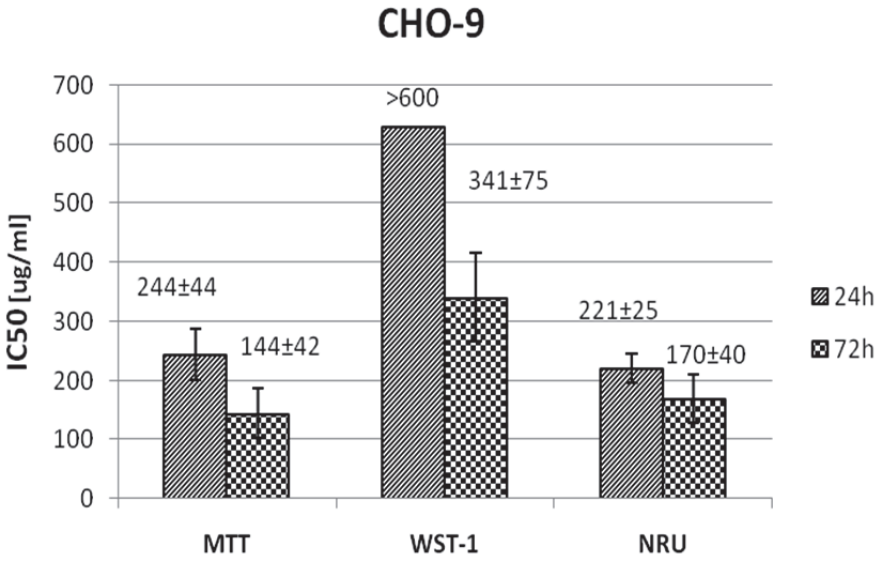


**Fig. 2**. The cytotoxicity of $CeO_2$NPs (< 25 nm) on CHO-9 cells after 24 h and 72 h exposure. Each bar represents an average value of $IC_{50}$ doses ± SD ($n \geq 9$)
**Rys. 2.** Porównanie cytotoksycznego działania $CeO_2$NPs (< 25 nm) na komórki CHO-9 po 24 h i 72 h czasie narażania. Każdy słupek reprezentuje wartość średnią dawek $IC_{50}$ ± odchylenie standardowe (SD) ($n \geq 9$)

### 3.3. Clonogenic assay (CFE assay)

As shown in Fig. 3, $CeO_2$NPs were able to significantly decrease the clonogenic survival and cell proliferation in dose of about 50 µg/ml. The 50% reduction of the clonogenic potential (SF = 0.5) was observed for about 100 µg/ml. At the concentrations of about 400 µg/ml, only 10-25% of the cells had the ability to form colonies (SF=0.1).

### 3.4. Alkaline comet assay

The extent of DNA damage in cells treated for 24h with $CeO_2$NPs is shown in Figure 4. The applied concentrations corresponded to cytotoxic doses (values close to $IC_{50}$ and lower) as determined in MTT and NRU assays. Exposure of CHO-9 cells to nanoceria caused a statistically significant increase in the level of DNA breakage (Single Strand Breaks, SSB) as compared to the control, at concentrations 10 μg/ml and above. The FPG-sensitive sites (SSB-FPG) significantly differed from the control at the concentrations about 100 μg/ml.

