Biological Analyses of the Effects of TiO_2 and PEG-b-PLA Nanoparticles on Three-Dimensional Spheroid-Based Tumor

Katarina KOŇÁRIKOVÁ[1], Gabriela Mária GIRAŠKOVÁ[1], Ingrid ŽITŇANOVÁ[1], Monika DVOŘÁKOVÁ[1], Eva ROLLEROVÁ[2], Soňa SCSUKOVÁ[3], Jozef BIZIK[4], Mária JANUBOVÁ[1], Jana MUCHOVÁ[1]

[1]Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic, [2]Slovak Medical University, Faculty of Public Health, Department of Toxicology, Bratislava, Slovak Republic, [3]Institute of Experimental Endocrinology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovak Republic, [4]Institute of Experimental Oncology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Received June 7, 2023
Accepted June 27, 2023

Summary

The aim of our study was to monitor the antiproliferative/ cytotoxic and genotoxic effects of both, poly(ethylene glycol)-block-poly(lactic acid) (PEG-*b*-PLA) and titanium dioxide (TiO_2) nanoparticles on the tumor (HT-29, MCF-7, U118MG) and healthy (HEK-293T) cell lines during 2D cultivation and during cultivation in the spheroid form (3D cultivation). Cells or spheroids were cultivated with nanoparticles (0.01, 0.1, 1, 10, 50, and 100 μg/ml) for 72 hours. The cytotoxic effect was determined by the MTT test and the genotoxic effect by the comet assay. We found that 2D cultivation of tumor cell lines with PEG-*b*-PLA and TiO_2 nanoparticles had an anti-proliferative effect on human colon cancer cell line HT-29, human breast cancer cell line MCF-7, human glioma cell line U-118MG during 72h cultivation, but not on control/healthy HEK-293T cells. At the concentrations used, the tested nanoparticles caused no cytotoxic effect on tumor cell lines. Nanoparticles PEG-b-PLA induced significant damage to DNA in HT-29 and MCF-7 cells, while TiO_2 nanoparticles in MCF-7 and U-118MG cells. Only PEG-*b*-PLA nanoparticles caused cytotoxic (IC_{50} = 7 μg/ml) and genotoxic effects on the healthy cell line HEK-293T after 72h cultivation. The cells which were cultivated in spheroid forms were more sensitive to both types of nanoparticles. After 72h cultivation, we observed the cytotoxic effect on both, the tumor and healthy cell lines.

Keywords

Nanoparticles • Tumor cell lines • 3D cell culture • Cytotoxicity • DNA damage

Corresponding author

K. Koňáriková, Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Sasinkova 2, 813 72, Bratislava, Slovak Republic. E-mail: katarina.konarikova@fmed.uniba.sk

Introduction

The use of nanotechnology in medicine, especially drug delivery systems, has been spreading rapidly [1]. In recent years, the search for new and more effective delivery systems is very intensive. Although nanoparticle (NP) research has been ongoing for more than 30 years, methods and standard protocols to ensure their safety for human use are still in development [2]. Nanoparticles as delivery systems, have been used to reduce toxicity and side effects of anticancer drugs. They can be designed to identify and target cancer cells without influencing normal cells. This is called active targeting where the drug carrier system is conjugated to a specific ligand on the cancer cells [3]. The targeted ligands should be in the form of small molecules such as antibodies, peptides, or designed protein and nucleic acid aptamers [4,5]. Last but not least, NPs must be nontoxic for healthy cells and must not affect drug delivery [6]. Nanoparticles have been studied for their safety examining their cell toxicity, immunotoxicity, and genotoxicity before their use as carrier systems of drugs [7].

The first polymeric nanoparticle design was revealed in 1970s [8]. Poly(ethylene glycol)-block-

poly(lactic acid) (PEG-*b*-PLA) as a commonly used copolymer, has been chosen to design micellar formulations. PEG-b-PLA is based on biodegradable and biocompatible poly(lactide) (PLA) and poly(ethylene glycol) (PEG). PEG-*b*-PLA is able to improve the drug's aqueous solubility, decrease the puncture effect as well as prolong the residence time of drugs in the *in vivo* systems. PEG-b-PLA can protect the residence of drugs in the *in vivo* systems against opsonization and phagocytosis by the organism [9]. The advantage of the PEG-b-PLA is in its ability to cross the blood-brain barrier into the brain parenchyma [10,11].

Metal and metal oxide NPs are the most often used nanomaterials. The metallic NPs can be distributed throughout the body and primarily accumulate in organs such as liver, spleen, kidney, and lymph nodes due to nonspecific uptake by reticuloendothelial cells. Metallic NPs could persist in the body for more than 6 months and can induce pathological damage to the organs [12,13]. Because TiO_2 NPs or metallic NPs (≤100 nm) can cross the blood-brain barrier, they should be good drug carrier systems for brain tumors [13].

The main mechanism underlining the toxicity potentially triggered by TiO_2 NPs seems to involve the reactive oxygen species (ROS) production, resulting in oxidative stress, inflammation, genotoxicity, metabolic change, and potential carcinogenesis. The extent and type of cell damage strongly depend on the chemical and physical characteristics of TiO_2 NPs, including size, crystal structure, and photo-activation [14].

In our study we have examined the effects of two types of NPs (TiO_2 and polymeric PEG-*b*-PLA NPs) on healthy and cancer cell lines. We have monitored their antiproliferative/cytotoxic and genotoxic effects during 2D and 3D cultivation.

Methods

Cell cultures (2D cultivation)

Human colon cancer (HT-29), breast cancer (MCF-7), glioblastoma (U-118MG), and noncancer (HEK-293T) cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Rockville, MD) containing 10 % fetal bovine serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin G at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air.

