

Original Research Article

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Zinc Oxide Nano Particle (ZnO NPs) and Innate Immunity: New Perspectives in Immunotoxicity

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ABSTRACT

In the contemporary, application of zinc oxide nanoparticles (ZnO NPs) is growing exponentially in food industry and allied sciences. Hence public concern about their potential adverse effect is increasing. ZnO NPs with their unique physiochemical properties are thought to underlie their exploitable immunological activities. A study was conducted to investigate effects of ZnO NPs with innate immune cells. To achieve this aim, amongst three experimental groups, two group received (5.0 and 50 mg/kg b.w), ZnO NPs was dispensed, subsequent analysis of the body, and different organs weight, cytotoxicity were done using (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT), Water-soluble tetrazolium salts (WST), Nitric oxide (NO), phagocytosis. The levels of NO yield, phagocytosis, and weight of spleen increased (*P<0.05) due to ZnO NPs at dose of 50 mg/kg b.w., which caused adverse effect on functionality of macrophages. ZnO NPs release of Zn²⁺ ions and produced reactive oxygen species and caused dose dependent toxicity. The results demonstrate that the high dose of ZnO NPs caused toxicity which is analysis by body and liver, kidney, and spleen weight, cell viability (MTT and WST), nitric oxide (NO) level, phagocytosis assay. The levels of NO production, phagocytic activity, and spleen weight increased significant (*P<0.05) while decrease in NO production and phagocytosis was observed. ZnO NPs (5.0 mg/kg b.w) caused significant increased phagocytosis, cell viability, therefore high at dose ZnO NPs caused immunocytotoxicity. Overall, nanotoxicology of ZnO NPs and their effect requires more study to fill up gap of immunotoxicity.

Keywords

Zinc oxide nanoparticle, Macrophages, Nitric oxide, Immunotoxicity, Phagocytosis

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Introduction

Nanotechnology is an emerging technology, which can lead to a new revolution in every field of science (Rico *et al.*, 2011), and widely used in different areas of science. Nanomaterials have become an essential part of our day to day lives because of their special properties including small size, high specific surface area and reactivity. Nanoparticles (NPs) have peculiar physiochemical properties and used in different fields such as in industrial catalyst, nanomedicine (Meng *et al.*, 2012), sensors, nanofertilizer (Lopez-Moreno *et al.*, 2010), diagnostic imaging tools in health sector (Yoo *et al.*, 2011). However, safety level knowledge about biological effects (immunological), potential health risks and toxicities of the widely exposure to nanomaterials still remain unclear.

Zinc Oxide nano particle (ZnO NPs) may enter the human as well as the animal body via food chain or directly ingestion of food items, inhalation, drugs, and nano vaccine or through the skin cosmetics and sun cream (Wang *et al.*, 2007). Once entered in body systems, they circulate in biological milieu, consequently come into contact with different of biomolecules including proteins and lipids as well dissolve in the body fluids such as blood, lymph and interstitial fluids. The biological molecules immediately coat the surface of Zinc Oxide nano particle (ZnO NPs) and form "corona" (Lundqvist *et al.*, 2011), which dictate the biological uniqueness of the ZnO NPs (Lynch *et al.*, 2008) and phagocytes through Innate immune cells include mononuclear phagocytes (monocytes, macrophages, and dendritic cells), granulocytes (neutrophils, eosinophils, basophils) and other cells. Mononuclear phagocytes, first identified by Metschnikoff over 100 years ago, are present in all adult mammalian tissues and play key roles in host defence system. However, acute or prolonged

use of ZnO NPs in different consumer materials, drugs and cosmetics items may produce severe impediment of immunity (Peng XH, Qian X, Mao H *et al.*, 2008). ZnO NPs affinity with macrophages depends upon interaction with bio molecules and chemical instability. Most important evidence implies that the toxicity of ZnO NPs could be affected by size and/or electrostatic charge or dose. ZnO NPs immunotoxicity was defined by the dose of ZnO NPs that produced a 50% reduction of cell viability (Puzyn *et al.*, 2011). Instability of the ZnO NPs is quantified as the enthalpy of formation of a gaseous cation (ΔH_{ME+}) that had the same oxidation state (charge) as the nanoparticles (NPs) solid state (Puzyn *et al.*, 2011). The relationship between cytotoxicity and ZnO NPs instability (ΔH_{ME+}) was defined as

