Zinc oxide nanoparticles induce toxicity in H9c2 rat cardiomyoblasts

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Research Article

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Abstract

Background

Zinc oxide nanoparticles (ZnO NPs) are widely used in the cosmetic industry. They are nano-optical and nano-electrical devices, and their antimicrobial properties are applied in food packaging and medicine. ZnO NPs penetrate the body by inhalation, oral and dermal exposure and spread through circulation to various systems and organs. Since the cardiovascular system is one of the most vulnerable ones, in this work we studied ZnO NPs toxicity in H9c2 rat cardiomyoblasts.

Methods

Cardiac cells were exposed to different concentrations of ZnO NPs and then measured morphology, proliferation, viability, mitochondrial membrane potential ($\Delta \Psi_m$), redox state and protein expression.

Results

Transmission electronic microscopy (TEM) and hematoxylin/eosin (H/E) staining showed strong morphological damage. ZnO NPs were not observed inside cells, suggesting Zn$^{2+}$ ions were internalized, causing the damage. ZnO NPs strongly inhibited cell proliferation and MTT reduction at 10 and 20 µg/cm$^2$ after 72 h of treatment. ZnO NPs at 20 µg/cm$^2$ elevated DCF fluorescence indicating alterations in cellular redox state, associated with changes in $\Delta \Psi_m$ and cell death. ZnO NPs also reduced troponin I and atrial natriuretic peptide expression.

Conclusions

ZnO NPs are toxic for cardiac cells, therefore consumption of products containing them could cause heart damage and develop cardiovascular diseases.

Background

Zinc oxide nanoparticles (ZnO NPs) constitute a white thermostable material and the most widely manufactured nanoparticles for personal care products, pigments, coatings, sensors, antibacterial creams, electronic gadgets, catalysts, food additives, and biomedical applications, among others [1, 2]. Due to their small size (< 100 nm), ZnO NPs can enter the human body by inhalation, ingestion, and dermal contact. Inhalation is the primary route in the occupational environment [3]. Dermal exposure is by skin contact with cosmetic products [4], and oral exposure occurs through water and food containing ZnO NPs either released into the environment or used as additives [5]. After exposure, ZnO NPs spread through systemic circulation into multiple tissues and organs. Nanoparticles have been detected in liver, spleen,
lungs, kidneys, and heart [6]. *In vivo* and *in vitro* studies show several toxic effects of ZnO NPs, including zinc ions release, generation of reactive oxygen species (ROS), mechanical damage of cells, cytotoxicity, genotoxicity, and neurotoxicity, as well as developmental toxicity [2].

Since the main route of nanoparticle distribution is through systemic circulation accounting for the heart accumulation of ZnO NPs, exposure to this material has been widely correlated with cardiovascular diseases [7]. Oral administration of ZnO NPs in rats had cardiotoxic effects increasing cardiac injury markers in serum such as troponin-T, creatine kinase-MB, and myoglobin, and pro-inflammatory markers as tumor necrosis factor-α, interleukin-6, and C-reactive protein [8]. ZnO NPs also elevated cardiac calcium concentration, induced DNA oxidation, and stimulated caspase-3 activity [8]. In an *in vitro* study ZnO NPs caused dysfunction of human cardiac microvascular endothelial cells increasing membrane permeability and inflammation [9]. Because there are few studies related to the effects of ZnO NPs on the heart, the underlying mechanisms remain unknown. Further studies are needed to confirm ZnO NPs cytotoxicity in cardiac cells. Here we studied the cellular internalization of ZnO NPs and their impact on morphology, proliferation, redox state, and viability of H9c2 rat cardiomyoblasts.

