

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

**DETERMINING DNA DAMAGE AND REPAIR
IN HUMAN CELLS EXPOSED TO
METABOLITES CHARACTERISTIC FOR
TYROSINEMIA**

ETRESIA VAN DYK Hons. B.Sc

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Supervisor: Prof P.J. Pretorius

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POTCHEFSTROOM CAMPUS

'EK IS TOT ALLES IN STAAT DEUR HOM WAT MY KRAG GEE'
-Filippense 4:13

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ABSTRACT

Hereditary Tyrosinemia (HT1) is an autosomal recessive disorder caused by a deficiency of fumarylacetoacetate hydrolase (FAH) but the mechanism by which the hepatic and renal symptoms of HT1 arise is largely unknown. The current hypothesis is that the final metabolites of tyrosine catabolism (maleylacetoacetate and fumarylacetoacetate, and their derivatives, succinylacetone and succinylacetoacetate) are toxic, and can possibly act as alkylating agents and/or disrupt sulphhydryl metabolism. In addition, aminolevulinic acid (ALA) accumulates under pathological conditions. Development of hepatic tumours is a characteristic of this inherited disease.

The aim of this study was to use the comet assay (single cell gel electrophoresis) with isolated lymphocytes and primary hepatic cells to study the genotoxicity of the accumulating metabolites to contribute towards a better understanding of the underlying mechanisms responsible for the pathophysiology of this disease.

From the results it was seen that the exposure of both isolated lymphocytes and hepatocytes to SA, ALA and pHPPA separately, caused DNA damage but a high degree of DNA repair in the exposed cells was also observed. Upon further investigation it was seen that the damage caused by SA and ALA inhibited the cells' capacity to repair damaged DNA but some repair was still possible as was reflected in the low calculated repair capacity. The marked increase in DNA damage in isolated lymphocytes and the much lower calculated repair capacity in the hepatocytes, however, suggested that specific damage caused by pHPPA may have had an effect on the repair mechanisms of the cell. Contrary to previous reports (Mitchell *et al*, 2001), pHPPA could have a definitive role in contributing to the clinical features such as the hepatocarcinogenesis characteristic of HT1.

OPSOMMING

Tyrosinemie tipe 1 (HT1) is 'n autosomale resessieve afwyking van die fumarielasetoasetaat hidrolase ensiem, maar die meganisme verantwoordelik vir die hepatiese en renale simptome van HT1 is egter grootliks onbekend. Die hipotese is dat die finale metaboliete van tirosienkatabolisme (maleïelasetoasetaat, fumarielasetoasetaat, suksinielasetoasetaat en suksinielasetoon (SA)) toksies is en moontlik as alkilerende agente kan optree of die sulfhidrielmetabolisme verstuur. Bykomend tot die bogenoemde akkumuleer δ-aminolevuliensuur (ALA) ook onder patologiese toestande. Die ontwikkeling van hepatiese tumors is karakteristiek van hierdie aangebore siekte.

Die doel van hierdie studie was om die genotoksisiteit van die akkumulerende metaboliete in geïsoleerde limfosiete en primêre hepatosiete met behulp van die komeetanalise (SCGE) te bepaal, om daardeur 'n beter begrip te verkry van die onderliggende meganismes verantwoordelik vir die patofisiologie van hierdie siekte toestand.

Vanuit die resultate is dit duidelik dat die blootstelling van geïsoleerde limfosiete en hepatosiete aan onderskeidelik SA, ALA en pHPPA DNA skade veroorsaak, maar 'n groot mate van DNA herstel in blootgestelde selle is wel waargeneem. Na verdere ondersoek is egter waargeneem dat die blootstelling aan SA en ALA die sel se vermoë om beskadigde DNA te herstel inhibeer, maar soos gereflekteer in die lae berekende herstel kapasiteit was herstel steeds moontlik. Die stelselmatige verhoging in DNA skade in geïsoleerde limfosiete en die aansienlik verlaagde berekende herstelkapasiteit in hepatosiete dui egter daarop dat die spesifieke skade deur pHPPA veroorsaak 'n effek kon hê op die herstelmeganismes van die sel. In teenstelling met vorige verslae (Mitchell et al, 2001), kan pHPPA dus moontlik 'n bydrae lewer tot die kliniese beeld bv. lewerkanker, kenmerkend van HT1.

LIST OF SYMBOLS:

$^{\circ}\text{C}$:	Degrees Celsius
μl :	Microlitre
μM :	Micromolar
α :	Alpha
β :	Beta
Δ or δ :	Delta
$\%$:	Percentage

LIST OF ABBREVIATIONS:

A:

- A: Adenine
AIP: Acute intermittent porphyria
ALA: δ-aminolevulinic acid
ANON: Anonymous
AP: Abasic site

B:

- BSA: Bovine serum albumin
BER: Base excision repair

C:

- C: Cytosine
CRIM: Cross-reactive immunologic material
Cu: Copper

D:

- ddH₂O: Double distilled water
DHPY: 3,6-Dihydropyrazine-2,5-dipropanoic acid
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic acid
DOPA: Dihydroxyphenylalanine
DOVA: 4,5-Dioxovaleric acid
DSB's: Double strand breaks

E:

EDTA:	Ethylenediamine tetraacetic acid
EGTA:	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ERK:	Extra cellular signal-regulated protein kinase
<i>Et al:</i>	Et Alii/Alia (Latin: And others)
EtOH:	Ethanol

F:

FAA:	Fumarylacetoacetate
FAH:	Fumarylacetoacetate hydrolase
FBS:	Fetal bovine serum
Fe:	Iron
FPG:	Formamido-pyrimidine glycosylase

G:

g:	Gram
G:	Guanine
GF:	Growth factor
GGR:	Global genomic repair
GSH:	Glutathione

H:

H ₂ O ₂ :	Hydrogen peroxide
HBSS:	Hank's balanced salts
HD:	Heavily damaged
HEPES:	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HMPA:	High melting point agarose
HR:	Homologous repair
HT1:	Hereditary tyrosinemia 1

I:

i.e.: id est (that is)

K:

KCl: Potassiumchloride

(KH₂P)O₄: Potassiumdihydrogen orthophosphate

L:

LMPA: Low melting point agarose

M:

ml: Millilitre

mM: Millimolar

M: Molar

MAA: Maleylacetoacetate

MMR: Mismatch repair

N:

N.A. Not applicable

NaCl: Sodium chloride

NaHCO₃: Sodium bicarbonate

NaOH: Sodium hydroxide

NER: Nucleotide excision repair

NHEJ: Non-homologous end joining

NTBC : 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

O:

OH: Hydroxy radical

P:

PBG:	Porphobilinogen
PBGD:	Porphobilinogen deaminase
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
pH:	Potential of Hydrogen
pHPPA :	p-Hydroxyphenylpyruvic acid
PKU:	Phenylketonuria

R:

ROS:	Reactive oxygen species
RPM:	Revolutions per minute

S:

SA:	Succinylacetone
SCGE:	Single cell gel electrophoresis
SSB:	Single strand break

T:

T:	Thymine
TCR:	Transcription coupled repair
TrisHCl:	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride

U:

U:	Uracil
UV:	Ultra violet

W:

WE:	Williams' Medium E
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CHAPTER 1

INTRODUCTION

Tyrosinemia type 1 (HT1, McKusick 276700) is an autosomal recessive disease caused by a deficiency in fumarylacetoacetate hydrolase (FAH, E.C.3.7.1.2), the last enzyme of the catabolic pathway of tyrosine (Mathews *et al*, 2000). In the absence of FAH, metabolites such as maleylacetoacetate (MAA), fumarylacetoacetate (FAA), and succinylacetone (SA) and to a lesser extent p-hydroxyphenylpyruvic acid, p-hydroxyphenyllactic acid and p-hydroxyphenylacetic acid accumulate (Mitchell *et al*, 2001).

The accumulation of SA inhibits aminolevulinic acid dehydratase (E.C.4.2.1.24) resulting in the accumulation of ALA (δ -aminolevulinic acid), which is one of the main features of acute intermittent porphyria (AIP). Figure 1.1 shows this interrelationship between the tyrosine and heme metabolism.

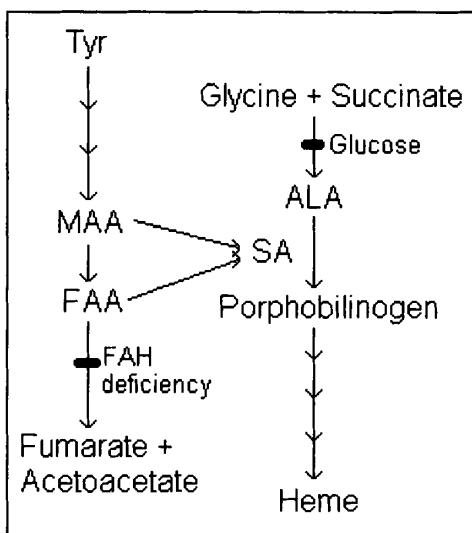


Figure 1.1. The interrelationship between tyrosine and heme metabolism (Mitchell *et al*, 2001)
ALA = δ -Aminolevulinic acid; FAA = Fumarylacetoacetate; FAH = Fumarylacetoacetate hydrolase; MAA = Maleylacetoacetate; SA = Succinylacetone; Tyr = Tyrosine

Over the past 20 years our laboratory has been involved in the identification and characterisation of inherited metabolic diseases and a shift towards the further molecular characterizations of the more prevalent diseases in the South African population became imperative to give further depth to this program. One such disease that lends it in particular for this purpose is HT1, because of the presence of scientifically relevant unresolved features.

For this study it was decided that the genotoxicity of SA and ALA will be investigated, since SA is the main accumulating metabolite and ALA is subsequently elevated. In addition to these metabolites it was decided to include p-hydroxyphenylpyruvic acid since it is one of the diagnostic markers of HT1. The aim of this study was to use the Comet Assay or SCGE (Single Cell Gel Electrophoresis) with lymphocytes and primary hepatic cells to study the genotoxicity of these metabolites. With this we envisaged to contribute towards a better understanding of the underlying mechanisms responsible for the pathology of this disease.

The study was designed to determine the baseline DNA damage in lymphocytes caused by the respective metabolites, but as this gives only a one-dimensional view of the genotoxicity of these metabolites, the effect on the repair capacity of the cell was also determined via additional exposure of the cells to H₂O₂ (Collins, 2004). During the execution of these tests, however, it was seen that pHPPA had a severe effect on the cells' capacity to repair DNA damage. As this is in contrast to what is suggested in Mitchell *et al* (2001) it was then tried to elucidate the type of damage caused by pHPPA. All these tests, however, were performed on lymphocytes, and since the liver is the main affected organ in HT1 and AIP (Baggott & Dennis, 1995; Mitchell *et al*, 2001), the effects of the metabolites were subsequently done with primary hepatocytes.

In chapter 2 a review is given of the relevant literature on HT1, AIP and DNA damage and repair, followed by the methodology in chapter 3. The results and a

discussion of these results are given in chapter 4 and a final summary and conclusion in chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Inherited metabolic disorders such as tyrosinemia (HT1) are rare as even the most prevalent disorder (PKU) affects fewer than 1 in 12,000 persons. These disorders are usually severe and can result in neurological impairment, mental retardation, and death. Fortunately, many of these effects can be reduced or avoided by early diagnosis and sustained dietary intervention (Levy *et al*, 2002).

Inherited metabolic diseases are identified by characteristic metabolic profiles, which have its origin in the block in a metabolic pathway due to a defective enzyme. This deficiency prevents the normal metabolism of a nutrient and can result in the accumulation of the nutrient or its metabolites to toxic levels. The accumulation of these metabolites gives rise to the specific metabolic profile. Each of the inherited metabolic diseases has clinical features by which they can be characterized, but the mechanism(s) responsible for the clinical features are largely unknown.

2.2 HEREDITARY TYROSINEMIA 1

2.2.1 Introduction

L-Tyrosine is derived either from hydrolysis of dietary or tissue protein or from hydroxylation of dietary or tissue phenylalanine and is therefore non-essential (Mitchell *et al*, 2001). Tyrosine is the starting point of synthetic pathways leading

to catecholamine, thyroid hormone, the melanin pigments, neurotransmitters including, dihydroxyphenylalanine (DOPA), norepinephrine and epinephrine (Di Pasquale, 2002). Quantitatively the major fates of tyrosine are incorporation into proteins or degradation.

