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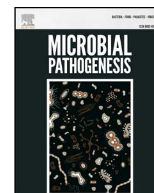

Green synthesis of anisotropic zinc oxide nanoparticles with antibacterial and cytofriendly properties

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ABSTRACT

Zinc oxide nanoparticles (ZnONPs) exhibit abundant biomedical applications. Anisotropic ZnONPs with a defined shape and size were synthesized using *Bacillus megaterium* (NCIM 2326) cell free extract as a bio-reductant. The study investigated the multidimensional effect of ZnONPs on *Helicobacter pylori* strains and assessed its biosafety in normal human mesenchymal stem cells (hMSc). The highly stable ZnONPs were produced using *B. megaterium* and Zinc nitrate as a precursor. The phase of ZnONPs formation and structural characterization were performed by UV- visible (UV-Vis), Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and Field Emission Scanning electron microscopy (FESEM) analysis. Furthermore, the ZnONPs exhibited higher biocompatibility against human mesenchymal stem cells (hMSC) and proved to be potentially safe in mammalian cells. Corroborating the current investigation, we described the anti-*H. Pylori* dosage of ZnONPs was safe to hMSC and could efficiently use as nano-antibiotic.

1. Introduction

Green chemistry procedure for metal and metal oxide nanoparticle synthesis was specially designed to reduce the environmental toxicity or eliminate the environmental pollution. The nanoparticle synthesis has rapidly evolved when compared to the early part of the century [1]. Earlier, physio-chemical synthesis involved the usage of hazardous toxic materials later seemed to be toxic and non-biodegradable to the environment [2]. ZnO plays a vital role in the daily life of the third highest global production volume only after SiO_2 and TiO_2 among the safest metal to use [3,4]. Recent studies have shown that ZnONPs have potent toxicity to bacteria but exhibit minimal effects on human cells [5,6]. The bio-compatibility and the bio-safety of the ZnO nanoparticles depend on the unique properties such as particle size and shape, aspect ratio and morphology [7]. The most notable features of ZnO nanomaterials are eco-friendly and biodegradability properties. Zinc oxide (ZnO) has the proven biocompatibility profile that "generally recognized as safe" (GRAS) material to the human and animal system by the USFDA (21CFR182.8991). Hence, it has been used widely in the combination of antimicrobial coatings, biosensors, biocompatible scaffolds and healthcare products [8–10]. ZnO nanoparticles have prominent antimicrobial activities (< 100 nm) due to large surface volume ratio facilitate the better dissolution and penetration of the bacteria [11,12]. The cause for the ZnONPs antibacterial efficiency was mainly from the nanoparticles compared to the release of free Zn^{2+} ions which is very low [13] and also reported that 12 nm ZnONPs was inhibited efficiently the bacterial growth where there is no significant inhibition on 212 nm particles. The actual process underlying their antibacterial behavior of ZnONPs is not extensively studied; moreover, it is an unopened subject of bacteriologically synthesized ZnONPs response to *H. pylori*. Metal oxide nanoparticles potentially induce injury through the production of reactive oxygen species (ROS) and oxidative stress [14].

In other hand, there is a controversial finding raised by the European consumer product that nano and microparticles of ZnO unlikely cause health issues when it is direct contact with the skin in the form of sunscreen [15,16]. The biological systems ultimately involved in multiple pathways of toxicity into a limited number of pathological outcomes, including inflammation, apoptosis, necrosis, fibrosis, hypertrophy, metaplasia, and carcinogenesis [17]. The accumulation of

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Zn ions exposure is high for the smaller nanosized particles, where lesser Zn was exposed from ZnO microparticles [18,19] While it is noteworthy that different sized biologically derived nanoparticles also excrete the same level of toxicity. Thus, the theme to facilitate the bioactivity and toxicity level of anisotropic ZnONPs, we have expressed the comprehensive in-vitro toxicity studies includes oxidative stress, apoptotic induction, LDH assay and biocompatibility levels against normal hMSCs cells. The term toxic oxidative stress or cytotoxicity and apoptosis caused by high oxidative stress which undergoes a perturbation of cellular electron transfer and the lock/key status of the mitochondrial permeability transition pore (PTP) [20]. For instance, we have demonstrated that the submicron anisotropic ZnONPs toxicity is mainly associated with the generation of oxidative stress. To make enhanced use of ZnONPs in the antibacterial application and to assist in the development of nontoxic, strong antimicrobial derivatives, it is necessary to identify the molecular bioactivity mechanism of ZnONPs in the further study.

2. Materials and methods

2.1. Materials

Zinc nitrate ($ZnNO_3.5H_2O$) was purchased from Sigma–Aldrich, USA. All other reagents were of analytical grade, and we used deionized water for the study. Nutrient agar and Chocolate blood agar (CBA) were used to cultivate the bacteria were obtained from (Oxoid, UK). Bacteria and cell culture information are included in the supplemental information S1.

2.2. Biosynthesis of ZnONPs

Freshly grown (24 h) *B. megaterium* cell-free supernatant was obtained by centrifugation at 8000 rpm for 8 min. Then 100 mL of supernatant was introduced with 1 mM final concentration of aqueous $ZnNO_3.5H_2O$. The reaction solution was placed in a Shaker Incubator at 37 °C for 48 h. The supernatant without the addition of $ZnNO_3.5H_2O$ precursor was maintained as a control.

