NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

A STUDY OF THE GENOTOXICITY OF TYROSINE METABOLITES IN RAT PRIMARY HEPATOCYTES

HM BHABHA Hons.BSc

Dissertation submitted in partial fulfillment of the requirements for the degree Master of Science in Biochemistry at the North-West University

Supervisor: Prof PJ Pretorius

Potchefstroom Campus
2008

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I wish to express my gratitude and sincerest thanks to the following individuals who enabled this document to be timeously completed:

Firstly, I would like to thank our Almighty God for giving me strength to face all conditions through His power (Philippians 4:13), I will always proclaim His greatness (Psalms 145).

Secondly, I would like to thank my mentor, the project supervisor Professor P.J Pretorius, for giving me the opportunity to conduct this study in the department of Biochemistry and for his guidance and suggestions regarding all aspects of the study.

Special thanks go to the Vet Lab technologists Mr. Cor Bester and Mrs. Antionette Fick from the animal center lab.

I am sincerely grateful to the Mitochondrial Lab staff for allowing me to use their tissue culture laboratory and for their assistance

I would like to offer sincere thanks to Miss Hlengiwe Mbongwa. Thanks for your input, support and the moments we shared in the office. It was great working with you.

To Miss Agnes Tlhabanelo, thanks for your love, laughter, and support and for always being there for me through thick and thin. I appreciate everything you have done for me.

This study would not have been possible without financial assistance and my sincerest thanks go to the National Research Foundation (NRF)

Most importantly, to my family, my mom Winnie, my son Ofentse, my sister Harriet and my two brothers Headley and Teboho Bhabha, not forgetting the Mokale family, thanks a lot for taking a parental role for my child (Ofentse) while I was studying.

To Miss Chrisna van Heerden, although you caught the tail-end of this endeavor you were very helpful when my ship was sinking. Thank you so much for everything you have done.

Finally, thanks to all other staff and students in the Biochemistry Department and throughout the North West University (Potchefstroom Campus). Many of you will have contributed in some way towards this study and although I have not mentioned you all, know that any assistance received was truly appreciated.

HM Bhabha

ABSTRACT

Toxicity of tyrosine metabolite has been hypothesiszed, but not proven, to play a role in the ethiopathogenesis of hepatic alterations found in hereditary tyrosinemia type1 (HT1), a metabolic disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH). A deficiency of this enzyme results in liver failure, hepatocellular carcinoma (HCC) and renal tubular dysfunction. Two of tyrosine intermediate metabolites (pHPPA) and succinyl acetone (SA) were tested on the rat primary hepatocytes. DNA damage, DNA repair capacity and DNA methylation status of the liver cells treated with these two metabolites were measured. Comet assay was used to measure the DNA damage and the repair capacity, DNA methylation was measured with the cytosine extension assay. Experiments showed that pHPPA had impairment on the DNA repair capability of treated cells and also increased the absolute % unmethylated Sample revealing the hypomethylation potency of this metabolite. SA caused damage but the treated cells were able to repair the damage inflicted on them, the results showed no significant effect on the global DNA methylation status of these SA treated cells.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

A:

A	adenine

B:

BER	base excision repair

C:

C	cytosine
CCGG	cytosine/cytosine/guanine/guanine
CpG	C (Cytosine) and G (Guanosine) are connected by a phosphodiester bond
CYP	cytochrome P_{450}

D:

ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	double strand break

E:

Et al	Latin: and others

F:

FAA	fumaryl acetoacetate
Fah $^{-1}$	fumarylacetoacetate hydrolase deficiency
FAH	fumarylacetoacetate hydrolase

G:

g	gravity
G	guanine

H:

H_2O	water
H_2O_2	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGD	homogentisic acid dioxygenase
HMPA	high melting point agarose
HpaII	a gene from *H. parainfluenzae* (ATCC 49669)
HPD	Hydroxyphenylpyruvate dioxygenase
Hpd^{-1}	hydroxyphenylpyruvic acid dioxygenase deficiency
HR	homologous repair
HT1	hereditary tyrosinemia type 1

I:

i.e.	that is
IEM	inborn errors of metabolism

K:

kb	kilobase
kDa	kiloDalton
K_m	substrate concentration

L:

LMPA	low melting point agarose

M:

MA	maleylacetate
mA	milliAmperes
MAAI	Maleylacetoacetate isomerase
MAI	maleylacetoacetate isomerase
$MgCl_2$	magnesium chloride
MMR	mismatch repair
MspI	a gene from *Moraxella* species (ATCC 49670)
MTT	Methylthiazol tetrazolium

N:

NaCl	sodium chloride
NER	nucleotide excision repair
NHEJ	nonhomologous end joining
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

P:

p	para
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pHPPA	p-hydroxyphenylpyruvic acid

R:

redox	reduction/oxidation
ROS	reactive oxygen species
rpm	revolutions per minute

S:

SA	succinyl acetone
SAM	S-adenosylmethionine
SCGE	single cell gel electrophoresis

T:

T	thymine
TAT	tyrosine amino transferase
TrisHCl	2-Amino-2-(Hydroxymethyl)-1,3-propandiol-hydrochloride
TTN	transient tyrosinemia of the newborn

U:

UV	ultraviolet

V:

V	Volts

LIST OF SYMBOLS

%	percent
µl	microliter
[^{3}H]dCTP	deoxy[1',2',5-^{3}H]cytidine 5'-triphosphate triethylammonium salt
4 – HPPD	4-hydroxy phenylpyruvate dioxygenase
5'	five prime
ml	milliliter
mM	milliMolar
mm^3	cubic meters
ng	nanograms
°C	degrees Celcius

ASOKA LANGUAGE EDITING

सत्यमेव जयते

DECLARATION

This is to certify that the following dissertation has been submitted for English Language editing

A study of the Genotoxicity of Tyrosine Metabolites in Rat Primary Hepatocytes

Candidate name: HM Bhabha

Degree: M.Sc. (Potchestroom)

Prof. D. Schauffer SATI member no.1001872

CHAPTER 1

INTRODUCTION

L-tyrosine is a semi-essential amino acid derived from hydrolysis of dietary or tissue protein or from hydroxylation of phenylalanine (Russo *et al*, 2001). Tyrosine is essential in improving concentration, mood and attention. It reduces appetite and delays fatigue. Hiraku *et al* (2006) reported that certain tyrosine metabolites are known to be carcinogenic or mutagenic which impaires degradation of the aromatic amino acid tyrosine leading to several acquired and genetic liver disorders (Grompe, 2001). Among liver disorders, tyrosinemia type 1 (HT1) is caused by deficiency of the enzyme fumarylacetoacetate hydrolase (FAH), the last step in tyrosine catabolism (Overturf *et al*, 1996). Since hereditary Tyrosinemia type 1 (HT1) causes liver diseases (Grompe, 2001) the main focus of the project was on the molecular effects of accumulating tyrosine metabolites in HT1.

The aim of the study was to measure DNA damage, DNA damage repair and DNA methylation in isolated hepatocytes treated with tyrosine intermediate metabolites. Since the main affected organ in HT1 is the liver, the effects of these metabolites were measured with primary hepatocytes. The single cell gel electrophoresis or Comet Assay, a simple, rapid and sensitive technique for analysing and quantifying DNA damage in individual mammalian cells was used to measure DNA damage and DNA repair capacity. Another molecular event investigated in tyrosinemia was whether or not changes in DNA methylation took place, a feature found in hepatocellular carcinoma (HCC), which is a frequent complication in HT1 (Tangkijvanich *et al*, 2007). Methylation has long been known to act as hotspots for mutations due to the high rate of spontaneous deamination of bases (Walsh *et al*, 2006), since some of the tyrosine

intermediate metabolites act as natural alkylating agents or disrupt sulfhydryl metabolism they may play a role in hepatocarcinogenesis or even HCC in affected people (Vogel *et al*, 2004).

The structure of the study

Part of this work was presented at the annual symposium of the Society for the Study of Inborn Errors of Metabolism in September 2007. This study has five chapters in addition to this introductory chapter which gives the background, motivation and aim. The second chapter provides a review of literature which focuses on the concept of the Inborn Errors of Metabolism (IEM) and their consequences. Chapter two concludes with more detailed information on a specific amino acid (Tyrosine) metabolism. Furthermore, all aspects of chapter two will ultimately be integrated into a consideration of the focus of the study namely the effect of tyrosine intermediate metabolites on primary rat hepatocytes. Effects measured are DNA damage, DNA repair and DNA methylation.

The methodology used for the study is described in chapter three. This includes ethical approval, methods, study materials, procedures and data analysis. Results are presented and discussed in chapter four. In chapter five a summary and a conclusion based on the results are drawn.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Inborn Errors of Metabolism (IEM) are hereditary affections resulting from incompetence in enzymatic reactions of intermediary metabolism due to enzyme deficiency and to its low activity or stability. As shown in figure 2.1, a blockage in a metabolic pathway generally causes an increase in the precursor for the compromised metabolic stage and lack of subsequent intermediaries or activation of alternate routes leading to the production of toxic substances (de Oliveira *et al*, 2001). In some disorders, the primary cause of disease is accumulation of a normally minor metabolite produced in excess by a reaction that is usually of trivial metabolic importance (Clarke, 2005).

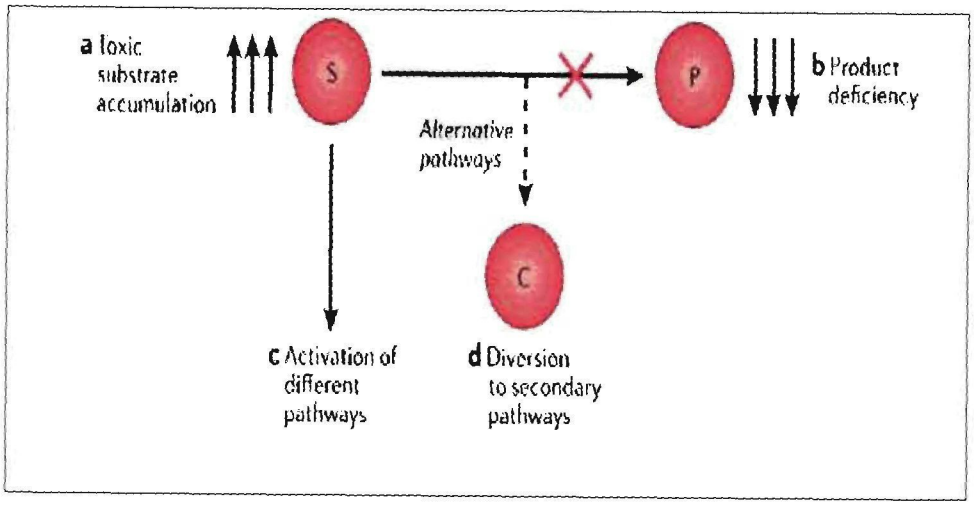

Figure 2.1: The pathogenetic mechanisms in inborn errors of metabolism: **a** direct toxicity of the accumulating upstream substrate (S); **b** deficiency of the downstream product (P); **c** activation of alternative pathways; **d** diversion of metabolic flux to secondary pathways and alternative metabolites (C) production. Large-molecule diseases often arise from the aberrant synthetic or degradative processing of polymeric molecules (Lanpher *et al,* 2006).

In hypertyrosinemia, for example, when tyrosine is not properly metabolized its accumulation leads to neurologic dysfunction and mental retardation (Levy, 1989). These errors occur in the metabolism of all organic compounds both in anabolism and catabolism and in energy production with a great number of diseases resulting from these metabolic disturbances. Several hereditary metabolic disturbances are known at present, many of which correspond to illness that frequently evolve to death or cause important sequels, especially mental deficiency (de Oliveira *et al*, 2001).

Some clinical investigations suggest that tyrosine metabolites are toxic to hepatocytes and renal tubular epithelial cells, therefore the use of animal models to investigate these diseases is important (Endo *et al*, 2003). In this regard a tyrosinemic mouse model with fumarylacetoacetate hydrolase (*Fah- / -*) and 4-hydroxyphenylpyruvic acid dioxygenase ($Hpd^{-/-}$) deficiencies has been developed to study the pathophysiologies of tyrosinemias (Endo *et al*, 2003).

2.2 TYROSINEMIA

2.2.1 Introduction

Tyrosinemia is a genetic disorder characterized by elevated blood levels of tyrosine and is caused by deficiency in fumarylacetoacetate hydrolase (FAH) the last enzyme in the tyrosine catabolic pathway (Jorquera and Tanguay *et al*, 2001). If untreated, tyrosine and its metabolites, fumarylacetoacetate (FAA) and maleylacetate (MAA) and their derivatives like succcinylacetone (SA), accumulate in tissues and organs, which leads to serious medical problems (Russo *et al*, 2001). There are three types of tyrosinemia, i.e type I, type II and type III (Table 2.1), each with distinctive symptoms and caused by deficiency in different enzymes (Endo *et al*, 2003). Hereditary tyrosinemia type I (HT1) is the most common of the three known diseases caused by defects in tyrosine

metabolism and it has the highest risk for primary liver cancer of any human disease (Vogel *et al*, 2004).

2.2.2 Clinical Manifestations

Elevated blood tyrosine levels are present in several clinical entities. The term tyrosinemia or hypertyrosinemia was first given to a clinical entity based on observations that have proven to be common to various disorders, including transient tyrosinemia of the newborn (TTN), hereditary infantile tyrosinemia (tyrosinemia I), Richner-Hanhart syndrome (tyrosinemia II), and tyrosinemia III (Roth, 2004).

Clinically HT1 is characterized by progressive acute or chronic liver damage and/or hepatocellular carcinoma, renal tubular dysfunction or neurological crises (Couce Pico *et al*, 2006). Hiraku *et al* (1998) demonstrated a high incidence of hepatocarcinoma in patients with the chronic form of HT1.

Depending on the underlying disease, patients with high risk (chronic viral hepatitis B or C and tyrosinemia) and those with low risk (morbus Wilson, primary biliary cirrhosis, primary sclerosing cholangitis) for the development of hepatocellular carcinoma can be identified. The prognosis of patients with hepatocellular carcinoma is independent of the underlying disease, but it does depend on the liver function and the tumor stage (Kubicka *et al*, 2003).

Three therapeutic strategies available for tyrosinemia treatment are: 1) dietary intervention which limits the precursor amino acids phenylalanine and tyrosine to minimize the amount of excess tyrosine that needs to be metabolized, 2) orthotopic liver transplantation, and 3) metabolic inhibition of the proximal tyrosine pathway with the use of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) to prevent the formation of succinylacetone. Higher levels of tyrosine may be associated with the deposition of tyrosine crystals in the cornea and symptoms of photophobia (Overturf *et al*, 1996; Russo *et al*, 2001).

2.2.3. Tyrosine Metabolism

In mammals, tyrosine is both ketogenic and glucogenic (Mitchell *et al*, 2001). Its degradation is catalyzed by a series of enzymes yielding acetoacetate (ketogenic) and the Krebs Cycle intermediate fumarate (glucogenic). The hepatocyte and renal proximal tubules are the only two cell types that express the complete pathway and contain sufficient quantities of all enzymes required for tyrosine catabolism (Fernández-Cañón *et al*, 2002).

