UNIVERSITI PUTRA MALAYSIA

ACUTE AND CHRONIC PULMONARY RESPONSES TO INTRATRACHEALLY INSTILLED BENZO(A)PYRENE IN RATS

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By

MAZLINA BINTI MAZLAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirements for the Degree of Master of Science

March 2010

Dedicated with love to:

My beloved family for their undying love and support

&

Dearest Dr. T. Tiagarajan for his unfailing encouragement and love

Thank you…

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Master of Science

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March 2010

Chairman : Noordin Bin Mohamed Mustapha, PhD

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Air pollution has been reported to exert serious health effects to all living things in both short and long term exposures. These allegations have been proven by mounting epidemiological data and studies which consistently showed clear evidences. However, the precise mechanisms behind the association between airborne particulates and the evidences have yet to be fully comprehended.

Benzo(a)pyrene (BaP) is a main constituent of haze and notably known to be the cause of mutagenicity and carcinogenicity in experimental animals. Despite the notorious reputation of BaP, no conclusive study has been conducted to assess the effects of this compound at such a low dose as the one that Malaysians encountered during the haze episode in 1997. Thus, this study was designed to assess the oxidative stress in rats intratracheally

exposed to BaP, and also to correlate these findings with the morphology of the lungs.

This study involved a total of 70 rats, divided into groups of 5 rats each and randomly assigned to different treatment groups. A total of 50 rats and 10 rats were assigned to the acute and chronic responses, respectively. The remaining 10 rats were used as controls. The rats that were assigned in acute study were instilled intratracheally with a single dose of 13.8 ng which is equivalent to 7 µl of BaP. In contrast, the rats destined to receive BaP in the chronic response were given 2 doses of equal amountd which have been divided equally (3.5 µl) with the second dose administered at 1 week post instillation (p.i). All the rats were alternately subjected to blood sampling *via* intracardiac puncture at hours (hr) 0, 1, 8, 16, 24, 32, 72 (acute) and weeks (wk) 1, 3, 6, 9, 12 (chronic) p.i. However, 5 rats in the treated group were euthanized at 1, 8, 16, 32 and 72 hr p.i. for the acute response study. Rats in the chronic response and the control groups were only sacrificed at 12 wk p.i. At necropsy, lung samples were collected for morphological studies. The antioxidant and lipid peroxidation assays were conducted on blood, lungs and liver samples.

Acute response revealed a marked inflammatory response characterized by predominantly alveolar macrophages and mild neutrophils infiltration at 8

hr and 16 hr p.i. in the BaP group. These lesions corresponded to the high malondialdehyde (MDA) and superoxide dismutase (SOD) with low glutathione peroxidase (GSH-Px) and glutathione S transferase (GST) concentrations in the blood, lungs and liver.

The chronic response revealed that the treated group demonstrated mild inflammatory reactions with persistent hyperplasia, metaplasia and dysplasia of the pneumocytes with presence of granuloma at 12 week p.i. Diffused emphysema of the lungs was the obvious lesion seen in the chronic study. The MDA and SOD activities in the peripheral blood, lung and liver of treated rats were significantly ($P<0.05$) high at 12 weeks p.i. On the contrary, the level of GSH-Px of treated rats in all of the samples (blood, lungs and liver) and was significantly ($P<0.05$) lower at 12 wk p.i. Likewise, the GST level in the lung and liver of treated rats were significantly ($P<0.05$) lower than the normal rats at 12 wk p.i.

It was concluded that even at minute levels, BaP is able to exert deleterious effects on tissue morphology via oxidative stress as evident from the disruption in the lipid peroxidation (MDA level) and antioxidant status (SOD, GSH-Px and GST levels) in blood, lungs and liver.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

GERAK BALAS PULMONARI AKUT DAN KRONIK TERHADAP BENZO(A)PIRENA YANG DIINSTILASIKAN SECARA INTRATRAKEA PADA TIKUS

Oleh

MAZLINA BINTI MAZLAN

March 2010

Pengerusi : Noordin Bin Mohamed Mustapha, PhD

Fakulti : Perubatan Veterinar

Pencemaran udara telah dilaporkan menyebabkan kesan yang serius pada kesihatan kepada semua benda hidup akibat pendedahan dalam jangka masa pendek dan panjang. Dakwaan-dakwaan ini telah pun dibuktikan dengan wujudnya begitu banyak data dan kajian epidemiologi yang secara konsisten menunjukkan bukti yang jelas. Namun begitu, mekanisma yang persis yang terlibat masih belum dapat difahami.

Benzo(a)pirena adalah unsur yang utama dalam jerebu and telah dikenalpasti sebagai penyebab kepada kemutagenan dan kekarsinogenan yang dilihat hasil dari kajian dalam haiwan. Walau BaP terkenal kerana kesan kemudaratannya tetapi masih tiada kajian yang konklusif dijalankan

untuk mengkaji kesan bahan tersebut pada dos yang begitu rendah seperti mana yang dihadapi oleh penduduk Malaysia ketika episod jerebu pada tahun 1997. Oleh itu, kajian ini dijalankan untuk menilai tekanan oksidatif dalam tikus yang didedahkan kepada BaP secara intratrakea dan untuk mengaitkan penemuan tersebut dengan morfologi peparu.