2.3. Biophysical characterization of ZnONPs

The as-synthesized ZnONPs were characterized by using UV-vis spectrophotometer (Lambda 45, Perkin-Elmer, USA), at the wavelength scanning range of 200 and 650 nm along with control. FTIR spectrum was recorded, to detect functional groups present in the cell filtrate as well as the obtained ZnONPs (Cary 660, Agilent Technologies, USA). XRD measurement was carried out in a Goniometer = PW3050/60 (Theta/Theta) using Cu kα radiation which works at 40 KV recorded at 2θ range of 20–80 °C. The structural morphology and elemental analysis of the ZnONPs was recorded by FESEM (FEI Quanta, USA) equipped with EDX.

2.4. Anti-Helicobacter pylori effect of ZnONPs

To determine the MIC of ZnONPs, a standard of Clinical and Laboratory Standards Institute (CLSI) broth microdilution method was followed. The freshly grown *H. pylori* UM158 and UM67, broth cultures were adjusted to obtain $\sim 10^8$ CFU/mL. The bacterial suspension culture was dispersed in each well of 96-well plate containing various concentrations of ZnONPs (3.125–100 μg/mL) resulting $\sim 10^5$ CFU/mL. Microtiter plate wells containing the brain heart infusion (BHI) broth used as a negative control, and bacterial culture without ZnONPs exposure served as a positive control.

The morphological changes of bacteria caused by ZnONPs were examined using FESEM. *H. pylori* strains UM67 and UM158 were exposed to the particles at 17 μg/mL was incubated for 60 min at 37 °C under microaerophilic condition. Then the cells were centrifuged and washed twice with 1% PBS. Further sample preparation procedure was followed by our earlier published procedure [21]. The samples were then viewed under FESEM (JEOL JSM-7001F, Germany) to study the bacterial morphological changes before and after ZnONPs treatment.

2.5. Cytocompatibility - LDH and Alamar blue assay of ZnONPs

Cellular cytotoxicity of as-synthesized ZnONPs against the hMSCs was evaluated using Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher) protocol. The hMSCs were seeded on the well plate after 24 h, optimum density of cells was exposed to an increased dosage of (3.125–100 μg/mL) ZnONPs for 24 h. After the treatment period, the medium was removed and subjected to manufacturer instruction procedure, and the absorption was measured at 490 and 680 nm. Likewise, other sets of the experiment the ZnONPs-exposed cells were rinsed with 1% PBS. 1.25% Alamar blue was added to each well and incubated for 30 min in the dark at 37 °C supplied with 5% CO_2. Subsequently, the fluorescence intensity was measured with excitation and emission wavelength of 532 and 590 nm using citation 5 reader. Cells without ZnONPs exposure served as control and cells treated with 0.1% Triton x-100 served as positive control.

2.6. Generation of reactive oxygen species (ROS) and FDA staining

To determine the nontoxic dosage of as-synthesized ZnONPs the hMSCs were exposed with the above increasing concentration of ZnONPs for 24 h. After that, (Fluorescein diacetate) FDA solution (30 μg/mL in 1X PBS) was flooded and imaged under a fluorescent microscope with a 20× objective.

The experimental conditions of cell seeding and ZnONPs treatment as described above and the cells were washed twice and flooded with 37 °C prewarmed DMEM media. Nextly, appropriate volumes of 20 μM DCFH-DA solution was prepared in serum, and phenol red free MEM added to each well and incubated for 15 min in the dark at 37 °C and 5% CO_2. Then the solution was discarded, and cells were washed twice with PBS. Then the fluorescence intensity was recorded at the excitation and emission wavelength of 488 and 530 nm filters respectively [22]. The ROS levels were calculated as described previously [23].

2.7. Apoptosis detection by Annexin-V/Propidium Iodide

ZnONPs apoptotic toxicity to the hMSCs was examined using (Tali apoptosis kit) containing green fluorescent (Annexin V- Alexa Flour 488) and red stain Propidium Iodide (PI). The cells were treated with ZnONPs at the dosage of 3.125–100 μg/mL for 24 h. Then, the cells were trypsinized and centrifuged at 3000 g for 5 min, and the pellet was re-suspended in 100 μL of binding buffer (ABB) followed by stained with Annexin-V and kept for 20 min under dark condition. Then, the cells were centrifuged at 3000 g for 5 min and resuspended in binding buffer followed by PI staining for 5 min in the dark at 37 °C. The stained cells were loaded into the slide chamber through capillary action, and Tali image-based cytometer analyzed the slide.

3. Results and discussion

3.1. Eco-friendly synthesis of ZnONPs using B. megaterium

The study reports the formation of ZnONPs after the *B. megaterium* cell-free supernatant exposed with 1 mM aqueous $ZnNO_3.5H_2O$ arising in the formation of a white precipitate. After 12–48 h of incubation the reaction mixture was observed to have evident coalescent white clusters of ZnONPs deposited at the bottom showed in the supplemental information (Fig. S1). Centrifugation separated the resultant materials and repeatedly washed with water and ethanol to obtain pure white precipitates.

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