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ANTIVIRAL EFFECT OF CERIUM DIOXIDE NANOPARTICLES ON THE MODEL OF THE CAUSATIVE AGENT OF BOVINE VIRAL DIARRHEA

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Cerium dioxide nanoparticles (CeO_2) have a wide range of biological properties, including antiviral activity. Preparations based on cerium oxide nanoparticles can be effective against animal RNA viruses, which have the greatest epizootic significance for Ukraine and potentially against human viruses, including SARS-CoV-2. In our studies, we determined the effect of cerium dioxide nanoparticles on bovine viral diarrhea virus (BVDV), a pestivirus that is the etiological agent of bovine viral diarrhea. To determine the antiviral efficacy of CeO_2, a cytopathogenic strain of BVDV "BK-1" was used as an etiological agent of bovine viral diarrhea, which induces a cytopathic effect on cow embryo lungs (CEL) cell culture. When evaluating the antiviral efficacy of CeO_2, it was determined that the MPC for CEL cell culture is 0.057 μg/cm^3, and the CC_{50} is 0.077 μg/cm^3. The antiviral activity of CeO_2 was evaluated by the IC_{50} index, which was 0.036 μg/cm^3 and the selectivity index, which after co-administration of CeO_2 with BVDV was 2.14. There was a decrease in the titer of infectious activity of the virus during treatment with CeO_2 by 2.09 lg TCD_{50}/cm^3.

Keywords: Bovine viral diarrhea virus, nanoparticles, cerium dioxide, bovine fetal lung (BFL) cell culture, antiviral activity.

Given the problem of significant prevalence in the world of viral etiology diseases caused by pathogens that have significant homology to animal viruses, the fight against them requires new approaches. The most resonant in this aspect were the diseases of severe acute respiratory syndrome or atypical pneumonia (SARS-CoV, 2002–2003) [1], Middle Eastern coronavirus respiratory syndrome (MERS-CoV, 2012–2015) [2] and coronavirus COVID-19 infection (SARS-CoV-2, pandemic since 2019) [3].

That is why the availability of highly effective therapeutic agents with proven antiviral activity, which would stop the spread of viruses, their possible emergence, and the main reduction in mortality from the disease is a topical issue today [4–7].

In order to determine the effectiveness of a therapeutic agent, their investigations is carried out on biological models of *in vitro* cell cultures [8–11], which allows to obtain preliminary data for further clinical study of substances. Therapeutic drug should have a pronounced antiviral effect, moderate toxicity, if possible should affect the non-specific part of the immune system, and be available.

Given the complex epidemiological situation around the world regarding COVID-19 pandemic, working with viral pathogens that pose a threat to humans is associated with significant biological risks, so the use of animal viruses for studying the antiviral efficacy of various substances will minimize these problems.

Taking into account considerable relevance of the problem of viral pneumo-enteritis of cattle to the livestock of Ukraine and the EU [12, 13], introduction of new methodological approaches to combating and eliminating viral pathogens, which circulates in cattle herds, acquires special significance. One of the most etiologically meaningful pathogens, which causes cattle disease with respiratory syndrome, is a bovine viral diarrhea virus (BVDV) [14, 15]. In addition, the defined virus is the most common contamination of

biological preparations (cell cultures, fetal bovine serum, embryos, vaccines, etc.) [16, 17]. The greatest importance in supporting the endemicity of the cattle regarding BVDV has persistently infected calves born by cows infected with non-cytopathogenic biotype in the early timing of pregnancy [18, 19].

In addition to the immunosuppressive state of infected animals, which leads to secondary infectious and non-infectious diseases in cattle [20], the virus may cause damage to the respiratory tract, abortion, infertility, mucous diseases, thrombocytopenia and acute inflammation of the gastrointestinal tract [21, 22].

Based on the above, the *in vitro* study of cerium dioxide nanoparticles antiviral effectiveness against viral animal pathogens, in particular BVDV, will allow to get the first responses about the substance effectiveness against viruses and determine the expediency of further research in this direction.

