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Repeated Inhalation Exposure of Manufactured Fine and Ultrafine Aluminum Oxide Particles in Mice

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ABSTRACT
This study evaluated and compared the pulmonary responses in mice upon repeated inhalation exposure of manufactured fine (150 nm) and ultra fine (10 nm) aluminum oxide particles. Swiss albino mice were exposed with UFAOP in nose-only inhalation chambers at concentrations of 0, 50, 100, and 160 mg/m³ and with FAOP at concentrations of 0, 140, 300, and 500 mg/m³ for 6h per day on 5 days. Bronchoalveolar larvae analysis done on different post exposure days of 0, 3, 7, 14 and 28 showed particle induced cytoxicity and pulmonary inflammation. Lung burden analysis showed percentage of clearance for FAOP is less when compared to UFAOP. Histopathological analysis showed the inflammatory reactions and mild inflammatory response characterized by the inflammation in the mice treated with UFAOP, well developed emphysema and anucleated macrophages in the mice treated with FAOP. Based on these results we conclude that FAOP induced concentration dependant toxicity when compared to UFAOP at the above concentrations tested.
Key words: *Inhalation, Inflammation, Nanoparticles*

INTRODUCTION

Manufactured nanoparticles of aluminum oxide (nano-alumina) have been widely used in the environment; however, their potential toxicity provides a growing concern for human health [3]. Studies in the recent past indicate that cerebral vasculature can be affected by nano-alumina which may be due to the alterations of mitochondrial functions may be the underlying mechanism of nano-alumina toxicity [3]. Nano-alumina nano particles can elicit a pro inflammatory response and thus present a cardiovascular disease risk [6]. Nano-alumina impairs neurobehavioral functions, including lengthened escape latency. It induces cell necrosis and apoptosis, which were likely mediated by the reduction of MMP and ROS, and the induction of the caspase-3 gene [19]. In the view of above the OECD in its testing programme has provided a list of representitative manufactured nano materials which includes nano alumina to support measurement, toxicology and risk assessment of nano materials [7]. This study focused to evaluate and compare the pulmonary responses induced in mice upon repeated inhalation exposure of agglomerated UFAOP and FAOP.

MATERIALS AND METHODS

Fine and Ultrafine Particle Source

Two different sizes of Aluminum oxide (Al_2O_3) ultrafine particles (Aluminum oxide (gamma), 10 nm; purity ≥ 99%; Stock No. #:1041HT) and Aluminum oxide (Al_2O_3) fine particles (Aluminum oxide (alpha), 150 nm; purity ≥ 99.97%; Stock No. #: 1005MR) were purchased from Nanostructured and Amorphous Materials, Inc. Houston, TX, USA. Manufacturer size specifications were confirmed using scanning electron microscope. Zeta potential measurements were done to know about the surface charge properties using Malvern zetasizer. Particle size measurements in solution were determined with dynamic light scattering (DLS) as described in Malvern Zetasizer Nano ZS 2000.

Animals

Six to seven weeks old Swiss albino mice purchased from Charles River, USA and bred at animal house facility, IIBAT, Padappai, Kancheepuram District, India were used for the study. The environmental conditions were set at a temperature of 21 ± 2°C, relative humidity of 55 ± 5%, and

a 12 h light/dark cycle. The number of animals used in this research is monitored by the Institutional Animal Ethics Committee, IIBAT.

Study design

Fifty mice (25 Male and 25 Female) per group were exposed with UFAOP in nose-only inhalation chambers to targeted concentrations of 0, 50, 100, and 160 mg/m^3 for 6h/day for 5 days. Similarly another four groups of mice were exposed with FAOP to targeted concentrations of 0, 140, 300, and 500 mg/m^3. The concentrations were selected based on the MMAD and GSD values. Post exposure sacrifices commenced on target days 0, 3, 7, 14 and 28. On each sacrifice 10 mice per group were used for lung lavage, lung burden analysis and histopathology. Clinical observations were performed on individual mice before and after exposure. Body weights were recorded twice weekly during the exposure period and once weekly thereafter.

Exposure technique and atmosphere generation.

The test material was dispersed into inhalation chambers using a Wright-Dust-Feeder (BGI, Inc., Waltham, MA). Aerosol concentration was measured gravimetrically by placing membrane filters (Durapore membrane filters, 0.47 µm) with inline filter holders connected with calibrated critical orifices. Aerodynamic particle size distribution was determined by using a Mercer seven stage impactor (In-Tox Products, USA) operated at 0.5 L/min.

Air flows were monitored and controlled by calibrated Rota meters. Temperature, humidity and oxygen concentration at the breathing zone was monitored regularly and recorded continuously.

Broncho alveolar Lavage fluid (BALF)

Broncho alveolar lavage fluid was obtained by lavaging the right lung five times with equal volumes of cold sterile ($Ca2^+$ plus $Mg2^+$)-free PBS (0.15 M, pH 7.2). Approximately 2 ml was harvested from mice, and this was centrifuged at 3000 rpm for 10 min. Cells were spun onto slides using cytofunnels (Shandon, Thermo Corp., Pittsburgh, PA). Cell differential counts were performed on Wright-Giemsa–stained cyto centrifuge slide preparations. Cell viability was determined using haemocytometer and trypan blue dye exclusion. Lactate dehydrogenase (LDH), total protein (TP) and gamma-glutamyl transpeptidase (γ-GT) levels were quantitated spectrophotometrically using a Seimens Dimension Xpand plus fully automated biochemistry analyzer (Siemens, USA).

