

The Histochemistry of Particulate Emissions on the Lung Tissues of Albino Mice

Joshua Kibet ^{1*}, Josephate Bosire ¹, Thomas Kinyanjui ¹, Benson Githaiga ¹

¹ Egerton University, KENYA

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ABSTRACT

Background: Particulate emissions produced from the combustion of diesel, tyre burning and forest fire is known to contain organic toxicants and a variety of reactive radical species which may cause serious respiratory health problems such as asthma.

Materials and methods: The particulate emissions from tyre burning, vehicular exhaust, and simulated forest fire were exposed to an inhalation cage. To simulate environmental exposure conditions, 12-week old albino mice were exposed to particulate emissions at a rate of $\sim 250 \mu\text{g m}^{-3}\text{day}^{-1}$ and their lung tissues were extracted for bioassay analyses. Comparisons were made between the lung tissues of mice exposed to the three types of particulate emissions, and the control mouse in order to determine the biological impact of particulates on the functioning of the lung tissues.

Results: Accordingly, there was swelling and shrinking of lung tissue cells as a result of exposure to tyre and diesel exhaust particulate emissions which caused disconnection of tissues and damage to the blood capillaries within the lung alveoli. **Conclusion:** Simulated forest fire particulates caused minimum damage to the lung tissues whereas particulate emissions from diesel and tyre caused grave damage to the lung system of the mice.

Keywords: lungs, histochemistry, particulate emissions, toxicity

INTRODUCTION

The principal focus of this investigation is to examine the respiratory injury of the lung tissues of albino mice as a result of exposure to particulate emissions from various combustion sources. Particulate matter is defined as an aggregate of small particulates, liquid droplets, and vapors that have been linked to respiratory and cardiovascular morbidity and mortality in susceptible populations (Lippmann and Schlesinger, 2002). Ambient particulate matter (PM) of various particle sizes from different sources has been found to originate from a number of human activities (i.e. tire destruction, diesel combustion and forest fire) and natural sources (Kelly and Fussell, 2012). During incomplete combustion under air-limited conditions at moderately high temperatures ($\sim 800\text{-}1000^\circ\text{C}$), PM emissions are more dominated by solid carbon aggregates (soot) (Gwaze et al., 2006). The adverse health effects of inhaled particles are highly dependent on the deposition and retention of particles in the lung (Kreyling et al., 2006). The deposition probability and deposition site of particles are governed by their aerodynamic properties, such as size, density, and shape, but also by other physicochemical properties such as hygroscopicity (i.e. water uptake) (Bølling et al., 2009). Experimental studies have identified a range of physicochemical properties that influence the toxic and inflammatory potential of PM, and possibly particle-induced health effects (Schwarze et al., 2006).

Particulate emissions from combustion of the tire, diesel, and other petroleum fuels is a dominant contributor to (PM_{2.5}) and (PM₁₀) particulates and contains emissions of carbonaceous particles with fused and free polycyclic aromatic hydrocarbons (PAHs) (Xi and Zhong, 2006). These fuels generate long chain hydrocarbons which further undergo degradation to generate free radicals which are considered biologically hazardous. PAH and long chain hydrocarbons metabolism in the body generates electrophilic and reactive metabolites that have been indicated as inducers of pulmonary cytochrome P450 s in diesel exhaust particles (Murphy Jr et al., 2008). Furthermore, ambient soot particulates contain persistent free radicals and reactive oxygen species (ROS) implicated in the generation of

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✉ jkibet@egerton.ac.ke (*Correspondence) ✉ bosire27@gmail.com ✉ tkinyanjui@egerton.ac.ke

✉ gikaru@egerton.ac.ke

cellular oxidative stress (Li et al., 2008). The persistent oxidative stress contributes to decreased lung function and increased diagnosis and exacerbation of asthma, bronchitis, and pneumonia in people living in areas of high levels of particulate air pollution (Perez-Padilla et al., 2010; Ko and Hui, 2012). Although exposure to particulate emissions has been linked to diminished lung development and function in children, the underlying biological mechanism responsible for enhanced susceptibility is largely unknown (Jayaraman et al., 2010).

