BIOLOGICAL EFFECTS OF METAL NANOPARTICLES AFTER EXPOSURE OF MAMMALIAN CELLS

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Abstract

Investigations of the biological effects of nanomaterials are important for assessing the risk of nanotechnology products. The present study includes the comparative investigation of the localization of core-shell gold hybrid nanostructures and native titanium dioxide nanoparticles. Changes in biochemical parameters of the treated rats in comparison to control animals were observed depending on the type of nanoparticles used. The exposure did not cause lethality, substantial behavior deviations, water and food consumption, pathomorphology of the internal organs. Besides, this study includes the investigation of biological effects of metal nanoparticles after exposure of mammalian cells. Dose- and time-dependent changes in cell viability after exposure to gold hybrid nanostructures and native titanium dioxide nanoparticles were estimated by MTT test.

Keywords: Metal nanoparticles, in vitro toxicity, cell viability, MTT-test

1. INTRODUCTION

Metal nanoparticles are nowadays one of the most widely used nanomaterials having a number of production and practical applications [1-4]. Among them, gold hybrid nanostructures (GHNs) and titanium dioxide (IV) nanoparticles (TiO_2 NPs) can be mentioned as promising materials used for various biomedical purposes including targeted delivery systems, photothermal therapy, implantation, drug and vaccines development, etc. [5-9]. Using of nanoparticles as therapeutic agents raises the issue regarding their biosafety toward humans. The toxicity mechanisms of nanoparticles is now intensively investigated by a scientific community [10-12]. For this purpose, both *in vivo* and *in vitro* biological models are used [12-14].

Biological models, alternative to *in vivo* test systems, are widely used in the study of the toxicity of various chemical compounds [15]. One of the effective approaches for toxicological studies is the use of cell cultures (*in vitro* test systems). Although *in vitro* experiments cannot completely replace *in vivo* studies, they can contribute to assessing the safety of engineered nanoparticles, including hybrid nanostructures. As a rule, *in vivo* experiments are carried out taking into account the initial information about the toxicity of nanoparticles. In addition, animal models are used to study indicators that are not available for *in vitro* systems (distribution of nanomaterials in organs and tissues, their metabolism and excretion, etc.). However, *in vitro* systems can be effectively used as indicators of the toxicity of nanoparticles and for conducting mechanistic investigations.

A number of undoubted advantages characterizes *in vitro t*est systems based on human and animal cell cultures: the simplicity of cell cultivation by proven methods, the possibility of visual detection of viable cells during the whole experiment using microscopic methods, and a higher reproducibility in comparison to *in vivo* test systems. Cost-effective and not time-consuming *in vitro* models allow decreasing the number of experimental animals or a complete replacing of *in vivo* test systems [16].

MTT test the is one of the most widely used methods for assessing cytotoxicity towards a large number of living cells of different cell lines exposed to xenobiotics [17]. This test is proved to be a classical approach for toxicological assessment of various compounds, including nanomaterials. The MTT assay estimates the mitochondria activity and therefore can be used for determination of the amount of living cells. The principle of the MTT test is based on the ability of the succinate dehydrogenase contained in the mitochondrial membrane of a mammalian cell to reduce the yellow salt of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to violet formazan crystals accumulated in the cytoplasm of living cells as a result of this reaction. Therefore, the rate of formazan crystals' accumulation in the cytoplasm represents the level of mitochondrial respiration of the cell, which is an indicator of its viability. The amount of formazan formed in the cell monolayer is proportional to the number of living cells. The effect of nanomaterials is evaluated by their inhibition of the proliferation and viability of exposed cells. The MTT test has such advantages as rapidity, high reproducibility, lack of sophisticated analytical equipment, and low cost.

This manuscript includes the results obtained as a part of the whole study directed to *in vivo* and *in vitro* investigation of biological effects of nanomaterials. Murine embryonal fibroblast (NIH-3T3) and murine monocyte/macrophage (P388-D1) cell lines were used to evaluate the cytotoxicity of core-shell GHNs and native TiO_2 NPs by MTT test.

2. EXPERIMENTAL

2.1. Materials

TiO_2 NPs of anatase form (<25 nm), chloroauric acid, bovine serum albumin, MTT, trypsin, streptomycin sulfate, dimethyl sulfoxide, Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich, USA. Goat polyclonal antibodies against mouse immunoglobulins (IgG) were purchased from Arista Biologicals, USA.

