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THE EFFECT OF N-NITROSODIMETHYLAMINE ON ENZYME ACTIVITIES AND HISTOLOGY IN THE MOUSE

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By

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LIST OF ABBREVIATIONS

%	-	percentage
^{o}C	-	degree Celsius
g	-	gram
mg	-	milligram
mL	-	milliliter
mM	-	millimolar
mw	-	molecular weight
M	-	molar
Nm	-	nanometer
µl	-	microliter
µg	-	microgram
C	-	control
NC	-	normal saline
NDMA	-	N-nitrosodimethylamine
GSH	-	glutathione (reduced form)
GSSG	-	glutathione disulphate (oxidised)
GST	-	glutathione S-transferase
GSH-px	-	glutathione peroxidase
GSH-r	-	glutathione reductase
γ-GT	-	γ-glutamyl transpeptidase
UDPGT	-	uridyl diphosphoglucuronyl
BSA	-	bovine serum albumin
NADPH	-	β-nicotinamide adenine dinucleotide phosphate
H_2O_2	-	hydrogen peroxide
CDNB	-	1-chloro-2,4-dinitrobenzene
K_2HPO_4	-	potassium hydrogen phosphate
KH_2PO_4	-	potassium dihydrogen phosphate
H_3PO_3	-	orthophosphoric acid
KCl	-	potassium chloride
EDTA	-	ethylenediaminetetra acetic acid
NaN_3	-	sodium azide
NaOH	-	sodium hydroxide
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
H & E	-	hematoxylin & eosin
LD_{50}	-	lethal dose 50
i.p	-	intraperitoneal injection
U	-	unit
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
x *g*	-	gravity

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

THE EFFECT OF N-NITROSODIMETHYLAMINE ON ENZYME ACTIVITIES AND HISTOLOGY IN THE MOUSE.

By

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July 1999

Chairman : Associate Professor Johari Ramli, Ph.D.

Faculty : Science and Environmental Studies

This thesis studied the effects of acute, sub-chronic and chronic exposures of NDMA on the enzyme activities of five tumour markers namely: glutathione S-transferase (GST), gamma glutamyl transpeptidase (γ-GT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R) and uridyl diphosphoglucuronyl transferase (UDPGT) in the liver and kidney of male mice were. Forty-eight male mice ICR strain, *Mus musculus* (26-38g, 8 weeks old) were divided into four groups. A single batch consisted of 12 mice; one-half of them for enzyme activities and the other one-half for histological study. Acute group (n=12) were injected with a single dose of 0.5 mg/kg b.w. of NDMA. Sub-chronic group received 0.25 mg/kg b.w. of NDMA with twice i.p. injection over a one month

period. Chronic group (n=12) were injected with 0.05 mg/kg b.w. of NDMA given four times over a period of four month. The mice were sacrificed by cervical dislocation after treatment. In the liver, acute, sub-chronic and chronic exposure to NDMA showed increased enzyme activities (GST, GSH-R, GSH-Px, γ-GT and UDPGT: except γ-GT at acute exposure) in comparison with control groups ($P<0.05$). In the kidney, however the effect of NDMA exposure was rather inconsistent. All the enzyme activities increased in the acute exposure. Meanwhile, in the sub-chronic and chronic exposure some enzyme activities increased (e.g.: GST, γ-GT and UDPGT) and some enzyme activities (e.g.: GSH-R) did not change significantly. Administration of NDMA to mice by intraperitoneal injection over various periods of times to a total of 0.05- 0.5 mg/kg body weight caused the development of the tumour in the liver and kidney. Single acute dose (0.5 mg/kg b.w. of NDMA) produced anaplastic tumour and tubular degeneration in the kidney. Prolonged exposure (chronic) of mice to NDMA produced a high incidence of hepatocellular adenoma and also necrosis in the liver. This study demonstrated that there is a very strong correlation between enzyme activities and cell damage. Therefore, it can be concluded that some enzyme activities especially GST, γ-GT and UDPGT can be used as tumour markers.

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

KESAN N-NITRODIMETILAMINA (NDMA) KE ATAS AKTIVITI ENZIM DAN HISTOLOGI PADA MENCIT.

