Research Article

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Comparative toxic effect of bulk zinc oxide (ZnO) and ZnO nanoparticles on human red blood cells

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Abstract: Metal oxide nanoparticles (NPs) are a member of nanomaterials family that have been produced on a large scale and their toxicity affected by their high surface-to-volume ratio. The hemolytic toxic effects of metal oxide NPs may be completely different from those observed in their bulk counterparts. So, the main purpose of this research was to measure the difference between the hemolytic effects of ZnO (NPs and bulk) on isolated human red blood cells (RBCs). Hemolysis was measured after incubation of human RBCs with 0.01–1 mM of ZnO (bulk and NPs) for 6 h. For measurement of other variables, human RBCs were treated with 0.1 mM of ZnO (NPs and bulk) for 1, 2, and 3 h. Our results demonstrate that bulk ZnO did not show any toxic effects in the concentrations tested, while ZnO NPs caused toxic hemolytic effects through formation of ROS, lipid peroxidation, and glutathione depletion.

Keywords: zinc oxide, erythrocytes, nanoparticles, oxidative stress, hemolysis

1 Introduction

Metal oxide nanoparticles (NPs) are a member of nanomaterials family that have been produced on a large scale for both household and industrial applications. ZnO NPs have wide applications in paints, pharmaceutics, cosmetics, food additives, and dietary supplement industries, owing to their high stability and low cost (Nohynek et al., 2007; Rincker et al., 2005; Steele et al., 2009). Reasonably, such rapid growth in usage of these nanomaterials increases the likelihood of human exposure. Otherwise, nanomaterials exhibit distinguished toxicological effects, due to their extraordinary chemical properties in comparison to their bulk counterparts (Dingman, 2008; Oberdörster et al., 2005). It has been shown in several toxicity studies that ZnO NPs adversely affect many *in vivo* and *in vitro* systems (De Berardis et al., 2010; Osman et al., 2010; Sharma et al., 2012a, 2012b; Wahab et al., 2011). Although the production and application of ZnO NPs keep growing, the toxicological information of these NPs is still incomplete. Investigations about ZnO NPs interaction with blood cells are of great importance, because most of the medicinal applications of these NPs are based on administration through intravenous/oral routes (Mocan, 2013).

Following the hemolysis of red blood cells (RBCs), hemoglobin released into the surrounding environment can be measured spectroscopically. The evaluation of nanomaterials' hemolytic effects is an important issue for nano and pharmaceutical science because they could simply enter into human body and cause substantial hemolysis of RBCs (Neun et al., 2018). The hemolytic effects of ZnO (NPs and bulk) have not been investigated in a comparative study; therefore, this research attempts to compare the toxicity of ZnO (NPs with bulk) on isolated human RBCs. Present research attempts to further understand how ZnO NPs affect human cells and provides additional data about toxicity of ZnO NPs on human RBCs, which contribute to our understanding of difference between toxicity of nano and bulk ZnO counterpart. Considering the oxygen (O_2) delivery role of RBCs, toxic damage to these cells can disturb oxygen delivery to the body tissues. For assessment of hemolytic effects, RBCs were treated with 0.01–1 mM of ZnO (bulk and NPs) for 6 h. The mechanisms underlying the hemolytic effects were evaluated following incubation of human RBCs with 0.1 mM of ZnO (NPs and bulk) for 1, 2, and 3 h. Consequently, the ZnO NPs were found to show significant toxicity in concentrations, while bulk ZnO did not show any toxic effects.

