ASSESSMENT OF AIR QUALITY AND CARCINOGENIC CAPACITY OF AIR POLLUTANTS FOUND IN KANDY CITY AREA

by

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DECLARATION

I, Nirodha Abayalath, declare that the work presented in this thesis are my own and, it has been generated by me as the result of my own original research. I confirm that where I have consulted the published work of others, this is always clearly attributed. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work. No part of this thesis has been submitted earlier or concurrently for a degree or any other qualification at this University or any other institution.

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This is to certify that this thesis has been prepared by Nirodha Abayalath under my supervision.

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Assessment of Air Quality and Carcinogenic Capacity of Air Pollutants Found in Kandy City Area

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ABSTRACT

Air pollution has become a major public health hazard in many countries, including Sri Lanka, and Kandy city air has been categorized as highly polluted. Particulate matter (PM), polycyclic aromatic hydrocarbons (PAHs), and heavy metals (HMs) are major air pollutants causing health issues. The purpose of the current study was to analyze 12 priority PAHs, toxic HM, and other elements of ambient air PM_{10} in Kandy city air and find whether exposure to PM_{10} can induce respiratory tract malignancies, including carcinogenesis. PM Samples were collected and analyzed for PAHs and HMs by gas chromatography coupled mass spectrometry (GC-MS) and inductively coupled plasma mass spectrometry (ICP-MS). The biological effects of particle-bound compounds were assessed using primary porcine airway epithelial cell culture, treated with extracted particle concentrations for 24 and 48 hours. Cytotoxicity, DNA damage, and relative gene expressions of several pro-inflammatory cytokines, xenobiotic metabolizing

enzyme CYP1B1, growth factors, tumor suppressor p53, and prostaglandin pathway gene PGES were evaluated. Results showed that the total PM_{10} and PAH concentrations ranged from 85.648 to 289.352 $\mu g/m^3$ and 3.062 to 36.887 ng/m^3 respectively, in the two selected sampling sites. No significant variations in PAH or PM concentrations between the two sampling sites were observed. PAHs with high molecular weight (rings ≥ 4) were found in a high percentage among the 12 PAHs analyzed. Toxic HM concentrations were significantly higher than the existing ambient air quality guidelines. Highly carcinogenic As, Cd, Cr, Ni, and probable carcinogens, Pb, Co, were recorded in elevated concentrations compared to the other cities in the world. The bioassays indicated significant acute cytotoxicity ($P < 0.05$) and DNA damage induction in the treated cells. The present data revealed alterations of gene expression, DNA damage, and cytotoxicity can occur in respiratory tract cells due to the pollutants in the Kandy ambient air leading to respiratory track elated disease condition and malignancies. Therefore, immediate attention is needed to rectify the air quality in Kandy city.

Keywords: Polycyclic aromatic hydrocarbons (PAHs), Particulate matter (PM), Heavy metals, Atmospheric pollution, Cancer risk, Sri Lanka

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LIST OF ABBREVIATIONS

Acp: Acenaphthene

Acpy: Acenaphthylene

ANOVA: Analysis of Variance

Ant: Anthracene

AQI: Air Quality Index

ATP: Adenosine triphosphate

BaA: Benz[a]anthracene

BaP: Benzo[a]pyrene

BbF: Benzo[b]fluoranthene

BghiP: Benzo[ghi]perylene

BkF: Benzo[k]fluoranthene

cDNA: Complementary DNA

Chr: Chrysene

COPD: Chronic Obstructive Pulmonary Disease

COX-2: Cyclooxygenase 2

CYP1B1: Cytochrome P450 1B1

DahA: Dibenz[a,h]anthracene

DCM: Dichloromethane

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic Acid

EGFR: Epidermal Growth Factor Receptor

EPA: Environmental Protection Agency

EtBr: Ethidium Bromide

FBS: Fetal Bovine Serum

Flt: Fluoranthene

Flu: Fluorene

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GC-MS: Gas Chromatography-Mass Spectrometry

GFF: Glass Fiber Filter

HBSS: Hank’s Balanced Salt Solution

HM: Heavy Metal

HMW: High Molecular Weight

HPLC: High-performance Liquid Chromatography

IARC: International Agency for Research on Cancer

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

IL-6: Interleukin 6

IL-8: Interleukin 8

Ind: Indeno[1,2,3-cd]pyrene

LMA: Low-melting temperature Agarose

LMW: Low Molecular Weight

M199: Medium 199

Nap: Naphthalene

NMA: Normal-melting Temperature Agarose

NSCLC: Non-small Cell Lung Cancer

P/S: Penicillin and Streptomycin

p53: Protein 53 (TP53)

PAH: Polycyclic Aromatic Hydrocarbon

PBS: Phosphate-buffered Saline

PCR: Polymerase Chain Reaction

PGES: Prostaglandin E Synthase

Phe: Phenanthrene

PM: Particulate Matter

Pyr: Pyrene

qPCR: Quantitative Real-time Polymerase Chain Reaction

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SD: Standard Deviation

TAE: Tris-Acetate-EDTA

TGF-β1: Transforming Growth Factor-beta 1

TNFα: Tumor Necrosis Factor-alpha

UK: United Kingdom

USA: United States of America

USEPA: United States Environmental Protection Agency

UV: Ultraviolet

VOC: Volatile Organic Compound

WHO: World Health Organization

CHAPTER 01

INTRODUCTION

This thesis is divided into five main chapters. The first chapter presents highlights of the history of air pollution studies, the physicochemical characteristics of atmospheric particles, their

sources, and their impact on human health. A section of this chapter devoted to the description of air pollution and its impact on health in Kandy city area. The second chapter is an in-depth review of the impact of air pollution on human health based on the literature while highlighting the hazardous air pollutants, particulate pollution (particle bound organic and inorganic components) and global assessment of particle pollution. Finally, the literature review focuses on the situation in Sri Lanka, to justify the objectives and methodology applied in this project. The third chapter includes a detailed explanation of the study procedure, including sample collection, chemical analysis of air samples, porcine primary airway epithelial cell culture model establishment, cell culture-based bioassays and gene expression analysis. The fourth chapter deals with all the results generated throughout the whole research project. The fifth and the final chapter discusses the results of the project extensively. The discussion chapter also elaborates the change of the hazardous components with time in Kandy city area and particle pollution associated carcinogenic capacity. Finally, this thesis ends with a general conclusion and the perspective of this study.

Clean air plays the most crucial role in keeping all the living systems on this planet. Association between human health risks and air pollution exposure has been recognized for over 700 years. King Edward 1 of England punished people by death who burned coal in 1306 because of his mother, Queen Eleanor's sickness as a result of coal fumes from the town at the castle. It has been confirmed that significant emissions of air pollutants from a combination of various natural (e.g., volcanos, wildfire) and anthropogenic activities affect negatively human health as well as

cause some serious environmental problems such as the greenhouse effect, acid rains, rise of global temperature, smog, haze, reduce agricultural productivity and degrading the surface water quality.

People became more aware and concerned about air pollution-related health problems due to the high air pollution episodes recorded globally within the last 90 years. One of the earliest acute episodes of lethal smog occurred in early December 1930 in Meuse Valley, Belgium. Hundreds of people were shown symptoms of severe respiratory diseases, and more than 60 people died from the event (Nemery et al., 2001). In October 1948, another air pollution episode was recorded from a small town called Donora in southwestern Pennsylvania, United States of America (USA). Twenty people died within the week of the Donora event (Ciocco and Thompson, 1961; Schrenk et al., 1949). London smog, or the great smog, is the turning point of environmental pollution history. In early December 1952, Londoners experienced lethal smog, and it was associated with an unusually high rate of respiratory deaths for 2.5 months (Bell and Davis, 2001).

Extreme smog events are likely to occur in the Asian region because of rapid urbanization and industrialization, especially megacities like in China and India. This rapid urbanization and industrialization added the highest amount of pollutants and toxic substances to the environment within the last decade. Starting from early 2013, China experienced a series of large-scale smog episodes, and the death rate was unprecedented (Cheng et al., 2017; D. Zhao et al., 2019; Zhou

et al., 2015). Delhi experienced several dangerous smog events. In 2016, another smog event affected over 25 million people around Delhi city. In 2017, the thick haze was strong enough to affect the test cricket match played between Sri Lanka and India (Sawlani et al., 2019). In November 2019, a high air pollution event was recorded in Sri Lanka, and according to the authorities, this could have been a result of the rising level of Delhi air pollution and wind. The pollution level was more than double the average level in Colombo city within the period. People were warned to limit prolonged outdoor activities within those few days, especially for people with respiratory conditions and children. If Colombo event was categorized as "Unhealthy" and possible health effects for everyone according to the Air Quality Index (AQI) values, still no clinical data or analyzed air quality data were published (USEPA, 2014b). Modern-day air pollution might not be the same as the London killer smog because the fuel source is shifted from coal to more harmful oils and gases. Therefore, future events will probably be much more lethal and rise immature deaths and morbidity.

Accumulation of airborne suspended particulate matter (PM) is one of the major factors contributing to all those air pollution events, which is made up of solid and liquid particles. Both natural and anthropogenic sources can cause the arising of these PMs, such as sea spray, volcanic ash, wind-blown dust, soil particles, fungal spores, pollen, ashes, and other products from forest fires, combustion of fossil fuel especially in power plants and vehicles, construction works and cigarette smoking. Diesel exhaust derived from diesel engines is the largest single human-made PM source (Riedl and Diaz-Sanchez, 2005; Zhang et al., 2010). The size and the

composition of these PMs vary with the source. Three major categories are defined according to the aerodynamic diameter of these particles: ultrafine, fine, and coarse. Ultrafine particulates are less than 0.1 µm in diameter. Fine particulates, known as $PM_{2.5}$, are less than 2.5 µm in diameter and released to the air directly from the source, such as primary combustions. Fine and ultrafine PM are getting the most interest due to deeper level penetration to the human respiratory tract and reportedly known for their high health risk. Coarse fraction (diameters between 2.5 and 10 µm, PM_{10}) includes the most visible forms of particle pollution such as black smoke, mechanically generated particles, soil, dust particles, pollen, other plant parts, and risk is mainly upon the upper respiratory tract.

This respirable atmospheric PM is a complex mixture of different chemical components because PM can absorb and adsorb toxic pollutants such as polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), heavy metals (HMs), ammonium nitrates, sulfates, and mineral dust (Hueglin et al., 2005). If many epidemiological and toxicological assays have been done to find the genuine contribution of these components to human health, it is challenging due to this high complexity. However, among all those components, particle-bound PAHs are one of the first atmospheric pollutants identified and proven that are hazardous to human health and considered as potential mutagenic and carcinogenic matter (Karlsson et al., 2004; Motta et al., 2004). PAHs are semi-volatile complex organic compounds which are having at least two benzene rings mainly produced by the major anthropogenic sources such as incomplete combustion of automobiles/fossil fuel, and other industrial, agricultural, domestic and natural

sources (Ravindra et al., 2008). PAHs which have 2-3 aromatic rings (low molecular weight, i.e., naphthalene, acenaphthylene, fluorene) mainly present in the gas phase, while heavier PAHs with four or more aromatic rings (i.e., pyrene, benzo[a]anthracene, benzo[b]fluoranthene) usually exist in PM-bound phase in air. Carcinogenicity of PAHs increases with the molecular weight (Gregoris et al., 2014; Kim et al., 2012). Of the hundreds of identified PAHs, 16 are designated as high priority pollutants with the number of benzene rings ranging from two to six due to their high toxicity to humans and other organisms by the United States Environmental Protection Agency (USEPA) (USEPA, 1986) (Fig. 1.1).

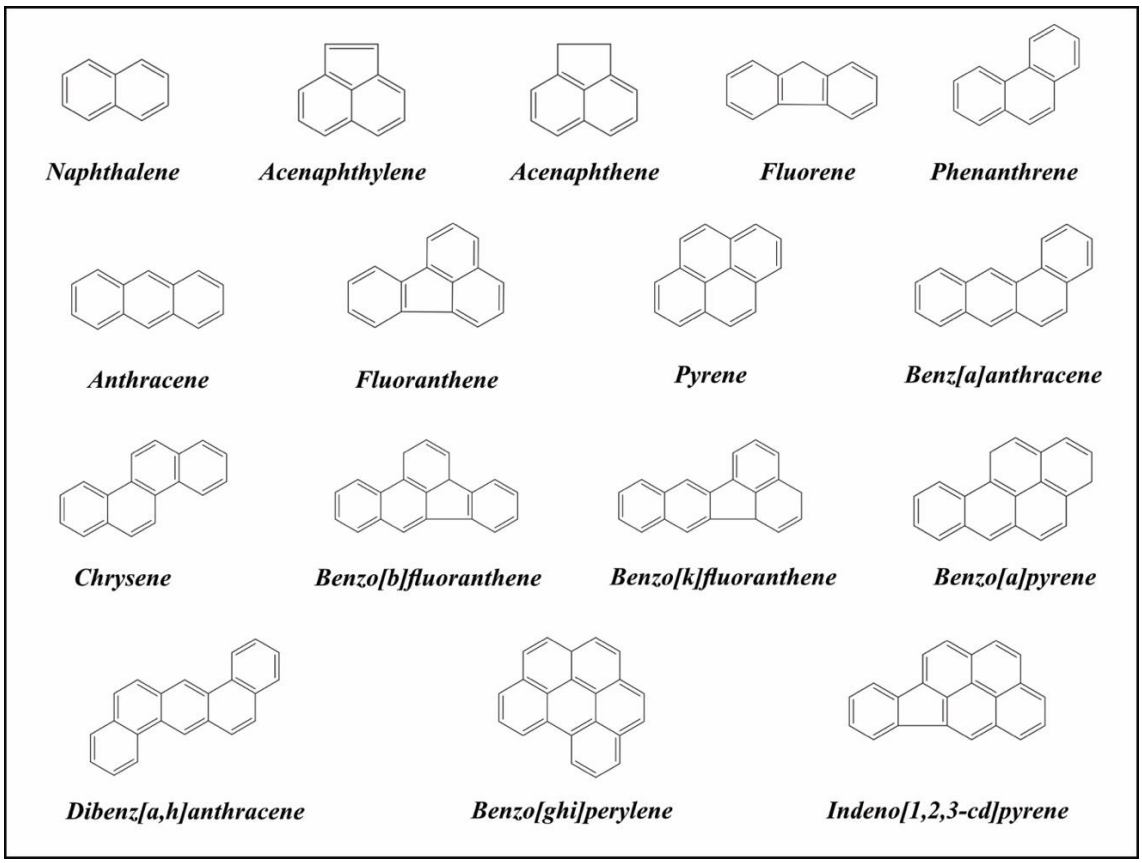


Fig. 1.1. EPA priority listed PAHs (Source: USEPA).

International Agency for Research on Cancer (IARC) classified chemical substances into four categories considering their human carcinogenicity (IARC, 1982) (Table 1.1). After reviewing all the experimental data, benzo[*a*]pyrene is classified as a group 1 carcinogen. dibenz[a,h]anthracene is classified as a probable carcinogen to humans (group 2A) and chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene are classified as possibly carcinogenic to humans (group 2B) (IARC, 2010, 2012). Studies have shown that long-term exposure to air with particle-bound PAHs increases lung cancer risk, because they can enter the lungs through the respiratory tract and damage numerous cell functions (biochemical disruptions) in important organs by penetrating through cell membranes (Etchie et al., 2018; Jiang et al., 2014; Liao et al., 2011; Xia et al., 2013; Zhang and Tao, 2009). Short-term exposure to elevated levels of these air pollutants can cause respiratory and cardiovascular diseases such as asthma, bronchitis, chronic obstructive pulmonary disease (COPD). Some studies have been able to find an association between PM exposure with the increasing number of strokes reported, reproductive defects, premature deaths in patients suffering from lung or heart disease, early pregnancy-related problems, liver cancer, and risk of developing brain tumors (Cohen et al., 2017; Hansen et al., 2008; Pan et al., 2016; Poulsen et al., 2016). Although the pollution level is often higher in the developing area than the developed countries, very few studies have been conducted in developing countries compared to the

western world. However, now growing interest in developing regions can be seen (Chang et al., 2019; Mohanraj et al., 2012).

Table 1.1. The carcinogenicity and number of benzene rings of 16 EPA PAHs

Components	**Abbreviation**	**Number of rings** (Kim et al., 2012)	**Carcinogenicity** (IARC, 2010, 2012)
Naphthalene	Nap	2	2B
Acenaphthylene	Acpy	3	Unclassified
Acenaphthene	Acp	3	3
Fluorene	Flu	3	3
Phenanthrene	Phe	3	3
Anthracene	Ant	3	3
Fluoranthene	Flt	4	3
Pyrene	Pyr	4	3
Benz[a]anthracene	BaA	4	2B
Chrysene	Chr	4	2B
Benzo[b]fluoranthene	BbF	5	2B
Benzo[k]fluoranthene	BkF	5	2B
Benzo[a]pyrene	BaP	5	1
Dibenz[a,h]anthracene	DahA	5	2A
Benzo[ghi]perylene	BghiP	6	3
Indeno[1,2,3-cd]pyrene	Ind	6	2B

Not only the particle-bound PAHs, nowadays, more than 40 chemical elements are also measured in atmospheric PM and consider those as air quality indicators (Yin et al., 2005). With a large amount of data over the last few decades, it has been proven that most PM associated metals play a considerable role in aerosol toxicity and human health. Almost all HMs induce various diseases such as lung diseases and cancer (Bellinger, 2005; Li et al., 2015). In particular, a few HMs such as As, Cd, Cr, and Ni are categorized as carcinogenic substances to humans by the IARC. Moreover, Pb and its compounds, Sb_2O_3, and Co are characterized as probable carcinogens (IARC, 2012). Oxidative stress caused by reactive oxygen species (ROS) is a well-known mechanism of heavy metal-induced damage (Borošková et al., 1993; Rönkkö et al., 2018; Stern, 2010). Due to the carcinogenic effects and non-degradable nature of HMs, the dissolved form of those can be accumulated via circulation in the biosystem, including the food chain, and finally end up in very high concentrations in plants and animals (El-Khatib et al., 2019; Houri et al., 2019; Liu et al., 2007; Turna Demir and Yavuz, 2020). Jan and colleagues had published a detailed review and covered HMs, the mechanisms involved in eliciting their toxicity, carcinogenicity, and hazardous effects within the body of living organisms (Jan et al., 2015). Furthermore, particle-bound metallic pollutants can be considered as a good indicator of anthropogenic activities and can be used to identify specific sources of air pollutants. In urban environments, rubber tyre wear, motor oil, and most importantly, fossil fuel combustion release HMs such as Zn, Pb, and Cd to the ambient air. Barium can be used as a marker of road transport emissions (Jandacka and Durcanska, 2019). Based on the knowledge of the chemical

composition and the presence of the element in the collected samples can be used for backward identification of the source and important recommendations such as importing cleaner fuel, applying cleaning systems for exhaust gas, discarding older engines, encouraging fuel-efficient vehicles (electric or hybrid), etc. can be made upon valid scientific data.

A vast number of air quality and health-related studies have been conducted in many cities in the world. Many have used cultured cells or animal models to prove the mutagenic and carcinogenic effects of short-term exposure to air pollutants. From the published data, we might say that the health burden of air pollution is different from place to place due to many responsible factors such as fuel consumption level, fuel quality, vehicular traffic, population number, industries found in the place, thermal power plants, geographical location, temperature, and other climatic parameters, etc. (Nandasena, A. R. Wickremasinghe, et al., 2012). As a developing Asian country, Sri Lanka faces the same air pollution-related health problems due to rapid urbanization, population growth, road traffic, and heavy use of poor-quality fuel (Gunasekera et al., 2018; Kumarihamy and Tripathi, 2019). Compared to other Asian countries, this field is neglected for years, and very few researches have been published based on the data from Sri Lankan cities. Still, we were unable to find a proper cohort study from Sri Lanka on this matter (Danansuriya et al., 2015; Karunasekera et al., 2005). Due to the lack of continuous air quality and health monitoring systems and well-planned researches, awareness of air pollution is in a primitive stage in Sri Lanka (Nandasena et al., 2010; Wickramasinghe et al., 2012).

As the first step, Kandy city was selected to conduct this study because Kandy is a major tourist and religious destination and a world heritage site. This unique heritage city has been identified as one of the most polluted cities in the country. Kandy is a small (26 km^2) but great city characterized by highly dense residential (6000 inhabitants/km^2) and commercial premises. It is observed that Kandy has about 200,000 daily mobile community and almost all schools, hospitals, and other public working places in the city are located near the main roads with a very high volume of vehicular traffic. Moreover, lung cancer and other respiratory diseases have become a critical issue in Kandy (Wickramasinghe et al., 2011). National Hospital in Kandy has recorded a very high number of lung cancers and other reparatory diseases compared to the other cities (National Cancer Control Programme, 2011). Exposing to the high level of air pollutants and smoke which emit from old buses (incomplete combustion of diesel engines is a primary source of carcinogenic PAHs) in the city area might be the reason for that (Liao et al., 2011; Wickramasinghe et al., 2012). Therefore, addressing the issues of air pollution in Kandy city is becoming increasingly important nowadays.

However, no bioassay-based research has been conducted to assess the effects of air pollutants in any city of Sri Lanka yet. Therefore, to fill that knowledge gap, the present study incorporated an *in vitro* cell culture model and several bioassays to assess whether the pollutants found in Kandy air are having any possible effect on airway epithelial cancer induction. To improve the air quality and protect public health, important recommendations and policies can be made upon the results of this study. Furthermore, by generating hard scientific evidence with the extension

of this research to the *in vivo* animal model and to other smaller cities, new national ambient air quality standards can be implemented.

To accomplish the objectives of this study, air samples were collected from several locations in Kandy city using a high-volume air sampler and depositions were extracted for chemical analysis (PAHs, HMs) and tested on a cultured porcine airway epithelial cell model to detect the impact through analysis for several biomarkers specific to the respiratory diseases and lung cancer. Moreover, high-throughput mass spectrometry (MS) was used to analyze the constituents in the collected samples as well.

1.1. Objectives

Broadly, the present investigation was undertaken to analyze the pollutants present in Kandy air and to assess the possible health impact of air pollutants in the Kandy city air.

To fulfill the broad objective, below specific objectives were followed through in this research.

I. To analyze/identify the levels of PAHs in PM_{10} presence in Kandy city air
II. To analyze/identify the levels of HMs present in Kandy city air and make a correlation with respiratory diseases.
III. To establish an *in vitro* cell culture-based model to investigate the effects of air pollutants found in Kandy air on the induction of carcinogenesis in airway epithelial cells.

IV. To assess selected molecular signaling pathways, which are important in carcinogenesis in airway epithelial cells upon exposure to the Kandy city air pollutants.

CHAPTER 02

REVIEW OF LITERATURE

2.1. Air Pollution

Air pollution is one of the rapidly growing problems of today's world. World Health Organization (WHO) defined air pollution as contamination of the indoor or outdoor environment by any chemical, physical, or biological agent that modifies the natural characteristics of the atmosphere (WHO, 2005). A mixture of solid particles and gases in the air is considered air pollution, according to the United States Environmental Protection Agency (USEPA) (USEPA, 2014a). According to many researchers in this field, air pollution is simply known as exceeded levels of various gases and fine particles that the environment can dissipate, absorb, and dilute (Kelly and Fussell, 2020; Laden et al., 2006; Schikowski et al., 2005). Hazardous air pollutants result in safety, health, and other welfare issues for all living organisms. Moreover, air quality is critical for the healthy living conditions of food animals, and other resources we extract from the environment, such as water and minerals (Ni et al., 2020).

The first air pollution records go back to the nineteenth century that the industrial revolution became significant. Dust and fumes generated from ancient Rome's mines were the leading causes of that air pollution episode (Stromberg, 2013). Then the chronic air pollution started mainly from industrialized countries such as the USA and Europe. Then this air pollution came

to the developing countries at a rapid pace. As a result of this unbearable speed of air pollution, we have witnessed several heavy air pollution episodes such as the valley of the Meuse (Belgium) in 1930, Donora (USA) in 1948, Poza Rica (Mexico) in 1950, London, United Kingdom (UK) in 1952, Cincinnati (USA) in 1968, New York (USA) in 1953, 1962-1963, 1966 (Bell and Davis, 2001; Ciocco and Thompson, 1961; Greenburg et al., 1962; Nemery et al., 2001; Schrenk et al., 1949). Most of these air pollution episodes critically affected human health. Thus, a significant number of deaths and hospitalizations were recorded. Nearly 4,000 deaths were recorded from the London smog event (December 1952), which is one of the most significant air pollution episodes in history lasting for five days (Logan, 1953; Polivka, 2018). Another analysis was done nearly five decades later. The authors have claimed that more than 12,000 deaths were actually caused by the event (Bell and Davis, 2001). When considering the current death rates directly and indirectly related to air pollution, 40,000 deaths per year in Austria and Switzerland and 48,000 deaths/year in France are recorded (Cohen et al., 2005; Samet et al., 2000). In the Arab region, hundreds to thousands of annual premature deaths and illnesses are emerging every year due to the exceeded levels of air pollutants of WHO guidelines (Gibson and Farah, 2012). Asian region also shows no significant difference than the air pollution-related deaths and other chronic diseases due to rapid industrialization, especially in China and India. More than 50% of all deaths in China due to cardiovascular and respiratory diseases (Ruan et al., 2019; Zhao et al., 2021). With the rapid increase in energy consumption of the 1.3 billion population, India is becoming the most polluted country in the world. Thus,

approximately one million premature deaths were attributable to air pollution in 2015 (Thurston et al., 2016; Yuan et al., 2019). According to the WHO report published in 2014, seven million annual premature deaths are attributable to air pollution. It is almost one in eight of the total global deaths. All these findings confirm that air pollution is the most critical and the heaviest health risk burden the world has to face today (WHO, 2014). Therefore, in this section, critical areas of air pollution such as particulate pollution, health burden in different regions, economic and aesthetic effects, etc., in the world were reviewed to expand the understanding of air pollution.

2.2. Hazardous Components of Air Pollution

Mainly two sources are considered as air pollutant emitters: anthropogenic and natural. Various human activities produce anthropogenic air pollution sources such as fossil fuel combustion, emissions from transportation methods, emissions from various industries (cement factories, oil refineries), emissions from thermal power plants, agriculture activities, mining, etc. (Sanchez et al., 2020; Von Schneidemesser et al., 2015). Natural sources are mainly linked to the earth's processes, such as volcanic eruptions, dust storms, and forest fire. These natural emission sources can release significant quantities of pollutants into the air within a very short period. Biological activities such as vegetation and microbial activities in the soil can also be identified as natural sources because those processes emit gases and chemicals into the air (Han et al., 2019).