**Results**

**ZnO NPs characterization**

Table 1 summarizes physicochemical characteristics of ZnO NPs. Zetasizer determinations showed differences depending of the suspension medium. When ZnO NPs were suspended in DMEM plus FBS a smaller hydrodynamic size (350.4 ± 28.9 nm) was observed compared with ZnO NPs suspended in NHB (448.8 ± 42 nm) (Table 1). Zeta potential was -20.93 ± 0.25 mV for nanoparticles suspended in culture medium and -4.30 ± 1.15 mV for those suspended in NHB. No change in polydispersity index was obtained comparing both suspension media.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Vehicle</th>
<th>Hydrodynamic size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Polydispersity Index</th>
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<tr>
<td>ZnO (20 μg/ml)</td>
<td>DMEM plus 10% FBS</td>
<td>350.4 ± 28.9</td>
<td>-20.93 ± 0.25</td>
<td>1 ± 0.0</td>
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<tr>
<td>ZnO (20 μg/ml)</td>
<td>NaCl-HEPES buffer</td>
<td>448.8 ± 42</td>
<td>-4.30 ± 1.16</td>
<td>0.96 ± 0.003</td>
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</tbody>
</table>

**Table 1.** ZnO NPs physicochemical characteristics. ZnO NPs were suspended in DMEM plus 10% FBS and NaCl-HEPES buffer (NHB) at 20 mg/mL, vortexed at 60 Hz for 10 min for subsequent reading on the Zetasizer Nano-ZS90 equipment.
**ZnO NPs were not observed inside cells**

H9c2 cells were exposed to different concentrations of ZnO NPs for 24 h and cellular uptake was evaluated by TEM. Cellular structures such as the nucleus, nucleoli, mitochondria, and endoplasmic reticulum showed no alterations in non-exposed cells. Nanoparticles were not observed inside cells; however, important morphological changes were perceived from 5 mg/cm$^2$ (Fig. 1). Multiple vacuole formation was detected at 5 mg/cm$^2$. At higher concentrations (≥ 10 mg/cm$^2$), numerous large vesicles containing dense material were observed. Organelle disintegration was detected in mitochondria and endoplasmic reticulum, as well as disruption of the cytoplasmic membrane and collapsed nuclei with low density of nuclear material. These results indicate ZnO NPs induced strong cellular damage.

**ZnO NPs altered cell morphology**

ZnO NPs-derived changes in cellular structure were identified by Hematoxylin-Eosin (HE) staining. Non-exposed cells displayed fusiform morphology of epithelial appearance with elongated and abundant cytoplasm, central and round nuclei, and several mitoses (Fig. 2). An important reduction in cell number was observed from 5 mg/cm$^2$ ZnO NPs. The most significant morphological changes were obtained at 20 mg/cm$^2$, including compact nuclei, cytoplasm with limited stellar extensions, plasma membrane rupture, and cytoplasm leakage, indicating strong cellular damage.

**ZnO NPs decreased cell proliferation and MTT reduction**

Since ZnO NPs induced strong morphological changes in cells, which could be associated with inhibition of cell proliferation or loss of viability, these events were determined by crystal violet and MTT reduction, respectively. An important decline in cell proliferation was observed at ≥10 mg/cm$^2$ ZnO NPs after 48 h of treatment with maximum effect (>50% of inhibition) at 72 h (Fig. 3A). Lower MTT reduction of about 50% was obtained after 72 h exposure with 10 and 20 mg/cm$^2$ (Fig. 3B).

**ZnO NPs altered cellular redox state**

Since ZnO NPs decreased cell proliferation and MTT reduction, changes in cellular redox state were examined (Fig. 4). After 1 h of treatment with all used concentrations of ZnO NPs, DCF fluorescence was increased, indicating an augment of reactive oxygen species (ROS) production and an alteration in the cellular redox state. Positive cells percentage to DCF increased and it was concentration-dependent, where the maximal number of positive cells was observed at 20 µg/cm$^2$. When longer exposure times were used (3, 6, and 24 h), the cell damage was so strong that the cellular redox state could not be determined (data not shown).

