MEDICAL AND BIOLOGICAL ASPECTS RELATED TO ASSESSMENT OF IMPACTS EXERTED BY RISK FACTORS

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Research article

ON ASSESSING THE POTENTIAL RISK OF DOSE-DEPENDENT HEPATOTOXIC EFFECTS OF SELENIUM OXIDE NANOPARTICLES

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Selenium nanoparticles (Se NPs) have found wide application in many human economic activities. Therefore, it is necessary to predict and assess emerging potential health risks. Nanotoxicants can affect the body causing negative effects that have a non-linear dependence on the dose of a toxic substance. There is no consensus on the LD_{50} of Se NPs. Recent data on the dose-dependent liver response to different exposures of selenium nanoparticles are contradictory.

The aim is to study and characterize potentially adverse dose-dependent effects in the liver under exposure to selenium oxide nanoparticles in a subchronic experiment using mathematical models.

Exposure was modeled on male rats aged 3 to 4 months, 12 animals in each group. We used three levels of selenium nanoxide doses for subchronic exposure: 3.6, 18, and 36 mg/kg. The research was approved by the Local Ethics Committee of the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers (Protocol No. 2 of April 20, 2021).

We observed an atypical dose-response relationship between selenium nanooxide exposure and hepatic changes. The negative effects included pronounced changes in mitochondria of liver cells as well as an imbalance of blood enzymes and cellular composition of the liver, which may indicate damage to the organ and impaired secretory functions following the exposure to low and moderate concentrations of SeO nanoparticles.

Our findings can be used for determining chemical safety standards for selenium oxide nanoparticles and assessing their health risks.

Keywords: *nanoparticles, in vivo, dose-effect, selenium oxide, hepatotoxicity, Kupffer cells, hepatocytes, health risk.*

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Selenium nanoparticles (Se NPs) are generated in the course of human economic activities: in the metallurgical and chemical industries [1], in production of ceramics, glass, and electronics. Use of Se NPs in enrichment of food products and supplements [2], as drug carriers [3], antibacterial and anticancer drugs [4–7], for disease prevention in farm animals [8, 9] and in plant cultivation can pose health risks from excessive accumulation of selenium compounds in the body not only to industrial workers but also to the general population. When analyzing biological effects of nanoselenium, it is necessary to take into account toxicity of NPs determined by physical properties and specific characteristics of the chemical elements that form them. Se NPs exhibit pro- or antioxidant activity depending on the dose and exposure duration [4, 5, 10]. It has been experimentally proven that smaller Se NPs (sized 6.8 nm) have greater penetrating ability and accumulation in organs, higher activity in replacing sulfur in sulfur-containing proteins when participating in the synthesis of selenoproteins [4, 11]. Bypassing the blood–brain barrier with small Se NPs (6.8 nm) leads to a decrease in the number of astrocytes [4], while larger Se NPs exhibit neuroprotective properties by increasing the number of neurons [10]. Adverse toxic effects of Se NPs are known to date. Oral administration of Se NPs to rats at the dose of 0.5 mg/kg body weight (b.w.) per day for 28 days induced local alopecia, a decrease in the body weight gain, and an increase in the relative weight of the liver [12]. Young rats demonstrated more intense accumulation of Se NPs, mainly in the liver, kidneys, and testicles, compared to adult rodents [13]. Se NPs accumulate in the liver [4, 14, 15], kidneys [4, 15], muscles, stomach, and blood [15]. They are potentially toxic to reproduction [16]. The liver is one of the key target organs for Se NPs. It facilitates conversion of Se NPs into selenocysteine and selenomethionine and their incorporation into enzymes [17]. Both pro-oxidant [18] and antioxidant activity of Se NPs [19] cause bioaccumulation of lipid peroxidation products in the liver [15]. The results of studying the dose-dependent effect of Se NPs on the liver seem contradictory: the functional activity and histological picture of the liver change ambiguously. In some cases, the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin in the blood remained unchanged, as in the 90-day experiment on fish at the doses of 0.25, 0.5, and 1 mg Se NPs/kg feed [9]. No differences were observed in the activity of AST and alkaline phosphatase (ALP) in rats following intake of Se NPs containing 0.5, 1.5, 3.0, and 5.0 mg Se/kg feed for 28 days; a significant decrease in ALT activity was found in all exposed groups compared to the controls and a decrease in superoxide dismutase activity in the liver in the group receiving Se NPs at 5 mg Se/kg (929 ± 103 U/mL) [14]. Several studies have noted a similar decrease in ALT, alone or in combination with AST [20, 22]. An increase in transaminases was noted in 28-day experiments on mice with oral administration of Se NPs sized 70–90 nm at 1 mg/kg feed and 4 mg/kg b.w. [23] and that by gavage at the doses of 1/10–1/5 LD_{50} (LD_{50} = 88.76 Se mg/kg b.w.) [24]. An increase in transaminases [16, 25] and in alkaline phosphatase activity [15] under effect of Se NPs was reported in earlier studies.

