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ОЦЕНКА БИОЦИДНОГО ДЕЙСТВИЯ НАНОЧАСТИЦ МЕТАЛЛОВ И БИОЭЛЕМЕНТОВ В ОДНОКЛЕТОЧНОЙ ЭУКАРИОТИЧЕСКОЙ ТЕСТ-СИСТЕМЕ

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Представлены результаты исследования биоцидных свойств наночастиц серебра, меди и диоксида кремния. Рассмотрены вопросы о безопасности использования нанокомпонентов в связи с неизученным воздействием их на экологию. Для оценки биоцидного действия наночастиц благородных металлов и биоэлементов использована одноклеточная эукариотическая тест-система, представляющая собой реснитчатый протистный микроорганизм *Paramecium caudatum*, обитающий в прудовых водоемах. Установлено, что растворы наночастиц благородных металлов и биоэлементов не являются биоинертными и биостимулирующими. Коллоидные растворы наночастиц серебра, меди и кремния диоксида имеют биоцидное воздействие, проявляют схожий дозозависимый эффект при наличии одинаковых концентраций наночастиц в исходных коллоидных растворах (300 мкг/мл). Коллоидный раствор серебра характеризуется более выраженной токсической активностью в одноклеточной протистной биологической модели, так как полная биоцидность в отношении парамеций обеспечивается разведениями коллоидного раствора наночастиц до значения 1 : 6 от исходного. По сравнению с ним у растворов наночастиц меди и оксида кремния показатель биоцидности 100% достигается только в значениях двух- и трехкратного разведения исходного раствора. Коллоидные растворы наночастиц в концентрациях, не вызывающих полной гибели инфузорий (1 : 5 от исходной для наночастиц меди и оксида кремния и 1 : 7 от исходной для наночастиц серебра), угнетают интенсивность их размножения приблизительно на одинаковую величину в 55–61% (индекс интенсивности размножения парамеций от 0,455 до 0,390 соответственно).

Ключевые слова: наночастицы серебра, наночастицы меди, наночастицы кремния диоксида, биоцидность, инфузория-туфелька, *Paramecium caudatum*

ESTIMATION OF BIOCIDAL EFFECT OF METAL AND BIOELEMENT NANOPARTICLES IN A UNICELLULAR EUKARYOTIC TEST SYSTEM

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The results of the study of biocidal properties of silver, copper and silicon dioxide nanoparticles are presented. Questions about the safety of nanocomponents in connection with their unstudied impact on the environment are considered. To evaluate the biocidal effect of noble metal nanoparticles and bioelements, a unicellular eukaryotic test-system, represented by a ciliated protist microorganism Paramecium caudatum inhabiting pond water bodies, was used. It was found that solutions of noble metal nanoparticles and bioelements are not bioinert and biostimulating. Colloidal solutions of silver, copper and silicon dioxide nanoparticles have a biocidal effect and show a similar dose-dependent effect if the concentration of nanoparticles in the initial colloidal solutions is the same (300 μg/ml). The colloidal silver solution is characterized by a more pronounced toxic activity in a unicellular protist biological model, since full biocidal activity against paramecium is provided by dilutions of the colloidal solution of nanoparticles to the value 1: 6 of the initial one. Compared to it, solutions of copper nanoparticles and silicon oxide have a biocidal index of 100% achieved only in values of two- or three-times dilution of the initial solution. Colloidal solutions of nanoparticles in concentrations that do not cause complete mortality of the infusoria (1: 5 of the original for copper and silicon oxide nanoparticles and 1: 7 of the original for silver nanoparticles) inhibit their

reproduction intensity by approximately the same value of 55-61% (paramecium reproduction intensity index of 0.455 to 0.390 respectively).

Keywords: silver nanoparticles, copper nanoparticles, silicon dioxide nanoparticles, biocidal activity, ciliates, *Paramecium caudatum*

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INTRODUCTION

Nanotechnology has greatly expanded the use of materials based on nanocomponents, which inevitably leads to their impact on the environment. To date, the impact of nanoparticles on biosphere objects remains unstudied, since many substances can become biologically active if used in the form of nanoparticles [1, 2]. Studies of water-dwelling eukaryotes have shown that nanocomponents can disturb aquatic ecosystems [3].

