



UNIVERSITI PUTRA MALAYSIA

STUDIES ON THE EFFECT OF BENZO(a)PYRENE IN RATS

THORIA ALI ABDUL HAMEED DRAGH

FPV 2001 15

STUDEIES ON THE EFFECT OF BENZO(a)PYRENE IN RATS

By

THORIA ALI ABDUL HAMEED DRAGH

**Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of
Veterinary Science in the Faculty of Veterinary Medicine
Universiti Putra Malaysia**

March 2001



DEDICATION

To my wonderful family



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

STUDIES ON THE EFFECT OF BENZO(a)PYRENE IN RATS

By

THORIA ALI ABDUL HAMEED DRAGH

March 2001

Chairman: Dr. Noordin Mohamed Mustapha

Faculty: Veterinary Medicine

Benzo(a)pyrene [B(a)P] is a carcinogen found in high concentrations during the Malaysian haze episodes. In view of developing strategies in alleviating deleterious effects of haze in humans, the effect of acute and chronic exposure to B(a)P was studied in rats.

In the acute exposure studies, an evaluation of apoptosis following treatment with B(a)P was assessed. The B(a)P treated rats received 24 ng (13 μ L) of B(a)P instilled intratracheally, while similar volumes of tricaprilyn (Tr) was administered to rats from the Tr group. Rats not receiving any treatment served as controls. An assessment of apoptosis was made on haematoxylin and eosin



(H&E) stained histologic sections, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis and DNA laddering of lung samples. Rats in the B(a)P group were killed at 1, 2, 4, 8, 16, and 24 hours post-instillation (p.i.) and rats from the control and Tr groups were only killed 24 hours p.i.

Apoptosis at different stages was found in the pneumocyte and bronchial epithelium of B(a)P-treated rats killed 8, 16 and 24 hours p.i. This was also confirmed positive by TUNEL analysis and DNA laddering.

In the chronic exposure studies, changes in the lung of B(a)P-induced rats during a three month period encompassing p53 expression, proliferating cell nuclear antigen (PCNA) expression, immune response (IgA, IgG levels and alveolar macrophage activity), levels of glutathion s-transferase (GST) marker enzyme and the effect of raw garlic as an anti-tumour agent were studied.

Rats from the control, B(a)P and Tr group were daily fed on a commercial basal diet while rats from the garlic (G) and [B(a)P+G] group were fed the basal ration containing garlic incorporated at the rate equivalent to an intake of 80mg/kg bodyweight/rat/day.

The results showed growth disturbances in pneumocytes and bronchial epithelium of rats from the B(a)P group. Apoptosis was detected in four rats from the B(a)P+G group. The PCNA positive areas were only found in hyperplastic



areas in the lungs of rats from the B(a)P-treated group. In addition, rats treated with B(a)P and B(a)P+G had lower levels of IgA, IgG, alveolar macrophages activities and glutathione S-transferase in the lung.

In conclusion, either short or long term exposures to B(a)P produce detrimental changes to lungs of rats and garlic has great potential in alleviating the chronic effects.



Abstrak tesis telah dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains Veterinar

KAJIAN KEATAS KESAN BENZO(a) PIREN PADA TIKUS

Oleh

TIHORIA ALI ABDUL HAMEED DRAGII

March 2001

Pengerusi: Dr. Noordin Mohamed Mustapha

Fakulti: Perubatan Veterinar

Kepekatan bahan karsinogen, benzo(a)piren [B(a)P] yang tinggi telah dikesan pada episod jerebu di Malaysia. Kajian mengenai kesan akut dan kronik pendedahan kepada B(a)P telah dijalankan ke atas tikus bagi merancang strategi mengurangkan kesan merbahaya terhadap manusia.

Dalam kajian pendedahan akut, penilaian terhadap apoptosis secara histologi, analisis pelabelan potongan hujung dUTP-biotin deoksinukleotid perantara transferase (TUNEL) dan tangga DNA pada lavaj bronkiol-alveolus. Tikus dari kumpulan B(a)P menerima sebanyak 24 ng (13 μ l) B(a)P yang diberi secara



intratrakea, manakala isipadu tricaprylin (Tr) yang sama telah diberi kepada tikus dari kumpulan Tr. Tikus yang tidak menerima apa-apa rawatan bertindak sebagai kawalan. Tikus dari kumpulan B(a)P telah dibunuh pada 1, 2, 4, 8, 16 dan 24 jam pasca-pemberian (p.i) manakala tikus daripada kumpulan lain hanya dibunuh selepas 24 p.i.

