



UNIVERSITI PUTRA MALAYSIA

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

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

By

MOHD NAZIL SALLEH

**Thesis submitted in Fulfilment of the Requirements for the Degree of Master
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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF PLATES	ix
LIST OF ABBREVIATIONS	xi
ABSTRACT	xiii
ABSTRAK	xvi
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	5
Chemical Carcinogenesis.....	5
Chemical Carcinogen	6
Mechanisms of Carcinogenesis	7
Initiation	7
Promotion	6
Progression	10
N-nitroso Compounds	10
N-nitrosodimethylamine	12
Absorption and Uptake of NDMA	15
Mechanism of Metabolic Reaction	16
Markers Enzyme	18
Hepatic Lesions	19
Liver Necrosis	20
Tissue Lesions: Kidney Damage	22
Enzymes Involved in Detoxification Reaction	22
Glutathione S-Transferase	22
Metabolism of Carcinogenesis by GST	24
γ -glutamyl Transpeptidase	26
UDP-glucuronosyl Transferase	28
Selenium-dependant Glutathione Peroxidase	30
Glutathione Reductase	32
The Glutathione Redox Cycle.....	34



III	MATERIALS AND METHOD	
	Chemicals	36
	Animals and Experiments	37
	Study Design.....	38
	Preparation of NDMA solution	38
	Intraperitoneal injection	39
	Preparation of cytosol and microsome	41
	UDP glucuronosyl transferase assay	42
	γ -glutamyl Transpeptidase assay	43
	Glutathione S-Transferase assay	44
	Glutathione Peroxidase assay.....	44
	Glutathione Reductase assay	45
	Protein determination	45
	Histological studies.....	46
	Lesion scoring.....	47
	Statistical Analysis	
IV	RESULTS AND DISCUSSIONS	
	Effects of NDMA on the activity of GST at different time exposure in the liver and kidney	48
	Comparison of GST enzyme activity between treatment groups.....	52
	Effects of NDMA on the activity of GSH-R at different time exposure in the liver and kidney	52
	Comparison of GSH-R enzyme activity between treatment groups....	54
	Effects of NDMA on the activity of GSH-Px at different time exposure in the liver and kidney	54
	Comparison of GSH-Px enzyme activity between treatment groups...	56
	Effects of NDMA on the activity of GGTP at different time exposure in the liver and kidney	56
	Comparison of GGTP enzyme activity between treatment groups...	58
	Effects of NDMA on the activity of UDPGT at different time exposure in the liver and kidney	58
	Comparison of UDPGT enzyme activity between treatment groups...	58
	Liver histology of mice after treatment with NDMA.....	62
	Kidney histology of mice after treatment with NDMA.....	62
	Lesion scoring	68
	Overall Discussion	70
	The Effects of NDMA on GST Enzyme Activities for Acute, Sub- chronic and Chronic Exposure	72

	The Effects of NDMA on GSH-R and GSH-Px Enzyme Activities at Acute, sub-chronic and chronic Exposure	74
	The Effects of NDMA on γ -GT Enzyme Activities at Acute, sub-chronic and chronic Exposure	75
	The Effects of NDMA on UDPGT Enzyme Activities at Acute, sub-chronic and chronic Exposure	77
	Correlation Between enzyme activities and Cell Damage	78
	Dose effect and Dose Rate on Induced Tumours	79
	Histological Evaluation of Liver and Kidney	80
v	CONCLUSION	84
	BIBLIOGRAPHY	85
	APPENDIX	
	I	100
	II	101
	III	102
	IV	103
	VITA	104



LIST OF TABLES

<i>Table</i>		<i>page</i>
1	Characteristics of Initiation, Promotion and Progression	11
2	Effect of Difference Treatment Regime on the Specific Activity of Various Marker Enzymes in the Liver and Kidney of Mice.	49



