EFFECT OF CERIUM AND SELENIUM NANOPARTICLES ON FUNCTIONAL ACTIVITY OF NEUTROPHILS IN VITRO

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Abstract. Colloidal solutions of cerium and selenium nanoparticles were synthesized using the laser ablation method in deionized water. The resulting nanoparticle samples had a monomodal size distribution. The studied nanoparticles at a concentration of 1011 NPs/ml inhibit the peroxidase activity of neutrophil myeloperoxidase by approximately 10–15%. At the same time, the average fluorescence intensity of neutrophils, which exhibit both CD11b and CD66b on their surface, increases, which is a sign of degranulation of specific and gelatinase granules, as well as secretory vesicles. The studied particles of cerium and selenium have the ability to initiate secretory degranulation of neutrophils in a dose-dependent manner. After the addition of cerium nanoparticles to neutrophils, an increase in the production of hydrogen peroxide by neutrophils was recorded. At the same time, the assembly of NADPH oxidase was probably activated in neutrophils after they absorbed the nanoparticles. It has been shown that cerium and selenium nanoparticles are capable of initiating the formation of neutrophil extracellular traps. In general, the data obtained suggest that cerium nanoparticles in the considered range of concentrations contribute to a more pronounced activation of neutrophils under in vitro conditions compared to selenium nanoparticles.

Keywords: NPs, selenium nanoparticles, cerium nanoparticles, neutrophils, immunomodulation, degranulation, NETs formation, netosis, NADPH oxidase activity, pro-oxidant.

List of Abbreviations

NPs – nanoparticles

ROS – reactive oxygen species

PMA – phorbol ether; phorbol 12-myristate 13-acetate 4-O-methyl ether

MPO – myeloperoxidase

DPI – diphenyl iodonium

NETs – neutrophil extracellular traps

H2DCFDA, DCF – 2',7'-dichlorodihydrofluorescein diacetate

APC – allophycocyanin

Introduction

Currently, there is a rapid increase in the scale of the market for nanomaterials and the use of NPs in many industries (electronics, medicine, chemical pharmaceuticals, biology, etc.). At the same time, it's expected that in the next decade, nanotechnologies will be increasingly introduced into various spheres of human life (Lin *et al.*, 2018; Sudha *et al.*, 2018; Barhoum *et al.*, 2022). The nanomaterials most in demand in industry include: carbon NPs, including its various modifications (fullerenes, nanotubes, graphene), titanium oxide, zinc oxide, aluminum oxide, gold, iron, copper, cobalt, and some others (Piccinno *et al.*, 2012). Also, a wide range of application possibilities for selenium (Se NPs) and cerium (Ce NPs) NPs is reported. For Se NPs, the application of these nanomaterials in agricultural technologies and the food industry are of interest. (Garza-García *et al.*, 2021), including as a fertilizer (Gudkov *et al.*, 2020). The use of selenium-based nanomaterials in biomedical applications is considered (Vinković Vrček, 2018): creating nanocomposites for targeted drug delivery (Varlamova *et al.*, 2022; Varlamova *et al.*, 2023), as well as a number of other applications due to the possible cytoprotective effect (Turovsky *et al.*, 2022; Varlamova *et al.*, 2022; Varlamova *et al.*, 2023), immunomodulatory (Khabatova *et al.*, 2022; Mal'tseva *et al.*, 2022; Serov *et al.*, 2021), anticancer (Varlamova *et al.*, 2021; Geoffrion *et al.*, 2020; Martínez-Esquivias *et*

al., 2022), as well as antimicrobial (Serov *et al.*, 2023) and antifungal activity of Se NPs (Shahverdi *et al.*, 2010).

Cerium and cerium oxide NPs are another type of common nanomaterials that are of great interest and occupy a significant place among the total volume of produced nanomaterials (Tumkur *et al.*, 2021). Due to the presence of oxygen vacancies in cerium oxide, they can act as a solid electrolyte in fuel cells (Younis *et al.*, 2016). Cerium oxide NPs have received significant attention from researchers in nanotechnology field due to their applications as catalysts, fuel additives, and self-healing antioxidants (Xu & Qu, 2014). From the point of view of biomedical use, Ce NPs and cerium oxide NPs are characterized by antioxidant (Dhall & Self, 2018; Nelson *et al.*, 2016), radioprotective (Kadivar *et al.*, 2020; Zal *et al.*, 2018), anticancer as well as antibacterial activity (Arumugam *et al.*, 2015; Zhang *et al.*, 2019; Barker *et al.*, 2022). However, the currently available data on biocompatibility, geno- and cytotoxicity of cerium NPs are insufficient to confirm their absolute biosafety (Kalyanaraman *et al.*, 2019).

It is obvious that the high volume of production and the prevalence of application makes it inevitable that nanomaterials enter the environment and, as a result, the human body. NPs can enter the body in various ways: through the inhaled air, with food, and also through the skin (De Matteis, 2017). Therefore, for the safe use of Se NPs and Ce NPs, it is necessary to comprehensively study the effect of these NPs under *in vitro* conditions. Of particular interest is the reaction of isolated cultures of immune cells in response to the introduction of NPs (Koltakov *et al.*, 2022). It is known that among all human leukocytes, neutrophils are the most numerous group. Neutrophils are phagocytes of the "first line" of defense when exposed to xenobiotics (Nauseef & Borregaard, 2014). In this regard, it is extremely important to study the reactions of neutrophils *in vitro* in response to the effects of Se NPs and Ce NPs: to evaluate changes in NADPH oxidase activity, degranulation, and netosis.

