Research article

EFFECTS OF SELENIUM OXIDE NANOPARTICLES ON THE MORPHOFUNCTIONAL STATE OF THE LIVER: EXPERIMENTAL DATA

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Copper smelters are the sources of emission of complex aerosols containing, inter alia, selenium-containing nanoparticles (NPs). It is very difficult to adequately estimate the hazard posed by such particles since available data on them are scarce and have been obtained in comparatively few experimental studies with rather contradicting results.

The aim of our study was to determine toxic health effects of selenium-containing nanoparticles more precisely with a focus on liver as a target organ.

Liver toxicity following exposure to suspended selenium oxide nanoparticles was investigated in a sub-chronic experiment on outbred male albino rats. The suspension was prepared by laser ablation of 99%-pure selenium plates. We examined ultrastructural changes by electron microscopy, did cytological and histological analyses of the liver, biochemical blood testing and metabolomic blood screening.

We observed lesions in the liver and inhibited secretory functions at various levels, from molecular to organismic, in the exposed animals. The microscopic examination showed that the number of normal and normal-vesicular mitochondria in liver cells went down by 7.78 %, p < 0.05; the metabolomic screening established lower levels of glycocholic acid in blood serum, p < 0.001; levels of alanine aminotransferase in blood serum grew by 30 %, p < 0.05; the number of acaryotic hepatocytes demonstrated a 3.1-fold increase, p < 0.05, according to the results of histological assessment of liver specimens. The touch smears of the liver examined showed a 2.2-fold increase in the number of degenerated hepatocytes (p < 0.05).

These experimental data can be used to estimate a potential hazard of selenium-containing nanoparticles within social-hygienic monitoring and biomedical predictions of health damage caused by exposure to such NPs. Altered levels of lysophosphatidylinositol can be a marker of exposure to the examined NPs and necessitate the search for early diagnostic predictors of associated health disorders.

Keywords: toxicity, hazard assessment, nanoparticles, selenium, liver, mechanism of action, in vivo, experiment.

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Copper smelters are sources of emission of complex aerosols containing, *inter alia,* selenium-containing nanoparticles (NPs). The latter become airborne when copper is refined either by electrolysis or in the melt flow. Workers of copper smelting, electrolysis or chemical metallurgy workshops are exposed to these aerosols.

It is very difficult to adequately estimate the hazard posed by selenium-containing nanoparticles since available data on them are scarce and have been obtained in comparatively few experimental studies with rather contradicting results. Toxic effects produced by nanoparticles are determined not only by their physical properties but also specific features of the basic chemical element [1]; obviously, consideration of data on biological activity of selenium should not be limited to its nano-sized form.

Adverse health effects of selenium are widely known. Since this trace element is biologically close to sulfur, it is able to replace the latter in chemical compounds [2, 3]. Selenium is a known pro-oxidant [4]. Hydrogen selenide, a selenium metabolite, is able to inactivate metal-containing enzymes, primarily oxidases [5]. Some studies report a negative impact of selenium on carbohydrate metabolism, its potential role in the development of type 2 diabetes mellitus [6], and contribution to cognitive impairments [7, 8]. Selenium affects multiple organs, with liver being its major target [9, 10].

Harmful effects of selenium-containing NPs have been described elsewhere [11] but the state of the liver has been addressed only in few experiments and *in vivo* studies showing inconsistent findings. Thus, authors describe rather ambiguous changes in activity of so-called 'liver' enzymes in blood serum: alanine transaminase became either more [12–14] or less active [15, 16] while aspartate transaminase became more active [17], less active [16], or its activity did not change [12, 15]. As for alkaline phosphatase, its activity in blood serum grew unambiguously [15, 17] following the exposure to selenium NPs. Histological assessment of the liver did not establish any apparent dystrophic changes though it was noted that exposure to high doses of selenium-containing NPs (5 [15] and 8 [17] mg Se/kg body weight) induced destruction of hepatocytes [15, 17]. Besides, accumulation of Se NPs in the body led to a significant increase in the selenium content in the liver [15, 16, 18, 19]. It is noteworthy that both selenium as a trace element and selenium-containing NPs in higher concentrations are pro-oxidants able to induce production of reactive oxygen species [20], and the process may be aggravated by their bioaccumulation in the liver [11].

The aim of our study was to determine toxic effects of selenium oxide nanoparticles on liver in experimental animals.

