COMPARATIVE IN VITRO ESTIMATES OF INHALATION TOXICITY OF SELECTED NANOPARTICLES

by

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Abstract

Airway inflammation is characterized by the release of pro-inflammatory cytokines (IL-6) and chemokine (IL-8) from airway epithelial cells. To screen for the potential inhalation toxicity as inflammation, we tested exposure of metal oxide nanoparticles (NPs)-Titanium dioxide, Magnesium oxide, FastAct and Titanium Silicon Oxide-Manganese (TSO-Mn)-Aerogel to BEAS 2B human bronchial epithelial cells. A monolayer of cells having 80 – 90% confluence was treated with different concentrations of the NPs and feedlot dust as positive control for inflammatory processes. Releases of IL-6 and IL-8 into the culture supernatant fluid were measured by sandwich enzyme-linked immunoassay (ELISA). Characterization of NPs such as solubility and agglomeration in cell culture media were carried out to predict the effect of these properties in cellular responses. Feedlot dust increased the release of both IL-6 and IL-8 by 3 to >5 fold, suggesting an inflammatory effect while NPs did not show any effect either at increasing the dose or duration of the incubation with cell. The NPs at higher doses reduced the total IL-6 and IL-8 released, suggesting that the NPs may have bound with the cytokine and chemokine or somehow interfered with their function. The inert activity of NPs was further investigated by inspecting cell morphology, counting viable cells and assessing mitochondrial membrane potential. Concentrations at 1000 mg/L of TiO_2 and 250 mg/L TSO-Mn-Aerogel could apparently limit lung epithelial cell multiplication by partially occupying the intercellular spaces, qualitatively increasing the number of cell pores and resulting in less recovered cells after 12 hours of incubation. Cells exposed to feedlot dust and titanium NPs were less viable as indicated by propidium iodide staining, but cells exposed to TSO-Mn-aerogel were more apoptotic as indicated JC-1 staining. These changes occurred at projected inhalation exposure levels > 40-100 fold above the nuisance dust level for TiO_2 and permissible exposure limit for Manganese. No MgO exposures reduced apparently recovered cells to < 50% as indicated by manual hemocytometer counts (+ 15-25% variability). The lack of toxicity was most likely reflected from the high MgO solubility in the incubating media, and the relative non-toxicity of MgO.

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CHAPTER 1 - Nanoparticles and Toxicity

1.1 Introduction

Nanotechnology is an emerging science that deals with structures of at least one dimension 100 nanometers (nm) or smaller. It involves manufacturing materials or devices within this range. Principally, "make it small" is the way of making "nanoparticles (NP)" and there are two methods to do it – Top-Down and Bottom-Up Methods (Whitesides and Love 2001). In the top-down method, the bulk material is ground in a high- tech instrument to smaller and smaller particles until the desired size is reached. In the bottom-up method, a few thousand molecules are assembled to form the desired material. An aerosol generation technique is a variety of top-down approach which may involve thermal decomposition of the bulk material (Dhaubhadel 2007). Whatever method is used in manufacturing, engineered NPs differ from bulk material in both physical and chemical properties because of their large surface areas and pore volumes. For example, the nanosized gold changes color and is no longer yellow but purple; 1 gm of nanosized gold has surface area (500 m^2) sufficient to cover ¼ of a football field; and it is more reactive than macrocrystalline (conventionally sized) gold. These unique features in metal oxide NPs give advantage in interaction to various industrial applications. They have been utilized in manufacturing paints, coatings, cosmetics, catalysts polymer composites, self-cleaning glass, industrial lubricants, advanced types, semiconductors, electronic components to target drug delivery and gene therapy (Aitken, Chaudhry et al. 2006). It has been estimated that the global market for nanotechnology products will increase to 2.6 trillion dollars by 2014 and that will create 10 million new jobs in the manufacturing sector of nanotechnology (Hullmann 2006) . At the same time, the rapid and widespread industrial applications of these unique materials have raised considerable concern about the potential for toxicity of NPs to ecosystems and to human health.

In the environment, it has been assumed that similar to the long-established organic and inorganic pollutants, NPs can be released from industrial or household products into and bind to the soil, water and air by human activities. NPs can find their way into a variety of organisms and have potential to cause toxicity to an individual or a population which can be deleterious to ecosystem (place and community of organisms) integrity (Biswas 2005). The public health

concern is that the commercial use of NPs can possibly evolve into particulate pollution in indoor or workplace air predisposing to respiratory distress. This concern is comparable to occupational exposures to ultrafine particulates (UFP)[1] (Gwinn and Vallyathan 2006). However, exposure of NPs via the skin or routes other than the lung cannot be ruled out depending upon the condition of the environment to which an individual is exposed, as well as the condition of the exposed individual(s). Thus, studies evaluating the toxicity of these novel materials contribute indispensable knowledge to understanding their safe handling and that of any commercial products made out of them.

It is thought that NPs have existed in nature since near the beginning of the earth as they have been found in volcano dust, natural waters, soils and sediments as non-anthropogenic (man-made) source (Handy 2008). However, engineered NPs are placed in new category of toxic substances because they have the possibility of being more toxic to the organism than natural NPs, conventional pollutants or ordinary materials similarly in high doses. The reasons for this assumption are many: For example, we may inadvertently introduce something in high concentration that we do not know is toxic; there is a complex relationship between toxicity and particle characteristics. A little variation in size, shape or other properties render a NPs unique material; NPs will behave differently in an organism compared to another different sized or shaped particle of same chemical composition (Hoet 2004). For example, if carbon nanotubes reach the lung, they might be more toxic than ordinary carbon black and more toxic than ordinary quartz (Lam, James et al. 2004), because of differences is size, shape or surface areas. Thus the safety evaluation of each type of engineered NM requires studying toxicity in its own physical and chemical properties even though similar particles or particles in bulk are safe (Hoet 2004). This concept is the current premise of the emerging field of nanotoxicology.

We evaluated in vitro cytotoxicity to BEAS 2B human bronchial epithelial cells from several different NPs made up of metal oxides of titanium, and magnesium as well as silica-titanium-manganese. Brief descriptions of physical/chemical properties and health/ environmental hazards of each of the metal oxide when they are in ordinary bulk form are as follows.

[1] The fine particles in air pollution studies are classified on the basis of their aerodynamic diameter and described by PM size. $PM_{0.1}$ or Particles less than 100 nm are called UFP; PM_1 or particles less than 1micrometer ($\leq$ 1000nm) and $PM_{2.5}$ or particulates less than 2.5 μm are called fine particles; PM_{10} or particles less than 10 μm and greater than 2.5 μm are called coarse particles

Titanium dioxide (TiO_2) is one of the top fifty molecules used commercially worldwide, presumably because of its durability. It is a naturally occurring oxide of titanium and extracted from titanium ores as Anatase. It is white crystalline powder with a very high refractive index, which makes it an excellent source of white pigment in industrial products and paints. For this reason, it is used in sunscreens, toothpaste, paints, and coatings as well. It is also added in food to make it whiter. Other essential applications of TiO_2 come from its efficient photocatalytic activity. Photocatalysis is the ability of a molecule to create electron holes and give up free radicals when exposed to the white light or UV light. Photocatalytic activity of TiO_2 has been extensively studied because of its potential application to sterilization, sanitation, or degradation as well as environmental remediation of hazardous wastes.

Regulatory agencies which are charged in safeguarding public health consider TiO_2 to be an inert, non-toxic molecule. As a food color with identification number E-171, the Food and Drug Administration has not forbidden its use but requires it to be limited to 1 percent of the food weight (www.foosafety.gov). However, current studies reveal that it is not as safe as thought and suggest new investigations of its toxic profile. The International Agency for prevention of cancer research (IARC; internationale agencie pour de la recherché au contraire cancer) placed TiO_2 in a Group 2B category in 2006 suggesting that it can be possibly carcinogenic to human beings. With this new designation, TiO_2 is now considered to be a potential carcinogen just like some forms of as Nickel or dichlorodiphenyltrichloroethane (DDT) (www.iarc.fr).

Magnesium oxide (MgO) is a very useful white hygroscopic powder. In medicine, it has wide use as an antacid for heartburn and upset stomach (Milk of Magnesia). It is also used as short-term laxative to improve the symptoms of indigestion, and as mineral supplement for animal feed. In libraries, it is sprinkled over books because of its hygroscopic properties to protect them from ambient moisture. It is used as insulator in cables; basic refractory materials in crucibles; and fire proofing agent in building material. Inhalation of magnesium fumes while heating or welding it results in metal fume fever, which has self-limiting (1-2 days) flu-like symptoms. But inhalation of magnesium particles by human beings has been reported to be non-toxic. In a controlled experiment, six healthy human volunteers inhaled high levels (~ 130 mg/ m^3) fine and ultrafine magnesium oxide and there were no significant differences in BAL inflammatory cell concentrations, BAL IL-1, IL-6, IL-8, tumor necrosis factor, or pulmonary

function compared with controls (Kuschner 1997). Thus, current literature suggests that high-dose fine and ultrafine magnesium oxide particle exposure does not produce measurable pulmonary inflammatory responses.

Silica is the most abundant mineral in the earth's crust and is found as sand or different forms of quartz. In one of the NPs in this study, silica was covalently coupled with titanium and manganese. Si is a major compound in all types of glass. Exposure to fine, alpha crystalline silica in very small quantities (0.1 mg/m^3) over time can lead to silicosis bronchitis and is an important occupational health hazard for people working in sandblasting (www.osha.gov). Following inhalation exposure with animals, some fine particles of SiO_2 are more inflammatory than TiO_2 (Driscoll 1991).

Manganese is a gray-white metal, physically resembling iron. It is hard, very brittle and easily oxidized. Mn is extracted from manganese dioxide, which is a black or brown inorganic solid material occurring naturally in the mineral pyrulusite. It is widely used as a component of steel and aluminum alloys. It has important biological significance as it is a trace element in all forms of life. It occurs as a co-factor in many enzymes. Manganese compounds are less inflammatory to biological organisms than those of nickel and copper. But inhalation of manganese particle and their dissolution can cause neurodegeneration and it can be an occupational health hazard for people working in mines and smelters.

1.2 Literature Review

1.2.1 Pulmonary inflammation: primary response of respiratory system to particulates

In particulate air pollution, size is the most important determinant of deposition site for particles in the lung. For example, a larger mass of dispersed ultrafine particles will deposit in lung parenchyma than will the mass of coarse or agglomerated particles. Inhaled coarse particles interact with nasal fluid and epithelial lining fluid and, either disperse, or travel upward on the mucociliary escalator and are often expectorated by the body or coughed up and swallowed. But fine and ultrafine particles escape the physical defenses of the upper respiratory tract, settle over the bronchial epithelium, can reach deeper into the alveolar epithelium and interstitial spaces, access the blood through endothelium and reach the vital organs of the body. An airway inflammation, characterized by redness, swelling of the bronchial lining and difficulty in breathing is an early response of bronchial epithelium to the fine and ultra fine particles. When

particles confront bronchial epithelium integrity by physically being deposited over it, the epithelium may secrete proinflamatory cytokines (notably interlukin-6), or affect mitochondrial pores (Nel 2006). The other cytokine, a chemotactic chemokine, interlukin-8, is found to be secreted by the epithelium concurrently to maintain as well as amplify the inflammatory events by recruiting neutrophils (Veranth 2004). These inflammatory responses in the lungs are elicited and maintained by the alveolar macrophages. Thus, these two interleukins, as well as other mediators of inflammation such as IL-1 and TNF-alpha and chemokine have primarily been studied widely as pulmonary inflammogens after the inhalation of fine and ultrafine particles in both in-vivo and in-vitro studies (Clapp 1994; Wang 1996; Wyatt 2007; Bailey 2008).