Lung burden analysis

Aluminum burden analysis of lung was performed according to the method of Levine et al 2003. After necropsy the lung and lymph nodes tissues of rat were collected and stored frozen until the burden analysis. The tissues were thawed, weighed, dried over night in a muffle furnace at 37°C, desiccated and weighed again. Dry tissue samples were digested overnight in a mixture of Nitric acid and Hydrofluoric acid and then further digested in a microwave oven. The sample digests were diluted with deionized water to 25 ml. Samples were analyzed using an Atomic Absorption Spectrophotometer VARIAN Model SPECTRAA 220. Data for lung tissue are expressed as milligrams of UFAOP per gram dry lung. For lymph node tissues, the data are expressed as the total micrograms of UFAOP.

Histopathological examination

Paraffin embedded left lung tissues were sectioned at 5 µm and stained with hematoxylin and eosin (Leica ST5020–Autostainer, Germany) evaluated through light microscopy (Nikon 50i, Japan) and images captured through an image analyzer (Q–Imaging systems, Canada).

Statistical Analysis

Statistical analyses were conducted with NCSS 2007 software. Data were expressed as means ± SD (n=10). Student's t-test was carried out to illustrate the significant difference between the treated groups and the respective control groups and levels of significance were represented for each result.

RESULTS

Characterization of UFAOP and FAOP

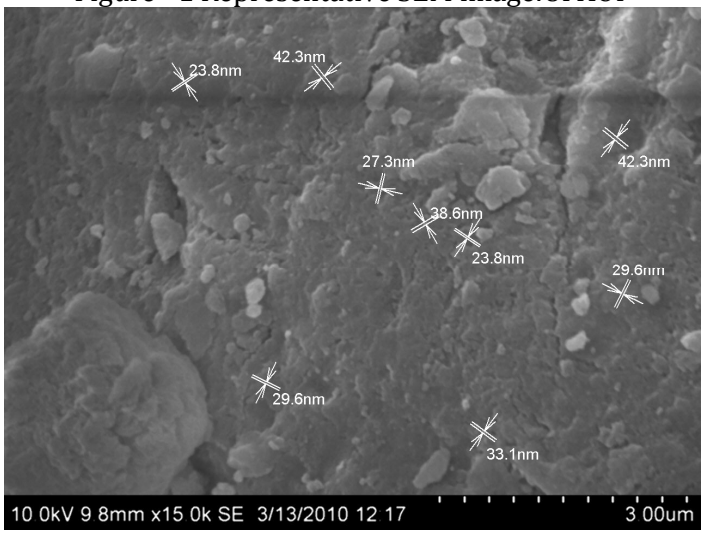
Prior to the conduct of study, characterization of ultrafine aluminum oxide particles was performed using SEM, DLS and Zeta potential methods which provided information on the size and surface charge respectively (Fig. 1&2 and Table 1). The average size obtained in SEM analysis of UFAOP is 29 nm and of FAOP is 133-155 nm. The UFAOP and FAOP are spherical in shape and agglomerated.

The DLS results illustrate the particles in solution do no retain their nano size. The average values in solution were 577.1 nm and 191.8 nm for UFAOP and FAOP with high poly disperse index (PDI) readings. The zeta potential for UFAOP and FAOP were found to be -2.28 mv and -40.6 mv.

Table 1:-Characterization Details of UFAOP and FAOP

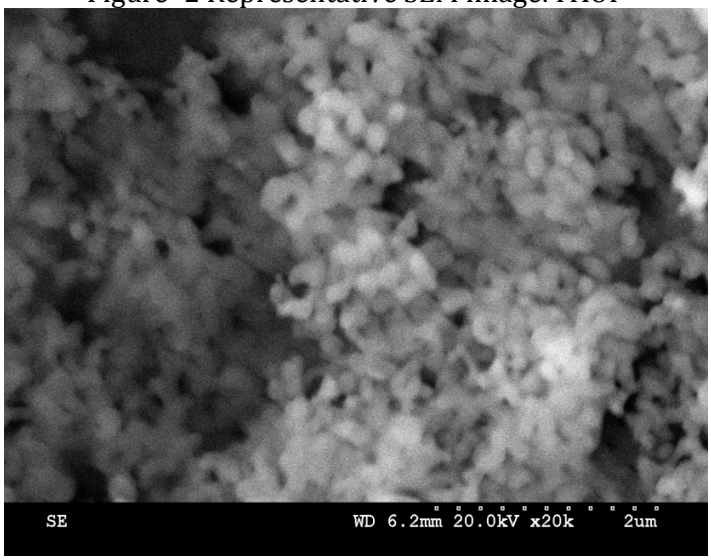
Particle	Particle size measured by SEM	Average size in solution	Polydispersity index (PDI)	Zeta potential	Specific surface area
UFAOP (10 nm)	29 nm	577.1	0.815	-2.28	>160 m^2/g
FAOP (150 nm)	135 -155nm	191.8	0.298	-40.6	5-15 m^2/g

Figure - 1 Representative SEM image:UFAOP



The above photograph is the SEM of Al_2O_3 of 10 nm nanoparticle.