**ZnO NPs did not induce mitophagy but affected mitochondrial integrity**

ZnO NPs did not increase LTR-lysosomes levels, indicating that mitophagy did not occur, but severely affected nuclear and mitochondrial integrity through loss of Hoechst and MitoTracker stainings,
respectively, from 10 and 20 µg/cm² (Fig. 5). Since MitoTracker is lost during cell death due to the collapse by ΔΨm depolarization, this result could indicate a lower mitochondrial function which might compromise cell viability.

ΔΨm status was verified using the Rh123 fluorescent probe (Fig. 6). ZnO NPs significantly reduced fluorescence intensity by 33% and 51% at 10 and 20 mg/cm², respectively, after 1h exposure, indicating lower mitochondrial transmembrane inner potential, and subsequent mitochondrial damage. Taken together, these results indicate other mechanisms associated with dysfunctional oxidative phosphorylation may be involved in cardiomyocyte death.

**ZnO NPs induced apoptotic and necrotic death**

To evaluate whether morphological changes induced by ZnO NPs, inhibition of cellular proliferation and metabolic activity as well as mitochondrial damage are associated with cell death, apoptosis and necrosis were quantified by annexin-V and propidium iodide staining. Control cells presented about 5-10% death at baseline. ZnO NPs increased levels of apoptotic cells in a concentration-dependent manner at all concentrations measured by fluorescence microscopy after 24 and 48 h, with maximum effect at 48 h (Fig. 7A and B). Necrotic cell number increased from 5 mg/cm². The 20 mg/cm² concentration was the most effective to induce death with 65.3% of apoptotic and 28.8% of necrotic cells (Fig. 7B). Flow cytometric analysis showed ZnO NPs increased apoptotic death by 7-fold at 10 and 20 mg/cm² after 24 h of treatment compared with untreated cells (Fig. 7C). After 48 h of exposure from 5 mg/cm² an 8-fold increase in apoptosis was detected.

**ZnO NPs inhibited cardiac proteins expression**

Since ZnO NPs caused strong damage to cardiac cells, the expression of biomarkers of cardiac muscle damage such as troponin I, and atrial natriuretic peptide were evaluated. ZnO NPs (10 mg/cm²) significantly reduced intracellular troponin-I and atrial natriuretic peptide expression by 27% and 20%, respectively (Fig. 8). It is important to mention that 20 mg/cm² ZnO NPs was so damaging to cells that concentration of protein was not enough to perform the determination of levels of these proteins.

**Discussion**

ZnO NPs enter the body by different routes including dermal, oral, and inhalation, then due to their nanometric size, NPs spread through systemic circulation reaching different organs and cell types. Given the increasing trend of using ZnO NPs in the manufacture of products for human consumption, their potential toxicity has become an issue of debate. The cardiovascular system is one of the most vulnerable, therefore in this work, ZnO NPs toxicity was evaluated in H9c2 rat cardiomyoblasts. These cells constitute a suitable model for studying cardiac damage since their hypertrophic responses are similar to those of primary cardiomyocytes [10].
In order to determine ZnO NPs behavior in different suspension media, we evaluated physicochemical characteristics of NPs dispersed in medium and saline solution. Results showed ZnO NPs dispersed in cell culture media supplemented with FBS displayed smaller hydrodynamic and agglomerate sizes compared with NPs suspended in NHB (Table 1). Agglomerate size is influenced by components of suspension media [11, 12]. Serum decreases agglomerate size preventing high aggregation due to steric stabilization, and improving the dispersion stability of NPs [13]. We also determined the zeta potential of ZnO NPs, a key parameter measuring repulsive electrostatic forces between particles. The higher the zeta potential the longer the range of repulsive force; therefore, suspension stability is enhanced by increased zeta potential [14]. We found ZnO NPs dispersed in cell culture media plus FBS displayed a zeta potential of -20.93 ± 0.25 mV, while ZnO NPs dispersed in NHB scored −4.30 ± 1.16 mV (Table 1). Literature shows dissolutions of NPs with zeta potential from −10 to +10 mV display neutral charges and low interaction with other molecules dispersed in media [15]. In addition, zeta potentials from −30 to +30 mV encompass NPs agglomerates with a negative charge that makes them able to interact with positively charged molecules, this may be the case for ZnO NPs dispersed in cell culture medium. The zeta potential of ZnO NPs was strongly influenced by FBS. This medium with serum makes it protein-rich, increasing the dispersion quality of NPs suspensions through steric effects [14]. ZnO NPs interact with proteins probably by electrostatic attraction. Plasma and blood proteins such as albumin, immunoglobulin, and fibrinogen bind gold nanoparticles [16]. The link between proteins and NPs enhances colloidal stability since proteins adsorbed on nanoparticle surfaces act like spring structures exerting repulsive forces and preventing contact between particles, thereby increasing colloidal stability. Smaller aggregates are more readily uptaken by cells. This could partially explain ZnO NPs toxicity after uptake by H9c2 cells, where NPs are dispersed in a medium containing serum, where agglomerates may interact with positively charged biomolecules, causing damage and disruption of cellular components. Other factors underlying NP toxicity include concentration, surface charge, and particle size, among others.