Materials and methods. Toxic effects of selenium nanoparticles (NPs) were investigated using selenium oxide nanoparticles (SeO NPs). The suspension of SeO NPs was prepared at the Ural Center for Shared Use "Modern Nanotechnologies" of the Ural Federal University, Yekaterinburg, Russian Federation, by pulsed laser ablation in sterile deionized water using thin selenium plates (99.99 %). Shapes and sizes of the analyzed particles were identified using scanning electron microscopy and described with a graph showing particle size distribution, the mean diameter equaling 51 ± 14 nanometers (Figure 1).

Stability of the suspensions was estimated by identifying their zeta potential and appeared to be quite high (up to 42 mV). This allowed us to raise particle concentrations in these suspensions to 0.25 mg/ml by partial water evaporation at 50 °C affecting neither size distribution nor chemical identity of NPs.

Health effects of SeO NPs were estimated in an experiment on 12 outbred male albino rats aged 4 months at the beginning of the experiment. The control group also included 12 rats, with their initial body weight of all animals varying between 200 and 270 grams (± 20 % of the mean). Subchronic poisoning was simulated by repeated intraperitoneal injections, thrice a week for 6 weeks (18 injections in total). Doses were selected based on the results of previous experimental studies. Solutions of the stable NP suspension were introduced as follows: 1 ml of the suspension in a single dose of 0.2 mg/kg and 1 ml of deionized water (SeO NPs 0.1 group);
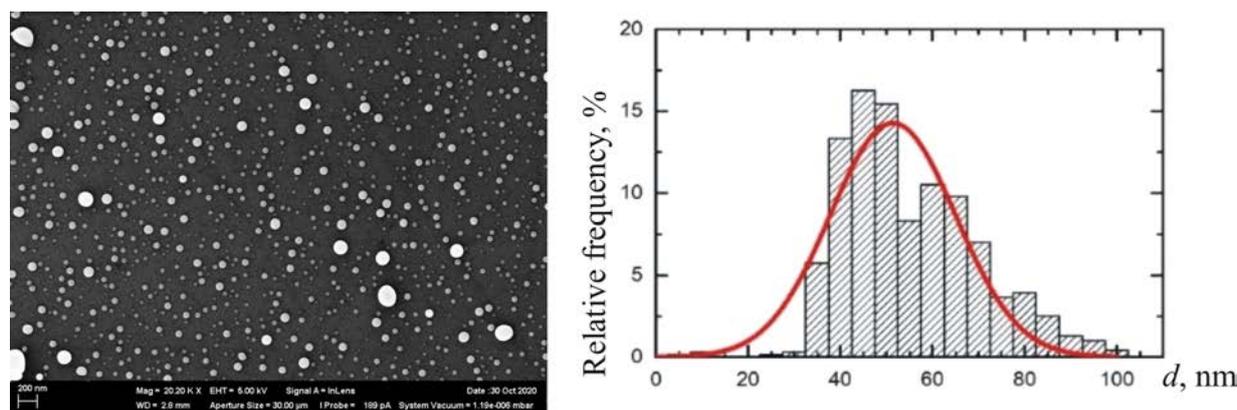
Figure 1. SEM-image of SeO NPs in a suspension prepared for the experiments (scanning electron microscopy, 20,200 × magnification) and a graph showing particle size distribution

2 ml of the suspension in a single dose 1 mg/kg (SeO NPs 0.5 group); 2 ml of the suspension in a single dose 2 mg/kg (SeO NPs 1 group); 2 ml of deionized water (Control). Animals were kept, bred and taken out of the experiment in accordance with conventional requirements. 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.

Following the exposure cessation, we did serum biochemistry using Cobas Integra® 400 plus analyzer (Roche Diagnostics GmbH, Germany) and relevant diagnostic sets. Activity of succinate dehydrogenase (SDH) in blood lymphocytes was identified and then used as an indicator of bioenergetic metabolism [21].

