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Zinc oxide nanoparticles mediated cytotoxicity, mitochondrial membrane potential and level of antioxidants in presence of melatonin

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are widely used in a variety of products and are currently being investigated for biomedical applications. However, they have the potential to interact with macromolecules like proteins, lipids and DNA within the cells which makes the safe biomedical application difficult. The toxicity of the ZnO NP is mainly attributed reactive oxygen species (ROS) generation. Different strategies like iron doping, polymer coating and external supply of antioxidants have been evaluated to minimize the toxic potential of ZnO NPs. Melatonin is a hormone secreted by the pineal gland with great antioxidant properties. The melatonin is known to protect cells from ROS inducing external agents like lipopolysaccharides. In the present study, the protective effect of melatonin on ZnO NPs mediated toxicity was evaluated using C6 glial cells. The Cytotoxicity, mitochondrial membrane potential and free radical formation were measured to study the effect of melatonin. Antioxidant assays were done on mice brain slices, incubated with melatonin and ZnO NPs. The results of the study reveal that, instead of imparting a protective effect, the melatonin pre-treatment enhanced the toxicity of ZnO NPs. Melatonin increased antioxidant enzymes in brain slices.

1. Introduction

ZnO NPs are semiconductor nanoparticles with unique properties, which are already exploited for industrial and biomedical applications. These nanoparticles are already present in commercial products like cosmetics and daily care products. The ZnO NPs are reported to have preferential toxicity for the cancer cells. Moreover, these particles are investigated for drug delivery applications. However, the high toxicity exhibited by these nanoparticles makes it difficult for the safe biomedical application of ZnO NPs.

The toxicity of ZnO NPs is attributed to its particle dissolution and ability to generate ROS. ROS generation can further lead to numerous secondary effects like alteration in mitochondrial membrane potential, endoplasmic reticulum stress and induction of apoptotic genes [1]. Cells have endogenous antioxidant mechanisms to mitigate the ROS generated inside the cells. But during oxidative stress, the cells antioxidant machinery fails to alleviate ROS, leading to ROS mediated cell and tissue damage [2]. The ZnO NPs are found to interfere with the antioxidant machinery of the cells and thereby inducing oxidative stress [3]. A number of strategies are adapted to reduce ROS generated by the nanoparticles which include polymer coating [4], metal doping [5] and the addition of antioxidants [6].

Antioxidants are mostly amino acid and protein derivatives that inhibit the oxidation of the other molecules. These molecules scavenge the free radicals generated by the oxidation reactions and thereby undergoing oxidation themselves. There are many naturally and artificially synthesized molecules which exhibit antioxidant properties. Organisms have its own antioxidant protective mechanism which protects the cells and tissue from reactive oxygen species generated as a metabolic by-product. One such molecule is melatonin, a hormone commonly found in animals, plants, fungi and bacteria. In higher forms of organisms, it is

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involved in the regulation of day-night cycle. In lower forms it mainly acts as an antioxidant [7,8]. In humans, this hormone is secreted by the pineal glands which control the sleep-wake cycles. Apart from its role in the maintenance of circadian rhythm, its antioxidant and anti-inflammatory effect thought to have a positive impact on clinical conditions like cancer, autism, gall bladder stone and infectious diseases [9–12]. Melatonin brings about its action either by acting directly as an antioxidant or by stimulating the antioxidant enzymes of the cells. The protective action of melatonin against ROS producing agents has already been reported [13,14]. Melatonin pre-treatment at μM range ameliorates β-amyloid induced oxidative stress and apoptosis in C6 glial cells. Hence it is hypothesised that the melatonin can scavenge the ROS induced by the nanoparticle there by protect the cells against ZnO NPs mediated toxicity. In the present study, effect of melatonin on the ZnO NPs induced ROS and nitric oxide release was analyzed *in vitro* using C6 cell lines. The effect of melatonin on the antioxidant machinery was analyzed using mice brain homogenate. Completely characterized ZnO NPs were used for the study.

2. Materials and methods

2.1. Chemicals and reagents

Phosphate Buffered Saline (PBS) and bovine serum albumin (BSA) were purchased from Gibco (Grand Island NY, USA). Melatonin, thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione (GSSG) and dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemicals Co. Ltd. (St. Louis, MO USA). Ethylene triamine tetra acetic acid (EDTA), disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from (Merck, Germany). All other chemicals were purchased locally from India and were of analytical grade.

2.2. ZnO NPs synthesis

ZnO NPs were synthesized by wet precipitation method using zinc nitrate ($Zn(NO_3)_2$) and sodium hydroxide (NaOH) as precursor. In brief, an aqueous solution of zinc nitrate (14.02 g) and sodium hydroxide (3.24 g) were prepared each in 100 ml of distilled water. The solution containing NaOH was heated to 55 °C and the temperature was maintained throughout the synthesis process. The solution of $Zn(NO_3)_2$ was added dropwise for 40 min to the heated NaOH solution with constant stirring at 800 rpm. The reaction was kept undisturbed for 2 h. The precipitated ZnO NPs were washed five times with deionized water followed by absolute ethanol (3 times). Freeze dried samples were characterized using several techniques.