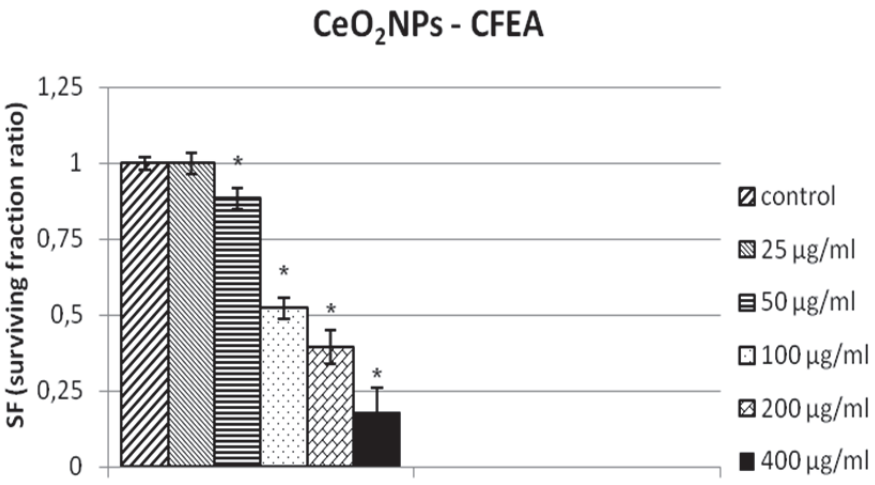


**Fig. 3.** Colony forming ability of CHO-9 cells treated with different concentrations of $CeO_2$NPs. Each bar represents the mean ± SD. * $p < 0.05$ vs. control

**Rys. 3.** Ocena zdolności komórek CHO-9 do tworzenia kolonii po narażeniu na różne stężenia $CeO_2$NPs. Każdy słupek reprezentuje wartość średnią ± SD. * $p < 0.05$ w stosunku do kontroli

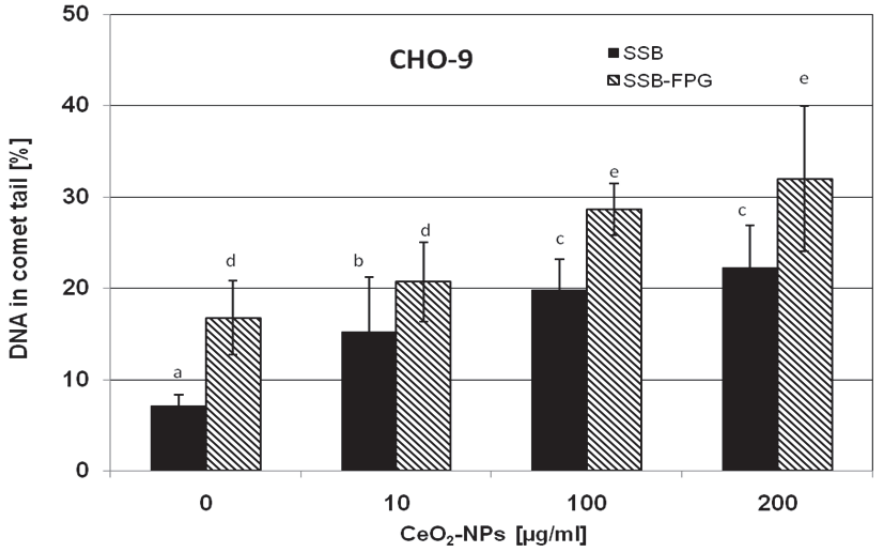


**Fig. 4.** Single strand breaks (SSB) and base damage recognized by formamidopyrimidine glycosylase (SSB-FPG) measured as percentage of DNA in the comet's tail in CHO-9 cells treated with $CeO_2$NPs for 24h at the concentrations indicated. The different letters over bars indicate statistically significant differences (Student's t-test, $p < 0.05$, $n = 5$)
**Rys. 4.** Wpływ $CeO_2$NPs na powstawanie jednoniciowych pęknięć DNA (SSB) oraz uszkodzeń oksydacyjnych DNA (SSB-FPG) po 24 h narażaniu komórek CHO-9. Różne litery nad słupkami oznaczają różnice istotne statystycznie (test t-Studenta, $p < 0.05$; $n = 5$)

## 4. Discussion

Characterization of nanomaterials, in particular, changes in particle size and particle size distribution, and formation of aggregates and agglomerates, have a crucial role in the interpretation of results being obtained in toxicological studies (OECD 2011). In this study, the $CeO_2$NPs existed in the form of single particles, as well as agglomerates, which were about 2-4 times larger than the nominal particle size. It has to be taken into consideration that aggregation is a natural process of nanoparticles, and in most studies on nanomaterials published, it was not possible to completely eliminate aggregates.

Cytotoxicity assessment of nanomaterials is a basic step in determining their potential effects on the cellular level. Cell viability assays

provide information not only on the cells survival, but also indicate the direction of toxic effects of nanoparticles. However, the results of the cytotoxicity assessment (the values of cytotoxic doses) depend on the cellular model and methods used for research. In this study we have used the CHO-9 which are a proven line with high sensitivity to xenobiotics, including metals (Zapór 2014).

