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PECULIARITIES OF SAFETY ASSESSMENT OF NANOSCALE MATERIALS (literature review)

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ABSTRACT. *The manifestation of the biological effects of nanoscale materials depends on many initial states as the NPs themselves (on their size and structural organization, on the physical nature, method of production and method of surface modification), and on the biological model under test and the following interfaces "nano-bio" after penetration into tissues and blood, so they are not predictable, and target organs and mechanisms of development of toxic effect can be varied, which is the basis for urgent need to improve scientific approaches to the assessment of toxicity and danger of nanomaterials.*
Key Words: *nanoscale materials, hazard, features of evaluation.*

The expansion of the production and use of various nanoscale materials requires addressing the issues of their harmful effects on humans. The basis and active agents of nanomaterials are actually nanoparticles having sizes smaller than 100 nm in diameter. The toxic effects of nanoparticles are manifested even when large portions of the relevant compounds are non-toxic [1] and can be largely corrected by their stabilization, modification and functionalization [2].

Reducing the size of particles of a substance to nanoscale sizes (10-9m) leads to changes in their physicochemical and biological properties, which can potentially and really be useful. But as a consequence, high reactivity of nanoparticles (NPs) can help to increase their toxicity. The potential toxicity of NPs cannot, theoretically, be predicted due to the toxicity of volumetric materials of the same chemical nature. According to the literature, nanomaterials (NMs) are practically always larger, especially with long-term admission to the body [3].

NPs toxicity is determined not only by their number, but also by their size, shape, and method of stabilization. Thus, NPs of the dendritic and fusiform forms have higher cytotoxicity than particles of spherical shape. The expressed toxicity is also characteristic of cationic NPs in comparison with anionic and neutral ones. In addition, the NPs entering the blood, lymph or any other biological fluid are covered with a layer of proteins that are constantly in solution and adsorbed on the surface of the particles. As a result, both the properties of the particles themselves and the proteins are modified [3, 4], which cannot be taken into account in standard toxicological studies, since most of them have been performed on a large number of different methods and test systems, the results of which are often not comparable [5].

In this connection, it is of fundamental importance to improve and develop a system of standard tests that will assess the risk of new NMs on their impact on the performance of selected biological systems, which may be pathogenic, conditionally pathogenic and symbiotic microorganisms, eutherian cell cultures, representative components of aquatic biocoenosis (crustaceans, fish), mammalian organisms. And the use of alternative in vitro approaches will allow to obtain information about the toxicity and danger of objects in less costly ways, in a shorter time and more humane from the standpoint of bioethics without the use of laboratory animals [6].

Consideration should be given to the absence of a monotonic dose-effect relationship for many known nanoscale materials and manifestations of high toxicity of low-slurry suspensions at low concentrations. In this regard, there are problems with the use of criteria based on the determination of the hazard class by the degree of dilution of the aqueous suspension or extract of the pollutant to a safe concentration [7].

The algorithm for toxicological studies of NPs depends on the alleged cause of the dangerous impact [8]. For preliminary assessments, it is recommended to use screening test kits. Their choice depends on knowledge of the data on the chemical, physical and biological properties of the studied nano-objects. In

screening and in the NP risk identification stage, in vitro studies as an early indicator of potential hazard may be informative.

While most chemicals cause cell damage through interaction with specific biomolecules, one type of NPs can be toxic as a result of a combination of different mechanisms [9, 10, 11, 12]. NPs can induce the formation of reactive oxygen species, be genotoxic, lead to morphological and immunological changes in the living organism [13, 14]. The mechanisms of the damaging effects of nanoscale substances may be: oxidative stress; dissolution and release of toxic metal ions; cationic damage to the surface membrane and organelles; profibrogenic responses to carbon nanotubes; inflammasome activation due to long proportion materials; photoactivation and band gap; interference of the zebrafish embryo; cell membrane lysis by surface reactivity.

