# รายงานการวิจัยฉบับสมบูรณ์

# ผลของอนุภาคฝุ่นขนาด 2.5-0.5 ไมครอนต่อการเกิดความเสียหายของดีเอ็นเอ และการเกิด 8-ไฮดรอกซี-2-ดีออกซีกวาโนซีน

# Effect of Particulate Matters 2.5-0.5 Microns on DNA Damage and 8-hydroxy-2-deoxyguanosine Formation


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## ABSTRACT

Occurrence of air pollution in Chiang Mai has reached crisis during the past few years, causing public worry and concern on the health impact among residents in the Chiang Mai-Lamphun valley. Acute effects of this crisis correlated prominently to an increased number of patients with respiratory diseases. However, chronic effects, especially genotoxic ones, after human exposure to fine particulate matter (PM) of 0.5, 1.0 or 2.5 microns in Chiang Mai, have not been reported. Therefore, the aim of this study was to investigate whether exposure to fine PM caused DNA damage and induced formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a by product of DNA damage resulting from hydroxyl radical production.

A high volume cascade impactor was installed in front of Saraphi Hospital in Chiang Mai to collect fine PM for 4 months from August to November, 2007. The PM was weighed and calculated for concentrations in five PM sizes before being extracted with ultrapure water and methanol for 60 minutes, and lyophilized to dryness. PM solution was prepared at each concentration before the A549 human lung epithelial cells were given a genotoxicity test. Comet assay was used to evaluate DNA strand break analysis and the enzyme immunoassay was used to quantitate 8-OHdG.

The results showed that concentrations of PM≥10, PM10-2.5, PM2.5-1.0, PM1.0-0.5 and PM≤0.5 were 283, 467, 280, 227 and 139 $\mu g/m^3$, respectively, in which PM10-2.5 had the highest proportion of total PM (33.4%). In addition, the amount of PM≥10, PM2.5-1.0, PM1.0-0.5 and PM≤0.5 was 20.3%, 20.1%, 16.2% and 10.0%, respectively.

DNA strand break analysis on the A549 human lung epithelial cells demonstrated that PM10-2.5 could significantly induce DNA damage at concentrations of 150, 300, 600, 1,200 and 2,400 μg/ml ($p \leq 0.001$). In addition, PM2.5-1.0, PM1.0-0.5 and PM≤0.5 could also induce DNA damage at concentrations of 75, 150, 300 and 600 μg/ml ($p \leq 0.001$), except for PM2.5-1.0 at the concentration of 75 μg/ml. When concentrations of PM1.0-0.5 and PM≤0.5 were decreased, in order to find the lowest one that could induce DNA damage, it was found that concentrations of 10, 20, 40 and 80 μg/ml could induce DNA damage significantly ($p \leq 0.001$). However, PM1.0-0.5 at the concentration of 10 μg/ml could not. Thus, the results showed that PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations of 150, 20 and 10 μg/ml, respectively, were the lowest concentrations of each PM that could induce DNA damage on A549 human lung epithelial cells. These low concentrations of PM were approximately the same as those that could be inhaled and accumulate in the human alveoli.

Aphidicolin (APC), a DNA repair inhibitor, was used at concentrations of 1.0 and 2.0 μg/ml, with PM2.5-1.0 at concentrations of 75 and 150 μg/ml in the comet assay. The results showed that the A549 human lung epithelial cells had greater DNA damage after treatment with PM2.5-1.0 and either concentration of APC than after experiments without APC. This meant that at a PM2.5-1.0 concentration of 75 μg/ml, which could not induce DNA damage, the cells may be protected by a repair mechanism within them.

Quantitation of 8-OHdG on the DNA of A549 human lung epithelial cells showed that only PM2.5-1.0 at concentrations of 75, 300 and 600 μg/ml, and PM≤0.5

at concentrations of 150 and 600 μg/ml could significantly elevate the 8-OHdG levels ($p \leq 0.05$). In contrast, PM1.0-0.5 at concentrations of 75, 150, 300 and 600 μg/ml could induce 8-OHdG formation on the A549 human lung epithelial cells ($p \leq 0.05$) at all concentrations.

In conclusion, fine PM (0.5, 1.0 and 2.5 microns) collected at Saraphi Hospital in Chiang Mai could induce DNA damage and increase 8-OHdG formation. The mechanism of DNA damage is possibly involved with the generation of hydroxyl radicals caused by fine PM itself, or chemical constituents that are adsorbed onto the PM and attack the guanine base on the DNA strand. The elevation of 8-OHdG might be implicated in the progression of lung cancer. Long term exposure to fine PM may be responsible for chronic adverse effects among Chiang Mai's inhabitants, who are reported to have the highest incidence of lung cancer in Thailand.

## บทคัดย่อ

การเกิดวิกฤตมลภาวะทางอากาศในจังหวัดเชียงใหม่ ในระยะ 2-3 ปีที่ผ่านมาเป็นเหตุให้เกิดความวิตกกังวลกับผลกระทบต่อสุขภาพของประชากรที่อาศัยในแอ่งจังหวัดเชียงใหม่-ลำพูนมาก ผลกระทบแบบเฉียบพลันทำให้มีจำนวนผู้ป่วยด้วยโรคระบบทางเดินหายใจสูงขึ้นชัดเจน แต่ผลกระทบแบบเรื้อรังโดยเฉพาะอย่างยิ่งต่อยีนหรือดีเอ็นเอหลังการสัมผัสอนุภาคฝุ่นละเอียดมากถึง 0.5, 1.0 หรือ 2.5 ไมครอนในจังหวัดเชียงใหม่ยังไม่เคยมีรายงานมาก่อน จึงเป็นวัตถุประสงค์ของงานวิจัยนี้ที่ต้องการศึกษาว่า อนุภาคฝุ่นละเอียดเหล่านี้ก่อให้เกิดความเสียหายต่อดีเอ็นเอ และเหนี่ยวนำให้เกิด 8-ไฮดรอกซี-2′-ดีออกซีกวาโนซีน (8-OHdG) ซึ่งเป็น by product ของการเกิดดีเอ็นเอบาดเจ็บ เนื่องจาก hydroxyl radical ที่เกิดขึ้นจากการได้รับอนุภาคฝุ่นขนาดเล็กหรือไม่

งานวิจัยนี้ได้ใช้ เครื่อง high volume cascade impactor ในการเก็บอนุภาคฝุ่นละเอียดต่าง ๆ โดยทำการติดตั้งที่บริเวณหน้าโรงพยาบาลสารภี ในจังหวัดเชียงใหม่ ในช่วงเวลา 4 เดือน คือ สิงหาคม กันยายน ตุลาคม และพฤศจิกายน 2550 และนำมาชั่งน้ำหนักเพื่อคำนวณหาความเข้มข้นของอนุภาคฝุ่นที่เก็บได้ 5 ขนาด จากนั้นนำมาสกัดด้วยน้ำและเมทานอล เป็นเวลา 60 นาที แล้วนำไปทำให้แห้งเพื่อเตรียมเป็นสารละลายความเข้มข้นของอนุภาคฝุ่นที่ความเข้มข้นต่าง ๆ ก่อนนำไปทดสอบความเป็นพิษต่อยีนในเซลล์เยื่อบุผิวของปอดคนชนิด A549 การวิเคราะห์การแตกหักของดีเอ็นเอใช้วิธีโคเมทแอสเสย์ และหาปริมาณ 8-OHdG ใช้วิธีเอนไซม์อิมมิวโนแอสเสย์

ผลการทดลองพบว่าความเข้มข้นของอนุภาคฝุ่นขนาดใหญ่ 10 ไมครอน, 10-2.5, 2.5-1.0, 1.0-0.5 และเล็กกว่า 0.5 ไมครอนมีปริมาณ 283, 467, 280, 227 และ 139 ไมโครกรัมต่อลูกบาศก์เมตร ตามลำดับ โดยอนุภาคฝุ่นขนาด 10-2.5 ไมครอนมีสัดส่วนทั้งหมดของฝุ่นมากที่สุดคิดเป็นร้อยละ 33.4 ของฝุ่นทั้งหมด และปริมาณอนุภาคฝุ่นขนาดมากกว่า 10 ไมครอน, 2.5-1.0, 1.0-0.5 และน้อยกว่า 0.5 ไมครอนร้อยละ 20.3, 20.1, 16.2 และ 10.0 ตามลำดับ

การวิเคราะห์การแตกหักของดีเอ็นเอในเซลล์เยื่อบุผิวของปอดคน พบว่าอนุภาคฝุ่นขนาด 10-2.5 ไมครอนที่ความเข้มข้น 150, 300, 600, 1,200 และ 2,400 ไมโครกรัมต่อมิลลิลิตร (มคก./มล.) ทำให้เกิดการแตกหักของดีเอ็นเอทุกความเข้มข้นอย่างมีนัยสำคัญทางสถิติ ($p \leq 0.001$) นอกจากนี้อนุภาคฝุ่นขนาด 2.5-1.0, 1.0-0.5 และน้อยกว่า 0.5 ไมครอนที่ความเข้มข้น 75, 150, 300 และ 600 มคก./มล. สามารถเหนี่ยวนำให้เกิดการแตกหักของดีเอ็นเอได้ ($p \leq 0.001$) ยกเว้นอนุภาคฝุ่นขนาด 2.5-1.0 ไมครอนที่ความเข้มข้น 75 มคก./มล. เมื่อลดความเข้มข้นของอนุภาคฝุ่นขนาด 1.0-0.5 และน้อยกว่า 0.5 ไมครอนลง เพื่อหาความเข้มข้นน้อยที่สุดที่ทำให้เกิดการแตกหักของดีเอ็นเอพบว่าความเข้มข้น 10, 20, 40 และ 80 มคก./มล. ทำให้เกิดการแตกหักของดีเอ็นเอได้ ($p \leq 0.001$) ยกเว้น

อนุภาคฝุ่นขนาด 1.0-0.5 ไมครอนที่ความเข้มข้น 10 มคก./มล. จากผลการทดลองแสดงให้เห็นว่าความเข้มข้นของอนุภาคฝุ่น 150, 20 และ 10 มคก./มล. เป็นความเข้มข้นต่ำสุดของอนุภาคฝุ่นขนาด 2.5-1.0, 1.0-0.5 และน้อยกว่า 0.5 ไมครอน ตามลำดับ ที่สามารถเหนี่ยวนำให้มีการแตกหักของดีเอ็นเอ และปริมาณอนุภาคฝุ่นที่ต่ำนี้เทียบได้กับปริมาณอนุภาคฝุ่นขนาดเล็กที่สามารถหายใจเข้าสู่ร่างกาย ไปสะสมในถุงลมปอดได้

ได้ทดลองให้ aphidicolin (APC) ซึ่งเป็นสารยับยั้งกระบวนการซ่อมแซมดีเอ็นเอที่ความเข้มข้น 1.0 และ 2.0 มคก./มล. ร่วมกับอนุภาคฝุ่นขนาด 2.5-1.0 ไมครอน ความเข้มข้น 75 และ 150 มคก./มล. ในการทดสอบโคเมทแอสเสย์กับเซลล์เยื่อบุผิวของปอดคน พบว่าเซลล์ที่ได้รับอนุภาคฝุ่นขนาด 2.5-1.0 ไมครอนและ APC ทั้งสองความเข้มข้นสามารถเกิดการแตกหักของดีเอ็นเอเพิ่มมากขึ้นเมื่อเปรียบเทียบกับการทดลองที่ไม่เติม APC แสดงให้เห็นว่าอนุภาคฝุ่นความเข้มข้น 75 มคก./มล. ที่ไม่เห็นผลการแตกหักของดีเอ็นเออาจเนื่องมาจากมีกระบวนการซ่อมแซมดีเอ็นเอภายในเซลล์ช่วยป้องกันไว้

ผลการทดลองหาปริมาณ 8-OHdG ในดีเอ็นเอของเซลล์เยื่อบุผิวของปอดคนที่สัมผัสอนุภาคฝุ่นขนาดต่าง ๆ พบว่า เซลล์ที่สัมผัสอนุภาคฝุ่นขนาด 2.5-1.0 ไมครอนความเข้มข้น 75, 300 และ 600 มคก./มล. และอนุภาคฝุ่นขนาดน้อยกว่า 0.5 ไมครอนความเข้มข้น 150 และ 600 มคก./มล. ทำให้ปริมาณของ 8-OHdG เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) ในทางตรงข้ามอนุภาคฝุ่นขนาด 1.0-0.5 ไมครอนที่ความเข้มข้น 75, 150, 300 และ 600 มคก./มล. สามารถเหนี่ยวนำให้เกิด 8-OHdG ในเซลล์เยื่อบุผิวของปอดคนได้ ($p \leq 0.05$) ทุกความเข้มข้น

สรุปผลการศึกษาครั้งนี้ได้ว่า อนุภาคฝุ่นละเอียดที่เก็บจากบริเวณหน้าโรงพยาบาลสารภี จังหวัดเชียงใหม่ สามารถเหนี่ยวนำให้เกิดการแตกหักของดีเอ็นเอและเพิ่มการสร้าง 8-OHdG โดยกลไกที่ทำให้เกิดการแตกหักของดีเอ็นเออาจเนื่องมาจากการกระตุ้นให้เกิด hydroxyl radical โดยอนุภาคฝุ่นเองหรือโดยสารที่ติดบนอนุภาคฝุ่นขนาดเล็กเข้าไปเกาะบนเบสกวานีนบนสายดีเอ็นเอ การเพิ่มขึ้นของระดับ 8-OHdG อาจเกี่ยวพันกับพัฒนาการของการเกิดโรคมะเร็งปอด การสัมผัสอนุภาคฝุ่นละเอียดเป็นระยะเวลานานอาจมีส่วนทำให้เกิดปัญหาสุขภาพแบบเรื้อรังของประชากรในจังหวัดเชียงใหม่ ที่มีรายงานว่ามีผู้ป่วยมะเร็งปอดเป็นอันดับหนึ่งของประเทศ

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2′-deoxyguanosine |
| AAQS | Ambient Air Quality Standards |
| APC | Aphidicolin |
| $\%B/B_0$ | Percent bound per maximum bound |
| C | Carbon |
| °C | Degree celsius |
| cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| dCTP | Deoxycytidine triphosphate |
| dtn | Determination |
| DNA | Deoxyribonucleic acid |
| DMSO | Dimethyl sulfoxide |
| ECD | Electrochemical detector |
| EDTA | Ethylenediaminetetraacetic acid |
| EIA | Enzyme immunoassay |
| g | Gram |
| µg | Microgram |
| GC | Gas chromatography |
| HBSS | Hank's buffered salt solution |

| | |
|---|---|
| HPLC | High-performance liquid chromatography |
| IgG | Immunoglobulin gamma |
| L | Liter |
| LC | Liquid chromatography |
| µl | Microliter |
| M | Molar |
| $m^3$ | Cubic meter |
| µM | Micromolar |
| µm | Micrometer |
| $\mu m^2$ | Square meter |
| mA | Milliampere |
| MEM | Minimum essential medium eagle |
| mg | Milligram |
| min | Minute |
| ml | milliliter |
| mM | Millimolar |
| mm | Millimeter |
| MMS | Methylmethane sulfonate |
| nm | Nanometer |
| MS | Mass spectrometry |
| NaCl | Sodium chloride |

| | |
|---|---|
| NaOH | Sodium hydroxide |
| NMPA | Normal melting point agarose |
| No. | Number |
| PACs | Polycyclic aromatic compounds |
| PAHs | Polycyclic aromatic hydrocarbons |
| PCD | The Pollution Control Department of Thailand |
| pg | Picogram |
| PM | Particulate matter |
| PUF | Polyurethane foam |
| rpm | Revolution per minute |
| ROS | Reactive oxygen species |
| TSP | Total suspended particles |
| UVC | Ultraviolet C |
| US.EPA | United States Environmental Protection Agency |
| WHO | World Health Organization |
| V | Voltage |
| % | Percent |

# CHAPTER I

# INTRODUCTION

## 1.1 Air pollution

Air pollution can be defined as the presence of substances in the air that cause adverse effects on the health of living organisms and the environment (Yu, 2004). For several decades, concerns over air pollution problems have increased, mainly in more-developed countries. Air pollutants differ in their chemical composition, reaction properties, emission, persistence in the environment, ability to be transported for long or short distances and their eventual impacts on human health (Kampa *et al.*, 2008). The adverse health effects of air pollution have been widely studied. Epidemiological studies have revealed that these effects create higher mortality rates, especially in the elderly and chronically ill. They exacerbate symptoms in elderly people with preexisting cardiopulmonary diseases, and patients with acute and chronic pulmonary disorders, and they also increase irritation of the eye and respiratory system, especially in asthma attacks when respiratory infection has been reported (Riedl, 2008).



## 1.2 Particulate matter

Particulate matter (PM) is the term used for a mixture of solid particles and liquid droplets suspended in the air. Their chemical and physical compositions depend on location, time and weather (Fierro, 2000). Major particles are divided into primary

and secondary particles. Primary particles are directly emitted into the atmosphere through man-made (anthropogenic) and natural processes. Secondary particles are formed in the air, usually by chemical change of gaseous substances. Products from the transformation of nitrogen oxides are mainly emitted by transportation, some industrial processes, and sulfur dioxide release from combustion of high sulfur-containing fuels. Secondary particles are mostly found in the fine PM fraction (Figure 1) (Kelly *et al.*, 2003).

