CAN EXISTING SOUTH AFRICAN AIR QUALITY STANDARDS BE APPLIED TO NANOPARTICLES? 2010 NACA CONFERENCE, POLOKWANE

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Abstract

Particulates less than 100 nm (in at least one diameter) are referred to as nanoparticles. The properties of nanoparticles may differ from those of larger particles from the same bulk material. These unique properties allow the use of nanoparticles for specific applications such as drug delivery within the human body, or to enhance or replace existing materials. For example, carbon nanotubes are six times stronger than steel. There is therefore a need for intentionally manufactured (engineered) nanoparticles. The manufacture and use of these particles will however inevitably lead to their release into the environment (air, water and soil). A primary concern is that very little is known about the effects of these nanoparticles on human health and the environment, especially because of their unique properties.

An *in vivo* study was conducted by the CSIR at the University of Pretoria Biomedical Research Centre at Onderstepoort, using the BALB/c murine model and nanoparticles engineered at the CSIR. These particles were engineered from titanium dioxide through a hydrothermal process, using potassium hydroxide. Animals were exposed to different concentrations of these particles in a whole-body inhalation chamber. The main aim of this study was to assess the degree of change, if any, to the weight, blood, and normal microscopic morphology of some organs, including the lungs.

The findings of the study indicate that existing air quality standards may not be adequate to protect human health following exposure to specifically engineered nanoparticles.

Keywords: titanium, dioxide, nanoparticles, toxicity, inhalation, BALB/c.

1. Introduction

Mankind has always been exposed to naturally occurring ultra fine particulate matter ($PM_{0.1}$: particles equal to or less than 0.1 µm in diameter), for example from volcanic eruptions. These ultra fine particles are also known as unintentionally formed nanoparticles (particles equal to or less than 100 nm in at least one diameter). However, since the dawn of the industrial revolution, man-made sources of primary unintentionally formed nanoparticles have increased, being emitted by motor vehicles and coal-fired power plants to mention just a few. In addition to these sources, secondary nanoparticles, such as sulphates and nitrates, may unintentionally form in ambient air through gas-to-particle conversion. Nanoparticles may even form unintentionally in an occupational environment, for example in welding fumes. The adverse health effects of these unintentionally formed nanoparticles have been well described in the literature through both animal and human studies (Smith *et al*., 2001; Biswas & Wu, 2005; Oberdöster *et al.,* 2005; Chow *et al.,* 2006).

The health effects of intentionally engineered nanoparticles are less known, although there are currently about 1000 consumer products that contain engineered nanoparticles (http:\\www.nanotechproject.org). There is however, a need for manufactured nanoparticles, because

they are engineered for specific applications including drug delivery within the human body, or to enhance or replace existing materials. Carbon nanotubes are for example used in cables as it is much stronger than steel, yet much lighter.

Titanium dioxide (TiO_2) is considered to be biologically inert (Kang *et al*., 2008) and this is most likely why titanium nanoparticles are widely used in consumer products such as paint, cosmetic products and sunscreen. These particles also have many applications, including their use in waste water treatment, because of unique properties such as the ability to act as a photo catalyst and generate anti-ultraviolet light (Liang *et al*., 2009).

The main concerns are that, due to the manufacture and wide commercial use of engineered nanoparticles, humans will inevitably be exposed (inhalation being one route of exposure) and the unique properties of these engineered particles could increase their toxicity. The benefits of the nanoparticle applications may therefore be short-term, while the detrimental effects may be long-term (Quadros & Marr, 2010).

The aim of this study was to examine the effects of different concentrations (10 mg/m^3 and 1 mg/m^3) of engineered nanoparticles in an *in vivo* study using BALB/c mice.