Multicellular spheroids (3D cultivation)

Human colon cancer (HT-29), breast cancer (MCF-7), glioblastoma (U-118MG), and noncancer (HEK-293T) cells were used to form multicellular spheroids. Briefly, U-bottomed 96-well plates were treated with 0.8 % low melting point agarose (Lonza, Basel, Switzerland) which was prepared in sterile water to form a thin film with a non-adhesive surface. Cells were seeded at 104 cells per well in 200 μL of Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Rockville, MD) containing 10 % fetal bovine serum, 100 μg/ml streptomycin, and 100 U/mL penicillin G. Spheroids were incubated for 14 days at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air. The culture medium was harvested and spun down to remove cell debris every 3 days.

PEG-b-PLA, preparations and characterization

Fresh nanoparticle micelles of PEG-*b*-PLA were prepared from copolymer PEG-b-PLA [CH3O(CH2CH2O)x(COCHCH3O)yH, PEG average Mn = 350 g/mol, PLA average Mn = 1000 g/mol, CAS 9004-74-4, Sigma-Aldrich, Steinheim, Germany] by modified solvent evaporation method [15].

Suspension of PEG-*b*-PLA was character-rized by the transmission electron microscopy (TEM; TE microscope JEM 1200; JOEL, Tokyo, Japan), electrophoretic light scattering (ELS; by Nicomp Submicron Particle Sizer Autodilute Model 380; Santa Barbara, CA, USA) and dynamic light scattering (DLS; NICOMPTM 380 ZLS Particle Sizer; Santa Barbara, CA, USA) methods. Zeta potential value measured in triplicate at pH 7.0 by the ELS method was 28.73 ± 1.44 mV. The size distribution of PEG-*b*-PLA was evaluated by DLS; micelles dispersion resulted in size distribution with two main peaks averaged as: 64.9 ± 10.5 nm and 911.4 ± 177.6 nm (for details see Rollerova 2015).

Titanium dioxide TiO2, preparation and characterization

Titanium (IV) oxide (mixture of rutile and anatase, 99.5 % trace metal basis, CAS No. 13463-67-7) was purchased in a powder form from Sigma-Aldrich (Munich, Germany). To prepare an experimental stock solution, 50 mg TiO_2 was mixed with 10 ml of the culture medium in a sterile glass conic flask and sonicated with a probe sonicator (MSE Ultrasonic Disintegrator, London, UK) for 15 min at 150 W [16]. The stock TiO_2 NPs suspension was subsequently diluted with the culture

medium to achieve the final assay concentration.

TEM revealed that TiO_2 NPs in solution were spherical and polydisperse with a primary particle size ranging from 20–50 nm accounting for 88 % and higher than 50 nm accounted for 12 % of the total particle count. DLS analysis of TiO_2 NPs solution showed 3 populations of particles: 339 nm (96.65 %), 4943 nm (5.45 %), and 72 nm (0.9 %), resulting in an average diameter of 310 nm. The particles were negatively charged with a zeta potential of -12.8 mV.

Cytotoxicity analysis (2D cultivation)

Effects of two types of nanoparticles (PEG-*b*-PLA, TiO_2) on viability of carcinoma cells (HT-29, MCF-7, U-118MG) and noncancer cells (HEK-293T) were determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Aldrich, Germany) colorimetric technique. Cells (8 × 103 cells/200 µL well) were placed in individual wells of 96-multiwell plates. Each concentration of nanoparticles was tested in triplicates. Nanoparticles were diluted with culture medium to final concentrations of 0.01, 0.1, 1, 10, 50, and 100 µg/ml and added to the cells. After 72h incubation (37 °C, humidified atmosphere of 5 % CO_2/95 % air), cells were treated with the MTT solution (5 mg/mL in PBS, 20 µL) for 3 h. The dark crystals of formazan formed in intact cells were dissolved in DMSO (200 µL). The plates were shaken for 15 min and the optical density was determined at 595 nm using a Microplate Reader (Biotek, USA).

Cytotoxicity analysis (3D cultivation)

The four multicellular spheroids of carcinoma cells (HT-29, MCF-7, U-118MG) and noncancer cells (HEK-293T) were placed into the wells of 24-multiwell plates. Plates were treated with 0.8 % low melting point agarose (Lonza, Basel, Switzerland) which was prepared in sterile water to form a thin film with a non-adhesive surface. Multicellular spheroids were affected with two nanoparticles (PEG-*b*-PLA, TiO_2) at the final concentration range of 0.01-100 µg/ml and cultivated for 72h (37 °C, humidified atmosphere of 5 % CO_2/95 % air). Diameters of the multicellular spheroids were measured every 24 h of the exposure in arbitrary units [17].

Comet assay

The DNA damage was measured using the alkaline comet assay according to Collins *et al.* [18].

Statistical analysis

Results are expressed as arithmetic means ± standard deviation (SD) of the means of three separate experiments (each experiment was performed with three parallels). The statistical evaluation was performed using a parametric unpaired t-test. When $P<0.05$ the finding was considered statistically significant.

Results

Cytotoxicity analysis (2D cultivation)

We have studied the effects of two types of tested nanoparticles (PEG-*b*-PLA, TiO_2) during 2D cultivation when cells form a monolayer on the bottom of the cultivation flask. Effects of tested nanoparticles on the human colon carcinoma cells HT-29, human breast carcinoma cells MCF-7, human glioblastoma cells U-118MG and the human noncancer cells HEK-293T were evaluated using MTT assay during 72h treatment. The concentration range of the nanoparticles was 0.01, 0.1, 1, 10, 50, and 100 µg/ml.

