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Three dimensional spheroid cell culture for nanoparticle safety testing

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ABSTRACT

Nanoparticles are widely employed for many applications and the number of consumer products, incorporating nanotechnology, is constantly increasing. A novel area of nanotechnology is the application in medical implants. The widespread use of nanoparticles leads to their higher prevalence in our environment. This, in turn, raises concerns regarding potential risks to humans. Previous studies have shown possible hazardous effects of some nanoparticles on mammalian cells grown in two-dimensional (2D) cultures. However, 2D *in vitro* cell cultures display several disadvantages such as changes in cell shape, cell function, cell responses and lack of cell-cell contacts. For this reason, the development of better models for mimicking *in vivo* conditions is essential.

In the present work, we cultivated A549 cells and NIH-3T3 cells in three-dimensional (3D) spheroids and investigated the effects of zinc oxide (ZnO-NP) and titanium dioxide nanoparticles (TiO_2-NP). The results were compared to cultivation in 2D monolayer culture. A549 cells in 3D cell culture formed loose aggregates which were more sensitive to the toxicity of ZnO-NP in comparison to cells grown in 2D monolayers. In contrast, NIH-3T3 cells showed a compact 3D spheroid structure and no differences in the sensitivity of the NIH-3T3 cells to ZnO-NP were observed between 2D and 3D cultures. TiO_2-NP were non-toxic in 2D cultures but affected cell-cell interaction during 3D spheroid formation of A549 and NIH-3T3 cells. When TiO_2-NP were directly added during spheroid formation in the cultures of the two cell lines tested, several smaller spheroids were formed instead of a single spheroid. This effect was not observed if the nanoparticles were added after spheroid formation. In this case, a slight decrease in cell viability was determined only for A549 3D spheroids. The obtained results demonstrate the importance of 3D cell culture studies for nanoparticle safety testing, since some effects cannot be revealed in 2D cell culture.

1. Introduction

Interest in nanoparticle research has increased dramatically over the last decades due to the wide range of applications of nanoparticles in everyday objects such as sun screens and paints. Nanoparticles are also intensively studied as possible candidates for the coating of medical implants. Such coatings can reduce the risk of bacterial contamination and can improve implant biocompatibility. Since nanoparticle safety issues are not fully clarified, further investigations are needed to characterize the effects of nanoparticles on human health and the environment. Nanotoxicological, nanotherapeutics and drug screening studies are still primarily based on two dimensional (2D) cell culture or, in the later stages, on complex animal *in vivo* models (da Rocha et al., 2013). Although 2D cell culture is a robust, well-established and reproducible technique of *in vitro* testing, the use of 2D cultures often yields results with large discrepancies relative to *in vivo* animal models. This is not surprising, since 2D cell culture represents an environment, remarkably distinct from the *in vivo* situation, where the majority of tissues are three-dimensional (3D) (Lee et al., 2009; Fennema et al., 2013; Li and Cui, 2013).

In the last few years, many new drugs have been withdrawn during animal trials because previous *in vitro* toxicity testing failed to identify their hazards (Astashkina et al., 2012). 2D cell cultures cannot represent the complex native tissue environment with the extracellular matrix (ECM) and the totality of intercellular interactions which are very important for cell physiology and behavior (da Rocha et al., 2013; Li and Cui, 2013). Moreover, gene expression

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analysis of diverse cell lines has revealed hundreds of genes which are expressed differently in 2D and in 3D cultures (Li and Cui, 2013; Luca et al., 2013). Therefore, various 3D models have been developed to better mimic the conditions and properties of *in vivo* tissue (Kim et al., 2013). In this regard, *in vitro* studies of nanoparticles effects in a 3D cell culture model seem to be more appropriate compared to 2D monolayer systems, since toxicity results can be more strongly influenced by the cell microenvironment (Drewitz et al., 2011; Mueller et al., 2014).

Spheroids (cell aggregates) represent a simple 3D system since no scaffold or supporting material is required for 3D cell growth (Fennema et al., 2013). It has been shown that cellular adhesion regulates cell migration, proliferation and apoptosis in spheroids (Li and Cui, 2013). Different techniques are used to generate spheroids such as hanging drop or low-attachment plates (Haycock, 2011). The size of the cell aggregates (spheroids) plays a crucial role and impacts cell responses (Asthana and Kisaalita, 2012; Fennema et al., 2013). Small-sized spheroids do not display the complexity of real tissue, whereas larger spheroids demonstrate diffusion limitations for oxygen and nutrients (Asthana and Kisaalita, 2012). These limitations often induce necrotic core formation and reduce the viability of the cells (Asthana and Kisaalita, 2012). In addition, various cell types show differences in their cell packing density (Griffith and Swartz, 2006). Some cells form very compact structures whereas others display low cell density in aggregates (Griffith and Swartz, 2006; Friedrich et al., 2007). Literature suggests that the ideal size for spheroids ranges from 100 to 600 μm (Griffith and Swartz, 2006; Friedrich et al., 2007; Lee et al., 2009; Asthana and Kisaalita, 2012; Gao et al., 2013). The spheroid critical size can vary, depending on cell type and cell packing density, thus a separate investigation for each cell line used is recommended. In cell spheroids, the ECM and the dense cell packing act as transport barriers, hindering nanoparticle penetration to the spheroid core (Goodman et al., 2007) and only a subset of the cells are exposed to the tested substance (*e.g.* nanoparticles) (Lee et al., 2009). Moreover, the penetration of nanoparticles in spheroids can be limited by nanoparticle-cell interaction (Gao et al., 2013).