Air pollutants can migrate away from their source. Until about twenty years ago, air pollution was thought to be just an arising local problem. However, with the new data, it has come to our understanding that air pollutants can migrate across continents and ocean basins. Thus, mainly three types of air pollutants are distinguished based on the scale of study: local, regional, and global (Ramanathan and Feng, 2009). The local air pollution occurred mainly due to anthropogenic activities and encountered in the immediate vicinity of the pollutant emission source (less than a few kilometers). The characteristic air pollutants of the local air pollution are, suspended particulate matter (PM), sulfur dioxide (SO_2), carbon monoxide (CO), nitrogen oxides (NOx), volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), elemental carbon, and metals (Quintela-del-Río and Francisco-Fernández, 2011). The regional pollution spreads over a larger area from a few kilometers to a few hundred kilometers from the air pollutant emission source. Physico-chemical transformation of primary pollutants to secondary pollutants is mainly a product of this regional pollution. One of the best examples of this regional air pollution is the secondary pollutant, ozone (O_3), formed by the presence of NO_x and VOCs. Other most common examples are nitric acid (HNO_3) and sulfuric acid (H_2SO_4) are formed when NO_x and SO_2 present in the air. Oxidizing nitrogen dioxide (NO_2) forms HNO_3, while SO_2 is oxidized in the atmosphere to form H_2SO_4 (Haskins et al., 2019). Global-scale air pollution contributes to climate change, global warming due to the accumulation of greenhouse gases such as carbon dioxide (CO_2) and ultimately affects the stratospheric ozone layer (Luderer et al., 2019; Ramanathan and Feng, 2009).

Today, air pollution is dominated by a few components mainly emitted from anthropogenic sources. These naturally non-existent components are in different concentrations in the air and critical for human health. Mainly, air pollutants can be encountered in two forms: gases and particles. These air pollutants are made of CO, NOx, SO_2, O_3, heavy metals (HMs), PAHs, VOCs, polychlorinated dibenzo-*para*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and PM (Adams et al., 2015). All these air pollutants have various complex interactions depending on the environmental conditions such as temperature, humidity, wind, photochemistry, etc. Due to these complex interactions, air pollutants are categorized as primary and secondary pollutants. Interactions between primary pollutants produce secondary pollutants, which modify the physicochemical characteristics of the atmosphere and lead to global deaths and diseases (Saxena and Sonwani, 2019; Shen et al., 2020).

2.3. Particulate Pollution

Ambient PM scientifically defined as liquid or solid particles suspended in a carrier gas or a combination of solid and liquid particles in the air, and the term “aerosols” also used (Abbas et al., 2018; Harrison, 2020). PMs classify mainly based on its aerodynamic properties and formation process (chemical composition, physical and biological properties). Based on the formation process, these particles are classified as primary and secondary aerosols. Primary aerosols are emitted directly from the source in particulate form, and secondary aerosols are

identified as particles, generated in the atmosphere due to the evolution of the primary particles (Arruti et al., 2012).

The most common PM classification method is based on the aerodynamic diameter. This classification method is important over other classification methods; thus, the aerodynamic properties of the PM govern their deposition and penetration through biological barriers such as cell membranes and respiratory tract. Most importantly, these properties can be used to identify the chemical composition, source, transport, and removal of these particles from the air (Clements et al., 2014). Three major PM categories are defined according to the aerodynamic diameter: coarse, fine, and ultrafine. PM of 10 μm or less in diameter (2.5 to 10 μm) is considered as PM_{10} (coarse fraction). They mainly affect the upper part of the respiratory tract and cause severe respiratory problems. The fraction of fine particulate matter ($PM_{2.5}$) is lesser than 2.5 μm in size. Ultrafine particulates are less than 0.1 μm in diameter (Coronas et al., 2009). These fine and ultrafine particles can easily penetrate to the deeper levels of the human respiratory system. Thus, this fraction is the most harmful among all air pollutants and causes various adverse health effects such as cardiovascular problems, respiratory diseases, adverse birth outcomes, mutagenicity, DNA damage, inflammatory responses, and lung cancer. The PM's toxicity depends on their composition, size, and shape (Hoek et al., 2013; Laden et al., 2006). According to many findings and available data, $PM_{2.5}$ is identified as a high-risk factor. In an extensive study conducted on the global burden of disease in 1990 - 2010, $PM_{2.5}$ placed on the ninth position out of all health risk factors (Lim et al., 2012). Apart from the adverse

health effects, PM is also responsible for various atmospheric effects such as reducing the atmospheric visibility, deposition of PM on plant leaves can reduce the ability of photosynthesis, affecting meteorological processes due to the deposition of metals and minerals on soil (Grantz et al., 2003; Von Schneidemesser et al., 2015).

Both natural and anthropogenic activities are responsible for the mass emission of primary aerosols globally, but natural sources belong to a significant PM fraction. Sea salt, soil erosion, volcanic eruptions, wildfires, and biogenic sources are the most significant natural PM sources. Among all these sources, sea salt is the biggest contributor; thus, about 75% of the earth's surface is covered by the ocean (Horemans et al., 2009). Sea salt particles are released into the air when the sea waves create and burst air bubbles on the surface of the water. Size and chemical reactions in the atmosphere depend on the salt composition (NaCl, Na_2SO_4, K_2SO_4, $MgSO_4$) of the sea salt particles (Kardel et al., 2020). Soil erosion is another main natural source, that happens primarily due to wind action through the dry regions. This is how crustal elements (Al, Si, Fe, Ti, Ca, Na, Mg, and K) emits into the air with soil particles. Geological characteristics, soil erosion conditions, wind direction, and many other important air pollution data of a specific region can be determined by understanding the elemental composition of the ambient air particles (Tian et al., 2019; Vasilatou et al., 2017). Volcanic eruptions form ash, dust, as well as gases (SO_2, CO_2, H_2O, H_2S) and contribute significantly to the particle burden of the global air due to the release of pollutants at high altitude (Searl et al., 2002). It is hard to make a clear boundary between natural and anthropogenic when considering wildfires. Chemical and

physical characteristics of the particles mainly depend on the burnt plant timber (Cascio, 2018). Biogenic particles have a great variation in origin. Pollen and fragments of plant materials are the main sources of biogenic particles. Other than that, seeds, spores, and microorganisms (bacteria, algae, fungi, and viruses) are also contributing as natural PM sources. Studies show that some airborne PMs contain even pathogenic microorganisms, which are severe threats to human health (Marone et al., 2020; Polymenakou et al., 2008). Environmental conditions such as humidity, rainfall, sunlight, etc., behave as the main controlling factors of the particle quantity produced by these sources (Choi et al., 2016).

The transportation sector is responsible mainly for the anthropogenic emission of PM. Industrial processes, incineration plants, petroleum products, and agricultural activities also play a huge role in emitting PM into the air. Today, almost all transportation systems use fuel. Thus, exhaust gas and wear of tires and brakes mainly responsible for many types of particles (Davis et al., 2001). Waste incineration, wood, and coal combustion (cooking, heating) emit organic compound-rich particles into the air (Mestl et al., 2007; Reche et al., 2012). Industrial dust is mainly responsible for HM pollution. HM is one of the most important components of air and critical in health. Therefore, HM emissions and the health effects are reviewed in a separate section. Sulfur and Nitrogen based gases (SO_2, NO, NO_2) are emitted into the atmosphere due to the burning of fossil fuel and biomass (Greaver et al., 2012). Animal husbandry, agricultural activities, and biomass burning are responsible for the emission of NH_3 (Phairuang et al., 2017;

Shen et al., 2018). Those gases, which are formed mainly via anthropogenic activities, facilitate the formation of secondary particles.

2.4. Global Status of PM

Exposure to air pollutants is a major health risk throughout the world. Compared to the developed countries, the risk is higher in developing countries (India, Indonesia, Egypt, Sudan) due to the high population density, weak environmental legislation, poverty, and poor use of modern technology (Briggs, 2003). Many developed countries have developed enforced emissions standards to improve air quality and reduce the emission of man-made hazardous pollutants. That is not the story of many developing countries. According to the World Bank, China alone had 16 (out of 20) of the world's most air-polluted cities (Xu et al., 2017). The rapid growth of industrialization is the major cause of air pollution in China. Most Chinese cities exceed the PM_{10} annual mean concentration standards implemented by Chinese Ambient Air Quality Standards (Miao et al., 2016). According to the annual distribution of the PM_{10} over the world (from 2008 - 2015), Eastern Mediterranean cities such as Riyadh, Ma'ameer, Dora, and Abu Dhabi were observed with very high average concentrations (>200 $\mu g/m^3$). Cities of Southeast Asia such as Delhi, Dhaka, Colombo, and Karachi, showed the second highest levels with high annual average concentration (150 - 200 $\mu g/m^3$). Concerning the PM_{10} pollution, Delhi, the capital of India, is found to be the most polluted city. PM_{10} concentration of Delhi was 11 times higher than the WHO annual mean standard level (20 $\mu g/m^3$) (Saxena and

Sonwani, 2019). Many cities in India are facing this particulate pollution problem. Most of the cities are exceeding the national and international PM standard values. According to the WHO, Delhi is identified as the most polluted city in the world (WHO, 2016). Another review also showed that particle pollution in Pakistan and other South Asian countries is very high (Waheed et al., 2006). According to an evaluation of air quality of eighteen main cities under the WHO guidelines, they recommend that Dhaka, Beijing, Cairo, and Karachi urgently need a reduction of air pollution. Only five megacities were classified as having fair air quality out of all eighteen (Gurjar et al., 2008). Bangkok and Thailand (from 1999 to 2003) also showed 2.6 times the annual mean PM_{10} concentration of WHO standards (Kan et al., 2010). In Korea, PM_{10} concentrations of seven major cities were monitored from 1996 to 2010. It revealed a significant decrease in PM_{10} concentrations in most of the cities after 2000. However, the values were still above the WHO levels and their national standards (Sharma et al., 2014). Slightly low average levels were shown in the Western Pacific region, but the concentrations lay within the range of 100 - 149 $\mu g/m^3$. America and Europe showed the lowest PM_{10} levels than the other regions (Saxena and Sonwani, 2019).

2.5. PM Composition and Health Effects

Atmospheric particles are generally composed of three major fractions: organic fraction, inorganic fraction, and biologic fraction. Only a few major components, including PAHs (organic) and HMs (inorganic), which are critical for human health, were reviewed here.

However, the chemical composition of atmospheric particles is not distributed equally in all size ranges; thus, it depends on the emission source (Zheng et al., 2014).

2.5.1. PAHs and Health

PAHs are known as a large group of organic compounds with two or more aromatic rings. Among all chemical and biological compounds of aerosols, PAHs are considered the most crucial group due to their confirmed adverse health effects. In the atmosphere, PAHs exist in both vapor and particle phases. Generally, light PAHs (low molecular weight) with less than three aromatic rings exist in the vapor phase, whereas heavy PAHs or multi-ringed PAHs (five or more aromatic rings) exist in the particle phase. Intermediate molecular weight PAHs (with four aromatic benzene rings) are partitioned between these two phases (Ravindra et al., 2008). Most importantly, more than 90% of carcinogenic PAHs are in the particulate phase. However, these harmful PAHs exist in minimal quantities but in high concentrations (Hayakawa et al., 2016). PAHs have high melting and boiling points, very low aqueous solubility, and low vapor pressure. With the addition of aromatic rings, the aqueous solubility reduces while PAHs are highly soluble in organic solvents due to their high lipophilic capacity. These characteristic features are usually used to identify the PAHs and their health effects (Akyüz and Çabuk, 2010; Masih et al., 2010).

Anthropogenic activities release PAHs into the air. Incomplete combustion of organic materials such as coal, oil, and wood are identified as the single largest contributor of PAHs to the

environment (Zhang and Tao, 2009). Although these PAHs are in high concentrations in the ambient air, none of the PAHs do not chemically synthesize for industrial purposes, but a few of them are used as intermediaries in pesticides, pharmaceuticals, agricultural products, manufacturing pigments and dyes, electronic products, photographic products, functional plastics, lubricating materials, and other chemical industries (Zheng et al., 2011). PAHs are formed via natural biological processes such as vegetation degradation as well as certain plants and bacteria. However, very few researches have been conducted on the PAHs related to the biological processes (Seo et al., 2007). Volcanos, forest fires, petroleum seeps, vegetation decomposition, erosion of petroleum hydrocarbons containing sediments, and bacterial and algal synthesis processes are the main natural sources of PAHs in the air (Zhang and Tao, 2009).

According to the USEPA, among all PAHs, 16 are classified as priority pollutants based on their toxicological profile, available data, the greater chance of exposure to these PAHs, and high concentrations in the air. But the health effects of all these 16 priority PAHs are not exactly alike (IARC, 1982). The 16 PAHs: Naphthalene (Nap), Acenaphthylene (Acpy), Acenaphthene (Acp), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Flt), Pyrene (Pyr), Benz[a]anthracene (BaA), Chrysene (Chr), Benzo[b]fluoranthene (BbF), Benzo[k]fluoranthene (BkF), Benzo[a]pyrene (BaP), Dibenz[a,h]anthracene (DahA), Benzo[ghi]perylene (BghiP), Indeno[1,2,3-cd]pyrene (Ind) are one of the first atmospheric pollutant group identified as suspected carcinogen (USEPA, 1986). The International Agency for Research on Cancer (IARC) classifies these PAHs as known, possibly, or probably carcinogenic to humans (Group

1, 2A, or 2B). Based on the available data, BaP is classified as a group 1 carcinogen. DahA is classified as a probable carcinogen to humans (Group 2A), and Nap, Chr, BaA, BbF, BkF, Ind are classified as possibly carcinogenic to humans (Group 2B) (IARC, 2010, 2012). Among all these PAHs, some are well known as carcinogens and mutagens. Therefore, exposure to these PAHs has a great threat to human health (Kim et al., 2013). The general population exposes to PAHs mainly (routes of exposure) by breathing ambient air and indoor air, smoking cigarettes, or eating PAHs containing foods (Yu et al., 2015). The main route of exposure of non-smokers is through food because some crops such as wheat, rye, and lentils synthesize PAHs or absorb them via water, air, or soil (Ciecierska and Obiedziński, 2013). Cooking foods at high temperatures is a major source of generating PAHs (Chen and Chen, 2001). PAH intake may occur via ingestion, inhalation, or dermal (skin) exposure in both occupational and non-occupational settings (Wang et al., 2012).

Both short term (acute) and long-term (chronic) health effects may occur with exposure to these hazardous PAHs. The acute effects of PAHs on human health mainly depend on the extent of exposure, PAHs concentration during exposure, the toxic capacity of the exposed PAHs, and the route of exposure. In addition to these pre-existing health conditions, age, and sex are also directly related to acute health effects (Ravindra et al., 2008). It has been reported that occupational exposures to high levels of pollutants containing hazardous PAHs have resulted in symptoms such as eye irritation, nausea, vomiting, diarrhea, and confusion (Ivy et al., 2008). However, it is not clear that which components of the mixture were responsible for those effects.

Other compounds that are commonly found in PAHs may be responsible for those symptoms. Skin irritation and inflammation are the other most common symptoms that have been recorded caused by the mixture of PAHs. Thus, Ant, BaP, and Nap are direct skin irritants and cause an allergic reaction in the skin (Callesen et al., 2014; Factor et al., 2011). Chronic or long-term health effects of exposure to PAHs might be responsible for a series of adverse health problems such as redness and skin inflammation, breakdown of red blood cells, decreased immune function, cataracts, kidney and liver damage (e.g., jaundice), breathing problems, asthma-like symptoms, lung function abnormalities, cancers (skin, bladder, gastrointestinal and lung cancers) (Diggs et al., 2012; Olsson et al., 2010). It has been reported that cancers can be formed with the long-term exposure of low levels of some highly carcinogenic PAHs (BaP) in laboratory animals (Diggs et al., 2012). Adverse reproductive and developmental effects have also been proven in animal models, but it has not shown the same effect on humans (Wells et al., 2010). Unfortunately, except for a few accidental naphthalene contacts, no oral or inhalation exposure studies have not been done on humans. The majority of the published studies are occupationally exposed to PAHs via inhalation. Therefore, even if some PAHs are proved to be highly carcinogenic to lab animals, it is not fair to adapt the conclusions of those studies to humans. Moreover, numerous authors have demonstrated that long-term exposure to PAHs may affect DNA damage, cell damage, and cardiopulmonary mortality (García-Suástegui et al., 2011; John et al., 2009; Kuo et al., 2003).

Carcinogenicity, teratogenicity, and genotoxicity of PAHs have been studied for decades. Most of the studies are based on occupational exposure studies. Reactive metabolites of some PAHs are one of the major causes of biochemical disruption and cell damage due to their high potential to bind to cellular proteins. Ultimate results might be mutations, malformations in the developmental process, tumors, and cancer (Bach et al., 2003; Misaki et al., 2016). Researchers have revealed that the mixture of PAHs is more harmful to human health than individual PAHs. Therefore, some PAH rich mixtures are classified as carcinogens to humans (Pushparajah and Ioannides, 2018). Epidemiological studies have shown that gas workers, coke-oven workers, and workers exposed to diesel exhausts have the highest rate of lung cancer and skin cancer. Even if the increased cases of cancer with occupation show a direct relation, all pieces of evidence showed that those working environments produce not only PAHs but also a complex mixture of pollutants that have carcinogenic potency (Kamal et al., 2015; Stenehjem et al., 2017; Wang et al., 2015; Zhang et al., 2012). When considering the long-term exposure studies conducted using animals, three main exposure types were identified: inhalation, ingestion, and skin contact exposure. Most of the studies have demonstrated that the long period of inhalation exposure developed lung cancer (Courter et al., 2008), stomach cancer from ingestion exposure via food, and skin cancer from skin contact (Helleberg et al., 2001; Knafla et al., 2006). Animal studies also showed that certain PAHs could affect the hematopoietic and immune systems. In addition, BaP is the first chemical carcinogen to be discovered and the most common PAH to cause cancer in animals (Feng et al., 2016; Latif et al., 2010). Embryotoxic effects of PAH

exposure were also described primarily using animal models. Ingestion of high levels of BaP during pregnancy resulted in birth defects, including decreased body weight, premature delivery, and heart malformations in the offspring (Chu et al., 2013; Kamelia et al., 2019; Wassenberg and Di Giulio, 2004).

Genotoxicity of PAHs is another great health problem. Various types of genotoxic effects of PAHs are also detected by both *in vitro* and *in vivo* (rodent and mammalian cell lines) DNA damage, chromosomal damage, gene mutations, and tumor formation (de Oliveira Galvão et al., 2019; IARC, 2010). Reactive oxygen species (ROS), which are formed by the metabolism of PAHs, can induce single and double-strand breaks. Both single and double-strand breaks can be measured using the comet assay (Matzenbacher et al., 2016). Carcinogenicity of PAHs is also related to Genotoxicity (McCarrick et al., 2019). Some PAHs such as BaP, Chr, Flt, benzo[ghi]fluoranthene, benzo[j]fluoranthene, dibenzo[a,l]pyrene, and triphenylene show high genotoxicity when incubated in the presence of exogenous metabolic activation mixture (Topinka et al., 2012; White, 2002). Genotoxicity of each PAH is not appropriately quantified yet with the available data because most of the data was generated with *in vitro* models. Therefore, to understand the genotoxicity of the PAHs, further *in vivo* studies must be done.

2.5.2. Inorganic Elements and Health

Another major fraction of the PM is trace elements and HMs. Al, Ca, Fe, K, Mg, and Na are the main elements adsorbed on the particles as well as the trace elements such as Ag, As, Ba, Cd,

Ce, Co, Cr, Cu, La, Mn, Nb, Ni, P, Pb, Rb, Sb, Sc, Se, Sn, Sr, Te, Ti, V, and Zn are also abundant on the surface of the particles (Guendouzi et al., 2020). Soluble ions such as F^-, Cl^-, NO^{3-}, $SO4^{2-}$, $PO4^{3-}$, and NH^{4+} could also be adsorbed and play an important role in human health. About 70%-90% of HMs are contained in PM_{10} (coarse fraction of the particles) (Mohanraj et al., 2004). However, most of the poisonous and harmful HMs such as Pb, Cd, Ni, Mn, V, Zn, etc., are mainly adsorbed by fine particles (Duan and Tan, 2013). Vast majority of HMs release by the industries and the types of HMs emit to the ambient air depend on the nature of the industry. Therefore, HM profiling can be used as a source identification method in all air pollution studies. However, most industries emit not just a single element but also a complex mixture of pollutants, including HMs (Ergenekon and Ulutaş, 2014). For example, As, and Se mainly emit from certain glass production industries, steel and ceramic related industries are responsible for the Cd release into the air, Cr, and Ni are mainly due to the ferrous metal production, chemical production and cement related industries responsible for the release of Hg; Pb and Zn come from the ferrous metallurgy (Illi et al., 2017; Yamazaki et al., 2014). However, the industries are not solely responsible for HM emission sources. Some HMs, which play a huge role in human health, such as Pb, Cu, Zn, Mn, Co, Ni, and Cd, are released by the abrasion of the mobile part (brakes, tires) of automotive (Zhang et al., 2018). The source of Antimony is identified as the excessive use of vehicle brakes (Grigoratos and Martini, 2015). Another most important source of HM pollution is fossil fuel burning. The rapid growth of population in the world is directly related to high energy consumption. Emission of HMs such as Ni and V is directly

related to fossil fuel burning (Al-Momani and Massadeh, 2005; Pyta et al., 2020), and petrol burning was the main reason for high concentrations of Pb in the air of major cities in the past. After implementing the EPA's regulatory guidelines on air quality, the Pb level was significantly declined by 94% between 1980 and 1999. Today, the major source of Pb is metal processing plants and aircraft engines, which are operated with leaded gasoline (Safar and Labib, 2010).

Exposure to environmental toxic metals has become a global health concern for a few decades (Fawell and Nieuwenhuijsen, 2003). Numerous health problems such as lung and cardiopulmonary diseases are associated with the HM absorbed inhalable particulates (Fei et al., 2017; Xie et al., 2016). HMs are also crucial for biological systems in many ways because they affect cellular components and organelles such as nuclei, lysosomes, cell membrane, mitochondria, and endoplasmic reticulum. In addition to that, HMs affect some cellular processes such as enzymes involved in metabolism, detoxification, and damage repair (Wang and Shi, 2001). HMs may cause DNA damage, cell cycle modulation, carcinogenesis, and apoptosis via interacting with cell components like DNA and nuclear proteins (Beyersmann and Hartwig, 2008). Due to the non-degradable nature of HMs, bioaccumulation occurs in plants and animals. This bio-system circulation and accumulation often happen via the dissolved form of HMs (Houri et al., 2019; Liu et al., 2007). According to the WHO and IARC, Cr, Ni, As, Cd, Pb, and its compounds, mercury, Sb_2O_3, and Co are confirmed carcinogens ("known" or "probable" human carcinogens) while As, and Cd are classified as group I human carcinogens.

Chronic exposure to high levels of toxic HMs, including As and Cd, is associated with various health risks, especially cancers in the bladder, kidney, liver, skin, and lung (IARC, 2012). Shreds of evidence have been shown that possible cancer risk even at lower concentrations (Järup, 2003). In addition to this, As and some other toxic elements may be an independent risk factor for cardiovascular disease (Chowdhury et al., 2018; Jomova and Valko, 2011; Jomova et al., 2011). Experimental studies have revealed that oxidative stress caused by ROS production plays a major role in the toxicity and carcinogenicity of HMs such as As, Cd, Cr, Pb, and Hg. ROS generation can be caused by the depletion of protein, trigger DNA damage, lipid peroxidation, and several other effects (Borošková et al., 1993; Stern, 2010). However, heavy metal-induced toxicity and carcinogenicity depend on many factors and complex cellular processes. Most of those complex processes have not been clearly understood yet. However, a few reviews have provided an extensive analysis on the available data of each toxic HMs and covered the areas of their emission sources, environmental occurrence and human exposure, characteristic features, physic-chemical properties, production and use, toxicological mechanisms, molecular mechanisms, genotoxicity, and carcinogenicity (Jan et al., 2015; Tchounwou et al., 2012).

2.6. Local Context: Sri Lanka

With the rapid growth of industries, population density, and an increasing number of motor vehicles and traffic congestion, air pollution is becoming a major concern in Sri Lanka. Motor vehicle emissions accounted for 55-60% of air pollution, while 20-25% was due to industries,

and 20% was from domestic sources in Sri Lanka during 2011 (Wickramasinghe et al., 2012). Within the last two decades, the number of motor vehicles has increased a considerable number. From 2000 to 2020, the number of motorcycles is increased five times, the number of cars has increased by 300%. This rapid growth pattern can be seen in other vehicle types. Therefore, this uncontrolled increase of vehicles is becoming a huge burden on the country's air quality (Ileperuma, 2020). People face huge traffic jams, stressed metal conditions and loss of productivity, and most importantly, respiratory-related diseases in the main cities such as Colombo and Kandy due to the road development pace is not synchronizing with the rate of registering the new vehicle in the country. On the other hand, with some good air quality regulatory mechanisms, leaded fuel was discontented in 2002, and atmospheric lead levels were reduced by around 80% in the Colombo city (Senanayake et al., 2001). PM levels were decreased by 10 - 20% by introducing the vehicular emission testing (VET) program (Premasiri et al., 2015).

Very few quantitative air quality measurement studies have been published in Sri Lanka. According to Ileperuma, (2020), the first air quality measurements are gone back to 1994; thus, the University of Colombo studied lead concentrations in ambient air in Colombo. Results of that study showed that the ambient lead concentration in a residential area of the Colombo urban environment was about 200 $\mu g/m^3$ and about 400 $\mu g/m^3$ in the vicinity of the main road. These values are far greater than the threshold values (2 $\mu g/m^3$ for a 24-hour sampling period) of ambient lead published by the Central Environmental Authority. Sri Lankan annual air quality

standard of PM_{10} is 50 μg/$m^{3,}$ and WHO recommends that the average annual ambient PM_{10} level be less than 20 μg/$m^{3,}$ but Colombo Fort air quality monitoring station showed steady variations within a range of 50-120 μg/m^3 within the period of 1998-2016. This might have happened due to traffic congestion in the area (Ileperuma, 2020).

