NUKLEONIKA 2024;69(1):13–22
doi: 10.2478/nuka-2024-0002

ORIGINAL PAPER

sciendo

No evidence of the long-term *in vitro* toxicity of Aeroxide P25 TiO_2 nanoparticles in three mammalian cell lines despite the initial reduction of cellular mitochondrial activity

Sylwia Męczyńska-Wielgosz, Teresa Bartłomiejczyk, Iwona Grądzka, Sylwester Sommer, Aneta Węgierek-Ciuk, Anna Lankoff, Katarzyna Sikorska, Maria Wojewódzka, Małgorzata Dobrzyńska, Marcin Kruszewski

Abstract. We studied the effects of Aeroxide P25 titanium dioxide nanoparticles (TiO_2 NPs) with a diameter of 21 nm on induction of DNA damage and long-term survival of three human cell lines: hepatocellular liver carcinoma HepG2, colorectal adenocarcinoma HT29 and lung carcinoma A549. The endpoints examined were DNA breakage estimated by the comet assay and oxidative base damage recognized by formamide-pyrimidine glycosylase (FPG) estimated with the FPG^+ comet assay, frequencies of histone γH2AX foci and micronuclei, apoptosis, cell metabolic activity measured by mitochondrial activity (MTT) assay and long-term survival measured by colony-forming ability. Each cell line had a different pattern of DNA breakage and base damage vs. nanoparticle (NP) concentration and treatment time. There was no increase in the frequencies of histone γH2AX foci and micronuclei as compared to those in the untreated cells. In parallel with these results, no induction of apoptosis has been found in none of the cell lines tested. The reported experiments provided no evidence of the long-term *in vitro* toxicity of Aeroxide P25 TiO_2 NPs, despite a slight decrease in mitochondrial activity and cell survival during the first 72 h.

Keywords: γH2AX assay • Caspase 3 activity • Comet assay • Micronucleus assay • MTT assay • Apoptosis

S. Męczyńska-Wielgosz✉, T. Bartłomiejczyk, I. Grądzka, S. Sommer, K. Sikorska, M. Wojewódzka
Centre for Radiobiology and Biological Dosimetry
Institute of Nuclear Chemistry and Technology
Dorodna 16 St., 03-195 Warszawa, Poland
E-mail: s.meczynska@ichtj.waw.pl

A. Węgierek-Ciuk
Institute of Biology, Jan Kochanowski University
Kielce, Poland

A. Lankoff
Centre for Radiobiology and Biological Dosimetry
Institute of Nuclear Chemistry and Technology
Warszawa, Poland
and Institute of Biology, Jan Kochanowski University
Kielce, Poland

M. Dobrzyńska
Department of Radiation Hygiene and Radiobiology
National Institute of Public Health – National Institute of Hygiene, Warszawa, Poland

M. Kruszewski
Centre for Radiobiology and Biological Dosimetry
Institute of Nuclear Chemistry and Technology
Warszawa, Poland
and Department of Molecular Biology and Translational Research, Institute of Rural Health, Lublin, Poland

Received: 8 March 2023
Accepted: 27 November 2023

Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in medicine, the food-processing industry and cosmetics as well as a photocatalyst in air and water cleaning [1]. Although nanoparticles have a great potential to improve our life, their interaction with the environment is inevitable, and the consequences of their use for human health and the ecosystem are still not fully characterized.

Although TiO_2 NPs belong to the NP most often studied, there are numerous discrepancies in the reports on their toxicity and the extent of induced DNA damage in various *in vitro* studies (for a recent meta-analysis of TiO_2 NPs genotoxicity, see Ref. [2]). For example, no genotoxicity of anatase TiO_2 NPs was reported in TK6 human lymphoblasts [3], CHO-K1 cells [4] and rutile TiO_2 NPs in Syrian hamster embryo cells [5]. More recently, Kazimirova *et al.* [6] reported a lack of Aeroxide P25-induced DNA damage in peripheral blood lymphocytes and TK6 cells. In contrast, anatase TiO_2 NPs and Aeroxide P25 were genotoxic in Syrian hamster embryo cells [5] and SHSY5Y human neuronal cells [7].

Some of these discrepancies may be due to the different properties of TiO_2 NPs preparations, such as their crystalline form, size, surface coat and

agglomeration. Additionally, differences in experimental methodology, and treatment protocols affect obtained results. Finally, cell lines differ in their ability to respond to oxidative stress, repair DNA damage, or transmit cellular signals that may change their response to TiO_2 NPs treatment.

There are relatively little data on TiO_2 NPs genotoxicity concerning clonogenicity loss. Therefore, we undertook a detailed study of unfunctionalized TiO_2 NPs action on three mammalian cell lines: human hepatocellular liver carcinoma HepG2, human lung carcinoma A549 and human colorectal adenocarcinoma HT29. All cell lines were treated for 2 h or 24 h with TiO_2 NPs. The primary endpoint examined in this report was the induction of DNA breaks and oxidative damage to DNA bases recognized by formamide-pyrimidine glycosylase (FPG) estimated with the comet assay ± FPG. In addition, the frequencies of histone γH2AX foci and micronuclei, apoptosis, metabolic activity 3-[4,5-dimethyldiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT assay) and short- and long-term survival (clonogenic capacity) were estimated.

Materials and methods

Nanoparticle preparation

TiO_2 NPs powder of nominal size 21 nm, anatase (80%)/rutile (20%) polymorphs (Aeroxide P25), factory-made by Degussa-Evonik (Essen, Germany) was kindly delivered by the European Commission Joint Research Center (EC JRS) depository. Nanoparticles stock solution was prepared at 2 mg/mL NPs in distilled water with sonification at 420 J/cm^3. Directly after sonication, 100 μL of BSA and 100 μL of a 10× concentrated phosphate-buffered saline (PBS) were given to 800 μL aliquots of suspension (Protocol 1 described in Ref. [8]). The hydrodynamic diameter and zeta potential (ζ) analysis was performed using dynamic light scattering (DLS) with the Zestasizer Nano ZS system (Malvern, UK) at 25°C with a scattering angle of 173°. Stock solutions were diluted at 1:8 in a complete culture media and analysed in triplicate with 14-sub runs. The pH of suspensions was 7.4. Zeta potentials were calculated using the Smoluchowski limit for the Henry equation with a setting calculated for practical use (f(ka) = 1.5).

