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PHOTODYNAMIC TREATMENT OF TITANIUM DIOXIDE NANOPARTICLES IS A CONVENIENT METHOD OF ADENOVIRAL INACTIVATION

Today, the search for safe ways to inactivate pathogens is becoming especially relevant in connection with the coronavirus pandemic. Standard methods using chlorides and ultraviolet irradiation have disadvantages related to toxicity and low efficiency. Photodynamic inactivation involving nanoparticles is already used to disinfect water and air from microorganisms and enveloped viruses such as human herpes simplex virus, vesicular stomatitis virus, human immunodeficiency virus, and hepatitis B and C viruses. The ***aim*** *of this work was to evaluate the possibility of the inactivation of human adenovirus type 5 in an organic medium using titanium dioxide irradiated with ultraviolet light.* ***Methods.*** *The nanosized titanium dioxide material was obtained by the thermal decomposition of a suspension of hydrated titanium dioxide $TiO(OH)_2$ (metatitanic acid). The analysis of the morphology of the TiO_2 nanopowder was carried out using electron scanning microscopy (SEM), which showed that TiO_2 nanopowder contains soft aggregates of nanoparticles mostly 20–30 nm in size. Cytotoxicity, virulicidal and antiviral action of titanium dioxide were determined by standard methods using (3-(4,5-dimathylthiazol-2-yl)-2,5-dipheniltetrazolium bromide (MTT). The titanium dioxide suspension was irradiated at a distance of 20 cm from 1 to 30 min with a bactericidal UV lamp (OBB15P, BactoSfera, Poland (254 nm). The concentration of nanoparticles for irradiation was 1.0 mg/mL. Adenovirus suspension with titer 6.0 $\log_{10}$ $TCID_{50}$ /mL was added to the nanoparticles immediately after irradiation. The titer of virus synthesized in the presence of titanium dioxide was determined by the end of the virus dilution, which causes 50% of the cytopathic effect of the virus on cells. All studies were performed in three replicates; the number of parallel determinations was three.* ***Results.*** *A dose-dependent effect of titanium dioxide nanoparticles on the viability of Hep-2 cells was revealed. At the NPs concentration of 1 mg/mL, quite a low cell viability was observed (32—39%), with a decrease in concentration to 0.1 and 0.01*

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mg/mL, the NPs were less toxic (cell viability was in the range of 62—90%). The TiO_2 NPs dissolved in glycerin-water had no virulicidal effect, as the virus titer was similar to the control values. Instead, NPs dissolved in propanediol-ethanol reduced the infectious titer of the virus by 6.0 log_{10}, which indicates their high virulicidal effect. The absence of an antiviral effect was shown when NPs were added to infected cells. A decrease in the virus titer by 4.5–5.0 log_{10} was recorded uponitsinteracting with irradiated NPs for 1–30 min. The effect persisted for 3 h after exposure to NPs. ***Conclusions****. The cytotoxic, virulicidal, and antiviral effects of optically active TiO_2 nanoparticles were determined in optimal conditions. Regardless of the solvent, NPs had low toxicity at a concentration of 0.1 mg/mL. The TiO_2 NPs dissolved in glycerin-water had no virulicidal effect; but dissolved in propanediol-ethanol reduced the infectious titer of the virus by 6.0 log_{10}, which indicates its high virulicidal effect. NPs in a propanediol-ethanol solution, irradiated with UV for 1–30 min, completely inhibited adenovirus reproduction. NPs in a glycine-water solution reduced the virus titer by 0.5 log_{10}. The control with NPs without irradiation slightly reduced the virus titer (by 0.45 log_{10}). The ability of NPs to completely inactivate adenovirus was maintained for 3 h. It was shown for the first time that the non-enveloped HAdV5 virus could be efficiently inactivated by UV-induced TiO_2 photocatalysis.*

