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COMPARATIVE TOXICOLOGICAL-HYGIENIC ASSESSMENT, STRUCTURAL-MORPHOLOGICAL, PHYSICOCHEMICAL CHARACTERISTICS, AND VIRUCIDAL PROPERTIES OF NEW NANOPOWDER MATERIALS TiO_2 AND TiO_2@Ag

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Ключові слова: *наноматеріали, електронна мікроскопія, токсикологія, цитокіни, окиснювальні ензими, гігієнічне нормування, цитотоксичність, аденовірус*

Abstract. Comparative toxicological-hygienic assessment, structural-morphological, physicochemical characteristics, and virucidal properties of new nanopowder materials TiO_2 and TiO_2@Ag. Yavorovsky O.P., Riabovol V.M., Zinchenko T.O., Zahornyi M.M., Ragulya A.V., Tyschenko N.I., Povnitsa O.Yu., Artiukh L.O., Zahorodnia S.D., Ostapiv D.D. *In order to address safety concerns related to the acquisition and utilization of TiO_2 and TiO_2@Ag nanomaterials, as well as to investigate their disinfectant and biological effects, the structural-morphological, morphometry, toxicological, cytotoxic, and virucidal properties of these specified nanomaterials have been studied through experiments conducted on laboratory animals and in vitro. It has been demonstrated that the TiO_2@Ag nanocomposite exhibited distinct physicochemical characteristics: it consisted of TiO_2 nanoparticles ranging in size from 13 nm to 20 nm and Ag nanoparticles ranging from 35 nm to 40 nm with 4.0 wt% of silver localized on the surface of titanium dioxide. The purity of the modification of synthesized nano-TiO_2 and nano-TiO_2@Ag has been confirmed. Acute intraperitoneal administration of nanopowders revealed LD_{50} values of 4783.30 mg/kg for nano-TiO_2 and 724.44 mg/kg for nano-TiO_2@Ag. A slight accumulation was observed upon repeated (28-fold) intragastric administration of nano-TiO_2. The cumulative dose administered, which equated to 15.9 multiples of the LD_{50} (76040 mg/kg), did not result in animal mortality but led to retardation in body weight gain. TiO_2 and TiO_2@Ag nanopowders do not irritate the skin, induce mild conjunctival irritation, and may exhibit a weak sensitizing effect. Nano-TiO_2 and nano-TiO_2@Ag powders accumulate in the tissues of internal organs and cause damage to the liver, kidneys, and lungs of laboratory animals upon*

intraperitoneal administration. The most characteristic morphological signs of the toxic effect of nano-TiO_2 on liver tissue were observed at a level of 67.7% (cytoplasmic vacuolization in hepatocytes), while in the case of nano-TiO_2@Ag initial necrotic changes were at a level of 70.0% (hepatocytes with pyknotic nuclei). Immunoassay analysis has demonstrated that TiO_2@Ag and TiO_2 nanomaterials at concentrations of 30 μg/ml can enhance the functional activity of peripheral blood mononuclear cells in vitro by increasing the production of cytokines IL-1, IL-6, TNF-α, and IL-4 in donors ($p<0.05$). This indicates the potential for chronic inflammation and allergic reactions among synthesis operators. In the study of the impact of nanomaterials on murine germ cells, it has been established that they affect the activity of mitochondrial enzymes and exert a damaging effect on mitochondrial membranes and overall cell integrity. Estimated approximate safe exposure levels in the workplace air are 0.3 mg/m^3 for nano-TiO_2 and 0.2 mg/m^3 for nano-TiO_2@Ag. Nano-TiO_2@Ag and nano-TiO_2 at a concentration of 100 μg/ml exhibit pronounced extracellular virucidal activity against human adenovirus serotype 2. The TiO_2@Ag nanocomposite has a less damaging effect on Hep-2 cells compared to nano-TiO_2.

Реферат. Порівняльна токсиколого-гігієнічна оцінка, структурно-морфологічні, фізико-хімічні характеристики та віруліцидні властивості нових нанопорошкових матеріалів TiO_2 та TiO_2@Ag. Яворовський О.П., Рябовол В.М., Зінченко Т.О., Загорний М.М., Рагуля А.В., Тищенко Н.І., Повниця О.Ю., Артюх Л.О., Загородня С.Д., Остапів Д.Д. *Для вирішення безпекових питань одержання і застосування наноматеріалів TiO_2 і TiO_2@Ag, а також вивчення їх знезаражувальної і біологічної дії вивчені структурно-морфологічні, морфометричні, токсикологічні, цитотоксичні та віруліцидні властивості зазначених наноматеріалів в експериментах на лабораторних тваринах та in vitro. Показано, що нанокомпозит TiO_2@Ag характеризується фізико-хімічними особливостями: складається з наночастинок TiO_2 розміром від 13 нм до 20 нм і Ag – від 35 нм до 40 нм, срібло локалізується на поверхні діоксиду титану, вміст наносрібла відносно титану становить 4,0 мас.%. Підтверджена чистота модифікації анатазу синтезованих нано-TiO_2 і нано-TiO_2@Ag. При гострому внутрішньоочеревинному введенні нанопорошків установлено, що LD_{50} для нано-TiO_2 дорівнює 4783,30 мг/кг, LD_{50} для нано-TiO_2@Ag – 724,44 мг/кг. При повторному (28-кратному) внутрішньошлунковому введенні нано-TiO_2 відзначена слабо виражена кумуляція. Сумарна введена доза, яка дорівнювала 15,9 одноразових LD_{50} (76040 мг/кг), не призводила до загибелі тварин, але зумовлювала відставання в прирості маси тіла. Нанопорошки TiO_2 і TiO_2@Ag не подразнюють шкіру, викликають слабке подразнення кон'юнктиви і можуть зумовлювати слабко виражену сенсибілізуючу дію. Нанопорошки TiO_2 і TiO_2@Ag накопичуються в тканині внутрішніх органів та пошкоджують печінку, нирки й легені лабораторних тварин при внутрішньоочеревинному введенні. Найбільш характерними морфологічними ознаками токсичної дії нано-TiO_2 на тканину печінки були дистрофічні зміни на рівні 67,7% (цитоплазматична вакуолізація в гепатоцитах), тоді як у випадку нано-TiO_2@Ag – початкові некротичні зміни були на рівні 70,0% (гепатоцити з пікнотичними ядрами). За результатами імуноферментного аналізу доведено, що наноматеріали TiO_2@Ag і TiO_2 у концентраціях 30 мкг/мл здатні підвищувати функціональну активність мононуклеарних клітин периферичної крові в умовах in vitro за продукцією цитокінів IL-1, IL-6, TNF-α і IL-4 у донорів ($p<0,05$), що свідчить про потенційне хронічне запалення та алергічні реакції в операторів синтезу. При вивченні впливу наноматеріалів на статеві клітини кнурів показано, що вони впливають на активність мітохондріальних ензимів і справляють ушкоджуючу дію на мембрани мітохондрій і загалом клітин. Розраховані орієнтовні безпечні рівні впливу в повітрі робочої зони становлять для нано-TiO_2 – 0,3 мг/м^3, а для нано-TiO_2@Ag – 0,2 мг/м^3. Наноматеріали TiO_2@Ag і TiO_2 у концентрації 100 мкг/мл мають виражену позаклітинну віруліцидну дію на аденовірус людини 2 серотипу. Нанокомпозит TiO_2@Ag порівняно з нано-TiO_2 має меншу ушкоджуючу дію на клітини Нер-2.*

Today, optically active metal oxide nanocomposites with Ag, Au noble metals have been engineered and applied to improve the efficacy of the diagnosis and treatment of diseases. Nanomaterials based on titanium dioxide, with high photocatalytic activity, hold promising potential for organic pollutant degradation, disinfection, and creation of antimicrobial and self-cleaning surfaces [1, 2, 3]. It is known that photocatalytic activity can be enhanced by incorporating specific amounts of noble metals into titanium dioxide (anatase) [1, 2]. In particular, nanopowders based on titanium dioxide are synthesized at the Frantsevich Institute for Problems of Materials Science NASU using their original method – thermal decomposition of metatitanic acid with the addition of silver in various ratios relative to titanium. This article presents research on a nanocomposite material of titanium dioxide that contained 4 wt% silver nanoparticles.

