



## Urban particulate air pollution induces lung inflammation in albino mice

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The present study was designed to evaluate urban air pollution status and the effects of fine particulate matter (PM<sub>2.5</sub>) on mice lung inflammation under controlled exposure conditions. Three weeks old mice were exposed six hours per day via whole-body inhalation of suspended particulate matter (SPM) and grouped according to different exposure durations of 5, 15, 21, 30 and 90 days. Particle characterization showed that SPM sampled in urban traffic area are rich in PM<sub>2.5</sub> and contain 50 to 60% black carbon, adequate amounts of silica and other metal elements. Bronchial biopsies studies have demonstrated that using bronchoalveolar lavage the elevated expressions of inflammatory mediators such as neutrophils, eosinophils, mast cells, monocytes and lymphocytes were found in the respiratory tract of mice after exposure to SPM. Experimental evidence of lung inflammation has been shown with the increase of SPM exposure. Our data supports the concept that levels of PM<sub>2.5</sub> may increase the risk of developing pulmonary injury.

### INTRODUCTION

A new study of global air pollution shows that India is now surpassing China as the deadliest in the world. The number of premature deaths in China caused by dangerous air particles has stabilized globally in recent years but has risen sharply in India. India's rapidly worsening air pollution is causing about 1.1 million people to die prematurely each year, according to the report issued by an international collaboration of the Indian Institute of Technology (Bombay), the Health Effects Institute (Boston) and the Institute of Health Metrics and Evaluation (Seattle).<sup>1</sup> India has registered an alarming increase of nearly 50 percent in premature deaths from particulate matter (PM) between 1990 and 2015.

Prolonged exposure to urban particulate air pollution was associated with significant increase in lung cancer mortality.<sup>2</sup> Toxicity effects of airborne PM can be explained by the carcinogenic mutagenic polycyclic aromatic hydrocarbons (PAHs) adsorbed to its surface. Inhalable fine PM were distinguished by their larger surface areas which may have more amount of inert toxic substances.<sup>3</sup> Other important concerns are the oxidative damage of DNA caused by metals and/or benzene attached in the PM and the inflammation induced by airborne particles.<sup>2,4</sup>

Another study has previously showed that chronic exposure to air pollution promotes a higher number of lung tumor nodules in mice.<sup>5</sup> Here the objective was to evaluate the effects of urban, traffic-related particulate matter with size of 2.5 micron in aerodynamic diameter (PM<sub>2.5</sub>) on mice lung inflammation under controlled exposure

conditions. The present study also aimed to investigate the physical and chemical properties of PM, such as size and elemental composition, which may have worse effects on lung injury.

### MATERIALS AND METHODS

#### The Study Area

Mysore city is one of the largest districts in the state of Karnataka. It is located at 135 km south of Bangalore metropolitan city and lies at 12° 18' 25" N latitude and 76° 38' 58" E longitude. Altitude of the city is 765 m above mean sea level. The city is well connected to the neighbouring states of Kerala and Tamil Nadu through road transport and rail network. Mysore city has a warm, cool and salubrious climate throughout the year with temperature range from 15 to 35°C. Mysore gets most of its rain during the monsoon between June and September with an annual average of 782 mm.<sup>6</sup>

