Cytotoxic, Immuno-Boosting and Antibacterial Impacts of Assorted Ratios of Zinc oxide and Titanium Oxide Nanoparticles to Some Mammalian and Bacterial Cells (Vitro Study)

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Abstract

The fast-growing utilization of Nanoparticles (NPs) in countless industries unlocked the potential for more research on NPs possible toxicity of both normal and pathological cells. In the current study. We focused on the cytotoxic effect of Zinc Oxide NPs (ZnO NP), Titanium dioxide NPs (TiO_2 NP) and the mixture of both NPs with three different molar ratios of ZnO / TiO_2 1:1,1:2 and 2:1 to different types of cells (African green monkey kidney cells, human lung fibroblast and macrophagic cells differentiated from rat's blood monocytes and epithelial carcinogenic cells. The NPs were prepared with average sizes < 25 nm and characterized by X-ray Diffraction (XRD), High-Resolution Transmission Electron Microscope (HRTEM), Fourier Transform Infrared (FT-IR). Our results showed that Zinc Oxide provided a high toxic effect on normal cells and epithelial carcinoma, while Titanium dioxide showed slight cytotoxicity. Adding a mixture of both NPS with different ratios caused various toxic effects on both normal and cancer cells depending on their ratio. Addition of Titanium dioxide to Zinc oxide reduced the toxicity of zinc oxide in normal cells and improved the cell immune response. i.e. Adding Titanium dioxide to Zinc Oxide had improved its characteristics in cancer cell control depending on the ratio added. On the other hand, Nanomaterials have distinct features matched to their bulk counterparts. A current feature that these NPs exhibit an antimicrobial behavior against pathogenic bacteria. In this study, we also investigate the antimicrobial vigor of ZnO, TiO_2, and their various ratios NPs against certain Gram-positive and Gram-negative bacteria.

Keywords: Cytotoxicity, immuno-stimulant, ZnO NPs , TiO_2 NPs and antibacterial activity.

Introduction: Nanoparticles(NPs), including metal oxides, are promising materials for applications in many fields such as cosmetic and other industrial products as well as in medicine. ZnO NPs are characterized by their photocatalytic and photo-oxidizing ability against chemical and biological species and have been used as a main component in many cosmetic and other

industrial products. Recently, ZnO NPs proved to play an important role in cancer therapy [1]. Likewise; TiO_2 NPs nowadays is used in many products such as paints and coatings, plastics, paper, inks, fibers, foods, pharmaceuticals, toothpaste and cosmetics. Continuous exposure to such NPs especially, the occupational exposures of workers and researchers in Nanotechnology field, has a great influence on human health. Some studies showed that certain nanomaterials, including metal oxide NP, can induce spontaneous reactive oxygen species(ROS) production, which results in cell apoptosis [2,3,4]. Stephan Hackenberg [5] showed that the genotoxic effect of zinc oxide in nasal mucosa are antagonized by titanium dioxide NPs. Similarly, it was observed that, cytotoxicity of ZnO toward the human cancer cell line was significantly higher than that observed on the corresponding primary cells, suggesting selective toxicity of the ZnO to cancer cells [6,7]. On the other hand, the up growth of multidrug-resistant bacteria has become a prime public health issue worldwide. This is not only complicating anti-infective therapeutics, but also boosts the demands for evolving substitutional access to more efficient infectious diseases control. Recently, there has been a shooting concern in surveying the antimicrobial properties of engineered nanoscale metal particles [8]. In the present work, three different ratios of titanium dioxide NP to zinc oxide NP were prepared and their effects on both different types of normal cells and cancer cells were studied, during the study, we tried to find out the answer for the following questions: whether the ratio of NP TiO_2 added to ZnO affect the percentage of ZnO toxicity? Whether these ratios affect both normal and cancer cell by the same amount? Which is the best ratio that can deeply affect cancer cells and has a minimal possible effect on the normal cells? Moreover, the antibacterial effects of these NPs and their innovated mixed ratios had been investigated as a part of the application of our study into a different applied biological field.

Materials and Methods:

1) Nanoparticles (NPs) preparations and characterization:

1.1 Preparation of TiO_2 (NPs):

Titanium dioxide NPs were prepared by the sol–gel method [9]. In typical method Titanium tetrachloride ($TiCl_4$) was dissolved in distilled water at 5 °C then added drop by drop into ethanol by ratio (1:10) at room temperature. The solution is stirred until the solution is

gelatinized. Then, the gel solution is dried at 100 °C and calcined at 500°C for about 3 hours to obtain TiO_2 NPs.