Figure -2 Representative SEM image: FAOP



The above photograph is the SEM of Al_2O_3 of 150 nm nanoparticle

Exposure atmosphere characterization

The mean gravimetric concentrations obtained for UFAOP were 50 ± 0.72, 100 ± 0.65 and 160 ± 1.52 mg/m^3 respectively. The average MMAD values at above concentrations were 3.80 ± 0.2 m, 3.10± 0.22 m and 2.21 ± 0.3 m respectively and the GSD values were 2.80 ± 0.3, 2.22 ± 0.11 and

1.92 ± 0.25. The mean gravimetric concentrations obtained for FAOP were 140 ± 0.54, 300 ± 0.49 and 500 ± 1.64 mg/m^3 respectively. The average MMAD and GSD values at above concentrations were 2.21 ± 0.2 m, 2.12± 0.15 m and 2.42 ± 0.24 m, 2.54 ± 0.4, 2.46 ± 0.19 and 2.13 ± 0.24 respectively.

Broncho alveolar lavage Fluid (BALF) analysis.

Cell viability decreased at day 0 in the mice exposed with UFAOP and FAOP across the groups in both sexes and increased by the end of day 28 post exposure. Significant increase in the total cell count was observed across the groups on all the post exposure days up to day 28 when compared to the control group of mice. Males exposed with FAOP of all the groups showed significant increase where as female showed gradual increase at all the exposure concentrations. The percentage of neutrophils increased significantly in the mice exposed with UFAOP and FAOP on all the post exposure days. Alveolar macrophages showed significant increase upto day 28 post exposure in either sex across the groups of mice exposed with UFAOP and FAOP. Significant increases over corresponding controls of LDH, total protein, gamma glutamyl transpeptidase were measured in BALF of treated mice on all the post exposure days .

Lung Burden Analysis

The amount of lung burden retained for UFAOP and FAOP exposed mice at the end of post exposure across the groups was 23.67, 36.67, 68.33 and 73.67, 150.67, 280.07 per gram lung in males and 25.33, 41.67,65.67 and 81.00, 166.67,307.33 mg per gram lung in female respectively.

Translocation of particles to the draining lymph nodes occurred to a minimum extent following the exposure period. The high concentration group male and female showed 1.1 and 0.9 mg UFAOP and 1.3 and 1.11 mg FAOP on day 28 of post exposure period.

Gross Pathology

Gross pathology examination conducted on all the mice at each sacrifice day did not reveal any gross lesions.

Histopathology

UFAOP treated group mice showed particle-induced lesions that were both concentration and time dependent Fig. 4&5. On microscopic examination none of the organs other than the lung and tracheo bronchial lymph nodes revealed any related toxic response of the UFAOP. In the high concentration group the ultra fine particles appeared as transparent agglomerated particles engulfed by the phagocytic alveolar macrophages in bronchiolar lining epithelium Fig.4A&B. Medullary sinuses of tracheobronchial lymph nodes showed the presence of UFAOP laden macrophages in all the days of post exposure.

FAOP treated group mice showed particle-induced lesions that were both concentration and time dependent Fig.5. In the high concentration group, deposition of particle laden inflammatory cells that are trapped in mucus secreted by bronchiolar epithelium, anucleated macrophages was observed on day 0 of post exposure Fig.5.A. Particle induced focal pulmonary emphysema was also observed Fig.5 B&C. On day 14 and 28 well developed diffused emphysema were observed Fig. 5C. Tracheo-bronchial lymph nodes showed FAOP laden macrophages at day 7 post exposure Fig. 5D. The increased lung burden caused the translocation of particles into lymph nodes was evident by the presence of particle laden macrophages in the lymph nodes. Non-phagocytosed free UFAOP, FAOP were not found in the lung parenchyma at any dose level and dose interval. The other extra pulmonary organs collected and examined did not show any pathological changes or the presence of the particles.

DISCUSSION

The rapid use of nanotechnology in treatments, diagnosis, monitoring, and controlling of biological systems generated concerns related to the potential toxicity of nano compounds to human health [2,9,10,11,12,17] . Increasingly, research is ongoing to investigate on how they interact with biological systems. The purpose of this study is to evaluate and compare the pulmonary toxicity of inhaled UFAOP and FAOP in mice upon exposure for 6h/day for five days.

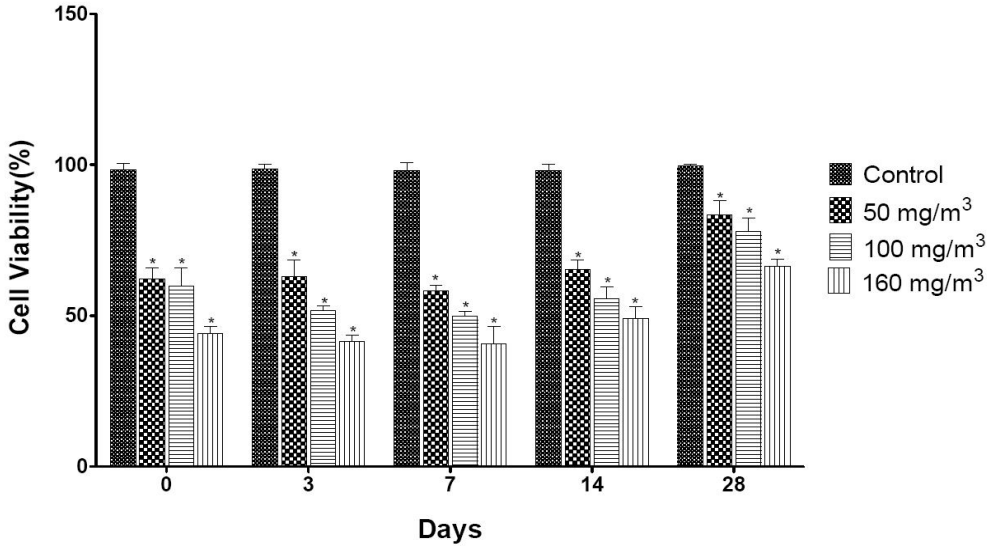
A key element of any nanomaterial toxicity screening strategy is a detailed and comprehensive physicochemical characterization of the test material being studied [8]. This is a critical factor for correlating the nanoparticle surface characteristics with any measured biological/toxicological responses, as well as to provide an adequate reference point for comparing toxicity results[4].