Earlier studies evaluated the ecotoxic effects of xenobionic substances on multicellular test objects such as insects, fish and even rodents [4]. Nevertheless, protist organisms should be recognized as the most suitable test system for the evaluation of ecotoxic effects. This is especially true for aquatic environments [5], since protozoa are natural aquatic inhabitants. Protozoa have a high potential of bioadaptation to toxicants, they do not require the creation of special conditions of maintenance.

At present, only a limited number of scientific publications are available on the evaluation of the biocidal effect of nanoparticles in a single-cell test model [6-8]. It should be acknowledged that there is actually no unified methodology for the toxic evaluation of nanoparticles on unicellular eukaryotes. Many authors often use their own adaptations and variations of this technique.

In our experiments, the free-living ciliate infusoria *Paramecium caudatum* was used to study the eco- and cytotoxic effects of colloidal solutions of metal nanoparticles and bioelements. This representative of ciliates belongs to the number of highly organized protozoa and is a widespread inhabitant of freshwater reservoirs. This free-living prostite is often used to assess the toxicity of many natural and artificial compounds [9]. On the one hand, this microorganism has all the structural features of a cell, has autonomous existence in the external environment, and reacts to external stimuli as an independent organism. On the other hand, *Paramecium caudatum* infusoria is not demanding to the conditions of cultivation, so a large amount of laboratory data can be obtained using it [10].

Three types of nanoparticles were used as test substances: silver, copper and silicon dioxide. The first of them belongs to the category of noble metals, the others to the class of bioelements. This choice was determined by their relatively wide use in veterinary and medical practice. In addition, such a set of nano-substances most widely reflects the different classes of tested nanocomponents: silver is a noble metal, copper is a biometal, silicon dioxide is a bioelement from among the non-metals. The ecotoxicity of their nanoscale forms has not been previously studied, although their biocidal properties have been investigated [6, 11].

The purpose of the study is to evaluate the biocidal properties of silver, copper and silicon

dioxide nanoparticles on the unicellular eukaryotic test object *Paramecium caudatum*.

The objectives of the study are to carry out a rapid assessment of the biological activity of colloidal solutions of nanoparticles of metals and bioelements, determine the biological impact of the tested colloidal solutions of nanoparticles on the resistance of paramecium to negative external influences, evaluate their ecotoxicity by the intensity of suppression of paramecium reproduction.

MATERIAL AND METHODS

The evaluation of the biocidal effect of colloidal solutions of silver, copper and silicon dioxide nanoparticles on the free-living *Paramecium caudatum* infusoria was performed according to the methodological recommendations[1]. Parameciums were cultured in Lozina-Lozinsky medium (pH 6.2-7.8) and at 20-26 °C (control medium). *Rhadotorula gracilis* yeast with the addition of rice grains served as the food for the test object.

In the experiments, the integrated effect of various nanomaterials on a living protist cell was evaluated; therefore, the studies included three stages. At the first stage, an express-evaluation of the biological activity of colloidal solutions of silver, copper, and silicon dioxide nanoparticles was performed. All colloids had an initial concentration of nanoparticles of about 300 μg/ml.

4.2 ml each of *Paramecium caudatum* infusoria culture in the stationary phase of growth were poured into 54 test tubes. A well-known bactericide (norfloxacin) and an adaptogen (eleutherococcus) were used as controls. Equal volumes of colloidal solutions of nanoparticles were added to the first test tube, followed by stirring and obtaining a twofold dilution of their solution (1: 2). For mathematical recording and subsequent statistical processing, a 1 × n-1 record was used, where n denotes the degree of dilution, i.e., it represents the denominator in absolute terms (e.g., 1 × 2-1 for a 1: 2 dilution). By further adding the solvent (distilled water), dilutions of 1: 3; 1: 4, 1: 5, 1: 6, and 1: 7 nanoparticles, or in our mathematical notation from 1 × 3-1 to 1 × 7-1, were obtained. The test tubes were then placed for 24 h in a thermostat at 22 °C. The contents from each tube in the amount of 0.1 ml were transferred to microaquariums for microscopic evaluation. During microscopy, the number of paramecia was not less than 100.