1.2 Preparation of ZnO NPs: Zinc oxide NPs were prepared according to Khorsand *et. al* [10]. with some modifications. In typical method 0.5 M $Zn(CH3COO)_2 2H_2O$ and 1M Na OH were dissolved in 50 ml deionized water, Na OH solution was added dropwise with vigorous stirring on zinc acetate solution. Then it centrifuged and washed with deionized water several times. The white precipitate dried at 100°C and calcinated at 400°C for 3 hrs.

1.3 Preparation of ZnO / TiO_2 NPs:

Zn O/ Ti O_2 NPs with 1:1, 1:2 and 2:1 molar ratio was prepared by grinding each mixture separately in a ceramic mortar for about 15 min then transferred into a beaker, 10 ml of deionized water was added then sonicated for 1 hour and finally dried at 40 °C for 24 hrs.

1.4 Measurement Techniques: X-ray Diffraction (XRD) was performed using PA Analytical X'Pert Pro Target Cu-Kα with secondary monochromator Holland radiation (λ= 0.1540 nm, the tube operated at 45kV, scans were collected over a 2θ range of 5-60°). High – Resolution Transmission Electron Microscope (HRTEM) was performed by JEM-2100F electron microscope with accelerating voltage of 200 kV. Fourier Transform Infrared (FT-IR) measurements were taken using JASCO, FT/IR-6100 in the spectral range of 4000-400 cm^{-1}.

1.5 NPs Characterization:

Fig. 1 shows the XRD pattern for ZnO NPs (a) and XRD pattern for TiO_2 NPs (b) the identification of the hexagonal phase of ZnO is according (JCPDS card file No. 361451) [11], while the tetragonal phase anatase TiO_2 is according to (JCPDS Card No. 83-2243) [12].

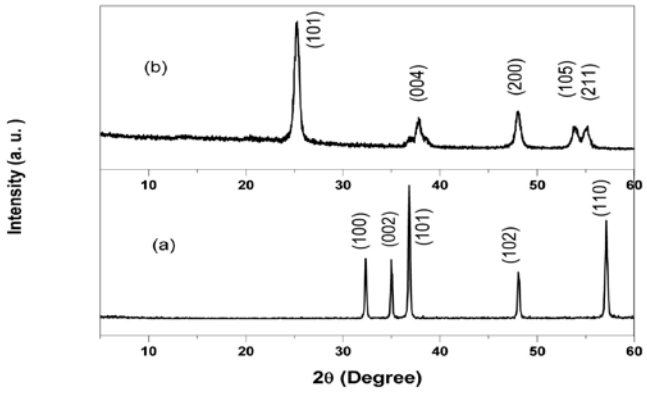

Fig. 1. XRD patterns of (a)ZnO and (b) TiO_2 nanoparticles.

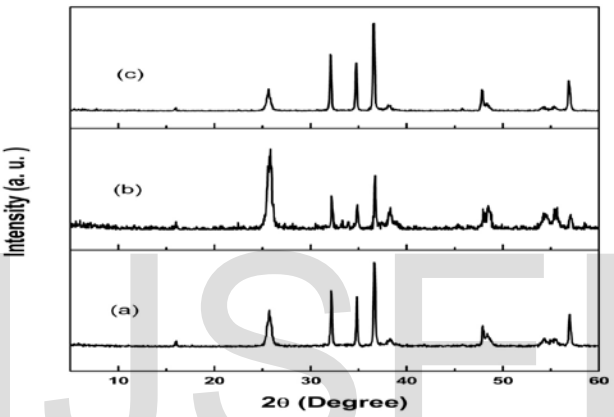

Fig. 2. XRD patterns of ZnO / TiO_2 Nano-particles with (a) 1:1, (b) 1:2 and (c) 2:1 weight percentage

Fig. 2 represent the XRD for different ZnO/ Tio_2 ratios (1:1 (a), 1:2 (b) and 2:1 (c)). The resultant figures reveal that the ZnO and the Tio_2 exist in the mixtures and the relative peaks intensity matched with their ratios.