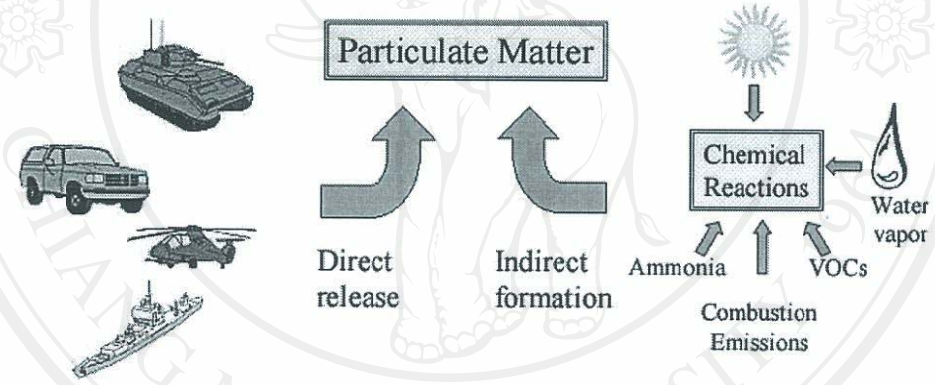


**Figure 1** Primary and secondary sources of particulate matter (Kelly *et al.*, 2003).

The particles vary in size, composition and origin. Their properties are summarized according to their aerodynamic diameter. The coarse fraction is called PM10 (particles with an aerodynamic diameter smaller than 10 μm). A smaller or fine fraction is called PM2.5 (with an aerodynamic diameter smaller than 2.5 μm). The size of the particles also determines the time they can spend in the atmosphere. PM10 can flow for a short time and PM2.5 for a long time (days or a few weeks) (World Health Organization, 2005).

The parameters that play an important role for eliciting health effects are the components of particles, size and surface areas (Kampa *et al.,* 2008). The chemical composition of PM varies and depends on many factors such as combustion sources, climate, seasons and type of urban or industrial pollution (Valavanidis *et al.,* 2008). The major components are organic compounds adsorbed onto particles, which can be volatile or semivolatile organic species, transition metal, ions, reactive gases, particle core of carbon materials, materials of organic origins and minerals (Rehwagen *et al.*, 2005; Whittaker *et al.*, 2006; Sevastyanova *et al.*, 2008).

The particle number and surface area are important factors of PM toxicity. The correlation between particle number and surface area is shown in Table 1. As the particle's size decreases, it corresponds to the ratio of surface area to volume (mass) of the particle increase (Carnelly *et al.*, 2001).

**Table 1** Characteristics of monodispersed particles of unit density at a mass concentration of 10 $\mu g/m^3$ (Oberdorster *et al.*, 1995)

| Diameter (μm) | No. per $m^3$ | Surface area ($\mu m^2$ per $m^3$ air) |
|---|---|---|
| 0.10 | $191{,}000 \times 10^6$ | $600 \times 10^6$ |
| 0.50 | $153 \times 10^6$ | $120 \times 10^6$ |
| 1.00 | $19 \times 10^6$ | $60 \times 10^6$ |
| 2.50 | $1.2 \times 10^6$ | $24 \times 10^6$ |

As is readily apparent, 10 $\mu g/m^3$ of particles in the size range of 0.1 to 0.5 μm diameter have more potential for interacting with respiratory tract cells than the

relatively lower concentration of particles at 2.5 μm, which would represent an equivalent mass (Gardner *et al.*, 1999). Unfortunately, only limited studies have been conducted to investigate the importance of particle number as a determinant of PM toxicity.

In 1971, the United States Environmental Protection Agency (US.EPA) established the first PM national ambient air quality standard. PM10 has an annual average concentration of 50 $\mu g/m^3$ and a 24 hour maximum of 150 $\mu g/m^3$. Next, the US.EPA concluded that PM2.5 has an association with mortality and morbidity rates greater than that of PM10. On this basis, the US.EPA established an annual PM2.5 standard level of 15 $\mu g/m^3$ and a 24 hour maximum of 65 $\mu g/m^3$ (Table 2) (Fierro, 2000).

**Table 2** US.EPA ambient air quality standard for PM (Adapted from California Environmental Protection Agency, 2008)

| Particulate matter | Particulate matter concentration ($\mu g/m^3$) | |
|---|---|---|
| | Annual average | 24-hour average |
| PM10 | 50 | 150 |
| PM2.5 | 15 | 65 |



The Pollution Control Department of Thailand (PCD) has set an acceptable level for the concentration of total suspended particles and PM10 in ambient air. A total suspended particle is an annual average concentration of 100 $\mu g/m^3$ and a 24 hour maximum of 330 $\mu g/m^3$. PM10 has an annual average concentration of 50 $\mu g/m^3$ and

a 24 hour maximum of 120 $\mu g/m^3$. Other pollutants are determined and noted in Table 3 (PCD, 2004).

**Table 3** Ambient air quality standard for pollutants (Adapted from PCD, 2004)

| Pollutants | Ambient Air Quality Standards (AAQS) | | | | |
|---|---|---|---|---|---|
| | 1-hour average | 8-hour average | 24-hour average | 1-month average | 1-year average |
| Carbonmonoxide ($mg/m^3$) | 34.2 | 10.26 | - | - | - |
| Nitrogen dioxide ($\mu g/m^3$) | 320 | - | - | - | - |
| Ozone ($\mu g/m^3$) | 200 | 140 | - | - | - |
| Sulfur dioxide ($\mu g/m^3$) | 780 | - | 300 | - | 100 |
| Lead ($\mu g/m^3$) | - | - | - | 1.5 | - |
| PM10 ($\mu g/m^3$) | - | - | 120 | - | 50 |
| Total suspended particle (TSP) ($\mu g/m^3$) | - | - | 330 | - | 10 |

### 1.3 Route of exposure

Inhalation is the main route of exposure that is of concern in relation to the direct effects of PM on human health (WHO Regional Office for Europe, 2000). The respiratory system can be divided into three regions (Figure 2) that are different in structure, airflow patterns, function, retention time and sensitivity to deposited particles. These regions are;

(i) the extrathoracic or nasopharyngeal region, which includes the nose, mouth, pharynx, and larynx,

(ii) the tracheo-bronchial region, which includes the trachea subdividing into increasingly smaller branches, and

(iii) the alveolar region, where gas exchange takes place.

In the functional role of the respiratory tract, main consideration is typically given to movement of gases to the alveoli and exchange of gases. Other functions include its role in clearing particle deposits, as an elaborate biochemical system that aids in detoxification or activation of inhaled chemicals (Gardner *et al.*, 1999).

Large quantities of air enter the body to provide adequate gas exchange. Typically, an adult individual takes about 15 breaths per minutes at rest, and with each breath inspires about half a liter of air. Individuals engaged in hard work have increased breathing rates and air intake volumes. Taking into account the various types of activities, individuals inhale 15 to 20 $m^3$ of air each day (International Committee on Radiological Protection, 1994). The volume of air inhaled is a key determinant of the amount of PM that is deposited and retained in the respiratory tract.

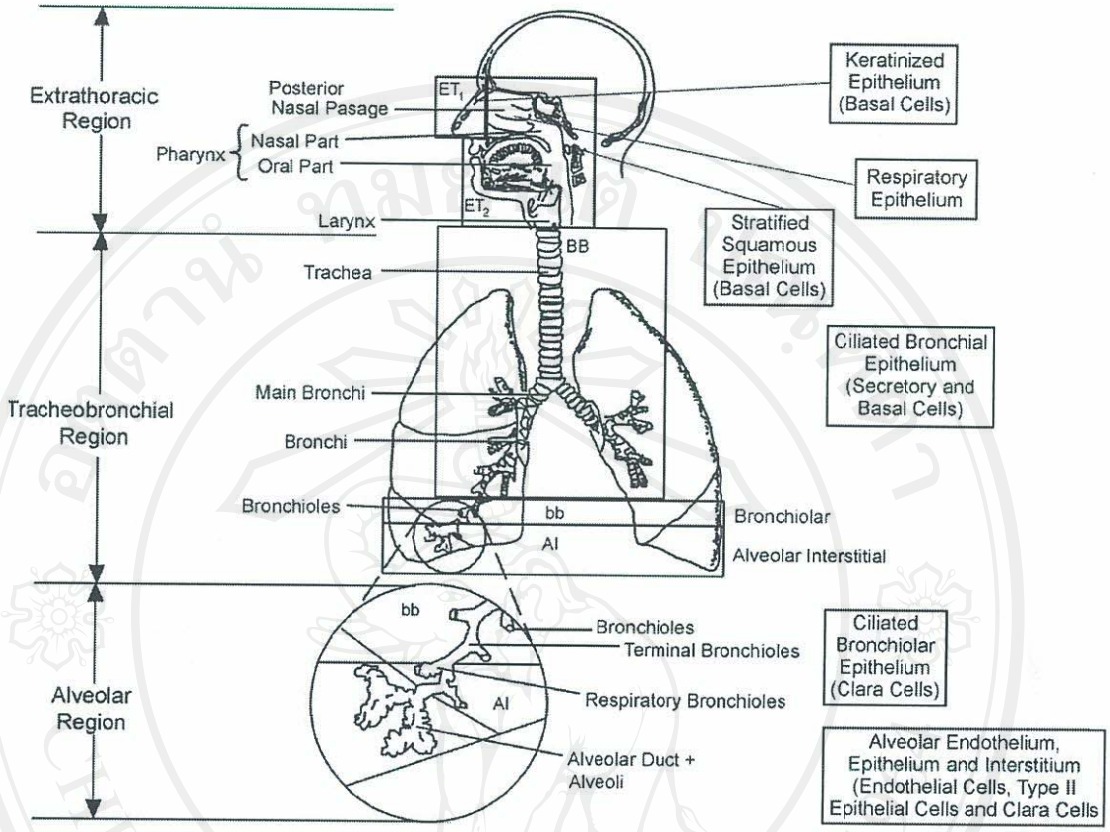


**Figure 2** Human respiratory tract that can be divided into three regions: extrathoracic, tracheobronchial and alveolar region.

(http://www.fysik.lu.se/... /swietlicki-airpolife-copenhagen-20060321-respi.ppt)

Particles can penetrate into the respiratory airways depending on the aerodynamic diameter, as shown in Table 4. The large particles (>10 μm) are filtered in the nose, naso and oropharynx, and the larynx. Only those particles smaller than 10 μm penetrate into the lung, and the smaller their aerodynamic diameter, the deeper they penetrate. Particles of approximately 5–8 μm are deposited in the tracheobronchial tree and those of 1–5 μm deposit primarily in the respiratory bronchioles and alveoli. Interestingly, particles of approximately 0.5 μm penetrate to

the alveoli during inhalation, but their diffusiveness and settling velocities are so low that they remain mostly airborne, and are exhaled rather than significantly deposited in the alveoli (Squadrito *et al.*, 2001).

**Table 4** Penetration, deposition and clearance of inhaled particles according to their aerodynamic diameter (Squadrito *et al.*, 2001)

| Aerodynamic diameter | Main site of deposition | Comments |
|---|---|---|
| > 10 μm | Nose, naso and oropharynx, and larynx | Cleared by mucocialiary transport |
| 5-8 μm | Tracheobronchial tree | Cleared by mucocialiary transport |
| 1-5 μm | Respiratory bronchioles and alveoli | Slow clearance |
| 0.5 μm | Deposit with difficulty and appear in mouth during exhalation | Particles remain airborne during inhalation due to low diffusiveness and settling velocity |
| < 0.5 μm | Alveoli | Exceedingly small particle mass |



It is thought that smaller particles have a peak value for deposition in the alveoli because smaller particles are typically more numerous and have larger surface areas (Salvi *et al.*, 1999).

The three major deposition mechanisms affecting inhaled PM are inertial impaction, sedimentation and diffusion (Martonen *et al.*, 2003). Deposition of inhaled PM in various regions within the respiratory system is an important aspect that determines the potential for the damage it can cause. The mechanism that clears particles in the respiratory system has several patterns. The particles deposited in the trachea and bronchioles rise on the mucociliary ladder, and are expelled by coughing or swallowing. Particles deposited beyond the terminal bronchioles are cleared largely by lung macrophages that, in turn, transport the ingested particles onto the mucociliary ladder or into the lymphatic system. A small fraction of these distally deposited particles migrate through alveolar tissue directly into the lymphatic circulation (Dockery *et al.*, 1994).

**1.4 Health effects of particulate matter**

Several studies have linked both PM10, and especially PM2.5, with significant health problems. PM2.5 is of specific concern because it contains a high proportion of various toxic metals and acids, and especially, it can penetrate deeper into the respiratory tract (Fierro, 2000).

Over the last ten years, several studies have linked PM concentration to increased death rates, and incidence of asthma, and adverse cardiac effects. In addition, PM from combustion sources contains polycyclic aromatic hydrocarbons (PAHs), which are reasonably anticipated to be carcinogens and elemental carbon linked to adverse cardiac effects (Kelly *et al.*, 2003).

Short term effects of PM are lung function changes, respiratory symptoms and mortality due to respiratory causes (Annesi-Maesano *et al.*, 2006). Studies of long-term health effects caused by PM have been conducted by many organizations, including the American Cancer Society, Harvard University, and the Health Effects Institute. These studies suggest that PM does affect long-term lung function, especially in the young and elderly, although this generally occurs only after very long periods (years) of exposure (US Army Center for Health Promotion and Preventive Medicine, 2002).

Pope *et al.* (2002) provide the strongest evidence that long-term exposure to fine PM, common to many metropolitan areas, is an important risk factor in cardiopulmonary mortality. Elevated exposure to fine PM exposures has been associated with all-cause, lung cancer and cardiopulmonary mortality. Each 10 $\mu g/m^3$ elevation was associated with approximately 4%, 6% and 8% of increased risk of all-cause, cardiopulmonary and lung cancer mortality, respectively. Measures of coarse fraction and total suspended particles were not consistently associated with mortality.

**1.5 Mechanism of DNA damage by particulate matter**

The DNA molecule is essentially a polynucleotide or polymer chain formed by phosphate diester groups joining β-D-deoxyribose sugars and phosphate through their 3' and 5' hydroxyl groups. The DNA model is a two-stranded helical structure, in which the two chains are held together by hydrogen bonds between the purine (A,G) and pyrimidine (T,C) bases (Figure 3) (Bansal, 2003).

The most important pathophysiological mechanism, proposed to explain the associations of PM exposure and health effect, is oxidative stress through the generation of reactive oxygen species (ROS), and formation of bulky DNA adducts by organic compounds such as PAHs (Risom *et al.*, 2005; Schins *et al.*, 2007; Valavanidis *et al.*, 2008).

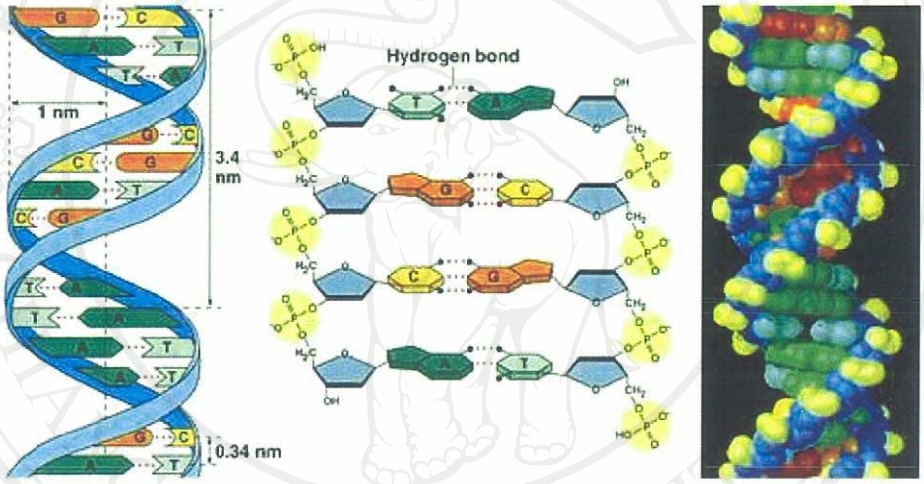


**Figure 3** The structure of DNA consists of base, sugar and the phosphate group. (http://academic.brooklyn.cuny.edu/.../16-05-doublehelix.jpg)

The oxidative stress mediated by PM may arise from a mixed source involving (Figure 4) (Risom *et al.*, 2005);

(i) direct generation of ROS from the surface area of particles,

(ii) soluble compound such as transition metals or organic compound,

(iii) altered function of mitochondria of NADPH-oxidase and

(iv) activation of inflammatory cells capable of generating ROS and reactive nitrogen species.