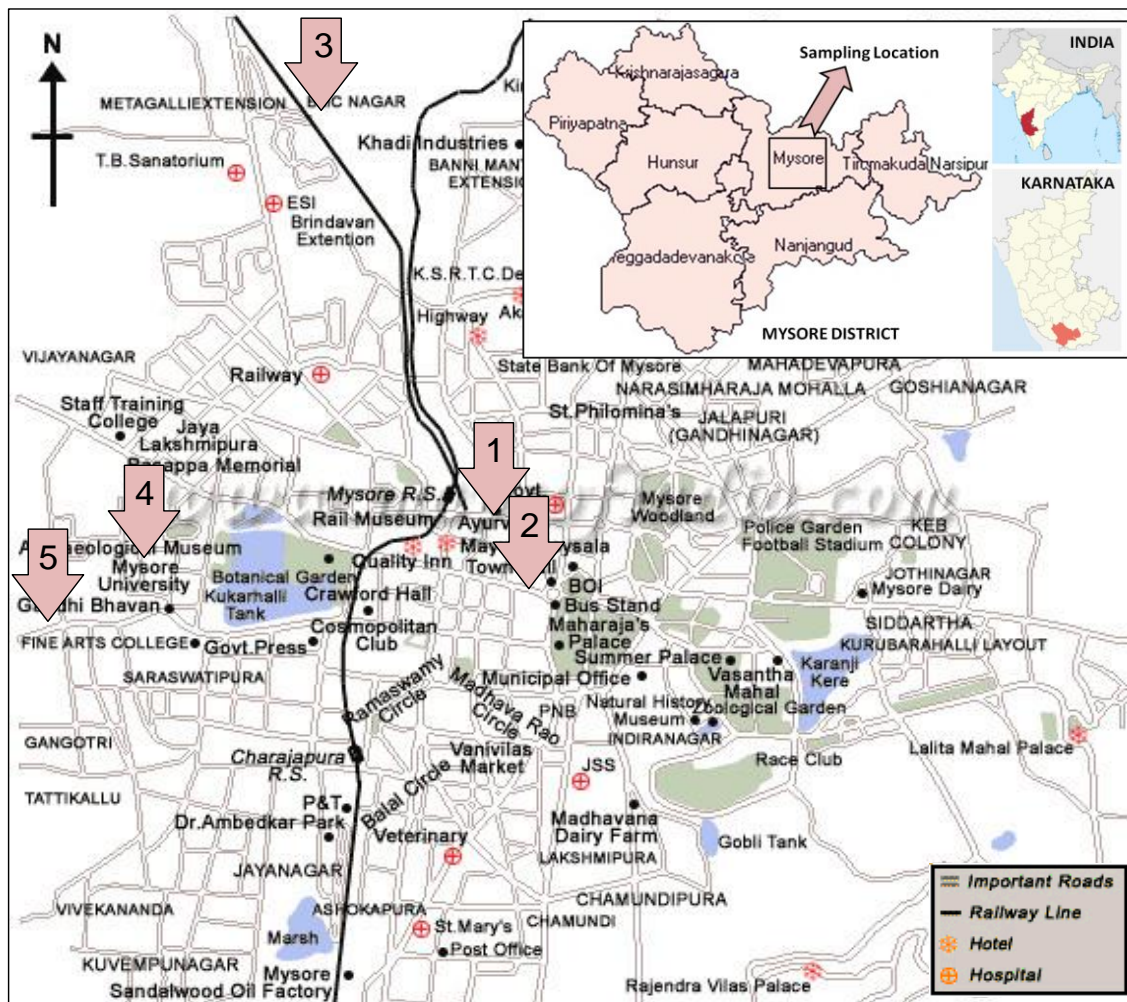
Like many other Indian cities, Mysore city has also a high vehicular growth and emissions problem, particularly PM. It had over 523 thousand vehicles registered in the city in 2015 and is projected to expand about 120% in 2020.<sup>7</sup> Irwin Road (Site 1) and Gandhi Square (Site 2) are the highly congested traffic lanes which are near the KSRTC bus stand in Mysore city and have been perfectly selected as study site for PM collection followed by moderate traffic area of Metagalli industrial estate (Site 3) and low traffic area of Mysore University campus (Site 4) and Vijayanagara residential area (Site 5) (Fig. 1).

#### PM Analysis

The particulate matter was collected with a vacuum air sampler at the exposure site during different seasons. The characteristics of the suspended particulates were then analyzed according to their physical (size distribution) and chemical (elemental composition) properties. Particle size distribution was determined using Dynamic Light Scattering (DLS) method. The technique measures the random changes

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**Figure 1** Study area consisting of five sampling sites of different traffic characteristics

in the intensity of light scattered from a suspension. Elemental composition of PM was analyzed using Energy Dispersive X-Ray (EDX) method. The data generated by EDX consists of spectra showing peaks corresponding to the elements. The height of the peaks represents relative abundance of X-rays emitted by the elements that indicating the abundance of such element present in the sample.