A full characterization of the TiO_2 NPs is given (*ibid.*). Briefly, the particle size distribution of TiO_2 NPs was narrow and uniform, with an average hydrodynamic diameter of 132 nm, depending on the culture medium. The ξ-potential was negative, on average about (–33 mV), depending on the culture medium.

Cell culture and treatment

The cell lines investigated were human colorectal adenocarcinoma HT29, human epithelial cell line A549 and human hepatic cell line HepG2; all three were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD, USA) and maintained according to ATCC protocols.

Suitable numbers of cells were seeded on 5 cm Petri dishes and left to settle down without nanoparticles for 24 h to achieve optimal cell attachment to the plastic surface. Afterwards, the nanoparticle suspension was added to the cell cultures to obtain the final concentrations of 10 μg/mL, 50 μg/mL and 100 μg/mL for 2 h or 24 h. After incubation, the medium containing NPs was removed; for comet assay and γH2AX foci experiments, the plates were washed twice with 5 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4). After washing, the cells were trypsinized and suspended in a fresh culture medium.

The positive controls in micronuclei and γH2AX foci experiments were NP-untreated cells X-irradiated with the dose of 2 Gy. X-irradiation was performed in an ice water bath, using a Smart200 (Yxlon) defectoscope operating at 200 kV and 4.5 mA, with 3 mm Al filtration, at a dose rate of 1.14 Gy/min.

Alkaline comet assay

The comet assay (single-cell gel electrophoresis) was performed as described in Refs. [9, 10]. The Comet Assay IV Image Analysis System (Perceptive Instruments, UK) performed an image analysis of the data. One hundred randomly selected comets per slide were analysed. The percentage of DNA in the comet's tail was used in this study to measure DNA damage.

Immunofluorescence labeling of γ-H2AX foci

An estimation of the γ-H2AX foci number in NP-treated cells was carried out as described in Ref. [11]. In short, cells were fixed in methanol at –20°C for 5 min and rehydrated in PBS for 3 × 10 min. Unspecific binding was blocked overnight in the refrigerator in a blocking buffer (2% BSA, 10% dried milk powder) in KCM+T buffer (120 mM KCl/20 mM NaCl/10 mM Tris·HCl/0.5M EDTA/0.1% Triton X-100, pH 8.0). Samples were treated with anti-phospho-histone H2AX (Ser 139) antibody (1:200; Upstate, Millipore) in a blocking buffer for 1 h at room temperature and washed in KCM+T buffer for 3 × 10 min. Thereafter, they were then incubated with Alexa Fluor 488 goat-anti-mouse IgG secondary antibody (1:200; Molecular Probes, Eugene, OR, USA) in a blocking buffer for 1 h at room temperature. The cells were washed in KCM+T buffer (4 × 10 min) and mounted with Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Foci were counted under a fluorescence microscope (Zeiss Axioplan-2) by the automated image-analysis system (MetaSystems, Altlussheim, Germany). In a single experiment, at least 2 × 200 cells were analysed per dose point. To test the reproducibility of the analysis, three independent experiments were performed.

Micronuclei frequency determination

After completing treatment with NP, the cells were washed thrice with a forward scatter (FCS)-free culture medium and left in a full culture medium for 24 h in the incubator. Afterwards, the cells were fixed with cold methanol:glacial acetic acid 3:1 for 15 min, washed with distilled water, air-dried and stained with 4′,6-diamidino-2-phenylindole (DAPI) according to Ref. [12]. To determine the number of cells with micronuclei, at least 2000 cells were analysed under a fluorescence microscope (Zeiss Axioplan-2) at 400× magnification. Objects close to mitotic cells or adhering to nuclei were excluded from the analysis. Since each cell line had characteristic morphologic features, microscopic preparations of X-irradiated (2 Gy) cells served as standards.

Flow cytometry determination of apoptosis and necrosis

HepG2, HT29 and A549 cells were incubated with Aeroxide P25 TiO_2 NP for 2 h and 24 h as described above. Staurosporine (for HepG2 – 1 μM; and for A549 and HT29 – 2 μM) served as the positive control. The frequencies of viable, apoptotic and necrotic cells were detected with the Annexin V-FITC apoptosis detection kit I (BD Pharmingen, USA), according to the manufacturer's recommendations. The fluorescence was measured by flow cytometry (LSR II flow cytometer [Becton Dickinson]). A computer system BD FACS DiVa (version 6.0, Becton Dickinson) was used for data acquisition and analysis.

Caspase 3 activity determination

After treatment with NPs, cell lysates were prepared as follows. The culture medium was removed and a culture plate (about 5×10^5 cells) was rinsed three times with 5 mL PBS. Cells were detached in 1 mL of lysis buffer containing the following: 20 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4, 250 mM NaCl, 1% Nonidet P-40, 5% glycerol, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM sodium ortovanadate, 50 mM NaF, 1 mM PMSF, 20 mM β-glycerophosphate, 30 mM sodium pyrophosphate and protease inhibitor cocktail (Roche Diagnostics Indianapolis, IN, USA), and lysed on the plate for 20 min. The resulting mixture was centrifuged for 20 min, 20,000 ×*g*. All steps were performed on ice using pre-cooled buffers. The supernatant (cell lysate) was collected, frozen in small aliquots and stored at –80°C. Protein content in the lysate was determined by Bradford's method. All lysates were standardized for the protein content of 2 mg/mL. The caspase 3 activity was measured by the enzyme-linked immunosorbent assay (ELISA) with the Human Caspase-3 (active) test (Invitrogen Corporation, Camarillo, CA, USA), according to the manufacturer's guidelines.

MTT assay

Mitochondrial activity (MTT) assay was used to measure the activity of mitochondrial dehydrogenases and was carried out as described in Ref. [13].

Neutral red assay

The assay was carried out according to Ref. [14].

Clonogenic survival

To assess the effect of NP on cell survival, a clonogenic assay was used. The stock suspension of NP, 2 mg/mL, was freshly prepared as described above. Appropriate volumes of NP were mixed with cell suspensions and the cells were seeded onto 60 mm Petri plates at a density of 700–1000 per plate in three replicates. The cells were growing in the presence of NP until visible clones were formed (usually 8–14 days); they were then fixed with formaldehyde and Giemsa stained. Clones were counted and survival was calculated as a percentage of the clone number in the untreated (control) cell sample. Individual points in the survival curves were shown as means + standard deviation (SD) of three to five independent experiments.