Studies that have been done epidemiologically have found that there is an association between ambient air PM concentrations and health effects. PM, in general, has been mostly associated with excess cardiovascular mortality (Brunekreef and Forsberg, 2005), lung cancer (O'Connor et al., 2008; Sexton et al., 2007) and cardiac arrhythmias (Delfino et al., 2005). All these effects vary in strength with sampling location, type, and season and size fraction. However, granular particulates have been mostly associated with respiratory disease and cardiopulmonary morbidity (Brunekreef and Forsberg, 2005; Brook et al., 2010). Toxicological studies have confirmed the organic credibility of the findings of epidemiology studies and demonstrated that PM-induced toxic effects are often dependent upon PM size and composition (Mirowsky et al., 2013). Moreover, if the origin of the smoke particulate emissions is of organic nature, then the smoke may cause a myriad of symptoms, including central nervous system breakdown, lung inflammation, and mucous membrane irritation in addition to other ailments (Chaturvedi, 2010). The oxidative potential of airborne particles is yet to be understood, however, some studies have established that fine PM has been linked to the generation of high levels of reactive oxygen species. Epidemiological studies have demonstrated that PM-induced toxic effects are often dependent upon PM size and composition (Mirowsky et al., 2013).

This study investigates the health impacts of various types of particulate emissions on the lung tissues of male albino mice with the aim of deriving the pathological impacts of inhaling such particulates by higher-order animals such as man. This contribution is also necessary in understanding the health consequences of airborne particles especially from vehicular soot, indiscriminate tyre burning, and disasters such as fireworks and forest fires. The information obtained will assist clinicians, biomedics, and environmentalists to make informed decisions concerning environmental pollution from the thermal degradation of organic matter. Generally, carbon is probably the most abundant of particulate matter primarily in the form of organic volatiles considered detrimental to biological health.

MATERIALS AND METHODS

SEM Characterization of Particulate Emissions

Soot samples from the tire and simulated forest fire sources were collected using a clean glass surface and carefully transferred into crimp top amber vials using a special brush free of impurities. The simulated forest fire was a result of burning of ~ 20 different trees and shrubs in a laboratory fabricated reactor. Approximately 5 mg of sample was added to 1 mL methanol and gold grids were dipped into the prepared sample. Twisters were used to pick the gold grids from the sample. The grids were allowed to dry in the open before putting them into the analysis chamber of the SEM (JEOL JMS 7100F) (Konert and Vandenberghe, 1997). The sample was analyzed under high vacuum to ensure no interference of air molecules during analysis. The SEM machine was then switched on and imaging of the sample conducted. The lens was varied at various resolutions until a clear focus of the sample was observed (Poynton et al., 2014). *Image J* computer program was used to determine the size of the soot particles and a distribution curve of soot size was then determined using *Igor* computer software. The mean sizes of the soot particles at 500 and 700 °C were reported and presented as a Gaussian distribution where the peak of the curve shows the average of the particle size.

Mice Handling Particulate Exposure Protocol

All experiments were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with international guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care (Goodman, 2015). Twelve week old male mice were purchased from Kenya medical research institute (KEMRI) and were housed in a pathogen-free environment (i.e. 20 x 43 x 18 cm polycarbonate cages with wire-top system containing Tech pellet/corn-cob bedding) at 19~26 °C and 50 ± 10% relative humidity with a 12-hour-light/12-hour dark cycle. The mice were provided with standard chow and they were allowed to acclimate for one week prior to the particulate exposure. The exposure protocol was conducted in such a way that it simulated approximate environmental air pollution conditions in polluted cities around the world.

During exposure periods, the wire-top lid was replaced with a sealed top and high-efficiency airflow system to deliver gaseous particulates to the mice in their cages at a rate of ~ 250 µgm⁻³day⁻¹. Mice were exposed to particulates from the different sources continually in order to mimic exposure of PM in the natural environment.

During exposure, the mice were provided with food pellets and water. Pulmonary response to exposure was evaluated after each soot exposure protocol was complete.

Histochemical Investigation

Within 2 hours of completing the exposure protocol, mice (control, and the mouse exposed to simulated forest fire) were sacrificed by euthanizing with sodium pentobarbital/chloroform. This was followed by dissection in order to remove and analyze the organs of interest (Pryor et al., 1998). The trachea was exposed, cannulated, and secured with a suture. The lungs were immediately removed from the thorax and frozen in liquid nitrogen. After twenty-four hours, a portion of the lung tissue was homogenized in 0 °C Tris-hydro-chloride (HCl) buffers (25 mM Tris, 1 mM EDTA). The lungs were lavaged four times with a single volume of phosphate-buffered saline. Then the samples were analyzed for biological damage and any pathological changes caused by particulate emissions was noted.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to test if there was a significant difference in the mean diameters of the particulate from forest fire and tyre sources.

