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Embryotoxicity of Poorly Soluble Nanoparticles at Various Stages of Zebrafish Development

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Abstract. The biological effects of the poorly soluble nanoparticles (NPs) of different chemical nature and structural characteristics were evaluated. It was established that the Zebrafish test response to contamination of aqueous medium with nickel NPs (nNi), platinum (nPt), zinc oxide (nZnO) and cerium oxide ($nCeO_2$) depends on the physicochemical properties of the NPs and embryo development stage. The concentrations of NPs not causing disruptions in embryonic development of Zebrafish were determined. The smallest impact on embryogenesis was exerted by $nCeO_2$: coagulation of a small number of embryos was observed only at C = 20.0 mg/L. The same effect was observed when exposed to lower concentrations of nPt (C = 5.0 mg/L) and nNi (C = 0.1 mg/L).The greatest number of coagulated embryos was observed when grown in the DS nZnO: 37.5% of embryos died at the DS concentration of C = 0.1 mg/L. Zebrafish cultivation in the DS with low concentrations ($C \leq LC_{10}$) of nNi and nZnO caused distortions in the development of embryos: development of scoliosis, malformation of somites, inhibited mobility.

INTRODUCTION

In the last decade along with the study of commercially demanded nanomaterials (NM) and promising means of diagnostics, treatment, and selective drug delivery active attention has been paid to the development of a new generation of antitumor drugs based on NPs (nanoparticles). However, preclinical NPs toxicity tests are extremely rare, and the literature on toxicity and accumulation of NPs is limited and highly contradictory. The current situation can be explained by the absence of standard research protocols (in particular, by the lack of adjustment of changing concentrations given the aggregation of NPs), the use of non-standardized NPs by the authors, as well as a variety of model test organisms and criteria. In the last two decades, the most popular *in vivo* model in biomedical research for discovering new medicines and assessing their safety has been Zebrafish [1, 2, 3, 4]. More than 400 laboratories all over the world use Zebrafish in fundamental and applied research now [5].

Over recent years oncology researchers have become more interested in such NPs as gold (nAu), copper (nCu), silver (nAg), platinum (nPt), iron oxide (Fe_2O_3) and a number of others [6, 7, 8]. The range of applications of nAu is expanding rapidly because NPs Au are nontoxic according to a number of authors and can be used for targeted drug delivery. The absence of pathological changes in the development of Zebrafish, including cardiotoxicity, is shown by Asharani et al. [7] (concentrations from 0.002 to 0.02 mg/L, particle size from 15 to 35 nm coated with polyvinyl alcohol), Bar-Ilan O.R. [9] (concentrations from 0.05 to 50 mg/L; NPs from 3 to 100 nm), Belyaeva N.F. [10] (concentrations from 13.0 to 58.0 mg/L, NPs dia. is from 10.5 to 17.1 nm). However, there are singular data on pathological changes during the embryonic development of Zebrafish [7]. Liu et. al. [8] assessed the toxic effects of four different NPs, namely CuO, ZnO, TiO_2 and Au (20 nm to 50 nm) on the development of Zebrafish embryos. It

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has been established that the tested NPs inhibit body growth, hatching speed of larvae, cause spinal malformations and pericardial edema, where smaller NPs cause greater toxicity to embryos.

Thus, the biological activity of a number of NPs with respect to the embryonic development of Zebrafish is well established. However, their activity at low concentrations and tissue-specificity of the effect has not been investigated in depth.

The discrepancy in the data, the limited research, and the lack of a single methodological approach determined the purpose of this research: studying of embryotoxicity of poorly soluble NPs at different stages of development of Zebrafish; and defined the research objectives:

1. Determine pathological changes in the development of embryos at different stages of embryogenesis in an aquatic environment contaminated with nPt, nNi, nZnO, $nCeO_2$.
2. Determine the toxicity index (I %).
3. Determine $L(E)C_{10,}$ $L(E)C_{20}$ и $L(E)C_{50}$ of the tested NPs.

MATERIALS AND METHODS

The biological effects and ecotoxicity of the tested NPs were determined using 14 indicators of Zebrafish embryotoxicity [11, 12, 13]. Five sets of experiments were carried out on 816 test-systems. Four types of NPs were studied.