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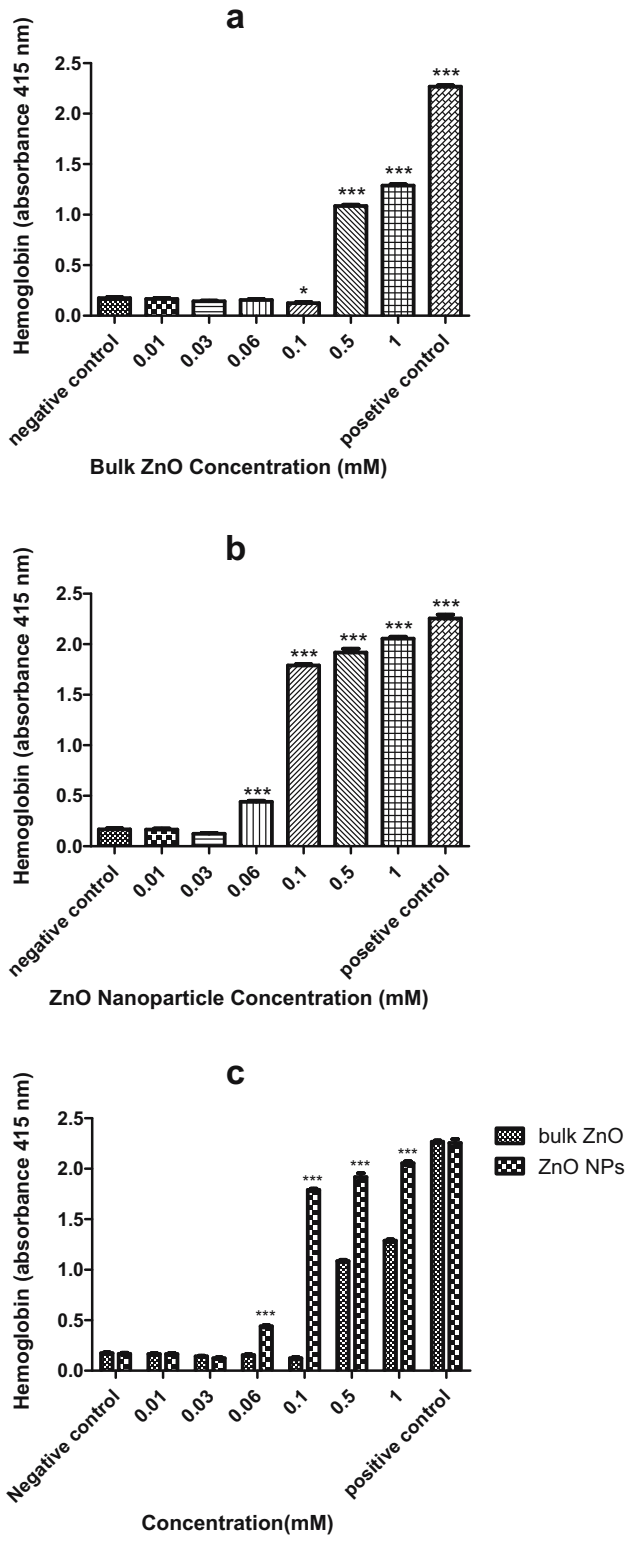

Figure 1: Hemolysis of human red blood cells following treatment with ZnO for 6 h. ZnO NPs induced hemolytic effects in a dose-dependent manner and this effect is significant at concentration higher than 0.03 mM (b) but such effect only observed by 0.5 and 1 mM of bulk ZnO (a). Comparison between concentrations of ZnO NPs and bulk ZnO (c). Negative control: DPBS without ZnO, and positive control: Triton X-100. *$P < 0.05$ and *** $P < 0.001$.

2 Results

2.1 Hemolytic effects of ZnO (bulk and NPs)

Following 6 h treatment, only 0.5 and 1 mM of bulk ZnO significantly ($P < 0.05$) disrupt human RBCs in comparison with negative control (Figure 1a). However, ZnO NPs induced significant ($P < 0.05$) hemolysis in RBCs at concentrations higher than 0.03 mM and this hemolytic effect increased in a concentration-dependent manner (Figure 1b). Further evaluation demonstrated that interaction between concentrations and form of Zn significantly ($P < 0.05$) affect ZnO hemolysis (Figure 1c). Hemolysis results were verified by positive (Triton X-100) and negative (DPBS without ZnO) control included in our experiments.

