



**UNIVERSITI PUTRA MALAYSIA**

**THE EFFECT OF BENZO (A) PYRENE (BAP) ON THE  
RESPIRATORY TRACT OF DOGS**

**HAZILA WATI HAMZAH, D. V.M**

**FPV 2000 3**

**THE EFFECT OF BENZO(A)PYRENE (BAP) ON THE RESPIRATORY  
TRACT OF DOGS**

**By**

**HAZILAWATI HAMZAH, D.V.M**

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

**September 2000**



## **DEDICATION**

**This thesis is dedicated with appreciation to my  
Husband, father and mother, father and mother- in- law, Wan,  
Abang, Along, Mie, Kak Yan, Dr. Lan, Dr. Rina, Imah, Ayo, Ijam and Adik Wan,  
who provide my inspiration,  
and also not forget to  
Nabilah Huda, Nurul Farahana Hazira, Nurul Hanis Fazliana and Mohd. Afiq:  
"May the understanding of these impacts reduce the  
burden they impose on all our lives".**

**-WATI-**



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

**THE EFFECT OF BENZO(A)PYRENE (BAP) ON THE RESPIRATORY  
TRACT OF DOGS**

**By**

**HAZILAWATI HAMZAH, D. V. M.**

**September 2000**

**Chairman: Dr. Noordin Mohamed Mustapha**

**Faculty: Veterinary Medicine**

The global impact of air pollution encompasses the population health and the economic status of a nation. Air pollution or 'haze' contains a variety of noxious agents including benzo(a)pyrene (BaP). This compound is known to induce acute or chronic deleterious effects. The objectives of the study were to determine the effect of BaP on the physiology, defense mechanism and pathology of the lung, to suggest sensitive diagnostic techniques for the diagnosis of early carcinogenesis and to recommend preventive measures to minimise the effect of BaP.



An experiment was conducted in 27 dogs that are allocated to nine groups simulating different environmental condition and health status. The groups comprising of three dogs each were as follows: control, BaP, cyclosporine (Cyclo), Selenium (Se), BaP+Cyclo, BaP+Se, BaP+Cyclo+Se and Tricaprylin (Tri). Benzo(a)pyrene was given at the dose of 120  $\eta$ g/dog intratracheally twice, six week apart, Se 20  $\mu$ g/dog/day and cyclosporine at the dose 50 mg/m<sup>2</sup>. The tidal volume (Vt) and whole blood glutathione peroxidase (GSH-PX) activity was analysed weekly for 12 weeks. While at necropsy, bronchoalveolar lavage (BAL) cytology, alveolar macrophage (AMØ) activities, BAL immunoglobulin (Ig) G and Ig A level, gross and histopathology of lungs were also analysed.

The finding revealed that the tidal volume (Vt) remain unchanged in all groups during the experimental period. The pulmonary immune response includes AMØ number, phagocytic and intracellular killing activities, and Ig A level in bronchoalveolar lavage (BAL) that was markedly suppressed in the BaP, Cyclo and BaP+Cyclo groups. Subsequently, the BaP and BaP+Cyclo+Se group exhibited gross and microscopic appearance of tumorigenesis, which was diagnosed as pulmonary adenocarcinoma with expression of mutant p53 protein while the BaP+Cyclo and BaP+Se had atypical adenomatous hyperplasia (AAH).

Based on the finding, exposure to BaP can lead to pulmonary immuno-suppression and tumorigenesis in dogs. It is also showed that during haze episode, immuno-stressed

individuals are more prone to the development of pulmonary immuno-suppression and tumorigenesis. Selenium supplementation or cyclosporine has great potential in combating these deleterious effects. However, simultaneous supplementation of Se together with cyclosporine during haze is not advised, since this will promote tumorigenesis in the lung.

In conclusion, intratracheal instillation (twice, six week apart) of 120  $\eta$ g BaP/dog causes insignificant reduction of  $V_t$ , pulmonary immunosuppression and pulmonary carcinogenesis. The immunocytochemical detection of p53 can be used as a sensitive diagnostic technique for the diagnosis of early pulmonary carcinogenesis. Daily oral administration of Se as a supplement has great potential in minimising the adverse effect of BaP.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Mater Sains Veterinar.