Histopathological studies demonstrated the dose-dependent state of the liver parenchyma varying from the absence [12] or mild dystrophic changes in the groups of rats receiving Se NPs at the doses of 0.5–3.0 mg Se/kg b.w. for 28 days [14] and 2.0 and 4.0 mg/kg b.w. for 14 days [16], to mild multifocal autolytic lesions with signs of congestion in the group receiving Se NPs at the dose of 5.0 mg Se/kg [14], and hepatocyte death [16].

Nanosized selenium compounds have different degrees of toxicity [26], and this is the reason for differences in experimental findings relating to body weight gain, activity of liver and selenium-dependent enzymes, changes in the antioxidant system of the body, and the severity of histological damage.

The question about the median lethal dose of Se NPs remains open, possibly due to the choice of different biological objects for research (fish, turkeys, mice, rats), but even for animals of the same species LD_{50} is uncertain: in mice, it ranges, for instance, from 61.6 mg Se/kg b.w. for SPF ICR mice [27] to 2,000 mg/kg b.w. [4].

The study aimed to establish dose-dependent hepatic effects of subchronic exposure to selenium oxide nanoparticles posing potential health hazard using mathematical models.

Materials and methods. *Description of nanoparticles and the suspension.* Selenium oxide nanoparticles (SeO NPs) in the form of a water-based suspension were generated at the Ural Center for Collective Use "Modern Nanotechnologies" of the Ural Federal University named after the First President of Russia B.N. Yeltsin. Scanning electron microscopy was used to confirm a nearly spherical shape and the size range of 37 to 65 nm (Figure 1). The nanoparticle concentration in the suspension (0.25 mg SeO/mL) was validated at a high zeta potential of up to 42 mV using a Zetasizer Nano ZS analyzer (Malvern Panalytical Ltd., UK).

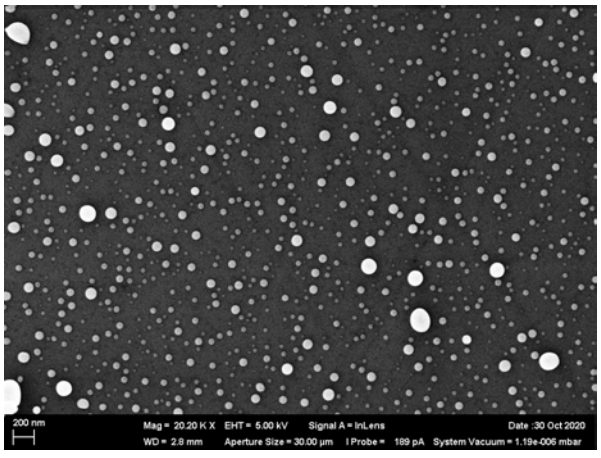

Figure 1. Suspended SeO nanoparticles (SEM image at 20,200 × magnification)

Laboratory animals and experimental exposure. The study was conducted using outbred 3-4-month-old male rats with a body weight of 200 to 270 g. We divided the animals into a control group and three experimental groups of 12 rats each, the latter being exposed to different doses of SeO NPs. Subchronic exposure was modeled over 6 weeks by successive intraperitoneal injections of a stable suspension of SeO NPs made thrice a week. The "SeO NPs 3.6" group was administered the total dose of SeO NPs equal to 3.6 mg/kg b.w., the "SeO NPs 18" group – 18 mg/kg b.w., the "SeO NPs 36" group – 36 mg/kg b.w., and the control group received injections of deionized water at the same intervals. Since there is no consensus on the LD_{50} of selenium NPs, the selection of doses and route of administration was carried out in a pilot experiment taking into account the use of chemically pure SeO NPs to obtain a suspension similar to that used in our previous works [28]. Our findings are comparable with those of other research teams [21, 25].