Direct particle generation of ROS can occur through the presence of free radicals and oxidants on the particle surface. The fine fraction has a significantly higher hydroxyl generation capacity than the coarse fraction (Shi *et al.*, 2006). Oxidative attack of DNA results from the surface area of the particle and may contain soluble transition metals such as iron, copper, chromium and vanadium that can generate ROS through Haber-Weiss reaction (Donaldson *et al.*, 1997). Ferrous iron ($Fe^{2+}$) reduces hydrogen peroxide ($H_2O_2$) with the formation of hydroxyl radical and oxidation of ferrous to ferric iron ($Fe^{3+}$). This reaction can be recycled by reductants such as superoxide anions, glutathione and ascorbic acid by reducing $Fe^{3+}$ to $Fe^{2+}$ (Risom *et al.*, 2005).

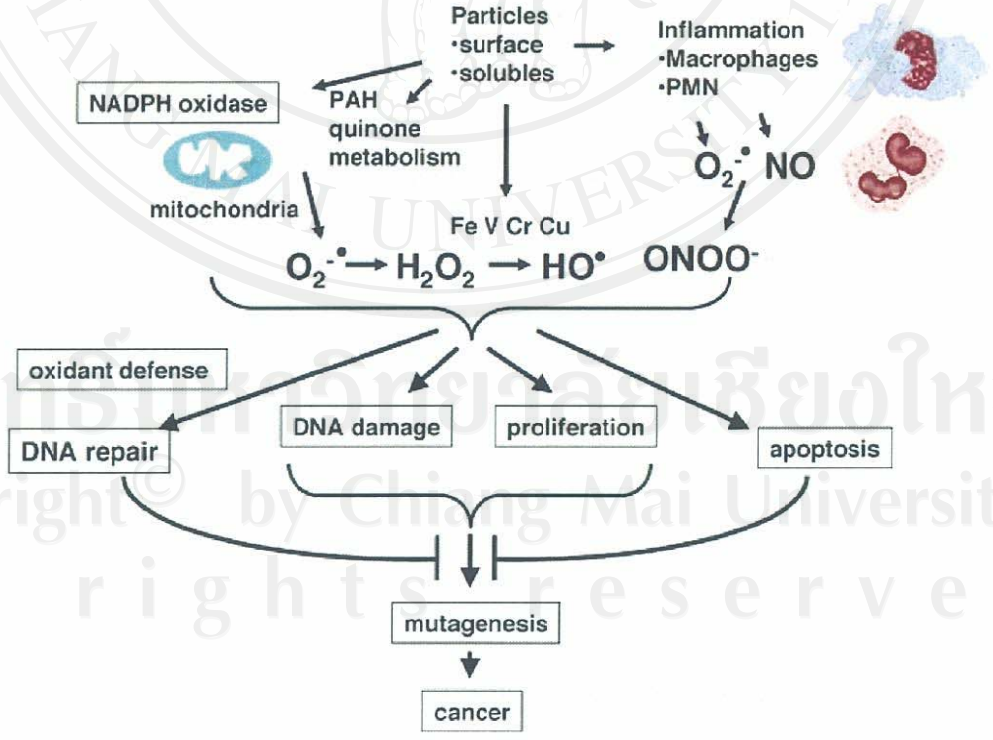


**Figure 4** Possible mechanisms for induction of oxidative stress and DNA damage by air pollution particles and their role in carcinogenesis (Risom *et al.*, 2005).

In addition, organic compounds associated with particles, such as PAHs, can be converted into redox-cycling semiquinone radicals upon metabolic activation (Squadrito *et al.*, 2001). The particle-elicited activation of the NAD(P)H-like enzyme system and particle-induced disturbance of mitochondria can generate intracellular ROS in target cells. Ultramicroscopic visualization of human macrophages and BEAS-2B cells incubated with organic diesel exhaust particle extracts showed that the appearance of apoptotic bodies were accompanied by change in mitochondrial morphology, including mitochondrial swelling and a loss of cristae (Li *et al.*, 2008).

Moreover, ROS is generated during particle-elicited inflammation. Neutrophils and alveolar macrophages play a key role in the defense reactions against foreign compounds and infectious agents in the lung. Alveolar macrophages are capable of phagocytosis of poorly soluble particles and they participate in the initiation of inflammatory responses in the lung. These are associated with excessive formation of ROS and reactive nitrogen species in the inflamed lung (Becker *et al.*, 2002).

The ability of organic compounds, such as PAHs, have led to DNA adduct formation that depends on desorption or leaching of these compounds from the particles in a biological environment. The ability of adsorbed PAHs and other constituents, which are released from the particle core, depends on the physicochemical properties of the particles (e.g. surface area, porosity), as well as the composition of organic fractions and the concentrations of its individual constituents. Meanwhile, particles and their chemical constituents may also elicit various other effects, for example via generation of ROS. This may lead to altered leaching and biotransformation of PAHs, as well as the induction of cell processes, including

proliferation apoptosis and possibly DNA adduct repair (Figure 5) (Schins *et al.*, 2007). The formation of bulky DNA adducts has been specially investigated *in vivo* and *in vitro*, in order to determine the significance of PAHs and related organic constituents in carcinogenesis. Soluble and insoluble PM caused by DNA adduct-forming PACs (polycyclic aromatic compounds) and oxidizing substances may contribute to the genotoxicity (Karlsson *et al.*, 2006; Sevastyanova *et al.*, 2008).

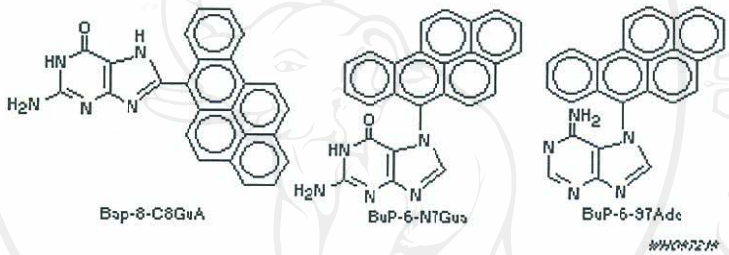


**Figure 5** Benzo(a)pyrene adduct is covalently linked to the C8 and N7 positions of guanine and the N7 position of adenine.
(http://www.inchem.org/documents/ehc/ehc/v202eh09.gif)

### 1.6 High volume cascade impactor

The high volume cascade impactor collects highly concentrated samples of PM for toxicological studies and the analysis of organic aerosols. It provides users with a selection of different size ranges of PM to be sampled. The collecting substrate is polyurethane foam (PUF), which has a large loading capacity that allows multi-day and even multi-week sampling. The system is made up of two major components: the sampler and high-capacity pump (Thermo Fisher Scientific Inc, 2008).

Each impaction stage is made of an acceleration and collection platform housed inside a modular shell. The stages are arranged in order from the largest to smallest diameter cut point to successively collect smaller particles as the sample stream passes through the sampler. In the acceleration platform, the sample stream flows through a circular nozzle that accelerates the air flow (Figure 6A). PM was sampled on the PUF, which had been placed on a collection platform, and it resided in the collection platform located beneath the acceleration platform (Figure 6B). The size of all the critical dimensions has been validated by developers of the ChemVol system, Harvard School of Public health (Rupprecht & Patashnick Co Inc, 2003).

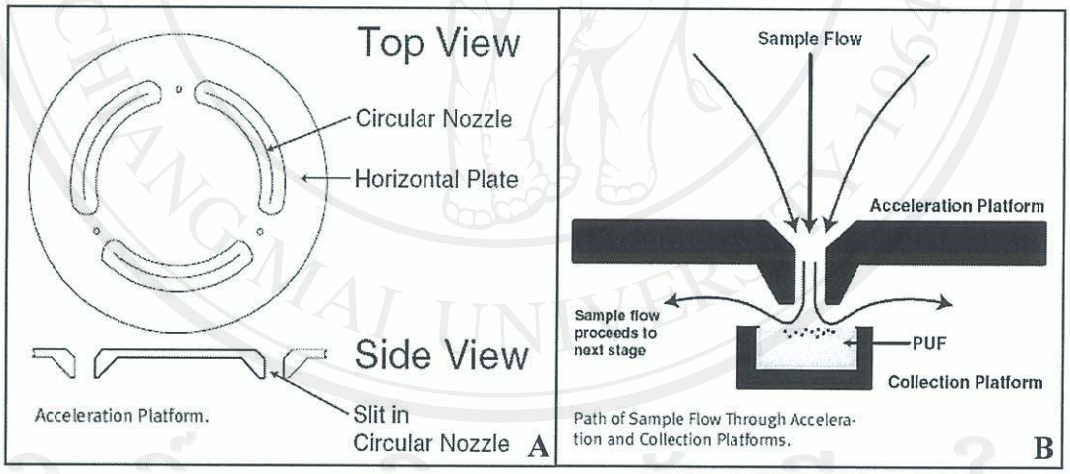


**Figure 6** A) The top and side views of the acceleration platform. B) The path taken by the sample stream as it passes through the acceleration platform and by the collection substrate (http://j.b5z.net/i/u/1640578/f/2400_brochure.pdf).



The PUF was used as an impaction substrate. This is a polymeric material with stable physical characteristics, low chemical background (when cleaned properly), and high collection efficiency characteristics. Another advantage is its ability to

collect large amounts of particles per surface area (Kavouras *et al.*, 2000). A previous study reported that the use of PUF substrates improves the performance of inertial impactors by minimizing bounce-off and re-entrainment losses, as compared to coated and uncoated filter flat plate substrates (Kavouras *et al.*, 2001). This increases the sensitivity of toxicological and chemical characterization studies. Moreover, the thickness of the PUF for all stages ensures that particles do not penetrate through the PUF and reach the substrate holder (Salonen *et al.*, 2000).

**1.7 Genotoxicity test**

In toxicology, the term "genotoxicity" usually refers to any type of damage to genetic material, the genome, just as cytotoxicity designates injury to the cell. The differentiated genome of the somatic cells, which constitute an organism, provides information for the synthesis of most cellular components and, hence, the maintenance of cellular integrity (Williams, 1989). Genotoxicity tests are an important part of cancer research and risk assessment of potential carcinogens. A wide variety of genotoxicity assays has been developed to identify gene mutations, chromosomal mutations or aneugenic effects and a broad spectrum of chemicals. The genotoxicity tests that have been applied in particle research (Schins, 2002) can be subdivided into;

(i) acellular system,

(ii) *in vitro* tests using bacterial strains, freshly isolated cells or cell lines,

(iii) *in vivo* studies usually in mice or rats, and

(iv) biomarker studies in humans with exposure to particles.

*In vitro* studies have been used with several particles and in a variety of tests. These studies have yielded relevant information in relation to the mechanism used with these particles. Examples of the tests used as follows: alkaline unwinding, alkaline single cell gel electrophoresis or comet assay, chromosome aberration test, micronucleus test and 8-hydroxy-2-deoxyguanosine (8-OHdG) formation. Cells or cell lines that have been used include lymphocytes, murine macrophage and epithelial cell lines (Schins, 2002).

**1.8 Single cell gel electrophoresis**

The evaluation of DNA damage by comet assay was developed to a standard method of genotoxicity test from the International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC, on March 25-26, 1999. The advantage of the technique includes its demonstrated sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, short time needed to complete a study and ease of application (Tice *et al.*, 2000).

The concept of microgel electrophoresis was first introduced by Ostling and Johanson as a method to measure DNA single-strand breaks that caused relaxation of DNA supercoils (Ostling *et al.*, 1984). In 1990, a modification of Ostling and Johanson's original method was introduced and named the "comet assay" after the appearance of the DNA from individual cells. The comet head containing the high-molecular-weight DNA and the comet tail containing the leading ends of migrating fragments were measured in real time from digitized images using specific software. Tail moment, a measure of both amount of DNA in the tail and distribution of DNA

in the tail, became a common descriptor along with tail length and percentage of DNA in the tail (Olive *et al.*, 1990.).

The principle of the method is based on the migration of DNA damage through an electrophoretic field. First, individual cells or nuclei are embedded on a microscope slide, which is coated with agarose gel, and the cells are lysed to expose their DNA. Afterthat, DNA is treated with alkaline solution to denature and relax the double strand to single strand, and electrophoresed to separate the DNA strand. The slide is visualized by ethidium bromide or fluorescent dyes. Damaged DNA containing strand breaks migrates farther in the gel than intact DNA, creating an image resembling a celestial comet. The length and fragment content of the tail is directly proportional to the amount of DNA damage (Figure 7) (Tice *et al.*, 2000).

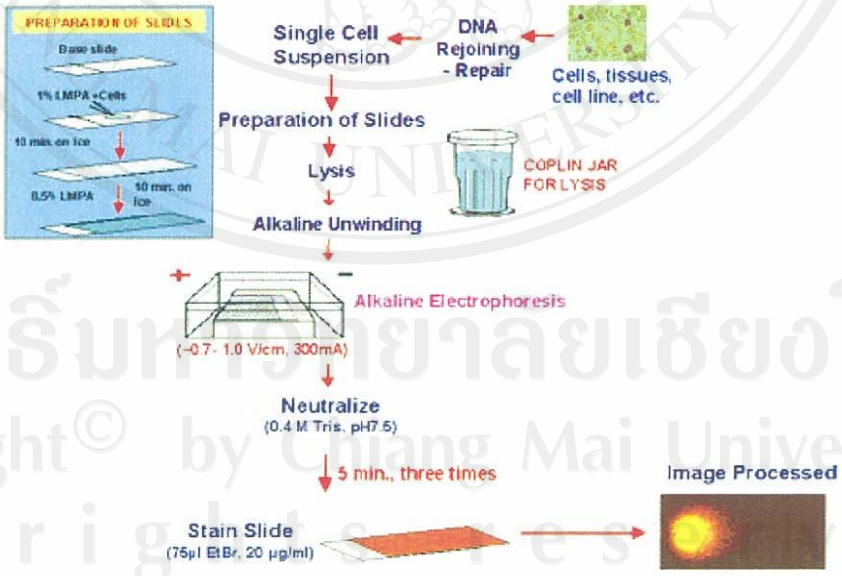


**Figure 7** General protocol of the comet assay.

(Adapted from www.cometassayindia.org/2.gif)

Depending on pH conditions for lysis and eletrophoresis, the sensitivity of the technique can change. Employing neutral conditions for both variables allows detection of DNA double strand breaks, but pH 12.3 detects single strand breaks and delays DNA repair sites, while at pH13 the sensitivity allows evaluation of alkali labile sites, single strand breaks and delay repair sites of DNA (Rojas *et al.*, 1999).

**1.9 Detection of 8-OHdG**

Regarding the ROS, the highly reactive hydroxyl radical (°OH) reacts with DNA by adding to double bonds of DNA bases. Hydroxyl radical can add to C4, C5 and C8 positions of purine bases generating °OH adduct radicals. The hydroxyl radical added to the C8 position of the guanine base is namely, 8-OHdG (Figure8).

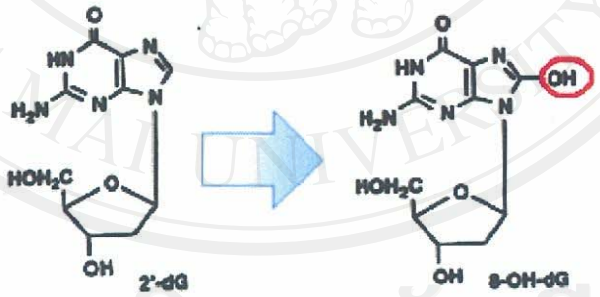


**Figure 8** The oxidaition of 2'dG by hydroxyl radical to form the 8-OHdG.

(http://www.abcysonline.com/Images/Oxidative_a.jpg)



The 8-OHdG is the product most frequently measured as an indicator of oxidative DNA damage. In the *In vitro* DNA synthesis assay, 8-OHdG has been demonstrated as a potential mutagen, due to misreading (producing a point mutation) of 8-OHdG during DNA replication. It has been shown that oxidized guanine bases in

replicating DNA lead to G-T and A-C transversion in an *in vitro* model and mammalian cell. The 8-OHdG has led to its proposed potential as an intermediated marker of a disease endpoint such as lung cancer. Support of this proposal is the finding that GC-TA transversion potentially derives from 8-OHdG, and has been observed *in vivo* in the *ras* oncogene and p53 tumor suppressor gene in lung cancer (Cooke *et al.*, 2003). Analytical approaches for 8-OHdG quantitaion (Peoples *et al.*, 2005) include the following;

(i) $^{32}P$ and fluorescent method of labeling nucleotides,

(ii) separation techniques such as high performance liquid chromatography, gas chromatography and capillary electrophoresis, and

(iii) immunoassay.

Key objectives have focused on achieving sensitive detection levels and improving sample preparation procedures. The normal levels of 8-OHdG in biologival materials (low nanomolar) have nescessitated the use of capable detectors such as laser-induced fluorescence, electrochemical and mass spectrometry (Peoples *et al.*, 2005).

Enzyme immunoassay (EIA) has become a household name for medical laboratories and manufacture's of *in vitro* diagnostic products. Immunoassay is generally termed as an assay that employs antibodies to detect and quantify antigens. The main component of immunoassay is the antibody that specifically binds a target molecule. The antibodies used in immunoassay belong to the immunoglobulin gamma (IgG), especially the IgG monoclonal antibody derived from hybridoma cells (Plaza *et al.*, 2000).