### Animal Model

The mice received care in compliance with the “Laboratory Animal Care” formulated by the ethical committee at the University of Mysore and in the “Guiding Principles in the Care and Use of Animals” approved by our Institutional Animal Care and Ethical Committee. Sixty ( $n=60$ ), 3-week-old mice (body weight 8-14 g) were assigned to five groups. This protocol was conducted for a period of 90 days in different intervals of five days a week. Animals were fed ad libitum with water and commercial pellet food. This study was approved by the Ethics Committee for Research of the Mysore University at the number UOM/IAEC/05/2013.

### Animal Exposure

Exposures were performed from February to August 2015 using the inhalation chambers installed on a busy traffic road of Mysore city. The exposure chamber consisted of cuboidal plastic structures measuring 37 cm (length) x 26 cm (width) x 23 cm (height). Air was forced into the chamber from the top, uniformly distributed throughout the chamber,

and finally exited at the bottom where there was opening. Two stages of filters were in line; the first (cotton bag filter) eliminated large particles and the second (plain screen filter) trapped fine particles.

Three-week-old mice were exposed via whole-body inhalation to suspended particulate matter in different intervals 6 hr/d for 5,15,21,30 and 90 days, respectively. The average total air pressure concentration was maintained at 25 L/m. Eight animals were included in each group and two sets of exposure for each group were performed; control animals were kept at the laboratory. Following exposure, samples of bronchoalveolar lavage (BAL) and lung tissues were collected for analysis.

### BAL Fluid Analysis

Bronchoalveolar lavage (BAL) of lungs was performed on half of the mice from each study group. Immediately after the respiratory mechanics assessment, BAL was performed by introducing 1 mL sterile phosphate-buffered saline (PBS) into the lungs via a tracheal cannula, and the recovered fluid was kept in a test tube on ice. This procedure was repeated 3 times. The fluid collected was centrifuged at 1000 rpm for 10 minutes at 5°C to separate cells from the supernatant. The cell pellet was resuspended in 300  $\mu$ l PBS. A volume of 100  $\mu$ l of resuspended pellet was removed and stored in an Eppendorf tube with 100  $\mu$ l PBS. Total cells were counted using an improved Neubauer’s haemocytometer chamber and an optical microscope with a 40 $\times$  zoom. Preparation of BAL was done following Harrod’s protocol with some

modifications.<sup>8</sup> Within 2 hr following the end of particle exposure (5, 15, 21, 30 and 90 days) mice were anesthetized using chloroform. Total cell count and viability were determined by Neubauer's haemocytometer. A minimum of  $10^6$  cells was used to prepare slides in duplicates by cytopspin of 100  $\mu$ l cell suspension at 1000 rpm for 5 min. Cells were stained with Giemsa to determine the proportion of macrophages, monocytes, lymphocytes, and neutrophils.

### Histology and Histopathology

The left lungs were fixed by intratracheal instillation of formalin (10% formaldehyde in 2% buffer) at a pressure of 20 cmH<sub>2</sub>O for 24 h. Longitudinal lung sections were paraffin embedded and 5-mm-thick histological sections were stained with haematoxylin and eosin for qualitative and morphometric analyses. Histological slides were coded for blind analysis. Morphometric measurements were also performed by the same observer.

### Statistical Analysis

Data of BAL fluid analysis are presented as mean  $\pm$  standard error (SE) per each group. Comparison between body weight of control and of treated mice was performed using regression analysis from SPSS version 16. The level of significance was set at 5%.

## RESULTS

### Air Pollution Status

The ambient air quality of Mysore city has been continuously monitored by the Karnataka State Pollution Control Board (KSPCB) as depicted in Figure 2. The figure shows that SO<sub>2</sub> and NO<sub>2</sub> concentrations were observed within the permissible limits of 50 and 40  $\mu$ g/m<sup>3</sup>, respectively, and there were no much variation in the monthly concentrations. Only PM<sub>10</sub> concentration showed undulating trend and at some particular events closed to or even exceeded the national standard that stands at 60  $\mu$ g/m<sup>3</sup>. However, if referred to the updated WHO guidelines<sup>9</sup> that require PM<sub>10</sub> at 20  $\mu$ g/m<sup>3</sup>, the PM status of Mysore may cross far away from the allowable limit.

