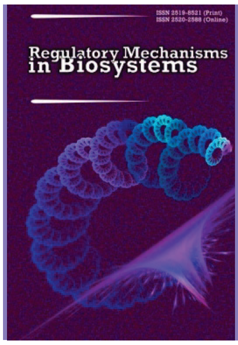

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Toxicological properties of mixtures of binary silver-copper, silver-zinc, and copper nanoparticles on cell culture model and laboratory animals

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The development, testing, and implementation of antimicrobial agents necessitates the determination of their toxicological characteristics. The objective of our research was to ascertain the direction and magnitude of the impact of experimental samples of disinfectants based on binary silver-copper, silver-zinc, and copper nanoparticles on biological entities of disparate levels of organization (cell culture, laboratory animals). The culture of calf coronary vessel cells (CVCs), 220 mature white laboratory rats, and 45 Chinchilla rabbits were used for the study. Mixtures of binary nanoparticles D1: MeNPs content – 5.4 mmol/L; D2: MeNPs content – 4.9 mmol/L were used as antimicrobial compounds. Toxic effects on cell culture were determined by the percentage of monolayer integrity, and biological effects in animals were determined by determining acute and subacute toxicity by clinical and biochemical parameters. The results of the culture studies demonstrated that the CC_{50} value of D2 was 6.2 times lower than that of D1, indicating a higher degree of cytotoxicity. No animal deaths were observed in the acute toxicity test (single intragastric administration to white rats at a dose of 30000 mg/kg body weight), which permitted the classification of the test samples as Class VI toxicity (relatively harmless) and Class IV hazardous (low-hazardous). When applied topically to rabbits' skin and mucous membranes, the experimental samples did not exhibit a pronounced irritant effect. A 30-day dermal application of the drugs to rats at doses of 0.5 and 5.0 mL/kg was conducted to determine the impact on hematological and biochemical parameters of a tenfold dose. Following the cessation of the administration of the aforementioned experimental disinfectant samples, the levels of all indicators were observed to return to the control levels within 14 days. The higher toxicity of the D2 drug for biological systems of different levels of organization, compared to the D1 drug, may be attributed to two factors: the higher concentration of AgNPs (2.4 times) and the potentiation of the toxic effect of two binary compounds (Ag-Zn and Ag-Cu) in its composition. Further research is needed to determine the biological impact of experimental samples of disinfectants based on silver, copper, and zinc metal nanoparticles on other functional systems of laboratory animals and the clinical and biochemical status of productive agricultural animals in production conditions.

Keywords: metal nanoparticles; AgNPs; CuNPs; ZnNPs; disinfectant; toxicity; laboratory animals; cell culture; blood.

Introduction

Despite the success achieved in ensuring the epizootic well-being of the Ukrainian livestock sector, sporadic cases of a number of economically significant and dangerous diseases of animals still occur today (Zubach et al., 2019; Rudoi et al., 2023). The most effective and cost-efficient method for the prevention and eradication of infectious diseases is the prompt and thorough disinfection of veterinary control facilities (Neat et al., 2021; Scollo et al., 2023). To this end, practitioners employ commercially available disinfectants, which exhibit differences in composition, properties, and spectrum of antimicrobial action (Wales et al., 2021; Houston et al., 2024; Paliy et al., 2024). Concurrently, it has been documented that the prolonged and uncontrolled utilization of disinfectants has resulted in the development of heightened resistance of microorganisms to their effects (Abdelaziz et al., 2019; Rozman et al., 2021). This, in turn, requires the improvement of existing formulations and the development of new ones for use in modern conditions (Alajlan et al., 2022; Eggers et al., 2023; Rutala et al., 2023).

Under contemporary standards, the development and subsequent introduction of an innovative antimicrobial agent necessitates a comprehensive and thorough preliminary evaluation (Tyski et al., 2022; Martin et al., 2023). In addition to exhibiting a broad spectrum of bactericidal activity, satisfactory physical and chemical characteristics, and environmental safety, an effective disinfectant should demonstrate minimal toxicity to macroorganisms (Christenson et al., 2021; Liu et al., 2022a). In this regard, the assessment of the toxicity of the developed antimicrobial compositions and their by-products represents a key stage in the design and testing of modern disinfectants (Pandian et al., 2022).

An analysis of scientific sources on the results of studies of antimicrobial activity and toxicity shows that metal nanoparticles (MeNPs) are more promising antibacterial agents in new disinfectants (Beyth et al., 2015; Anand et al., 2022; Skłodowski et al., 2023). It has been established that bacteria have a limited ability to develop resistance to nanomaterials due to various mechanisms of their antibacterial activity, which include the formation of reactive oxygen species, the release of metal ions, damage to bacterial membranes and cell walls, intracellular macromolecules such as proteins and DNA (Niño-Martínez et al., 2019). A variety of metal nanoparticles are used to inactivate infectious agents at various facilities (Lin et al., 2021; Franco et al., 2022). In particular, much attention is currently paid to AgNPs due to their excellent optical, chemical, electrical, and catalytic properties; these crystals are used in various sectors of the economy, including healthcare (Deshmukh et al., 2019; Xu et al., 2020). It is known that copper and silver nanoparticles are widely used in many fields, including catalysis, medicine, electronics, etc. Silver and copper are known for their antibacterial properties against both gram-positive and gram-negative bacteria (Bruna et al., 2021; Ma et al., 2022). Binary nanoparticles, in which atoms of one metal are homogeneously distributed among atoms of another metal, have properties that differ from those of individual components, and a synergistic effect can often be observed. It has been proven that binary Cu/Ag nanoparticles have better antibacterial properties against *Bacillus subtilis* and *Escherichia coli* than individual copper nanoparticles, individual silver nanoparticles, or a mixture of copper and silver nanoparticles (Fan et al., 2021; Vasiliev et al., 2023).

In most cases, the toxicity of metal nanoparticles to prokaryotes is attributed to the released metal ions, and the antimicrobial activity depends on their physicochemical properties, such as surface, size, and charge. The size of the particles is a significant factor as it determines whether they penetrate microbial cells and biofilms, thus increasing their toxicity (Amaro et al., 2021). Given the mechanisms of antimicrobial activity typical of nanoparticles (induction of oxidative stress, the release of metal ions, DNA damage, ATP depletion, non-oxidative pathways such as changes at the transcriptional and proteomic levels), bacteria are less likely to acquire resistance to such agents compared to conventional antimicrobials (Slavin et al., 2017; Lee & Jun, 2019), so the use of nano preparations is one of the strategies to combat antibiotic resistance in microorganisms.