Apoptosis pada pelbagai peringkat telah dilihat pada pneumosit dan epitelium bronkiol tikus dari kumpulan B(a)P yang dibunuh selepas 8, 16, dan 24 jam p.i. Ujian TUNEL dan tangga DNA memberikan keputusan yang sama.

Pada kajian pendedahan kronik, perubahan pada paru-paru tikus dikaji 3 bulan selepas menerima rawatan dengan B(a)P merangkumi p53, antigen pemroliferatan nuklias sel (PCNA), gerakbalas imun, aras enzim petunjuk dan kesan bawang putih mentah sebagai anti-tumor.

Tikus dari kumpulan kawalan, B(a)P dan Tr diberi makan makanan komersial manakala tikus daripada kumpulan bawang (G) dan B(a)P+G menerima makanan dengan penambahan bawang putih mentah pada kadar 80 mg/kg berat/tikus/hari.

Keputusan kajian menunjukkan gangguan tumbesaran pada pneumosit dan epitelium bronkiol pada tikus dari kumpulan B(a)P. Apoptosis telah dikesan pada empat ekor tikus dari kumpulan B(a)P+G. Tindakbalas positif PCNA hanya didapati

pada kawasan hiperplasia di paru-paru tikus dari kumpulan B(a)P sahaja. Tambahan lagi, paru-paru tikus yang diberi B(a)P dan B(a)P+G mempunyai aras IgA, IgG, aktiviti makrofaj alveolus dan glutathion S-transferase yang rendah.

Sebagai rumusan, pendedahan akut atau kronik B(a)P menjana perubahan membahayakan pada paru-paru tikus dan bawang putih mempunyai potensi besar untuk mengurangi kesan kronik B(a)P.

ACKNOWLEDGEMENTS

All praise to Almighty Allah, the Merciful and the Benevolent. Had it not been due to His will and favour, the completion of this study would not have been possible.

I would like to express my sincere gratefulness and appreciation to my supervisor, Dr. Noordin Mohamed Mustapha, who has devoted much of his time for invaluable guidance, advice, supervision, and support throughout the course of this study.

I wish to express my sincere gratitude to the Dean of the Faculty of Veterinary Medicine, Professor Dato' Dr. Sheikh Omar Abdul Rahman who is also my co-supervisor, for his invaluable advice, support and continuous encouragement towards the completion of this work.

Sincere thanks are also due to my other co-supervisors, Dr. Mohd Azmi Mohd Lila who has granted the use of his laboratory that have enlightened and improved this study.

I would like to express my gratitude to Dr. Daud Ahmad Israf Ali who has unselfishly provided facilities in his laboratory during the course of this study.



I would also like to express my heartfelt gratitude to Dr. Panayiotis Loukopoulos from the School of Veterinary Science, University of Queensland, Australia for his assistance in immunohistochemistry techniques.

I have also been very fortunate in receiving assistance from a number of my colleagues and friends. Many of them went out of their way to assist me and it would not be possible to name all of them. However, I would like to thank Ms. Abeer Hasan Sahtout, Mr. Isam Qudssiah, Puan Zunita Zakaria and Puan. Hazilawati Hamzah.

I am deeply indebted to the staff members of the post-mortem laboratory and Mr. Fauzi Che Yusof who were involved either directly or indirectly in sharing their knowledge and skilled assistance during this study.

Continuous moral support and encouragement from my mother-in-law during my study is deeply appreciated.

Grateful thanks are also conveyed to my supportive husband, Dr. Muthafar H. Al-Haddawi and children Zeinab, Hussain and Ridah who have long put up with my late nights and weekends at the office so that I could complete this work.