$$\text{Log}(1/EC_{50}) = 2.59 - 0.50 * \Delta H_{ME+}$$

Where, EC_{50} is the dose of nanoparticle that gives half-maximal response and (ΔH_{ME+}) is the NPs instability. Instability directly related to reactivity of nanoparticle with biological molecules and corona formation. Small size nanoparticle is more reactive because of high surface area to volume ratio. Zinc oxide nanoparticle ionize to form Zn^{2+} ions; positive charged ZnO NPs could be more toxic than negative charge nanoparticles due to more interaction with the negative charge of acidic acid (sialic acid) on the surface of macrophages (Dwivedi *et al.*, 2009). Number of cationic charge on the nanoparticle is inversely proportional to the cytotoxicity of ZnO NPs. When nanoparticle enters inside the body, absorbed and translocates to tissues and, as like any foreign substance, is likely to encounter the innate immune system. Inflammation is the first immune response produced by innate immune system. The inflammatory process begins by innate immune cells present in all tissues mainly resident as macrophages, dendritic cells,

neutrophils and mast cells. Nanoparticles produce a many of chemical factors that induce vasodilatations and favor chemo taxis of phagocytes. ZnO NPs generate reactive oxygen species (ROS) when interact with immune cells causes accumulation of oxidized glutathione (GSSG). The amount of the generated oxidative stress determines the pathogenicity or cell death of nanoparticle (Nel *et al.*, 2006).

Withaferin A was the first withanolide to be isolated from Withaferin A (Glatter, 1991; Kupchan *et al.*, 1965). Withaferin A is mainly present in roots, leaves, and stems, of *W. somnifera* and accounts for about 1.6 % of the dry weight (Gupta *et al.*, 1996; Mirjalil *et al.*, 2009). Withaferin A is more reactive because of ketone presenting different rings, unsaturated A ring, the epoxide in B ring and unsaturated lactone ring. It was shown to have anti-inflammatory, antitumor, anti-stress, antioxidant, immuno-modulatory, hematopoietic, and rejuvenating properties as well as benefiting the endocrine, cardiopulmonary, and central nervous systems (Mishra *et al.*, 2008). In this study, we explored the *in vivo* immunotoxicity of ZnO NPs on peritoneal macrophages, and effect of ZnO NPs on the functionality of macrophages using *Balb/c* mice. Further, we investigated the Competency of Withaferin A on immunotoxicity.

Materials and Methods

All chemicals were purchased from Sigma and Aladdin reagent Company

Preparation ZnO NPs suspensions

Zinc oxide Nanoparticle (Cat.Nr.PL-ZnO-25-25g) was purchased from the Plasma Chem. GmbH Rudower Chaussee 29, D-12489, Berlin (Germany). The ZnO NPs used in this study were sized at 25 nm (240 mg and 2400

mg dry powder of ZnO NPs was dissolved into 14.4 mL reverse osmosis (RO) water for dose 5.0 and 50 mg / kg body weight respectively. The ZnO NP suspension was Vortexed for 5 minutes at room temperature and then kept at room temperature until use. Before using, the suspension was sonicated at 4 °C for 10-15 minutes with a sonicator (Branson Sonic Power Company, Danbury).

Particle characterization

The suspension of ZnO NPs in RO water was sonicated (Branson Sonic Power Company, Danbury) for 10 min. The hydrodynamic diameter was determined by Zeta sizer Nano-ZS (Malvern instruments Ltd., Malvern, UK). The average size of ZnO NPs (100 µg/mL) in Milli-Q water was determined by transmission electron microscopy (TEM). (Tale1)

Maintenance of animals and treatment

Six-week-old Male *Balb/c* mice, supplied by the laboratory animal house, CSIR-Indian institute of integrative medicine, Jammu, India were used in this study (n=6). The initial body weight 25 ± 1.5 g (mean \pm standard error of mean, SEM) of mice were housed at room temperature (22-25 °C) in a 12 hr light and 12-hr dark period with an *ad libidum* feed and RO (drinking) water and were maintained in a pathogen-free condition. The animals for the research have been approved by institutional ethical committee, ICAR-NDRI, Karnal - 132001, India.