Accumulation of air pollutants such as priority PAHs and PM_{10} in Kandy city happens mainly due to its isolated geographic conditions and a higher level of traffic congestion. According to the available research data, the mean PM_{10} concentration in Kandy city was 129 μg/$m^{3,}$ which exceeds the Sri Lanka standard PM_{10} concentration (Wickramasinghe et al., 2012). Another study that has determined PM_{10} from 25 sites in Kandy revealed that 13 out of 25 sites had exceeded the current 24 h standard of 100 μg/m^3. Due to the high traffic density (2640 vehicles/h), the Katugastota bridge showed the highest PM_{10} concentration, which is 340 μg/m^3. Besides, most of the locations with high traffic congestion showed over twice the national air quality standard level of PM_{10} (Elangasinghe and Shanthini, 2008). On the other hand, PAHs are also present in very high concentrations in Kandy city (Wickramasinghe et al., 2012). The mean total concentration of 16 priority PAHs in Kandy ranged from 57.43 to 1246.12 ng/$m^{3,}$ with a mean of 695.94 ng/m^3 in urban heavy traffic locations. Rural high firewood burning area and rural low firewood burning area showed 192.48 ng/m^3 and 100.31 ng/m^3 average concentration, respectively (Wickramasinghe et al., 2012). With these results, it can be determined that the high traffic volume and incomplete fossil fuel burning are responsible for the high PAH levels in urban areas of Kandy while firewood burning in rural areas.

Medical surveys have shown that Sri Lanka might become a country with a higher aging population by 2030 and have around 22% of the population over 60 years old. Approximately a thousand deaths have occurred due to outdoor air pollution in Sri Lanka, according to the estimations of WHO in 2004 (Premasiri et al., 2010). However, in Sri Lanka, no long-term epidemiological studies have been published to assess air pollution and health but only a few brief reports (Galappaththi, 2020). When considering the air pollution and respiratory health in Sri Lanka, since 1995, respiratory disease has ranked as the second highest cause for hospitalization. It is among the first five leading causes of death in most of the age groups. Asthma might be considered as the most important respiratory disease due to a large number of hospitalizations and hospital deaths. Nasopharyngeal cancers have also become one of the major health issues in Sri Lanka, especially in Kandy (Nandasena, A. R. Wickremasinghe, et al., 2012).

Children's respiratory health must be given a high priority in any country because immature respiratory systems are more prone to diseases due to air pollution than mature adult respiratory systems. Very few studies have been done in Sri Lanka focused especially on children's respiratory diseases in relation to air pollution. The majority of those have been done around Colombo and Kandy due to their high population density and high air pollution level records (Galappaththi, 2020). A study was published in 2001, which was done to find the correlations between hospital attendance for acute wheezing and air pollution in Colombo. According to their findings, hospital attendance (Lady Ridgeway Children's Hospital, Colombo) in 1998 for wheezing needing nebulizer therapy significantly correlated with the pollutant levels recorded

for ambient air in Colombo. The highest and lowest hospital attendance rates were directly associated with the highest and the lowest pollutant levels (Senanayake et al., 2001). Dharshana and Coowanitwong, (2008) have revealed that PM_{10} is the most dominant air pollutant in the Colombo ambient air; thus, nearly 20% of asthma patients at the Lady Ridgeway Children's hospital could be due to exposure to high PM_{10} levels in 2005. They have also observed that nearly 60% of the respiratory cases occurred at even below 80 μgm^{-3} PM_{10} concentration. In addition to that, their investigation showed that PM_{10} has strong associations with three types of respiratory illnesses: bronchitis, emphysema, and other chronic obstructive pulmonary diseases, especially among children. These disease categories showed a significant association with a correlation coefficient of 0.717 at 99% confidence. Another exposure study on children was carried out from 2009 to 2010 in urban and semi-urban settings in Sri Lanka. They have found that children from the urban setting (Colombo) had a significantly higher prevalence of wheezing than children from the semi-urban setting (Panadura), which is presumed to have a much lower ambient air pollution level. In addition to that, they have found that the highest $PM_{2.5}$ levels were reported from homes that burned biomass for cooking in the urban setting (Nandasena, A. Wickremasinghe, et al., 2012).

Another study was conducted in Kandy using school children (510 children) to find a relationship between ambient air pollution and school children's respiratory illnesses (Siritunga et al., 2006). For that, they have selected school children from rural and urban areas in the Kandy district, and their respiratory illnesses were recorded using the diary method. Average

concentrations of air pollutants such as SO_2, NO_2, and O_3 were three to five times higher in the city school premises compared to the rural areas. Moreover, they have found that the respiratory symptoms such as the occurrence of cough were 1.8 times higher, nasal discharges were 1.4 times higher, and throat irritation was 1.8 times higher in the schools in the city (urban area). These respiratory problems might have a correlation with the high ambient air pollution in the city. Moreover, deadly respiratory diseases such as lung cancer, severe asthma attacks, and other respiratory diseases are becoming critical issues in Kandy (Wickramasinghe et al., 2011). Compared to the hospitalized cases (National Hospital in Kandy) of other cities, lung cancers and other respiratory illnesses are very high in Kandy (National Cancer Control Programme, 2011). Exposure to the smoke emissions of old buses, which are abundant in carcinogenic PAHs, might be the reason for this (Wickramasinghe et al., 2012). A study which was done in Ratmalana (a suburb in the Colombo district) also has revealed bus drivers are the most affected by NO_2 (57.36 $\mu g/m^3$) and SO_2 (82.70 $\mu g/m^3$) among three-wheeler drivers, street vendors, and shopkeepers. Besides, a high prevalence of respiratory illnesses was also reported among bus drivers (Perera et al., 2007). After reviewing a few more epidemiological studies, the necessity of having larger epidemiological investigations to address the problems of measurement error, reduce uncertainties in risk estimates, and identify the determinants of exposures have been raised (Nandasena et al., 2010).

The economic value of preventing adverse air pollution-related health effects is a huge burden to the government. The potential financial loss due to outdoor air pollution in Colombo owing

to $PM_{2.5}$ has been estimated to be around Rs.774 million, and due to PM_{10}, it is about 26 billion per annum (Chandrasiri, 2006). Due to traffic congestion in Colombo, the economic loss has been estimated to be around Rs.32 billion annually. This financial loss might be able to reduce significantly by developing the road network at least in major cities and improving the public transport system is one of the most efficient methods to reduce air pollution. Therefore, continuous monitoring and understanding of air pollution and public health are vital for future plans.

CHAPTER 03

MATERIAL AND METHODS

3.1. Description of Sampling Site

The study was performed on urban particulate matter (PM_{10}) of the city of Kandy (7°17′47″N; 80°38′6″E), which lies in the middle of the Kandy plateau, Sri Lanka, about 500 m above mean sea level. Kandy city, which is considered as the cultural center of the country and the administrative capital of the central province, expanded over 28 km^2 with over 170,000 permanent population and around 100,000 daily transient population, as suggested by the National Census data in 2012 (Census, 2012). The region is characterized by the humid tropical climate, and the average daytime ambient temperature is in the range of 28-32°C, monthly rainfall is in the range of 52-398 mm, while the daytime relative humidity is in the range of 63-83% (Wickramasinghe et al., 2011). Thus, the limited land area of the city has heavy traffic flow (estimated 24 h traffic flow is 1,06,000 via four main entrance roads), and the mountains surrounding the city result in exceptionally high vehicular emissions (Wickramasinghe et al., 2011).

Sampling was performed in two sites, and the sites were selected considering the parameters such as traffic flow, school children's health, and public transport. Fig. 3.1 shows the locations of the two sampling sites, and the sites were designated as Mahamaya Girls' College (MGC)

and the Provincial Department of Education, Central Province (PDE). MGC is located at the edge of the Sangaraja Mawatha, which was affected by the daily heavy traffic. National Hospital Goods Shed bus terminal, railway station, main post office, and municipal central market are within a 200 m radius of the PDE site. There was no other significant contamination source of PAHs near both sampling sites.

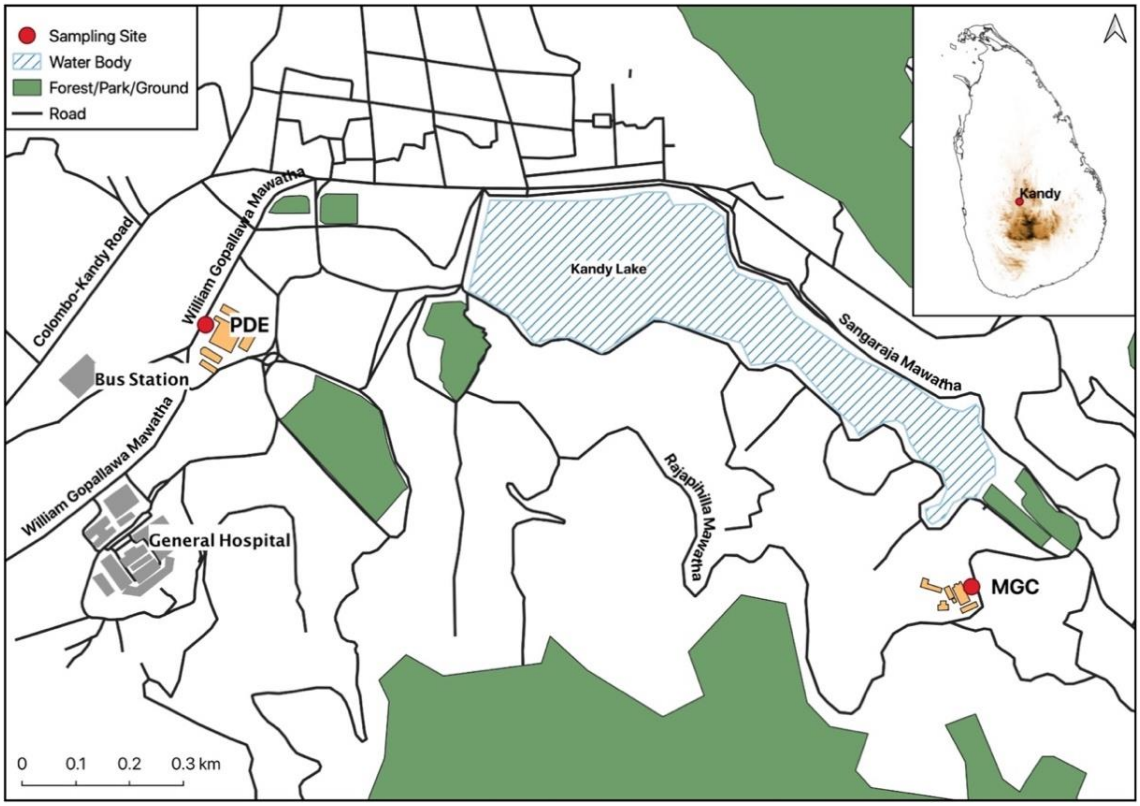


Fig. 3.1. Map of the study area and locations of the two sampling sites (PDE and MGC). Site map was prepared in QGIS version 3.8 (http://www.qgis.org).

3.2. PM_{10} Sampling

PM_{10} in ambient air was sampled using a high-volume Respirable Dust Sampler (Model: APM 460, Envirotech, India) operated at a constant flow rate of ~1.2 m^3/min with glass microfiber filter paper with the size of 20.3 cm x 25.4 cm (Whatman EPM 2000, USA) (Wickramasinghe et al., 2011). The air sampler collected particles with aerodynamic diameter less than or equal to 10 μm. The filter papers were weighed with an analytical balance (AE 240, METTLER, USA) under controlled temperature and humidity conditions (25°C, 50% RH) before sampling. Samples were collected in 14 days from February to October 2017 (only daytime). All the samples were collected during ordinary days avoiding high humidity, heavy rains, thunderstorms, and school vacations.

Atmospheric particles were collected by running the sampler for six hours each day (between 7 am to 6 pm covering the whole period). The sample collection starting time was changed to represent the whole day, including peak traffic times and weekends. The air sampler was placed about 1.5 m above the ground to simulate the human breathing zone and at least 2 m from any obstacle. In the sampling process, human activities at the sampling sites were normal as usual. Loaded filter papers were wrapped carefully in aluminum foil at the sampling site and transported to the laboratory. Then the filter papers were weighed again under similar conditions (before used in the field) for gravimetric determination of PM_{10} and stored at -20°C laboratory

freezer (BLCF, EVERmed, Italy) until subjected to the extraction procedure (Gao et al., 2015; USEPA, 1999).

3.3. Analysis of PAHs

3.3.1. Sample Preparation

To guarantee the accuracy of the experiment, aerosol glass fiber filter (GFF) samples from Kandy city were analyzed at the State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, China and kept at -20°C until analysis. High-performance liquid chromatography (HPLC) grade chemicals were used throughout the whole process. Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich, USA. Sample collection, preparation, and analysis were based on USEPA Compendium Method TO-13A (USEPA, 1999). Pieces of the GFF sample (diameter: 45mm) were cut for analysis of PAHs. The GFF sample was wrapped with filter paper and extracted with 140 ml dichloromethane (DCM) for 24 hours using Soxhlet extraction following the addition of 200 ng deuterated PAHs mixture (naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, and perylene-d12) as surrogates. The filter paper was cleaned with DCM and methanol for 24 hours before use. Sample extracts in flasks were reduced to 2 ml by rotary evaporation (Zhao et al., 2015). The extracts were then transferred to a 10 ml glass vial. The flasks were rinsed three times with 1 ml DCM for each time. The DCM, which was used to rinse the flask also transferred to the 10 ml glass vials. The volume of the extracts was reduced to 0.5

ml by gentle nitrogen (purity: 99.999%) blowing. 1000 ng hexamethylbenzene was added into the samples as an internal standard.

3.3.2. Sample Analysis

Samples were analyzed using a gas chromatography-mass spectroscopy (GC-MS) (GCMS-QP2010, Shimadzu, Japan) fitted with a capillary column (DB-5MS, Agilent Technologies, USA) (Initial diameter 0.25 mm, length 30 m and thickness 0.25 µm) in electron ionization (EI) selected ion monitoring (SIM) mode which gives more sensitivity. GC inlet temperature was 290°C. The oven temperature program was as follows: initial 60°C for 1 min, 4°C/min until 100°C, 10°C/min until 295°C and held for 20 minutes (Tham et al., 2008). Molecular ions of USEPA PAHs were used as quantifiers. One quantifier was also monitored for each compound. A calibration curve, including six levels (0.02-0.20 ng/µl), was used for PAH quantitation. The correlation coefficient of the calibration curves was more than 0.997 ($r^2 > 0.997$) for a linear least square fit of the data. Two batches of samples were analyzed. Three blanks were carried out, and duplicates were run for each batch. Concentrations were determined for 12 PAHs in all samples as follows: Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Flt), Pyrene (Pyr), Benz[a]anthracene (BaA), Chrysene (Chr), Benzo[b]fluoranthene (BbF), Benzo[k]fluoranthene (BkF), Benzo[a]pyrene (BaP), Dibenz[a,h]anthracene (DahA), Benzo[ghi]perylene (BghiP), and Indeno[1,2,3-cd]pyrene (Ind). The four most volatile PAHs (naphthalene, acenaphthylene, acenaphthene, and fluorene) are not reported here because of low recovery (Choi et al., 2007).

3.4. Metal Analysis

Metal analysis of the Kandy city aerosol GFF samples was also conducted at the State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, China. The element analysis experiment process was previously described by Gao et al., (2015). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (ICP-MS X Series 2, Thermo Scientific, USA) was used to identify the concentrations of the trace metals (Na, Mg, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Y, Cd, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Pb, Th, U, Al). GFF sample (7.948 cm^2) was cut with a clean scissor and digested using 5 ml concentrated nitric acid under 120℃ for 10h. The digestion was carried out in a Teflon vessel using a digester (Multiwave 5000, Anton Paar, Germany). The sample was then transferred to a 50 ml centrifuge tube and rinsed the Teflon vessel three times by using ultra-pure water and transferred to the same centrifuge tube. Final volume of the digestion solution was fixed to 45 ml. The tube was centrifuged at 1200 circles/min for 10 minutes. 10 ml of the solution from the upper transparent layer was taken and measured metals using ICP-MS. For quality control of element analysis, one duplicate was included in every ten measurements. Samples were analyzed more than twice to check reproducibility and reduce errors. The error of duplicate measurement was below 5%. Samples were prepared and tested as specified in USEPA Method 200.8 (USEPA, 1994).

3.5. Particle Extraction for Cell Culture Treatments

All chemicals used for particle extraction from the filter papers were purchased from Sigma-Aldrich, USA, unless otherwise mentioned. To obtain particles for *in vitro* exposure, the sonication extraction method was used (Chen and Chen, 2011; De Martinis et al., 1999). First, quartz fiber filters were cut into 1 cm x 1 cm pieces and soaked with a small amount of 75% alcohol. Then the soaked filter papers were sonicated for 30 minutes in an ultrasonic bath sonicator (DC-200H, mrc, UK), which was filled with ice-cold water. Then, each filter paper was treated with 5 ml of ultra-pure water and sonicated again for 15 minutes, and suspensions with detached particles were collected. To extract the maximum volume of particles from the filter paper, each group was sonicated three times. The detached particles were then dried out by lyophilization using a freeze dryer (CHRIST-ALPHA 1-4 LD plus, Germany) and resuspended in ultra-pure water to prepare the 3mg/ml stock solution. Stock solutions were then stored in -80°C ultralow freezer (Thermo Fisher Scientific, USA) until further use.

3.6. Cell Culture

To better understand the possible biological effects associated with PM_{10} in the city of Kandy, a primary porcine airway epithelial cell culture was established with modifications to a previously described method (Wu et al., 1985). The cell culture grade chemicals were purchased from Sigma-Aldrich, USA, unless otherwise mentioned, and the whole cell culture process was done in an aseptic environment.

3.6.1. Reagents

Hank's Balanced Salt Solution (HBSS) (cat number: H6136), Dulbecco's Modified Eagle's Medium (DMEM/ F-12) (cat number: D8900), L-glutamine (cat number: G7513), Fetal Bovine Serum (FBS) (cat number: F7524), Penicillin and Streptomycin (P/S) (cat number: P4333), Gentamicin solution (cat number: G1397), 0.25% Trypsin-EDTA solution (cat number: T4049), Collagen from rat tail (cat number: C7661), Protease from *Streptomyces griseus* (cat number: P5147), Trypan Blue solution (cat number: T8154). All above chemicals were purchased from Sigma-Aldrich Co., St. Louis, USA. Amphotericin B solution (MP Biomedicals, LLC, catalog number: 1672348), Phosphate-buffered saline (PBS) (Gibco, USA, catalog number: 10010-023), Dimethylsulfoxide (DMSO), RNase-free DNase, 100% Isopropyl alcohol, Glacial acetic acid, Sodium bicarbonate ($NaHCO_3$), 1N Hydrochloric Acid (HCl), 1N Sodium Hydroxide (NaOH).

3.6.2. Equipment

Class II safety cabinet (Safe flow 1.2, EuroClone S.P.A., Italy), autoclave (SX-500, Tomy Digital Biology, Japan), centrifuge (Z 513 K, HERMLE Labortechnik GmbH, Germany), vortex mixer (SA8, Stuart, UK), dissecting tools, water bath (YCW-010E, Gemmy Industrial Corp., Taiwan), pH meter (E163694, inoLab, Germany) micropipettes (Nichipet EX II, Nichiryo, Japan), haemocytometer (FSI-Brighton 87325, Weber Scientific, England), inverted fluorescence microscope system (IX73, Olympus, Japan), computer and image capturing

software (OLYMPUS cellSens Standard, version 1.17), syringe filter units (pore size 0.22 µm, diam. 33mm; MILLEX®GP, Merck, Ireland), Freezing container (Mr. Frosty™, Thermo Scientific, USA), cryovials (Biologix, USA) cell scrapers (Biologix, USA, cat number: 70-1250), plastic culture plates and flasks (Life Technologies, USA and Iwaki, Japan), 15 ml/ 50 ml autoclavable tubes and tips (Life Technologies, USA) and glassware (SciLabware Ltd, Germany).

3.6.3. Media Preparation (HBSS, DMEM/ F12)

One liter of HBSS solution was prepared by dissolving one bottle of HBSS powder and 0.35 g of $NaHCO_3$ in 970 ml of distilled water. Then the pH was adjusted to 7.2-7.4 using concentrated HCl or NaOH. The prepared medium was filtered under the Ultraviolet (UV) sterilized laminar flow using syringe-driven filters into autoclaved media bottles. Finally, the HBSS medium was supplemented with 1% P/S and stored in a 4°C refrigerator until needed. HBSS was used for sample transportation and washing purposes only. For DMEM/ F12 medium preparation, the same protocol was used as HBSS; instead, 1.2 g of $NaHCO_3$ was used. Nutrient-rich DMEM/ F12 was used as the primary culture medium for porcine airway epithelial cell culture (Lechner and LaVeck, 1985).

3.6.4. Culture Model Establishment

3.6.4.1. Tissue Sample Collection

Tissue samples of the respiratory tract were extracted from healthy, untreated and freshly slaughtered pigs. Those pigs were reared in an isolated location of Livestock Field Station, Faculty of Agriculture, University of Peradeniya, Uda Peradeniya, Kandy, which has no heavy vehicular moments. The adherent structures and excess connective tissues around the distal airways were dissected off and extracted segments (~ 5 cm) of the respiratory tract were washed several times with HBSS (with 1% P/S and 2.5 µg/ml Amphotericin B) at the abattoir. Then, those samples were transported to the laboratory in ice-cold HBSS medium.

3.6.4.2. Isolation of Primary Porcine Epithelial Cells for Culture

Isolation of normal porcine airway epithelial cells was performed as previously described by Wu and colleagues in 1985 with minor modifications (Wu et al., 1985). Media and other chemicals were pre-warmed to 37°C in a water bath. Samples that had been brought into the laboratory were washed several times with fresh HBSS supplemented with Penicillin 100 I.U./ml, Streptomycin 100 µg/ml, and 2.5 µg/ml Amphotericin B, and then all specimens were cut into 1 x 2 cm pieces. A tissue digestion media was prepared using HBSS supplemented with 1.5 mg/ml Protease, 10 µg/ml DNase, 1% P/S (100 I.U./ml Penicillin and 100 µg/ml Streptomycin), 2.5 µg/ml Amphotericin B and 1% L-glutamine 2mM in a 50 ml falcon

tube/plastic bottle and placed all pieces in it. After 24 hours of incubation at 4°C, the tube was vortex vigorously for a few minutes to detach the cells from the luminal surface of the tissue (Fulcher et al., 2005; You et al., 2002) (Fig. 3.2). Cells were then collected by centrifugation (1000 x g for 10 min each) and resuspension in HBSS with antibiotics. Cell palette was resuspended in complete culture medium (DMEM/ F12 supplemented with 1% P/S, 2.5 µg/ml Amphotericin B and 1% L-glutamine 2mM and 10% FBS) and planted onto non-coated culture flasks and incubated at least two hours in a CO_2 incubator (5%) at 37 °C. This was done to remove the fibroblasts by differential adherence to the plastic. Then the remaining non-adherent epithelial cells were collected and resuspended in the same complete culture medium described above and seeded into 6-well culture plates (each well was treated with 3 ml of cell suspension) pre-coated with rat tail collagen. Then incubated at 37°C, 5% CO_2 environment, and cultures were routinely maintained in the same culture (media changes were done every 48 h) until cells reached 70-90% confluence.

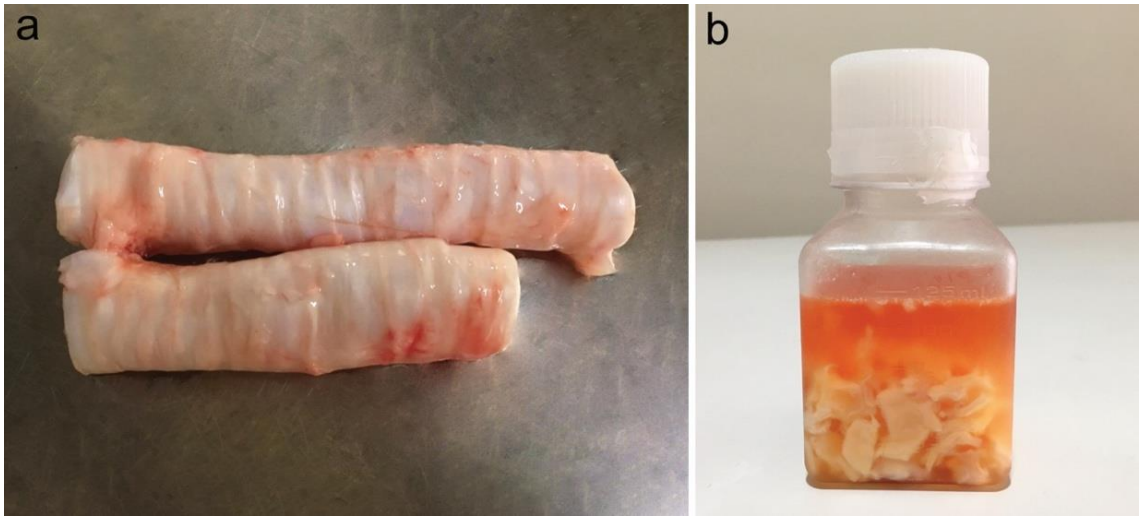


Fig. 3.2. Porcine airway epithelial cell extraction. (a) Tracheal samples (~ 5 cm) extracted from freshly slaughtered pigs. (b) Trachea pieces placed in the digestion media (HBSS, 1.5 mg/ml Protease, 10 µg/ml DNase, 1% P/S (100 I.U./ml Penicillin and 100 µg/ml Streptomycin), 2.5 µg/ml Amphotericin B, L-glutamine 2mM).