I certify that an Examination Committee met on 16th March 2001 to conduct the final examination of Thoria Ali Abdul Hamced Dragh on her Master of Veterinary Science thesis entitled “Studies on The Effect of Benzo(a)Pyrene in Rats” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follow:

Daud Ahmad Israf Ali, DVM., MS., Ph.D
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Noordin Mohamad Mustapha, DVM, MS., Ph.D
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Dato’ Sheikh Omar Abdul Rahman, B.V.Sc., M.V.Sc., M.R.C.V.S
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Mohd Azmi Mohd Lila, DVM, MBA, Ph.D
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)



MOHD GHAZALI MOHAYIDIN, Ph.D
Professor
Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 04 MAY 2001



This thesis submitted to the senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Sciences.

AINI IDERIS, Ph.D,
Professor
Dean of Graduate School,
Universiti Putra Malaysia

Date:



I hereby declare that the thesis is based on 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 UPM or other institutions.



Thoria Ali Abdul Hameed Dragh, BVM&S

Date: 4.5.2001

TABLE OF CONTENTS

DEDICATION	ii
ABSTRACT	iii
ABSTRAKT.....	vi
ACKNOWLEDGEMENTS	ix
APPROVAL SHEETS.....	xi
LIST OF TABLES.....	xvi
LIST OF PLATES	xvii
LIST OF ABBREVIATIONS	xix

CHAPTER	Page
I INTRODUCTION.....	1
II LITERATURE REVIEW.....	4
Air pollution.....	4
Haze Episodes in Malaysia.....	7
Benzo(a)Pyrene.....	8
Physical and Chemical Properties of Benzo(a)Pyrene.....	8
Metabolism and Toxicity of Benzo(a)Pyrene.....	8
Benzo(a)Pyrene as Immunosuppressor.....	11
Apoptosis.....	13
Benzo(a)Pyrene as Carcinogen.....	14
Type of Cancer Induced by Benzo(a)Pyrene.....	17
Strategies in alleviating Benzo(a)Pyrene-induced injury.....	19
Garlic.....	19
Constituents and Beneficial Effect of Garlic (<i>Allium Sativum</i>)... 20	20
Anticarcinogenic and Antitumor potential of garlic	22
Toxicity of Garlic.....	26
III ACUTE EXPOSURE TO BENZO(a)PYRENE	
Introduction.....	28
Materials and Methods.....	29
Animals and Management.....	29
Inoculum.....	29
Experimental Design.....	29
Pathology.....	30
Detection of DNA Laddering.....	32
Detection of p53 Tumour Suppressor Gene.....	34
Results	36
Discussion.....	40



IV	CHRONIC EXPOSURE TO BENZO(a)PYRENE	
	Introduction.....	43
	Materials and Methods.....	46
	Animals.....	46
	Inoculum.....	46
	Garlic.....	46
	Experimental Design.....	46
	Immunohistochemistry.....	47
	Immunology.....	48
	Glutathion S trnsferase Estimation in Lung Tissue.....	51
	Results.....	52
	Clinical Signs.....	52
	Pathology.....	52
	Immunohistochemistry.....	58
	Immunology	60
	Glutathion S-transferase Activity.....	61
	Discussion	62
V	GENERAL DISCUSSION AND CONCLUSIONS.....	70
	REFERENCES	74
	APPENDICES.....	89
	BIODATA OF THE AUTHOR	93



LIST OF TABLES

Table	Page
1. The Major and Important Trace Flavour Compounds Found in Garlic.....	20
2. The Experimental Design of Acute Exposure to Benzo(a)Pyrene in Rats....	30
3. The Experimental Design of Chronic Exposure to Benzo(a)Pyrene in Rats..	47
4. The level of (OD) IgA and IgG in the lung lavage of rats at necropsy (Mean±SD).....	60
5. The level (OD) of IgG in the serum of rats at necropsy (Mean±SD).....	60
6. The alveolar macrophage activities in the lung lavage of rats (Mean±SD).....	61
7. The activity (OD) of GST in lung lavage of rats at necropsy (Mean±SD).....	62