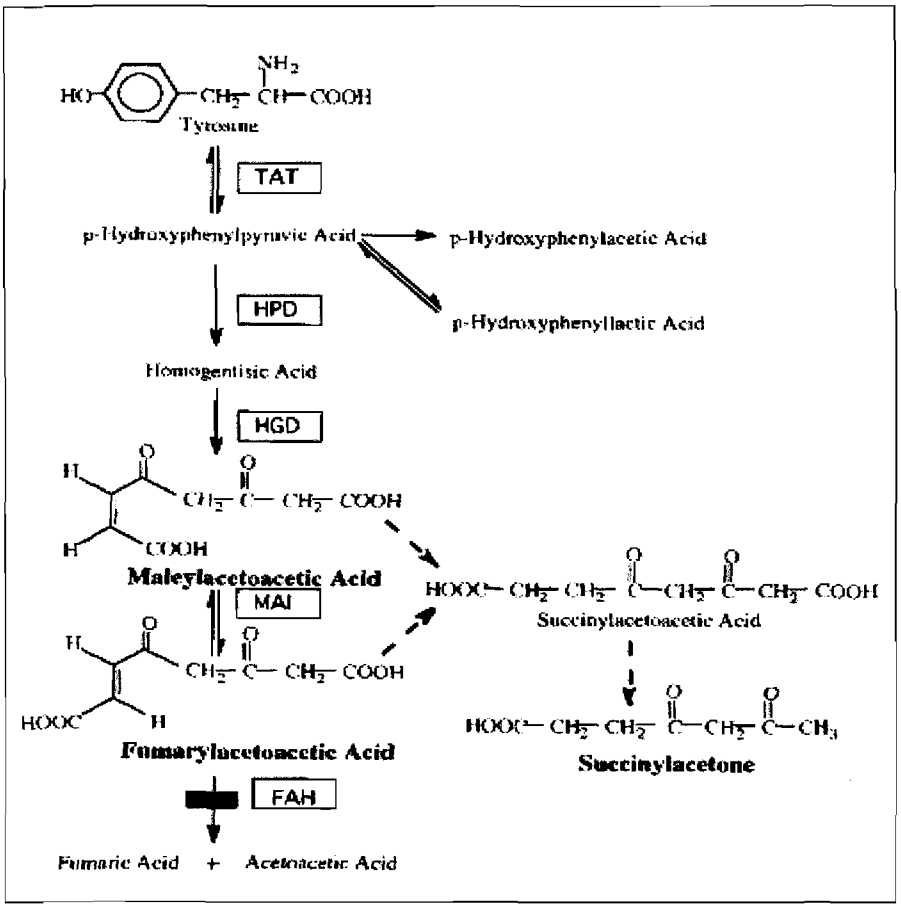

Figure 2.2: The tyrosine catabolic pathway. Tyrosine amino transferase (TAT), 4-Hydroxyphenylpyruvate dioxygenase (HPD), homogentisic acid dioxygenase (HGD), maleylacetoacetate isomerase (MAI), Fumarylacetoacetate hydrolase (FAH) (Grompe, 2001, Fernandez-Canon *et al*, 2002).

The main catabolic pathway of tyrosine begins with transamination by hepatic cytosolic TAT yielding *p*-hydroxyphenylpyruvate (Ohisalo *et al*, 1982).This enzyme is under strict hormonal control and is inducible by corticosteroids, glucagons, catecholamines, and by tyrosine in rats. The rest of the enzymes in the pathway are not known to be induced by hormones (Ohisalo *et al*, 1982).

2.2.3.1 Enzymology

Enzymes are proteins that control the rate of chemical reactions in the cell. In general, each enzyme controls the rate of only one or a few reactions. Enzymes function by binding to the substrate and altering their chemical bonds producing products. Enzymes are often linked in multistep pathways, such that the product of one reaction becomes the substrate for another. In this way, a simple molecule can be changed, step by step into a complex one, or vice versa. In addition, the multiple steps provide additional levels of regulation, and intermediates, can be shunted into other pathways to make other products. When all the enzymes in a pathway are functioning properly, intermediates rarely build up to high toxic concentrations (Berg *et al*, 2001; Clarke, 2005). The metabolism of tyrosine is one such metabolic pathway (Figure 2.2). The different enzymes involved in this pathway, will be described briefly.

Tyrosine amino transferase (TAT)

TAT the first enzyme of tyrosine catabolism, has been studied extensively in rodents because of its hormone-development and tissue specific pattern of expression and its role as the rate-determining step of tyrosine catabolism since it is developmentally regulated. Given that expression of TAT is strictly limited to the cytoplasm of hepatocytes, its activity is a useful marker of hepatocytic differentiation (Mitchell *et al*, 2001).

4-Hydroxy phenylpyruvate dioxygenase (4-HPPD)

This enzyme catalyzes the formation of homogentisate from 4-Hydroxyphenylpyruvate and molecular oxygen. This reaction proceeds through

an oxidative decarboxylation of the 2-oxoacid side chain of the substrate, which is accompanied by hydroxylation of the aromatic ring. The purified enzyme was shown to contain nonheme-reduced iron, which is essential for catalytic activity and in most organisms this enzyme activity is involved in catabolism of aromatic amino acid tyrosine (Garcia *et al*, 1999).

Homogentisate oxidase (HGD)

This cytoplasmic dioxygenase enzyme is found in liver and kidney and it mediates the cleavage of the aromatic ring of homogentic acid. A deficiency in this enzyme is not associated with hypertyrosinemia (Mitchell *et al*, 2001).

Maleylacetoacetate isomerase (MAAI)

Maleylacetoacetate isomerase (MAAI) is a key enzyme in the metabolic degradation of phenylalanine and tyrosine that catalyzes the glutathione-dependent isomerization of maleylacetoacetate to fumarylacetoacetate (Polekhina *et al*, 2001). A deficiency along the degradation pathway leads to serious diseases (Polekhina *et al*, 2001).

Fumarylacetoacetate hydrolase (FAH)

Fumarylacetoacetate hydrolase (FAH) mediates the last step of tyrosine catabolism, i.e the hydrolytic formation of fumarate and acetoacetate (Mitchell *et al*, 2001). Loss-of-function and mutations in the gene-coding for fumarylacetoacetate hydrolase are associated with the severe metabolic disorder HT1. Acute HT1 is characterized by complete fumarylacetoacetate hydrolase deficiency with rapid liver failure and neonatal death. Chronic HT1 is characterized by partial loss of fumarylacetoacetate hydrolase activity with hepatocellular carcinomas and nephropathies (Fanconi's syndrome) (Lantum *et al*, 2003).

2.2.3.2 Defects of tyrosine catabolism

The causes of enzyme defects are mostly through genetic mutations that affect the structure and/or regulation of the enzyme protein, this usually creates problems with the transport, processing and/or binding of co-factors. In general, the consequences of enzyme deficiency are that of natural cellular chemistry disturbances because of 1) either a reduction in the amount of an essential product, 2) the accumulation of a toxic intermediate or 3) the production of a toxic side-product (Berg *et al*, 2001; de Oliveira *et al*, 2001).

Most inborn errors of tyrosine catabolism produce hypertyrosinemia. Hypertyrosinemia is also encountered in various acquired conditions, in particular severe hepatocellular dysfunction. There are three types of tyrosinemia, each with distinctive symptoms and caused by the deficiency of a different enzyme, see table 2.1 below:

Table 2.1: Enzymatic defects and major manifestations in tyrosinemia

Enzyme	Defect	Major manifestation
Tyrosine aminotrasferase	Tyrosinemia **type II** (oculocutaneous tyrosinemia)	Corneal thickening, developmental delay, hyperkeratosis of palms & soles.
4-Hydroxyphenylpyruvate dioxygenase	Tyrosinemia **type III** Transient tyrosinemia of the newborn. Hawkinisinuria	Transient immaturity of enzyme, usually resolves spontaneously.
Homogentisate oxidase	Alcaptonuria	Arthritis in older patients Dark urine when exposed to air.
Maleylacetoacetate isomerase		Reported in two siblings with liver failure and renal disease.
Fumarylacatoacetate hydrolase	Tyrosinemia **type I** Hepatorenal tyrosinemia	Hepatocellular carcinoma, renal and neurologic disease

(Russo *et al*, 2001)

2.2.4 Genetics of HT1

HT1 is inherited as an autosomal recessive disorder; it is caused by deficiency of enzyme fumarylacetoacetate hydrolase (FAH) which is coded by the 35 kb gene localized at q23-q25 on chromosome 15, which contains 14 exons. Furthermore, it has been reported that, the expressed protein forms a homodimer within the cytoplasm of 46.3 kDa and it does not require co-factors and is primarily expressed in liver and kidney (Scott, 2006). It is expressed at very low levels in most tissue (King *et al*, 2006).

Missense, nonsense and splice consensus site mutations compromised in 11 different mutations have been identified in HT1 patients. A missense mutation has recently been identified and it has also been reported that it causes a reduction of FAH activity, thus leading to accumulation of alkylating metabolites causing liver damage (van Amstel *et al*, 1996).

2.3 ETIOLOGY OF HEPATOCARCINOMA

The liver plays a central role in the pathophysiology of many inborn errors of metabolism because it is a major site of catabolic, synthetic and detoxification reactions (Overturf *et al*, 1996). Hepatocytes are polarized cells arranged in bicellular plates with the basal membrane abutting the perisinusoidal space of Disse. They are the site of primary injury in many metabolite liver disorders and they are vulnerable because of: 1) their diverse metabolic activity, 2) their unique vascular arrangement and pericentral hypoxia, 3) the presence of cytochromes P450 which generate reactive metabolites, 4) exposure to gut-derived nutrients, 5) toxins and xenobiotics, and 6) synthesis and excretion of bile (toxic detergent) into canaliculi between adjacent cells (Tanner, 2002). Many of the known enzyme deficiency disorders are treatable by liver transplantation and therefore would potentially be amenable to liver gene therapy (Overturf *et al*, 1996).

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant liver diseases worldwide and according to Wang *et al* (2002) it ranks fourth in mortality rate, behind lung, stomach and colon cancers. HCC is unusual among cancers because the specific causative factor can be identified in most patients. Common risk factors include chronic viral hepatitis (HBV = Hepatitis B viral and HCV = Hepatitis C virus) and underlying liver disease in the form of cirrhosis (Di Bisceglie, 2002). Right upper quadrant pain and weight loss are typical symptoms of HCC, and physical examination usually reveals enlarged, hard and irregular liver (Di Bisceglie, 2002).

It is hypothesized that oxidative stress and generation of reactive oxygen species (ROS) can cause mutations in cancer-related genes or alter the function of important proteins regulating DNA repair, the cell cycle and apoptosis (Wang *et al*, 2002). A multi-step accumulation of genetic alteration has long been proposed as one of the major mechanisms underlying HCC (Pang *et al*, 2006). The inactivation of p53, which is a tumor suppressor gene, either through mutations or by binding to other viral and cellular onco-proteins, is the most common event in human cancers (Wang *et al*, 2002).

Since HCC is an aggressive tumor associated with dismal prognosis, Pang *et al* (2005) demonstrated that surgical resection and liver transplantation are two curative treatments for HCC, but are applicable to only a small proportion of patients with early tumors. Currently there is no proven effective systemic chemotherapy for HCC except alternative treatment strategies such as transcatheter arterial chemo-emobilization, percutaneous intratumoral ethanol injection and radio-frequency ablation (Di Bisceglie, 2002; Pang *et al*, 2005).

2.3.1 DNA Damage and hepatocarcinogenesis

There are several types of DNA damage, e.g. base modification (methylation, oxidation and adduct formation), mispairs (mistakes in DNA synthesis) and cross-linked nucleotides (intrastrand, interstrand covalent links and strand breaks). DNA can be modified by a variety of damaging agents originating from both exogenous and endogenous sources (Waschsmann, 1997). DNA damage plays a major role in mutagenesis, carcinogenesis and ageing. The majority of mutations in human tissues are certainly of endogenous origin (De Bont *et al*, 2004).

Unsuccessful repair of these modifications can generate genomic mutations and result in the expression of proteins showing a spectrum of altered functional properties (Waschsmann, 1997). The chemical events that lead to DNA damage include hydrolysis, exposure to ROS and other reactive metabolites (De Bont *et al*, 2004) which are potent chemical species that are able to damage DNA, thereby producing highly mutagenic modified bases (Wheelhouse *et al*, 2003)

2.3.2 Oxidative DNA damage

Oxidative stress or damage may affect a number of cell targets including DNA and it has been hypothesized as the most important cause of cellular genotoxicity which is thought to be the cause of diseases such as cancer and neurological disorders (Choudhury *et al,* 2003). Oxidative mechanisms have been demonstrated to possess a potential role in the initiation, promotion, and malignant conversion (progression) stages of carcinogenesis (Cooke *et al*, 2003).

Changes in DNA structure such as base modification, rearrangement of DNA sequences, miscoding of DNA lesions, gene duplications and the activation of oncogenes may be involved in the initiation and promotion of various cancers (Waris *et al,* 2006). On the other hand the role of ROS in the progression stage

of carcinogenesis is evident from the fact that a number of different free radical-generating compounds enhance the malignant conversion of benign papillomas into carcinoma which is the progression in carcinogenesis (Athar, 2002).

2.3.3 DNA Methylation

DNA methylation is an epigenetic change that is heritable, which can result in changes in chromatin structure often accompanied by modified patterns of gene expression (Waschman *et al*, 1997). DNA methylation involves a co-valent addition of a methyl group (CH_3) to the 5' position of cytosine that is followed by guanosine in the DNA sequence known as CpG islands. This is referred to as epigenetic because this modification does not change the coding sequence of DNA (Davis *et al*, 2004).

The functions of methylation include genome defense and/or protection and regulation in gene expression (Robertson *et al*, 2000). For example, human DNA and bacterial DNA (foreign DNA) are methylated differently, the defense mechanism will allow only foreign DNA to be destroyed by the endonuclease (Robertson *et al*, 2000)

The degree of DNA methylation can be regulated by three mechanisms each with distinctive DNA methyltransferase enzymes (Dnmt), *de novo* methylation of unmethylated cytosines (Dnmt 3a and 3b), maintenance of methylation after DNA replication (Dnmt 1) and lastly the loss of DNA methylation of methylated cytosines enzymatically through a demethylation process (Dnmt 2) (Watson *et al*, 2002).

In a study done by Watson (2002), it was shown that altered DNA methylation contributed to carcinogenesis and developmental disorders through:
1) hypomethylation of promoter regions leading to over-expression of oncogenes, 2) hypermethylation of promoter regions leading to suppression of

tumor suppressors, 3) hypermethylation leading to an increased deamination of 5-methylcytosine to thymine, and 4) alteration of imprinted gene regulation which is common in certain types of cancers.

2.3.4 Effect of chemicals on DNA integrity

Any unprogrammed change in the structure of DNA molecule structure especially from genotoxicants may pose serious biological problems particularly those with cancer potency (Shugart, 2000). Different genotoxicants have broad structural diversity and their genotoxic mechanisms are different see, table 2.2 below (Islaih *et al*, 2005). Possible genotoxic activity of chemicals and additional information on the relationship between chemical structure and ability to induce DNA lesions is important (Mattioli *et al*, 2004).

Furthermore, it has been shown that DNA lesions, many of which are potentially mutagenic, can interfere with the ability of DNA to serve as a substrate for Dnmt, which can result in a generalized hypomethylation. Also, abasic sites, single-stranded and products of oxidative and alkylation products can reduce the methyl-accepting ability of DNA. (Waschman, 1997).It has also been projected that polyploid cells that arise from exposure to genotoxicants, typically in the liver, become aneuploid through genetic instability, which contributes to or even drives cell death or mutation (Cantero *et al*, 2006).

Table 2.2: Structural modifications caused by genotoxicants on DNA

Genotoxicant	Type of modification	Mechanism
Physical	Thymine-Thymine dimer	Pyrimidine base dimerization
	Strand breakage	Free radical formation
Chemical	Adduct	Covalent attachment of genotoxicant
	Base alteration	Chemical modification of existing bases
	Abasic site	Loss of unstable adduct or damaged base
	Strand breakage	Breakage of phosphordiester bonds due to free radical and abasic sites formation
	DNA Hypomethylation	Postreplication interference
	Mutation	DNA repair interference

(Shugart, 2000)

2.3.5 Pathophysiology of tyrosine intermediate metabolites

p-Hydroxyphenylpyruvic acid (pHPPA)

According to Endo *et al* (2003) pHPPA is a keto acid that causes no apparent visceral damage and its accumulation in body fluids does not cause any specific pathology. However, it was found recently that exposure of isolated lymphocytes to pHPPA did cause DNA damage, but that its main effect was the inhibition of the DNA repair capacity (Van Dyk & Pretorius, 2005).