However, it has been shown that nanoparticles of some metals are also toxic to eukaryotic cells (Vimbela et al., 2017). Given this fact, it is crucial to develop nanoparticles that are selectively toxic to prokaryotic cells while maintaining a dose-response balance between efficacy and toxicity. Therefore, the determination of the degree of toxicity of new nanodisinfectants is a necessary step in the development of effective and environmentally safe antimicrobial agents, which in turn opens up new perspectives in the fundamental understanding of their impact on the state and functional activity of cells.

The study aimed to establish the toxicological characteristics of disinfectants containing AgNPs, CuNPs, and ZnNPs by determining the peculiarities of their effect on biological objects of different levels of organization (cell cultures, laboratory animals).

Materials and methods

The research employed metal nanoparticles (AgNPs, CuNPs, and ZnNPs) prepared at the Research and Technology Complex "Institute of Single Crystals" of the National Academy of Sciences of Ukraine. These nanoparticles were obtained through a joint sequential reduction of the salts of the aforementioned metals in an aqueous medium with sodium borohydride and ascorbic acid. This method allows the production of stable suspensions with a nanoparticle size of 30–40 nm (Sofronov et al., 2013).

Toxicological characteristics *in vitro* and *in vivo* were determined in experimental drugs:

D1: binary Ag nanoparticles (0.7 mmol/L) – Zn^{2+} (2.2 mmol/L) and Cu nanoparticles (2.5 mmol/L), stabilizers: Na citrate (15 mmol/L), cetyltrimethylammonium bromide (CTAB) (1.0%), ascorbic acid (0.2 mmol/L). The total content of MeNPs is 5.4 mmol/L.

D2: binary nanoparticles Ag (0.7 mmol/L) – Zn^{2+} (2.2 mmol/L) and Ag (1.0 mmol/L) – Cu (1.0 mmol/L), stabilizers: Na citrate (15 mmol/L), sodium dodecyl sulfate (SDS) (0.85%), oleic acid (0.4%), ascorbic acid (0.085 mol/L). The total content of MeNPs is 4.9 mmol/L.

Experimental studies to determine the toxicity of the nanoparticle complex on biological systems of different levels of organization (cell cultures and laboratory animals) were conducted at the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine".

The cytotoxic effect of nanometal-based drugs was determined *in vitro* by applying them in different concentrations to a monolayer of a continuous culture of calf coronary vessel cells (CVCs). This cell culture is sensitive to bovine coronavirus, which will be used in the future to determine the antiviral efficacy of the drugs. The inoculation concentration of the cells of the continuous line was 3×10^5 cells/cm^3. DMEM medium (BioWest, France) with 10% fetal bovine serum (FBS, BioWest, France) and antibiotics (penicillin 100 units/cm^3 and streptomycin 100 μg/cm^3) were used as a growth medium.

The cytotoxic effect of the experimental drugs D1 and D2 was determined in the following dilutions: native, 1:2, 1:4, 1:10, 1:20, 1:30, 1:40, 1:50 and 1:100 by applying the indicated dilutions to the formed monolayer of cells. For this purpose, the growth medium was removed from the formed monolayer and replaced with a maintenance medium containing drugs based on metal nanoparticles. For each dilution of the drug, 5 samples of the continuous culture of CVCs were used. As a control, a cell culture (5 samples) with a formed monolayer on the maintenance medium that did not contain the test drugs was used. The cytotoxic effect of different concentrations of the test drugs was evaluated after 24, 48, and 72 hours using an inverted microscope, taking into account changes in cell morphology and disruption of the monolayer integrity. The integrity of the cell membrane was determined using a 0.2% trypan blue solution (in the absence of cell staining) (Crowley et al., 2016; Asuzu et al., 2022). To assess cytotoxicity by probit analysis, a regression curve was built using SPSS, and the cytotoxic concentration of the drug was determined, which led to the death of 50% of monolayer cells (Liu et al., 2022b). The magnification (bars) of the presented objects (Figs. 1 and 2) is 100 μm.

In vivo experiments were performed on white rats and rabbits. The acute and subacute toxicity of the experimental samples of mixtures of metal nanoparticles was determined for internal and external administration. The experimental groups of laboratory animals were formed on the principle of analogs, taking into account the weight and sex of the animals.

All experimental studies were conducted following modern methodological approaches and in compliance with generally accepted standards, in particular, they met the requirements of DSTU ISO/IEC 17025:2005 (2006). Animals were kept and all manipulations were carried out under the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Simmonds, 2017). Laboratory animals (white rats, rabbits) were kept under optimal vivarium conditions: the room temperature was 20 ± 2 °C, relative humidity was 55–65%, the day-night lighting cycle was 14–10 hours during the experiment, and the air volume in the vivarium room was changed 10 times per hour. The density of cages for rats was 160 cm^2/animal and for rabbits – 2000 cm^2/animal. For feeding the animals, we used complete animal feed for each rodent species. The animals had free access to water and food. The rats were euthanized after deep inhalation anesthesia with chloroform.

The study of the parameters of acute toxicity of metal nanoparticles under intragastric administration to white rats was carried out on 100 female nonlinear white rats of 3–4 months of age and weighing 220–250 g. Right before the introduction of the experimental samples of metal nanoparticles, each animal was weighed, and the doses administered were calculated individually, according to the weight of the rat. The volume of the drug administered intragastrically at one time did not exceed 2.5 cm^3. In the case of two- and three-dose administration, the time between drug administration was 60 minutes. The study was conducted in five stages, gradually increasing the dose of nanocomposite mixtures: 5000, 10000, 15000, 20000, and 30000 mg/kg body weight. Each subsequent stage was started 7 days after the previous one. The clinical condition of the experimental animals was monitored for 14 days. During the clinical examination of rats, attention was paid to behavior, reaction to external stimuli, appetite, skin condition, color of mucous membranes, respiratory and defecation rates, changes in color and consistency of feces, etc. On the 14th day after the start of the experiment, the animals of the experimental and control groups (n = 15) were euthanized and their pathological necropsy was performed. The macroscopic method of research was used to determine pathological changes.