Measurements of reactive oxygen species (ROS) formation

Intracellular ROS formation was studied based on intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH--DA; Sigma Aldrich) to undergo conversion into the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Cells were plated in 6-well plates at a density of 2.5×10^5 cells per well and cultured for 24 h. Aliquots of NP suspensions increasing in concentration were added to the wells, and they were treated for 2 h. After that time, the cells were detached, resuspended in the fresh medium and DCFH-DA solution was added to the final concentration of 5 μM. After 30 min incubation, FCS, side scatters (SSC) parameters and fluorescence of cells were measured with a BD LSR Fortessa cytometer (BD Biosciences).

Statistical evaluation

Statistical analysis of the obtained data was performed using Statistica 7.1 software (StatSoft Inc., Tulsa, OK, USA). The data were expressed as mean ± SD of at least three independent experiments. To calculate the significance of a particular experimental variable, an analysis of variance (ANOVA) was performed, followed by a *post hoc* Tukey's test. The indicated statistical significance was evaluated using pairwise comparison by Student's *t*-test. Differences were considered statistically significant when the *P*-value was less than 0.05.

Table 1. Hydrodynamic diameter and zeta potential of Aeroxide P25 TiO_2 NPs in water, EMEM, F12 and DMEM media

Medium	Hydrodynamic diameter (nm)	Polydispersity index (PDI)	Zeta potential 7.4 (mV)
Water	356 ± 1	0.457 ± 0.04	–15.3 ± 0.5
EMEM	246 ± 1	0.193 ± 0.07	–25.5 ± 0.6
F12	295 ± 3	0.220 ± 0.05	–28.7 ± 2.5
DMEM	300 ± 2	0.280 ± 0.07	–27.5 ± 0.2

The data provided are in the form mean ± SD.
n denotes the number of experiments; n = 3.

Results

Analysis of the size and zeta potential of Aeroxide P25 TiO_2 NPs in different culture media

Analysis of the size and zeta potential of Aeroxide P25 TiO_2 NPs in different culture media used in this study revealed no major differences (Table 1). The hydrodynamic size of NPs in all media was in the range of 250–300 nm, whereas the zeta potential was about 25–29 mV. The values of the polydispersity index below 0.3 indicate acceptable homogeneity of all NPs suspensions.

DNA breakage and base damage

The formation of DNA single-strand breaks (SSB) and net formamide-pyrimidine glycosylase (FPG) sites in cells treated for 2 h or 24 h with Aeroxide P25 TiO_2 NP at three concentrations is shown in Fig. 1. In A549 cells the TiO_2 NP induced a statistically significant increase in both SSB and base damage induction, except the lowest NPs concentration. After the 24 h treatment, the FPG sites did not differ from the control, whereas a statistically significant difference was found in the extent of SSB in cells treated with 50 mg/mL. No significant difference in the induction of DNA damage was found in HT29 despite the treatment time and NPs concentration. A similar pattern was observed in HepG2 cells. HepG2 cells respond to 2 h TiO_2 NP treatment with the lowest DNA damage level (both SSB and base damage). However, 24 h TiO_2 NP treatment with the highest concentration induced marked, statistically significant base damage.

Micronuclei

The effect of 2 h and 24 h treatment on micronuclei frequency using the same NP concentrations as those used for comet assay is shown in Table 2. The effect of X-irradiation with 2 Gy is given in the same figure for comparison (a positive control). There were no statistically significant differences in micronuclei frequencies between the control and the treated cells in the three cell lines examined.

Induction of γ-H2AX foci

The same table shows the mean number of γH2AX foci per nucleus after 2 h and 24 h treatment of the cells with the same NP concentrations as those used for comet assay measurements. The effect of X-irradiation with 2 Gy is given for comparison (a positive control). There were no differences in γH2AX foci numbers between the control and the treated cells in the three cell lines examined.

Apoptosis and necrosis

The results of apoptosis induction in Aeroxide P25 TiO_2 NP-treated cells are shown in Table 3. The staurosporine-induced apoptosis is shown for comparison (a positive control). Though an increase in the number of apoptotic cells was observed in all cell lines for almost all treatment regimes, the increase was very weak, as compared to the effect of staurosporine. For example, treatment of the cells with 100 μg/mL, Aeroxide P25 TiO_2 NP for 24 h resulted

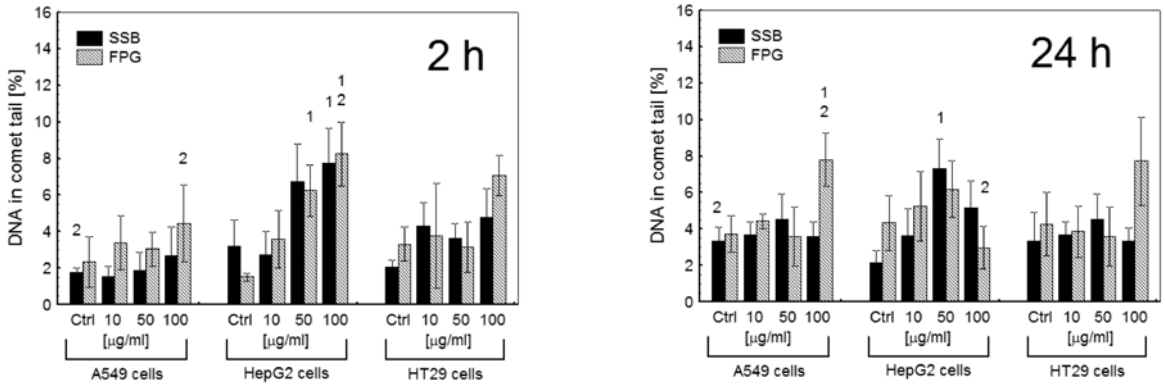

Fig. 1. SSB and base damage recognized by FPG in A549, HepG2 or HT29 cells treated with Aeroxide P25 TiO_2 NP for 2 h (left panel) and 24 h (right panel) at concentrations indicated. Numbers (1, 2) denote results of Tukey's test *post hoc* analysis, indicating a significant difference of the means from the control when NP concentration was considered (1) or a significant difference of the means between the same NP concentration, but different treatment times (2).