## **KESAN BENZO(A)PYRENE KE ATAS SISTEM PERNAFASAN ANJING**

**Oleh**

**HAZILAWATI HAMZAH, D. V. M.**

**September 2000**

**Pengerusi: Dr. Noordin Mohamed Mustapha**

**Fakulti: Perubatan Veterinar**

Kesan global pencemaran udara memudaratkan kesihatan populasi dan juga status ekonomi negara. Pencemaran udara atau 'jerebu' mengandungi berbagai jenis bahan-bahan merbahaya termasuk benzo(a)pyrene (BaP). Bahan ini diketahui boleh menyebabkan kesan akut dan kronik yang merbahaya. Tujuan ujikaji ini dijalankan adalah untuk menentukan kesan BaP ke atas fisiologi, mekanisma pertahanan dan patologi paru-paru, untuk mencadangkan teknik diagnosis yang sensitif untuk diagnosis awal barah paru-paru dan mencadangkan untk mencadangkan langkah-langkah pencegahan bagi meminimumkan kesan BaP.



Ujikaji telah dijalankan pada 27 ekor anjing yang telah dibahagikan kepada sembilan kumpulan berdasarkan ke atas simulasi persekitaran dan status kesihatan yang berbeza. Kumpulan-kumpulan tersebut yang masing-masing mempunyai tiga ekor anjing adalah: kontrol, BaP, siklosporin (Cyclo), Selenium (Se), BaP+Cyclo, BaP+Se, BaP+Cyclo+Se dan Trikaprilin (Tri). Benzo(a)pyrene (BaP) telah disuntikkan pada dos 120  $\eta$ g/anjing secara intratrakea sebanyak dua kali berselang enam minggu. Isipadu tidal dan aktiviti glutathion peroksidase (GSH-Px) darah telah dianalisis setiap minggu selama 12 minggu. Sitologi dan basuhan bronkiol alveolus (BAL), aktiviti makrofaj alveolus (AMØ), tahap immunoglobulin (Ig) G dan Ig A dalam BAL, patologi makro dan mikro paru-paru telah dianalisis semasa nekropsi.

Hasil kajian menunjukkan isipadu tidal (Vt) kekal tidak berubah di dalam semua kumpulan sepanjang jangkamasa ujikaji. Tindakbalas keimunan paru-paru termasuk jumlah AMØ, aktiviti fagositosis dan pembunuhan intrasel, dan paras Ig A di dalam BAL menunjukkan perubahan yang sangat ketara dalam kumpulan BaP, Cyclo dan BaP+Cyclo. Seterusnya, kumpulan BaP dan BaP+Cyclo+Se menunjukkan pembentukan barah paru-paru secara kasar dan mikroskopi, yang mana telah didiagnosis sebagai adenokarsinoma pulmonari dengan kemunculan protin mutan p53, sementara itu kumpulan BaP+Cyclo dan BaP+Se mempunyai hiperplasia atipikal seperti adenoma (AAH).

Berdasarkan kepada penemuan ini pendedahan kepada BaP boleh menyebabkan penurunan keimunan pulmonari dan pembentukan barah pada anjing. Ini juga



menunjukkan bahawa semasa jerebu, individu yang mempunyai tahap keimunan yang rendah lebih mudah terdedah kepada penurunan keimunan pulmonari dan pembentukan barah. Pengambilan Se atau siklosporin mempunyai potensi yang besar untuk melawan kesan bahaya ini. Walau bagaimanapun, pengambilan Se serentak bersama siklosporin semasa jerebu adalah sangat tidak digalakkan kerana ia akan merangsangkan pembentukan barah di dalam paru-paru.

Kesimpulannya, suntikan BaP secara intratrakea (dua kali, berselang enam minggu) pada dos 120  $\eta$ g/anjing menyebabkan penurunan Vt yang tidak ketara, penurunan mekanisme pertahanan paru-paru dan pembentukan barah pulmonari. Pengesanan p53 secara immunositokimia boleh digunakan sebagai satu teknik diagnosis yang peka bagi karsinogenesis awal. Pengambilan tambahan Se secara oral setiap hari mempunyai potensi yang besar untuk meminimumkan kesan buruk BaP.

## ACKNOWLEDGMENTS

I am especially grateful to my supervisors, Dr. Noordin Mohamed Mustapha, Prof. Dato' Dr. Sheikh Omar Abdul Rahman, and Dr. Daud Ahmad Israf Ali for their help in many ways and for their consistent advice, encouragement, moral support and excellent supervision throughout the course of the study.

