UNIVERSITI PUTRA MALAYSIA

EFFECTS OF NITROSAMINES ON HEPATIC ENZYMES ACTIVITIES AND HISTOPATHOLOGICAL STUDIES OF WHITE MICE (MUS MUSCULUS)

JEEVEN A/L KARRUPPAN.

FBSB 2005 5

EFFECTS OF NITROSAMINES ON HEPATIC ENZYMES ACTIVITIES AND HISTOPATHOLOGICAL STUDIES OF WHITE MICE (*MUS MUSCULUS*)

By

JEEVEN A/L KARRUPPAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2005

DEDICATION

To the memory of my late grandfather Chadayan and my father Karruppan, to my mother Kamatchi , my dear wife Janagiammal and my son Logganaath who were the source of inspiration and encouragement throughout the period of this study.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

EFFECTS OF NITROSAMINES ON HEPATIC ENZYMES ACTIVITIES AND HISTOPATHOLOGICAL STUDIES OF WHITE MICE (*MUS MUSCULUS*)

By

JEEVEN A/L KARRUPPAN

December 2005

Chairman: Associate Professor Johari Ramli, PhD

Faculty: Biotechnology and Biomolecular Sciences

Dietary and environmental hepatocarcinogens will be metabolized to active compounds and it must be detoxified in order to maintain liver integrity. In this study the feeding of nitrosamines and their effects on tumour marker enzymes Alkaline Phosphatase (ALP), Gamma-Glutamyl Transpeptidase (GGT), Glutathione S-transferase (GST) and Uridyl diphospho-glucuronosyl transferase (UDPGT) were analyzed in mice liver.

The initial work involved homogenization of liver samples with different buffers at various concentrations. Results with ALP and GGT shows highest specific activities for liver samples extracted with 0.01M Tris-HCl at pH 7.5. Further work on the use of different solvents, surfactants and detergents to optimize the extraction of alkaline phosphatase and gamma-glutamyl transpeptidase were

conducted. The results obtained showed that 0.01M Tris-HCl buffer at pH 7.5 alone is sufficient to extract these membrane bound enzymes.

Acute studies were conducted by feeding mice with 2-20% of LD_{50} of N-Nitrosodimethylamine (NDMA) and N-Nitrosodiethylamine (NDEA) and mice were killed at 24^{th}, 36^{th}, 48^{th}, 60^{th} and 72^{nd} hours and the liver ALP and GGT were assayed for their activities. Mice fed with 5mg of NDMA/kg of body weight dose for 36 hours showed highest and significant ($p<0.05$) activation of liver ALP and GGT compared to respective controls suggesting that feeding of NDMA had activated liver marker enzymes activities. The enzyme activities of ALP and GGT for treated mice were 4.215 IU/g protein and 0.656 IU/g protein respectively and in the control liver ALP activity were 1.084 IU/g protein and GGT activity were 0.375 IU/g protein.

Chronic toxicity study was conducted with oral feeding of 5mg NDMA/kg of body weight on weekly basis for 20 weeks. The control and treated mice were sacrificed every fortnight. The severity of neoplasia was studied by histological evaluations and the activity of ALP, GGT, GST and UDPGT were assayed. Studies on these enzymes show significant elevation at ($p<0.05$) for ALP, GGT and GST compared to respective controls. UDPGT does not show any changes in control and treated mice. ALP and GST was significantly ($p<0.05$) elevated compared to control at 2^{nd}, 16^{th} and 20^{th} week and GGT was significantly higher than control at week 8^{th}, 10^{th}, 16^{th} and 20^{th}. The highest enzymes activities

measured for the three enzymes were on the 20th week of experiment. The activities of liver enzyme in treated mice were 5.63 IU/g protein, 1.55 IU/g protein and 2.55 µmole/min/mg protein respectively for ALP, GGT and GST and the activities in control mice during the same week was 1.27 IU/g protein for ALP, 0.376 IU/g protein for GGT and 1.39 µmole/min/mg protein for GST. Histological evaluations through Hematoxilin and Eosin (H&E) staining and Transmission Electron Microscopy (TEM) obtained showed chronic ingestion had caused loss of normal cell organization in liver. Observation with H&E staining and TEM also showed the shrinking of nucleus, cellular and vacuolar degeneration and paler hepatocytes. From 10th week onwards significant ($p<0.05$) increase in lesion score in liver compare to control liver was observed in slides stained with H&E. The present result suggests even at low dose and at weekly feeding to mice, NDMA is capable in elevating tumour marker enzymes in liver and this compound also caused disruption to the normal cell organization of the liver.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN NITROSAMINA KE ATAS AKTIVITI ENZIM HATI DAN KAJIAN HISTOPATOLOGI PADA MENCIT (*MUS MUSCULUS*)