Despite ZnO NPs agglomerates dispersed in the culture medium were smaller than those dispersed in saline solution, TEM analysis did not show NPs inside cells; however, a strong morphological damage to the whole cell dependent of concentration was detected (Figs. 1 and 2). We previously showed that food-grade titanium dioxide E171 alters F-actin distribution in H9c2 cells [17]. Actin, the major cytoskeletal protein of most cells, is responsible for mechanical support and determines cell shape and movement. ZnO NPs may alter actin filaments causing morphological changes in membranes and intracellular structures, mitochondrial injury, and outflow of organelles [18–20].

There are two ways for ZnO NPs entry into cells: 1) internalization as whole NPs and 2) internalization as free Zn\(^{2+}\) ions [2]. Since ZnO NPs were not detected inside H9c2 cells, we hypothesize free Zn\(^{2+}\) ion uptake may underlie the cellular toxicity of ZnO NPs. Many studies illustrate ZnO NPs fragmentation releasing Zn\(^{2+}\) ions into extracellular fluids which diffuse passively across the plasma membrane, as a critical step for cell toxicity [21, 22]. One study on mouse macrophage Ana-1 showed dissolved Zn\(^{2+}\) ions play a key role in ZnO NPs toxicity [23]. Likewise, antimicrobial activities of ZnO NPs evaluated through
bioluminescence of *Photobacterium phosphoreum* were due solely to released Zn$^{2+}$ ions [24]. These results highlight Zn$^{2+}$ ions as the main factor for ZnO NPs toxicity in H9c2 cells.

Morphological alterations induced by ZnO NPs were linked to decreased cell proliferation and MTT reduction (Fig. 3), oxidative stress (Fig. 4), mitochondrial dysfunction (Figs. 5 and 6), and cell death (Fig. 7). Similar outcomes derive from other investigations on normal cells. In RAW 264.7 macrophages, ZnO NPs elevated intracellular Zn$^{2+}$ ion concentration, generating intracellular ROS, plasma membrane leakage, mitochondrial dysfunction, and cell death [25]. In mouse-derived spermatogonia (GC-1 spg cells), ZnO NPs induced apoptosis and autophagy through oxidative stress [26].

Oxidative stress has been described as a key component of ZnO NPs toxicity [27]. ZnO NPs entry into cells triggers a defense mechanism that generates ROS surpassing the antioxidant systems eventually leading to inflammation [28]. Inflammation affects the mitochondrial electron transport chain at the internal membrane and injures membrane and cellular components such as DNA ultimately causing cell lysis, and death [29–33]. Another mechanism involved in ZnO NP toxicity is endoplasmic reticulum (ER) stress. In HUVEC cells, ZnO NPs triggered ER stress responses followed by apoptosis, suggesting ER stress can be an earlier end-point for ZnO NPs toxicity [34]. We observed very early toxic effects of ZnO NPs on H9c2 cells, indicating oxidative stress could be involved.