Table 2. Frequency of micronuclei and mean number of γ-H2AX foci per nucleus after 2 h and 24 h treatment with TiO_2 NP concentrations indicated, and with the effect of X-irradiation with 2 Gy shown for comparison

Micronuclei frequency							
Treatment	Dose	Cell line/time of treatment					
		A549		HepG2		HT29	
		2 h	24 h	2 h	24 h	2 h	24 h
Control		22.9 ± 9.5	31.5 ± 2.0	34.6 ± 3.4	26.3 ± 7.8	5.9 ± 3.2	7.5 ± 1.8
X-ray	2 Gy	80.1 ± 5.2[a]		155.2 ± 7.7[a]		45.5 ± 0.3[a]	
TiO_2 NP 21 nm	10 μg/mL	33.2 ± 6.9	26.8 ± 7.5	22.3 ± 8.8	27.0 ± 9.5	6.8 ± 1.0	8.5 ± 6.6
	50 μg/mL	22.5 ± 9.6	25.2 ± 3.7	24.6 ± 4.5	23.0 ± 4.4	5.5 ± 0.9	7.8 ± 1.6
	100 μg/mL	25.5 ± 5.8	30.0 ± 8.2	24.5 ± 12.5	23.5 ± 6.4	6.1 ± 2.8	10.0 ± 7.2
The mean number of γ-H2AX foci per nucleus							
Treatment	Dose	Cell line/time of treatment					
		A549		HepG2		HT29	
		2 h	24 h	2 h	24 h	2 h	24 h
Control		10.8 ± 5.7	10.7 ± 3.0	23.6 ± 10.8	7.2 ± 2.1	25.5 ± 10.1	26.4 ± 10.1
X-ray	2 Gy	78.2 ± 10.2[a]		87.2 ± 10.0[a]		87.9 ± 3.4[a]	
TiO_2 NP 21 nm	10 μg/mL	11.2 ± 6.8	10.5 ± 3.1	21.1 ± 6.0	9.7 ± 4.0	25.4 ± 10.0	25.0 ± 9.4
	50 μg/mL	10.3 ± 3.5	7.8 ± 1.5	19.3 ± 5.6	11.4 ± 2.7	26.6 ± 6.8	23.7 ± 8.8
	100 μg/mL	10.6 ± 4.1	6.4 ± 2.5	18.3 ± 8.0	9.5 ± 3.2	28.0 ± 5.0	24.5 ± 10.5

The data provided are in the form mean ± SD.
n denotes the number of experiments; n = 3.
[a]denotes statistically important difference from control (Student's *t*-test, $P < 0.05$).

in less than 10% of apoptotic cells, whereas treatment of the same cells with staurosporine revealed 50–60% of apoptosis.

These results were further confirmed by analysis of caspase 3 activation in treated cells (Table 4). Following induction of apoptosis, elevated caspase 3 activity was observed only in HepG2 and HT29 cells treated with the highest dose of the nanomaterial. Though statistically significant, the caspase 3 activation by Aeroxide P25 TiO_2 NP was very low, for example, two orders of magnitude lower than that induced by staurosporine in Jurkat cells.

Mitochondrial activity

The effect of different concentrations of Aeroxide P25 TiO_2 NPs on mitochondrial homoeostasis was determined by MTT assay. A549 cells appeared to be the most vulnerable to Aeroxide P25 treatment, as a slight decrease in mitochondrial activity was observed even 24 h after treatment. However, a decrease in mitochondrial activity higher than 15% was observed in neither A549 cells nor HT29 cells. No changes in mitochondrial activity were observed in HepG2 cells (Fig. 2).

Short-term survival

Short-term survival (up to 72 h) after 24 h treatment with Aeroxide P25 TiO_2 NP was measured by neutral red assay (Fig. 3). A slight decrease in cell survival was observed for all cell lines 48 h and 72 h after treatment with the highest doses of Aeroxide P25. However, in line with a decrease in mitochondrial activity, the decrease in cell survival was not higher than 15%.

Clonogenic survival

In opposition to the MTT assay and neutral red assay results, the determination of the cloning ability of cells continuously treated with TiO_2 NP revealed no apparent toxicity of Aeroxide P25 (Fig. 4). The most susceptible cell line was HT29 with a survival rate of 80% or higher despite the NPs concentration. Survival rates of the two remaining cell lines (A549 and HepG2) were around 100%, indicating no toxicity of the tested compound.

Formation of reactive oxygen species

The analysis of Aeroxide P25 TiO_2 NP-induced ROS formation revealed that the highest concentration of nanoparticles significantly elevated ROS in all cell lines (Fig. 5). The highest production of ROS was observed for HepG2 cells.

Discussion

It is generally agreed that oxidative stress is the main cause of NP-induced effects at the cellular level, including genotoxicity, mutagenicity, disturbed mitochondrial respiration, impaired proliferation

Table 3. The extent of apoptosis after 2 h and 24 h treatment with TiO_2 NP at indicated concentrations