Higher concern arises on PM air quality status due to it has high annual and seasonal variation as well as crucial quality level compared to the allowable standards. Our previous study<sup>10</sup> obtained important findings that ambient PM concentration was considerably correlated with outdoor temperature, and was significantly correlated with traffic density and estimated vehicular emissions of PM<sub>10</sub> and PM<sub>2.5</sub>.

### Particle Characterization

The results from DLS analysis showed that ambient particulates entrapped in the sampling chamber were fine particles (PM<sub>2.5</sub>) with a size less than 2.5  $\mu$ m or 2500 nm.<sup>10</sup> About 90% of particles were sized above 200 nm (Fig. 3). The larger the sizes, the less percentage of particles were identified. The size was certainly higher for particles in ultrafine category or PM<sub>0.1</sub> (size <100 nm) and much lower for PM<sub>10</sub> category (size 2.5 to 10  $\mu$ m). The EDX analysis showed that roadside PM collected on summer and monsoon season mainly consists of carbon (C) about 56.38% and 58.13%, respectively.<sup>10</sup> Other metal and metalloid elements such as Si, Fe, K, Ca, Na, Mg, Al, etc. present in a smaller fraction (Fig. 4). In nature, these elements may present as single elements like C, Fe and Al, or as chemical compounds in combination of different elements, such as Si presents as silica, Fe as rust, Al as alumina, Ca as lime or gypsum, Na and K present as marine salts, or other possible forms of compounds. Ambient particles rich in black

carbon indicated that sources of the airborne particles entrapped in the sampler are mainly generated from fossil fuelled vehicles which often emit carbonaceous materials as waste products of fuel combustion.

### Effects of SPM Exposure on Body Weight

After 2-month exposure to air pollution, the treated mice either males or females have higher increase in body weight about two grams than the control. Continued to the next month, the treated animals still weigh higher than the control, but the treated males were heavier than the females (Fig. 5) due to the higher food consumption of males as compared to the females which is normal to most of the mammals and humans. High degree of stress in polluted and noisy environment had made the treated animals to have higher appetite than the untreated ones.<sup>11</sup>

### Effects of SPM Exposure on Respiratory System

Biochemical parameters of BAL fluid on mice after PM<sub>2.5</sub> exposure at urban traffic areas in Mysore city for 5, 15, 21, 30 and 90 days are presented in Table 1. There was reduction of BAL lymphocyte after 5, 15, and 21 days exposure. Inflammation has elevated the expression of neutrophils, monocytes, and eosinophils compared to control. Percentage viability of leucocytes has decreased and differential cell of macrophages (98.10 $\pm$ 1.37) and neutrophils (1.34 $\pm$ 0.17) were increased following mice exposure to PM for 90 days. The total cell concentration in the BALF increased after exposure to PM compared to control.

### Histopathology of Mice Lungs

The morphologic lesions in lung of mice exhibits allergic bronchiolitis and epithelial lesions were more in proximal bronchioles (Fig. 6B) compared to distal terminal bronchioles was characterized by peribronchiolar edema associated with inflammatory cell influx of eosinophils, mast cells. Peribronchiolar inflammation was located in the subepithelial interstitial tissues (e.g. lamina propria and submucosa) on the surface epithelium (Fig. 6C). In 30 days exposed mice lung shows a mucous cell metaplasia with increased amount of mucus substances on the surface epithelium (i.e. intraepithelial mucus substances) including the proximal and distal axial airways (Fig. 6D) in compared to the control mice. There was no significant difference in the amount of intraepithelial mucus substances between 5, 15 days treated and control mice (Fig. 6A). The lung parenchyma of mice exposed to 90 days was characterized by accumulations of large numbers of alveolar macrophages with lesser numbers of lymphocytes cells, in the alveolar airspace (Table 1).

The alveolar septa in the areas of alveolitis were thickened due to type II pneumocyte hyperplasia, accumulation of inflammatory cells followed by capillary congestion (Fig. 7A and B). The pathological characteristics of the exposed mice were observed: an extensive bronchial-associated lymphoid tissue (BALT) at many bifurcations of the airways, and a minimal to moderate perivascular infiltrate of lymphocytes. Small to large alveolar foci were seen in septa with increased cellularity and an influx of alveolar macrophages. It is noticeable in many experimental animals that small haemorrhages were present in alveolar spaces, followed by a minimal to moderate inflammatory foci, with interstitial pneumonia and alveolitis, consisting of macrophages and some neutrophilic granulocytes (Fig. 7C and D).