The toxic effects of metal nanoparticles are associated with their nanoscale size and high surface activity, which allows them to penetrate cells and interact with biological structures. This can lead to the generation of reactive oxygen species, oxidative stress, DNA damage, disruption of cell membranes, organ and tissue dysfunction, inflammation, immunotoxicity, and the development of long-term consequences such as cancer, neurotoxicity, reproductive disorders, and other adverse effects [3, 4].

However, information about the morphometrics, toxicological and virucidal activity (model adenovirus HAdV2) of TiO_2 nanopowders and its

composite modified with 4 wt% of silver nanoparticles, synthesized by the thermal decomposition of metatitanic acid in a multi-section rotary kiln, was not available in the sources of scientific literature accessible to us at the beginning of our research.

To address safety concerns related to the production and application of these nanomaterials, as well as to study their disinfection properties, it was necessary to obtain information about the structure, morphometry, physicochemical properties, toxicological characteristics of nano-TiO_2 and nano-TiO_2@Ag, as well as their biological activity.

The purpose of the study was the comparative assessment of structural-morphological, morphometrics, toxicological, cytotoxic, and virucidal properties of nano-TiO_2 and nano-TiO_2@Ag in experiments on laboratory animals and *in vitro*.

MATERIALS AND METHODS OF RESEARCH

The morphology of nano-TiO_2 and nano-TiO_2@Ag, as well as the structure of their surfaces, were investigated using scanning electron microscopy (SEM). For this purpose, a Mira 3 Tescan microscope (Czech Republic) with a ZEISS EVO 50 XVP energy-dispersive spectrometer (United Kingdom) was employed. Powders weighing 10 mg were applied onto polished Si plates (5×5 mm) with subsequent sputtering of gold-palladium (1:1) with a thickness of 30 nm for 15 minutes.

The morphology and structure of the investigated samples were also determined using a transmission electron microscope JEM-1400 (JEOL, Japan) at instrumental magnifications ranging from 2000 to 100000 and an accelerating voltage of 80 kV [3].

The structure and crystalline phase of nano-TiO_2 and nano-TiO_2@Ag were investigated using an X-ray diffractometer DRON-3M [1].

The size of nanoparticle agglomerates in the dispersion medium was determined using laser granulometry on the Analysette 12 DynaSizer instrument.

The assessment of toxicity of titanium dioxide nanopowders (nano-TiO_2) and titanium dioxide-nanosilver composite (nano-TiO_2@Ag) was conducted through acute and subacute experiments on mice, guinea pigs, and rabbits using commonly accepted toxicological methods.

The irritation effects on the eye mucosa of rabbits and the skin of guinea pigs were investigated according to the methods [5, 6]. Sensitizing effects of the nanopowders were studied using the method [7]. General toxicity was studied under conditions of intraperitoneal administration of nanopowder suspensions to mice, followed by the calculation of LD_{50} using the Probit analysis method in the modification by V.V. Prozorovsky. Cumulative properties of TiO_2 nanopowder were investigated using the method of Lim et al. [8] under conditions of repeated oral administration to rats. The titanium and silver content in the liver, kidneys, spleen, heart, lungs, and brain tissues of the test mice were determined by inductively coupled plasma optical emission spectroscopy. Microscopic preparations of liver tissue were examined using an Olympus BX51 light microscope.

The impact of nanomaterials, nano-TiO_2, and nano-TiO_2@Ag, on the immune system was studied using the enzyme-linked immunosorbent assay (ELISA) method, measuring the functional activity of peripheral blood mononuclear cells from healthy donors in an *in vitro* setting. Cytokine production, including interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), was assessed. Mononuclear cells were incubated with phytohemagglutinin mitogen (FGA), nano-TiO_2, and nano-TiO_2@Ag at a concentration of 30 μg/mL, both with and without the stimulating agent (spontaneous production).

The effects of nano-TiO_2 and nano-TiO_2@Ag on cellular oxidative metabolism were investigated using freshly obtained sperm from boar (n=6). Parameters such as respiratory activity (ng-atom O_2/0.1 ml per min), succinate dehydrogenase (SDH) activity (units/g of tissue x 0.1 ml per hour), cytochrome oxidase (COX) activity (units/g of tissue x 0.1 ml per hour), and sperm survival (hours) were measured. The experimental samples were exposed to nanomaterials at doses of 1/100 LD_{50}, 1/10 LD_{50}, and 1.0 LD_{50}, which were calculated in terms of milliliters of sperm. The calculated quantitative LD_{50} doses per milliliter of sperm were 47 μg/mL for nano-TiO_2 and 7 μg/mL for nano-TiO_2@Ag.

The conditions for keeping and using laboratory animals complied with the rules and provisions of the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986). The research was conducted in accordance with the principles of bioethics and was approved by the Commission on Bioethical Expertise and Ethics of Scientific Research at the Bogomolets National Medical University, protocol number 128 dated December 23, 2019.

For the assessment of biological activity, a cell culture of Hep-2 cells (human laryngeal carcinoma cells) and human adenovirus serotype 2 (HAdV2) were used. Cells were cultured in a mixture of DMEM (Biowest, France) and RPMI 1640 (Biowest, France) media with 10% fetal bovine serum (Sigma, USA). Adenovirus was cultured according to standard methodology [9].

Cell viability was assessed based on mitochondrial activity using the MTT solution (NeoFroxx, Germany) following standard methodology [10]. To

determine the virucidal activity, a virus suspension was mixed with an equal volume of nanomaterial suspension and incubated at 37°C for 1 hour. A series of 10-fold dilutions were prepared, and sensitive cells were infected with 50 µl of the mixture in each well of a 96-well plate. The virus was adsorbed at 37°C for 1 hour, followed by the addition of 150 µl of serum-free medium per well. The plate was maintained at 37°C with 5% CO_2 until pronounced cytopathic effects of the virus appeared (up to 4 days). The MTT solution was used for spectrophotometric analysis at a wavelength of 540 nm on a Multiskan FC plate reader (Thermo Fisher Scientific, USA). The virus's infectious titer was determined using Microsoft Excel software for Pentium Pro's predictive function. A reduction in the infectious titer of the virus by 2 $\log_{10}$ $TCID_{50}$ or more, compared to the virus control, indicated a virucidal effect [11].