1.2.2 Characterization of NPs is important in toxicity testing

With the assumption that unique characteristics of engineered NPs can induce toxicity, the conventional toxicological studies that consider dose (mass at target organ receptors) as a single factor in producing response have limitations in evaluating toxicity of NPs. Thus, the toxicity of chemically active NPs more directly depends upon how they interact with target organ receptors and somewhat less on the mass near the receptors. The extent to which this interaction depends upon characteristics of the material such as size (size distribution and agglomerated particle size), shape (and other morphological features e.g. crystallinity, porosity, surface roughness), surface area, solubility in physiological media, state of dispersion, surface chemistry, volumetric lung burden and physiochemical properties is currently being studied (Oberdorster 1996; Powers 2005). For example, in evaluating chronic inhalation toxicity of titanium dioxide NPs, it was found that inflammatory responses and development of lung tumors were best correlated with the large surface area of the particle retained in the interstitial spaces and, presumably, a proportionate reaction with critical target tissue receptors (Oberdorster 1996). Also important were the breakdown of the agglomerated particles into smaller units and the rate of movement from alveolar spaces to interstitial spaces, again, presumably increasing the interaction with these critical receptors(Oberdorster 1996). Taking to these characteristics as factors in inducing toxicity, we assessed solubility and particle agglomeration in cell culture media.

1.2.3 Production of ROS is the basis of Nanotoxicity

Reactive oxygen species (ROS) or free radicals are molecules that carry unpaired electrons. The cellular ROS is composed of oxygen ions ($O2^-$) and peroxides of both inorganic and organic molecules. In a normal cell, ROS are unavoidable byproducts of cellular respiration, which involves reduction of oxygen to water. In each step of the electron transport chain, electron leaks from the transport chain molecule and are captured by cellular oxygen that forms oxygen ions(Turrens 2003).

The exact mechanisms of cell responses to NPs are still unknown. There is a knowledge gap in explaining how NPs interact with signaling biomolecules (such as β1 integrin and epithelial growth factor receptor [EGF-R]) at the surface of the cell or sub-cellular organelle if they are internalized by the cell. However, studies so far confirm that the basis of nanotoxicity is the induction of ROS in the cell; this proposed mechanism is believed to correspond well to the high surface area and high surface interaction of NPs. Therefore, more details of how ROS are generated during normal cellular respiration and how cells maintain homeostasis for reactive oxygen species will allow understanding of a possible mechanism by which cells respond to the effect of NPs.

Briefly, electron transport chains are arrays of protein molecules, which are designated as Complex-I through IV molecules, along the inner membrane of mitochondria. As the name implies, these complexes are capable of accepting electrons and passing them to the next complex. The ultimate goal of electron transport is to produce high energy phosphate (ATP) from the byproducts of the Kreb's cycle such as reduced nicotine adenine dehydrogenase (NADH) but molecules in transport chain do not perform the operation directly. The transport chain proteins pass electrons sequentially which are finally taken up by oxygen to form water. When the byproducts of the Kreb's cycle and beta-oxidation, notably NADH, reduced flavin adenine dinucleotide ($FADH_2$) and succinate, accumulate in the mitochondrial matrix, the transport chain molecule is charged with electrons as NADH donates the first electron to the transport chain molecule. This donation leaves behind NADP and a proton (H+) inside the mitochondria. While the transport chain protein deals with the electron, the proton passes through the membrane of the mitochondrion into the cytoplasm. This results in accumulation of protons (energy) in the cytoplasm just like water behind a dam. The protons flow back by the concentration gradient to the mitochondrial matrix and are used in the phosphorylation of ADP

to ATP. In this way, molecules of NADH yield three molecules each of ATP through the electron transport chain. During this process, electron transport chain molecules leak a few electrons which add to the oxygen forming free radicals.

Considering all physical and chemical characteristics of engineered NPs and potential interactions with physiological solutions, three possible mechanisms have been postulated on how the engineered NPs can produce ROS in a cell when it interacts with biological tissue (Nel 2006).

First, NPs can directly donate electrons to cellular oxygen. The NPs have discontinuous engineered crystals and defects to enhance activity, so that the notch or spikes in them generate active electronic configurations. When NPs come in contact with the cell membrane, the active sites of NPs pass electrons to the receptor molecules that flow into the cell and attack the cellular oxygen converting it to a charged molecule.

Secondly, the coating metals or organics in NPs , which enhance its reactivity due to positive charge on it or electron holes, simulate the Fenton reaction by capturing electron from cellular oxygen or H_2O_2 and produces OH^- . In a typical Fenton reaction, positively charged iron radicals (Fe^{++}) act as catalysts to destroy the organic molecule by oxidation. The metal oxide NPs typically act this way. A study with zinc oxide and cerium dioxide NPs showed that they were capable of inducing spontaneous ROS production (Zinc) or protecting against it (Cerium). (Xia, Kovochich et al. 2008). Such a phenomenon has been observed with manganese and silver NPs as well (Hussain 2006). To generate ROS by passing electrons from the active site as in first mechanism or drawing electron into the electron holes as in second mechanism, NPs do not need to be taken into the cell, rather the extracellular accumulation NPs will create more oxidative stress to the cell. Interestingly, manganese seems to heighten mitochondrial toxicity of our metal oxide NPs by this mechanism. The degree to which the mechanism is related to internalization has not been determined by this work. NPs such as titanium dioxide, which is activated by UV light creating electronic holes in it, can induce ROS if the interaction with cell happened to take place in the presence of light.

Third, ROS production takes place when the NM is transported into the cell. This would be more devastating if it bears reactive sites as mentioned in first and second mechanism. Cellular uptakes of NPs are facilitated if they were soluble in physiological solution or media.

For instances, hydrophobic NPs can more readily pass through lipid bilayer of cell membrane than hydrophilic substances.

1.2.4 Cellular responses to NPs -The Hierachial oxidative stress model

Under normal conditions, the mitochondrial production of ROS from the electron transport chain molecules is scavenged by antioxidant enzymes- superoxide dismutase (SOD), catalase and Glutathione S-transferase(Azbill, Mu et al. 1997). Accumulation of ROS inside the cell produces varying amounts of deleterious effects to the cell components which were termed oxidative stress.

Excess of ROS oxidizes and inactivates antioxidants, such as glutathione (GSH) reducing the cell's ability to deal with free radicals. ROS causes depletion of glutathione (GSH) and accumulation of oxidized glutathione (GSSG). Therefore, decrease of GSH to GSSG ratio is a sensitive measurement of oxidative stress (Flora 1999).

The cellular response to oxidative stress has been assumed to occur in three tiers (Nel 2006). A tier 1 response is indicated by the production of Phase II enzymes, activating phase-II reactions such as conjugation reactions involving methylation (methyltransferase), sulphation (glutathione S-transferase) acetylation (N-acetyltransferase). These enzymes are synthesized through nuclear regulatory factor (Nrf)-2 signaling pathways in order to maintain immediate redox homeostatis. In a tier 2 reponse, which occurs at increasing stress ROS, cell responds by secreting inflammatory cytokines such as IL-1, TNF-α, IL-6 and chemokine such as IL-8. A tier 3 response related to mitochondrial toxicity pores and suggests apoptosis or necrosis, which occurs at higher levels of oxidative stress.

1.2.5 Cellular uptake of NPs

The engineered NPs, by definition, have sizes that are somewhat comparable to the cellular proteins. Eukaryotic cells measure about 10 μm in diameter. A typical nanoparticle of 10nm dimension has an eight hundred times smaller diameter (~500,000,000 smaller volume or mass) than a mammalian red blood cell. Theoretically engineered NPs are comfortably accommodated inside a eukaryotic cell. But size is not a single determinant of uptake of NPs by the cell. Parameter such as surface coatings and surface area also also attributable for the transport of NPs across cell membranes. Studies with gold NPs have indicated that uptake is dependent on physical characteristics such as surface area (Chithrani 2006). Cancer

chemotherapies or drug delivery technique involves proper surface coating of the NPs with specific proteins that recognizes the membrane receptor facilitating their uptake into the cancer cells (Gupta and Gupta 2005).

1.2.6 Engineered NPs of Titanium induces Oxidative stress of varying degree

There is no evidence of the incidence of titanium dioxide toxicity in human population. IARC (Institute for Aerosol Research against Cancer) has designated titanium dioxide as carcinogen in 2006 based on studies with animals. However, epidemiological studies in population of North America and Europe did not show evidence of association between occupational exposure of TiO_2 and risk of cancer (Lee 1985; Siemiatycki, Weiderpass et al. 2001; Baan and Baan 2006).

Engineered nanosized titanium dioxide was widely studied to guarantee the safety of its wide application in industry and environmental remediation program. At the same time studies on titanium dioxide are intended to explore the mechanism of cellular responses to the engineered NPs.

Studies in rodents showed that clearance and inflammatory responses of inhaled titanium NPs depended on age, bodyweight/size, strain of the rodents, and the method of instillation and size of particles. For example, particle burden or retention of TiO_2 was higher when it was intratracheally instilled than normal whole-body-inhalation (Osier 1997). Hamsters were less affected by particle burden (Bermudez 2004). Ultrafine particles of TiO_2 were slowly cleared and induced more inflammation than their fine counterparts (Penney, Oberdrster et al. 1992). Since clearance kinetics was driven by alveolar macrophage, slowly clearing lung particles resulted from impaired phagocytosis and sequestration of particles in the alveolar interestium (Renwick 2001). In addition to this, TiO_2 induced varying degree of inflammation of the respiratory tract and led to associated effects such as lung injury, cholesterol granulomas and fibrosis (Schapira 1995; Park 2009).

In vitro studies attempting to understand the cellular basis of toxicity indicate that both fine and ultrafine TiO2 can cause DNA damage by producing reactive oxygen species but that the effect is stronger for ultrafine particles. This effect is enhanced by exposure to simulated sunlight or ultraviolet light as it creates electron holes (Wei, Yin et al. 1997; Hirakawa 2004).

1.3 Research Objectives and Hypothesis

The objectives of this study were twofold. The first objective was to assess physical characteristics of the NPs such as solubility and particle agglomeration when NPs are incubated in cell culture media simulating the lung environment. In the previous study, we found that the presence of bicarbonates in the lung simulant fluid dissolved more magnesium but not titanium dioxide. Therefore, we were interested to know whether magnesium pulled titanium into solution if these two materials were combined in their nano-size forms as in FastAct. The second objective was to evaluate the toxicity of metal oxide NPs to a lung epithelial cell by comparing them with exposure to feedlot dust (positive control) which was found to be inflammatory in our previous studies. Thus we hypothesized that NPs of titanium dioxide; Magnesium dioxide and manganese-titanium-silicon oxides are soluble in lung simulant fluid and induces proinflammatory cytokine (IL-6) and chemokine (IL-8) release from bronchial epithelial cells. In other words, these NPs are capable of inducing Tier-2 oxidative stress to the cell.

CHAPTER 2 - Materials and Methods

2.1 Materials

Four different types of NPs were used in this study. Titanium dioxide, TiO_2 (NanoScale Corporation. Manhattan, KS) is a nanocrystalline metal oxide. The bulk material has a surface area of 500 m^2/g (as measured by the BET method; a porosity of 0.3 cc/g). Magnesium oxide, MgO (NanoScale Corporation. Manhattan, KS) is also nanocrystalline metal oxide which is made out of hexagonal platelets with the thickness of 7 nm and 100-200 nm length and width that aggregate into very strongly bound particles with an average particle size of 4-8 micrometers (volume mode diameter = 8 micrometers) and has surface area equal to or greater than 300 m2/g. Nanocrystalline titanium dioxide NPs are comprised of 4-7 nm crystallites that also aggregate into micron size particles (main volume mode = 4-5 micrometers). FastAct® (NanoScale Corporation. Manhattan, KS) was a combination of 2 parts MgO and 1 part TiO_2 (6-8 micrometers). TSO-Mn-Aerogel (Kennedy K. Kalebaila and Kenneth J. Klaubunde, Chemistry Department, Kansas state university, KS) is a TiO_2/SiO_2 metal gel doped with manganese or iron and has surface area as high as 330-550 m^2/g.