Materials and Methods

Synthesis and characterization of Se NPs and Ce NPs

Ce NPs and Se NPs were obtained by laser ablation in deionized water (Gudkov *et al.*, 2022). A solid target (selenium or cerium) was placed at the bottom of the cuvette under a thin layer of water (Sdvizhenskii & Lednev, 2022). In this state, a solid target was irradiated with a laser beam (λ = 1064 nm; T = 4–200 ns; f = 20 kHz; P = 20 W; Ep = 1 mJ). The laser beam was moved over the target surface using a TM 2D galvanomechanical scanner (Ateko, Russia). Depending on the parameters of laser radiation, the speed and trajectory of the laser beam, as well as the time of exposure, using laser ablation, it is possible to obtain colloidal solutions of NPs with specified characteristics (Ashikkalieva *et al.*, 2022; Simakin *et al.*, 2021). When changing the parameters of laser radiation pulses, it is also possible to achieve thermal and non-thermal effects on the target (Matveeva *et al.*, 2022). The procedure for obtaining and characterizing selenium NPs is described in detail earlier (Turovsky *et al.*, 2022). The concentration of NPs and the hydrodynamic size were evaluated using Zetasizer Ultra Red Label (Malvern, UK).

Peroxidase activity of myeloperoxidase assessment

Myeloperoxidase (MPO) peroxidase activity was recorded by spectrophotometric method by the rate of o-dianisidine (*o*-DA) oxidation. A standard sample for spectrophotometric determination of MPO peroxidase activity (2.5 nM) contained: phosphate-buffered saline (PBS) containing 8.3 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 123 mM NaCl and 2.7 mM KCl (pH 7.4), 50 μM H_2O2, *o*-DA substrate (380 μM), and test NPs at various concentrations. The reaction was started by adding 50 μM H_2O_2, and the optical density of the sample was determined in the kinetic mode for 6–8 min at a wavelength of 460 nm (OD_{460}). The measurements were carried out at 23 °C on a PB 2201 spectrophotometer (SOLAR, Minsk, Belarus).

Secretory neutrophil degranulation assessment

Secretory degranulation of neutrophils was assessed by flow cytometry as described previously (Panasenko *et al.*, 2022). Neutrophils (1×10^6 cells/mL in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) were incubated with the studied NPs at the required concentration or with the standard neutrophil activator, PMA (50 nM), for 20 min at 37 °C. Then, a marker of exocytosis of the contents of specific granules, antibodies against CD66b conjugated with FITC, and a marker for secretion of gelatinase granules and secretory vesicles, antibodies against CD11b conjugated with APC, were added to the cell suspension at a concentration of 1% of the volume and incubated for 5 min at room temperature in darkness. The resulting cell suspension was analyzed using a CytoFocus flow cytometer (Healicom, China). A laser with a wavelength of 488 nm was used to excite FITC fluorescence, and a 520 ± 20 nm filter was used for registration; APC excitation was carried out by a laser with a wavelength of 638 nm, for registration — a filter at 660 ± 20 nm. At least 50.000 neutrophils were recorded in each sample, and the data obtained was analyzed using the software supplied with the flow cytometer. The average fluorescence intensity of neutrophils carrying FITC-conjugated anti-CD66b antibodies or APC-conjugated anti-CD11b antibodies was used as a quantitative characteristic characterizing secretory degranulation. Background fluorescence intensity was subtracted from all measurements.

Assessment of the activity of NADPH oxidase of neutrophils

Neutrophil NADPH oxidase activity was assessed by the production of H_2O_2 by the fluorescent method using scopoletin, as shown earlier (Gorudko *et al.*, 2011). Scopoletin is a fluorescent substrate of horseradish peroxidase, the oxidation of which in the presence of H_2O_2 produces a non-fluorescent product. To 1 ml suspension of neutrophils (106 cells/mL in PBS with 1 mM/L $CaCl_2$, 0.5 mM/L $MgCl_2$, 37 °C) containing 1 μM/L scopoletin, 20 μg/mL horseradish peroxidase and 1 mM/L NaN_3, NPs were added, and the kinetics of scopoletin oxidation was recorded by a decrease in fluorescence intensity at a wavelength of 460 nm (excitation at a wavelength of 350 nm) on a computerized spectrofluorimeter CM 2203 (SOLAR, Minsk, Belarus). The rate of H_2O_2 production by the cells was determined as the slope of the linear section of the kinetic curve, which reflects the decrease in the fluorescence intensity of scopoletin as a result of its oxidation by H_2O_2. As a rule, to standardize the experimental conditions, the reaction mixture was preliminarily prepared by mixing aqueous solutions of 0.2 mM/L scopoletin, 2 mg/mL horseradish peroxidase, and 0.1 M/L NaN_3 in a ratio of 1:2:2; μL of this solution to 950 μL of cell suspension.

Intracellular production of reactive oxygen species (ROS) by neutrophils was assessed using the H2DCFDA fluorescent probe by flow cytometry. Neutrophils (1×10^6 cells/mL) were loaded for 10 min at room temperature with a fluorescent H2DCFDA probe (2.5 μM), after which the cells were incubated with test NPs at a concentration of 5% by volume or PMA (50 nM) for 15 min at 37 °C. DCF fluorescence intensity was assessed using a CytoFocus flow cytometer (Healicom, China), using a 488 nm laser for fluorescence excitation and a 520 ± 20 nm filter (FITC channel) for registration. At least 50.000 neutrophils were recorded in each sample, and the data obtained was analyzed using the software supplied with the flow cytometer.