The conditions for biotesting are presented in Table 1.

TABLE 1. Bioassay conditions.

Test-organism	t, °C	pH	Photo-period	Lighting	Content O_2/CO_2	Control culture medium	Duration experiment	Refe-rences
Zebrafish (Embryo-toxicity)	26±1.0	7.0-8.2	(12/12) h	1000 lux	6 mg O_2/dm^3	Water, SAN PIN 2.1.4.1074-01	96 h	[11, 12, 13]

The study of embryotoxicity of poorly soluble NPs was carried out according to the self-developed methodology "Methods for determining toxicity of nanomaterials and highly dispersed materials based on mortality of fish embryos Danio rerio" [14] at different stages of development of Zebrafish. The methodology was developed on the basis of method OECD [11], updated and certified.

In Figure1 scheme of the procedure for acute toxicity of embryo Zebrafish is presented.

The toxicological effects and presence of pathological changes in the development of embryos at different stages of embryogenesis of Zebrafish (24, 48, 72 and 96 hours post fertilization) in the aqueous medium contaminated with nPt, nNi, nZnO, $nCeO_2$ were determined according to the following metrics:

- the max concentration not causing death during testing;
- the min concentration causing 100 % death during testing;
- the total death count for each concentration for recommended period of observation;
- LC_{50} values in 96 hpf;
- the curve “concentration-death“ at the end of the test;
- the death count in the controls (negative control, internal control in the plate, and positive control);
- the results for each of the four main observations;
- the frequency of occurrence and description of morphological and physiological anomalies, if any.

The toxicity level was measured in accordance with the criteria of the certified methodologies and normative documents [21, 22]. The toxicity of NPs was measured according to the generally accepted parameter in nanoecotoxicology, – toxicity index (I, %).

Using the probit method, the values $L(E)C_{10,}$ $L(E)C_{20}$ и $L(E)C_{50}$ were calculated.

NPs nNi (Δ50 = 5 nm, Ssp = 80-100 m^2/g), nPt (Δ50 = 5 nm, Ssp = 36 m^2/g), nZnO (Δ50 = 12 nm, Ssp = 47 m^2/g) and $nCeO_2$ Δ50 = 16 nm, Ssp = 20-40 m^2 g) were obtained by laser ablation in distilled water from bars of corresponding high purity material [15]. When a metal bar was exposed to radiation by the pulse Nd-YAG laser, LS-2134UTF (LOTIS TII, Belarus, Japan), the target material ablated and spattered into the environment. The thickness of the removed layer did not exceed several tens of nanometres’. Outside the target, the removed material formed NPs. The ablation was continued up to a concentration of NP 50 mg/L.