2. Materials and methods

2.1. Particles used in the study

Two types of nanoparticles were donated by the Nanocentre, CSIR for use in this study. The first type was commercially available and the second type engineered at the Nanocentre. The commercially available material was TiO_2 nanoparticles (Degussa P25). This material is uncoated and consists largely of anatase (Long *et al*., 2007). The characteristics of the Degussa P25 (first type) particles are described in Grassian *et al*., (2007). Transmission electron microscopy (TEM) analysis done during the current study showed that the particles were spherical in form while X-Ray diffraction showed that the particles consisted of 84% anatase and 16% rutile. The Brunauer Emmitt Teller (BET) surface area was measured to be 42.6 ± 3.5 m^2/g. BET is an analysis technique used to determine a specific surface area of a material.

These P25 particles were then used to engineer the second type of (rod-shaped) particles through a hydrothermal process using potassium hydroxide (KOH). A semi-quantitative determination of the rod-shaped particles estimated that it consisted of potassium tetratitanate ($K_2Ti_4O_9$) (70%) and anatase (30%). TEM analysis of these particles showed nanosized rod-shaped particles, with the BET surface area being 188.9 ± 2.1 m^2/g.

Before use, the different concentrations (10 mg/m^3 and 1 mg/m^3) of the nanoparticles were suspended in phosphate buffered saline (PBS) and sonicated in a sonicator bath for 20 minutes. It was decided to use the South African occupational standard for inhalable particles of TiO_2 (10 mg/m^3) (Government Gazette, 2006) as the higher level of exposure. Inhalable particles are between 2.5 and 10 µm in diameter (DEA, 2009). An uncertainty factor of 10 was applied to this concentration to determine the lower level (1 mg/m^3) of exposure The uncertainty factor was applied because these particles are in the nanosize range and thus potentially able to penetrate deeper into the lung.

2.2. Animals used in the study

Six-week-old female BALB/c mice, each of average weight (about 20g) were used. They were maintained at the University of Pretoria Biomedical Research Centre (UPBRC) and provided with OVA-free food and water *ad libitum*. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee (ethics approval number H002-09). Mice were divided into test groups as indicated in Table 1.

Each test group was exposed by means of a nebuliser-venturi unit, in a whole body inhalation chamber (Glas-Col model 099C A4212), to 1.27 m^3 air per hour.

The exposure frequency was one hour per day, four days per week, for a duration of six weeks (days 0 to 39).

2.3 Observation and weighing of animals

The animals were assessed daily for possible signs of pain and distress. Animals were also weighed before the start of the study and thereafter twice a week. The final weights were recorded the day prior to termination. Should any signs of pain and distress be observed or a body weight loss of more than 10% recorded, the specific animals would have been terminated, and the organs histopathologically examined.

2.4 Preparation of tissue samples for transmission electron microscopy (TEM)

Organs were removed and fixated in a 2.5% formaldehyde/gluteraldehyde solution. Each organ tissue block from each animal was finely sliced using a scalpel. The tissue of each organ prepared in this way, was pooled to contain tissue of each organ from half of the animals in each of the exposure groups. Thus there were two (pooled) samples of lung tissue for the animals exposed to the high concentrations and two (pooled) samples of lung tissue for the group exposed to the low concentrations of particles.

For preparation of the tissue samples for TEM examination, the samples were immersed in the fixative for 1 hour, after which it was rinsed three times in 0.075M sodium potassium phosphate buffer (pH 7.4) for 15 min, before being placed in a secondary fixative of 1% osmium tetra-oxide (OsO_4) solution for 1 hr. Following fixation, the tissue samples were rinsed again as described above. The samples were then serially dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. Samples were subsequently embedded in quetol resin, after which ultra-thin sections (80-100 nm) were cut with a diamond knife using an ultra microtome. Samples were contrasted with uranyl acetate for 15 minutes, then washed with distilled water, followed by 10 minutes of contrasting with lead citrate; again washed with distilled water, after which they were allowed to dry for a few minutes before examination with a JEOL TEM (Model JEM 2100F).

2.5 Preparation of blood smears for differential white blood cell counts

On the day of termination three blood smears, prepared for each animal in every group, were stained with QuickDiff and then histologically investigated to determine white blood cell count differences.