Hypothesis. Preparations based on cerium dioxide nanoparticles can be effective against RNA animal viruses that have the largest epizootic value for Ukraine (pestivirus, causative agent of BVDV) and potentially against human viruses, including SARS-COV-2.

The aim of our research was to establish the antiviral activity of cerium dioxide nanoparticles on a model of pestivirus of cattle – BVDV.

Materials and Methods

Strain of virus and cell culture

BVDV causes viral diarrhea disease – mucous disease (VD-MD). This single-chain RNA-containing virus with a diameter of 40–60 nm, is a representative of the *Pestivirus* genus of *Flaviviridae* family. The disease in cattle is caused by two genotypes of the virus (BVDV1 and BVDV2), each of them have cytopathic and non-citopathic biotypes [21].

BVDV is largely manifested by crosslinking, that is, reproduced in various organs and tissues of cattle, especially cells that are rapidly multiplied – embryo cells, breeding organs, respiration and immune system, and gastrointestinal tract. "VK-1" BVDV strain (obtained from the Depositary of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC "IECVM"), Kharkiv) was used in the work after the restoration of infectious activity during 6 consecutive passages.

Cow embryo lungs (CEL) cell culture sensitive to the viral diarrhea virus, obtained from the cryobank of NSC "IECVM" was used in the work. The initial concentration of the cells was 3×10^5 cells/cm^3; DMEM and 199 (LLC "RE Veterinary Medicine", Ukraine) were used as a growing nutrient media in equal ratios with 10 % of the native inactivated cattle serum and antibiotics (penicillin 100 units/cm^3 and streptomycin 100 μg/cm^3) [23].

Identification and establishment of virus infectious activity

According to the results of the virus reproduction on the sensitive cell culture (typical cytopathic effect), the virus-containing material was obtained, the presence of BVDV was confirmed by the immunofluorescence method in accordance with the manufacturer's instruction (LLC "RE Veterinary Medicine", Kharkiv) and its genetic material – by means of a polymerase chain reaction (PCR) using primer systems 324 (ATGCCC(*T/A*)TAGTAGGACTA) and 326 (TCAACTCCATGTGCCATGTAC) [24, 25]. The establishment of infectious activity of BVDV strain "VK-1" (before and after the treatment with CeO_2) was carried out by detecting a cytopathic effect (CPE) in infected culture of CEL. The calculation of the virus infectivity titre was carried out according to Rid and Mench [26] method and was expressed in tissue cytopathic doses of 1.0 cm^3, causing a loss of 50 % of infected cell culture (TCD_{50}/cm^3).

Obtaining of cerium dioxide nanoparticles

Cerium dioxide aqueous solution obtaining was performed at the Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine by dissolving of 3.73 g of cerium chloride heptahydrate (III) and 2.0 g of citric acid in 20 mL of distilled water. This solution with a continuous mixing was rapidly added to an aqueous ammonia solution made by mixing of 10 g of concentrated ammonia ("Sigma", USA) and 100 mL of distilled water. The solution was stirred for 5 hours, followed by boiling, resulting in obtaining of 100 mL of 0.1 M cerium dioxide nanoparticles.

Investigation of cerium dioxide nanoparticles

The optical spectrum of cerium dioxide was determined on the OceanOptics QE 65000 spectrometer using a single-beam circuit. Radiation was provided by deuterium halogen lamp DH 2000 and xenon lamp HPX 2000. Optical absorption spectra were further used to calculate the energy of *EG* of CeO_2 nanoparticles. The size of cerium dioxide nanoparticles was determined by the method of transmission electron microscopy (TEM) on electron microscope LEO 912 AB Omega at 100 kV. The hydrodynamic diameter of citrate-coated CeO_2 was measured by a dynamic light

scattering method (DLS) using Malvern Zetasizer Nano ZS analyzer. The sol was diluted with distilled water before measuring. An analysis of powder X-ray diffraction (XRD) of cerium nanoparticles obtained by centrifugation of sol was performed using Rigaku D/MAX 2500 diffractometer (CuK_a radiation, an instrumental extension of 0.10 ± 0.012q). The speed of the goniometer rotation was 2°2q/min. The size of crystallite (D) of nanocrystalline cerium was calculated according to the Scherrer formula, where the anisotropy coefficient was equal to 1. Linear profiles for reflections (111) and (200) were corrected according to the Voigt profile.