Table 2.1. Comparison of different types of Tyrosinemia

	TYROSINEMIA 1	TYROSINEMIA 2	TYROSINEMIA 3
ENZYME DEFECT	Fumarylacetoacetate hydrolase	Tyrosine aminotransferase	4-Hydroxyphenyl-pyruvic acid dioxygenase
GENE	15q23-q25	16q22.1-q22.3	12q24-qter
SYMPTOMS	Acute liver failure, cirrhosis, hepatocellular carcinoma, renal Fanconi syndrome, glomerulosclerosis, crises of peripheral neuropathy	Palmaplantar keratosis, painful corneal erosions, mental retardation	Intermittent ataxia, neurological abnormalities
TREATMENT	NTBC, hepatic transplantation, dietary restriction of tyrosine and phenylalanine	Dietary restriction of tyrosine and phenylalanine	
ALTERNATIVE TITLES	Hepatorenal tyrosinemia, Fumarylacetoacetate deficiency, FAH deficiency,	TAT deficiency, Keratosis palmoplantaris with corneal dystrophy, Richner-Hanhart syndrome, Oregon type tyrosinemia, Oculocutaneous type tyrosinosis	4-Hydroxyphenyl-pyruvic acid oxidase deficiency, 4-Hydroxyphenylpyruvate dioxygenase deficiency

(Mitchell *et al*, 2001; McKusick 276700)

Tyrosine degradation occurs primarily in the cytoplasm of hepatocytes and is both glucogenic and ketogenic. Under most circumstances the rate of tyrosine

degradation is determined by the activity of tyrosine aminotransferase (E.C.2.6.1.5) (Mitchell *et al*, 2001).

2.2.2 Clinical background

Hereditary tyrosinemia 1 (HT1) is an autosomal recessive disorder and the distribution between sexes are thus equal. This disorder is caused by a deficiency in the fumarylacetoacetate hydrolase enzyme, FAH (E.C.3.7.1.2), the last enzyme in the tyrosine catabolism pathway, as shown in figure 2.1 (Jorquera & Tanguay, 1999). As a result the levels of tyrosine in the blood are elevated. Affected patients have a sudden and severe onset that lead to rapid development of hepatic cirrhosis and liver failure. Most patients present with symptoms within the first few months of life (Mitchell *et al*, 2001).

A failure to thrive precedes the appearance of more dramatic findings. In such cases, a history of diminished nutritional intake and anorexia is present. Vomiting and diarrhoea follows which rapidly progress to bloody stool, lethargy and jaundice. At this stage the cabbage-like odour is present. The acute onset may be dramatic with hepatomegaly, jaundice, epistaxis, melena, purpuric lesions, marked oedema and a cabbage-like odour (Roth, 2003).

Because of the inhibiting effect of SA on heme biosynthetic pathway, patients with the chronic form present polyneuropathy and painful abdominal crises. Survivors may evidence hepatic nodules and cirrhosis, and these nodules may be indicative of hepatocellular carcinoma (Berger, 1996; Mitchell *et al*, 2001; Roth, 2003).

2.2.3 Genetic background

The sole explanation for HT1 is a genetic mutation in homozygous form, as heterozygotes are entirely unaffected. The gene contains 14 exons and spans

approximately 35 kb of DNA (Labelle *et al*, 1993). According to the Human Gene Mutation Database the gene maps to chromosome 15q23-q25 and approximately 42 distinct mutations, including 26 missense/nonsense, 12 splice site mutations, two small deletions, one gross deletion and one complex rearrangement have been reported. The enzyme FAH is expressed predominantly in the liver but is also found in a wide range of tissues and cell types, e.g. the brain, kidney and fibroblasts (Berger, 1996).

There is no clear relationship between genotype and phenotype as patients presenting either the acute or the chronic form revealed identical phenotypes (Aponte *et al*, 2001; Roth, 2003). Kvittingen *et al* (1994) reported a mosaic pattern in FAH expression. Further investigation showed reversion of the primary point mutation to the normal nucleotide in one allele. They proposed that nodule formation was due to a growth advantage of the reverted cells. Demers *et al* (2003) found that reversion of FAH mutations is a frequent event in livers of HT 1 patients. They suggested that the extent of the reversion is positively correlated with a lower clinical severity. According to Poudrier *et al* (1998) and Demers *et al* (2003) the varying extent of reverted cells contributes to the different phenotypes observed for the same FAH mutation. Poudrier *et al* (1998) also suggested that the time of life when the reversion occurs can have an effect on the varying phenotypes.

DNA damage can occur by spontaneous DNA replication errors, base deamination or oxidation and environmentally induced alkylation (Teis, 1999; Jackson, 2004). Repair of these damages and correction of replicative errors are critical to maintain the genome integrity and DNA-repair deficiency syndromes have an increased risk of developing cancer (Allinen, 2002). Therefore, the high cancer incidence occurring in HT1 patients, the high hypersensitivity of established HT1 cells to several DNA-damaging agents, lead to the hypothesis that enzymes involved in DNA repair and/or replication could be altered in HT1 cells (Prieto-Alamo & Laval, 1998).

2.2.4 Damage-causing metabolites in HT1

(A limited amount of literature is available on this subject. The following is a summary from this literature.)

The FAH deficiency results in an accumulation of two abnormal tyrosine metabolites, FAA and MAA (figure 2.1). It has been suggested that FAA and MAA possibly act as natural alkylating agents and/or disrupt sulphhydryl metabolism (Prieto-Alamo & Laval, 1998). Both of these metabolites however have never been isolated as circulating or excreted metabolites (Mitchell *et al*, 2001), which suggests that they are transformed rapidly to SA (Prieto-Alamo & Laval, 1998), and SA concentrations from 6 to 43 µM were measured in the plasma of HT1 patients (Mitchell *et al*, 2001).

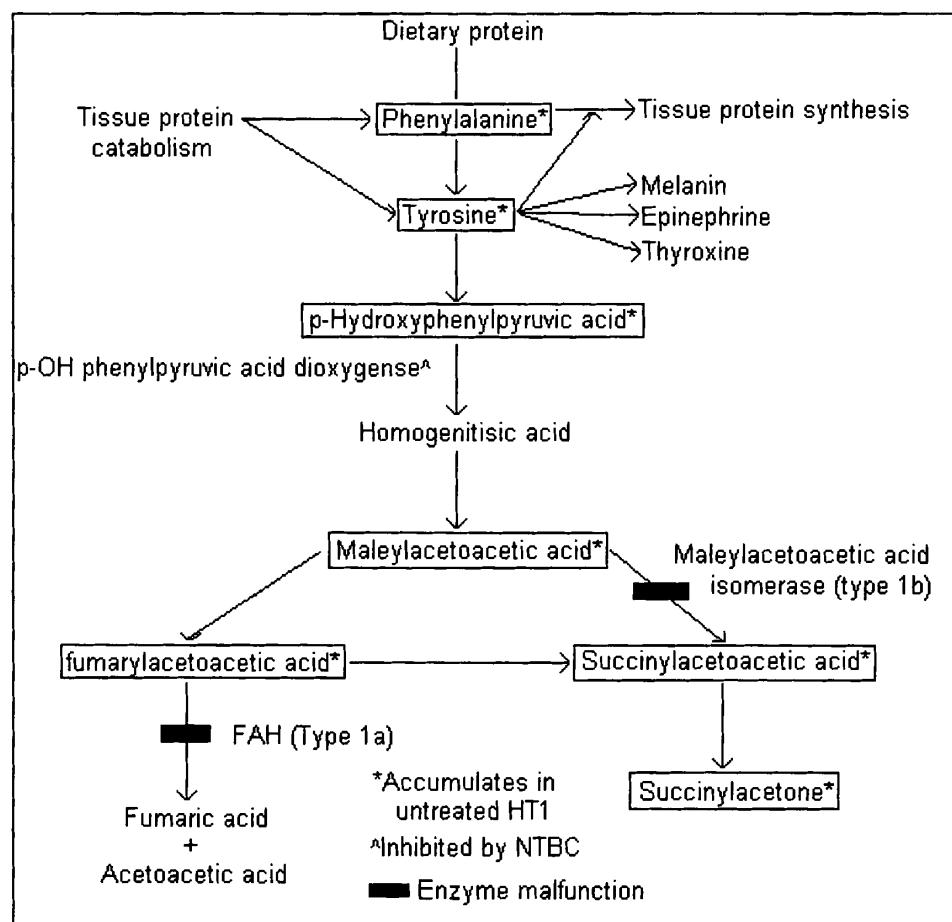


Figure 2.1. Different outcomes of tyrosine metabolism. FAH = Fumarylacetoacetate hydrolase; HT1 = Hereditary tyrosinemia type 1; NTBC = 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

SA is a decarboxylation product of succinylacetone derived from the tyrosine catabolic intermediate FAA. It was inferred that the enzymatic defect might reside in deficiency of fumarylacetone, which mediates production of fumaric acid and acetone (Prieto-Alamo & Laval, 1998; Jorquera & Tanguay, 1999).

SA reacts with the amino acids lysine, glycine, methionine, phenylalanine, serine, alanine and glutamine and proteins to form stable adducts via Schiff base formation, with lysine being the most reactive amino acid (Manabe *et al*, 1985). SA could thus react with proteins involved in DNA metabolism and preferentially with proteins whose active site includes a lysine residue. The active sites of DNA-ligases, which are involved in the rejoining of strand interruptions formed transiently during replication and recombination, have such a lysine residue (Prieto-Alamo & Laval, 1998).

A high level of chromosomal breakage is observed in HT1 cells, suggesting a defect in the processing of DNA (McKusick 276700). The overall DNA-ligase activity can in addition account to only about 20% of the normal value and the Okazaki fragments are rejoined at a reduced rate than in normal fibroblasts (Prieto-Alamo & Laval, 1998).

It was illustrated *in vitro* that SA inhibits the overall DNA-ligase activity present in normal cell extracts. As shown in figure 2.2 SA inhibited the activity of purified T4 DNA-ligase, whose active site is also a lysine residue, in a dose-dependant manner. This suggests that accumulation of SA reduces the overall ligase activity in HT1 cells and indicate that metabolism errors may play a role in regulating enzymatic activities involved in DNA replication and repair (Prieto-Alamo & Laval, 1998).

SA is an inhibitor of the enzyme d-aminolevulinic acid dehydratase (E.C.4.2.1.24) which mediates the formation of porphobilinogen, the cyclic precursor of

porphorins in the heme biosynthetic cycle (Deepali *et al*, 2004). Excretion of this metabolite decreases after orthoptic liver transplant, although excretion persists at lower levels than before the transplant (Roth, 2003).

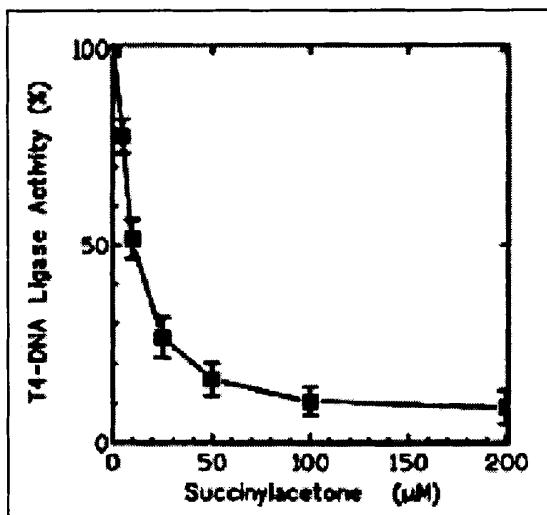


Figure 2.2. T4 DNA-ligase activity in the presence of SA. (Prieto-Alamo & Laval, 1998)

FAA, the mutagenic metabolite accumulating in HT1, is a powerful glutathione depletor. Its mutagenicity is potentiated by depletion of cellular glutathione. FAA induces dose dependant cell cycle arrest and apoptosis in human (Hepg2) and rodent (Chinese hamster V79) cells (Jorquera & Tanguay, 1999, 2001).

FAA and MAA contain α,β -unsaturated carbonyl compound structures that confer electrophilic properties and potentiate biological activities (Jorquera & Tanguay, 2001). These metabolites can also alkylate cellular macromolecules, such as DNA, and/or disrupt essential sulphydryl reactions by forming complexes with proteins of glutathione (GSH) (Jorquera & Tanguay, 1999). FAA arrests the cell cycle in G2/M phase, and the arrested cells then undergo apoptosis. This causes ERK activation through thiol-regulated, a tyrosine kinase dependant but GF receptor and protein kinase C-independent, pathway. DNA methylation plays an important role in controlling important cellular functions. GSH depletion alters DNA methylation and this is a common finding in cancer cells (Lertratanangkoon

et al, 1997). Replenishment of GSH abolishes ERK activation and reduces chromosomal instability induced by FAA by 80 % (Jorquera & Tanguay, 2001).