Nanoparticles did not cause cytotoxic effects on the tested cancer cell lines during 72h incubation (Table 1). IC_{50} was higher than the highest concentration used (100 µg/ml).

Table 1. Growth inhibitory concentrations IC_{50} (µg/ml) of PEG-*b*-PLA and TiO_2 nanoparticles for the human cancer cell lines HT-29, MCF-7, U-118MG, and human noncancer cells HEK-293T in the monolayer during 72h of the nanoparticles influence

IC_{50} (µg/ml)	**PEG-*b*-PLA**				**TiO_2**			
Time	HT-29	MCF-7	U-118MG	HEK-293T	HT-29	MCF-7	U-118MG	HEK-293T
24h	>100	>100	>100	>100	>100	>100	>100	>100
48h	>100	>100	>100	>100	>100	>100	>100	>100
72h	>100	>100	>100	7 ± 0.7	>100	>100	>100	>100

Results are expressed as the mean ± standard deviation from triplicates.

Human noncancer cells HEK-293T were sensitive to PEG-b-PLA nanoparticles after 72h incubation (IC50 = 7 μg/ml) but were not sensitive to TiO_2 nanoparticles (IC50 > 100 μg/ml).

Cytotoxicity analysis (3D cultivation)

We have examined the influence of tested nanoparticles (PEG-*b*-PLA, TiO_2) on human carcinoma cells and human noncancer cells during 3D cultivation when cells form a spheroid model.

We evaluated the diameter of the spheroid during 72h treatment. The concentration range of the nanoparticle was 0.01-100 μg/ml.

Our results indicate that nanoparticles caused cytotoxic effects on each of the tested cancer cell lines except MCF-7 in the case of PEG-*b*-PLA nanoparticles (IC_{50} >100) during 72h influence (Table 2). PEG-*b*-PLA nanoparticles exhibited the highest effectivity against U-118MG cells (IC_{50} = 40 μg/ml) compared with the effect on HT-29 and MCF-7. TiO_2 nanoparticles exhibited the highest effectivity also against U-118MG cells (IC_{50} = 8 μg/ml).

Both nanoparticles caused cytotoxic effects on the noncancer cells HEK-293T (Table 2).

Comet assay

We examined the DNA damage induced by two nanoparticles, PEG-*b*-PLA and TiO_2 (0.01 - 100 μg/ml), after 24h incubation. DNA damage was monitored by the comet assay which detects DNA strand breaks forming the tail of comets visualized by fluorescence microscopy.

We have found dose-dependent DNA damage in cancer cell lines HT-29, MCF-7, and U-118MG induced by tested nanoparticles. Figures 1, 2, 3, and 4 show the effects of two types of nanoparticles, PEG-b-PLA and TiO_2, on the induction of DNA damage in cancer cells HT-29, MCF-7, U-118MG, and non-cancer HEK-293T cells. PEG-b-PLA nanoparticles induced statistically significant DNA damage (*$p<0.05$) to the cells HT-29 at concentrations of 50 and 100 μg/ml (Fig. 1). In this cell line, PEG-b-PLA nanoparticles induced a 1.71-fold (at the concentration of 50 μg/ml) and 1.77-fold (at the concentration of 100 μg/ml) increase in DNA damage compared to control cells (cells without the treatment). TiO2 nanoparticle induced statistically significant DNA damage (*$p<0.05$) to the cells HT-29 at concentrations of 0.01 and 10 μg/ml (Fig. 1).

Table 2. Growth inhibitory concentrations IC_{50} (μg/ml) of PEG-*b*-PLA and TiO_2 nanoparticles against the human cancer cell lines HT-29, MCF-7, U-118MG MCF-7 and human noncancer cells HEK-293T in the spheroid form during 72h influence with nanoparticles

IC_{50} (μg/ml)	**PEG-*b*-PLA**				**TiO_2**			
Time	HT-29	MCF-7	U-118MG	HEK-293T	HT-29	MCF-7	U-118MG	HEK-293T
24h	>100	>100	>100	>100	>100	>100	>100	>100
48h	>100	>100	>100	>100	>100	>100	>100	>100
72h	71±0.4	>100	40± 0.8	45 ± 0.02	90±0.3	84±0.2	8±0.01	48±0.3

Results are expressed as the mean ± standard deviation from four parallels.

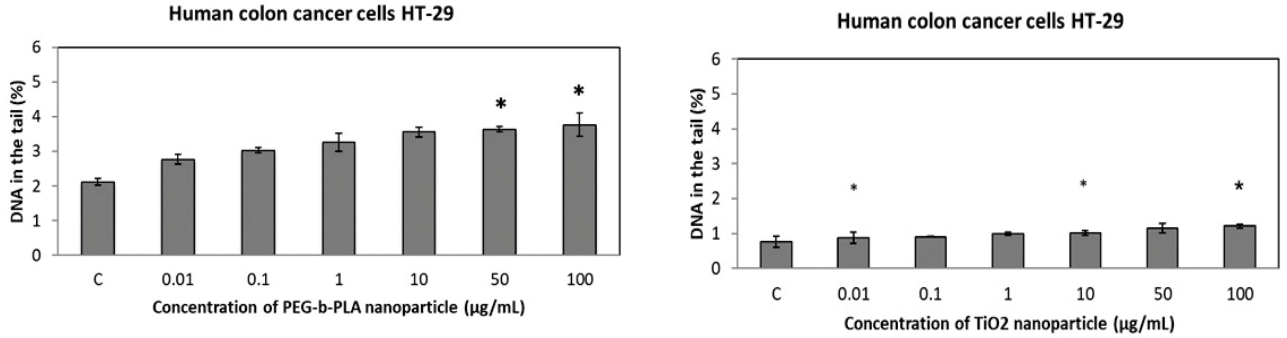

Fig. 1. Genotoxic effects of PEG-*b*-PLA (**a**) and TiO_2 (**b**) nanoparticles against HT-29 cells shown as a percentage of DNA in the tail caused by nanoparticles after 24h incubation. C – control cells without treatment. Each value represents the arithmetic mean ± SD of three separate experiments (n = 3): *$p<0.05$.