Although EIA offers simplicity, all immunoassay-based methods are limited by the selectivity of the antibody. It has been shown that the sample matrix may contain interferences capable of competing for antibody recognition (Peoples *et al.*, 2005). The EIA system consisting of the antibody was immobilized on a solid surface, such as on the internal walls of the wells in the microtiter plate. The level of antibody present is limiting, and labeled and unlabelled antigen compete with each other for binding. The labelled antigen (antigen-enzyme conjugate) is retained by the immobilized antibody. After reaching equilibrium (antibody-antigen binding), unbound antigen is removed by a washing step. The amount of enzyme-labelled antigens retained is detected by enzymatic activity (Plaza *et al.*, 2000).

The 8-OHdG EIA kit (Stressmarq Biosciences Inc., Canada) is a competitive assay. It is based on the competition between 8-OHdG and a 8-OHdG-acetylcholinesterase conjugate (8-OHdG tracer) for a limited amount of 8-OHdG monoclonal antibody. The concentration of the 8-OHdG tracer, which is able to bind to the 8-OHdG monoclonal antibody, is inversely proportional to the concentration of 8-OHdG in the well. This antibody 8-OHdG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to acetylcholine, is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-OHdG tracer bound to the well, which is inversly porportional to the amount of free 8-OHdG present in the well. A schematic of this process is shown in Figure 9.

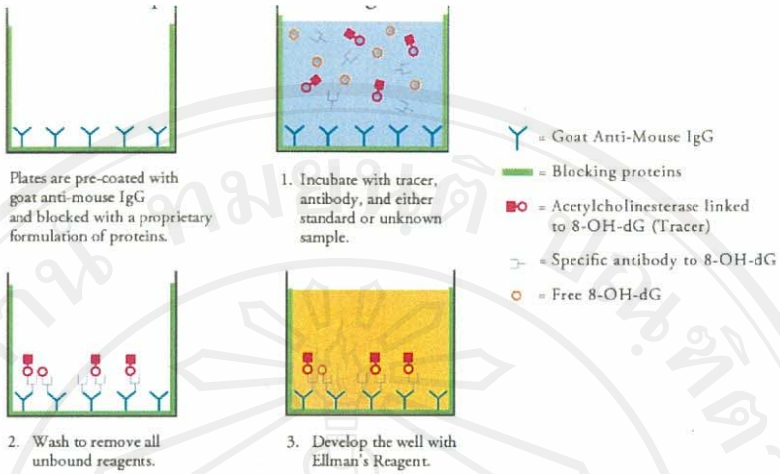


**Figure 9** Schematic of the process for 8-OHdG quantitaion using the 8-OHdG EIA kit.

### 1.10 Statement and significance of the problem

Chiang Mai is one of the fastest growing cities in Thailand. Growing urbanization, traffic volume, and commercial and industrial activities have resulted in an increased concentration of airborne particulate matter and other gaseous pollutants (Tippayawong *et al.*, 2006). The PCD has announced that since 1977 Chiang Mai has been the fourth ranked province after Samut Prakarn, Saraburi and Lampang for PM levels exceeding the acceptable level of 120 $\mu g/m^3$ per 24 hours for 176 days (PCD, 2009).

The study of air pollution at Warorod and Hang Dong markets during 2002-2003, found levels of TSPs in the range of 229 – 433 $\mu g/m^3$ and 322 – 470 $\mu g/m^3$, respectively, during the day, and 136 – 357 $\mu g/m^3$ and 160 – 360 $\mu g/m^3$, respectively, at night. These PM showed the genotoxic effect on lymphocyte cells and induced abnormal pulmonary values of inhabitants who lived at the Warorod site (Mokmeud,

2004). Organic extract of PM2.5 collected from the urban area of Chiang Mai showed direct mutagenicity in the *Salmonella typhimurium* strains, TA98 and TA100, as well as indoor PM2.5 (Chunram *et al.*, 2007).

Sopajaree *et al.* (2007) found that PM10 and PM2.5 levels, which were collected at Saraphi district during 2005-2006, had higher levels than in other sites in the Chiang Mai-Lamphun valley. Particulate matter at Saraphi district consisted mostly of PAHs, which might associate with high numbers of lung cancer patients among Saraphi inhabitants. Moreover, the extracted PM had genotoxicity to the alveolar type II cells, alveolar macrophage and bronchial epithelial cells. During an air pollution crisis in 2007, the PCD monitored PM10 at Yupparaj Witthayalai School station and found that its level was higher than the acceptable level of the US.EPA by approximately three times (maximum level was 382.70 $\mu g/m^3$). This climate crisis affected the visibility and health of inhabitants who lived in this area.

The incidence rate of lung cancer in Chiang Mai seems to have risen continuously since the year 2000. Saraphi district has been reported to have a high incidence of lung cancer patients (Vatanasapt *et al.*, 2002), which may be associated with toxic agents in the ambient air, especially increases of PM10 and PM2.5.

Many studies have suggested a link between the concentration of particulate matter and genotoxic effect. However, the genotoxic effect on human lung epithelial cells of fine PM below 2.5 microns in Chiang Mai has not been studied or reported.

### 1.11 Objectives of the study

The aims of this study were to investigate the genotoxicity of fine particulate matter of 2.5-0.5 microns collected at Saraphi district on an induction of DNA damage and hydroxyl radical formation in A549 human lung epithelial cells. The results of this study might be helpful in clarifying a mechanism of fine particulate matter of 2.5-0.5 microns, which causes DNA damage and may associate with the progression of lung cancer in local inhabitants.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Apparatus and chemicals

### 2.1.1 Apparatus

1) High volume cascade impactor, Rupprecht & Patashnick Co., Inc., U.S.A.

2) Ultrasonic bath, Branson Cleaning Eqiupment Company, U.S.A.

3) Analytical balance, Model AG285 (five digit), Mettler Toledo, U.S.A. and Adventuer OHAUS (four digit), U.S.A.

4) Rotavapor RE120, BUCHI, Switzerland

5) Lyophilizer, Christ, Germany

6) Class II bioharzard safety cabinet, Model LA2-4A1, ESCO, Singapore and Nuaire, U.S.A.

7) $CO_2$ incubator, Shel-lab, U.S.A.

8) Inverted microscope, Nikon TMS, Japan

9) Hemacytometer, Boeco, Germany

10) Refrigerated centrifuge, Model 5920, Kubota, Japan and microcentrifuge, Model 4214, ALC, U.S.A.

11) Water bath 1245PC, Shel-lab, U.S.A.

12) Hot plate, Torrey Pines Scientific, U.S.A.

13) Electrophoresis set EPS301, Amersham Biosciences, Sweden

14) Fluorescent microscope, Carl Zeiss, Germany

15) Slide and coverglasses, D.A.T. Scientific Co.Ltd., Thailand

16) DNA biophotometer, Eppendrof, Germany

17) Pipette aid, Drummond, U.S.A. and autopipette, Socorex, Switzerland

18) Culture flask, Corning Incorporated, U.S.A.

19) 96, 24 Well plate, Corning Incorporated, U.S.A.

**2.1.2 Chemicals**

The A549 human lung epithelial cell line was purchased from American Type Culture Collection, Manassas, VA, U.S.A.. All reagents for cell culture were purchased from Gibco, U.S.A. (tissue culture grade). The enzyme immunoassay kit for 8-hydroxy-2′-deoxyguanosine quantitation was purchased from StressMarq Bioscience Inc., Canada. Other chemicals and reagents were analytical grade. The details of reagents and preparation are in Appendix A and B.

**2.2 Particulate matter collection**

A high volume cascade impactor was used for particulate matter collection. The collection substrate was a polyurethane foam (PUF) material, which had a high sampling capacity and was easily extracted with methanol and/or water (Halinen *et al.*, 2005). The PUF was used to collect PM≥10, 10-2.5, 2.5-1.0, 1.0-0.5 and ≤0.5 microns separately. The machine was operated for 48 hours and stopped for 24 hours throughout the four months of August, September, October and November, 2007. The machine was set up in front of Saraphi Hospital, Saraphi district, Chiang Mai province, Thailand, due to this area having a high incidence rate of lung cancer (Vatanasapt *et al.*, 2002). The machine was set up in accordance with the environmental engineering guideline, in which the machine had to be more than 3 meters away from the building (Figure 1). The machine was operated at a flow rate of

760 ± 40 liters per minute, which was checked before and at the end of each collection day to ensure a constant flow rate throughout the sampling period.

**Figure 10** A ChemVol®Model 4200 high volume cascade impactor, set up in front of Saraphi Hospital, Chiang Mai, to collect particulate matter for four months.

**2.3 Particulate matter quantitation**

The PM quantitation was performed according to the guidance of the ChemVol® Model 2400 High Volume Cascade Impactor. Before collection, the PUF was cleaned by sonication in ultrapure water at room temperature for 60 minutes, then dried in a clean-air hood for 24 hours and finally wrapped with aluminum foil to protect against light. The PUF was kept in a desiccators at 25 ± 3°C and 50 ± 5% humidity before weighing. It was weighed in an analytical balance, placed in a controlled cabinet at 25 ± 3°C and 50 ± 5% humidity. The PUF was placed at the center of the balance five times and calculated for average weight, before putting into the high volume cascade

impactor ready for PM collection. The PM concentration was calculated using the following formula:

$$C\ (\mu g/m^3) = \frac{Wf \times 10^6}{SLPM \times 1.44}$$

Where; C = PM concentration ($\mu g/m^3$)

Wf = the difference of the PUF weight before and after sample collection (g)

SLPM = standard flow through the sampler (L/min)

1.44 = conversion factor of L/min to $m^3$/day.

The SLPM was calculated using the flowmeter calculation system. Four parameters; ambient temperature, absolute pressure, pressure of the atmosphere and pressure across the orifice, were calculated. Ambient temperature and absolute pressure were provided by the Thai Meteorological Department. The pressure of the atmosphere and pressure across the orifice were recorded before and at the end of each collection day, as the example shown below:



Flowmeter Calculation System

| | | |
|---|---|---|
| Input Ambient Temperature: | 28.3 | Celsius |
| Input Absolute Ambient Pressure: | 29.75 | Inches Hg |
| Input the □P w.r.t the atmosphere: | 90 | Inches of $H_2O$ |
| □ P across the orifice: | 5.00 | Inches of $H_2O$ |
| Raw Flow = | 636.3 | lpm |
| Temperature correction factor= | 1.034 | |
| Pressure correction factor= | 1.110 | |
| Standard flow through the sampler= | 730.2 | SLPM |
| Actual flow through the sampler= | 766.3 | ALPM |

**2.4 Particulate matter extraction**

Fine PM was removed from the PUF according to the EPA-USA guideline (U.S.EPA, 1999). The extract was suitable for analysis of the water-soluble components. The PUF was cut into 24 pieces using stainless-steel scissors and put into an Erlenmeyer flask. Then, 10 ml of methanol was added into the flask, which was then shaken gently to wet the PUF pieces evenly with methanol. One hundred and ninety milliliters of ultrapure water were added and sonicated for 60 minutes at a temperature below 30°C. The solution was then filtered through a Whatman filter No. 4. The filtrate was evaporated for 15 minutes at 60°C in order to remove the methanol, and then lyophilized for 48 hours. The residue was dissolved in ultrapure water and stored as a stock solution at the concentration of 20 mg/ml in the dark before use in the genotoxicity assay.

**2.5 Human lung epithelial cell preparation**

The A549 Human lung epithelial cell line was cultured, following a method modified from that of Tice *et al.* (2000) and Shi *et al.* (2006). A monolayer in Minimum Essential Medium Eagle (MEM) was supplemented with 5% heat-inactivated fetal calf serum, sodium pyruvate and 50 μg/ml of gentamycin at 37°C, 5% $CO_2$ in a humidified atmosphere. The cells were split every 3-5 days by trypsinization and refreshed with new medium one day before the experiments. To trypsinize the cells, the culture medium was discarded and 1 ml of 0.005% trypsin-EDTA solution was added to the culture flask and incubated at 37°C, 5% $CO_2$, for 5 minutes. The flask was tapped to detach the adherent cells to single cell suspension. For the experiments, the cells were trypsinized and harvested in a 15 ml Falcon tube

and washed with HBSS (Hank's Buffered Salt Solution) by centrifugation at 1,200 rpm and 4°C for 5 minutes. The pellet was resuspended in MEM medium, counted and adjusted to 2 x $10^6$ cells/ml, before the cells were treated with extracted fine PM. For the comet assay, methylmethane sulfonate (MMS) was used as a positive control. For solvent control, the extract of a clean PUF with all solvents was in the experiment. Aphidicolin (APC), a DNA repair inhibitor, was used to investigate the repair mechanism of the DNA in the A549 cells. Concentrations of APC at 1.0 and 2.0 μg/ml were selected to treat the cells according to the previous experiments of Speit *et al.* (2004).

**2.6 DNA strand break analysis**

DNA strand break analysis in the A549 cells was determined by the alkaline comet assay, which was modified from the methods of Tice *et al.* (2000), Brits *et al.* (2004), Speit *et al.* (2004), Karlsson *et al.* (2006) and Shi *et al.* (2006). The cells were embedded in agarose placed on a microscope slide and lysed by lysis solution. Then the liberated DNA was electrophoresed under alkaline conditions. Cells with an increased frequency of DNA strand break would display an increased migration of DNA toward the anode. The migrating DNA was quantitated by staining with ethidium bromide and the intensity measured by fluorescence microscope.

**2.6.1. Agarose - slide preparation**

Microscope slides were precoated with 1% normal melting point agarose (NMPA) and stored at 4°C for 10 minutes. After the cells were treated with fine PM and incubated at 37°C, for 3 hours in 5%$CO_2$, they were washed with HBSS by centrifugation at 1,200 rpm and 4°C for 5 minutes, and resuspended in MEM

medium. The cells viability was evaluated using the trypan blue dye exclusion method. Ten microliters of cell suspension (approximately 2 x $10^6$ cells/ml) were mixed with 80 µl of 1% low melting point agarose and added to the slide for the second layer. The slide was stored at 4°C for 10 minutes. Subsequently, the slide was immersed in the lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% TritonX, 10% DMSO; pH 10.0) at 4°C for 1 hour.

**2.6.2. Alkaline electrophoresis**

The slides were immersed in electrophoresis buffer (1 mM EDTA, 300 mM NaOH; pH>13.0) at 4°C for 20 minutes and placed in an electrophoresis chamber (17V/cm, 300 mA) filled with new fresh electrophosis buffer for 25 minutes and washed three times with neutralization buffer (0.4M Tris pH7.4). All steps had to be performed in a dark room to prevent additional DNA damage.

**2.6.3. Evaluation of DNA damage**

The slides were stained with 20 µl of ethidium bromide solution. The comet phenomenon was observed under 20X objective on a fluorescence microscope connected to the computerized image analysis system, Metasystem Comet Imager 1.2, as shown in Figure 11. Generally, fifty randomly selected cells were scored on each slide (two slides per sample). The comet parameters, tail moment and tail length, were calculated to demonstrate the genotoxic effect of the PM.



$$\text{Tail moment} = \frac{\text{Tail length x Tail intensity}}{100}$$

Tail length = the length of the comet tail

Tail intensity = the intensity of the comet tail

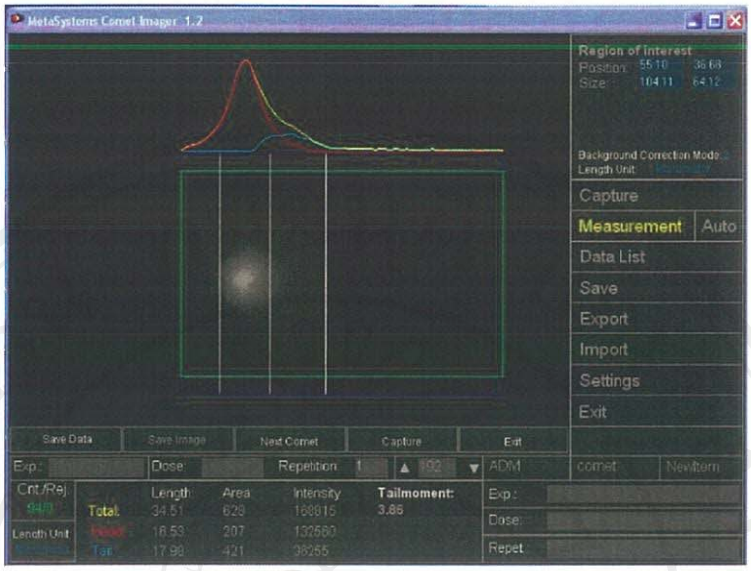


**Figure 11** Metasystem Comet Imager 1.2 program showing the calculated tail moment of A549 cells treated with methylmethane sulfonate concentration of 80 μM.