Investigation of cytotoxic concentration (CC_{50}) of cerium dioxide

Determination of CC_{50} of the preparation that contained $CeO_2$was carried out on CEL cell culture at its final concentration of 0.172 μg/cm^3, 0.086 μg/cm^3, 0.057 μg/cm^3, 0.043 μg/cm^3, and 0.034 μg/cm^3. Working dilutions of the preparation of CeO_2 were prepared using supporting nutrient medium 199. Determination of the cytotoxic action of the preparation was carried out by removing a growth nutrient medium from a formed monolayer and replacing it to supporting, which contained the corresponding concentration of CeO_2. 5 samples of CEL cell culture were used for studying of each dilution of the drug.

Cell culture (5 samples) with a formed monolayer on a supporting nutrient medium without CeO_2 was used as a control. The cytotoxic effect of the various concentrations of cerium dioxide was evaluated visually after 24, 48 and 72 hours using inverted microscope, taking into account changes in cell morphology and violation of monolayer integrity. The integrity of cell membranes was determined using 0.2 % solution of trypan blue (in the absence of cell color) [23]. The degree of manifestation of CeO_2 cytotoxic effect was evaluated in crosses (from "++++" to "¾"), where "¾" – a complete absence of cells degeneration, "+" – violation of cells morphology and integrity of no more than 25 % of the monolayer, "++" – no more than 50 % of cells monolayer,"+++" – no more than 75 % of cells monolayer and "++++" – complete degeneration of cells and destruction of the monolayer. CC_{50} of CeO_2 was determined as its concentration, which led to the destruction of 50 % of cells monolayer due to cytotoxicity.

To the study the antiviral efficacy, we used CeO_2 in concentrations (taking into account the maximum allowable concentration) which did not cause cell death in the monolayer of the CEL cell culture and did not cause morphological changes in cells.

Determination of antiviral efficacy of cerium dioxide nanoparticles on the model of BVDV

After determining the maximum allowable concentration of CeO_2, non-toxic for CEL cell culture, the antiviral efficacy of the drug was determined by applying of its different concentrations simultaneously with BVDV strain "VK-1" after their co-incubation [27]. 100 TCD_{50}/cm^3 of BVDV were used to standardize the viral suspension and infect cells monolayer [28]. "VK-1" strain was previously treated with different concentrations of CeO_2 in a ratio of 1:1. For this purpose, a mixture of different concentrations of CeO_2 and BVDV strain "VK-1" with a final infectious activity of 100 TCD_{50}/cm^3 was prepared and then incubated for 40 minutes at a temperature of 37 ^{0}C. The growth medium was removed from CEL cell culture with the 100 % monolayer formed, monolayer was washed three times with DMEM medium, and then 1:10 of the drug-virus mixture volume was added. The samples were incubated for 60 minutes at 37 ^{0}C, and then the virus-drug mixture was removed from the monolayer, then it was washed three times with DMEM medium and a growth support medium was added. Incubation of samples was performed at a temperature of 37 °C.

We used 5 samples of CEL cell culture for each dilution of the drug. The following control samples (n = 5) were used to determine the efficiency of CeO_2 at different concentrations: control of CEL cell culture on maintenance medium (CC); control of 100TCD_{50}/cm^3 of BVDV strain "VK-1" on CEL cell culture (VC); control of cytotoxic effect of the corresponding concentrations of CeO_2 on CEL cell culture.