All other chemicals were of HPLC gradient grade (Sigma-Aldrich, USA, and Panreac, Spain). Milli-Q deionized water (Millipore, USA) was used for preparing solutions.

The optical density (OD) was measured at 570 nm with a Zenyth 3100 microplate photometer (Anthos Labtec Instruments, Austria).

2.2. Obtaining of GHNs

GHNs were obtained on the base of gold nanoparticles (GNPs) of 30 nm diameter and goat polyclonal antibodies (IgG). Conjugation GNPs with IgG was carried out as described in [18]. To obtain GHNs, the IgG concentration of 10 μg/mL was selected.

2.3. Characterization of nanomaterials by transmission electron microscopy

TiO_2 NPs and GHNs were dispersed in deionized water at a concentration of 0.1 mg/ml. The aliquot of the obtained dispersion was dropped onto a Formvar film-coated grid and studied by transmission electron microscopy (TEM) on a JEM-100C microscope (Jeol, Japan) at an accelerating voltage of 80 kV.

2.4. Nanomaterials dispersion protocol

TiO_2 NPs and GHNs were diluted to concentrations of 250, 100, 50, 25, 10, 5, 1, 0.5, and 0.1 μg/mL with DMEM. The resulting mixtures were stirred vigorously for 10 min at room temperature.

2.5. Exposure of murine embryonal fibroblast cells (NIH-3T3) and murine monocyte/macrophage cells (P388-D1) to TiO_2 NPs and GHNs

NIH-3T3 and P388-D1 cells were cultured in DMEM supplemented with 10% (vol./vol.) heat-inactivated fetal calf serum and streptomycin sulfate in concentration of 100 µg/mL. An environment of humidified air containing 5% CO_2 was maintained at 37 °C. At 85% confluence, cells were harvested using 0.25% trypsin and were subcultured into 60 mm dishes, 6-well plates or 96-well plates. Cells were allowed to attach the surface for 24 h prior to treatment. TiO_2 NPs and GHNs were diluted to appropriate concentrations by suspending in cell culture medium. Then, the dilutions of nanomaterials were sonicated using an ultrasound bath (100 W, 40 kHz) at room temperature for 10 min to avoid their aggregation prior to administration to cells.

2.6. Cytotoxicity evaluation experiment

To evaluate the cytotoxicity of TiO_2 NPs and GHNs, cells in the log phase were seeded onto a 96-well culture plate with 5000 cells per well and incubated at 37 °C in a CO_2 incubator for 24 h until the cells adhered to the plate. Then, TiO_2 NPs or GHNs in concentrations of 250, 100, 50, 25, 10, 5, 1, 0.5, and 0.1 µg/mL were added. After 24, 48, 72 h incubation, cell viability was measured using the MTT assay. As a control, cell media without nanomaterials was added. For this assay, 100 µL of MTT solution in concentration of 0.5 mg/mL (prepared in medium without serum) was added to the cells and incubated at 37 °C in a CO_2 incubator for 4 h. Dimethyl sulfoxide was then used to dissolve the formazan crystals. Finally, the OD was measured at 570 nm using a microplate reader.

2.7. Data analysis

The number of living cells (as a percentage of control cells not exposed to nanomaterials) is determined by the following formula:

$OD_{570exp}/OD_{570control} \times 100$ %,

where:

OD_{570exp} is an OD at 570 nm for cells treated with nanomaterials

$OD_{570control}$ is an OD at 570 nm for control cells.

3. RESULTS AND DISCUSSION

3.1. Characterization of TiO_2 NPs and GHNs by TEM

The size and aggregation state of the TiO_2 NPs and GHNs were determined using TEM. Both samples contained electron-dense particles with well-developed facets (**Figure 1**).