Evaluation of the formation of neutrophil extracellular traps (NETs, netosis)

NETs formation was recorded by flow cytometry. Neutrophils were incubated with the test compounds at various concentrations for 30–60 min at 37 °C, and then, if necessary, the standard NETs formation inducer, PMA (25 nM), was added and incubated for another 30 min at 37 °C. Next, SYTOX Green (50 nM), which binds to nucleic acids when the cell plasma membrane is damaged, and anti-MPO antibodies conjugated with the Cy5 fluorescent label were added to the neutrophil suspension and incubated for 5 min at room temperature in the dark. To excite SYTOX Green fluorescence, a laser with a wavelength of 488 nm was

used; for registration, a filter of 520 ± 20 nm (FITC channel) was used; Cy5 excitation was carried out with a laser with a wavelength of 638 nm; for registration, a filter at 660 ± 20 nm (APC channel) was used. The fluorescence intensity of neutrophils stained with SYTOX Green and Cy5-conjugated anti-MPO antibodies was assessed using a CytoFocus flow cytometer (Healicom, China). At least 30.000 neutrophils were recorded in each sample, and the data obtained were analyzed using the software supplied with the flow cytometer. The following quantitative characteristics characterizing netosis were chosen: % of SYTOX Green-positive neutrophils, average fluorescence intensity of neutrophils stained with SYTOX Green, average fluorescence intensity of neutrophils carrying Cy5-conjugated anti-MPO antibodies on their surface.

Results

Colloidal solutions of cerium (Ce1, Ce2) and selenium (Se) NPs were synthesized using the laser ablation method in deionized water. The NPs samples had a monomodal size distribution. The average hydrodynamic diameter of cerium NPs is about 185 and 280 nm for Ce1 and Ce2 NPs, respectively. The average size of Se NPs is about 130 nm. At concentrations up to 10^{11} Se NPs/ml, there was no aggregation in the colloidal solution. The maximum distribution of the zeta potential of colloidal solutions of NPs was 19.7, 1.2, and 16.6 mV for Ce1, Ce2, and Se NPs, respectively.

According to the results of the study of the effect of cerium and selenium NPs on the peroxidase activity MPO (cell-free medium), it was found that the studied NPs suppress the peroxidase activity of MPO by approximately 10–15% (at a concentration of 10% by volume), and, therefore, in this concentration range do not have a pronounced antioxidant activity (Fig. 2).

Analysis of the effect of Ce and Se NPs on neutrophils degranulation showed that after cell incubation with PMA (50 nM, 20 min at 37 °C, positive control), the average fluorescence intensity of neutrophils exhibiting both CD11b and CD66b on their surface increases (Fig. 3), which confirms that PMA causes degranulation of specific and gelatinase granules, as well as secretory vesicles.

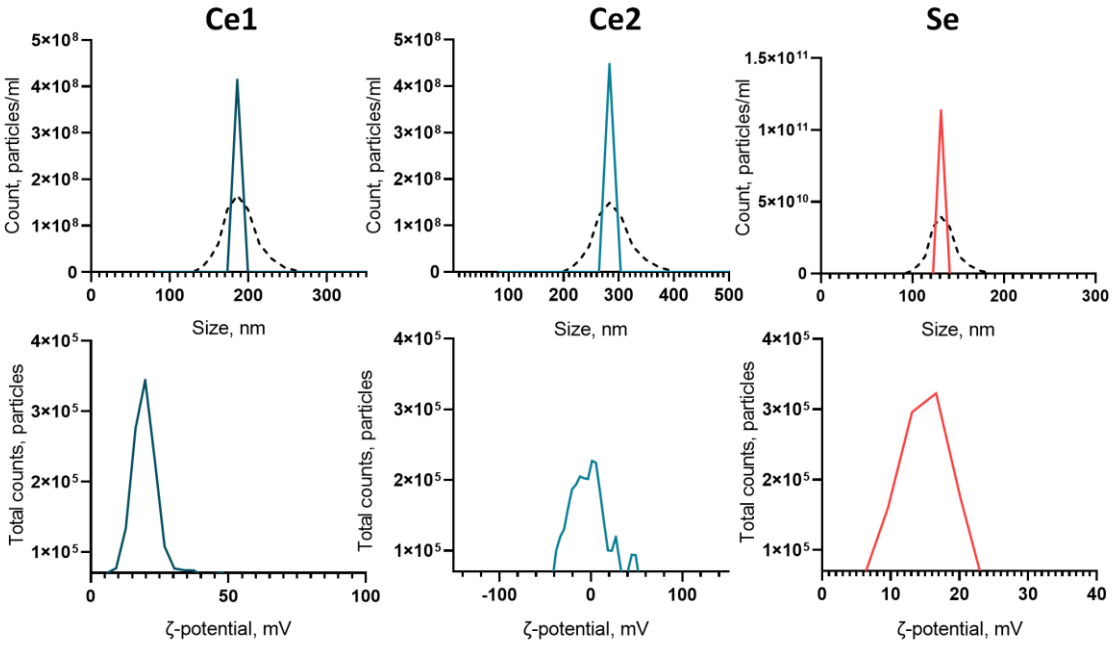

Fig. 1. Characteristics of the synthesized samples of Ce NPs and Se NPs. Concentrations and size distributions of NPs (top row of graphs) and NP zeta potential (bottom row of graphs)