The results of the study were recorded according to the results of 100% CPE of BVDV strain "VK-1" in CEL cell culture monolayer in samples of 100 TCD_{50}/cm^3 virus control (VC) [29].

Determination of the antiviral efficacy of CeO_2 was performed by establishing IC_{50} – the concentration of the drug that inhibits virus-induced CPE in 50 % of cells monolayer [30]. The state of the monolayer, its integrity, changes in cell morphology in the appropriate controls and test samples containing different concentrations of the drug were taken into account. Evaluation of virus CPE, by analogy with the cytotoxic concentration, was performed in crosses (from "++++" to "¾"). Cell viability was determined by the integrity of

their membrane using vital staining with 0.2 % of trypan blue solution (no cell staining).

After cytotoxic and inhibitory concentrations of CeO_2 investigation, the selectivity index (SI) was calculated as the ratio of CC_{50} to IC_{50} to determine the antiviral efficacy of the drug.

Statistical data processing

Statistical processing of data from preclinical studies was performed by nonparametric analysis (presented in the form of medians and interquartile intervals of the sample) [31, 32]. Data were visualized in boxplot graphs through nonparametric Kruskal-Wallis ANOVA analysis and median test. Data processing was performed using the "Statistica" software and online calculator of CC_{50} and IC_{50} AAT Bioquest [33].

Results

Identification and restoration of infectious activity of bovine viral diarrhea virus

Restoration of biological properties of BVDV strain "VK-1" after storage at a temperature of minus 70 ^{0}C in the Depository of NSC "IECVM" was carried out on CEL cell culture during 6 consecutive passages.

The presence of genetic material of BVDV in aliquots after storage in the Depository of NSC "IECVM" and after restoration of its biological properties during 6 consecutive passages was confirmed by PCR (the formation of amplicon at 324/326 base pairs was observed) and its antigens – by immunofluorescence reaction (a specific luminescent glow of antigens of BVDV

Table 1

Cytotoxicity of cerium dioxide nanoparticles on CEL cell culture

Concentration of CeO_2, μg/cm^3	Indicator	**Affected cells,%**
0.172	M ± m*	70.0±5.00
	$Q_2[Q_1 – Q_3]$**	75.0***
0.086	M ± m*	50.0±7.91
	$Q_2[Q_1 – Q_3]$**	50.0***
0.057	M ± m*	4.0±2.45
	$Q_2[Q_1 – Q_3]$**	0 [0–10.0]
0.043	M ± m*	0
	$Q_2[Q_1 – Q_3]$**	0
0.034	M ± m*	0
	$Q_2[Q_1 – Q_3]$**	0

*M ± m, where M is the mean value, m is the arithmetic mean error; **Q_2 [$Q_1 – Q_3$], where Q_2 is the median (50 % of the sample), where Q_1 and Q_3 are the lower and upper quartiles (25 % and 75 % of the sample); ***Q_2 corresponds to the interval [$Q_1 – Q_3$].