High performance liquid chromatography – mass spectrometry (HPLC-MS) was used for metabolomic screening. Chromatographic separation was accomplished using a liquid chromatographer with C18 column in gradient elution mode and detection was performed with a quadrupole time-of-flight mass spectrometer. A set of value pairs 'm/z – signal intensity' was obtained for each experimental group. These values corresponded to individual metabolites in blood. A mean or a median (in case of a non-normal distribution) value of a signal was established for each substance in the experimental group. The data obtained for each group were then compared to establish statistical differences. The ultimate results included the values demonstrating more than a two-fold change before/after

comparison of the experimental results. Generalization as per an exact mass and fragmentation spectra was then performed for the selected m/z values; these spectra were obtained by repeated analysis using a mass spectrometer in tandem mode with different energy levels. The resulting spectra were analyzed using free-access databases (HMDB, MoNA, METLIN, MassBank EU).

Cell ultrastructure was estimated using a Hitachi REGULUS SU8220 scanning electron microscope in the STEM mode. Mitochondria were classified in accordance with Sun et al. [22] based on the topology of mitochondrial inner membrane (matrix density and homogeneity, the number of cristae) [22]. In calculations, type A mitochondria (normal) and type B mitochondria (normal-vesicular) were considered normal whereas type C (vesicular), type D (vesicular-swollen), and type E (swollen) were considered pathologically altered.

For imprint cytology, we did touch smears of cross-sections of the liver, kidneys, spleen, and mesenteric lymph nodes, let them dry at room temperature, and then stained using Leishman's staining method. Cell composition and signs of cell damage were estimated using a Carl Zeiss Primo Star light binocular microscope with USCMOS video camera at 100× and 1000× magnifications.

We examined histological changes in the liver of rats from the SeO NP exposure and control groups. Acaryotic hepatocytes and Kupffer cells were quantified by morphometric analysis using Avtandilov mesh.

The statistical significance of differences in mean values of indicators describing toxic effects was estimated by using Student's t-test with a correction for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

Results and discussion. The data obtained by metabolomic blood screening were analyzed using the principal component technique. The analysis revealed certain clusterization of samples in the test groups before/after comparison thereby indicating substantial changes in blood of the experimental animals (Figure 2). Samples of the animals from the control group created one cluster excluding one animal; obviously, it had certain deviations in its blood composition.

Only some of the selected substances had enough intensity of an analytical signal to obtain informative fragment spectra; therefore, generalization was not possible for all the metabolites in the groups. The metabolites identified belonged to lipids and phospholipids (Table 1).

Levels of acylcarnitines and their derivatives (decanoylcartinine, hydroxydecenoylcarnitine, hydroxyhexadecanoylcarnitine, tetradecadienoylcarnitine, see Table 1, Nos. 3–6) increased in all the groups.

On the one hand, these metabolites transport fatty acids through the carnitine shuttle in mitochondrial inner membranes for further beta-oxidation; on the other hand, they transport excessive products of this process from mitochondria to the extracellular space since excessive quantities of oxy-Acyl-CoA derivatives disrupt mitochondria functions up to initiation of apoptosis [23–25].

Variations in contents of the aforementioned substances indicate ongoing changes in beta-oxidation of fatty acids that takes place in mitochondria; this was previously shown for exposure to selenium salts [26] and for the first time has been demonstrated in our study for exposure to selenium NPs.

The assumed damage to mitochondria mediated by their functional disorders was confirmed microscopically. Ultrastructural investigation established a decrease in the proportion of normal mitochondria (type A and B as per Mei G. Sun [22], 87.44 ± 1.14 % in the SeO NPs 1 group against 94.82 ± 0.95 % in the control group, $p < 0.05$) in liver cells.

Pathological changes in mitochondria, so-called 'energy stations' within a cell, were identified by metabolomic analysis and visualized with electron microscopy. These changes led to a decrease in their energy potential. The latter was indirectly evidenced by a statistically significant, monotonic and dose-dependent decline in activity of succinate dehydrogenase in blood lymphocytes following the exposure to SeO NPs (517.50 ± 2.58 formazan granules in 50 lymphocytes in the SeO NPs 0.1 group against 575.78 ± 6.10 formazan granules in 50 lymphocytes in the control group, 495.14 ± 6.91 formazan granules in 50 lymphocytes in the SeO NPs 0.5 group and 484.00 ± 7.14 formazan granules in 50 lymphocytes in the SeO NPs 1 group against the control group and the SeO NPs 0.1 group, $p < 0.05$). This decline in the activity of succinate dehydrogenase can be attributed to the ability of selenium to replace sulfur in compounds as described elsewhere [3].