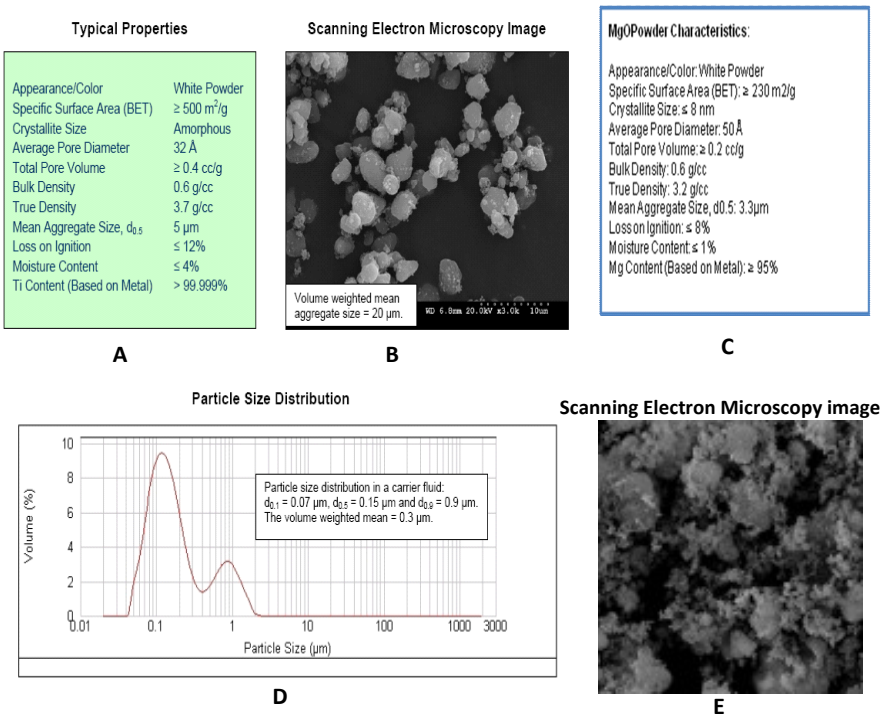


Figure 1 Properties of Nanoparticles A. Typical properties of Titanium dioxide NPs. B. Scanning electron microscopy image of Titanium dioxide powder of aggregate size 20µm. C. Typical properties of MgO NPs. D. Particle size distribution of TiO_2 NPs in carrier fluid showing mean particle diameter 0.3µm. E. Scanning electron microscopy image of Magnesium oxide powder in aggregate (With permission from Olga Koper, Nanoscale corp. www.nanoscalecorp.com)

Lung simulant fluid resembled the composition of Broncho-alveolar Lavage (BAL) fluid particularly in terms of bicarbonate composition. Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich) and Dulbecco's Modified Eagle's Medium with low glucose (DMEM, Invitrogen) that were used as fluids to lung epithelial cells, served as a model of epithelial lining fluid (ELF). Both fluids were capable of supporting lung epithelial cell growth, but had different bicarbonate concentrations that resembled the fluctuation in bicarbonate between normal inspiration and expiration.

2.2 Methods

2.2.1 Solubility Measurement

Dissolution of NPs was carried out in aqueous media and culture media. Titanium dioxide, magnesium oxide and mixture-FASTACT were weighed and 50mg/L and 250 mg/L

concentration were prepared. De-ionized water (22 ºC ± 2 ºC) was used as solvent in the first trial. HBSS and DMEM (5% CO_2 and 37ºC) were used in second and third trial. A shaker was fitted into the cell-culture incubator. Gentle agitation was applied in a shaker to mimic the action of the lungs. Sampling was done at 0.5 hour to 48 hour in 6 different points. Four ml of each material was taken and centrifuged at 13,200 rpm for 20 min (264,000 g-minutes and the supernatant was taken as the material that was solubilized. The dissolved Titanium and Magnesium were measured using inductively coupled plasma Mass Spectrometer (ICP-MS; Elan 9000, Perkin Elmer, Shelton, CT).

2.2.2 Cell preparation

The normal human bronchial cell line, BEAS-2B (American Type Tissue Culture Collection, Rockville, MD) was used in this study. This cell line, isolated from bronchi, was obtained from autopsy of a normal, non-cancerous individual and used as a surrogate of upper airway respiratory cells. It was infected with an adenovirus 12-SV40 virus hybrid. Such cells would be expected to resist senescence and be maintained simply in routine tissue culture which could extend up to and beyond 48 hours. The BEAS 2B cell retained many of the characteristics of the normal type II epithelial cells, such as surfactant production and squamous cell differentiation in response to serum. Cells were grown in the Dulbecco's modification of Eagles Minimal Essential Media (EMEM; DMEM) (Lonza Walkersville Inc, MD) containing L-glutamine was supplemented with 1% fetal bovine serum. Two antibiotics, streptomycin and penicillin (InVitrogen Inc, CA) was also added in the media, to minimize microbial growth. After incubating for 24 hours in a 75 cm^2 flask (Corning Inc, NY), the cells were transferred into 96 well- or 6 well-plates in accordance with the design for cell treatment. For such transfer, the old media was discarded, cell-layer over the flask was washed with PBS, treated with Trypsin (InVitrogen Inc, NY) and incubated for five minutes. Then the trypsinized suspension was diluted and counted (manual counts in a hemocytometer (+ 15-25% reproducibility) to obtain the number of cells in the suspension. Finally the suspension was diluted to contain $3x10^5$ cells / ml for seeding into the wells of culture plate (Nunc Inc., IL). Following overnight incubation the cells were treated with different concentrations of the NPs and feedlot dust extract at the stage when the cells reached 80-90% confluence as a monolayer. Prior to subsequent assays, cells were manually counted in a hemocytometer (+ 15-25% reproducibility).

2.2.3 Preparation of NPs in solution

NPs were weighed, mixed with cell culture media and resuspended by agitation (Fisher, Genie-2) and used immediately for cell treatment. 1mg, 5mg and 20 mg of different NPs were weighed and mixed with 20 ml of culture media making a concentration of 50 mg/L 250 mg/L and 1000mg/L solution. These were labeled as mixture (centrifuged) or uncentrifuged. Since we detected particles in the supernatant fluid even if the solution of the particle was centrifuged, we concluded that NPs had small enough masses to minimize the effect of centrifugal force. Three other concentrations were prepared by centrifugation from the mixture. For this, 10 mL from each uncentrifuged solution was transferred into a microcentrifuge tube and centrifuged at 13,000 rpm for 20 min at 240C. The supernatant fluid was collected and labeled. Thus the wells containing monolayer of cells were treated with 6 different concentrations – 3 were uncentrifuged and other 3 were centrifuged.

2.2.4 Preparation of the Feedlot Dust Extract

Dried manure mat was received from Professor Ronaldo G. Maghirang of Biological and Agricultural Engineering Department at Kansas State University, Manhattan, KS. It was ground with mortar and pestle for half an hour making the dust as fine as possible. 1 g was weighed and mixed with 20 ml of culture media making 5% dust suspension to extract. The dust was incubated with the media for one hour. Over that period it was agitated (Vortex) for 10 minutes in the beginning and allowed to stand for the rest of the time. It was centrifuged at 4,000 rpm for 10 minutes and the supernatant was filtered through 0.22 μm sterilized syringe filter (Millipore, Billerica, MA). The final solution was subsequently diluted to 3% and 1% by adding 6 ml and 2 ml of 5% of extract to 4ml and 8ml of the media respectively (Wyatt 2007)

2.2.5 Measurement of Particle size

The different concentration of NPs, both centrifuged and uncentrifuged, was taken for the measurement of the agglomerated hydrodynamic size of the particle in culture media. It was the same solution used for the cell treatment reaction. Diffraction light Scattering (DLS) was measured in an apparatus available in the Department of Physics. The solution was agitated (Vortex) for 10 minutes and allowed to settle down for 30 minutes assuming the NPs form a colloidal solution when the larger particle settle down. Briefly, 3 ml of solution was taken in a clean transparent test tube and placed inside the apparatus. A beam of x-ray of wavelength of

632 nm was passed through the colloidal solution and the correlation time was measured. The diameter of the suspended particle was estimated from the correlation time as described by the following formulae (www.proteinchemist.com)

The diffusion coefficient is

$$D = \frac{kT}{6\pi\eta R}.$$

The scattering wave vector is

$$q = 4\pi n \lambda^{-1} \sin\frac{\theta}{2}.$$

The correlation time is

$$t_c = \frac{1}{2Dq^2} = \frac{3\eta\lambda^2 R}{16\pi n^2 kT \sin^2\frac{\theta}{2}}.$$

So

$$R = \frac{16\pi n^2 kT \sin^2\frac{\theta}{2}}{3\eta\lambda^2} \cdot t_c$$

Set λ=632nm, Boltzmann Constant k=1.38*10-23J/K, Room Temperature T=295K

2.2.6 Cytokine Release Assay

The cell culture supernatant was collected at 4 different time points after 3, 6, 12 and 24 hours of treatment. In the subsequent repetition of the study, only 6 and 12 hr time points were considered for 3 hours was too short and 24 hours was too long to see viable cells. The collected supernatant fluid was stored in microtubes at 4-8^{0}C. Two cytokines, IL-6 and IL-8, were quantified in these samples by sandwich ELISA assay (R &D Systems, Minneapolis, MN)

2.2.7 Interleukin-6 (IL-6) Assay

The capture antibody (mouse anti-human IL-6) was diluted in physiologically buffered saline (PBS) to a working concentration of 2µg/mL and coated immediately on a 96-well microplate (Coster). It was sealed and incubated overnight at room temperature. The following day, each well was aspirated and washed with wash buffer and blocked with 300µL of reagent

diluents and incubated at room temperature for 1 hour. The wells were washed second time as before, 100µL sample or standard were added per well and incubated two hours at room temperature. Following third wash, 100µL of detection antibody (biotinylated goat anti-human IL-6) diluted to working concentration of 200ng/mL in reagent diluents, was added per well and incubated for 2 hours. Following another wash as before; 100µL of Streptavidin-HRP diluted to working concentration of 1:200 (R&D system) – was added and incubated for 2 minutes. The wells were again aspirated and washed with buffer, as before. Then 100µL of substrate solution was added per well and incubated for 20 minutes. Finally the reaction was stopped by adding 50 µL of stop solution containing 2N H_2SO_4 (R&D system). The plates were gently tapped to ensure thorough mixing. The optical density of each well was determined by using a microtiter plate reader at 450nm wavelength.

2.2.8 Interleukin-8 (IL-8) Assay

The capture antibody (mouse anti-human IL-8) was diluted in PBS to a working concentration of 4µg/mL and coated immediately on a 96-well microplate (Coster). It was sealed and incubated overnight at room temperature. The following day, each well was aspirated and washed with wash buffer (R&D system) and blocked with 300µL of Reagent Diluent (R&D system) and incubated at room temperature for 1 hour. The wells were washed second time as before, 100µL sample or standard were added per well and incubated two hours at room temperature. Following third wash, 100µL of detection antibody (biotinylated goat anti-human IL-8) diluted to working concentration of 20ng/mL in reagent diluents as indicated in the product (R&D system), was added per well and incubated for 2 hours. Following, as before; 100µL of Streptavidin-HRP (R&D system) diluted to working concentration of – was added and incubated for 2 minutes. The wells were again aspirated and washed with wash buffer, as before. Then 100 µL of substrate solution was added per well and incubated for 20 minutes. Finally the reaction was stopped by adding 50 µL of stop solution containing 2N H_2SO_4 (R&D system). The plates were gently tapped to ensure thorough mixing. The optical density of each well was determined by using a microtiter plate reader at 450nm wavelength.

2.2.9 Cell Morphology and viability with Propidium iodide (PI)

After 12 hours of incubation with the different concentration of the NPs, we observed the cells in an inverted phase microscope (Nikon, Eclipse TS100). Pictures 100 and 200 times magnified were taken to see the morphology and pattern of BEAS 2B cell growth.

Then the culture supernatant was removed from the wells and the cell layer was washed with phosphate buffered saline (PBS), trypsinized and harvested in a conical centrifuge tube. It was gently swirled in finger vortex (Fisher, Genie-2) sufficiently to make a uniform suspension. About 50 µL of this suspension was mixed with same volume of 0.4% Trypan blue (Sigma-Aldrich Inc, MO) in a micro centrifuge tube. Approximately 20 µL of the suspension was used to charge (fill) the hemacytometer. When it filled the chamber of hemocytometer, the cells were viewed under the microscope at 100X magnification. Then total number of recovered cells determined by counting the cells overlying four 1mm square areas of the counting chamber and converting to total cell number (www.cascadebio.com)

The cell viability test was carried out by staining with Propidium Iodide, PI (InVitrogen Corp, Carlsbad, Ca). The culture supernatant was removed from the wells of a six well microtiter plate and washed with PBS, trypsinized with 200 µL of trypsin and then neutralized for the effect of trypsin by adding 800 µL of culture media. The mixture was pelleted at 3,000 rpm for 5 min and suspended in 1 ml of a warm phosphate buffered saline. To each sample, 1 µL PI solution was added. Then the dye added cells were incubated on ice for 20-30 minutes. As soon as possible after the incubation period, the stained cells were analyzed in a flow cytometry using UV/488 dual excitation.