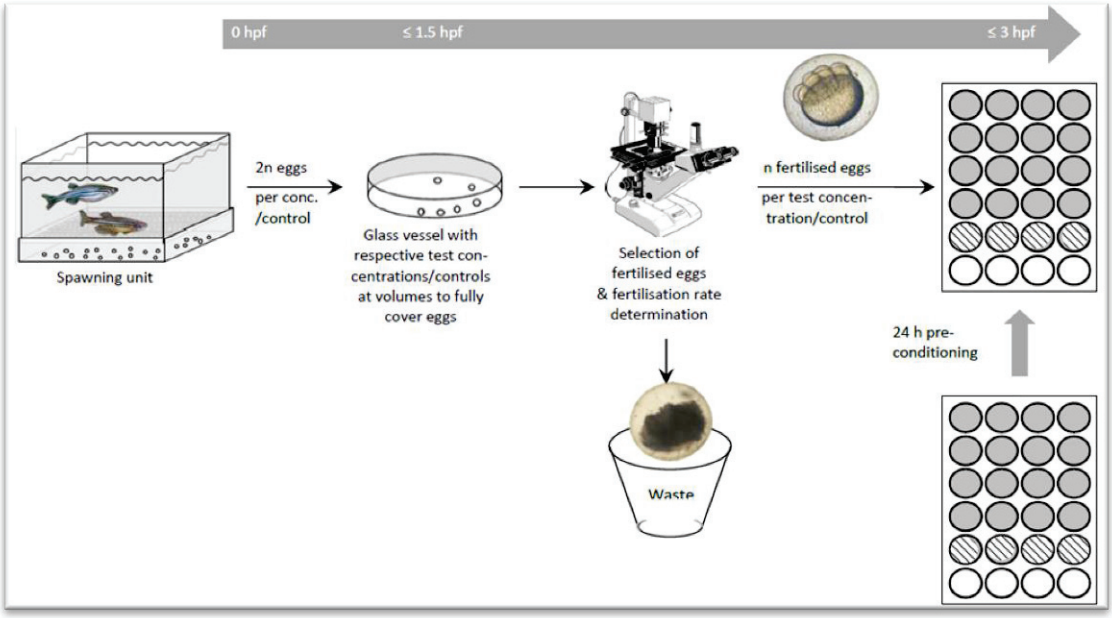

FIGURE 1. Scheme of the zebrafish embryo acute toxicity test procedure in our modification (from left to right): production of eggs, collection of the eggs, pre-exposure immediately after fertilization in glass vessels, selection of fertilized eggs with an inverted microscope or binocular and distribution of fertilized eggs into 24-well plates prepared with the respective test concentrations/controls, n - number of eggs required per test concentration (here 16), hpf – hours post fertilization

Eggs are distributed to well plates in the following numbers [13]:
– 16 eggs on one plate for each test concentration;
– 16 eggs as positive control on one plate;
– 4 eggs in dilution water as internal plate control on each of the above plates;
– 16 eggs in dilution water as a negative control on one plate.

The properties of formed NPs were verified by TEM (Phillips CM-12, France), by the method of dynamic light scattering ("Zetasizer Nano ZS", USA), by the method of BET (TriStar 3000, USA).

The DS NPs were prepared using a self-developed methodology [14], including dilution of the initial DS cultivation medium for Zebrafish and ultrasonic redispersion (30 W/l for 5 minutes). The biological effects and ecotoxicity of NPs were studied in the concentrations (C) 0.1 mg/L, 1.0 mg/L, 5.0 mg/L, 10.0 mg/L, 20.0 mg/L. The DS NPs remained stable during the entire incubation period. A decrease in the concentration of suspended particles did not exceed 5%.

The Excel 2010 software was used for statistical processing of the obtained data. The accepted significance value is $p < 0.05$. All equipment used for testing was properly calibrated.

RESULTS AND DISCUSSION

Currently in connection with the discovery of oncotherapeutic properties of some NPs, an active study of antitumor drugs of a new generation created using nAu, nCu, nAg, nPt, iron Fe_2O_3 and others is being conducted [3, 6,16,17,18]. The data presented in the literature on the preclinical toxicity tests of NP-based drugs are scarce, contradictory and difficult to compare due to the use by various authors of different test organisms, which undoubtedly have different sensitivity.

Based on the data available in the literature it can be concluded that the Zebrafish model does not replace the classical models of mammals, but it can serve as the first step in the development of medicines and simulation of human diseases. In Figure 2 it is shown schematic diagram of various stages of development of Zebrafish and their relevance for nanotoxicological studies [19].

Zebrafish embryo develops rapidly. Only three days after fertilization it has a functioning heart, blood, and nervous system. Four days later young fish appear able to eat and swim. The epithelial cells of the intestinal tract

release digestive enzymes, hepatocytes secrete bile, pancreatic cells produce insulin and carboxypeptidases [3, 18]. Zebrafish embryos easily absorb low-molecular compounds from water through skin and gills and after 7 days of development the absorption occurs through the mouth (and not through the skin).