The statistical analysis of the obtained results was conducted considering the normal distribution of data using the Shapiro-Wilk test . Parametric statistical criteria were employed for analysis, including the Student's t-test, analysis of variance (ANOVA), and the Scheffe multiple comparison method. Non-parametric methods used included the Wilcoxon W-test, the Kruskal-Wallis rank one-way analysis of variance, and multiple comparisons using Dunn's test. Statistical significance was considered at $p<0.05$. The data were presented as the mean value ± confidence interval at a confidence level of 95%. Data analysis was performed using the MedStat v.5.2 software package (Copyright © 2003-2019) [12].

RESULTS AND DISCUSSION

Morphology and structural characteristics of nanotitanium dioxide and nanotitanium dioxide, modified with nanosilver. Results of the study scanning electron microscopy (SEM) of nano-TiO_2 are shown on the electron microscopically in Fig. 1. It was established that TiO_2 nanopowder contains mainly nanoparticles with a size of 21÷28 nm. TiO_2 nanopowder has developed surface structure due to mesopores (pores 2÷50 nm) and specific surface area 50.84 m^2/g. SEM study of nano-TiO_2@Ag (Fig. 2) its dimensions are established – 17÷22 nm. The features of morphological and structural characteristics (SEM and TEM) of titanium nanodioxide supplemented with nanosilver are presented by us in previously published works [1, 3]. As "ball-shaped" silver particles in the investigated nanocomplex localized on the surface of titanium dioxide particles were noted. Sizes of nanoparticles Ag was 35-40 nm, and the size of TiO_2 nanoparticles was 13-20 nm in composite material.

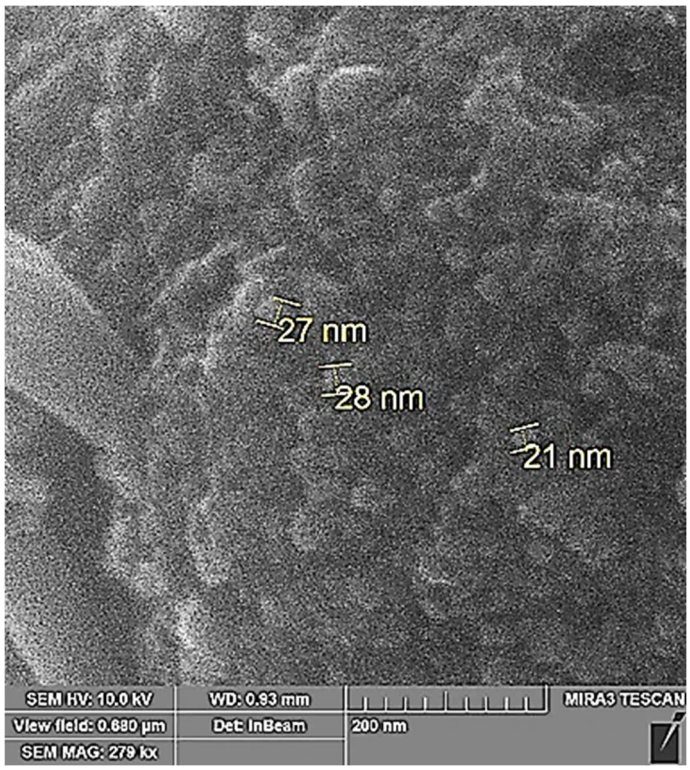


Fig. 1. Scanning electron microscope image of nano-TiO_2 at ×279000 magnification, a scale bar 200 nm

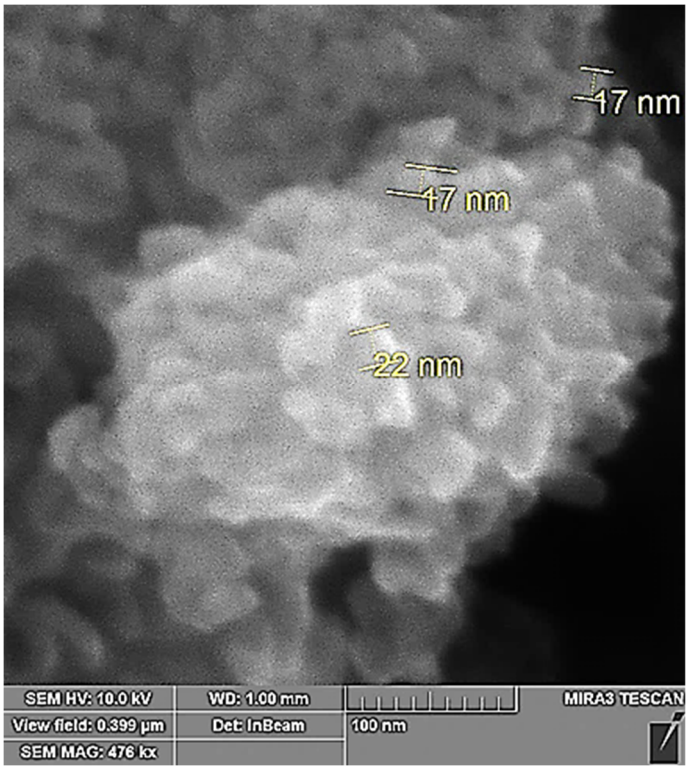


Fig. 2. Scanning electron microscope image of nano-TiO_2@Ag at ×476000 magnification, scale bar 100 nm

X-ray diffraction analysis (XRD) was utilized for the identification of the crystalline phase type of TiO_2 and TiO_2@Ag nanopowders (rutile, anatase, brookite). The obtained values of crystal lattice parameters and crystallite size for both TiO_2 and TiO_2@Ag indicate the tetragonal anatase modification of the metal oxide, even in the presence of silver nanoparticles located on the surface layer [1, 13].

To investigate the size of agglomerates, the samples of powders were dispersed in stabilizing media of various natures. The maximum reduction in agglomerates is observed for the nano-TiO_2 composite with silver, especially in a mixture of citrate with glucose, which contributes to the reduction of the size of nano-TiO_2 particle agglomerates (Fig. 3). The stabilization of the nanopowder suspension with a glucose-citrate buffer (4 g of glucose, 1 g of sodium citrate, 100 ml of distilled water) significantly contributed to the reduction of agglomerated average sizes of complexes' resulting in 325.53 nm for TiO_2 and 166.76 nm for TiO_2@Ag agglomerates.

Toxicological characteristics of TiO_2 and TiO_2@Ag nanomaterials were assessed through intraperitoneal administration at various doses after preliminary determination of an effective range. Specifically, nano-TiO_2 was administered within the range of 3000 mg/kg to 11000 mg/kg (7 doses), while nano-TiO_2@Ag was administered within the range of 1000 mg/kg to 10000 mg/kg (4 doses). Clinical signs of acute toxicity were observed when administering nano-TiO_2 at doses of 5000 mg/kg or higher, and for nano-TiO_2@Ag at doses of 1000 mg/kg or higher. These signs included decreased interest in food and water, reduced motor activity, lethargy, and depression in the animals. Over a two-week observation period, mouse mortality was noted to increase with higher administered doses. The maximum tolerated dose for nano-TiO_2 was found to be 4000 mg/kg, while for nano-TiO_2@Ag, the tolerated dose was below 1000 mg/kg. The median lethal doses (LD_{50}) calculated using the Prozorovsky method were as follows: for nano-TiO_2 – LD_{50} =4783.30 mg/kg, and nano-TiO_2@Ag – LD_{50} = 724.44 mg/kg.