Experimental design and Grouping of animals

Balb/c mice were divided into three groups with six animals each (Table 2). Normal control group and which given RO water and two experimental groups were treated with two doses of ZnO NPs. The ZnO NP suspension was orally administered in the

Balb/c mice with the dosage of 5 mg/kg and 50 mg/kg alone, every day continuously for 28 days. All experiments were approved by the Institutional Animal Institutional Ethical Committee, ICAR-NDRI, Karnal 132001, India.

The animals were administered with Zinc oxide nanoparticle (ZnO NPs) 5.0 mg/kg b.w and 50 mg/kg b.w to investigate the effect of ZnO NPs on macrophage functionality. After 28 days of administration, animals were euthanized and the peritoneal fluid was collected in fresh DMEM media for experiment and different organs were collected in PBS for organ index measurement.

Collection of peritoneal fluid

The Balb/c mice were euthanized by diethyl ether and the peritoneal fluid was collected by injecting 6 mL of DMEM Hams F-12 medium (without phenol red) in peritoneal cavity by following a gentle massage of the abdomen (Perdigon *et al.*, 1986). A portion of the peritoneal exudates cell suspension was used for MTT assay and WST assay. The cells in peritoneal fluid were counted using Neuters' chamber and 1×10^6 cells/ mL was taken further.

Evaluation of macrophage functions

Balb/c peritoneal macrophage was cultured in chemically define Dulbecco's Modified Eagle's Medium Hams F-12 DMEM; (Sigma-Aldrich, St Louis, MO, USA) without phenol red, supplemented with 1.2 g/ L of sodium bicarbonate, 0.1% bovine serum albumin followed by addition of penicillin (200U/ mL) and streptomycin (50 µg/ mL). The pH of the medium was adjusted to 7.2 using 1.0 N HCl or NaOH and then sterilized by passing through 0.2 µm Millex-GV filter unit (Millipore).

Isolation and enrichment of macrophages(*in vitro* culture)

The remaining portion of peritoneal fluid was distributed into 35 mm culture grade petri dishes and incubated in a humidified atmosphere of 5 % CO₂ in air at 37 °C for 2 h to allow attachment of adherent cells according to Kumangi, 1979. Non-adherent cells were removed by washing two times with DMEM. The adherent cells were collected by jetting chilled 1ml DMEM onto the cells with a pipette and cells were counted again using Neubauer's chamber under phase contract microscope and final concentration was adjusted to 1.0×10^6 cells/ mL

Macrophage culture (*in vitro*)

One fraction of above enriched macrophages were cultured in DMEM Hams F-12 medium and incubated in a humidified atmosphere of 5 % CO₂ for 18 h at 37 °C. At the end of the incubation period, phagocytosis assay was done. Other fraction of enriched macrophages (5×10^5 cells/ml) suspended in DMEM supplemented with heat-inactivated Fetal calf serum 10% (FBS, Hyclone Laboratories Inc.), were plated 300 µl/well in 24 well cell culture plates in presence of *E. coli* (serotype 055:B5) lipopolysaccharide (LPS) (1 µg/ml final concentration) and incubated in CO₂ incubator perfused with 5 % CO₂ in air at 37 °C for 24 h. The cell free supernatant collected at 24 h was analyzed for NO (nitric oxide) production.

Study of macrophage viability

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide) assay

The MTT assay was employed to evaluate the cytotoxic effect of ZnO NPs. The MTT assay was done according to Mosman., 1983, with some modifications. Peritoneal macrophages were isolated from the peritoneal fluid of mice

in 96-well plates (1.0×10^6 macrophage/well), 10 μ l MTT (5.0mg/ml) reagents was added per well, then incubated for 4h at 37 °C humidified incubator with 5 % CO₂. The cells were removed from incubator and 100 μ l DMSO (dimethyl sulfoxide) was added to it. After 10 minutes, Absorbance (A) of plate was read by an ELISA reader at 570 nm. (Beckman Coulter, Fullerton, CA, USA). The percentage of cell viability was calculated by the following formula Macrophage cell viability (%) = (mean absorbency in test wells)/ (mean absorbency in control wells) $\times 100$. The viability of the macrophages used in all the experiments was higher than 98%, as measured by the 0.4 % trypan blue exclusion method

Nitric oxide assay

This assay was done for measurement of nitric oxide (NO). Nitric oxide production in the peritoneal macrophages culture medium was quantified spectrophotometrically using the Griess reagent G4410-10G. The nitrite (NO₂⁻) present in the supernatant of culture macrophages (*M ϕ*) was used as an indicator of NO. Nitrite is a stable degradation product of NO. Nitric oxide (NO) production was determined by measuring the stable end-products of NO metabolism *i.e.* nitrite and nitrate. Nitrate present in the sample was reduced to nitrite using vanadium chloride, which was then estimated using Griess reagent.