2.3 INTERMITTENT PORPHYRIA

2.3.1 Introduction

Acute intermittent porphyria (AIP) disorders are characterized by an overproduction and accumulation of ALA, especially in the liver. Lead poisoning and HT1 also increase urinary excretion of ALA and should be taken into account when diagnosing AIP (Baggott & Dennis, 1995).

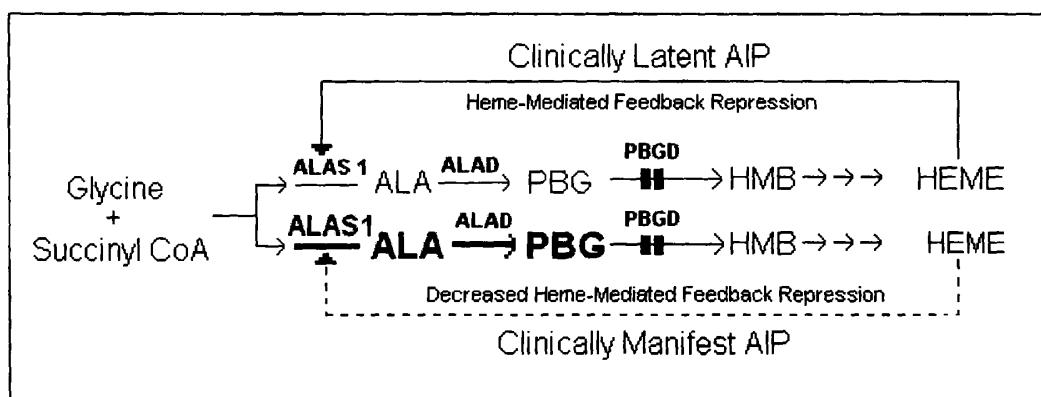


Figure 2.3. Enzymatic block in AIP. (Kappas *et al*, 2001) AIP = acute intermittent porphyria; ALAD = δ -aminolevulinic acid dehydratase; ALAS1 = ALA synthase; HMB = hydroxymethylbilane; PBG = porphobilinogen; PBGD = porphobilinogen deaminase.

The δ -aminolevulinic acid synthase (E.C.2.3.1.37) reaction occurs in the mitochondria as can be seen in figure 2.3 (Kappas *et al*, 2001). This is the rate limiting step of the heme synthesis reaction and is thus tightly regulated. Pyridoxal phosphate is an essential cofactor for this reaction and is therefore sensitive to a nutritional deficiency of Vitamin B6 or drugs that is antagonistic to pyridoxal phosphate (Baggott & Dennis, 1995).

2.3.2 Clinical background

Acute intermittent porphyria is inherited as an autosomal dominant disorder and is characterized by recurrent attacks of abdominal pain, gastrointestinal dysfunction, neurological disturbances, and excessive amounts of aminolevulinic acid and porphobilinogen in the urine (Kappas *et al.*, 2001). AIP results from an error in pyrrole metabolism due to a deficiency of porphobilinogen deaminase (E.C. 4.3.1.8), the third enzyme in the heme biosynthetic pathway. As a result, the porphyrin precursors, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) can accumulate. The free heme pool would consequently decrease and the P450 and other important antioxidant enzymes would be adversely affected, which can lead to impaired hepatic detoxification reactions (De Siervi *et al.*, 2002)

A characteristic of this disorder is an acute episode of a variety of neuropathic symptoms, between which the patient is healthy. According to the American Porphyria Foundation (2005) abdominal pain is the most common symptom during acute attacks and is usually accompanied by constipation, urinary retention, and nausea and vomiting. Neuromuscular weakness, which may progress to quadriplegia and respiratory failure, is the most prominent and potentially lethal neurological manifestation (James & Hift, 2000). Other phenomena, including seizures, psychotic episodes, and hypertension may occur during acute attacks (Kappas *et al.*, 2001). Acute attacks rarely occur before puberty or after 40 (James & Hift, 2000; DeLoughery, 2004), but they can be precipitated by porphyrinogenic drugs such as barbiturates and sulphonamides, some of which are known to induce the earlier rate-controlling step in heme synthesis, the delta-aminolevulinic acid synthase reaction (Anon 1, 2004).

The most acute attacks, if correctly recognized, settle with supportive treatment; dextrose infusion and high carbohydrate intake may be helpful. Successful treatment by infusion of haematin, which is a specific feedback inhibitor of heme

synthesis, has been reported, but haematin is neither readily available nor very soluble and its use may carry a risk of renal damage (Anon 1, 2004).

Immunologic studies revealed three subtypes of PBGD mutations of the mutant enzyme proteins in erythrocytes, according to Kappas *et al* (2001). Type 1 is the largest category of mutations (~85%) and is cross-reactive immunologic material (CRIM) negative mutations. They render the enzyme protein unstable as there are a 50 % decrease in the PBGD activity and enzyme protein in all tissues. Type 2 mutations are also CRIM negative. This includes 5 mutations that can result in the absence of the housekeeping isozyme, but normal levels of the erythroid-specific isozyme. These patients thus have normal erythrocyte PBGD activity, while the activity in other tissues and cells is half-normal. Type three mutations are CRIM positive and include mutations that decrease PBGD activity, but don't alter the mutant enzyme's stability.

It was suggested in Mustajoki (1981) that in one variant of acute intermittent porphyria the enzyme defect is not expressed in red cells. A reduced activity is a consistently found during or between acute attacks, and characterizes latent AIP which is inherited as an autosomal dominant trait. Most enzymopathies are however recessively inherited because only a few enzymes are so rate limiting that it would cause a serious reduction in the rate of a metabolic pathway when the enzyme has 40 to 60 % normal activity (Anon 1, 2004).

2.3.3 Genetic background

AIP is caused by any of 237 mutations on chromosome 11q23.3. A search of the human gene mutation database revealed that of these mutations 104 are missense/nonsense, 58 are splicing, 44 are small deletions, 24 are small insertions, and there are 2 small indels, 3 gross deletions and 2 gross insertions and duplications.

In AIP and several other genetic porphyrias the enzyme defects are deficiencies rather than absolute deficits (James & Hift, 2000) as it manifests in the heterozygous, single gene dose, a few homozygotes for porphobilinogen deaminase deficiency have been reported. Homozygosity for null alleles in the heme biosynthesis pathway might be lethal because of the essentiality of heme not only in haemoglobin but also in cytochrome P450 enzymes, catalyze, etc. In those conditions that do represent the homozygous state, such as congenital erythropoietic porphyria and acute hepatic porphyria, and in those dominantly inherited porphyries for which the homozygous state has also been observed, the mutations may be leaky (Anon 2, 2004).

Major gene deletions are unlikely to be present in more than a small proportion of the commonest type of AIP, the CRIM-negative form (Anon 2, 2004).

Puy *et al* (1998) stated that 135 different mutations had been reported in the PBGD gene in cases of AIP; however, only 3 mutations, all located in exon 1 and the surrounding intron/exon junction, had been characterized in the nonerythroid AIP variant. In classic AIP, both the housekeeping and the erythroid-specific isoforms of the enzyme have half-normal activities in erythroid and nonerythroid tissues, whereas in the variant form of the disease, representing 2 to 5% of cases, the housekeeping enzyme has half normal activity, while the erythroid-specific PBGD isozyme has normal activity. Clinical characteristics in the 2 forms are identical; diagnostic methods based on the level of enzyme in erythrocytes are ineffective.

2.3.4 Damage causing metabolites in AIP

In plasma, during acute attacks of AIP, the heme precursors, delta-aminolevulinic acid and porphobilinogen are increased. During these acute attacks up to 100mg of δ-aminolevulinic acid is excreted in urine per day, as compared to 1.5-7.5mg per day in healthy subjects (Burcham, 1999). It was noted by James & Hift

(2000) and Anon 1 (2004) that the pathophysiology of this disorder is poorly understood. There is evidence in a porphyric rat model of increased plasma concentration and brain uptake of tryptophan and of increased synthesis of serotonin in the nervous system. The increased concentration of tryptophan and serotonin can be partly due to the hepatic heme deficiency decreasing the activity of the liver cytosolic enzyme heme-dependant tryptophan pyrolase.

In women with AIP there is a characteristic rise in serotonin and plasma tryptophan during the attacks, whereas both daytime and night time melatonin concentrations are dramatically decreased, although melatonin is produced from tryptophan. Injection of heme lowered heme precursors, tryptophan, and serotonin to normal levels but did not increase melatonin. It was suggested that delta-aminolevulinic acid is responsible for decreased production of melatonin by the pineal gland (Anon 1, 2004).

The heme precursor 5-aminolevulinic acid (ALA) accumulates in both inborn and acquired hepatic porphyria such as AIP (Onuki *et al*, 2002). This has been previously linked to an enhanced production of reactive oxygen species generated by a metal-catalyzed ALA oxidation process, which was shown to cause DNA single-strand breaks and guanine oxidation within both isolated and cellular DNA. It was established that the final oxidation product of ALA, 4,5-dioxovaleric acid, is an efficient alkylating agent of the guanine moieties within both nucleoside and isolated DNA. Adducts were produced through the formation of a Schiff base involving the N2-amino group of 2'-deoxyguanosine (dG) and the ketone function of DOVA, respectively (Douki *et al*, 1998)

5-Aminolevulinic acid is able to undergo enolization and to be subsequently oxidized in a reaction catalyzed by iron complexed yielding 4,5-dioxovaleric acid (DOVA). The released superoxide radical (O_2^-) is involved in the formation of reactive hydroxyl radical ($\cdot OH$) or related species arising from a Fenton-type reaction mediated by Fe^{II} and Cu^I . This leads to DNA oxidation. The metal

catalyzed oxidation of ALA may be exalted by the O_2^- and enoyl radical-mediated release of Fe^{II} ions from ferritin. (Di Mascio *et al*, 2000)

It was suggested that other effects of ALA can arise from its dimerization product (Butler *et al*, in Onuki *et al*, 2002). At elevated concentrations, two ALA molecules can condense through an amino-carbonyl reaction to DHPY. It was reported that some dihydropyrazine derivatives are able to give rise to DNA strand breaks in plasmid DNA through the generation of ·OH radicals and carbon-centred radicals. It was demonstrated that DHPY is able to induce strand breaks in isolated DNA and to increase the formation of 8-oxodG in solution and it was suggested that the formation of DHPY could contribute to the mechanisms of DNA damage promoted by ALA, since micromolar concentrations of DHPY is sufficient to induce DNA lesions (Onuki *et al*, 2002).

2.4 DNA DAMAGE

2.4.1 Introduction

According to Voet & Voet (1995) DNA isn't the inert substance that is supposed from the naïve consideration of the genome's stability. Human genetic material is exposed daily to a number of physical and chemical substances, both intracellular and environmental of origin, which can cause direct or indirect damage to DNA. These substances include UV-radiation, X-rays and chemical reactive species (Friedberg, 1985). DNA damage can be divided into two groups, namely spontaneous (endogenous) and environmentally (exogenous) induced. Temporary changes in DNA can lead to permanent changes (mutations) in the genetic material (Martin, 2001). Through the inactivation or deregulation of control proteins, these mutations can have a great effect on normal biological functioning and may lead to cell death or cellular dysfunction. Oxidative modifications can be in proteins, lipids and DNA, with the most important

modifications, the modifications in DNA, as it can become permanent through the forming of mutations and other genomic instabilities (Friedberg, 1985; Christman *et al*, 2003). Any DNA damage must thus be repaired, for the genetic message to maintain its integrity.

2.4.2 DNA Damage and HT1

(A limited amount of literature is available on this subject. The following is a summary from this literature.)

HT1 is an autosomal recessive disease caused by the deficiency of fumarylacetoacetate hydrolase. Symptoms include acute liver failure, cirrhosis, hepatocellular carcinoma, renal Fanconi syndrome, and glomerulosclerosis (Mitchell *et al*, 2001).

DNA damage occurs by spontaneous base deamination, alkylation or oxidation by endogenous or environmental exposure to various compounds. Repair of these damages and correction of replicative errors are critical to maintain the genome integrity, as DNA-repair deficiency syndromes have an increased risk of developing cancer (Prieto-Alamo & Laval, 1998). According to Gilbert-Barnes *et al*, as quoted by Prieto-Alamo & Laval (1998) the high cancer incidence occurring in HT1 patients, the high level of chromatid breaks observed in HT1 cells and the hypersensitivity of established HT1 cells to several DNA-damaging agents lead to the hypothesis that enzymes involved in DNA repair and/or replication could be altered in HT1 cells.