The state of protist microorganisms was evaluated according to the following indicators: PN - indifferent (infusoria exhibit a markedly uniform Brownian motion, indistinguishable from that of the control); BA - bioactivity (increased intensity of infusoria movement); BC50 - biocidity (death of about half (50 ± 5%) of the infusoria population); BC100 - biocidity (death of almost the entire (90 ± 10%) population of infusoria); and control samples should contain not less than 100 infusoria performing a uniform Brownian motion.

At the next stage, we determined the biological activity of the colloidal nanoparticle solutions tested by the toxic load method after exposure to superhypertensive (with tenfold excess of isotonicity) sodium chloride solution (8%). For this purpose, we used four test tubes with 1 ml of control paramecium culture where 0.3 ml of 8% sodium chloride solution was added, causing the death of all infusoria within 5 min. Paramecium death was recorded in microaquariums under a light microscope using a minute timer in seconds. Then, we performed a similar experiment with cultures of parameciums of the first stage (after daily exposure of nanoparticles). Several observations were performed to obtain reliable experiment data.

The biological activity index (BAI) was calculated according to the formula

[1]Shabunin S.V. Screening of biostimulants and biocides (adaptogens, bactericides and other drugs): guidelines. Moscow; Voronezh: All-Russian Research Veterinary Institute of Pathology, Pharmacology and Therapy, 2006. 51 p.

$$IBA = \frac{TO}{TK}, \quad (1)$$

where BAI is the index of biological activity of the studied substance; TO is the life expectancy (in seconds) of parameciums under the action of 0.3 ml of 8% sodium chloride solution after their incubation for 24 h in Lozina-Lozinsky medium with the studied concentration of the nano-substance; TK is the life expectancy (in seconds) of paramecia under the action of 0.3 ml of 8% sodium chloride solution after incubation for 24 h in control medium (Lozina-Lozinsky medium).

After calculating the BAI index, we evaluated it. When the BAI value was 1.000 ± 0.1000, we considered the nanosubstance to be bioinert. A bioactivity index value higher than 1.000 ± 0.1000 indicated a positive effect of the tested substance on the resistance of paramecium to hypertensive shock. A bioactivity index value of less than 1.000 ± 0.1000 indicated a negative effect of the tested substance on the viability of parameciums.

At the last stage of the study we evaluated the biological activity of colloidal solutions of silver, copper and silicon dioxide nanoparticles by the intensity of paramecium reproduction after the exposure to sublethal (slightly below lethal) concentrations of nanoparticles. For this purpose, we used a culture of parameciums in the active growth phase after contact with the colloidal solution of nanoparticles under study. At the beginning of the experiment, the inoculum density (the number of parameciums in 1 ml of medium) was established. For this purpose, the number of infusoria cells in 1 ml of culture was determined: 20 microliters of 5% alcohol iodine solution was added to 1 ml of paramecium. Then, the contents were stirred, placed in a Fuchs-Rosenthal chamber, and the number of parameciums in 10 squares was counted, determining the average number of cells in one square. The volume of one square was one ten thousandth of a milliliter.

After incubating the test tubes in the thermostat at 22 °C for 72 h, the density of the inoculum was determined in each test tube.

The calculations were performed according to the formula

$$RRI = \frac{DIEA \times DICB}{DICA \times DIEB}, \quad (2)$$

where RRI is the paramecium reproduction rate index; DIEA is the density of the inoculum in the experiment after 72 h of incubation; DICB is the density of the inoculum in the control before incubation; DICA is the density of the inoculum in the control after 72 h of incubation; DIEB is the density of the inoculum in the experiment before incubation.

After calculating the index of paramecium reproduction rate, the cytotoxicity of the medium was evaluated based on the following values: if the index of paramecium reproduction rate was 1.000 ± 0.100, the chemical nanosubstance was considered biologically inert. A paramecium breeding intensity index value higher than 1.000 ± 0.100 indicated a stimulating effect of the nanosubstance on paramecium. When the value of the paramecium reproduction rate index less than 1.000 ± 0.100 was obtained, the inhibition of paramecium reproduction by the chemical nanosubstance was concluded.