RESULTS

Particulate Emission Characterization Using Scanning Electron Microscopy (SEM)

At a magnification of x400, the particulate soot of tire and simulated forest fire soot were examined. The micrographs of forest fire soot were remarkably interesting although it was quite difficult to get a clear image of forest fire soot in the whole range of magnification.

Nevertheless, after several attempts a fairly neat image was obtained. *Image J* was used to measure the sizes of the soot particles and size distribution was determined using *Igor* software. The mean diameter of the soot was found to be $11.51 \pm 4.9 \mu\text{m}$ (cf. **Figure 1**). The particulate size of soot from tire burning was quite large ($16.23 \pm 3.36 \mu\text{m}$), **Figure 2**, *vide infra*. Notably, to obtain enough data points to plot size distribution curves, several micrographs taken at the same magnification were used. From the micrographs reported in this work, simulated forest fire particulates appear smaller than those from tyre burning. Nonetheless, the two types of emissions fall within the PM₁₀ category of airborne particulates. Although, these particulates are fairly, large and may not be inhaled deeper into the respiratory system, their deposition on the respiratory surface for instance the lung tissues (macrophages and alveoli) may inflict serious biological damage. These deposits may act as growth centers of tumours, accelerate cancer, oxidative stress, and cardiopulmonary death. Based on the results reported in this work, PM₁₀ are equally toxic in comparison to PM_{2.5} and other ultrafine particulates. Nonetheless, the particulate size may not be enough reason for the observed inflammations in the lungs of albino mice exposed to various soot particulates. There must be other organic toxics and probably intermediate reactive species including organic radicals capable of causing cell injury and eventually the growth of tumours. These proposed predictions are clearly displayed in **Figure 4**, *vide infra*.

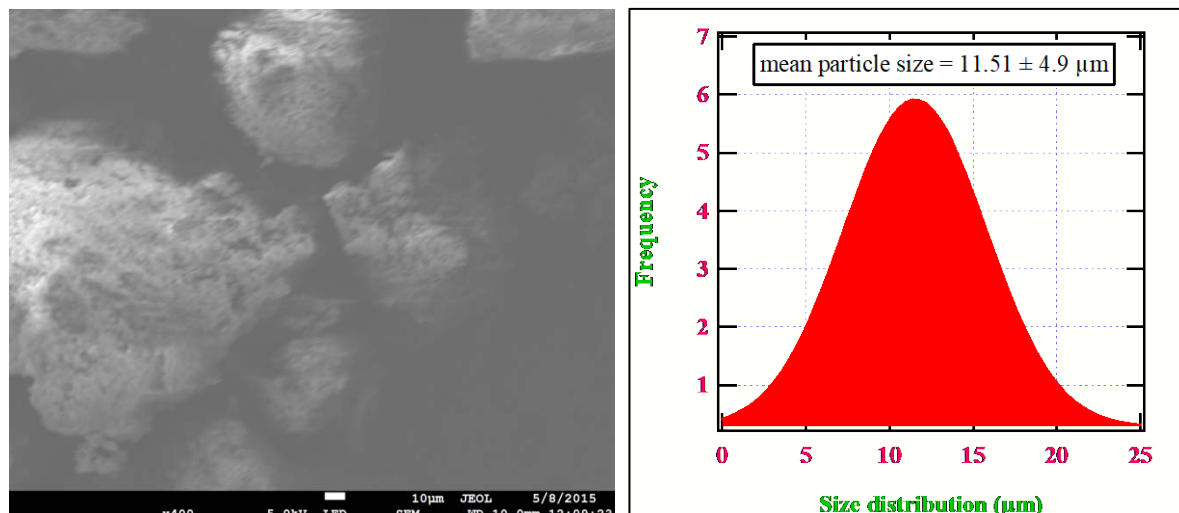


Figure 1. SEM image and particle size distribution (Gaussian red) of soot particles from simulated forest fire