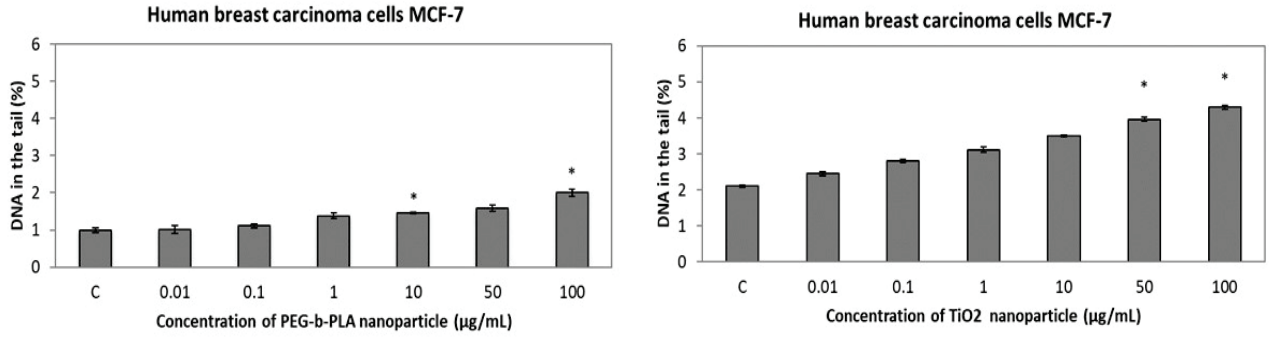

Fig. 2. Genotoxic effects of PEG-b-PLA (**a**) and TiO_2 (**b**) nanoparticles against MCF-7 cells shown as a percentage of DNA in the tail after 24h incubation. C – control cells without treatment. Each value represents the arithmetic mean ± SD of three separate experiments (n = 3): *p<0.05

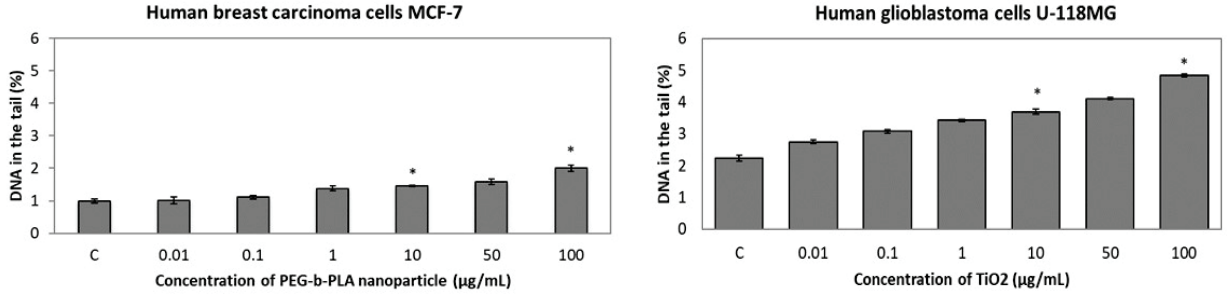

Fig. 3. Genotoxic effects of PEG-*b*-PLA (**a**) and TiO_2 (**b**) nanoparticles against U-118MG cells shown as a percentage of DNA in the tail caused by nanoparticles after 24h incubation. C – control cells without treatment. Each value represents the arithmetic mean ± SD of three separate experiments (n = 3): *p<0.05

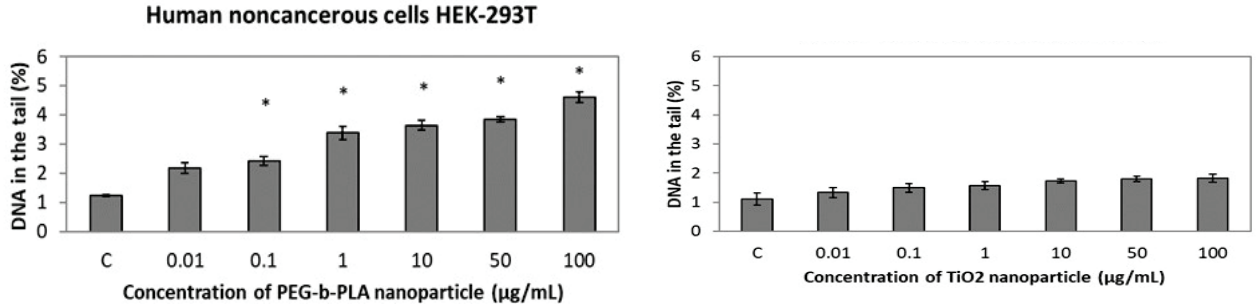

Fig. 4. Genotoxic effects of PEG-*b*-PLA (**a**) and TiO_2 (**b**) nanoparticles against HEK-293T cells shown as a percentage of DNA in the tail caused by nanoparticles after 24h incubation. C – control cells without treatment. Each value represents the arithmetic mean ± SD of three separate experiments (n = 3): *p<0.05

Nanoparticles PEG-*b*-PLA induced significant DNA damage (*p<0.05) to cancer cell line MCF-7 at the concentrations of 10 and 100 μg/ml and TiO_2 nanoparticles induced significant DNA damage (*p<0.05) in the same cell line at concentrations of 50 and 100 μg/ml (Fig. 2).