Keywords*: titanium dioxide nanoparticles, photoinactivation, adenovirus.*

Currently, the search for safe ways to inactivate pathogens is becoming especially relevant in connection with the coronavirus pandemic. Standard methods using chlorides and ultraviolet irradiation have disadvantages related to toxicity and low efficiency. Chlorination in the presence of organic substances leads to the formation of mutagenic compounds [1–3]. Pathogens have different sensitivities to ultraviolet (UV) irradiation, and hard UV radiation is harmful to all living organisms, including humans. Oxide semiconductors can oxidize pathogens to CO_2 and water in air (or in liquid) under UV or visible light. After oxidation, the pathogens turn into stable harmless inorganic compounds, such as water, carbon dioxide, etc. Due to their unique properties, the use of titanium dioxide nanoparticles (TiO_2 NPs) as disinfectants is widely researched [4–6]. For future commercial use, it is important that TiO_2 photocatalysis not require the addition of chemicals and not generate hazardous waste [2]. In nature, TiO_2 exists in three crystal modifications – anatase, rutile, and brookite. It has been shown that the crystalline form of anatase nano-TiO_2 is more toxic compared to the mixture of anatase and rutile [7, 8]. It has been shown that TiO_2 NPs enter cells by endocytosis, they are not absorbed by organelles, and do not undergo biodegradation inside the cell. In the cell, nano-TiO_2 causes a nonspecific increase in the concentration of intracellular reactive oxygen, which leads to cell apoptosis and/or the initiation of an inflammatory reaction [8]. The photocatalytic properties of TiO_2 are used to destroy pathogenic bacteria, fungi, and viruses in water and air environments [9–11]. A huge advantage of the method is the universal possibility of disinfection for viruses and pathogenic microorganisms. Viruses from the adenovirus family (Adenoviridae) are double-stranded viruses with linear DNA, without an envelope, and have the ability to infect highly differentiated post-mitotic cells such as skeletal muscle, lung, brain, and heart [12]. The safest way to fight viral infections to prevent infection is exposure to the extracellular virus. In practice, hard UV radiation is widely used to disinfect surfaces, air, and liquids. Adenoviruses are among the most resistant viruses to UV disinfection [13, 14]. Therefore, there is growing concern about human infection with adenoviruses through drinking water and air. The coronavirus pandemic has demonstrated an urgent need for the development of disinfection methods. Due to the high prevalence of adenoviruses and their ability to persist in the environment for a long time, there is also a growing concern about human contamination through drinking water and air. Coronaviruses, like adenoviruses, are able to spread through air, liquids, and surfaces, so the environment needs

effective continuous disinfection. Photocatalysts based on TiO_2 are just such inexpensive, environmentally friendly, and effective antimicrobial/antiviral agents with the possibility of indoor use [15]. Antiviral activity with complete inactivation of the virus SARS-CoV-2 was observed upon photoactivation of TiO_2 under the action of LED light 405 nm [16].

The aim of our study was to first determine the prospects of using ultraviolet treatment of TiO_2 NPs for inactivation of human adenovirus type 5 (HAdV5).

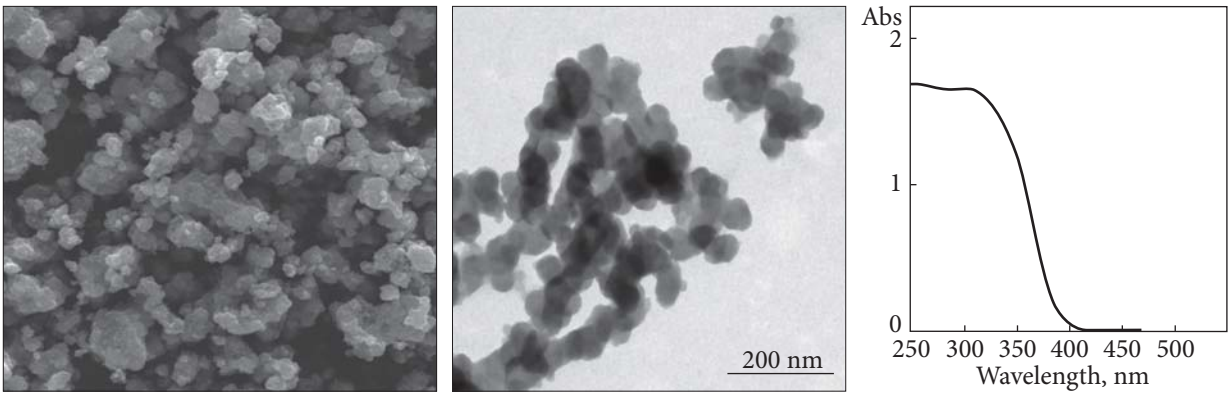
Materials and methods. *The manufacture of TiO_2 NPs.* The nanosized titanium dioxide material was synthesized at the Frantsevich Institute for Problems of Materials Science, NAS of Ukraine (IPMS). TiO_2 was obtained by the thermal decomposition of a suspension of hydrated titanium dioxide $TiO(OH)_2$ (metatitanic acid), a product of titanium concentrates and slags («Sumykhimprom», Ukraine). The suspension was heated to 600 °C with a heating rate of 5 °C/min to obtain anatase. This production method promotes the formation of additional active centers on the TiO_2 surface. This process improves the optical and photocatalytic properties of anatase. It has been stated that due to the mesoporous and crystalline structure, the modification of anatase TiO_2 has photocatalytic activity [17]. The visualization of TiO_2 nanoparticles was performed using scanning electron microscopy (SEM). Electronic images of samples were recorded on a MIRA3 TESCAN unit (Tescan, Brno, Czech Republic). In addition, the morphology of TiO_2 under study was presented using a JEM-1400 electron microscope (JEOL, Japan).