3.6.4.3. Cell Culture Passaging

Once confluent, cells were Trypsinized into the next passage or cryopreserved for long-term storage (Delgado-Ortega et al., 2014). Culture plates were washed several times with PBS (or serum-free DMEM because serum blocks the action of trypsin) to remove dead cells, debris, metabolic wastes, and serum. The wells were then treated with 300 µl Trypsin solution (0.25%) for each and placed in the CO_2 incubator for about 2 minutes. Cells were observed under the microscope to ensure that all cells are detached and floating. The side of the plate was gently tapped to detach the remaining attached cells. Then the detached cells were collected to a 15 ml tube and treated with DMEM with 10% FBS to stop the action of trypsin. Centrifuge the cell suspension, and the cell pellet was resuspended in full culture medium as described earlier. Live cells were counted using a haemocytometer and 0.4% Trypan Blue solution (described in 3.7), cell concentration was adjusted to $1x10^5$ cells/ml by multiplying the average number of viable cells (viable cells/number of squares counted in the haemocytometer) by dilution factor and 10^4 (constant). Cells were then seeded in collagen-coated plates, as described above. All the PM_{10} exposure treatments were done on second passaged cells.

For cryopreservation, this process is similar until Trypsinization to cell counting. To achieve good recovery after freezing, cell viability should be at least 90% for cryopreservation. Instead of seeding the cells in new culture plates, the remaining medium was removed by centrifugation at 150 x g for 5 minutes, and the pellet was resuspended in cryopreservation medium (90% FBS and 10% DMSO) (C. Wang et al., 2017; Wu et al., 2012) that the volume required to maintain the cell density to $1x10^6$ cells/ml. 1 ml of cell freezing medium was added to the cryovials immediately and transferred to the freezing container filled with 100% isopropyl alcohol. The freezing container was put in -80°C overnight to decrease the temperature steadily 1°C/min, and the frozen ampules were transferred to storage boxes and stored -80°C. When re-culturing the preserved cells, cryopreserved vials were taken out from the freezer and thawed in a 37°C water bath. Gently pour into a flask with a pre-warmed full culture medium and placed in the CO_2 incubator. Media change was done as soon as the cells were attached to the bottom of the flask to remove the DMSO in the medium.

3.6.4.4. Collagen Coating

The most suitable collagen concentration for primary porcine airway epithelial cells was determined by culturing the extracted cells on culture plates pre-coated with rat tail collagen (30 µg/ml collagen solution). Collagen was dissolved in 0.02 N glacial acetic acid, and 300 µl of the solution was pipetted into each well of the 6-well plate. Collagen was spread on the well

bottom using a pipette and let stand in the laminar flow under the UV light overnight. The collagen coating was done 24 h before seeding (Fulcher et al., 2005; Horani et al., 2013).

3.6.5. Exposure of Culture Cells to PM_{10}

To expose the cells to PM in the air samples, a suspension treatment approach was used as described previously (Leung et al., 2014). The cells were seeded on 6-well collagen-coated plates (two sets for 24 h and 48 h), and extracted particles were prepared in three different concentrations (3 µg/ml, 30 µg/ml, and 300 µg/ml) using the 3 mg/ml stock solution. Aliquots of PM_{10} suspensions were dissolved in culture medium (DMEM/ F12 supplemented with 1% P/S, 2.5 µg/ml Amphotericin B, and 1% L-glutamine and 1% FBS) under aseptic conditions. Culture media tubes supplemented with PM_{10} were sonicated for 10 minutes to distribute the particles evenly and then applied to the cultured cells. Three wells were taken as replicates in each treatment group (n=5). Ultra-pure water was used for the control group. One set of the treated plates was incubated for 24 hours, and the other was incubated for 48 hours in the humidified atmosphere of the incubator at 37°C temperature and 5% CO_2 and 95% RH conditions before subjected to the assays.

3.7. Cell Viability Assay

Cell viability assay was performed using the Trypan Blue dye exclusion method (Strober, 2015). Live cells possess intact cell membranes that exclude dye and can observe in colorless under the

microscope, whereas dead cells do not. Therefore, in this assay, viable cells can be easily identified with clear bright cytoplasm while non-viable cells have blue cytoplasm. Before preparing cells for the viability assay, cells were exposed to PM_{10} treatments for 24 h, and 48 h then observed under the microscope for any morphological changes, and photographs were captured for further analysis. The cells were then rinsed with PBS (or serum-free media) several times, and cells were incubated ~2 minutes with Trypsin solution (0.25%) as described earlier in the 3.6.4.3. Cells in each treatment group were collected separately and added media with serum and centrifuged. Supernatant was discarded, and the cell pellet was resuspended in 1 ml of PBS. 20 µl of the concentrated cell suspension and an equal amount of Trypan Blue (1:1 dilution) were mixed by pipetting up and down. The mixture was incubated for less than three minutes at room temperature. Hemocytometer with the coverslip already in place was filled with the Trypan Blue-treated cell suspension (each side of the filling area takes 10-20 µl). Hemocytometer was placed on the inverted light microscope, and cells were counted (both live and dead cells). Cell viability percentages were calculated by dividing the number of viable cells by the number of total cells and multiplying it by 100. This calculation was for each treatment group separately (Control, 3 µg/ml, 30 µg/ml, 300 µg/ml: n=5). Potential contribution of the filter leachate to cell toxicity was assessed by treating the extracts from clean glass microfiber filter paper with the same cell culture. To assess whether the effects are cell type specific to primary porcine cell culture, the same set of experiments were conducted on hTERT-hNOF cell lines under culture conditions as previously published (Illeperuma et al., 2011).

3.8. Alkaline Comet Assay

Comet assay (single cell gel electrophoresis) is a widely used biomonitoring tool for DNA damage because it is relatively simple to perform but a sensitive method (Valverde et al., 1999). The comet assay technique has been modified many times and extensively validated over the years (McKelvey-Martin et al., 1993; Singh et al., 1988). The detailed protocol is given below. This study also examined whether the extracted total particulate matter in Kandy city air induces DNA damage in airway epithelial cells. For this, cultured porcine airway epithelial cells were exposed to the same particulate matter concentration series as before (Control group, 3 µg/ml, 30 µg/ml, 300 µg/ml) and used a slightly modified version of the comet assay protocol previously published by Singh et al., (1988).

3.8.1. Reagents

Low-melting temperature agarose (LMA) (Promega, USA, cat number: V2111), Normal-melting temperature agarose (NMA) (Sigma-Aldrich, cat number: A9539), PBS (Ca^{2+}- and Mg^{2+}-free), DMSO, 100mM ethylenediaminetetraacetic acid (EDTA), 2.5 M sodium chloride (NaCl), NaOH, Triton X-100, 10 mM Trizma base (Sigma-Aldrich, cat number: T-8524), Absolute ethanol, Trypsin-EDTA, Ethidium Bromide solution (EtBr)

3.8.2. Equipment

Microscope glass slides (better agarose adhesion can be acquired using fully frosted slides) (Sail Brand, China, catalog number: 7101), Microscope coverslips (22 x 40 mm), centrifuge, water bath, haemocytometer, glass Coplin jars (Marienfeld, Germany), electrophoresis chamber and power supply, same inverted fluorescence microscope system, computer and image capturing software used for cell culture.

3.8.3. Slide Preparation

When slide preparation, one to three agarose gel layers are used. In this experiment, the three-layer procedure was used. A cell containing 0.5% LMA layer was placed on 1% normal agarose precoated slides. Then another layer of 0.5% LMA was placed on the second agarose layer to fill any residual holes in the second gel layer and increase the distance between cells. It is necessary to have a good distance between cells to see the comet clearly and analyze the length of the comet. Glass slides were dipped in absolute ethanol and burned them over a blue flame to clean the oils and dust. Then 50 ml of 1% NMA was made (500 mg agarose dissolved in 50 ml PBS) in a beaker, heated until near boiling in a microwave oven. Labeled slides were dipped in the molten agarose beaker while it was hot and gently removed. Undersides of the slides were wiped and placed on a flat surface air dried to a thin gel layer. Coated slides were stored at room temperature until needed. Usually, precoating was done at least 24 h before the experiment. The whole process was conducted in a low-humidity environment to ensure agarose adhesion.

3.8.4. Cells Isolation

Before isolating the cells from the cultured palates, 0.5% LMA (125 mg LMA dissolved in 25 ml PBS) was prepared by carefully heating the bottle in a microwave at low power. Vigorous boiling was avoided and ensured all agarose was dissolved and let it settle in a 40°C water bath until use. Culture media was removed from the treated cell culture plates and washed several times with PBS (37 °C) to remove the serum in the media, particles, and leachate from the filter extracts. Then cells were added 0.005% Trypsin-EDTA and kept 5 minutes in a 37°C incubator to detach and isolate the cells. A deficient concentration of trypsin solution was used because higher concentrations might increase the DNA damage (Szeto et al., 2005). Detached cells were collected in a 15 ml plastic tube and added an equal amount of FBS to quench trypsin, and the resulting cell suspension was centrifuged at 1000 x g, 4°C for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 200 µl of chilled PBS. Then, ~10,000 cells in 10 µl (cell density was adjusted using a hemocytometer and Trypan blue dye) were mixed rapidly with 75 µl of LMA, which was placed in the water bath. 80 µl from the cell mixture was pipetted out and layered onto the pre-coated slide immediately, and a coverslip was placed on it. Then the slide was put in a 4°C refrigerator for 5 to 10 minutes to solidify the gel layer. For applying the third gel layer, the coverslip was removed gently. 65-90 µl of 0.5% LMA and coverslips were added as done in the second gel layer. Again, the slide was put in the refrigerator for 5-10 minutes to set the gel fully and removed the coverslip carefully.

3.8.5. Cell Lysis

Slides were placed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, pH 10) for at least 1 hour at 4 °C. To maintain the stability of the gel layers, the lysis solution was chilled (refrigerated 30 minutes prior to use). Alkaline conditions were used instead of neutral conditions in both cell lysis and electrophoresis.

3.8.6. Electrophoresis

Prior to electrophoresis, slides were transferred into Coplin jars containing freshly prepared and chilled electrophoresis buffer (1 mM EDTA and 300 mM NaOH, with pH > 13.0) and incubated at least 20 min in 4°C for unwinding of the DNA. Then the slides were transferred to the electrophoresis tank and filled with electrophoresis buffer until the liquid level completely covered the slide and proceeded to electrophoresis at 25 V and 300 mA for 45 minutes.

3.8.7. Neutralization

After electrophoresis, the alkali condition in the gels was neutralized by rinsing the slides in neutralization buffer (Tris Buffer at pH 7.5), which was also chilled prior to use. Five washes were done for 5 min each to remove the high background is seen during the comet scoring.

3.8.8. Slide Staining

Rinsed slides were placed on a flat surface and incubated the slides with 50 µl of 20 µg/ml EtBr in water for 5 minutes. Then the excess stain was removed by dipping the slides in chilled distilled water and placed a coverslip on the gel layer for immediate scoring.

3.8.9. Image Analysis

Immediately after staining, slides observed under the inverted fluorescence microscope and comet images were captured. To analyze the microscopy images of the comet assay, OpenComet plug-in (v 1.3.1) (Gyori et al., 2014) for a popular open source image processing platform ImageJ (NIH, USA v 2.0.0-rc-68/1.52g) was used. The OpenComet plugin has an automated scoring system and a user-friendly interface. Measure of the percentage of DNA in the tail was used as the DNA damage in different groups. 50-100 randomly selected comets were analyzed from each sample and avoided analyzing doublets or comets at the slide edges.

3.9. Gene Expression Analysis

Primary porcine cells were grown up to 70% confluence in 6-well culture plates as described above and then treated with 3, 30, 300 µg/ml PM_{10} under standard culture conditions. After 24 and 48 h of incubation, the media were removed, and the cell monolayer was then rinsed once with ice-cold PBS. 500 µl of Trizol® reagent (Life Technologies, USA, cat number: 15596026) was added to each well, and scrape the wells with cell scrapers to detach the cell sheath from

the bottom. Then the cells suspended in Trizol® were collected in 1.5 ml autoclaved microcentrifuge tubes and stored at -80°C until further use.

3.9.1. RNA Isolation

Total RNA was extracted from the samples in Trizol® following the manufacturer's instructions. Briefly, the samples were incubated for 5 minutes at room temperature, and the cell lysate was passed through the pipette several times. Then the tube was vortexed thoroughly. Each tube was added 200 µl of chloroform per 1 ml of Trizol® reagent, an insufficient amount of Trizol® may cause the DNA contamination of isolated RNA (Krebs et al., 2009), and the sample tubes were capped securely to the vortex vigorously for 15 seconds. Tubes were incubated 2-3 minutes at room temperature. Then the samples were centrifuged at 12,000 x g for 15 minutes at 4 °C (Biocen 22 R, Ortoalresa, Spain). Phase separation was done due to the centrifugation. There were three clearly visible phases, such as lower red, phenol-chloroform phase, interphase, and colorless upper aqueous phase. RNA was concentrated exclusively in the upper aqueous phase. Therefore, the upper aqueous phase was transferred carefully into a new tube without disturbing the interphase, and the rest was discarded. 500 µl of 100% isopropyl alcohol was added to each tube and mixed well. Tubes were incubated about 10 minutes at room temperature, and the tubes were then centrifuged 12,000 x g for 10 minutes at 4 °C. RNA precipitate was formed on the wall and bottom of the tube. The supernatant was eradicated, and the RNA pellet was washed by adding 1 ml of 75% ethanol to each tube. Tubes were vortexed briefly and centrifuged again

at 7,500 x g for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was air-dried for 5-10 minutes. The RNA pellet was dissolved in 30 µl of DEPC-treated water by passing the solution a few times through a pipette tip. Tightly closed tubes were then kept in a pre-heated heat block at 55°C for 10 minutes. Then the isolated RNA was then stored at -80°C until further use.

3.9.2. RNA Quantification

RNA was quantified using Qubit® 3.0 Fluorometer and NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, USA) (Walton and O'Connor, 2018). The purity and integrity were evaluated by checking the absorbance ratio at 260-280 nm (A260/A280 > 1.8).

3.9.3. First-strand cDNA Synthesis

Two hundred nanograms of RNA were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA, cat number: K1622) following the manufacture's protocol. Briefly, components of the kit were thawed, mixed gently, and stored on ice. The total volume of 12 µl per reaction was prepared by mixing the template RNA (calculated volume for 200 ng), nuclease-free water, and Random Hexamer primer (1 µl) in a PCR tube. After the tubes were mixed and centrifuged, briefly incubated at 65°C for 5 using a pre-heated heat block) minutes. The mixture was then chilled on ice immediately, spin down, and placed the vial back on ice until the reverse transcription reaction mix was added. A total volume of 8 µl of reverse

transcription reaction mix was prepared by mixing 4 µl of 5x Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix and 1 µl of RevertAid M-MuLV RT (200 U/µl) per reaction in this order. This mix was then added to the previously prepared 12 µl of RNA mix, and tubes with 20 µl of total volume were mixed gently and centrifuged briefly. Then the tubes were placed in a programmable thermal cycler (BOECO TC-PRO Thermal Cycler, Germany), and RT reaction conditions for cDNA synthesis were 25°C for 5 minutes followed by 42°C for 60 minutes. The reaction was terminated by heating at 70°C for 5 minutes and 4°C for 10 minutes. Reaction products were stored at -70°C until used for the PCR amplifications.

3.9.4. Polymerase Chain Reaction (PCR) to Check the Quality of Synthesized cDNA

Prior to proceeding for qPCR analysis, to make sure cDNA has been synthesized properly and the internal control gene β-Actin (*ACTB*) was amplified. Prepared cDNA was amplified by PCR to detect the amplification of β-Actin, which was purchased from IDT®, USA (F-GGACCTGACCGACTACCTCATG, and R-CGACGTAGCAGAGCTTCTCCTT) (Messina et al., 2012). The purpose of doing the PCR for *ACTB* was to see if there is any error in RNA quantification and cDNA synthesis process prior to the qPCR. For the PCR amplification, 10 µl Master Mix (GoTaq® Green Master Mix, Promega, USA, cat number: M7122), was mixed with 1 µl of each primer, 7 µl of nuclease-free water, and 1 µl of the template cDNA per reaction. The thermal cycling parameters for *ACTB* were initial hot start 2 min, 95°C; then 35 cycles of: 40 sec, 95°C; 35 sec, 58.5°C; 45 sec, 72°C; followed by 10 min, 72°C. PCR products were later

resolved in a 2% agarose gel as described below. PCR was performed on the same thermal cycler (BOECO TC-PRO Thermal Cycler, Germany) used for cDNA synthesis. The whole process of cDNA synthesis and PCR was done in a PCR cabinet (PCR-01, BIOBASE, China).

3.9.5. Agarose Gel Electrophoresis

Normal agarose gel electrophoresis was followed, and after PCR, the samples were run in 2% agarose gel (in 1x TAE buffer-Tris, Acetic Acid, and EDTA, pH 8.5) containing 1 µl/30ml EtBr. 10 µl of PCR products were loaded into wells in the agarose gel along with the 100bp DNA ladder mixed with the loading dye. Then the electrophoresis tank was filled with 1x TAE buffer until the liquid level completely covered the gel, and electrophoresis was done at 80 V for 90 minutes. Separated DNA bands were observed under UV transilluminator (Fusion SL3, Vilber Lourmat, France).

3.9.6. Quantitative Real-time Polymerase Chain Reaction (qPCR)

RT-PCR reaction mixtures were diluted four times using in nuclease-free water, and qPCR was performed using SYBR® Green PCR master mix (Thermo Fisher Scientific, UK, cat number: 4309155) for all target genes (Table 3.1). The manufacturer's protocol was adapted with a few modifications. Briefly, a total volume of 19 µl (per reaction) of the reaction mixture was prepared by mixing the components in the kit (10 µl of SYBR® Green, 7 µl of nuclease-free water, 1 µl of forward primer, and 1 µl of reverse primer). Then the reaction mixture was mixed

gently, and 19 µl was dispensed into each well of 96-well qPCR plate (Biologix, USA). 1 µl of cDNA was added as template per well. Reactions were performed in triplicate for each sample with negative controls for each gene. The plate was sealed tightly and was run in an ABI 7500 Real-Time PCR system (Applied Biosystems, Singapore). Cycling conditions were 2 min, 50°C; 10 min, 95°C (holding stage) followed by 40 cycles of 15 sec, 95°C; 1min, annealing temperature. ROX is pre-mixed with SYBR green as a passive reference dye.

3.9.7. qPCR Data Analysis

Relative gene expressions were calculated using the method previously described $2^{-\Delta\Delta Ct}$ relative quantification method (Schmittgen and Livak, 2008). Threshold Ct Mean of the target genes and the Ct Mean of the reference gene were calculated from the triplicated wells. The expression levels of target genes were normalized to GAPDH (reference gene) expression, and the change in gene expression (fold change relative to the expression in control) were calculated using the following equation (Schmittgen and Livak, 2008).

$$\Delta\Delta CT = (Ct\ target - Ct\ control)_{treatment} - (Ct\ target - Ct\ control)_{control}$$

$$Fold\ change = 2^{-\Delta\Delta CT}$$

Table 3.1. Primer sequences and annealing temperatures (T/°C) used for qPCR

Gene	Sequence F-Forward, R-Reverse primer	T/°C	Reference
IL-6	F-AACGCCTGGAAGAAGATGCC R-CTCAGGCTGAACTGCAGGAA	61	(Vezina et al., 1995)
IL-8	F-AAGCTTGTCAATGGAAAAGAG R-CTGTTGTTGTTGCTTCTCAG	60	(Petrov et al., 2014)
TNFα	F-AACCTCAGATAAGCCCGTCG R-ACCACCAGCTGGTTGTCTTT	60	(Petrov et al., 2014)
CYP1B1	F-CCATGCGCTTCTCTAGCTTTG R-AGCCTAAGACCGAGGCATTG	60	(Messina et al., 2009)
EGFR	F-CAGCATGCATACCAGCAGCCTTTA R-TTTCAAGAGCAGCTTCCGTTTCGC	61	(Kim et al., 2011)
TGF-β1	F-CGATTAAAGGTGGAGAGAGGACTG R-AATGAATGGTGGACAGACACAGG	56	(Leydon et al., 2014)
P53	F-GGAACAGCTTTGAGGTGCGTGTTT R -ATACTCGCCATCCAGTGGCTTCTT	60	(Hamm et al., 2014)
PGES	F-ACGCTGCTGGTCATCAAGA R-CACACACACAGGCCCACTG	60	(Waclawik et al., 2006)
GAPDH	F-CTTCACGACCATGGAGAAGG R-CCAAGCAGTTGGTGGTACAG	63	(Delgado-Ortega et al., 2014)

3.10. Statistical Analysis

All results represent at least three independent experiments and values obtained as mean ± standard deviation (SD). Multiple group means were first compared by one-way ANOVA followed by Dunnett's test for mean comparisons. All statistical analyses were conducted using Minitab software (version 17) and Microsoft® Excel for Mac (version 16.17). A probability level of $p \leq 0.05$ was considered statistically significant.

CHAPTER 04

RESULTS

4.1. PM_{10} and PAH Concentrations

A total of 13 ambient air samples were collected from two sampling locations in Kandy city area, Sri Lanka used in the analysis. Each air filters collected was undergone standardized Soxhlet extraction for PAHs and performed chemical analysis. The weights of PM_{10} collected on the filters were measured. The concentrations of 12 EPA PAHs were measured in PM_{10} per m^3 airflow of the two selected sampling sites in the Kandy city area (Fig. 4.1).

Fig. 4.1. Ambient air sample collection using high-volume respirable dust sampler operated at a constant flow rate of ~1.2 m3/min with glass microfiber filter paper. (a) Air sampler located

at the MGC sampling site. (b) Dark color deposition on the clean and white filter paper after operating for 6 hours at the same site.

According to the GC-MS results, the six-hour average concentrations of total PAHs bound to PM_{10} ($\sum_{12}$ PAHs) and PM_{10} from the two sampling sites of Kandy city are given in Table 4.1. In general, the mean $\sum_{12}$ PAHs of PDE and MGC sites are 17.044 $\pm$ 6.735 ng/m^3 and 23.324 $\pm$ 8.116 ng/m^3, respectively. Within the PDE and MGC sites ranged from 3.062 ng/m^3 to 22.864 ng/m^3 and 15.795 to 36.887 ng/m^3 respectively. When considering both sampling sites, 3.0624 ng/m^3 is the lowest, and the highest value is 36.887 ng/m^3, but overall, particle-bound $\sum_{12}$ PAHs concentrations are not significantly different between the sites (Fig.4.2).

Table 4.1. Six hours average concentrations of PM_{10} and total PAHs bound to PM_{10} ($\sum$12 PAHs) in the two sampling locations

Location	**Sample**	**$\sum PM_{10}$ (µg/m^3)**	**$\sum_{12}$ PAHs (ng/m^3)**
PDE	01	208.3333	17.9338
	02	192.1296	22.0815
	03	261.5741	17.9334
	04	101.8519	22.8643
	05	85.6481	14.9808
	06	148.1481	20.4532
	07	97.2222	3.0624
MGC	08	162.0370	28.2287
	09	289.3519	16.3723

10	141.2037	36.8871
11	138.8889	15.7947
12	192.1296	23.3766
13	118.0556	19.2860

The mean PM_{10} concentrations of PDE and MGC sites are 156.415 $\pm$ 66.567 µg/m^3 and 173.611 $\pm$ 61.992 µg/m^3, respectively. While the lowest concentration, 85.648 µg/m^3 records from the PDE, and the highest value of 289.352 µg/m^3 records from the MGC. In contrast, all mean concentration values of PM_{10} and particle-bound $\sum_{12}$ PAHs were slightly higher in the MGC sampling site but not significantly, and the highest concentrations of PAHs and PM_{10} were recorded from the samples which were collected from 7 am to 1 pm in both sampling sites.

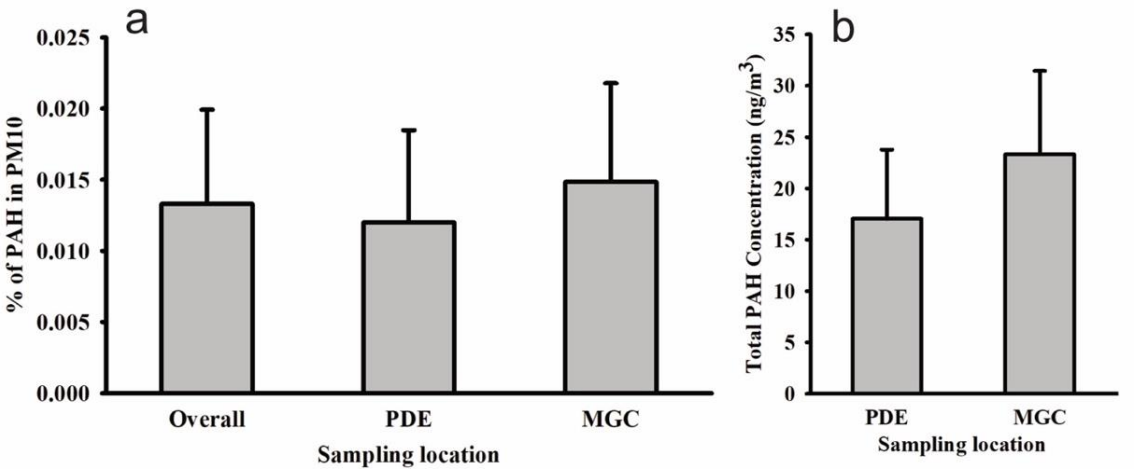


Fig. 4.2. PAH concentrations in PM_{10}. (a) % PAH concentrations in total PM_{10} from the two sampling sites. (b) Total PAH concentrations in PM_{10}.

4.2. Variation of Individual PAH Concentrations

Except for naphthalene (Nap), acenaphthylene (Acpy), acenaphthene (Acp), fluorene (Flu), all other 12 USEPA PAHs out of 16 were detected in the filter samples of the two sampling sites. When considering the molecular weight of the compounds, two 3-ringed compounds, four 4-ringed compounds, four 5-ringed compounds, and two 6-ringed PAHs are measured (Fig. 4.3).