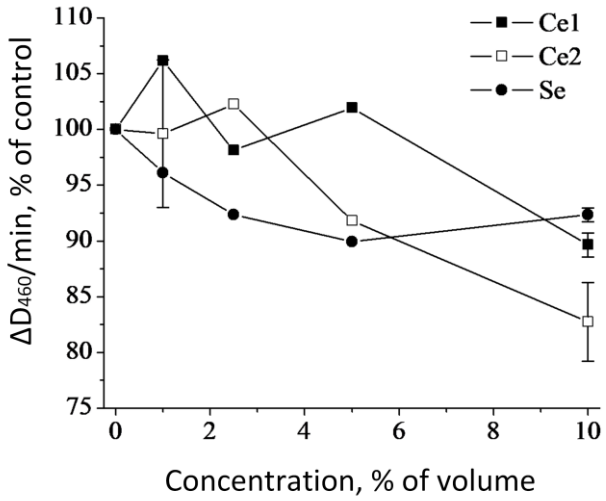

Fig. 2. Effect of Ce and Se NPs on peroxidase activity evaluated by spectrophotometric method from the oxidation rate of orthodianisicin (*o*-DA)

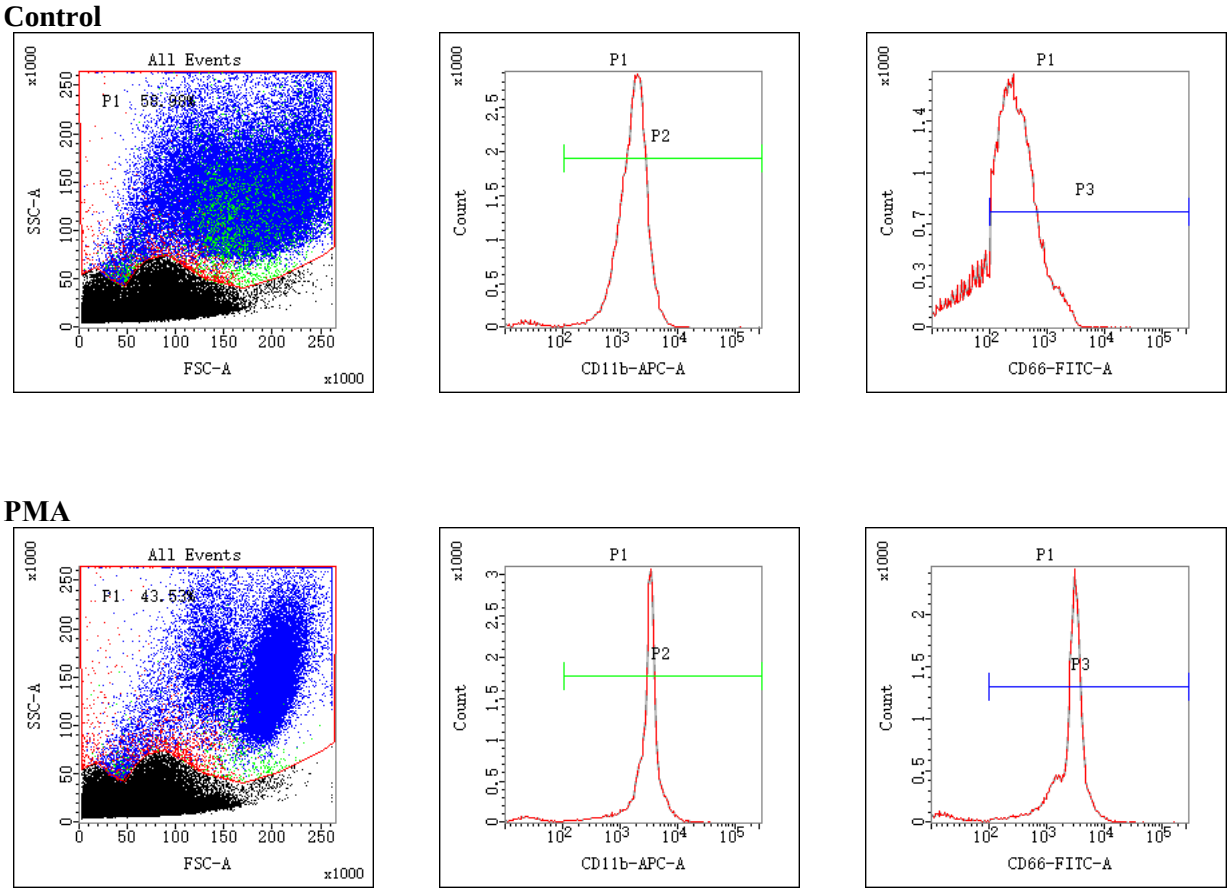

Fig. 3. Histograms of forward and side scatter light and fluorescence intensity of neutrophils incubated with APC-conjugated anti-CD11b and FITC-conjugated anti-CD66b antibodies in control (upper row of images) and after incubation with PMA
(lower row of images)

The studied Ce and Se NPs have the ability to initiate secretory degranulation of neutrophils in a dose-dependent manner. The maximum effect (for a concentration of 7.5% by volume) was provided by Ce2 NPs (Fig. 4).