2.2 Oxidative stress in human RBCs

Following treatment of isolated human RBCs, 0.1 mM ZnO NPs caused a significant ($P < 0.05$) increase in oxidative stress at 1, 2, and 3 h and this increase was time-dependent. However, treatment of human RBCs with 0.1 mM of bulk ZnO for 3 h did not cause any significant ($P < 0.05$) increase in oxidative stress generation (Figure 2).

2.3 Lipid peroxidation

Lipid peroxidation significantly ($P < 0.05$) increased after treatment of isolated human RBCs with 0.1 mM ZnO NPs

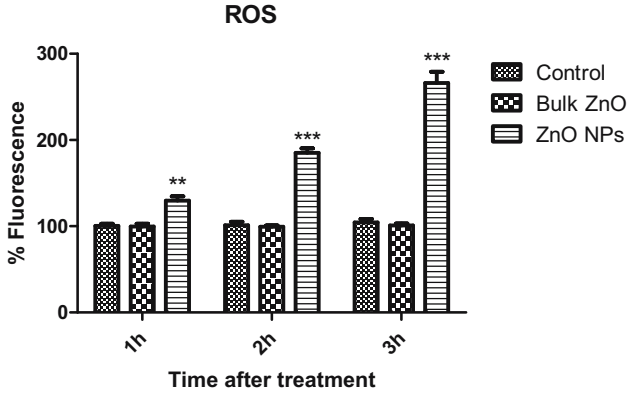

Figure 2: ROS generation in human RBCs after incubation with ZnO. Induction of ROS by ZnO NPs (0.1 mM) was significant ($P < 0.05$) at 1, 2, and 3 h time intervals. However, bulk ZnO ROS-generation was not significant ($P < 0.05$) in human RBCs after 3 h treatment. **$P < 0.01$ and ***$P < 0.001$.

for 1, 2, and 3 h. ZnO NPs induced oxidative stress in human RBCs as a function of time (Figure 3). Nevertheless, bulk ZnO (0.1 mM) did not cause any significant ($P < 0.05$) increase in lipid peroxidation following 1, 2, and 3 h of treatment (Figure 3).

2.4 Change in glutathione (GSH) and GSSG content

Isolated human RBCs were treated with ZnO (bulk and NPs) and GSH and GSSG levels (Figure 4a and b) were measured 1, 2, and 3 h following treatment. ZnO NPs (0.1 mM) caused a significant ($P < 0.05$) decline in GSH (Figure 4a) and increase in GSSG (Figure 4b) in a time-dependent manner. However after 1, 2, and 3 h of exposure to human RBCs, the bulk ZnO did not cause any significant ($P < 0.05$) changes in intracellular GSH and extracellular GSSG (Figure 4a and b).

3 Discussion

Due to their high surface-to-volume ratio, ZnO NPs are able to interact with biomolecules such as proteins and lipids (Casciano and Sahu, 2009). Oxygen derivatives such as hydroxyl radicals, superoxide anions (O_2^-), and hydrogen peroxide are called reactive oxygen species (ROS), and are formed during induction of toxic effects in live organisms. During interaction with biomolecules,