The limitation of our study was the use of animals of the same species and sex.

The study was approved by the Local Ethics Committee of the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers (protocol No. 2 of April 20, 2021).

After exposure cessation, we performed biochemical testing of blood serum on a Cobas Integra 400 plus Roche analyzer (Switzerland) using ready-made diagnostic kits for determining transaminases and alkaline phosphatase.

Morphological studies of liver cells and tissue. Cytological preparations were tested to identify post-exposure morphologic hepatic changes in all groups of animals. After Leishman staining, liver smears were examined by light microscopy at 100× and 1000× magnifications using a Primo Star microscope (Carl Zeiss, Germany) with a USCMOS visualization video camera for 300 cells and the percentage composition of cells and the number of damaged cells were calculated.

We scrutinized the histological picture of the liver of the control rats and those in the SeO NPs 36 exposure group receiving the maximum dose. Morphometric studies of

enucleated hepatocytes (cytoplasts) and Kupffer cells were performed using the Avtandilov grid.

Electron microscopy. Ultrastructural characteristics of cell damage were determined according to the classification by Sun [13] using a high-resolution scanning electron microscope Hitachi REGULUS SU8220 (Hitachi High-Technologies Corp., Japan) in the STEM mode. Based on the topological characteristics of the inner mitochondrial membrane (number of cristae, homogeneity and density of the matrix), we distinguished normal mitochondria (type A) and a variant of normal vesicular mitochondria (type B), as well as pathological forms, including vesicular (type C), vesicular swollen (type D), and swollen (type E) ones.

Mathematical modeling and statistical analysis. Based on the obtained values of generally accepted indicators reflecting changes in liver functioning, we constructed the relationship between the total dose of SeO NPs and its toxic effects on the liver using the following functions:

- The modified Hill function (1) introduced by Panov et al. [29]:

$$y = \left(b_0 + \frac{b_1 + b_2 x^{b_3}}{1 + \left(b_4 x\right)^{b_5}} \right) \left(1 + b_6 x^{b_7}\right), \qquad (1)$$

where b_0, …, b_7 are the parameters determined by the least squares method from experimental data;

- The hyperbolic function (2) related to the Michaelis-Menten equation, which is used, for instance, to describe the rate of enzyme reactions [15]:

$$y = \frac{b_0 + b_1 x}{b_2 + b_3 x} \qquad (2)$$

- The linear combination of Chebyshev polynomials (3). Chebyshev polynomials of the first kind are defined by the following equality:

$$T_n\left(x\right) = \sum_{k=0}^{[n/2]} C_n^{2k} (x^2 - 1) x^{n-2k}, \qquad (3)$$

Where [n/2] is the integer part of the number n/2 and C_n^{2k} is the number of combinations of n by 2k; and

- The modified Johnson – Lovett dose-response model [29, 30]:

$$y = b_0 + \frac{b_1}{1 + b_2 e^{b_3 x}} + \frac{b_4}{1 + b_5 e^{b_6 x}}. \qquad (4)$$

The statistical significance of intergroup differences in the mean values of all quantities was assessed using the Student's t-test ($p < 0.05$) and the Mann – Whitney U test.

Results and discussion. Damaging effects of SeO NPs on the liver were analyzed at the subcellular, cellular, tissue, and organ levels.

Ultrastructural examination of liver cells using electron microscopy revealed a decrease in the number of type A and B mitochondria, attributed by Sun to the normal morphotype [13], ranging from 94.82 ± 0.95 % in the control to 87.44 ± 1.14 % in the SeO NPs 36 group ($p < 0.05$).

We detected changes at the cellular and tissue levels in rats after SeO NP exposure. Microscopy of histological liver preparations showed an increase in the proportions of cytoplasts and Kupffer cells (KC) in the SeO NPs 36 group ($p < 0.01$).

One of the manifestations of the damaging effect of SeO NPs was an increase in the number of degenerated hepatocytes (Figure 2). The increase in their percentage in line with an increase in the exposure dose is described by a graph using a linear combination of Chebyshev polynomials (Formula 3).