The study of the effect of Ce and Se NPs on the NADPH oxidase activity of neutrophils showed that after adding Ce1 NPs to the suspension of neutrophils (Fig. 5.), a dose-dependent significant decrease in the intensity of scopoletin fluorescence was recorded, which indicates the production of H_2O_2 by neutrophils. In the presence of diphenylene iodonium (DPI), an inhibitor of flavin enzymes, no decrease in the intensity of scopoletin fluorescence in the neutrophil suspension was observed after the addition of Ce1 NPs, which indicates activation of the NADPH oxidase assembly of neutrophils after the addition of NPs. A dose-dependent activation of NADPH oxidase was also observed after the addition of Ce2 NPs to the suspension of neutrophils (Fig. 6).

Assembly of NADPH oxidase can be activated in neutrophils after they have taken up the NPs, since H_2O_2 production wasn't detected when neutrophils were pretreated with cytochalasin B (2.5 μg/mL) (Fig. 7).

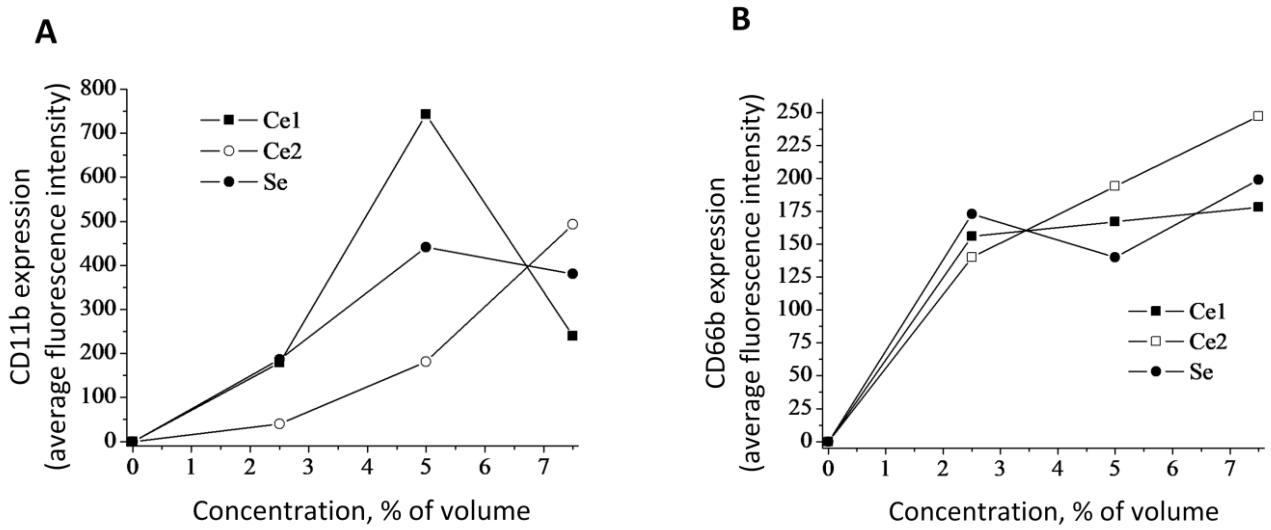

Fig. 4. The effect of Ce (Ce1 and Ce2) and Se NPs on neutrophils degranulation evaluated by flow cytometry by exposure on the surface of CD11b (A) and CD66b (B) cells

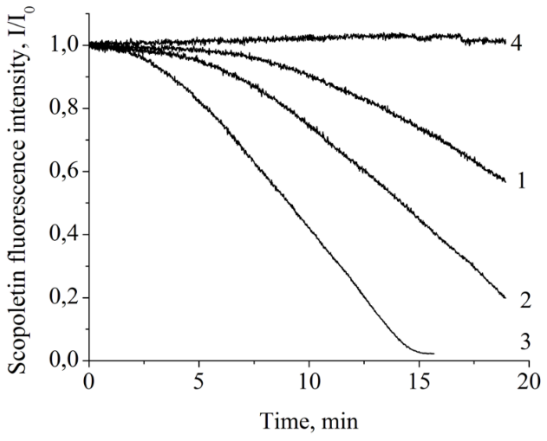

Fig. 5. Typical kinetic curves of scopoletin oxidation by neutrophils after the NPs addition: Ce1 NPs (1.25% by volume) (1), Ce1 NPs (2.5% by volume) (2), Ce1 NPs (5% by volume) in the absence and presence of DPI (100 μM) (3 and 4, respectively). Suspension of neutrophils (2×10^6 cells/mL) in PBS contained 1 mM/L $CaCl_2$, 0.5 mM/L $MgCl_2$, 1 μM/L scopoletin, 20 μg/mL horseradish peroxidase and 1 mM/L NaN_3