The high levels of toxicity observed for NPs are due to such factors as the small size of NP, allowing it to penetrate the external and internal barriers of the organism, large specific surface area (high ratio of surface area ofNP to volume), high surface reactivity of NP [14, 15]. In addition, most NPs are unstable in dispersion, prone to aggregation and sedimentation, which significantly affects the NP uptake process and their toxicity [16].

Another problem in assessing the toxic effect of NPs is the different approach to estimating NP concentration. It is shown that at equal concentrations, expressed as mass per unit volume, small NPs can cause more severe toxic reactions than larger NPs of the same nature [17, 18]. It is known that toxicological reactions depend on the surface properties of NPs and that the surface area increases exponentially with decreasing NP size. Therefore, a number of researchers propose to express the concentration of NPs as the surface area of NP per unit volume [19].

Contradictions arising from the assessment of NP toxicity may be the result of the use of different techniques for the assessment of toxicity. This may relate to the time and conditions of sample incubation from the NPs used in the dose range.

Choosing an NP dose in an experiment that mimics human impact is not an easy task, since general exposure involves different ways of getting into the body – through the respiratory system, the gastrointestinal tract, the skin [20, 21].

Another important factor in the occurrence of differences in the assessment of NP toxicity is the use of different environments in which the latter are dispersed. The environment can cause NP aggregation, which in turn determines NP behavior in dispersion, as well as in NP uptake and toxicity development processes [22, 23]. By assessing the toxicity of chemicals soluble in the cellular environment, it is possible to accurately indicate the concentration of the substance in solution. With NP, such estimation is impossible, since NP in dispersion is prone not only to diffusion, but also to processes of sedimentation, agglomeration and aggregation [22].

Practically most nanomaterial toxicity results are obtained with single or short-term administration to laboratory animals. It was found that many of the tested materials did not have acute toxicity. But the effects of chronic exposure to NPs have not yet been sufficiently studied, although they may be significant, especially for individuals with a long-life cycle, including for humans. Almost nothing is known about the accumulation of nanomaterials in various organs and tissues, especially in chronic admission to the body [24].

Another important feature of the biological environment of the NPs is that when they get into the blood, lymph, gastric juice or any other biological fluid, they are covered by a kind of «crown» – a layer of proteins that are constantly in solution and adsorbed on the surface of the particles.As a consequence of the mutual influence, the properties of the particles change under the action of the «crown», and the proteins with which the particle comes into contact, can be modified. The binding process also affects the behavior of the particle inside the body. The amino acids and proteins that cover the surface of the NPs form a “crown” around the particle and change the surface properties, namely, they are responsible for the efficiency of nanoparticles. Surface modification can potentially reduce or, conversely, increase the toxicity of particles.

Due to their small size, NPs may not be recognized by the body's defense systems, undergo biotransformation and have a long half-life. Once in the body, NPs are capable of damaging biomembranes, disrupting the function of biomolecules, in particular molecules of the cell's cellular apparatus and cellular organelles, leading to disruption of regulatory processes and cell death [25].

Thus, the degree of danger of nanomaterials depends on a large number of NP characteristics and environmental factors. In turn, the number of potential combinations of different material properties can reach tens of thousands, which is unrealistic to consider in an animal experiment.

Traditional approaches for assessing the biological effects of NM in laboratory animal experiments are not only time-consuming and costly, but are not always feasible, since particle size and surface area can be crucial, while increasing NP concentrations may not be dose-dependent.

At present, test objects such as insects (Drosophila melanogaster) and hydrobionts (Brachydanio rerio) are gaining increasing popularity in in vivo toxicological studies, although their use, while assessing the risks of using nanomaterials for subsequent generations of organisms, is not sufficient for prediction of danger to humans.

For nanomaterials characterized by medium hazard, the scope of the planned studies should be substantially expanded to assess the impact on critical functions of the laboratory animal. In turn, for nanomaterials with a high degree of potential danger, toxicological and hygienic characteristics should be carried out in full with the use of special types of studies (embryotoxicity, teratogenicity, mutagenicity, carcinogenicity, etc.). It is possible to conduct experiments throughout the life of laboratory animals (2-3 years) or even for several generations [26, 27].