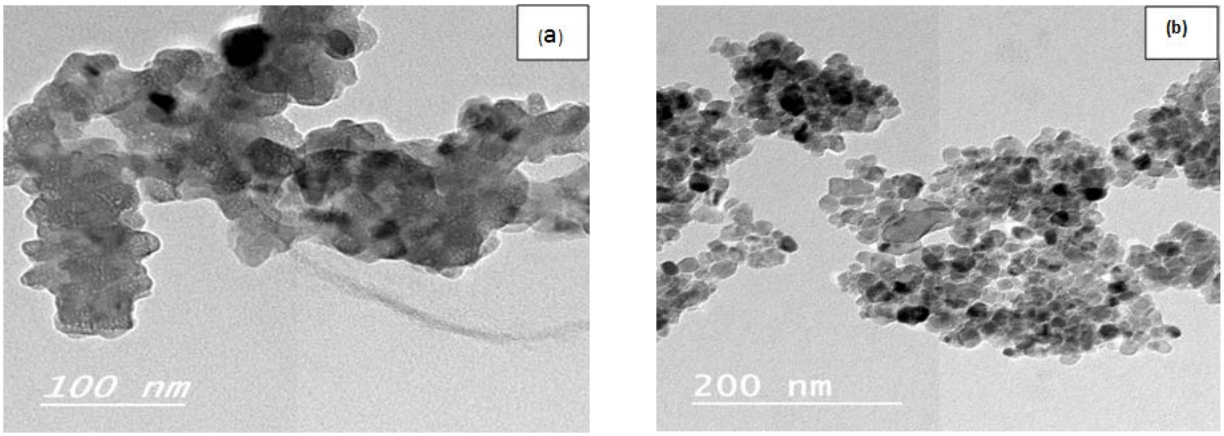

Fig. 3. HRTEM images of (a) ZnO and (b) TiO_2 Nano-particles.

The HRTEM images for ZnO (a) and TiO_2 (b) represented in fig. 3 the size graph bars support the Nano-size of the prepared particles with some aggregation.

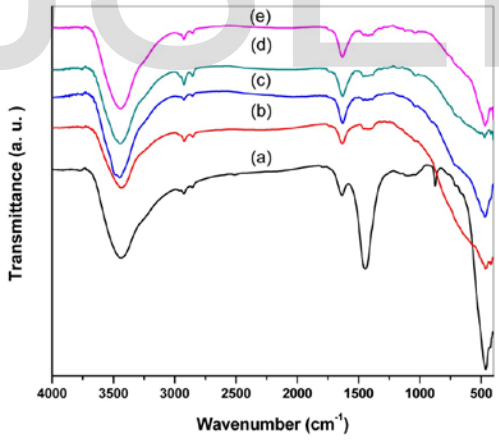

Fig. 4. FT-IR spectra of (a) ZnO NPs, (b) TiO_2 NPs, (c) 1:1, (d) 1:2 and (e) 2:1 weight percentage of ZnO / TiO_2 NPs.

Fig. 4 shows the FTIR for the NPs and the different their ratios. For the line (a) which represent the pure ZnO spectrum the strong band at 400 cm-1 is attributed to stretching vibrations of Zn-O bonds while for the line (b) which represent the TiO_2 the weak band at 435 cm^{-1} can be due to the

Ti-O stretching vibrations of anatase TiO_2 [13]. In addition for the all spectrum from (a) to (e) the absorption bands in at 1500 cm^{-1} , which are due to the oxygen-metal linkages and The absorption bands in 3600-3200 cm^{-1} connected to the stretching vibrations of hydroxyl groups, while the band at 1700 is related to C = O stretching vibrations.

2) Biological samples preparations:

Three cell lines; "Vero cells" isolated from African green monkey kidney epithelial cells, and WI-38 cell line extracted from human lung fibroblast, and epithelioid carcinoma (purchased from VACSERA Giza) were harvested from 25 cm^2 culture flasks using trypsinization by the addition of 100 µl trypsin-EDTA (trypsin, 250 mg/L and EDTA, 100 mg/L phosphate-buffered saline). Once the cells were dissociated from the bottom of the flask, 100 µl trypsin-inhibitor (fetal calf serum) was added, and cells were subsequently added in 96-well culture plates with 200 µl MEM medium counted with a Haemo-cytometer and incubated at 37 °C in 5% CO_2.

Five test tubes containg 1ml of the solvent (DEMSO) were prepared and 1mg of each of the five nanoparticles samples (ZnO, TiO_2 and ZnO/TiO_2: 1/1, 1/2, 2/1) were added to the solvent separately , the concentration of each of the nanoparticles samples used is 1mg/1ml.

2.1. MTT cytotoxicity: The MTT assay was performed basically as described, Sieuwerts *et al*. [14] with some modifications [15]. The quantity of formazan was measured in absorbance at 540 nm were read immediately thereafter on an automatic microplate reader (ELx 800 UV (Bio-Tek). The cell viability was determined as the ratio of the optical density (OD) of the sample to the OD of the control solution and expressed as a percentage. If the percentage was greater than 60%, the sample will have no cytotoxicity, and if it was less than 60%, the sample will be highly cytotoxic.