The irritating effect of metal nanoparticles on the skin and mucous membrane of the rabbit eye was determined on 45 rabbits of the Chinchilla breed aged 3.0–4.0 months and weighing 3.1–3.4 kg. To study the irritant effect of the drugs, one control (n = 15) and two experimental groups were formed, 15 rabbits in each. The day before the experiment, the hair was removed from the intended application site by carefully clipping it with scissors. In addition, the animals were wearing protective collars to prevent licking of the drug. The drug application was performed in the morning before feeding. The drugs were applied evenly to a 6×6 cm area of rabbit skin. Each experimental sample of metal nanoparticles was applied to the skin of rabbits in two doses (by absolute weight) – 3000 and 6000 mg/kg of body weight. The observation of experimental animals was conducted over 14 days. We assessed the general condition of the animals, the nature of the skin lesions at the application site, and the time of death or recovery of the animals. To ascertain the irritant effect on the mucous membrane of the eye of rabbits of experimental samples D1 and

D2, two experimental groups (n = 15) were constituted. A quantity of 0.1 g of nanocomposites was introduced into the conjunctival sac of the left eye of the animals. After treatment, the eyes were examined thoroughly in one, 24, 48, 72, 96 hours and up to 14 days. The irritant effect on the ocular mucosa of the experimental samples was determined by the presence (absence) of conjunctival hyperemia, blood vessel injection, the condition of the sclera, cornea, and eyelids and was evaluated using a 3-4 points system.

The study of the toxicity of metal nanoparticles in white rats (subacute toxicity at dermal application) was carried out on 120 sexually mature nonlinear male rats weighing 255–270 g. For the experiment, one control and four experimental groups of 24 rats were formed. Solutions of metal nanoparticles D1 and D2 were applied at doses of 0.5 and 5.0 mL/kg body weight to the skin for 30 days, then the animals were observed for another 14 days. In the dynamics of the experiment (daily), integral indicators (animal behavior, appearance, reactions to external stimuli, water and feed consumption), as well as indicators characterizing the functions of organs and systems were studied in rats. Blood samples for hematological and biochemical studies were collected from 8 animals from each group on the 15th, 30th and 45th day of the experiment. The following were determined in the stabilized blood; the number of red blood cells, leukocytes and total hemoglobin content. The activity of indicator enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was determined in the blood serum using kits from Reagent PJSC on a Shimadzu UV-1600 spectrophotometer, gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, lactate dehydrogenase (LDH), total lipids, triglycerides, cholesterol, calcium, phosphorus and magnesium were determined by conventional methods.

The results are presented as the mean ± standard deviation (x ± SD). Analysis of variance (ANOVA) was used to compare the difference in the means between experimental and control groups. The significance of differences between individual groups was determined by a posteriori Tukey test. The $P < 0.05$ was considered a significant value.

Results

According to the results of determining the cytotoxicity of the combined drug D1, it was found that in native dilution and dilution 1:2 it led to a violation of the morphology of 65.2 ± 12.1% and 60.0 ± 4.6% of monolayer cells, respectively (Table 1), with their subsequent detachment from the glass. The use of this combination of the drug in dilutions of 1:4 and 1:10 was characterized by slightly lower cytotoxicity, but still led to destructive changes in the monolayer cells at the level of 20.4 ± 8.4% and 5.4 ± 2.6%, respectively. The use of D1 in dilutions of 1:20 – 1:100 had no negative effect on cell morphology and membrane integrity, which was characterized by the viability and preservation of the monolayer of all CVC cells (Fig. 1). In control cell cultures in a maintenance medium, there were no signs of cytotoxicity, viability, and cell survival were 100%.

Table 1
Cytotoxicity of combined drugs of metal nanoparticles for cells of the continuous CVC line (%, n = 5, x ± SD)

Sample	Native drug	Dilution							
		1:2	1:4	1:10	1:20	1:30	1:40	1:50	1:100
D1	65.2 ± 12.1*	60.0 ± 4.6*	20.4 ± 8.4*	5.4 ± 2.6*	0	0	0	0	0
D2	100*	100*	100*	100*	20.2 ± 7.4*	10.4 ± 4.3*	0	0	0

Note: * – $P < 0.05$; changes are statistically significant compared to the indicators of control cell cultures.

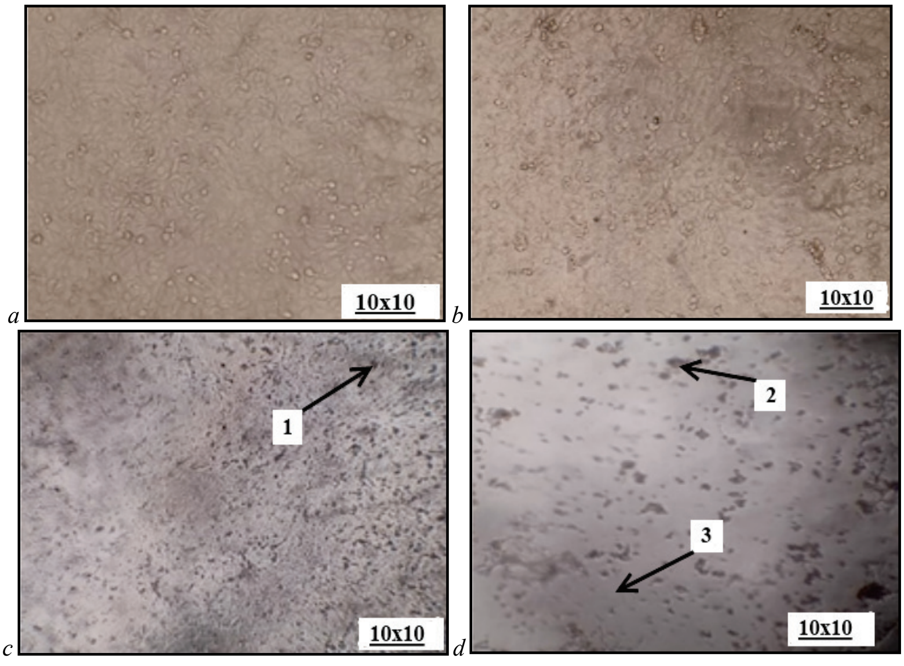

Fig. 1. Morphology of CVC cells under the impact of D1 drug: *a* – control (without monolayer destruction); *b* – maximum permissible dilution 1:20 (without monolayer destruction); *c* – dilution 1:4 (monolayer destruction of 25%); *d* – native drug (monolayer destruction of 100%); cell morphology disorders and monolayer destruction: *1* – rounding of cells; *2* – conglomeration of cells; *3* – detachment of cells from the monolayer

According to the results of determining the cytotoxic effect of the complex drug D2 on the cells of the continuous culture of CVCs, it was found (Table 1) that it had a slightly higher toxicity compared to the drug D1, which was reflected in the morphology of cell cultures (Fig. 2). It was found that the introduction of D2 in its native form and dilutions of 1:2–1:10 led to a change in the morphology of 100% of cells and, accordingly, to a violation of the integrity of the monolayer of the continuous culture of CVCs. The use of the drug at a dilution of 1:20 and 1:30 on the monolayer of CVC cells was characterized by a change in morphology and membrane integrity of 20.0 ± 9.35% and 10.0 ± 6.12% of cells, respectively, which led to detachment of the monolayer from the surface. On the contrary, the use of the test drug at a dilution of 1:40–1:100 had no negative effect on the cell morphology and integrity of the monolayer of the continuous culture of CVCs (Fig. 2). Using probit analysis, we performed a regression analysis and obtained regression curves (Fig. 3), which allowed us to determine the CC_{50} for each drug under study. Thus, for nanocomplex D1 it was 0.231 ± 0.026 mg/mL, and for D2 – 0.037 ± 0.053 mg/mL.