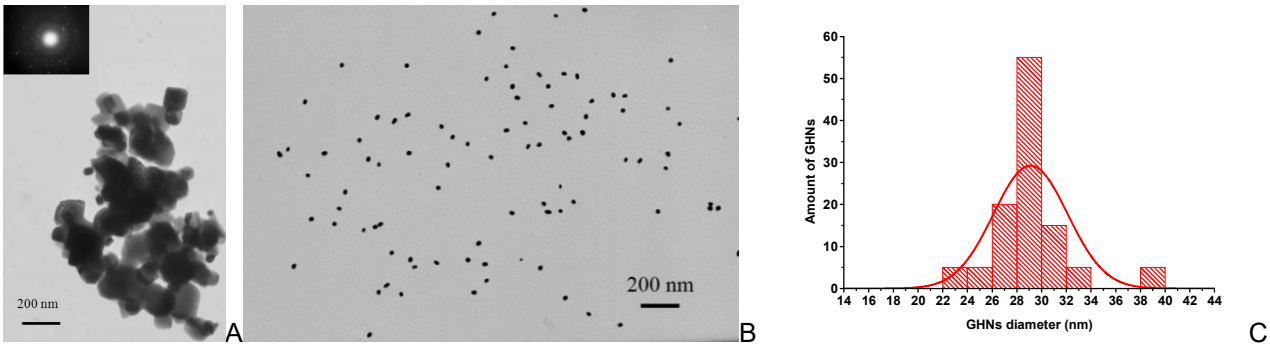

Figure 1 Microphotographs of TiO_2 NPs (A), GHNs (B), and GHNs size distribution (C)

TiO_2 NPs was characterized by a clear contour and a high electron density. Individual *spherical TiO_2 NPs had a diameter of approx. 20-25 nm and tended to form aggregates of a diameter larger than 100 nm in an aqueous medium (**Figure 1A**)*. The GHNs sample contained homogeneous spherical non-aggregated nanoparticles with the average diameter of 33,7 ± 8,9 nm and the mean ellipticity of 1,1 ± 0,4 (**Figures 1B, C**).

3.2. The dose- and time-dependent cytotoxicity of TiO_2 NPs

For evaluation of nanomaterial-induced cytotoxic effects toward cells an MTT test was used. Cell viability was assessed after incubation of NIH 3T3 and P388-D1 cells with TiO_2 NPs and GHNs in concentrations of 250, 100, 50, 25, 10, 5, 1, 0.5 and 0.1 μg/mL during 24, 48 and 72 h as the ratio (%) of OD values of treated cells to that of untreated ones. The results of cytotoxicity assessment after exposure to TiO_2 NPs are presented in **Figure 2**.

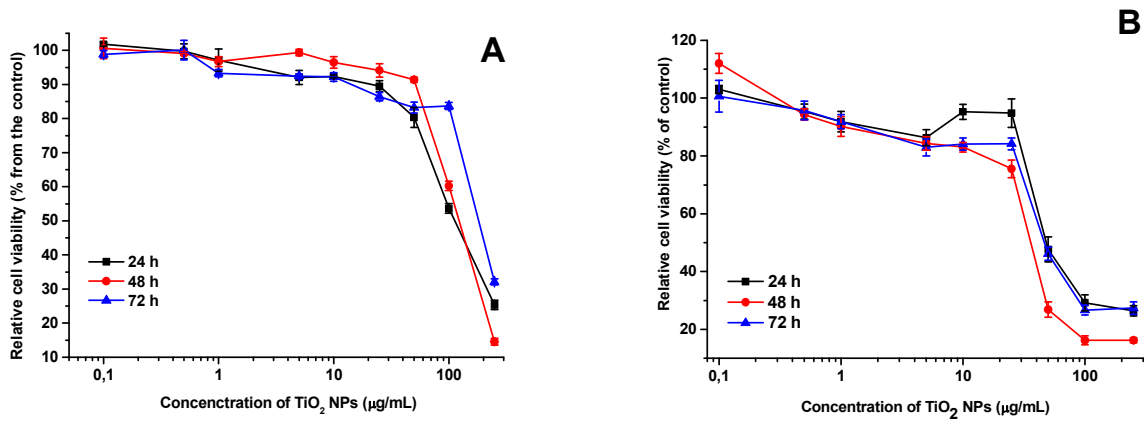

Figure 2 Viability of NIH-3T3 cells (A) and P388-D1 cells (B) after 24, 48 and 72 h of exposure to different concentrations of TiO_2 NPs evaluated with MTT assay. Values are mean ± standard deviation (SD) from five independent experiments

As it is evident from **Figure 2A**, the cytotoxic effect of TiO_2 NPs towards fibroblasts increased dose-dependently. The manner of this effect is similar for 24, 48, and 72 h exposition. NIH 3T3 cells' viability was about 80 % and more after the incubation with TiO_2 NPs in a concentration range of 0.1-25 μg/mL. At higher TiO_2 concentrations (100 and 250 μg/mL) cell viability dramatically decreased down to 20 % and less and was characterized by the same values for different times of cell treatment. For monocyte/macrophage cells, the observed dependency was quite comparable (**Figure 2B**). Cell viability was above 80 % after exposition to TiO_2 NPs in a concentration range of 0.1-50 μg/mL. Higher concentrations (100 and 250 μg/mL) cause extensive cell death resulting in only 10 - 30 % of live monocytes/macrophages. The described effect was similar for all incubation periods.