Treatment	Dose	Cell line/time of treatment					
		A549		HepG2		HT29	
		2 h	24 h	2 h	24 h	2 h	24 h
Control	Viable	99.16 ± 0.14	98.36 ± 0.16	97.63 ± 0.29	96.34 ± 0.43	98.03 ± 0.55	97.04 ± 0.69
	Early apoptosis	0.54 ± 0.21	1.34 ± 0.26	2.20 ± 0.30	3.22 ± 0.29	1.80 ± 0.56	2.61 ± 0.43
	Late apoptosis and necrosis	0.30 ± 0.10	0.30 ± 0.08	0.17 ± 0.15	0.44 ± 0.17	0.17 ± 0.06	0.35 ± 0.09
Staurosporine/24 h[b]	Viable	32.48 ± 8.88[a]		23.70 ± 5.33[a]		20.20 ± 3.20[a]	
	Early apoptosis	7.98 ± 2.33[a]		48.20 ± 3.22[a]		58.03 ± 4.98[a]	
	Late apoptosis and necrosis	59.54 ± 3.99[a]		28.10 ± 2.56[a]		21.77 ± 2.37[a]	
TiO_2 NP 10 mg/mL	Viable	93.07 ± 0.81[a]	93.30 ± 1.20[a]	93.20 ± 0.26[a]	92.93 ± 0.64[a]	93.13 ± 0.25[a]	92.50 ± 1.46[a]
	Early apoptosis	5.10 ± 0.44[a]	4.70 ± 0.75[a]	5.03 ± 1.03	4.53 ± 0.40[a]	4.43 ± 0.75	4.17 ± 0.79
	Late apoptosis and necrosis	1.83 ± 0.40[a]	2.00 ± 0.46[a]	1.77 ± 0.81	2.53 ± 0.26[a]	2.43 ± 0.50[a]	3.33 ± 0.81[a]
TiO_2 NP 50 mg/mL	Viable	92.10 ± 0.80[a]	93.93 ± 1.75[a]	93.97 ± 0.65[a]	91.13 ± 1.37[a]	93.17 ± 0.78[a]	91.83 ± 1.60[a]
	Early apoptosis	5.97 ± 0.76[a]	3.83 ± 1.33[a]	3.97 ± 0.61	6.00 ± 1.15[a]	3.70 ± 0.60	5.17 ± 1.40[a]
	Late apoptosis and necrosis	1.93 ± 0.15[a]	2.23 ± 0.42[a]	2.07 ± 0.15[a]	2.87 ± 0.35[a]	3.13 ± 0.25[a]	3.00 ± 0.26[a]
TiO_2 NP 100 mg/mL	Viable	93.63 ± 1.20[a]	91.87 ± 1.10[a]	93.73 ± 0.64[a]	90.87 ± 0.96[a]	92.23 ± 1.46[a]	90.57 ± 0.23[a]
	Early apoptosis	3.97 ± 0.75[a]	5.90 ± 1.47[a]	4.27 ± 0.40[a]	6.33 ± 0.91[a]	4.70 ± 0.79[a]	6.17 ± 0.65[a]
	Late apoptosis and necrosis	2.40 ± 0.46[a]	2.23 ± 0.40[a]	2.00 ± 0.26[a]	2.80 ± 0.26[a]	3.07 ± 0.81[a]	3.27 ± 0.47[a]

The data provided are in the form mean ± SD.
n denotes the number of experiments; n = 3.
[a]denotes statistically important difference from control (Student's *t*-test, $P < 0.05$).
[b]denotes the effect of staurosporine being shown for comparison (positive control).

Table 4. Caspase 3 activity after 2 h and 24 h treatment with TiO_2 NP at indicated concentrations

Treatment	Cell line/time of treatment					
	A549		HepG2		HT29	
	2 h	24 h	2 h	24 h	2 h	24 h
Control	0.12 ± 0.05	0.05 ± 0.01	1.02 ± 0.03	1.42 ± 0.02	0.26 ± 0.02	0.42 ± 0.03
TiO_2 NP 10 mg/mL	0.10 ± 0.02	0.06 ± 0.01	0.98 ± 0.02	1.43 ± 0.09	0.31 ± 0.01	0.41 ± 0.06
TiO_2 NP 50 mg/mL	0.13 ± 0.02	0.07 ± 0.01	1.08 ± 0.07	1.37 ± 0.05	0.51 ± 0.05[a]	0.63 ± 0.05[a]
TiO_2 NP 100 mg/mL	0.11 ± 0.01	0.07 ± 0.01	1.39 ± 0.06[a]	1.39 ± 0.03	0.37 ± 0.02[a]	0.55 ± 0.04[a]
Positive control: Jurkat cells treated with 1 mM staurosporine for 24 h: 39.89 ± 3.82[a]						

The effect of staurosporine is shown for comparison.
The data provided are in the form mean ± SD.
n denotes the number of experiments; n = 3.
[a]denotes statistically important difference from control (Student's *t*-test, $P < 0.05$).

and apoptotic death *in vitro* [15–17] and *in vivo*, e.g. Refs. [18, 19]. These effects may be a potential cause of toxicity of NPs and are sufficient to raise concern for human health and the environment [20].

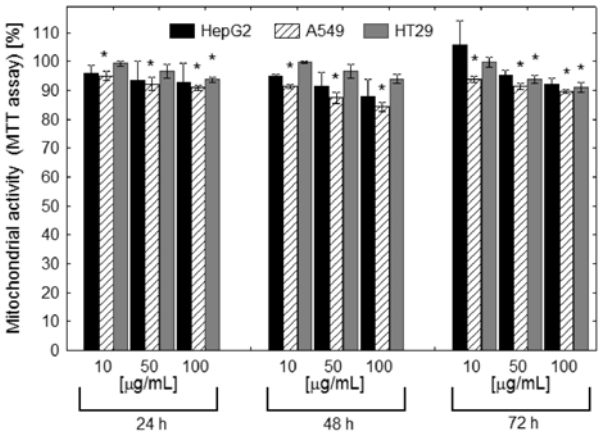

Fig. 2. Mitochondrial activity (MTT assay) of cells treated with Aeroxide P25 TiO_2 NP for 24 h and measured 72 h after treatment expressed as a percent of the control (untreated cells). The asterisk denotes a statistically significant difference from the control. Two-way ANOVA with Tukey's HDS *post hoc* comparison. $P < 0.05$.

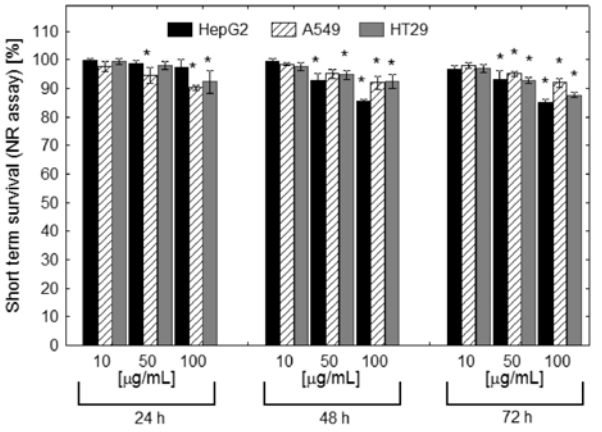

Fig. 3. Short-term survival of cells treated with Aeroxide P25 TiO_2 NP for 24 h, as measured by neutral red assay. The asterisk denotes a statistically significant difference from the control. Two-way ANOVA with Tukey's HDS *post hoc* comparison. $P < 0.05$.