A   *B*   *C*   *D*
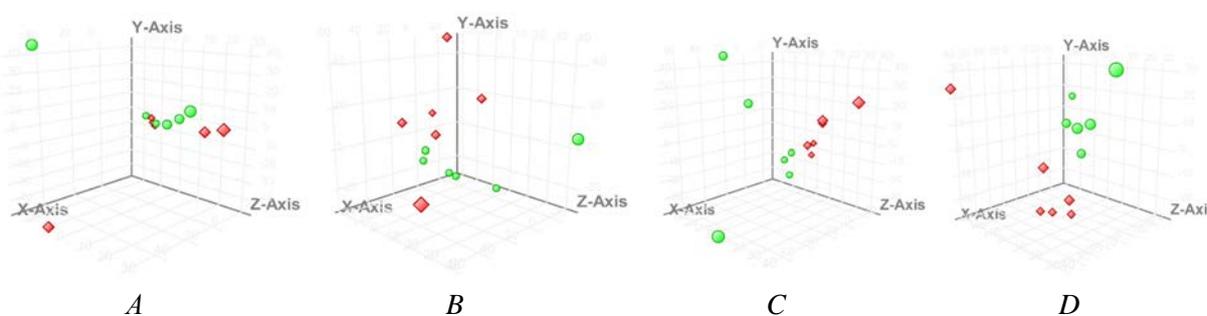
Figure 2. Results obtained by Principal Component Analysis of HPLC-MS spectra of blood samples (● pre-exposure, ▢ post-exposure): A is the control, B is SeO NPs 0.1 group, C is SeO NPs 0.5 group, C is SeO NPs 1 group

T a b l e 1

Metabolomic screening of rats' blood following sub-chronic exposure to selenium oxide nanoparticles

No.	Substance	Groups and changes in metabolite levels, before/after comparison					
		SeO NPs 0.1	*p*	SeO NPs 0.5	*p*	SeO NPs 1	*p*
1	Bile acid	-		↓	0.029	-	
2	Glycocholic acid	-		↓	< 0.001	-	
3	Decanoylcartinine	-		↓	0.008	-	
4	Hydroxydecenoylcarnitine	-		↓	< 0.001	↓	0.006
5	Hydroxyhexadecanoylcarnitine	↓	0.041	↓	0.005	↓	0.048
6	Tetradecadienoylcarnitine	-		↑	< 0.001	↑	0.007
7	Tetracosahexaenoic acid	-		↓	0.043	-	
8	Methyl arachidonate	-		↑	0.003	↑	0.001
9	Methyl hexadecanoic acid	-		↑	0.004	↑	< 0.001
10	Methyl linoleate	↑	< 0.001	↑	< 0.001	↑	< 0.001
11	Methyl-[10]-gingerol	↑	0.004	↑	0.002	-	
12	13'-hydroxy-alpha-tocopherol	↑	0.026	-		↑	0.020
13	Phosphorylcholine	↑	0.008	-		↑	0.006
14	PC(16:1/2:0)	-		-		↓	0.029
15	PC(3:0/2:0)	-		↑	< 0.001	-	
16	LPC(9:0)	↓	0.008	-		-	
17	LPC(18:2)	↑	0.008	-		-	
18	LPC(18:3)	-		↑	0.008	-	
19	LPC(20:4)	↑	0.007	↑	0.046	-	
20	LPC(28:6)	-		↓	0.006	-	
21	2-(9-Oxononanoyl)-glicero-3-phosphocholine	-		-		↓	0.006
22	LPE(18:2)	-		↑	0.045	-	
23	PS(3:0/2:0)	-		↑	0.046	-	
24	LPI(18:0)	-		-		↑	0.040
25	LPI(20:4)	↑	< 0.001	-		-	

Notes: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; LPI, lysophosphatidylinositol. The first figure in brackets stands for a carbon chain length of a fatty acid fragment in a compound and the second one stands for the number of double bonds in it. The symbol '↑' means growing intensity of an analytical signal of a metabolite mass in before/after comparison; the symbol '↓', declining intensity; '-' means no significant changes in contents were identified for this substance in the given experimental group.

NPs are easily transported along the bloodstream and penetrate into cells of various organs [27]; this capability, together with the fundamental role of succinate dehydrogenase in the chain for mitochondria electron transportation, determines functional changes in vital organs and systems. The liver is the first organ to be affected, which is quite logical with its barrier function borne in mind. Besides, toxic effects of nanoparticles depend on their chemical essence [1] and the liver is the major depot [10] and target organ for toxic effects of selenium [9].