Therefore, the unified toxicological characterization of nanomaterials involves both in vitro and in vivo studies, including animal experiments, which can last longer than 9-12 months. All this indicates the practical impossibility of characterizing in the near future the biological effects of all-important nanomaterials due to unacceptable labor and material resources [28].

At present, methods that are alternative to classical tests on experimental animals are widely used in the evaluation of toxicity – these are models using cell cultures. The use of cell cultures not only solves the ethical problems associated with the use and death of laboratory animals during experiments, but also significantly reduces the cost of previous toxicity studies of new materials. In addition, the terms of the full cycle of toxicological studies are reduced [9, 10].

In vitro screening reactions are usually performed on cells and cell cultures from eight representative organs, which may be affected by exposure to the NPs by oral, inhalation and parenteral administration. There are such phenomena as oxidative stress, inflammation, immunotoxicity, cytotoxicity, genotoxicity [9]. As an additional indicator of the real hazard criterion of the NPs, a hazard index has been developed that characterizes their hazard compared to the native substance.

Another toxicity test is to measure the activity of lactate dehydrogenase enzyme in cell culture medium. This enzyme is localized in the cytoplasm of a living cell and released into the incubation medium as a result of disruption or destruction of cell membranes. The overall toxicity estimate is based on the determination of the amount of enzyme released into the environment. This test can be performed in real time and allows to determine the dependence of the toxic effect of NPs on exposure time.

Cytotoxicity can be determined by staining individual cells in a colony. The technique is that cells are introduced into the culture medium in low concentration, whereby individual cells form separate colonies. NP treatment is carried out either before insertion of cells into the incubation medium or after colony formation. Colonies are stained with dye and quantitatively characterized and sized [29].

In vitro toxicity assessment is performed not only on cell cultures, but also on sections of high precision cultures obtained from homogeneous tissues under sterile conditions. Such systems have several advantages over homogeneous cell cultures. Cross-sections of cultures present all cell types of the organ under study, in addition to maintaining an intercellular communication system that allows to assess the extent of the effect of NPs on specific cells. A significant drawback of high precision culture slices is their rapid degeneration. The life span of such systems is no more than 24 hours.

In order to overcome the above difficulties, attempts are constantly being made to develop new testing methods. Because NPs can cause different effects through different mechanisms, a multivariable toxicity assessment method has been proposed [12, 13]. This method involves determining a number of parameters in different ways and using several

types of cells from different organisms, which significantly increases the efficiency and reliability of the results of studies of the toxic effect of NPs [30]. The assessment parameters were chosen: acute toxicity, induction of reactive oxygen species, morphological changes, genotoxicity, NP degradation [11, 30].

As with other man-made materials, in vitro and in vivo experiments should be performed to assess the toxicity of NPs [31]. It is believed that in vitro experiments may be the basis for predicting the potential toxicity of nanoparticles and reducing the number of animals used [32]. Many in vitro models outperform animal-based toxicity studies that have traditionally been used in toxicology. Animal models are not only inhumane, but often not reliable enough to predict effects in humans.

According to the literature [20, 33, 34, 35, 36], in studies on the toxicity of a number of nanomaterials, in in vitro reactions open systems and organelles are commonly used, for example, inhibition of mitochondrial activity, lactate dehydrogenase activity (for CdO, Ag, MoO_3, MnO_2, Fe_3O_4, Al, W);inhibition of V. Fischer bacteria growth when exposed to TiO_2, ZnO, CuO.The cytotoxic effect of Fe_3O_4, Cs, Si, As, CdTe, CdSe / ZnS points coated with a polyacrylic acid polymer was studied on lung epithelial cells, BRL 3A cell cultures (ATCC, CRL-1442), rat liver cells, precursors of alveolar macrophages, Xenopus blastomeres. Studies on hydrobionts (crustaceans, fish) for fullerenes revealed lethal effects for Daphnia magna, t. Platyurus Micropterus salmoides. And inhibition of growth of Pseudo-kirchneriella subcapitata for TiO_2, ZnO, CuO was studied at microalgae.