Prieto-Alamo & Laval (1998) stated that as diketones, from tyrosine, react with the ϵ -amino group of lysine, among the possible targets for the formation of a Schiff-base with SA, was the lysine residue present in the active site of mammalian ligases. Prieto-Alamo & Laval (1998) also showed that the overall DNA-ligase activity measured in cells from unrelated HT1 patients corresponds to about 20% of the activity present in normal human fibroblasts. SA reduces the activity of purified T4 DNA-ligase, whose active site is also a lysine residue,

which strongly suggests that this compound is responsible for the low activity measured in HT1 cells, Prieto-Alamo & Laval (1998) reported that Wagner *et al* suggested that the extent of DNA-ligase I deficiency tolerable to mammalian cells is low and that ligases I, II, and III are unable to compensate for each other. Hence, the overall ligase deficiency observed in HT1 cells probably plays a key role in the symptoms associated with this disease.

A human cell line (46BR) sensitive to killing by DNA-damaging agents, shows defective rejoicing of Okazaki fragments, and possesses reduced DNA-ligase I activity. It was also reported that HT1 results in a slow rejoicing of Okazaki fragments in HT1 cells, probably plays a role in the completion of DNA repair processes, and might result in genomic instability. Prieto-Alamo & Laval (1998) reported that sequencing of PCR-amplified ligase I cDNA revealed mutations in 46BR cells, carried in different alleles. As contrasted to 46BR cells, no mutation were detected in the ligase I cDNA of HT1 cells and the level of transcription of this gene were identical in normal and HT1 cells. Although this activity is reduced in both 46BR and HT1 cells, the clinical symptoms associated with the diseases are different, suggesting that besides its inhibitory effect on DNA-ligases, SA may have other deleterious effects on the cellular metabolism.

2.4.3 DNA Damage and AIP

Acute intermittent porphyria is and autosomal, dominant acute hepatic porphyria that results from the half-normal activity of porphobilinogen deaminase. Urinary excretion of ALA and PBG (porphobilinogen) is markedly increased (Kappas *et al*, 2001). ALA has a pro-oxidant potential and is able to promote the formation of DNA lesions such as strand-breaks and oxidized bases *in vitro* and *in vivo* (Onuki *et al*, 2002).

The genotoxicity of δ-aminolevulinic acid are poorly understood, but a role for oxidative mechanisms seems likely. Δ-Aminolevulinic acid generates reactive

oxygen species under physiological pH conditions, due to a tendency to undergo metal-dependant enolization reactions (Burcham, 1999).

It was reported that the *in vitro* incubation of DNA with δ-aminolevulinic acid and transition metals produces some of the hallmarks of oxygen radical-induced genetic damage, including 8-oxo-dG and strand-nicking (Fraga *et al* in (Burcham, 1999)). There was a 4.5 fold elevation in the 8-oxo-dG content in hepatic DNA, in rats after chronic, rather than a single, administration with a toxic dose of δ-aminolevulinic acid.

It was suggested that Cu^{II} seems to be a more efficient catalyst of autoxidation of δ-aminolevulinic acid than Fe^{II}, although *In vivo* Fe^{II} would be the more likely catalyst, since hepatic iron mobilization accompanies chronic exposure to δ-aminolevulinic acid, which also promotes iron release from ferritin *in vitro* (Burcham, 1999).

Calf thymus DNA exposed to different concentrations of ALA in the presence of Fe^{II} or ferritin led to the formation of 8-oxodG, but was ferritin dose dependant and it was stated, that the 8-oxodG lesion could participate in the carcinogenic process as a pre-mutagenic lesion (Onuki *et al*, 2002).

The relevance of δ-aminolevulinic acid autoxidation during porphyric carcinogenesis extends beyond that of oxygen radical generation. The major nonradical product of δ-aminolevulinic acid autoxidation is 4,5-dioxovaleric acid, DOVA, which is structurally related to the various 2-ketoaldehydes formed during glycoxidation reactions (Burcham, 1999). In an analogous reaction to the formation of N²-(1-carboxyethyl)-Gua from methyl glyoxal, 4,5-dioxovaleric acid reacts with the N² exocyclic primary amine of dG, forming a Schiff base adduct. A high concentration of DOVA also leads to the efficient formation of DNA strand breaks (Burcham, 2002).

In conclusion, in AIP, ALA is overproduced, as a result of a deficiency in PBGD activity. ALA then accumulates for years in the liver, probably in the mitochondria. The increased amount of free iron in the hepatocytes of AIP patients is then likely to participate in ALA-mediated oxidation processes. DHPY is also generated at elevated levels of ALA. These systems together produce H₂O₂ that leads to the generation of ·OH radicals *in situ*, which promotes nuclear and mitochondrial DNA attacks. As a result this gives rise to increased nucleobase oxidation, strand-breaks, and DNA adducts. The increased DNA lesions could lead to mutations in genes involved in cell cycle regulation, initiating a carcinogenic process (Onuki *et al*, 2002).

2.5 DNA REPAIR

2.5.1 Introduction

The preservation of genomic integrity is very important, therefore organisms posses a variety of defence and repair mechanisms to preserve its genomic integrity. These mechanisms must first recognize the type of DNA damage, then remove the damaged DNA and then finally replace the removed segment with the correct DNA. (For recent extensive reviews see Jackson, 2002; Mitra *et al*, 2002; Christmann *et al*, 2003)

2.5.2 Repair mechanisms

There are five major DNA repairing mechanisms, namely:

- Base excision repair (BER),
- Mismatch repair (MMR),
- Nucleotide excision repair (NER), and
- Homologous recombination repair (HR),
- Non homologous end joining (NHEJ).

The following is a short description of each of the repair mechanisms.

Base excision repair (BER)

In BER damaged DNA bases, which are recognized by specific enzymes (DNA glycosylases), are removed. The lesions repaired by BER include oxidized DNA bases e.g. 8-oxoG, arising spontaneously within the cell during inflammatory responses or from exposure to exogenous agents, and DNA alkylation induced by endogenous alkylating species and exogenous carcinogens.

The mechanism for the repairing of both modified bases and AP sites, by BER, can be divided into five steps, namely:

- Recognition, base removal and incision,
- Nucleotide insertion,
- Decision between short- and long-patch repair,
- Strand displacement and DNA-repair synthesis by long-patch BER, and
- Ligation.

MTH1 is an enzyme reducing the level of oxidized bases, but are not directly involved in the BER. This enzyme prevents 8-oxoG form being incorporated into DNA by hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP, and thereby removing it from the nucleotide pool.

Mismatch repair (MMR)

Spontaneous and induced base deamination, oxidation, methylation and replication errors are removed by the MMR system. G/T, G/G, A/C and C/C are the main targets for mismatch repair. MMR does not only bind to spontaneously occurring base mismatches but also a variety of other chemically induced DNA lesions.

The three steps by which the MMR proceeds are as follows:

- Recognition of DNA lesions,

- Strand discrimination, and
- Excision and repair synthesis.

Nucleotide excision repair (NER)

NER repair bulky DNA adducts, such as UV-light-induced photo lesions, intra-strand cross-links, large chemical adducts generated from aflatoxine, benzo[a]pyrene and other genotoxic agents.

The two pathways of NER, global genomic repair (GGR) and transcription-coupled repair (TCR) proceed as follows:

- DNA damage recognition,
- DNA unwinding,
- Excision of the DNA lesion, and
- Repair synthesis.

The two main repair ways for DNA DSB's are HR, which is error-free, and NHEJ, which is error-prone.

Homologous recombination:

In Homologous recombination, which is error-free, the damaged chromosome comes in physical contact with an undamaged DNA, with which it share sequence homology, and uses it as a template for DNA repair. The stages of HR are as follow:

- Processing of DNA ends, 5'-3' resection,
- Strand exchange,
- DNA synthesis, and
- DNA ligation, branch migration and resolution of Holliday junctions.

Non Homologous End Joining:

The error-prone, NHEJ repair pathway ligates the two ends of a DNA DSB without the requirement of sequence homology between the two DNA ends. This process occurs mainly in the G0/G1 phase of the cell cycle and proceeds as follow:

- Recognition and binding to damaged DNA,
- Processing of DNA ends, and
- Ligation.

2.6 AIMS AND APPROACH OF STUDY

2.6.1 Aim of study

The general aim of this study was to investigate the effect of SA, ALA and pHPPA on DNA damage and repair in human cells in order to contribute towards a better understanding of the pathophysiology of Hereditary Tyrosinemia Type 1.

2.6.2 Approach of study

The following approach was formulated for this study:

The Comet Assay was used to determine DNA damage and repair in:

1. Isolated human lymphocytes,
2. Isolated rat hepatocytes harvested by collagenase perfusion, and
3. Isolated rat hepatocytes harvested with a mincing solution

after exposure to:

1. Succinylacetone (SA),
2. δ-Aminolevulinic acid (ALA), and
3. p-Hydroxyphenylpyruvic acid (pHPPA).

CHAPTER 3

MATERIALS AND METHODS

3.1 ETHICAL APPROVAL

Ethical approval was obtained from the Ethical Committee of the North-West University under the title *Determining DNA damage and -repair in primary hepatocytes exposed to metabolites characteristic of tyrosinemia and galactosemia*. Ethic approval number: 04D11.

3.2 COMET ASSAY

3.2.1 Introduction

Conventional methods for evaluating genetic damage includes measuring chromosomal aberrations, micronucleus assay, and sister chromatid exchange. These techniques are time consuming, resource intensive and require proliferating cell population (Dhawan, 2004).

The comet assay or SCGE (Single Cell Gel Electrophoresis assay) is a rapid and sensitive technique for analysing and measuring DNA damage and repair *in vitro* and *in vivo* at individual cell level (Anon 3, 2004). This technique is a powerful tool to study factors modifying mutagenicity and carcinogenicity and has gained importance in the field of genetic toxicology and human biomonitoring. It can also be used for ecological monitoring and measuring of DNA repair. (Collins, 2004; Dhawan, 2004).

The comet assay measures double strand breaks (DSB's), single strand breaks (SSB's), and alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-drug cross linking and DNA repair (Singh *et al*, 1988; Dhawan, 2004).

The comet assay works on the principle that strand breakage of the super coiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis (Singh *et al*, 1988). The denaturation and unwinding of the duplex DNA and expression of alkali labile sites as single strand breaks occur under highly alkaline conditions. Comets then form as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. According to Seth (2003) there are two principles in the formation of comets, namely:

1. DNA migration is a function of both size and the number of broken strands of the DNA.
2. Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoretic conditions, not the size of fragments.

3.2.2 Materials

All materials used were of the highest purity available and were obtained from Sigma-Aldrich, unless stated otherwise. See Appendix A for the preparation of the following solutions: Lysis buffer, electrophoresis buffer, neutralizing buffer, phosphate buffered saline, staining solution, high melting point agarose, low melting point agarose, enzyme buffer.

3.2.3 Method

3.2.3.1 Preparation of microscope slides

Frosted microscope slide (figure 3.1) was coated with 300 μ l HMPA. The slide was then set on ice, to keep cool until the sample was added.

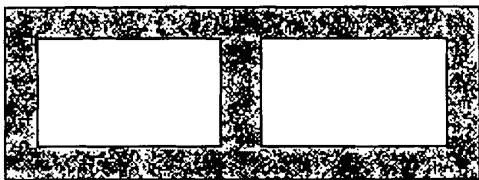


Figure 3.1. Frosted microscope slide

3.2.3.2 Isolation of lymphocytes

Heparinized blood was collected and 2ml of this blood was collected and added on top of 2ml of Histopaque® in a Falcon tube. This was centrifuged at 5500rpm for 30 minutes at 25°C. After centrifugation the plasma layer was discarded and the buffy coat was collected and aspirated into a 2ml Eppendorf® tube. Cells were then washed by adding 250µl of PBS and centrifuging for 3 minutes at 3000rpm at 4°C. The supernatant was discarded and 250µl PBS was added to the pellet. The cell suspension was again centrifuged for 3 minutes at 3000rpm at 4°C. The supernatant was removed and 500µl HAMS F10 was added to the pellet.

3.2.3.3 Cell counting and viability

Cell counting was done by using the Trypan Blue Staining method. For each sample 40µl of cell suspension was combined with 40µl Trypan Blue Staining solution. From this combination 10µl was placed on a haemocytometer and the cells were counted under a light microscope. The volume of the cell suspension was then adjusted so that there would be approximately 1000 cells per sample.