## DISCUSSION

The current status of air pollution in Mysore city is still showing safe level in terms of SO<sub>2</sub> and NO<sub>2</sub>, but an alarming level was found in the

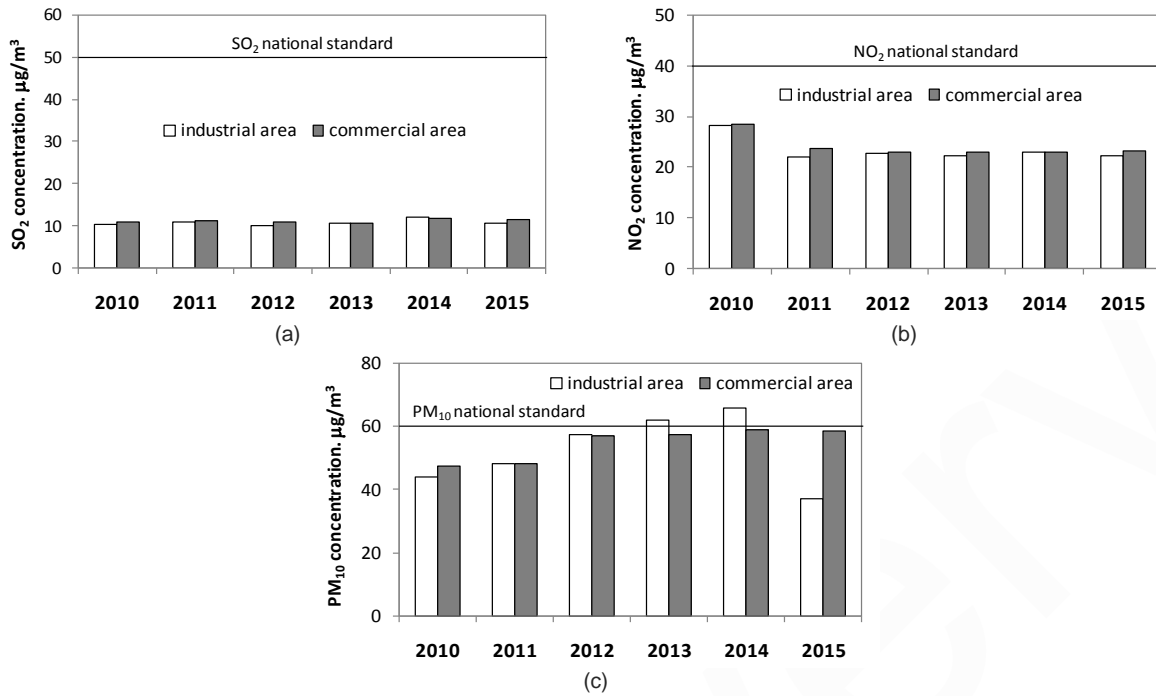


Figure 2 Annual average concentration of (a) SO<sub>2</sub> (b) NO<sub>2</sub> (c) PM<sub>10</sub> compared to the National Ambient Air Quality Standards

