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In vitro cytotoxicity of hydrothermally synthesized ZnO nanoparticles on human periodontal ligament fibroblast and mouse dermal fibroblast cells

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ABSTRACT

The use of metal oxide nanoparticles (NPs) in industrial applications has been expanding, as a consequence, risk of human exposure increases. In this study, the potential toxic effects of zinc oxide (ZnO) NPs on human periodontal ligament fibroblast cells (hPDLFs) and on mouse dermal fibroblast cells (mDFs) were evaluated *in vitro*. We synthesized ZnO NPs (particle size; 7–8 nm) by the hydrothermal method. Characterization assays were performed with atomic force microscopy, Braun–Emmet–Teller analysis, and dynamic light scattering. The hPDLFs and mDFs were incubated with the NPs with concentrations of 0.1, 1, 10, 50 and 100 μg/mL for 6, 24 and 48 h. Under the control and NP-exposed conditions, we have made different types of measurements for cell viability and morphology, membrane leakage and intracellular reactive oxygen species generation. Also, we monitored cell responses to ZnO NPs using an impedance measurement system in real-time. While the morphological changes were visualized using scanning electron microscopy, the subcellular localization of NPs was investigated by transmission electron microscopy. Results indicated that ZnO NPs have significant toxic effects on both of the primary fibroblastic cells at concentrations of ∼50–100 μg/mL. The cytotoxicity of ZnO NPs on fibroblasts depended on concentration and duration of exposure.

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1. Introduction

The unique size-dependent properties of nanomaterials make them very attractive for potential applications within the biomedical, commercial and environmental sectors (Aili and Stevens, 2010; Harishchandra et al., 2010). Due to their superior electrical, thermal, mechanical, and imaging properties, engineered nanomaterials have found applications such as in cell imaging (Zhong et al., 2012), drug delivery (Panyam and Labhasetwar, 2012), cancer therapy (Xiao et al., 2012) and piezoelectric (Seker et al., 2010), electronic and optical (Lia et al., 2010) sensor systems. As the use of nanomaterials increase worldwide, the risk on human health and environmental nanomaterials increases. It is, therefore, necessary to assess the potential adverse effects of exposure, on human health and the environment.

Metal oxide nanoparticles (NPs) have been widely used in nanotechnology-based applications, such as catalysis, sensors, environmental remediation and in consumer products. In particular, zinc oxide (ZnO), gold (Au), platinum (Pt) and titanium dioxide (TiO_2) NPs find application in personal care products, especially sunscreens and toothpastes (Fröhlich and Roblegg, 2012), in coatings and paints, due to their UV absorption efficiency and transparency to visible light (Franklin et al., 2007). Despite many advantages in the wide applications of NPs, nanomaterials may cause serious environmental and health problems. Ever increasing use of the nanotechnology products requires their elaborate toxicological evaluations. Many types of NPs have proven to be toxic to human tissue and cell cultures. In particular, the respiratory and intestinal tracts and skin are in direct contact with the environment. Adding to these, NPs can translocate from these routes via different pathways and mechanisms. It is very important to characterize

Abbreviations: NP, nanoparticle; ZnO, zinc oxide; hPDLF, human periodontal ligament fibroblast; mDF, mouse dermal fibroblast; AFM, atomic force microscopy; BET, Braun–Emmet–Teller; DMEM, Dulbecco's modified Eagle's medium; SEM, scanning electron microscopy; TEM, transmission electron microscopy; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; LDH, lactate dehydrogenase; ROS, reactive oxygen species; carboxy-H_2DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate.

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NPs throughout their interaction with the biological system and to accurately determine nanoparticle uptake site and concentration (Maurer-Jones et al., 2010). Although studies regarding the environmental toxicity and potential harm on human health have increased during the last course of the decade, there is still a significant lack of knowledge related to the impact of nanomaterials on human health and the environment (Böhmert et al., 2012).

Nanotoxicology studies performed directly on model animals or cultured cells provide significant information on the effects it might have on human and other species (Suh et al., 2009). In particular, *in vitro* methods are generally utilised for investigating NP interactions with the biological systems (Arora et al., 2012; Hillegass et al., 2010). For example, cytotoxic responses such as changes in cell morphology, cell adhesion, cell–cell interactions and cell proliferation can be monitored in real-time by using the electrical impedance-based high-throughput method without using any label (McGuigan and Li, 2014).