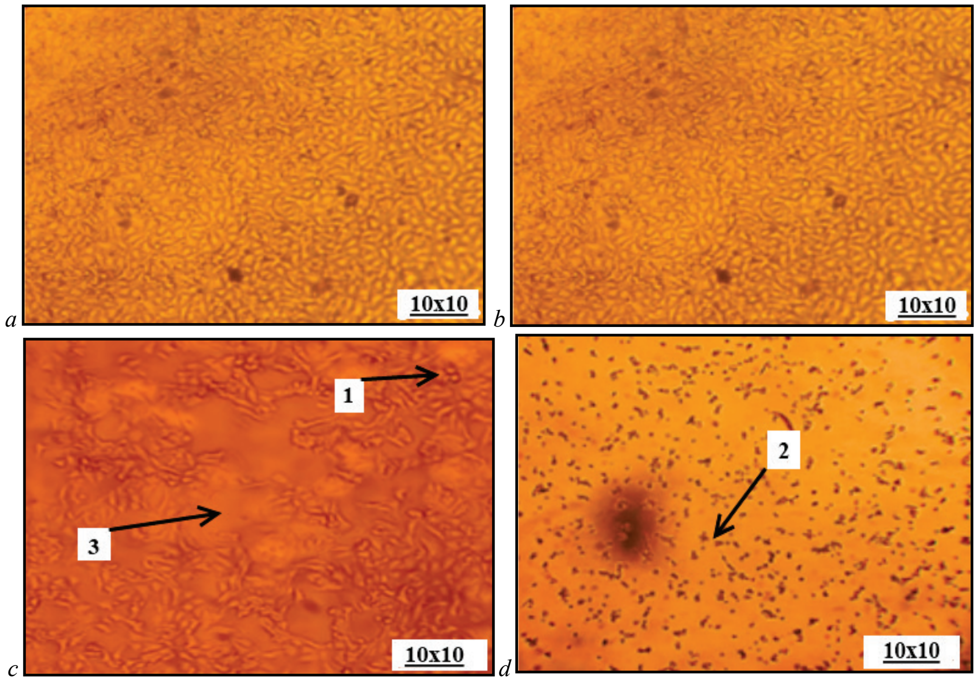

Fig. 2. Morphology of CVC cells under the influence of the combined drug D2: *a* – control (no monolayer destruction); *b* – maximum permissible dilution 1:40 (no monolayer destruction); *c* – dilution 1:20 (monolayer destruction of 25%); *d* – minimum dilution 1:10 (monolayer destruction of 100%); cell morphology disorders and monolayer destruction: *1* – rounding of cells; *2* – conglomeration of cells; *3* – detachment of cells from the monolayer

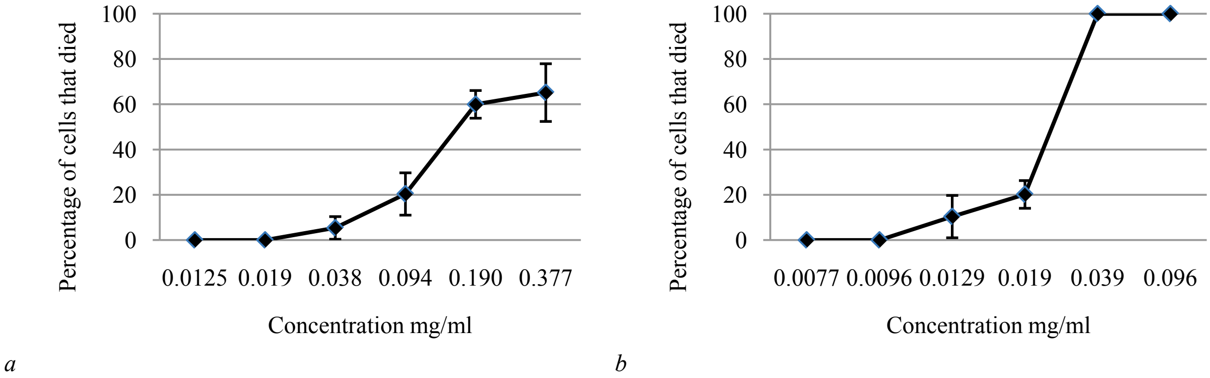

Fig. 3. Curves dose–cytotoxicity of CVC cells under the impact of experimental disinfectant samples: *a* – drug D1; *b* – drug D2

During the *in vivo* study of the acute toxicity parameters of experimental samples of metal nanoparticle compositions D1 and D2 at a single intragastric administration to white rats, no animal death was observed at any stage of the experiment, but clinical signs of their toxic effect were recorded when administered intragastrically at a dose of 30,000 mg/kg of body weight, which corresponds to a dose of metal nanoparticles of 11.31 and 11.58 mg/kg, respectively: at autopsy, 25% of animals in group D1 showed foci of catarrhal inflammation of the mucous membrane in the small intestine, and 30% of rats in group D2 showed catarrhal inflammation and foci of hemorrhagic inflammation of the small intestinal mucosa (Fig. 4). Since no rat deaths were recorded in the experiment, it is not possible to calculate the LD_{50} for experimental samples of metal nanoparticles under conditions of intragastric administration to laboratory rats, but this figure will obviously be higher than 30,000.0 mg/kg body weight.

In determining the irritant effect of experimental samples of metal nanoparticle compositions on the skin of rabbits, it was found that there were no manifestations of their irritant effect at a dose of 6,000.0 mg/kg of animal body weight. No skin edema, crusting, or cracking of the skin was detected, indicating that there were no signs of dermatitis or irritant effects on the skin. It should also be noted that none of the experimental animals died during the experiment.

After applying the experimental samples of the test preparations to the mucous membrane of the rabbit eye (Fig. 5), during the first or second day after application, in rabbits of group D1, slight hyperemia of the ocular mucosa (1 point) and discharge (1 point) were observed (Fig. 5b), which disappeared on the second day of the experiment. Animals of group D2 developed more pronounced hyperemia of the ocular mucosa (2 points), eyelid edema (1 point), and discharge (3 points) (Fig. 5c). These changes completely disappeared on the fourth day after application.