3.3. The dose- and time-dependent cytotoxicity of GHNs

The cytotoxic effect of GHNs against fibroblasts and monocytes/macrophages evaluated by MTT test are presented in **Figure 3**.

The data presented in **Figure 3A** demonstrate the dose-dependent cytotoxic effect of GHNs with respect to NIH-3T3 cell line. A decrease in cell viability was observed at all exposure times studied. In the concentration range of 0.1-10 μg/mL, the amount of living fibroblasts was 80 % and even higher (up to 100 %). After exposition of cells to GHNs at the middle dose range (25-50 μg/mL), the percentage of living cells was lower: ~55 - 75 % of cells remained viable after the exposure. At high concentrations of GHNs (100 and 250 μg/mL), cell viability declined sharply to 20 - 30 % or less.

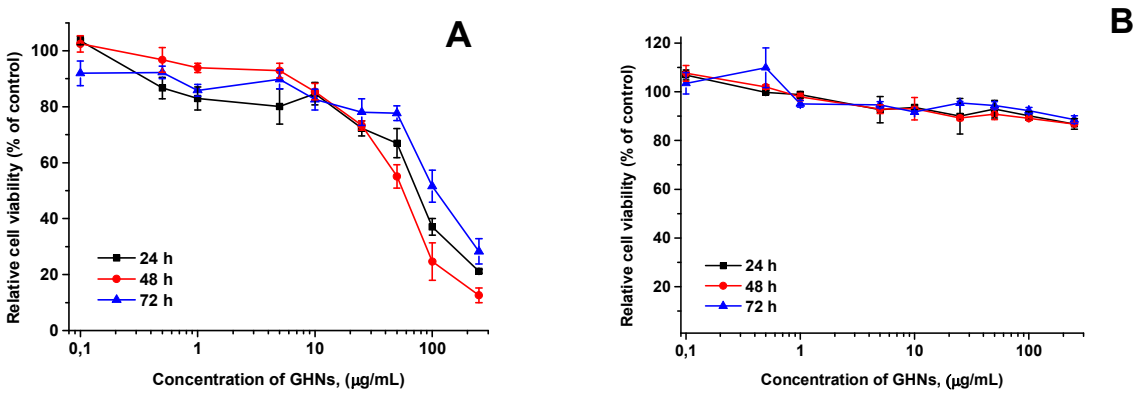

Figure 3 Viability of the NIH-3T3 cells (A) and P388-D1 cells (B) after 24, 48 and 72 h of exposure to different concentrations of GHNs evaluated with MTT assay. Values are mean ± standard deviation (SD) from five independent experiments

For GHNs studied, a time-dependent cytotoxic effect was investigated in 24-48-72 h time interval. The percentage of living cells was reduced mainly upon incubation in the 24-48 h interval (**Figure 3A**). It should be noted that for middle and high doses (25 μg/mL and more), this effect was most pronounced. The cytotoxicity of GHNs after exposure at low doses didn't have a time-dependent manner: the amount of survived cells reached 100 %.

For the P388-D1 cell line, the observed cytotoxic effects differed from those detected for the NIH-3T3 cell line (**Figure 3B**). As it can be seen from the presented data, after exposure to GHNs in the low and middle concentration ranges (0.1-50 μg/mL), the cell viability level was about 90% and higher and practically did not depend upon the exposure time (24-48-72 h). Incubation with GNHs at higher concentrations (100 and 250 μg/mL) caused an increase in cell death, but the level of living cells dropped to only 80 %. The described effect was observed for all time periods of exposure. Therefore, after exposure to GHNs the survival rate of murine monocyte/macrophage cells was higher than that of murine fibroblast cells. The time-dependent cytotoxic effect was less pronounced than for fibroblasts.

4. CONCLUSION

Overall, the cytotoxic effect of GHNs and native TiO_2 NPs on mammalian cells of two cell lines was studied. A dose- and time-dependent effect on cell viability was demonstrated using the MTT test. Difference in the sensitivity of cell lines to the studied nanomaterials was demonstrated. A safe *in vitro* dosage was determined for both nanostructures.

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