Homogentisate

The first steps of tyrosine degradation lead to the formation of homogentisate, in animals this is then sequentially acted on by homogentisate dioxygenase (HGD), maleylacetoacetate isomerase (MAAI) and fumarylacetoacetate hydrolase (FAH)

to generate fumarate and acetoacetate (Dixon *et al*, 2006). It has been shown that administration of homogentisic acid induced apoptosis of hepatocytes and there was an onset of acute liver failure (Kubo *et al*, 1998). In plants, homogentisate is used to generate the essential redox metabolites tocopherol and plastoquinone, which effectively act as an alternative metabolic fate for tyrosine (Dixon *et al*, 2006).

Maleylacetoacetate (MAA)

The accumulation of electrophilic intermediates such as maleylacetoacetate and maleylacetone results in a high level of oxidative stress (Blackburn *et al*, 2006). MAA can also alkylate cellular macromolecules such as DNA and/or disrupt essential sulfhydryl reactions by forming complexes with glutathion (GSH) proteins (Jorquera and Tanguay, 1999).

Fumarylacetoacetate (FAA)

It has been shown that a sub-apoptogenic dose of FAA the metabolic metabolite accumulating in HT1 induces spindle disturbances and segregational defects in both rodents and human cells (Jorquera *et al*, 2001). FAA, with its alkylating potential, attacks membranes of various cellular components, including direct interaction with the mitochondria and induction of the release of cytochrome c, which is an essential macromolecule that initiates activation of the caspase cascade leading to fragmentation of the nucleus. FAA seems to be a major metabolite responsible for the cell death signal via mitochondria leading to apoptosis (short-term effect) and to DNA damage (long-term effect) (Kubo *et al*, 1998).

Succinylacetone (SA)

SA excreted in the urine is a decarboxylation product of succinylacetoacetate and it is derived from the tyrosine catabolic intermediate fumarylacetoacetate. SA has been demonstrated in the kidney to be a mitochondrial toxin, that inhibits substrate-level phosphorylation by Krebs cycle. This compound also causes

membrane transport dysfunction in normal rat kidneys, altering membrane fluidity and possibly disrupting normal structure. It can cause renal tubular dysfunction in normal rat kidneys (Roth *et al*, 2003)

2.4 DNA REPAIR

The human genome (DNA), compromising three billion base pairs coding for 30 000-40 000 genes is constantly attacked by endogenous reactive metabolites, therapeutic drugs and a surplus of environmental mutagens that have an impact on its integrity, therefore the stability of the genome must be under continuous surveillance (Christmann *et al*, 2003).

This is accomplished by DNA repair mechanisms (Islaih *et al*, 2005), which have evolved to remove or tolerate pre-cytotoxic, pre-mutagenic and pre-clastogenic DNA lesions. The importance of DNA repair is illustrated by DNA repair deficiency and genomic instability syndromes which are characterized by increased cancer incidence and multiple metabolic alterations (Christmann *et al*, 2003). Next is the outline and brief discussion on the DNA repair mechanisms.

2.4.1 Mismatch repair (MMR)

The mismatch repair (MMR) system is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, DNA methylation and replication errors. Steps by which MMR proceeds are as follows: recognition of DNA lesions, strand discrimination and excision and repair synthesis (Christmann *et al*, 2003).

2.4.2 Nucleotide excision repair (NER)

NER is a versatile repair pathway involved in the removal of a variety of bulky DNA lesions; a complex process which includes recognition of DNA lesion, separation of the double helix at the DNA lesion site, single strand incision at both sides of the lesion, excision of the lesion-containing single stranded DNA

fragment, DNA repair synthesis to replace the gap and finally the ligation of the remaining single stranded nick (Mullenders *et al*, 2001).

2.4.3 Homologous recombination (HR)

Chromosomal double-strand breaks (DSBs) stimulate homologous recombination by several orders of magnitude in mammalian cells, but the efficiency of recombination decreases as the heterology between the repair substrates increases (Elliot *et al*, 2001).

2.4.4 Nonhomologous end joining (NHEJ)

This pathway ligates the two ends of a DNA double strand breaks (DSBs) without the requirement of sequence homology between the two DNA ends. It proceeds in the following steps: recognition and binding to damaged DNA, processing of DNA ends and finally ligation (Elliot *et al*, 2001).

2.4.5 Base excision repair (BER)

The main lesions subjected to BER are oxidized DNA bases, arising spontaneously within the cell during inflammatory response or from exposure to exogenous agents. It proceeds in the following steps: recognition, base removal and incision; nucleotide insertion; decision between short and long patch repair; strand replacement and DNA repair synthesis by long-patch BER and finally the ligation (Christmann *et al*, 2003).

2.4.6 Fanconi Anemia "pathway"

The mechanism on how the Fanconi Anemia chromosome stability pathway functions to cope with inter-strand cross-links and other DNA lesions has been elusive (Thompson *et al*, 2005).

2.5 PROBLEM STATEMENT

Tyrosine is a semi-essential amino acid derived from hydrolysis of dietary or tissue protein or from hydroxylation of phenylalanine. It is degraded through a cascade of enzymes to produce intermediate metabolites that are used in protein synthesis. Deficiency of one of the enzymes can lead to accumulation of these intermediate metabolites, and the buildup of these metabolites can lead to serious medical problems or genetic disorders like tyrosinemias.

2.6 STUDY AIM

To investigate the effects of tyrosine intermediate metabolites on genotoxicity and repair capacity and DNA methylation on rat hepatocytes *in vitro*

2.7 APPROACH

Rat hepatocytes will be isolated and then treated with tyrosine intermediate metabolites before assessing genotoxicity, DNA repair capacity and DNA methylation using:

- Single-cell gel electrophoresis (Comet assay)
- MTT assay
- DNA extraction using Nucleon Genomic DNA extraction kit (BACC1)
- Agarose gel electrophoresis
- DNA methylation using the cytosine extension assay

CHAPTER 3

MATERIALS AND METHODS

Ethical approval: This study was approved by the Ethics Committee of the North West University. Approval number 04D11.

3.1. PREPARATION OF LIVER CELLS WITH A MINCING SOLUTION

The 10 weeks old male Sprague-Dawley rats were sacrificed by decapitation and the liver was minced into large pieces and submerged in 3ml mincing solution (Appendix A). This was done to remove all blood from the liver to prevent contamination with blood cells e.g. lymphocytes, erythrocytes, platelets etc. After 20 minutes the mincing solution was aspirated and the liver further minced into smaller pieces and submerged in 3ml fresh mincing solution for 30 minutes at room temperature. Subsequently 1ml of the mincing solution, in which the liver pieces were submerged, was removed and 250µl PBS added. The cell suspension was centrifuged for 5 minutes at 5500rpm (1902xg) at 4°C. The supernatant was discarded and 250µl PBS was added to the pellet and centrifuged at the same specification as the previous step. The supernatant was again discarded and the cells were re-suspended in 500µl HAMS F10 and kept on ice.

3.2 CELL COUNTING AND VIABILITY

This was accomplished with the trypan blue dye exclusion assay. The principle behind the assay is that viable cells exclude the dye, whereas non-viable cells

stain blue due to a breakdown of their cell membranes. The following protocol describes steps for cell counting and viability:

The haemacytometer and cover slip were cleaned with 70% ethanol. Twenty microlitre of cell suspension was mixed with 20µl Trypan blue, using the pipette. 10µl of the cell suspension was transferred to the edge of the haemacytometer and allowed to spread evenly by capillary action.

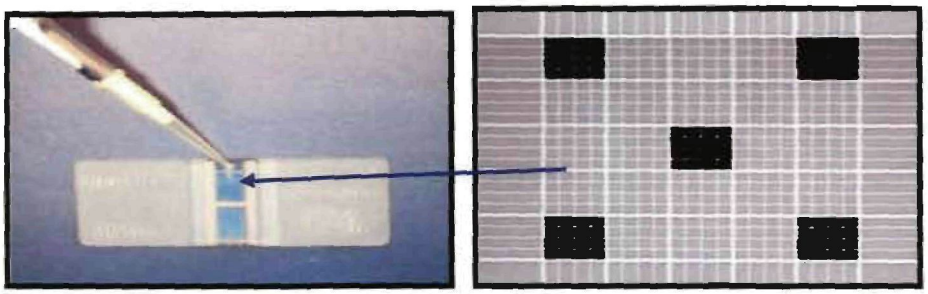

Figure 3.1: Haemacytometer. A specially designed glass slide with a 0.1 mm^3 chamber and a counting grid used for cell count and cell viability (www.edu-graphics.com/.../Haemacytomters.html)

The grid lines in the chamber were focused using the 10X objective lens. All four corner grids and the one in the middle were counted (figure 3.1), this prevents cells from being counted twice.

To calculate the number of cells the following formula was used :

Cells/ml=Average number of cells x dilution factor x 1000

and for cell viability

$$\% \text{ cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100\%$$

Cells were diluted with 1000µl HAMS F10 to a final concentration of 1 X 10^6 cells /ml

3.3 COMET ASSAY

3.3.1 Background

The comet assay is a simple, sensitive, and versatile method for the detection of DNA damage in individual cells. The assay can be applied to cells collected from virtually any eukaryotic organism, and can be used to detect DNA damage resulting from exposure to a broad spectrum of genotoxic and cytotoxic compounds *in vitro*, *in vivo*, and *in situ* (Cavallo *et al*, 2002). The assay is attractive because of its simplicity, sensitivity, versatility, speed, and low economical costs. The comet assay not only provides an estimate of how much damage is present in cells, but also the type of damage. Although it is essentially a method for measuring DNA breaks, the introduction of lesion-specific endonuclei allows detection of, for example, ultraviolet UV-induced pyrimidine dimers, oxidized bases, and alkylation damage (Collins *et al*, 2004).

3.3.2 Chemicals and Reagents

Hanks Balance Salt Solution, Phosphate Buffered Solution, Ficol Histopaque, Hydrogen peroxide (H_2O_2), high melting point agarose (HMPA), Low melting point agarose (LMPA), and ethidium bromide (staining dye) were purchased from Sigma Company, Johannesburg, South Africa. Lysis buffer, electrophoresis buffer and neutralization buffer were freshly prepared before use.

3.3.3 Materials.

Pipette tips, small test tubes and microscopic slides were purchased from merck, Johannesburg, Pasteur pipettes from AEC Armersham and disposable conical tubes from Separations.

3.3.4 Instrumentation.

- **Electrophoresis tank:** A horizontal self-circulation buffer electrophoresis tank. The tank accommodates 10 microscope slides.
- **Power supply:** A microcomputer electrophoresis power supply.

- **Fluorescence microscope:** Equipped with 10X, 40X and 50X objective lenses, a fluorescent burner, blue and green excitation filters and CCD camera.

3.3.5 Method

3.3.5.1 Effect of damage-causing metabolites on DNA repair capacity

A control sample of 20µl was taken from freshly prepared cells and mixed with 150µl Low melting point agarose (LMPA), 130µl of cell suspension was spread on a microscope slide coated with high melting point agarose (HMPA). For treated samples, metabolite (50µM SA; 100µM pHPPA) was added to the remaining cell suspension and incubated for 1 hour at 37°C. After incubation 250µl PBS was added to the cell suspension and this was centrifuged at 3000rpm (845xg) for 5 minutes at 4 °C to remove the metabolite before the 20 minutes and 40 minutes incubation allowed for DNA repair to take place. The supernatant was discarded and 300µl HAMS F10 was added to the pellet. Twenty microlitres of cells was mixed with 150µl LMPA and 130µl of cell suspension was spread on the microscope slide coated with high melting point agarose. The remaining cell suspension was incubated for 20 and 40 minutes, respectively to allow for DNA repair to take place

The microscopic slides were immersed overnight in the lysis buffer at 4°C. The following day the slides were rinsed in ddH_2O before incubation in electrophoresis buffer for 30 minutes and electrophoresed for 20 minutes at 30V and 270mA at 4°C in the electrophoresis buffer. After electrophoresis the slides were rinsed with ddH_2O before incubating in Tris buffer for 15 minutes, and were then stained for 15 minutes in the ethidium bromide solution at 4°C and finally rinsed in ddH_2O. The last step was comet scoring, whereby comet pictures were taken and cells were scored with Comet IV. This software measures various parameters of the comets.

Comet assay IV program

Comet Assay IV's unique single-click scoring method and ultra-fast live video picture make it the most efficient and easy-to-use system available for measuring DNA damage using single cell gel electrophoresis. There are no complicated parameters to adjust and no hardware to install. Simply connect the camera and start scoring (http://www.perceptive.co.uk/cometassay/)

Simply use the mouse to select a cell and Comet Assay IV will instantly calculate all measurement parameters and then add the data for the cell to your list of results. Click on the next cell to be scored and the process is repeated. Each scored cell within the field of view is marked with a cross to help prevent rescoring the same cell twice.

This program measures the amount of DNA in the comet tail, which correlates with the amount of DNA damage. The extent of DNA damage was grouped into classes according to the amount of DNA in the tail of the comets and is illustrated in figure 3.2.

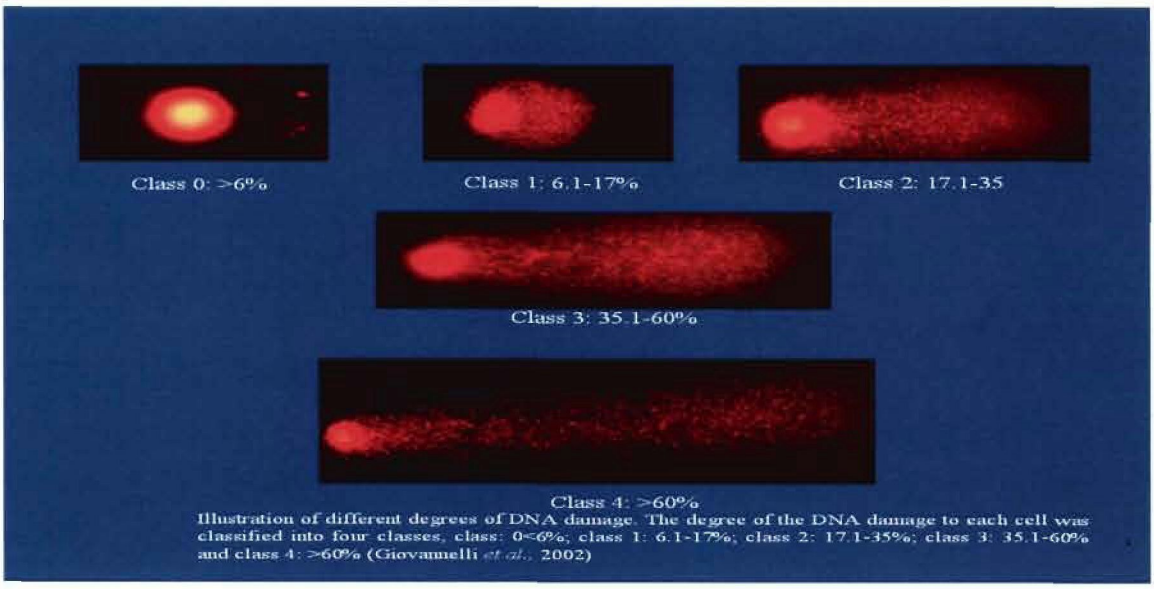

Figure 3.2: The different classes of comets (Giovannelli *et al*, 2002)

3.3.5.2 Effect of damage-causing metabolites on DNA repair capacity after oxidative damage induction

For oxidative DNA damage induction, the final concentration of 0.00588 M hydrogen peroxide (H_2O_2) was added to the treated cells (section 3.3.5.1) and then incubated for 30 minutes at 37°C After the incubation, cells were washed twice with phosphate buffered saline (PBS) to remove the excess H_2O_2. Twenty microlitre of cells was mixed with 150µl LMPA and 130µl of cell suspension was spread on the microscope slide coated with HMPA after treatment with hydrogen peroxide for 30 minutes at 37°C. The remaining cell suspension was incubated for 20 minutes to allow repair and another 20 minutes. Formula for repair capacity

$$RC = 1 - \frac{\%\text{Tail DNA (40 minutes)}}{\%\text{Tail DNA } (H_2O_2)}$$

and normal ranges for repair are between 0.4-0.5.