2.3. ZnO NPs characterization

SEM imaging accompanied by EDX analysis was performed with scanning electronic Microscope JEOL JSM6400F equipped by Oxford Instruments EDS analyzer. The sample was prepared by evaporating a diluted suspension of NPs in ethanol solution on a carbon-coated silicon substrate.

Transmission Electron Microscopy (TEM) characterizations were performed using a JEOL JEM-2100F microscope operating at 200 kV (point to point resolution of 0.19 nm).

carbon-coated copper grid (carbon Film – 300 mesh, EMS, Hatfield, PA).

Specific surface area (SSA) of nanoparticles was analyzed by Brunauer, Emmett and Teller theory (BET), with the help of a Micromeritics Tristar II apparatus. Samples were outgassed in situ under a pressure of 20 mTorr and at 100 °C. The measurements were performed at liquid N_2 temperature with N_2 adsorbing gas.

Powder X-Ray Diffraction (XRD) patterns were collected using a D8 Advance diffractometer with Cu $K\alpha_1$ ($\lambda\alpha_1 = 1.5406$ Å). The phase identification was done by comparison of the diffraction patterns with the reference cards of the ICDD Powder Diffraction File. The data analysis was carried out with Topas® software. Rietveld method was used to obtain lattice parameters and mean crystallite size.

A Discovery Thermo-Gravimetric Analysis (TGA) TA Instrument with an air flow rate of 25 ml min^{-1} and a temperature ramp of 5 °C/min from 25 °C to 800 °C was used to analyze powders.

X-ray Photoelectron Spectroscopy (XPS) measurements were collected with a PHI 5000 Versaprobe instrument equipped with a Al Kα monochromated radiation ($EK\alpha_{(Al)=}$1486.7 eV with a 200 μm diameter spot size). Powders were pressed on an indium sheet in order to immobilize NPs during measurements. Data were analyzed with CasaXPS processing and MultiPak software. A neutralization process has been applied in order to avoid a charge accumulation on the surface of samples. The carbon C1 s peak at 284.5 eV was used as reference. A Shirley background was subtracted and Gauss (70%) – Lorentz (30%) profile was used. Full Width at Half Maximum (FWHM) were fixed between 1.5 and 2.0. Quantitative analysis has been realized with the help of MultiPak software.

ζ-potential and Dynamic Light Scattering (DLS) measurements were performed at 25 °C with a Malvern Nano ZS instrument supplied by DTS Nano V7.02 software. Prior to measurement, suspensions were diluted in an aqueous solution containing NaCl (10^{-2} M). Samples were filtered (0.45 μm filter) in order to remove eventual pollutant or large agglomerates. DLS curves were derived from intensity calculations.

2.4. Cell culture and particle treatment

C6 cells were grown in monolayer to 70–80% confluency in a cell culture flask containing DMEM F12 supplemented with 10% FBS, 1% antibiotic- antimycotic solution, 1% glutamax and 1 mM sodium pyruvate. Freshly prepared stock solutions of melatonin (100 mM) were used in all the experiments. Cells were treated with melatonin 1 h prior to ZnO NPs exposure. Before each experiment, the suspension of ZnO NPs (in water) was sonicated for 1 h and working stocks were prepared in complete cell culture medium.

2.5. Cell viability- MTT assay

MTT assay was carried out to determine the dose response of melatonin, ZnO NPs and for the combination treatment of melatonin and ZnO NPs. The concentration ranges of melatonin included 1, 10, 100, 200, 400, 800 and 1600 μM. The ZnO NPs concentrations were 5, 10, 20, 40 and 80 μg/ml. Briefly, cells were seeded at a density of 1×10^4 cells/well and kept overnight for attachment. They were incubated with different concentrations of melatonin or ZnO NPs or with a combination of both for 24 h. Cells were then incubated with MTT (100 μl/well) for 3 h. The formazan crystals formed were solubilized using DMSO and absorbance was measured at 540 nm using multiwell plate reader (Bio-TekWinooski, USA) [15].

2.6. ROS generation- DCF-DA assay

Cells were pre-treated with melatonin (100 μM) and later with ZnO NPs (5, 20, 40 μg/ml) for 4 h. The cells were harvested by trypsinisation and centrifugation. The pellets were resuspended in DCFDA working solution (0.1 μM) for 30 min at 37 °C. After washing with PBS, the pellets were resuspended in PBS and were immediately analyzed using flow cytometry (BD FACSAria™, BD