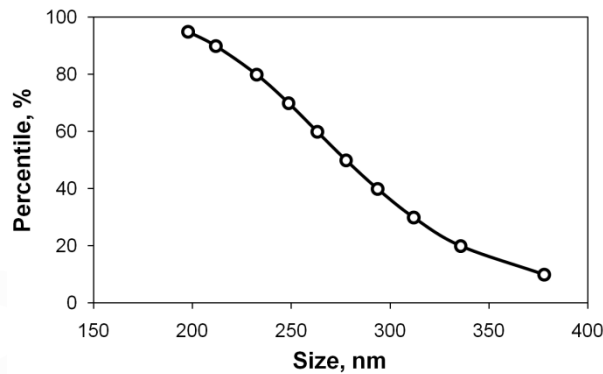


Figure 3 Size distribution of urban traffic PM

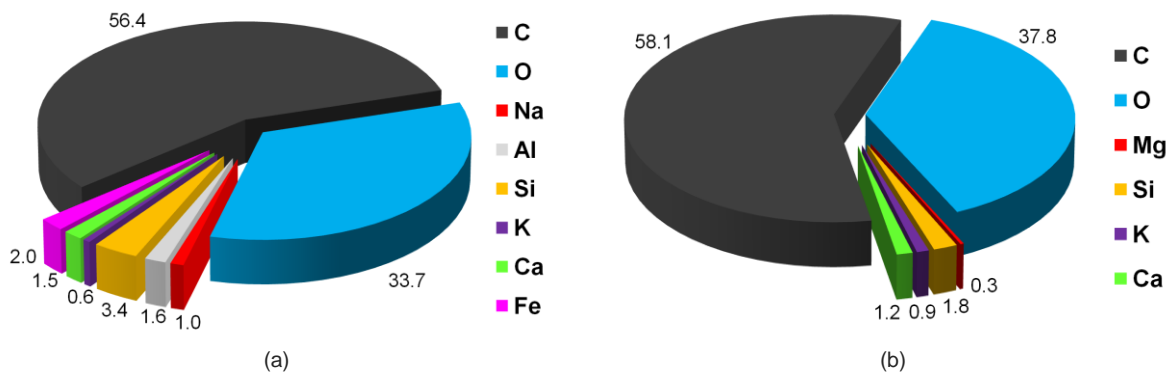
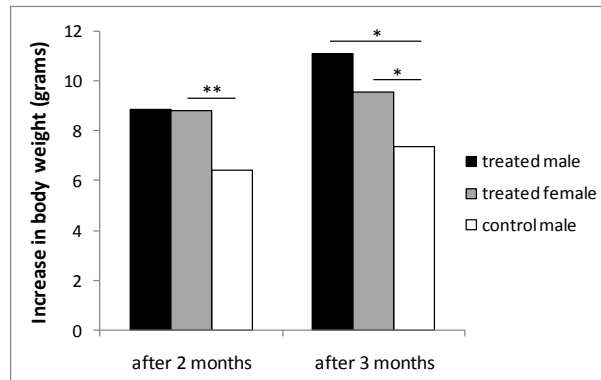


Figure 4 Elemental composition (%wt.) of particulate matter collected on (a) summer and (b) monsoon season