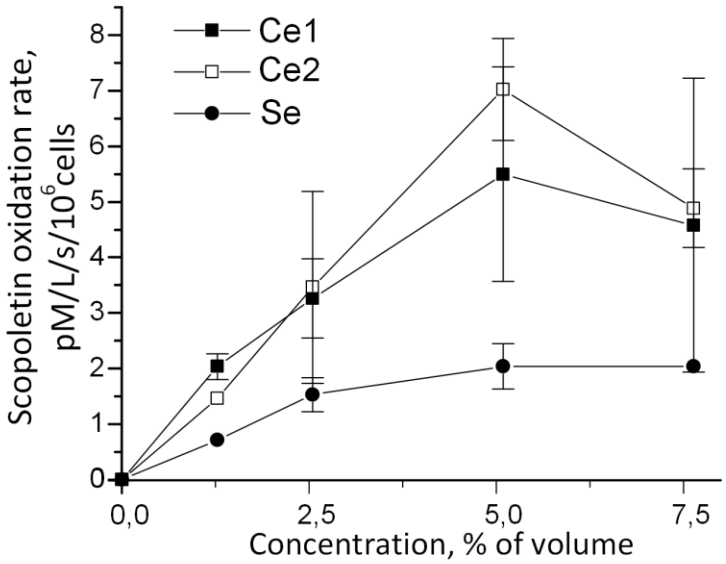

Fig. 6. Influence of Ce and Se NPs on the production of H_2O_2 by neutrophils

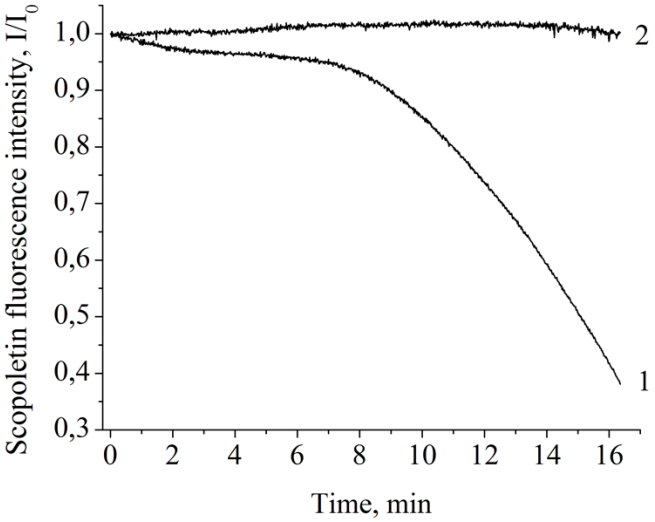

Fig. 7. Typical kinetic curves of scopoletin oxidation by neutrophils after the addition of Ce1 NPs (5% by volume) in the absence (1) and presence of cytochalasin B (2.5 μg/ml), respectively. Suspension of neutrophils (2×10^6 cells/mL) in PBS contained 1 mM/L $CaCl_2$, 0.5 mM/L $MgCl_2$, 1 μM/L scopoletin, 20 μg/mL horseradish peroxidase and 1 mM/L NaN_3

Further, the effect of NPs on the intracellular ROS production was studied by flow cytometry using H2DCFDA. After cell incubation with PMA (50 nM, 15 min at 37 °C, positive control), the mean fluorescence intensity of neutrophils loaded with H2DCFDA increased, indicating intracellular ROS production (Fig. 8).

Ce NPs activated intracellular ROS production in neutrophils. At the same time, Ce2 NPs had the greatest stimulating effect (Fig. 9).

The effect of Ce1, Ce2 and Se NPs on the formation of neutrophil extracellular traps (NETs, netosis) was studied. The results obtained indicate that after cell incubation with PMA (25 nM, 30 min at 37 °C, positive control), the number of SYTOX Green-positive events and the intensity of fluorescence from the Cy5 label conjugated with anti-MPO antibodies increase (Fig. 10). In the presence of Ce1 and Ce2 NPs, the number of SYTOX Green-positive neutrophils increased com-

pared to the control, which indicates the ability of the above NPs to initiate the formation of NETs (Fig. 11). This effect seems to be associated with the ability of the NPs to activate the respiratory burst of neutrophils, followed by the production of ROS. In addition, it was found that Ce2 NPs had a priming effect on PMA-induced netosis, which was detected by exposure of anti-MPO antibodies on NETs.

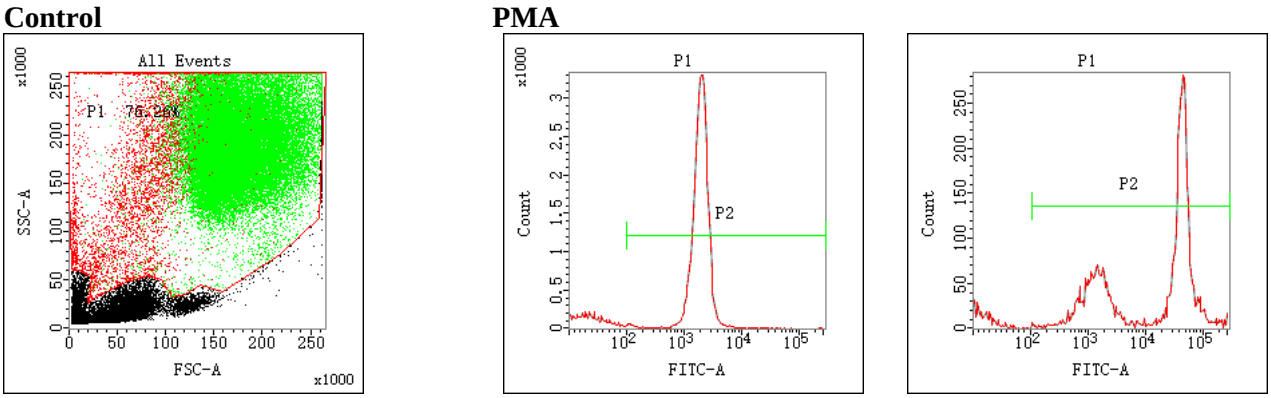

Fig. 8. Histogram of forward and side scatter, as well as fluorescence intensity of neutrophils loaded with H2DCFDA (FITC channel) in control and after incubation with PMA (50 nM)