Cell culture and viruses. Reference strain of human adenoviruses 5 serotypes (HAdV5), supported by the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, were cultured in human laryngeal carcinoma cells Hep-2 (ECACC N86030501). Cell and virus cultivation was carried out according to standard methods [18].

The cytotoxicity of TiO_2 NPs was tested *in vitro* by the MTT method [19]. Cells in a 96-well plate were incubated with serial 10-fold dilutions of TiO_2 NPs for 48 h, and 20 μL/well of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, USA) was added at a concentration of 5 mg/mL and cultivated for 3 h at 37 °C in an atmosphere of 5% CO_2. The culture medium was removed, and 100 μL/well of 96° ethanol was added. The research was carried out in three repetitions. The optical density of the samples was determined spectrophotometrically on a Multiskan FC reader (Thermo Fisher Scientific, USA) at a wavelength of 538 nm. The percentage of living cells was calculated for each concentration of substance compared to control cells (not added with NPs).

The study of the virulicidal effect of TiO_2 NPs was carried out according to the classical scheme [20]. A solution of TiO_2 at the maximum non-toxic concentration with an equal volume of undiluted virus was mixed and incubated at 37 °C for 1 h. Next, the monolayer of Hep-2 cells in 96-well plates was infected with serial 10-fold dilutions of the virus/TiO_2 suspension (50 μL/well) in three replicates. A mixture of the virus with an equal volume of supportive medium was maintained as a control under the same conditions. 150 μL of the supportive medium was added to the wells, and the plates were cultured at 37 °C in an atmosphere of 5% CO_2. After 4–5 days of cultivation, with the appearance of pronounced cytopathogenic action (CPA) of the virus in the control, further processing was performed according to the standard method of MTT. Virus titers were determined at the end point of the dilution, which causes 50% CPA. Using the prediction function of Microsoft Excel, virus titers and indexes of inhibition of virus reproduction (IRI) were calculated as follows:

$$\text{IRI} = \text{Virus titer control } (\log_{10}) - \text{Virus titer experiment } (\log_{10})$$



a *b* *c*

Fig. 1. (a) SEM and (b) TEM of TiO_2 NPs; (c) optical absorbance of TiO_2 NPs. An UV ViS_NIR Spectrometer tested TiO_2 NPs. TiO_2 anatase can absorb light with wavelengths below 400 nm [17]

A decrease in the infectious titer of the virus by 4 $\log_{10}$ or more, compared to the control of the virus, indicates a virulicidal effect [21].

Photoactivation of TiO_2 NPs. A bactericidal UV lamp (OBB15P, BactoSfera, Poland) with a power of 15 W, frequency of 50 Hz, wavelength of 254 nm, and irradiation time of 1–30 min was used for photoactivation of TiO_2 NPs. Samples were placed in plastic cryotubes perpendicularly to the lamp at a distance of 20 cm. The concentration of nanoparticles for irradiation was 1.0 mg/mL. Adenovirus suspension with titer 6.0 $\log_{10}$ $TCID_{50}$/mL was added to the nanoparticles immediately after irradiation. As controls, virus with non-irradiated NPs and virus without adding NPs were used. A series of 10-fold dilutions of controls and studied suspensions were prepared, and Hep-2 cells were infected. After 4 days of cultivation of cells using MTT analysis, virus titers were determined using the prediction function of Microsoft Excel, and the indexes of inhibition of virus reproduction (IRI) were calculated. *Statistical processing of results.* All studies were performed in three experiments; the number of parallel determinations was three. Mean values, standard deviation, and mean error were calculated. Differences in the means were considered significant at $p \leq 0.05$. The research results were processed using Microsoft Office Excel 2010.