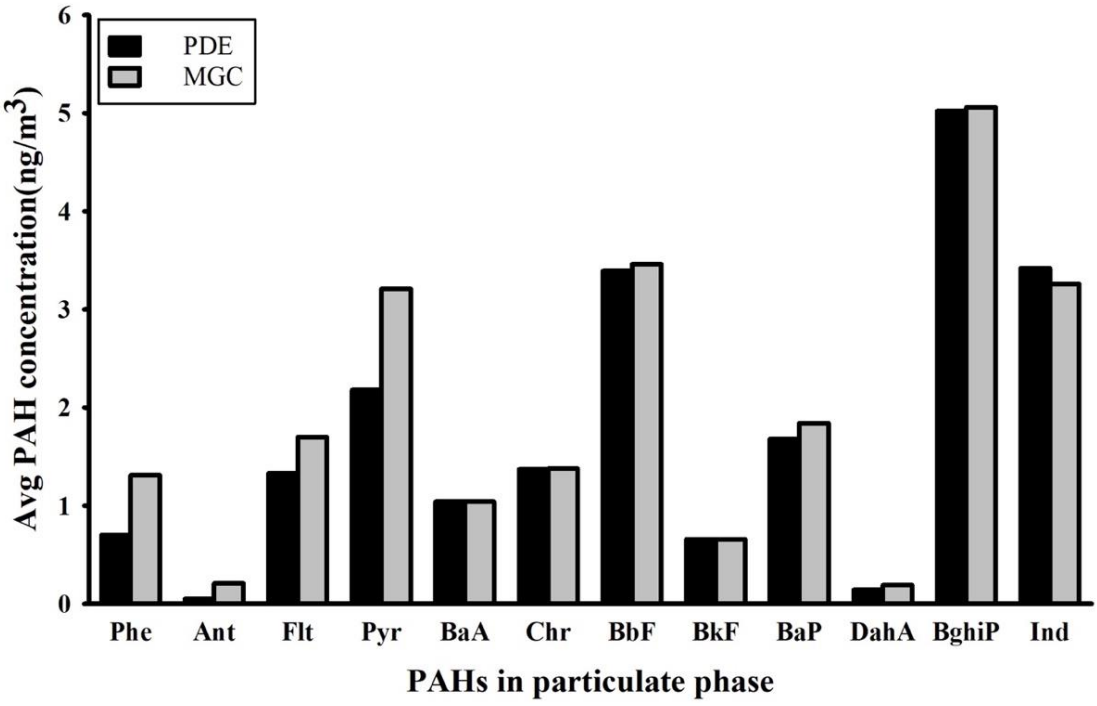
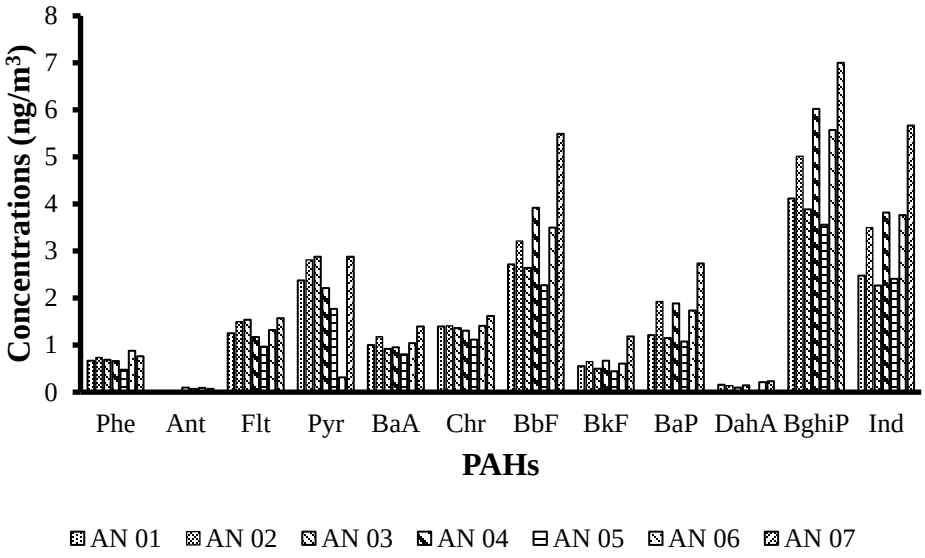


Fig. 4.3. Distribution of mean concentrations of individual PAHs of two sampling sites.

The 6-ringed and heaviest benzo[ghi]perylene and indeno[1,2,3-cd]pyrene were recorded as the highest concentrations and anthracene, and dibenz[a,h]anthracene were found to be the least

abundant among 12 PAHs measured, ranging from 0.0464 ng/m^3 to 5.0611 ng/m^3. When considering both sampling sites, except indeno[1,2,3-cd]pyrene, all other 11 PAHs have a slight increase in the MGC sampling site.



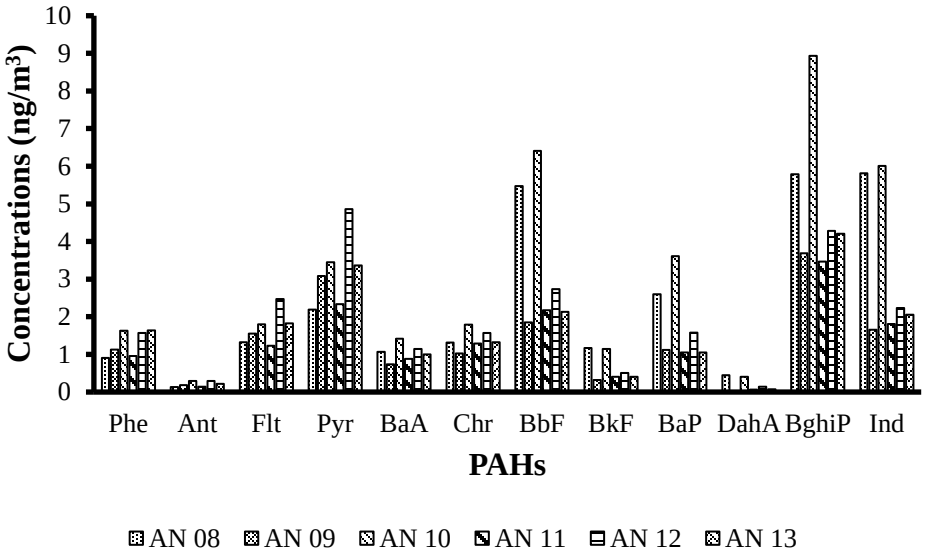


Fig. 4.4. PAH concentrations of individual samples from different times at the PDE (AN 01 - AN 07) and MGC (AN 08 - AN 13).

According to the PAH concentrations of the individual samples collected, sample number 07, and 10 from the PDE and MGC show the overall highest concentrations, respectively (Fig. 4.4). Both samples were collected from the time period of 7 am to 1 pm.

4.3. Distribution of Particle-bound $\sum_{12}$ PAHs on Molecular Weight Basis

PAHs are divided into two major groups based on the number of fused benzene rings. They are known as low molecular weight (LMW) and high molecular weight (HMW) PAHs. PAHs with two to three benzene rings (Nap, Acpy, Acp, Flu, Phe, Ant) categorized as LMW whereas HMW

PAHs, (Flt, Pyr, BaA, Chr, BbF, BkF, BaP, DahA, BghiP, Ind) consist of more than three benzene rings.

Analyzed PAHs were categorized according to the number of rings/molecular weight (3, 4, 5, and 6 rings). In both sampling stations, compounds with 6 rings gave the highest contribution; PDE, 40.2%, and MGC, 35.7%, followed by 4 rings > 5 rings > 3 rings. Compounds with 4 and 5 rings have almost equal contributions to both sampling sites. In general, HMW compounds were dominant in both sampling sites, while LMW compounds have less than 10% contribution to the total (PDE, 3.5% and MGC, 6.5%) (Fig. 4.5).

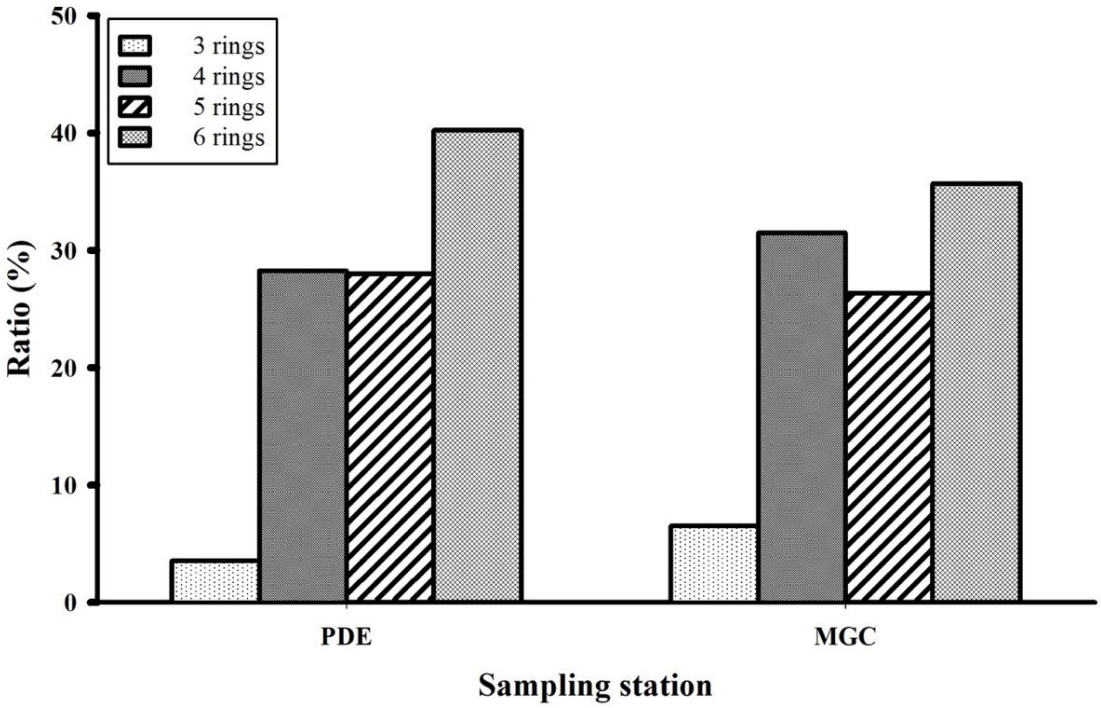


Fig. 4.5. Percentage contribution of different MW groups of PAHs over the two sampling stations.

4.4. Toxic Heavy Metal Concentrations

Collected filter papers were analyzed for 34 elements, including ten highly toxic HMs (V, Cr, Mn, Co, Ni, Cu, Zn, As, Cd, Pb) which have confirmed as adversely affect on human health, other abundant metals such as Na, Mg, Al, K, Ca, Ti, Fe, 14 metals from the Lanthanoid series of the periodic table (Lanthanoids), Y, Th, and U. When considering the metals which are summarized in Table 4.2, concentrations show a vast variation ranging from 0.0408 ± 0.0118 to $101343.9696 \pm 14732.5777$ ng/m^3.

Table 4.2. Other metal concentrations at the two sampling locations in Kandy city. Metals in bold are non-Lanthanoid metals

Element (ng/m^3)	**PDE**		**MGC**	
	Mean	**SD**	**Mean**	**SD**
La	2.3019	0.4171	1.9122	0.6343
Ce	4.4386	0.6896	3.8945	1.3159
Pr	0.5471	0.1011	0.4505	0.1425
Nd	2.0166	0.3173	1.7044	0.5125
Sm	1.7699	0.1428	1.8196	0.2628
Eu	0.9525	0.0957	1.0011	0.1305
Gd	0.5664	0.0670	0.4705	0.1177

Tb	0.0950	0.0095	0.0743	0.0176
Dy	0.5728	0.0530	0.4480	0.0966
Ho	0.1133	0.0069	0.0879	0.0177
Er	0.3068	0.0286	0.2503	0.0462
Tm	0.0472	0.0079	0.0408	0.0118
Yb	0.3411	0.0365	0.2753	0.0481
Lu	0.0515	0.0059	0.0418	0.0067
Y	3.8980	0.2458	3.0510	0.5053
Th	0.7337	0.7663	0.4030	0.1321
U	0.2360	0.0241	0.1827	0.0259

Among the selected toxic HMs from both sampling sites, Zn was the most concentrated metal (385.0203 $\pm$ 65.1822 ng/m^3), followed by Cu > Pb > Mn > Cr > Cd > Ni > V > Co > As (0.9861 $\pm$ 0.3505 ng/m^3). Zn, Cu, and Pb are the only metals recorded over 100 ng/m^3 within the toxic HM group from both sampling sites (Fig. 4.6).

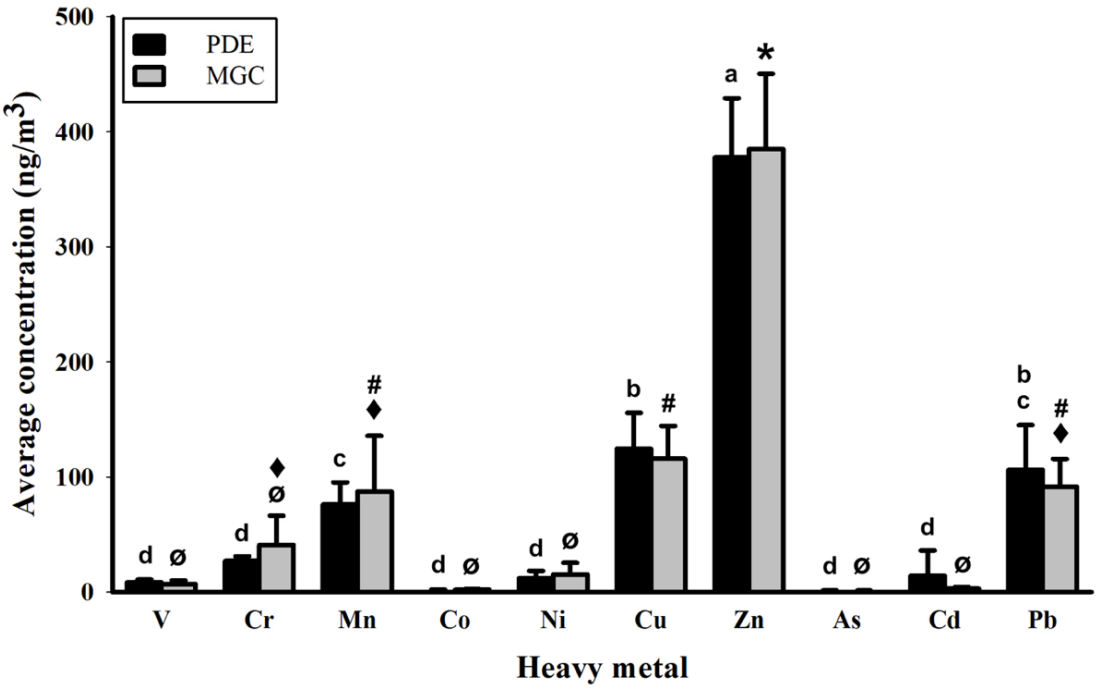


Fig. 4.6. Mean concentrations of toxic HMs at the two sampling sites of Kandy city (different simple letters denote significant differences from each other at $P < 0.05$ for PDE and different symbols denote significant differences from each other at $P < 0.05$ for MGC).

4.5. Other Element Concentrations

Except for the highly toxic HMs, 24 other elements have also been analyzed, including abundant elements and very rare lanthanoids.

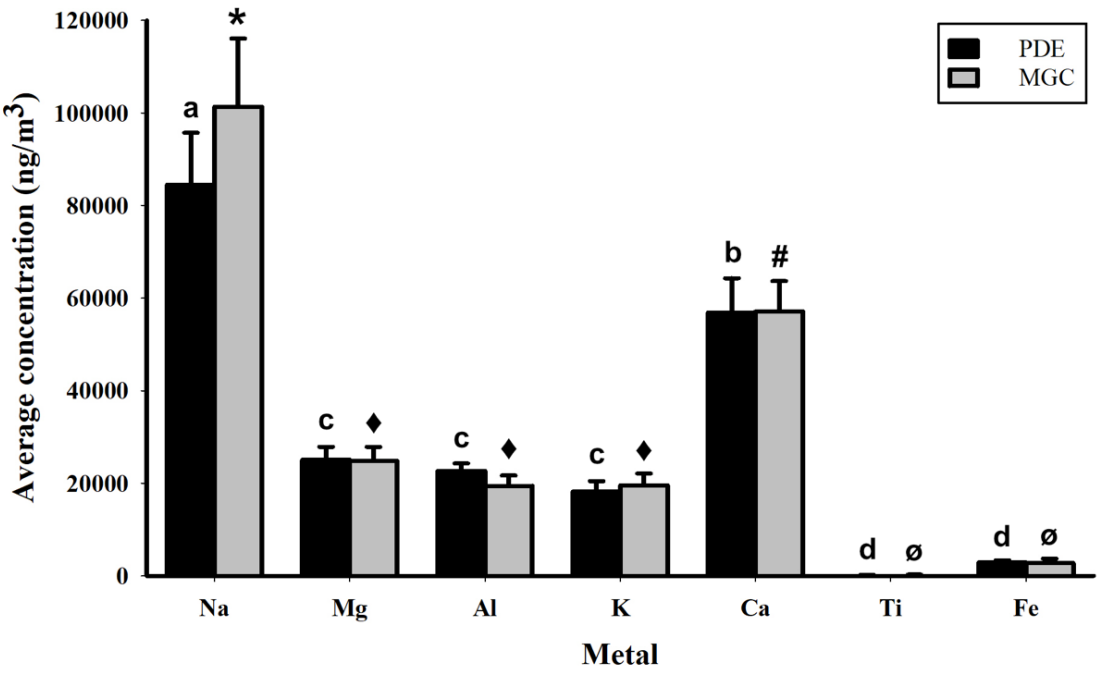


Fig. 4.7. The most abundant elements in PM_{10} (different simple letters denote significant differences from each other at $P < 0.05$ for PDE and different symbols denote significant differences from each other at $P < 0.05$ for MGC).

Na was the most concentrated element (101.3440 $\pm$ 14.7326 µg/m^3) among all the measured elements, followed by Ca > Mg > Al > K > Fe > Ti (0.2004 $\pm$ 0.0347 µg/m^3) (Fig. 4.7). When considering the Lanthanoids, Ce was the most concentrated element (4.4386 and 3.8945 ng/m^3 while Tm shows the lowest of 0.0472 and 0.0408 ng/m^3 in PDE and MGC, respectively. A slightly higher concentration was recorded from PDE in most of the lanthanoid metals.

4.6. Cell Culture Model

To evaluate the functional significance of PM_{10} bound PAHs and HMs on respiratory epithelial cells, a primary porcine airway epithelial cell culture was successfully established (Fig. 4.8). According to the observation, cells were adhered to the bottom and grown fast in 30 µg/ml collagen-coated flasks. Therefore, 30 µg/ml collagen solution was used throughout the research and maintained the cell culture at 37 °C, 5% CO_2, and 95% RH conditions.

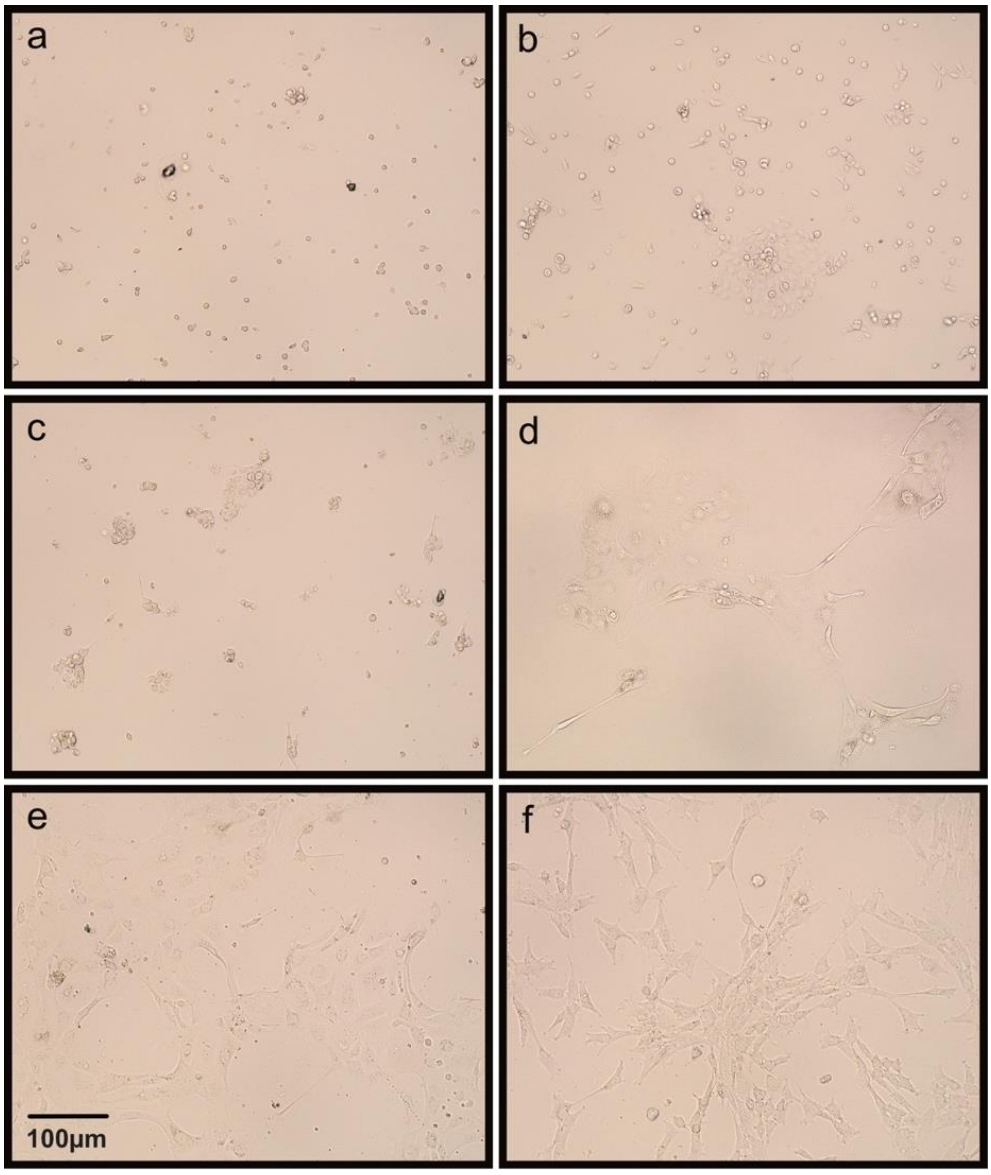


Fig. 4.8. Growth and development of primary porcine airway epithelial cells from day 0 to 10 (x 200). Cell culture became 70% confluent on day 10 - 14. (a) Day 0. (b) Day 2. (c) Day 3. (d) Day 5. (e) Day 7. (f) Day 10.

4.7. Cell Morphological Changes and Viability After Exposure to PM_{10}

To investigate the potential cytotoxic effects of the PM_{10} extracted from the Kandy air samples, primary porcine airway epithelial cells were incubated with various concentrations (Control, 3, 30, 300 µg/ml: n=5) of PM_{10} for 24 and 48 hours initially. After 24 or 48 hours of exposure, morphological changes of the cell-matrix such as shape changes of the cell-matrix (Cell elongation), cell-matrix shrinkage, and dissociated cell clumps were observed in the 30 µg/ml and 300 µg/ml treatment groups. After 48 hours of exposure to 300 µg/ml, cell-matrix detached areas of the culture wear bottom were observed, and the cells were loosely attached to the bottom (Fig. 4.9 e, f). It was effortless to detach the whole cell-matrix with a few taps on the side of the culture ware. These effects were shown to be increased in both dose and exposure time-dependent manner.

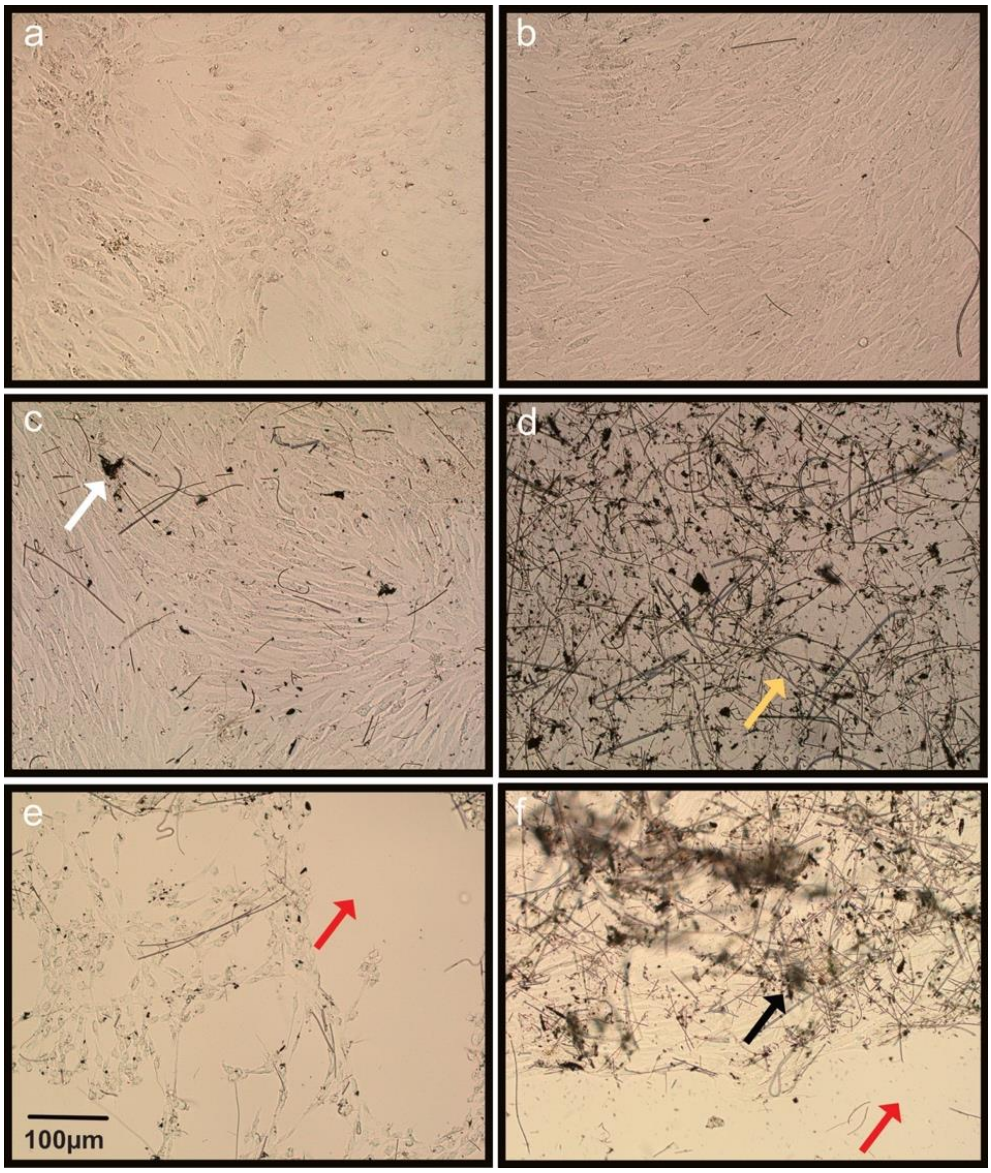


Fig. 4.9. Primary cell cultures exposed to extracted total PM and morphological changes of the cell-matrix after 24 or 48 h (x10 x 20). (a) Control group. (b) 3 µg/ml treatment group. (c) 30 µg/ml treatment group. (d) 300 µg/ml treatment group. (e, f) shrunk cell monolayer (e and f were taken after removing the media and washed using PBS). The white arrow indicates the extracted

PM_{10} particles, while the yellow arrow indicates the fibers coming from filter papers. The red arrows indicate the cell detached area of the cell-matrix. The black arrow indicates settled and adherent PM_{10} particles and fibers to the cell layer.