Oleh

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Hasil daripada kesan akut, sub-kronik dan kronik akibat daripada pendedahan NDMA ke atas aktiviti enzim penanda tumor; glutation S-transferase (GST), gama-glutamiltransferase (γ-GT), glutation peroksidase (GSH-Px), glutation reduktase (GSH-R) dan uridil difosfoglukuronil transferase (UDPGT) di dalam hati dan ginjal mencit. Empat puluh lapan (n=48) mencit jantan *Mus musculus* ICR strain (26-38g berat badan; berusia 7-8 minggu) telah dibahagikan kepada 4 kumpulan. Dalam satu kumpulan mengandungi 12 ekor mencit ; 6 ekor untuk aktiviti enzim dan 6 ekor lagi bagi tujuan histologi. Kumpulan akut (n=12) mencit menerima sekali suntikan di bahagian kaviti peritonial pada kadar dos 0.5 mg/kg NDMA mengikut berat badan. Bagi kumpulan sub-kronik mencit menerima dua kali suntikan sepanjang tempoh 4 minggu dengan kadar dos 0.25 mg/kg NDMA

mengikut berat badan. Manakala kumpulan kronik menerima empat kali suntikan sepanjang tempoh 16 minggu dengan kadar dos 0.05 mg/kg NDMA mengikut berat badan. Kesemua mencit dibunuh secara dislokasi servikal selepas tempoh kajian. Kesemua aktiviti enzim didapati meningkat (GST, GSH-R, GSH-Px, γ-GT and UDPGT; kecuali γ-GT pada akut) pada hati mencit apabila di bandingkan dengan kumpulan kawalan ($p<0.05$). Manakala pada ginjal mencit kesan pendedahan NDMA adalah tidak stabil. Kesemua aktiviti enzim meningkat pada tempoh akut. Pada tempoh sub-kronik dan kronik kebanyakan aktiviti enzim meningkat (contoh: GST, γ-GT dan UDPGT) dan ada juga aktiviti enzim tidak menunjukan signifikans (contoh: GSH-R). Penyuntikan NDMA ke atas mencit pada pelbagai tempoh masa dengan jumlah akhir 0.05 - 0.5 mg/kg berat badan menyebabkan pertumbuhan tumor ke atas hepar dan ginjal. Penyuntikkan kadar dos yang tinggi (0.5 mg/kg berat badan) pada tempoh akut menghasilkan anaplasia tumor dan inflamasi tubul pada ginjal. Pemanjangan tempoh pendedahan (kronik) Pada mencit memberi kesan yang tinggi ke atas pembentukan nekrosis dan ademomas tumor pada hati. Kajian ini menunjukkan terdapat ikatan yang kuat di antara aktiviti enzim dan kerosakan sel. Oleh yang demikian ianya boleh disimpulkan; sebahagian aktiviti enzim mampu menjadi penunjuk kehadiran tumor yang baik (GST, γ-GT dan UDPGT)

CHAPTER I

INTRODUCTION

Mankind have been plagued by diseases throughout the history of civilisation. Through advances in medical science, most of these diseases have been overcome. In the twentieth century, cancer has become one of the most important killer diseases in the world. Only in this century, the population has become acutely aware of its significance. Being one of the major human health problems, it has received enormous biomedical attention over the past few decades. Approximately a hundred types of human cancer have been recognised. Cancer is the second leading cause of death after heart disease. From epidemiological studies, about 35% of cancer are related to diet (Hodgson & Levi, 1994).

Cancer can be characterised as the loss of normal growth regulation. As we know, when normal stability of the organisation of tissues and organs are disturbed, a variety of diseases will arise. It is also characterised as groups of cells that arise from a single cell (Pitot *et* al., 1989). It is general knowledge that the body is developed from a single cell formed by the union of the male sperm and

the female egg. This cell divides into two and these two divides again. Divisions of their progeny continue generation after generation and the cell formed undergo progressive alterations to fit them for the various roles they are destined to play. Cells, even when they are fully grown, continue to divide, replacing worn out tissues, repairing injuries and healing wounds.

Cancer cells are different from normal cells. They divide excessively to form lumps or tumour. The excessive growth of cells is due to the failure of cell proliferation control (disturbance of the control mechanism). Normal cells stop growing when they touch each other, a phenomenon known as contact inhibition. Cancer cells however are under no such control and therefore form lumps of cells called nodules. In other words cancer is the 'outlaw cell'.

It is well documented that cancer development in organs and tissues is induced by different types of carcinogenic stimuli, including chemicals, viruses and radiation (Scott , 1979). The first indication that exposure to chemical agents might cause cancer was when Percival Pott (1775) noted that chimneysweepers, exposed to soot, were liable to scrotum cancer (IARC, 1991). Chemicals have been shown to exhibit carcinogenic activity in experimental animals.

In the modern industrialised world, cancer has become the most feared of all diseases. It is a progressively fatal disease for which no treatment has yet been discovered.

Studies on cancer have been carried out, histologically (Kessler *et* al., 1992), histochemically (Ogawa *et* al., 1980) and cytologically (Cameron *et* al., 1988). However, very few studies have been done on the correlation between biochemical assay and histological effects. The carcinogenic process in the mice liver and kidney was evaluated by the determination of the marker enzymes; glutathione S-transferase, glutathione reductase, glutathione peroxidase, γ-glutamyl transpeptidase and uridine diphosphate glucuronyl transferase.