Introduction of TiO_2 and TiO_2@Ag nanoparticles in their native form into the conjunctival sac of rabbits at a dose of 10 mg caused mild irritation of the conjunctiva, which was assessed on a scale of 1 to 3 points. However, when cream was prepared by mixing nanomaterials TiO_2 and TiO_2@Ag (nanopowder in a 1:1 vaseline mixture) and applied to the shaved skin of guinea pigs, no irritation was observed.

Sensitization of guinea pigs through intracutaneous administration of nanopowder suspension at a dose of approximately 200 mg in a physiological solution, followed by observation of skin changes using provocation tests, revealed that about half of the guinea pigs in the experimental groups showed a

positive provocation test in the form of ear swelling. After sensitization with nanomaterial TiO_2, the swelling was scored as 1 (0.03-0.07 mm), while after sensitization with the nanocomposite nanomaterial TiO_2@Ag, the swelling was scored as 2 (0.08-0.12 mm). Thus, both nanomaterial TiO_2 and nanomaterial TiO_2@Ag can cause weakly expressed sensitization, with slightly stronger effects observed for nanomaterial TiO_2@Ag.

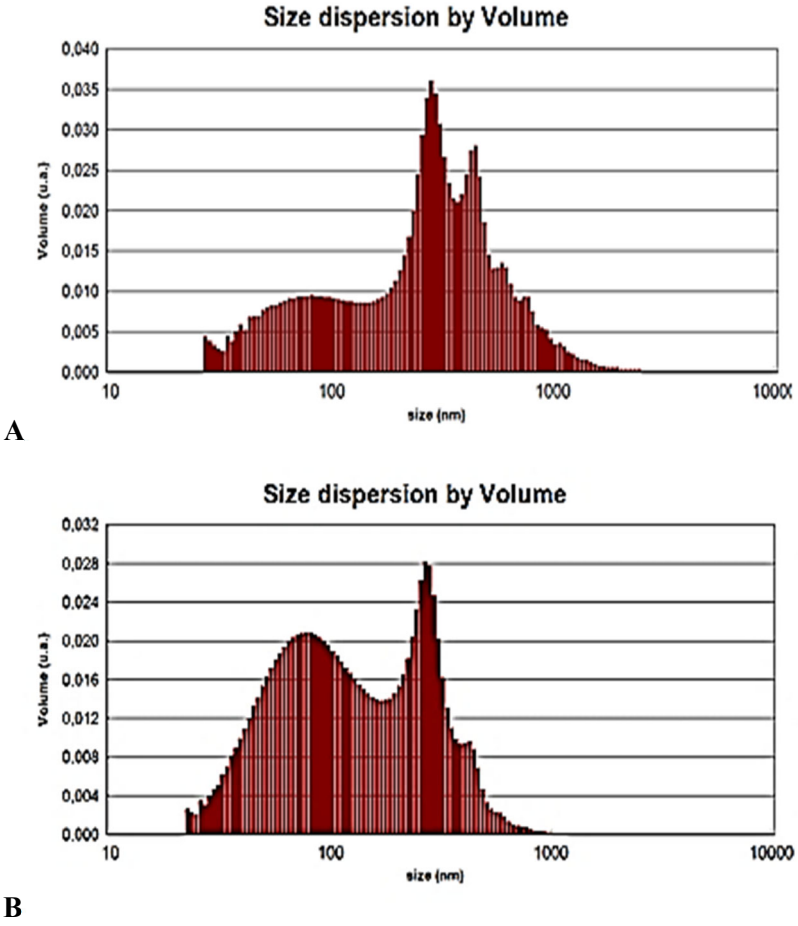


Fig. 3. Distribution of agglomerates by size for compounds TiO_2 in citrate with glucose medium. A –TiO_2, D_{mean}=325.53 nm; B –TiO_2@Ag, D_{mean}=166.76 nm

After repeated (28-fold) intragastric administration of titanium dioxide nanoparticles to rats, starting from 1/10 LD_{50} and increasing by 1.5 times every fifth day, with a cumulative dose of 15.9 LD_{50} (76040 mg/kg), no fatalities occurred among the animals. However, growth retardation was observed in the experimental group compared to the control group.

The determined median lethal doses for nanomaterial TiO_2 were 4783.30 mg/kg and for nanomaterial TiO_2@Ag, it was 724.44 mg/kg. According to the classification by Sidorov K.K., intragastric administration of nanomaterial TiO_2 corresponds to class VI (relatively harmless), while nanomaterial TiO_2@Ag corresponds to class IV (slightly toxic). According to the classification "Hygienic regulations for chemical substances in the air of the working zone" (order of the Ministry of Health of Ukraine No. 1596 dated July 14, 2020) [14], based on the LD_{50} values, nanomaterial TiO_2 falls into the 4th class (moderately hazardous), and nanomaterial TiO_2@Ag belongs to the 3rd class (moderately hazardous).

After a single intraperitoneal administration of high doses ranging from 4000 mg/kg to 10000 mg/kg of the investigated nanopowders, the organs with the highest accumulation of nano-TiO_2 and nano-TiO_2@Ag in mice were found to be the liver, kidneys, lungs, and spleen ($p<0.05$). In liver tissue exposed to nano-TiO_2, predominantly dystrophic histological changes were observed (cytoplasmic vacuolization in hepatocytes) – at a level of 67.7%. Under the influence of nano-TiO_2@Ag, initial necrotic changes prevailed (hepatocytes with pyknosis of nuclei) – at a level of 70%. It should be noted that less frequently, the toxic effect of nano-TiO_2 and nano-TiO_2@Ag

manifested as focal necrosis and inflammatory reactions (focal infiltration). In isolated cases, adaptive changes were observed, characterized by an increase in the number of binuclear hepatocytes. Scanning electron microscopy in combination with morphometry in liver samples revealed aggregates of foreign material (crystalline inclusions), which were spectroscopically examined and showed a high content of titanium (Ti). Morphometry stated (Fig. 4) that the sizes of nanoparticle aggregates ranged from 80 nm to 20 mm. Renal tissue damage manifested as an enlargement of the urinary space in renal corpuscles and dystrophic changes in the epithelium of various degrees of the tubules. In the lungs, dystrophic changes, initial necrotic changes, and hemorrhagic tissue infiltration were observed.