The results indicate that $CeO_2$NPs caused a disturbance in metabolic activity of the cells and cells membrane permeability, in dose and time dependent manner. The toxic doses were dependent on the kind of method used. Both MTT and NRU tests gave repetitive $IC_{50}$ values. There were any interference between the nanoparticles and testing reagents, as was noted by other researchers (Herzog et al. 2007, Kroll et al. 2012). On the other hand, in the case of the WST-1 assay, the $IC_{50}$ values were much higher than those calculated in MTT or NRU tests. After 24-h time of exposure, they were extrapolated because the range of concentrations used was exceeded. The use of higher concentrations disturbed the colorimetric measurements. The $IC_{50}$ values obtained in 24-h experiment should be considered as high, which indicates weak cytotoxicity of nanoceria; however it is worth noting that they decreased with the time of exposure.

Most of *in vitro* assays determine toxic effects of xenobiotics in the short time (24-72 h). In order to study possible long-term toxic effects of nanoceria the clonogenic assay was used, which is performed within 7 days. This test is used to detect cells that retained the capacity for producing a large number of progeny after treatments that can cause reproductive death as a result of damage to chromosomes, apoptosis, etc. (Herzog et al. 2007). Recently, the clonogenic assay has been considered a promising test to study toxicity of nanomaterials, as it makes use of no cellular dyes, which have been found to be a possible reason for invalid results due to their biochemical interaction with the nanomaterials tested (Herzog et al. 2007, Ponti et al. 2010). $CeO_2$NPs were able to significantly decrease cell proliferation in the doses over 50 μg/ml, which suggest potential chronic toxicity of $CeO_2$NPs.

In the literature data, there are significant discrepancies in respect to the level of cytotoxic potency of nanoceria. Generally, these results are agree – in terms of the level of cytotoxic doses – with the data obtained by others authors in studies on the human cells such as: hepatoma (Cheng

et al. 2013), bronchial epithelial cells (Park et al. 2008), dermal fibroblast (Auffan et al. 2009), endothelial cells (Strobel et al. 2014).

The doses used in this study may be considered high, but are likely to be reached following long time of exposure, or after acute accidental exposure in occupational conditions. Also, it has to be taken into consideration that nanoceria is biopersistent and can accumulate in many tissues of a body. It has been shown that $CeO_2$NPs can be primarily retained in the lungs, spleen, liver, bone marrow, and also brain (Geraets et al. 2012, Yokel et al. 2012). There is some evidence that nanoceria released from industrial processes undergo bioaccumulation and biomagnification in the terrestrial food chain (Majumdar et al. 2015).

The recent ecotoxicity studies also indicate that nanoceria in low doses can exhibit toxic effects on water organism. For instance, an effective concentration of $CeO_2$NPs that inhibits the cellular function of interest by 50% ($EC_{50}$) for microalgae reached values below 10.0 mg/l (Rogers et al. 2010, Manier et al. 2013) or 2.4-29.6 mg/l range (Rodea-Palomares et al. 2011).

It has been reported that nanoparticles attack mitochondria, which are redox active organelles, and induce reactive oxygen species (ROS) formation (OECD 2011). In a cell, ROS may damage proteins, lipids which are components of cell membranes, and the genetic material (DNA). One of the sensitive and widely employed in nanotoxicology research methods for assessment the extent of DNA damage is the comet assay (Karlsson 2010). In order to analyse DNA damage caused by ROS, the comet assay is performed using specific endonucleases e.g. formamido-pyrimidine glycosylase (FPG) applied for the detection of oxidative damage to purine bases of DNA, including 8-oxoguanine.

The results obtained from comet assay demonstrated that $CeO_2$NPs caused a statistically significant increase in the level of DNA damage at concentrations lower than cytotoxic doses. Likewise the FPG-sensitive sites were significantly different from the control at the same concentrations, which indicates the oxidative reason of DNA damages.