In the cell line MCF-7, PEG-*b*-PLA nanoparticles induced a 1.47-fold (at the concentration of 10 μg/ml) and 2.02-fold (at the concentration of 100 μg/ml) increase in the DNA damage compared to control cells (cells without nanoparticle treat -ment). Nanoparticles TiO_2 induced a 1.88-fold (at a concentration 50 μg/ml) and 2.04-fold (at a concentration of 100 μg/ml) increase in the DNA damage compared to control cells (cells without nanoparticle treatment).

Nanoparticles PEG-*b*-PLA did not induce significant DNA damage in cancer cell line U-118MG during 24h incubation at the concentration range used (Fig. 3). On the other hand, nanoparticles TiO_2 induced significant DNA damage (*p<0.05) at the concentrations of 10, 50, and 100 μg/ml and this DNA damage was 1.65-fold (at the concentration of 10 μg/ml), 1.84-fold (at the concentration of 50 μg/ml) and 2.16-fold (at the

concentration of 100 μg/ml) increased compared to control cells (cells without nanoparticle treatment).

In noncancer cells HEK-293T, nanoparticles PEG-*b*-PLA (concentration range 0.1-100 μg/ml) induced significant DNA damage (*p<0.05) (Fig. 4) at the concentrations of 0.1, 1, 10, 50, and 100 μg/ml. The lowest concentration of nanoparticles 0.1 μg/ml caused a 1.97-fold increase in DNA damage compared to control cells (cells without treatment).

Nanoparticles TiO_2 in the concentration range of 0.01-100 μg/ml caused no significant DNA damage (Fig. 4) during 24h cultivation with the noncancer cells.

Discussion

Nanoparticles are used in many fields of everyday life including therapeutic and diagnostic purposes [19]. Local delivery of chemotherapeutic drugs by nanoparticles to a tumor offers treatment advantages compared to standard systemic chemotherapy [20]. We have been interested in the biological effects of nanoparticles with no-loaded drugs on several cancer and noncancer cell lines. In this study, we have tested two types of nanoparticles (TiO_2 and PEG-b-PLA) on human cancer (colon cancer HT-29; breast cancer MCF-7; glioblastoma U-118MG) and noncancer cell lines (embryonic kidney HEK-293T) in the concentration range of 0.01 - 100 μg/ml. Our results indicate that TiO_2 nanoparticles had no cytotoxic effects on cancer (HT-29, MCF-7, and U-118MG) as well as noncancer (HEK-293T) cells grown in 2D cultivation. TiO_2 nanoparticles appear to be a good (inert) carrier system for drug delivery. Kumar *et al.* (2018) used nanoparticles as a carrier of phloroglucinol drugs. They reported a dose-dependent cytotoxic effect on MCF-7 cells with an inhibitory concentration of about 78.03 ± 0.23 μg/ml [21]. This effect was caused by the intracellular damage due to the smaller size (10–50 nm) of nanoparticles [21].

We have reported no cytotoxic effect of TiO_2 nanoparticles against human colon cancer cells HT-29. However, another type of human colon cancer cell line - HCT116 was reported to have selective bio-effects with dose- and cell-dependent influence on viability [22]. The growth and viability of these cells were inhibited and authors observed increased P53, Bax, and Caspase 3 expression and decreased BcL-2 levels. The ratio of Bax/Bcl-2 was down-regulated. The authors demonstrated apoptosis in HT29 cells and also up-regulated P53 and Bax at mRNA level, increased Bax / Bcl-2 ratio, and ultimately up-regulated caspase 3 by TiO_2 nanoparticles.

Similarly, as with HT-29, TiO_2 showed no cytotoxic effects against another cancer cell line that we have tested - glioblastoma cells U-118MG. Gliomas are the most common primary brain tumors [23]. The problem with brain tumor treatment is the existence of the blood-brain barrier. This barrier restricts the delivery of drugs into the brain. Nanoparticles offer a good way how to transport effectively anticancer drugs into a tumor [24]. A wide variety of nanoparticles have been made to allow the transport of therapeutic drugs across the blood-brain barrier. Recent studies showed that biodegradable polymers or copolymers of nanoparticles and metal nanoparticles have been attractive vehicles for carrying drugs across the blood-brain barrier to treat human gliomas [24]. The results of Glaser *et al.* (2017) suggest that TiO_2, after irradiation, would induce an increase in free radical production and cell death in glioblastoma. Titanium dioxide caused membrane damage and DNA fragmentation, which is characteristic of apoptosis [25]. Markowska-Szczupak confirmed that reactive oxygen species are responsible for this mechanism [26]. Recent studies point to the mechanism by which nanoparticles seek and destroy brain tumor cells without destroying healthy tissue. TiO_2 nanoparticles covalently conjugated with antihuman-IL13α2R via DOPAC (3,4-dihydroxyphenlacetic acid) linker after exposure to visible light, initiated production of ROS, which damaged brain tumor cells and induced programmed cell death [27]. Other studies point to the possibility that TiO_2 nanoparticles can damage DNA and thus increase the risk of cancer by a mechanism associated with oxidative stress [28]. After the influence of HepG2 cells with TiO_2 nanoparticles, expression of Nrf2 mRNA was significantly increased and down-regulated genes were activated, including NQO1, HO-1, and GCLC. Reduction of Nrf2 resulted in increased sensitivity to DNA damage and, conversely, an increase in Nrf2 resulted in reduced sensitivity to DNA damage. These processes were associated with changes in oxidative stress conditions [29]. However, this effect has been demonstrated after the application of nanoparticles to mice, in blood and tissues, but not in the brain in connection with healthy tissue [30].