We did not detect mitophagy after ZnO NPs exposure (24 h), however, severe damage overcame cellular defenses resulting in nuclear and mitochondrial alterations causing apoptosis and necrosis. Interestingly, fluorescence microscopy revealed apoptosis and necrosis whereas flow cytometry detected only apoptosis (Fig. 7). The difference between these techniques is that flow cytometric analysis relies on cell suspensions. Necrotic cells are washed away during trypsinization, therefore pass undetected. Therefore, fluorescence microscopy is the best choice to evaluate both apoptosis and necrosis.

Markers of cardiac injury include the natriuretic peptides released from cardiac atria and ventricles from myocytes in response to increased wall tension and stretch to reduce blood pressure, cardiac hypertrophy, and ventricular fibrosis [35, 36]. On the other hand, cardiac troponin I is a structural peptide released after cardiomyocyte damage [37]. Therefore, we measured levels of troponin I and atrial natriuretic peptide proteins in H9c2 cells exposed to ZnO NPs (Fig. 9). Total protein expression dropped at 10 µg/cm$^2$, thus intracellular levels decreased while secreted protein increased. This result supports the strong injury caused by ZnO NPs in cardiac cells.

Remarkably, ZnO NPs concentrations of 10 and 20 µg/cm$^2$ (33 and 66 µg/mL) were most toxic to H9c2 cells. A pharmacokinetic study of rats orally exposed to single-dose ZnO NPs showed 50 mg/Kg ZnO NPs displayed maximum plasma concentration near 100 µg/mL after 6 hours [38]. Despite concentrations tested *in vitro* differ from those *in vivo*, ZnO NPs levels in our *in vitro* model are within the range of concentrations achieved *in vivo* during acute exposure. We hypothesize cellular damage exerted by ZnO NPs in organisms derives from accumulation over time during chronic exposure. However, there is no conclusive data on the total urinary and fecal excretion of NPs [38].
Conclusions

ZnO NPs induced severe damage to cardiomyocytes characterized by lower cell proliferation, higher oxidative stress, cell death, and strong morphological changes with cellular disruption, therefore consumption of products containing ZnO NPs could develop organic failure and cardiovascular diseases.

Methods

Materials

Cell culture reagents including Dulbecco's modified Eagle's medium (DMEM) high glucose, Fetal Bovine Serum (FBS), antibiotic-antimycotic solution (Anti-Anti 100X), and 0.25% trypsin-EDTA solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Western blot reagents were acquired from Bio-Rad (Hercules, CA, USA). Antibodies against b-actin, troponin I, and atrial natriuretic peptide were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The autophagy assay kit was supplied by ABCAM (Cambridge, UK). Annexin-V-FLUOS staining kit was provided by Roche (Nonnenwald, Germany). Annexin-V-FITC was provided by Biolegend (San Diego, CA, USA), Annexin-V buffer and Propidium Iodine were supplied by BD (New Jersey, USA). Sterile plastic material for tissue culture and 8-well tissue culture chambers were obtained from Sarstedt (Nümbrecht, Germany). MitoTracker Green FM and LysoTracker were from Invitrogen (Carlsbad, CA, USA). ZnO nanopowder < 50 nm particle size (BET), >97% (cat. No. 677450), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Cells derived from embryonic rat ventricular tissue (H9c2) were obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM high glucose supplemented with 10% FBS and Anti-Anti solution (1X). Cells were maintained at 37°C under a humidified atmosphere with 5% CO₂.

ZnO NPs characterization

For the hydrodynamic diameter, zeta potential, and polydispersity index measurements, ZnO NPs powder was suspended both in DMEM medium supplemented with 10% FBS and in 0.9 % NaCl-HEPES buffer (NHB). Both suspensions were vortexed at 60 Hz for 10 min for subsequent reading on the Zetasizer Nano-ZS90 equipment.

