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Cytotoxic Effects of Zinc Oxide on Human Periodontal Ligament Fibroblasts *In Vitro*

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ABSTRACT

Zinc oxide (ZnO) has been widely used in wound dressing, food additive and root canal filling material owing to its antibacterial and antifungal properties. This study describes potential cytotoxic effect of ZnO on human periodontal ligament fibroblasts (hPDLFs) in vitro. ZnO was characterized using BET (Braun-Emmet-Teller) and DLS (dynamic light scattering) analyses. The hPDLFs were treated with 0.1, 1, 10, 50 and 100 µg/mL of ZnO for 6 h, 24 h and 48 h durations. After exposure to ZnO, phase contrast microscopy, transmission electron microscopy (TEM), mitochondrial function (MTT) and lactate dehydrogenase (LDH) assays were used to evaluate cell morphology, particle uptake, cell viability and membrane leakage. In addition, real time cell responses to ZnO exposure were monitored by impedance measurements. Results indicated that ZnO exposure caused toxic effect in a dose- and time-dependent manner when compared to control group, especially at 50 and 100 µg/mL.

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INTRODUCTION

Zinc oxide (ZnO) is used extensively in various fields, ranging from rubber, ceramics, food and coating to cosmetic, medical and pharmaceutical industry due to its unique properties such as antibacterial, antifungal, anti-corrosive, low electrical conductivity and high heat resistance [1, 2]. ZnO particles have been incorporated into various polymers to improve the antimicrobial properties of materials for food packaging [3] and wound dressing [4]. Furthermore, antibacterial and antifungal activities of ZnO have received significant interest in endodontics as a filling material in root canal sealers [5, 6]. ZnO together with eugenol, called as zinc oxide-eugenol (ZOE), has been used as a filling material for many years in clinical dentistry. Nowadays, new endodontic sealers containing ZnO are still being developed to improve the bioactivity and antibacterial activity of cementation materials [7, 8].

The evaluation of the possible toxic effects with regard to exposure to chemical or biological agents is crucial for maintaining human health. Therefore, a better understanding of their toxic effects on human health will provide significant insights into their safe use [9]. In order to evaluate the potential toxic effect of a substance, a variety of *in vitro* assays (e.g., MTT, LDH and ROS) have been performed. These methods are based on evaluation of changes in cell growth, membrane leakage or reactive oxygen species production, which allow determination of cell responses at a specific time point. In recent years, impedance-based systems began to gain importance in toxicological studies due to providing label-free and real time monitoring of cell adhesion, proliferation, and death [10]. One of these, real-time cell analysis (RTCA) system uses an electrode-plates (E-plate) with gold microelectrode incorporated in the bottom of the well of culture plates. The microelectrodes are connected to a computer that measures electrical impedance changes in response to an applied voltage. The changes in electrical impedance are converted into cell index (CI) [11]. The impedance in culture medium without cell results from the ion environment at the electrode/culture medium interface. When the cell attaches onto the E-plate, the ionic environment at the electrode/culture medium interface changes, causing higher impedance. The increase in cell number attached to the E-plate leads to larger increase in electrode impedance. The electrode impedance can also be affected by the degree of interaction between electrode surface and cell. The strong attachment of cell to the electrode surface leads to a larger increase in electrical impedance [12]. By this means, the change in CI

gives particular information about cell viability, morphology, migration and adhesion in real time.

The aim of this study is to evaluate the toxic effects of ZnO on human periodontal ligament fibroblast using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, LDH assay and real-time impedance-based assay. The ZnO characterization was performed using BET (Braun-Emmet-Teller) and DLS (dynamic light scattering) analyses. The cellular uptake of ZnO within cells was evaluated by transmission electron microscopy (TEM).

MATERIALS AND METHODS

Chemicals and reagents

ZnO powder, MTT and LDH assay kits were purchased from Sigma (St. Louis, MO, USA). DMEM, L-glutamine and penicillin/streptomycin were purchased from Lonza (Biowhittaker, Verviers, Belgium). All other chemicals were obtained from Sigma.

Braun-Emmet-Teller (BET) analysis

BET analysis was used to measure the mean specific surface area of ZnO. The measurements were carried out using Quantachrome NOVA 2200e (Instruments, Boynton Beach, FL, USA).

Zeta potential and DLS analysis

Before measurements, ZnO suspension (100 µg/mL) was prepared in the cell medium and sonicated in an ultrasonic bath for 5 minutes to obtain homogenous dispersion. Then, thirteen measurements were carried out by Malvern's NanoZetasizer-ZS (Worcestershire, UK) at 25°C.

hPDLF culture

Cells were purchased from ATCC, defrost and adapted to culture conditions, and were used in all experiments. Briefly, the frozen cells in the cryovials were thawed and cultured in DMEM including 10% FBS, 100 U/mL penicilin, 100 µg/mL streptomycin and 1% non-essential amino acids under standard culture conditions (5% CO_2 at 37°C in a humidified atmosphere).