## 2.7 Quantitation of 8-OHdG

### 2.7.1 DNA extraction

The DNA was isolated using the Flexigene DNA kit (QIAGEN Inc.), which provided good yield of high-purity DNA. After the cells were treated with fine PM and incubated at 37°C for 3 hours in 5%$CO_2$, they were washed with HBSS by centrifugation at 1,200 rpm and 4°C for 5 minutes. Three hundred microliters of the buffer FG1 were added to the cell pellet and mixed by pipetting until the cells were resuspended, then 300 μl of the buffer FG2/QIAGEN protease were added and the tube was inverted three times before placing in an incubator at 65°C for 10 minutes. Six hundred microliters of Isopropanol were added and mixed thoroughly by inversion until the DNA precipitate became visible as threads or a clump. The tube was centrifuged at 16,400 rpm for 3 minutes and the supernatant was discarded before adding 600 μl of 70% ethanol and centrifuging at 16,400 rpm for 3 minutes. The

supernatant was discarded and the tube inverted onto a clean piece of absorbent paper for at least 5 minutes. The DNA pellet was dried under room temperature until all the liquid had evaporated. Finally, 200 µl of the buffer FG3 was added and mixed for 5 seconds before incubating at 65°C for 30 minutes.

DNA concentration was measured by spectrophotometer at the absorbance of 260 nm. To be accurate, the absorbance reading at 260 nm had to fall between 0.1 to 1.0.

**2.7.2 Quantitaion of 8-OHdG**

The amount of 8-OHdG in the A549 cells after treating the PM on the cell culture was quantitated using the 8-OHdG EIA kit (StressMarq Biosciences Inc.). This assay was based on the competition between 8-OHdG and 8-OHdG-acetylcholinesterase tracer conjugate (8-OHdG-AchE tracer) for a limited amount of 8-OHdG monoclonal antibody. The product of this enzymatic reaction had a distinct yellow color and absorbed strongly at 412 nm. The intensity of this color was proportional to the amount of 8-OHdG-AchE tracer bound to the well, which was inversely proportional to the amount of free 8-OHdG in the sample.

Double strand DNA of the A549 cells were converted to single strands by incubating the sample at 95°C for 10 minutes and rapidly chilling it on ice. The DNA was digested using 5 units of S1 nuclease, and incubated at 37°C for 2 hours. One unit of the calf intestinal alkaline phosphatase was added to the DNA, incubated at 37°C for 1 hour, boiled for 10 minutes and placed on ice until use.

One hundred microliters of the enzyme immunoassay buffer were added to the non-specific binding (NSB) well and 50 µl to the maximum binding (B0) well. Then, 8-OHdG standard was added to the standard wells at concentrations of 10.3, 23.1,

52.0, 117.1, 263.4, 592.6, 1,333 and 3,000 pg/ml for the standard curve. Fifty microliters of the DNA were added to the sample wells and assayed in triplicate. Fifty microliters of the 8-OHdG-AchE tracer were added to each well, except the total activity (TA) and blank (Blk) wells. Then, the 8-OHdG monoclonal antibody was added at 50 µl to each well, except the total activity (TA), non-specific binding (NSB) and blank (Blk) wells. The plate was incubated at 4°C for 18 hours before rinsing five times with washing buffer, and adding Ellman's reagent at 200 µl into each well to develop color reaction. Optimum developed reaction was obtained using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark for 90 minutes. This color reaction was measured by spectrophotometer at the absorbance of 412 nm. The amount of 8-OHdG in the A549 cells were calculated using the 8-OHdG standard curve.

**2.8 Statistical analysis**

Mann-Whitney U Test statistics were used to compare the genotoxicity of the experimental groups. One-way ANOVA and Independent-Samples T test were also used to compare 8-OHdG quantitation.

# CHAPTER III

# RESULTS

## 3.1 Particulate matter concentrations

PM concentrations obtained from August to November, 2007, at Saraphi Hospital, Saraphi district, Chiang Mai province, Thailand are shown in Table 5 and Figure 12. They revealed that PM10-2.5 was the highest concentration of PM when compared to PM≥10, PM2.5-1.0, PM1.0-0.5 and PM≤0.5, consecutively. Concentrations of the PM obtained in each 48 hour period varied from 2.46 to 33.64 $\mu g/m^3$. Figure 13 shows the percentage of each total PM concentration at 33.44% of PM10-2.5, 20.30% of PM≥10, 20.06% of PM2.5-1.0, 16.24% of PM1.0-0.5 and 9.96% of PM≤0.5.

## 3.2 DNA strand break analysis

Tail moment, the parameter of DNA damage, was calculated by the Metasystem Comet Imager 1.2 program. Undamaged and damaged DNA of the cells showing tailing of the DNA strand break (resembling a comet) after treatment with fine PM are shown in Appendix C. The tailing was directly proportionate to the severity of the DNA damage. The cytotoxicity test (trypan blue exclusion) showed cell viability of up to 95%. The genotoxicity of PM10-2.5 at concentrations of 75, 150, 300, 600, 1,200 and 2,400 μg/ml is shown in Table 6 and Figure 14. The DNA damage on the cells treated with that PM was dose-dependent. The tail moment of the cells treated with PM10-2.5 at all concentrations was significantly greater than that in the solvent

**Table 5** Details of PM concentrations ($\mu g/m^3$) collected from August to November, 2007, at Saraphi Hospital, Chiang Mai.

| dd/mm/yy | Particulate matter concentrations ($\mu g/m^3$)* | | | | |
|---|---|---|---|---|---|
| | PM≥10 | PM10-2.5 | PM2.5-1.0 | PM1.0-0.5 | PM≤0.5 |
| 20/08/07 – 21/08/07 | 2.46 | 10.39 | 6.02 | 5.54 | 4.06 |
| 23/08/07 – 25/08/07 | 14.64 | 19.32 | 9.44 | 7.12 | 3.91 |
| 27/08/07 – 29/08/07 | 19.02 | 15.83 | 12.95 | 10.95 | 7.79 |
| 31/08/07 – 02/09/07 | 5.29 | 15.08 | 6.96 | 5.83 | 3.74 |
| 04/09/07 – 06/09/07 | 15.22 | 11.01 | 3.51 | 4.61 | 3.02 |
| 08/09/07 – 10/09/07 | 18.87 | 28.76 | 20.46 | 6.60 | 10.08 |
| 12/09/07 – 14/09/07 | 12.99 | 24.62 | 9.50 | 11.88 | 6.04 |
| 16/09/07 – 18/09/07 | 16.91 | 27.65 | 11.24 | 12.22 | 6.73 |
| 20/09/07 – 22/09/07 | 18.21 | 34.25 | 22.23 | 17.54 | 11.39 |
| 24/09/07 – 26/09/07 | 17.32 | 32.97 | 26.28 | 22.19 | 15.10 |
| 28/09/07 – 30/09/07 | 6.21 | 18.12 | 6.80 | 7.11 | 5.20 |
| 02/10/07 – 04/10/07 | 24.59 | 33.64 | 23.57 | 17.05 | 10.60 |
| 06/10/07 – 08/10/07 | 16.07 | 30.82 | 10.34 | 9.58 | 6.34 |
| 10/10/07 – 12/10/07 | 16.46 | 21.30 | 12.81 | 10.02 | 5.82 |
| 14/10/07 – 16/10/07 | 17.50 | 25.83 | 14.66 | 11.11 | 5.53 |
| 18/10/07 – 20/10/07 | 15.28 | 32.22 | 23.00 | 19.53 | 9.47 |
| 22/10/07 – 24/10/07 | 17.14 | 32.49 | 30.73 | 26.23 | 12.86 |
| 30/10/07 – 01/11/07 | 17.29 | 31.71 | 26.16 | 17.80 | 8.43 |
| 03/11/07 – 05/11/07 | 11.96 | 20.76 | 3.43 | 3.82 | 2.92 |
| Total ($\mu g/m^3$) | 283.43 | 466.77 | 280.09 | 226.73 | 139.03 |

* Ambient temperature = 26.21 ± 1.51°C

Rainfall = 5.29 ± 8.87 mm

Relative humidity = 85.32 ± 5.05%

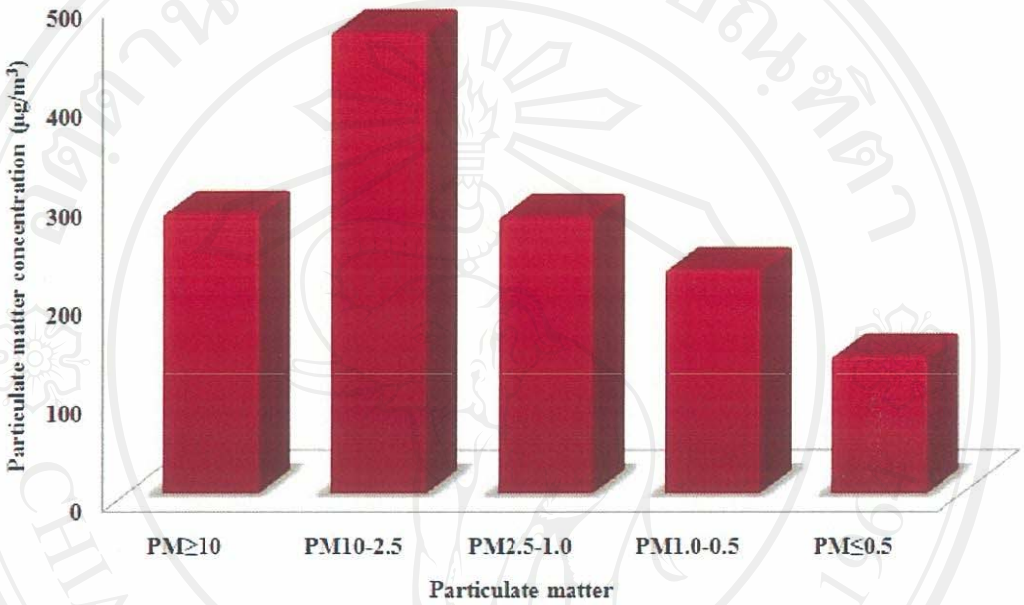


**Figure 12** Total PM concentrations of PM10-0.5 collected for 37 days during August to November, 2007, using a high volume cascade impactor, operated for 48 hours and stopped for 24 hours throughout the four months.

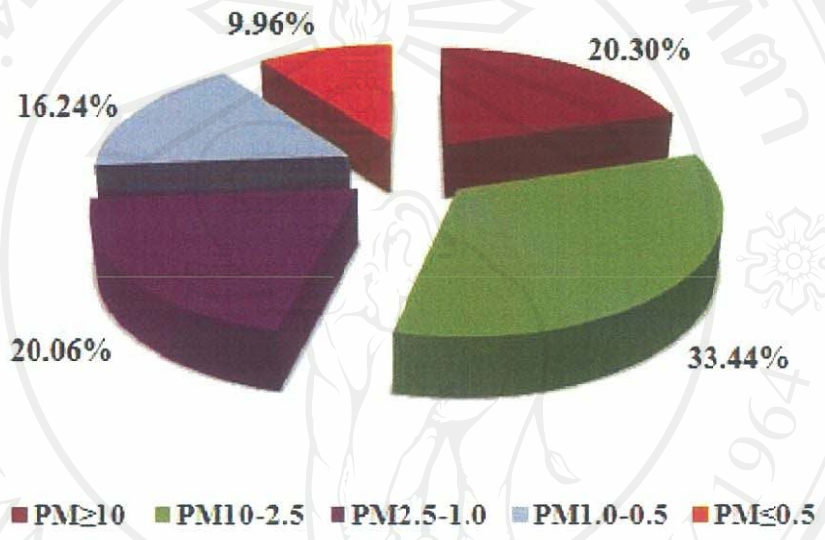


**Figure 13** Percentage of each total PM concentration, collected from August to November, 2007, showing the highest and lowest total concentration of PM10-2.5 and PM0.5, respectively, by high volume cascade impactor.

**Table 6** Average tail moment ± standard error of mean of treated A549 human lung epithelial cells with PM10-2.5 at concentrations between 75 – 2,400 µg/ml

| Concentrations (µg/ml) | n | Tail moment (µm) (mean ± S.E.M.) |
|---|---|---|
| 75 | 100 | 6.78 ± 1.52 |
| 150 | 100 | 16.05 ± 2.37* |
| 300 | 100 | 17.73 ± 2.23* |
| 600 | 100 | 23.23 ± 2.28*** |
| 1,200 | 100 | 25.12 ± 2.06*** |
| 2,400 | 100 | 26.53 ± 2.10*** |
| Solvent control [a] | 100 | 6.76 ± 1.03 |
| Positive control [b] | 100 | 28.81 ± 1.08 |

[a] Experiments without PM10-2.5.

[b] Experiments with 80µM methylmethane sulfonate.

*$p \leq 0.05$, *** $p \leq 0.001$ compared to the solvent control.

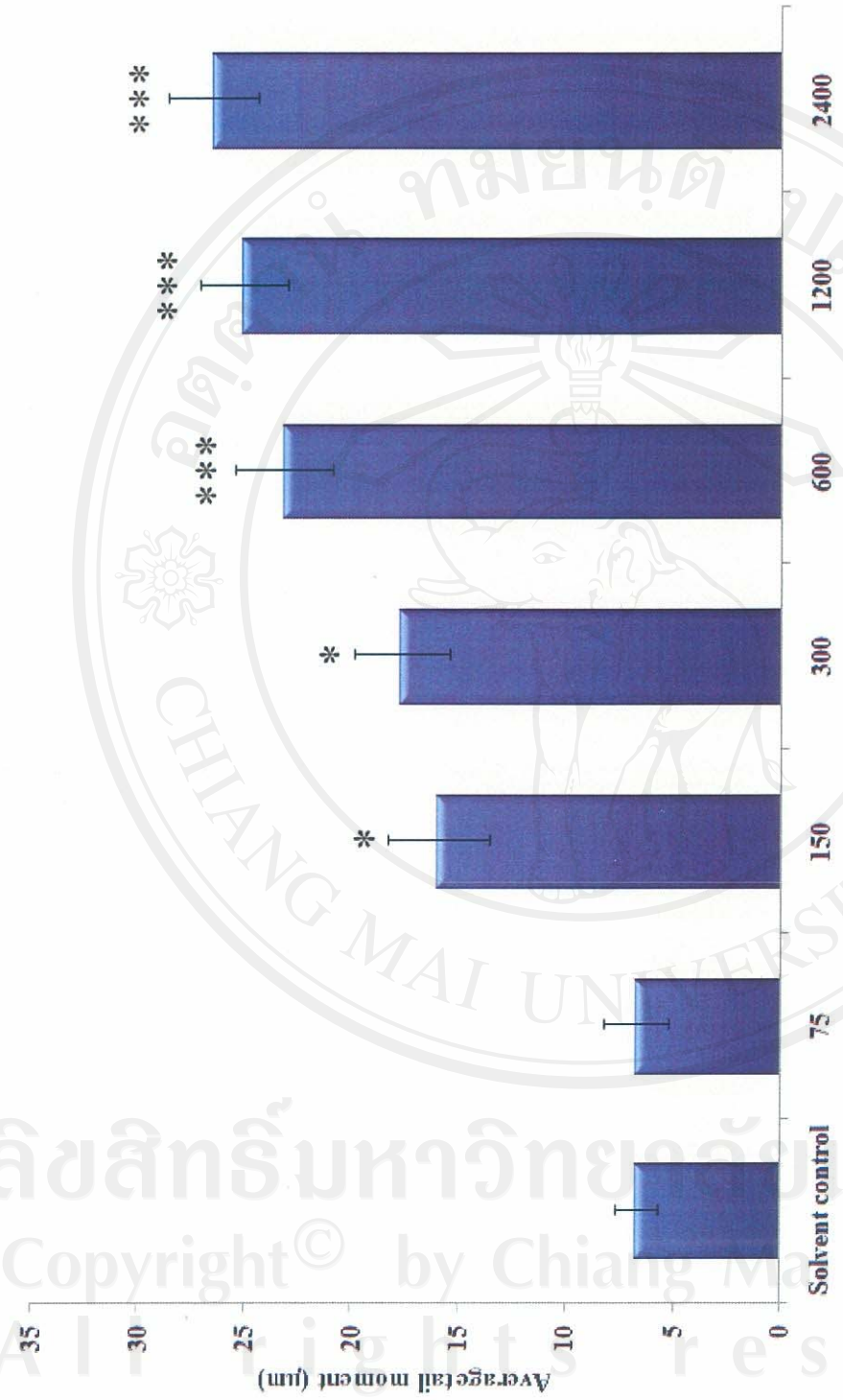


**Figure 14** Mean ± S.E.M. of the tail moment of A549 human lung epithelial cells treated with the PM10-2.5.

*$p \leq 0.05$, ***$p \leq 0.001$ compared to the solvent control.

control, except at the concentration of 75 μg/ml. The severity of the DNA damage at concentrations of 600, 1,200 and 2,400 μg/ml was greater than the damage caused by PM10-2.5 at concentrations of 150 and 300 μg/ml. Therefore, a concentration of 600 μg/ml was chosen for the next experiment.

Genotoxicity of PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations of 75, 150, 300 and 600 μg/ml is shown in Table 7 and Figure 15. The DNA damage on the cells treated with those PM was also dose-dependent. The tail moment of the cells treated with PM2.5-1.0, PM1.0-0.5 and PM≤0.5, at all concentrations, was significantly greater than that in the solvent control, except for PM2.5-1.0 at the concentration of 75 μg/ml. Therefore, 150 μg/ml was the lowest concentration of PM2.5-1.0 that could induce DNA damage.