In the study of the toxicity of experimental samples of compositions based on metal nanoparticles in white rats (subacute toxicity when applied dermally), it was found that their use at a dose of 0.5 and 5.0 mL/kg body weight did not cause significant changes in the behavior and appearance of experimental animals compared to the control. During the entire observation period (45 days), the animals were active, had a satisfactory appetite, responded well to sound and light stimuli, and maintained reflex excitability; no respiratory, urinary, or defecation disorders were observed.

In the determination of hematological parameters in the blood of rats (Table 2), on the 15th day of the experiment in rats of experimental groups D1 (5.0 mL/kg) and D2 (5.0 mL/kg), an increase in hemoglobin level by 10.6% ($P \leq 0.05$) and 13.5% was found, on the 30th day the difference was 8.6% and 12.9%, respectively, compared to the control group. Also, an increase ($P \leq 0.05$) in the number of red blood cells by 12.0% and 18.9% was recorded in D1 (5.0 mL/kg) and D2 (5.0 mL/kg) rats on day 15 of the experiment, and on day 30 the difference ($P \leq 0.05$) was 15.2% and 24.0%, respectively. An increase in the number of leukocytes was found: on day 15 – by 19.8% ($P \leq 0.05$) and 10.4%, respectively, on day 30 – an increase of 19.8% ($P \leq 0.05$) in rats of group D1 (5.0 mL/kg). In 15 days after the cessation of drug administration, the level of indicators of the experimental groups was close to the control values.

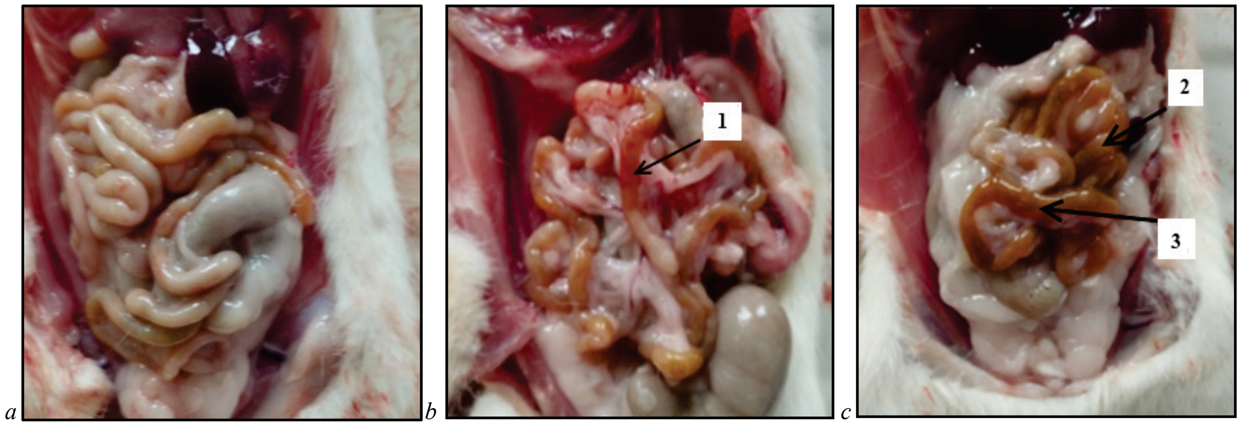

Fig. 4. The small intestine of female rats in the experiment to study the acute toxicity of experimental samples of metal nanoparticles: *a* – control group; *b* – experimental group D1; *c* – experimental group D2; *1* – focus of catarrhal inflammation of the small intestine; *2* – catarrhal inflammation of the small intestine; *3* – focus of hemorrhagic inflammation of the small intestine

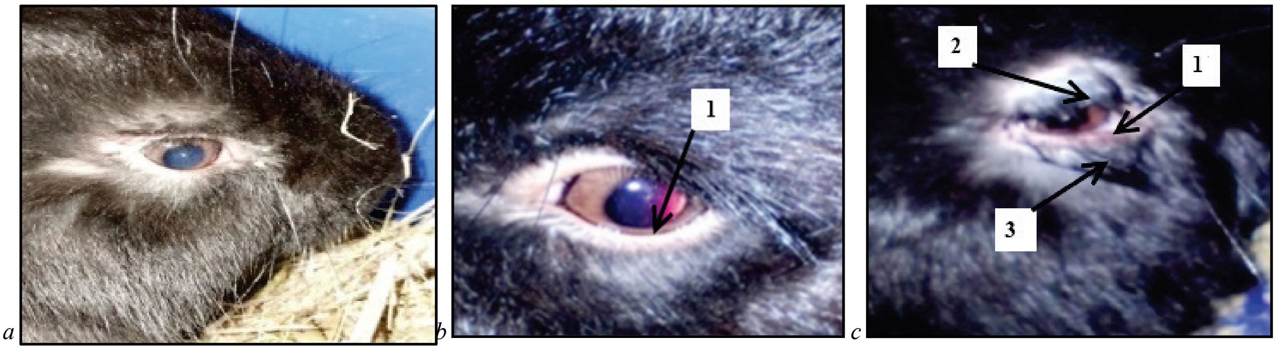

Fig. 5. Signs of irritating effect of experimental disinfectant samples: *a* – control group; *b* – experimental group D1; *c* – experimental group D2; *1* – hyperemia of the ocular mucosa; *2* – swelling of the upper eyelid; *3* – moistening of the coat from eye discharge

Table 2
Clinical blood parameters in rats after administration of experimental disinfectant samples (x ± SD, n = 8)

Experimental groups		Indicator	Study period, day		
Drug	Dose, mL/kg		15	30	45
D1	0.5	Total hemoglobin (HGB), g/dm^3	124.56 ± 2.12^{a}	127.13 ± 2.06^{a}	123.73 ± 1.89^{a}
	5.0		139.22 ± 2.34^{c}	136.31 ± 5.40^{b}	126.37 ± 2.46^{bc}
D2	0.5		128.13 ± 2.05^{b}	126.69 ± 2.65^{a}	125.37 ± 3.61^{abc}
	5.0		142.81 ± 2.09^{d}	141.75 ± 2.11^{c}	126.56 ± 2.67^{bc}
Control			125.82 ± 1.29^{a}	125.53 ± 4.03^{a}	124.46 ± 2.23^{abc}
D1	0.5	Erythrocytes (RBC), 10^{12}/dm^3	6.68 ± 0.16^{b}	6.54 ± 0.14^{b}	6.47 ± 0.17^{ab}
	5.0		7.29 ± 0.11^{c}	7.28 ± 0.12^{c}	6.66 ± 0.16^{cd}
D2	0.5		6.52 ± 0.14^{ab}	6.48 ± 0.05^{ab}	6.63 ± 0.12^{bcd}
	5.0		7.74 ± 0.16^{d}	7.84 ± 0.12^{d}	6.71 ± 0.04^{bcd}
Control			6.51 ± 0.15^{ab}	6.32 ± 0.11^{a}	6.56 ± 0.19^{acd}
D1	0.5	Leukocytes (WBC), 10^9/dm^3	10.35 ± 0.14^{b}	11.21 ± 0.13^{c}	10.70 ± 0.31^{a}
	5.0		11.85 ± 0.56^{d}	13.12 ± 0.21^{d}	10.74 ± 0.12^{a}
D2	0.5		10.43 ± 0.24^{b}	10.65 ± 0.42^{ab}	10.86 ± 0.27^{a}
	5.0		10.92 ± 0.38^{c}	10.40 ± 0.55^{a}	10.89 ± 0.19^{a}
Control			9.89 ± 0.23^{a}	10.95 ± 0.37^{bc}	10.71 ± 0.14^{a}