Zinc oxide (ZnO) and titanium dioxide nanoparticles (TiO_2-NP) are widely used for many applications. For ZnO-NP several cytotoxic effects have been reported on different cell types in 2D, whereas only a few studies have been published in 3D. It was reported that ZnO-NP decreased cell viability (Heng et al., 2010; Hsiao and Huang, 2011; Taccola et al., 2011; Zhao et al., 2012; Li et al., 2013; Sahu et al., 2013), induced oxidative stress (Fukui et al., 2012; Sahu et al., 2013), lead to DNA damage (Ng et al., 2011; Sahu et al., 2013) and induced apoptosis (Meyer et al., 2011; Ng et al., 2011). In contrast to ZnO-NP, the toxicity of TiO_2-NP is still a point of discussion. The differences in the reported results of TiO_2-NP toxicity may occur due to the divergence in used cells, size of nanoparticles and applied assays (Demir et al., 2014). Several working groups revealed toxic effects of TiO_2-NP such as induced generation of reactive oxygen species (ROS) (Liu et al., 2010), up-regulated apoptosis markers (Srivastava et al., 2013) and decreased cell viability (Aueviriyavit et al., 2012; Marquez-Ramirez et al., 2012; Hou et al., 2013). In addition, genotoxic effects of TiO_2-NP on NIH-3T3 cells were detected by the comet assay at the highest dose of 1.000 μg/ml TiO_2-NP (Demir et al., 2014). Other groups reported absence of toxic effects of TiO_2-NP in 2D cultures (Wagner et al., 2008; Fujita et al., 2009; Hsiao and Huang, 2011; Fisichella et al., 2012).

In few published studies on the effects of nanoparticles in 2D and 3D cell cultures particles were added after spheroid formation. Kim et al. revealed similar cell proliferation of A549 cells in 2D and 3D cell cultures treated with ZnO-NP, whereas molecular markers for oxidative stress were significantly reduced only in 2D cell culture (Kim et al., 2014). Cell viability in C6 rat glioma spheroids was not decreased in the presence of TiO_2-NP (Yamaguchi et al., 2010). Lee and colleagues showed a reduced toxic effect in HepG2 spheroids for cadmium telluride (CdTe) and gold nanoparticles in comparison to the 2D cell culture (Lee et al., 2009). Interestingly, the EC_{50} values obtained for some drugs in 3D cell culture were in the same range as those obtained in *in vivo* experiments (Mueller et al., 2014). This clearly demonstrates that *in vitro* 3D nanoparticle safety testing could bridge the gap between conventional 2D cell cultures and complex animal studies (da Rocha et al., 2013). By using valid *in vitro* models which mimic the complexity of tissues, the number of whole-animal studies can be minimized (Friedrich, 2007) and costs can be reduced (Drewitz et al., 2011; da Rocha et al., 2013). Although being very advantageous for simulating the *in vivo*-like cell microenvironment, 3D cell cultures still need further improvement and validation, since viability assays were initially established for monolayer 2D cell culture (Fennema et al., 2013; Li and Cui, 2013). Moreover, the influence of the size and density of cell aggregates, as well as the effect of the time point of drug or nanoparticles application during spheroid formation need further investigation.

The skin and the respiratory tract are important pathways of possible nanoparticle adsorption into the human organism. Therefore, in the present work we used two model cell lines – human lung carcinoma A549 cells and murine fibroblasts NIH-3T3 cells. ZnO-NP and TiO_2-NP were chosen as nanoparticle models because both nanoparticles are extensively used for different applications and their effects on 2D monolayer cultures has been described in the literature. Cytotoxic effects of ZnO-NP were reported for both A549 (Hsiao and Huang, 2011; Fukui et al., 2012) and NIH-3T3 cells (Li et al., 2013) in 2D cultures. TiO_2-NP were shown to be non-toxic to NIH-3T3 cells (Wagner et al., 2008; Demir et al., 2014) and to A549 cells (Wagner et al., 2008). Some working groups, however, reported toxic effects of TiO_2-NP on A549 cells (Aueviriyavit et al., 2012; Srivastava et al., 2013) in 2D cell cultures. The aim of this work was to study the influence of the time point of application of nanoparticles on the cell viability in 3D spheroids. In addition, a validation of two different cell viability assays for such cultivation systems was carried out.

2. Materials and methods

2.1. Cell culture

Both used cell lines A549 human lung carcinoma cells (DSMZ no.: ACC 107) and NIH-3T3 mouse fibroblasts cells (DMSZ no.: ACC 59) were purchased from the German Collection of Microorganisms and cell cultures (DSMZ). All cell lines were cultivated in Dulbecco's Modified Eagle's medium (DMEM)(D7777 Sigma–Aldrich, Steinheim, Germany) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml antibiotics (penicillin/streptomycin) in a humidified environment at 37 °C/5% CO_2. Cells were sub-cultivated every 3 or 4 days when the cultures reached 70–80% confluence. All used cells had a passage number of less than 20.

2.2. Nanoparticles

TiO_2-NP (Hombikat XXS 700) were obtained from Sachtleben, Duisburg, Germany. According to the data sheet the primary particle size of TiO_2-NP is 7 nm (REM) in the anatase form. The ZnO-NP (with 0.1% Ru) used for the cytotoxicity tests were synthesized and characterized by Bloh et al. (2012, 2014). For the ZnO-NP a BET surface of 6.54 m^2/g were measured with a particle size of 50 ± 10 nm (X-ray) by Bloh et al. (2014). To measure the hydrodynamic diameter and the distribution of the ZnO-NP and TiO_2-NP in water and in the used cell culture media, dynamic light scattering measurements were performed. Suspensions (1 ml) with a concentration

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