I am also particularly grateful to Dr. Panayiotis Loukopoulos, Dr. Ng Kok Han, Mrs. Nor Azura Salim, Mrs. Azlina Mohd Salim, Mrs. Hartina Abdul Khan, Mr. Hari Govindan, Mrs. Azimah and Mrs. Sakdiah for their kind guidance in immunology and special staining technique. Thank is also extended to the excellent help of Tuan Haji Mohamad Nor and Mr. Jamil for their guidance in processing and preparation of histology specimens. Special thanks to Dr. Shizhen Zhang, Dr. Thoria, Dr. Muthafar, Dr. Goh Yong Meng, Mr. Ghazali Yusof, Mr. Noraziman Sulaiman, Mr. Apparao a/l Somanaidu, Chamadre a/l Vengadasamy, Miss. Maizatul Akmal Moktar, all the staff of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (U.P.M) for their technical support and to all the staff of the Dog Unit, Dewan Bandaraya Kuala Lumpur, for providing healthy dogs for this study.

Sincere gratitude is also conveyed to the Ministry of Science, Technology and the Environment of Malayisa for the provision of the IRPA grant (06-02-04-0071) and the National Sciences Fellowship for the scholarship.



Last but not least, the consistent moral and technical support, patience and understanding of my loving husband, Dr. Mohd Rosly Shaari throughout the course of the study will always be remembered and appreciated.



## TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL SHEETS	xi
DECLARATION FORM	xiii
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
LIST OF PLATES	xxi
LIST OF ABBREVIATIONS/NOTATIONS/GLOSSARY OF TERMS	xx
 <b>CHAPTER</b>	
I	
INTRODUCTION	1
Air Pollution	1
Haze	1
Haze Episodes in Malaysia	2
II	
LITERATURE REVIEW	7
The Physiology and Anatomy of the Respiratory System	7
The Conducting Airway of the Respiratory Tract	8
The Gaseous Exchange Portion	10
The Pathophysiology of the Lung during Air Pollution	12
Pulmonary Function Test	12
Clinical Signs	14
Functional Changes	15
Patterns of Disordered Lung Function	17
The Pulmonary Defense System during Air Pollution	19
Pulmonary Defense Mechanism	20
Pulmonary Immunosuppression	23
Prevention and Treatment	26
Benzo(a)pyrene (BaP)	31
Pathological Changes of the Lung during the Influx of Polluted Air	37
Pathological Changes of Lung Injury by pollutants	37
Classification of Lung Cancer	37
Influence of Lung Tumour on Proto-oncogenes, p53 and PCNA	38
General Summary	44



III	METHODOLOGY	48
	Experimental Design	48
	Assessment of Lung Function	49
	Measurement of the Physiological Lung Function	49
	Bronchiol Alveolar Lavage (BAL)	50
	Bronchiol Alveolar Lavage (BAL) Cytology	50
	Acridine Orange Chemilumescence (AO) Assay	51
	Immunoglobulin (Ig) Assay	53
	The Measurement of the GSH-Px Activity (DTNB Direct Method)	54
	Examination of Pathological Changes of the Lung	55
	Histopathological Examination of the Lung	55
	Immunocytochemistry of the Tumour Suppressor Gene and Proliferating Cell Nuclear Antigen (PCNA)	56
	Statistical Analysis	59
	Discussion	60
IV	THE EFFECT OF BENZO(A)PYRENE (BAP) ON THE PULMONARY PHYSIOLOGY OF DOGS	64
	Introduction	64
	Materials and Methods	64
	Results	65
	Clinical Signs	65
	Functional Changes	65
	Discussion	66
V	THE EFFECT OF BENZO(A)PYRENE (BAP) ON THE PULMONARY DEFENSE SYSTEM OF DOGS	68
	Introduction	68
	Materials and Methods	69
	Results	69
	The Glutathione Peroxidase (GSH-Px) Activity	69
	Bronchiolalveolar Lavage (BAL) Cytology Examination	71
	An of alveolar Macrophages (AMØ) Activities	72
	Immunoglobulin (Ig) Levels in Bronchoalveolar Lavage (BAL)	74
	Discussion	75