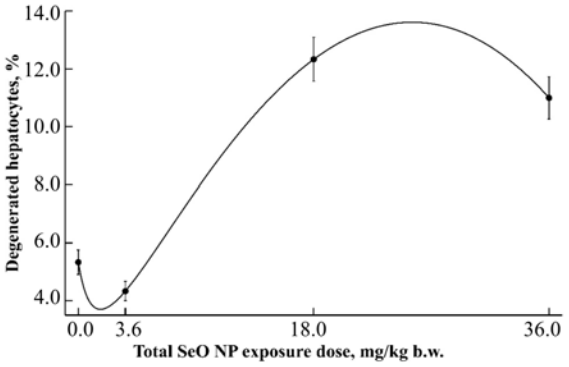

Figure 2. Changes in the proportion of degenerated hepatocytes in liver smears following SeO NP exposure (mean ± error of the mean)

At the lowest SeO NP exposure dose of 3.6 mg/kg b.w., we observed a slight decrease in the percentage of degenerated hepatocytes, but at 18 mg/kg b.w., we detected the maximum number of damaged hepatocytes ($p < 0.05$), which decreased insignificantly at the highest exposure dose of 36 mg/kg b.w. Similar non-monotonicity of the response of biological objects was also noted by other authors [31–34].

The non-linearity of the graphs of the relationship between SeO NP concentrations and the amount of degenerated hepatocytes is explained by toxic damaging mechanisms of SeO NPs in the liver. At low doses (like 3.6 mg/kg b.w. chosen for the experiment), SeO NPs can be consumed for the synthesis of selenium-containing enzymes, including thioredoxin reductase, phospholipid hydroperoxide glutathione peroxidase, and glutathione peroxidase [17], and pose no serious threat. Yet, a multiple (here, 5-fold) increase in the SeO NP exposure causes damage to mitochondria, disruption of the genetic apparatus of cells, damage to cells by oxidative stress products, and early apoptosis [15, 35].

At the same time, a decrease in the reparative potential of the liver is observed. Liver regeneration involves hepatocytes, which make up more than 60 % of the liver cell population [36], sinusoidal cells, 50 % of which are represented by Kupffer cells and leukocytes, connective tissue cells and the extracellular matrix [37].

The ability of the liver to regenerate was assessed by the proportions of Kupffer cells, leukocytes and binuclear hepatocytes (BH) in smears (Figures 3–6). The obtained function (Formula 4) for BH based on the modified Johnson–Lovett model [30] demonstrates a reduced reparative potential against the background of SeO NP accumulation. According to modern concepts, the appearance of binuclear cells occurs as a result of hepatocyte mitosis without cytotomy or amitotic division of hepatocytes and accompanies reparative regeneration of the damaged liver [38].

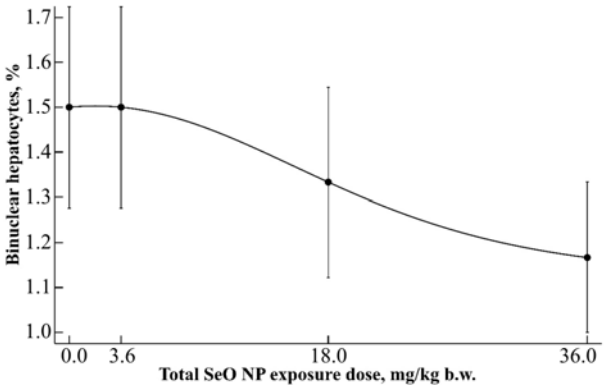

Figure 3. Changes in the proportion of binuclear hepatocytes in liver smears following SeO NP exposure (mean ± error of the mean)

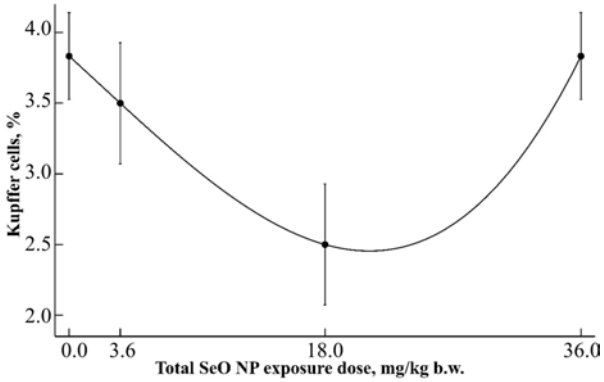

Figure 4. Changes in the proportion of Kupffer cells in liver smears following SeO NP exposure (mean ± error of the mean)

In the absence of a response to the low dose of SeO NPs, we revealed a stable tendency towards a decrease in the proportion of binuclear hepatocytes with a multiple increase in the total dose of NPs to 18 and 36 mg/kg b.w.