Kajian ini melibatkan sejumlah 70 ekor tikus yang telah dibahagikan sama rata kepada kumpulan yang terdiri dari 5 ekor tikus bagi setiap kumpulan dan tikus-tikus tersebut telah secara rawak diagihkan kepada kumpulan rawatan yang berlainan. Sejumlah 50 ekor tikus dan 10 ekor tikus yang lain masing-masing telah ditugaskan dalam kajian gerak balas akut dan kronik. Sepuluh ekor tikus yang selebihnya pula telah digunakan sebagai kawalan. Kesemua tikus yang telah diagihkan ke dalam kumpulan rawatan untuk kajian gerak balas akut menerima satu dos yang mengandungi 13.8 ng atau bersamaan dengan 7 μl BaP secara intratrakea. Berbeza dengan tikus-tikus yang telah ditentukan menerima BaP dalam kajian gerak balas kronik telah diberikan 2 dos yang sama jumlahnya yang telah dibahagikan sama rata (3.5 μl) di mana dos kedua telah diberikan pada minggu pertama pasca pemberian (p.i.). Kesemua tikus secara bergilir telah diambil sampel darah melalui intrakardiak pada jam 0, 1, 8, 16, 24, 32, 72 (akut) dan minggu 1, 3, 6, 9, 12 (kronik) p.i. Walau bagaimanapun, 5 ekor tikus dalam kumpulan rawatan telah dikorbankan pada 1, 8, 16, 32 dan 72 jam p.i. dalam kajian

gerak balas akut. Tikus-tikus dalam kajian gerak balas kronik dan kumpulan kawalan pula hanya dikorbankan pada 12 minggu p.i. Ketika nekropsi, sampel peparu telah diambil bagi tujuan kajian morfologi. Asai peroksidasi lipid dan antioksida telah dijalankan pada sampel darah, peparu dan hati.

Gerak balas akut mendedahkan gerak balas keradangan yang sangat jelas yang bercirikan penyusupan oleh makrofaj alveolus secara pradominan dan neutrofil yang sederhana pada 8 dan 16 jam p.i. dalam kumpulan yang menerima rawatan BaP. Lesi-lesi ini berpadanan dengan penemuan konsentrasi malondialdehid (MDA) dan superoksid dismutase (SOD) yang tinggi bersama dengan glutation peroksidase (GSH-Px) dan glutatione S-transferase (GST) yang rendah dalam darah, peparu dan hati.

Gerak balas kronik pula mendedahkan bahawa kumpulan rawatan menunjukkan tindak balas keradangan yang sederhana diikuti dengan hiperplasia, metaplasia dan displasia pneumosit berterusan bersama dengan kehadiran granuloma pada 12 minggu p.i. Selerakan emfisema pada peparu adalah lesi yang paling ketara ditemui dalam kajian gerak balas kronik ini. Aktiviti MDA dan SOD dalam darah dari periferi, peparu dan hati dari tikus-tikus dalam kumpulan rawatan adalah ketara tinggi ($P<0.05$) pada 12 minggu p.i. Sebaliknya, paras GSH-Px dari tikus yang menerima rawatan dalam kesemua sampel (darah, peparu dan hati) adalah rendah yang ketara

($P<0.05$) pada minggu ke-12 p.i. Begitu juga bagi paras GST dalam hati dan peparu dalam tikus dari kumpulan rawatan yang menunjukkan paras yang lebih rendah secara ketara ($P<0.05$) berbanding tikus normal pada 12 minggu p.i.

Secara kesimpulannya, ternyata bahawa pada kadar yang sangat rendah pun BaP masih boleh mengakibatkan kesan yang mudarat pada morfologi tisu melalui tekanan oksidatif yang dibuktikan oleh perubahan pada paras peroksidasi lipid (paras MDA) dan status antioksida (paras SOD, GSH-Px dan GST) dalam darah, peparu dan hati.

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I certify that a Thesis Examination Committee has met on 15 March 2010 to conduct the final examination of Mazlina Binti Mazlan on her thesis entitled "Acute and Chronic Pulmonary Responses to Intratracheally Instilled Benzo(A)Pyrene in Rats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment for the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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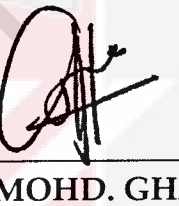