One way of intensifying tests and reducing their cost may be the use of accelerated toxicological studies on simple biological systems. In this regard, the development and implementation of alternative methods in vitro have become one of the leading areas of toxicological studies of NM [5].

It should be noted that the use of in vitro systems for the assessment of NP toxicity has been approved by the European Center for the Validation of Alternative EU Methods [37]. The proposed methods of in vitro diagnostics cover different areas: reproductive toxicity, assessment of potential carcinogenicity, transfer through various barriers (skin, vascular epithelium, blood-brain barrier, etc.). There are several model systems for the evaluation of NM toxicity in vitro: cell lines, gene expression, oxidative stress, mitochondrial damage, DNA, cellular dysfunctions, absorption. In vitro systems allow the control of such parameters of cytotoxicity as cell morphology, their viability, proliferation, inflammatory processes, oxidative stress. In vitro studies have shown the dependence of toxicity on the size, shape and concentration of NPs [38], as well as some mechanisms of toxic action (oxidative stress, apoptosis) [39]. In general, the results of the studies revealed a high sensitivity of alternative models and their ability in the acute experiment to establish a toxic effect of doses that cause a similar effect in animals only in the conditions of long-term experiments. This fact is extremely important because the use of alternative approaches will allow to obtain a screening assessment of the toxicity of the investigated NPs or nanoproducts, which is very relevant in the context of increasing volumes of their production and use.

It is believed that traditional approaches for the study of chemical toxicity are not sufficient for the study of objects in the nanoscale [40]. Nanomaterial and nano-product hazard assessments by conventional classical toxicological methods have also been complicated by the large volume of research.

Also, the characteristics of absorption, distribution, deposition and accumulation of nanoparticles and nanomaterials in the body are only possible when using in vivo studies. According to the developed systems after synthesis and study of the physical and chemical properties of the new nanomaterial, it is recommended to evaluate acute and chronic toxicity, cumulativeness, genotoxicity and cytotoxicity, immunotoxicity, carcinogenic properties, study of metabolism in the body, as well as biotransformation. The main problem of toxicity of nano-objects is dosimetry, which depends on the time of introduction and concentration of the preparations used, the number of nanoparticles and their morphological parameters (size, shape, density, state of agglomeration, as well as surface charge) [41].

The difficulty of choosing the appropriate model for testing lies in the correct selection of the cell line. Also, in vitro models cannot provide an estimate of the risks of nanoparticles being used for subsequent generations of cells or organisms.

The main in vitro methods for assessing nanotoxicity include [42, 43, 44, 45]:

1. Assessment of cell viability by the respiratory activity of mitochondria, redox potential of cells, cell proliferation.

2. Cell apoptosis – morphological changes of cells, DNA damage, analysis of caspases, detection of apoptotic cells.

3. Cell necrosis by the absorption of dyes neutral red, trypan blue, lactate dehydrogenase activity.

4. Analysis of inflammation by definition of antibodies or antigens.

5. Characterization of oxidative stress – by lipid peroxidation, oxidative protein modification, determination of reactive oxygen species, superoxide dismutase enzyme and glutathione content.

Most researchers argue that in vitro methods are highly specific, do not require significant costs for reagents and devices, allow to exclude from the model system third-party factors that can affect the research processes, allow for quantitative evaluation of the effects and simultaneous screening of a large number of research objects [46].

Test objects for in vitro toxicity studies may be cell lines, microorganisms, biochemical processes with establishment of cytotoxic, absorption, biokinetics, intracellular transport, systemic toxicity, genotoxicity, oxidative stress, sensitization, toxicity, reproductive toxicity, metabolism [6].

In fact, alternatives to experimental animal studies have been used to assess the toxicity of chemicals since the 1950s and 1960s. According to the REACH European Legislative Program on the Registration, Expertise and Authorization of Chemicals, which has been operational in Europe since 2007, the toxicity and risk management scheme of chemical compounds is required to assess in vitro toxicity of substances prior to animal and can be carried out on cell cultures and other alternative objects.