Total nitric oxide production was determined by the method of Miranda *et al.*, 2001. Briefly, 100 μ l of vanadium chloride and 50 μ l of Griess reagent were added to 100 μ l cultures supernatant and incubated at 37 °C for 40 min. The absorbance was measured at 550 nm and the concentration of nitrite (indicator of nitric oxide) was determined from the standard curve generated from serial dilutions of NaNO₂, (3.12 μ M in DMEM).

Macrophages phagocytosis assay

Phagocytosis is a two stage process involving binding (adherence) of foreign particle to macrophage followed by ingestion of the former by the later. Yeast were dissolved in DMEM media and sonicated gently to disrupt clumps and further diluted to 1.0×10^8 cells/mL. For phagocytosis assay peritoneal fluid (1×10^6 cells/mL) were incubated at 37 °C for 2 h in 35 mm Petri-dishes. The medium was removed and the adhered cells were washed twice with DMEM Hams F-12. Then 1 ml of DMEM and 100 μ l of yeast cells (1×10^8 cells/ml) was added and incubated for 1 h at 37 °C. The supernatant was removed; the cells were washed with DMEM and air dried. The cells were then stained with May Grunwald stain freshly diluted with Giemsa buffer (1:2) for 2 min and then washed with PBS and dried. Phagocytosis was observed at 1000X magnification under oil immersion microscope (Olympus Optical Co. Ltd, Japan) and following observations were recorded.

Phagocytosis (%) = No of macrophages with yeast cells internalized (N) per 100 Macrophages

Evaluation of phagocytic activity of macrophages

The phagocytic activity of macrophages was evaluated by counting phagocytic cells (macrophages) after their identification on Neubauer Chamber, stained with 0.4 % trypan blue. A minimum of 100 phagocytic cells were counted in each field, together with the number of ingested yeast beads, using a 1000 X magnification under oil immersion microscope (Olympus Optical Co. Ltd., The percentage (%) of macrophages showing phagocytic activity and the number of yeast contained within macrophages cells was determined. Phagocytic activity was expressed as phagocytic index (PI), calculated using the following formula (Barbuddhe *et al.*, 1998)

Statistical analysis

In this study, all data are presented as the mean \pm standard error mean. The mean values among different groups were analyzed and compared using one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey) with Graph Prism (Graph Pad Software, La Jolla, CA, USA) version 5 software packages. One-way ANOVA with repeated measurements followed by Tukey's test was applied to test the influence of ZnO NPs on bodyweight gain, spleen weight, cell viability, phagocytosis %, nitric oxide production, and relative mRNA expression. A $P \leq 0.05$ and $P \leq 0.1$ was considered to be statistically significant.

Results and Discussion

ZnO-NPs Characterization

The average particle size of ZnO NPs measured by dynamic light scattering (DLS) were 52.80 nm (Table 1 and Fig. 1) while manufacture size was 25 nm. ZnO NPs structure was spherical.

Effects on body and organ weight gain study

The effect of ZnO NPs on the body and organ weight was examined by weighing balance. ZnO NPs at the dose 5.0 mg/kg b.w and 50 mg/kg b.w had no effect on the body, liver, and kidney weight after 28 day of exposures. Although there was a significant increase in the spleen weight at a dose 50 mg/kg b.w. A, increased the spleen weight ($P < 0.05$) compared to the ZnO NPs 50 mg/kg group (Fig. 2).

Assessment of macrophages viability

The cell viability assessment was done by MTT and WST assay to evaluate the

cytotoxicity of ZnO NPs in Macrophages. When mice were treated with various concentrations of ZnO NPs (5.0 and 50 mg/kg body weight) for 28 days, macrophages viability was significantly influenced as compared to control. The effect on viability in *Balb/c* mice macrophages is shown in Table 3, 4 and Figure 3.