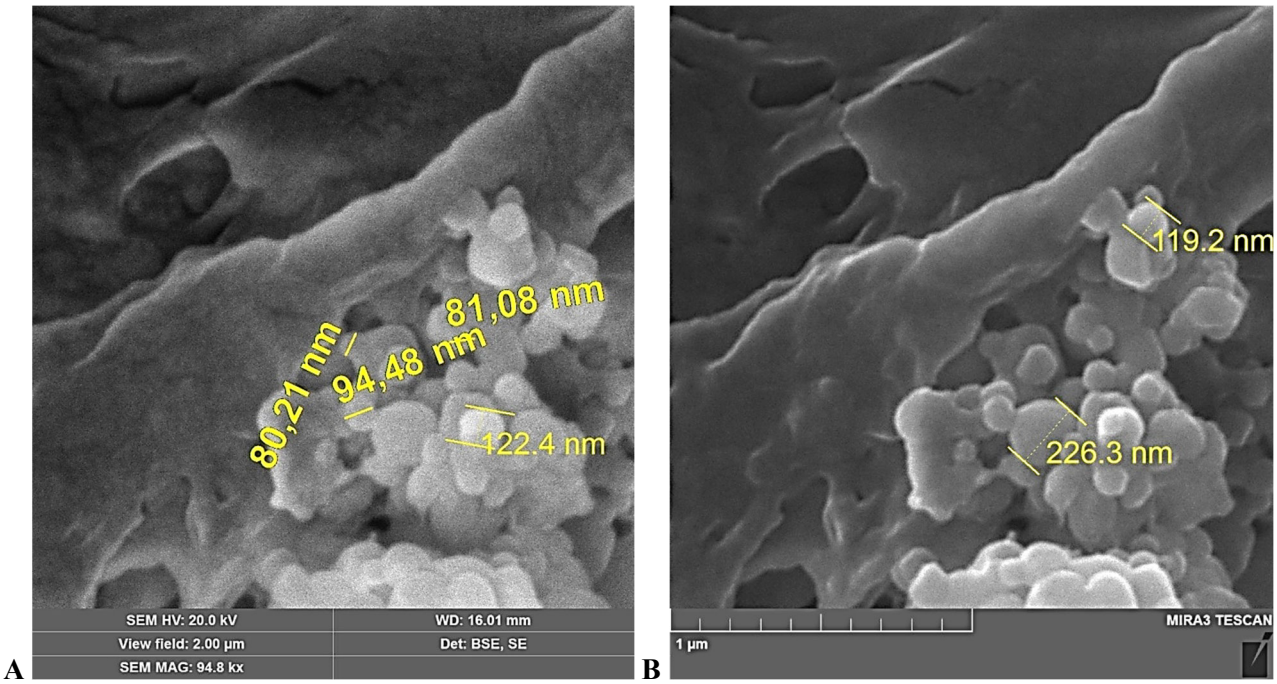
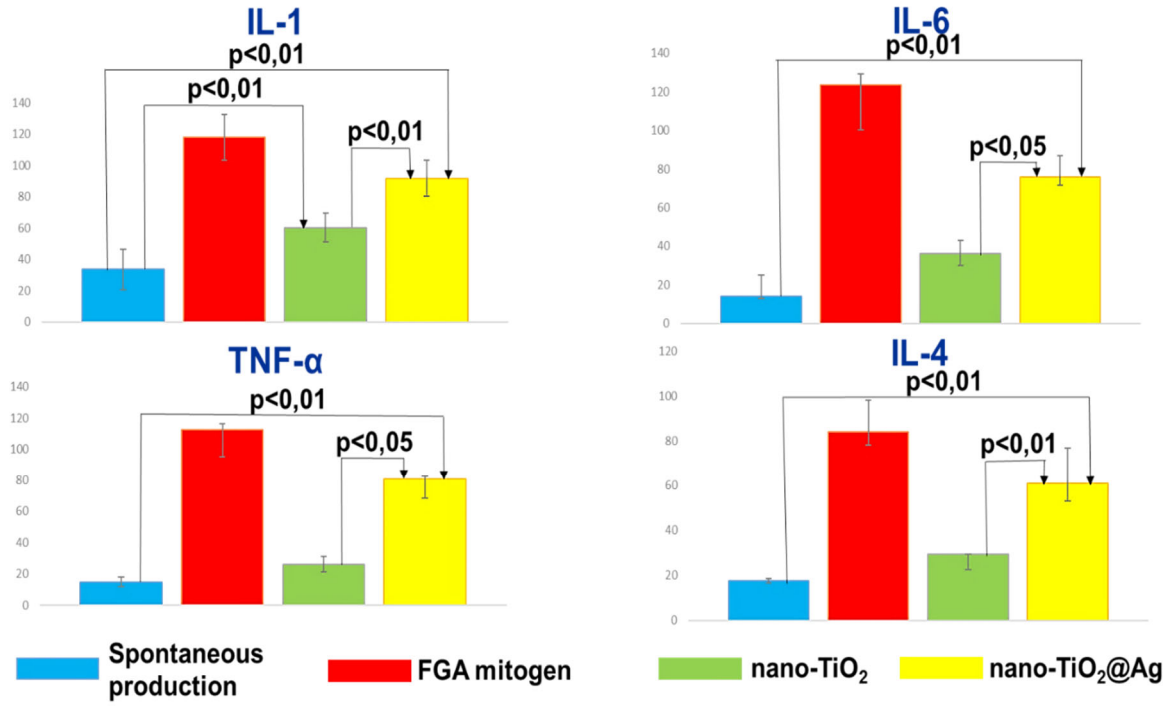


Fig. 4. Results of SEM with nano-TiO_2 morphometry application.
Agglomerate of nano-TiO_2 in the liver of mice 1 day after intraperitoneal administration.
A – BSE (backscattered electron) imaging mode; B – SE (secondary electron) imaging mode

The summarized results of the influence of TiO_2 and TiO_2@Ag nanomaterials at concentrations of 30 µg/ml on the *functional activity of mononuclear cells* from donors in terms of cytokine production (IL-1, IL-6, TNF-α, IL-4) under in vitro conditions are presented in Figure 5. In terms of conversion, this concentration corresponded to the dose of nanoparticles that accumulate in the body of operators over a 20-year work experience in real production conditions.

As evident from Figure 5, upon stimulation of mononuclear cells, the production of IL-1 increased to 60.38±9.04 pg/ml (1.79-fold increase; $p<0.01$) for nano-TiO_2, and to 91.75±11.49 pg/ml (2.73-fold increase; $p<0.01$) for nano-TiO_2@Ag, compared to spontaneous production. IL-1 production in response to nano-TiO_2@Ag increased by 1.52 times ($p<0.01$) compared to nano-TiO_2. Changes in IL-6 and TNF-α production are shown in Figure 5. Upon stimulation of mononuclear cells, IL-4 production increased to 60.8 (95% CI 52.8-77.6) pg/ml (3.45-fold increase; $p<0.01$) for nano-TiO_2@Ag compared to the control. When comparing the two, the effect of nano-TiO_2@Ag increased the specified parameter by 2.07 times ($p<0.01$) compared to nano-TiO_2. Thus, nano-TiO_2 and nano-TiO_2@Ag at concentrations of 30 µg/ml under *in vitro* conditions can enhance the functional activity of peripheral blood mononuclear cells in terms of cytokine production (IL-1, IL-6, TNF-α) ($p<0.05$), indicating their potential impact on chronic inflammation formation, and increase IL-4 production ($p<0.05$), suggesting a potential allergenic effect for synthesis operators. The functional activity of peripheral blood mononuclear cells in cytokine production increases more intensively under the influence of nano-TiO_2@Ag than nano-TiO_2, indicating a relatively greater potential immunotoxicity risk of nano-TiO_2@Ag for synthesis operators.



Notes: spontaneous production – spontaneous cytokine production; FGA mitogen – cytokine production under stimulation with phytohemagglutinin mitogen.