Hence, ultrafine aluminum oxide particles characterization was performed using SEM, DLS and Zeta potential methods to confirm the size and surface charge.

Table 2 :- Clearance Of UFAOP And FAOP From mice Lung During 28 Day Post Exposure

Group	Time after exposure days	Lung clearance					
		male			Female		
		mg deposited	mg cleared	% cleared	mg deposited	mg cleared	% cleared
UFAOP 160 mg/m³	0	86.67	18.34	21.16	91.33	25.66	28.09
	28	68.33			65.67		
FAOP 500 mg/m³	0	344.33	63.66	18.48	302.00	5.33	1.93
	28	280.67			307.33		

A



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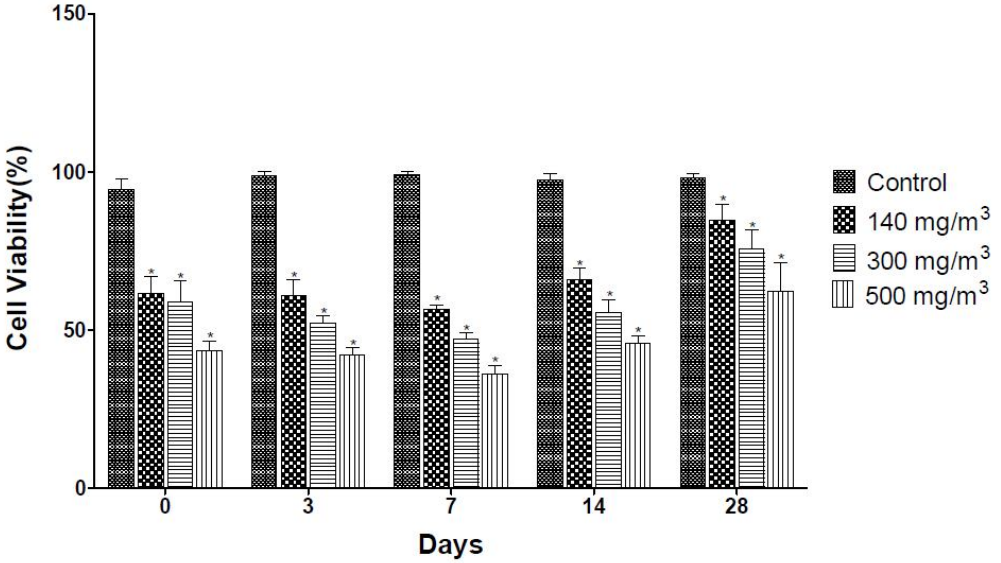
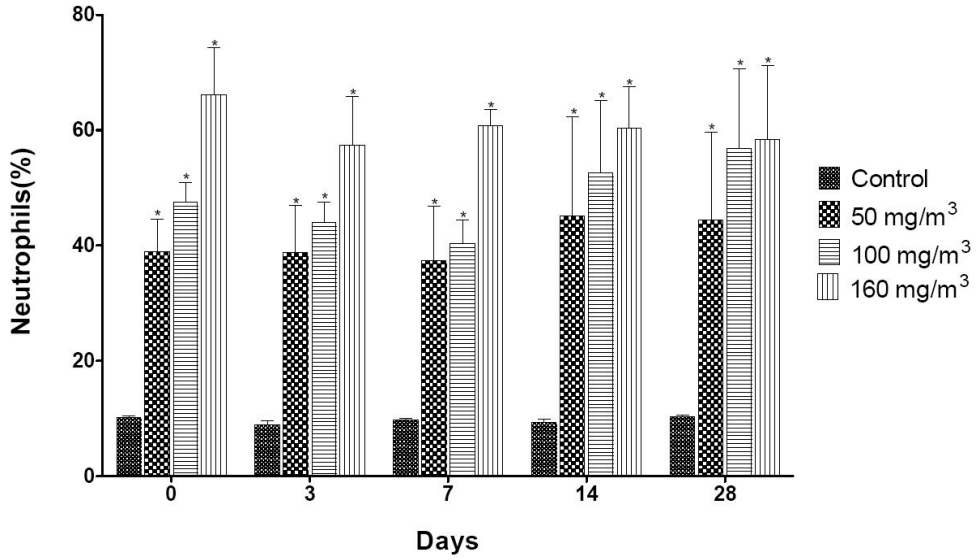


Figure 3a. Changes in cell viability (A-UFAOP; B-FAOP) in BALF of mice at the designated days of post inhalation exposure**.** Values are mean ± S.D (10animals/group). Significantly different from respective control group: (* - $P < 0.05$, ANOVA).