Nitric oxide assay

Nitric oxide (NO) is a reactive form of nitrogen and is an important cellular signaling molecule involved in different biological processes, and moreover, serves as one of the key mediators of immune defense. To further explore the impact of ZnO NPs on macrophages and immune defense, we measured the level of macrophages NO production after 28 days treatment. As shown in figure 4, and Table 5, a significant decrease in NO level occurred after administration 50 of ZnO NPs, mg/kg b.w. However, there was no substantial difference of NO level in ZnO NPs, 5 mg/kg b.w fed *Balb/c* mice as compared with the control. This decrease may result from more reactive oxygen species generation resulting in decreased iNOS activity (Kim *et al.*, 2015).

Phagocytosis assay

Because ZnO NPs show cytotoxic effect via enhancing oxidative stress, which may cause cell apoptosis; we evaluated the phagocytosis activity of macrophages. *Balb/c* mice were treated with ZnO-NPs. Our result showed that treating mice with ZnO NPs (5.0 mg/kg) enhanced phagocytic activity. However, phagocytic activity significantly decreased in mice treated with ZnO NPs (50 mg/kg) (Figure 7. Higher dose of Zinc oxide nanoparticle produce more free radicals leading to oxidative stress of immune cells (Lin *et al.*, 2014). As shown in Figure 5 and Table 6.

Table.1 Particle characterization of ZnO NPs. Size and structure was determined by DLS and high resolution transmission electron microscope (HR-TEM) Tecnai G2 20 twin

Characterization	Mean diameter Size (nm) in MilliQ water	Crystalline structure
Manufacture size	25.00	spherical
DLS	52.8	spherical
HR-TEM	25 -30	spherical

Table.2 Different experimental groups and treatment (28 day) to *Balb/c* mice

Groups	Treatment
Control	Rivers osmosis (RQ) Drinking water @ 300 µl/mice
ZnO NPs 5	5.0 mg/kg b.w ZnO NPs @300 µl/mice
ZnO NPs 50	50 mg/kg b.w ZnO NPs @300 µl/mice

Table.3 Cytotoxicity of ZnONP on macrophages viability by MTT assay

Treatment group	Viability (%)
Control	100 ± 0.0
ZnONP5(5.0 mg/kg)	128.9 ± 9.1
ZnONP 50(50 mg/kg)	102.9 ± 8.1

. **P* < .05 and. ***P* < .01 (mean ± standard error of mean (SEM) and were analysed using one way ANOVA for cell viability)

Table.4 Cytotoxicity of ZnONPs s on macrophages viability by WST assay

Treatment group	Viability (%)
Control	100 ± 0.0
ZnONP5(5.0 mg/kg)	124 ± 18
ZnONP50(50 mg/kg)	86 ± 1.29

. **P* < .05 and. ***P* < .01 (mean ± standard error of mean (SEM) and were analysed using one way ANOVA for cell viability)

Table.5 Effect of ZnONPs on Nitric oxide production by macrophages

Treatment group	Nitric Oxide conc.(n.mole)
Control	148.33 ± 5.67
ZnONP5(5.0 mg/kg)	180 ± 21
ZnONP50(50 mg/kg)	55 ± 18.1

P* < .05 and. *P* < .01 (mean ± standard error of mean (SEM) and were analysed using one way ANOVA for nitric oxide production)

Table.6 Effect of ZNFs on phagocytosis of macrophages (%)

Treatment group	Phagocytosis (%)
Control	34.66 ± 3.74
ZnONP5(5.0 mg/kg)	53.0 ± 0.0
ZnONP50(50 mg/kg)	16.66±0.20

P* < .05 and. *P* < .01 (mean ± standard error of mean (SEM) and were analysed using one way ANOVA for phagocytosis assays).