ZnO NPs internalization

ZnO NPs internalization was detected by TEM. Cells were cultured with ZnO NPs (2.5, 5, 10, and 20 mg/cm²) for 24 h. After treatment, H9c2 cells were fixed with 2.5% glutaraldehyde-paraformaldehyde in phosphate buffer solution (PBS) (pH 7.2) for 45 min. After fixation cells were placed in 1% osmium tetroxide for 1 h. Then, cells were dehydrated through graded series of alcohols and embedded in Epon 812 epoxy resin. Then 60 nm-thin sections were obtained with a diamond knife on Ultracut-R ultramicrotome, mounted on copper-grids, and impregnated with heavy metals, lead nitrate, and uranyl
acetate. Grids were examined in a JEOL 10/10 transmission electron microscope at 60 kV and AMT Camera System.

**Cell morphology**

For morphological analysis, H9c2 cells (20 × 10^3) were seeded on 8-well tissue culture chambers, exposed to 2.5, 5, 10, and 20 mg/cm^2^ over 48 h, then stained with hematoxylin/eosin (H/E), analyzed with a microscope Olympus BX51 with 20x objective and photographed with a camera Q Imaging MicroPublisher 5.0 RTV.

**Cell proliferation and viability**

Proliferation and viability were evaluated by crystal violet staining and MTT reduction respectively, as previously described by our work group [39]. H9c2 cells (3 × 10^3/well) were cultured in 96-well plates and exposed to different concentrations of ZnO NPs (2.5, 5, 10, and 20 mg/cm^2^) over 24, 48, and 72 h. Non exposed cells were used as negative control. After exposure, cells were fixed with 1.1% glutaraldehyde for 10 min, washed twice with water, and stained with 0.1% crystal violet solution for 20 min. Incorporated crystal violet was solubilized with 100 ml/well acetic acid (10%) and optical density at 590 nm was measured in a microplate spectrophotometer (Benchmark Plus, BIO-RAD). To assay viability, 20 ml/well of MTT (5 mg/ml) were added, and cells were incubated for 4 h at 37°C. Then the medium was removed, formazan crystals were dissolved with acid isopropanol (0.04 N HCl) and optical density was read at 570 nm.

**Oxidative stress**

The cellular redox state was measured by oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) into 2,7-dichlorodihydrofluorescein (DCF) which is a redox indicator probe [40]. H9c2 cells (200 × 10^3) were cultured without or with ZnO NPs (5, 10, 20 mg/cm^2^) for 1, 3, and 6 h in 56-mm glass Petri dishes. After treatment, cells were trypsinized and incubated with H_2DCFDA (10 mM) for 30 min and washed twice with PBS. Fluorescence was quantified in a FACSARia flow cytometer (Becton-Dickinson, CA, USA).

Oxidative stress was also tested by ΔΨm changes based on the fluorescent dye rhodamine 123 (Rh123). To this, cell suspensions (1 × 10^6) were treated without and with ZnO NPs (5, 10, 20 mg/cm^2^) for 1 h. Then cells were washed with PBS and incubated with Rh123 (5 mg/mL) for 15 min. After incubation, cells were washed with PBS and analyzed in a FACSARia flow cytometer (Becton-Dickinson, CA, USA). Data were processed using FlowJo 8.7 software (Stanford University).

**Mitophagy assay**

To assay mitophagy (selective removal of damaged mitochondria by autophagosomes and lysosomes), H9c2 rat cardiomyoblasts (50 x 10^4) were cultured in 35-mm glass-bottomed Petri dishes (MatTek,
Ashland, MA, USA) in absence or presence of ZnO NPs (2.5, 5, 10 and 20 μg/cm²) for 6 h. To detect nucleus, mitochondria, or lysosomes, cells were pre-incubated with 0.4 μM Bis-Benzimide H 33342 trihydrochloride (Hoechst), 0.5 μM MitoTracker Green (MTG), and 0.5 μM LysoTracker Red (LTR), respectively, for 30 min at 37°C in DMEM without phenol red. Epifluorescence images were taken with the EVOS FL (Thermo Fisher Scientific Waltham, MA, USA) cell imaging microscope at 60x magnification.