Oleh

JEEVEN A/L KARRUPPAN

Disember 2005

Pengerusi: Profesor Madya Johari Ramli, PhD

Faculti: Bioteknologi dan Sains Biomolekul

Proses detoksifikasi adalah amat penting dalam penyingkiran bahan terkumpul akibat metabolisma bahan karsinogen dari persekitaran atau pun dari permakanan dan proses ini dapat memelihara organ hati. Dalam kajian ini kesan nitrosamina terhadap hati mencit telah dilakukan. Pemberian nitrosamina dan perubahan pada enzim-enzim penanda tumor Alkaline Phosphatase (ALP), Gamma-Glutamyl Transpeptidase (GGT), Glutathione S-transferase (GST) and Uridyl diphospho- glucuronosyl transferase (UDPGT) telah diselidiki pada hati mencit.

Kajian awal dilakukan melibatkan penghomogenanan sampel hati dengan dengan penimbal-penimbal yang berbeza kepekatan dan pHnya. Pengukuran aktiviti spesifik ALP dan GGT adalah tinggi dengan apabila hati diempar dengan penimbal 0.01M Tris-HCl pada pH 7.5. Seterusnya solven, surfaktan dan

detergen ditambah pada sample hati dan dihomogenisasikan bersama penimbal 0.01M Tris-HCl pada pH 7.5 untuk memaksimakan pengekstrakan ALP dan GGT dari hati. Selepas pengemparan, didapati tiada kesignifikan pada aktiviti enzim ALP dan GGT apabila dibandingkan dengan peggunaan penimbal sahaja maka penimbal 0.01M Tris-HCl (pH 7.5) sudah memadai untuk pengekstraksian maksima enzim-enzim ini.

Penyelidikan seterusnya ditumpukan kepada kesan akut pemberian nitrosamina NDMA dan NDEA sebanyak 2-20% daripada LD_{50} pada kumpulan mencit dan haiwan ini dibunuh pada 24, 36, 48, 60 dan 72 jam dan enzim-enzim ALP dan GGT diasai pada sampel hati tersebut. Mencit yang diberi dos 5mg/kg berat badan NDMA menunjukkan aktiviti yang paling tinggi serta signifikan pada $p<0.05$ berbanding dengan hati kawalan. Aktiviti spesifik hati pada mencit yang dirawat ialah 4.215 IU/g protin untuk ALP, 0.656 IU/g protin untuk GGT manakala untuk kawalan aktiviti ALP ialah 1.084 IU/g protin dan GGT pula 0.375 IU/g protin.

Seterusnya kajian melibatkan kajian ketoksikan kronik selama 20 minggu. Dos rawatan ialah 5mg NDMA/kg dari berat badan mencit dan dos diberikan setiap minggu. Mencit dibunuh setiap dua minggu dan enzim penanda hati ALP dan GGT GST dan UDPGT diukur aktivitinya. Analisis enzim-enzim penanda ALP, GST dan GGT ini menunjukkan aktiviti spesifik yang signifikan ($p<0.05$) berbanding dengan sampel kawalan. UDPGT pula tidak menunjukkan sebarang