Toxic properties of nanomaterials depend on a variety of parameters such as surface charge, chemical composition, quantity, resolution, shape and surface area. Besides, impurities subsided during NP production may be involved in material toxicity. Different biological systems may show distinct sensitivities to nanomaterials (Oberdörster et al., 2004). ZnO is one of the most widely used semi-conducting oxide (wide direct band gap of 3.37 eV) in electrical (Gulino and Fragala, 2002), electrochemical (Yumak et al., 2011), magnetic (Norton et al., 2003), and optical (Fujihara et al., 2001) applications. ZnO NPs have received considerable attention for years due to their wide range of possible technological applications such as solar cells (Jiang et al., 2007), photovoltaic devices (Ravirajan et al., 2006), varistors (Pillai et al., 2004) and sensors (Lin et al., 1998; Muti et al., 2010). Various methods (chemical precipitation, microwave technique, solvothermal, sol–gel, hydrothermal and solid state) have been used to synthesize NPs. In this work, hydrothermal method was chosen to synthesize ZnO NPs owing to particular advantages such as, allowing superior compositional and morphological control, and neither requiring calcination nor milling steps.

In this study, the physicochemical properties of hydrothermally synthesized ZnO NPs were characterized through zeta potential, particle size distribution via dynamic light scattering (DLS) and Braun–Emmet–Teller (BET) analyses. Biological responses of periodontal ligament fibroblasts (cells of the supportive tissue of the teeth) and dermal fibroblasts (cells of the skin tissue) against ZnO NPs were determined by transmission electron microscopy (TEM). Degree of intracellular reactive oxygen species (ROS) generation, cell impedance, mitochondrial activity and membrane leakage were also measured. Our findings demonstrated that ZnO NPs at 50–100 μg/mL were significantly cytotoxic to primary hPDLF and mDF cell cultures.

2. Materials and methods

2.1. Synthesis and characterization of NPs

All chemicals used for NP synthesis were of analytical grade. Triton X-100, zinc acetate and ethanol were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. We synthesized ZnO NPs by using the hydrothermal method as previously described (Sinag et al., 2011). Detailed characterization including the XRD analysis of the ZnO NPs is reported elsewhere (Sinag et al., 2011).

2.2. Atomic force microscopy (AFM)

AFM was used to investigate further the morphology and size distribution of the nanosized ZnO, coated onto polished gold surface. Study was performed on an NI–AFM model atomic force microscope (Nanomagnetics Inst., Ankara, Turkey), operating in dynamic mode in air (Seker et al., 2010). A Tap300A1 model cantilever (Budget Sensors, Innovative Solutions, Sofia, Bulgaria) with a resonance frequency of 320 kHz was used. The coated surfaces were scanned with 40 N/m force constant and imaged at a scan area of 2 μm × 2 μm. The NP solution was prepared (100 μg/mL) in dichloromethane (Sigma) at 25 °C and dispersed using a Fisher FB15060 model sonicator (Fisher Scientific, Schwerte, Germany) for an hour. For AFM evaluation, thin films on polished gold surfaces were prepared at 3000 rpm by a Primus SB15 model spin coater (Singen, Germany), then the samples were dried in air at room temperature.

2.3. BET analysis

The Braun–Emmet–Teller (BET) surface area of the ZnO NPs was determined using a NOVA 2200e volumetric gas adsorption instrument (Quantachrome Instruments, Boynton Beach, FL, USA), according to the procedures described elsewhere (Yagmur et al., 2008).

2.4. hPDLF cell isolation and culture

Periodontal ligament fibroblast cells were isolated from periodontal ligament tissue from the root surfaces of premolar teeth, following review board approval and informed consent before extraction (Albandar, 2005; Inanc et al., 2007). Briefly, periodontal ligament fibroblast tissue from the middle third of the tooth roots were scraped with sterile blades, minced and transferred to culture flasks containing DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acid stock solution, and 2 mM L-glutamine (all from Sigma). The explants were cultured at 37 °C, 5% CO_2 and 95% humidity until the fibroblast cells became confluent. hPDLF cells between passages 8–10 were used in the experiments.

2.5. mDF cell isolation and culture

Dermal fibroblast cells were isolated from the tail skin of 8–10 weeks old adult mice according to the standards of international regulations, using the method described elsewhere (Lichti et al., 2008). Briefly, mouse was anesthetized with intraperitoneal injection of avertin and under anesthesia, the tail was disinfected with ethanol 70%. Tails of ten to fifteen animals were cut at the base and the skin was sectioned and removed. After cutting into square pieces along with strong antisepsis and washing, the fragments were transferred to a petri dish (60 × 15 mm) containing 20 ml of 0.5% trypsin solution and stored overnight at 4 °C to separate the epidermis from the dermis. Then, the dermal explants were cultured and grown in fibroblast medium [DMEM high-glucose medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicilin, 100 μg/mL streptomycin and 1% non-essential amino acids (all from Sigma, St. Louis, MO)] at 37 °C, humidified atmosphere with 5% CO_2. After 48 h, the non-adherent cells were removed and the adherent cells were cultured. Culture medium was changed twice a week.

2.6. Preparation of ZnO NP dispersion for cell culture

The nano sized ZnO was sterilized using UV irradiation (254 nm) for 30 min before use. The stock solution of ZnO NPs was prepared in cell culture medium and dispersed by using a sonicator for 30 min. Then, the stock solution was diluted to different concentrations in cell culture medium completed by 10% fetal

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