Morphofunctional changes in the liver were established by cytological examination of touch smears of the liver, histomorphological analysis of liver tissues, biochemical testing of blood serum, and metabolomic analysis.

The cytological examination of touch smears of liver showed an increase in the proportion of degenerated hepatocytes (16.33 ± 0.92 % in the SeO NPs 0.5 group and 17.33 ± 0.99 % in the SeO NPs 1 group against 6.33 ± 0.49 % in the control group and 6.83 ± 0.79 % in the SeO NPs 0.1 group).

Histomorphology revealed a drastic growth in the number of acaryotic hepatocytes (27.60 ± 1.46 cells in the SeO NPs 1 group against 8.90 ± 0.56 cells in the control group, $p < 0.05$) and Kupffer cells (20.50 ± 0.71 cells

in the SeO NPs 1 group against 11.50 ± 0.58 cells in the control group, $p < 0.05$). The liver structure of the rats from the control group corresponded to its histological standard: hepatocytes forming liver plates and portal tracts were intact; there were apparent dystrophic changes in hepatocytes and acaryotic hepatocytes in the liver of the rats from the SeO NPs 1 group (Figure 3).

Alanine transaminase (ALT) became more active in blood serum of the rats from the SeO NPs 1 group against the controls and the difference was statistically significant. Levels of alkaline phosphatase (AP) went down in all the animals exposed to selenium nanoparticles and this decrease was dose-dependent but statistical significance was established only for the difference between the SeO NPs 1 group and the controls (Table 2).

All these trends combined, including an increase in ALT activity, lower AP levels (Table 2), degenerative changes in hepatocytes (Figure 3), lower levels of bile and glycocholic acid (Table 1, Nos. 1–2), that were identified in the rats exposed to SeO NPs can indicate that exposure to SeO NPs damages the liver and impairs its secretory functions [26, 28]. The latter is consistent with the less intensive analytical signal of lysophosphatidylcholines LPC (9:0) and LPC (28:6) (Table 1, Nos. 16, 20) with short and very long fatty acids in their structure since these substances are synthesized predominantly in the liver, participate in transportation of fatty acids, and are precursors of membrane phospholipids [29]. On the other hand, an increase in the levels of lysophosphatidylcholines LPC (18:2), LPC (18:3) and LPC (20:4) (Table 1, Nos. 17–19) can indicate that inflammatory processes have intensified in the experimental animals [30]. The aforementioned substances, being transporters of linoleic, linolenic and arachidonic acids, are precursors of eicosanoids, or inflammation mediators, and can be synthesized directly from membrane phospholipids due to effects of phospholipase A [31].
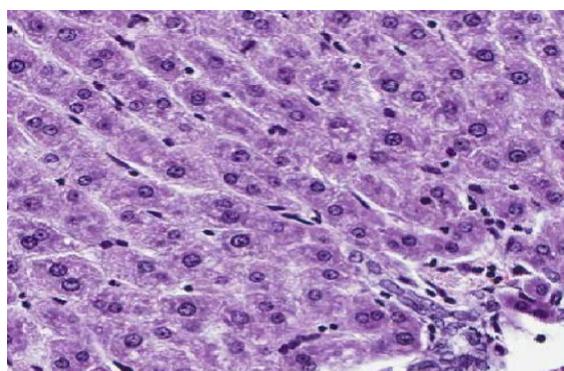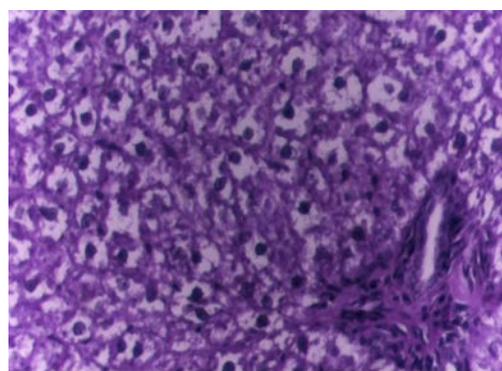
A   *B*

Figure 3. A rat's liver (stained with hematoxylin – eosin, 100× magnification):
A is the control group, B is the SeO NPs 1 group

T a b l e   2

Indicators describing the state of the liver of rats following subchronic exposure to selenium oxide nanoparticles

Indicators	Control	SeO NPs 0.01	SeO NPs 0.5	SeO NPs 1
ALT in blood serum, U/l	42.96 ± 2.55	**56.64 ± 3.47 ***	56.44 ± 4.95	46.58 ± 3.76
AP in blood serum, U/l	199.28 ± 9.45	189.87 ± 15.45	175.05 ± 10.08	**127.76 ± 12.37 *♦●**

Notes: * statistically different from the control groups; ♦ from the SeO NPs 0.1 group; ● from the SeO NPs 0.5 group ($p < 0.05$, Student's t-test).