Fig. 5. Comparative cytokine production by mononuclear blood cells *in vitro* in donors under the influence of nanomaterials, pg/ml

The results of the study of the damaging effect of nano-TiO_2 and nano-TiO_2@Ag on the reproductive cells of boars based on indicators of respiratory activity, mitochondrial enzyme activity (succinate dehydrogenase and cytochrome oxidase), and sperm survival *in vitro* in a comparative aspect are shown in Figure 6. These data is a fragment of the study of the damaging effect of four samples of obtained nanopowders based on titanium on germ cells of wild boars, were previously published by us [15].

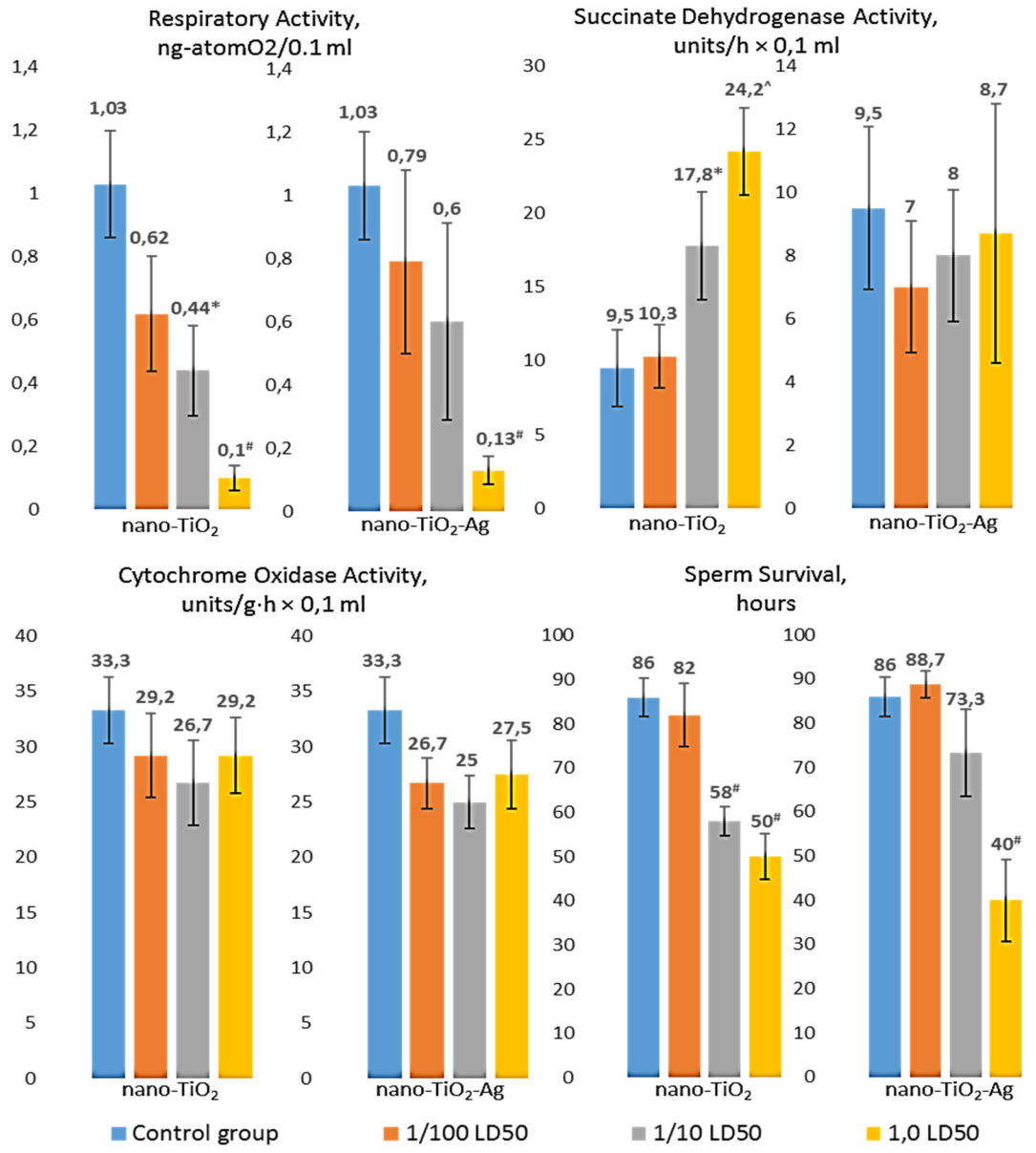
In particular, the respiratory activity of spermatozoa under the influence of titanium dioxide nanoparticles was significantly reduced compared to the control, starting from a dose of 1/10 LD_{50} and higher. Specifically, the respiratory activity value under the influence of nano-TiO_2 significantly reduced by 57.3% compared to the control ($p<0.05$). At a dose of 1.0 LD_{50}, both nanopowders had a significant impact on sperm respiratory activity: the respiratory activity decreased by 90.3% ($p<0.001$) under the influence of nano-TiO_2, and by 87.4% ($p<0.001$) under the influence of nano-TiO_2@Ag compared to the control. Suppression of sperm respiratory activity due to TiO_2-based nanoparticle exposure is likely attributed to disruption in substrate utilization and electron transport in the mitochondrial respiratory chain of cells, which is characterized by changes in mitochondrial enzyme activity.

The research findings indicated that under the influence of 1/10 LD_{50} nano-TiO_2, the activity of succinate dehydrogenase (SDH) increased by 87.4% ($p<0.05$) in samples, and at 1.0 LD_{50}, it increased by 154.7% ($p<0.01$) relative to the control. Nano-TiO_2@Ag did not significantly affect the level of the mentioned enzyme.

It was determined that both nanopowders at a dose of 1/10 LD_{50} had a clear impact on the magnitude of the physiological survival indicator. Upon the addition of nano-TiO_2@Ag, survival decreased by 14.8%, and under the influence of nano-TiO_2, it decreased by 34.6% compared to the control ($p<0.001$). When applying the maximum dose (1.0 LD_{50}), the sperm survival in all samples was likely lower than in the control. Under the influence of nano-TiO_2, the indicator value decreased to 50.0±5.23 hours (by 41.9%; $p<0.001$), and under the influence of nanoTiO_2@Ag, it decreased to 40.0±9.24 hours (by 53.5%; $p<0.001$).

Thus, titanium dioxide compounds (nano-TiO_2 and nano-TiO_2@Ag), characterized by similar morphological characteristics, starting from a dose of 1/10 LD_{50}, exhibit a damaging effect on boar reproductive cells, manifested by reduced sperm respiratory activity and increased succinate dehydrogenase activity. Since SDH is an intramitochondrial enzyme, it is evident that nano-TiO_2, compared

to nano-TiO_2@Ag, exerts a more pronounced damaging effect on cell membranes, including mitochondria. Increased organelle membrane permeability leads to easier substrate access to the enzyme, resulting in its increased activity. The most sensitive marker turned out to be sperm survival, which significantly decreased under the influence of nano-TiO_2 at a dose of 1/10 LD_{50}, as a consequence of disruption in mitochondrial enzyme activity and, consequently, ATP resynthesis.



Notes: statistical significance compared to control: * – $p<0.05$; ^ – $p<0.01$; # – $p<0.001$.