Notes: means in each column followed by different letters are significantly different from one another on the results of comparison using the Tukey test ($P < 0.05$).

The determination of the activity of serum indicator enzymes (Table 3) revealed a decrease in ALT activity by 18.4% ($P \leq 0.05$) in rats of group D2 (5.0 mL/kg) on day 30 of the experiment. During this period of the study, AST activity tended to increase by 12.3%. On the 15th day of the experiment, the activity of GGT decreased by 35.0% ($P \leq 0.05$) in D1 rats (5.0 mL/kg) compared to the control. Also, in rats of group D1 (5.0 mL/kg) on the 15th and 30th day of the experiment, a decrease ($P \leq 0.05$) in the activity of alkaline phosphatase by 56.2% and 46.8% was noted. On the 30th day of the experiment, a decrease in the activity of alkaline phosphatase by 19.8% ($P \leq 0.05$) was found. The activity of LDH in the blood serum of rats of group D1 (5.0 mL/kg) on the 15th and 30th day of the experiment was reduced ($P \leq 0.05$) by 13.4% and 17.0%, and in rats of group D2 (5.0 mL/kg) a decrease in activity by 12.5% ($P \leq 0.05$) was determined on the 30th day of the experiment.

According to the results of the study of the level of lipid metabolism in the blood serum of rats in the dynamics of the experiment on the 30th day, an increase in the level of total lipids by 11.5% ($P \leq 0.05$) and 7.3% in rats of groups D1 (5.0 mL/kg) and D2 (5.0 mL/kg), respectively, was noted. When determining the content of triglycerides, a decrease in this indicator by 33.3% ($P \leq 0.05$), 48.5% ($P \leq 0.05$) was found on day 15 of the experiment in rats of groups D1 (5.0 mL/kg) and D2 (5.0 mL/kg), respectively. On the 30th day of the experiment, an increase in triglyceride content by 15.1% was observed only in rats of group D1 (5.0 mL/kg). On the 30th day of the experiment, a tendency to decrease the cholesterol content was observed in rats of group D2 (5.0 mL/kg).

On the 30th day of the experiment, a tendency to increase in the level of calcium in rats of group D1 (5.0 mL/kg) and a reliable increase of 22.8% in rats of group D2 (5.0 mL/kg) was observed. At the same time, under the influence of the experimental sample D2 at a dose of 5.0 mL/kg, the level of inorganic phosphorus decreased by 23.5% ($P \leq 0.05$) on day 30, and magnesium level also tended to decrease.

It is important to note that 14 days after the cessation of the application of the studied experimental samples of disinfectants, the level of all the above indicators was restored to the control level.

Table 3
The activity of hepatospecific enzymes in the blood serum of rats after administration of experimental disinfectant samples (x ± SD, n = 8)

Experimental groups		Indicator	Study period, day		
Drug	Dose, ml/kg		15	30	45
D1	0.5	Alanine aminotransferase, µmol/h×cm^3	4.33 ± 0.02^{b}	4.42 ± 0.01^{c}	4.48 ± 0.05^{bc}
	5.0		4.35 ± 0.06^{bc}	4.23 ± 0.01^{b}	4.38 ± 0.07^{a}
D2	0.5		4.39 ± 0.02^{c}	4.22 ± 0.02^{b}	4.42 ± 0.09^{abc}
	5.0		4.37 ± 0.06^{bc}	4.06 ± 0.01^{a}	4.35 ± 0.05^{ab}
Control			4.25 ± 0.05^{a}	4.31 ± 0.02^{d}	4.51 ± 0.02^{c}
D1	0.5	Gamma-glutamyl transpeptidase, µmol/h×cm^3	2.28 ± 0.12^{bc}	2.30 ± 0.09^{b}	2.38 ± 0.06^{a}
	5.0		2.33 ± 0.21^{ac}	1.48 ± 0.01^{a}	2.36 ± 0.11^{a}
D2	0.5		2.18 ± 0.06^{abc}	2.33 ± 0.14^{b}	2.38 ± 0.07^{a}
	5.0		2.15 ± 0.11^{ac}	2.32 ± 0.12^{b}	2.35 ± 0.05^{a}
Control			2.22 ± 0.09^{ac}	2.28 ± 0.05^{b}	2.34 ± 0.04^{a}
D1	0.5	Alkaline phosphatase, U/L	95.46 ± 2.34^{b}	155.68 ± 2.39^{c}	162.38 ± 1.25^{a}
	5.0		42.48 ± 1.98^{a}	84.66 ± 4.98^{a}	161.54 ± 1.47^{a}
D2	0.5		100.02 ± 2.06^{c}	157.25 ± 1.43^{c}	162.24 ± 1.59^{a}
	5.0		104.62 ± 3.32^{d}	127.81 ± 4.97^{b}	161.89 ± 1.22^{a}
Control			97.11 ± 2.54^{b}	159.36 ± 0.97^{d}	166.85 ± 1.36^{b}
D1	0.5	Lactate dehydrogenase, mmol/h×cm^3	1.68 ± 0.06^{b}	1.73 ± 0.08^{c}	1.79 ± 0.02^{ab}
	5.0		1.48 ± 0.04^{a}	1.46 ± 0.04^{a}	1.82 ± 0.03^{b}
D2	0.5		1.76 ± 0.03^{c}	1.72 ± 0.04^{c}	1.82 ± 0.08^{ab}
	5.0		1.86 ± 0.06^{d}	1.54 ± 0.06^{b}	1.79 ± 0.07^{ab}
Control			1.71 ± 0.02^{b}	1.76 ± 0.06^{c}	1.81 ± 0.05^{ab}

Notes: means in each column followed by different letters are significantly different from one another on the results of comparison using the Tukey test ($P < 0.05$).