2.2.10 Measurement of membrane potential and cellular health

We used JC-1 kit (InVitrogen Corp, Carlsbad, Ca) to estimate the J-aggregates in a cell that has high membrane potential. After 12 hours of incubation with different concentration of NPs and feed lot dust as a positive control, cells from 6 well microtiter plates was trypsinized and suspended in fresh medium to neutralize the effect of trypsin. The suspension was pelleted at 3000 rpm for 5 minutes and suspended in 1 ml warm phosphate buffered saline (PBS). For each control, 1 µL of CCP (Carbonyl cyanide 3-chlorophenylhydrazone) was added to induce nearly total apoptosis which was used as positive control. In each sample 10µL of JC-1 was added and

incubated at 37^0C, 5% CO_2 for 15-30 minutes. It was pelleted again as above and suspended in 500μL of fresh PBS and analyzed on a flow cytometer with 488 nm excitation.

CHAPTER 3 - Results

Data were analyzed using Microsoft Excel 2007 and statistical program for social scientists (SPSS) 12.0 for windows statistical program. We used Mann-Whitney U test, the ANOVA F-test and Bonferroni t-test, wherever appropriate, to compare the treatments with control. The results are summarized below.

3.1 Dissolution (Solubility) of metal oxide NPs

Magnesium Oxide was soluble in water as well as in cell culture media or lung simulant fluid. Solubility of MgO was media dependent that is it was related to the bicarbonate concentration.

Table 1 Solubility of MgO

		Magnesium dioxide	
		mg/L	Ppm
MgO Alone	Distilled Water	33.33	20.7**
	HBSS	33.3	30.4±9.2*
Mg in Mixture (Fast Act)	Distilled water	33.3	17.2**
	HBSS***	33.3	17.9±0.2[B]
	DMEM***	33.3	35.0**[B]
MgO Alone	Distilled water	166.7	46.3**
	HBSS	166.7	69.6±4.4[B]
MgO in Mixture (Fast Act)	Distilled water	166.7	56.4**
	HBSS	166.7	74.1±8.1[B]
	DMEM	166.7	103.2±0.7[B]

***Corrected arithmetically to either 33.3 or 166.7 (250 mg/L), to this equimolar basis.**

****p ≤ 0.05 significant linear regression correlation coefficient; or highest value chosen when values were concentration dependent.**

*****HBSS and DMEM are abbreviations of Hanks Balanced Salt Solution and Dulbecco's Modified Equivalent Medium, respectively.**

[B] p < 0.05 significant difference between HBSS and DMEM (Student t or Mann-Whitney U statistic for central tendency)

Thus, DMEM which had 350 mM bicarbonate dissolved more magnesium than did HBSS which contained 35 mM bicarbonate ($p < 0.05$). Both dissolved more than distilled water which contained minimal or no bicarbonate ($p < 0.05$). Time dependence was greatest when magnesium was extracted most slowly, that is in distilled water. Lung simulant fluid extracted it more quickly so dissolution was more compressed in time and less clearly linearly related to time. In these cases a peak extraction was indicated in this table**.

Table 2 Solubility of Titanium dioxide

		Titanium dioxide	
		mg/L	Ppm
TiO_2 Alone	Distilled Water	16.7	2.4±0.1*
	HBSS***	16.7	0.7±0.1*
TiO_2 in Mixture (FastAct)	Distilled water	16.7	1.6±0.3
	HBSS	16.7	1.6±0.3
	DMEM***	16.7	0.6±0.1
TiO_2 Alone	Distilled water	83.3	7.5±0.9*
	HBSS	83.3	4.1±3.4*
TiO_2 in Mixture (FastAct)	Distilled water	83.3	1.5±0.4
	HBSS	83.3	6.0±3.1
	DMEM	83.3	0.7±0.1

***Corrected arithmetically to either 33.3 or 166.7 (250 mg/L), to this equimolar basis.**

****p < 0.05 significant linear regression correlation coefficient; or highest value chosen when values were concentration dependent.**

*****HBSS and DMEM are abbreviations of Hanks Balanced Salt Solution and Dulbecco's Modified Equivalent Medium, respectively.**

[A] p < 0.05 significant difference between water and media (Student t or Mann-Whitney U statistic for central tendency)

[B] p < 0.05 significant difference between HBSS and DMEM (Student t or Mann-Whitney U statistic for central tendency)

Unlike magnesium, titanium dioxide NPs had minimal dissolution. Specifically, in only a few instances were measured dissolutions different from no dissolution. There were no difference in dissolution between HBSS and DMEM media, suggesting the lack of any media or bicarbonate effect on the minimal dissolution. There were no differences related to concentration, as indicated by samples designated as (83.3 mg/L) being approximately the same as those labeled (16.7 mg/L), five-fold lower. There were no differences related to time over the duration of 0.5-48 hours, suggesting that none of these factors had significant influence(s) on dissolution of titanium. NPs remain visible in the media even after the centrifugation at 13,200 rpm for 20 mins (264,000 g-minutes), suggesting that the particles were sufficiently small that this centrifugal force could not precipitate them.

3.2 Effect of centrifugation on agglomeration and hydrodynamic size

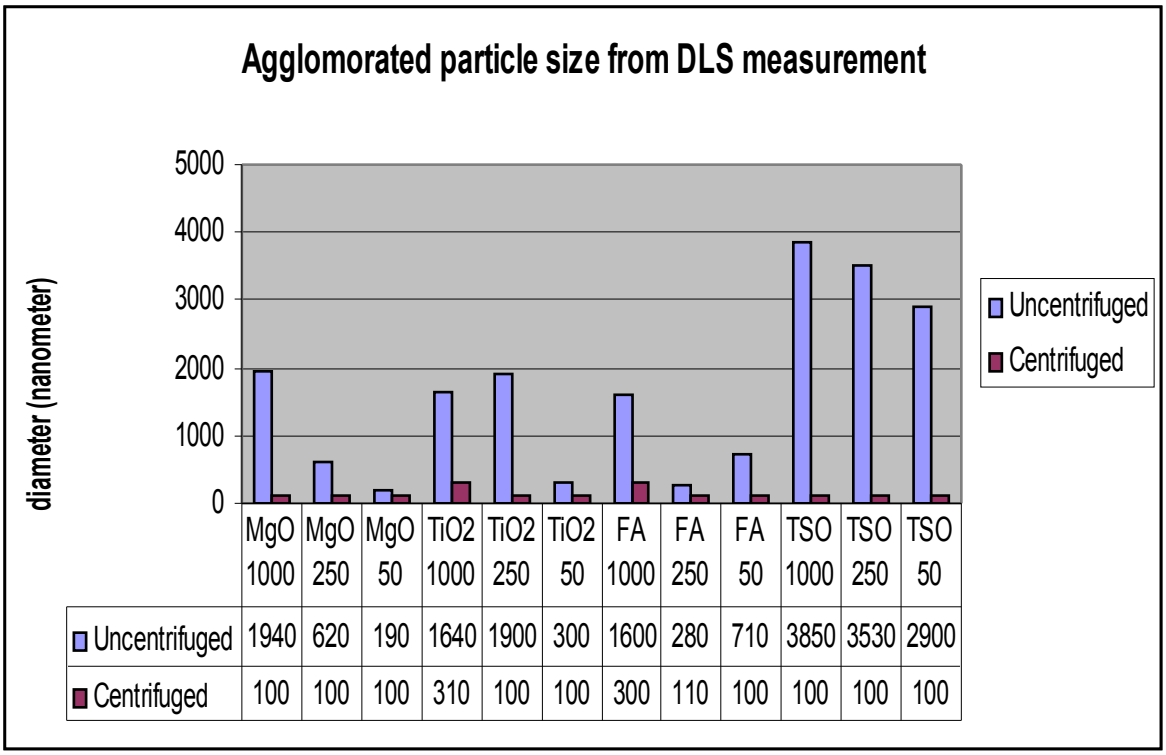
NPs in uncentrifuged-high concentrated solution agglomerated to near micron size in cell culture media (Figure 2) as compared to centrifuged suspension of NPs in media that were less than 300 nm. As diffuse light scattering (DLS) detected the particles even in supernatant of centrifuged sample, we considered that centrifugation at 13,000 g for 20 minutes was not sufficient to remove all the NPs. To confirm, we made a 500mg/L suspension of TSO-Mn-Aerogel NPs in culture media or water, spun them at different centrifugation speed and analyzed in ICP-MS for the presence of manganese (Table 3).

Table 3 Presence of Mn in centrifuged sample

	Media (mg/L)			**Water (mg/L)**		
Force*	**260,000g**	**15,000g**	**0.00g**	**260,000g**	**15,000g**	**0.00g**
Reading-1	1.1	1.2	6	0.8	0.8	9.3
Reading-2	1.1	1.1	7	0.8	0.8	9.5
Reading-3	1.2	1.1		0.8	0.9	
Average**	1.1	1.1	6[#]	0.8	0.8	9.4[#]

*** Force means centrifugal force of a centrifuge machine.260, 000 g means centrifugation force generated in 13000 rpm for 20 minutes. 0.00g indicates uncentrifuged sample. Each readings was corrected by subtracting from blank**
****It is average of 2 or 3 readings as shown in the table.**
#The uncentrifuged samples showed large variations, could be due to non-uniform suspension of TSO-Mn-Aerogel in media or water.

The result of centrifugation showed that almost 1/6 proportion of NPs remain in the supernatant even at 260,000 g-min (Table 3). The suspended smaller particle in centrifuged sample could have sufficiently low mass, density, or an as yet undiscovered property that might have resisted the centrifugation of this force. The large agglomerated particles in high concentrated solution, which were measured in a range of 1.5 to 2.5 μm, could be considered as fine particles. The biggest agglomerated particle was found in highest concentrated solution of TSO-Mn-Aerogel. They were larger than 2.5 μm (2,500 nm) allowing them to be considered as coarse particles. While centrifuged counterparts of all concentrations of all types of NPs, which measured less than 50 nm, have size in range of ultrafine particles. Thus, the solution of NPs prepared to induce inflammation in BEAS 2B cells has mixture of ultrafine, fine and coarse particles.



	MgO 1000	MgO 250	MgO 50	TiO2 1000	TiO2 250	TiO2 50	FA 1000	FA 250	FA 50	TSO 1000	TSO 250	TSO 50
Uncentrifuged	1940	620	190	1640	1900	300	1600	280	710	3850	3530	2900
Centrifuged	100	100	100	310	100	100	300	110	100	100	100	100

Figure 2 Size of the NPs in the solution as measured by DLS (single measurement). The bar represents diameter of agglomerated particles in DMEM. The horizontal bar shows four types of NPs with different concentration (e.g. MgO 1000 refers to 1000mg/L). NPs were mixed in DMEM media and each concentration was centrifuged at 13,000 rpm for 20 minutes to remove bigger agglomerated particles. The uncentrifuged or mixture at higher concentration has bigger size (as big as 3.5 µm) but the centrifuged samples have particles in 100-300 nm ranges.

3.3 Pattern of Cytokine release

Titanium and magnesium oxides did not significantly induce increased total amounts of pro-inflammatory cytokines (IL-6) and Chemokine (IL-8) over the period of 24 hours from BEAS 2B cells when treated with different concentrations of particles. The release of both IL-6 and IL-8 was neither dose-dependent nor there was any pattern with respect to time at 3 hours, 6 hours, 12 hours and 24 hours time points. In fact, the release of IL6 was decreased for magnesium, titanium and a mixture of the two at 1,000 mg/L. However, at 50 and 250 mg/L IL6 total release was decreased only for titanium ($p < 0.05$; Mann-Whitney U Statistic). Centrifuged solution, which constitutes more fine particles, was unchanged relative to control BEAS 2B cells

(Table 3). IL8 release from BEAS 2B lung cells was unchanged by any metal oxide NPs studied (Table 4).