To reduce the volume and accelerate the research of nanomaterials, it is proposed to use a priority scale of their degree of danger, according to which they are divided into low-, medium- and high-risk [47].It is established by the results of preliminary assessment of the degree of danger of nanomaterials: on the basis of an array of scientific information, all known properties that influence the potential danger of nanomaterials are considered, namely: geometric characteristics, physicochemical properties, interaction with biomacromolecules, influence on cells, organism, ecological characteristics according to screening tests on cell cultures, bacterial cultures, plants and hydrobionts.

Thus, for low-risk objects, only certain, critically important test studies are recommended, and the same criteria and approaches as for "traditional" analogs obtained without the use of nanotechnology may be used in the future. For example, the risk assessment of nanomaterials without the use of animals can be based on Monte Carlo simulations [27].

In assessing the dangers of nanomaterials, their impact on such biological characteristics as biomembrane permeability, genotoxicity, activity of redox processes, including lipid peroxidation, biotransformation and elimination from the body, must first be established [26, 48]. It should be noted [3] that:

— toxicity of nanomaterials cannot be deduced in comparison with analogues in macrodispersed form or as solid phases, because their toxicological properties result not only from chemical composition, but also a variety of other features, such as surface characteristics, size, shape, composition, chemical reactivity, etc.;
— the available toxicological methodologies are based on the determination of the toxicity of substances with respect to mass concentration, which is not acceptable for nanomaterials (for which the size of the surface area may be the main determining properties);
— there are no standardized indicators of toxicity of nanomaterials, which must necessarily take into account the contribution of such characteristics as surface properties, size, shape, composition of compounds, chemical reactivity of their constituent particles;
— there is no convincing data on the target organs of the action of specific nanomaterials;
— methods for the detection, identification and quantification of nanomaterials in environmental objects, foodstuffs and bio-environments that could reliably distinguish them from chemical analogues in macrodispersed form, poorly worked out.

It is believed that models of cytotoxicity assessment of substances in vivo do not allow

to study in detail those possible reactions of mammalian cells, tissues and organs, which should be known when using NPs as drug components and in other biotechnological fields. For this reason, it is of utmost importance to have methods of studying the toxic properties of NPs in vitro, in which the very cells that have contact with the NPs under study will act as models. At the same time, in vivo studies do not allow or complicate the assessment of responses to NPs of individual cells and tissues and such parameters as the effect of NPs on cell proliferation, reproductive potential, assessment of carcinogenic properties, the ability to absorb NPs by different cell types and determine the pathways of absorption. In addition, their costly, more time consuming and often contrary to bioethical requirements. Therefore, when analyzing the cytotoxic properties of NPs, from an economic point of view, from the standpoint of bioethics and scientific approach, it seems appropriate to use cell cultures and other objects for in vitro studies in the first stages of screening.

The main advantages of using in vitro systems are that they provide scientifically sound results that can be applied not only in practice but also in the development of fundamental foundations [6].

Although in vitro experiments cannot replace full-fledged animal studies, their use is the basis for assessing the dangers of nanoparticles of a man-made nature. This approach is important, taking into account the unique properties of NPs: large surface area, large surface area / mass ratio, small dimensions that can facilitate their penetration through cell membranes, epithelial or endothelial barriers and reach internal organs.

Thus, the toxic effects of nanomaterials depend on many initial states, both of the NPs themselves (on their size and structural organization, on the physical nature, method of production and method of surface modification), and on the biological model under test and subsequent interfaces "nano-bio" after penetration into tissues and blood, so they are not predictable, and the target organs and mechanisms of development of toxic effect – can be varied.

Despite more than fifteen years of worldwide research, existing data do not allow us to conclude conclusively on the dependence of biological effects not only on the structure and levels of action, but also on changes in the characteristics and properties of NMs that occur in use procedures and standard toxicological research.

It should be noted that alternative in vitro models have demonstrated a high sensitivity and ability to detect the toxic effect of doses that cause similar changes in animals only in long-term experiments and require the use of highly sensitive special equipment. This fact is extremely important as alternative methods allow to obtain a screening assessment of the toxicity of the investigated NMs or nanoproducts, which is relevant in the context of increasing production volumes.