VI	THE PATHOLOGY OF BENZO(A)PYRENE (BAP)- INDUCED LUNG INJURY IN DOGS	86
	Introduction	86
	Materials and Methods	86
	Results	87
	The incidence of tumour-like lesions	87
	Gross Pathology	87
	Microscopic Pathology	88
	Immunocytochemistry and special stain	95
	Discussion	96
VII	GENERAL DISCUSSION AND CONCLUSION	105
	REFERENCES	110
	APPENDICES	125
	BIODATA OF THE AUTHOR	138



## LIST OF TABLES

- Table 2.1: Histologic classification of lung tumours
- Table 3.1: The experimental design and its analog to the experimental and health status
- Table 4.1: The average body weight (kg), and tidal volume ( $V_t$  mL/ breath) for the 12 week-period (mean  $\pm$  SD), correlation of tidal volume ( $V_t$ ) on body weight, and comparison of linear regression.
- Table 5.1: The average activity of whole blood Glutathione Peroxidase (GSH-Px) of dogs for the 12 week-period.
- Table 5.2: Bronchiol Alveolar Lavage (BAL) of dogs at necropsy (mean  $\pm$  SD).
- Table 5.3: Alveolar Macrophage (AM $\emptyset$ ) activities in dogs at necropsy (mean  $\pm$  SD).
- Table 5.4: Levels of Immunoglobulin (Ig)G and IgA in the Bronchiol Alveolar Lavage (BAL) of dogs at necropsy (mean  $\pm$  SD).
- Table 6.1: The presence of tumour-like lesions in the lung of dogs at necropsy.
- Table 6.2: The microscopic presence of tumour-like lesions in the lung of dogs.
- Table 6.3: The scoring of tumour-like lesions in the lung of dogs.
- Table 6.4: The percentage of Atypical Adenomatous Hyperplasia (AAH) in the individual lung lobe of dogs.
- Table 6.5: The immunocytochemistry and special staining of tumour-like lesions in the lung of dogs.
- Table 6.6: The scoring of nuclear p53 in the lung of dogs.
- Table 6.7: The scoring of cytoplasm p53 in the lung of dogs.



## LIST OF FIGURES

Figure 1.1: Spirometric volumes.

Figure 5.1: The Glutathione Peroxidase (GSH-Px) of dogs during the experimental period.





## LIST OF PLATES

- Plate 5.1: The photomicrograph of an AMØ in Bronchiol Alveolar Lavage (BAL) with phagocytosed bacteria.
- Plate 6.1: Photomicrograph, lung, dog from BaP group. Normal and Atypical Adenomatous Hyperplasia (AAH) of the Lung (x 120, H & E).
- Plate 6.2: Photomicrograph, lung, dog from BaP group. Atypical Adenomatous Hyperplasia (AAH) of the lung (x 250, H & E).
- Plate 6.3: Photomicrograph, lung, dog from BaP group. Adenocarcinoma of the lung (x 120, H & E).
- Plate 6.4: Photomicrograph, lung, dog from BaP group. Adenocarcinoma of the lung (x 250, H & E).
- Plate 6.5: Photomicrograph, lung, dog from BaP group. Adenocarcinoma of the lung (x 250, H & E).
- Plate 6.6: Photomicrograph, lung, dog from BaP. Adenocarcinoma of the lung (x 500, H & E).
- Plate 6.7: Photomicrograph, lung, dog from BaP group. Adenocarcinoma of the lung (x 500, H & E).
- Plate 6.8: Photomicrograph, lung, dog from BaP group. Adenocarcinoma of the lung (x 500, H & E).
- Plate 6.9: Photomicrograph, lung, dog from BaP group. Atypical Adenomatous Hyperplasia (AAH) of the lung (x 250, Alcian Blue).
- Plate 6.10: Photomicrograph, lung, dog from BaP group. The cytoplasmic staining of p53 (x 400, p53 Immunocytochemistry Stain).



## LIST OF ABBREVIATIONS/NOTATIONS/GLOSSARY OF TERMS

AAH	atypical adenomatous hyperplasia
ADCC	antibody-dependent cellular cytotoxicity
AHH	aryl hydrocarbon hydroxylase
AMØ	alveolar macrophage
AO	acridine orange
APC	antigen presenting cell
BAC	bronchoalveolar carcinoma
BAL	bronchiol alveolar lavage
BALT	bronchial associated lymphoid tissue
BaP	benzo(a)pyrene
BSA	bovine serum albumin
CO <sub>2</sub>	carbon dioxide
COPD	chronic obstructive pulmonary disease
CSI	cytoplamic staining intensity
Cyclo	cyclosporine
CV	crystal violet
DA <sub>2</sub> PL+CPV	distemper adenovirus type 2 parainfluenza leptospira + canine parvo virus
DAB	diaminobenzidin
Dm	capillary membrane
DMBA	dimethylbenz(a)pyrene
DNA	deoxyribonucleic acid
DTNB	dithio-bi-nitrobenzoic acid
ELISA	enzyme link immunosorbent assay
EM	electron microscope
ERV	expiratory reserve volume
FCS	fraction of cytoplasmic staining
FRV	functional reserve volume