Table 4 Effect of NPs in releasing IL-6 from BEAS 2B cells

	IL-6 pg/mL (Mean of 6 observation ±SE)					
Dose	**Titanium dioxide**		**Magnesium Oxide**		**Mixture (MgO and TiO2)**	
	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged
50 mg/L	560±50	670±90	890±110	1150±210	1040±190	1040±150
250 mg/L	560±90	1060±160	680±100	1440±330	830±90	1150±110
1000 mg/L	610±80	1000±140	370±80	1050±220	650±70	1400±250
Control*	**1000±125 (18)**		**1000±125 (18)**		**1000±125 (18)**	
Sig**	**<0.05**		**<0.05**		**<0.05**	

*** Uninoculated control cultures from 18 observations**

**** Mann-Whitney U Statistic, Uncentrifuged vs centrifuged.**

Table 5 Effect of NPs in releasing IL-8 from BEAS 2B cells

	IL-8 pg/mL (Mean of 8 observation ±SE)					
Dose*	**Titanium dioxide**		**Magnesium Oxide**		**Mixture (MgO and TiO2)**	
	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged
50 mg/L	3720±110	3890±380	4130±290	3980±250	3680±230	4040±200
250 mg/L	3650±260	3311±200	4050±170	4290±290	3620±90	4410±360
1000 mg/L	3810±320	3370±170	3190±510	3280±140	4100±560	4100±230
Control**	**3630±100 (18)**		**3630±100 (18)**		**3630±100 (18)**	
Sig***	**≥0.05**		**≥0.05**		**≥0.05**	

*** Uninoculated control cultures from 18 observations**
**** Mann-Whitney U Statistic, Uncentrifuged vs centrifuged**

3.4 Release of Cytokines: Comparison of Titanium NPs with feedlot dust

Feedlot dust exposure included as positive cytokine controls lead to the release of more total quantities of IL6 and IL8 than control BEAS 2B lung cells (Figure-3, Figure-4). Feedlot dusts were potent inducers of inflammatory cytokines which released tenfold more IL-6 than controls after 12 hours of incubation to 1 and 3% feedlot dust extracts. Although the increases were less from 5% feedlot dust extract than from the 1% and 3% extracts; increased pores were observed in the cells.

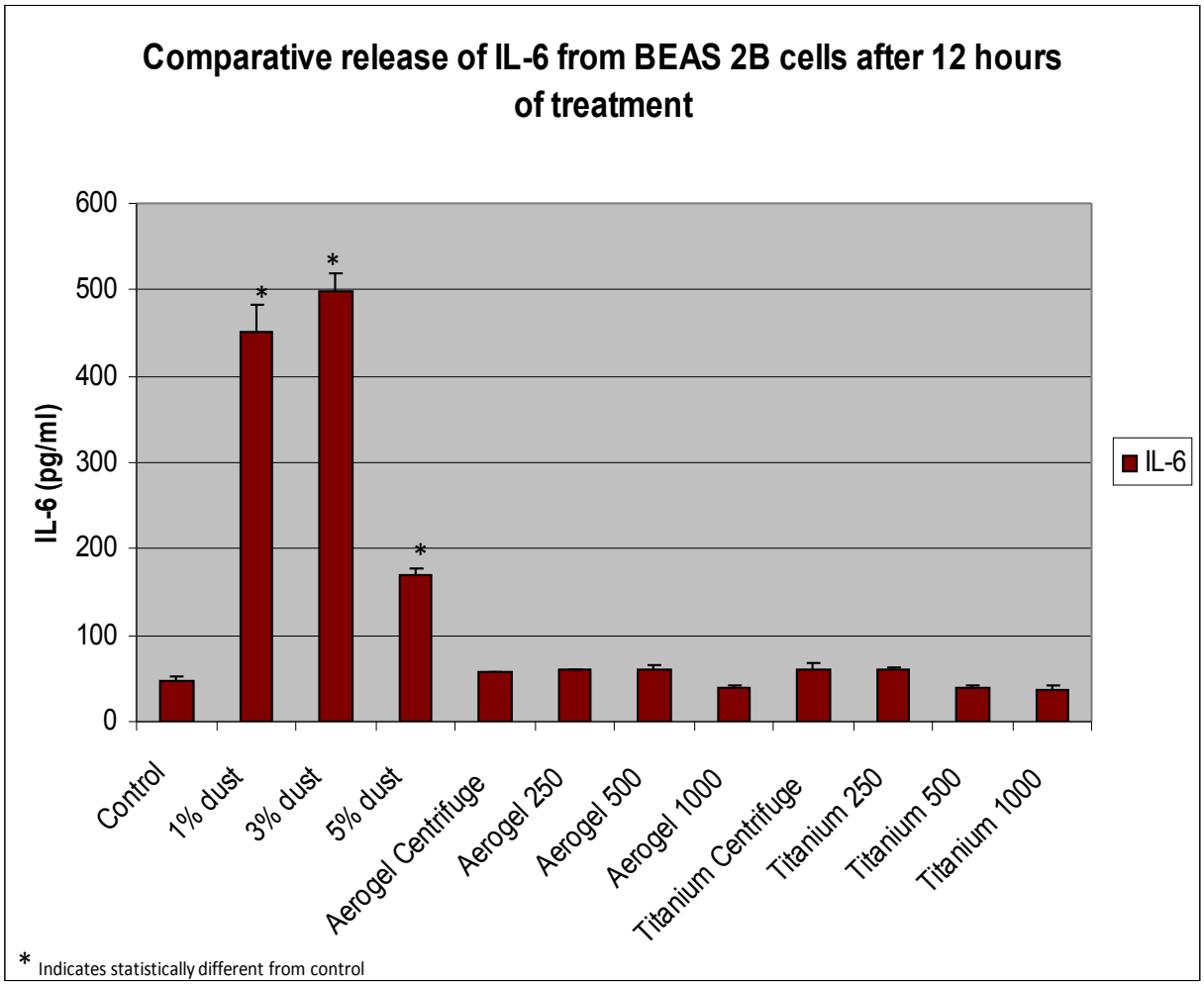


Figure 3 Bars represent the average IL-6 release from BEAS 2B cells when treated with different concentration of manure mat dust and NPs after 12hours of incubation. Error bars represent the standard error of mean (Mean ± SEM, n=6). Analysis of Variance (ANOVA) with 12 different treatment factors shows that they were statistically different from each other (F=201.9, v1=11, v2=60, p=0.00 at 5% confidence interval). Further, Bonferroni t-test statistics were calculated to compare the mean difference between individual treatment group and control when multiple comparisons were made. Manure mat dust extract 1%, 3% and 5% were significantly different from control (Bonferroni t-test, n=6, p=0.000, SE=16.35). Manure mat dust of 3% extract induced peak production of IL-6. Effect of metal oxide naoparticles was not statistically difference from control at 5% confidence interval (Bonferroni t-test).

Significant, but modest increases (15-20%) of total IL 8 releases were seen in the 1 and 3% dust extracts (Figure-4). Almost all concentrations of metal oxide NPs did not significantly affect total IL 8 releases. The only exception was the highest concentration of 1000 mg/L of Aerogel, which released more than 5-fold less total IL 8. This aerogel may have contained

sufficient material to be toxic to BEAS 2B cells (Figure-8). When one takes the more than 15 fold reduction in cells recovered at 12 hours, and calculates IL 8 released on a per cell basis, more IL8 was released per cell, perhaps reflecting cytotoxicity of this high level of exposure concentration. Titanium and magnesium oxides had relatively little effect on the release of total IL8.

The greatest changes in IL8 release were caused by exposure to centrifuged feedlot dust particles. Metal oxide NPs appeared to cause release of higher IL8 amounts per cell only with very high concentrations of uncentrifuged NPs. Releases of increased amounts of IL8 required exposures to higher concentrations of metal oxide NPs than were required for released of increased amounts of IL6. Marked cytotoxicity from metal oxide NPs elicited increased releases of metal oxide NPs on a per cell basis. By comparison exposure of cells to centrifuged feedlot dust particles, even at modest concentrations elicited increased release of IL6 and lesser increased releases of IL8.

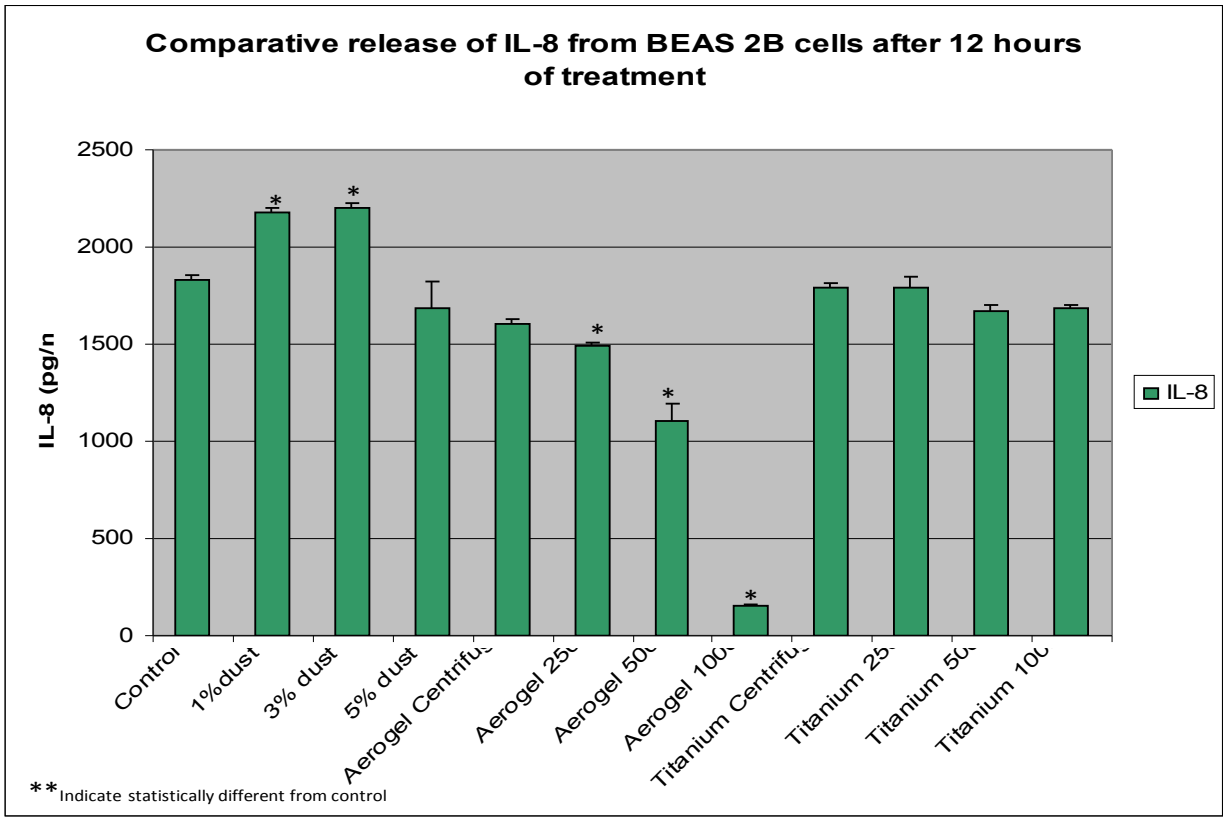


Figure 4 Bar represents the average IL-8 release from BEAS 2B cells when treated with different concentration of manure mat dust and NPs after 12hours of incubation. Error bar represents the standard error of mean (Mean±SEM, n=6). Analysis of Variance (ANOVA)

with 12 different treatment factors shows they are statistically different from each other (F=107.12, v1=11, v2=60, p=0.00 at 5% confidence interval). Bonferroni t-test statistics was calculated to compare the mean difference between individual treatment group and control. Manure mat dust 1% and 3% extract induced IL-8 production (Bonferroni t-test, SE=73.66, p<0.01). Aerogel at concentration of 250 mg, 500 mg and 1000 mg reduce the amount of IL-8 (Bonferroni t-test, SE=73.66, p<0.01). Effect of other metal oxide NPs and 5% manure mat dust extract was not statistically different from the control (Bonferrroni t-test).