LIST OF PLATES

Plate		Page
1	Photomicrograph, lung, rat from the B(a)P group killed 8 hrs p.i. Note apoptotic cell (arrow head) with chromatin condensation and compaction of cytoplasm by development of vacuoles. H&E x 500.....	37
2	Photomicrograph, lung, rat from the B(a)P group killed 16 hrs p.i. Note apoptotic cell (arrow head) with peripheral and margination of chromatin along the nuclear membrane. H&E x 500.....	37
3	Photomicrograph, lung, rat from the B(a)P group killed 16 hrs p.i. Note the apoptotic cell (arrow heads) yellowish green in colour and the normal cells red in colour (arrow). Fluorescein stain x 500.....	38
4	Agarose (1%) gel electrophoresis of PCR products of DNA of rats inoculated with B(a)P and control, lanes 1 and 2 are amplified DNA of rats killed 2 and 4 hrs p.i., Lane 3 is a molecular weight marker (100bp), lane 4 represents a DNA of a rat killed 16 and 24 hrs p.i. lane 5 represents a DNA of rats killed 8 hrs p.i. and lane 6 represents a DNA of rats from control group.....	39
5	Photomicrograph, lung, from the B(a)P treated group killed 24 hrs pi. Note the faint blue staining of the nuclei indicating the absence of p53 protein. (H&E x 500).....	39
6	Photograph, lung of rat from the B(a)P group killed 90 days p.i. Note the presence of pin-point whitish nodules (arrow).....	52
7	Photograph, lung of rat from the B(a)P group killed 90 days p.i. Note the presence of haemorrhagic spots on the right apical lobe (arrow).....	53
8	Photomicrograph, lung, from the garlic group killed at necropsy. Note hyperplasia of bronchial associated lymphoid tissue (BALT) with extension of lymphoid tissue toward the epithelial lining of bronchus. H&E x 370.....	54



9	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note proliferation of mononuclear cells (predominantly lymphocytes and macrophages) in lamina propria of trachea. H&E x 370.....	54
10	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note increase in alveolar wall thickening due to epithelisation (arrow). H&E x 140.....	56
11	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note large proliferated polygonal cells with pale and vacuolated cytoplasm. H&E x 370.....	56
12	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note presence of large cells with scanty acidophilic cytoplasm and some of which appeared with two nuclei (arrow). H&E x 370.....	57
13	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note presence of cluster of cuboidal cells forming glandular structure (arrow head). The area surrounding these cells was infiltrated with mononuclear cell (predominantly lymphocytes). H&E x 370.....	57
14	Photomicrograph, lung, from the B(a)P group killed at necropsy. Note the PCNA positive cells with yellowish brown nuclei (arrow heads) and those of negative cells were faint blue (arrow). H&E x 100.....	60



List of Abbreviations

AFC	Antibody-forming cells
AmØ	Alveolar macrophage
ANF	α -naphthoflavn
B(a)P	Benzo(a)pyrene
BALT	Bronchial associated lymphoid tissue
bp	Base pair
B-PBS	Bovine serum-phosphate buffered solution
BPDE	Benzo(a)pyrene-7,8-diol-9,10-epoxide
Cx	Control
DAB	3'3'diaminobenzidinc-tetrahydrochloride
DAS	Diallyl sulfide
DCNB	1,2 dichloro-4nitrobenzene
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
ELISA	Enzyme linked immunosorbant assay
g	Grams
G	Garlic
GST	Glutathion S-transferase
H&E	Hematoxylin and eosin
hr	Hour
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
KCl	Potassium chloride
M	Molar
MNU	Methylnitrosurea
ng	Nano-gram
p.i	Post instillation
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered solution
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PM	Particulate matter
RBC	Red blood cells
TNF- α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidal transferase-mediated DUTP-biotin nick end labeling
μ g	Microgram
μ l	Microliter



CHAPTER I

INTRODUCTION

Airborne pollutants produce a wide variety of harmful effects on humans, animals and plants. Chemicals are released due to coal combustion, gasification, emissions, biomass burning, coal and tobacco smoke that may interact with different metabolic systems in human tissues and cell and eventually cause cancer (Tokiwa *et al.*, 1998). Among these chemicals is a group of multi-ring hydrogen and carbon compounds known as polycyclic aromatic hydrocarbons (PAHs), which are commonly found in the emissions from burned plant and petroleum products. Deteriorating air quality due to an increase in PAH concentration will cause increasing adverse effects on human health. (Omland *et al.*, 1994; Hock *et al.*, 1997; Scarlett *et al.*, 1996) One of the most studied of the PAHs is benzo(a)pyrene [B(a)P], which is an ubiquitous environmental pollutant present in coal tar, cigarette smoke and biomass burning (Yang *et al.*, 1997).