The concentrations of PM1.0-0.5 and PM≤0.5 were varied to 10, 20, 40 and 80 μg/ml in order to find the lowest concentration of PM that could induce DNA damage. The results are shown in Table 8 and Figure 16. The DNA damage was dose related. Tail moment of the cells, after treatment with PM1.0-0.5 and PM≤0.5 at all concentrations, was significantly greater than that in the solvent control, except for the PM1.0-0.5 at the concentration of 10 μg/ml. However, the severity of DNA damage from PM≤0.5 at the concentration of 10 μg/ml was lower than that from other PM concentrations. It was concluded that PM1.0-0.5 and PM≤0.5 at the low concentrations of 5 and 10 μg/ml, respectively, were able to induce DNA damage.

**Table 7** Average tail moment ± standard error of mean of treated A549 human lung epithelial cells with PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations between 75 – 600 μg/ml (n = 100 cells)

| Concentrations (μg/ml) | Tail moment (μm) (mean ± S.E.M.) | | |
|---|---|---|---|
| | PM2.5-1.0 | PM1.0-0.5 | PM≤0.5 |
| 75 | 6.48 ± 1.44 | 8.19 ± 1.25*** | 12.13 ± 1.55*** |
| 150 | 8.89 ± 1.35*** | 10.50 ± 1.37*** | 13.02 ± 1.67*** |
| 300 | 11.55 ± 1.55*** | 11.93 ± 1.16*** | 19.62 ± 2.17*** |
| 600 | 15.82 ± 2.09*** | 17.75 ± 2.17*** | 23.28 ± 2.23*** |
| Solvent control [a] | 2.54 ± 0.63 | 3.26 ± 0.84 | 5.50 ± 1.04 |

[a] Experiments without PM.

Tail moment of the positive control (80 μM MMS) is 29.69 ± 1.24 μm.

*** $p \leq 0.001$ compared to the solvent control.

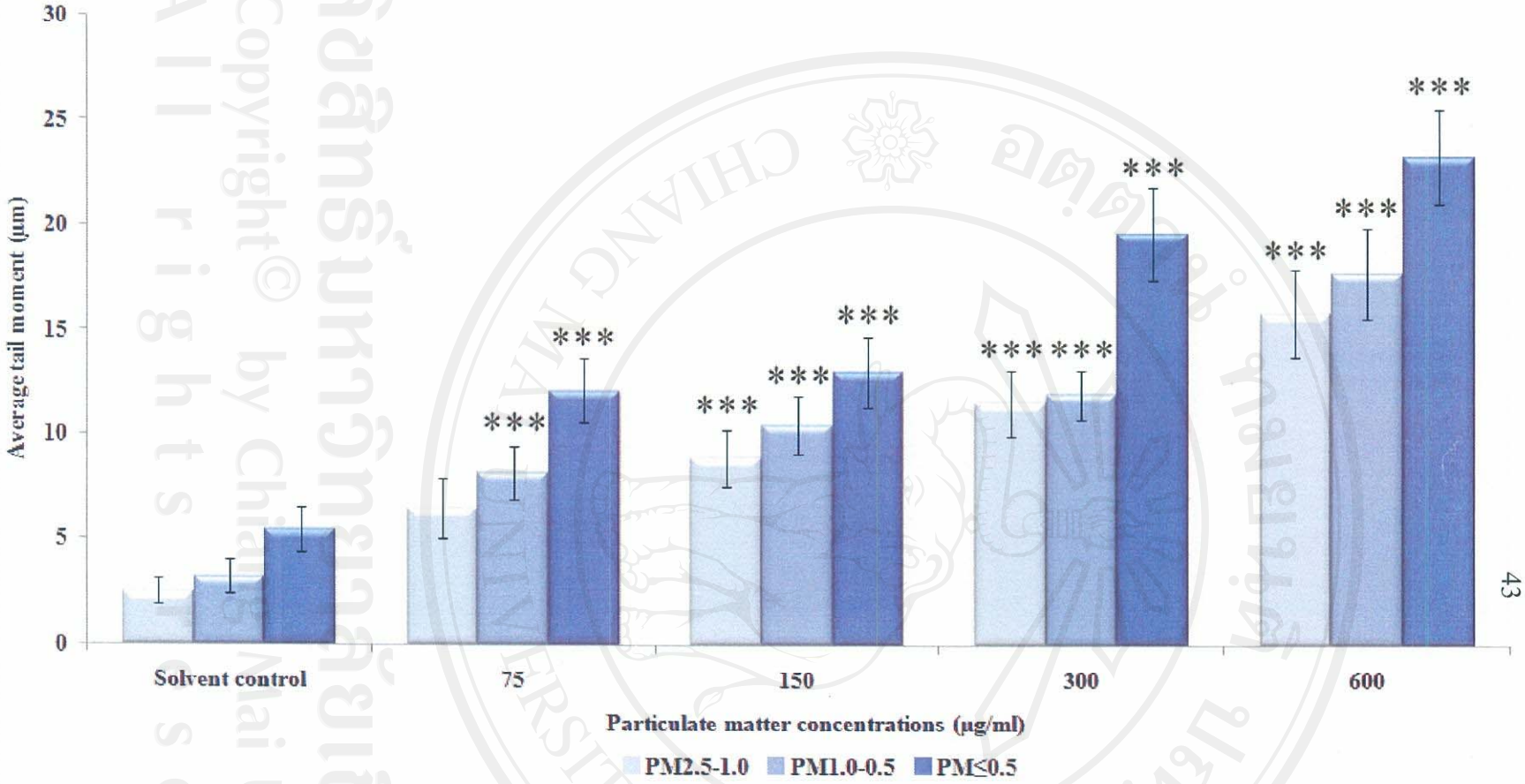


**Figure 15** Mean ± S.E.M. of the tail moment of A549 human lung epithelial cells treated with PM2.5-1.0, PM1.0-0.5 and PM≤0.5.

***p≤0.001 compared to the solvent control.

**Table 8** Average tail moment ± standard error of mean of treated A549 human lung epithelial cells with PM1.0-0.5 and PM≤0.5 at concentrations between 10 - 80 μg/ml (n = 100 cells)

| Concentrations (μg/ml) | Tail moment (μm) (mean ± S.E.M.) | |
|---|---|---|
| | PM1.0-0.5 | PM≤0.5 |
| 10 | 10.76 ± 1.27 | 10.17 ± 1.36* |
| 20 | 13.68 ± 1.66* | 21.17 ± 2.00*** |
| 40 | 14.86 ± 1.75* | 20.52 ± 1.61*** |
| 80 | 18.66 ± 1.98*** | 28.08 ± 2.02*** |
| Solvent control [a] | 7.78 ± 1.00 | 7.79 ± 1.13 |

[a] Experiments without PM.

Tail moment of the positive control (80 μM MMS) is 38.73 ± 1.37μm.

*p≤0.05, *** p≤0.001 compared to the solvent control.

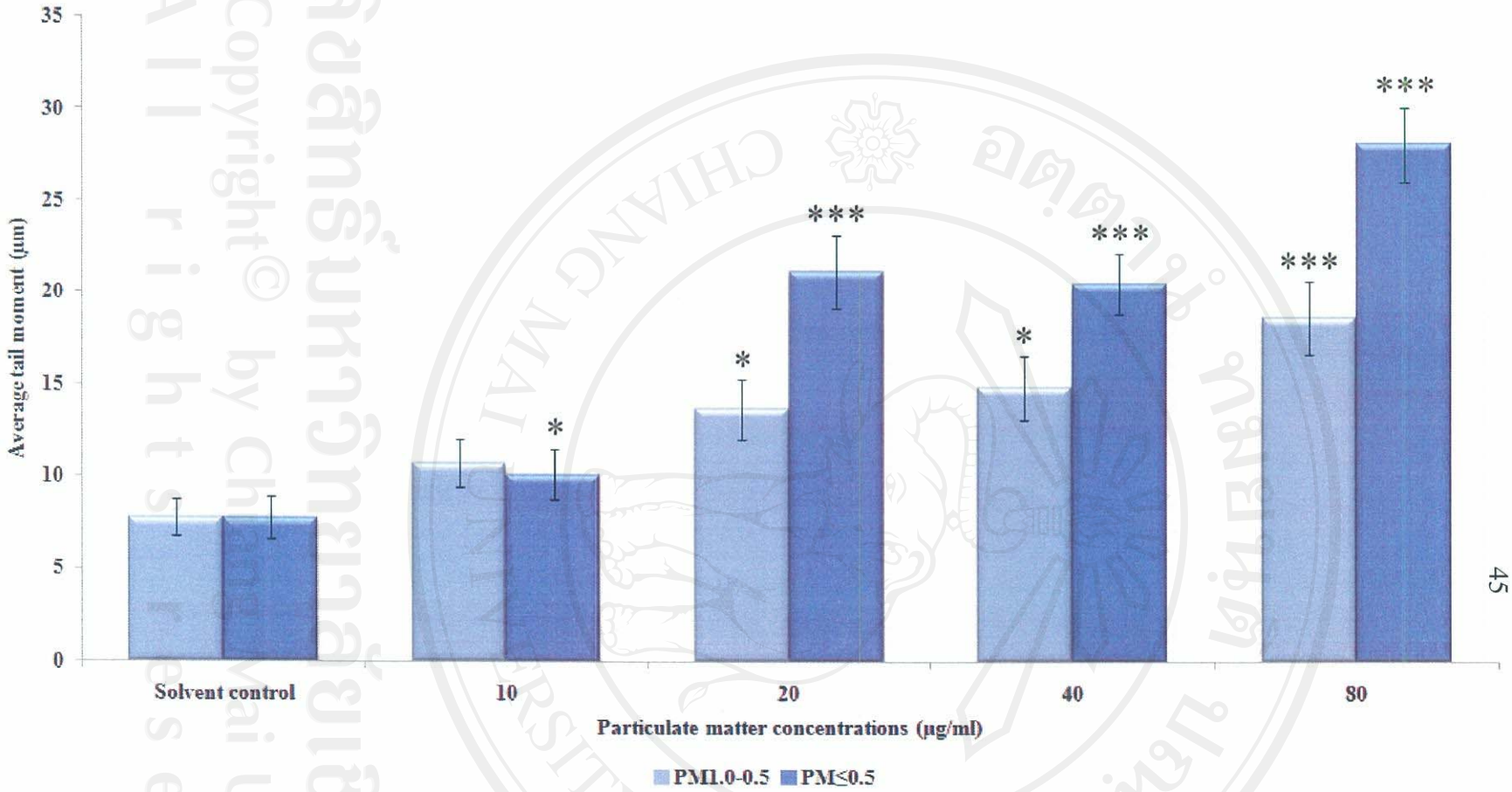


**Figure 16** Mean ± S.E.M of the tail moment of A549 human lung epithelial cells treated with PM1.0-0.5 and PM≤0.5.

*p≤0.05, ***p≤0.001 compared to the solvent control.

Aphidicolin (APC), a DNA repair inhibitor, was selected to investigate the repair mechanism of the DNA in some experiments, in which a low concentration of PM could not induce DNA damage. APC was treated at concentrations of 1.0 and 2.0 μg/ml with PM2.5-1.0 at the concentration of 75 and 150 μg/ml. The results are shown in Table 9 and Figure 17. The APC did not significantly induce DNA damage at the concentration of 2.0 and 1.0 μg/ml when compared to the solvent control. Tail moment of the cells after treatment with PM2.5-1.0 at the two concentrations of APC were significantly greater than damage to the cells treated with the PM2.5-1.0 only. Therefore, the APC could elevate DNA damage in the experimental culture cells.

**3.3 Quantitation of 8-OHdG**

The linearity of the standard curve of 8-OHdG is shown in Figure 18. The regression line equation was $y = -2.052x + 4.146$, where y was a logit $(B/B_0)$ and x a log concentration of 8-OHdG. The correlation coefficient (r) was 0.9965. The amount of 8-OHdG was determined in the DNA of the cells treated with PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations of 75, 150, 300 and 600 μg/ml. The results are shown in Table 10 and Figure 19. There was a significant increase in the level of 8-OHdG after treating the cells with PM2.5-1.0 at concentrations of 75, 300 and 600 μg/ml, and PM≤0.5 at concentrations of 150 and 600 μg/ml, whereas all concentrations of PM1.0-0.5 were able to induce the 8-OHdG level significantly greater than the solvent control. In conclusion, fine PM caused a formation of 8-OHdG in the A549 human lung epithelial cells.

**Table 9** Average tail moment ± standard error of mean of treated A549 human lung epithelial cells with PM2.5-1.0 at concentration 75 and 150 μg/ml and APC (n = 100 cells)

| Samples | Tail moment (μm) (mean ± S.E.M.) | |
|---|---|---|
| | APC 1.0 μg/ml | APC 2.0 μg/ml |
| PM 75 μg/ml [c] | 5.96 ± 1.25 | 5.17 ± 1.15 |
| PM 75 μg/ml + APC | 8.19 ± 1.29* | 10.49 ± 1.74* |
| PM 150 μg/ml [c] | 8.75 ± 1.57 | 9.33 ± 1.52 |
| PM 150 μg/ml + APC | 12.84 ± 1.61* | 13.35 ± 1.71* |
| Solvent control [a] | 4.34 ± 0.91 | 4.25 ± 0.91 |
| APC control [b] | 5.06 ± 0.88 | 5.72 ± 1.14 |

[a] Experiments without PM.

[b] Experiment with APC at two concentrations (1.0 and 2.0 μg/ml).

[c] Experiment without APC treatment.

*p≤0.05 compared to PM2.5-1.0 without APC treatment.

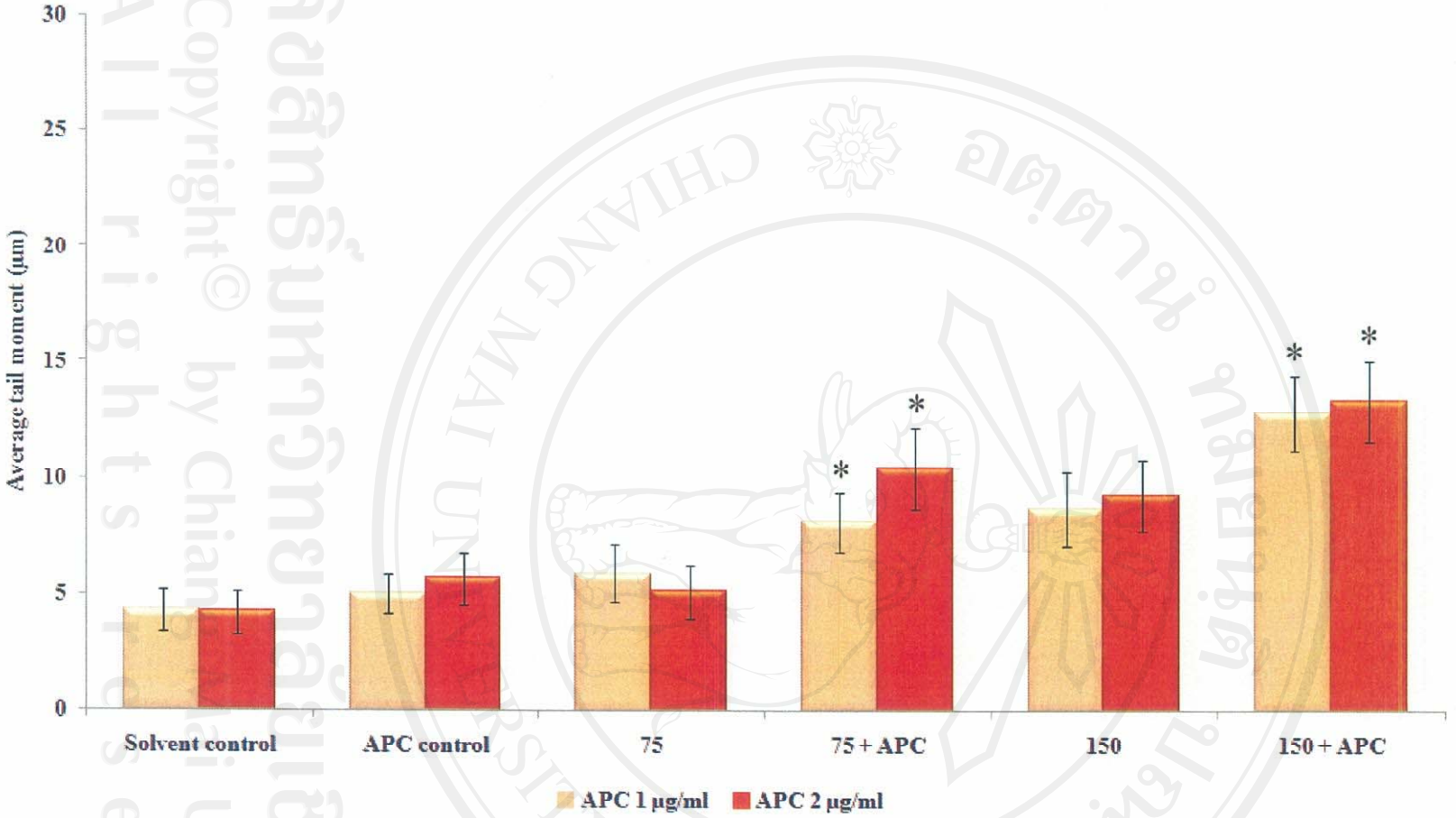


**Figure 17** Mean ± S.E.M. of the tail moment of A549 human lung epithelial cells treated with PM2.5-1.0 and aphidicolin (APC).