3.5 Reduction of IL-6 by NPs in a cell free media

NPs may have bound, temporarily reduced function of, or otherwise inactivated cytokines and reduced the amount of interleukins that were measurable in the tissue supernatant. Alternatively, they may have bound molecules important to production or release of the cytokines. The Il-6 that is released may be inflammatory. Reductions in measurable IL6 in the supernatant fluid were manifested when known amounts of standard IL-6 were added into different NPs solutions and measured using an IL 6 ELISA to determine apparent concentrations. Soluble magnesium oxide NPs had less effect on apparent releases than did nearly insoluble titanium NPs (Figure 5). TSO-Mn-Aerogel particles which had more surface area than TiO_2 NPs produced more decrease in apparent IL-6 quantity than did TiO_2 NPs; the effect was dose related. Thus, these data suggested that increasing surface area and concentration of uncentrifuged larger aggregated NPs were capable of binding, reducing function of, or otherwise inactivating the known concentrations of IL-6 from BEAS 2B bronchial epithelial cells, possibly by binding them or otherwise interfering with their function. Since all these effects were reductions in apparent release of IL-6 our reported values may have underestimated the release of IL-6 from BEAS 2B bronchial epithelial cells. Statistically significant effects occurred only at the highest exposure levels for exposures from uncentrifuged (larger aggregated) Mg and Ti oxide NPs, but at all exposure levels to uncentrifuged (larger, aggregated) TSO-Mn-Aerogel NPs with greater surface area, suggesting that many conditions in which IL-6 was inactivated might have impaired function of cultured BEAS 2B bronchial epithelial cells.

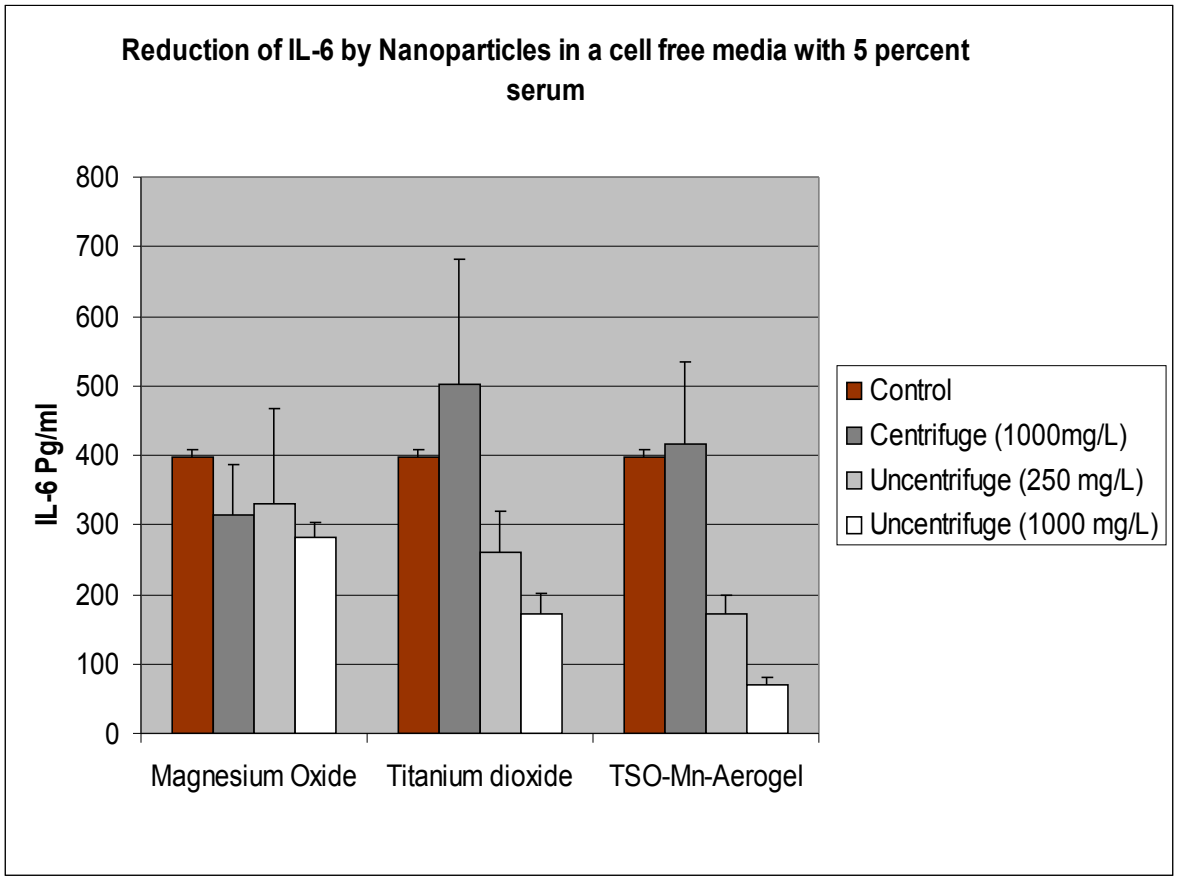


Figure 5 Each bar represents average amount of IL-6 measured by ELSA when 350 pg of IL-6 was incubated in 1 ml of solution containing different concentration of NPs (Mean±SEM, n=4). Centrifuged sample did not reduce as much as uncentrifuged sample. Uncentrifuged sample of TSO-Mn-Aerogel reduced 5.7 fold at 1000mg/L concentration and 2.31 fold at 250 mg/L concentration. TiO_2 at 1000mg/L concentration reduced 2.3 fold while MgO at highest concentration reduce 1.4 fold.

3.6 Cell Morphology

The inert activity of titanium and magnesium NPs in releasing interleukin from BEAS 2B Cells were further investigated by visual inspection of cell morphology under an inverted microscope (Nikon, Eclipse TS100) at 200 times magnification after 12 hours of treatment with NPs.

BEAS 2B bronchial epithelial cells treated with higher doses of feedlot dust extract or NPs were unable to form confluent monolayer in the incubating media (Figures 6-8). They had a shrunken outline and looked unhealthy. Cells treated with 1% and 3% dust extract concentrations resembled the control cells, morphologically (Figure 6), suggesting any effect on BEAS 2B bronchial epithelial cells from the lower concentration of feedlot dust extract was minimal. Both 1 and 3% concentrations of feedlot dust extract had about the same number of cells recovered as estimated by manual counts with hemocytometer chamber. Cells treated with 5% dust extract had a large number of cell pores (presumably from interrupting or reducing integrity of the confluence monoplayer) (Figure 6), suggesting considerable cell death or apoptosis. With exposure to 5% feedlot dust extract only 25% of the cells in the control sample were recovered after 12 hours of culture (all sample counts were given one count, with + 10-25% reproducibility).

Cell morphology after 12 hours of incubation with different concentration of titanium dioxide NPs is shown in the incubating media in Figure 7. Beas 2B bronchial cells exposed to 250 mg/L and 500 mg/L TiO_2 did not appreciably change the morphology of the cells relative to control cells which received no exposure and recovered cells after 12 hours of culture were > 50% of control levels. Cells exposed to 1,000 mg/L were visibly condensed and shrunken and recovered cells were < 10% of control cells recovered.

Morphology of BEAS 2B bronchial epithelial cells after 12 hours incubation with different concentration of TSO-Mn-Aerogels is shown in the incubating media in Figure 8. Beas 2B bronchial epithelial cells exposed to the centrifuged preparation of TSO-Aerogels were not morphologically different from cells exposed to control exposure levels, and > 50% of control cell numbers were recovered after 12 hours of culture. All cells exposed to uncentrifuged aggregated TSO aggregated aerogel particles at 250, 500 and 1,000 mg/L were progressively more condensed and fragmented (Figure 8) and had < 30-40% of control levels of cells recovered after 12 hours of incubation.

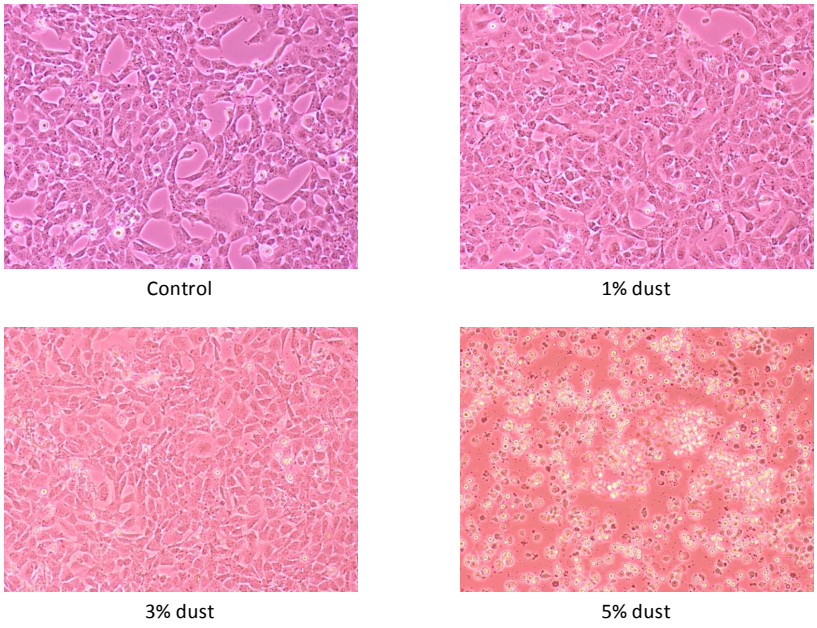


Figure 6 Cell morphology after 12 hours of incubation with different concentration of dust particle. BEAS 2B bronchial epithelial cells that received 1% and 3% dust extract concentration were not clearly distinguishable from the morphology of cells that received only a control exposure. Cells exposed to a 5 % dust extract had a large number of cell pores, and considerably less viable cells.

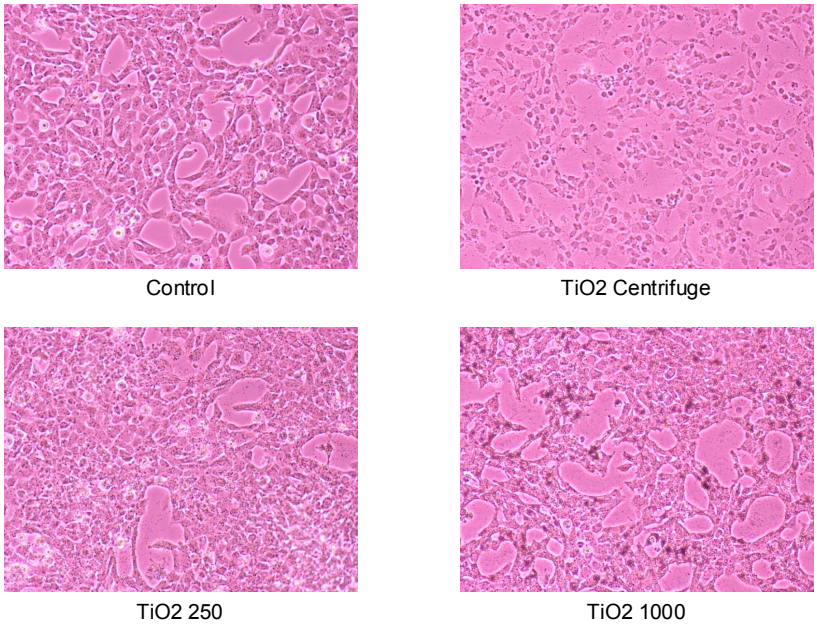


Figure 7 Cell morphology 12 hours of incubation with different concentration of titanium dioxide NPs. BEAS 2B bronchial epithelial cells exposed to 250 mg/L and 500 mg/L were not morphologically different from cells that received only a control exposure. Cells exposed to 1,000 mg/L were visibly condensed and shrunken.