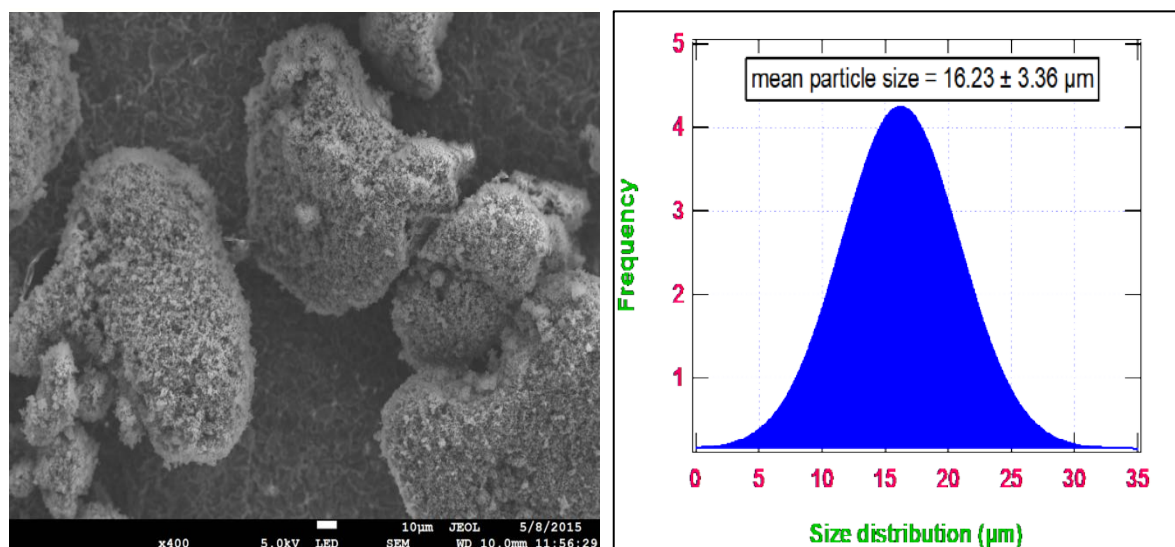


Figure 2. SEM image and particle size distribution (Gaussian blue) of soot particles from tire burning

Since the principal source of airborne fine particles from combustion carry with them free radicals, it is evident that free radicals may induce cellular and alveolar damage and ultimately causing tumors, cancer, and death (Dellinger et al., 2001; Pryor et al., 1998) not only in research animals but also higher order animals including man. In general, the surface area of inhaled particulates is more decisive of their toxicity than their size, and smaller particles tend to have a larger relative surface area, for instance, those sized 10microns or less.

Examination of Lung Damage after Particulate Exposure

In order to understand the inhalation toxicity of particulates, lungs of albino mice were examined by a light microscopy interfaced with a computer to assess. Figure 3 presents the extracted lungs from albino mice exposed to various types of soot particulates.

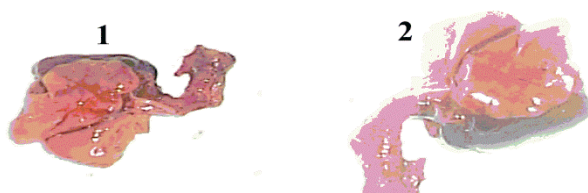


Figure 3. 1 is the lung exposed to diesel while 2 is the lung exposed to tyre soot

