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The Effect of Bulk ZnO and ZnO Nano-particle Dispersions (cationic, anionic and non-ionic) on THP-1 Production of IL-8

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ABSTRACT

The responses of THP-1 cell (human monocytic cell lines) to nano ZnO with dispersant of different charges (cationic, anionic and non-ionic) and bulk ZnO was compared by observing interleukine-8 concentration of the cells. Treatment of the cell lines with different dispersants resulted to clustering of the cells and degree of clustering was shown to differ with the various treatments (bulk ZnO and ZnO nano-particle dispersions). The cationic treated cells showed a higher degree of agglomeration. The cell membranes were generally disrupted in all cases giving rise to irregular unbounded cells. The effect of ZnO nano-particles on THP-1 IL-8 production was higher than that of bulk ZnO irrespective of the nano-particle charge. THP-1 cells produced the highest concentration of IL-8 when treated with anionic (AN) nano-particle, this was closely followed by non-ionic (NI) and then cationic which produced the least of IL-8 from treatedTHP-1 cells. There was significant difference between the control and cationic ($p<0.0022$), control and anionic ($p<0.002$), control and non-ionic ($p<0.00023$) and control and bulk ($P<0.09389$). Similarly, there was a significance difference in IL-8 concentration between cationic and anionic ($p<0.01153$) and between cationic and non-ionic ($p<0.01136$)

INTRODUCTION

Zinc oxide nano-particles (ZnO NPs) have found usefulness in diverse and wide technological applications such as in the area of biomedical and cancer treatment, environmental protection, textiles, renewable energy, pharmaceuticals, personal care, food, etc (Hong *et al.*, 2011; Nag *et al.*, 2011; Rasmussen *et al.*, 2010; Yang and Xie, 2006 and Tsuzuki, 2009). There is a growing trend in the current and potential application of nano-particles that covers a wide range of markets

and industries; this has led to the public being exposed to a large quantity of nano-materials, hence, raising safety concerns (Prach *et al.*, 2012). The knowledge of how toxic these materials are is really lacking behind the rate at which they are being produced (Prach *et al.*, 2012).

Experimental studies using non-particulate (bulk) form of zinc oxide and titanium have implicated these compounds as having few adverse effects in animals (Yu and Li, 2011). On this basis, however, zinc oxide has been classified as GRAS (Generally recognised as safe) for use as food additive by FDA. On the other hand, products with ZnO nano-particles such as personal care products are extensively used for skin protection against harsh UV rays in human and others used as antibacterial agent in consumables such as surgical instruments. However, there is no regulation specific for the use of these chemicals in nano-particle format (Yu and Li, 2011) since it is not clear whether or not ZnO containing materials are hazardous to human health or safe enough to be allowed for health related uses (Morag *et al*, 2012). Several studies have provided evidence that nano-particles exhibits size-dependent properties when they are compared with their bulk type for example, ZnO NPs (zinc oxide nano-particles) showed more cytotoxicity than the bulk. In the present study, the responses of THP-1 cells (human monocytic cell lines) to nano ZnO with dispersions of different charges (cationic, anionic and non-ionic) and bulk ZnO was compared by observing interleukine-8 concentration of the cells.

MATERIAL AND METHODS

Cell Culture

The human monocytic cell line THP1 was provided by the immunology Department of Edinburgh Napier University, Scotland, UK and was used as an in vitro model for this study. RPMI 1640 medium which was supplemented with 10% heat inactivated foetal bovine serum (FBS), streptomycin (100 µg/ml), L glutamine (2 Mm) and penicillin (100 U/ml) was used to maintain the cell line. Culture conditions for cell lines were done at 37^{o}C in a humidified environment that had 5% CO2. Using 24 or 96 well plates with final volume of 1 ml or 100 µl per well, respectively, cell lines were seeded at 1 x 106 cells/ml to

obtain a suspension cell line. The cells were subsequently treated using the particles in concentrations that range between 0 to 300 μg/106 cells for 24 h. The cells were immediately (within 30 min) treated after the bulk ZnO and nano-particulate ZnO had been prepared. To assess the interference of the particles with the various assays, cell free controls were included.

THP-1 Cell Cytospin and Diff-quik Staining

A THP-1 cell suspension was provided by the immunology laboratory, Edinburgh Napier University, Scotland, UK, at a density of approximately 1x106 cells/ml for the cytospin preparation. A cytocentrifuge smear was then prepared by adding 160μl culture medium and 40μl of cell suspension at a concentration of $2x10^6$ cells/ml to a fully assembled sample chamber. Cells were then centrifuged at 110xg for 5 minutes in a Shandon cytospin 3 one after the other (control, LPS, bulk ZnO(bZnO), cationic (cat), anionic (An) and non-anionic (NI) ZnO).Slides were left to air dry and labelled with initials and date.