2.2. Macrophage Isolation and differentiation: Rats were sacrificed, and blood was collected in heparinized tubes, leukocytes were isolated from blood, and adjusted to 2×10^6 viable cells per milliliter, according to Hogan *et al*. [16]. Differentiation was induced using 40 ng/ml PMA (phorbol 12-myristate 13-acetate; Sigma-Aldrich). Cells were dispensed into 6 well plates (1.0 X 10^6 cells/ml) and incubated with PMA for 24 h at 37 °C in a 5 % CO_2 humidified chamber. After incubation of macrophages and opsonized zymosan with different treatments for 2 hours. The following assays were applied: -

2.3. Measurement of Nitric oxide (NO) production: The nitric oxide molecule is microbicidal agent, produced from immune phagocytic cells and can be assayed according to the method described by Ramadan *et al*. [17], with some modification [18]. The Optical density was determined at 540 nm, following method described by Tafalla and Novoa [19] with some modification.

2.4. Hydrogen peroxide production assays: The total peroxidase content in the media was measured according to Quade and Roth [20] and referred to hypochlorous radicals. The absorbance was recorded at 450 nm.

3. Antibacterial measurement: The synthesized NPs were screened for their antibacterial activity using agar well diffusion method against clinical methicillin resistant Gram-positive (*Staphylococcus aureus*) and multidrug Gram-negative enteric bacteria (*Escherichia coli* and *Salmonella enteritidis*) according to Jones *et al*. [21]

Results:

Cytotoxicity:

Control	2.908		2.914		2.881	
Zinc oxide	0.961	33%	1.304	44.7%	1.765	61.3%
Titanium dioxide	1.840	63.3%	1.971	67.6%	2.223	77.2%
1:1 ($ZnO/TiO_{2)}$	1.745	60%	1.904	65.3%	2.134	74.1%
1:2 (ZnO/TiO_2)	2.533	87.1%	2.418	83%	2.529	87.8%
2:1 (ZnO/TiO_2)	1.408	48.4%	1.792	61.5%	1.016	35.3%

Table (1): MTT Cytotoxicity for each treated group

The results showed that ZnO is highly toxic while TiO_2 is slightly toxic to both normal and cancer cells; adding titanium to zinc with the ratios1:1 ,1:2 ,2:1 decreases the cytotoxicity of the cells in a rate depends on the amount of titanium added. Also, we noticed that the different types of cells showed different cytotoxic response to the same ratio of the NPs added.

Macrophage peroxide and NO productions:

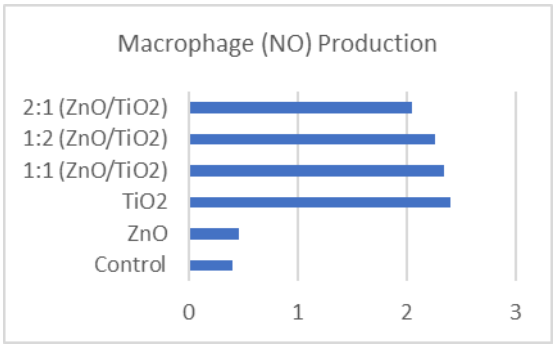

Figure (5): Macrophage NO production in micromoles µM

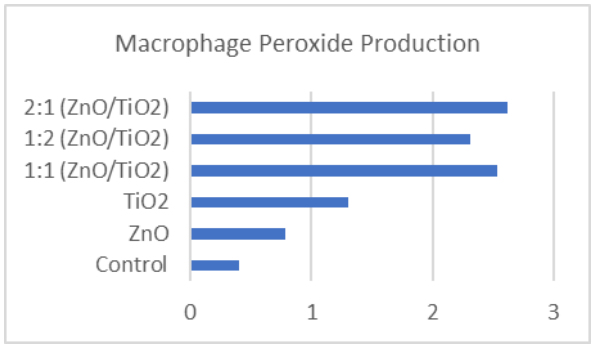

Figure (6): Macrophage peroxide production in nanomoles nM

It can be noticed from the above figures that adding TiO_2 to ZnO increases the production of Nitric oxide(NO) and stimulate more hydrogen peroxide production.

Antibacterial assay: Antibacterial activity results which represented in table no (1) revealed that the addition of TiO_2 to ZnO NPs increased the degree of antibacterial activity against both Gram-positive and Gram-negative bacteria when compared to ZnO NPs.