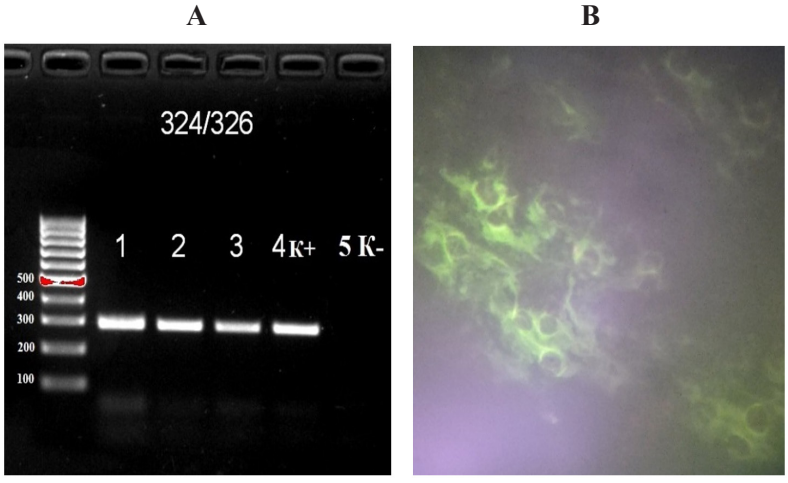

Fig. 1. Identification of BVDV in the test material at 6 passages by immunofluorescence reaction (a), as well as before 1st and after 6th passages by PCR (b). Note: 1 – after storage in the Depository of NSC "IECVM"; 2, 3 – after 6 passages on the CEL cell culture; 4K+ – positive control; 5K– negative control.

in the infected monolayer of CEL cell culture was observed after treatment of the test sample with specific immunofluorochrome) (Fig. 1).

According to the results of the restoration of BVDV biological properties in CEL cell culture, it was found that the titer of infectious activity of the pathogen at the level of 6 passages was 6.87 lg TCD_{50}/cm^3. This indicator was chosen as the starting point in the study of cerium dioxide nanoparticles efficiency. Taking into account the infectivity titer of BVDV strain "VK-1" on CEL cell culture, the standard dose of infection was determined when studying the antiviral efficacy of CeO_2 (100 TCD_{50}/cm^3).

Determination of cytotoxic action of cerium dioxide nanoparticles

Determination of cytotoxic effect of cerium dioxide nanoparticles was performed on CEL cell culture, using the final concentration of the drug from 0.172 μg/cm³ to 0.034 μg/cm³ (Table 1).

According to the results of studying the cytotoxic effect of CeO_2 on CEL cell culture, it was determined that the use of the drug in a final concentration of 0.172 μg/cm³ led to changes in morphology and reduced viability of 50–75 % of cells. The use of the drug at a final concentration of 0.086 μg/cm³ was characterized by changes in morphology and disruption of membrane integrity of 25–75 % of cells. Instead, the use of cerium dioxide nanoparticles in the nutrient medium at a final concentration of 0.057 μg/cm³ did not have a significant effect on cell morphology and the integrity of the monolayer of CEL cell culture.

Based on the results of the studies, the CC_{50} (Fig. 2) and the maximum allowable concentration (MAC) of cerium dioxide nanoparticles were determined, this concentration did not affect cell viability and monolayer degeneration (preservation of cell monolayer at 90–100 %) and was 0.057 μg/cm³.

Visualization of data on the cytotoxic concentration of CeO_2, which leads to the death of 50 % of CEL cell culture monolayer was performed using non-parametric Kruskal-Wallis ANOVA analysis and median test.

The concentration of CeO_2 that leads to the death of 50% of cells monolayer of CEL cell culture is 0.077 μg/cm³.

Determination of antiviral effect of cerium dioxide nanoparticles on BVDV model

It was found that 100 % manifestation of the cytopathic effect of BVDV strain "VK-1" on CEL cell culture (control samples) was observed 24 hours after application of virus in 100 TCD_{50}/cm^3. This time was chosen to take into account the results of experimental and control samples. During this time, the morphology of cells and the integrity of the monolayer of control samples of cell culture and control samples of determining the cytotoxicity of different concentrations of CeO_2 remained unchanged.

The use of cerium dioxide nanoparticles at a final concentration of 0.057 μg/cm³ inhibited the manifestation of virus-induced CPE on CEL cell culture, maintaining cell morphology and monolayer integrity at 75.0–100.0 % (Table 2).

Further reduction of the final concentration of cerium dioxide to 0.043 μg/cm³ for virus treatment led to the preservation of morphology and viability of cells monolayer at the level of 50.0–100.0%.