Results. When studying disinfection properties, UV light from mercury lamps of low and medium pressure with continuous radiation is often used. An alternative to this can be a pulsed xenon arc or xenon flash. However, even under these conditions, inactivation of the adenovirus occurred only by 1.0 $\log_{10}$ after 10 pulses. Adenovirus requires 100 pulses to decrease the titer by approximately 3.0 $\log_{10}$ and 200 pulses to decrease by more than 4.0 $\log_{10}$ [22]. Today, there is great research interest in photoactivated nanomaterials [3, 9–11, 17, 24]. Among many photocatalysts, TiO_2 nanomaterial is one of the best. Under UV light, free radicals are formed, which have a bactericidal and antiviral effect against various microbes and viruses [11, 15]. The antiviral activity of TiO_2 against SARS-CoV-2 has been investigated [16].

In this work, we determined the cytotoxic, virucidal, and antiviral effects of optically active TiO_2 nanoparticles. The nanosized titanium dioxide was obtained by the thermal decomposition of a suspension of hydrated titanium dioxide $TiO(OH)_2$ (metatitanic acid). The morphology of the TiO_2 nanopowder was examined using SEM and TEM analyses, which showed that TiO_2 nanopowder contains soft aggregates of nanoparticles mostly 20–30 nm in size (Fig. 1). In addition, it exhibited a developed

surface structure due to the presence of mesopores (2–50 nm) and a specific surface area of 50.84 m^2/g [8, 17].

Since the antiviral activity of NPs was investigated *in vitro* in cell culture, the study of their cytotoxic effect was carried out as well (Fig. 2). A dose-dependent effect of TiO_2 NPs dissolved in both types of solvents on the viability of Hep-2 cells was revealed. At the NPs concentration of 1 mg/mL, quite a low cell viability was observed (32—39%). With decreasing concentration to 0.1 and 0.01 mg/mL, the NPs became less toxic (cell viability was in the range of 62—90%). It should be noted that TiO_2 NPs dissolved in propanediol-ethanol were more toxic.

It is known that an extracellular virus can be the target of antiviral drugs, therefore, in our studies, the virulicidal effect of nanoparticles was determined [21]. In these experiments, NPs were used in predetermined non-toxic concentrations of 0.1 mg/mL. The TiO_2 NPs dissolved in glycerin water had no virulicidal effect, as the virus titer was similar to control values. Instead, NPs dissolved in propanediol-ethanol reduced the infectious titer of the virus by 6.0 $\log_{10}$, which indicates their high virulicidal effect (Fig. 3).

Nanoparticles can affect the newly formed virus and its exit from the cell, thus its infectivity can be significantly reduced. That is why, the effect of TiO_2 NPs on HAdV5 replication in Hep-2 cells when NPs were added immediately after virus adsorption was studied. However, it was found that NPs did not show anti-adenoviral, since the decrease in virus titer did not exceed 0.8 $\log_{10}$ (data not shown).

The anti-adenoviral effect of photoactivated TiO_2 nanoparticles was studied. The suspension of TiO_2 in propanediol-ethanol and TiO_2 in glycerin-water (1 mg/mL) were irradiated with UV light (254 nm) for 30 min. Complete suppression of reproduction of HAdV5 by UV irradiated TiO_2 NPs dissolved in propanediol-ethanol was shown (Fig. 4).