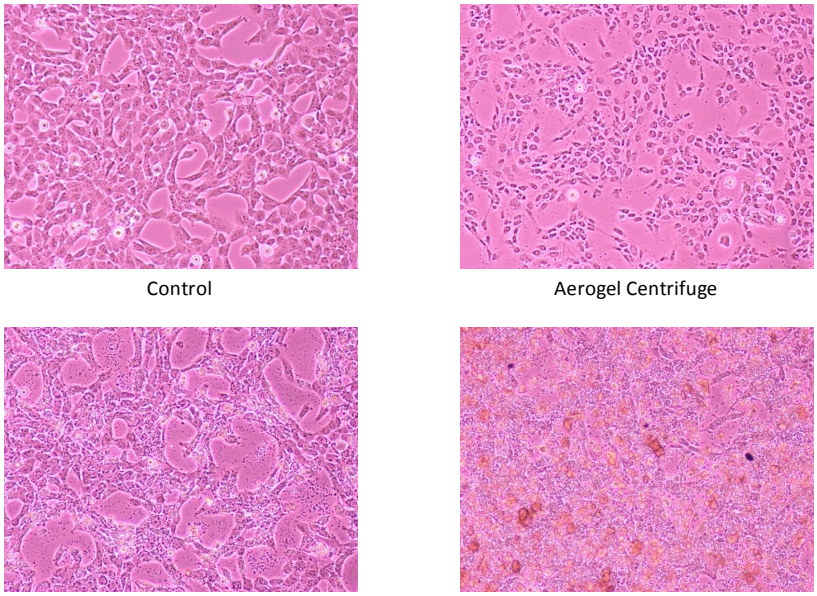


Figure 8 Morphology of cells after 12 hours incubation with different concentration of TSO Mn-Aerogels. Beas 2B bronchial epithelial cells exposed to the centrifuged preparation of TSO-Aerogels were not morphologically different from cells exposed to control levels. All cells uncentrifuged, exposed to the aggregated TSO aerogel particles at 250, 500 and 1,000 mg/L were progressively more condensed and fragmented.

3.7 Cell Viability using Propidium Iodide (PI)

To further define cell changes, we first investigated samples with the greatest effects, that is where < 40% of cells recovered after 12 hours of incubation. Viable and dead cells were counted by staining with a PI florescence dye and detecting them in a Flow Cytometer. PI only permeates and stains dead cells, by staining the nucleic acid. The result shows that nearly fifty percent of cells die in 5% dust treated sample. NPs at 250mg/L killed about the same number of cells as at 1000 mg/L.

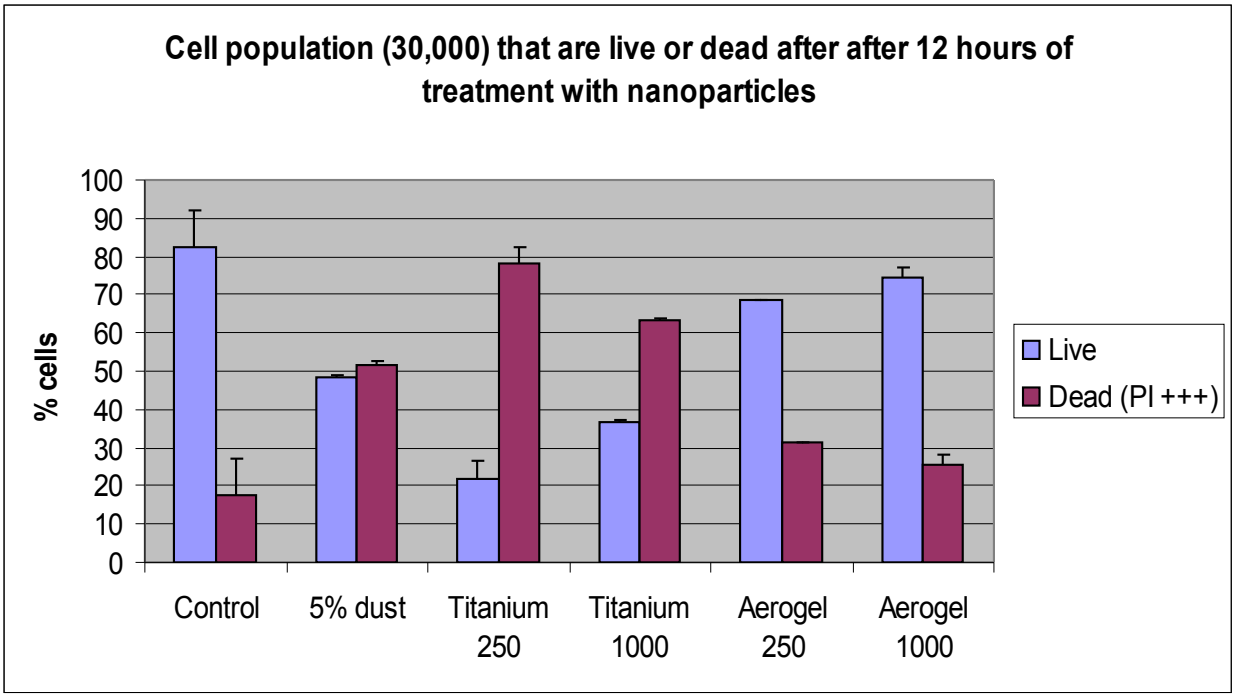


Figure 9 Viability of Cells after 12 hours of incubation with different NPs and Feed lot dust as analyzed in Flow cytometry. TiO_2 and 5% manure feedlot dust extract killed more cells than did exposure to TSO-Mn-Aerogel particles. Viable and dead cells were the averages of three separate trials (Mean±SEM).

TiO_2 and 5% manure feedlot dust extract showed a trend toward killing more cells than did exposure to TSO-Mn-Aerogel particles. One reason could be that particles from TSO-Mn-Aerogel, had low density was floated into the culture media and less interaction occurred with the cells on the bottom of the microtiter plate. On the other hand, Titanium deposited evenly all over the monolayer cell and tended to aggregate and agglomerate.

3.8 Apoptosis

To detect the extent to which the NPs causes apoptosis due to increased reactive oxygen species (oxidant load), mitochondrial membrane potential was examined by staining with JC-1 florescent dye. Integrity of mitochondrial membrane potential is considered as an identical feature of healthy cells. During programmed cell death or apoptosis, key events occur at mitochondria, for instances, release of caspase activators and cytochrome C, changes in the electron transport molecules or loss of mitochondrial membrane potential. Loss of mitochondrial membrane potential is an indication of accumulation of ROS(Chung 2001; Xia, Kovochich et al. 2008). In healthy cells, with high membrane potential JC-1 forms complexes known as J-aggregates in cell, which appears as red fluorescence which can be analyzed in a Flow Cytometry with 488 nm excitation. Thus, JC-1 has been extensively used to estimate cell health or oxidative stress due to ROS. The apoptotic cells or cells with low membrane potential (called JC-1 low fraction) cannot form such aggregates. When high number of cells in a population has low JC-1, it indicates apoptosis particularly due to ROS.

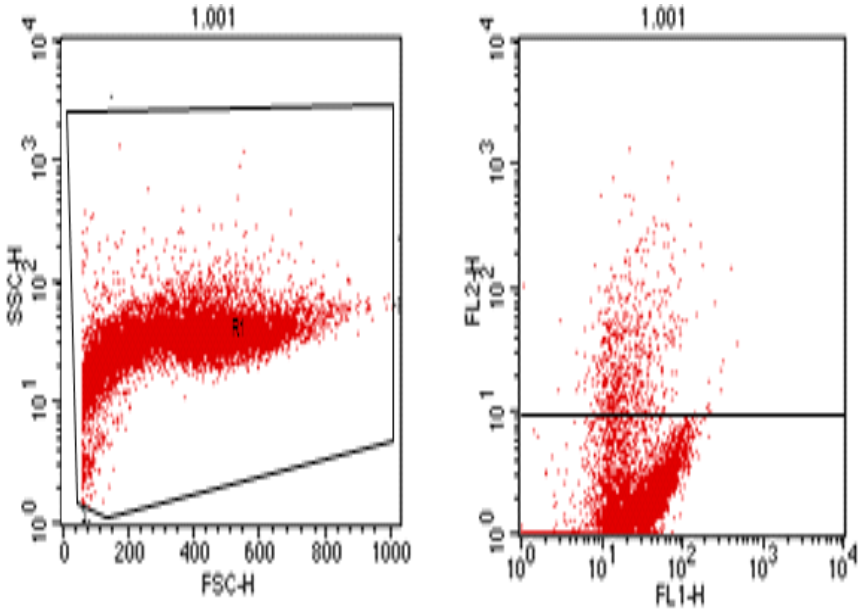


Figure 10 Flow Cytometry result for JC-1 in a CCP treated sample (positive control). Lower quadrant represents JC-1 low cells (JC-1 green). Upper quadrant represents cell population with high JC-1 (JC-1 red). In this particular picture above 90 percent cell out of 10,000 counts are JC-1 low as CCP induces apoptosis

These analyses focused on samples with < 40% recoverable cells relative to control cell sample (Figures 6-8). We found that exposure to feedlot dust at highest concentration (5% solution) and titanium dioxide induced less ROS production in BEAS 2B cells than did exposure to TSO-Mn-Aerogel particles (Figure 11). Titanium dioxide at highest concentration (1,000 mg/ L) produced about the same ROS as the lower concentration (250 mg/L). Since the population of JC-1 low cells in titanium treated sample even at its higher doses was not significantly different from control (Bonferroni t-test, $p>0.05$), it can be considered as a trend toward elevation, but perhaps, not significantly less safe, as described elsewhere in literature. But the effect of TSO-Mn-Aerogel at higher concentration was equal to the positive control and significantly different from negative control ($p < 0.005$), so that at this level, it can be said to have a higher chance of causing unhealthy apoptic cells among the cells that remain alive. Thus TSO-Mn-Aerogel particles were capable of inducing oxidative stress in cells with production of reactive oxygen species leading to apoptosis. In brief, it can be seen that these NPs are more capable of inducing apoptosis by ROS than manure dust even if they do not induce inflammatory cytokines.

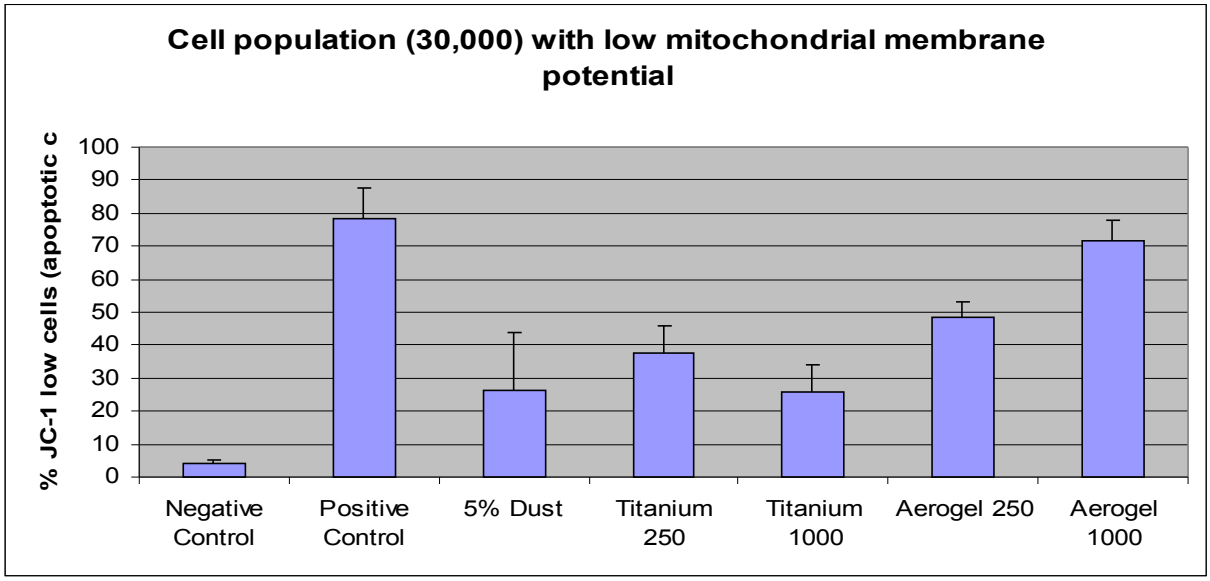


Figure 11 Bar represents the percentage of cells that take low JC-1 red fluorescene (Mean ±SEM, n=3). Analysis of Variance (ANOVA) shows signgnificant F-statistics at 5 % confidence intervcal (F=8.32, v1=6, v2=14, p=0.003). Further, Bonferroni t-statiscs was used to compare the individual treatment with negative control. The positive is a control in which apoptosis is induced by CCP (a cynide molecule that comes along with JC-1 kit).Only Positive control and Aerogel were statistically different from control (Bonferroni t-test, p<0.005)

CHAPTER 4 - Discussion and Summary

4.1 Discussion

In this work, we hypothesized that magnesium oxide would be more soluble than titanium dioxide, based on aqueous solubility data, in distilled water. Data from Table 1 clearly illustrate than MgO is quite soluble in bicarbonate buffers, roughly proportionate to the concentration of bicarbonate. Although soluble in water, MgO NPs are less soluble than in a bicarbonate buffer. There is a qualitative increase from increasing concentration that is less than linear. Any effect from NPs surface area was minimal relative to solubility in the bicarbonate buffer. Titanium (Table 2) on the other hand is minimally soluble. Bicarbonate, time and concentration have minimal if any effect on solubility. Thus, magnesium oxide is more soluble than titanium dioxide. Solubility was dependent on bicarbonate concentration, concentration and time. There was no effect on titanium or magnesium solubility by the other, titanium or magnesium being present in a mixture of the two oxides.