3.2.3.4 Effect of metabolites on DNA and repair of damaged DNA

A control sample of 40µl was taken from the cell suspension (see 3.1.3.2) and added to 150µl LMPA. This was vortexed for one second and the previously prepared frosted microscope slide (see 3.1.3.1) was coated with 130µl of the sample. The metabolite (SA 50µM; ALA 3mM; pHPPA 100µM) was added to the remaining cell suspension and incubated for 60 minutes at 37°C. After 60 minutes 250µl PBS was added to the cell suspension and this was centrifuged at 3000rpm for 5 minutes at 4°C. The supernatant was discarded and 500µl of HAMS F10 was added to the pellet. 130µl of the 40µl cell suspension and 150µl

LMPA was used to coat a previously prepared frosted microscope slide. The remaining cell suspension was incubated for 20 minutes after which a 40 μ l sample was taken and added to 150 μ l of LMPA to coat the slide. This was repeated twice more, at 20 minute intervals. The slides were then immersed in lysis buffer.

3.2.3.5 Effect of metabolite on DNA repair capacity

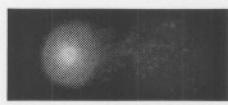
A control sample of 40 μ l was taken from the cell suspension (see 3.1.3.2) and added to 150 μ l LMPA. It was vortexed for one second and 130 μ l of this was used to coat the HMPA coated slide. 40 μ l of the original cell suspension was used for cell viability tests. The metabolite was added (SA 50 μ M; ALA 3mM; pHPPA 100 μ M) and the slides were coated. This was incubated for 60 minutes at 37°C. After 60 minutes 250 μ l of PBS was added to cell suspension and centrifuged at 3000rpm for 3 minutes at 4°C. The supernatant was removed and 400 μ l HAMS F10 was added to the pellet and a sample was taken. Thereafter 15 μ l of H₂O₂ was added to the cell suspension and incubated for 20 minutes at 37°C. After 20 minutes the H₂O₂ was removed by adding 250 μ l of PBS and centrifuging for 3 minutes at 3000rpm. The supernatant was discarded and 400 μ l HAMS F10 was added to the pellet. From this cell suspension 40 μ l was taken to coat the next slide. The cell suspension was then incubated for 20 minutes. After the 20 minutes a slide was coated with 130 μ l of the 40 μ l cell suspension and 150 μ l LMPA. The cell suspension was then again incubated for another 20 minutes where after the slide was coated.

3.2.3.6 Buffer solutions and processing of results

After the slides were submerged in lysis buffer (overnight at 4°C), to remove cellular proteins, the slides were placed in an electrophoresis tank, containing the electrophoresis buffer, for 30 minutes, before electrophoresis at 30V for 45 minutes at 4°C. After electrophoresis the slides were washed for 15 minutes in TrisHCl, pH 7.5. The slides were then stained with Ethidium Bromide for 30 minutes at 4°C, before being washed for 10 minutes in ddH₂O. After the slides were washed with ddH₂O, pictures were taken of the comets by using the Olympus IX70 fluorescence microscope (200x).

For statistical significance all experiments were done in duplicate and 50 comets were scored per sample with the CASP© (comet assay software project) program. This program measures the amount of DNA in the comet tail, which correlates with the amount of DNA damage. The data was then further processed in Microsoft Excel®. Afterwards the extent of DNA damage was grouped into classes.

Table 3.1. Illustration of different classes of comets.

Class	Picture	Class	Picture
Class 0 0 - 6 %		Class 1 6.1 - 17 %	
Class 2 17.1 - 35 %		Class 3 35.1 - 60 %	
Class 4 60.1 - 100%		Heavily damaged	

3.2.3.7 Treatment of samples with Fpg, EndoIII, Mspl and Hpall

After the overnight exposure of the samples to the lysis buffer, the samples were transferred to the respective enzyme buffers for 10 minutes. In this time an enzyme reaction buffer was prepared. The excess enzyme buffer was dabbed off the sample with tissue paper. Each sample was then coated with 50µl of the enzyme reaction buffer (see Table 3.2). The slides were then incubated for 30 minutes at 37°C. After incubation the slides were placed in the electrophoresis tank and the rest of the standard procedure was followed.

Table 3.2. Composition of enzyme reaction buffer for different enzymes.

	Fpg	EndoIII	MspI	HpaII
Enzyme Buffer	5µl	5µl	5µl	5µl
Enzyme	0.25µl	0.25µl	0.1µl	0.2µl
ddH₂O	44.75µl	44.75µl	44.9µl	44.8µl
Total	50µl	50µl	50µl	50µl

3.3 COLLAGENASE PERFUSION

3.3.1 Introduction

According to the Hyperdictionary-Medical (2003), perfusion is a procedure in which a catheter is placed into the artery that provides blood to the liver, and another catheter is placed into the vein that takes blood away from the liver.

As per product information by Roche collagenase perfusion of rat liver delivers a high yield of hepatocytes which may be exposed to test compounds in order to assess their effects on cell viability and enzyme leakage. This technique is relatively fast and reproducible compared to other techniques, e.g. slicing and enzymatic or mechanical dispersion and cell damage is minimized as there is no mechanical force (Jurgen, 1991). Hepatocytes are usually obtained after a two-step collagenase action. This disrupts intercellular contacts and communication systems and the cells lose their polar character and changes shape. Proteolysis also damages the enzyme and receptor apparatus of cells and impairs the biophysical characteristics and transport capabilities. The cells are nevertheless capable of repairing membrane defects and may preserve the majority of their functions. However the utilization of hepatocytes in suspension is limited by their survival period during which they can exhibit metabolic activity. This is why cell suspension can be used only for a period of four to six hours (Cervenkova *et al*, 2001).

Perfusion can be used for many types of *in vitro* assay, such as the determination of cytotoxicity, metabolism, transport of xenobiotics, etc. It can be used for initial screening of substances for potential hepatotoxicity, or for research into molecular mechanisms of drug action (Jurgen, 1991).

The advantage of using isolated cells is that a number of experiments can be performed on just one rat liver, thus reducing the number of animals required.

3.3.2 Materials

All materials used were of the highest purity available and were obtained from Sigma-Aldrich, unless stated otherwise. See Appendix A for the preparation of the following solutions: William's Medium E (WE), WE for Perfusion, WE + 10% FBS, Hank's Balanced Salts, Hank's Wash and Hank's Perfusion.

3.3.3 Method

3.3.3.1 General preparations for perfusion

The day before the perfusion were performed, all the reagents were prepared. On the day of perfusion the water baths was set on 37°C and Hank's Perfusion Solution and WE for Perfusion was placed in the water bath. The perfusion pump was first rinsed with 70% EtOH and then with ddH₂O after which pumping of Hank's Perfusion solution was started.

3.3.3.2 Perfusion

Rats, massed between 150 and 300g, were anaesthetized. The pump speed was reduced to 0.04 ml/min. The rat was sprayed with 70% EtOH and two lengths ($\pm 10\text{cm}$) of catgut were cut. A big area of abdominal skin was removed and all instruments were rinsed to remove loose hair. The abdominal muscle was carefully cut open up to the diaphragm and the intestines removed with gauss swaps. The catgut was loosely bound around the exposed portal and aorta veins. The perfusion needle was inserted into the portal vein and catgut was tied tightly around the needlepoint. The pump speed was increased to

7.6ml/min and the aorta catgut string was tightened. The thorax was opened and the heart punctured after which the liver was perfused for 10 minutes. Collagenase and CaCl_2 was added to the WE for perfusion. The perfusion pump was stopped and the tube placed in the WE for perfusion. The pump was started and the timer started after a darker pink was observed in the tube. The liver was digested for approximately 15 minutes at a pump speed between 4.00 and 5.00 ml/min. After digestion the pump was stopped and the needle removed. The liver was held over a petri dish and cut loose and WE for Perfusion were poured over the liver.

3.3.3.3 Cell harvesting and growth

The liver capsule was removed with tweezers and the cells gently freed from the capsule. These cells were filtered through sterile single layer cheesecloth into a 50ml blue cap Falcon tube. A small volume of WE + 10% FBS was used to rinse the cells. The cell suspension was centrifuged for 10 minutes at 450rpm and the supernatant was removed. Afterwards the tube was filled with 20ml WE + 10% FBS for a 5ml pellet. The tube was gently shaken to resuspend the cells in solution. The cell suspension was filtered through double layer cheesecloth into another 50ml tube, but the cheesecloth was not rinsed. This solution was used for viability determinations.

3.3.3.4 Viability Determinations

Ensuring a 12X dilution, 10 μl cells suspension, 90 μl WE and 20 μl Trypan Blue were added together. Ten μl of this solution was placed in the haemocytometer and cells in four squares were counted and the mean calculated. The living and the dead cells were counted and the percentage viability was calculated.

% Viability: dead cells/living cells X 100

$$= (100 - \text{answer})$$

$$= \% \text{ viable}$$

Value above 90 % is very good and preferable

3.4 PREPARATION OF LIVER CELLS WITH A MINCING SOLUTION

3.4.1 Materials

Hank's Balanced Salt solution (HBSS), EDTA and DMSO were obtained from Sigma-Aldrich and were of the highest purity available. The mincing solution was prepared as described by Tice & Vazquez (1999).

3.4.2 Method

The research animal was sacrificed, and the liver was minced into large pieces and submerged in 3ml mincing solution. 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 1 ml of the mincing solution in which the liver pieces is submerged was removed and 250 μ l PBS added. The cell suspension was centrifuged for 5 minutes as 5500rpm at 4°C. The supernatant was discarded and 250 μ l PBS was added to the pellet and centrifuged at the same specifications as the previous step. The supernatant was again discarded and 500 μ l HAMS F10 was added to the pellet and the cell suspension was used for further investigations as described in 3.2.3.4 and 3.2.3.5.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ESTABLISHING CONCENTRATION VALUES AND TIME DEPENDANCY

The repair of damaged DNA is critical to maintain genomic integrity. Thus, increase of succinylacetone (SA) as a result of a defective FAH enzyme, followed by the resulting increase of δ -aminolevulinic acid due to the inhibitory effect of SA on the δ -aminolevulinic acid dehydratase enzyme, can cause DNA damage (Prieto-Alamo & Laval, 1998). Patients with hereditary tyrosinemia type 1 (HT1) have increased plasma and urine levels of SA and pHPPA (Mitchell *et al*, 2001). In view of this, we investigated the effect of these metabolites with the comet assay to attempt to clarify the mechanisms responsible for the clinical features of HT1. In view of the effect of increased levels of SA on the δ -aminolevulinic acid dehydratase (Roth, 2003), we also assessed the effect of ALA.

4.1.1 Succinylacetone

SA inhibits T4-DNA ligase activity in a dose dependant manner. In our studies SA concentrations of up to $50\mu\text{M}$ reduced T4-DNA ligase activity to approximately 15% (figure 2.2). Concentrations higher than $50\mu\text{M}$ did not inhibit further. Therefore, we decided to expose the cells to $50\mu\text{M}$ SA (Prieto-Alamo & Laval, 1998).

4.1.2 Δ -Aminolevulinic acid

In several studies concentrations ranging between 2 and 5mM of ALA were used to asses the effect of the metabolite on DNA. In each case, marked DNA damage was observed. Based on these published information we used 3mM ALA to expose the cells to (Di Mascio *et al*, 2000; De Siervi *et al*, 2002; and Onuki *et al*, 2002).

4.1.3 pHPPA

There is not much information available with respect to the kind of DNA damage that pHPPA causes. This may be explained by the fact that pHPPA only occurs in trace amounts in plasma and urine. It was probably deemed unnecessary to assess the effect of this metabolite on DNA. We therefore did a dose-response study series to assess the effect of pHPPA on DNA. Lymphocytes were incubated for 30, 60 and 120 minutes with 25, 50, 100 and 200 μ M of pHPPA respectively.

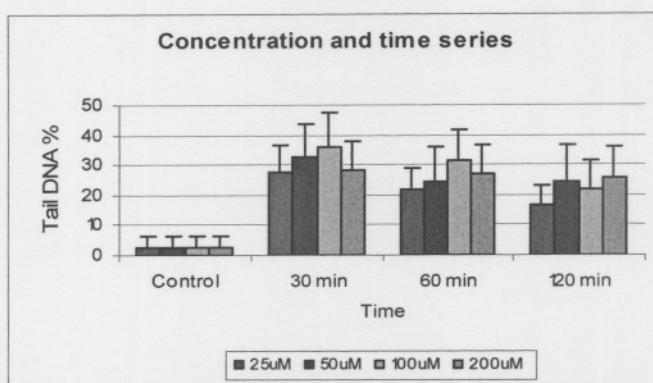


Figure 4.1. The effect of pHPPA on DNA damage. Lymphocytes were exposed to 25, 50, 100 and 200 μ M of pHPPA at 37°C for 60 and 120 minutes.