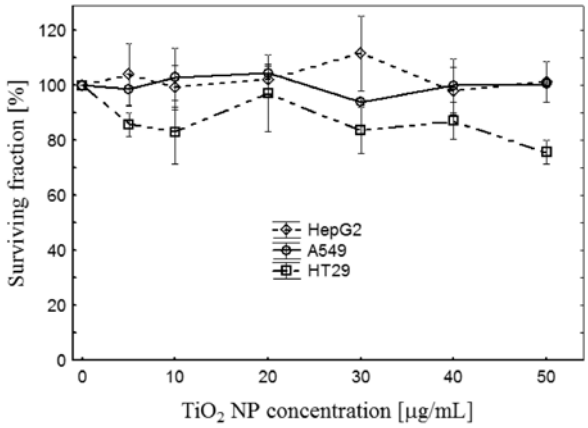

Fig. 4. Cloning ability determination of A549, HT29 and HepG2 cells growing in the presence of Aeroxide P25 TiO_2 NP.

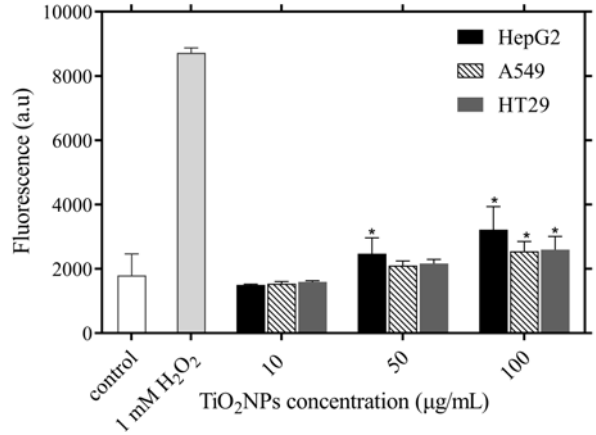

Fig. 5. Generation of ROS in HepG2, A549 and HT29 cells incubated with Aeroxide P25 TiO_2 NP. H_2O_2 in a concentration of 1 mM served as a positive control. The data provided are in the form mean ± SD. n denotes the number of experiments; n = 3. The asterisk denotes statistically significant difference from unexposed control, $P < 0.05$.

However, cellular biology aspects of examination of the genotoxic effect of NP pose considerable difficulty. Such factors as NP uptake into the cell, their crystalline form, the status of cellular antioxidative defence and/or DNA repair systems, and/or cellular signaling and proneness to apoptosis may decide upon the cell's fate. This is apparent in the case of

TiO_2 NPs. Though this type of NPs is among the most intensively studied during the last decades, many contradictory papers have been published so far, some confirming TiO_2 NPs toxicity, whereas others indicating a lack of toxicity [21]. Nevertheless, a recent review and meta-analysis summarizing the works completed till 2020 concluded that short-term exposure to TiO_2 NPs can cause genotoxicity *in vitro* [2]. Though it seems that the genotoxicity of TiO_2 NPs is well documented, there are still gaps in our knowledge of its cytotoxicity, especially in the context of long-term survival. The majority of papers dealing with TiO_2 NPs cytotoxicity utilize short-term assays based on cellular ability to reduce tetrazolium salts, such as MTT, XTT, WST or resazurin that give very limited information on the cytotoxic potential of the tested compound.

In this paper, we used three human cell lines of different origins to compare the relationship between DNA damage inflicted by short-term treatment with TiO_2 NPs and short-term *in vitro* cytotoxicity (MTT and neutral red assays) or long-term *in vitro* cytotoxicity expressed as clonogenic ability resulting from continuous exposure to NPs that is a more realistic scenario in case of environmental exposure. A common practical problem in toxicology is the use of the toxic agent at different concentrations adapted to the endpoint examined. Hence, DNA damage was estimated after treatment with 10 μg/mL, 50 μg/mL, and 100 μg/mL, whereas the concentration range of TiO_2 NP for clonogenic survival was 5–50 μg/mL.

In the present study, Aeroxide P25 TiO_2 NPs induced DNA damage only in the highest concentrations used (50 μg/mL and 100 μg/mL); however, the pattern of damage differed between cell lines (Fig. 1). The most susceptible were HepG2 cells showing a marked increase in the formation of DNA SSB and net base damage (FPG sensitive sites) after 2 h treatment. However, the induction of DNA damage was mitigated after 24 h treatment with NPs, indicating effective DNA repair or induction of antioxidant defence. A similar pattern of induction of DNA damage was observed in HepG2 cells treated with silver nanoparticles (Ag NPs). The damage revealed after 24 h treatment was less pronounced than the damage after 2 h treatment [11]. This indicates either the effective repair of the damage or the induction of an antioxidant defence that efficiently diminishes the intensity of the damage generation. Indeed, we have previously shown that, in HepG2 cells, Ag NPs induced NFκb-dependent genes, likely including those taking part in cellular antioxidant defence, to a much higher extent than in A549 cells [22]; thus, these cells are much better responders to the oxidative stress condition than A459 cells. Indeed, analysis of ROS induction revealed that HepG2 cells were the ones most prone to the formation of ROS (Fig. 5). In the other two cell lines, Aeroxide P25 TiO_2 expressed negligible genotoxicity without any obvious pattern of damage induction. Despite the oxidative DNA damage, Aeroxide P25 TiO_2 NPs treatment did not induce any increase in the number of micronuclei nor γ-H2AX foci per nucleus in either of the three cell lines tested (Table 2). This is in concordance with the lack of any increase in the number of micronuclei nor γ-H2AX foci induced in these cells by silver NPs reported in our previous work [11]. The inability of Aeroxide P25 TiO_2 NPs to induce the formation of micronuclei or γ-H2AX foci suggests its inability to induce toxic DNA double-strand lesions and cell death as a consequence.

This was further reflected in the ability of Aeroxide P25 TiO_2 NPs to induce apoptosis. The NPs turned out to be a very weak inducer of apoptosis, either measured as annexin V manifestation on cellular membranes (Table 3) or activation of caspase 3 (Table 4), although all three cell lines entered apoptosis upon staurosporine treatment (Table 3). Although significant, the Aeroxide P25 TiO_2 NPs treatment resulted in barely higher induction of apoptosis; this was observed in untreated control and did not suggest any massive cell loss. It should be added that HepG2 cells were relatively apoptosis-prone in comparison to the other two cell lines examined which is following the formation of DNA damage.