The second tested nanoparticle in our study was PEG-*b*-PLA nanoparticle. These polymeric micelles function as inert carriers for hydrophobic anticancer agents such as paclitaxel (PTX) and doxorubicin (DOX) [31]. In the literature, there are no results on toxic effects

caused by PEG-*b*-PLA nanoparticles (without drugs) against cancer or noncancer cells. We have also found no cytotoxic effects on cancer (HT-29, MCF-7, and U-118MG) and noncancer (HEK-293T) cells grown in 2D cultivation.

Recently, spheroids (3D cultivation) have been more often used to answer a wide range of questions in clinical and biomedical research. 3D cell cultures have several *in vivo* attributes of tumors, e.g. cell-cell interaction, hypoxia, penetration of drugs, response and resistance, and production or deposition of extracellular matrix [32,33]. Tumor cells respond differently to various stimuli when are grown in 2D versus 3D tissue environments [34]. For this reason, we have decided to compare results from cytotoxic analyses obtained from 2D and 3D cultivations. Using a modification of existing methods, we have prepared tumor-fragment spheroids. Spheroids appear to simulate characteristics of the original tumor and may be used to assess critical therapy-modulating features of the microenvironment. Our tested nanoparticles were titanium dioxide (TiO_2) and copolymer PEG-*b*-PLA [$CH_3O(CH_2CH_2O)$ x $(COCHCH_3O)$yH] with size <100 nm. We have found that PEG-*b*-PLA nanoparticles exhibited the highest effectivity against U-118MG cells (IC_{50} = 40 μg/ml) in the spheroid formation compared with its effect on HT-29 (IC_{50} = 71 μg/ml) and MCF-7 (>100 μg/ml) both in the spheroid formation. Similarly, TiO_2 nanoparticles exhibited the highest effectivity also against glioma cells U-118MG (IC_{50} = 8 μg/ml) in the spheroid formation compared to MCF-7 cells (IC_{50} = 84 μg/ml) and HT-29 (IC_{50} = 90 μg/ml). The effects of both nanoparticles on noncancer cells HEK-293T were similar (Table 2).

Authors Danny Jian Hang Tang *et al.* (2015) discovered that the MIA PaCa-2 cell line showed the strongest affinity for 110 nm hybrid polymeric nanoparticles (PLGA and mPEG-DSPE) compared with 65 nm and 85 nm nanoparticles. In the spheroid formation of the MIA PaCa-2 cell line, 65 nm nanoparticles produced the greatest therapeutic effect [35].

Cultivation of nanoparticles with cells in the spheroid form showed cytotoxic effects after 72h. This effect was different depending on the cancer cell lines and nanoparticles used. However, the effect of both types of nanoparticles on control HEK-293T cells was comparable.

In the second part of our study, we detected DNA damage in human cancer cells after incubation with tested nanoparticles. We have examined the effect of two types of nanoparticles, PEG-*b*-PLA and TiO2, on DNA by the single cell gel electrophoresis – comet assay using human cancer cells (HT-29; MCF-7; U-118MG) and human noncancer cells (HEK-293T). For this experiment, we have used cells from 2D cultivation because the spheroid formation is the complex (entirety) of cells and we had to classify separate cells into the four classes [18]. According to our results, the most sensitive cells against PEG-*b*-PLA nanoparticles (0.1-100 μg/ml) were HEK-293T cells. On the other hand, HEK-293T cells were not significantly sensitive against TiO_2 nanoparticles.

A recent study indicates the genotoxic effects of metal nanoparticles in different human cancer and noncancer cells mainly through the generation of oxidative stress in cells [36].

In the literature, there are no results on DNA damage caused by PEG-*b*-PLA nanoparticles against cancer or noncancer cells. As a negative attribute of this nanoparticle, Xiao *et al.* (2010) reported that PLA applications are limited due to its weak hydrophilicity, excessively long degradation time, and low loading of polar drugs. Thus, in low concentrations, the copolymer is nontoxic and not accumulative *in vivo* [37]. The degradation products of PEG–PLA block copolymer can enter the tricarboxylic acid cycle or be eliminated by the kidney [38,39].

Our results indicate the toxic effects of PEG-*b*-PLA nanoparticles at higher concentrations (10, 50, 100 μg/ml) on cancer cells. On the other hand, cultivation with the noncancer cells HEK-293T shows DNA damage at concentrations 0.1, 1, 10, 50, and 100 μg/ml.

Conclusion

Our *in vitro* results highlight the necessity to study the toxic effects of nanoparticles without drugs, and detailed molecular mechanisms of their action in two types of conditions (monolayer 2D- and spheroid forming 3D- cultivation of cells). Understanding the biology of sphere-forming cells may contribute to the identification of novel therapeutic opportunities.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This paper was financially supported by the Operational

Program Integrated Infrastructure for the project: Research and development in medical sciences - the way to personalized treatment of serious neurological, cardiovascular and cancer diseases (ITMS code: 313011T431), co-financed from the resources of the European Regional Development Fund.