Exposure of isolated lymphocytes to different concentrations of pHPPA caused marked damage to DNA (figure 4.1). It seems that exposure to 100 μ M pHPPA was responsible for the most severe degree of damage after 30 and 60 minutes. One must also bear mind that significant damage occurred at 25 μ M pHPPA. Since the damage to DNA after exposure to 100 μ M pHPPA did not differ significantly between 30 and 60 minutes, we decided to expose the cells for 60 minutes in order to simplify the experimental design and to make the results comparable with the results obtained from exposure of lymphocytes to SA and ALA.

4.2 DNA DAMAGE AND REPAIR IN LYMPHOCYTES

4.2.1 Repair after exposure to different metabolites

SA, ALA and pHPPA cause damage to DNA (Prieto-Alamo & Laval, 1998; Di Mascio *et al*, 2000). The extent and duration is, however, not known.

4.2.1.1 Succinylacetone

Isolated lymphocytes were exposed to 50 μ M SA at 37°C for 60 minutes whereafter the metabolite was removed. Repair of DNA was then followed for 60 minutes. The percentage of tail DNA, measured with the CASP program, was used as an indication of the extent of DNA damage. The comets were also grouped in classes according to the extent of DNA damage.

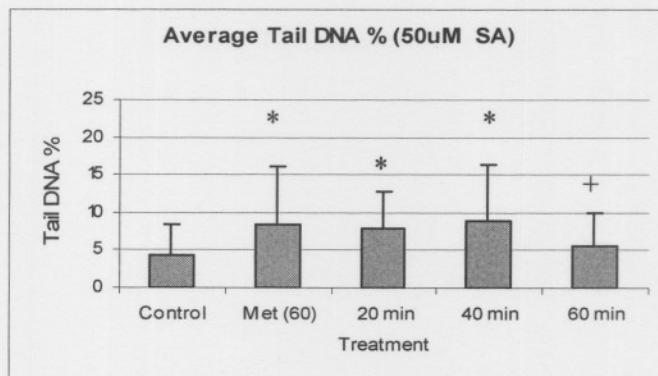


Figure 4.2. DNA damage and repair after exposure of lymphocytes to 50 μ M SA. [Met (60) is the sample after 60 minutes of incubation with SA; 20 min, 40 min and 60 min is the time allowed for DNA repair] *(p<0.05) versus control; + (p<0.05) versus Met(60)

There was significant DNA damage ($p<0.05$) following exposure of lymphocytes to 50 μ M SA for 60 minutes (figure 4.2). After the metabolite was removed, 60 minutes was allowed for DNA repair. During the first 40 minutes, there appeared to be no significant repair since there was no significant difference between the values at met(60), and 20 and 40 minutes. After 60 minutes, some DNA repair could be observed. This is also evident in the calculated repair capacity of 0.33. Thus, 50 μ M SA did cause marked DNA damage, and the repair capacity of the cell was also impaired, i.e. only after 60 minutes could some repair be observed.

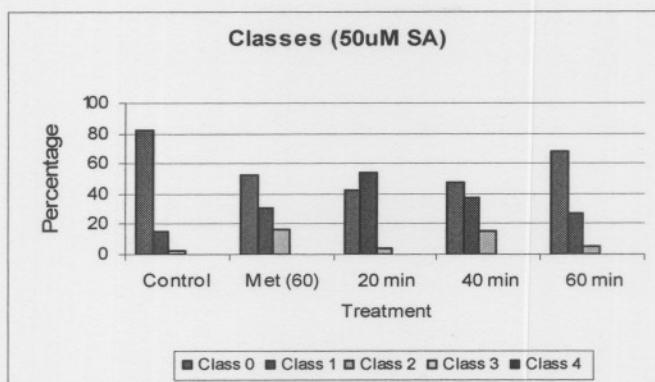


Figure 4.3. Class distribution of comets after exposure to SA and the various repair times.

Figure 4.3 is the distribution of the comets after exposure of the cells to SA. It is obvious that, although there was DNA damage after exposure of the cells to SA for 60 minutes, the damage was minimal since the highest percentage of comets were distributed in classes 0 to 2. Thus DNA damage was less than 35%. The comet distribution also showed that repair was minimal during the first 40 minutes of exposure. Thereafter the pattern of the classes began to resemble that of the control, indicating repair.

4.2.1.2 Δ -Aminolevulinic acid

The effect of ALA on DNA in isolated lymphocytes was determined by exposing the lymphocytes to 3mM ALA for 60 minutes at 37°C. Following exposure, the metabolite was removed to measure the repair capacity of DNA for 60 minutes. Following exposure, DNA damage increased slightly (figure 4.4). The relatively large standard deviations around the mean clearly indicate a large variation in DNA damage.

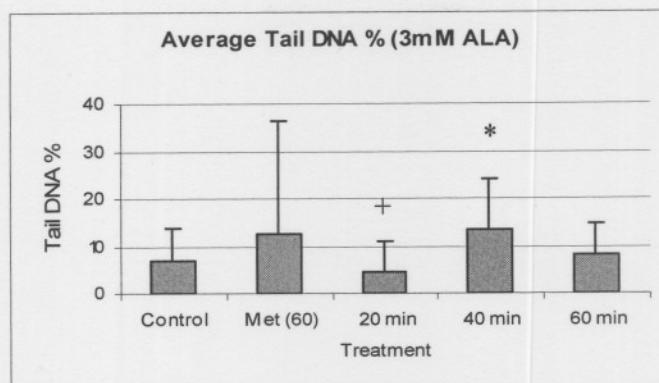


Figure 4.4. The extent of DNA damage after exposure to 3mM ALA. DNA repair was followed for 60 minutes after exposure to ALA. *($p<0.05$) versus control; + ($p<0.05$) versus Met(60).

This is confirmed by the comet distribution (figure 4.5). The extent of damage caused by ALA was also larger than in the case with SA (8%). DNA repair also appeared to be slightly better because the calculated repair capacity was 0.36.

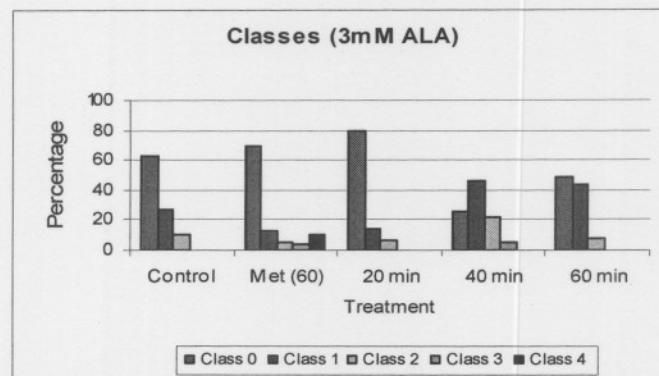


Figure 4.5. Distribution of DNA comets after exposure to 3mM ALA for 60 minutes at 37°C.

Although the extent of DNA damage was still low, comet distribution indicated some comets in class 3 and 4. Thus, in spite of the relatively low level of damage, damage was 100% in some cases. There was less comets in class 1 after 60 minutes of repair than directly after exposure. There were also no more comets in class 3 and 4, which may confirm the slightly higher calculated repair capacity than in the case of SA.

4.2.1.3 pHPPA

The effect of pHPPA on DNA in lymphocytes was determined by exposing the cells to 100 μ M of the metabolite for 60 minutes at 37°C. Similarly to SA and ALA,

pHPPA was removed after 60 minutes and the repair of the DNA was assessed for 60 minutes.

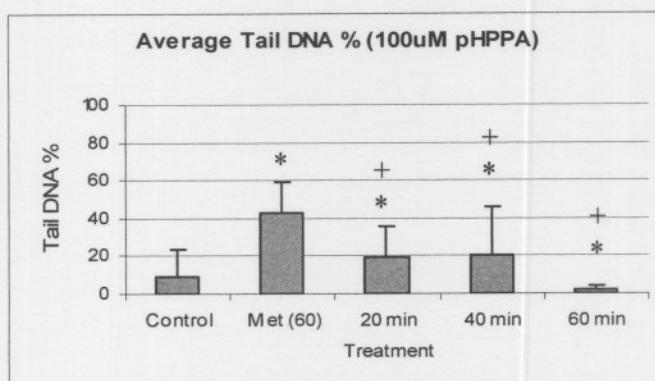


Figure 4.6. The extent of DNA damage in isolated lymphocytes following exposure to 100 μ M pHPPA. *($p<0.05$) versus control; + ($p<0.05$) versus Met(60)

Following exposure of the cells, damage to DNA increased by approximately 32% (figure 4.6). This was considerably higher than that caused by SA or ALA. The observed rate of DNA repair was rapid during the first 20 minutes (20 min), and then reached a plateau from 20 - 40 min before further repair took place from 40 - 60 min. Similar to SA, DNA repair seemed to be slow, but in contrast to SA the calculated repair capacity was almost double, i.e. 0.622. It is therefore not unlikely that complete repair may be possible following exposure to pHPPA. The distribution of comets (figure 4.7) supports this.

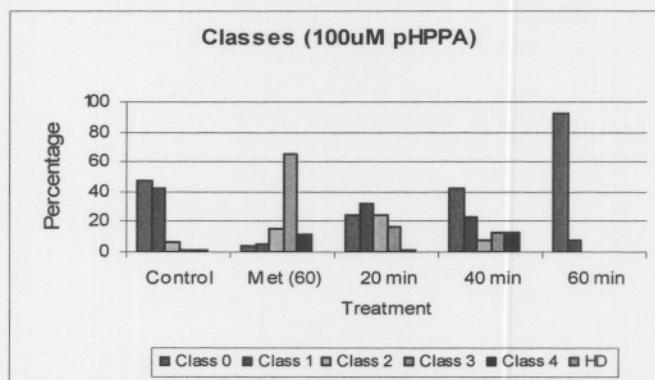


Figure 4.7. Distribution of comets after exposure to 100 μ M of pHPPA for 60 minutes at 37°C.

The majority of comets following exposure to pHPPA (figure 4.7) were in class 3, in contrast to SA and ALA which was in class 1. DNA damage was therefore up

to 60 %. The distribution of comets also suggests that repair took place at a rapid rate following 40 minutes. After 60 minutes almost all the comets was in class 1.

Our results indicate that isolated lymphocytes react differently to the exposure to different metabolites. The extent of DNA damage and DNA repair varied. Exposure of isolated lymphocytes to SA slightly inhibited the capacity of the cell to repair their DNA. Almost similar results were observed following exposure to ALA. pHPPA caused more DNA damage but repair was more rapid than in the case of SA and ALA.

4.2.2 The effect of addition oxidative stress on DNA repair

Although the SA, ALA and pHPPA caused DNA damage the effect on the repair capacity of the cells under stress was not as clear. Since repair of cells is generally linked to its susceptibility to cancer (Martin, 2001; Christmann *et al*, 2003), it is important to investigate this. DNA repair capacity of a cell can be measured by inflicting oxidative DNA damage with H₂O₂ and then monitor the rate with which the lesions are repaired (Collins, 2004).

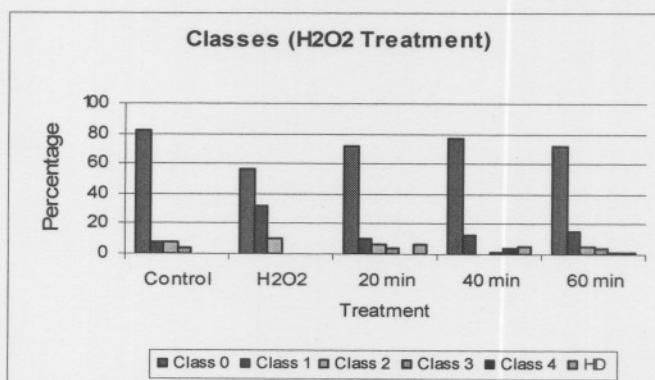


Figure 4.8. Distribution of comets after the lymphocytes was exposed to H₂O₂ for 20 minutes at 37 °C. The repair of DNA was followed for 60 minutes.