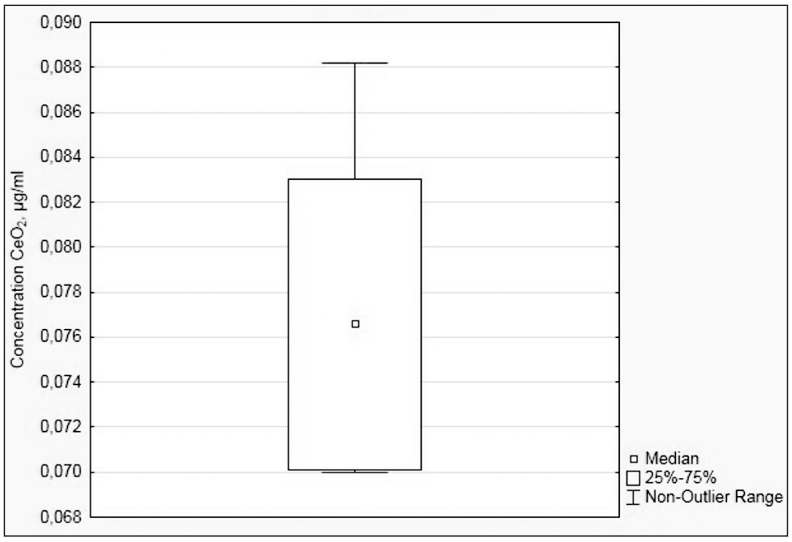

Fig. 2. Median and interquartile range of CC_{50} of CeO_2 visualized through non-parametric Kruskal-Wallis ANOVA analysis and median test (boxplot)

Table 2

The effectiveness of CeO_2 in preventing the manifestation of BVDV-induced cytopathic effect on CEL cell culture

Concentration, μg/cm³	Indicator	Cells without changes in morphology and membrane integrity, %
0.057	M ± m*	90.0±6.12
	Q_2 [$Q_1 – Q_3$]**	100.0 [75.0–100.0]
0.043	M ± m*	75.0±7.91
	Q_2 [$Q_1 – Q_3$]**	75.0***
0.034	M ± m*	50.0±11.18
	Q_2 [$Q_1 – Q_3$]**	50.0 [25.0–75.0]

*M ± m, where M is the mean value, m is the arithmetic mean error; **Q_2 [$Q_1 – Q_3$], where Q_2 is the median (50 % of the sample), where Q_1 and Q_3 are the lower and upper quartiles (25 % and 75 % of the sample); ***Q_2 corresponds to the interval [$Q_1 – Q_3$].

BVDV treatment with CeO_2 in concentration of 0.034 μg/cm³ before CEL cell culture monolayer infection ensured the safety of 25.0–75.0 % of cells monolayer.

Visualization of data on the antiviral efficacy of the drug, which provides protection (inhibition of virus-induced CPE) in 50 % of CEL cell culture monolayer (IC_{50}) using non-parametric Kruskal-Wallis ANOVA analysis and median test is shown in Fig. 3.

The concentration of CeO_2, which provides protection (inhibition of virus-induced CPE manifestation) 50 % of CEL cell culture monolayer (IC_{50}) is 0.036 μg/cm³.

Evaluation of the inhibitory activity of CeO_2 *in vitro* was performed according to SI, which was 2.14 for these nanoparticles.

In order to determine the effect of cerium dioxide nanoparticles on the infectious activity of BVDV, titration of the pathogen obtained after the above studies was performed. The titer of infectious activity of BVDV strain "VK-1" on CEL cell culture obtained after treatment with CeO_2 was reduced compared to the control depending on the concentration of the drug (Fig. 4).

The use of cerium dioxide nanoparticles at a final concentration of 0.057 μg/cm³ under conditions of its simultaneous application with BVDV to CEL cell culture led to a decrease in the titer of infectious activity of the pathogen by 2.09 lg TCD_{50}/cm³ compared to control. A further decrease in the concentration of CeO_2 to 0.043 μg/cm³ and 0.034 μg/cm³ led to a decrease in the titer of infectious activity of the pathogen by 1.31 lg TCD_{50}/cm³ and 0.87 lg TCD_{50}/cm³, respectively.