RESULTS AND DISCUSSION

In the course of studies to assess the toxic effect of colloidal solutions of nanoparticles of metals and bioelements, initially qualitative results were obtained (see Table 1). They were evaluated by the characteristics of the Brownian motion of parameciums, and the toxic effect of the preparation was expressed in the slowing of protist movement, and the inhibition increased with prolongation of the exposure, but the final toxicity evaluation was performed after 24 h of exposure to the tested substance.

The results show that all tested colloidal solutions of nanoparticles have different biocidal activity: all tested samples of nanoparticles showed a high level of biocidal activity at the minimum dilution (1: 2), determined by

Табл. 1. Дозозависимое действие нановеществ на парамеции при экспозиции 24 ч (по критерию «концентрация – эффект»)

Table 1. Dose-dependent effect of nano-substances on parameciums at 24 h exposure («concentration-effect» criterion)

The substance under study	Biocidity in dilutions $1 \times n^{-1}$					
	n					
	2	3	4	5	6	7
Control	–	–	–	–	–	–
Eleutherococcus	–	–	–	–	–	–
Norfloxacin	+	+	±	±	±	±
Silver nanoparticles	+	+	+	+	+	±
Copper nanoparticles	+	+	±	±	–	–
Silicon dioxide nanoparticles	+	+	±	±	±	–

Note. Dash - no biocidal activity (NB): "±" - biocidal to 50% (less than BC50); "+" - biocidal to 100% (about BC100).

Табл. 2. Влияние нановеществ на резистентность парамеций (по критерию «концентрация – сопротивляемость токсической нагрузке»)

Table 2. Effect of nano-substances on the resistance of parameciums (according to the "concentration-resistance to toxic load" criterion)

The substance under study	Biological activity index in dilutions $1 \times n^{-1}$					
	n					
	2	3	4	5	6	7
Control	1,000	1,000	1,000	1,000	1,000	1,000
Eleuthero-coccus	1,480	1,560	1,918	1,334	1,016	1,002
Norfloxacin	–	–	0,698	0,854	0,957	1,000
Silver nanoparticles	–	–	–	–	–	0,499
Copper nanoparticles	–	–	0,589	0,655	0,789	0,856
Silicon diox-ide nanopar-ticles	–	–	0,455	0,578	0,675	0,745

Note. Dash - biocidal effect.

the state of activity of infusoria. The level of biocidal activity of silver nanoparticles was significantly higher compared to bioelements (copper and silicon dioxide) nanoparticles. The dilutions of silver nanoparticles solution 1: 2-1: 6 unambiguously demonstrated high biocidal effect against infusoria, only in the last dilution (1: 7) the biocidity fell below the BC50 value.

Solutions of copper nanoparticles caused complete death of infusoria only in small dilutions (1: 2-1: 3), with increasing dilution the bioactivity decreased sharply. Silicon dioxide nanoparticles had comparable biocidal activity, as paramecium mortality was observed in similarly high concentrations of nanoparticles (dilutions 1: 2-1: 3). The known biocide norfloxacin had comparable cytotoxicity. The solutions of bioinert nanoparticles at the highest dilution (1 × 7-1, or 1: 7) had no anti-protist effect, while silver nanoparticles at the highest dilution still failed to achieve bioinertness (mortality of infusoria at less than 50% of the population was observed).

At the second stage, the biological activity index of the tested nanoparticle samples was determined by the toxic load method (after adding a hypertonic sodium chloride solution, which has a membrane-damaging effect), in which the valeonegative (reducing the survival and viability of bioorganized systems) effect was evaluated. The results of these studies are shown in Table 2.