In heavily polluted regions, it has been found that air pollution can cause congenital anomalies especially those who are exposed to high levels of PM₁₀, pollutant particles of less than 10 micrometers in diameters. The DNA damage by PAHs has been reported (Leadon *et al.*, 1995) to be due to adduction of that can lead to mutations and increase the probability of developing cancer (Nakanishi *et al.*, 1997).



Studies in animals have shown that dermal exposure to B(a)P and PAH can cause skin cancer. Effects from breathing or ingestion were also reported to draw a connection with other kinds of cancer. Animal studies have shown B(a)P to be teratogenic, embryotoxic, and mutagenic (Lu *et al.*, 1986; Nesnow *et al.*, 1998a). Other animal tests have shown that exposure to B(a)P may cause reproduction difficulty (Zenzes *et al.*, 1999).

Although acute toxicity appears to be low for humans, sub-chronic and chronic toxic effects exist. Some PAHs such as B(a)P are known potent immunosuppressors (White and Holsapple, 1984; Thomas *et al.*, 1987).

There is a growing concern about the health effects of air pollution in the world (Scarlett *et al.*, 1996). Many epidemiological and experimental studies have been conducted to examine the effect of air pollution on health (Dockery and Pope, 1994; Pope *et al.*, 1995; Scarlett *et al.*, 1996).

In Malaysia there has been considerable concern about the effect of pollution arising from haze. In 1997, Malaysia and other Southeast Asia countries have been exposed to a severe haze episode. However, no studies have examined the effect of air pollution on lung function in human or animals.

The aim of this study was to determine the effect of haze on health by using rats as models for humans. Likewise, it is also aimed at evaluating

environmental pollution caused by haze and the relationship between the concentration of B(a)P during the haze episode and the outcome of lung injury.

The objectives of this study were:

- i. to determine the manner of cell death during acute exposure to B(a)P
- ii. to determine the development and type of tumour induced by B(a)P
- iii. to determine the sensitive and reliable indicators of tumours induced by B(a)P
- iv. to assess the efficacy of garlic in alleviating B(a)P induced injury

CHAPTER II

LITERATURE REVIEW

Air Pollution

Undoubtedly, over decades air pollution is a problem of growing national and international interest. Air pollution can be defined as any atmospheric condition when excessive substances are present above normal ambient levels to produce measurable effects on man, animals, vegetation or materials. Substances refer to any natural or man-made chemical elements or compounds capable of being airborne. These substances may exist in the gaseous, liquid or solid states in the atmosphere (Painter, 1974).

The earliest pollutants noted in the atmosphere were probably of natural origin. Man, plants, animals, and the act of nature would contribute to pollution (Ottar, 1987).

The origin of air pollution varies between smoke, fumes, ashes and gases from volcanoes and forest fires, sand and dust from wind storms in arid region, fog in humid, low-lying areas and natural trepan hazes from pine trees in mountainous regions (Ross, 1972).

Haze is an atmospheric phenomenon caused by the presence of tiny particulate suspended in air. These particulate, which are molecular in size, scatter and absorb sunlight resulting in diminished visibility giving the atmosphere a characteristic opalescent appearance (Hassan *et al.*, 1998).

Particulate matter (PM) which is produced by the burning of wood, diesel and other fuels by agriculture activities and industrial processes is made up of ash, smoke, soot, dust, fibers and liquid droplets. Apart from impairing ambient visibility, the smaller PM can be inhaled and over an extended period of time can injure the respiratory system.

Particulate matter can be defined as any material that can remain in the atmosphere or gas stream at standard conditions in the solid or liquid states. The term PM_{10} and $PM_{2.5}$ are primary particulate referred to during the occurrence of an air pollution. The PM_{10} is a particle with an aerodynamic diameter less than or equal to $10\mu m$ and $PM_{2.5}$ has an aerodynamic diameter less than $2.5\mu m$. These particles represent health hazard, enhance atmospheric chemical reactions, and environmental response as well as negatively affecting aesthetic appearances (Heil, 1998).

Despite a poorly understood mechanism, epidemiological studies have documented the association between fine particulate air pollution especially particles of the size of PM_{10} or less with the development of pulmonary diseases. Inhalable particles with a diameter over $10\mu m$ are predominantly deposited in the