A



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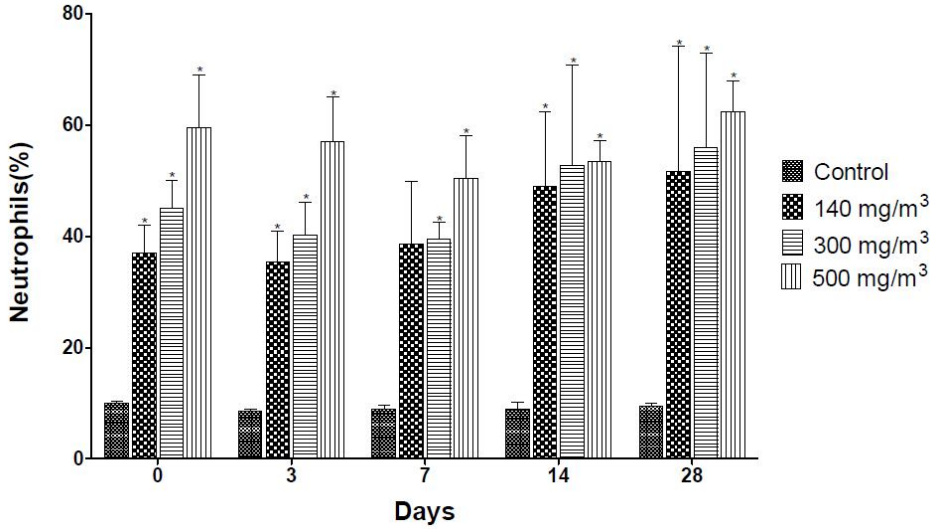
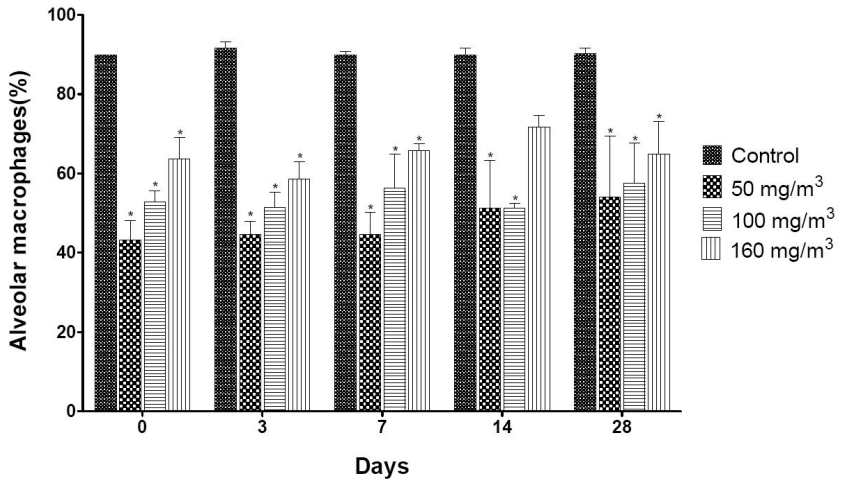


Figure 3 b. Changes in neutrophil percentage (A-UFAOP; B-FAOP) in BALF of mice at the designated days of post inhalation exposure**.** Values are mean ± S.D; 10 animals/group. Significantly different from respective control group. (*-P<0.05, ANOVA).

A



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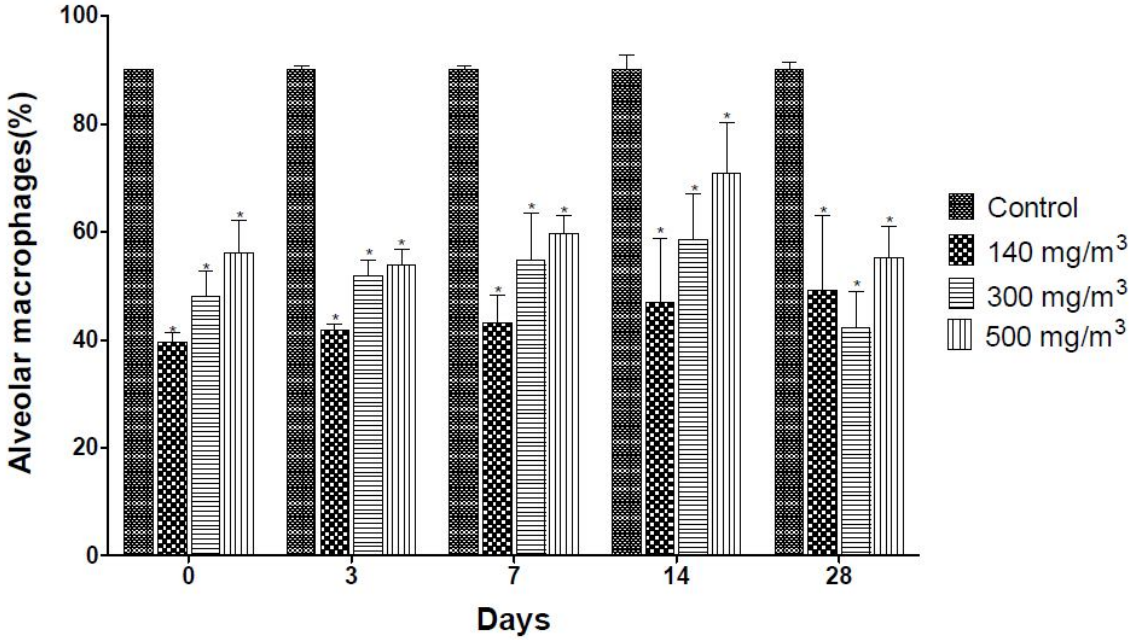