	Staphylococcus aureus	*Escherichia coli*	*Salmonella enteritis*
ZnO NPs	15 mm	12 mm	10 mm
TiO_2 NPs	15 mm	15 mm	15 mm
ZnO: TiO_2 (2:1) NPs	20 mm	15 mm	20 mm
ZnO: TiO_2 (1:1) NPs	25 mm	20 mm	25 mm
ZnO: TiO_2 (1:2)	30 mm	30 mm	30 mm

NPs			

Table 2: Zones of inhibition (10-15 mm weak +ve) (15-20 mm medium +ve) (> 20 mm strong +ve).

Discussion

It has been proved that elevated levels of ROS induce intracellular oxidative stress, lipid peroxidation, cell membrane leakage, and oxidative DNA damage and can induce cell apoptosis [22,23,24]. In vitro the induction of oxidative stress is the most expected mechanism underlying ZnO NPs toxicity [25]. In the present work, it was observed that ZnO produced the highest toxic effect on both epithelioid carcinoma and normal kidney cells (33% and 44.7%, respectively) while it was less toxic to human lung fibroblast (61.3%). Meanwhile TiO_2 showed less toxic effect on both cancer and normal cells, Table (1). The toxic effect of the NPs is attributed to excess in ROS secretions; e.g. hydrogen peroxide and superoxide anions [26], ZnO NP has toxic effect on mammalian cells, which is caused by production of Zn^{2+} from NP dissolution outside the target cell, which can be clearly observed in figure (6), in which ZnO and TiO_2 with different ratios induced the secretions of hydrogen peroxide. It can be observed from Figure (5) the excessive macrophage production of NO in case of TiO_2 and the low NO production in case of ZnO; and as known NO production is related to cell immune response [27], which indicates that TiO_2 is a strong immune-stimulant. By comparing the ratios of ZnO to TiO_2, we found that when the ratio of Zinc to Titanium is 2:1 the mixture showed high cytotoxic effect on cancer cells and human lung cells but slightly toxic to kidney cells. When the ratio of ZnO to TiO_2 changed to be 1:2, the mixture showed less cytotoxic effect on cancer cell, normal lung and kidney cells. While when the ratio of Zinc to Titanium was1:1, the mixture showed cytotoxic effect on epithelioid carcinoma cell (60%) and at the same time demonstrated less cytotoxicity to kidney cells (65.3 %) and was significantly less toxic to lung fibroblast (74.1%). Figure (5 and 6) showed that adding Titanium to Zinc with 1:1 ratio significantly increased the macrophage production of NO as well as production of hydrogen peroxide compared to other ratios of Zinc to Titanium {(1:2) and (2:1)}, these results could be an indicative mark in cancer treatment. On another sight, the antibacterial results demonstrated that NPs have promising antibacterial activity against both Gram negative as well as Gram positive MDR bacteria. There are many proposed mechanisms of NPs action against bacteria; attachment to bacterial cell wall via hydrophobic, electrostatic, and

receptor ligand interactions which leads to cellular damage and eventual death. Another concept, NPs generate reactive oxygen species which interact with bacterial cell membrane [28].

Conclusion: Achieved results might lead to partial answers to our questions in which some are concurring and substantiating findings from other previous studies. ZnO NPs is a cytotoxic substance to both normal and cancer cells, while TiO_2 could possibly decrease the toxicity of ZnO as well as has immune-stimulatory effect. Adding TiO_2 to ZnO by a ratio 1:1 was considered the optimum mixture as it stimulated cell secretions of microbicidal agents, i.e. despite of its cytotoxicity to cancer cell line it could be nontoxic to normal cell. Higher titanium ratio than zinc (1:2) improves the immune response of normal cell against the toxic effect of ZnO. Different examined types of biological cells showed different degrees of cytotoxicity after exposure to zinc oxide and/ or titanium oxide, these results could be a spark of hope in cancer treatment as it opens the door for applying innovated mixtures of NPs in cancer treatment, however further work and research are essential to be followed to imply a complete picture of such field. Further, ZnO NPs alone were most efficient against Gram-positive bacterial strains matched to Gram-negative bacterial strains. Our findings pointed that, the addition of TiO_2 NPs boosted the immunity, and enforced the antibacterial activity of ZnO NPs especially against Gram-negative bacterial strains. As, the investigations on bio-medical implementations of NPs have yet recently launched, more researches will be needed to value their performance in vivo.

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Conflict of Interests: "The authors declare that no competing interest exists".

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