Fig. 6. Damaging effect of nano-TiO_2 and nano-TiO_2@Ag on boar reproductive cells in experiment *in vitro*

The estimated safe levels of exposure (ESLE) for nano-TiO_2 and nano-TiO_2@Ag in the work area air were calculated based on the data obtained in the toxicological experiment. The ESLE values were calculated using three mathematical equations that take into account the median lethal dose LD_{50} of the nanopowder, the molecular weight of the compound, the number of metal atoms in the compound, and safety and margin factors [16]. The ESLE was determined as the average value obtained from the three mathematical equations used for each nanopowder.

For nano-TiO_2, the calculated ESLE is 0.3 mg/m^3, and for nano-TiO_2@Ag, it is 0.2 mg/m^3. It is worth noting that the National Institute for Occupational Safety and Health (NIOSH) has stated ESLE for TiO_2 nanoparticles at 0.3 mg/m^3 value. The ESLE values proposed by us are approximate to the NIOSH standard for titanium dioxide nanoparticles.

Biological activity study of TiO_2 and TiO_2@Ag nanopowders. The cytotoxic effect of nanoparticles was investigated in permissive for adenoviruses Hep-2 cells. As shown in Figure 7, at a concentration of 100 µg/ml, the studied nanomaterials were toxic to the cells and led to partial cell death. In further dilutions, nano-TiO_2@Ag (4 wt%) exhibited no toxicity, while TiO_2 remained partially toxic even in subsequent dilutions. Using the predictive function of Microsoft Excel 2010, cytotoxic concentration indicators-CC_{50} – were determined. Thus, the CC_{50} values for nano-TiO_2 and nano-TiO_2@Ag for Hep-2 cells were 15 µg/ml and 64 µg/ml, respectively.

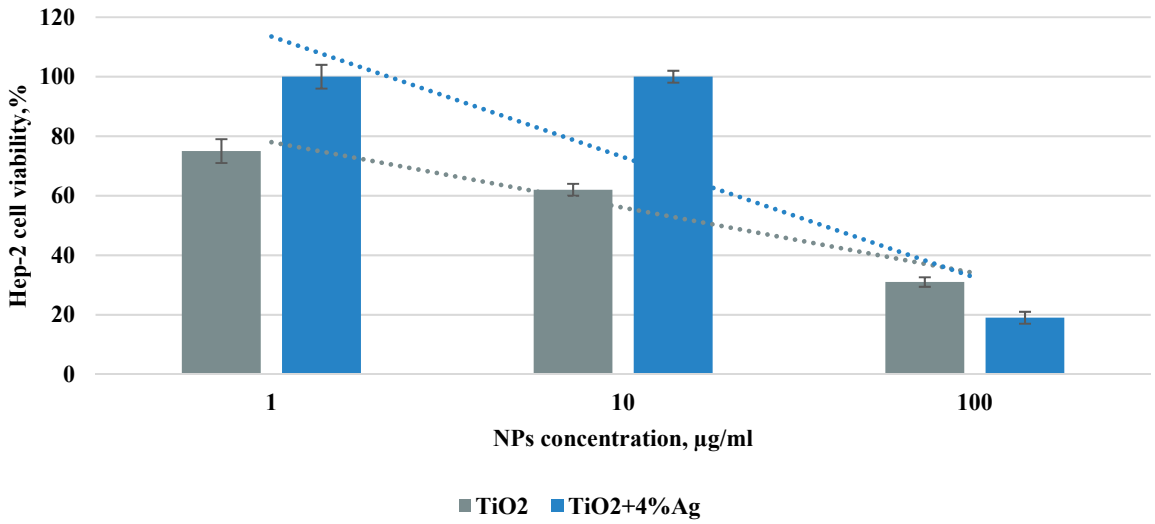


Fig. 7. Cytotoxic effect of nano-TiO_2 and nano-TiO_2@Ag in Hep-2 cell culture

The determination of virucidal activity is based on the interaction of the test sample with the extracellular virus and its ability to inactivate its infectivity [17, 18]. In our the experiment, nano-TiO_2 and nano-TiO_2@Ag were used at concentrations of 100 µg/ml. The obtained results indicate that both titanium dioxide and silver-modified titanium dioxide (4 wt%) exhibit equally pronounced virucidal activity against adenovirus. When the nanoparticles (final concentration 50 µg/ml) were in contact with the adenovirus for 1 hour, they completely inactivated its infectivity (Fig. 8). The reduction in virus infectivity titer for both types of nanoparticles was 7.5 $\log_{10}$ $TCID_{50}$/ml.

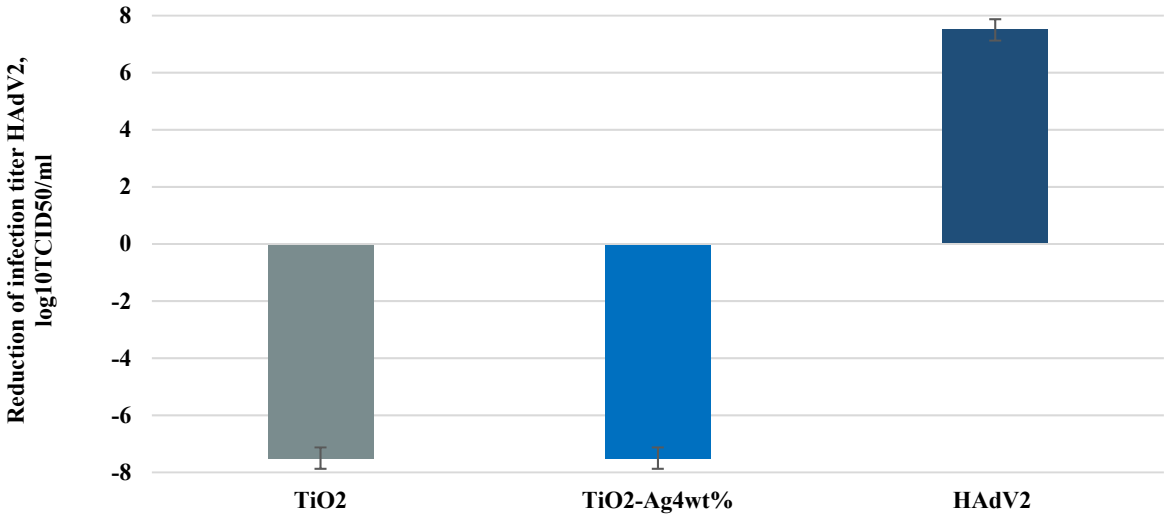


Fig. 8. Virucidal effect of nano-TiO_2 and nano-TiO_2@Ag against HAdV2, contact time with the virus 1 hour. The virus control titer was 7.5 $\log_{10}$ $TCID_{50}$/ml

The impact of nano-TiO_2 and nano-TiO_2@Ag on virus reproduction within cells was investigated by introducing them into cells immediately after virus adsorption. It has been demonstrated that the antiviral activity of both nanomaterials was absent (not illustrated). We note, that a successfully reduction in the infectious herpes virus titer by 5.0 $\log_{10}$ $TCID_{50}$/ml was observed after 1 h of contact, and a decrease of 7.5 and 7.9 $\log_{10}$ TCID50/ml titer for influenza type A virus was achieved for nano-TiO_2 and nano-TiO_2@Ag, respectively [18].

Thus, the new TiO_2 and TiO_2@Ag nanopowders exhibit structural features that distinguish them from other metal nanomaterials. For the first time, it has been demonstrated that in the nano-TiO_2@Ag composite, silver is localized on the surface of titanium dioxide, allowing us to associate the influence of this phenomenon with the biological activity of TiO_2 nanoparticles [3, 18].