Available information on the genotoxicity of $CeO_2$NPs is inconsistent, and the results of researches are largely dependent on the cellular system tested. For example, nanoceria induced strong DNA lesions at low concentrations i.e. 0.05-20 μg/ml in human dermal fibroblasts (Auffan et al. 2009). In the follicular cells and mouse oocytes, a significant

DNA damage was observed in the concentration range of 2-100 μg/ml **(**Courbiere et al. 2013), whereas in the case of A549, CaCo2 and HepG2 cells – in the concentration range of 0.5-500 μg/ml (DeMarzi et al. 2013). In turn, 48 hour-long exposure to concentrations of up to 100 μg/ml did not cause any measurable genotoxic effects in human lens epithelial cells (Pierscionek et al. 2012).

## 5. Conclusion

The reported studies demonstrated that $CeO_2$NPs caused cytotoxic effects in the cells which increases with the time of exposure of the cells. Prolonged exposure (7 days) caused the cell proliferation disorder, which suggest potential chronic toxicity of $CeO_2$NPs. Considering the results of comet assay, it cannot be ruled out that nanoceria may cause long-term harmful effects related with the DNA damages and oxidative base of these lesions.

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## Ocena wybranych parametrów toksycznego działania nanocząstek ditlenku ceru na komórki CHO-9

## Streszczenie

Nanometryczny tlenek ceru ($CeO_2$NM) ma szerokie zastosowanie w wielu sektorach przemysłu, co stwarza możliwość emisji do środowiska (zarówno środowiska naturalnego, jak i środowiska pracy). Stosowany jest przede wszystkim jako katalizator paliwa w silnikach Diesla, elektrolit w stało tlenkowych ogniwach paliwowych (SOFC, solid oxide fuel cells), materiał polerski, barwnik plastiku, warstwa buforowa dla nadprzewodników, powłoka dla filtrów podczerwieni, pochłaniacz UV oraz jako przeciwutleniacz w biomedycynie. Pomimo, że produkcja $CeO_2$NM jest wielkotonażowa, to istniejące dane toksykologiczne, z uwagi na niespójny obraz toksycznego działania, uznane zostały za niewystarczające do oceny zagrożeń, jakie może stwarzać dla ludzi i środowiska.

Celem badań była ocena toksycznego działania $CeO_2$NM o nominalnej wielkości cząstek: < 25 nm na komórki układu rozrodczego (CHO-9), po krótkotrwałym (24 lub 72 h) i długotrwałym (7 dni) narażeniu komórek. Badano wpływ $CeO_2$NM na integralność błon komórkowych (test NRU), aktywność metaboliczną komórek (test MTT, test WST-1) oraz zdolność komórek do proliferacji (test wydajności tworzenia kolonii - CFEA). Oceniono również wpływ $CeO_2$NM na uszkodzenia DNA w komórkach poprzez oznaczenie metodą kometową (SCGE w warunkach zasadowych) jednoniciowych pęknięć DNA (SSB) oraz oksydacyjnych uszkodzeń zasad rozpoznawanych przez glikozylazę formamido-pirymidynową (SSB-FPG).

$CeO_2$NM powodował zależne od stężenia i czasu narażenia działanie cytotoksyczne. Kierunek zmian toksycznych obejmował zarówno zmiany w przepuszczalności błon komórkowych, jak i zaburzenia aktywności metabolicznej mitochondriów. Zakres stężeń cytotoksycznych wynosił 100-400 μg/ml zależnie od ocenianego skutku cytotoksycznego. Długotrwałe narażenie komórek na $CeO_2$NM powodowało utratę zdolności komórek do proliferacji w stężeniach ok. 50 μg/ml. $CeO_2$NM powodował zależny od stężenia istotny statystycznie wzrost poziomu jednoniciowych pęknięć DNA oraz oksydacyjnych uszkodzeń DNA rozpoznawanych przez FPG w narażanych komórkach już w najniższych ze stosowanych stężeń (10-100 μg/ml). Wyniki testu kometowego i klonogennego wskazują na potencjalną toksyczność przewlekłą $CeO_2$NM.

**Słowa kluczowe**:
nanocząstki ditlenku ceru, cytotoksyczność, genotoksyczność

**Keywords**:
cerium dioxide nanoparticles, nanoceria, cytotoxicity, genotoxicity