LIST OF FIGURES

<i>Figure</i>		<i>Page</i>
1	A Model of the Process of Multistage Carcinogenesis showing the Three Stages of Initiation, Promotion and Progression.	8
2	Structures of Different Classes of Nitrosamine	13
3	Decomposition of NDMA. The reaction of NDMA requires enzymatic activation and generates a methylating agent which reacts with DNA as indicated.	17
4	The Involvement of Phase I and II Enzymes in Carcinogenesis. some of the tumour marker enzymes measured in this study are the phase II enzymes.	19
5	Glutathione Conjugation and Mercapturic Acid Biosynthesis.	25
6	Scheme for Association of γ -GT with the Membrane.	27
7	The γ -Glutamyl Cycle.	27
8	Glucuronidation of a Hydroxyl Functional Group.	29
9	Mechanism of Glutathione Reductase Activity	33
10	Mechanism of Glutathione Redox Cycle.	35
11	Experimental Design.	38
12	Summary Diagram of the Methods used to Prepare the Samples for Measuring the Enzyme Activity and Histological Study.	40
13	Effect of NDMA on the Enzyme Activity of GST at Different Exposure in the Liver and Kidney.	50
14	Effect of NDMA on the Enzyme Activity of GSH-R at Different Exposure in the Liver and Kidney.	52
15	Effect of NDMA on the Enzyme Activity of GSH-Px at Different Exposure in the Liver and Kidney.	54
16	Effect of NDMA on the Enzyme Activity of γ -GT at Different Exposure in the Liver and Kidney.	56

17	Comparisons of Percentage Changes of Enzyme Activities in the Liver and Kidney for Acute Exposure.	58
18	Comparisons of Percentage Changes of Enzyme Activities in the Liver and Kidney for Sub-Chronic Exposure.	58
19	Comparisons of Percentage Changes of Enzyme Activities in the Liver and Kidney for Chronic Exposure.	59
20	Effect of NDMA on the Enzyme Activity of UDPGT at Different Exposure in the Liver and Kidney.	60
21	Changes of Preneoplastic Marker Enzymes During Chemical Hepatocarcinogenesis.	76



LIST OF PLATES

Plate		<i>Page</i>
3.1	Liver section from Control Mice (received 0.9% normal saline) showing Normal Liver Histology. This photomicrograph shows normal appearing hepatocyte. Magnification 120x.	62
3.2	A Photomicrograph of the Liver of a Mice which received a single acute dose of 0.5 mg/kg b.w of NDMA. This photomicrograph shows the hepatocyte are slightly enlarged. There are also evidences of degeneration and necrosis. Magnification 120x.	62
3.3	A Photomicrograph of the Liver of a Mice which received 0.25 mg/kg b.w of NDMA, twice i.p. injection over a period 4 weeks. This photomicrograph shows some necrosis of hepatocyte and enlarged Kupffer cells. Magnification 100x.	63
3.4	A photomicrograph of the Liver of a Mice which received 0.05 mg/kg b.w of NDMA, four times i.p. injection over a period 16 weeks. This photomicrograph shows that clear evidences of necrosis . The hepatocyte shows pyknotic nucleus and the Kupffer cells enlarged. Magnification 120x.	63
3.5	A Photomicrograph of the Liver of Mice. This photomicrograph shows clear evidence of necrosis. The hepatocyte shows pyknotic nucleus and enlarged Kupffer cells. Magnification 100x.	65
3.6	A Photomicrograph of the Liver of Mice. This photomicrograph showing hepatocellular necrosis in the centrilobular region of the liver parenchyma with marked venous congestion. Magnification 100x.	65
3.7	Kidney Section from Control Mice (received 0.9% normal saline) showing Normal Kidney Histology. Magnification 100x.	66
3.8	A Photomicrograph of the Liver of a Mice which received a single acute dose of 0.5 mg/kg b.w of NDMA. Kidney section showing proximal tubular damage and anaplastic tumour. Magnification 140x.	66
3.9	A Photomicrograph of the Liver of a Mice which received 0.25 mg/kg b.w of NDMA, twice i.p. injection over a period 4 weeks. This photomicrograph shows tubular degeneration and enlarged. Magnification 120x	67



- 3.10 A photomicrograph of the liver of a mice which received 0.05 mg/kg b.w of NDMA, four times i.p. injection over a period 16 weeks. This photomicrograph show hepatocellular adenoma with tubular degeneration and enlarged. Magnification 140x. 67
- 3.11 A Photomicrograph of the Kidney of Mice. This photomicrogrpah shows an evidence of tubular degeneration. However, the lesion is very mild and in general, the cells look healthy. Magnification 100x. 68
- 3.12 A Photomicrograph of the Liver of Mice. This photomicrograph shows enlarged Kupffer cells and evidences of degenerated and necrotic cells. Magnification 100x. 68



LIST OF ABBREVIATIONS

%	-	percentage
°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 ₂ O ₂	-	hydrogen peroxide
CDNB	-	1-chloro-2,4-dinitrobenzene
K ₂ HPO ₄	-	potassium hydrogen phosphate
KH ₂ PO ₄	-	potassium dihydrogen phosphate
H ₃ PO 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.