It can be clearly observed that glass fibers released to the extract from the filters when using the sonication extraction method. Therefore, the toxicity of the filter leachate by treating the extracts from clean glass microfiber filter papers on the same cell culture was assessed and found that the fibers do not have significant effects on cell viability or morphological changes. A progressive decrease in cell viability was observed with increasing concentrations of PM_{10} and time dependently, but a significant viability change ($p < 0.05$) compared to controls was observed only in 300 μg/ml treatment groups exposed for 24 and 48 h (Fig 4.10).

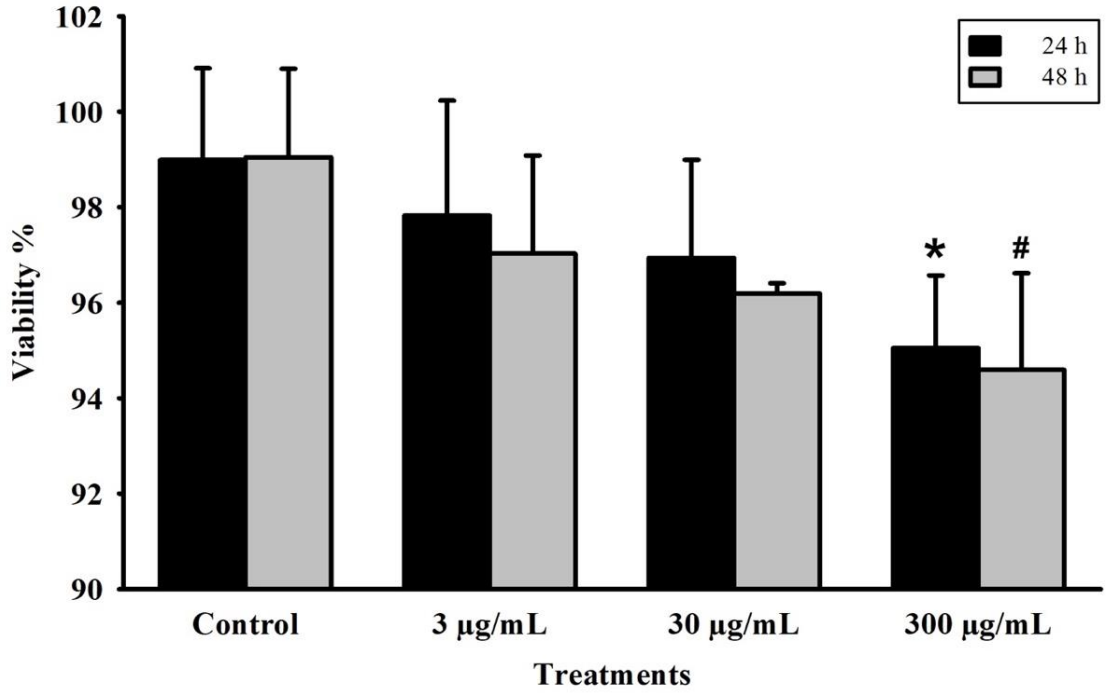


Fig. 4.10. Results of cell viability, PM_{10} exposure for 24 and 48 h (*P < 0.05 vs 24 h Control, #P < 0.05 vs 48 h Control).

4.8. Comet Assay and DNA Damage

Extent of DNA damage was assessed using comet assay by analyzing 100 cells in each treatment and the control group replicates after 24 and 48 h exposure to the extracted PM_{10} for tail percentage DNA, tail DNA length, and Olive tail moment (Table 4.3).

Table 4.3. Mean tail DNA length and Olive tail moment values for 24 and 48 hours

Treatment	Tail DNA length		Olive tail moment	
	24 h	48 h	24 h	48 h
Control	16.15 ± 5.79	15.98 ± 7.27	2.37 ± 1.05	1.98 ± 1.05
3 µg/mL	82.9 ± 28.62	120.87 ± 31.78	22.02 ± 10.23	43.2 ± 16.72
30 µg/mL	80.91 ± 15.38	37.16 ± 21.15	20.54 ± 5.12	5.79 ± 3.88
300 µg/mL	125.47 ± 26.26	112.72 ± 22.71	33.19 ± 10.12	40.38 ± 13.07

In the control group, DNA damage was negligible while all other treatment groups had a prominent comet/tail and significantly higher levels of different comet parameters compared to the control ($P < 0.05$), suggesting increased DNA damage in the airway epithelium (Fig. 4.11)

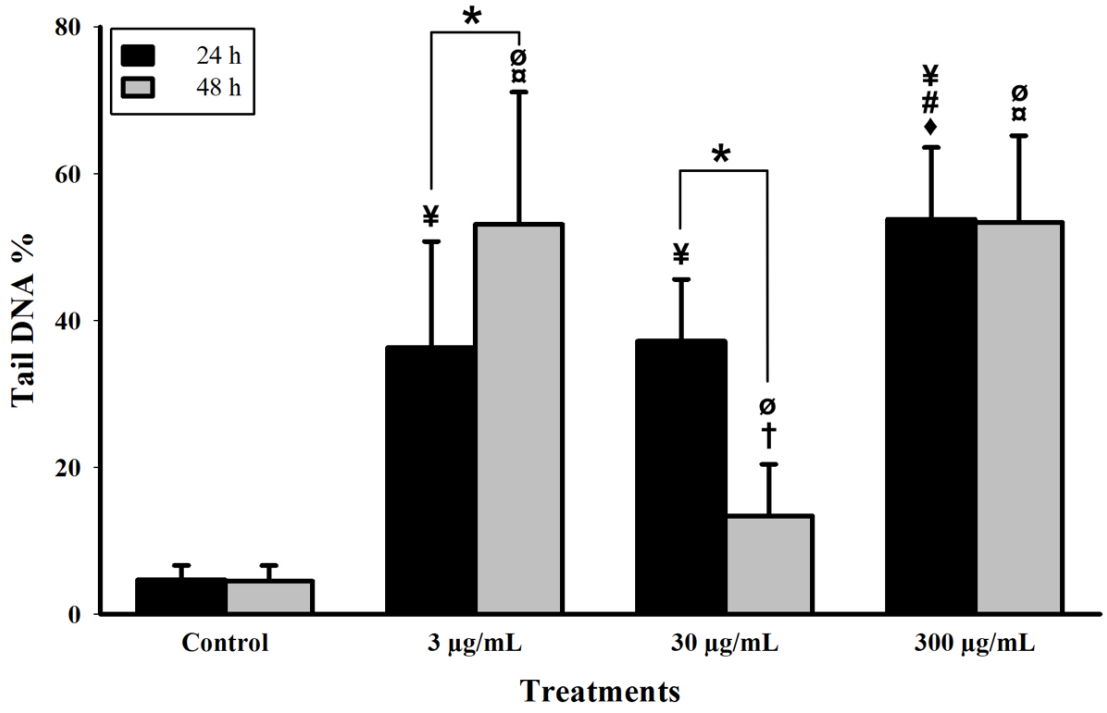


Fig. 4.11. Changes in comet tail percentage DNA (¥P < 0.05 vs Control, #P < 0.05 vs 3 µg/ml, ♦P < 0.05 vs 30 µg/ml for 24 h group, øP < 0.05 vs Control, †P < 0.05 vs 3 µg/ml, ¤P < 0.05 vs 30 µg/ml for 48 h group, *P < 0.05 24 h vs 48 h in each treatment).

Due to the OpenComet being an automated cell analyzing system, it is possible to occur some errors during the analysis process. Difficulties such as selecting wrong cells (cells on the edges), analyzing cell clusters as one, different shaped cells, identifying debris as cells, etc. were overcome by carefully extracting only the appropriate data for the analysis (Fig. 4.12).

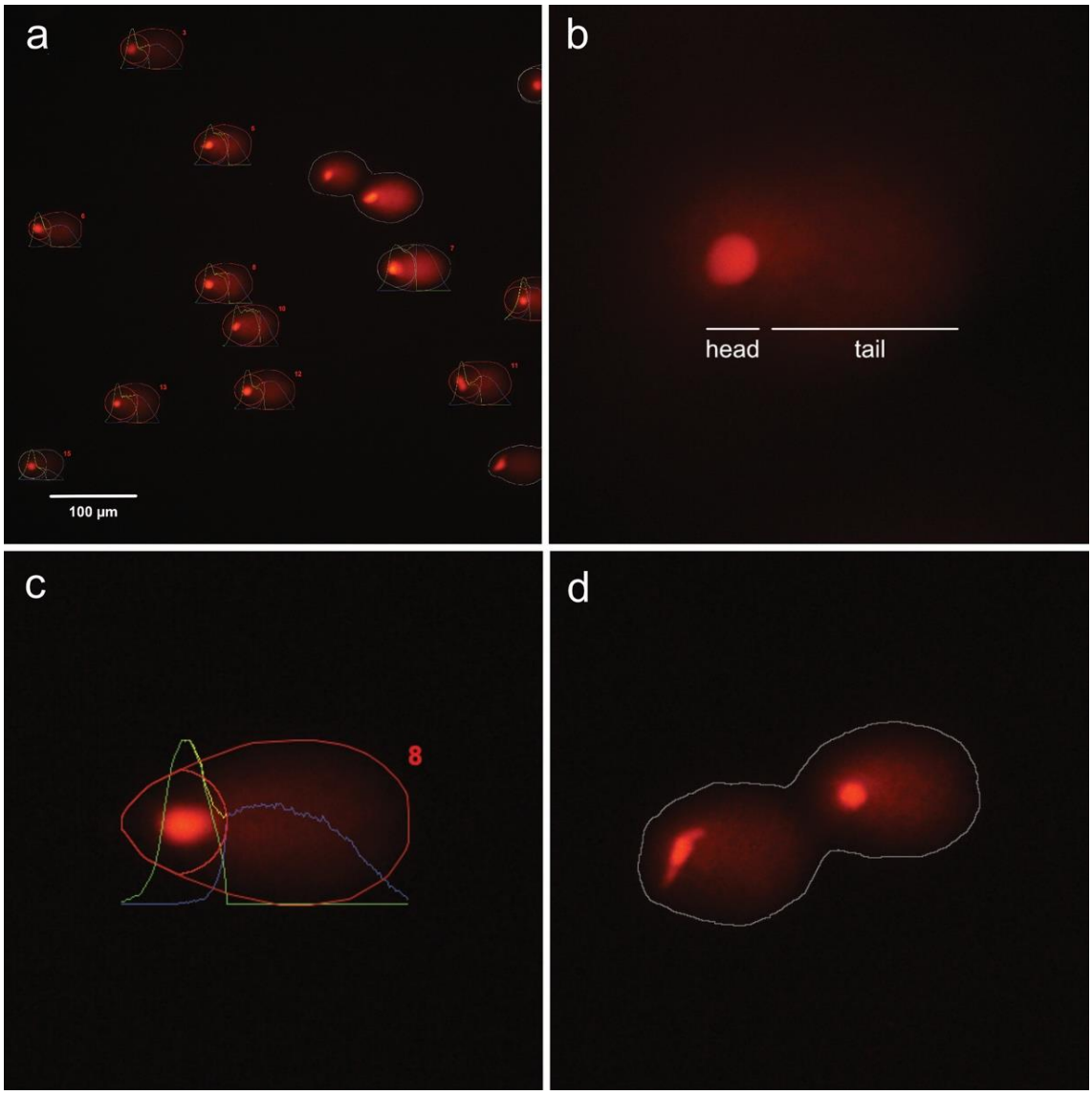


Fig. 4.12. Comet assay microscopic images analyzed using OpenComet plug-in (v 1.3.1) for the popular open-source image processing platform, ImageJ. (a) OpenComet output image of 300 µg/ml treatment group exposed for 48 h. (b) Defined head and tail of the comet image. (c) analyzed comet as a valid comet. (d) eliminated comet as an invalid comet.

Different degrees of DNA damage can be identified easily from photomicrographs of comets taken using a fluorescence microscope. Growing comet tails were clearly visible in the order of 3, 30, and 300 µg/ml treatment groups compared to the control after 24 hours of exposure (Fig. 4.13). The 48 hours exposure group was also shown visually distinguishable comet tails. However, 30 µg/ml treatment group showed shorter comet tails compared to the other treatment groups but still longer than the control group.

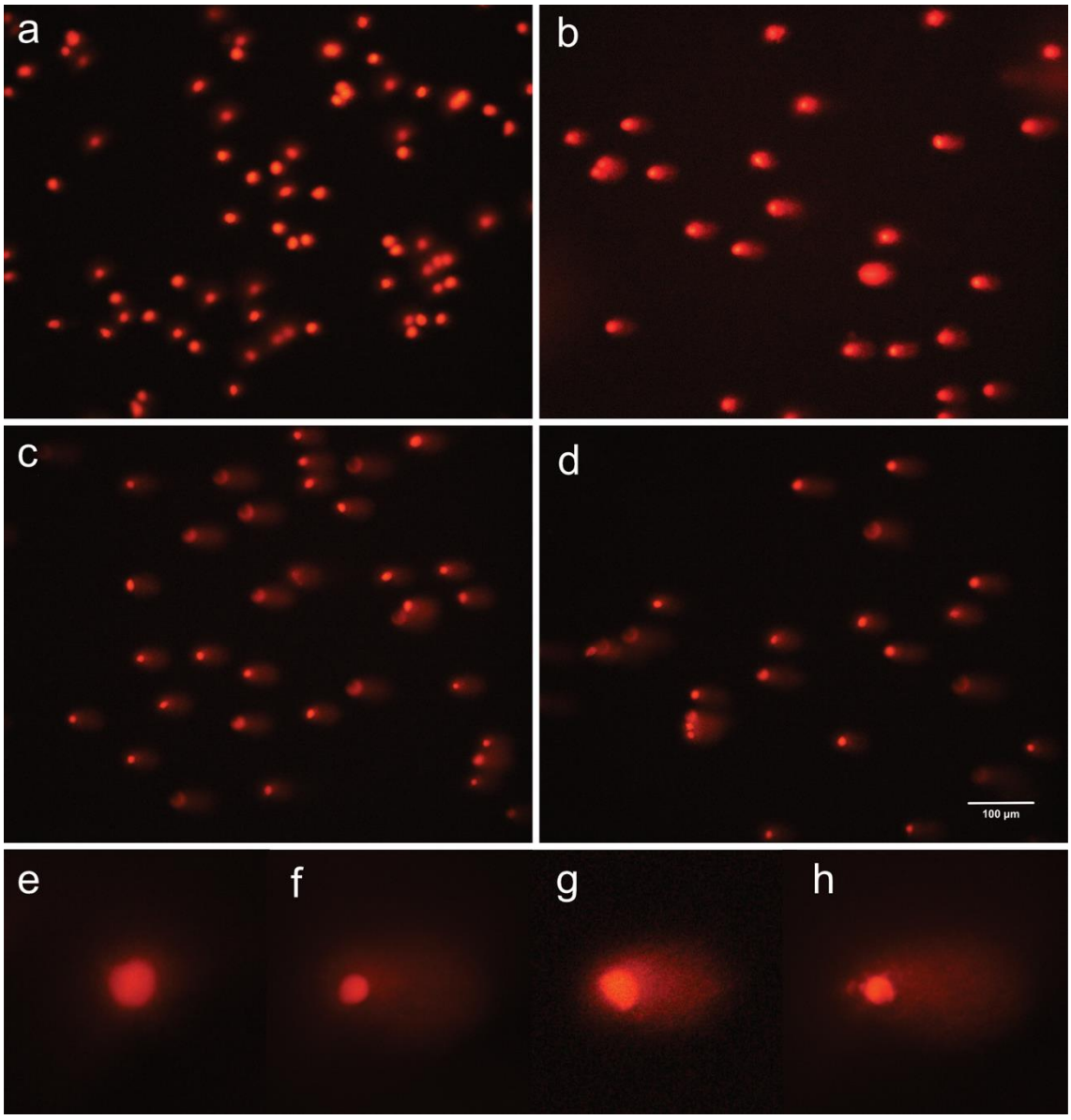


Fig. 4.13. Representative photomicrographs show different degrees of DNA damage in the comet assay of airway epithelial cells in all treatment groups after 24 h exposure (x10 x 20). (a,

e) Control group. (b, f) 3 µg/ml treatment group. (c, g) 30 µg/ml treatment group. (d, h) 300 µg/ml treatment group.

4.9. Gene Expression

4.9.1. Gene Expression After 24 Hours Exposure

Primary porcine airway epithelial cells were used to determine the gene expression (mRNA expression) levels of pro-inflammatory cytokines (IL-6, IL-8, and TNFα), xenobiotic metabolizing enzyme (CYP1B1), growth factors, and a few oncogenes (EGFR, TGF-β1, p53, and PGES). Cells were exposed for 24 h to the extracted PM_{10}.

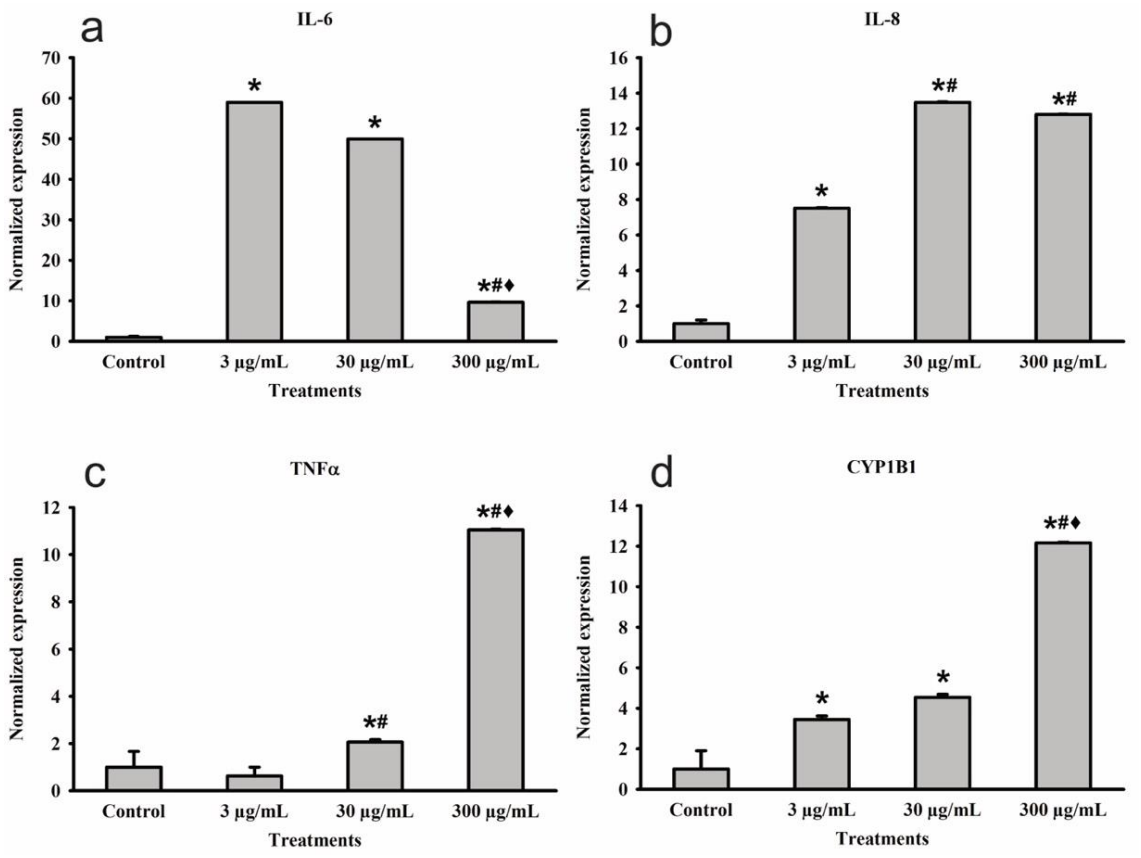


Fig. 4.14. Gene expression of pro-inflammatory cytokines and xenobiotic metabolic enzymes upon PM_{10} treatment for 24 h in porcine primary airway epithelial cells. (a) Gene expression of IL-6. (b) Gene expression of IL-8. (c) Gene expression of TNFα. (d) Gene expression of CYP1B1(*$p < 0.05$ vs Control, #$p < 0.05$ vs 3, ♦$p < 0.05$ vs 30).

Interleukin-6 and 8 (IL-6 and IL-8) expressions were significantly up-regulated ($p < 0.05$) in all treatment groups as compared to the control. For IL-6, the highest normalized expression was recorded in 3 µg/ml treatment (Fig. 4.14 a, b). Gene expression of tumor necrosis factor-alpha

(TNFα) was significantly up-regulated ($p < 0.05$) in the 30 and 300 µg/ml treatment groups compared to control (Fig. 4.14 c). Cytochrome p450 enzyme (CYP1B1) expression was significantly ($p < 0.05$) up-regulated in all treatment groups compared to the control (Fig. 4.14 d).

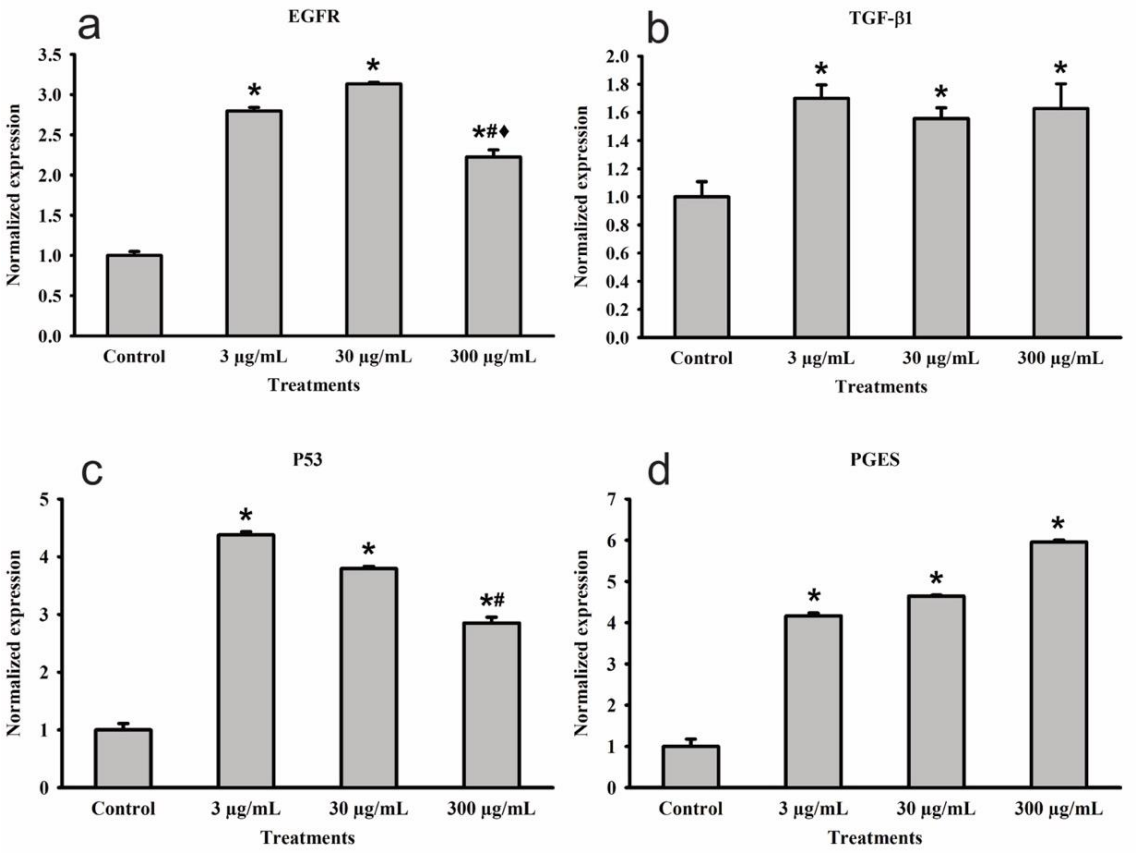


Fig. 4.15. Gene expression of cancer-related growth factors and oncogenes upon PM_{10} treatment for 24 h in porcine primary airway epithelial cells. (a) Gene expression of EGFR. (b) Gene

expression of TGF-β1. (c) Gene expression of p53. (d) Gene expression of PGES (*p < 0.05 vs Control, #p < 0.05 vs 3, ♦p < 0.05 vs 30).

Gene expression of epidermal growth factor (EGFR) was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control (Fig. 4.15 a). Transforming growth factor-beta 1 (TGF-β1) expression was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control, but no significant difference among different treatment groups (Fig. 4.15 b). Gene expression of tumor suppressor protein gene (p53) was significantly up-regulated ($p < 0.05$) by PM_{10} exposure in all treatment groups (Fig. 4.15 c). Prostaglandin E synthase (PGES) expression was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control (Fig. 4.15 d).

4.9.2. Gene Expression After 48 Hours Exposure

Identical cell cultures were used to determine the expression of the same genes (IL-6, IL-8, TNFα, CYP1B1, EGFR, TGF-β1, p53, and PGES). This time, cells were exposed for 48 h to the extracted PM_{10}.