The microscopic slides were then treated as described above (section 3.3.5.1)

3.4 MEASUREMENT OF GROWTH AND CYTOTOXICITY USING THE TETRAZOLIUM SALT BIOREDUCTION ASSAY

3.4.1 Background

Tetrazolium salts are extensively used for a variety of research applications, including cell proliferation and cytotoxicity assays. In these assays, the tetrazolium salts are reduced to blue insoluble formazan dye crystals, (Berridge *et al*, 1996). In 1983 Mosmann introduced the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as the prototype tetrazolium salt used in cellular bioassays. The reduction of tetrazolium salts is regarded as a convenient test for measurement of the cellular activity and proliferation by incorporation of MTT (Bank *et al*, 1991). The general principle behind the assay is that formazan

crystals are formed by mitochondrial succinate dehydrogenase in living and early apoptotic cells, but not in dead cells (Berridge *et al*, 1996).

3.4.2 Method

Hepatocytes were isolated (according to section 3.1) and treated with (50μM, 100μM and 150μM) SA and (50μM, 100μM and 150μM) pHPPA for 1 hour. Following this incubation period, the cells were centrifuged at 1600g for 20 minutes, the supernatant was discarded and then the MTT cell viability bioassay was conducted. Three wells per treatment were performed and conducted in triplicates. Wells were treated with 5mg/ml MTT salt for 4 hours in the cell culture environment. Following incubation, the plates were centrifuged at 1600g for 20 minutes the supernatants were carefully aspirated with a sterile micropipette and discarded to remove the salt. Cells were incubated for 60 minutes with dimethyl sulfoxide (DMSO) to allow for solubilisation of the formazan product. Spectrophotometric analysis was conducted using Bio-Rad multiwell plate reader at 596 with a reference wavelength of 655nm.

3.5 AGAROSE GEL ELECTROPHORESIS OF DNA

3.5.1 Background

Gel electrophoresis based separation methods have been used for analysis of important biological polymers including proteins, DNA etc (Guttman and Ronai, 2000). It is one of the most commonly used separating techniques in the modern biology laboratory, due to its simplicity and versatility (Basim and Basim, 2001). Electrophoresis is used to separate the DNA fragments by size. Negatively charged DNA fragments are separated in an agarose gel bed by subjecting them to an electric field (Guttman and Ronai, 2000; Ye *et al*, 1999).

3.5.2 Method

The liquidized gel mixture (Appendix E) was poured into the molder, a comb was placed in to make wells and the gel was left to solidify. The combs were gently

removed and the mold was placed into the tank filled with buffer. Samples (Section 3.4.2) were loaded into the wells (3µl treated DNA sample + 2µl loading dye) with 2µl DNA ladder in the first well. The tank was closed and the power supply was switched on for 10 minutes at 20V and then increased to 60V for 1 hour. After 1 hour the power was switched off, the gel was removed from the tank and then viewed with the Gene snap program.

3.6 GLOBAL DNA METHYLATION PATTERN USING THE CYTOSINE EXTENSION ASSAY

3.6.1 Background

Changes in genomic DNA methylation patterns are an early and consistent hallmark of disease (Pogribny *et al*, 1999). Cancer cells are considered to have global hypomethylation and regional hypermethylation (Zambrano *et al*, 2005). Different methods have been developed to determine changes in global and regional DNA methylation status but none of them permits the simultaneous assesment of both global and CpG island methylation density in one assay (Pogribny *et al*, 1999).

Despite the fact that global and regional DNA methylation is frequently observed in cancer cells, mechanisms underlying these changes remain unclear (Pogribny *et al,* 2004).The cytosine extension method is based on the selective use of methylation-sensitive restriction enzymes that leave a 5' guanine overhanging after DNA cleavage followed by single nucleotide primer extension with [^{3}H] dCTP. It is adaptable to screening DNA from lymphocytes, tissue sections and biopsy samples for rapid and sensitive detection of early instability in DNA methylation patterns (Pogribny *et al*, 1999). Cytosine extension assay has several advantages over existing methods because radiolable incorporation is independent of the DNA integrity, methylation detection does not require PCR amplification and it is applicable to nanogram (ng) quantities of DNA (Pogribny *et al*, 1999).

3.6.2 Extraction of DNA from rat hepatocytes using the Nucleon Genomic DNA extraction kit (BACC1) for cytosine extension assay

Isolated and treated (pHPPA and SA) (Section 3.4.2) hepatocytes were collected by centrifugation at 600g for 5 minutes at 4°C. The supernatant was discarded without disturbing the pellet and cells were resuspended in 1ml of Reagent A and then left on ice for 5 minutes. The suspension was centrifuged at 1300g for 5 minutes at 4°C and the supernatant was discarded. For cell lysis, 350µl of Reagent B was added to the pellet and briefly vortexed to resuspend the pellet. The suspension was transferred to a clean 1.5ml microtube. For deproteinization, a solution of 100µl sodium perchlorate was added and then mixed by inverting at least 7 times It is strongly recommended that this is done by hand. Six microlitres of chloroform was added and then mixed by hand inversion 7 times to emulsify the phases.

A 150µl aliquot of Nucleon resin was added and without remixing the phases the suspension was centrifuged at 350g for 1 minute. Without disturbing the Nucleon resin layer (brown in colour), the upper phase (450µl) was transferred to a clean 1.5 microtube. Two volumes (900µl) of cold absolute ethanol was added and the hand inversion was done several times until the DNA was precipitated. This was followed by centrifugation at 4000g for 5 minutes to pellet the DNA then the supernatant was discarded, 1ml of cold 70% ethanol was added and hand inversion was done several times. The suspension was re-centrifuged and the supernatant was discarded and the pellet was air-dried for 10 minutes ensuring that all the ethanol has been removed. The DNA was re-dissolved in 250µl TE buffer.

3.6.3 Chemicals and Reagents

Nucleon Genomic DNA extraction kit (Nucleon BACC 1 for blood and Cell Cultures) was purchased from Amersham bioscience, Methylation-sensitive restriction endonucleases, 1X PCR buffer II, 1.0mM $MgCl_2$, 0.25 units of AmpliTaq DNA polymerase were purchased from Promega. Radio-labelled [^{3}H]-

dCTP was purchased from AEC-Amersham and Whatman DE-81 ion-exchange filters from Merck.

3.6.4 Method

3.6.5.1 Cytosine Extension Assay

Generation of DNA methylation standard: For the methylated control (positive control), the genomic DNA was treated with M.Sss I enzyme in the presence of a methyl donor S-adenosylmethionine (SAM). This enzyme then methylated the CpG sites resulting in a theoretically 100% methylated DNA control. For the unmethylated control (negative control), the genomic DNA was amplified via whole genome amplification which resulted in an unmethylated DNA control.

Liver cells were isolated (Section 3.1) and treated with tyrosine intermediate metabolites (pHPPA and SA). One microgram of isolated genomic DNA was digested for two hours with the methylation-sensitive Hpa II restriction endonuclease and its isochizomer Msp I at 37°C, respectively, in two different tubes. The single nucleotide extension reaction was performed in a 25µl reaction mixture containing 0.5µg DNA, 1X PCR buffer (Promega), 1.0 mM $MgCl_2$, 0.5 units GoTaq Flexi DNA polymerase (Promega), 0.1µl of [^{3}H]-dCTP (specific activity of 58.0Ci/mmol) and incubated at 56°C for 1 hour. Background incorporation was tested with time zero controls for each sample. Samples were then applied to DE-81 ion-exchange filters and washed two times with phosphate buffered saline (PBS) at room temperature. Filters were then air-dried and processed for scintillation counting with Liquid Scintillation Analyzer (TRU-CARB 2100TR) purchased from Packhar Bioscience Company.

The [^{3}H]-dCTP incorporation into DNA was expressed as mean disintegrations per minute (dmp) after subtraction of dmp incorporation in time zero samples (background incorporation). The scintillation counter was allowed to run three times. The absolute percent of double strand unmethylated CCGG sites was

calculated as [dmp incorporation after Hpa II / dmp incorporation after Msp I] X 100 (Pogribny *et al*, 1999).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The effect of tyrosine metabolites on DNA damage and repair in hepatocytes

4.1.1 Control experiments

Single cell gel electrophoresis (SCGE or comet assay) is a rapid and very sensitive fluorescent microscopic method to examine DNA damage and repair at individual cell level (Marlin *et al*, 2004). The DNA repair capacity of cells can also be measured by inducing oxidative DNA damage with H_2O_2 and then monitoring the lesion repair rate. To determine the effect on DNA integrity of the accumulating metabolites characteristic for tyrosinemia type1, primary liver cells were prepared as described in section 3.1. Approximately $1x10^6$ cells/ml could be isolated and the viability was above 90% which is enough for the comet assay (van Dyk and Pretorius, 2005). In this study, after exposing the isolated liver cells to tyrosine intermediate metabolites (SA and pHPPA), cells were subsequently exposed to H_2O_2. This was done to determine the effect of the metabolites on the DNA repair capacity of the liver cells.

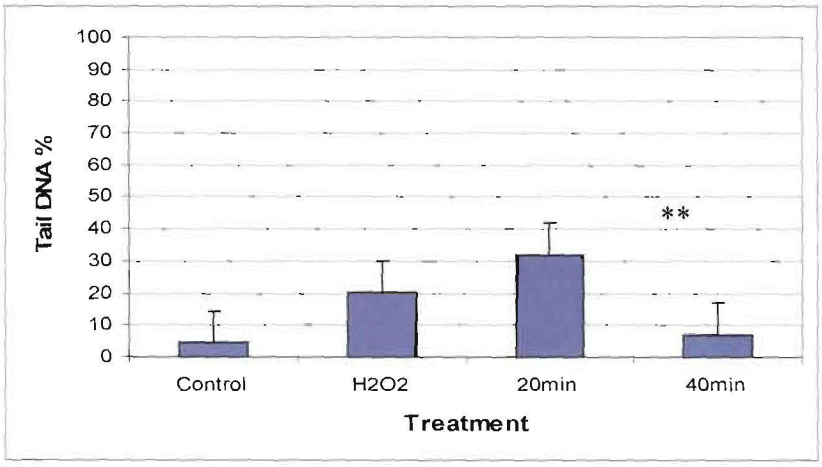

Figure 4.1: DNA repair in isolated liver cells. Asterisks indicate (**) significant difference relative to control $P< 0.05$.

To establish the feasibility of this cellular system for performing the proposed experiments, freshly prepared hepatocytes were exposed to 0.0058M H_2O_2 for 30 minutes at 37°C to induce oxidative DNA damage. The cells were then given time for DNA repair to take place and then the lesion repair rate by the cells was monitored with the comet assay. As shown in figure 4.1 there was an increase in tail DNA % relative to the control indicating the DNA damaging effect of H_2O_2. A further increase took place in the comet tail DNA % up till 20 minutes of repair time but thereafter the tail length decreased to a level slightly higher than the initial level. This transient increases in tail DNA % measured after 20 minutes repair time can be ascribed to the fact that the comet assay under alkaline (pH>13) conditions exposes the single strand breaks that is part of the initial stages of the DNA repair processes before the lesions are sealed during the ligation phase (Cipollini *et al*, 2006). Substantial DNA repair was apparent after another 20 minute repair time, and this is evident from the calculated repair capacity of 0.4 (Section 3.3.5.2).

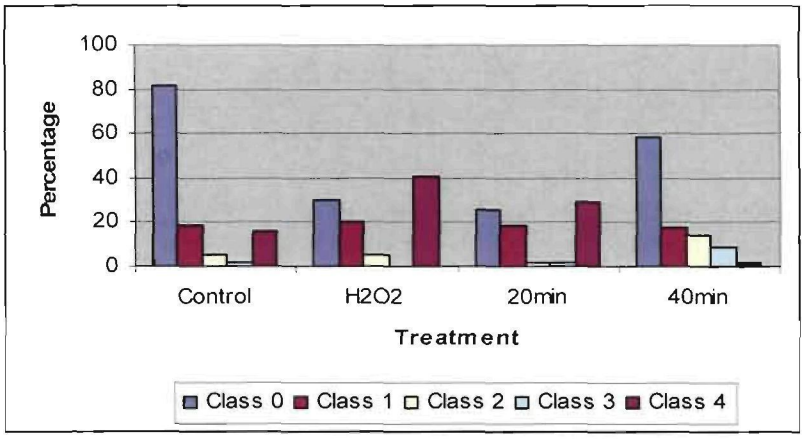

Figure 4.2: Comet distribution after H_2O_2 exposure and during repair time.

To allow a more detailed interpretation of the extent of DNA damage and repair, the comets were grouped into the different classes (Section 3.3.5.1, Figure 4.2). Most comets (40%) were present in class 4 and after H_2O_2 (Section 3.3.5.2)

treatment indicating severe DNA damage. Although there were still more comets (81%) in class 0 in the control cells compared to the situation after 40 minutes repair time (about 60%), it suggests a significant level of DNA repair and that the time allowed for DNA repair was long enough to obtain significant results. From these results it is clear that H_2O_2 did induce DNA damage but that the hepatocytes were able to repair this damage to a large extent.

This hepatocyte model seems, then, to be appropriate to be used to study the effect of accumulating intermediary metabolites of tyrosine metabolism on DNA integrity and DNA repair. Dixon *et al* (2004) has postulated that cells respond to DNA damage by activating a variety of DNA damage response pathways but if the damage is excessive cells die through induction of apoptosis and in the absence of DNA repair, the DNA damage results in mutations.

The next two sections give the results of experiments in which the effect of two such intermediates, i.e pHPPA and SA on DNA damage and repair were investigated.

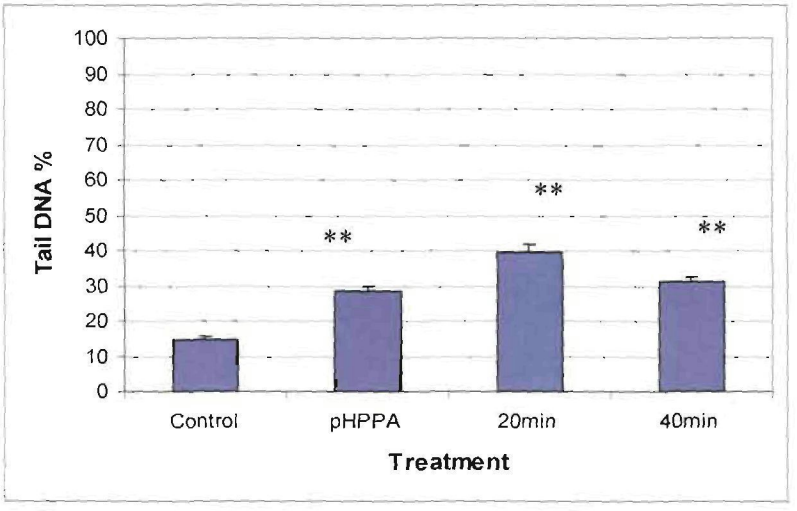

Figure 4.3: Effect of 100µM pHPPA on DNA integrity and repair after exposure in rat primary hepatocytes. Asterisks (**) indicate significant difference relative to control $P< 0.05$.

4.1.2 *para*-Hydroxyphenylpyruvate (pHPPA) treatment

Isolated hepatocytes were exposed to 100µM pHPPA for 60 minutes at 37° C then the metabolite was removed and the cells were allowed 20 minutes and 40 minutes time to repair damage inflicted to the nuclear DNA. There was an increase in the tail DNA % after metabolite exposure demonstrating the effect on the DNA integrity (Figure 4.3). An increase in the tail DNA % after 20 minutes repair time can be ascribed to the nicks created by the repair process as alluded to above. However, the tail DNA % after 40 minutes repair time did not decrease to the same level as was reported in figure 4.1. This means that some DNA repair did take place but not to the same level as was the case for the cells not exposed to this metabolite (Figure 4.1). A more detailed analysis of the comet distribution after exposure of the isolated liver cells to pHPPA was subsequently performed and the results are given in figure 4.4.