Marker enzyme activities are expected to show a rise because of the change in the enzyme synthesis rate, principally to the increase in the number of cells synthesising the enzymes. During carcinogenesis, cancer cells undergo abnormal cell growth and the number of cells increase very rapidly. These enzymes can be used as marker enzymes and diagnostic tool for cancer (Moss, 1987).

Lesion scoring is very important in order to determine the severity of tumours (Mostofi & Dans, 1998). The microscopic changes in the liver and kidney tumours are studied to create the lesions scoring for liver and kidney tumours. The pathologic and inflammatory changes in the liver and kidney tumours are different among the same species of animals or humans (Henry *et* al., 1997). Hence, the microscopic lesions scoring system outlined in this study was developed to evaluate and classify the severity of liver and kidney lesion.

The aim of this study is to investigate the effect of chemical carcinogen (N-nitrosodimethylamine) on tumour marker enzyme activities by chemical carcinogenesis (N-nitrosodimethylamine) in the liver and kidney of male mice, mainly to :

I) Determine enzyme activities of GST, GSH-Px, GSH-R, γ-GT and UDPGT in the liver and kidney.

II) Study of liver and kidney histology after treatment with NDMA

III) Study the correlation between cell damage and enzyme activities

IV) Histological study and lesion Scoring

CHAPTER II

LITERATURE REVIEW

Chemical Carcinogenesis

Approximately 80-90% of cancer incidence in human beings is caused by environmental factors. These environmental factors include diet, chemicals, biological agents and ionising radiation. Chemical carcinogenesis is a process that consists of many steps with exposure to complex compounds in the environment. Carcinogenic agents, which enter the body, compete with the active metabolism pathway and detoxification. Most of the exogenous chemicals will be metabolised and excreted through various specific steps, which is also involved in the formation of reactive mediating agent. This reactive species will interact covalently with macromolecules within the cell and has the potential to start carcinogenesis and mutagenesis (Philips *et* al., 1985). Most industrial chemicals in our environment cause cancer. Carcinogenic substance was shown to be involved in the formation of free radicals (T'sao & Caspary, 1977).

Carcinogenesis is caused through the production of free radicals. Free radicals are produced through oxidising metabolism, mainly through peroxidation of polyunsaturated fatty acids (Carpenter, 1991) and it is also a reactive species of oxygen, as well as play an important role in the promotion of tumour (Sharma *et al.,* 1994). These free radicals activate the proto-oncogen. Free radicals which are produced in the oxidation process attack cells, cellular proteins, enzymes, lipid membranes and DNA strands. When the carcinogen activates the proto-oncogen, it will increase the production of proteins to abnormal levels.

Chemical Carcinogen

Polycyclic aromatics, aromatic amine, aflatoxin and N-nitrosamines have been identified as carcinogens (Weston & Harris, 1991). Chemical carcinogens can be divided into two major categories: genotoxic agents which produce alterations in the genetic material of the host and epigenetic agents which do not alter the primary sequence of DNA. The genotoxic agents possess cancer initiating activity whereas the epigenetic compounds possess cancer promoting activity (Hodgson & Levi, 1994).

A chemical carcinogen only becomes active after being metabolised to the 'ultimate' carcinogen form (the neoplasia-initiating derivative) but there are exceptions for alkylating agents. The 'ultimate' carcinogen form also has a strong electrophilic reaction. The 'ultimate' carcinogen consists of electron starved atoms and can react with nucleophilic site which can be found in abundance in

DNA, RNA and protein, including some oxygen and nitrogen atoms in protein (Miller, 1978). The chemical interaction of carcinogen with DNA causes the symptoms of cancer or a genetic illness. This illness will be transferred down from the parent cell to the progeny in every cell cycle. This is linked to the change in the nucleotide in DNA and the change in the gene transcription (Miller & Miller, 1981).

Mechanism of Carcinogenesis

Carcinogenesis is a multistage process driven by carcinogen induced genetic and epigenetic damage in susceptible cell that gains a selective growth activation of proto-oncogens or inactivation of tumour suppressor genes (Harris, 1991).

The development of a single cell into malignant tumour occurs in three stages as shown in Figure 1:-

Initiation

The first stage of carcinogenic process is tumour initiation (Tappel, 1988). It involves exposure of normal cells to chemical, genetic, physical or microbial carcinogens that cause genetic changes providing the initiated cells with both an altered responsiveness to their micro environment and exerts a selective clonal expansion advantage when compared to the surrounding normal cells (Yuspa & Harris, 1982). The initiated cells may have a decreased response to the intra- and