References

1. Kavoosi F, Modaresi F, Sanaei M, Rezaei Z. Medical and dental applications of nanomedicines. J Pathol Microbiol Immunol 2018;126:795-803. https://doi.org/10.1111/apm.12890
2. Hofmann-Amtenbrink M, Grainger DW, Hofmann H. Nanoparticles in medicine: Current challenges facing inorganic nanoparticle toxicity assessments and standardizations. Nanomedicine: Nanotechnology, Biology and Medicine, 2015;11:1689-1694. https://doi.org/10.1016/j.nano.2015.05.005
3. Rizvi SAA and Saleh AM. Applications of nanoparticle systems in drug delivery technology. Saudi Pharm J, 2018;26:64-70. https://doi.org/10.1016/j.jsps.2017.10.012
4. Liu J, Jiang X, Ashley C, Brinker CJ. Electrostatically mediated liposome fusion and lipid exchange with a nanoparticle-supported bilayer for control of surface charge, drug containment, and delivery. J Am Chem Soc 2009;10;131:7567-7569. https://doi.org/10.1021/ja902039y
5. Friedman AD, Claypool SE, Liu R. The Smart Targeting of Nanoparticles. Curr Pharm Des 2013;19:6315-6329. https://doi.org/10.2174/13816128113199990375
6. Czajka M, Sawicki K, Sikorska K, Popek S, Kruszewski M, Kapka-Skrzypczak L. Toxicity of titanium dioxide nanoparticles in central nervous system. Toxicol In Vitro, 2015;29:1042-1052. https://doi.org/10.1016/j.tiv.2015.04.004
7. Bahadar H, Maqbool F, Niaz K, Abdollahi. Toxicity of Nanoparticles and an Overview of Current Experimental Models. Iran Biomed J, 2016; 20:1-11.
8. Lakkireddy HR and Bazile D. Toxicity of nanoparticles and an overview of current experimental models. Iran Biomed J, 2016; 20:1-11.
9. Mohamad NH, Morsi MM, Ahmed AE, Sherif K. Microwave-assisted preparation of Nano-hydroxyapatite for bone substitutes. Ceramics International, 2016;42:3725-3744. https://doi.org/10.1016/j.ceramint.2015.11.044
10. Tam YT, To KK, Chow AH. Fabrication of doxorubicin nanoparticles by controlled antisolvent precipitation for enhanced intracellular delivery. Colloids Surf B Biointerfaces 2016;139:249-58. https://doi.org/10.1016/j.colsurfb.2015.12.026
11. Saucier-Sawyer JK, Deng Y, Seo YE, Cheng CJ, Zhang J, Quijano E, Saltzman WM. Systemic delivery of blood-brain barrier targeted polymeric nanoparticles enhances delivery to brain tissue. J Drug Target 2015; 23: 736-749. https://doi.org/10.3109/1061186X.2015.1065833
12. Shi H, Magaye R, Castranova V, Zhao J. Titanium dioxide nanoparticles: a review of current toxicological data. Part Fibre Toxicol 2013;10:15. https://doi.org/10.1186/1743-8977-10-15
13. Lin Z, Monteiro-Riviere NA, Riviere JE. Pharmacokinetics of metallic nanoparticles. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2015;7:189-217. https://doi.org/10.1002/wnan.1304
14. Grande F, Tucci P. Titanium dioxide nanoparticles: a risk for human health? Mini Rev Med Chem 2016;16:762-769. https://doi.org/10.2174/1389557516666160321114341
15. Rollerova E, Jurcovicova J, Mlynarcikova A, Sadlonova I, Bilanicova D, Wsolova L, Kiss A, Kovriznych J, Kronek J, Ciampor F, Vavra I, Scsukova S. Delayed adverse effects of neonatal exposure to polymeric nanoparticle poly(ethylene glycol)-block-polylactide methyl ether on hypothalamic-pituitary-ovarian axis development and function in Wistar rats. Reprod Toxicol 2015;57:165-175. https://doi.org/10.1016/j.reprotox.2015.07.072
16. Volkovova K, Handy RD, Staruchova M, Tulinska J, Kebis A, Pribojova J, Ulicna O, Kucharská J, Dusinska M. Health effects of selected nanoparticles in vivo: liver function and hepatotoxicity following intravenous injection of titanium dioxide and Na-oleate-coated iron oxide nanoparticles in rodents. Nanotoxicology 2015;1:95-105. https://doi.org/10.3109/17435390.2013.815285