2.6 Preparation of fibrin clots for scanning electron microscopy (SEM)

On day 43, during termination, about 100 µℓ blood was drawn from each of the mice in every group and 11 µℓ citrate added for every 100 µℓ blood. The blood from each group was pooled. Blood was centrifuged at 1000 rpm for two minutes to obtain platelet rich plasma (PRP).

Human thrombin (provided by the South African National Blood Services) was used to prepare fibrin clots (Pretorius *et al*., 2006). The thrombin concentration was 20 U/mℓ and was prepared in a biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor, are released into the coagulum.

Ten micro litre (10 µℓ) mouse PRP was mixed with 10 µℓ human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 µm Millipore membrane to form the coagulum (fibrin clot) on the membrane. This Millipore membrane was then placed in a Petri dish on filter paper damped with PBS (to create a humid environment) and kept at 37 ℃ for 10 min. This step was followed by a washing process during which the Millipore membranes with the coagula were placed in PBS and magnetically stirred for 20 min. This step is necessary to remove any blood proteins trapped within the fibrin network (Pretorius *et al.,* 2006).

Washed fibrin clots were fixed in 2.5% formaldehyde/gluteraldehyde in 0.075 M phosphate buffer solution at a pH of 7.4 for one hour. Each fibrin clot was rinsed three times in phosphate buffer for 5 minutes before being fixed for 1 hour with 1% osmium tetra oxide (OsO_4). The samples were rinsed three times with phosphate buffer for 5 minutes and were then dehydrated serially in 30%, 50%, 70%, 90% ethanol and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting and examining the tissue with a Zeiss Ultra Plus FEGSEM.

The purpose of this analysis was to compare platelets and fibrin networks of the control group with those of the exposed groups as well as to previously studied platelet and fibrin morphology of asthmatic animals.

Table 1. Different experimental groups, levels of exposure, types of particles and number of animals used in the study.

Group description	Exposure level	Type of nano-particle	Number of subjects
Control	None	None	6
High concentrations	10 mg/m^3	P25 spherical particles	12
Low concentrations	1 mg/m^3	P25 spherical particles	12
High concentrations	10 mg/m^3	Rod-shaped particles	12
Low concentrations	1 mg/m^3	Rod-shaped particles	12

3. Results and discussion

Regular observations and weighing of animals did not show any signs of pain and distress or a body weight loss of more than 10% in any of the exposure groups. It was therefore not necessary to terminate the experiment before the planned date (day 43).

Although lung fibrosis were observed in all of the exposure groups, it was considerably worse in the groups exposed to spherical particles (TiO_2). Thus, TiO_2 nanoparticles may cause lung fibrosis, even at concentrations ten times lower than the occupational standard for inhalable particles.

As far as the differential white blood cell counts are concerned, results were obtained for the group exposed to the rod-shaped nanoparticles. It was found that eosinophils were statistically significant elevated in the group exposed to 1 mg/m^3, indicating an inflammatory response.

Pretorius et al (2007) were the first to use the BALB/c asthma model to study ultrastructure and changes in platelet ultrastructure during asthma. Pretorius & Oberholzer (2009) showed that similar platelet and fibrin network ultrastructure is found in uncontrolled human asthmatic subjects and BALB/c asthmatic animals. The challenge when using animal models is always whether the model adequately mimics the human disease. Pretorius & Oberholzer therefore presented morphological support for the use of the animal model in the study of asthma (Pretorius & Oberholzer, 2009).

A typical fibrin network consists mainly of major, thick fibres with minor, thin fibres dispersed among the thick ones, while a typical platelet aggregate shows a smooth membrane surface with pores and pseudopodia being visible.

In asthma (human and animal models), minor thin fibres form a thin, netlike layer over the major fibres, and the platelets do not form a typical bulbous aggregate with pseudopodia.

Preliminary results showed that in both concentrations of rod-shaped nanoparticles, the thin fibres had a thickened netlike appearance and platelets did not aggregate in the same way as seen in the controls, suggesting that these rod-shaped nanoparticles may have the potential to cause a reaction that show attributes similar to asthma.