Inhibition of liver secretory functions is also evidenced by accumulation of fatty acid esters in blood. These esters are decomposed into very low density lipoproteins by liver phospholipases with a simultaneous decrease in levels of tetracosahexaenoic acid, which may occur when sulfur is replaced with selenium in lipases with a relevant decline in their activity [3].

In addition, we should note that lysophosphatidylethanolamines are known to be able to inhibit phospholipase synthesis in the liver but the mechanism of action has not been clarified yet [32]. Elevated LPE (18:2) levels in the SeO NPs 0.5 group are well in line with the highest concentrations of fatty acid esters identified in blood of the rats from this group.

A statistically significant increase in the levels of lysophosphatidylinositols, which are primarily synthesized in the liver (80 % of the total pool) [33], was observed in the SeO NPs 0.1 group and SeO NPs 1 group (Table 1, Nos. 24, 25). We did not identify it in the SeO NPs 0.5 group probably because the liver functions were the most inhibited in this group due to changes in levels of bile acid and fatty acid esters described above. Lysophosphatidylinositols act as precursors for synthesis of phosphatidylinositol di – and triphosphates, cell membrane modifiers able to change its fluidity and thereby promote changes in activity of membrane channels [34]. Fusion and decomposition of phospholipid membranes are known to be associated with the latter being enriched with LPI derivatives [35].

On the other hand, these substances are ligands for GPR55 [36], a receptor conjugated with G-protein, which mediates intracellular signal cascades and stimulates several processes including exocytosis–secretion of insulin and neuromediators; production of pro- and anti-inflammatory interleukins, phospholipase–synthesis of pro- and anti-inflammatory eicosanoids; cell proliferation and migration; stimulation of angiogenesis under artificial LPI introduction was shown in tumor cells [35, 37–39].

Changes in the levels of lysophosphatidylinositols have never been established previously in studies addressing metabolome responses of the body to effects produced by selenium compounds including salts.

Conclusion. Barrier functions performed by the liver as well as the liver being the major depot and target organ for toxic effects of selenium determine a cascade of impairments at all levels. At organismic level, contents of alanine transaminase and alkaline phosphatase in blood serum were established to have changed by 30 % and 57 %, respectively, $p < 0.05$. At cellular and tissue levels, the number of degenerated hepatocytes was established to have grown by 2.2 times, $p < 0.05$; at sub-cellular level, the proportion of normal mitochondria (types A and B as per Mei G. Sun) went down by 7.78 % in the liver of exposed animals, $p < 0.05$. For the first time, changes were identified at molecular level following the exposure to SeO NPs; specifically, there were changes in beta-oxidation of fatty acids occurring in mitochondria due to effects produced by selenium-containing nanoparticles. They were estimated by changes in levels of acylcarnitines and their derivatives in rats' blood. We also established a decrease in cell energy potential, which was indirectly evidenced by a 16 % decrease in activity of succinate dehydrogenase, $p < 0.05$. Besides, damage to the liver and inhibition of its secretory functions can be further confirmed by accumulation of fatty acid esters in blood, elevated levels of lysophosphatidylethanolamines, lower levels of bile and glycocholic acid.

These experimental data can be used to estimate a potential hazard of selenium-containing nanoparticles as a chemical risk factor, both environmental and occupational one, within socio-hygienic monitoring and biomedical predictions of health damage related to exposure to such NPs. Such assessment should consider the established capability of selenium-containing nanoparticles to affect the metabolome profile and produce subchronic toxic effects on warm-blooded animals.

Changes in the levels of lysophosphatidylinositol that have been established in our

study for the first time in response to selenium exposure can be used as a starting point in searching for early diagnostic predictors of health disorders induced by exposure to the examined NPs.

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