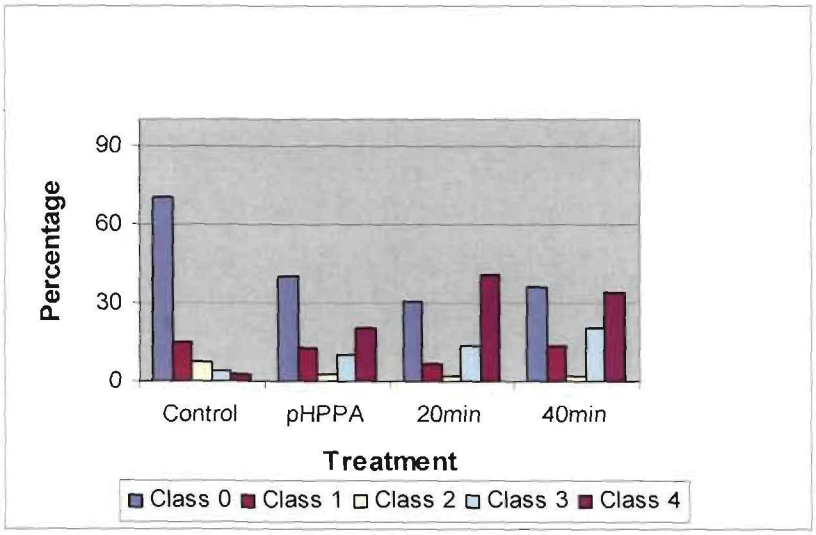

Figure 4.4: Comet distribution after exposure of the liver cells to 100µM pHPPA for 60 min at 37° C

Markedly more comets were present in classes 3 and 4 after pHPPA treatment of the liver cells in comparison to the unexposed cells. Comets were distributed in a similar pattern after 20 and 40 minutes of DNA repair time, but a further increase in the number of comets in these classes became apparent. These observations can be explained as follows: exposure of the liver cells to this metabolite caused

DNA damage that the cells were not able to repair during the allowed DNA repair time, because the number of comets in classes 3 and 4 slightly increased during repair time and the distribution of the comets stayed virtually the same during this time (Figure 4.4). As mentioned above, the increase in the number of comets in classes 3 and 4 can not be ascribed to nicks created by the repair process since this phenomenon is more transient in character (Cipollini *et al*, 2006), which is clearly not the case here. To ascertain whether pHPPA has a more direct effect on the activity of the DNA repair processes hepatocytes were treated with pHPPA for 60 minutes and then treated with H_2O_2 for 30 minutes at 37° C. The result and discussion is given in figure 4.5.

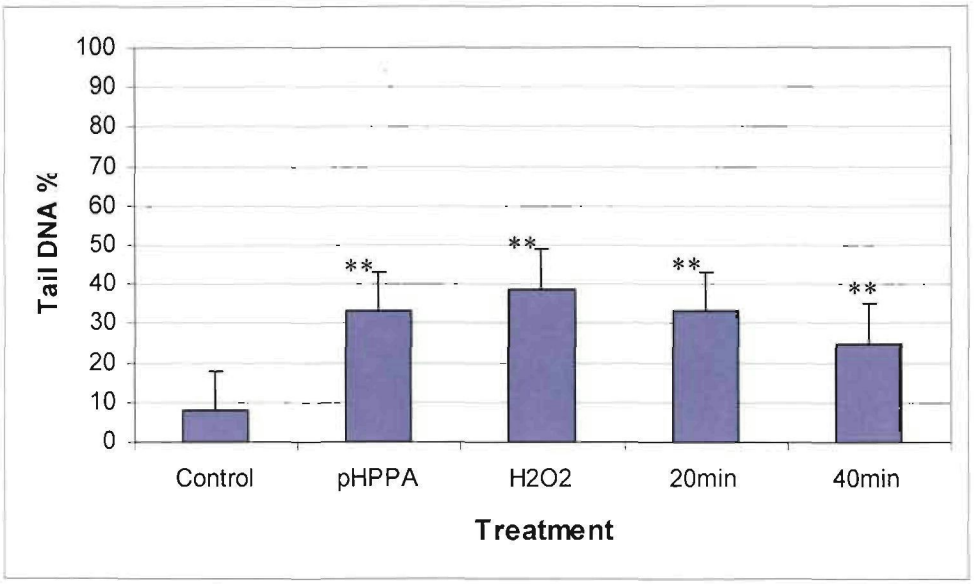

Figure 4.5: Effect of additional oxidative stress on DNA repair in pHPPA exposure liver cells. Liver cells exposed to100µM pHPPA were treated with 0.02% H_2O_2 as described in materials and methods. Asterisks (**) indicate significant difference relative to control $P<$ 0.05.

Additional oxidative stress imposed on the cells did cause further DNA damage as is evident from the slight increase in the tail DNA % after treatment of pHPPA exposed cells with H_2O_2. The decrease in the tail DNA % during the 20 and 40 minutes repair time suggests that some DNA repair did take place. However, the absence of the above-mentioned increase in the tail DNA % during the initial

phase of DNA repair (Cippolini *et al*, 2006) suggests that the DNA repair process was impaired by the exposure of the liver cells to pHPPA.

This result is supported to a great extent when the distribution of the comets in the various damage classes is compared (Figure 4.6). Treatment of the pHPPA exposed cells with hydrogen peroxide resulted in markedly more comets in class 4 than after the initial metabolite exposure. During the DNA repair time allowed, the number of comets in this class initially decreased by more than ten percentage points but beyond the 20 minutes repair time virtually no decrease in the number of comets in this class was seen. This decrease in the number of comets in class 4 as well as in the other classes took place in favour of class 0.

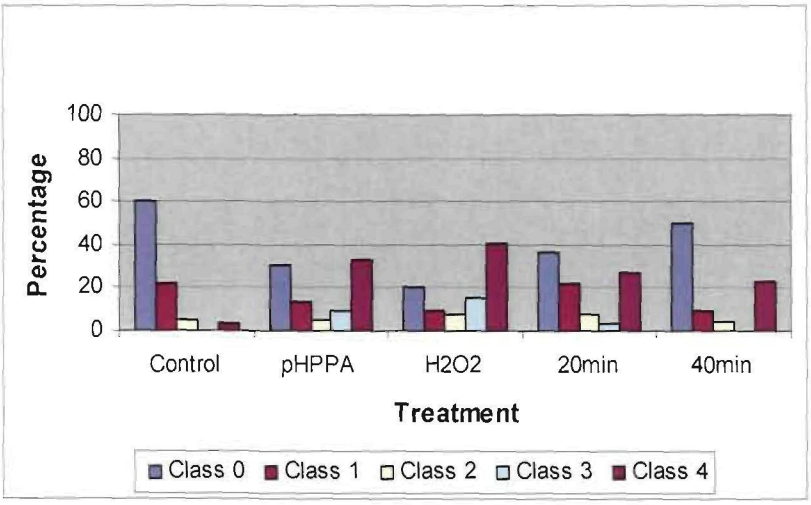

Figure 4.6: Comet distribution after 100μM pHPPA treated hepatocytes were exposed to H_2O_2 for 30 min at 37° C

Comparing these observations with the results given in figure 4.2, it looks as if DNA was repaired to the same level as was the case in control experiments; however, a substantial number of comets was still present in class 4. Although this substantial number of comets was still present in class 4 after 20 minutes repair time in the control experiments (Figure 4.2), this number decreased to almost zero after a further 20 minutes repair time. This was not the case when the cells were exposed to pHPPA (Figure 4.6). In one report numerous

mechanisms of induction of genetic instability have been identified, abnormalities of DNA repair enzymes being one of them (Bignold, 2004). The only conclusion to be made from these observations is that the pHPPA in some way hindered the DNA repair processes. This means that DNA damage by this metabolite, beyond a certain level, could not be repaired by the metabolite exposed liver cells.

4.1.3 Succinylacetone (SA) treatment

After exposing the hepatocytes to 50µM SA for 60 minutes at 37°C the metabolite was removed and the cells were allowed to repair for 20 minutes and 40 minutes. The columns in figure 4.7 show an increase in tail DNA % after SA exposure demonstrating the DNA damaging effect of the metabolite in the liver cells.

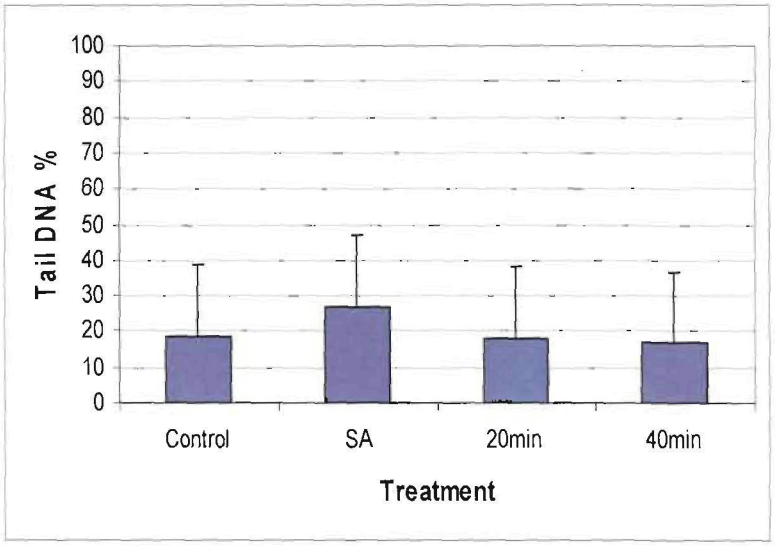

Figure 4.7: Effect of 50µM SA on DNA damage and repair in rat primary hepatocytes.

DNA repair did take place after 20 minutes as demonstrated by a decrease in the tail DNA % and after 40 minutes there was a further slight decrease in the tail DNA % observed. These results show a difference in DNA repair rate between liver cells treated with H_2O_2 (Figure 4.1) and SA compared to those treated with pHPPA (Figure 4.3). In the case of the SA treatment of the liver cells, no transient increase in the number of comets was apparent after 20 minutes of

repair time following exposure to the metabolite. Although this metabolite had a damaging effect on the DNA it was not statistically significant.

The analysis of the comet distribution after exposure of the isolated liver cells to SA was also performed. The results given in figure 4.8 show the highest number of comets in class 0 in the control cells with almost no comets in classes 3 and 4. After the cells were exposed to SA the number of comets in class 0 decreased and there was an increase of comets in class 3 and 4. After 20 minutes the comets in class 0 started to increase again and the number of comets in class 3 and 4 decreased showing the beginning of repair and after 40 minutes the comets were distributed in a similar pattern to that in the control cells.

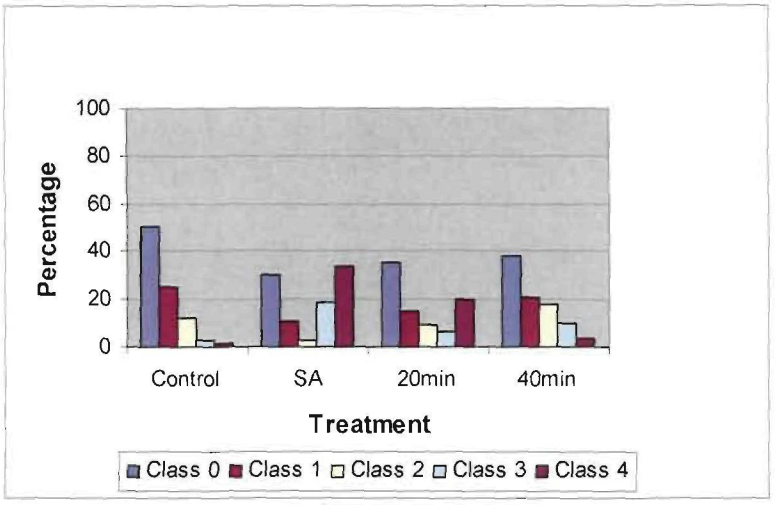

Figure 4.8: Comet distribution after exposure of the liver cells to 50μM SA exposure for 60 min at 37° C

These results show that the time given was sufficient for the liver cells to repair their DNA almost completely. This confirms the result given in figure 4.7.

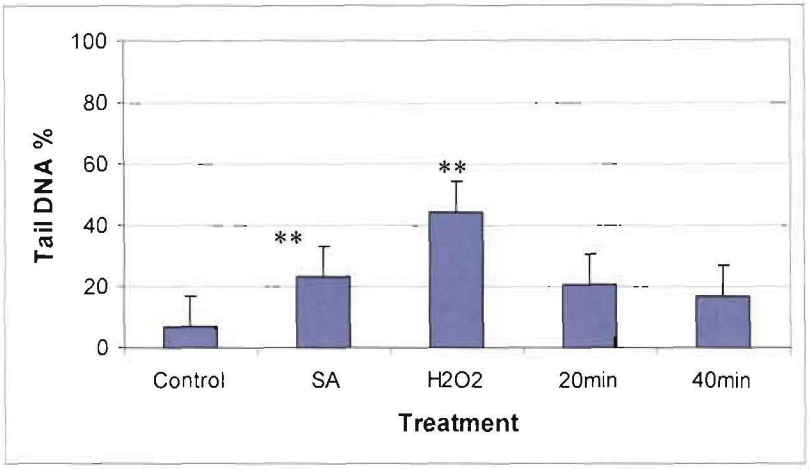

Figure 4.9: Effect of SA exposure on DNA repair in liver cells. Liver cells treated with SA were exposed to 0.00588M H_2O_2. Asterisks (**) indicate significant difference relative to control $P< 0.05$.

To ascertain whether SA has a more direct effect on the activity of the DNA repair processes, liver cells were treated with SA for 60 minutes and then treated with H_2O_2 for 30 minutes at 37°C. Additional oxidative stress inflicted on the cells did cause further DNA damage as was demonstrated by the increase in the tail DNA % after treatment of SA exposed cells to H_2O_2. The results given in figure 4.9 suggest that the DNA repair was not impaired by the exposure of the liver cells to SA This is evident from the decrease in tail DNA % after 20 minutes and 40 minutes. Given enough time, this would eventually reach the control value.

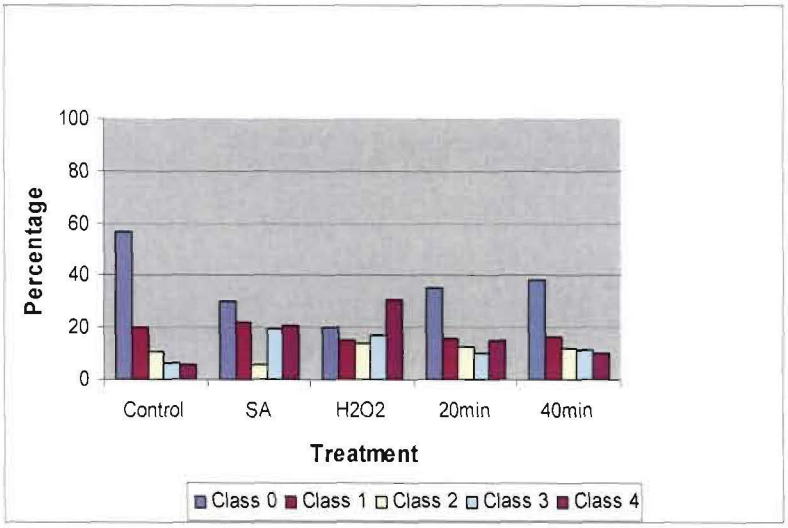

Figure 4.10: Comet distribution after exposure of SA treated hepatocytes with H_2O_2 for 30 min at 37° C

A closer look at these results confirms this notion. Treatment of the SA exposed cells with H_2O_2 resulted in more comets in class 4 than after SA exposure. During the DNA repair time allowed, the number of comets in class 0 increased and comets 3 and 4 started to decrease indicating continuous DNA repair. The results show that the DNA repair capacity was not significantly affected.