When standards are set based on toxicological study results, uncertainty factors are applied to the no observed adverse effect level (NOAEL), or in the absence of that, the lowest observed adverse effect (LOAEL) found in the study. A factor of 10 is normally applied for each uncertainty, for example when extrapolating from animals to humans or from acute or intermediate exposure to chronic exposure.

When this method is applied to the current study, the LOAEL of 1 mg/m^3 will have to be divided by at least 10 to account for animal to human extrapolation.

The current findings of this study therefore support the conclusion that existing air quality standards may not adequately protect human health from exposure to specifically engineered nanoparticles.

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References

Biswas P. & Wu C. 2005, 'Critical Review: Nanoparticles and the Environment', *Journal of the Air and Waste Management Association* **55:** 708-746

Chow J.C., Watson J.G., Mauderly J.L., Costa D.L., Wyzga R.E., Vedal S., Hidy G.M., Altshuler S.L., Marrack D., Heuss J.M., Wolff G.T., Arden Pope III C. & Dockery D.W. 2006, '2006 Critical Review Discussion Health Effects of Fine Particulate Air Pollution: Lines That Connect' *Journal of the Air and Waste Management Association* **56**: 1368-1380

DEA 2009, State of Air Report, Pretoria, South Africa

Government Gazette 2006. Gazette number 29276 of 5 October 2006

Grassian V.H., Adamcarcova-Dodd A., Pettibone J.M., O'Shaughnessy T.O. & Thorne P.S. 2007, 'Inflammatory Response of Mice to Manufactured Titanium Dioxide Nanoparticles: Comparison of Size Effects through Different Exposure Routes' *Nanotoxicology* **1**(3):211–226

Kang J.L., Moon C., Lee H.S., Lee H.W., Park E. M., Kim H.S. & Castranova V. 2008, 'Comparison of the biological activity between ultrafine and fine titanium dioxide particles in RAW 264.7 cells associated with oxidative stress'. *J. Toxicol. Environ. Health A* **71**, pp. 478-485.

Long T.C., Tajuba J., Sama P., Saleh N., Swartz C., Parker J., Hester S., Lowry G.V. & Veronesi B. 2007, 'Nanosize Titanium Dioxide Stimulates Reactive Oxygen Species in Brain Microglia and Damages Neurons *in vitro*' *Environmental Health Perspectives* **115** (11):1631-1637

Oberdöster G., Oberdöster E. & Oberdöster J. 2005, 'Nanotoxicology : An Emerging Discipline Evolving from Studies of Ultrafine Particles' *Environmental Health Perspectives* **113**(7): 823-839

Pretorius E., Briedenhann S, Marx J. & Franz R.C. 2006, 'Structural Changes in the Fibrin Network of a Pretoria Family with Dysfibrogenaemia: A Scanning Electron Microscopical Study' *Ultrastructural Pathology* **303:**167-176.

Pretorius E., Ekpo O.E., Smit E. 2007, 'Comparative ultrastructural analyses of platelets and fibrin networks using the murine model of asthma'. *Exp Toxicol Pathol* **59**: 105-114.

Pretorius E, & Oberholzer H.M. 2009, 'Ultrastructural changes of platelets and fibrin networks in human asthma: a qualitative case study' *Blood, Coagulation and Fibrinolysis* **20**(2):146-149.

Quadros M.E. & Marr .L.C. 2010. 'Environmental and human health risksof aerosolized silver nanoparticles'. *Air and Waste Management Association* **60**: 770-781.

Smith S., Chen Y.S. & Yeh H.C. 2001, 'Deposition of Ultrafine Particles in Human Tracheobronchial Airways of Adults and Children' *Aerosol Science and Technology* **35**: 697-709.

Liang G., Pu Y., Yin L., Liu R., Ye B., Su Y., & Li Y. 2009, 'Influence of Different Sizes of Titanium Dioxide Nanoparticles on Hepatic and Renal Functions in Rats with Correlation to Oxidative Stress' *Journal of Toxicology and Environmental Health, Part A*, **72**: 740–745