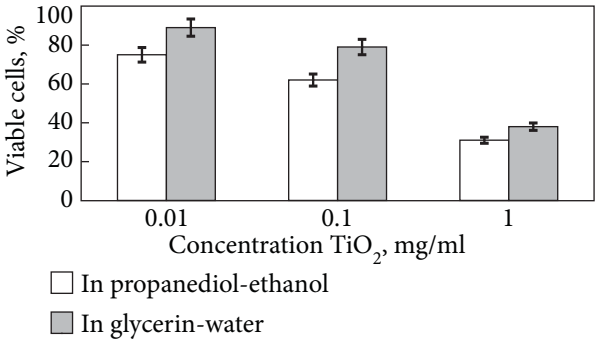


Fig. 2. Influence of TiO_2 NPs on Hep-2 cell viability and mitochondrial activity. Cell growth after 72 h exposure to different concentrations of the NPs was monitored by a colorimetric MTT assay. Control untreated cells — 100% viability

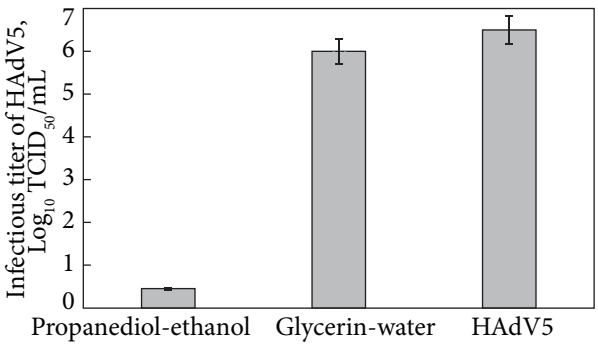


Fig. 3. Virulicidal effect of TiO_2 NPs after 1 h contact with viruses, evaluated by a colorimetric MTT assay after 72 h p.i. of cells with HAdV5

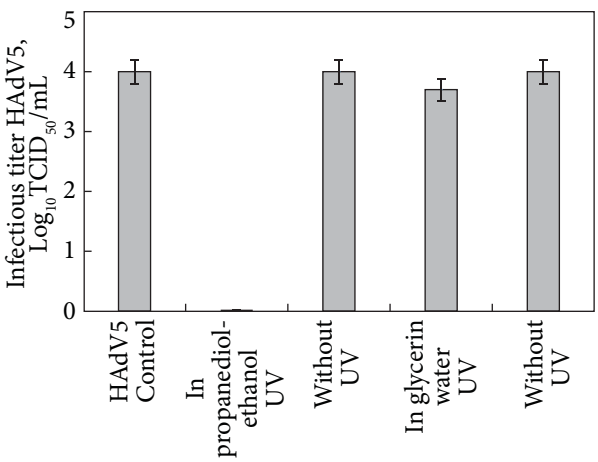


Fig. 4. Infectious titer of HAdV5 under the action of UV-photoactivated TiO_2 NPs (254 nm, 30 min)

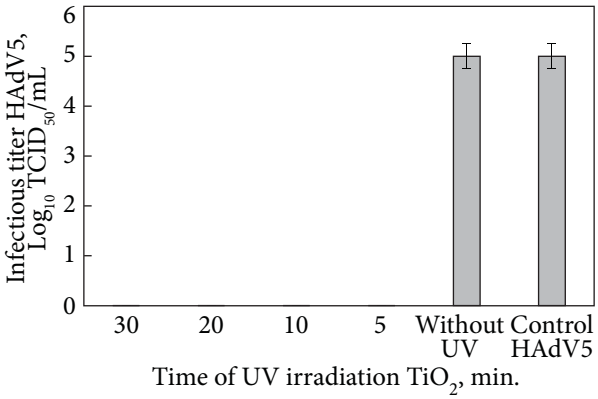


Fig. 5. Infectious titer of HAdV5 in the presence of TiO_2 NPs photoactivated with UV for 5—30 min. NPs were dissolved in propanediol-ethanol

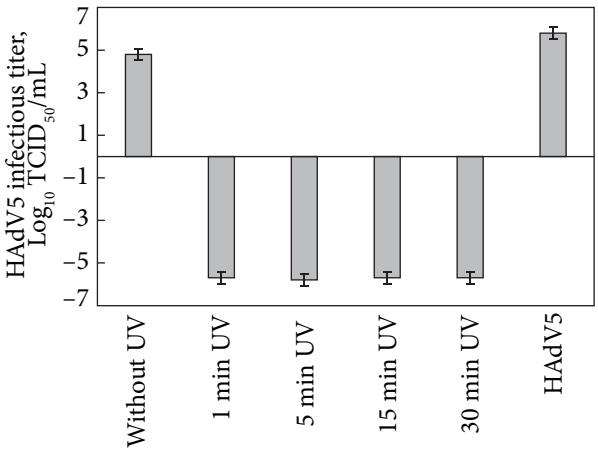


Fig. 6. Reduction of the infectious titer of HAdV5 in contact with UV-activated NPs 3 h after photoactivation

Photoactivated NPs in the propanediol-ethanol solvent for 1—30 min at a concentration of 1 mg/mL when added with an equal volume of HAdV5 suspension with a titer of 6.0 log_{10} $TCID_{50}$/mL completely inhibited adenovirus reproduction. Non-photoactivated nanoparticles did not change the virus titer (Fig. 5). Also, the duration of preservation of the effect of UV activation of TiO_2 NPs suspensions was investigated (Fig. 6).