ZnO exposure

The ZnO powder sterilization was performed using UV irradiation for 30 minutes. The sterile stock suspension was prepared in culture media and sonicated for 30 min. Then, the stock solution was diluted to desired concentrations (0.1, 1, 10, 50 and 100 µg/mL) in cell culture medium. In ZnO exposure experiments, the hPDLF cells were seeded at a density of $1.0x10^4$ cells/well into 96-well plates and cultured for 1 day. Then, the culture medium was replaced with the ZnO-containing medium. The cells were cultured with ZnO suspensions for 6 h, 24 h and 48 h. Control cells were cultured with cell culture medium without any particle.

The morphology of human PDLFs was examined after ZnO exposure using an inverted microscope (Zeiss, Jena, Germany). Before imaging, the cells were washed carefully with PBS to remove excess particles.

TEM analysis

The subcellular localization of ZnO in hPDLF cells was observed by TEM (transmission electron microscopy). After 48 h of exposure to ZnO, the cells were washed and harvested by centrifugation. Sample preparation for TEM was performed according to a previously reported method [13]. The cell sections were imaged under a JEOL 100 CX TEM (Tokyo, Japan).

MTT assay

After 6 h, 24 h and 48 h of exposure to ZnO, cell viability was determined by MTT assay. Control cells were incubated in normal culture medium without ZnO. Optical density at 570 nm was detected using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

LDH assay

Membrane leakage assay was performed using a LDH Toxicology Assay kit (Sigma), as described in detail elsewhere [13]. LDH leakage was normalized to positive control cells as 100%.

Real-time impedance measurements

Impedance measurements were carried out using the xCELLigence Real-Time Cell Analyzer (RTCA) DP (Roche, Basel, Switzerland) in real time. For that purpose, the hPDLF cells were seeded in the wells of E-plates at a density of $12.5x10^3$ cells/well, and cultured at 37°C and 5% CO_2 for 15 h. Thereafter, ZnO suspensions were added into each well of E- plate (final concentration of 0.1, 1, 10, 50 and 100 µg/mL). As a control, the cells were cultured with cell medium without ZnO. The CI changes were continuously monitored at 15 min intervals for 100 h. To determine whether particle interference had occurred, the CI measurement was also carried out with ZnO solutions (in culture medium) with the highest concentrations used in this study.

Statistical analysis

Data were collected from three independent experiments, displayed as the mean±SD, and analysed by two-way ANOVA with Bonferroni post-test using GraphPad Prism software version 5.00 (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Particle characterization

The physicochemical properties of a substance such as surface area, surface charge, and hydrodynamic diameter have major effects on its toxicity, fate and mechanisms in cells [14, 15]. The particle surface area obtained from BET analysis was

8.886 m^2/g. The particle size distribution, zeta (ζ) potential and polydispersity index (PDI) of the ZnO were determined by DLS measurements (Table 1). The average hydrodynamic diameter of ZnO was 842.4 nm (Figure 1 and Table 1). The particle size distribution measurements retrieved from the DLS analysis indicated that ZnO might have a tendency to aggregate in cell culture medium.

Table 1. Characterization of ZnO

BET results (m2/g)	*8.886*
Average diameter in DLS (nm)	*842.4*
Polydispersity index	*0.540*
Zeta potential (mV)	*13.6*

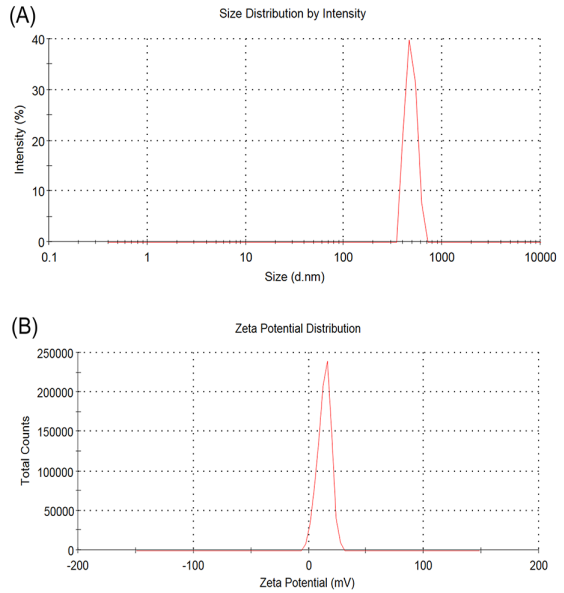

Figure 1. Particle size distribution (A) and zeta potential (B) results of the ZnO.