With gloved hands, lids were taken off all Coplin staining jars and were placed in the tray. The slides were immersed in a jar containing solution A fixative (methanol) for 30 seconds one after the other and were all drained off the excess by touching the edge of each slide on the side of the jar. Each of the slides was then immersed into eosin solution B for 15-30 seconds by slowly agitating the slides in the solution. Excess was drained off on the jar. Slides were transferred to the haematoxylin solution C without rinsing and staining was repeated as for solution B. Subsequently, slides were immersed in a jar of rinsing buffer and placed in a second tray to air dry. The dried cytospin slides were observed under the microscope and images seen were photographed.

Pre-Coated ELISA Plate

Appropriate pre-coated ELISA plate (IL-8 capture antibody) was collected from the laboratory bench and the coating antibody solution from the ELISA plate shaken into the sink. ELISA plate was washed 4 times using a Wash Buffer in a spray bottle and it (ELISA plate) was transfer to 4 sheets of blue roll on the bench. Plates were turned upside down

on the sheets of paper roll and banged dried. To each well of the plate, 300µl of Assay Buffer was added to block the plate and this was incubated at room temperature for one hour.

Standard/Samples with Blocked ELISA

Assay Buffer from the ELISA plate was shaken into the sink after one hour incubation and the Wash Buffer in the spray bottle was used to wash the ELISA plate 4 times. ELISA plate was transfer to 4 sheets of blue roll on the bench, turned upside down on the sheets of paper and banged until the plate was dry. A tube of IL-8 standard (stock standard, 800pgml^{-1}) was collected from the Laboratory bench and was double diluted in duplicate in Assay Buffer. Using eppendoff tubes, serial dilution was carried out by adding 300µl of Assay Buffer to each pair of eppendoffs (14 in total) which were labelled accordingly as 400, 200, 100, 50, 25, 12.5, and 6.2pgml^{-1}. The serial dilution was carried out starting with 800pgml^{-1} by transferring 300µl of each standard to the next tube and vortexing each tube. 100µl of the standards, samples and blanks (Assay Buffer) was added as in the template.

50µl detection Antibody was added to all wells and plate was labelled with initials and incubated for 2 hours.

Streptavidin-HRP Conjugate

The detection Antibody from the ELISA plate was shaken off into the sink and the Wash Buffer in spray bottle was used to wash the ELISA plate 4 times and was subsequently transfer to 4 sheets of blue roll on the bench. The plate was turn upside down on the sheets of paper and banged until it was dry. 100µl of Streptavidin-HRP conjugate collected from the Laboratory bench was added to each well and incubated for 20mins at room temperature. The plate was kept to avoid direct light.

Substrate Solution

The ELISA plate was shaken off of the Avidin-HRP conjugate into the sink and Wash Buffer from spray bottle was used to wash the ELISA plate 4 times and transfer to 4 sheets of blue roll on the bench. The plate (ELISA) was turned upside down on the sheets of paper and banged till plate was dry. 100µl Hydrogen peroxide (H2O2) and Tetramethylbenzidine (TMB) liquid substrate was added to each well and incubated for

10mins at room temperature. 50µl stop solution (2N H2SO4) was added to each well and absorbance at 450nm (reference absorbance 650nm) with an ELISA plate reader was measured and results read and printed.

Particles Preparation and Scanning Electron Microscopy (SEM)

ZnO NPs dispersion which was included in 70 nm APS; 50% IN water, colloidal dispersion with non-ionic, anionic and cationic dispersants and bulk ZnO (-325 mesh powder = < 44µm) were obtained from Alfa Aesar (ZnO Nanoshield™ ZN-2000, ZN-3014A and ZN-3008C respectively and provided by the immunological laboratory of Edinburgh Napier University, Scotland, UK. To measure the endotoxin levels, the in vitro limulus amoebocyte lysate (LAL) assay (E-TOXATE, Sigma, UK) was used.

The prepared particle dispersions were centrifuged onto glass cover slips through the use of a Shadon cytospin 3.Before they were mounted to the SEM stubs all particles were allowed to air dry on the cover slips. A Hitachi S4800 ultra High Resolution Field Emission Scanning Electron Microscope (10 KV accelerating Voltage: sputtering coating using gold) was used to obtain images.

Dissolution: Zinc Analysis

The content of zinc in bulk and nano ZnO dispersions in the cell culture media which consist of RPMI 1640 supplemented with L-glutamine (2nM), penicillin (100 µg/ml) and streptomycin (100 µg/ml) +/- 10%foetal bovine serum) was determined. This was determined by flame atomic absorption spectroscopy (AAS) that use a perkin Elmer AAnalyst 200.