At the lowest tested dose of 3.6 mg/kg b.w., the percentage of BH remained similar to that in the control group. With an increase in the dose to 18 and 36 mg/kg b.w., an increase in the percentage of DH (Figure 2) was accompanied by a decrease in the percentage of BH (Figure 3), indicating a decrease in the reparative ability of the liver ($p < 0.01$).

In response to liver cell damage, macrophages migrate to the inflammation zone. Kupffer cells, as well as monocytes and neutrophils coming with the bloodstream, are the representatives of the macrophage community. To describe the dose-dependent effect of SeO NPs on the proportion of Kupffer cells in

liver smears, we applied the variant with a linear combination of Chebyshev polynomials (Formula 3).

The percentage of Kupffer cells in liver imprint smears decreased significantly following SeO NP exposure at 3.6 mg/kg b.w. and was more pronounced at 18 mg/kg b.w. ($p < 0.05$); however, upon reaching the concentration of 36 mg/kg b.w., the amount of those cells returned to its initial values, comparable with the control ones (Figure 4). Quantitative restoration of Kupffer cells is explained by *in situ* proliferation [39], the influx and differentiation of blood monocytes into tissue macrophages [40, 41]. When cells are damaged, Kupffer cells and blood leukocytes (monocytes, neutrophils, and eosinophilic leukocytes) migrate to the site of inflammation and play an important role in the macrophage destruction of damaged cells and the replacement of degenerative cells with new cells and even other tissues [38]. Minor damage for a prolonged period can induce chronic inflammation and tissue replacement with connective tissue with fibrosis zones. That is why predicting damaging effects is so important.

When comparing the graphs for degenerated hepatocytes (Figure 2) and Kupffer cells (Figure 4), it becomes obvious that fluctuations in the proportion of the latter can be associated with the destruction of hepatocytes by SeO NPs and reparative processes in the liver, which are supported by the proliferation of Kupffer cells and the influx from outside. During liver regeneration, polymorphonuclear leukocytes, Kupffer cells, and endothelial cells secrete metalloproteinases (collagenases, gelatinases, elastases, and other proteinases), changing the density of the extracellular matrix to deliver regulatory signals to all liver cells (cytokines) thereby [42, 43]. The maximum decrease in the proportion of Kupffer cells upon exposure to SeO NPs at 18 mg/kg b.w. can be associated not only with the direct toxic effect of NPs on cells, but also with their migration to the lymph nodes associated with the antigen-presenting function.

In case of macrophage deficiency, blood cells, specifically neutrophils and eosinophilic leukocytes, join the process of removing damaged cells, the change in the percentage of which is associated with the activation of the inflammatory degenerative process in the liver. Neutrophils are attracted to the damaged liver by inflammation mediators [38]; yet, they themselves secrete a sufficient amount of chemoattractants to induce other cells to migrate towards them. The organism maintains the necessary and sufficient population of neutrophils to participate in inflammation.

To describe the relationship between SeO NP exposure and the proportion of neutrophils found in imprint smears (Figure 5), we used a model [29] based on the Hill function (Formula 1), which reflected the tendency towards a decrease in the proportion of neutrophils in the smear under the toxic effect of NPs.

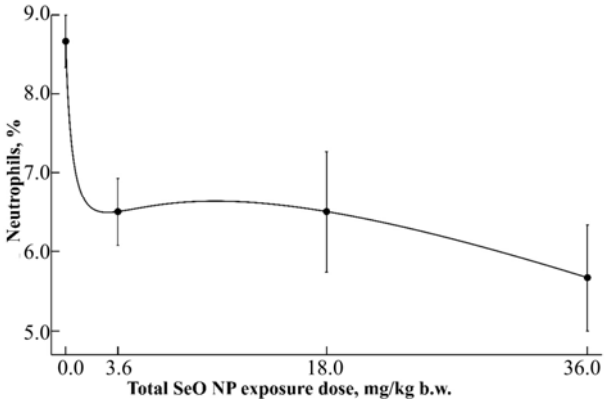

Figure 5. Changes in the proportion of neutrophils in liver smears following SeO NP exposure (mean ± error of the mean)

A comparable plateau-like decrease in the percentage of neutrophils in liver smears occurs at low (3.6 mg/kg b.w.) and moderate (18 mg/kg b.w.) exposure doses of SeO NPs and is aggravated by the high one (36 mg/kg b.w.) ($p < 0.05$).