Figure 3 c. Changes in alveolar macrophage percentage (A-UFAOP; B-FAOP) in BALF of mice at the designated days of post inhalation exposure of UFAOP**.** Values are mean ± S.D.; (10 animals/group). Significantly different from respective control group (* - P < 0.05, ANOVA).

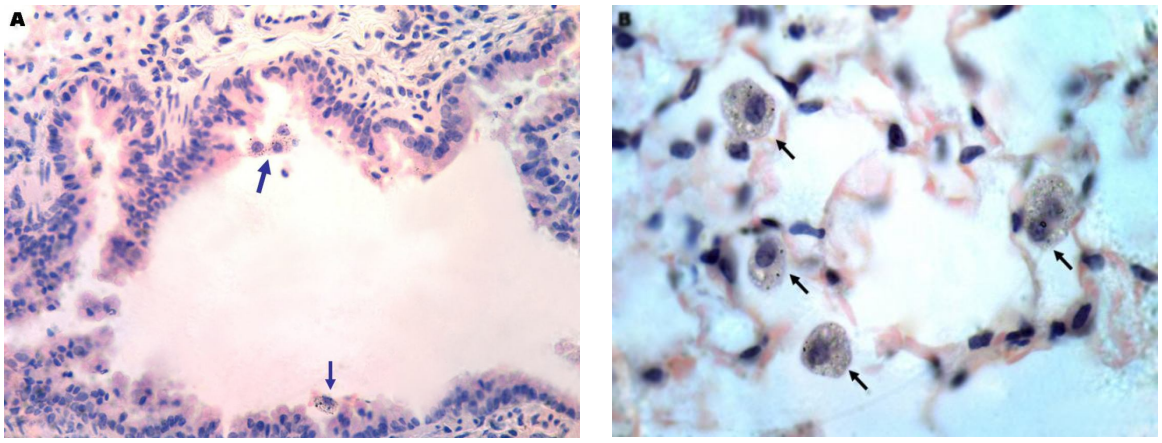
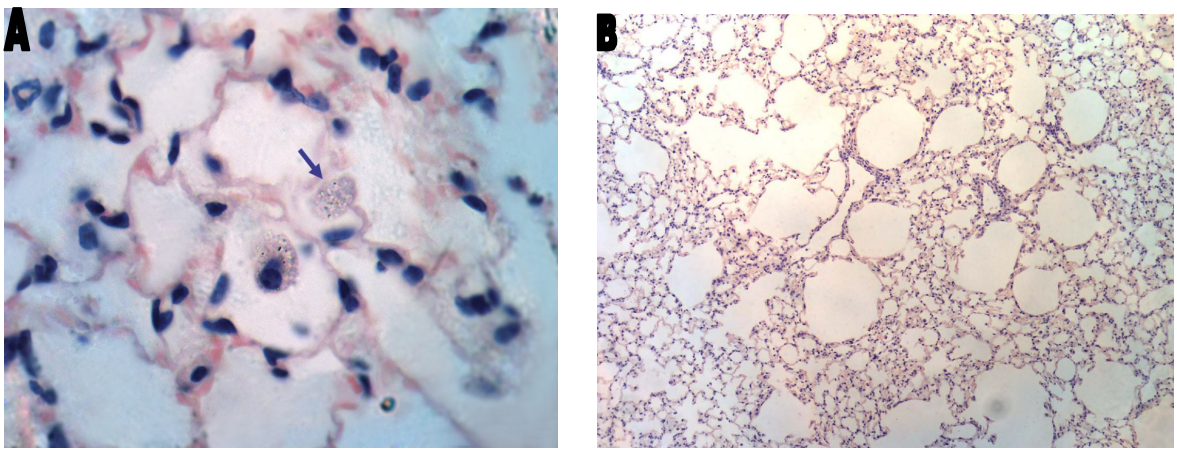


Figure 4. Photomicrograph of mice lung in (A) showing UFAOP laden macrophages in bronchiolar lining epithelium (H&E -400-) (B) UFAOP laden foamy macrophages in alveolar lumen (H&E - 1000-)