Statistical Analysis

Obtained data were subjected to statistical analysis using t-test for level of significance between certain treatments. IL-8 concentration was calculated for each treatment using the standard curve equation y=0.0015x-0.0016

RESULTS

Cell Morphology

Observation of cell under a light microscope (x40 magnification) showed cells clustering together. The degree of clustering however differs with the various treatments (bulk ZnO and ZnO nano-particle dispersions). From figure 1 below, the cationic treated cells showed a higher degree of agglomeration.

The cell membranes were generally disrupted in all cases giving rise to irregular unbounded cells.

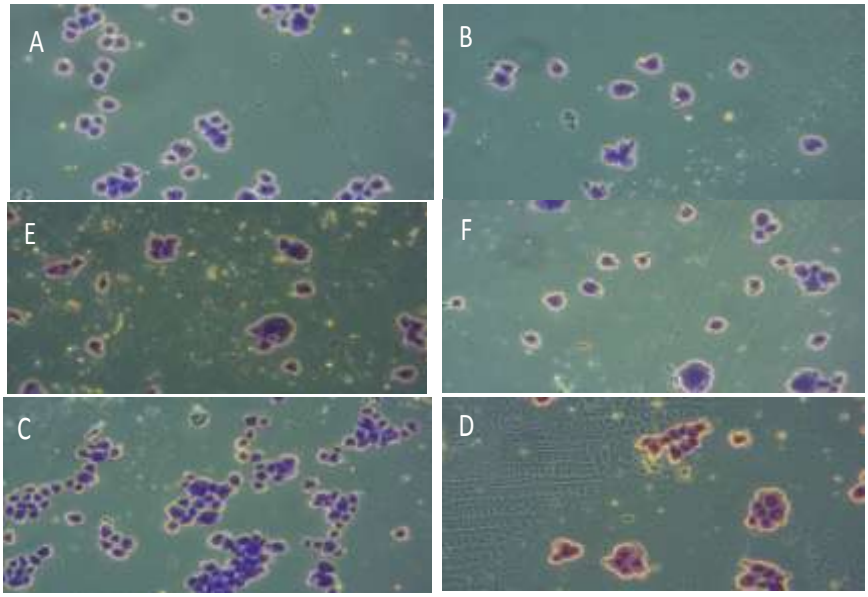

Fig.1: Representative images of THP-1 treated with bulk (F) ZnO and ZnO nano-particles dispersions: cationic(C), anionic (D), non-ionic (E), control (A) and lipopolysaccharide (B) as observed under a light microscope (x40).

ZnO bulk and ZnO Nano-particle Dispersions (cationic, anionic and non-ionic) effect on THP-1 Production of IL-8

The effect of ZnO nano-particles on THP-1 IL-8 production was higher than that of bulk ZnO irrespective of the nano-particle dispersions (Fig. 2). There were however, differences in THP-1 production of IL-8 when treated with different ZnO nano-particle dispersions. THP-1 cells produced the highest concentration of IL-8 when treated with anionic (AN) nano-particle, this was closely followed by non-ionic (NI) and then cationic which produced the least of IL-8 from treatedTHP-1 cells. There was significant difference between the control and cationic ($p<0.0022$), control and anionic ($p<0.002$), control and non-ionic ($p<0.00023$) and control and bulk ($P<0.09389$). Similarly, there was a significance difference in IL-8 concentration between cationic and anionic ($p<0.01153$) and between cationic and non-ionic ($p<0.01136$) (Fig.2).

The Effect of Bulk ZnO and ZnO Nano-particle Dispersions (cationic, anionic and non-ionic) on THP-1 Production of IL-8

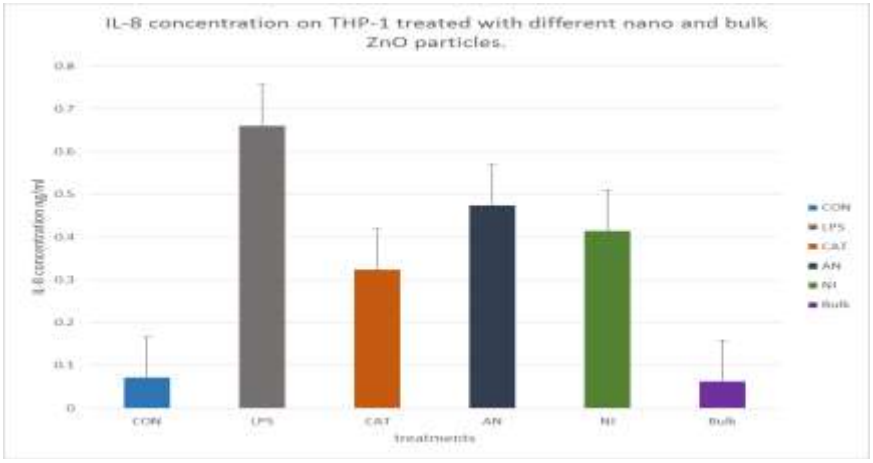

Fig.2: Effect of ZnO (BULK) and ZnO nanoparticle dispersions: CAT (cationic), AN (anionic), NI (non-ionic) and LPS (lipopolysaccharide) on IL-8 production of THP-1

DISCUSSION

Results from the microscopic photograph showed disruption of the THP-1 cells membrane which could cause an influx of the ZnO particles. Such membrane disruption had also been observed by previous Researchers (Roiter *et al.,* 2008 and Slowing *et al.,* 2009).