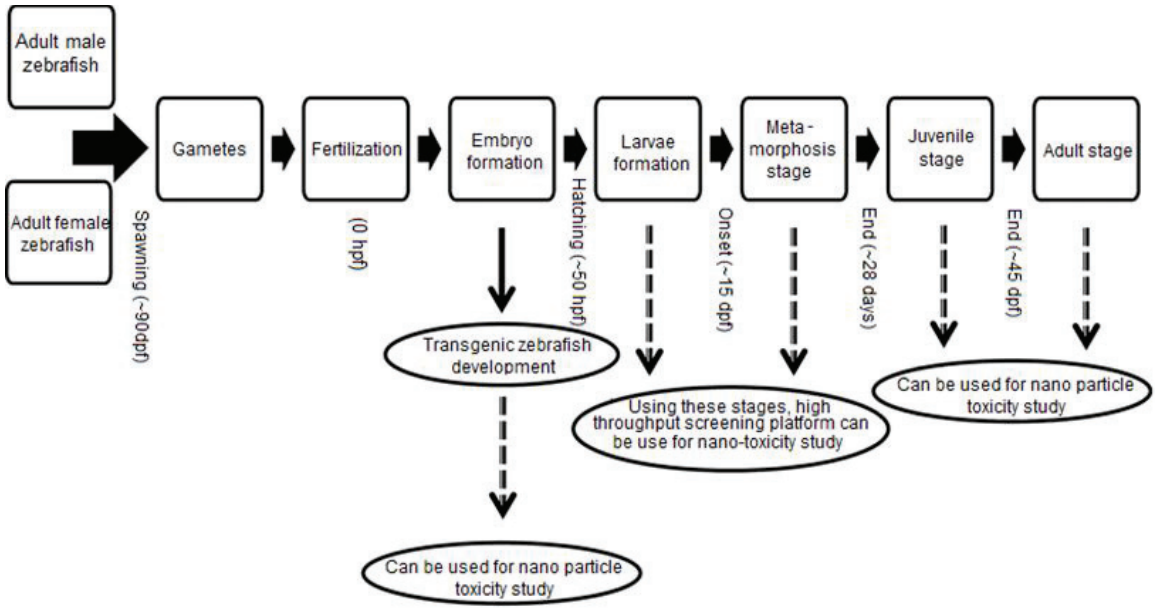

FIGURE 2. Schematic diagram of various stages of development of Zebrafish and their relevance for nanotoxicological studies [19]

We studied Zebrafish embryotoxicity for four types of poorly soluble NPs: nPt, nNi, $nCeO_2$ and nZnO. None of the controls required by the procedure (negative control, internal control in the plate) showed coagulation of embryos. Embryo sensitivity to the toxicant was assessed in separate plates, coagulation of embryos corresponded to the procedural requirements (< 30 %) [11,12,13].

Table 2 shows survival rates, Figure 3 shows mortality rates of Zebrafish embryos depending on concentrations 24, 48, 72 and 96 hpf (hours post-fertilization) when placed in the DS with different concentrations of nPt.

TABLE 2. Survival of embryos at different stages of development Zebrafish under the influence of nPt..

Concentration, mg/L / **Hours post-fertilization**	**Control**	**0.1**	**1.0**	**5.0**	**10.0**	**20.0**
24 h	16	16	16	14	14	16
48 h	16	16	16	14	14	14
72 h	16	16	16	14	14	14
96 h	16	16	16	14	14	14
Overall survival of embryos	16	16	16	14	14	14

It is shown that exposure of Zebrafish embryos in the DS nPt with NP concentration range of 0.1-1.0 mg/L does not cause embryo coagulation within 72 hours. An increase in concentration to C > 5.0 mg/L causes insignificant (12.5 %) mortality of embryos 24 hpf. Further increase of the experiment duration and concentration of the DS nPt did not cause an increase in the total coagulation of the embryos.

Table 3 shows the survival rates of Zebrafish embryos 24, 48, 72 and 96 hpf when embryos were exposed in the DS nNi of different concentrations.

TABLE 3. Survival of embryos at different stages of development Zebrafish under the influence of NPs Ni.

Hours post-fertilization \ Concentration, mg/L	Control	0.1	1.0	5.0	10.0	20.0
24 h	16	16	12	14	10	14
48 h	16	16	12	14	10	12
72 h	16	16	12	12	10	12
96 h	16	14	12	12	10	12
Overall survival of embryos	16	14	12	12	10	12

The data of the conducted experiments showed that the exposure of Zebrafish embryos in the DS nNi at a concentration of NPs 0.1 mg/L caused mortality of 12.5 % embryos at the end of the experiment (96 hpf). In the DS nNi at C = 1.0 mg/L 25.0 % of coagulated embryos were observed in 24 hpf; at C = 5.0 mg/L – 12.5 % coagulation 24 and 48 hpf, and 25.0 % coagulation in 72 and 96 hpf; at C = 10.0 mg/L – 37.5 % coagulation 24 hpf; and C = 20.0 mg/L caused mortality of 25.0 % embryos 24 hpf.