A decrease in the percentage of neutrophils at low exposure doses leads to mobilization of the body expressed by a slight increase in the proportion of eosinophils

(Figure 6) in liver smears at higher SeO NP exposure doses (18 and 36 mg/kg b.w.). The dose-response relationship [30] for EL is shown in Figure 6.

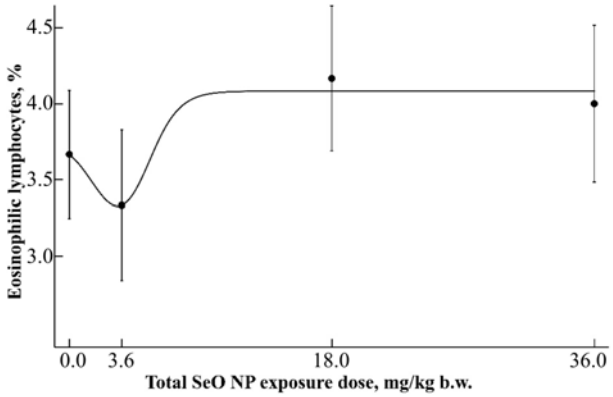

Figure 6. Changes in the proportion of eosinophilic lymphocytes in liver smears following SeO NP exposure (mean ± error of the mean)

The graph of dose-dependent changes in the EL proportion in liver smears is noteworthy for its fluctuations related to inflammatory response development. The destruction of EL at the lowest exposure dose of SeO NPs (3.6 mg/kg b.w.) with the release of cytotoxic EL granules contributes to tissue destruction, but, on the other hand, the release of interleukin-4 (IL-4) stimulates liver regeneration [44, 45]. These findings presented by nonlinear functions are explainable in terms of liver process regulations by migrating cells of the blood and immune system [38]. By producing cytokines (interleukins, chemokines, growth factors) and through direct contact, these cells control the expression of receptors of various liver cells, predetermining its regeneration process [46].

Health and even life threatening disruption of vital organ and system functioning is associated with the ability of NPs to penetrate the bloodstream and cells of various organs [47]. The liver that is well-known for its detoxifying abilities and high blood supply is a target organ for SeO NPs [35]. Functional changes in the liver are observed in animals exposed to SeO NPs, as shown, for instance, by serum enzymes. Maintaining the proportion of degenerated hepatocytes at the level of 11.0 to 12.33 % creates the prerequisites for an increased release of enzymes into the blood. When cells are damaged, enzymes first leak into the intercellular fluid from the cytosol and lysosomes and then, in case of deeper damage, from mitochondria, ribosomes, and nucleus of these cells. The larger the lesion and depth of damage, the greater the enzyme concentration that enters the intercellular space and blood.

To assess the effect of SeO NPs on the activity of ALT and ALP in blood serum, we used the modified Johnson–Lovett model [30] for ALT (Figure 7) and a model close to the Michaelis–Menten equation for ALP when constructing the dose-effect relationship (Figure 8).

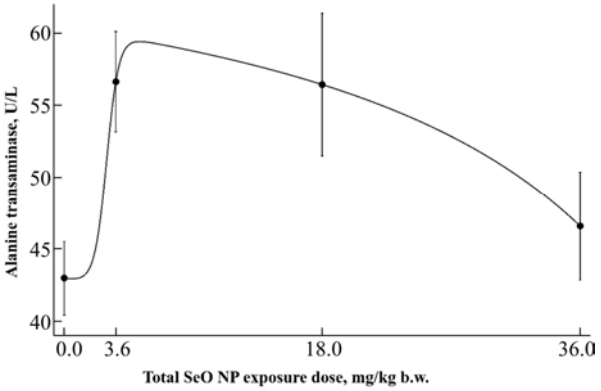

Figure 7. Changes in ALT activity in the blood following SeO NP exposure (mean ± error of the mean)