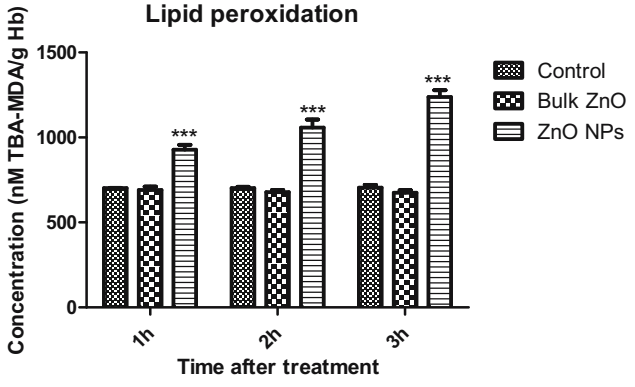

Figure 3: Induction of lipid peroxidation in human red blood cells after incubation with ZnO. At 1, 2, and 3 h following treatment, ZnO NPs (0.1 mM) significantly ($P < 0.05$) increased MDA concentrations in human RBCs, but significant ($P < 0.05$) rise in MDA was not observed with 0.1 mM of bulk ZnO at different time intervals. ***$P < 0.001$.

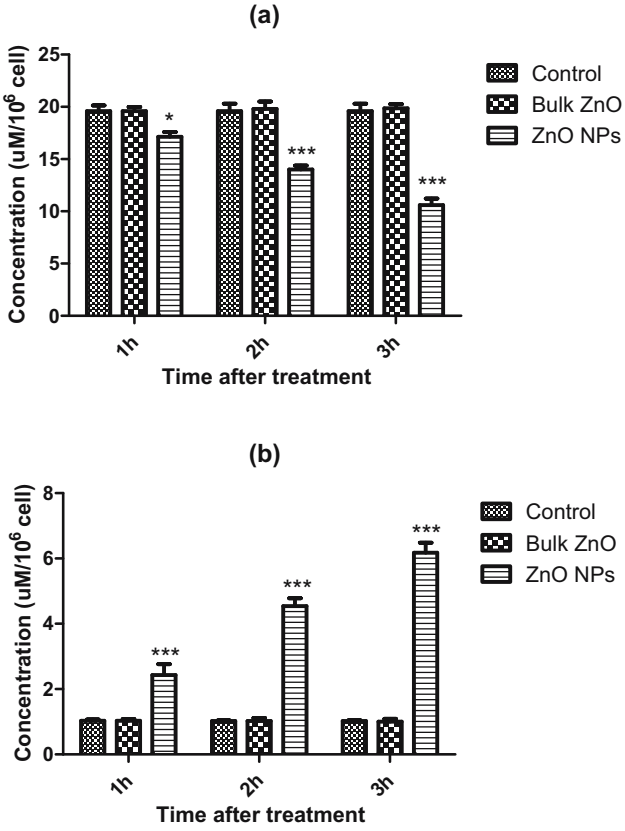

Figure 4: Influences of ZnO treatment on levels of intracellular GSH and extracellular GSSG. As demonstrated in (a) and (b), significant ($P < 0.05$) intracellular GSH decrease and raise in RBCs extracellular GSSG were found at 1, 2, and 3 h after treatment with ZnO NPs, but these effects were not observed in case of bulk ZnO (with same concentrations), respectively. *$P < 0.05$ and ***$P < 0.001$.

NPs increase superoxide radical (O_2^-) formation, which leads to ROS generation and oxidative stress (De Berardis et al., 2010). Induction of oxidative stress following ROS generation is the major mechanism of NPs toxicity. Low level of CuO or ZnO NPs can generate large amounts of ROS (Toduka et al., 2012). In the present study, we observed that 0.1 mM of ZnO NPs can induce oxidative stress in RBCs. Reversely, bulk ZnO did not trigger oxidative stress generations at concentrations tested in our study. Induction of oxidative stress following convenient crossing through RBCs membrane is a key mechanism in ZnO NPs toxicity. Oxidative stress in RBC impairs oxygen delivery and induces RBC aging. NPs-triggered ROS generation can cause a range of toxic effects that determined the cellular pathways and antioxidant response involved in ROS generation, and the quantity of ROS generation (Xia et al., 2008). The results of the studies in zebrafish cells showed that the relative abundance of hydroxyl radical (˙OH) and therefore the oxidative stress and oxidative damage in metal oxide NPs'-exposed organism were