**Cell death**

The annexin-V-Fluos staining kit was used to determine cell death by microscopy. H9c2 cells (50 × 10⁵/well) were cultured in 6-well plates and treated with 2.5, 5, 10, and 20 mg/cm² ZnO NPs for 24 and 48 h. After exposure cells were analyzed by fluorescence microscopy in a Floid Cell Imaging Station (Life Technologies, Carlsbad, CA, USA). To this, the culture medium was discarded, cells were washed with PBS and subsequently incubated with 100 mL of annexin-V-Fluos labeling solution (20 mL propidium iodide plus 20 mL annexin-V-Fluos in 1 mL of incubation buffer) for 15 min and analyzed immediately.

Apoptosis and necrosis were also examined by flow cytometry using dual staining with annexin V-FITC and propidium iodide according to the manufacturer’s instructions. Briefly, 1 × 10⁶ cells in 100 ml annexin buffer were stained with propidium iodide staining solution and FITC annexin V staining solution. Cells were incubated at room temperature in the dark for 15 minutes and acquired in a FACSaria flow cytometer (Becton-Dickinson, CA, USA). To flow cytometry, cells were washed with PBS, trysinized, incubated with 100 mL of annexin-V-Fluos labeling solution and immediately analyzed. Data were processed using FlowJo 8.7 software (Stanford University).

**Protein expression**

Western blot analysis was performed as described previously by our work group [41]. Cells were exposed to ZnO NPs (5, 10, 20 mg/cm²) for 48 h and total proteins were isolated with a lysis extraction buffer. Proteins were quantified using Bio-Rad protein assay dye reagent concentrate. Proteins (30 mg) were separated by electrophoresis using 8% SDS-polyacrylamide gels and transferred to PVDF membranes, blocked, and incubated with primary antibodies against troponin I, atrial natriuretic peptide and β-actin as a load control (diluted 1:2500, 1:000, and 1:2500, respectively) overnight. After, membranes were washed three times with TBS-T, incubated with the secondary antibody (diluted 1:2500) for 1 h, and washed again three times with TBS-T. Proteins were detected with the SuperSignal® system and the ChemiDocTM MP Imaging System (Bio-Rad). Densitometric analysis for protein quantification was carried out with the Image Lab™ V 4.0 Software (Bio-Rad).

**Statistical Analysis**

To quantify and compare the patterns of actin structures a non-parametric Kruskal-Wallis test was performed using the Prism 7.0a software (GraphPad software, Inc.). In order to determine statistical differences in all assays, Student’s t-tests were performed and p<0.05 was considered significant. For
western blot analysis, multiple comparisons were based on one-way analysis of variance (ANOVA) followed by Turkey's pairwise comparison as post hoc test in Prism 5.01.

**Abbreviations**

ZnO NPs, zinc oxide nanoparticles; H9c2, rat cardiomyoblasts; TEM, transmission electron microscopy;

**Declarations**

**Ethics approval and consent to participate:** Not applicable

**Consent for publication:** Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Competing interests:** "The authors declare that they have no competing interests"

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**Authors' contributions:**

FIMM and KAVD carried out cell culture, measured ΔΨm, and western blots; MAHR performed cell proliferation and MTT-reduction assays; ZCV, performed western blot analysis and graphical abstract; MPRG described cell morphology by TEM; ACM evaluated cell morphology through HE and Trichrome stains and quantified cell death; CMM and AVM determined oxidative stress and cell death; DXRC evaluated mitophagy; NLDB and YIC characterized ZnO NPs; RLM designed and financially supported the project and wrote the manuscript. All authors read and approved the final manuscript.