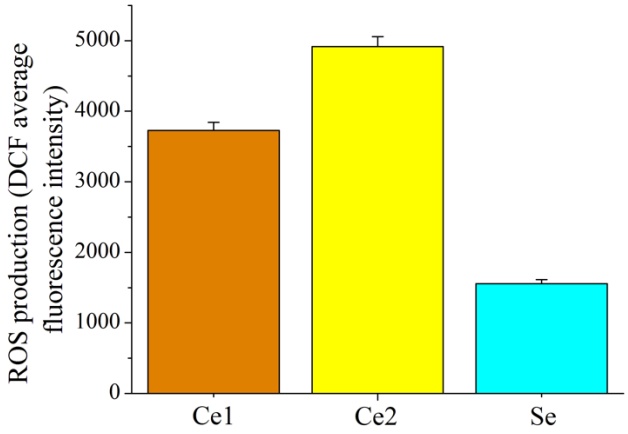

Fig. 9. Influence of Ce1, Ce2 and Se NPs at a concentration of 5% by volume on intracellular production of ROS by neutrophils, evaluated by flow cytometry using H2DCFDA

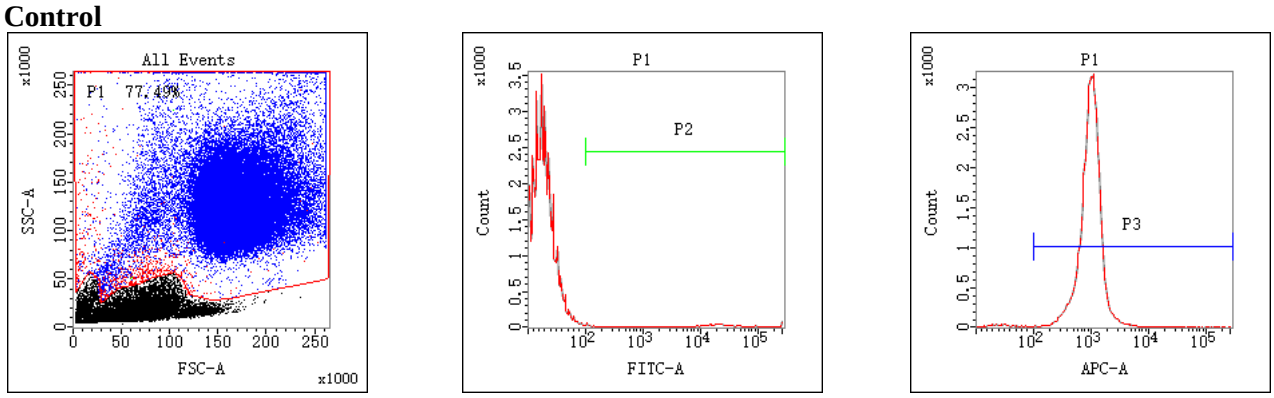

Fig. 10. Histograms of forward and side scatter light and fluorescence intensity of neutrophils stained with SYTOX Green (FITC channel) and Cy5-conjugated anti-MPO antibodies (APC channel) in control (upper row of images) and after incubation with PMA (lower row of images). Figure continued on page. 32

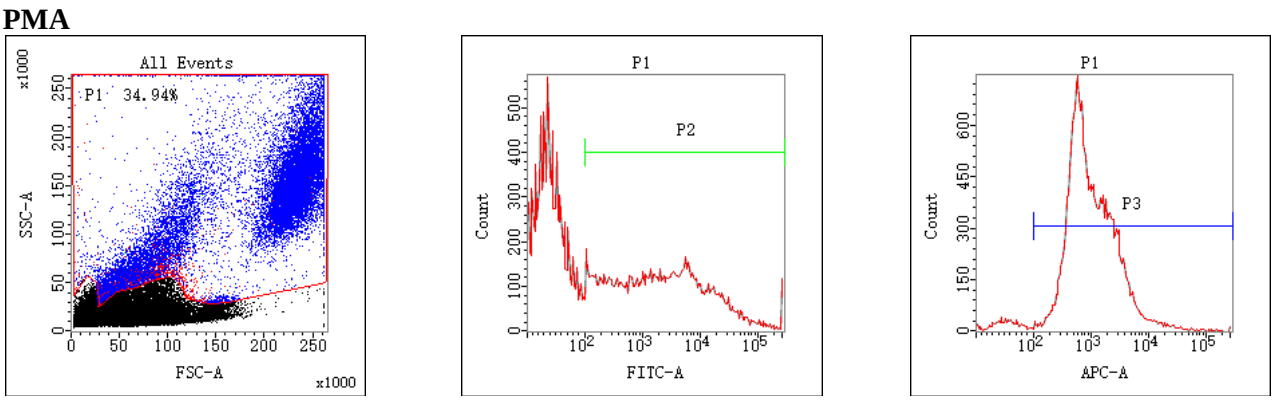

Fig. 10. Histograms of forward and side scatter light and fluorescence intensity of neutrophils stained with SYTOX Green (FITC channel) and Cy5-conjugated anti-MPO antibodies (APC channel) in control (upper row of images) and after incubation with PMA (lower row of images)