The value $L(E)C_{50}$ exceeds (Table 8) the limit ($L(E)C_{50}$>100 mg/L) therefore nNi does not produce the acute toxic effect on Zebrafish embryos. However, it is a biologically active substance causing significant mortality of fish embryos, which in natural conditions can affect the number of next populations.

Table 4 shows the survival rates of Zebrafish embryos 24, 48, 72 and 96 hours post fertilization when embryos were exposed in the DS at various concentrations of $nCeO_2$.

Less significant impact than nNi on embryonic development of Zebrafish is contamination of the aqueous environment with NPs CeO_2: coagulation of 12.5 % of Zebrafish embryos was registered only at C = 20.0 mg/L at the end of the 96-hour period past fertilization.

TABLE 4. Survival of embryos at different stages of development Zebrafish under the influence of NPs $nCeO_2$.

Hours post-fertilization \ Concentration, mg/L	Control	0.1	1.0	5.0	10.0	20.0
24 h	16	16	16	16	16	16
48 h	16	16	16	16	16	16
72 h	16	16	16	16	16	16
96 h	16	16	16	16	16	14
Overall survival of embryos	16	16	16	16	16	14

The studies have shown that unlike nPt, nNi and $nCeO_2$, NPs ZnO causes more serious disruptions in the development of Zebrafish embryos: the total coagulation of embryos 96 hpf was 37.5 % in the DS C = 0.1 mg/L and 1.0 mg/L; 12.5 % in the DS C = 5.0 mg/L and 10.0 mg/L and 25.0 % at C = 20.0 mg/L. Notably, the manifestation time of the effect was contingent on NPs concentration.

Table 5 shows survival rates of *Zebrafish* embryos 24, 48, 72 and 96 hours post fertilization when embryos were exposed in the DS nZnO of various concentrations.

TABLE 5. Survival of embryos at different stages of development Zebrafish under the influence of NPs nZnO.

Hours post fertilization \ Concentration, mg/L	Control	0.1	1.0	5.0	10.0	20.0
24 h	16	16	16	14	14	12
48 h	16	14	10	14	14	12
72 h	16	10	10	14	14	12
96 h	16	10	10	14	14	12
Overall survival of embryos	16	10	10	14	14	12

The symptoms of abnormal development of embryos are a malformation of somites, development of scoliosis and lack of detachment of the tail-bud from the yolk sac, no heartbeat, and slowness of movement of embryos. In the DS nPt we observed the slowing of movement of embryos 48 and 72 hpf; in the DS nNi, $nCeO_2$ and ZnO the slowing of movement of embryos was observed 48 and 72 hpf in the range of tested concentrations and development of scoliosis in the DS 20.0 mg/L. We registered no abnormalities in formation of somites, detachment of tail-bud from the yolk sac, slowing or absent heartbeat in surviving embryos (the data from the observations are given in Table 6).

TABLE 6. Apical observations of acute toxicity of NPs ZnO at a concentration of 1.0 mg/L in embryos of Zebrafish 24 - 96 hours post fertilization.

Indicators	Exposure times			
	24 hrs	48 hrs	72 hrs	96 hrs
Coagulated embryos	-	+	-	-
Lack of somite formation	-	-	-	-
Non-detachment of the tail	-	-	-	-
Lack of heartbeat		-	-	-

Note:
+ - Indicator is present;
- - No indicator

An important toxicity indicator of NPs and NM [our methodology] and chemical substances [OECD] according to the test procedure is the maximum concentration of the DS NPs that does not result in mortality in the test. It was established that the maximum concentration of the DS nPt not causing death within 96 hours of testing is 1.0 mg/L, nNi is < 0.1 m/L, $nCeO_2$ is 10.0 mg/L and nZnO is < 0.1 mg/L.

The maximum toxicity is registered for nNi and nZnO: I_{max} = 37.5 ± 4.4 % (Table 7). NPs CeO_2 and nPt did not cause significant disruptions of embryonic development of Zebrafish: I_{max} = 12.5 ± 1.9 %.

The summarized data on toxicity parameters of the investigated nanoparticles are presented in Table 7 and Fig.3.