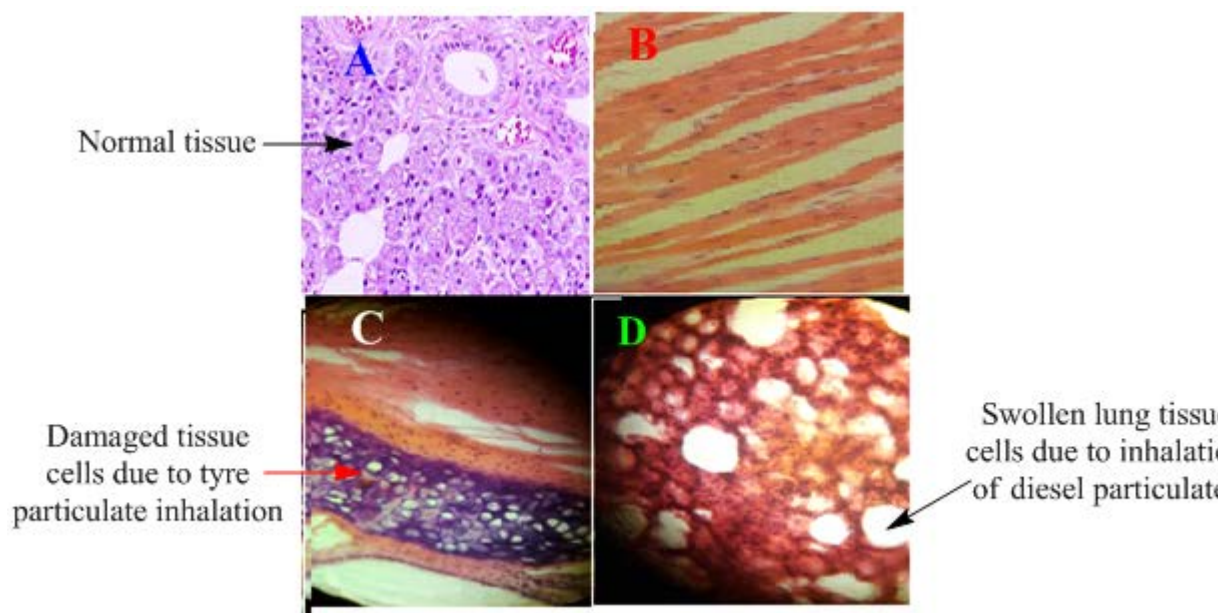


Figure 4. Lung tissue photographs obtained from Light microscope at X200 magnification; **A**-control, **B**- forest fire exposed tissue, **C**- tyre exposed tissues, and **D**- vehicular (diesel) exposed tissue

After sections of 3~4- μm thick were cut and stained with hematoxylin and eosin (H&E), they were examined histologically. Two exposure-related findings were apparent in comparison to the control lung sample (**Figure 4A**, *vide infra*) i.e.

- i. Maximal to mild yellow-brown to black pigmentation within the alveolar macrophages following a 2-day exposure of tyre and 3 days exposure of diesel combustion exhaust.
- ii. Minimal increase in alveolar macrophages (histiocytosis) following exposure to simulated forest fire soot.

The alveolar histiocytosis was characterized by slightly increased number of alveolar macrophages that in general were diffusely distributed throughout the lung. The pigmented particulates found in the alveolar macrophages from the recovery animals were more commonly observed in punctate aggregates, possibly reflecting continued cellular processing of the material. Histopathological effects in the lung following exposure to the three combustion sources shows that the control mouse exposed to ambient air for 5 days had normal alveolar air spaces and septa (**Figure 4A**).

Air spaces contain scattered pulmonary interstitial macrophages. The mice exposed to forest fire soot (**Figure 4B**) showed minimally disturbed airways. However, the mice that were exposed to the diesel soot (**Figure 4D**, died after 3 days of exposure) indicated that alveolar air spaces contained increased macrophages with minimal to mild pigmentation while those exposed to tyre emissions (**Figure 3C**) died after two days. Clearly, as observed in **Figure C**, pulmonary alveolar macrophages contained dark yellow to black intracytoplasmic pigments.

DISCUSSION

The respiratory tract epithelium which normally begins at the nostril and extends to the alveoli, its cellular composition varies greatly by function and structural site along the respiratory path. Volatiles carried into the respiratory tract first pass over epithelial cells in the nasal cavity (Barile, 2013). These cells represent a natural block and aid in the removal of inhaled foreign substances. They are most likely the primary cells to exhibit the adverse consequences of exposure to inhaled particles and thereby may serve as a sensitive and direct measure of particle

toxicity (Landsiedel et al., 2014) as observed in **Figure 4C** and **Figure 4D**. Epithelial cells lining the tracheobronchial tree, central acini, and alveoli of the lungs also serve as sensitive indicators of the pattern of induced injury due to soot emissions deposition and subsequent particle-mediated events in these regions. Two important sites for particle deposition in the lungs are the respiratory bronchioles and central acini. These two sites showed significant damage as evidenced in **Figure 4**. These structures form the transitional zone between the air conduction path and gas exchange alveoli. In mice, the transition from the terminal bronchiole to the alveolar duct is abrupt (Townsend, 2012). In contrast, humans possess a more complex transitional zone, with one to three generations of airways. Because the respiratory bronchioles and central acini are primary sites for lung injury after exposure to airborne pollutants (Adamson et al., 1999; Pinkerton and Joad, 2000), it is critical to understand the effects of particle deposition on structural and functional changes in this region.