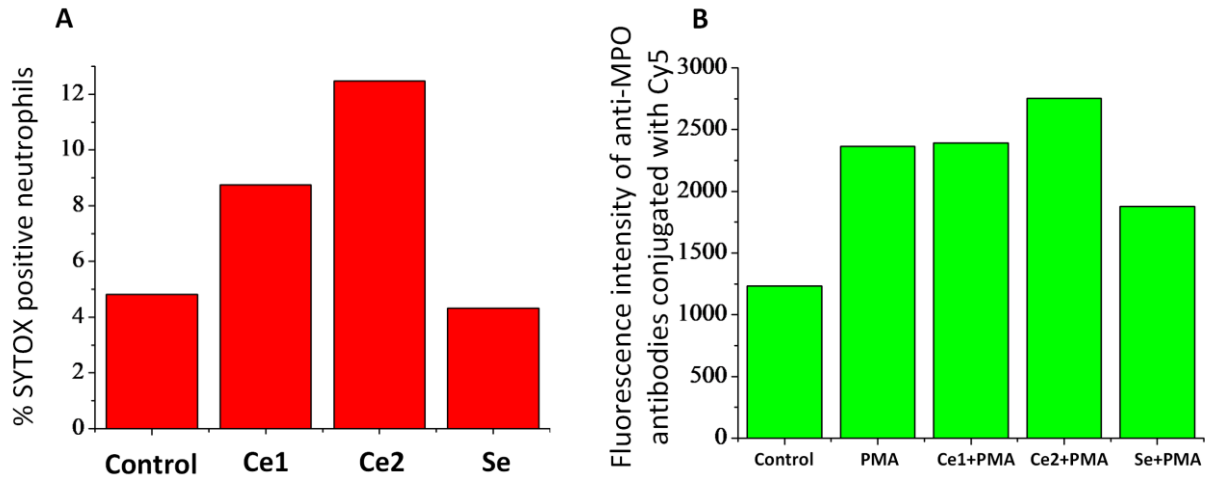

Fig. 11. The number of SYTOX-Green-positive neutrophils after incubation with Ce1, Ce2 and Se NPs at concentration of 5% by volume (A); as well as the average fluorescence intensity of neutrophils carrying Cy5-conjugated antibodies against MPO on their surface in samples incubated with the studied NPs (5% of the volume, 30 min) and PMA (25 nM, 30 min) (B)

Discussion

Ce NPs (Ce1, Ce2) and Se NPs samples obtained by laser ablation had a monomodal distribution of hydrodynamic diameter, which was 185 and 280 nm for Ce NPs (Ce1 and Ce2, respectively) and 130 nm for Se NPs. The zeta potentials of colloidal solutions were 19.7, 1.2, and 16.6 mV for Ce1, Ce2, and Se NPs, respectively. The characteristics of the obtained Se NPs are comparable with the samples used in the previous work (Mal'tseva *et al.*, 2022).

According to the results obtained, cerium and selenium NPs didn't have antioxidant activity in the considered range of concentrations (Fig. 2). The application of Ce and Se NPs promoted neutrophil degranulation, which was manifested in a significant increase in expression of CD11b and CD66b on the neutrophils surface (Fig. 3). The effect was dose-dependent and was most pronounced when cerium NPs were added at higher concentrations (7.5% by volume) (Fig. 4). The addition of Ce1 and Ce2 NPs contributed to an increase in the activity of

NADPH oxidase of neutrophils, which manifested itself in a significant increase in the production of H_2O_2 (Fig. 5-7).

A dose-dependent formation of intracellular ROS in neutrophils was also observed with the addition of Ce1 and Ce2 NPs (Fig.8,9). Along with other mechanisms of ROS generation in aqueous media (Shcherbakov, 2022), the activity of NPs against cellular NADPH oxidase is considered as one of the key possible mechanisms for generating high concentrations of intra- and extracellular ROS (Petty, 2016). A number of studies have shown that NPs-mediated hyperactivation of NADPH oxidase contributes to the development of oxidative stress (Meghea, 2020; Rozhkova *et al.*, 2015). In addition, it was found that the addition of Ce1 and Ce2 NPs stimulated neutrophil netosis (Fig. 10). Notably, no significant increase in NETs formation was observed with Se NPs (Fig. 10). Ce2 NPs also had a priming effect on PMA-induced netosis, which was expressed in an increase in the expression of anti-MPO antibodies on NETs (Fig. 11).

Conclusion

Thus, the synthesized samples of cerium and selenium NPs stimulated the activation of neutrophils with subsequent degranulation and exocytosis of specific and gelatinase granules. At the same time, cerium NPs had a more pronounced prooxidant activity, which increased with an increase in the concentration of NPs; stimulated the activation of NADPH oxidase of neutrophils *in vitro* and increased the production of intracellular ROS, and also stimulated netosis in neutrophils. Our data suggest that cerium NPs in the concentration range under consideration contribute to a more pronounced activation of neutrophils under *in vitro* conditions compared to selenium NPs.

Acknowledgements

This work was supported by a grant of the Ministry of Science and Higher Education of the Russian Federation (075-15-2022-315) for the organization and development of a world-class research center “Photonics”.

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