TABLE 7. The mortality of embryos Zebrafish (%) under the influence of different NPs.

Concentration, mg/L / Nanoparticles	Control	0.1	1.0	5.0	10.0	20.0
nPt	0	0	0	12.5±1.9	12.5±2.0	12.5±1.9
nNi	0	12.5±3.9	25.0±4.4	25.0±5.1	37.5±4.4	25.0±3.4
$nCeO_2$	0	0	0	0	0	12.5±1.9
nZnO	0	37.5±2.8	37.5±4.4	12.5±3.6	12.5±1.9	25.0±2.0

The values of $L(E)C_{10}$, $L(E)C_{20}$, and $L(E)C_{50}$ for the tested NPs after a 96-hour period are given in Table 8.

TABLE 8. Criteria of NPs effects on Zebrafish by embryotoxicity at 96 h.

Criteria	NPs			
	Pt	Ni	ZnO	CeO_2
$L(E)C_{10}$ [mg/L]	10.61±1.30	0.015±0.003	1.23±0.22	>100
$L(E)C_{20}$ [mg/L]	25.18±4.10	0.705±0.120	3.76±0.73	>100
$L(E)C_{50}$ [mg/L]	>100	>100	12.93±1.62	>100

If $L(E)C_{50}$ value for a substance exceeds the limit for determining the class of toxicity ($L(E)C_{50}$ > 100 mg/L), then this substance does not produce a chronic toxic effect on Zebrafish embryos, however is a biologically active substance causing embryo coagulation that slightly exceeds their mortality in the control which is permissible in the standard case.

The revealed deviations in the development of Zebrafish embryos in an aqueous environment contaminated with NPs Ni, Pt, ZnO, and CeO_2 depend on the physicochemical properties of NPs and sensitivity of embryos at different stages of embryogenesis.

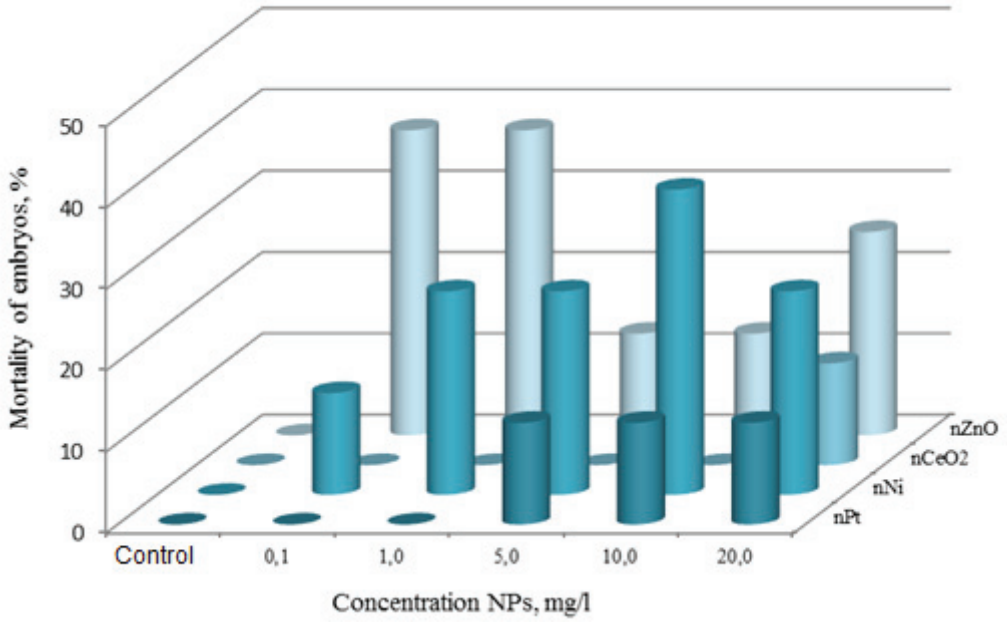

FIGURE 3. Mortality of Zebrafish embryos (%) exposed to different NPs.
All non-zero values significantly differ from zero. Statistical deviations is shown in Table 7

The obtained data are difficult to compare with the published sources since the literature on the effects of NPs (in increasing concentrations) on embryogenesis of Zebrafish is scarce and contradictory.