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Date : 12 August 2010

DECLARATION

declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

MAZLINA BINTI MAZLAN

Date: 15 March 2010

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LIST OF ABBREVIATIONS

Ahr	Aryl/aromatic hydrocarbon receptor
ANOVA	One-way analysis of variance
AM	Alveolar macrophage
AP-1	Activator protein-1
API	Air pollution index
ARB	California Air Resources Board
ATSDR	Agency for Toxic Substances and Disease Registry
BALT	Bronchus associated lymphoid tissue
BaP	Benzo[a]pyrene
BSA	Bovine serum albumin
CAT	Catalase
CuZnSOD	Copper-Zinc-containing-SOD
dH_2O	Distilled water
DIW	Deionised water
DOE	Department of Environment, Malaysia
DTNB	Ellman's solution/5,5-dithiobis-2-nitrobenzoic acid
EA	United Kingdom Environmental Agency
ECSOD	Extracellular SOD
EEPSEA	Economy and Environment Program for Southeast Asia
EPA	United States Environmental Protection Agency

GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSH-Rx	Glutathione reductase
GSSG	Reduced glutathione
GST	Glutathione transferase
H & E	Haematoxylin & Eosin
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HPO3	Meta-phosphoric acid
hr	Hour
IARC	International Agency for Research on Cancer
MDA	Malondialdehyde
MnSOD	Manganese-containing-SOD
Na_2HPO_4	Disodium phosphate
Na_2WO_4	Sodium tungstate
NaCl	Sodium chloride
NFκB	Nuclear factor κB
p.i.	Post instillation
PAH	Polycyclic aromatic hydrocarbon
PHG	Public Health Goal
PM	Particulate Matter
PMN	Polymorphonuclear

Pr	Protein
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SE	Standard error
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
Tr	Tricaprylin
Tris-HCl	Tris hydrochloride
wk	Week
WWF	World Wide Fund for Nature

CHAPTER 1

INTRODUCTION

Air pollution has become a major environmental health problem affecting the global population. It is the result of steady change in the atmosphere's composition that began ever since the onset of the industrial revolution. This is mainly due to the use of fossil fuel combustions for transportation and generation of the much needed energy.

There has been mounting concern over the adverse health effect of air pollution. Clear association exists linking the increased in hospital admissions, cardiovascular morbidity, poor lung function and even mortality to airborne concentrations of photochemical and particulate pollutants (Risom *et al.*, 2005; Kelly, 2003).

Besides the serious health impacts of air pollution, reduced visibility has affected the Malaysian economy whereby land, air and marine traffics were restricted. There was a tremendous decline in the tourism revenues, industrial and also fishing as well as agricultural activities. (EEPSEA and WWF, 2003; Hassan *et al.*, 2000).

Numerous epidemiologic studies have consistently shown clear evidences of adverse health effects related to particulate air pollutants even at low levels beyond the National Ambient Air Quality Standards. However, the precise biologic mechanisms behind the association between airborne particulates and the evidences have yet to be fully established (Martin *et al.*, 1997).

So far, the multi-pathogenic mechanisms of how the damages linked to particulate matter (PM) are brought about are not well understood. Although there are various research done on the ambient air PM inducing oxidative DNA damage in *in vitro* systems, there is scarcity of research done *in vivo* (Lin *et al.*, 2009).

Lung suffers a significant impact of haze as it encounters direct exposure to ambient air and experiences enhanced oxidative stress invoked by environmental contaminants that lead to generation of excessive free radicals (Kinnula and Crapo, 2003).

Benzo(a)pyrene (BaP), a recognized polycyclic aromatic hydrocarbon (PAH) notoriously known for its carcinogenic and mutagenic properties (ATSDR, 1995; 1990; Faust, 1994; IARC, 1983), was found to be one of the PM with the highest concentration during the devastating haze that hit Malaysia in 1997 (Zakaria *et al.*, 1998). It is believed that upon exposure, PM could have been

the main culprit for the occurrence of inflammation and oxidative stress (Risom *et al.*, 2005).

A dose-related study conducted revealed an increase in the incidence of malignant lung tumour after injection of BaP in tricaprylin (Tr) or beeswax into the lung tissue of rats (IARC, 2006). Garcon *et al.* (2001) stated of a possibility of BaP inducing oxidative stress leading to injury in the respiratory system.

Although much epidemiological reports exist on the adverse health effects of BaP present in soot, tar and oil, these data are considered as insufficient in assessing the carcinogenicity of BaP itself (ARB, 1994). Moreover, most of the studies of BaP either singly or in combination with other substances in animal models used higher concentrations of BaP than that of the one the public was exposed to during the severe and intense haze episode in 1997 (IARC, 2006; Sigma-Aldrich, 2006; Faust, 1994; EPA, 1991; ATSDR, 1990).

Therefore, the aim of this study was to determine the effect of haze, particularly BaP, primarily on the respiratory system and the mechanisms behind the cause and effect in a rat model.

It is hypothesized that BaP is potent in exerting deleterious effects on the pulmonary system even at a very minute dose either for a short duration or even chronically. Hence, this study was conducted to assess the effect of BaP exposure on rats based on the following objectives:

i) to assess the antioxidant and lipid peroxidation status during BaP-induced lung injury

ii) to evaluate the morphological changes in lungs after administration of BaP

iii) to explain the mechanism and pathway of BaP-induced lung injury

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