The current development of nanotechnology is ahead of the development of approaches to the toxicity and hazard assessment ofNPs and NMs. Due to the high level of complexity and uncertainty of many aspects in this field, the risk assessment and scientific justification of the respective strategies and rules are associated with some obstacles. One way of intensifying tests and reducing their cost may be the use of accelerated toxicological studies on simple biological systems (models). In this regard, the development and implementation of alternative in vitro methods has become one of the leading areas of NM toxicology research.

Various model test systems, such as protozoa, microorganisms, cell lines and subcellular structures (mitochondria, microsomes, DNA), hydrobionts, plants, insects, cattle, are offered,vessels of chick chorioallantoic membrane (CAM) and others.

It should be noted that alternative methods of toxicological research at the international level are being developed through the combined efforts of various organizations, in particular, the Organization for Economic Cooperation and Development (OECD) has created a database on nanomaterial safety research. Recommended toxicity assessment methods involve the use of different models for studies other than mammals. At the same time, it is emphasized that among the existing methods and test systems, the ones that would be the most informative, standardized, have an objective digital evaluation of the results and are well correlated with the data obtained from animals [36].

Despite the large number of proposed test systems for screening the impact of nano-sized objects, it is urgent to choose the most sensitive

ones, depending on the goals and objectives of the study [49, 50]. And the toxicity of NMs has an ambiguous dependence on their size and can be caused by both the physicochemical properties and size of the NP, as well as the carrier phase and stabilizers. However, there are no general laws regarding their effect on the toxic properties of NPs, so these relationships should be established on a case-by-case basis. At the same time, the information obtained from in vitro experiments can be used to screen NM toxicity as a «vector» for in-depth in vivo experimental studies.

Conclusion

In the context of increasing production of nanomaterials, the assessment of their danger by conventional classical toxicological methods is complicated by the ambiguity of the results obtained and the considerable amount of research. A significant problem in evaluating the effects of nanoproducts is the instability of nanoparticles and the unpredictability of changes in their parameters, characteristics and properties, and therefore, pronounced structural-dose biological effects. Therefore, there is an urgent need to improve scientific approaches to the evaluation of the toxicity and danger of nanomaterials.

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ОСОБЛИВОСТІ ОЦІНКИ НЕБЕЗПЕЧНОСТІ НАНОРОЗМІРНИХ МАТЕРІАЛІВ
(Огляд літератури)

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РЕЗЮМЕ. *Прояв біологічних ефектів нанорозмірних матеріалів залежить від багатьох вихідних станів як самих НЧ (від їх розмірів та структурної організації, від фізичної природи, способу отримання та способу модифікації поверхні), так і від біологічної моделі, на якій проводяться випробування, і наступних інтерфейсів «нано-біо» після проникнення в тканини і кров, тому вони не передбачувані, а органи-мішені і механізми розвитку токсичного ефекту можуть бути різноманітними, що є підставою нагальної необхідності вдосконалення наукових підходів з оцінки токсичності та небезпеки наноматеріалів.*

Ключові слова: *нанорозмірні матеріали, небезпечність, особливості оцінки*

ОСОБЕННОСТИ ОЦЕНКИ ОПАСНОСТИ НАНОРАЗМЕРНЫХ МАТЕРИАЛОВ
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РЕЗЮМЕ. *Проявление биологических эффектов наноразмерных материалов зависит от многих исходных состояний как самих НЧ (от их размеров и структурной организации, от физической природы, способа получения и способа модификации поверхности), так и от биологической модели, на которой проводятся испытания, и последующих интерфейсов «нано-био» после проникновения в ткани и кровь, поэтому они не предсказуемы, а органы-мишени и механизмы развития токсического эффекта могут быть различными, что является аргументом для совершенствования научных подходов в оценке токсичности и опасности наноматериалов.*

Ключевые слова: *наноразмерные материалы, опасность, особенности оценки.*

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