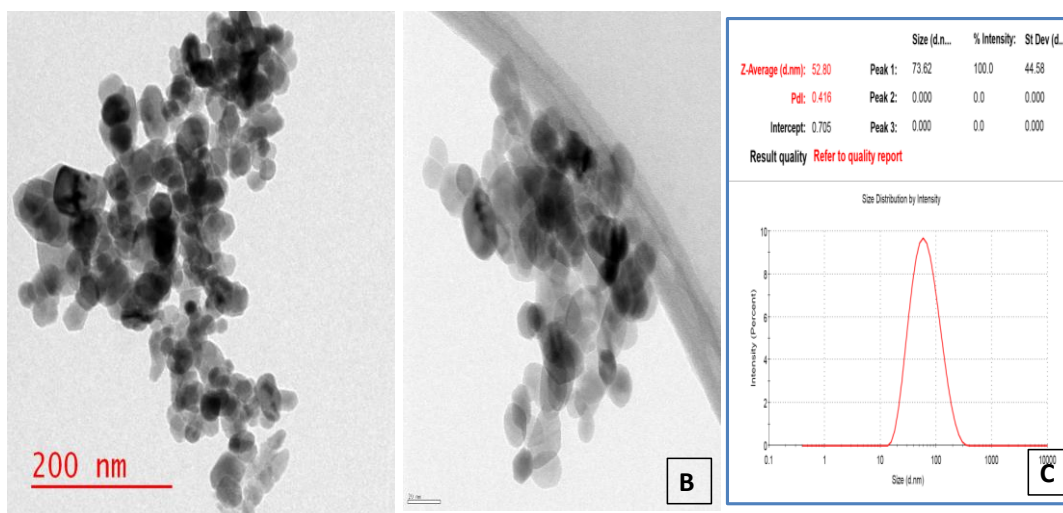


Fig. 1. (A) Particle size determined by high resolution transmission electron microscope (HR- TEM) Tecnai G2 20 twin and shape(size 25-30nm (B) Manufacture image -TEM images of ZnO NPs (100 µg/mL) in Milli-Q water(size 25 nm) (B) DLS showing size (52.80 nm)

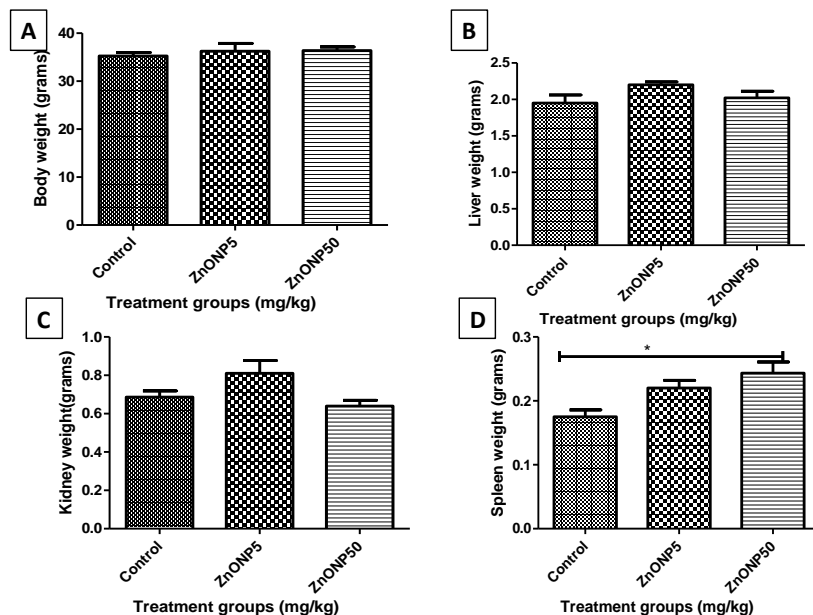


Fig 2. Effect of increasing dose of zinc oxide nanofertilizer on (A) Body weight, (B) Liver weight, (C) Kidney weight and (D) Spleen weight of *Balb/c* mice. Values are mean \pm SEM, (n=6) and significantly different within the group (* $P < 0.05$).