Before the effect of oxidative stress and exposure to the different metabolites could be determined, we have to ensure that H₂O₂ did not damage the

lymphocytes irreparably. Our results clearly showed that H₂O₂ caused some DNA damage (figure 4.8). There were fewer comets in class 0 and more comets in class 1 and 2. It appeared that damage increased slightly in the first 20 minutes after the H₂O₂ was removed. There was an increase in comets in class 3. In addition we also saw heavily damaged comets i.e. comets with so much DNA damage that the CASP program could not score them so they had to be scored manually. However, almost complete repair followed, i.e. the percentage of comets in class 0 increased and the percentage of comets in class 4 and the percentage of heavily damaged comets decreased significantly.

In summary, H₂O₂ did cause DNA damage, but the damage was repairable. In addition, the H₂O₂ allowed us to cause additional DNA damage in order to study the added effect of oxidative stress in conjunction with the exposure of the lymphocytes to the metabolites.

4.2.2.1 Succinylacetone

Lymphocytes were exposed to 50 μ M SA for 60 minutes at 37°C whereafter the metabolite was removed and H₂O₂ added to the cell suspension. After an additional 20 minutes at 37°C the H₂O₂ was removed and repair of DNA allowed to take place for 40 minutes. The results are summarized in figure 4.9.

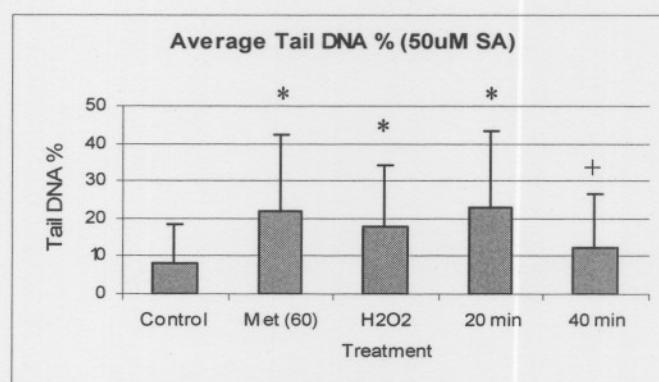


Figure 4.9. The effect of oxidative damage on DNA repair. After exposure to SA the lymphocytes were placed under oxidative stress to determine the repair capacity under these circumstances. *(p<0.05) versus control; + (p<0.05) versus Met(60)

Additional oxidative stress did not cause extra DNA damage. Repair of DNA was also not affected by H₂O₂, i.e. the repair capacity, 0.30, was not significantly different from that of SA alone.

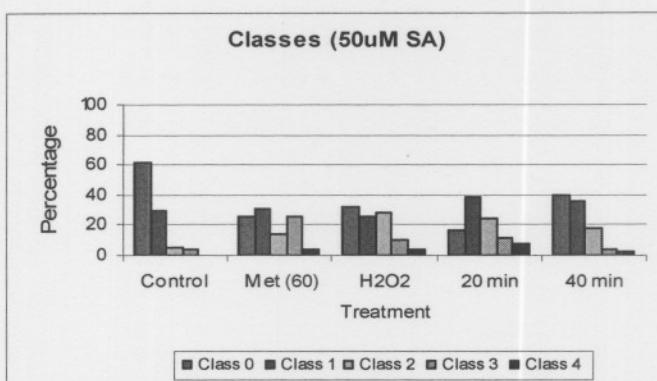


Figure 4.10. The distribution of comets after exposure of SA treated cells and oxidative stress

The grouping of comets into classes also indicated that exposure of the lymphocytes to SA for 60 minutes caused damage to the DNA (figure 4.10), i.e. more comets in classes 2 and 4. DNA damage is therefore slightly more extensive after the additional oxidative stress. A pattern of DNA repair was present since majority comets were present in classes 1 and 2 after 60 minutes repair time (figure 4.10).

4.2.2.2 Δ-Aminolevulinic acid

Isolated lymphocytes were exposed to 3mM ALA for 60 minutes, whereafter it was removed, and the cells then exposed to H₂O₂ for 20 minutes at 37°C. H₂O₂ was then removed and the repair followed for 40 minutes (figure 4.11).

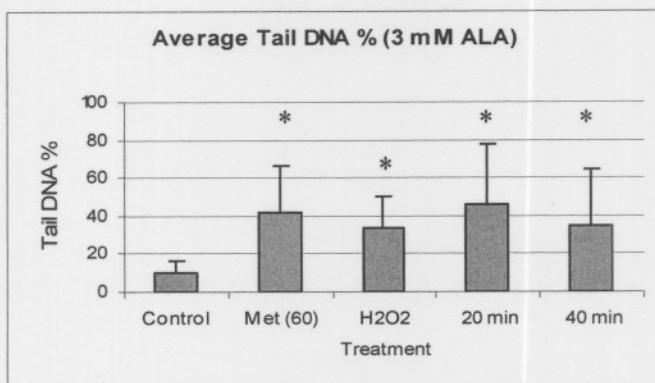


Figure 4.11. The extent of DNA damage and repair after exposure of ALA treated cells to oxidative stress. *($p<0.05$) versus control

ALA caused DNA damage in isolated lymphocytes similar to the results in figure 4.4. Oxidative stress appears to allow some DNA repair to take place. In contrast to SA, the additional oxidative stress after exposure to ALA slightly inhibited the repair capacity of the cells, i.e. from 0.36 to 0.18.

The distribution of the comets (figure 4.12) also shows that ALA caused damage to DNA, similar to results in figure 4.5.

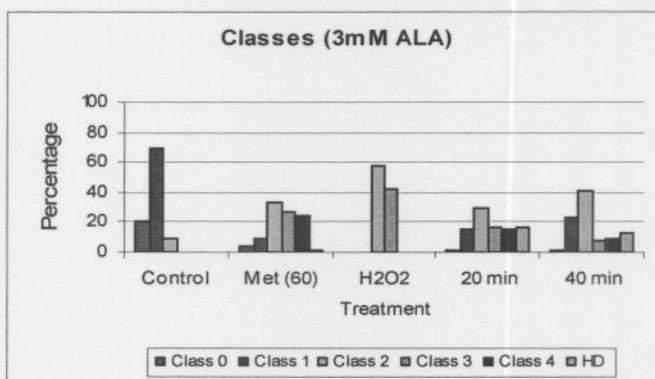


Figure 4.12. The distribution of comets after exposure of ALA treated cells to oxidative stress.

It is evident that very little DNA repair took place following exposure to H₂O₂, i.e. the comets were present mainly in class 2 and 3, indicating DNA damage of up to 60% (figure 4.12). After exposure of the cells to the additional oxidative stress, heavily damaged DNA was present, which emphasizes the apparent inhibitory effect on DNA repair in ALA exposed cells.

4.2.2.3 pHPPA

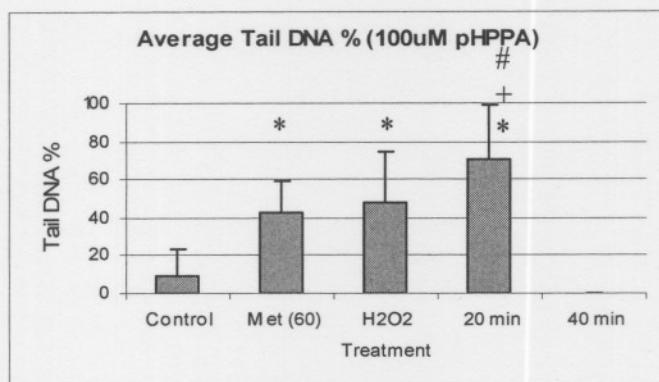


Figure 4.13. The extent of DNA damage and repair after exposure of pHPPA treated cells to oxidative stress. *(p<0.05) corresponding to the control; + (p<0.05) versus Met(60); #(p<0.05) versus H₂O₂.

Exposure of isolated lymphocytes to pHPPA for 60 minutes caused DNA damage (figure 4.6 and 4.13). Additional oxidative stress caused slightly more damage. In the case of both SA and ALA the additional oxidative stress did not cause measurably additional DNA damage, in contrast to accumulated damage of pHPPA exposed cells. The repair capacity of DNA was also more inhibited than in the case with ALA, since the calculated repair capacity of the cells after exposure to pHPPA and H₂O₂ was almost zero. All of the comets distributed into the heavily damaged class (figure 4.14). Viability tests using Trypan blue indicate that cell viability was in excess of 90% throughout the experiment. The heavily damage of the cells can thus not be due to cell death.

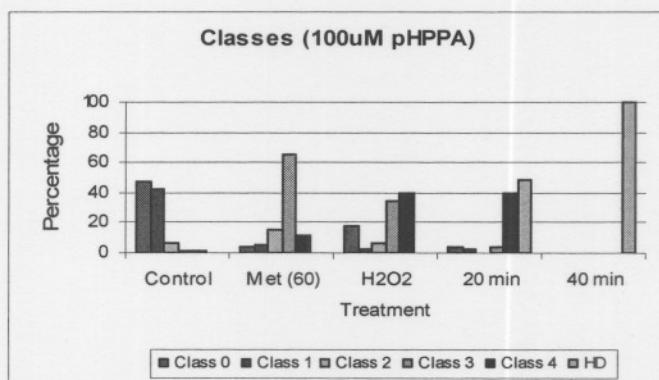


Figure 4.14. The distribution of comets after exposure of pHPPA treated cells to oxidative stress.

It is more obvious that no repair of damaged DNA took place after the additional exposure of the pHPPA treated cells to oxidative stress (figure 4.14), since there were more heavily damaged comets after 40 minutes repair time. From these results it is safe to say the DNA repair capacity of the lymphocytes was severely affected. Exposure to pHPPA and H₂O₂ therefore decreased the repair capacity of the cells markedly.

Exposure of the isolated lymphocytes to additional oxidative stress gave a better indication of the capacity of cells to repair damaged DNA. Exposure of the lymphocytes to oxidative stress after exposure to SA had no additional effect on the ability to repair their DNA. The almost unchanged calculated repair capacity supports this. Exposure to ALA affected the cell's ability to repair oxidative DNA damage. The calculated repair capacity was almost half that after exposure to ALA alone. pHPPA had an immense effect on the cell's ability to repair DNA damage after oxidative stress (figure 4.13 and 4.14). The damage increased markedly during the time allowed for DNA repair to take place. In order to elucidate the effect of pHPPA on the repair capacity of the cell it is necessary to first determine the type of DNA damage caused by pHPPA.

4.2.3 Type of DNA damage caused by pHPPA

DNA damage in cells can occur by spontaneous DNA replication errors, base deamination, oxidation or environmentally induced alkylation (Teis, 1999; Jackson, 2004). To determine what type of DNA damage occurred following exposure of isolated lymphocytes to pHPPA, it was necessary to introduce modifications to the comet assay to assess particular types of DNA lesions. Formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (EndoIII) are useful to determine the type of oxidative damage inflicted to DNA (Collins, 2004). Speit *et al* (2004) suggested that Fpg may also be sensitive to alkylated residues in DNA. This can be the cause if the type of DNA damage is not clear. The restriction enzymes Mspl and Hpall can be used to determine whether pHPPA affects methylation of the CCGG sequences in the genome of cells.

To determine the effect of these enzymes on the metabolite treated nucleoids, the same experimental design as in 4.2.1.3 and 4.2.2.3 were used.

4.2.3.1 Control

To understand and interpret the effect of the additional enzyme treatments samples a reference was needed. This was done by using the same experimental design to obtain the effect of the enzyme on the metabolite treated nucleoids, but without the addition of the enzyme. For proper comparisons, those results are included in the current figures.

4.2.3.2 Fpg

Fpg (also known as 8-oxoguanine DNA glycosylase) acts both as an N-glycosylase and an AP-lyase (product information supplied by NE Biolabs). The N-glycosylase activity releases damaged purines from double stranded DNA to generate an apurinic (AP site). The AP-lyase activity cleaves both 3' and 5' to the AP site to remove the AP site and leave a 1 base gap. The treatment of the samples with Fpg will therefore indicate if pHPPA cause any oxidative damage to purines (Albertini *et al*, 2000).

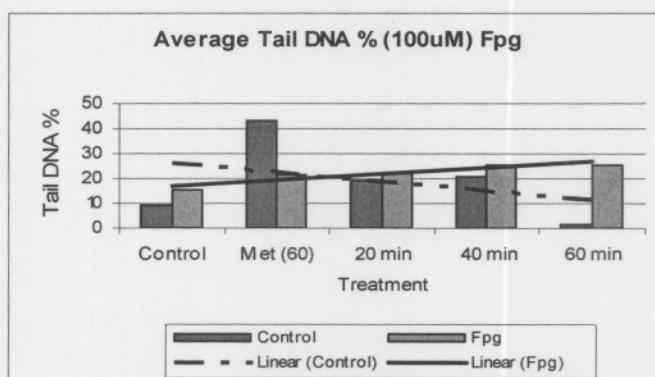


Figure 4.15. The extent of DNA damage after Fpg treatment of nucleoids from pHPPA-treated cells.