According to the results of the studies, it was determined that cerium dioxide nanoparticles when co-administered with BVDV strain "VK-1" to CEL

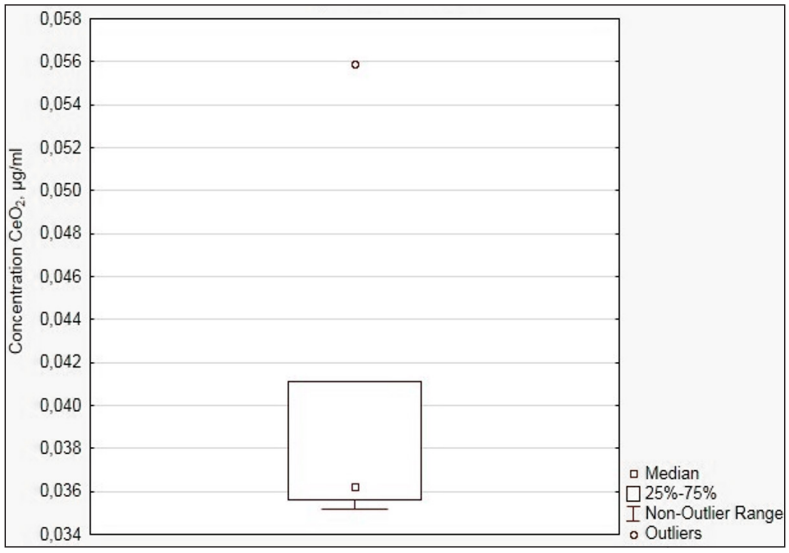

Fig. 3. Median and interquartile range of cerium dioxide IC_{50} on CEL cell culture, visualized by non-parametric Kruskal-Wallis ANOVA analysis and median test (boxplot)

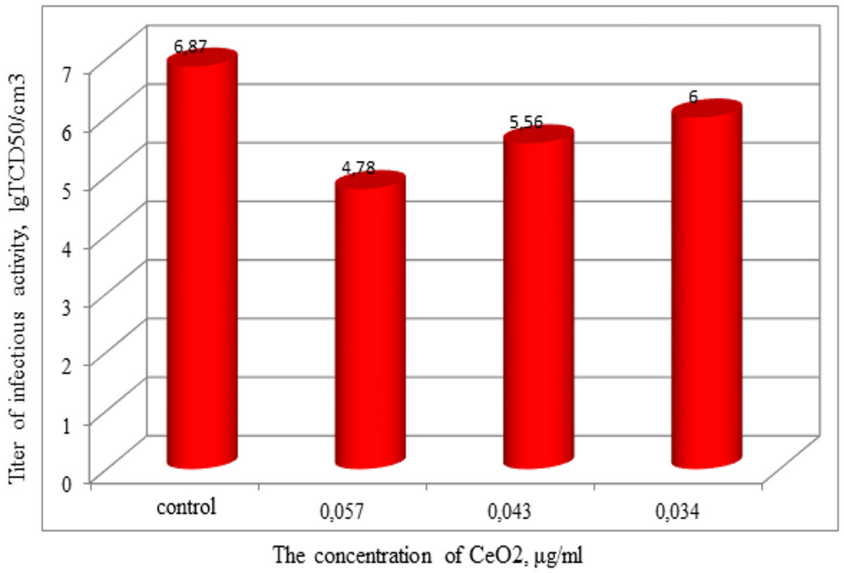

Fig. 4. Titer of BVDV strain "VK-1" infectious activity on the CEL cell culture after treatment with CeO_2 nanoparticles

cell culture exhibit antiviral activity – inhibit virus-induced cytopathic effect and reduce infectious activity of the pathogen. Generalized results of the study of antiviral activity of cerium dioxide nanoparticles are shown in Table 3.