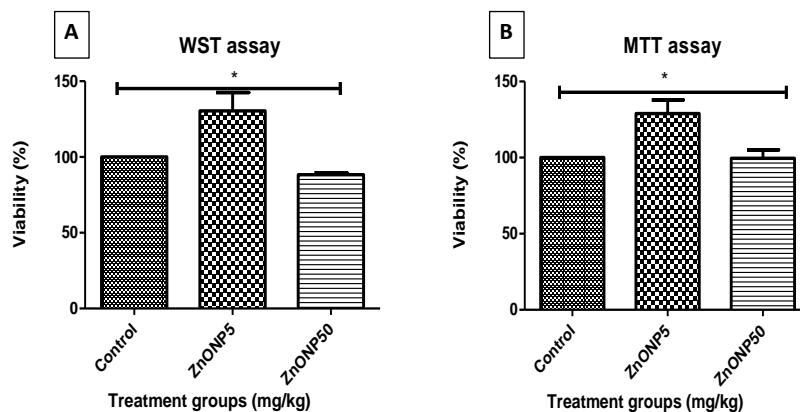


Fig 3. Effect of increasing dose of zinc oxide nanofertilizer on macrophage viability of Balb/c mice (A) WST assay and (B) MTT assay. Values are mean \pm SEM, n=6 Spleen weight of Balb/c mice. Values are mean \pm SEM, (n=6) and significantly different within the group (* $P < 0.05$).

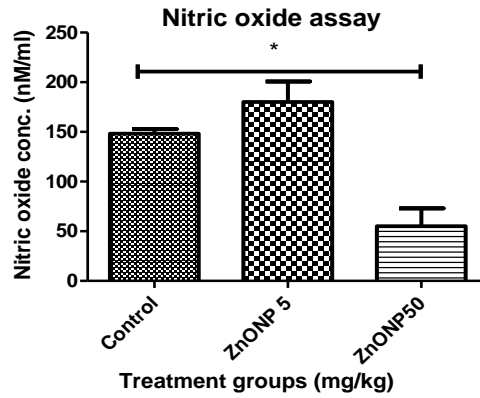


Fig 4 . Effect of increasing dose of zinc oxide nanofertilizer on nitric oxide production from macrophages of Balb/c mice . Values are mean \pm SEM, n=6 and significantly different within the group (* $P < 0.05$).

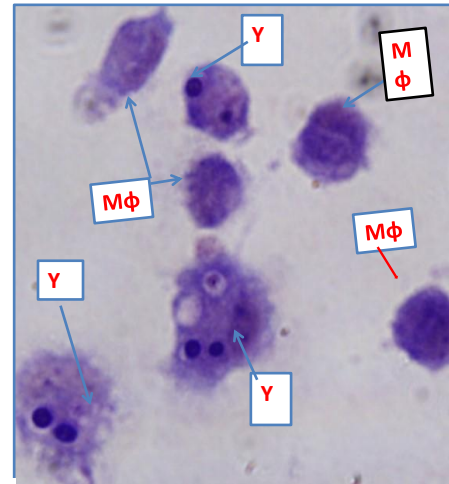
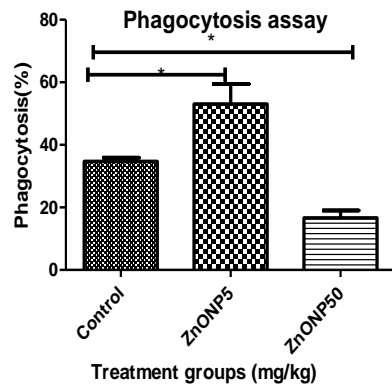


Fig 5 .Effect of increasing dose of zinc oxide nanofertilizer on macrophages Phagocytic activity of Balb/c mice. Values are mean \pm SEM, n=6 and significantly different within the group (* $P < 0.05$).

Fig 4.5 Phagocytosis of yeast cells by peritoneal macrophages, M ϕ = macrophages, Y=Engulfed yeast cells (1000x)