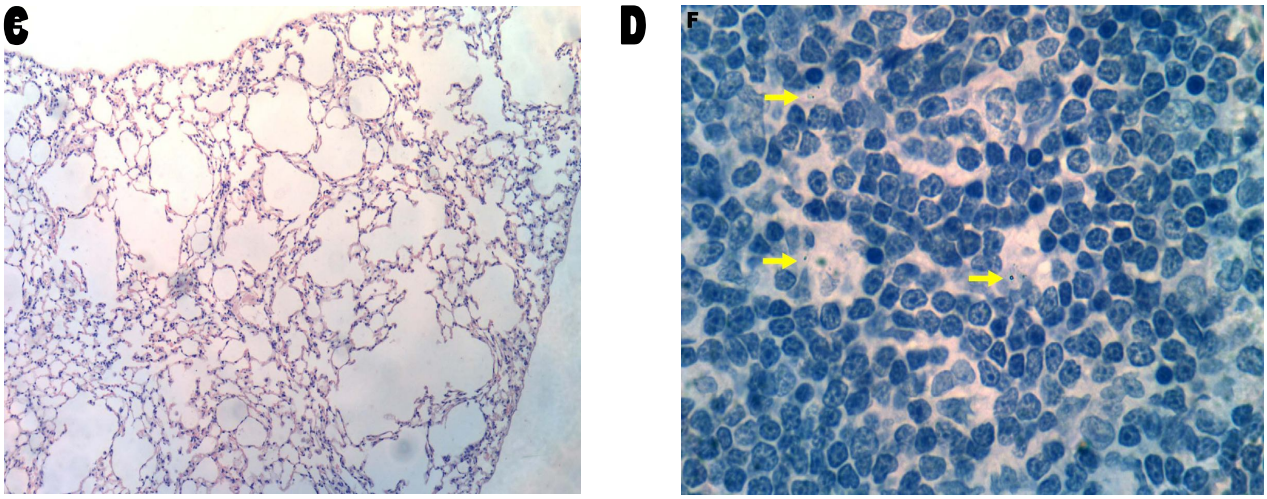


Figure 5. Photomicrograph of mice lung in (A) FAOP laden macrophages in the alveoli of the exposed mice at day 0 post exposure. Arrow points the AMs devoid of nucleus (H&E -400-) (B) Multifocal to diffuse emphysema at day 7 post exposure (H&E -200-) (C) multi focal to diffuse emphysema was observed on day 14 and was consistent up to 28 day post exposure. (H&E -200-) (D) Particle laden macrophages in tracheobronchial lymph node at day 28 post exposure (H&E -1000-).

As the UFAOP have shown agglomeration it was difficult to determine the individual size by SEM. The DLS results illustrate the particles in solution do no retain their nano size. The average values in solution showed a high poly disperse index (PDI) readings. The variation from the original size in the particle was due to the agglomeration of particles. The formation of agglomerates may be due to the particle–particle interactions known as van der Waals forces.

Zeta potential is a physical property which is exhibited by any particle in suspension. As the FAOP showed a larger zeta potential of -46.6 mv they are repelling each other and appeared to more free in the solution, where as the UFAOP showed a value of -2.28 mv there was no force to prevent them coming together and hence agglomerated. The agglomeration is evidenced by the larger size shown in the size measurements by DLS.

In this study different groups of mice were exposed with UFAOP and FAOP to targeted concentrations of 0, 50, 100, 160 mg/m^3 and 0, 140, 300, and 500 mg/m^3 respectively for 6h per day on five days. The concentrations were selected based on the concentration at which the MMAD and GSD are at the respirable region and on the results of 4 week inhalation study with aluminum oxyhydroxide [14,15] and a 13 week sub chronic inhalation toxicity study with ultra fine TiO_2 [1] Post exposure sacrifices were done on day 0, 3, 7, 14 and 28.

To determine the pulmonary inflammation and cytotoxicity BALF analysis was done. Studies in recent past show that ultra fine particles cause inflammation when composed of low toxicity materials. The mechanism of induction of inflammation would be via oxidative stress, Ca^{2+} signaling perturbations [5]. Significant decrease in cell viability in our results of BALF of mice treated with UFAOP and FAOP up to 14 days post inhalation exposure period, suggests that nano particles induced apoptosis. These results were further supported by elevated levels of LDH activity. LDH, a cytoplasmic enzyme is used as a primary cell injury indicator in nano particle exposed animals [16] was elevated significantly throughout the study in all the dose groups. Changes in lung permeability were determined as total protein in the BAL fluid. Our results also showed elevated levels of TP in high concentration group on all the post exposure days indicating an injury to the alveolar epithelial barrier. Repeated exposure of ultra fine aluminum oxy hydroxide caused in the increase in the levels of γ-GT [14]. Increased levels of γ-GT in our results indicate occurrence of epithelial cell damage. The total cell count in BALF was used as a primary indicator of the degree of inflammatory response to nano particle exposure [16]. Our results showed that the TCC levels increased in the high dose group on day 14 and 28 post exposure days as compared to control indicating the degree of inflammatory response stimulated by both UFAOP and FAOP in lungs. Our results suggest that the decrease in the cell viability may be due the cellular

stress caused by the UFAOP and FAOP due to the changes caused in the permeability of alveolar membranes due to the elevation of TP, LDH and γ-GT.

Lung burdens of UFAOP and FAOP increased in a concentration dependent manner. The increased burden is due o the inability of macrophages in clearing the particles. This is evidenced by the increased number of macrophages in the BALF upto 28 day post exposure in high dose groups. Presence of particle laden macrophages in the histological sections of lung upto 28 days post exposure proves the inability of macrophages to clear the particles which in turn lead to the lung burden. The increased burden is supported by the slow elimination of UFAOP by 21.15 % in males and 28.09% for females, similarly 18.49 % in males and 1.93 % in females for FAOP at the end of day 28 post exposure (Table 2). The half-time of poorly soluble particles for alveolar clearance in mice under non-overloading conditions has been reported to be in the range of 50–60 days. Longer elimination half-times occur when the intracellular particle volume exceeds approximately 6% of the phagocytic cell [13]. Aluminum being poorly soluble showed high biological half time. Assuming the zero - order clearance kinetics retention halftimes (biological half time) were predicted to be 66 days and 86 days post exposure for UFAOP and FAOP at concentration levels of 160 mg/m3 and 500 mg/m3. Translocation of particles to the draining lymph nodes occurred to a minimum extent following the exposure period.