The short-term viability (neutral red) and MTT tests revealed a moderate toxic effect of Aeroxide P25 TiO_2 NPs in all three cell lines tested (Figs. 2 and 3). The most susceptible were HepG2 cells, for which surviving fraction and mitochondrial activity dropped to about 85% 48 h after treatment with 100 μg/mL of NPs. In contrast, the clonogenic survival experiments revealed only small differences between the three cell lines after treatment with Aeroxide P25 TiO_2 NPs. No cytotoxicity was observed for HepG2 and A549, even for the highest dose tested (50 μg/mL). HT29 cells responded in a dose-independent mode, with an initial reduction of the survival to 85% of the control, followed by a plateau phase till 50 μg/mL.

Our results indicate that considerable differences may be expected between cytotoxicity estimations for short (few days) and long (2 weeks) treatment intervals. These differences would also depend on the applied method of cytotoxicity estimation. Interestingly, the moderate toxicity of Aeroxide P25 TiO_2 NPs suggested by the neutral red and MTT assays was completely contradicted by the results of long-term treatment (clonogenic assay).

There was also no consistent relation between cell sensitivity estimated from clonogenic ability and the levels of oxidative DNA damage. For example, the level of oxidative damage to DNA in the HT29 cell line that was relatively most sensitive to Aeroxide P25 TiO_2 NP in the clonogenicity test was the lowest of all three lines tested; and the opposite also held true – HepG2 cells that proved to be the most susceptible to Aeroxide P25 TiO_2 NP treatment in terms of induction of oxidative DNA damage were also completely resistant in terms of clonogenic survival (Figs. 1 and 4). The clonogenic survival of cells depends in the majority on their ability to repair DSB formed either by the direct action of xenobiotics or from SSB conversed into DSB (as observed during replication, e.g. Ref. [23]). Either no DSB formation resulted from Aeroxide P25 TiO_2 NP treatment or those formed were efficiently repaired, since

there was observed neither an increase in histone γH2AX foci nor micronuclei frequency (Table 2). Interestingly, other authors reported the presence of γH2AX foci in NP-treated cells [19, 24]. It should be stressed, however, that S-phase cells often exhibit a number of γH2AX foci due to the transient perturbation of DNA replication. An elevated number of foci may indicate the presence of DSB or a shift in the cell distribution in the cell cycle, with an increased S-phase compartment. In case of doubt, rigorous γH2AX foci determination should be carried out with the exclusion of S-phase cells from scoring [25]. Furthermore, cells undergoing perturbed base excision repair or early apoptosis also contain γH2AX foci [26, 27]. Therefore, the interpretation of the presence of γH2AX foci resulting from the induction of DSB by NPs must be treated with care.

Interestingly, short-term assays, such as MTT and neutral red assays, revealed slight cytotoxicity of Aeroxide P25 TiO_2 NP, especially corresponding to the usage of higher doses. The A549 cells were the most sensitive ones; however, these cells showed no susceptibility to Aeroxide P25 TiO_2 NP in long-term clonogenic assays (Figs. 2–4). Since the highest dose used in the clonogenic assay was similar to the lowest and middle doses used in the short-term assays were the same as those used in the clonogenic assay, this discrepancy did not result from the differences in the used doses. A possible explanation of this inconsistency may be a ROS-mediated stimulation of pro-survival signaling pathways [28] that might have a larger effect in the case of long-term survival than in the case of short-term survival.

Our data provided no evidence of the toxicity of Aeroxide P25 TiO_2 NP in three mammalian cell lines as measured by clonogenic assay *in vitro*. Despite initial induction of oxidative damage to DNA or even an initial decrease of mitochondrial activity and ability to uptake neutral red dye, the long-term survival of the cells was, in general, undisturbed. This, however, does not exclude the harmful effects of Aeroxide P25 TiO_2 NP at the higher level of organization, such as tissues, organs or whole organisms, that should be further investigated with diligence.

Conflict of interest statement. The authors declare that there are no conflicts of interest.

Acknowledgments. The work was supported by the Polish Norwegian Research Fund (PNRF-122-AI-1/07), National Science Centre grant N N404 316540 (MK) and National Science Centre grant No. UMO-2016/23/D/NZ7/03842 (SMW). The authors thank Ms. T. Iwaneńko for technical support.

ORCID

M. Dobrzyńska http://orcid.org/0000-0002-2185-3745
M. Kruszewski http://orcid.org/0000-0003-4554-8566
A. Lankoff http://orcid.org/0000-0002-6661-7949
S. Męczyńska-Wielgosz http://orcid.org/0000-0002-9475-983X
K. Sikorska http://orcid.org/0000-0001-8480-7722
S. Sommer http://orcid.org/0000-0001-8405-5460
A. Węgierek-Ciuk http://orcid.org/0000-0002-6527-7364
M. Wojewódzka http://orcid.org/0000-0003-0809-4614