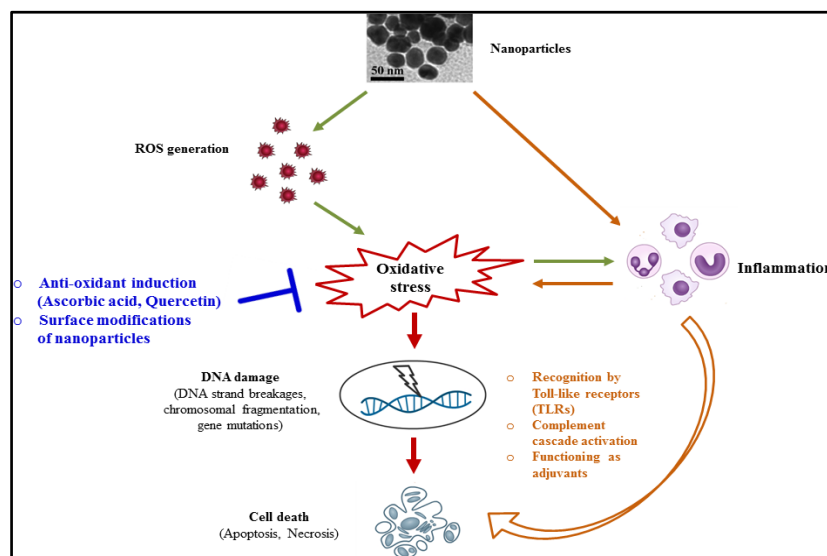


Fig.6 Nanoparticle interaction with the immune system and there mechanism

(Kononenko *et al.*, 2015)

In this research, ZnO NPs at dose 5.0 mg/kg, and 50 mg /kg alone. The effects induced by ZnO NPs included spleen enlargement, macrophages viability, nitric oxide production, peritoneal macrophages phagocytic activity statistically significant changes

In summary the adverse effect of ZnO NPs were tested in accordance with the OECD test guideline. Balb/c mice were orally treated with ZnO NPs for 28 days, with doses of 5 mg/ kg b.w and 50 mg/kg b.w. The results of this study suggested that the administration of 50 mg/kg b.w ZnO NPs to mice had adverse effect on functionality of macrophages. Based on the results of this study, a dosage of 5 mg/kg b.w of ZnO NPs was considered the no-observed-adverse-effect for immunotoxicity. There are different proposed mechanisms for ZnO NPs induced immunotoxicity. ROS generation and consequent OS are frequently observed and is the most discussed paradigm for NP toxicity (Nel., 2006). The Main mechanism of ZnO

NPs is oxidative stress could be attributed to the combination of more than one phenomenon including: generation of ROS on the surface of particles (Park *et al.*, 2011), physical interaction of ZnO NPs with the membrane wall leading to the deformation and rupture of membrane and dissolution and release of Zn²⁺ ions in the body (Li *et al.*, 2008). ROS generation mechanism varies according to NPs but exact mechanism are not understanding. Most important mechanism may be generation of ROS (relative oxygen species) by ZnONPs them self (Premanathan *et al.*, 2011). NPs dissolve in body fluid and subsequent release of Zn²⁺ ions that lead to ROS mediated cell death. OS and hydroxyl radical (OH[·]) is one of the main species responsible for macrophages damaging (Xia T *et al.*, 2006). To keep the redox equilibrium at an intermediate level of OS, pro-inflammatory pathways are activated by ZnO NPs and increase in pro-inflammatory cytokines TNF- α , IL-6, and IL-8. (Lipovsky *et al.*, 2011), further decreased phagocytosis (Liu H *et al.*, 2013).

Our research revealed a significantly decreased nitric oxide (NO) production in dose 50 mg/kg b.w treated mice while increase in 5.0 mg/kg b.w treated. ZnONPs induce general toxicity by increasing in the weight of spleen when orally exposed at a dose of 50 mg/kg body weight. ZnONPs could affect the functionality of innate immune cell macrophages. Nanoparticle interaction with the immune system and there mechanism shows in Figure 6.

In conclusion our research results shows that ZnONPs at a dose of 50 mg/kg b.w causes adverse effect on functionality of macrophages after 28 day exposes to *Balb/c* Mice while ZnONPs at the dose 5 mg/kg b.w have immunomodulatory effects such kind effect.

List of abbreviations

Nanoparticles (NPs), Zinc Oxide nano particle (ZnO NPs), instability (ΔH_{ME+}), EC₅₀ dose of nanoparticle that gives half-maximal response (EC₅₀), Reactive oxygen species (ROS), Reverse osmosis water (RO), Dulbecco's Modified Eagle's Medium Hams F-12 (DMEM), Fetal calf serum(FCS), Nitric oxide (NO), (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide(MTT), Dimethyl sulfoxide (DMSO), standard error of the mean (SEM), zinc oxide nanoparticle 5 mg/kg body weight (ZnONP5), zinc oxide nanoparticle 50 mg/kg body weight (ZnONP50).

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Conflict of Interest

The authors declare no any competing financial interest in this study.

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