4.1.4 Effect of tyrosine metabolites on cell viability

The question of what the effect of these DNA damaging metabolites was on the viability of the isolated liver cells, was subsequently investigated by performing the MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Upon addition of DMSO, crystals were solubilized, released into the extra-cellular space and then readily quantified colorometrically. The number of surviving cells is directly proportional to the level of the formazan product created (Bank *et al*, 1991; Berridge *et al*, 1996). For this study, before measuring DNA methylation after exposing isolated liver cells to the accumulating tyrosine metabolites, the MTT cell viability bioassay was conducted to measure the cytotoxicity of the accumulating metabolites.

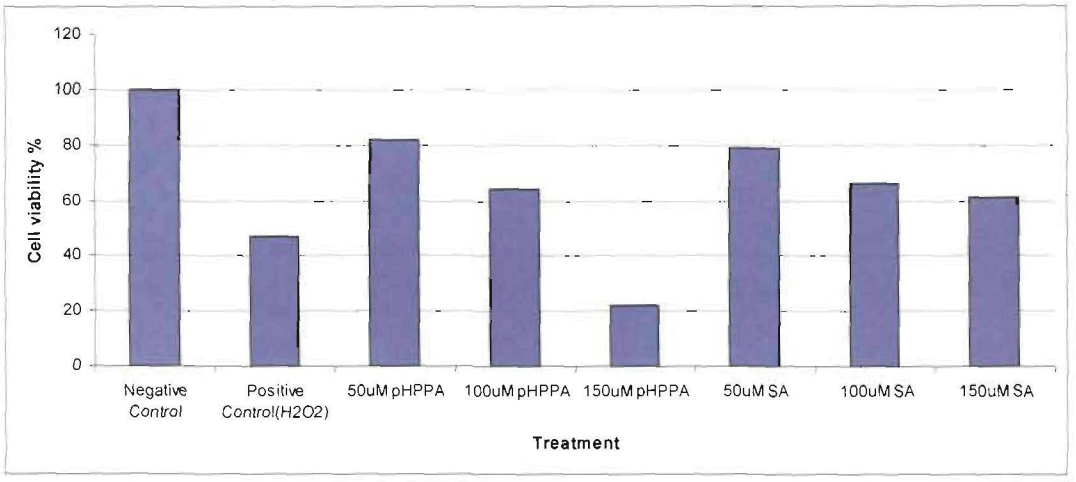

Figure 4.11: Cell viability following exposure of liver cells to HT1 metabolites. Isolated liver cells were exposed to increasing concentrations of p-hydroxyphenylpyruvic acid (pHPPA) and succinylacetone (SA), respectively, for one hour. Cells exposed to 0.00588M H_2O_2 were used as a positive control.

Hepatocytes were isolated (according to section 3.1) and treated with (50μM, 100μM and 150μM) SA and (50μM, 100μM and 150μM) pHPPA for 1 hour. Because the results would be represented clearly and simply as percentages, the untreated control was taken as 100% and the rest of the percentages were then calculated accordingly. A dose-dependent decrease in cell viability was observed at all concentrations of the metabolites studied (Figure 4.11). Cell viability decreased more significantly in the case of pHPPA than in the case of SA. After exposure of the cells to the highest concentration (150μM) of pHPPA only 22% of the cells were viable whilst in the case of SA, more than 60% of the cells were viable. From the results shown in figure 4.11 it is clear that high concentrations of these tyrosine metabolites caused cell damage and even cell death in the case of pHPPA.

4.2 The DNA methylation status of metabolite exposed liver cells

In order to measure the possible effect on the global DNA, methylation caused by pHPPA and SA hepatocytes were isolated (section 3.1), exposed to the respective metabolites and total DNA was extracted using the Nucleon Genomic

DNA extraction kit (BACC1 for blood and cultured cells) (Section 3.6.2) and the DNA concentrations and purity were determined spectrophotometrically.

Table 4.1: Yield and purity of the isolated DNA.

Treatment	[DNA] ng/μl	Purity (OD_{260}/OD_{280})	(Yield) from $1x10^6$ cells ng/μl
Control	92.2	1.81	100%
50 μM pHPPA	4.7	1.49	21%
100 μM pHPPA	4.4	1.57	11%
150 μM pHPPA	4.3	1.31	9%
50 μM SA	78.9	1.75	79%
100 μM SA	78.1	1.74	71%
150 μM SA	41.1	1.72	69%

A representative set of results is given in table 4.1. This experiment was repeated three times with great variation in the results but each time the same trend was observed, i.e. isolation of very small amounts of DNA from the pHPPA treated cells and for both metabolites a decrease in the DNA yield with increase in metabolite concentration. There was a drastic decrease in the quantity of isolated DNA isolated after the pHPPA treatment as was obvious from low values obtained with the NanoDrop spectrophotometer. From these results it is also evident that treatment of the cells with the metabolites also affected the DNA purity because pure DNA (OD_{260} /OD_{280}> 1.8) was isolated only from the untreated cells.

Agarose gel eletrophoresis analysis of the isolated DNA shows that virtually no DNA could be isolated from the pHPPA treated cells (Figure 4.12) at all the concentrations tested. Since only very faint bands are visible in lanes 1, 2 and 3, it is possible that no intact large molecular weight DNA could be isolated from these cells because of single and double strand breaks introduced by the pHPPA. This corresponds, to a large extent, with the results given in figure 4.3

where nicks were suggested to exist in the DNA after exposure of the cells to pHPPA.

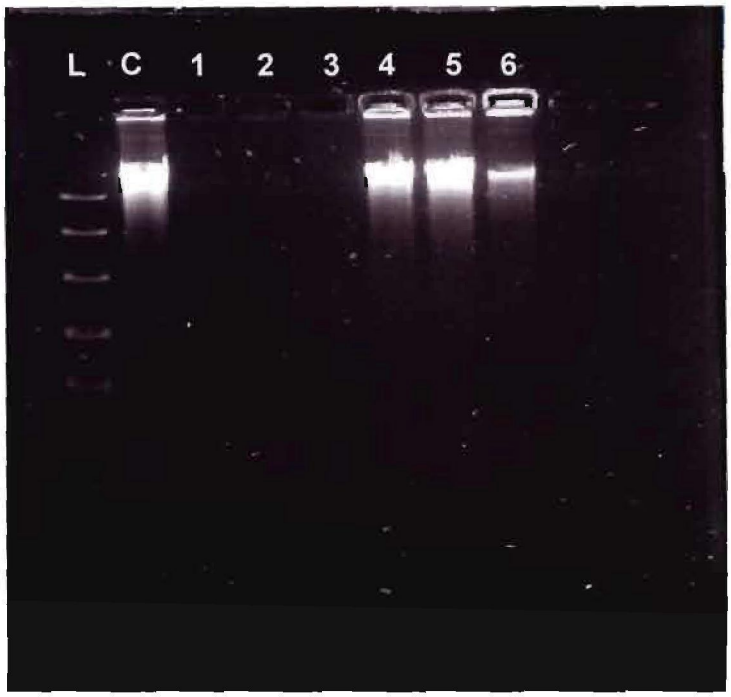

Figure 4.12: Gel electrophoresis of extracted genomic DNA. Each lane contains DNA after treatments at different concentrations of metabolites. **L**-ladder.(Fermentas Fast ruler DNA ladder high ruler), **C**-control, **1**-50μM pHPPA, **2**-100μM pHPPA , **3**-150μM pHPPA, **4**-50μM SA, **5**-100μM SA, **6**-150μM SA.

In the case of the SA treated cells from the highest concentration exposed cells, less DNA was observed in the gel but in all lanes it does appear that the DNA was not markedly more degraded by the exposure to this metabolite than the control DNA. This does not explain why DNA could not be isolated from the pHPPA exposed cells, except if the effect on the DNA by the two metabolites takes place through different mechanisms. Figures 4.3 to 4.6 show that enough cells could be seen as comets after treatment.

From the original cells source, DNA was extracted after the cells were treated with pHPPA and SA for the cytosine extension assay whose results are shown in Figure 4.13. It can clearly be seen that the higher the concentrations of the

treating metabolites, the cells yield decreased tremendously and DNA was undetectable at concentrations 100µM and 150µM pHPPA most probably because most cells died during the treatment stage of the experiment and the few left were lost during the washing stages of the DNA extraction method.

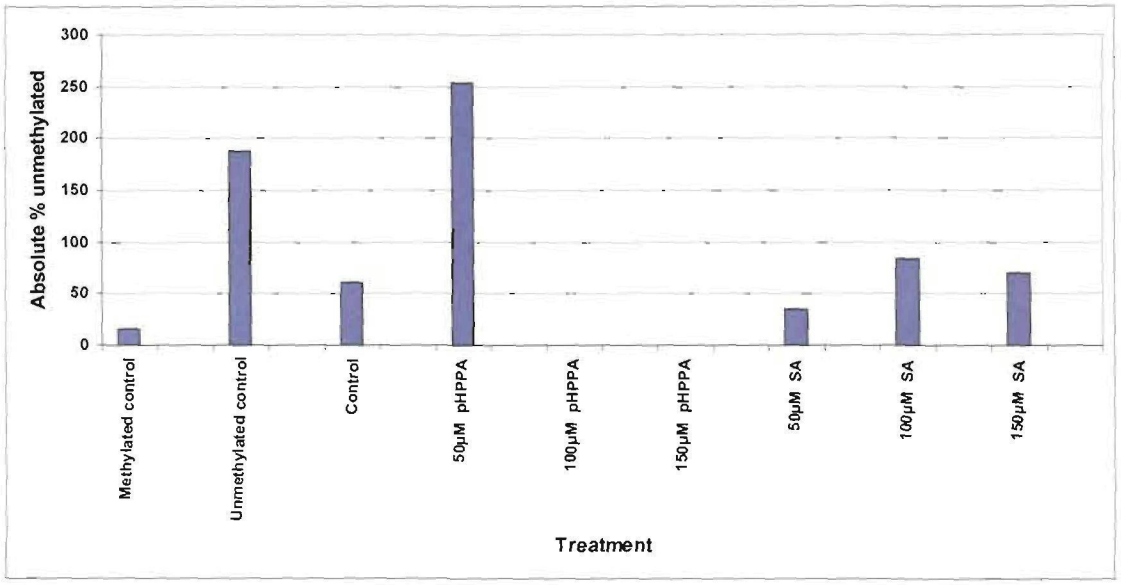

Figure 4.13: DNA methylation in metabolite exposed hepatocytes. The isolated liver cells were treated with pHPPA and SA for 1 hour at 37°C at the concentrations indicated. These are the results from one of two experiments that showed the same trend.

For the global DNA methylation assay, the single nucleotide extension reaction was performed in the presence of two controls, i.e. a methylated and unmethylated control substrate. Liver cells were isolated (section 3.1) and treated with tyrosine intermediate metabolites (pHPPA and SA). The cytosine extension assay was preformed with these DNA samples as substrates.

With this assay of the global DNA methylation levels, an almost two-hundred-fold difference was detected in the methylation status of the unmethylated and methylated controls (Figure.4.13). Thus, this assay seems to be suitable to measure the effect of these two metabolities on the global DNA methylation status in the isolated hepatocytes.

In the case of the hepatocytes exposed to 50µM pHPPA, the calculated % unmethylated DNA was almost five times higher relative to the unexposed cells. Compared to the controls, it seems as if hypomethylation occurred during exposure to the higher concentrations of this metabolite, however, this result is most probably due to the small amount of DNA available. In the cells treated with 50µM SA a decrease in absolute % unmethylated DNA compared to the unmethylated control, but higher that the methylated control, is evident. This could mean that at this concentration hypermethylation of the DNA occurred. At 100µM and 150µM there were increases in absolute % unmethylated value. This shows that the CCGG were starting to gain methyl groups.

These results suggest that exposure of the hepatocytes to pHPPA may induce demethylation of the DNA but that SA did not have a significant effect on the global DNA methylation status of these cells *in vitro*

In the case of pHPPA treatment, cells were found to be deficient in their overall levels of DNA methylation, which could also mean that the pHPPA could be a DNA methyltransferase (DMT) inhibitor. If this is the case, it suggests that pHPPA does not have a direct effect on the DNA methylation but rather an indirect effect via decreasing the activity of the DMT enzyme.

CHAPTER 5

SUMMARY AND CONCLUSION

Impaired degradation of the aromatic amino acid tyrosine is a feature of several acquired and genetic liver disorders and HT1 is one of the disorders known to cause liver dysfunctions (Grompe, 2001). HT1 is an autosomal recessive inborn error of tyrosine metabolism caused by deficiency of FAH (Figure 2.1) that produces liver failure in infancy or a more chronic course of liver disease with cirrhosis often complicated by hepatocellular carcinoma in childhood or early adolescence (Rootwelt *et al*, 1994; van Amstel *et al*, 1996; Kim *et al*, 2000).

In the case of HT1 a defective FAH leads to accumulation of alkylating tyrosine metabolites that causes hepatic damage (Rootwelt *et al*, 1994). HT1 can be distinguished into two major clinical forms, the acute and chronic forms based on the severity and rate of progression. The chronic form results in more gradual liver diseases which are normally complicated by HCC (Vogel *et al*, 2004).

The acute form is characterized by liver failure and death (van Amstel *et al*, 1996). High risk of cancer has been shown to be reflected in the high frequency of dysplasia, aneuploidy and the variable gene and protein expression encountered in tyrosinemic liver (Russo *et al*, 2001). It has also been shown that aberrant DNA methylation is also involved in the early event of tumour development in the general world of cancer (Brena *et al*, 2006). Current therapeutic strategies in HT1 are dietary restriction of amino acids tyrosine and phenylalanine, the use of NTBC and liver transplantation (Overturf *et al*, 1996).

There was a case study (Kim *et al*, 2000) done on a thirty-seven year old woman with HT1 whose severe liver disease in infancy and rickets during childhood was resolved with dietary therapy. From the age of 14 she resumed an unrestricted diet with the continued presence of the biochemical features of tyrosinemia, yet maintained normal liver function. In adult years she accumulated only small amounts of SA as was detected in her urine. Despite this evolution to a mild biochemical and clinical phenotype, the woman eventually developed HCC. This shows that HCC still occurred despite long-term survival in chronic tyrosinemia type 1.

In one report (Gilbert-Barness *et al*, 1994), HCC and renal tubular dysplasia were revealed in an autopsy of an HT1 patient. Cytogenic studies revealed greatly increased chromosome breakage (≈71% of the cells). This suggested the development of hepatoma which is frequent in HT1 and the presence of hepatic dysplastic changes in non-tumorous liver that are related to genetic instability caused by accumulation of tyrosine intermediates, which are mostly natural alkylating agents (Gilbert-Barness *et al*, 1994).

Tyrosine metabolites have been known to be causative agents of the pathologies observed in HT1 (Jorquera *et al*, 1997), but not much has been said about pHPPA being directly involved in the pathophysiology of HT1 except that its main effect is the long-term impairment of the DNA repair machinery, which could develop into HCC eventually (Van Dyk *et al*, 2007). It has been demonstrated that various amino acids metabolites induce oxidative DNA damage (Hiraku *et al*, 2006) and that enhancement by H_2O_2 might lead to further increase in damage.

pHPPA is a keto acid that causes no apparent visceral damage and its accumulation in body fluids does not cause any specific pathology (Endo *et al* , 2003), and SA is a decarboxylation product of succinylacetoacetate which is known to cause membrane transport dysfunction in normal rat kidneys, altering membrane fluidity and possibly disrupting normal structure (Roth *et al*, 2003)

To elucidate the possible contribution of tyrosine intermediate metabolites, that accumulate in HT1, to the development of hepatocellular carcinoma, DNA damage, repair and methylation were measured in isolated hepatocytes. This was done by treating primary rat hepatocytes with two of the known accumulating metabolites in the tyrosine catabolism (SA and pHPPA). The comet assay was used in the measurement of DNA damage and repair. The assay involves embedding cells in agarose, lysis of the cells and electrophoresis under alkaline conditions. Fluorescent staining revealed fragmented DNA that has migrated from nuclei of cells.

The principle behind this assay is that the distance and/or amount of DNA migration is indicative of the number of single and double DNA strand breaks (Collins, 2004). This assay was shown to be appropriate to measure the amount of DNA repair in isolated hepatocytes following exposure of the cells to hydrogen peroxide (Figure 4.1). This allowed us to investigate the effect of the above mentioned metabolites on the integrity of DNA in hepatocytes to determine their role in establishing the phenotype of HT1.