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Chairman : Associate Professor Johari Ramli, Ph.D.

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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.



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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; glutathione S-transferase (GST), gama-glutamyltransferase (γ -GT), glutathione peroxidase (GSH-Px), glutathione reductase (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 peritoneal 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 menunjukkan 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 adenomas 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 divide again. Divisions of their progeny continue generation after generation and the cells 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 tumours. 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-oncogenes 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

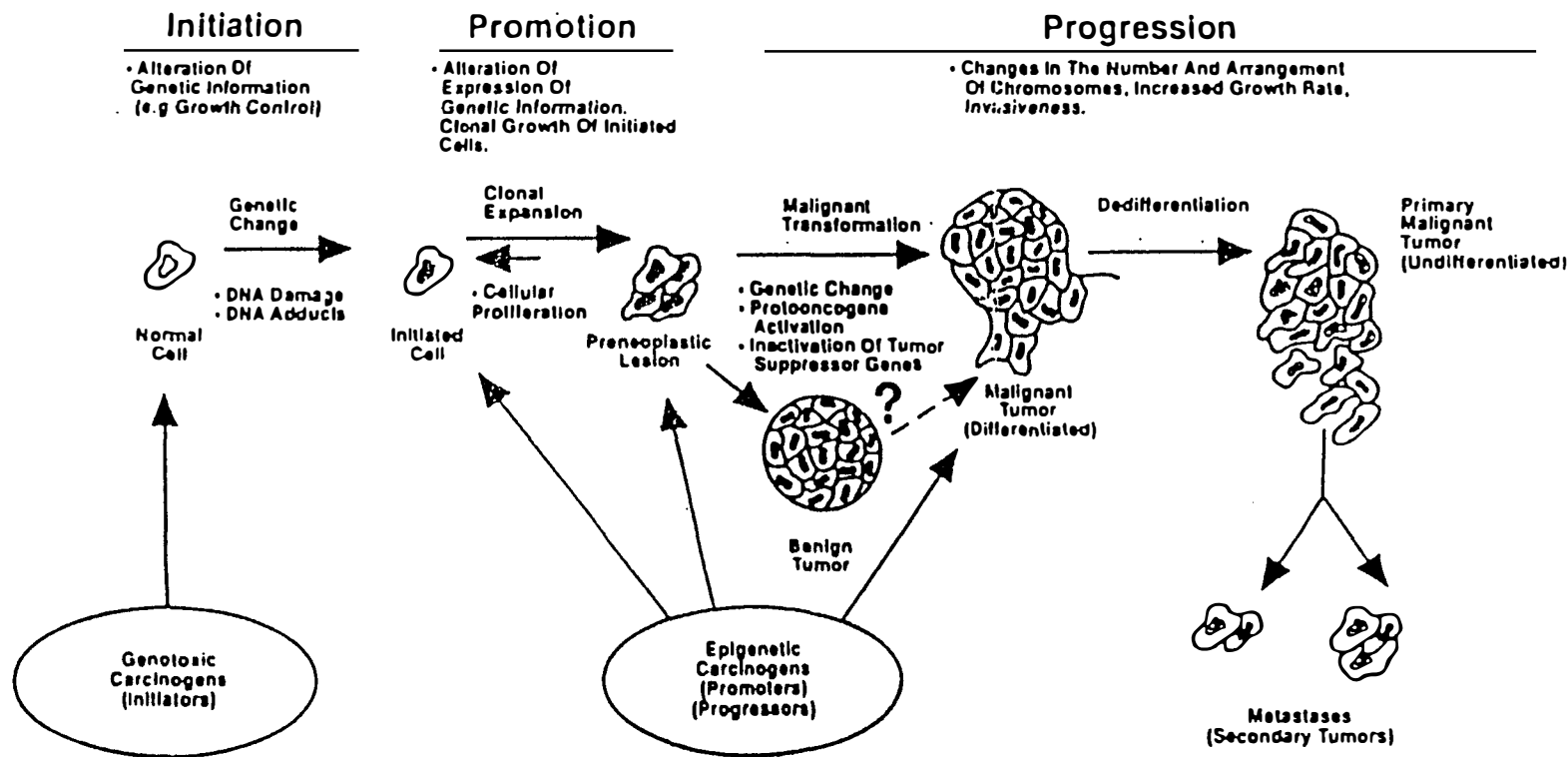


Figure 1 : A Model of the Process of Multistage Carcinogenesis showing the Three Stage Of Initiation, Promotion and Progression. Adapted from Farber, (1934).

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