As insoluble particles persist in the lung interstitial space, they can be a source of irritation and inflammation to lung tissues and cells. Inhalation exposure to insoluble particles, if sufficient amounts reach the lungs, may reduce lung particulate clearance. If the particle is toxic to lung cells, this may lead to lung cell dysfunction, and ultimately death. On the other hand, inhalation exposures to soluble particles may allow soluble metals to enter the circulation and be distributed throughout the internal organs(Dorman 2001). Since NPs of magnesium oxide have greater solubility than titanium dioxide, inhalation of TiO_2 particles would most likely result more persistence in lung tissue and a higher lung burden for titanium dioxide, following inhalation exposure into animals or man.

We hypothesized that metal oxide NPs can induce inflammation by releasing pro-inflammatory cytokine IL-6 and chemokine IL-8 in the same manner as our positive control of Feed lot dust. Alternatively, released IL-6 and IL-8 may be cytoprotective (reduce cell necrosis) when there is irritation to the cell (Waxman and Kolliputi 2009). We took feed lot dust as positive control as it has been extensively studied before. We also found in our previous study

that it was an inflammaogen. Lower doses of TiO_2 i.e.50 µg/ml (50 mg/L) have been reported not to lead to release of cytokines (Xia, Kovochich et al. 2008). Our 5 – 20 fold higher doses that produce proportionately higher responses seen by Xia et al.'s earlier work. We also expected TSO-Mn Aerogel particles to be more inflammatory and cytotoxic than titanium dioxide because they had Mn compounds doped into the particles, which could be more toxic as it could generate more electron holes (Nel 2006). Of the three tested NPs, MgO did not reduce recoverable cells after 12 hours incubation significantly (recoverable cells > 50% of control), while other exposures produced < 40% recoverable cells.

In a normal bronchial cell, induction of an irritant or stress caused a defensively increased nuclear regulatory factor (nrf) 2 indicating stimulation of dehydrogenases to help the cells overcome the effect of the stress (healing by primary intent – regeneration). This is called a tier I response; (Nel 2006; Xia, Kovochich et al. 2008). If this attempt at repair failed, the lung cells would attempt to resolve the injury by secondary intent (inflammation) by releasing inflammatory cytokines such as IL-6 and IL-8. In some instances, IL-6 can be cytoprotective. We call this a tier 2 response(Nel 2006; Xia, Kovochich et al. 2008). Feedlot dust, our positive control for release of inflammatory cytokines, caused the release of IL6 and IL8. Compared to feedlot dust extract, metal oxide NPs – titanium dioxide, magnesium oxide, FastAct, and TSO-Mn metal aerogel did not release excess total amounts of either IL 6 or IL 8. In fact high concentrations appeared to release less total IL-6; mostly total IL-8 was less affected than IL-6. This would suggest that feedlot dust causes tier-2 effect but the metal oxide NPs did not. They may not have been exposed to a level which was inflammatory, or alternatively, no such level may have existed. If one looks at approximate cell numbers recovered after 12 hours of incubation, the amount of IL-6 released per cell may actually have gone up. Such numbers should be interpreted with considerable caution, because the reproducibility of manual chamber counts is + 15-25%.

However, perhaps more importantly, concentrations IL-6 were either significantly bound by increased Ti or TSO-Mn-Aerogel surface area, and were not measurable, or the exposure concentration that increased BEAS 2B cell death after 12 hours of culture. These data are supported by finding morphological indications of cytotoxicity after metal oxide NPs exposures at very high levels, *in vitro*, and increased cellular pores after exposure to 5% feedlot dust extract.

MgO exposure at the highest level produced a marginally significant reduction of IL-6 release, apparently not sufficient to lead to more than a modest reduction in recoverable BEAS 2B cells after 12 hours of culture or any morphologic evidence of cytotoxicity (> 50% of control levels of cells recovered). We suspect that this lack of toxicity corresponds closely to the increased solubility of MgO in bicarbonate buffers.

It was likely that presence of less functional mitochondria as indicated by high percentage of JC-1 low cells in BEAS 2B cells signified a high level of oxidative stress that might lead to BEAS 2B cell death. Injury at sufficiently high levels might have caused toxic changes in these cells corresponding to the appearance of increased numbers of pores and morphologic indication of cytotoxicity (Figure 6-8). Changes in mitochondrial pores of LEC has been classified a Tier 3 response, an *in vitro* lung cell change (Nel 2006; Xia, Kovochich et al. 2008). Toxic involvement of mitochondrial pores may have reflected an inability of cells to balance shifting of mitochondrial pores between the nucleus and cytoplasm as indicated by the effects of cyclin D1 (De Falco 2004), or some other explanation.

Considering surface area, TiO_2 at ~230 m^2/g and 1,000 mg/L, we should have ~0.7 m^2 per well of a 6 well plate (1,000 mg/L X 3.0 ml X 230 m^2/g). Comparatively, a similar number for the Ti-Mn-Si oxide aerogel would be slightly > 1.2 m^2/well in a 6 well plate in a 1,000 mg/L plate, and 0.3 m2 per well in a 250 mg/L plate. Thus, the aerogel surface area would bracket the highest level of titanium. While this surface area would be expected to cover a 9.6 cm^2 well many times (300-1,200 times) over, it is ~ the 1,000 mg/L of MgO (1.8 m^2 surface area) that produced minimal to moderate reductions in recoverable BEAS 2B, possibly because it was dissolved and no longer particulate. Thus, we conclude that there must be at least some factors other than the large amount of surface area that binds IL-6 or somehow impairs the function of BEAS 2B cells. These comparisons say that the inherent toxicity of TiO2 and Mn-Ti-Si oxide NPs is > 10 times greater than that of MgO, yet the surface areas are not too different. Possibly MgO's greater solubility changes the form seen by the cells sufficiently to alter the response.

Although NPs can get in the way of cell processes (Moss 2006), how this getting in the way related directly to the increased cell death (TiO_2) or the oxidative stress of TSO-Mn-Aerogel in these studies is not clear. The responses in each case were elicited at levels modeling 5-20 times a nuisance dust level (TiO2) or PEL (Mn).

The results obtained from the in vitro studies with cultured bronchial epithelial cells are limited and should be extrapolated to the real world with considerable caution (Sydbom, Blomberg et al. 2001; Oberdorster and Finkelstein 2006; Phalen, Oldham et al. 2006). Firstly, these are exposures to BEAS 2B cells only, not to intact animals with lungs. Secondly, these are exposures at very high levels, specifically, 5-20 times the exposures modeled for a nuisance dust exposure (TiO_2) or the permissible exposure level (PEL) of Mn. Previous work has shown that TiO_2 was innocuous at levels modeling a nuisance dust level in the same cell system (Xia, Kovochich et al. 2008).

Attention and evaluation must be given to the effect of the type, size, concentration in the ambient environment, the likelihood of exposure to the particle makes the calculation of real level of exposure extremely complex. Additionally, there is complexity in how the respiratory system behaves toward the inhaled particles. The presence or absence of chronic obstructive pulmonary disease (COPD), the animal's age/ immune status are the primary factors that determine the pulmonary response to inhaled particles. In addition to this tracheo-broncial (TB) region should not be considered to be a uniform patch of the epithelial cells similar to the confluence monolayer of the cultured cells because there are hot spots like bronchial bifurcation where inhaled particles tend to deposit more(Phalen, Oldham et al. 2006). Despite these limitations, *in vitro* studies are important to understand mechanistic toxicology.

4.2 Summary

Magnesium is more soluble than Titanium; a bicarbonate buffer enhanced solubility of MgO. Insoluble particles can persist in the interstitial lung space as a continuing source of injury. Since MgO particles are more soluble, we would predict that they would be cleared from the lung, predominantly as soluble magnesium which is tightly regulated by the body (Bateman, 2006). Moreover, MgO has is called Milk of Magnesia and minimal toxicity, so we would predict it would have less toxicity than titanium or other metal oxides. In vitro, when BEAS – 2B cells were exposed to centrifuged (smaller unagglomerated particles) or uncentrifuged larger agglomerated particles of MgO, only minimal morphological changes in BEAS – 2B cells after 12 hours were noted. Moreover amounts of released less IL-6 were approximately control levels as total release. These data correspond to our prediction of minimal toxicity for MgO in vitro.

The lack reduction of cell numbers after 12 hours of incubation for magnesium at any concentration used in the conditions of our assay suggest that relatively non-toxic MgO NPs, even aggregated and agglomerated were less cytotoxic to lung cells than were TiO_2 or TSO-Mn-Aerogel particles. This latter effect may have been assisted by the > 75% solubility of MgO in bicarbonate buffers.

In contrast, TiO_2 is less soluble than MgO, in fact it has minimal solubility, even as a nanocrystalline structure. Bicarbonate does not enhance solubility, which is independent of time or concentration. MgO does not enhance the minimal solubility of TiO_2. When deposited in the lung, these relatively insoluble particles can persist in the lung as a continuing source of injury. While BEAS – 2B cells exposed to particles of TiO_2 at high and low doses did not induce release of increased total IL-6, reduction in viable cells and lowered membrane potential were taken as an indication of cytotoxicity at the extremely high doses of titanium dioxide.

The concentrations of large aggregated TiO_2 or TSO-Mn-NPs which cause cytotoxicity (reduction in viable cells and/or a high percentage of apoptotic cells as indicated by JC-1 dye, after 12 hours of incubation) are the same concentrations which bind or interfere with the release of IL-6 from BEAS – 2B lung cells. These data suggest that the NPs may have been present in sufficient numbers to get in the way of one or more of the BEAS – 2B cell functions, agreeing with the earlier work of Moss 2006. Large aggregated TiO_2 or TSO-Mn-Aerogel NPs in extremely high concentrations would be more likely to interfere structurally (Moss 2006) than would smaller, non-aggregated or the largely dissolved MgO particles.

The consistently greater responses for TSO-Mn-Aerogels led us on surmise that *in vitro* cytotoxicity was at least 4-fold greater for TSO-Mn-Aerogel than it was for TiO_2. This NP caused more apoptosis than TiO_2 suggesting that the presence of Mn in it creates electron holes inducing oxidative stress in cell.

The positive inflammatory control data from feedlot dust extract indicated that there may be a maximum level for the minimally cytotoxic effect of small unaggregated NPs. However, the greater reduction of viable cells caused by the 5% feedlot dust extract when coupled with the morphologic appearance of numerous cellular pores both indicate a cytotoxic response. Thus, 5% feedlot dust extract under these conditions appears more cytotoxic than 1-3% feedlot dust. Perhaps it is present in sufficient concentrations to get in the way (Moss 2006), although this must be demonstrated with further research.

CHAPTER 5 - References

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