17. Bizik J, Kankuri E, Ristimäki A, Taïeb A, Vapaatalo H, Lubitz W, Vaheri A. Cell-cell contacts trigger programmed necrosis and induce cyclooxygenase-2 expression. Cell Death Differ 2004;11:183-195. https://doi.org/10.1038/sj.cdd.4401317
18. Collins A, Dusinská M, Franklin M, Somorovská M, Petrovská H, Duthie S, Fillion L, Panayiotidis M, Raslová K and Vaughan N. Comet assay in human biomonitoring studies: Reliability, validation and applications. Environ Mol Mutagen 1997;30:139-146. https://doi.org/10.1002/(SICI)1098-2280(1997)30:2<139::AID-EM6>3.0.CO;2-I
19. Wolfram J, Zhu M, Yang Y, Shen J, Gentile E, Paolino D, Fresta M, Nie G, Chen C, Shen H, Ferrari M, Zhao Y. Safety of nanoparticles in medicine. Curr Drug Targets 2015;16:1671-1681. https://doi.org/10.2174/1389450115666140804124808
20. Rezazadeh M, Davatsaz Z, Emami J, Hasanzadeh F, Jahanian-Najafabadi A. Preparation and characterization of spray-dried inhalable powders containing polymeric micelles for pulmonary delivery of paclitaxel in lung cancer. J Pharm Pharm Sci 2018;21(1s):200-214. https://doi.org/10.18433/jpps30048
21. Kumar P, Yuvakkumar R, Vijayakumar S, Vaseeharan B. Cytotoxicity of phloroglucinol engineered silver (Ag) nanoparticles against MCF-7 breast cancer cell lines. Materials Chemistry and Physics 2018;220:402-408. https://doi.org/10.1016/j.matchemphys.2018.08.074
22. Kukia NR, Rasmi Y, Abbasi A, Koshoridze N, Shirpoor A, Burjanadze G, Saboory E. Bio-Effects of TiO2 nanoparticles on human colorectal cancer and umbilical vein endothelial cell lines. Asian Pac J Cancer Prev 2018; 19:2821-2829. https://doi.org/10.22034/APJCP.2018.19.10.2821
23. van Tellingen O, Yetkin-Arik B, de Gooijer MC, Wesseling P, Wurdinger T, de Vries HE. Overcoming the blood-brain tumor barrier for effective glioblastoma treatment. Drug Resist Updat 2015;19:1-12. https://doi.org/10.1016/j.drup.2015.02.002
24. Zhang F, Xu CL, Liu CM. Drug delivery strategies to enhance the permeability of the blood-brain barrier for treatment of glioma. Drug Des Devel Ther 2015;9:2089-2100. https://doi.org/10.2147/DDDT.S79592
25. Glaser T, Han I, Wu L, Zeng X. Targeted Nanotechnology in Glioblastoma Multiforme. Front Pharmacol 2017;8: 166. https://doi.org/10.3389/fphar.2017.00166
26. Markowska-Szczupak A, Ulfig A, Morawski W. The application of titanium dioxide for deactivation of bioparticulates: An overview. Catalysis Today 2011;169:249-257. https://doi.org/10.1016/j.cattod.2010.11.055
27. Rozhkova EA, Ulasov I, Lai B, Dimitrijevic NM, Lesniak MS, Rajh T. A high-performance nanobio photocatalyst for targeted brain cancer therapy. Nano Lett, 2009;9:3337-3342. https://doi.org/10.1021/nl901610f
28. Shi Z, Niu Y, Wang Q, Shi L, Guo H, Liu Y, Zhu Y, Liu S, Liu C, Chen X, Zhang R. Reduction of DNA damage induced by titanium dioxide nanoparticles through Nrf2 in vitro and in vivo. J Hazard Mater 2015;298:310-319. https://doi.org/10.1016/j.jhazmat.2015.05.043
29. Zhang R, Niu Y, Li Y, Zhao C, Song B, Li Y, Zhou Y. Acute toxicity study of the interaction between titanium dioxide nanoparticles and lead acetate in mice. Environ Toxicol Pharmacol 2010;30:52-60. https://doi.org/10.1016/j.etap.2010.03.015
30. Sugibayashi K, Todo H, Kimura E. Safety evaluation of titanium dioxide nanoparticles by their absorption and elimination profiles. J Toxicol Sci 2008;33:293-298. https://doi.org/10.2131/jts.33.293
31. Zhang Z, Xiong X, Wan J, Xiao L, Gan L, Feng Y, Xu H, Yang X. Cellular uptake and intracellular trafficking of PEG-b-PLA polymeric micelles. Biomaterials 2012;33:7233-7240. https://doi.org/10.1016/j.biomaterials.2012.06.045
32. Ong CS, Zhou X, Han J, Huang CY, Nashed A, Khatri S, Mattson G, Fukunishi T, Zhang H, Hibino N. In vivo therapeutic applications of cell spheroids. Biotechnol Adv 2018;36:494-505. https://doi.org/10.1016/j.biotechadv.2018.02.003
33. Zanoni M, Piccinini F, Arienti C, Zamagni A, Santi S, Polico R, Bevilacqua A, Tesei A. 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. Sci Rep 2016;6:19103. https://doi.org/10.1038/srep19103
34. Lao Z, Kelly CJ, Yang XY, Jenkins WT, Toorens E, Ganguly T, Evans SM, Koch CJ. Improved methods to generate spheroid cultures from tumor cells, tumor cells & fibroblasts or tumor-fragments: microenvironment, microvesicles and MiRNA. PLoS One 2015;10: e0133895. https://doi.org /10.1371/journal.pone.0133895

35. Tng DJH, Song P, Lin G, Soehartono AM, Yang G, Yang CH, Yin F,1 Tan CH, Yong KT. Synthesis and characterization of multifunctional hybrid-polymeric nanoparticles for drug delivery and multimodal imaging of cancer. Int J Nanomedicine 2015;10:5771-5786. https://doi.org/10.2147/IJN.S86468
36. Radhakrishnan VS, Dwivedi SP, Siddiqui MH, Prasad T. *In vitro* studies on oxidative stress-independent, Ag nanoparticles-induced cell toxicity of Candida albicans, an opportunistic pathogen. Int J Nanomedicine 2018; 13(T-NANO 2014 Abstracts): 91-96. https://doi.org/10.2147/IJN.S125010
37. Xiao RZ, Zeng ZW, Zhou GL, Wang JJ, Li FZ, Wang AM. Recent advances in PEG-PLA block copolymer nanoparticles. Int J Nanomedicine 2010;5:1057-1065. https://doi.org/10.2147/IJN.S14912
38. Shin HC, Cho , Lai C, Kozak KR, Kolesar JM, Kwon GS. Pharmacokinetic study of 3-in-1 poly(ethylene glycol)-block-poly(D, L-lactic acid) micelles carrying paclitaxel, 17-allylamino-17-demethoxygeldanamycin, and rapamycin. J Control Release 2012;163:93-99. https://doi.org/10.1016/j.jconrel.2012.04.024
39. Jong WHD and Borm PJA. Drug delivery and nanoparticles: Applications and hazards. Int J Nanomedicine 2008; 3:133-149. https://doi.org/10.2147/IJN.S596