*p≤0.05 compared to PM2.5-1.0 and no APC treatment.

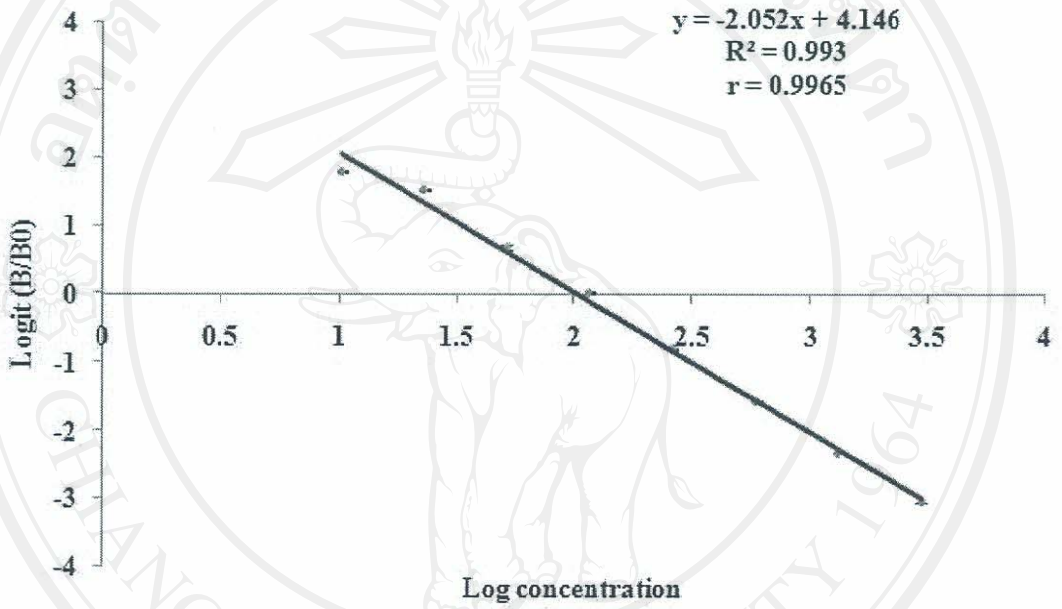


**Figure 18** The linearity of the standard curve of 8-OHdG at concentrations between 10.3 to 3,000 pg/ml, plotted as logit ($B/B_0$) versus log concentration in linear regression fit. The equation of the curve was y = -2.052x + 4.146, r = 0.9965.

**Table 10** Average amounts of 8-OHdG ± standard error of mean of treated A549 human lung epithelial cell DNA with PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations between 75 – 600 μg/ml (n = 3)

| Concentrations (μg/ml) | | 8-OHdG (pg/μg of DNA) | | | |
|---|---|---|---|---|---|
| | | I | II | III | Mean ± S.E.M. |
| PM2.5-1.0 | 75 | 274.15 | 394.30 | 337.14 | 293.64 ± 16.67* |
| | 150 | 256.04 | 256.04 | 247.67 | 253.25 ± 2.79 |
| | 300 | 356.54 | 394.30 | 337.14 | 362.66 ± 16.78* |
| | 600 | 334.51 | 331.91 | 394.30 | 353.58 ± 20.38* |
| PM1.0-0.5 | 75 | 342.51 | 350.82 | 362.42 | 351.92 ± 5.77* |
| | 150 | 326.81 | 324.30 | 377.81 | 342.97 ± 17.44* |
| | 300 | 374.65 | 329.34 | 353.66 | 352.55 ± 13.09* |
| | 600 | 483.27 | 408.36 | 419.46 | 437.03 ± 23.34* |
| PM≤0.5 | 75 | 266.67 | 300.85 | 100.73 | 222.75 ± 61.80 |
| | 150 | 451.62 | 384.27 | 447.38 | 427.75 ± 21.78* |
| | 300 | 242.85 | 272.25 | 298.65 | 271.25 ± 16.12 |
| | 600 | 305.32 | 305.32 | 314.58 | 308.41 ± 3.09* |
| Solvent control [a] | | 246.05 | 199.18 | 216.70 | 220.64 ± 13.67 |

[a] Experiment without PM.

*p≤0.05 compared to the solvent control.

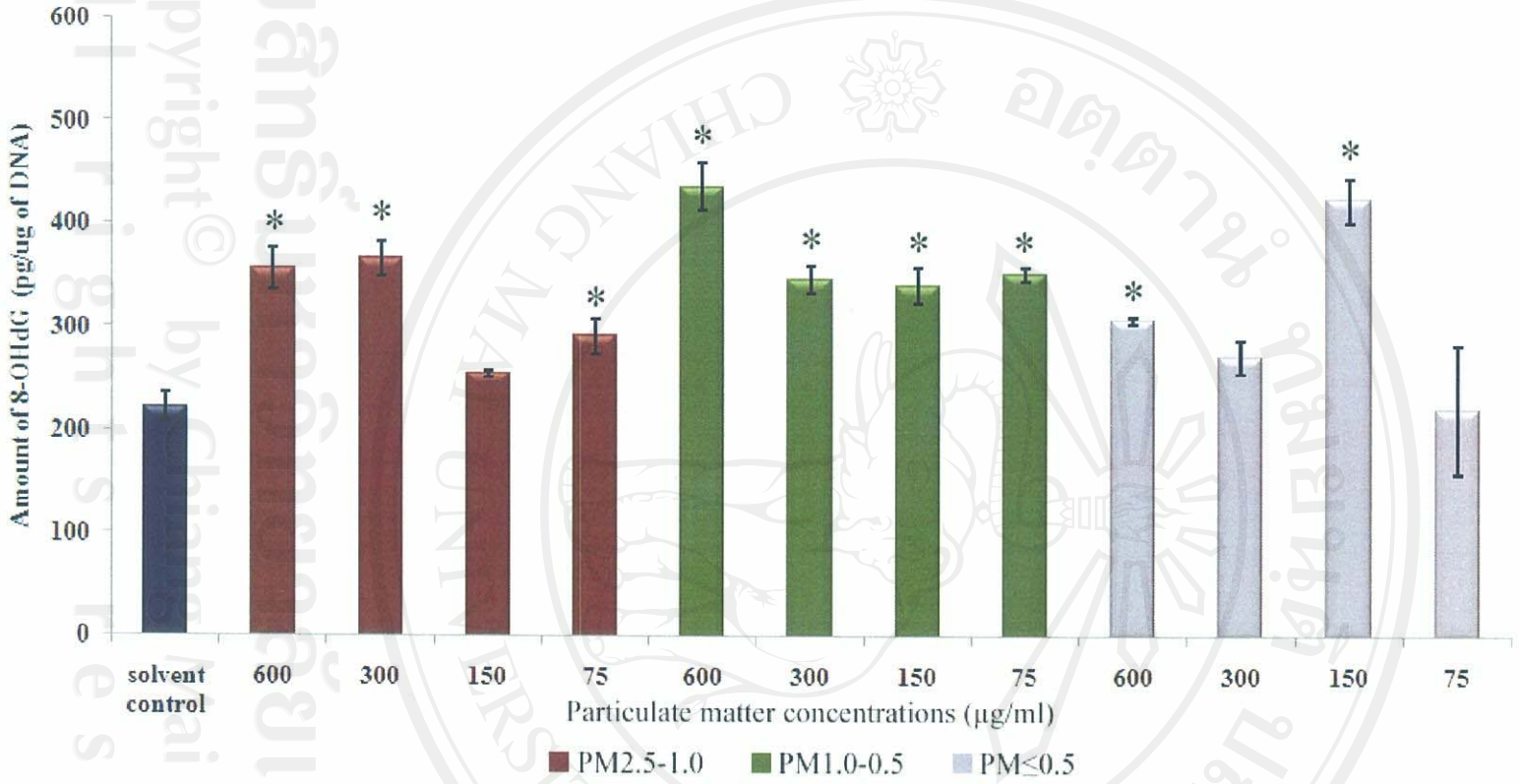


**Figure 19** Mean ± S.E.M. of the amount of 8-OHdG in the DNA of A549 human lung epithelial cells treated with PM2.5-1.0, PM1.0-0.5 and PM≤0.5.

*p≤0.05 compared to the solvent control.

# CHAPTER IV

# DISCUSSION AND CONCLUSION

Growing urbanization, traffic volume and industrial activities in Chiang Mai province have resulted in increased concentrations of PM and other air pollutants. The province is situated a valley surrounded by mountains in northern Thailand. Generally, the wind is not strong. The topography and climate of this area are important factors regarding the accumulation of PM in the atmosphere (Tippayawong *et al.,* 2006). The PCD of Thailand, which monitors the level of PM, showed that PM10 had a higher than acceptable level every year. This condition affected the visibility and health of inhabitants who live in the area. The fact that incidence rates of lung cancer in Chiang Mai have continuously increased since the year 2000 (Srisukho *et al.,* 2004), may be related to air pollutants, especially the increase of PM10 and PM2.5 levels.

Many studies have suggested a link between the concentration of PM and genotoxic effect, but the effects in Chiang Mai of fine PM below 2.5 microns on human lung epithelial cells has not been reported. Therefore, this study aimed to evaluate the genotoxicity of fine PM of 2.5-0.5 microns collected from Saraphi district, Chiang Mai province, to DNA strand break and 8-OHdG induct on A549 human lung epithelial cells.

The study area, Saraphi district, was selected based on reports of it having a high incidence of lung cancer patients (Vatanasapt *et al.,* 2002), which may be associated with the toxicity of PM. A high volume cascade impactor, the PM collection machine,

was set up in front of Saraphi Hospital throughout the four months of August, September, October and November, 2007. The meteorological data during this period concluded that the weather was rainy. This condition could influence the formation and transportation of PM2.5 (Degaetano *et al.,* 2004). The total PM concentrations were 283.43, 466.77, 280.09, 226.73 and 139.03 $\mu g/m^3$ of PM≥10, PM10-2.5, PM2.5-1.0, PM1.0-0.5 and PM≤0.5, respectively (Table 5, Figure 12). During June 2005 to June 2006, the daily level of the PM10 and PM2.5 collected in front of the Government Office of Saraphi had the highest level compared to the other sites studied in Chiang Mai (Sopajaree *et al.,* 2007).

In this study, the dispersion of PM showed that the percentage of PM10-2.5 had the highest concentration compared to other PM sizes (33.44%) (Figure 13). This is in contrast with the study of Sopajaree *et al.* (2007), who found that PM≥10 had the highest percentage (24-42%). The different sources of environment and seasons affected the dispersion of PM. Six urban areas in Europe found that PM1.0-0.2 dominated other types of PM during the autumn and winter. In contrast, PM10-2.5 dominated during the spring and summer (Pennanen *et al.,* 2007). The factor that affects the dispersion of PM may suggest different ambient air pollution sources in various seasons and the influence of meteorological conditions such as temperature, relative humidity and wind speed (Tippayawong *et al.,* 2006; Sevastyanova *et al.,* 2008). Kuhlbusch *et al.* (2001) observed significant diurnal variation of PM2.5 and PM10, which were generally higher in concentration during day than night at a rural site in Germany. The maximum concentrations released during daytime were from commuter traffic. Also, Gomiscek *et al.* (2004) studied the diurnal variation of PM10,

PM2.5 and PM1.0, and the maximum variation appeared in the summer around noon and in winter during the afternoon.

Source identification and apportionment of the PM10 in the Chiang Mai-Lamphun valley showed that natural gas combustion, coke oven and vegetative burning such as forest fire and agricultural waste burning were heavily loaded with PM10. Saraphi has about 195 factories, and these could perform industrial combustion (Pengchai *et al.,* 2008).

The genotoxicity of extracted fine PM on A549 human lung epithelial cells was evaluated. The characteristic of the cell lines was epithelial cell type II, which was selected to study PM toxicity, including interleukin and cytokine excretion (Karlsson *et al.,* 2006), gene expression and free radical induction (Shi *et al.*, 2006). This cell line is widely acknowledged as a suitable model for the study of environmental particle interaction with lung epithelial cells.

Using the comet assay, the genotoxicity was detected at single cell levels. In the literature reviewed, lymphocyte cells were chosen to demonstrate genotoxic effect, but a recent study selected A549 human lung epithelial cells in order to reflect the human respiratory condition when exposed to PM. Therefore, the condition for DNA strand break analysis was tested in order to find the optimal condition.

The genotoxicity of PM10-2.5 showed dose response relationships. The DNA damage of the cells treated at concentrations of 150, 300, 600, 1,200 and 2,400 μg/ml could significantly induce DNA damage (Table 6, Figure 14). These data are similar to the study of Puaninta *et al.* (2007), whose results showed that PM10 at the concentrations of 600, 1,200 and 2,400 μg/ml, collected in front of the Government Office of Saraphi, were presented by dense traffic in a rural area, and reports of

people suffering from respiratory disease, during the rainy and dry season, could have induced DNA damage in lymphocyte cells.

The particles and organic extracts of PM10 collected from urban, industrial and rural sources in Germany showed DNA damage, but no clear relationship of dose response. Particles from urban sources appeared to be toxic at the highest dose only. However, no differences were observed between particles from industrial and rural sources (Brits *et al.*, 2004).

The statistical level of tail moment for PM10-2.5 was greater at concentrations of 600, 1,200 and 2,400 μg/ml ($p \leq 0.001$) than at concentrations of 150 and 300 μg/ml ($p \leq 0.05$). Therefore, a concentration of 600 μg/ml was selected for the next experiments, due to its clear sight of direct genotoxicity.

To study the lowest concentration of each fraction of fine PM (PM2.5-1.0, PM1.0-0.5 and PM≤0.5), the fine PM had varied concentrations as a reference for the preliminary study of PM10-2.5. The genotoxicity of the fine PM elicited a dose response relationship. The PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations of 75, 150, 300 and 600 μg/ml could also induce DNA damage, except for PM2.5-1.0 at the concentration of 75 μg/ml (Table 7, Figure 15). When the concentration of PM1.0-0.5 and PM≤0.5 was decreased, in order to find the lowest concentration that could induce DNA damage, it was found that concentrations of 10, 20, 40 and 80 μg/ml could induce DNA damage significantly. However, PM1.0-0.5 at the concentration of 10 μg/ml could not (Table 8, Figure 16).

According to its concentration at 10 μg/ml, PM≤0.5 may not have the lowest concentration that induces DNA damage, since it still causes a genotoxic effect when

compared to the solvent control. Thus, there may be a lower concentration that can cause DNA damage.

Therefore, results showed that PM2.5-1.0, PM1.0-0.5 and PM≤0.5 at concentrations of 150, 20 and 10 μg/ml, respectively, were the lowest concentrations of each PM that were able to induce DNA damage on the cells. These low concentrations of PM were approximately the same as those of PM that could be inhaled and accumulate in the human alveoli.

Several studies reported the lowest concentration of PM2.5 that could induce DNA damage. Dong-Qun *et al.* (2004) found that PM2.5 at its lowest concentration of 5 mg/ml, collected from areas contaminated by coal-fuel pollution and vehicle exhausts, caused DNA damage of the human alveolar epithelium. Meng *et al.* (2007) studied the genotoxicity of dust storm PM2.5 on DNA damage. The results showed that three fractions of PM2.5: solvent extractable organics, PM2.5 suspension and water-soluble fraction, at the lowest concentrations of 25, 33.3 and 300 μg/ml, respectively, elicited DNA damage on rat alveolar macrophage. *In vivo* experiments, the PM2.5 suspension at the lowest concentration of 7.5 mg/kg body weight induced DNA damage in the lung cell of rats. The variability of the lowest concentration may be due to the different solvent extraction, cells type, incubation time and the source of the PM.

The comparative toxicity of PM2.5-1.0, PM1.0-0.5 and PM≤0.5 showed that the severity of DNA damage depended on the PM size, in which PM≤0.5 was more toxic than PM1.0-0.5 and PM2.5-1.0, consecutively. Because of the particles, size decreases correspond to the ratio of surface area to volume mass of the particle increase. When comparing particles that have a diameter of 2.5 and 1.0 μm, the

surface area increases to 2.5 times. Moreover, the surface area increases to 5 times when compared to a particle with a diameter of 0.5 μm (Oberdorster *et al.,* 1995). Therefore, smaller particles have larger surface areas that are porous and able to trap more chemical compounds, which elicit genotoxic effect to cells.

Moreover, the chemical compounds of fine PM induce genotoxicity, then the particle core of PM2.5-1.0, PM1.0-0.5 and PM≤0.5 probably involves the genotoxicity effect. Karlsson *et al.* (2004) showed that the insoluble particle core at the concentration of 100 μg/ml significantly induced DNA strand break when compared to control cells and all solvents tested. The native PM suspension at the highest concentration of 20 μg/ml significantly increased in DNA strand breakage. However, this was not observed for its particle-free filtrate, suggesting that the insoluble particle fraction contributes to PM-induced DNA damage (Knaapen *et al.*. 2002) or the particle core, when ingested by cells, causes mechanical damage to organelles that may lead to DNA damage. Thus, the results indicate that all of the chemical compounds and native particles of PM2.5-1.0, PM1.0-0.5 and PM≤0.5 could induce DNA damage on A549 human lung epithelial cells.