The results of this study show that in freshly isolated primary cultures of rat hepatocytes, DNA damage inflicted by exposure of the cells to H_2O_2 and the DNA repair could be measured by the comet assay In the control cells, with hepatocytes not exposed to metabolites, the H_2O_2 did induce DNA damage but the hepatocytes managed to repair these lesions showing that the H_2O_2 did not hinder or affect their DNA repair capacity (Figure 4.1). This system was therefore deemed suitable to study the effect of metabolites on DNA damage and repair.

To determine the possible detrimental effect of some of the metabolites accumulating in HT1 on the DNA repair capacity *per se*, cells were exposed to pHPPA and SA respectively and subsequently to H_2O_2. This experiment was done because chemical damage to DNA was implicated in the development of

HCC which is a feature of HT1 (Rootwelt *et al*, 1994; Wang *et al*, 2002). The results of the present study show that pHPPA (Figure 4.3) caused DNA damage that the cells were not able to repair during the allowed DNA repair time. This indicates that pHPPA affected the DNA repair capacity of the cells. To ascertain whether or not pHPPA has a more direct affect on the activity of the DNA repair process, additional oxidative damage (H_2O_2) was imposed on the cells and the damage increased even further and the DNA repair capacity was decreased (Figure 4.5), which was also demonstrated by Hiraku (2006).

It has been hypothesized that SA accumulation could play a role in the DNA metabolism of HT1 cells (Grompe, 2001). SA reacts nonenzymatically with proteins and free amino acids especially lysine producing adducts via Schiff base formation. SA reacts with proteins involved in the active site of DNA-ligases that are involved in DNA replication, repair and recombination (Prieto-Alamo *et al*, 1998). From the findings observed by Daza (1995) it is clear that DNA-ligases are SA targets This was confirmed by a high level of chromosomal breakage observed in the fibroblast from HT1 patients caused by the overall low DNA-ligase activity and the reduced rate at which the Okazaki fragments were rejoined. Cells deficient in DNA ligase have been shown to be hypersensitive to an alkylating agent that can induce chromosomal damage (Daza *et al*, 1995). However, our experimental results (Figure 4.7) showed almost complete DNA repair after SA treatment of the cells and treatment with SA plus H_2O_2 (Figure 4.9) DNA damage increased but there was a great deal of DNA repair observed. There were differences seen in all controls and that difference could be attributed to the fact that different rats were used each day an experiment was done and the average was used for the results.

In order to extend our observation on the effect of the metabolites in question, a cell viability assay was conducted. The MTT assay was used for assessing cell viability of the metabolite treated cells. There was lower cell viability in pHPPA treated cells (Figure 4.11) and with SA treatment the viability was much higher

than in pHPPA treated cells. This means that pHPPA had a more drastic effect on the isolated hepatocytes than SA. To investigate whether or not this is reflected in the integrity of the genetic material, DNA was then isolated from metabolite treated and untreated cells with the Nucleon Genomic DNA Extraction Kit (BACC1) (Section 3.6.2). The concentration and purity of the isolated DNA were measured with the NanoDrop spectrophotometer. Smaller amounts of DNA with less purity were isolated from cells treated with pHPPA than from cells treated with SA (Table 4.1). The smaller A_{269}/A_{280} ratios obtained in the case of the pHPPA treated cells, can most probably be ascribed to the inability of the NanoDrop to give a proper UV spectrum of the DNA at these low concentrations of DNA.

This nucleon kit has been known consistently to provide high yields of highly pure and largely intact DNA from a range of sample types (small blood volumes up to 1ml and $1X10^6$ to $3X10^6$ cultured cells, BACC1, *Blood and Cell Culture Manual*). According to the manual, during the extraction, the DNA is not bound to a solid surface at any point in the process. This avoids exposing the DNA to shearing forces and promotes the recovery of high yields of highly intact DNA. To check further the presence and nature of the isolated DNA, agarose gel electrophoresis (Figure 4.12) was conducted. The results (Figure 4.12) confirmed the results from the NanoDrop (Table 4.1). From the results given in table 4.1 and figure 4.12 it seems as if the DNA isolation kit could extract only the remaining intact DNA from treated cells. However, with the comet assay the cells could be read as comets because the DNA, although damaged, was still inside the intact cells and only released during the soaking of the slides in the lysis buffer.

Because alterations in the heritable patterns of DNA methylation are commonly observed in the malignant transformations usually observed in HT1 (Pogribny *et al*, 1999), its detection may be a useful clinical biomarker and warrant clinical intervention. It was therefore important to measure the methylation status of the

DNA of the liver cells treated with the tyrosine intermediate metabolites using the cytosine extension assay.

One study done by Ehrlich (2002) showed that, although malignant and normal samples display diversity in their DNA's genomic 5-methylcytosine content, a high percentage of malignant tumours have DNA with low genomic 5-methylcytosine contents relative to the normal, showing hypomethylation. In this study the DNA's global genomic 5-methyllcytosine content was measured on the treated rat liver cells with the cytosine extension assay.

In this assay, the absolute percent of methylated sites in DNA were determined by using HpaII and MspI–based cytosine extension assay with minor modifications that measured the proportion of CCGG that had lost methyl groups on both strands (Pogribny *et al*, 2004). With this assay the effect on the methylation levels of DNA caused by the metabolites under investigation in this study could be measured. Results (Figure 4.13) showed an increase in the absolute % unmethylated DNA after 50μM pHPPA treatment illustrating hypomethylation There were no results the 100μM and 150μM pHPPA because of absence of DNA. It is speculated that DNA was too fragmented. That is why the isolation was not successful in this regard. SA seemed to cause methylation because it had low absolute % unmethylation. These results suggest that pHPPA might have induced demethylation of the DNA but SA did not have a significant effect on the methylation status of these cells *in vitro*. pHPPA is known indirectly to be involved in the etiology of HT1 but it has the ability to promote cancer through effects on chromosomal stability because of its hypomethylation potency.

In another study done by Pogribny *et al* (1999), the damaged DNA template has been shown to interfere with DNA methyl acceptance therefore resulting in hypomethylation. This clearly shows the case of the pHPPA in which this metabolite caused the damage to the DNA template thus resulting in hypomethylation (Figure 4.13). Because hypomethylated DNA in preneoplastic

tissues often contains unrepaired lesions and because the lesions caused by pHPPA in this case were not significantly repaired, this could result in mutations that could lead to liver dysfunctions often complicated by hepatocellurcarcinoma seen in the chronic forms (Jorquera *et al*, 1997). SA did not have a significant effect on the global DNA methylation status of the treated cells (Figure 4.13).

Bignold (2004) concluded that DNA is a carcinogen target after some observations that showed strand breaks in the DNA of hepatocytes caused by carcinogens. It has been observed that strand breaks caused by hepatic carcinogens are slowly repaired in the liver, while breaks caused by non-hepatic carcinogens are rapidly repaired in this organ. The experimental results from this study suggest that pHPPA (Figure 4.3) could be an hepatic carcinogen as the breaks were slowly, if at all, repaired compared to the SA treated cells (Figure 4.7).

It has been hypothesized that accumulation of abnormal tyrosine intermediate metabolites produced in HT1 causes DNA damage, causing lesions on the DNA (Figure 4.3 and 4.7) (Gilbert-Barness *et al*, 1990). Failure to repair these lesions gives rise to cell death, chromosomal damage, mutations and even cancer (Kaina *et al*, 2007). The two tested metabolites (pHPPA and SA) showed that they could contribute to some of these outcomes mentioned above. Among other changes, global DNA hypomethylation is found in HCC as a common feature of HT1 (Tangkijvanich *et al*, 2007). This was demonstrated in figure 4.13 with two tested metabolites. Although SA did not have a significant effect on the methylation status of the treated cells, pHPPA caused demethylation of DNA.

The aims of this study were achieved to a large extent because in this study the putative genotoxic effects of these two metabolites, pHPPA and to a lesser extent SA, were confirmed to be involved in the development of HCC in HT1. Extending these findings, future studies can cover many endpoints to study the effect of combinations of these metabolites on DNA damage and repair and

epigenetic events including the use of the possibilities of conducting Comet assay with lesion-specific endonuclease enzymes like *Alk*A on the detection of damage by alkylating agents. Use of animal models and microarray techniques should be able to shed light on the study of new aspects of inborn errors of amino acid metabolism.

A limitation in this study could be the fact that primary cells were used and not cell lines. Although the primary cells closely mimic the *in vivo* state and generate more physiologically relevant results, their drawback is that they are different in nature for each preparation and one cannot do repeats from one source because of their relatively short life-span. Also, different animals were used in experiments performed on different occasions. Further studies should include the use of both primary cells and cell lines to compare the mechanisms and effects of these tyrosine intermediate metabolites.

REFERENCES

ATHAR M. 2002. Oxidative stress and experimental carcinogenesis, *Indian Journal of Experimental Biology* **40**(6): 656-67.

BANK U, REINHOLD D AND ANSORGE S. 1991. Measurement of cellular activity with the MTT test. Optimization of the method, *Allergie und Immunologie* **37**(3-4): 119-123.

BASIM EH AND BASIM H. 2001. Pulse field gel electrophoresis (PFGE) technique and its use in molecular biology, *Turkish Journal of Biology* **25**: 104-418

BERG J, TYMOCZKO J AND STRYER L. 2001.*Biochemistry,* 5th edition. New York:W. H. Freeman. The electronic version of this book can be found online at http://www.jiffynotes.com/a_study_guides/book_notes/gen_03/gen_03_00177.html

BERRIDGE MV, TAN AS, McCOY KD AND WANG R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4**: 14-19.

BIGNOLD LP. 2004. Carcinogen-induced impairment of enzymes for replicative fidelity of DNA the initiation of tumours, *Carcinogenesis* **25**(3): 299-307.

BLACKBURN AC, MATTHAEI KI, LIM C, TAYLOR MC, CAPELLO JY, HAYES JD, ANDERS MW AND BOARD PG. 2006. Deficiency of glutathione transferase zeta causes oxidative stress and activation of antioxidant response pathways; *Molecular Pharmacology* **69** (2): 650-657

BRENA RM, HERBERT A, KARL K, BJÖRN H, APARNA R, JOHN CB AND CHRISTOPH P. 2006. Accurate quantification of DNA methylation using combined bisulfite restriction analysis coupled with the Agilent 2100 Bioanalyzer platform; *Nucleic Acids Research* **34**(3): e17.

CANTERO G, PASTON N, MATEOS S, CAMPELLA C AND CORTES F. 2006. Cisplatin-induced endoreduplication in CHO cells: DNA damage and inhibition of topoisomerase II, *Mutation Research* **599**(1-2): 160-166.

CAVALLO D, TAMAO P, MARINACCIO A, PERNICONI B, ANDREA S, SILVANA P AND IAVICOLI S. 2002. Evaluation of DNA damage in flight personnel by Comet assay; *Mutation Research* **516**: 148-152.

CHOUDHURY S, ZHANG R, FRENKEL K, KAWAMORI TC , FUNG L AND ROY R. 2003. Evidence of alterations in base excision repair of oxidative DNA damage during spontaneous hepatocarcinogenesis in Long Evans Cinnamon rats. *Cancer Research* **63**: 7704-7707.

CHRISTMANN MS, TOMICIC TM, ROOS PW AND KAINA B. 2003. Mechanisms of human DNA repair; *Toxicology* **19**: 3-34.

CIPOLLINI M, HE J, ROSSI P, BARONTI F, MICHELI A, ROSSI AM AND BARALE R. 2006. Can individual repair kinetics of UVC-induced DNA damage in human lymphocytes be assessed through the comet assay? *Mutation Research* 1-12.

CLARKE TR. 2005. A Clinical Guide to Inherited Metabolic Diseases, 3rd Edition.

COLLINS RA. 2004. The comet assay for DNA damage and repair; *Principles, Applications and Limitations* **26**: 249-261.

Comet assay IV http://www.perceptive.co.uk/comet assay. Accessed on 12 May 2006

COOKE MS, EVANS MD, DIZDAROGLU M AND LUNEC J. 2003. Oxidative DNA damage: mechanisms, mutation and disease, *The FASEB Journal* **17**: 1195-1214.

COUCE PICO ML, DEL RIO PASTORI I, PICON COTOS M, BOVEDA FONTAN MD, COCHO DE JUAN JA AND FRAGA BERMUDEZ JM. 2006. Advances in the diagnosis and treatment of children with tyrosinemia type 1, *Pediatrics* **26**(1): 37-42.

DAVIS CD AND UTHUS EO. 2004. A methylation, cancer susceptibility, and nutrient interactions, *Experimental Biology of Medicine* **229**: 988-995.

DAZA P, ORTIZ T, PINERO J AND CORTES F. 1995 T_4 DNA ligase modulates chromosome damage induced by restriction endonucleaeses through an error-free, *Mutagenesis* **10**: 5, 399-402.

DE BONT R AND VAN LABEREKE N. 2004. Endogenous DNA damage in humans: a review of quantitative data, *Mutagenesis* **19**(3): 169-185.

DE OLIVEIRA AC, DAS SANTOS AMN, MARTINS AM AND D"ALMEIDA V. 2001. Screening for inborn errors of metabolism among newborns with metabolic disturbances and /or neurological manifestations without determined cause, *Sao Paulo Medical Journal* **119**(5): 160-164.

DI BISCEGLIE MA. 2002. Epidemiology and clinical presentation of hepatocellular carcinoma, *Journal of Vascular Interventional Radiology* **13**: S169-S170.

DIXON K AND KOPRAS E. 2004. Genetic alterations and DNA repair in human carcinogenesis, *Seminars in Cancer Biology* **14**: 441-448

DIXON P DAVID AND EDWARDS R. 2006. Enzymes of tyrosine catabolism in *Arabidopsis thaliana*, *Plant Science* **17**(3): 360-366.

EDEN A, GAUDET F, WAGHMARE A AND JAENISCH R. 2003. Chromosomal Instability and tumors promoted by DNA hypomethylation, *Science* **300**(5618): 455.

EHRLICH M. 2002. DNA methylation in cancer: too much, but also too little, *Oncogene* **21**: 5400-5413.

ELLIOTT B AND MARIA J. 2001. Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells; *Molecular and Cellular Biology* **21**(8): 2671-2682.

ENDO F, TANAKA Y, TOMOEDA K, TANOUE A, TSUJIMOTO G AND NAKAMURA K. 2003. Animals models reveal pathologies of Tyrosinemia, *American Society for Nutritional Science* **133**(6): 2063S-2066S.

FERNÁNDEZ-CAÑÓN JM, MANFRED WB, MILTON F, TERRY BURLINGAME K, GIBSON M AND MARKUS G. 2002. Maleylacetoacetate isomerase (*MAAI/GSTZ*)-deficient mice reveal a glutathione-dependent nonenzymatic bypass in tyrosine catabolism; *Molecular and Cellular Biology* **22**(13): 4943-4951.

GARCIA I, RODGERS M, REGIS P, TZUNG-FU H AND MATRINGE M. 1999. Characterization and subcellular compartmentation of recombinant 4-HPPD from Arabidopsis in Transgenic tobacco, *Plant Physiology* **119**(4): 1507-1516.

GILBERT-BARNESS E, BARNESS LA AND MEISHER LF. 1994, Chromosomal instability in hereditary tyrosinemia type I, *Pediatric Pathology* **6**(14): 1055-7.

GIOVANNELLI L, COZZI A, GUARNIERI I, DOLARA P AND MORONI F. 2002. Comet Assay as a novel approach for studying DNA damage in focal cerebral ischemia: effects of NMDA receptor antagonists and poly (ADP-Ribose) polymerase inhibitors, *Journal of Cerebral Blood Flow and Metabolism* **22**: 697-704.

GROMPE M. 2001. The pathophysiology and treatment of hereditary tyrosinemia type 1, *Seminars in Liver Disease* **21**(4): 563-571.