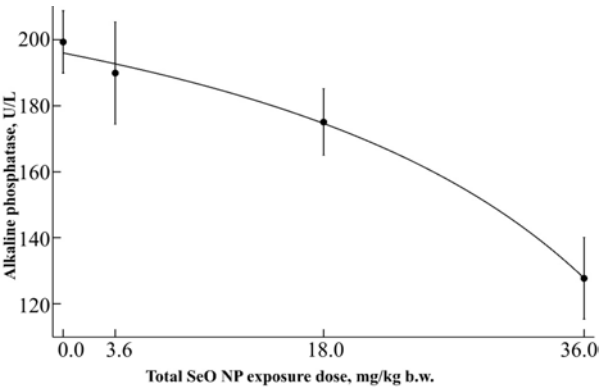

Figure 8. Changes in the activity of alkaline phosphatase in the blood following SeO NP exposure (mean ± error of the mean)

The dose-effect responses of the organism observed during the experiment with SeO NPs were non-monotonic, which was not surprising given the multicomponent nature of the biological system. When examining the charts of the proportion of degenerated hepatocytes and ALT activity, we noted a relationship between the increase in the SeO NP concentration and changes in the concentration of ALT and the above cell count. An insignificant decrease in the proportion of degenerated hepatocytes at low concentrations of the irritant led to a sharp increase in blood ALT concentrations. The observed non-monotonicity of both functions was associated with the damaging effect of SeO NPs on liver cells and the release of ALT, the marker for cytolysis. At the lowest dose of SeO NPs, however, a sharp increase in ALT activity was accompanied by an insignificant increase in the proportion of degenerated hepatocytes, whereas the dependencies of these indicators uniformly decreased at the moderate and high doses ($p < 0.05$). The sharp increase in ALT activity becomes clear when comparing the graphs of changes in the proportion of neutrophils and EL[1], Kupffer cells in liver smears and ALT (Figures 4–7). The contribution of these cells, or rather their destruction [20] due to the toxic effect of SeO NPs, increased the concentration of the cytosolic enzyme ALT at the low NP dose of 3.6 mg/kg b.w.

The toxic effect of SeO NPs on biliary epithelial cells was associated with a dose-dependent decrease in the concentration of alkaline phosphatase in the blood serum in all groups exposed to SeO NPs.

Despite the fact that destroyed epithelial cells of the biliary tract, intestine, brush border of the kidneys, and bone tissue contribute the most to the increase in ALP blood levels, it is important to highlight the role of myeloid cells, including neutrophils and EL, in amending blood ALP concentrations. The entry of ALP into the blood accompanying the destruction of biliary epithelial cells can be enhanced by the destruction of neutrophils and EL, which is manifested upon exposure to SeO NPs at 3.6 mg/kg b.w. (see Figures 5–6). The most pronounced decrease in ALP activity was observed at the highest tested SeO NP dose of 36 mg/kg b.w. ($p < 0.05$) (Figure 8). It is likely that the dose-dependent decrease in ALP activity with an increase in SeO NP exposure is associated with a deficiency in zinc and magnesium, which are part of the enzyme, but data on competitive interactions between magnesium/zinc and selenium are lacking.

Changes in the structure of liver cell mitochondria, an imbalance of blood enzymes and the cellular composition of the liver may indicate liver damage and dysfunction induced by SeO NP exposure. Mathematical modeling can be used to assess the dose-dependent general toxic effect at the level of cells, tissues, and organs and to analyze health risks.

Conclusions:

1. We revealed atypical dose-response relationships between SeO NP exposures and hepatic changes. They were nonlinear and described by non-monotonic functions for such parameters as the proportion of eosinophilic leukocytes, neutrophils, Kupffer cells, degenerated hepatocytes, and the activity of ALT and ALP enzymes.

2. The effectiveness of using mathematical models built on a linear combination of Chebyshev polynomials, the Michaelis–Menten equation, modified Hill functions, and the Johnson–Lovett model for describing dose-dependent adverse effects of SeO NP exposure was proven.

3. The highest health risks were posed by low (3.6 mg/kg b.w.) and moderate (18 mg/kg b.w.) doses of SeO NPs, as shown by changes in ALT and ALP activity and the percentage of degenerated hepatocytes and Kupffer cells.

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4. The effect of SeO NP exposure dose of 36 mg/kg b.w. on the percentage of degenerated hepatocytes and neutrophils in the liver was most pronounced.

When developing chemical safety standards and assessing health risks, it is therefore necessary to consider that, in case of subchronic exposure, the highest number of pronounced toxic effects and the risk of pathological disorders are observed at low and medium doses of SeO NPs tested.

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Conflict of interest statement. The authors have no conflicts of interest to declare.

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