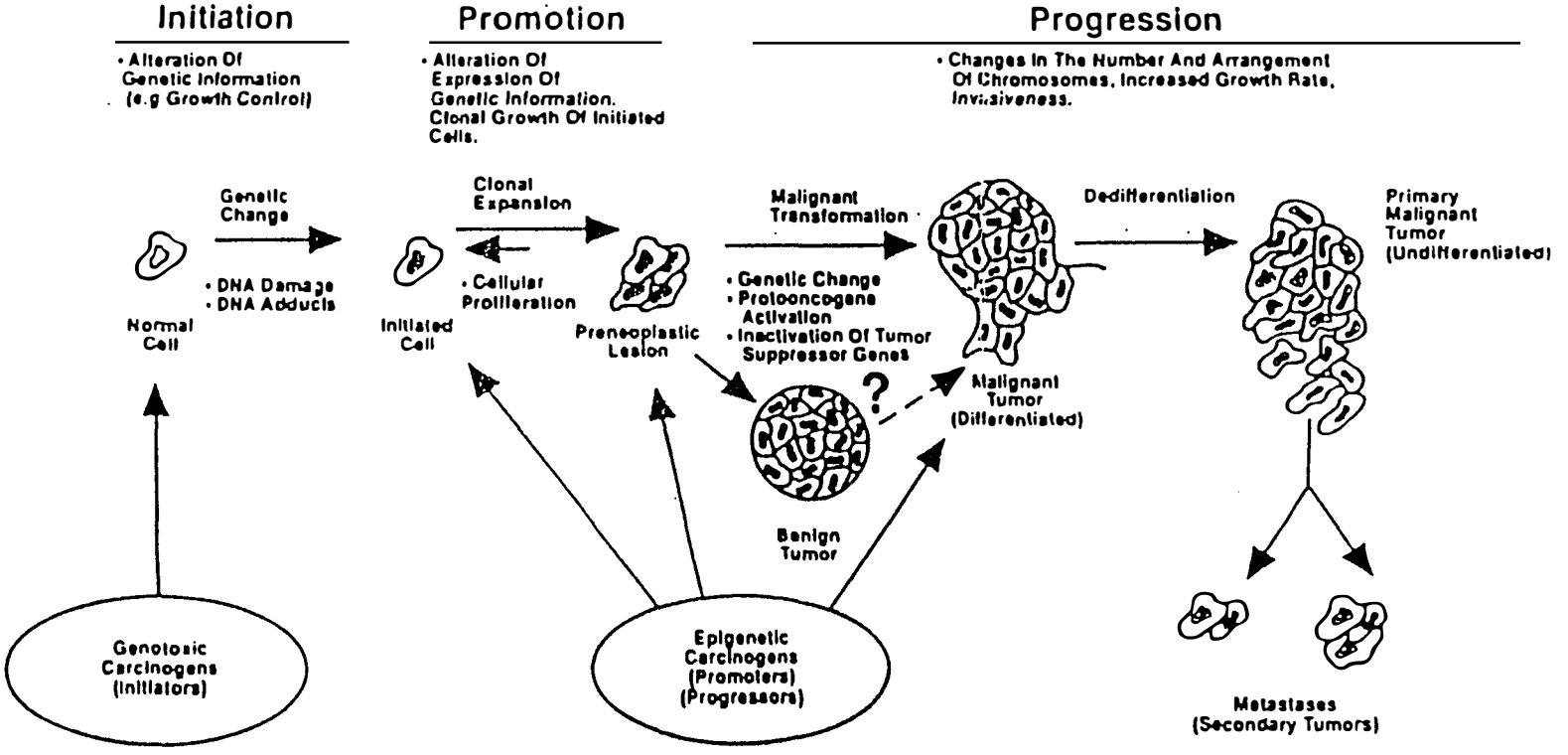

Figure 1 : A Model of the Process of Multistage Carcinogenesis showing the Three Stage Of Initiation, Promotion and Progression. Adapted from Farber, (1934).

inter-cellular signals that maintain normal tissue texture and regulate the homeostatic growth and maturation of cells (Weinstein *et al.*,1984). Initiated cells are also less responsive to negative growth factors.

Initiating agents are chemical, physical or biological agents, which are capable of directly altering the molecular structure of the genetic component (DNA) of the cell. The alteration may be a result of a covalent reaction of DNA with the initiating agents itself or with one of its metabolites. For example, during the administration of hepatocarcinogens (initiating agents) to an organism, several focal and nodular hyperplastic lesions develop in the liver cells. These focal and nodular hyperplasias are likely candidates for precursors of hepatic carcinoma. Hyperplastic nodules have two biological options after the 'initiation' process. The first option is to regress where a majority of the hyperplastic nodules will disappear in a process variously termed as regression (Farber, 1963) or 'remodelling' or 'phenotypic maturation' into normal structure of liver. The second option is, the hyperplastic nodules persist and undergo the second stage of neoplasm development.

Promotion

Tumour promotion results in the proliferation or survival of the initiated cells to a greater extent than normal cells and enhance the probability of additional genetic damage including endogenous mutations and accumulation in the expanding population of the cells (Weston & Harris, 1991). The probability of a