GUTTMAN A AND RONAI Z. 2000. Ultra-thin layer gel electrophoresis of biopolymers. *Electrophoresis* **21**: 3952-3964

HALLIWELL B AND GUTTERIDGE JM. 1989. Free Radicals in Biology and Medicine, 2nd edition, Claredon Press Oxford, 416-508.

HASEN PJ. 2002. Use of Hemacytometer, www.edugraphics.com/...Haemacytometer (Accessed on 10 June 2008).

HEMMINIKI K, KOSKINEN M, RAJANIEMI H AND ZHAO C. 2000. DNA adducts, mutations and cancer 2000, *Regulatory Toxicology and Pharmacology* **32**: 264-275

HIRAKU Y, YAMASAKI M AND KAWANISHI S. 1998. Oxidative DNA damage induced by homogentisic acid, a tyrosine metabolite, *FEBS Letters* **432**: 13-16.

HOWLETT GN; ZORICA S; ALAN DDA AND ROBERT HS. 2006. Impaired DNA double strand break repair in cells from Nijmegen breakage syndrome patients; *DNA repair* **5**(2): 251-257.

ISLAIH M, HALSTEAD BW, KADURA IA, LI B, REID-HUBBARD JL, FLICK L, ALTIZER JL, DEAHL JT, MONTEITH DK, NEWTON RK AND WATSON DE. 2005. Relationship between genomic, cell cycle and mutagenic responses of TK6 cells exposed to DNA damaging chemicals, *Mutation Research* **578**: 100-116.

JORQUERA R AND TANGUAY R.M. 1997. The mutagenicity of the tyrosine metabolite, fumarylacetoacetate, is enhanced by Glutathione depletion, *Biochemical and Biophysical Research Communications* **232**: 42-48.

JORQUERA R AND TANGUAY RM. 2001. Fumarylacetoacetate, the metabolite accumulating in hereditary tyrosinemia, activates the ERK pathway and induces mitotic abnormalities and genomic instability, *Human Molecular Genetics* **10**(17): 1741-1752.

KAINA B, CHRISTMANN M, NAUMANN S AND ROOS WP. 2007. MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents, *DNA Repair* **6**: 1079-1099.

KIM SZ, KUPKE KG, IERARDI-CURTO L, HOLME E, GRETER J, TANGUAY RM, POUDRIER J, D'ASTOUS M, LETTRE F, HAHN SH AND LEVY HL. 2000. Hepatocellular carcinoma despite long-term survival in chronic tyrosinemia 1, *Journal of Inherited Metabolic Diseases* **23**: 791-804.

KING SL, TRAHMS C AND SCOTT CR. 2006. Tyrosinemia Type 1. http://genetests.org/profiles/tyrosinemia/index.html [On line]-Accessed on (05/08/2006)

KUBICKA S, MANNS MP. 2003. Epidemiology and clinical presentation of hepatocellular carcinoma, *Chirurgische Gastroenteroenterologie mit Interdiszplinaren Gesprachen* **19**(3): 214-217.

KUBO S, SUN M, MIYAHARA M, UMEYAMA K, URAKAMI K. 1998. Hepatocyte injury in yyrosinemia type 1 is induced by fumarylacetoacetate and is inhibited by caspase inhibitors; *Medical Science* **95**(16): 9552-9557.

LANPHER B, BRUNETTI-PIERRI N AND LEE B. 2006. Inborn errors of metabolism: the flux from Mendelian to complex diseases, *Nature Reviews Genetics* **7**: 449-459.

LANTUM BM, HOFFMAN JC, PIERCE RH AND ANDERS MW. 2003. Perturbation of maleylacetoacetic acid metabolism in rats with dichloroacetic acid-induced glutathione transferase zeta deficiency; *Toxicological Sciences* **74**: 192-202.

LEVY HL. 1989. Nutritional therapy for selected inborn errors of metabolism, *Journal of American College of Nutrition* **8**: 54S-60S.

LUCZAK WM AND JAGODZINSKI PP. 2006. The role of DNA methylation in cancer development, *Folia Histochemica* **44**(3): 143-154.

MARLIN JD, JOHNSON L, KINGSTON AD, SMITH CN, DEATON CM, MANN S, HEATON P, VAN VUGT F, SAUNDERS K, KYDD J AND HARRIS AP. 2004.

Applications of the comet assay for investigation of oxidative DNA damage in enquine peripheral blood mononuclear cells, *American Society for Nutritional Sciences* 2133S-2140.

MATTIOLI F, GARBERO C, GOSMAR M, MANFREDI V, CARROZZINO R, MARTELLI A AND BRAMBILLA G. 2004. DNA fragmentation, DNA repair and apoptosis induced in primary rat hepatocyte by dienogest, dydrogesterone and 1,4,6- androstatriene-17 ß-ol-3-one acetate, *Mutation Research* **564**: 21-29.

MITCHELL GA, GROMPE M, LAMBERT M AND TANGUAY RM. 2001. The metabolic basis in Inherited diseases, Sciver CR Beudet AL, Valle D, ed New York, Mc Graw-Hill, 1777-1806.

MOSMANN T. 1983. Rapid colorimetric assay for cellular growth and survival: application and cytotoxicity assays, *Journal of Immunological Methods* **16**; 65 (1-2): 55-63.

MULLENDERS HFL, STARY A AND ALAIN S. 2001. Nucleotide excision repair; *Atlas of Genetics and Cytogenetics in Oncology and Hematology.*

OHISALO JJ, LASKOWSKA-KLITA T, AND ANDERSSON SM. 1982. Development of tyrosine aminotransferase and para-hydroxyphenylpyruvate dioxygenase activities in fetal and neonatal human liver; *Journal of Clinical Investigation* **70**(1): 198–200.

OVERTURF K; AL-DHALIMY M; TANGUAY R; BRANTLY M; CHING-NAN O; FINEGOLD M AND GROMPE M. 1996. Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinemia type I, *Nature genetics*. **12**: 266-273.

PANG R, TSE E AND POON RTP. 2006. Molecular pathways in hepatocellular carcinoma, *Cancer Letters* **240**: 157-169.

POLEKHINA G, BOARD PG, BLACKBURN AC AND PARKER MW. 2001. Crystal structure of MAAI/glutathione transferase zeta reveals the molecular basis for its remarkable catalytic promiscuity; *Biochemistry* **40**(6): 1567-1576.

PRIETO-ALAMO MJ AND LAVAL F. 1998. Decicient DNA-ligase activity in the metabolic disease tyrosinemia type1, *Proceedings of the National Academy of Sciences of the United States of America* **95**: 12614-12618.

PROGRIBNY I, YI P AND JAMES SJ. 1999. A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands, *Biochemical and Biophysical Research Communications* **262**: 624-628.

PROGRIBNY I, YI P, JAMES SJ, JERNIGAN STEFANIE AND POGRIBNA M. 2004. Genomic hypomethylation is specific for preneoplastic liver in folate/methyl deficient rats and does not occur in non-target tissues, *Mutation Research* **548**: 53-59.

ROBERTSON KD AND WOLFFE AP2000. DNA methylation in health and disease, Nature reviews **1**: 11-18.

ROOTWELT H, CHOU J, GAHL WA, BEGER R, COSKUN T AND BRODTKORB E. 1994. Two missense mutations causing tyrosinemia type1 with presence and absence of immunoreactive fumarylacetase, *Human Genetics* **93**: 615-619.

ROTH SK. 2003. Tyrosinemia, e-*Medicine instant access to the minds of medicine*.

RUSSO AP, MITCHELL AG AND TANGUAY R. 2001, Tyrosinemia; *Pediatric and Developmental Pathology* **4**: 212-221.

SCOTT CR. 2006. The genetic tyrosinemias, *American journal of Medical Genetics Part C*, **142 C**: 121-126

SEDGWICK B. 2004. Repairing DNA methylation damage, *Reviews* **4**: 148-156.

SHUGART LR. 2000. DNA damage as a biomarker of exposure, *Ecotoxicology* **9**: 329-340.

STRATHDEE G AND BROWN R. 2002. Aberrant DNA methylation in cancer: potential clinical interventions; *Expert Reviews in Molecular Medicine*.

TANGKIJVANICH P, HOURPAI N, RATTANATANYONG P, WISEDOPAS N, MAHACHAI V AND MUTIRANGURA A. 2007. Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma, *Clinica Chimica Acta* **379**: 127-133.

TANNER MS. 2002. Mechanisms of liver injury relevant to pediatric hepatology, *Critical Reviews in Clinical Laboratory Sciences* **39**(1): 1-61.

THOMPSON LH. 2005. Unraveling the fanconi anaemia-DNA repair connection, *Nature Genetics* **37**(9): 921-922.

UOTANI Y, NAKANO S, NAKASHIMA S, ANNO Y, FIJII M AND SUGIMOTO N.2003. Large stacking stability of a base pair mimic nucleotide on the DNA duplex, *Nucleic Acids Research Supplement* **No 3**: 79-80.

VAN AMSTEL JKP, BERGMAN AJI, VAN BEURDEN EACM, ROIJERS JFM, PEELEN T, VAN DEN BERG IET, POLL-THE BT, KVITTINGEN EA AND BERGER R. 1996. Hereditary tyrosinemia type1: novel missense, nonsense and splice consensus mutations in the human fumarylacetoacetate hydrolase gene, variability of the genotype-phenotype relationship, *Human Genetics* **97**: 51-59.

VAN DYK E AND PRETORIUS P. 2005. DNA damage and repair in mammalian cells exposed to *p*-Hydroxyphenylpyruvic; *Biochemical and Biophysical Communications* **338**: 815-819.

VOGEL A, VAN DEN BERG IET, AL-DHALIMY M, GROOPMAN J, OU C, RYABININA O, IORDANOVAM S, FINEGOLD M AND GROMPE M. 2004. Chronic liver disease in murine hereditary tyrosinemia type 1 induces resistance to cell death, *Hepatology* **39**: 433-443.

WALSH CP AND XU GL. 2006. Cytosine methylation and DNA repair, *CTMI* **301**: 283-315.

WANGX W, HUSSAINS P, HOU T, WU C, FORGUES M, HOFSETH LJ, BRECHOT C AND HARRIS CC. 2002. Molecular pathogenesis of human hepatocellular carcinoma, *Toxicology* **181-182**: 43-47.

WARIS G AND AHSAN H. 2006. Reactive Oxygen Species: role in the development of cancer and various chronic conditions, *Journal of Carcinogenesis* **5**: 14.

WASCHSMAN JT. 1997. DNA methylation and the association between genetics and epigenetic changes: relation to carcinogenesis, *Mutation Research* **375**: 1-8.

WATSON RE AND GOODMAN JI. 2002. Epigenetics and DNA methylation come of age in toxicology, Toxicological Sciences **67**: 11-16.

WHEELHOUSE. MN, CHAN Y, GILLIES ES, CALDWELL H, ROSS AJ, HARRISON JD AND PROST S. 2003. TNF-α induced DNA damage in primary murine hepatocytes. *International Journal of Molecular Medicine* **12**: 889-894.

YE X, SUEN CY AND WANG E. 1999. A recent development in image analysis of electrophoresis gel, *Trios-Revieres Canada* **2**(29): 1-7.

YU J, NI M, XU J, ZHANG H, GAO B, GU J, CHEN J, ZHANG L, WU M, ZHEN S AND ZHU J. 2002. Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis, *BMC Cancer* **2**(29): 1-14.

ZAMBRANO P, SEGURA-PACHECO B, PEREZ-CARDENAS E, CETINA L, REVILLA-VAZQUEZ A, TAJA-CHAYEB L, CHAVEZ-BLANCO A, ANGELES E, CABRERA G, SANDOVAL K, TREJO-BECERRIL C, CHANONA-VILCHIS J AND DUENAS-GONZÁLEZ A. 2005. A phase I study of hydralazine to demethylate and reactivate the expression of tumor suppressor genes. *Biomedcentral (BMC) Cancer* **5**(44). The electronic version of this article is the complete one and can be found online at: http://www.biomedcentral.com/1471-2407/5/44

APPENDICES

Appendix A: Preparation of Mincing Solution

HBSS (Ca^{++}, Mg^{++} free) with 20 mM EDTA and 10% DMSO: To 400 mL 1x HBSS (Ca^{++}, Mg^{++} free), add 3.72 g EDTA and 50 mL DMSO, adjust pH to 7.0 - 7.5., fill up to 500 mL, store at room temperature.

Appendix B: Preparation of Comet Assay solutions.

Phosphate buffered saline (PBS) – Dissolve 7.77g NaCl (133 mM, Mr: 58.44)
1.49g K_2HPO_4 (8.60 mM, Mr: 174.2)
0.20 KH_2PO_4 (1.5mM, Mr: 136.1)
in 1L dd H_2O, Ph 7.6.Store at 4°C. Prepare fresh once a month.

High melting point agarose (1% HMPA)- Dissolve 0.5 g HMPA in 50 ml ddH2O. Store at room temperature. Prepare fresh once a week.

Low melting point agarose (0.5 % LMPA)- Dissolve 0.25 g LMPA in 50 ml PBS. Store at room temperature. Prepare fresh once a week.

Lysis buffer-Reagents:

- NaOH-Dissolve 40.0g NaOH (Mr = 40.00) in ddH_2O (1M). Store at room temperature.
- Lysis buffer- Mix the following
  NaCl (2.5 M)-Dissolve 146.1g NaCl (Mr = 58.44) in 800 ml ddH_2O.
  Trizma base (10mM)- Add 1.2g to NaCl solution.
  EDTA (0.1 M)- Add 37.22g to NaCl solution.
  Stir mixture, add 8g NaOH and allow the mixture to dissolve (30 min). Adjust pH to 10 if necessary using concentrated HCl or NaOH and adjust volume to 890 ml with ddH_2O.
  Then add:

Triton X-100-10ml (1%)

DMSO-100ml (10%)

Store at 4°C. Prepare fresh once a week.

Electrophoresis buffer- Mix the following:

NaOH (0.3mM)- 300ml of 1 M solution (pH >13)

EDTA (1mM)- 0.372g

ddH_2O-Fill up to 1L

Store at 4° C. Prepare fresh once a week.

Neutralisation and staining- Reagents:

Tris buffer: Tris.HCl (0.4 M)- Dissolve 48.5g (Mr =121.1) in 500 ml ddH_2O.

Adjust pH to 7.5 with NaOH and fill up to 1L.

Store at 4° C. Prepare fresh once a month.

Ethidium bromide: Dissolve 4mg ethidium bromide (Mr = 394.31) in 1L ddH_2O (10μM). Keep covered with aluminium foil at 4°C. Prepare fresh once a month.

Appendix C: Preparation of MTT assay solution

For 5mg/ml MTT : Dissolve 5mg MTT salts in 1 ml phosphate buffered saline (PBS) and store at 4° C.

Appendix D: Contents of Nucleon BACC 1 for blood and cell cultures

Reagent A

Reagent B (Detergent)

Sodium perchlorate

Nucleon resin

Appendix E: Preparation of the Agarose gel electrophoresis solutions

Agarose gel (1%) preparation-

- For a 1 % gel, weigh 0.4 g agarose in 40 ml TAE buffer.
- Boil the suspension in a microwave oven until the agarose is melted.
- Let the gel cool to approximately 50°C, and add 10 µl ethidiumbromide.
- Mix well without shaking, by gently swirling the flask to avoid air bubbles.
- Pour the gel in the tray and remove any bubbles present and let the gel set completely before removing the comb very carefully.

!!! Ethidium bromide is a mutagen. Wear gloves during preparation and when handling the gel. Do not dispose of ethidium bromide in a drain or normal waste system!!!

TE Buffer-Mix the following

1 ml TE,

50 µl RNase,

Pinch of sucrose to make the solution heavy

35 µl Bromophenol blue

Appendix F: Cytosine extension assay solutions

Liquid scintillation cocktail components

Sonvent: Toluene, xylene, pseudodocume

Methylation-sensitive restriction enzymes (HpaII and its isoschizomer MspI)

PCR buffer

$MgCl_2$

Taq Flexi DNA polymerase

[^{3}H]-Dctp

Phosphate buffered saline