Discussion

The use of any disinfectant should be scientifically justified with a comprehensive assessment of potential risks for the environment (Paliy, 2018; Aranke et al., 2021; Ghafoor et al., 2021), particularly in the case of disinfectants based on MeNPs (Deshmukh et al., 2019). A review of the literature reveals that the interest in the biological action of metal nanoparticles, including silver, has persisted for the past two decades, particularly in the context of nanomedicine (Naeemi et al., 2020; Assar, 2022). The mechanisms of action of these nanoparticles exhibit general biological characteristics and are largely dependent on their size (Shi et al., 2018).

Furthermore, an examination of the available literature reveals a lack of data on the biological action of MeNPs mixtures, particularly those comprising silver and copper or silver and zinc in binary nanoparticles. Cell lines are an indispensable component of in vitro experiments, serving as models for a range of applications, from understanding fundamental cell functioning to drug discovery (Noufal et al., 2022; Arokia Femina et al., 2023). The results of our investigation into the cytotoxicity of experimental disinfectant samples with spherical nanoparticles of 30–40 nm in size on CVC cell culture demonstrated that the CC_{50} value of drug D2, which contained a higher concentration of AgNPs and two binary nanoparticles, was 6.2 times lower than that of drug D1. This indicates that drug D2 exhibits greater cytotoxicity.

According to Kumbıçak et al. (2014), Cu-ZnNPs with an average size of 200 nm significantly induced single- and double-stranded DNA damage and intracellular formation of peroxide radicals in BEAS-2B cells, and cell death occurred mainly due to necrosis. In experiments on the cytotoxicity of AgNPs of 15 and 10 nm size at a concentration of 5–50 µg/mL on BRL rat liver cells, Hussain et al. (2005) found a change in the functional state of mitochondria and the release of lactate dehydrogenase, in contrast to the effect of Fe_3O_4NPs (30 and 47 nm), AlNPs (30 and 103 nm), MgO_3NPs (30 and 150 nm) and TiO_2NPs (40 nm) at concentrations of 10–50 µg/mL, when no negative effect on the cellular state was recorded, indicating a high cytotoxicity of AgNPs. In addition, low doses of AgNPs induced the generation of reactive oxygen metabolites and a decrease in reduced glutathione, changes in cell size, and cell shrinkage. This led the authors to conclude that the cytotoxic effects of AgNPs in cell cultures are mediated by oxidative stress. In the study by Barbasz et al. (2021), as in our work, AgNPs were synthesized by chemical reduction using ascorbic acid, and their average size was 26 ± 6 nm. After exposure of human promyelocytic (HL-60) and histiocytic (U-937) lymphoma cells to AgNPs, changes in mitochondrial activity and secretion of inflammatory and apoptotic mediators (induction of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and caspase-9) were detected. Other researchers have stated that the hepatotoxic effect of AgNPs can be regulated by two mechanisms, involving the apoptotic/anti-apoptotic pathway through activation of the BAX gene and inhibition of Bcl-2 expression levels in a dose-dependent manner (Naeemi et al., 2020; Assar, 2022).

The spherical biosynthesized ZnNPs with sizes from 22.5 to 50.0 nm demonstrated cytotoxicity in lung cancer cell lines A549 and Calu-6 with inhibitory concentration (IC_{50}) values in the range of 2.25–12.4 µg/mL, 47 nm size against colon cancer cell lines HT-29 – IC_{50} 9.5 µg/mL, against epidermoid carcinoma cell lines A43 – IC_{50} 16.5 ± 1.6 µg/mL and against liver cancer cell lines Hep-G2 with an IC_{50} value of 14.1 ± 0.7 µg/mL (Andleeb et al., 2021). It was also found that ZnONPs are highly toxic to NIH/3T3 cell culture, causing loss of viability, membrane disruption, and morphological changes. It is believed that it is the released Zn from ZnOMeNPs that induces cytotoxicity. ZnO nanoparticles at a concentration of 20 µg/cm^2 also alter the state of redox processes in rat H9c2 cardiomyoblast cells, reduce intracellular troponin I expression, and cause cell death (Mendoza-Milla et al., 2022). Taken together, the results indicate that membrane damage and ROS production are also more strongly induced by copper nanoparticles and free or labile copper ions/complexes compared to copper bound to biomolecules (Hedberg et al., 2016).

Thus, the cytotoxic effects of metal nanoparticles, especially AgNPs, are due to the development of oxidative stress, damage to DNA or cellular proteins involved in cell signaling pathways, apoptosis, and autophagy by regulating the expression of key cell death proteins (Leon, 2017; Andleeb et al., 2021; Hu et al., 2024). However, an *in vitro* to *in vivo* eCVC mapping is needed to support the development of a next-generation risk assessment (NGRA) strategy for AgNPs (Jagiello & Ciura, 2022).

Extensive *in vivo* studies in laboratory animal models have shown fairly low rates of overall toxicity of MeNPs. For example, Majeed et al. (2020) and Tousson et al. (2022) found no death in determining acute toxicity after intragastric administration of different doses of AgNPs and ZnNPs for 14 days. The acute toxicity study of four Cu nanoparticles of various sizes (30, 50, 80, and 1000 nm) administered at a single equivalent dose (200 mg/kg) revealed that the LD_{50} values were 359.6, 1022, 1750, 2075, respectively, which was less than the regulatory limit of 5000 mg/kg (Tang et al., 2018). According to the results of our studies on the determination of acute toxicity in rats, experimental samples of disinfectants containing Ag, Cu, and Zn NPs at a dose of 30,000 mg/kg were also classified as toxicity class VI – relatively harmless substances (LD_{50} > 15000 mg/kg body weight), and in terms of hazard – class IV – low-hazardous substances (LD_{50} > 5000 mg/kg).

AgNPs have been found to have a low skin irritation potential, with eye irritation and some cases of allergic contact dermatitis reported, the most common being minor skin redness and redness or spots on the eyes. The toxicological safety of copper and zinc composite nanoparticles when applied to the skin has been proven (Majeed et al., 2020; Noga et al., 2023). Our studies also revealed the absence of acute dermal toxicity in rats and irritant effects on the skin of rabbits of experimental samples of disinfectants based on Ag, Cu, and Zn nanoparticles, but sample D2, which contained a higher concentration of AgNPs, caused a more pronounced and prolonged hyperemic reaction of the ocular mucosa. Summarizing the analyzed results, it can be concluded that MeNPs can be relatively safe when administered to the mouth, eyes, and skin of animal models for a short period (Hadrup et al., 2018).