FEV	force expiratory volume
FEV <sub>1</sub>	force expiratory volume in one second
FPN	fraction of positive nuclei
FPC	fraction of positive cytoplasm
GSH-Px	glutathione peroxidase
GST	glutathione S-transferase
GSTM1	glutathione S-transferase M1
LAL	left apical lobe
LALN	lung associated lymph node
LCL	left cardiac lobe
LDL	left diaphragmatic lobe
H & E	haematoxylin & Eosin
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
Ig	immunoglobulin
IL	Interleukin
IL-2R	Interleukin 2 receptor
ILDs	interstitial lung disease
IRV	inspiratory reserve volume
NAC	n-acetylcysteine
NCLC	non-small cell lung cancer
NO	oxide of nitrogen
NRC	National Research Council
NSI	nuclear staining intensity
PAH	polycyclic aromatic hydrocarbon
PAS	Periodic Acid-Schiff
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCO <sub>2</sub>	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PM	particulate matter
RAL	right apical lobe



RacL	right accessory lobe
RBC	red blood cell
RCL	right cardiac lobe
RDA	recommended daily allowance
RDL	right diaphragmatic lobe
RR	respiratory rate
RV	residual volume
rhIL2	recombinant human Interleukin 2
S	sulphur
SCLC	small cell lung cancer
SD	standard deviation
Se	selenium
SO <sub>2</sub>	sulphur dioxide
SPM	suspended particulate matter
SRBC	sheep red blood cell
Th	T helper
TLC	total lung capacity
Tri	tricaprylin
VC	vital capacity
V <sub>m</sub>	minute ventilation
V <sub>t</sub>	tidal volume
WHO	World Health Organization

# CHAPTER 1

## INTRODUCTION

### Air Pollution

Over centuries, the human population is very much concerned about air pollution, primarily in occupational settings and outdoors in urban areas that are mostly derived from automobile exhaust, industrial smoke and mines. Recently, air pollution becomes an important issue since its tremendous impacts are global, not only to animal and human health, but also to the economy of a nation. Nowadays, the major factor contributing to the air pollution phenomena termed 'haze', is large scale open burning of forest. It usually happened during recultivation in dry season for example at Kalimantan and Sumatra, Indonesia. Air pollutant components originating from biomass burning includes particulate matter (PM), polycyclic aromatic hydrocarbon (PAH), sulfur dioxide (SO<sub>2</sub>), oxides of nitrogen (NO) and formaldehyde (Usmani *et al.*, 1998).

### Haze

Haze is defined as suspended particles that are dispersed through a portion of the atmosphere. It is invisible to the naked eye and will grow in size as humidity increases.



The formation of a haze layer requires a source of haze particles and a relatively stable atmospheric condition in the lower layer of the atmosphere.

Atmospheric air PM with an aerodynamic diameter of  $2.5 \mu\text{m}$  and less, and  $2.5 \mu\text{m} - 10 \mu\text{m}$  is defined as PM<sub>2.5</sub> and PM<sub>10</sub>, respectively. A very significant increase in the atmospheric PM concentration, particularly the finest particle was observed during the haze which covered the Malaysia atmosphere from July to December 1997 (Khalid *et al.*, 1998).

Polycyclic aromatic hydrocarbon (PAH), which exists as colourless, white or pale yellow-green solids as a result of combustion and pyrolysis of organic substances is traditionally associated with PM includes benzo(a)pyrene (BaP), benzo(a)anthracene, pyrene, which may contribute to deleterious short and long-term health effects in human as well as animals.

### **Haze Episodes in Malaysia**

The visibility depends on suspended particulate matter (SPM), particle sizes and relative humidity. Slight hazy conditions are common in Malaysia with visibility often below than 10 km, especially during the period of August - September. The hazy situation becomes worst when the visibility range reaches to  $< 1 \text{ km}$ .