References

1. McIntyre, R. A. (2012). Common nano-materials and their use in real world applications. *Sci. Prog.*, *95*, 1–22. https://doi.org/10.3184/003685012X13294715456431.
2. Ling, C., An, H., Li, L., Wang, J., Lu, T., Wang, H., Hu, Y., Song, G., & Liu, S. (2021). Genotoxicity evaluation of titanium dioxide nanoparticles in vitro: a systematic review of the literature and meta-analysis. *Biol. Trace Elem. Res.*, *199*, 2057–2076. https://doi.org/10.1007/s12011-020-02311-8.
3. Woodruff, R. S., Li, Y., Yan, J., Bishop, M., Jones, M. Y., Watanabe, F., Biris, A. S., Rice, P., Zhou, T., & Chen, T. (2012). Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and comet assay. *J. Appl. Toxicol.*, *32*, 934–943. https://doi.org/10.1002/jat.2781.
4. Wang, S., Hunter, L. A., Arslan, Z., Wilkerson, M. G., & Wickliffe, J. K. (2011). Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells. *Environ. Mol. Mutagen.*, *52*, 614–622. https://doi.org/10.1002/em.20660.
5. Guichard, Y., Schmit, J., Darne, C., Gate, L., Goutet, M., Rousset, D., Rastoix, O., Wrobel, R., Witschger, O., Martin, A., Fierro, V., & Binet, S. (2012). Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in Syrian hamster embryo cells. *Ann. Occup. Hyg.*, *56*, 631–644. https://doi.org/10.1093/annhyg/mes006.
6. Kazimirova, A., Baranokova, M., Staruchova, M., Drlickova, M., Volkovova, K., & Dusinska, M. (2019). Titanium dioxide nanoparticles tested for genotoxicity with the comet and micronucleus assays in vitro, ex vivo and in vivo. *Mutat. Res.*, *843*, 57–65. https://doi.org/10.1016/j.mrgentox.2019.05.001.
7. Valdiglesias, V., Costa, C., Sharma, V., Kilic, G., Pasaro, E., Teixeira, J. P., Dhawan, A., & Laffon, B. (2013). Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. *Food Chem. Toxicol.*, *57*, 352–361. https://doi.org/10.1016/j.fct.2013.04.010.
8. Lankoff, A., Sandberg, W. J., Wegierek-Ciuk, A., Lisowska, H., Refsnes, M., Sartowska, B., Schwarze, P. E., Meczynska-Wielgosz, S., Wojewodzka, M., & Kruszewski, M. (2012). The effect of agglomeration state of silver and titanium dioxide nanoparticles on cellular response of HepG2, A549 and THP-1 cells. *Toxicol. Lett.*, *208*, 197–213. https://doi.org/S0378-4274(11)01606-7 [pii]; DOI: 10.1016/j.toxlet.2011.11.006.
9. Wojewodzka, M., Kruszewski, M., Iwanenko, T., Collins, A. R., & Szumiel, I. (1998). Application of the comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation. I. Strand breakage. *Mutat. Res.*, *416*, 21–35.
10. Kruszewski, M., Wojewodzka, M., Iwanenko, T., Collins, A. R., & Szumiel, I. (1998). Application of the

comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation. II. Base damage. *Mutat. Res*., *416*, 37–57.
11. Kruszewski, M., Gradzka, I., Bartlomiejczyk, T., Chwastowska, J., Sommer, S., Grzelak, A., Zuberek, M., Lankoff, A., Dusinska, M., & Wojewodzka, M. (2013). Oxidative DNA damage corresponds to the long term survival of human cells treated with silver nanoparticles. *Toxicol. Lett*., *219*, 151–159. https://doi.org/S0378-4274(13)00104-5 [pii]; DOI: 10.1016/j.toxlet.2013.03.006.
12. Eckl, P. M. (1995). Aquatic genotoxicity testing with rat hepatocytes in primary culture. II. Induction of micronuclei and chromosomal aberrations. *Sci. Total Environ*., *159*, 81–89.
13. Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol. Lett*., *160*, 171–177.
14. Repetto, G. P. A. del, & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc*., 3, 1125–1131.
15. Reeves, J. F., Davies, S. J., Dodd, N. J., & Jha, A. N. (2008). Hydroxyl radicals (*OH) are associated with titanium dioxide (TiO(2)) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. *Mutat. Res*., *640*, 113–122.
16. Liu, S., Xu, L., Zhang, T., Ren, G., & Yang, Z. (2010). Oxidative stress and apoptosis induced by nanosized titanium dioxide in PC12 cells. *Toxicology*, *267*, 172–177.
17. Shukla, R. K., Sharma, V., Pandey, A. K., Singh, S., Sultana, S., & Dhawan, A. (2011). ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. *Toxicol. Vitro*, *25*, 231–241.
18. Wang, J., Li, N., Zheng, L., Wang, S., Wang, Y., Zhao, X., Duan, Y., Cui, Y., Zhou, M., Cai, J., Gong, S., Wang, H., & Hong, F. (2011). P38-Nrf-2 signaling pathway of oxidative stress in mice caused by nanoparticulate TiO(2). *Biol. Trace Elem. Res*., *140*(2), 186–197. DOI: 10.1007/s12011-010-8687-0.
19. Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., & Schiestl, R. H. (2009). Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Res*., *69*, 8784–8789.
20. Singh, N., Manshian, B., Jenkins, G. J., Griffiths, S. M., Williams, P. M., Maffeis, T. G., Wright, C. J., & Doak, S. H. (2009). NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials*, *30*, 3891–3914.
21. Shi, H., Magaye, R., Castranova, V., & Zhao, J. (2013). Titanium dioxide nanoparticles: a review of current toxicological data. *Part Fibre Toxicol*., *10*, 15. https://doi.org/1743-8977-10-15 [pii]; DOI: 10.1186/1743-8977-10-15.
22. Stepkowski, T. M., Brzoska, K., & Kruszewski, M. (2014). Silver nanoparticles induced changes in the expression of NF-kappaB related genes are cell type specific and related to the basal activity of NF-kappaB. *Toxicol. Vitro*, *28*, 473–478. https://doi.org/S0887-2333(14)00011-3 [pii]; DOI: 10.1016/j.tiv.2014.01.008.
23. Neijenhuis, S., Verwijs-Janssen, M., Kasten-Pisula, U., Rumping, G., Borgmann, K., Dikomey, E., Begg, A. C., & Vens, C. (2009). Mechanism of cell killing after ionizing radiation by a dominant negative DNA polymerase beta. *DNA Repair*, *8*, 336–346.
24. Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S. M., Schlager, J. J., & Hong, J. (2008). DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. *Toxicol. Appl. Pharmacol*., *233*, 404–410.
25. Lobrich, M., Shibata, A., Beucher, A., Fisher, A., Ensminger, M., Goodarzi, A. A., Barton, O., & Jeggo, P. A. (2010). GammaH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization. *Cell Cycle*, *9*, 662–669.
26. Matsumoto, M., Yaginuma, K., Igarashi, A., Imura, M., Hasegawa, M., Iwabuchi, K., Date, T., Mori, T., Ishizaki, K., Yamashita, K., Inobe, M., & Matsunaga, T. (2007). Perturbed gap-filling synthesis in nucleotide excision repair causes histone H2AX phosphorylation in human quiescent cells. *J. Cell. Sci*., *120*, 1104–1112.
27. Rogakou, E. P., Nieves-Neira, W., Boon, C., Pommier, Y., & Bonner, W. M. (2000). Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J. Biol. Chem*., *275*, 9390–9395.
28. Zuberek, M., & Grzelak, A. (2018). Nanoparticles-caused oxidative imbalance. *Adv. Exp. Med. Biol*., *1048*, 85–98. https://doi.org/10.1007/978-3-319-72041-8_6.