At the same time, according to the literature, longer-term use of MeNPs can cause changes in the morphological and functional state of various organs and systems of laboratory animals. Thus, Yousef et al. (2022) found that AgNPs with a size of 7.8–28.4 nm when administered orally at doses of 1.0 and 2.0 mg/kg of live weight for 30 days caused an increase in the activity of some enzymes (AST, ALT), the level of lipid peroxidation metabolites (MDA) and cytokines (TNF-α and IL-6), which correlated with a decrease in the concentration of total protein, albumin, reduced glutathione and superoxide dismutase activity. Similar results were obtained with intraperitoneal administration of 12 nm AgNPs at a dose of 1.0 mg/kg body weight for 30 days: exposure to nanoparticles caused hematological changes – the development of microcytic hypochromic anemia and leukocytosis, and a shift in the leukoformula (Assar et al., 2022). The intravenous administration of 10 mg/kg and 20 mg/kg ZnONPs led to a decrease in the level of red blood cells, hemoglobin, and hematocrit, while the number of leukocytes and neutrophils was increased (Lee et al., 2016). According to the results of our studies, a pronounced effect on hematological parameters is exerted by the application of both variants of the drugs (D1 and D2) to the skin at a dose of 5 mL/kg – on the 15th day after the start of application, there was an increase in the level of hemoglobin, erythrocytes, a reliable increase in the number of leukocytes was caused by the use of drug D2.

According to Yousef et al. (2022), the introduction of low doses of AgNPs per os destroyed liver structure, caused a decrease in the number of normal and an increase in necrotic hepatocytes, and ultrastructural and molecular changes in the postsynaptic region of synapses were identified in the rat brain, where NMDA receptors are localized as a multiprotein complex, while significant changes in neurotransmitters and amino acids, decreased expression of several proteins, brain oxidative stress, and neuronal nitric oxide synthase (nNOS) were detected (Ahmed & Hussein, 2017; Dąbrowska-Bouta et al., 2021). Intragastric injection of CuNPs into rats caused a significant increase in biomarkers of increased oxidative and nitrosative stress in the liver with depletion of GSH levels in the liver, increased activity of hepatospecific enzymes in the blood serum (AST, ALT, and total bilirubin) and a significant decrease in total protein concentration. This was accompanied by a significant increase in lipoperoxidation and induced nitric oxide, copper content, and the level of apoptosis gene expression in the liver (Tang et al., 2018; Assar et al., 2022; Tousson et al., 2022). The intravenous administration of ZnONPs has been shown to reduce the activity of creatine phosphokinase and the level of phospholipids and inorganic phosphorus (Lee et al., 2016). ZnONPs also cause liver damage when administered orally, intraperitoneally, intravenously, and intratracheally, kidney damage (oral, intraperitoneal, and intravenous), and lung damage (intratracheal) (Fujihara & Nishimoto, 2024). It has also been shown that the toxic effect of chronic administration of a mixture of MeNPs at a dose of 4.0 mg/kg body weight in white rats is manifested by partial immunosuppression, severe hypoproteinemia, excessive formation of circulating immune complexes, and acute-phase mucoid proteins in the blood serum, and cytolytic damage to hepatocyte membranes. Their toxic effect is due to oxidative stress, which slows down lipoperoxidation along with an increase in the level of carboxylated proteins, depleting the body's antioxidant defenses (Romanko et al., 2023).

In our studies, we investigated the effect of MeNPs mixtures when applied by dermal application, as one of the possible ways of disinfectants entering the body of animals, on the state of hepatospecific enzymes, lipid and mineral metabolism in the blood serum. In the dynamics of the experiment, a significant decrease in the activity of such enzymes as GGT, ALT, and LDH was found when D2 was administered at a dose of 5.0 mL/kg body weight, which may indicate degenerative changes in the liver. The application of complex preparations with MeNPs to the skin caused shifts in lipid metabolism, which were dynamically manifested by fluctuations in the content of triglycerides, an increase in total lipids with the use of both drugs. The use of D2 at a dose of 5.0 mL/kg body weight led to a decrease in the content of magnesium and inorganic phosphorus and an increase in calcium levels. Possible reasons for the peculiarities of the effect of the experimental samples of disinfectants on biochemical parameters established by us are the route of administration (dermal) and the multidirectional impact of metal nanoparticles (Shehata et al., 2021), which are part of their composition, which requires further research.

It is important to emphasize that 14 days after the cessation of the application of the drugs, the level of all the above-mentioned hematological and biochemical parameters was restored to the control level. This may indicate the development of adaptive changes in the organism of laboratory animals when using the studied experimental samples of disinfectants based on a mixture of binary Cu-Ag nanoparticles. At the same time, the experimental sample of the drug D2 had a more pronounced toxic effect on the body of laboratory animals. The increased toxicity of preparation D2 for biological systems of different levels of organization, as compared to preparation D1, can be attributed to both the higher concentration of AgNPs (2.4 times) and the potentiation of the toxic effect of two binary compounds ZnNPs and CuNPs in its composition.

Analysis of the literature shows that the main general biological mechanisms of action on eukaryotic cells of both mono- and complex products based on metal nanoparticles are aimed at damaging DNA or cellular proteins involved in cell signaling pathways, generating reactive oxygen species (ROS), activating lipoperoxidation processes, inhibiting proteasemes, and cell death was mainly due to necrosis. There are reports that some disinfectants and their by-products also disrupt DNA integrity and cause cancer in humans (Evans et al., 2020). It is important that complex disinfectants are non-carcinogenic and environmentally safe and can be used for sanitation in direct contact with animals (Schmid et al., 2022). However, addressing the issue of carcinogenicity and embryo-oxidation of the developed innovative antimicrobial agents based on MeNPs still requires a more in-depth and reasoned approach (Chaves et al., 2019).

Conclusion

Experimental samples of disinfectants developed based on binary Ag-Zn^{2+} nanoparticles and Cu nanoparticles (total MeNPs content – 5.4 mmol/L), as well as binary Ag-Zn^{2+} nanoparticles and binary Ag-Cu nanoparticles (total MeNPs content – 4.9 mmol/L), diluted 1:4 and 1:10, exhibited cytotoxicity on CVC cell culture, and when diluted 1:40 – 1:100, there was no cytotoxicity. According to the results of the acute toxicity study in rats, the investigated drugs were classified as Class VI toxicity (relatively harmless substances – LD_{50} > 15,000 mg/kg body weight), and as Class IV hazardous substances (low hazardous substances – LD_{50} > 5,000 mg/kg). During a 30-day dermal application of the drugs to rats at doses of 0.5 and 5.0 mL/kg, the impact of a tenfold dose of the drugs on hematological and biochemical parameters was determined.

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