Cell morphology

The inverted microscopy images of control and ZnO-exposed cells are shown in Figure 2. As seen in the images, the control cells showed the characteristic fibroblastic morphology. The hPDLF cells exposed to low concentration of ZnO (0.1 µg/mL) showed no dramatic morphology changes. Whereas, at 100 µg/mL ZnO, the cells had a round morphology, showed weaker attachment to the surface. The results indicated that exposure of PDLFs to high concentration of ZnO resulted in dramatic changes in morphology.

Subcellular localization of ZnO

TEM analysis is one of the basic methods for determination of intracellular distribution of particles following uptake into cells [16]. TEM images of control and exposed cells are presented in Figure 3. The results revealed that ZnO particles were mostly located within cytoplasmic vacuoles, whereas, any particle was not observed in uniformly homogeneous vacuoles of control cell, as in previous studies [17, 18].

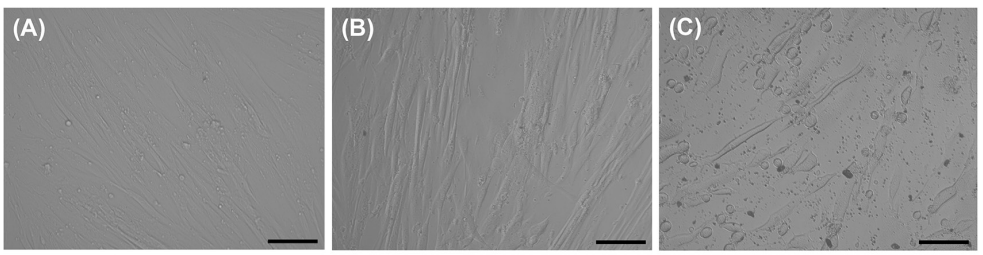

Figure 2. Inverted microscopy images of hPDLF cells. (A) Control cells. Cells exposed to (B) 0.1 µg/mL ZnO, and (C) 100 µg/mL ZnO for 48 h. Scale bars: 200 µm.

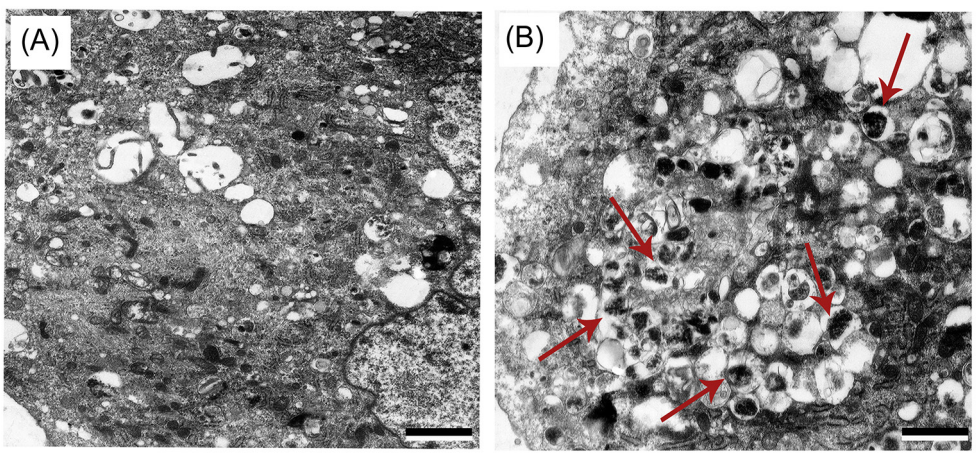

Figure 3. TEM micrographs of the hPDLF cells. (A) Control cells, and (B) cells exposed to ZnO. The arrows indicate the localization of ZnO particles in the vacuoles. Scale bars: 1 µm.

MTT results

hPDLF cells were treated with different concentrations of ZnO for 6 h, 24 h and 48h. Then, the cell viabilities were assessed by the MTT assay and data were normalized to unexposed cells. It is clearly seen from Figure 4, after 6 h of exposure, ZnO at concentrations below 50 µg/mL did not exhibit a significant toxic effect on hPDLFs. After 24 h and 48 h of exposure, ZnO at low concentrations (0.1, 1 and 10 µg/mL) resulted in ~50-60 % decrease in the cell viability, while the cell viability at all three time points significantly decreased at higher concentrations (50 and 100 µg/mL) of ZnO. These results showed that exposure to ZnO caused cell death in a concentration- and time -dependent manner.