Toxicological studies of nano-TiO_2 and nano-TiO_2@Ag revealed their lack of irritant effects on the skin, weak irritation of the conjunctiva, mild sensitizing effects, and for nano-TiO_2, weakly pronounced accumulation was also observed. It has been shown that nano-TiO_2 belongs to the 4th class (slightly hazardous), while nano-TiO_2@Ag belongs to the 3rd class (moderately hazardous). This is consistent with our previous research regarding nitride of titanium, chromium disilicide, lead sulfide compounds, and others [3, 19].

The observed effect of nano-TiO_2 and nano-TiO_2@Ag on oxidative-reductive processes in boar germ cells expand our understanding of the damaging effects of nanomaterials on living organisms [20].

The combined application of SEM and EDS (energy dispersive spectroscopy) methods made it possible to reliably establish the presence of TiO_2 inorganic inclusions in the organs (mainly in the liver). The appearance of crystal inclusions at the level of the capsule organs (liver and kidney) and in the parenchyma of organs was associated with intraperitoneal injection of nano-TiO_2.

In vitro, TiO_2 and TiO_2@Ag nanomaterials are capable of increasing the functional activity of peripheral blood mononuclear cells by producing cytokines IL-1, IL-6, TNF-α ($p<0.05$), indicating their potential impact on chronic inflammation formation, and they also increase the production of IL-4 ($p<0.05$), suggesting a possible allergic effect for synthesis operators. These findings are consistent with studies of other authors [19].

TiO_2 and TiO_2@Ag nanopowders exhibit a pronounced virucidal effect against adenovirus (HAdV2), which could be a promising step towards creating active antimicrobial materials based on TiO_2 with low cytotoxicity [17].

CONCLUSIONS

1. It has been observed that the nano-TiO_2 powder consists of nanoparticles ranging in size from 20 nm to 30 nm, prone to aggregation. The nano-TiO_2@Ag composite is composed of TiO_2 nanoparticles ranging from 13 nm to 20 nm and Ag nanoparticles ranging from 35 nm to 40 nm (the content of nanosilver compared to titanium is 4.0%); silver is localized on the surface of titanium dioxide. Both nanopowders exhibit an anatase crystalline structure; a developed surface structure with mesopores and a specific surface area of 50.84 m^2/g for nano-TiO_2 and 50.11 m^2/g for nano-TiO_2@Ag has been noted. Laser granulometry demonstrated that ultrasonic dispersion in glucose-citrate buffer allows for buffer desegregation of nano-TiO_2 and nano-TiO_2@Ag powder agglomerates.
2. In acute intraperitoneal administration, the stated LD_{50} is 4783.30 mg/kg for nano-TiO_2, and for nano-TiO_2@Ag it is 724.44 mg/kg. TiO_2 and TiO_2@Ag nanoparticles do not cause skin irritation, induce mild conjunctival irritation, and may have weak sensitizing effects. Toxicity and hazard were found to be higher when administering the nano-TiO_2@Ag composite as compared to nano-TiO_2 alone. According to the classification "Hygienic Regulations for Chemical Substances in the Air of the Working Zone," based on the LD_{50} values, nano-TiO_2 corresponds to Class 4 (low hazard), while nano-TiO_2@Ag falls into Class 3 (moderately hazard). Upon repeated (28-fold) intragastric administration of nano-TiO_2, a weakly pronounced accumulation was observed (the total administered dose amounted to 15.9 LD_{50} or 76040 mg/kg).
3. Nano-TiO_2 and nano-TiO_2@Ag nanoparticles accumulate in the liver, kidneys, lungs, and spleen of laboratory animals upon intraperitoneal administration. It has been observed that with an increase in the administered dose (from 4000 mg/kg to 10000 mg/kg), the accumulation of titanium (under the influence of nano-TiO_2) and titanium and silver (under the influence of nano-TiO_2@Ag) increases in the liver and other organs of laboratory mice. Microscopic signs of toxic effects of nano-TiO_2 and nano-TiO_2@Ag nanoparticles after intraperitoneal administration to laboratory mice include dystrophic changes in hepatocytes, and initial necrotic changes in liver parenchyma, while inflammatory reactions are less common. Kidney tissue damage is characterized by an enlargement of the urinary lumen in renal corpuscles and dystrophic changes in epithelial cells of varying degrees in renal tubules. In the lungs, dystrophic and necrotic changes, as well as hemorrhagic infiltration of tissue, have been detected.
4. In the *in vitro* conditions, it was demonstrated that nano-TiO_2 and nano-TiO_2@Ag nanoparticles at concentrations of 30 μg/ml are capable of enhancing

the functional activity of peripheral blood mononuclear cells by promoting the production of pro-inflammatory cytokines IL-1, IL-6, TNF-α, and IL-4 production in donors ($p<0.05$). This suggests a potential impact on the development of chronic inflammation and allergic reactions in operators involved in the synthesis and production of nanomaterials.

5. The influence of nano-TiO_2 and nano-TiO_2@Ag on male boar germ cells exerts a damaging effect: they inhibit respiratory activity, disrupt membrane integrity, and induce an imbalance in the activity of mitochondrial respiratory chain enzymes, leading to a disruption in ATP resynthesis. As a result of biochemical and structural changes, the addition of nanomaterials led to a significant reduction in one of the physiological characteristics – cell survival, which serves as a sensitive marker of the higher negative impact of TiO_2 (1/10 LD_{50}) compared to TiO_2@Ag (1.0 LD_{50}).

6. The calculated Occupational Exposure Limit Values (OELVs) were determined using mathematical equations that take into account the median lethal dose LD_{50} of the nanoparticles, the molecular weight of the compound, the number of metal atoms in the compound, and safety factors. The OELV for nano-TiO_2 is 0.3 mg/m^3, while for nano-TiO_2@Ag it is 0.2 mg/m^3.

7. It has been demonstrated that the introduction of silver nanoparticles led to a reduction in the toxicity of TiO_2 nanoparticles for Hep-2 cells. The incorporation of 4 wt% silver into the composite did not diminish its virucidal activity. Furthermore, due to the reduction in cytotoxicity, this approach could be a promising strategy for the development of active nanocomposite materials based on TiO_2 with low cytotoxicity. The study also revealed that the nanoparticles had no impact on adenovirus replication in cells when added after cell infection.

Contributions:

Yavorovsky O.P. – project administration, conceptualization, methodology, resources, writing – review & editing;

Riabovol V.M. – writing – original draft, methodology, investigation, formal analysis, writing – review & editing;

Zinchenko T.O. – methodology, investigation, formal analysis, writing – review & editing;

Zahornyi M.M. – writing – original draft, methodology, investigation, formal analysis, resources, writing – review & editing;

Ragulya A.V. – project administration, resources, supervision;

Tyschenko N.I. – methodology, investigation, formal analysis, resources;

Povnitsa O.Yu. – methodology, investigation, formal analysis;

Artiukh L.O. – methodology, investigation, formal analysis;

Zahorodnia S.D. – methodology, investigation, for mal analysis, resources, writing – original draft, supervision;

Ostapiv D.D. – methodology, investigation, formal analysis, resources.

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Conflict of interests. The authors declare no conflict of interest.

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