Use of the repair inhibitor, APC, should be useful in investigating the repair mechanism of DNA in some experiments, in which a low concentration of PM could not induce DNA damage. The results showed that the cells after treatment with PM2.5-1.0 and either concentration of APC had DNA damage greater than the experiments without APC (Table 9, Figure17). Speit *et al.* (2004) found that APC enhances the genotoxic effect of several mutagens in unstimulated and stimulated blood. The APC, tetracyclic diterpenoid, is a potent and specific inhibitor of DNA polymerase α, δ. The mechanism of action is its binding to the polymerase molecule

near the dCTP binding site, so that binding of the dCTP is blocked, which inhibits chain termination at the cytosine requiring sites. Inhibition of the repair mechanism of the cells resulted in accumulation of the DNA strand breaks (Oguro *et al.*, 1979; Sheaff *et al.,* 1991). It was concluded that PM2.5-1.0 at the concentration of 75 μg/ml could not induce DNA damage and the DNA may be protected by the repair mechanism within the cells.

Therefore, these results show that fine PM collected from Saraphi could induce DNA damage on human lung cells. However, the mechanism of fine PM that induces DNA damage is not fully understood. Therefore, another objective is to quantitate the 8-OHdG level, a by product of DNA damage resulting from hydroxyl radical production, in order to reveal the mechanism of DNA damage via this process.

The 8-OHdG reflects hydroxyl radical attack on the C8 position of the guanine base on the DNA strands. The DNA extraction for 8-OHdG quantitation, using the Flexigene DNA kit (QIAGEN Inc.), has a good yield of high purity DNA. A previous study showed that DNA isolation might also produce artifacts causing formation of 8-OHdG or other products in DNA (Collins *et al.,* 1997). Dizdaroglu *et al.* (2001) measured the 8-OHdG in DNA by HPLC-MS comparison with GC-MS using the commercial kit for DNA extraction. The result showed that isolation of DNA might be caused by a minimum amount of oxidation or no oxidation of guanine in the DNA. Recently, EIA has received significant attention as an alternative method for the analysis of 8-OHdG, due to the simplicity of the assay versus HPLC-ECD and LC/MS/MS methods. The study of Evans *et al.* (1999) found that measurement of the UVC-product, 8-OHdG, by EIA had a high correlation with HPLC-ECD. The EIA

detected greater levels than HPLC-ECD at a higher dose, because at higher doses the antibody may cross react with a molecular species similar to 8-OHdG.

The monoclonal antibody of the 8-OHdG EIA kit (Stressmarq Biosciences Inc., Canada) has more specificity of up to 100% 8-OHdG and cross-reactivity with 8-hydroxy guanosine, 8-hydroxy guanine and guanosine, which have about 23, 23, less than 0.01%, respectively.

The regression line equation of the 8-OHdG standard curve was $y = -2.052x + 4.146$, whereas the correlation coefficient (r) was 0.9965, which had high linearity (Figure 18). The method could detect 8-OHdG at the lowest concentration of 10.3 pg/ml. These results showed that PM2.5-1.0 at concentrations of 75, 300 and 600 μg/ml and PM≤0.5 at concentrations of 150 and 600 μg/ml could significantly elevate 8-OHdG levels. In contrast, PM1.0-0.5 at all concentrations, 75, 150, 300 and 600 μg/ml, could significantly elevate 8-OHdG levels more than the solvent control (Table 10, Figure19). The highest 8-OHdG level was 437.03 ± 40.42 pg/μg of DNA treated with PM1.0-0.5 at the concentration of 600 μg/ml. According to PM≤0.5, the result did not show a dose response relationship, as a concentration of 150 μg/ml showed the highest level. Also, Shi *et al.* (2003) showed that the fine and coarse fraction of PM at the concentration of 50 μg/ml elicited 8-OHdG formation. For extracting solvent, water could induce 8-OHdG greater than solvents (hexane, dichloromethane, acetone, dimethyl sulfoxide) by about 100 times (Karlsson *et al.*, 2004).

According to the size of fine PM, PM1.0-0.5 had a higher 8-OHdG level than PM2.5-1.0 and PM≤0.5. This was unexpected because PM≤0.5 has a higher surface area and should induce 8-OHdG more than other sizes of fine PM. Li *et al.* (2003)

found that ultrafine particles (diameter less than 0.1 micron) induced more cellular heme oxygenase expression, a sensitive marker for oxidative stress, than PM2.5 and PM10. This effect directly correlated with the high organic carbon and PAH contents of the particles. The oxidative stress, mediated by the PM, may arise from a mixed source, including direct generation of ROS from the surface area of particles, soluble compound such as transition metals or organic compound, altered function of the mitochondria of NADPH-oxidase, and activation of inflammatory cells capable of generating ROS and reactive nitrogen species (Risom *et al.,* 2005).

The correlation of 8-OHdG levels with lung cancer found that 8-OHdG levels in urine or DNA in cells correlated to cystic fibrosis, small cell carcinoma and lung cancer (Cooke *et al.,* 2003). Therefore, the elevation of 8-OHdG might implicate the progression of lung cancer. Not only 8-OHdG may relate to lung cancer, but also other markers.

In conclusion, the fine PM (0.5, 1.0 and 2.5 microns) collected at Saraphi Hospital in Chiang Mai province could induce DNA damage and increase 8-OHdG formation. The mechanism of DNA damage possibly involves with the generation of hydroxyl radical caused by the fine PM itself or chemical constituents, which are adsorbed onto the PM and attack the guanine base on the DNA strand. The elevation of 8-OHdG might implicate the progression of lung cancer. Moreover, the individual repair mechanism is probably one of the factors that help to protect the cells from DNA damage after exposure to low concentrations of fine PM. Long term exposure to fine PM may be responsible for chronic adverse effects among Chiang Mai inhabitants, who have been reported as processing the highest incidence of lung cancer in Thailand.

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ขจรศักดิ์ โสภาจารีย์, มงคล รายะนคร, พงศ์เทพ วิวรรธนะเดช, อุษณีย์ วินิจเขตคำนวณ และดวงจันทร์ อาภาวัชรุตม์ เจริญเมือง. ความรุนแรงของปัญหาฝุ่นละอองในบรรยากาศและผลกระทบต่อสุขภาพอนามัยของประชาชนในเชียงใหม่และลำพูน. สำนักงานกองทุนสนับสนุนการวิจัย. กรุงเทพมหานคร. 2550.

# APPENDICES

## APPENDIX A

### List of chemicals and reagents

| Chemicals and reagents | Sources |
|---|---|
| Alconox | Alconox Inc., U.S.A. |
| Absolute methanol (HPLC grade) | Fisher Scientific, UK |
| Aphidicolin | Sigma, U.S.A. |
| Calf intestinal alkaline phosphatase | Invitrogen, U.S.A. |
| Dimethyl sulfoxide (GC grade) | Sigma, U.S.A. |
| Ethidium bromide | Sigma, U.S.A. |
| Ethylenediaminetetraacetic acid | Sigma, U.S.A. |
| Flexigene DNA kit | Qiagen Inc., U.S.A. |
| Low melting point agarose | Sigma, U.S.A. |
| Normal melting point agarose | Sigma, U.S.A. |
| S1 nuclease | Invitrogen, U.S.A. |
| Sodium chloride | Merck, Germany |
| Sodium hydroxide | Merck, Germany |
| Sodium pyruvate | Gibco, U.S.A. |
| Trisma base | Sigma, U.S.A. |
| Triton X-100 | Sigma, U.S.A |

## APPENDIX B

### List of reagent preparations

**1. Minimum essential medium (MEM)**

| | |
|---|---|
| MEM | 500 ml |
| 50 µg/ml gentamycin | 0.3125 ml |
| Sodium pyruvate 100X | 5 ml |
| Heat-inactivated fetal calf serum | 25 ml |

The solution was filtrated with a 0.2 µm syring filter and kept at 4°C.

**2. Stock 1% Trypsin**

| | |
|---|---|
| Trypsin | 1 g |
| Hank's buffer salt solution (HBSS) | 100 ml |

The solution was mixed and kept at 4°C.

**3. Stock 1% EDTA**

| | |
|---|---|
| EDTA | 1 g |
| Hank's buffer salt solution (HBSS) | 100 ml |

The solution was mixed and kept at 4°C.

**4. 0.005% Trypsin-EDTA solution**

| | |
|---|---|
| Stock 1% Trypsin | 25 µl |
| Stock 1% EDTA | 25 µl |
| Hank's buffer salt solution (HBSS) | 500 ml |

The solution was filtrated with a 0.2 µm syring filter and kept at 4°C.

**5. Stock lysing solution**

| | |
|---|---|
| NaCl | 146.1 g |
| EDTA | 37.2 g |
| Trisma base | 1.2 g |
| Ultrapure water | 890 ml |

The solution was adjusted to pH10 using NaOH 8 g and kept at room temperature. The working lysing solution was prepared freshly by 1 ml of Triton X-100. Ten milliliters of DMSO were added in 89 ml of stock lysing solution and kept at 4°C for at least 60 minutes before use.

**6. Electrophoresis buffer**

| | |
|---|---|
| NaOH | 7.2 g |
| Ultrapure water | 600 ml |
| 200 mM EDTA | 3.0 ml |

The solution were mixed and kept at 4°C for at least 60 minutes before use.

**7. Neutralization buffer**

| | |
|---|---|
| Trizma base | 24.2 g |
| Ultrapure water | 500 ml |

The solution was mixed and kept at room temperature.

**8. Stock methylmethane sulfonate (MMS) 0.24M**

| | |
|---|---|
| MMS | 2.0 µl |
| Hank's buffer salt solution (HBSS) | 98 µl |

The solution was mixed and kept at -20°C. The working MMS (480 µM) was prepared by diluting stock solution of 0.24 M MMS 2 µl and HBSS was added to a final volume of 500 µl. The solution was mixed and stored at 4°C.

**9. 1% Normal melting point agarose (NMPA)**

| | |
|---|---|
| Normal melting agarose | 0.1 g |
| Phosphate buffer saline | 10 ml |

The solution was mixed and boiled until the agarose was melted.

**10. 1% Low melting point agarose (LMPA)**

| | |
|---|---|
| Low melting point agarose | 0.1 g |
| Phosphate buffer saline | 10 ml |

The solution was mixed and boiled until the agarose was melted. Before the experiment, LMPA was placed in the water bath at 37°C.

**11. Buffer FG2/QIAGEN Protease**

| | |
|---|---|
| Lyophilized QIAGEN Protease | 1 vial |
| Buffer FG3 | 0.3 ml |

The solution was mixed and kept at 4 °C

**12. EIA buffer**

| | |
|---|---|
| EIA buffer concentrate | 10 ml |
| Ultrapure water | 90 ml |

The solution was mixed and the EIA buffer vial rinsed to remove any salts that may have precipitated. This buffer was kept at 4 °C for about two months.

**13. Wash buffer**

| | |
|---|---|
| Wash buffer | 2.5 ml |
| Ultrapure water | 1 L |
| Tween 20 | 0.5 ml |

The solution was mixed and kept at 4 °C for about two months.

**14. 8-OHdG EIA standard 30 ng/ml**

| | |
|---|---|
| 8-OHdG standard 300 ng/ml | 100 µl |
| Ultrapure water | 900 µl |

The solution was mixed and kept at 4 °C. This solution is stable for approximately six weeks.

To prepare the standard for use in EIA: eight concentrations of it were prepared at 3,000, 1,333, 592.6, 263.4, 117.1, 52.0, 23.1 and 10.3 pg/ml. The diluted standards had to be stored at 4 °C for no more than 24 hours.

**15. 8-OHdG AChE tracer**

| | |
|---|---|
| 8-OHdG AChE tracer | 100 dtn |
| EIA buffer | 6 ml |
| Tracer dye | 60 µl |

The solution was mixed and kept at 4 °C (without freezing) and used within four weeks.

**16. 8-OHdG monoclonal antibody**

| | |
|---|---|
| 8-OHdG monoclonal antibody | 100 dtn |
| EIA buffer | 6 ml |
| Antiserum dye | 60 µl |

The solution was mixed and kept at 4 °C. This solution is stable for at least four weeks.



**17. Ellman's reagent**

| | |
|---|---|
| Ellman's reagent | 100 dtn |
| Ultrapure water | 20 ml |

The solution was mixed and had to be used on the same day that it was prepared. Reconstituted reagent was unstable and had to be protected from light when not in use.

## APPENDIX C

### Undamaged and damaged DNA of A549 human lung epithelial cells


Undamaged (A) and damaged DNA (B) of the cells showing tailing of the DNA strand break (resembling a comet) after treatment with fine PM.

# CURRICULUM VITAE

**รายละเอียดหัวหน้าโครงการ**

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ส่วนที่จะรับผิดชอบในโครงการวิจัยนี้ ให้คำปรึกษาการวางแผนงานวิจัยและการจัดเตรียมอุปกรณ์การวิจัยทั้งโครงการ ตลอดจนการวิเคราะห์ข้อมูล การรายงานผล และการนำเสนอผลงานนักศึกษา

ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่จะทำการวิจัยครั้งนี้

1. Puainta C, Pangjaidee N, Chantarasittipol, Wunnapak K, Petchsophonsakul W, **Ruangyuttikarn W**, et al. Genotoxicity of particulate matters in ampient air collected from different areas in Chiang Mai and Lumphun. Proceeding Anatomy 2007;95-6.

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7. Khacha-ananda S, Puaninta C, Sopajaree K, **Ruangyuttikarn W**. Detection of DNA damage by comet assay in A549 human lung epithelial cells exposed to fine particulate matters collected in Saraphi District, Chiang Mai, Thailand. Thai Journal of Toxicology. 2008: 23(2); 186.

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ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่จะทำการวิจัยครั้งนี้

1. Chewaskulyong B, Mokmued P, Ruangyuttikarn W, Mevatee U, Pothirat C, **Sopajaree K**. Pulmonary function of inhabitants from heavy traffic area in Chiang Mai. Chiang Mai Med Bull 2004; 43(3): 23.

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4. Khacha-ananda S, Puaninta C, **Sopajaree K**, Ruangyuttikarn W. Detection of DNA damage by comet assay in A549 human lung epithelial cells exposed to fine particulate matters collected in Saraphi District, Chiang Mai, Thailand. Thai Journal of Toxicology. 2008: 23(2); 186.

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ส่วนที่จะรับผิดชอบในโครงการวิจัยนี้ ให้คำแนะนำในการทดสอบความเป็นพิษของอนุภาคฝุ่นโดยวิธี comet assay รวมทั้งการวิเคราะห์ข้อมูล

ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่จะทำการวิจัยครั้งนี้

1. **Puaninta C**, Mevatee U, Vinitketkumnuen U, Suthakome N, Aungsuchawan S , Bumroongkit K. Effects of Lannate, Tamarons and Furadan on Human DNA as Evaluated of Chromosome Aberration and Comet Assays. Research abstract of 1st ASEAN conference on medical, Kota Bharu, Kelantan, Malasia. May 18-21, 2001.

2. **Puaninta C**, Pangjaidee N, Chantarasittipol K, Wunnapak K, Petchsopholsakul W, Ruangyuttikarn W, Mevatee U. Genotoxicity of Particulate Matters in Ambient air Collected from Different Areas in Chiang Mai and Lumphun. 30th Annual conference of the Anatomy Society of Thailand. May 2-4, 2007.

3. Khacha-ananda S, **Puaninta C**, Sopajaree K, Ruangyuttikarn W. Genotoxicity of fine particulate matter collected in Saraphi District, Chiang Mai, Thailand on A549 human lung epithelial cell. IWA DIPCON 2008 BOOK OF ABSTRACTS. 2008: 198.

4. Khacha-ananda S, **Puaninta C**, Sopajaree K, Ruangyuttikarn W. Detection of DNA damage by comet assay in A549 human lung epithelial cells exposed to fine particulate matters collected in Saraphi District, Chiang Mai, Thailand. Thai Journal of Toxicology. 2008: 23(2); 186.

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ส่วนที่จะรับผิดชอบในโครงการวิจัยนี้ เก็บตัวอย่างและหาปริมาณอนุภาคฝุ่น รวมทั้งการทดสอบความเป็นพิษของอนุภาคฝุ่นโดยวิธี comet assay และการหาปริมาณของ 8-ไฮดรอกซี-2-ดีออกซีกวาโนซีนโดยวิธี Enzyme immunoassay

ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่จะทำการวิจัยครั้งนี้

1. **Khacha-ananda S**, Puaninta C, Sopajaree K, Ruangyuttikarn W. Genotoxicity of fine particulate matter collected in Saraphi District, Chiang Mai, Thailand on A549 human lung epithelial cell. IWA DIPCON 2008 BOOK OF ABSTRACTS. 2008: 198.

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