The obtained data indicate that of all substances used in the experiments only eleutherococcus has a valeopositive effect as it increases the resistance of paramecium cells to the toxic effects of superhypertensive medium (8% sodium chloride solution): the index of biological activity of the preparation was higher than 1.000 (from 1.002 to 1.918), and the greatest biostimulating effect on paramecia was demonstrated in the dilution of 1 : 4. In the experiment, the BAI index above unity indicates an increase in the life span of paramecium cells under the influence of the preparation and is expressed in the values of more than 100% protective activity. This result is quite expected, since eleutherococcus is a well-known adaptogen. The concept of an adaptogen, first proposed by Soviet scientists in the late 1950s, states that an adaptogen is any substance that affects a biological object, correcting any of its dysfunction and causing no undesirable side effects. The adaptogen-containing plant *Eleu-*

therococcus senticosus, also called "Siberian ginseng", contains a large number of chemical substances that have a protective and/or inhibitory effect against free radicals, and the list of such substances contained in Eleutherococcus is not fully defined [12].

The other tested components, including the known biocidal agent norfloxacin together with colloidal solutions of nanoparticles, had a strong biodegrading effect (bioactivity index value below 1.000). The BAI values indicate that nanoparticles have a more pronounced negative effect on the resistance of parameciums, since the coefficient of increase of endurance of infusoria after their exposure was slightly lower than that of norfloxacin (see Table 2). Objective comparison of the toxicity of the antibiotic (norfloxacin) and nanoparticles in this experiment is not quite correct, since these drugs are of completely different classes and have different expression rates for their concentrations. We were able to assess the effect of silver nanoparticles on the endurance of paramecium only in the maximum dilution of their solution (1: 7), because its lower values had an antiprotist effect. When comparing the BAI values of nanoparticles, a higher cytotoxicity of noble metal nanoparticles (index 0.499) compared to bioelements (copper and silicon) nanoparticles (0.856 and 0.745, respectively) was confirmed.

At the last stage of the integrated biotesting of nano-substances, their evaluation was carried out according to the index of paramecium reproduction rate. For this purpose, its values at addition of sublethal concentrations of tested substances are calculated. For all of them, a dilution of 1: 5 was taken, since it exceeded the lethal concentration (1: 3) by two points in all cases (except for silver nanoparticles), except for the adaptogen eleutherococcus which actually had no such concentration. The results of determining the biological activity of the colloidal nanoparticles under study at the optimal sublethal concentration by the intensity of paramecium reproduction are given in Table 3.

Eleutherococcus solution at a concentration of 1: 5 increases the intensity of paramecium division almost 1.6-fold (infusoria reproduction rate index 1.589), which is quite understandable from the expected adaptogenic effect of this component (see Table 3). The studied biocides (norfloxacin and colloidal solutions of nanoparticles) in concentrations that did not cause complete death of the infusoria inhibited their reproduction intensity by approximately the same amount - 55-61% (paramecium reproduction intensity index from 0.455 to 0.390, respectively). All tested nanoparticles in sublethal concentrations reduced the propagation intensity of protists by at least a half. However, an objective comparison in this case is possible only for bioelements, because only they were biotested in the same dilution (1: 5), while silver nanoparticles are initially more cytotoxic and therefore were taken in the experiment in a higher dilution (1: 7).

CONCLUSIONS

1. Biotesting of nanoparticles of metals and bioelements is reliably and qualitatively carried out on a free-living infusoria *Paramecium caudatum*, as it is an inexpensive convenient model for evaluation of biocidal action.

2. None of the solutions of noble metal nanoparticles and bioelements used in the ex-

Табл. 3. Влияние изучаемых препаратов в сублетальной концентрации на размножение инфузорий

Table 3. Effect of the studied preparations at sublethal concentration on the reproduction of infusoria

The substance under study	Optimal concentration (dilution $1 \times n^{-1}$)	Infusoria rate of propagation index
Control	–	1,000
Eleutherococcus	5	1,589
Norfloxacin	5	0,450
Silver nanoparticles	7	0,390
Copper nanoparticles	5	0,462
Silicon dioxide nanoparticles	5	0,385

periment can be recognized as bioinert or biostimulating in relation to the eukaryotic test object *Paramecium caudatum*.

3. Colloidal solutions of silver, copper and silicon dioxide nanoparticles have a biocidal effect, each of which exhibits a dose-dependent effect, with the former characterized by a much more pronounced toxic activity in a single-celled protist biological model.

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