This study demonstrates that diesel exhaust inhalation and tire soot inhalation for a substantial period (i.e. two days for tire and three days for the diesel soot exposure) of time aggravates the neutrophilic lung swelling or proinflammatory chemokine expressions related to lung dysfunction. The mouse exposed to tire soot died after two days of exposure while that exposed to vehicular diesel soot died after 3 days of exposure. On the other hand, the mouse exposed to simulated forest fire soot did not die even after 5 days of exposure although it showed signs of weakening and watery eyes. Nevertheless, the lung pathology observed in the mice following the experimental exposures to the particulate emissions from different fuels (forest fire, tire, and diesel) was quite similar; airway epithelial, necrosis, and parenchymal congestion, edema, hemorrhage and inflammation (**Figure 4**). However, the microscopic examination of lung sections indicated marked differences between the lungs of exposed mice, and the control (**Figure 4A**). A great accumulation of soot particles was found surrounding the bronchioles in the lymphatics, with the surrounding alveolar wall structures frequently absent especially in the tire and diesel-exposed lungs. This condition is similar anatomically to that of the centrilobular emphysema (Witschi, 2000).

Particulate emissions generated by tire burning exhibited extensive destruction to the tissues which is revealed by significant damage to the cells, for instance, capillary disconnection and blockage of air pathways (**Figure 4C**). Exposure of mice to diesel soot particles also impaired cell proliferation in the alveolar region of the lung (**Figure 4D**). Clear evidence of inflammation was observed in both exposures, however; forest fire soot showed minimal damage (**Figure 4B**). Remarkably, in all exposures, the biological response in the airways and lung tissue to inhalation of soot particles was dependent on the source of particulate emissions. Focal airway epithelial necrosis was found in the trachea and with decreasing frequency distally not noticed in terminal bronchioles. The damage to the endothelium and the alveolar epithelium resulted in the creation of an open interface between the lung and the blood which facilitated the spread of soot particles from the lung systemically, and ultimately a systemic inflammatory response (Morrison and Bidani, 2002).

It is well established in literature that, the injury to epithelial cells handicaps the ability of the lung to pump fluid out of airspaces (Bhanothu et al., 2012). Fluid filled airspaces, loss of surfactant, micro-vascular thrombosis, and unsystematic repair reduces resting lung volumes, leading to increased ventilation-perfusion discrepancy from right to the left shunt resulting to breathing problems. In addition, lymphatic drainage of lung units appears to be reduced, i.e. dazed by the acute injury which contributes to the build-up of extravascular fluid (Luh and Chiang, 2007). Vessels were markedly congested while in others, vessels were distantly pulled apart (**Figures 4 C** and **D**). Edema was also observed around broncho-vascular trunks, **Figures B, C, and D**. The non-specificity of these changes mostly reflects the direct toxicity of many soot components, the effects of products released from locally altered or reactive inflammatory leucocytes, platelets and endothelial cells and changes in the flow of the lung blood (Morrison and Bidani, 2002). There were more convincing differences between the tire and diesel exposures, from the fact that the tyre soot caused sudden death (after 2 days) as compared to diesel soot (after 3 days of exposure). There was also swelling and shrinking of tissues cells i.e. the tyre and vehicular diesel soot volatiles caused disconnection of the connective tissues and blood capillaries within the lungs which evidently caused swelling and shrinking as noted in (**Figure 4C** and **Figure 4D-*vide supra***).

It is evident from this study that exposure to tyre and diesel exhaust particulates have the greatest impact on lung tissues. Forest fire soot, however, causes some health effects but requires a longer duration of exposure before the effects become significant. Moreover, there was a plausible degree of neutrophilic infiltration seen in the lung specimens in the diesel and tyre groups. Short-term exposure to increased ambient amounts of this soot is associated with an increase in morbidity and daily mortality for cardiopulmonary conditions, especially in industrialized cities (Schwarze et al., 2006). However, a probable biologic mechanism linking short-term soot exposure and pathophysiologic effects has not been fully established.

In conclusion, this study has revealed that combustion of tyre, simulated forest fire and diesel results in the emission of different sized particles which are considered responsible for lung inflammation. There may also be the production of irritant compounds such as PAHs as reported in literature surveys. These substances disrupt the normal lining of the respiratory tract and thus causing potential swelling, airway collapse, and respiratory distress. The responses experienced in this study were dependent on the chemical composition of emissions and a PM size,

which backs up previous studies that suggest mass concentration alone, may not be sufficient for evaluating the toxic responses to PM exposure. Evidently, exposure to tyre and vehicular diesel particulate emissions may cause serious biological ailments to higher order animals such as man.

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