much higher comparing to bulk formulations (Xiong et al., 2011). ˙OH can oxidize nearly all the cellular components and are generally known as one of the most dangerous ROS species (Yamakoshi et al., 2003). The metal oxide NPs-generated extracellular ˙OH might cause cell membranes' oxidative damage, which can affect the viability of the cells. In the present study, we evaluated the influence of ZnO-induced oxidative stress to mediate RBCs hemolysis and subsequent release of hemoglobin. ZnO NPs distinctly demonstrated higher hemolytic effects as compared to the bulk ZnO and this can be due to the higher ability of ZnO NPs for inducing extracellular ˙OH generation and subsequent oxidative damage to RBCs membrane.

Reaction of ROS with biomolecules can cause an imbalance between the generation of reactive oxygen and the biological system's antioxidant capacity to eliminate reactive intermediates or repair the resulting damage (Yang et al., 2009). Following passing the cell membrane, NPs may trigger intracellular oxidative stress through disturbing the balance between the generation of reactive oxygen species and the biological system's antioxidant capacity. Our results demonstrated that ZnO NPs cause significant ($P < 0.05$) collapse in GSH and increase in GSSG (opposite to the bulk ZnO). Collapse in GSH level, along with higher level of hydroxyl radical (˙OH) which are generally produced by metal oxide NPs, lead to oxidative damage and cell death. Unrestricted oxidative stress can also cause oxidation in nucleic acids, proteins, and lipids, which further motivates the antioxidant defense system or even leads to cell death. Incapacitation of RBCs antioxidant system and their rupture by ZnO NPs furthermore proved the higher potential of ZnO NPs for generation of thiobarbituric acid reactive substance in RBCs. There are several works which have been demonstrated: SnO_2-doped ZnO NPs/reduced graphene oxide nanocomposites (SnO_2–ZnO/rGO NCs), green stabilized Mo-ZnO/RGO NCs (Mo-ZnO/RGO NCs), and green synthesis of ZnO-reduced graphene oxide nanocomposites (ZnO-RGO NCs) using garlic clove extract have enhanced anticancer activity and better biocompatibility than those of pure ZnO NPs. These works have suggested a new approach to reduce the toxicity of ZnO NPs which can be the subject of future research works using ZnO NPs without any remarkable toxic effects (Ahamed et al., 2021, 2022a, 2022b).

4 Conclusion

The hemolytic effects of ZnO (NPs and its bulk counterpart) were investigated in the present study. Based on our observations, at concentrations of bulk ZnO which did not cause any significant hemolysis, ZnO NPs caused significant hemolytic effects in human RBCs. Based on the main results of the present research which show the distinct hemolytic effect of ZnO NPs, future works must focus on the reasons of ZnO NPs' higher toxicity and investigate other mechanisms that maybe involved in higher toxicity of ZnO NPs including difference between kinetic of ZnO NPs and bulk counterpart. Further *in vivo* and *in vitro* studies can characterize the difference between the hemolytic effects of ZnO NPs with green synthetized ZnO nanocomposites which has been shown to be more biocompatible.

Experimental

Chemicals

2′,7′-Dichlorofluorescin diacetate (DCFH-DA), *o*-phthalaldehyde (OPA), *N* ethylmaleimide (NEM), zinc oxide powder (catalogue no. 205532, size: <5 μm, 99.9%) and NPs (catalogue no. 544906, average particle size: < 100 nm, specific surface area ($m^2 \cdot g^{-1}$): 10–25), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany).

Isolation and treatment of human erythrocytes from whole blood

Blood samples (3 mL) were obtained from 20 volunteers (healthy and non-smoking) aged from 18 to 30 years old and collected in lithium heparin vacutainers (BD Biosciences, USA). 3 mL of blood samples were diluted to 15 mL with Dulbecco's phosphate buffered saline (DPBS, Mg++ and Ca++ free, Sigma-Aldrich, USA) and RBCs isolated using centrifugation (Jouan centrifuge, model number BR4i). Approximately 7×10^6 RBCs were suspended in 1 mL of DBPS, and after 6 h of treatment with 0.01–1 mM ZnO (NPs and bulk), hemolytic effect was assayed. Other parameters were measured following incubation of RBCs with 0.1 mM of ZnO (NPs and bulk) for 1, 2, and 3 h.