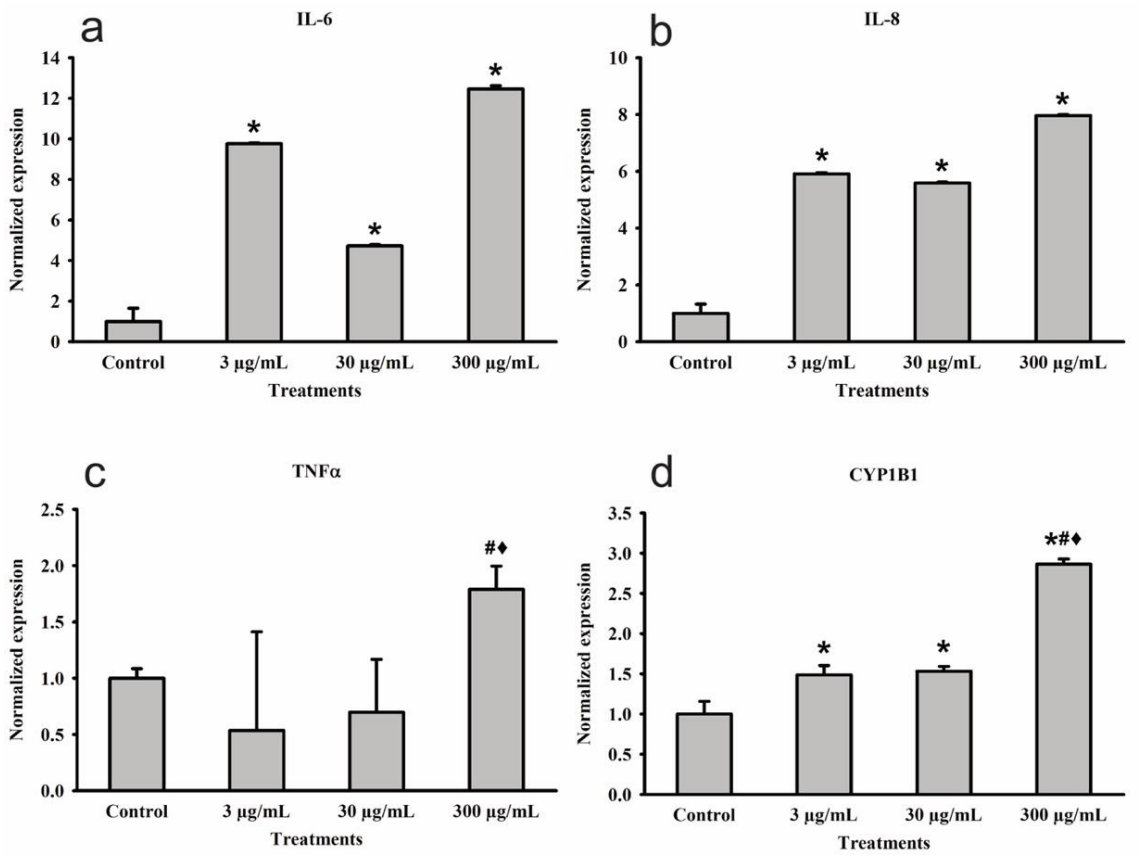


Fig. 4.16. Gene expressions of pro-inflammatory cytokines and xenobiotic metabolic enzymes upon PM_{10} treatment for 48 h in porcine primary airway epithelial cells. (a) Gene expression of IL-6. (b) Gene expression of IL-8. (c) Gene expression of TNFα. (d) Gene expression of CYP1B1 (*$p < 0.05$ vs Control, #$p < 0.05$ vs 3, ♦$p < 0.05$ vs 30).

IL-6 and IL-8 expressions were significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control (Fig. 4.16 a, b). TNFα expression was significantly up-regulated ($p <$

0.05) only in 300 µg/ml treatment group compared to the control (Fig. 4.16 c). Expression of CYP1B1 was significantly up-regulated ($p < 0.05$) in all the PM_{10} treatments (Fig. 4.16 d).

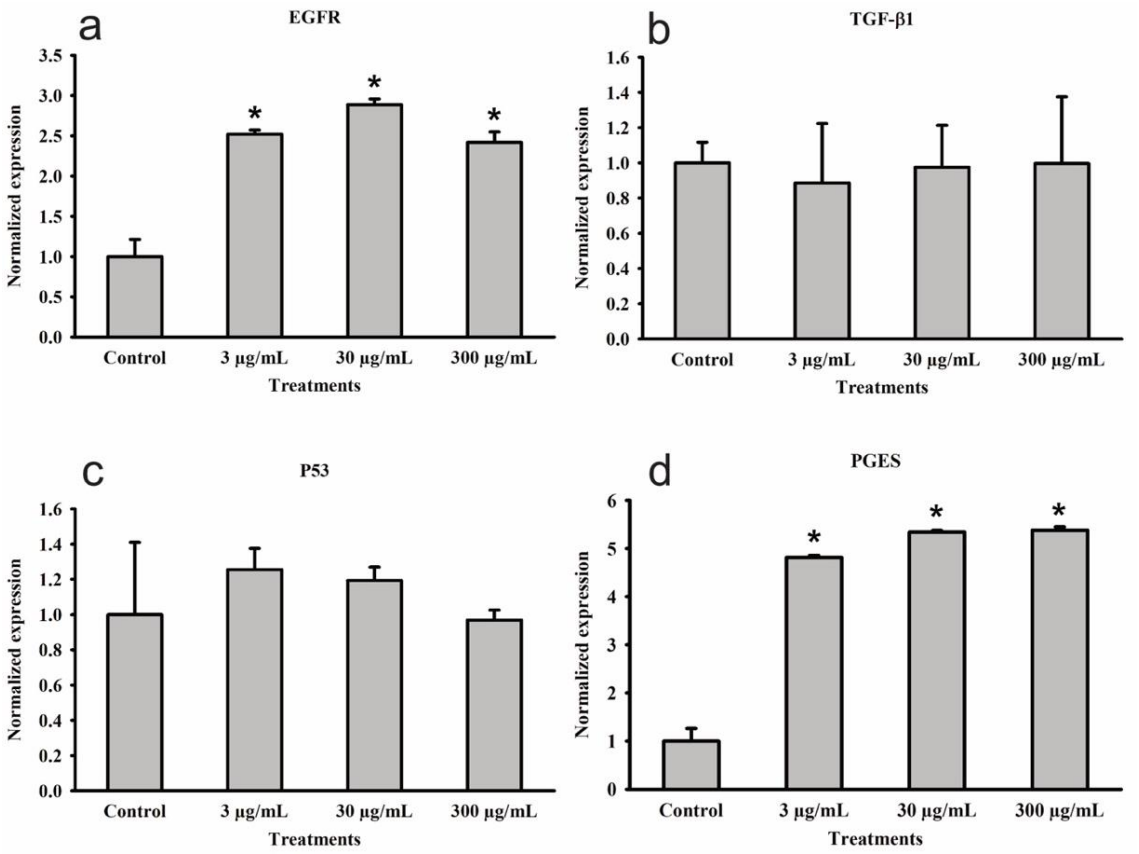


Fig. 4.17. Gene expressions of growth factors and oncogenes upon PM_{10} treatment for 48 h in porcine primary airway epithelial cells. (a) Gene expression of EGFR. (b) Gene expression of TGF-β1. (c) Gene expression of p53. (d) Gene expression of PGES (*$p < 0.05$ vs Control, #$p < 0.05$ vs 3, ♦$p < 0.05$ vs 30).

EGFR expression was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control (Fig. 4.17 a). TGF-β1 and p53 expressions did not show any significant changes ($p < 0.05$) in any treatment group compared to the non-treated control (Fig. 4.17 b, c). Expression of PGES was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control (Fig. 4.17 d).

CHAPTER 05

DISCUSSION

5.1. Discussion of Key Findings

Today, people are usually breathing a mixture of hazardous pollutants, including particles and PAHs emit mainly from motor vehicle exhaust. A growing number of epidemiological researches have reported that PM exposure is associated with many respiratory and several other critical diseases in humans (Kim et al., 2018). From the view of this health concern, quantification of the levels of particulate PAHs in urban areas is critical. To our knowledge, even if numerous studies have assessed the toxicological effects of exposure to PM in many cities (industrial and non-industrial) in the world, this study is the first study that attempted to identify the relationship between exposure to PM_{10} and carcinogenic effects *in vitro* especially in Sri Lanka. Therefore, this study revealed the levels of particle-bound 12 PAHs and 32 elements in Kandy air. Furthermore, exposure to three different doses of PM_{10} resulted aberrant expression of three pro-inflammatory cytokines, xenobiotic metabolic enzymes, cancer-related growth factors, and oncogenes upon 24 and 48 hrs exposure in a porcine primary airway epithelial cell culture-based bioassay which mimicked human airways cells.

Sampling sites were mainly selected considering the traffic flow, public transport, and school children's health. PDE was selected as the site with heavy traffic and within the optimal range

of the bus terminal. Moreover, the other most important factors to consider PDE as a crucial sampling spot was the location of Kandy National Hospital, railway station, Kandy main post office, and Kandy municipal central market within 200 m range of the sampling site. MGC is located at the edge of the Sangaraja Mawatha, Kandy, which is usually affected by the heavy daily traffic at the lake round of the Kandy. School children are the main exposing group for the air pollutants at the MGC sampling site since they stay around 7-8 hours on the regular schooling day. Moreover, this sampling location was selected to make a correlation between our PAHs and HM data targeting future work (Collaborated with the Respiratory unit, Kandy National Hospital) related to children's lung function and diseases. Moreover, most importantly, there was no other significant contamination source of PAHs near both the sampling sites, and Kandy city does not have significant industrial activities that could emit hazardous PAHs and HMs (Weerasundara et al., 2017). Therefore, it can be concluded that vehicular emissions (mainly buses) and other anthropogenic activities can be considered as the primary sources of the PAHs in the Kandy city air.

Mean $\sum_{12}$ PAHs, and PM_{10} concentrations showed a slight increase in the MGC sampling site but not significantly different between the two locations, and the highest concentrations were recorded in the samples collected from 7 am to 1 pm in both sampling sites. This may be due to the additional vehicles entering the area from school service vehicles, and Sangaraja Mawatha at MGC sampling site traffic is slow-moving and creates low air turbulence compared to the PDE site. Even if the PDE site is closer to the Good Shed bus terminal, the site has a large open

area that can facilitate the dispersion of pollutants, which can reduce the PAH concentrations. On the other hand, while traffic density strongly correlates with the PM and PAH concentrations, tree borders and green roofs act as passive filters of airborne PM (Freer-Smith et al., 2004; Qiu et al., 2018; Speak et al., 2012). Spacious surroundings in the vicinity and wind velocity affect airflow, and the diffusion of airborne particulates might have a significant role in lowering the pollutant concentrations (Yin and Xu, 2017). Therefore, the proportionate contribution of the sources cannot be decided solely based on the PM_{10} and PAH data of the current study.

In a previous study, Wickramasinghe et al., (2011) have selected Kandy bus stand as one of their leading sampling sites (as an Urban-Heavy Traffic site), which is very close to the PDE sampling site (within 200 m radius), but they have not collected samples close to MGC site. The mean $\sum_{12}$ PAHs of PDE and MGC sites were significantly lower than the previous study conducted in Kandy city (Wickramasinghe et al., 2011, 2012). This difference could be caused by geographic, climatic, and other factors such as changes in the traffic plans practiced since 2008, 2009 to 2017, improved quality of the imported fuel, number of old vehicles (mostly, old buses) operated in the area. Most importantly, Wickramasinghe et al., (2011) measured all 16 PAHs with 8 hours continuous sampling starting from 9 am, while the present study measured only 12 PAHs and 6 hours continuous sampling without fixing a specific starting time (sampled between 7 am to 6 pm). However, according to the present data, there is a high probability that

PM_{10} concentrations in Kandy city to exceed the annual (20 μg/m^3) and 24-hour (50 μg/m^3) exposure levels (WHO, 2005).

5.2. Kandy PAH Levels and Health Impacts

By investigating the PAH profiles, their emission sources can be identified and their behavior can be predicted because the ring number is also related to the source of pollutants (Hu et al., 2016; Ohura et al., 2005). From the health risk perspective, some of the PAH species are identified as animal and/or human carcinogens (IARC, 1982). Therefore, the individual evaluation of PAH profiles is essential. When samples were analyzed for individual PAH profiles, 12 PAHs were detected from both sampling sites. PAHs in ambient air can be found in both particulate and gaseous forms. PAHs found in particulates have a high molecular weight than the gaseous phase. The four most volatile PAHs (naphthalene, acenaphthylene, acenaphthene, and fluorene) were not reported here due to their undetectable levels (Choi et al., 2007). Filter sample transportation time from the Kandy city sampling sites to the laboratory at the University of Peradeniya and then to the laboratory at the Chinese Academy of Sciences where sample analysis was done for PM_{10} and PAHs might be the main reason for this low recovery of the most volatile PAHs though precautions were taken adequately to minimize the losses. Therefore, the quantification of only the least volatile PAHs with more than two rings provides the best results (Choi et al., 2007).

The heaviest PAHs with six rings identified such as benzo[ghi]perylene and indeno[1,2,3-cd]pyrene were found to be relatively the most abundant in both sampling sites while the benzo[ghi]perylene is known as a representative indicator of gasoline vehicle emission (Ho et al., 2002). Among the PAHs with five carbon rings detected (benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene), benzo[b]fluoranthene showed the highest concentration which is a representative indicator of firewood combustion or diesel combustion (Tobiszewski and Namieśnik, 2012). Since no known firewood combustion was available in the localities, the main source might be the diesel combustion mainly from passenger busses. Benzo[k]fluoranthene and benzo[a]pyrene are also indicative of diesel combustion (Ho et al., 2002). Among the recorded PAHs with four rings, chrysene is mostly emitted from gasoline/diesel engines and industrial oil burnings while benz[a]anthracene, indicative for petrol cars without or ill-functioning catalytic converters (Manoli et al., 2004). fluoranthene (4 rings) and phenanthrene (3 rings) are representative indicators of coal combustion (Kakareka et al., 2005). Considering the levels of 12 PAHs in the present study, the highest concentrations of most of the recorded PAH species have a strong relation either with diesel combustion or wood burning. Therefore, it can be suggested that vehicle emissions (gasoline/diesel) and very limitedly domestic wood burning are the probable emission sources of the hazardous PAHs in the Kandy ambient air. Previous research has concluded that diesel emissions with significant contribution from firewood combustions were the primary sources of particle-bound PAHs in Kandy, while the gasoline emissions have been very low and constant

(Wickramasinghe et al., 2011) but, we do not have vehicular traffic data to find the diesel emission dominance over gasoline today. Nevertheless, the primary source of PAHs in most Asian countries is traffic exhaust (Chang et al., 2006). However, previously recorded PAH concentrations in Kandy city were significantly higher than the present study (Wickramasinghe et al., 2011). The same reasons mentioned above may affect this difference in these two studies. When Kandy air particulate PAH concentrations were compared along with the results in major cities of Asia and other cities in the world, in general, Asian countries (India, Korea, Taiwan, China) have higher total PAHs concentrations than Europe (Italy, Belgium) (Fang et al., 2006; Kim et al., 2012; Srogi, 2007).

When considering the molecular weight of the PAHs and carcinogenicity, higher molecular weight usually means greater carcinogenicity (Chang et al., 2006). According to the IARC, benzo[a]pyrene is categorized as a group 1 carcinogen and dibenz[a,h]anthracene is categorized as the probable carcinogen to human. According to the results of contribution percentages of the PAHs, high molecular weight PAHs (6, 5, and 4 ring PAHs) gave the highest contribution in both sites, and all known carcinogenic compounds were recorded in higher concentrations compared to the non-carcinogenic compounds. If the contribution of the low molecular weight compounds, which have less carcinogenic or mutagenic properties, is meager, these lighter PAH compounds can react with other pollutants to form more toxic derivatives (Park et al., 2002).

Sampling and analysis of the PAHs can be affected by various reasons. Therefore, the comparison of many studies conducted around the world is a bit difficult due to the use of different analytical techniques and sample collection periods. Moreover, different techniques can cause incomparable results where some PAHs with deficient concentrations can only be detected using highly accurate and sensitive analytical methods and instruments. Most of the atmospheric particles and PAHs sampling approaches use filters and adsorbents as the sampling methods, which are not suitable for volatile species in the mixture and some species cannot be extracted entirely when adsorbed (Chang et al., 2006) and re-volatilization may happen from the deposition and variations among the different meteorological conditions and the ambient air contaminants.

5.3. Kandy Inorganic Element Levels and Health Impacts

After analyzing the PM_{10} bound PAHs, ten highly toxic HMs (V, Cr, Mn, Co, Ni, Cu, Zn, As, Cd, Pb), which were previously confirmed to impart a strong influence on human health were analyzed in the present study along with 22 other metals from the air samples (IARC, 2012). This was done mainly to find the other toxic components in Kandy ambient air, identify the potential emission sources, and it can also be directly linked with the genetic effects and cytotoxicity. Due to the unavailability of HM data in Sri Lankan cities, we compared the results with other major cities in Asia. However, as mentioned earlier, it is challenging to make a critical and accurate comparison of the results with the experiments done elsewhere due to the use of

different analytical techniques and sample collection periods. Consequently, we have to anticipate significant variations in results around the world.

In the samples, metals such as Na, Mg, Al, K, Ca, Ti, and Fe (Ca > Mg > Al > K > Fe > Ti) have relatively the highest concentrations. Interestingly, Al and Ca are the main elements in the Earth's crust (Ziyath et al., 2016). Therefore, the high atmospheric loading of these elements may be due to the airborne wind-blown soil dust and frictional work from construction sites in the ambient environment (Huang et al., 1994). Previous reports also confirmed that the coarse fraction of PM_{10} contains considerably more crustal elements than the fine fraction and possesses inflammatory potential (Gualtieri et al., 2010; Veranth et al., 2006). However, explaining the origin of many other elements that were recorded in deficient concentrations (Lanthanoid metals, Y, Th, and U) from the ambient air particles is difficult with the available data. These elements may come from the impurities in fossil fuel, motor oil, or motor vehicle composite materials (Zhou et al., 2015). To test this, a complete analysis of the components of imported fossil fuel (all different qualities) and vehicular emissions of individual vehicle categories should be done.

When considering the toxic HMs, Zn was found to be in the highest concentrations in the samples. Vehicle emissions are one of the essential sources of Zn in atmospheric depositions (Duan and Tan, 2013). Emissions from tyre and brake wear corrosion of galvanized automobile components, Zn coated roofing materials and galvanized building components in the sampling

area, re-entrainment by traffic of dust enriched with Zn and lubrication oil additives, electroplating industries and garages could also be attributed to this elevated Zn in Kandy city (Weerasundara et al., 2018). Cu, Zn, Cd, and Pb are considered metal markers for fuel combustion (Jandacka and Durcanska, 2019; Sansalone et al., 1996), while Zn is also considered as a useful marker of gasoline engine emissions (Vile et al., 2000). Pb is mainly an anthropogenic metal emits into the urban environment from fossil fuel burning. Although there are no dominant industrial activities in Kandy, toxic HM concentrations correspond to the heavily industrialized cities in India and China (Karar et al., 2006; Wang et al., 2006) and an elevated concentration of highly carcinogenic HMs (As, Cd, Cr, and Ni) and probable carcinogens (Pb and Co) compared to the other cities in the world (IARC, 2012). It may be due to the consumption of poor-quality fossil fuel (leaded fuel) and traffic-related activities.

Among all elements, exposure to the hazardous HMs should be the primary concern due to the critical health conditions influenced by those. Most of these elements play a crucial role in biological systems. HMs have been reported to affect several cellular components such as cell membrane, mitochondria, lysosome, endoplasmic reticulum, nuclei, and some enzymes involved in cellular processes such as detoxification and damage repair (Wang and Shi, 2001). Some metal ions can interact with nuclear proteins and cause DNA damages and cell cycle alterations, carcinogenesis, or apoptosis (Beyersmann and Hartwig, 2008). Over exposure to toxic HM such as Zn might causes pharyngitis, chest tightness, headache, increased indices of pulmonary inflammation, nausea, decrease in the activity of copper metalloenzyme, decreased

HDL-cholesterol level, immune toxicity, and gastrointestinal effects in humans (Bhattacharya et al., 2016). Cu within the human body beyond its permissible limit causes hematemesis, jaundice, melena, damage to the central nervous system, liver, and kidney problems (Gaetke et al., 2014; Pujol et al., 2016). Chronic Cu toxicity in the form of liver cirrhosis and damage to other organs is seen in the genetic abnormalities of Cu metabolism (Wilson's disease) (Pandit and Bhave, 2002). Human exposure to Pb is a serious health problem and Pb exposure occurs mainly via inhalation of lead-contaminated dust particles or aerosols. Lead poisoning directly affects the nervous system and several other organs in the body including kidney, liver, endocrine system, hematopoietic system, and reproductive system (Menezes-Filho et al., 2018). Several works have been published on the adverse health effects of Pb, especially in children. In children, diminished intelligence, lower intelligence quotient (IQ) level, speech and language handicaps, decreased hearing acuity, growth retardation, and anti-social and diligent behaviors were mainly identified (Factor-Litvak et al., 1998; Kaul et al., 1999). Decreased sperm count in men and spontaneous abortions in women have been associated with high lead exposure in the adult population (Apostoli et al., 1998). Mn disrupts Adenosine triphosphate (ATP) synthesis and mitochondrial respiration chain by accumulating in neuron mitochondria (Chen et al., 2001). Decreased intracellular ATP levels and the generation of free radicals, increase oxidative stress, which may lead to manganese cellular toxicity (Gunter et al., 2006). The primary target organ of Cr is the lung and the principal route of human exposure is through inhalation, but it has been reported that significant human exposure occurs through the skin (Costa, 1997). Some

individuals are extremely sensitive to Cr ingestion and occur severe allergic reactions. Epidemiological evidence strongly proves that Cr is an agent in carcinogenesis (Y. Wang et al., 2017). The other main adverse health effects of Cr are irritation and ulcers in the stomach and small intestine, anemia, sperm damage, and male reproductive system damage (Costa and Klein, 2006). Cadmium compounds are classified as human carcinogens by several regulatory agencies such as The IARC and the U.S. National Toxicology Program (IARC, 2012). Except for inhalation of ambient air, humans expose to Cd through several sources such as smoking cigarettes and eating contaminated food. Cd can be a fatal HM when inhaled or ingested and a severe pulmonary and gastrointestinal irritant. Studies have proven that chronic inhalation of cadmium causes pulmonary adenocarcinoma using rodents (Q. Zhao et al., 2019). Some studies have shown that occupational or environmental Cd exposure is also associated with cancer development of the stomach, prostate, kidney, liver, and hematopoietic system (Chen et al., 2016). Excess inhalation of Ni causes asthma, pneumonia, allergies, heart disorder, skin rashes, and miscarriage. Other than that, Ni has a potential for developing nose cancer, larynx cancer, and prostatic adenocarcinoma (Zhou et al., 2017). Moreover, Ni can induce carcinogenic processes by generating free radicals (Zambelli et al., 2016). Excessive exposure to V also can produce toxic effects in the respiratory, cardiovascular, urinary, blood, and digestive systems (Woodin et al., 2000). Furthermore, reproductive and developmental toxicities, and even cancer can be developed with overexposure to V (Ress et al., 2003). Exposure to the higher concentration of Co causes decreased pulmonary function, asthma, interstitial lung disease,

wheezing, and reduced pulmonary function (Leyssens et al., 2017). It is confirmed that individuals that expose to high arsenic concentrations suffer from cancers in the lung, liver, kidney, or bladder, and skin lesions (Smith et al., 2000). Even low concentration exposure of arsenic for a long period causes enlargement of kidney, histological changes, mitochondrial and liver damage in humans and other animals. Prolonged exposure to arsenic also affects the central nervous system. The severity of adverse health effects is related to the chemical form of arsenic is also time and dose-dependent (Bhattacharya et al., 2016). Although the carcinogenicity of As in humans seems to have strong evidences, the mechanism of tumor development in human body is yet to uncover (Yang et al., 2002).

Some toxic HMs such as As, Cd, Cr, Pb, Ni, and Hg are notorious for generating ROS. In this present study, except Hg, all other HMs were recorded in significant concentrations in this order; Pb > Cr > Cd > Ni > As. Over 100 ng/m^3 concentration was recorded in Pb, which is the highest among these five HMs. The ROS generation incorporates the death of neuronal cells, cognitive dysfunction, and Alzheimer's disease, and oxidative stress that play a vital role in toxicity and carcinogenicity (Landolph, 1994). Moreover, it has been speculated that the generation of ROS causes single-strand DNA damage through base pair mutations, deletion, or oxygen radical attack on DNA and disrupts the synthesis of nucleic acids and proteins (Mitra, 1984). Repetitive or long-term exposure to different HMs may lead to accumulation in the human body and induce severe cardiac and pulmonary diseases (Yedjou and Tchounwou, 2006). However, their toxicity depends on several factors such as dose, chemical species, age, gender, genetics, and nutritional

status of exposed individuals. Therefore, it is important to have biological data along with the chemical compositions of the air to build a proper correlation (Tchounwou et al., 2012). Since the unavailability of that kind of exposure data in Sri Lanka, the extracted total PM_{10} was exposed to an *in vitro* cell culture model to determine whether the cytotoxic effects, gene expression changes, and DNA damage produced in the cells related to specific compounds evaluated on the particles.

5.4. *in vitro* Exposure Study

A primary porcine airway epithelial cell culture model was established successfully for the exposure study. In here, primary porcine airway epithelial cells were considered as a model for human epithelial lung cells because comparative genetic maps indicate that more structural similarity between pigs and humans (Humphray et al., 2007). Though the porcine primary cell culture models were well established for *in vitro* toxicity studies, several optimizations had to be made to the available protocols due to a few difficulties faced throughout the process (Hauser et al., 2013; Wu et al., 1985). To find the optimum pre-coating collagen concentration for culture plates, several different concentrations were tested. Faster cell attachment and growth were observed in the plates that used 30 µg/ml collagen coating. Thus, 30 µg/ml collagen coating was used throughout the whole cell culture study. Collection of airway tissue samples from a healthy untreated adult and transport samples with the shortest possible time to the laboratory are vital factors to consider for this study. Therefore, samples were obtained only from the Livestock

field station, Uda Peradeniya, Kandy because it was operated under the University of Peradeniya, and the animals were not exposed to traffic-related air pollution due to the location of the piggery. Quick transportation is vital to maintain cell viability and reduce contamination. With the explained cell extraction procedure and the growth conditions (37°C 5% CO_2 environment and media changes were done every 48 h), up to 70% confluency was obtained within 10-14 and reached 100% confluency within 20 days. Nevertheless, cells in the second passage were used for treatment due to the fast growth of the second passage and it has less debris that comes while extracting the cells from the porcine airway like in the first passage of culture.