Previous studies with ultra fine particles in rats developed particle induced lesions that were time and concentration dependant [1]. Histopathological changes in this study were confined to lung and lymph nodes only. Lungs of Low and intermediate dose exposed groups of UFAOP showed particle laden macrophages. FAOP treated group mice showed particle-induced lesions that were concentration dependant. In the high concentration group exposed mice, deposition of particle laden inflammatory cells that are trapped in mucus secreted by bronchiolar epithelium, anucleated macrophages focal pulmonary emphysema was also observed. Well developed diffused emphysema were observed on day 14 and 28. Less clearance and higher biological half time of FAOP caused the severe lesions of emphysema and inflammation in FAOP treated mice.

In summary repeated exposure of FAOP in mice showed more toxicity than exposure to UFAOP. BALF analysis done on post exposure days showed decrease in cell viability and increase in the neutrophils, Alveolar macrophages up to day 28 post exposure indicating pulmonary inflammation. Lung burden analysis showed a less clearance of FAOP when compared to UFAOP. The biological half time calculated for FAOP is more than the UFOP suggesting its less clearance rate. Lung burden was higher for the FAOP. Histopathological examination of lungs of FAOP treated mice showed inflammation and pulmonary emphysema where as the lungs of mice treated with UFAOP showed only the particle laden macrophages.

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REFERENCES

1. Bermudez E, Mangum J B, Wong BA et al. (2004). Pulmonary responses of mice, rats, and hamsters to sub chronic inhalation of ultra fine titanium dioxide particles. *Toxicol. Sci.*; 77, 347–357.
2. Bianco A, Kostarelos K, Prato M. (2005). Applications of carbon nanotubes in drug delivery. *Curr Opin Chem Biol*.2005; 9:674–679.
3. Chen L, Yokel RA, Hennig B, Toborek M. (2008).Manufactured aluminum oxide nano particles decrease expression of tight junction proteins in brain vasculature. *J Neuroimmune Pharmacol.* 3(4):p286-95.
4. Christie MS, David BW. (2009). Characterization of nanomaterials for toxicity assessment. *John Wiley & Sons, Inc. WIREs Nanomed Nanobiotechnol*. 1 660–670.
5. Donaldson K, Stone V. (2003). Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann Ist Super Sanità*.; 39(3):405-410.
6. Elizabeth O, Nitin C, Vasileios G et al. (2008). Alumina nanoparticles induce expression of endothelial cell adhesion molecules. *Toxicology Letters.*; Volume 178, Issue 3, p160-166
7. Guidance manual for the testing of manufactured nanomaterials: (2009). Organisation for Economic Cooperation and Development's Sponsorship Programme, July 9.
8. Hueiwang AJ, James S. (2006). Toxicity of Metal Oxide Nanoparticles in Mammalian Cells. *Journal of Environmental Science and Health Part A*. 41:2699–2711.
9. Kralj M, Pavelic K. (2003). Medicine on a small scale. *EMBO Rep.* 4: 1008–1012.
10. Kreuter J. (2007).Nanoparticles—a historical perspective. *Int J Pharm*. 331:1–10.
11. Maynard AD, Aitken RJ, Butz T et al. (2006).Safe handling of nanotechnology. *Nature*; 444:267–269.

12. Moghimi SM, Hunter AC, Murray JC. (2005). Nanomedicine: current status and future prospects. *FASEB J*.19:311–330.
13. Morrow, P. 91988). Possible mechanism to explain dust overloading of the lungs. Fundam Appl Toxicol. **10:**369-384.
14. Pauluhn J. (2009). Pulmonary Toxicity and Fate of Agglomerated 10 and 40 nm Aluminum Oxy hydroxides following 4-Week Inhalation Exposure of Rats: Toxic Effects are determined by Agglomerated, not Primary Particle Size. *Toxicological Sciences*.109(1):p152-167.
15. Pauluhn J. (2009). Retrospective analysis of 4-week inhalation studies in rats with focus on fate and pulmonary toxicity of two nanosized aluminum oxyhydroxides (boehmite) and pigment-grade iron oxide (magnetite): the key metric of dose is particle mass and not particle surface area. *Toxicology*. 259(3): p140-8.
16. Renwick LC, Brown D, Clouter A, Donaldson K.(2004). Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle type. *Occupational Environmental Medicine*. 61: p442–447.
17. Service RF. (2004). Nanotoxicology. Nanotechnology grows up. *Science*. 2304:1732– 1734.
18. Uwatoku T, Shimokawa H, Abe K et al.(2003). Application of nanoparticle technology for the prevention of restenosis after balloon injury in rats. *CircRes*. 92:e62–e69.
19. Zhang QL, Li MQ, Ji JW et al.(2011). *In vivo* toxicity of nano-alumina on mice neurobehavioral profiles and the potential mechanisms. *Int J Immunopathol Pharmacol.*; Jan-Mar; 24 (1 Suppl):23S-29S

Conflict of Interest

None