perubahan pada aktiviti enzim pada hati yang dirawat dan kawalan. Aktiviti ALP dan GST adalah signifikan ($p<0.05$) berbanding kawalan pada minggu kedua, ke-16 dan ke-20 dan aktiviti GGT pula signifikan ($p<0.05$) berbanding kawalan pada minggu kelapan, ke-10, ke16 dan ke-20. Aktiviti spesifik yang paling tinggi diukur untuk kesemua enzim-enzim ini ialah pada minggu ke-20 dan aktivitinya pada sampel hati yang dirawat ialah, ALP sebanyak 5.63 IU/g protin, GGT pula 1.55 IU/g protin dan 25.55 µmole/min/mg protin ialah aktiviti GST. Pada tempoh yang sama aktiviti spesifik untuk kawalan ialah 1.27 IU/g protin, 0.376 IU/g protin dan 1.39 µmole/min/mg protin masing-masing untuk ALP, GGT dan GST. Pada sampel hati ini juga, kajian dilakukan dari segi histologi dengan teknik pencerapan Hematoxilin and Eosin (H&E) dan kaedah elektron mikroskop. Dari minggu ke-10 pada slaid yang dicerap dengan H&E, lesion neoplastik diadapati bertambah secara signifikan ($p<0.05$) berbanding pada hati kawalan. Keputusan melalui kedua-dua kaedah histologi menunjukkan di sel hati ada kekecutan pada nucleus, perubahan bentuk atau degenerasi pada sellular dan vakuol dan sel-sel yang pucat. Keputusan kajian ini menunjukkan bahawa walaupun NDMA diberi pada dos yang rendah dan hanya seminggu sekali, tetapi karsinogen ini mampu meningkatkan aktiviti enzim-enzim penanda dan kompaun ini juga menyebabkan kehilangan penyusunan sel-sel normal.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation and deepest gratitude to my supervisor, Assoc. Prof. Dr Johari Ramli and co-supervisors Prof. Dr. Mohd Arif Syed, Assoc. Prof. Dr. Juzu Hayati Arshad and Prof. Dr. Nor Aripin Shamaan, for their support throughout the course of study. Without their guidance, advice, suggestions and encouragement, I would not have been able to present this work as it is today.

I would also like to extend my heartfelt thanks to Dr. Abu Bakar bin Hj. Hussin, Director of Food Technology Centre, MARDI, Puan Normah Omar and Dr. Kamariah Long for providing facilities, suggestions and encouragement. Sincere thanks to Dr. Fauziah Othman for providing facilities as well as valuable guidance and assistance in the histological evaluations.

My sincere gratitude also goes to the technical staff from the Department of Biochemistry and Microbiology, UPM, my friends and colleagues in UPM and MARDI especially Dr.Yunus, Isam, Paricher, Arif Zaidi Jusoh, Liza, Sashikala and Sharizan for their help and assistance.

I would also like to take this opportunity to express deepest appreciation to my friends Raj Pacyappan, Janaki, Kumaran and Shashikumar for their unwavering

support during the years of studies. Thanks are due to all who have helped or contributed in one way or other towards the completion of this study.

Lastly I wish to express my deepest and heartfelt feelings to my mother for providing me with guidance and support since my childhood. My heartfelt appreciation to my wife Janagiammal for being supportive and understanding. Special thanks to my son Logganaath for the enjoyable moments.

I certify that an Examination Committee met on 13th December 2005 to conduct the final examination of Jeeven a/l Karruppan on his Doctor of Philosophy entitled "Effects of Nitrosamines on Hepatic Enzymes Activities and Histopathological Studies of White Mice (*Mus Musculus*)" 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 follows:

Marziah Mahmood, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abdul Manaf Ali, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Norhani Abdullah, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Azimahtol Hawariah Lope Pihie, PhD
Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(Independent Examiner)

HASANAH MOHD GHAZALI, PhD
Professor/ Deputy Dean
School Of Graduate Studies
Universiti Putra Malaysia

Date: 27 FEB 2006

The thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as a fulfilment of the requirement for the degree of Doctor of Philosophy. The members of Supervisory Committee are as follows:

Johari Ramli, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Juzu Hayati Arshad, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mohd Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Nor Aripin Shamaan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School Of Graduate Studies
Universiti Putra Malaysia

Date: 09 MAR 2006

DECLARATION

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 institution.

JEEVEN A/L KARRUPPAN

Date: 27th January 2006

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAF	2-Acethylaminofluorene
ALP	Alkaline Phosphatase
BSA	Bovine Serum Albumin
CDNB	1-chloro-2,4-dinitrobenzene
C.V.	Coeeficient of variation
DEN	diethylnitrosamine
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic Acid
GGT	Gamma Glutamyl Transpeptidase
GSH	Glutathione
GST	Glutathione S-tranferases
HCL	Hydrogen chloride
HCC	Hepatocellular carcinoma
H&E	Hematoxylin and eosin
NNC	N-nitrosocompounds
NDMA	N-Nitrosodimethylamine
NDEA	N-Nitrosodiethylamine
NPYR	N-Nitrosopyrolidine
PNP	p-nitrophenol
PNPP	p-nitrophenol phosphate
RER	Rough Endoplasmic Reticulum