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**References**


Figures

Figure 1

ZnO NPs uptake by H9c2 cells. Cells (50 × 10^3) were cultured in 6 well plates, exposed to 0, 2.5, 5, 10, and 20 mg/cm^2 ZnO NPs for 24 h, and analyzed by TEM with a JEOL 10-10 microscope and an ATM camera system. Images are shown at a direct magnification of 8000x and 12000x. Bars = 2 mm. A representative image of three independent experiments is shown.
Figure 2

ZnO NPs effects on cell morphology. Cells were exposed to 0, 2.5, 5, 10, and 20 mg/cm^2 ZnO NPs over 48 h. After treatment, cells were stained with hematoxylin-eosin (HE). Microphotographs were taken in an Olympus BX51 microscope at 20x magnification.
Figure 3

ZnO NPs effects on cell proliferation and viability. Cells (3 × 10^3/well) were exposed to 0, 2.5, 5, 10, and 20 mg/cm² ZnO NPs for 24, 48, and 72 h. Cell proliferation and viability were measured by crystal violet staining (A) and MTT reduction (B), respectively. Results were expressed as mean ± standard deviation of three independent experiments. * p<0.05 compared with control.
Figure 4

ZnO NPs effects on cellular redox state. Cells (3 × 10^3/well) were exposed to 0 (control), 5, 10, and 20 mg/cm^2 ZnO NPs for 1 h. The cellular redox state was evaluated by H₂DCFDA and analyzed by flow cytometry. In A, a representative experiment is showed where a continuous line represents control cells and a dashed line represents treated cells. In B, results were expressed as DCF positive cells (%) mean ± standard deviation of three independent experiments. * p<0.05 compared with control.
Figure 5

ZnO NPs effect on mitophagy. Cells were exposed to ZnO NPs (2.5, 5, 10, and 20 μg/cm²) over 6 h. Cells were cultured in DMEM in glass bottom culture dishes. Cells loading protocol was performed as indicated in Material and Methods section. Bars = 50 μm.
Figure 6

ZnO NPs effect on Δψm. Cells (1 × 10⁶) were exposed to 0 (Control), 5, 10, and 20 mg/cm² ZnO NPs for 1 h and Δψm was measured using Rh123 and flow cytometry. In A, a representative experiment is shown where the continuous line represents control cells and the dashed line represents treated cells. In B, results were expressed as mean ± standard deviation of three independent experiments, normalized vs. control cells set as 100%. * p<0.05 compared with control.

<table>
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<tr>
<th>Treatment</th>
<th>Intensity of Fluorescence (%)</th>
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<tr>
<td>Control</td>
<td>100 ± 2.6</td>
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<td>5</td>
<td>92 ± 6.7</td>
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Figure 7

ZnO NPs effect on cell death. Apoptotic and necrotic cells were detected using annexin-V and propidium iodide, respectively. Cells (1 × 10^6) were exposed to 0, 2.5, 5, 10, and 20 mg/cm^2 of ZnO NPs. In A, cell micrographs of a representative experiment after 48 h exposure to ZnO NPs were taken in a fluorescence microscope with a 20X objective. In B, percentage of apoptotic and necrotic cells after 48 h of exposure is shown. In C, flow cytometric analysis of cells is presented as mean ± standard deviation of three independent experiments. * p<0.05 compared with control.
Figure 8

ZnO NPs effect on cardiac protein expression. Cells ($1 \times 10^6$) were exposed to 5 and 10 $\mu$g/cm$^2$ over 48 h and the expression of troponin I (A) and atrial natriuretic peptide (B) was determined by western blot. A representative experiment is shown where β-actin was used as load control. Densitometric analysis of protein expression normalized against α-actin level is shown below and results are expressed as mean ± standard deviation of three independent experiments. * $p<0.05$ compared with control.

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