Liu et. al. [8] and Lopes S. et. al. [20] assessed toxic effects of nZnO with a particle size of 30 nm, 50 and 80-100 nm on the survival rate of *Daphnia m*. S. and development of fish embryos *Danio rerio*. The values of $L(E)C_{50}$ for nZnO corresponded to 1.02 ± 0.24 mg/L and 1.10 ± 0.05 mg/L which demonstrates high toxicity of these NPs. However, the authors do not exclude the possibility of Zn ions entering the environment (nZnO can act as a source of ions) causing negative consequences.

Zebrafish has recently become an attractive object used in simulations of various physiological and pathological processes. It is also used in pharmacological studies such as screening and studying the mechanism of biologically active compounds, pharmacogenomics, and toxicogenomics. According to numerous studies only tests that encompass all stages of Zebrafish life can provide an accurate assessment of acute and chronic toxicity of chemicals while exclusion of certain stages of the life cycle from the design of the experiment may result in an incorrect assessment of toxicity of a substance in question [19, 21]. With the development of modern technologies, this model has been intensively used in recent years to assess the toxicity of chemicals and can soon become a serious alternative to the mammalian models for the toxicological assessment of NM.

Heiden and colloboratos [22] believe that this model is ideal for determining the toxicity of new nanotherapeutic agents.

The authors used Zebrafish embryos to determine the toxicity of poly(amidoamine) (PAMAM) – dendrimers and their conjugates with RGD peptide. A study of the chronic and subchronic toxicity of various dendrimers showed that G3.5 dendrimers conjugated with RGD peptide exhibited the lowest toxicity. In 2007 the results of a study of transport and generation of nAg in the early stages of Zebrafish embryo development were published [23]. The unique optical properties of nAg make it possible to study the effects of some sedatives on the locomotor activity of Zebrafish larvae [24] and observe them inside a developing embryo in real time. It is shown that transport of nAg (5 to 46 nm in size) is done via passive diffusion through porous channels of the chorion, whose diameter is 0.5-0.7 μm. At concentrations up to 0.08 nM, nAg does not exert toxic effects on a developing embryo. With an increase in concentration of NPs to 0.19 nM the authors found no normally developing embryos. Rizzo, L. Y. [25], Browning, L.M. [26], Xina Qi [27] in a model experiment on Zebrafish embryos found the dependence of the degree of toxicity of nAg on the size of NPs. At the same time some disruptions in the development of Zebrafish

were observed: fin defected, tail curvature, edema in the heart, head and yolk sac, and disorders in the development of eyes. The exposure to nanodiamonds for a period of 7 days caused both lethal outcomes (LC_{50}=104±18 mg/L) and deviations in the development of Zebrafish.

Since Zebrafish embryos are transparent, their development can be observed visually with a microscope. The reliability of obtained results was provided by the use of the modern research methods such as TEM and SEM, RSS, optical spectroscopy, and application of the conventional methods for data analysis and reproducibility of results.

CONCLUSION

As a result of the conducted studies, we found that the smallest effect on embryogenesis is produced by $nCeO_2$: coagulation of a small number of embryos (12.5 %) was observed only at C = 20.0 mg/L. The same effect was observed for smaller concentrations of nPt (C = 5.0 mg/L) and nNi (C = 0.1 mg/L). However, with an increase of nPt concentration (up to C = 20.0 mg/L) the mortality of embryos does not increase while with increasing concentration of nNi the mortality of embryos goes up significantly, reaching 37.5 % (C = 10.0 mg/L) and then drops to 25.0% (C = 20.0 mg/L). The greatest number of coagulated embryos was observed in the DS nZnO: 37.5% of embryos died at the DS with a concentration of C = 0.1 mg/L. The number of dead embryos changes irregularly with increasing concentration of nZnO: 37.5 % (C = 1.0 mg/L), 12.5 % (C = 5.0 mg/L and C = 10.0 mg/L) and 25.0 % (C = 20.0 mg/L). It was also established that the growth of Zebrafish in the DS with low concentrations of nNi ($C \leq 0.015$ mg/L) and nZnO ($C \leq 1.233$ mg/L) caused disruptions in the development of embryos: development of scoliosis, malformation of somites, and inhibited mobility.

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