Conclusions. According to the results of the study, it was determined that cerium dioxide nanoparticles when co-administered with BVDV inhibit the manifestation of virus-induced CPE (IC_{50} 0.036 µg/cm^3) and reduce the titer of infectious activity of the pathogen by 2.09 lg TCD_{50}/cm^3.

Prospects for further research. Given the need for highly effective harmless antiviral drugs against the background of the spread of viral pathogens in humans and animals, including zoonoses, search for inorganic and organic substances is promising and requires further research. Studies have shown inhibitory effect of cerium dioxide nanoparticles on the manifestation of virus-induced cytopathic effect of bovine pestivirus *in vitro* and data on reducing the titer of infectious activity of the pathogen in CEL cell culture. In our opinion, the low index of selectivity calculated during the study, the optimal value of which should be SI≥10 [34, 35], is associated with the method of CeO_2introducing (simultaneously with the virus). Therefore, further studies of cerium nanoparticles antiviral activity should be under conditions of their introduction into the nutrient medium before and/or after infection of the cell culture with viruses [27], including bovine coronavirus.

Gratitude. The authors are grateful to employees of the Department of Molecular Epizootology, Diagnosis and Quality Control of Genetic Resources of NSC "IECVM" for assistance in conducting molecular genetic research.

Table 3

The results of the study of the CeO_2 antiviral efficacy in CEL cell culture on the model of BVDV

MAC, µg/cm^3	*CC_{50}, µg/cm^3	*IC_{50}, µg/cm^3	** Titer of infectious activity, lg TCD_{50}/cm^3	SI
0.057	0.077 [0.070 – 0.083]	0.036 [0.036 – 0.041]	Decrease by 2.09	2.14

* Q_2 [Q_1– Q_3], where Q_2 is the median (50 % of the sample), Q_1 and Q_3 are the lower and upper quartiles (25 % and 75 % of the sample), respectively; ** at CeO_2 concentration of 0.057 µg/cm^3.

ВИВЧЕННЯ ПРОТИВІРУСНОЇ ДІЇ ПРЕПАРАТУ НА ОСНОВІ НАНОЧАСТОК ДІОКСИДУ ЦЕРІЮ НА МОДЕЛІ ЗБУДНИКА ВІРУСНОЇ ДІАРЕЇ ВЕЛИКОЇ РОГАТОЇ ХУДОБИ

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Резюме

Наночастки оксиду церію (CeO_2) мають широкий спектр біологічних властивостей, в т.ч. противірусну активність. Препарати на основі наночасток оксиду церію можуть бути ефективні проти РНК-вірусів тварин, які мають найбільше епізоотичне значення для України та потенційно проти вірусів людини, в т.ч. SARS-CoV-2. В наших дослідженнях визначали дію наночасток оксиду церію на вірус вірусної діареї великої рогатої худоби (ВД ВРХ) – пестівірус, який є етіологічним агентом вірусної діареї ВРХ. Для визначення противірусної ефективності CeO_2 використовували цитопатогенний штам вірусу ВД «ВК-1» як етіологічний агент ВД ВРХ, який індукує прояв цитопатичного ефекту на перещеплюваній культурі легенів ембріону корови. При оцінці противірусної ефективності CeO_2 визначено, що ГДК для перещеплюваної культури клітин ЛЕК становить 0,057 мкг/см^3, а CC_{50} сполуки дорівнює 0,077 мкг/см^3. Противірусну активність CeO_2 оцінювали за показником IC_{50}, який становив 0,036 мкг/см^3 та індексом селективності, який при одночасному внесенні наноцерію з вірусом ВД ВРХ дорівнював 2,14. Відмічено зниження титру інфекційної активності вірусу при обробці CeO_2 на 2,09 lg $ТЦД_{50/см^3}$.

Ключові слова: Вірус, діарея великої рогатої худоби, наночастки, оксид церію, перещеплювана культура клітин легенів ембріону корови, противірусна активність.

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