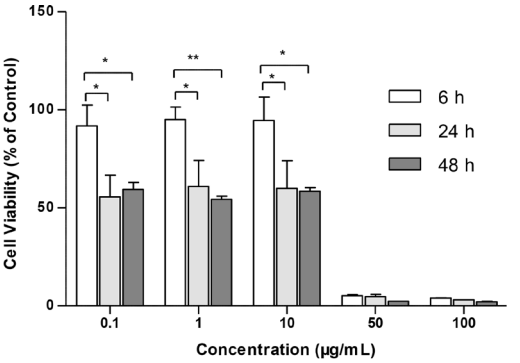

Figure 4. Cell viability results of hPDLFs exposed to 0.1-100 µg/mL of ZnO for 6 h, 24 h and 48 h. Data were expressed as the mean±SD, and conducted using the two-way ANOVA with Bonferroni post-test. Asterisks show significant differences between the groups: * = $p<0.05$, ** = $p<0.01$.

LDH results

The measurement of LDH activity has been widely used to determine the loss of membrane integrity related to cell death [19, 20]. In this study, cell membrane integrity was assessed by measuring the released LDH from cells with damaged membrane. As seen in the Figure 5, exposure to ZnO for 6 h resulted in no significant difference between exposed groups, regardless of the concentration used. After 24 h of exposure, LDH release significantly increased, especially at 50 and 100 µg/mL. The results indicated that exposure to ZnO at concentrations above 10 µg/mL caused membrane damage and LDH release in hPDLFs.

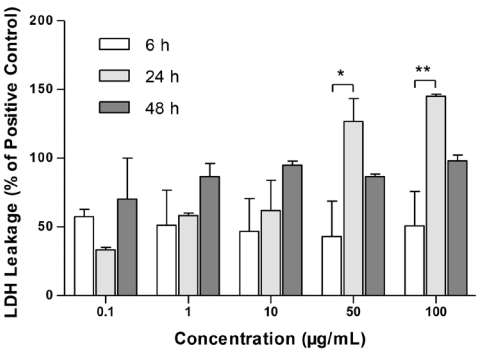

Figure 5. LDH release of hPDLF cells exposed to 0.1-100 µg/mL of ZnO for 6 h, 24 h and 48 h. Data were expressed as the mean±SD, and conducted using the two-way ANOVA with Bonferroni post-test. Asterisks show significant differences between the groups: * = $p<0.05$, ** = $p<0.01$.

Impedance profiles

Real-time cell responses to different concentrations of ZnO were monitored by impedance-based system. The normalized cell index (NCI) measurements of control and ZnO-exposed cells are shown in Figure 6. The results indicated that impedance profiles of cells exposed to 0.1, 1 and 10 µg/mL ZnO had similarity with the control cells. However, the NCI values at concentrations of 50 µg/mL and 100 µg/mL started to decrease at ~4 hours after exposure. After 12 hours, NCI values rapidly decreased to zero. The results of the real-time impedance measurements indicated that the presence of 50 and 100 µg/mL ZnO caused toxic effect on hPDLFs.

CONCLUSION

Endodontic sealers may cause inflammatory responses and periodontal tissue damage. Therefore, it is necessary to know their toxic effects on periodontal tissues [21]. *In vitro* toxicity assays used mammalian cell culture systems have been commonly performed to evaluate the biocompatibility of dental materials using cell lines (e.g., HeLa, 3T3, L929 cells) [22] or primary cells (e.g., PDLFs, dental pulp cells) [23]. In the study, hPDLF cells were exposed to different concentration of ZnO and the toxic effects of ZnO were evaluated using MTT, LDH and real-time impedance based assays. The results of this study demonstrated that the toxic effect is related to exposure concentration and time. Impedance-based assay could be implemented as an effective and sensitive tool for

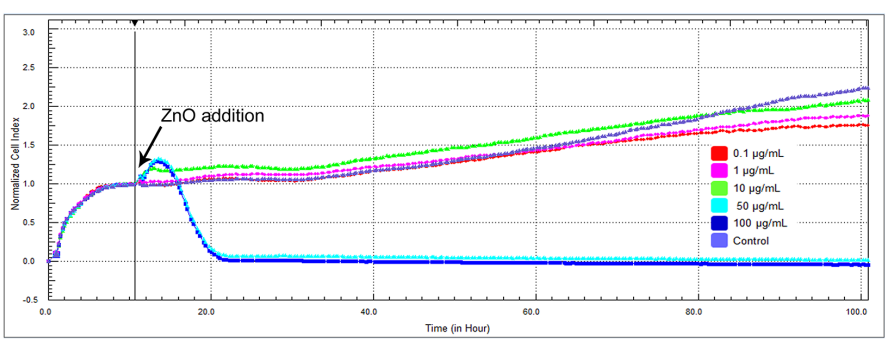

Figure 6. Real-time cell impedance profiles of hPDLF cells treated with different concentrations of ZnO.

long-term monitoring of hPDLF cell responses. Morever, the results indicated that ZnO at concentration below 10 µg/mL had no significant toxic effects on hPDLF cells.

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