Hemolysis assay

Human RBCs were treated with 0.01–1 mM of ZnO (NPs and bulk) at 37°C for 6 h during which time the samples

were shaken every 1 h. Supernatants were collected by centrifugation for 5 min at 573×*g*. 100 µL of supernatants were placed into wells of a 96-well plate in triplicate and hemoglobin absorbance was measured at 415 nm with an ELISA micro plate reader (Tecan, Rainbow Thermo).

Measurement of ROS

DCFH-DA was used for assessment of ROS. DCFH-DA is hydrolyzed to non-fluorescent DCFH by cellular esterases and after reaction with ROS generates fluorescent dichlorofluorescin (DCF). Human RBCs were treated with 0.1 mM concentrations of ZnO (NPs and bulk) for 1, 2, and 3 h and after washing twice were incubated with 500 µL of 10 µM DCFH-DA solution for 20 min at 37°C. Finally, samples' fluorescence was recorded at 495 and 530 nm excitation and emission wavelength using fluorescence spectrophotometer, respectively (Shimadzu RF5000U) (Pourahmad et al., 2011).

Lipid peroxidation measurement

Lipid peroxidation was measured based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA). ZnO (NPs and bulk)-treated RBCs were lysed with 2% triton and heated with 200 µL of TBA reagent (TBA 0.37%, trichloroacetic acid (TCA) 15%, and HCl 2.5 N) in hot water (90°C) for 60 min. TBA-MDA concentration was assessed based on samples absorbance at 532 nm (Beckman DU-7 spectrophotometer) and calibration curve of the TBA-MDA (Wasowicz et al., 1993).

GSH and GSSG assessment

Hissin and Hilf fluorimetric method with some adjustment was used to measure GSH (oxidized and reduced). Following treatment with 0.1 mM of ZnO (NPs and bulk) for 1, 2, and 3 h, human RBCs were lysed with TCA 10% (0.5 mL). Following dilution with phosphate-EDTA buffer, cell supernatants were incubated with 100 µL of the o-Phthalaldehyde (OPT) solution (15 min) for measurement of GSH. For GSSG measurement, NaOH 0.1 N solution was added to cell supernatants and before addition of OPT, diluted supernatants were incubated with 200 µL of NEM for 30 min at room temperature. Finally, GSH and GSSG concentrations were determined using samples' fluorescence intensity at 350 and 420 nM and GSH and GSSG calibration curve (Hissin and Hilf, 1976).

Statistical analysis

Statistical analysis of data was performed using GraphPad Prism5 (Graphpad software, La Jolla, CA). Data were evaluated and compared using one-way ANOVA followed by the post hoc Tukey test and two-way ANOVA followed by Bonferroni tests. Data are representative of at least three independent experiments and P value of less than 0.05 was reported as statistically significant. Results are demonstrated as the mean value ± SEM.

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Author contributions: Mohammad Hadi Zarei: project administration, writing – review and editing, methodology, study conception, and design; Maryam Salami and Maryam Khosravi: writing – original draft, formal analysis, and material preparation.

Conflict of interest: Authors state no conflict of interest.

Ethics approval and consent to participate: This research was done in Shahid Beheshti University of Medical Science (SBMU) at Faculty of Pharmacy and ethical approval was given by the research ethics committee of SBMU. After becoming aware of our investigation, donors were asked to fill out the approval form. Informed consent was obtained from all individual participants included in the study.

Consent for publication: The authors affirm that human research participants provided informed consent for publication of data.

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