A suspension of HAdV5 was added to TiO_2 NPs at 3 h after their UV-photoactivation, and the infectious titers of the virus were determined in experimental samples, virus control, and control with NPs that were not irradiated. Complete inhibition of the virus reproduction was detected even later than 3 h after photoactivation of NPs.

Discussion. Adenoviruses are among the most resistant viruses to UV disinfection [13, 14]. It is known that TiO_2 photocatalysts generate strong oxidizing power when illuminated with UV light of wavelengths under 385 nm [11]. In the case of penetration of the virus into the cells, an effective way to suppress it is to block its replication by suppressing the expression of specific enzymes involved in the replication of the nucleic acid of the virus [25]. Photocatalytic TiO_2 can be used in (i) powder form (e.g. Degussa P25), typically dispersed in aqueous solutions, (ii) film/coating applied to various substrates, or (iii) immobilized on surfaces [26]. The ability of TiO_2 to induce the photocatalytic degradation of almost any kind of organic and living pollutants, including bacteria and viruses, has fostered its use in purification technologies [11, 27]. The mechanism of the antiviral action is that OH and O_2 generated at the UV-activated TiO_2 surface are able to degrade the capsid and envelope proteins and phospholipids of non-enveloped and enveloped viruses, respectively. Besides, the leakage and consequent NPs degradation occur, ultimately leading to the inactivation of the viral particles. TiO_2 nanoparticles are most often used in the powder form, as a rule, dispersed in water or immobilized on the surface. UV-induced photocatalysis of TiO_2 inactivates a wide range of mammalian viruses, including poliovirus 1, avian and human influenza viruses, and the SARS coronavirus. The efficiency of UV-induced photocatalysis of TiO_2 varies depending on various parameters, namely the crystallinity and concentration of the photocatalyst, as well as the correct combination of light intensity and exposure time [11, 28]**.** Different TiO_2 nanostructures such as NPs, nanotubes (NTs) and nanowires (NWs),

etc. have also been studied and engineered for the enhanced photocatalytic antimicrobial and antiviral effect to inactivate viruses including the SARS-CoV-2 virus with great efficiency [11, 15]. Hamza et al. [28] studied the effect of TiO_2 NPs for the disinfection of SARS-CoV-2, which exhibited strong anti-SARS-CoV-2 activity at very low cytotoxic concentrations *in vitro* with excellent antiviral activity at a very low concentration. It was concluded that these TiO_2 nanostructures are suitable for coatings as a potent disinfectant to combat SARS-CoV-2. The authors recommend the use of TiO_2 NPs *in vitro* and in wall coatings as a potent disinfectant to combat SARS-CoV-2 with little irritation to host cells. To date, there are no publications on the use of UV-induced TiO_2 photocatalysis for adenovirus inactivation. Our results indicate the perspective of TiO_2 applications to the adenovirus inactivation in the development of non-toxic disinfection systems and use of them against air-borne or water-borne pathogens.

Conclusions. Thus, the cytotoxic, virulicidal, and antiviral effects of optically active TiO_2 NPs were determined in optimal conditions. Regardless of the solvent, NPs had low toxicity at a concentration of 0.1 mg/mL. The TiO_2 NPs dissolved in glycerin water had no virulicidal effect but dissolved in propanediol-ethanol reduced the infectious titer of the virus by 6.0 $\log_{10}$, which indicates their high virulicidal effect. NPs in a propanediol-ethanol solution, irradiated with UV for 1-30 min, completely inhibited adenovirus reproduction. NPs in a glycine-water solution reduced the virus titer by 0.5 $\log_{10}$. The control with NPs without irradiation slightly reduced the virus titer (by 0.45 $\log_{10}$). The ability of NPs to completely inactivate adenovirus was maintained for 3 h. It was shown for the first time that the non-enveloped HAdV5 virus can be efficiently inactivated by UV-induced TiO_2 photocatalysis.