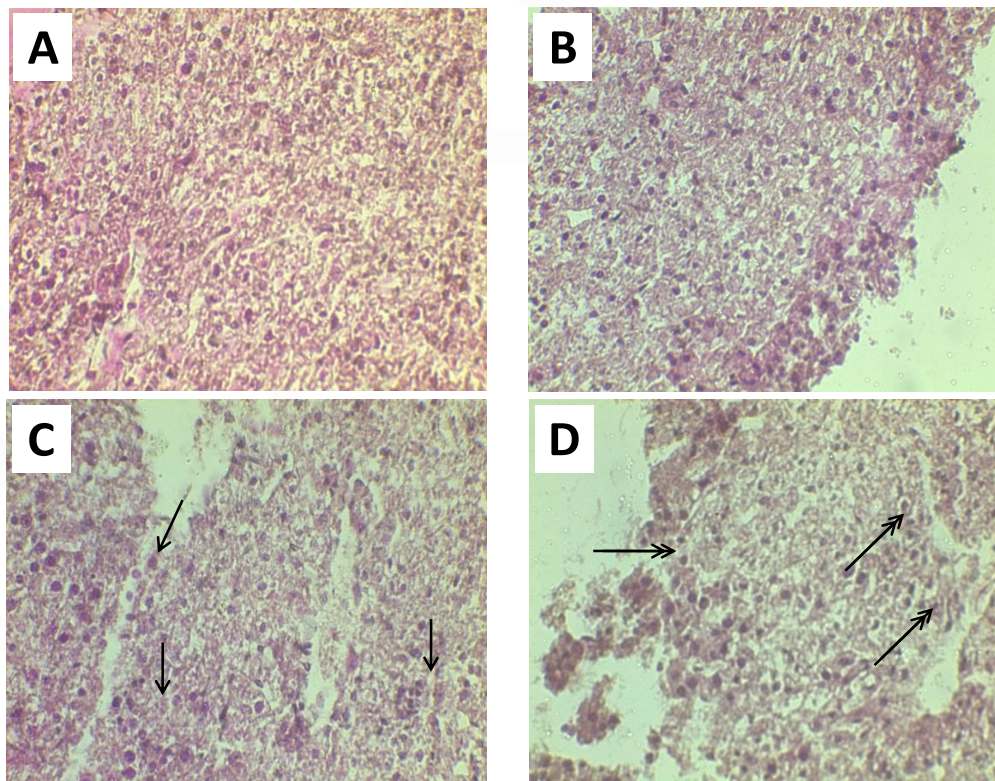


**Figure 5** Increase in mice body weight after exposure to PM as compared to control. \*  $p < 0.05$ , \*\*  $p < 0.01$

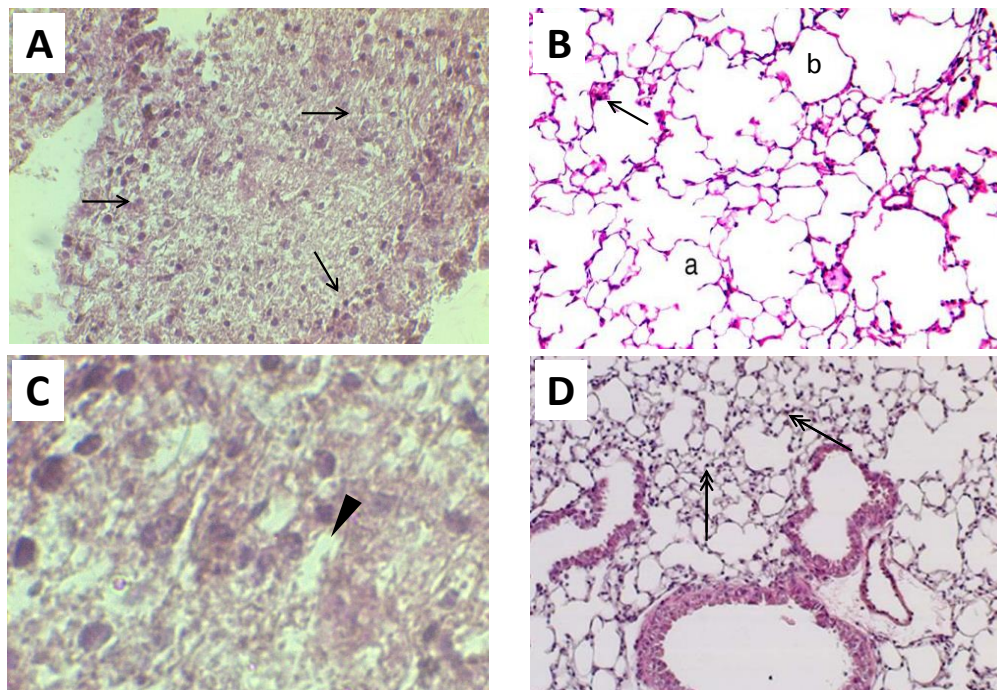
**Table 1** Changes in cell number, viability and differential cell count\* in BAL fluid of mice following exposure to PM at different intervals

Biochemical parameters	Control	5 days	15 days	21 days	30 days	90 days
Cells/ml, $\times 10^6$	2.24 $\pm$ 0.28	3.12 $\pm$ 0.31	2.51 $\pm$ 0.11	3.14 $\pm$ 0.23	4.70 $\pm$ 0.43	7.56 $\pm$ 2.47
Cell viability, %	82.24 $\pm$ 11.10	80.74 $\pm$ 20.32	79.43 $\pm$ 23.01	88.23 $\pm$ 39.22	70.25 $\pm$ 72.98	65.54 $\pm$ 28.22
Macrophages, %	96.46 $\pm$ 2.25	95.88 $\pm$ 3.23	98.73 $\pm$ 1.23	95.46 $\pm$ 3.21	97.12 $\pm$ 4.25	98.10 $\pm$ 1.37
Lymphocytes, %	2.34 $\pm$ 0.41	1.28 $\pm$ 0.27	0.62 $\pm$ 0.24	0.34 $\pm$ 0.21	0.64 $\pm$ 0.16	0.61 $\pm$ 3.10
Neutrophils, %	0.33 $\pm$ 0.23	1.09 $\pm$ 0.26	1.02 $\pm$ 0.17	1.97 $\pm$ 0.23	1.03 $\pm$ 0.10	1.34 $\pm$ 0.17
Eosinophils, %	0.03 $\pm$ 0.13	0.18 $\pm$ 0.17	0.33 $\pm$ 0.13	0.93 $\pm$ 0.42	0.79 $\pm$ 0.33	0.23 $\pm$ 0.14
Mast cells, %	0.20 $\pm$ 0.10	0.14 $\pm$ 0.13	0.03 $\pm$ 0.13	1.73 $\pm$ 0.77	0.43 $\pm$ 0.22	0.03 $\pm$ 0.13