Cells were exposed to PM_{10} extracts containing 3, 30, and 300 µg/ml particle concentrations that always induced a significant effect in previous studies (Alessandria et al., 2014; Schilirò et al., 2010). When using the sonication extraction method to extract particles from the filter papers, a high amount of glass fibers released to the extracts was observed, especially in the high concentration treatment group. To minimize the burden of filter leachate on the cultured cells, the filter extracts without air samples were incubated with cells to find whether there are any morphological and viability changes. A significant effect on cell viability or morphology could not be identified due to the presence of glass fibers. After 24 and 48 hours of treatments, morphological changes (shape changes of the cell-matrix, cell-matrix shrinkage, and dissociated cell clumps shrinkage in the cell-matrix) of the cell-matrix could be observed in the 30 and 300 µg/ml treatment groups in exposure time and dose-dependent manner compared to the control

group. The 3 µg/ml group remained unaffected with treatment. These morphological changes may occur due to DNA damages and activation of cell death pathways, which are triggered by cellular stress conditions in the cell culture caused by exposure to environmental genotoxic agents such as PAHs and HMs (Fulda et al., 2010; Orrenius et al., 2011). It has been demonstrated that a variety of environmental contaminants, including HMs (Cu, Cd, Pb), organotin compounds, and dithiocarbamates can cause apoptotic cell death using *in vitro* models (He et al., 2003). In this study, the cell viability decreased progressively in dose and exposure time-dependent manner, but a significant viability decrease was observed only in the 300 µg/ml group only compared to the control (~5% decrease). Many other *in vitro* PM exposure studies have carried out cytotoxicity assays and experienced a similar less impact on cell viability by the PM_{10} (Scapellato and Lotti, 2007; Wendy Hsiao et al., 2000). This reduced cell viability and morphological changes may be attributed to different air pollutants bound to PM_{10} such as PAHs, nitro-PAHs, and metals (Naimabadi et al., 2016). Toxic levels and triggering biological effects of PM depend on many parameters such as sampling site, size fraction, sampling time, and contaminant composition of the particles (Calcabrini et al., 2004; Frampton et al., 1999).

To find the genotoxic effects of the available PAHs and HMs in Kandy city air, the exposed cells were subjected to single cell gel electrophoresis which is known as alkaline comet assay. It is a rapid, simple, visual, and sensitive technique for measuring and analyzing DNA breakage in mammalian cells (McKelvey-Martin et al., 1993; Singh et al., 1988). The formation of a tail by the nuclear DNA is used to measure the level of DNA breakage, and it evaluates the extent

of DNA damage, and the smallest detectable size of migrating DNA was identified as Olive tail moment (Dusinska and Collins, 2008). Interestingly, the present data also demonstrate that PM can elicit DNA damage in porcine airway epithelial cells because comet formation significantly increased in all treatment groups compared to the control. This result clearly explained high particle-bound compound concentrations, especially carcinogenic HMs in Kandy air can induce DNA damage and impart genotoxic effect leading to aberrant gene expression and regulation plus genetic mutations without reducing the cell viability in greater detail. When DNA damaged cells survive, it leads to aberrant gene expressions, which were found in the present study leading to carcinogenesis. Numerous epidemiological studies are demonstrating a strong linkage between lung cancer mortality and particle pollution (He et al., 1991; Risom et al., 2005). It has been established that PM exposure can induce oxidative stress caused by ROS generation due to the presence of numerous direct and indirect DNA damaging agents such as PAHs (particle-bound BaP, DahA), HMs, free radicals, and oxidants adsorbed on their surface (Baulig et al., 2003; Pope III et al., 2002). As a result of ROS generation, different signaling pathways can be triggered related to cell proliferation, apoptosis, oncogene expression, and ultimately mutagenesis and carcinogenesis (Zhou et al., 2016). Among all agents, HMs play a crucial role in DNA damage and inducing cancer. Various reports have found that exposure to toxic HMs leads to disruption in tumor suppressor gene expression, cell enzymatic activity, and damage repair processes (Jan et al., 2015). According to molecular toxicity and pathway analysis data, all four conformed carcinogenic HMs, which are As, Cd, Cr, and Ni, interfere with DNA repair

processes, ROS generation, and they can bind DNA and interrupt cellular processes (Kim et al., 2015). The two most concentrated toxic HMs in Kandy air are Cu and Zn, which serve as a cofactor in a few hundred enzymes that are essential for intracellular processes and have DNA-binding domains that lead to cancers and other diseases (Romano et al., 2020; Sabuncuoglu et al., 2015). Numerous studies have shown that the HM-bound particles could penetrate the head airways and damage along the way through the tracheobronchial tree and then the alveolar region (Colt and Blair, 1998; Olawoyin et al., 2018). Based on the present findings, it is well evident that confirmed DNA damaging agents are present in high concentrations in the Kandy city air since particle-bound individual PAH and HM concentrations were significantly high compared to the other major cities in the world based on our analysis. It can be suggested that this may have a strong influence on the increasing number of lung cancers and other respiratory-related diseases in Kandy over other cities of the country within the last decade (Mukherjee et al., 2013; National Cancer Control Programme, 2011).

It has been reported that due to the high heterogeneity in PM composition in the air, exposure to these inhalable particles can induce cytotoxicity, morphological changes, functional alterations, and inflammatory responses in the lung cells (Calcabrini et al., 2004). These PM induced inflammation responses and alterations are responsible for different lung and cardiovascular diseases (Lodovici and Bigagli, 2011). *In vivo* exposure studies also reported that animals exposed to high levels of specific PAHs for an extended period under laboratory conditions show critical lung cancer development from inhalation and skin cancer from skin

contact from predominantly but also bladder and gastrointestinal cancers (Lippman and Hawk, 2009). Based on all this evidence and data from the present study, we can stipulate that pollutants in Kandy air can induce aberrant changes in gene expression that lead first inflammatory and later carcinogenic signaling pathways activation in lung cells with a less direct effect on cell viability. To determine the pathways involved in response to pollutants exposure, the porcine airway epithelial cell culture model was used in the present study, which has never been attempted in Sri Lanka. In the current study, cultured cells were exposed to 3, 30, and 300 µg/ml PM_{10} treatment for 24 and 48 h and gene expression levels of several pro-inflammatory cytokines (IL-6, IL-8, and TNFα), xenobiotic metabolizing enzyme (CYP1B1), growth factors and a few oncogenes (EGFR, TGF-β1, p53, and PGES) were investigated.

According to a few recent studies, PAHs, which are adsorbed to PM, can lead to pro-inflammatory responses (Abbas et al., 2019; Cachon et al., 2014). This inflammatory response initiation strongly relates to the induction of various cytokines and chemokines (Cachon et al., 2014; Dergham et al., 2012). Moreover, a robust correlation between up-regulated cytokine secretion and high concentrations of metals such as Cu, Fe, As, Zn, total carbon adsorbed on PM (urban traffic), and inorganic ions (e.g., sulfate) were already established (Perrone et al., 2010). A few studies have revealed that inflammatory cytokines might be strongly correlated with COPD and asthma (Halatek et al., 2011; Huang et al., 2016). Nevertheless, the exact mechanisms by which PM induced COPD and asthma are still unclear. Several previous studies suggest that ROS production plays a crucial role in the development of cardiovascular disorders.

Excessive generation of free radicals may lead to oxidative stress and inflammation (Rengarajan et al., 2015). However, we still need further investigations to delineate the detailed mechanism.

5.5. Gene Expression Study

The gene expression data of the present study demonstrated that the interleukin-6 (IL-6) and interleukin-8 (IL-8) expressions were significantly induced in all treatment groups after 24 h and 48 h exposure. IL-6 and IL-8 are considered as critical pro-inflammatory cytokines produced by the epithelial cells to initiate and stimulate inflammatory responses. Findings of previous studies confirmed that increased mRNA expression of IL-6 and IL-8 due to exposure to PM_{10} and $PM_{2.5}$ (Cachon et al., 2014; Gualtieri et al., 2011). Furthermore, according to their study, decreased cell viability has a strong correlation with the high secretion of all cytokines induced in cultured cells exposed to PM. Transcription of IL-8 switches on and increases the IL-8 secretion from airway epithelium and neutrophils by TNFα through activation of transcription factor and nuclear factor-kB. This is one of the key processes for protection against pathogens (Gualtieri et al., 2011). Nevertheless, adverse health effects can be induced by this excessive or prolonged recruitment of neutrophils (Kulkarni et al., 2006). Epidemiological studies suggest that the coarse fraction may have higher pro-inflammatory potential and lead to adverse pulmonary responses, which may be leading to hospital admission (Brunekreef and Forsberg, 2005). In accordance with that, significant up regulation of both IL-6 and IL-8 in treated cells compared to the untreated cells were observed. On the other hand, Cachon et al.,

(2014) have revealed a reliable correlation between mRNA expression levels of IL-6/IL-8 and CYP1B1 gene expression levels.

The tumor necrosis factor-alpha (TNFα) expression was found to be significantly induced in all treatment groups after 24 and but interestingly, the 300 µg/ml group showed a significant expression in 48 hours. TNFα is a cytokine expressed by many cell types and has the capacity to induce tumor regression via suppressing tumor cell proliferation. According to numerous reports, TNFα expression is increased in different pre-neoplastic and tumor tissues (Ahmed et al., 2001; Szlosarek et al., 2006). The aberrant expression of TNFα has been implicated in different human diseases, including diabetes, osteoporosis, cancer, and including pulmonary fibrosis (Piguet et al., 1993). Experiments were done by using pulmonary fibrosis animal models (*in vivo*), also have been shown an elevated level of TNFα in the lungs (Thrall et al., 1997). Therefore, TNFα mRNA expression and secretion could be used as a good indicator of the initiation of cardiovascular disease, pulmonary fibrosis, and lung cancer. Another study demonstrated that PM_{10} exposure significantly altered the oxidative stress status in rat heart tissue, which led to elevated TNFα (Radan et al., 2019). Moreover, TNFα mRNA expression and secretion could be used as a good indicator of the initiation of cardiovascular disease, pulmonary fibrosis, and lung cancer. It has been shown a strong positive association between COPD, asthma, and outdoor air pollution with a few comparative case studies done in Sri Lanka (Kumarihamy and Tripathi, 2019; Nandasena et al., 2010). For the years 2010 to 2014, a total of 33,384 respiratory disease incidences were recorded. The total asthma incidence is higher

than the total of COPD, as 19,909 and 13,475, respectively, in Kandy (Kumarihamy and Tripathi, 2019). In addition to Kandy, PM_{10} is the highest contributing air pollutant to respiratory-related hospital admissions/deaths in Colombo as well (Dharshana and Coowanitwong, 2008). Further, these epidemiological study-based data can be further explained with the significant elevation of mRNA expression of several pro-inflammatory cytokines in the present study. Thus, to have further knowledge about the exact behavior of TNFα under *in vitro* conditions, whole transcriptomic level data and proteomic approach might be needed.

The xenobiotic metabolic enzyme (CYP1B1) expression was also up-regulated by PM_{10} extract treatment at 24 and 48 hours. CYP1B1 pathway can also be induced by PAHs (Dergham et al., 2012; Stéphanie et al., 2011), and gene expression could also generate DNA adduct production. A positive correlation between PAH exposure and PAH-DNA adducts has been found in epidemiological studies. Unrepaired adducts may cause permanent mutations. (Tsay et al., 2013). If these mutations have occurred at critical sites of tumor suppressor genes or oncogenes, they might lead to a cellular transformation and tumor development (Olivier et al., 2010). Recent studies have revealed a significant up-regulation of genes involved in the metabolism of AhR (Aryl hydrocarbon receptor) ligands induced by PM (Gualtieri et al., 2011). In fact, it has been reported as the central element in the regulation of cytochrome metabolizing enzymes. Therefore, it regulates the CYP1B1 genes by AhR-ARNT complex and important in the initiation of lung cancer (Gualtieri et al., 2011). These PM generated inflammatory responses trigger the pathway of inflammation process enrichment of ROS that could lead to DNA

damage, regulate the expression of numerous genes related to the initiation, promotion, and progression of genetic diseases and lung cancer (Schins and Knaapen, 2007). According to the present data, strong up-regulation of IL-6, IL-8, TNFα, and CYP1B1 mRNA expression may trigger inflammatory response and cancer initiation if the human exposure happens for a prolonged period. Thus, it can be postulated that these *in vitro* results are applicable in explaining the causes of air pollution-induced lung inflammation and other diseases.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase protein that is expressed in some normal epithelial, mesenchymal, and neurogenic tissues (Bethune et al., 2010). It has been recorded that overexpression of EGFR is involved in the progression of many human malignancies and EGFR binding to its ligand triggers several signal transduction cascades (Inamura et al., 2010). Mutations in EGFR have been discovered in association with some lung cancers. In addition to mutated EGFR, evidence is emerging that increased EGFR gene copy number in cancers (Cappuzzo et al., 2005). Due to the overexpression of EGFR in the majority of patients with non-small cell lung cancer (NSCLC), it is considered an important target in cancer treatment (Tasdemir et al., 2017). Previous studies have shown that 22-34 percent of NSCLC patients show positivity in mRNA expression (Brabender et al., 2001). Not only human patients, mRNA overexpression of EGFR and its correlation with cancer has also been tested with *in vitro* cell culture-based models and *in vivo* animal (mice) models (Ishiguro et al., 2013; Liu et al., 2017). In relation to this previous literature, the present study demonstrated significant up regulation of mRNA expression for EGFR in all treatment groups

for 24 and 48 hours compared to the nontreated control group. Therefore, it can be suggested that PM_{10} from Kandy air might have a strong potential for lung cancer initiation. However, according to the many previous studies reviewed, the correlation among gene mutation, protein expression, and mRNA expression is still unclear and has significant variations in lung cancer patients (Amador et al., 2004). Some reports have shown that EGFR mutations and expression vary even within ethnic groups (Tasdemir et al., 2017). Due to all these uncertainties, it will be important to have a precise evaluation and interpretation of EGFR protein expression and mRNA expressions investigated with proper clinical data support.

In this present study, TGF-β1 has shown significant upregulation of gene expression compared to the control in 24 hours treatment group only. Transforming growth factor-beta 1 (TGF-β1) is secreted by epithelial cells, fibroblasts, macrophages, and platelets (Yamane et al., 2003). TGF-β1 plays a critical role in regulating cellular growth, alveolar epithelial cell differentiation, growth suppression of epithelial cells, fibroblast activation, adhesion, apoptosis, immune responses, and tumorigenesis in many biological systems (Aschner and Downey, 2016). Expression and activation of TGF-β1 via stimuli and pathogens induce multiple mechanisms in the lungs. TGF-β1 ligand upregulation was observed in all major pulmonary diseases such as including pulmonary fibrosis, emphysema, bronchial asthma, and lung cancer. Moreover, the deep involvement of TGF-β1 in asthmatic airway inflammation was also observed (Heldin et al., 1997). It has been shown that TGF-β1 knockout mice die shortly after birth due to the development of severe inflammation of the heart and lungs (Aschner and Downey, 2016). This

growth factor is also essential for the wound healing process. However, the previous report shows that TGF-β1 can be involved in the initiation of the fibrotic response (Leask and Abraham, 2004; Yue et al., 2010). TGF-β1 triggers Idiopathic pulmonary fibrosis, which is a lethal disease via fibroblast to myofibroblast differentiation (Checa et al., 2016). Numerous reports have shown that TGF-β1 has a strong relationship with cytokines and expression of pulmonary arterial hypertension, COPD, asthma, and several other diseases (Aschner and Downey, 2016). However, one relatively old research has shown that TGF-β1 was abundantly expressed both at the protein and mRNA level in human lung and no overexpression in asthmatic and COPD patients (Aubert et al., 1994). Although TGF-β1 expression is not significantly induced in asthma and COPD cases, the role of TGF-β1 in the pathogenesis of these cannot be ignored. Previous literature showed that increased TGF-β1 mRNA expression levels in the epithelium of bronchial biopsies have positive relationships with the expression of TGF-β1 protein (Xie et al., 2009). Furthermore, *in vivo* studies have revealed that TGF-β1 expression is positively correlated with the duration of exposure to and the amount of atmospheric PM in the environment (Furuyama et al., 2006; Líbalová et al., 2012). According to the present study, acute exposure to PM_{10} may provoke TGF-β1 mRNA expression compared to the untreated control, but in the 48 hours group, no significant expression can be seen, which may be due to the evaporation of volatile compounds, cells masking the effects of toxic compounds, maturation of the cells or secretion of the inhibitors of the expression of TGF-β1 with the continuous exposure.

The expression of p53 mRNA showed a significant up-regulation only in the 24 hours treatment group compared to the untreated control in the present study. After 48 hours, all three treatments had no significant effect compared to the non-treated control, which is similar to the TGF-β1 expression. Expression of p53 is very important because the p53 gene is a critical tumor suppressor gene involved in the majority of cancers (68% of NSCLC), and clinical data also show the same (Kong et al., 2019). mRNA overexpression of p53 activates by different stress and damage pathways and regulates cell biological responses, including cell cycle arrest, repair pathways, and apoptosis (López et al., 2015). The p53 tumor suppressor protein is activated in response to various cellular stresses such as DNA damage, unfolded protein response, and other stresses by inhibiting proliferation or inducing programmed cell death (Brown et al., 2009). In the process of cancer development, often, genomic aberrations can be seen (Elledge, 1996). In many types of cancers, p53 mRNA overexpression was reported. However, in some tumors, p53 mRNA expression was high, and some have only a slight increase due to tumor heterogeneity (El-Mahdani et al., 1997). Other than that, the mRNA overexpression of p53 can affect many different signaling pathways, and controlling p53 expression is critical for cell homeostasis and stress responses (Haronikova et al., 2019). With the present results, we cannot conclude that there is any relation between the present aberration of mRNA expression of p53 and cancer progression when exposing to PM_{10} without doing further investigations on gene mutations in tumors, signaling pathways, and protein expression, but it is safe to mention that this overexpression of p53, can be easily directed to many critical health problems. Though the

overexpression alone is not enough to make decisions on tumors, as a rapid method, mRNA quantification can be used (El-Mahdani et al., 1997). Furthermore, to have a better correlation of p53 mRNA expression with the tumors needs more analysis on p53 mutation and clinical data from patients with tumors.

Prostaglandin E synthase (PGES), which is an essential bioactive lipid, plays a fundamental role in various signaling pathways. In the present study, PGES expression was significantly up-regulated ($p < 0.05$) in all treatment groups compared to the control for both 24 and 48 h PM_{10} treatments. PGES is responsible for producing PGE_2, and it is a tissue-specific hormone (Yin et al., 2017). PGE_2 shows diverse physiological roles, and it can activate key downstream signaling cascades via transmembrane EP receptors located on the cell surface (Breyer et al., 2001). Elevated levels of COX-2 and concomitant overproduction of PGE_2 are often found in human cancers. Therefore, changes in the expression of PGES have potential involvement in pathological conditions such as angiogenesis, migration, and proliferation (Samuelsson et al., 2007). On the other hand, a high level of prostaglandins in the body leads to inflammation reactions (de Oliveira et al., 2008). Some groups have found that strikingly enhanced PGES expression due to the exposure of PM, suggesting that PGES might contribute to PM-related apoptosis and vascular inflammatory injury (Yin et al., 2017). PGES expression and its contribution to the pathogenesis of cancer was also investigated by many previous researches (Jakobsson et al., 1999). In the present study, significant mRNA up-regulation of PGES can be seen in both 24 h and 48 h treatments of PM_{10}. All the treatments showed a clear progressive

expression in a dose-dependent manner compared to the control. With this data, it can be suggested that that exposure to PM_{10} from Kandy air can alter the expression of PGES in porcine airway epithelial cells and may be initiated different cell signaling pathways that lead to inflammations and tumors. However, due to the lack of proper investigations done on variations of PGES secretions and mRNA expression in specific cells of the respiratory tract, we cannot identify the exact signaling pathways activated in the porcine airway epithelial cells.

5.6. Limitations and Future Directions of the Study

There were some limitations to the present study. With the available resources, collection and analysis of $PM_{2.5}$ were not possible, which is the most concerned particulate category these days due to its high penetration level and variation in chemical composition (Kampa and Castanas, 2008). As one of the cities with the most contaminated air in Sri Lanka, Kandy needs further investigation of the $PM_{2.5}$ composition profile and human health (Wickramasinghe et al., 2011). Moreover, the present study was done on the primary porcine airway epithelial cell culture model, while most of the cited *in vitro* exposure studies have been used BEAS-2B (Human bronchial epithelium, normal) or A549 (human lung, carcinoma) cell line. A recent report has shown that A549 cells are less sensitive to PM-induced effects and cell death stimuli induced by different compounds compared to the other lung cell lines (Gualtieri et al., 2010). Therefore, we have to be cautious extrapolating the results generated with the primary cell cultures and commercially produced cell lines. Significant variations in mRNA expression, DNA damage,

and cytotoxicity can be expected in human cells. On the other hand, the present study was conducted as an *in vitro* bioassay. To have complete knowledge of the biological processes induced by PM (ambient air) and its impact on human health, it is essential doing *in vivo* exposure experiments because biological responses to certain chemicals may be different during *in vitro* compared to *in vivo* experiments. However, despite these limitations, the present study was the first of its kind done in Sri Lanka to recognize the effects of airborne PM on gene expression levels. Therefore, the results of this study will help researchers to answer the emerging questions and design new experiments to correlate the PM induced DNA methylation, affected signaling pathways, and protein expression with clinical data.

CONCLUSIONS

Comprehensive chemical characterization of airborne PM_{10} was performed for samples from two sampling sites in Kandy city, Sri Lanka. 12 priority PAHs and 32 elements, including ten hazardous HMs were identified. Chemical analysis results showed higher levels of PAH and HM contamination in Kandy, indicating the pollution of the city is much more severe than other cities in the world and beyond all WHO guidelines of PM and PAH levels. All these pollutants might get accumulated in ambient air due to two primary sources. The crustal source is associated with wind-blown dust particles and anthropogenic sources related to vehicular traffic and wood-burning because the concentrations of most of the recorded PAHs and HMs have a strong relationship with diesel combustion, wood burning, and abundance of the crustal elements.

High molecular weight confirmed carcinogenic PAHs (IARC) such as benzo[a]pyrene and dibenz[a,h]anthracene recorded in very alarming concentrations, and PAHs with high molecular weight contributed the most compared to the low molecular weight PAHs in both sites. Highly carcinogenic HMs, As, Cd, Cr, Ni, and probable carcinogens, Pb, Co have also recorded in elevated concentrations compared to the other cities in the world. Within the Kandy city, there are no significant variations in PAH or PM concentrations between the two sampling sites. Concerning our *in vitro* exposure study, PM_{10} at low concentrations (3 and 30 μg/ml) induced no cytotoxicity on primary porcine airway epithelial cells but significant cytotoxicity in the

highest concentration (300 μg/ml). Due to the presence of DNA damaging agents adsorbed on their surface of the particles such as (PAHs and HMs) in high concentrations, significant DNA damage was identified in all treatment groups compared to the control. In the gene expression data obtained from the qPCR analysis, aberrant expression of all pro-inflammatory cytokines (IL-6, IL-8, and TNFα), xenobiotic metabolizing enzyme (CYP1B1) and other growth factors and oncogenes (EGFR, TGF-β1, p53, and PGES) show a strong connection with human health (COPD, asthma, inflammatory response, wound healing, pulmonary fibrosis, and cancer).

Therefore, based on all available PM_{10} bound PAHs, HMs, and gene expression data from the present study, it can be concluded that ambient air in Kandy consists of highly carcinogenic and mutagenic compounds which are emitted mainly from anthropogenic activities, accumulated at an alarming rate compared to the other cities in the world and exposure to Kandy air may trigger inflammation, mutagenesis, and carcinogenesis related signaling pathways in respiratory epithelial cells.

APPENDICES

Appendix 1. EPA divided AQI values into six categories for easy understanding the local air quality values means to human health

AQI Levels of Health Concern	AQI Value	Meaning
Good	0 to 50	Air quality is considered satisfactory, and air pollution poses little or no risk.
Moderate	51 to 100	Air quality is acceptable; however, for some pollutants there may be a moderate health concern for a very small number of people who are unusually sensitive to air pollution.
Unhealthy for Sensitive Groups	101 to 150	Members of sensitive groups may experience health effects. The general public is not likely to be affected.
Unhealthy	151 to 200	Everyone may begin to experience health effects; members of sensitive groups may experience more serious health effects.
Very Unhealthy	201 to 300	Health alert: everyone may experience more serious health effects.
Hazardous	301 to 500	Health warnings of emergency conditions. The entire population is more likely to be affected.

Appendix 2. Average blank and method detection limit (MDL). MDL is defined as the average blank plus three times of the SD of blanks

Compounds	Average blank (ng)	Method detection limit
Phenanthrene	2.29	4.22
Anthracene	1.17	5.90
Fluoranthene	0.39	1.19
Pyrene	0.53	1.07
Benz[a]anthracene	0.072	0.30
Chrysene	0.20	1.05
Benzo[b]fluoranthene	0.46	1.44
Benzo[k]fluoranthene	0.14	0.46
Benzo[a]pyrene	0.40	1.56
Indeno[1,2,3-cd]pyrene	0.40	0.87
Dibenz[a,h]anthracene	0.25	0.50
Benzo[ghi]perylene	0.20	0.75

Appendix 3. Recoveries (%) of PAHs surrogates

Sample	phenanthrene-d10	chrysene-d12	perylene-d12
AN 01	100.5	117.3	82.2
(duplicate 1/2)	80.5	106.6	77.7
AN 02	76	107.6	110.2
(duplicate 1/2)	96	80.6	86
AN 03	57.3	105.6	76.4
AN 04	96.4	107.6	52.3
AN 05	96.3	104.3	75.7
AN 06	98.5	104.7	74.4
AN 07	99.4	109.3	71.2
AN 08	100	110.5	75.6
AN 09	79.3	112.2	86.3
AN 10	98.9	105.2	65.9
AN 11	99.7	106.7	82.1
AN 12	91.6	111.9	83.1
AN 13	99.7	105.4	77.9

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