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Competing Interests. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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ФОТОДИНАМІЧНА ОБРОБКА НАНОЧАСТИНОК ДІОКСИДУ ТИТАНУ — ЗРУЧНИЙ МЕТОД ІНАКТИВАЦІЇ АДЕНОВІРУСУ

Сьогодні пошук безпечних способів інактивації збудників стає особливо актуальним у зв'язку з пандемією коронавірусу. Стандартні методи з використанням хлоридів і ультрафіолетового опромінення мають недоліки, пов'язані з їхньою токсичністю та низькою ефективністю проти безоболонкових вірусів. Фотодинамічна інактивація за участю наночастинок (НЧ) вже використовується для знезараження води та повітря від мікроорганізмів і вірусів із супероболонкою, таких як вірус простого герпесу людини, вірус везикулярного стоматиту, вірус імунодефіциту людини та віруси гепатиту В і С. **Метою** даної роботи було оцінити можливість інактивації аденовірусу людини типу 5 в органічному середовищі за допомогою діоксиду титану, опроміненого ультрафіолетовим світлом. **Методи.** Нанорозмірний діоксид титану отримано шляхом термічного розкладання суспензії гідратованого діоксиду титану $TiO(OH)_2$ (метатитанової кислоти). Наночастинки (НЧ) TiO_2 розчиняли в пропандіол-етанолі та гліцерин-воді. Аналіз морфології нанопорошку TiO_2 виконано за допомогою скануючої (SEM) та трансмісивної (TEM) електронної мікроскопії. Визначення цитотоксичної, віруліцидної та противірусної дії діоксиду титану проведено стандартними методами з використанням 3-(4,5-диметилтіазол-2-іл)-2,5-дифенілтетразолію броміду (МТТ). Для фотоактивації суспензію НЧ опромінювали на відстані 20 см від бактерицидної УФ-лампи (ОВВ15Р, BactoSfera, Польща (254 нм)) протягом 1–30 хв. Початкова концентрація НЧ для опромінення становила 1,0 мг/мл. Після фотоактивації НЧ до них додавали суспензію аденовірусу людини серотипу 5 із титром 6,0 $\log_{10}$ТЦД$_{50}$/мл. Визначали титр аденовірусу, синтезованого в присутності НЧ TiO_2, за кінцевою точкою розведення вірусу, що спричиняє 50% розвитку цитопатичної дії вірусу на клітини. **Результати**. Показано, що нанопорошок TiO_2 містить м'які агрегати наночастинок переважно розміром 20–30 нм. Виявлено дозозалежний вплив НЧ на життєздатність клітин Нер-2. НЧ TiO_2, внесені в пропандіол-етанол, знижували інфекційний титр аденовірусу на 6,0 $\log_{10}$, що свідчить про їх високу віруліцидну дію. НЧ TiO_2у воді-гліцерині не мали віруліцидної дії. За додавання до аденовірусу НЧ, фотоактивованих УФ протягом 1–30 хв, виявлено зниження титру вірусу на 4,5–5,0 $\log_{10}$. Такий ефект зберігався ще протягом 3 год після опромінення НЧ. Фотоактивовані НЧ у воді-гліцерині не впливали на інфекційність аденовірусу, максимальне зниження титру становило 0,5 $\log_{10}$. **Висновки.** Визначено цитотоксичну, віруліцидну та противірусну дію оптично активованих наночастинок TiO_2. НЧ мали низьку токсичність у концентрації 0,1 мг/мл. Розчинені в пропандіол-етанолі НЧ TiO_2 знижували інфекційний титр аденовірусу на 6,0 $\log_{10}$, що вказує на їх високу віруліцидну дію. Фотоактивовані протягом 1–30 хв НЧ повністю інактивували аденовірус та зберігали цю здатність протягом 3 год після опромінення. Таким чином, вперше показано, що фотокаталіз TiO_2, індукований УФ-променями, може ефективно інактивувати аденовірус людини, що належить до групи безоболонкових вірусів. Встановлено, що фотоактивований TiO_2 має пролонговану противірусну активність.

***Ключові слова**: наночастинки діоксиду титану, фотоінактивація, аденовірус.*