The aim of this present work was to study the cytotoxicity of both bulk and nano-particles of ZnO (size effect) and charge difference among the nano-particles (cationic, anionic and non-ionic) on THP-1 cells. From the results of this study, it is clear that toxicity of ZnO to THP-1 cells is size dependant as all particles of nano size irrespective of charge were more cytotoxic than the bulk ZnO. This agrees with the findings of Prach *et al* (2012) who also observed a similar trend in their study with ZnO particles and monocytes.

Between charge differences also exist as demonstrated by the results from this study. Cationic ZnO nano-particle showed a higher degree of toxicity making them more potent than the other nano-particles which suggest likely higher affinity to the membranes which are mostly negatively charged. From the results it is also obvious that charge plays a role in cytotoxicity of nano-particles. This also agrees with the findings of Prach *et al* (2012) who asserted that despite their extensive and rapid solubility, in cell medium, charge

can still influence their biological activities.

CONCLUSION

From the results of this study it is concluded that there is a significant difference in cytotoxicity between bulk and nano-particles of ZnO (size effect) and between the different charges of the nano-particles (charge effect). This agrees with the hypothesis of this experiment.

The study was unable to show what effect the up regulation of IL-8 production have whether it will be a positive or negative effect. There is also the need to know the dose of the nano-particles at which they are sublethal and when they become lethal. Finally, their mode of action should be investigated to know whether they act via the classic type 1 proinflammatory activation of innate immune cells.

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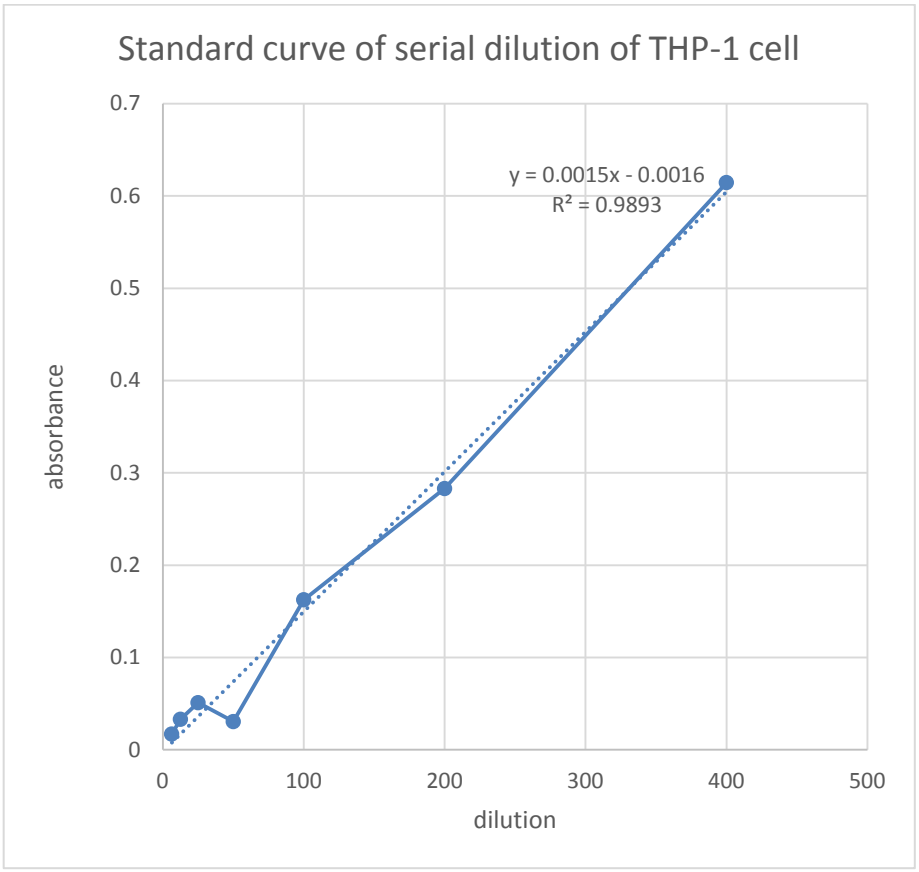
Appendix 1


Standard curve of serial dilution of THP-1 cell
y = 0.0015x - 0.0016
R² = 0.9893
absorbance
dilution
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
200
300
400
500