\* Values are mean  $\pm$  S.E. (n=8 per group)



**Figure 6** The photomicrograph of the respiratory epithelium (B) lining the proximal axial airways in the left lobe of control mice lung. Significant morphologic changes are present in mice after 21 and 30 days exposure (C and D). The most prominent histological changes due to particulate matter exposure included a thickened, hypertrophic, respiratory epithelium with increased numbers of mucous goblet cells, and a mixed inflammatory cell infiltrate (arrows) consisting of scattered eosinophils (double arrows) in the interstitium of the airway followed by vacuolization compared to the normal airway in the control mice (A). All tissues are stained with Hematoxylin and Eosin.  $\times 400$



**Figure 7** Histological distal lung samples (A) of 30 days exposed mice showing sparse, mild foci of macrophages (arrows) represents alveolar parenchyma. Lung parenchyma (B) of 90 days exposed mice showing the alveolar spaces that are enlarged, hypertrophy and irregular (a and b) when compared with control as result of incomplete alveolization (arrow). The mild foci of macrophage accumulations occurred followed by predominant vacuoles (arrow head) in the alveolar areas (C). Accumulation of particles within alveolar macrophages was readily apparent in lung parenchyma and in lymph nodes (C and D). It appeared that there were more particle laden macrophages (double arrows) found in lymph nodes (D) of 90 days exposed mice in comparison to control. All tissues are stained with Hematoxylin and Eosin.  $\times 400$  and  $\times 1,000$

concentration of PM. Therefore, the present study was focused only to identify the effects of PM on health aspects, particularly on respiratory system. Previous characterization of particle mass collected at the near-roadway environment carried out by combining EDX and DLS methods has shown that vehicular emissions and crust resuspension are the major  $PM_{2.5}$  components at this site.

The BALF parameters were statistically and significantly different in the overall analysis of the experiments. In addition, visible inclusions of PM in macrophages as well as increased neutrophils suggest that PM were deposited in the lungs of mice.<sup>12</sup> It was demonstrated that SPM significantly induced pulmonary cytotoxicity as indicated by differential count variations in BAL fluid, and that these continued for days after exposure.

In this study we have demonstrated that chronic exposure to  $PM_{2.5}$  particles trigger alterations in lung structure of the alveolar parenchyma, associated with cell inflammation. Particle retention in lung tissue results in a chronic, low-grade inflammatory response that may be pathogenetically important in the progression of lung disease. It is possible that longer exposures could have a more significant impact on lung mechanics or remodeling.<sup>13</sup>

The results suggest that increased exposure to  $PM_{2.5}$  may increase inflammation-induced changes in cell number, viability and differential cell count in BAL fluid. As such, mice exposed to urban, traffic-related  $PM_{2.5}$  during a 3-month period developed more lung injury and cell viability than those exposed to shorter duration. Although there is epidemiological evidence of an association between urban air pollution and lung tumorigenesis in urethane treated mice,<sup>5</sup> while in our present study it shows association between ambient levels of urban, traffic-related  $PM_{2.5}$  and lung injury development.

The study reports inflammatory effects of ambient concentration of air pollutants at selected urban city Mysore, in levels that are similar to

those observed in several cities across the globe. Our data support the concept that low levels of  $PM_{2.5}$  for one- and three-month exposures to mice may increase the risk of developing lung injury. Although we have not studied the specific components of the PM involved in inflammatory responses or the mechanisms related to this process, it is that, the specific influence of  $PM_{2.5}$  obtained from Mysore city's air pollution was related to lung injury promotion using a very simple experimental model assessing the relationship between lung inflammation and air pollution.

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