The tail DNA percentage of the control was slightly higher after treatment with Fpg (figure 4.15). This indicates that a small amount of damage to DNA can be ascribed to endogenous oxidative DNA damage. Exposure of the cells to pHPPA resulted in increased DNA damage. In contrast to the overall control where DNA

repair was observed after the removal of the metabolite (evident from the negative slope of the trendline), the percentage tail DNA increased at a slow rate after the removal of the metabolite (evident from the positive trendline). This indicates that, although there appeared to be repair of DNA after the metabolite was removed (control), there was still oxidative damage to the DNA (Fpg). The slow increase in DNA damage caused by pHPPA over time, even after the metabolite was removed, is also reflected in the distribution of the comets (figure 4.16).

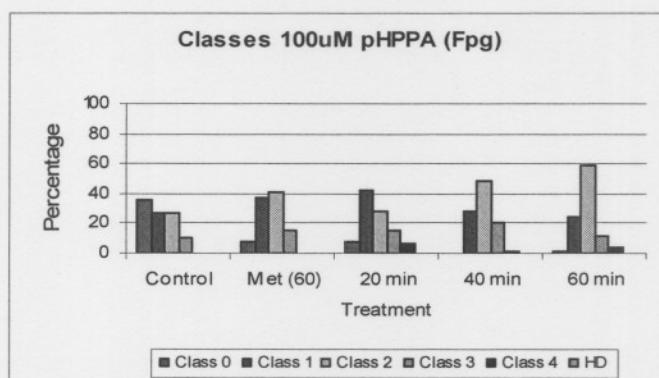


Figure 4.16. The distribution of comets after exposure of lymphocytes to $100\mu\text{M}$ pHPPA followed by treatment with Fpg.

The increase in DNA damage after exposure of the lymphocytes to pHPPA is reflected in the increase in the percentage of comets in classes 2 and 3. Following the removal of the metabolite some repairs of DNA seems to take place. This is not so, since there was an increase in the comets in class 4. After 40 minutes of repair time there were no comets in class 0 and the percentage of comets in class 1 and 2 increased. After 60 minutes the percentage of comets in class 2 have still increased and there was also an increase in the comets in class 4. This suggests a slow but steady increase in DNA damage over time, even after the metabolite was removed.

We then measured the effect on DNA damage and repair of an additional oxidative exposure of the metabolite-exposed cells.

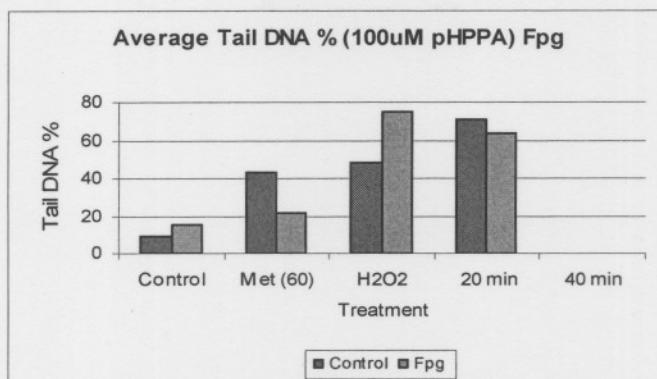


Figure 4.17. The extent of DNA damage after exposure to 100 μ M pHPPA for 60 minutes at 37°C. The repair was measured for 40 minutes after the cells were under oxidative stress. After lysis the samples were treated with Fpg enzyme.

A sharp increase in DNA damage (H_2O_2) occurred after the cells were exposed to H_2O_2 (figure 4.17) indicating that Fpg is highly sensitive to indicate oxidative damage to DNA (Speit *et al*, 2003; Collins, 2004). During the first 20 minutes after exposure to oxidative stress some repair of DNA appeared to take place. Similar to the results in figure 4.13 the tail DNA % bar at 40 minutes was absent, because all the comets indicated heavily damaged DNA and the CASP program was unable to score these comets. The distribution of the comets in different classes gave a much more accurate representation of the effect of H_2O_2 on the metabolite exposed cells. There was a marked increase in the percentage of comets in the heavily damage class (figure 4.18).

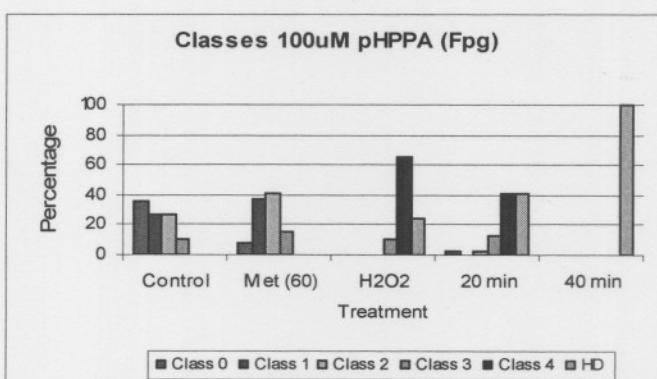


Figure 4.18. The distribution of comets after exposure to 100 μ M pHPPA for 60 minutes at 37°C followed by exposure to H_2O_2 . After lysis the samples were treated with Fpg enzyme.

After 40 min repair time no comets were present in classes 0 - 4 which means that no DNA repair took place. From the control studies it appeared as if the

damage caused by pHPPA is repaired over time, but the treatment of the DNA with Fpg showed that there were still oxidized purines present in the DNA and that these were not repaired. The exposure of cells to additional oxidative stress, via H₂O₂, either exceeded the ability of the isolated lymphocytes to repair the damage inflicted on their DNA or that pHPPA affected the repair mechanisms of the cells.

4.2.3.3 Endo III

Endonuclease III (Nth), a protein isolated from *E. coli*, acts as both an N-glycosylase and an AP-lyase (product information supplied by NE Biolabs). The N-glycosylase activity releases damaged pyrimidines from double-stranded DNA, generating an apurinic (AP site). The AP-lyase activity of the enzyme cleaves 3' to the AP site leaving a 5' phosphate and a 3' opened ring sugar. Thus, the results obtained from DNA samples treated with EndoIII would indicate oxidative damage to pyrimidine bases (Albertini *et al*, 2000).

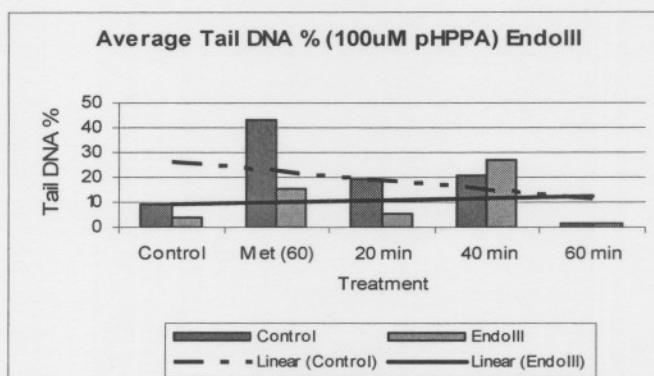


Figure 4.19. The extent of DNA damage after exposure to 100 μ M pHPPA for 60 minutes at 37°C. After lysis the samples were treated with EndoIII enzyme for 30 minutes at 37°C.

Damage occurred to pyrimidines (figure 4.19), but to a somewhat lesser extent than to the purines (figure 4.15). The control trendline had a negative slope, which indicated that some repair took place (figure 4.17 and 4.19). In contrast to the damage to purines, the damage to pyrimidines appeared to have undergone some repair. These observations are also reflected in the grouping of DNA damage in the different classes (figure 4.20)

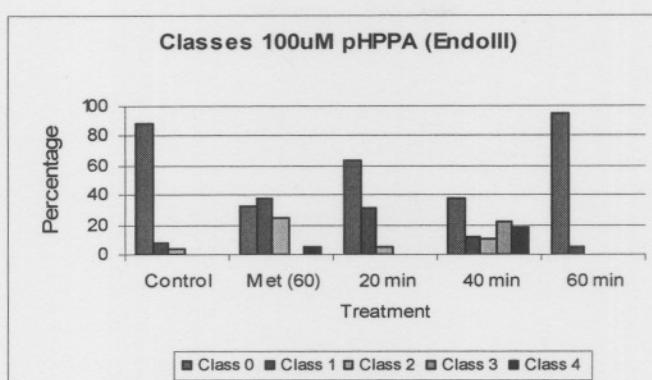


Figure 4.20. The distribution of comets after exposure to $100\mu\text{M}$ pHPPA for 60 minutes at 37°C and treatment with Endolll enzyme.

The high value at 40 min in figure 4.19 is repeated in figure 4.20 as there was an increase in the percentage of comets in class 1 – 4. However, the value at 60 min closely resembles the value at 20 min, which indicates that repair of DNA took place.

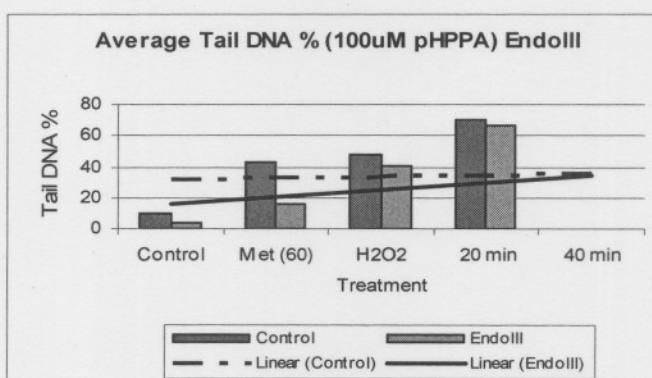


Figure 4.21. The extent of DNA damage after exposure to $100\mu\text{M}$ pHPPA for 60 minutes at 37°C . The repair was measured for 40 minutes after the cells were under oxidative stress. After lysis the samples were treated with Endolll enzyme.

Even though exposure of the isolated lymphocytes to pHPPA caused lesser damage to pyrimidines than to the purines (figure 4.19), a similar cumulative trend in DNA damage was observed after induced oxidative stress. This is evident from the positive slope of the Endolll trendline (figure 4.21).

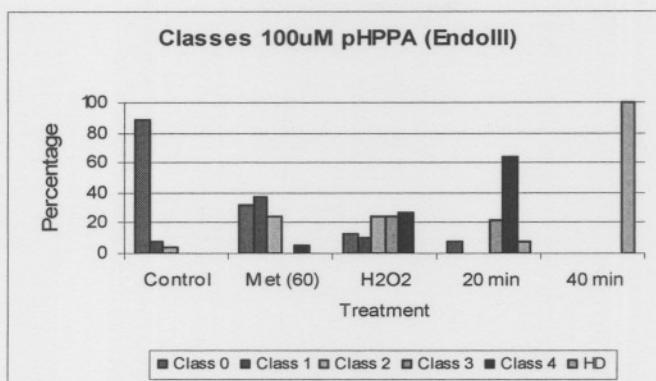


Figure 4.22. The distribution of comets after exposure to $100\mu\text{M}$ pHPPA for 60 minutes at 37°C and after oxidative stress. After lysis the samples were treated with EndoIII enzyme.

Analysis of the distribution of the comets clearly shows this cumulative trend in DNA damage. Immediately following exposure to additional oxidative stress (H_2O_2), less DNA damage occurred with the pyrimidines than with the purines. This is so because there were more comets in classes 2 to 4 than in the heavily damaged class (figure 4.18). However, in the time allowed for DNA repair to take place, the accumulation of DNA damage became more and more pronounced because of the shift of comets from class 2 – 4 to mainly the heavily damaged class.

4.2.3.4 Mspl and Hpall

Mspl is an isoschizomer of Hpall (product information supplied by NE Biolabs). When the external C in the sequence CCGG is methylated, Mspl and Hpall cannot cleave. However, unlike Hpall, Mspl can cleave the sequence when the internal C residue is methylated. The combination of these restriction enzymes is generally used to study CpG methylation levels in cells under various conditions (Kupper *et al*, 1997; Field, 2000).



Figure 4.23. Pictures of comets after DNA sample treatment with (A) Mspl and (B) Hpall.

Pictures of comets after treatment with the restriction enzymes clearly show the difference in the type of comet that was formed after treatment of the nucleoids with the respective enzymes. The heads of the comets after Mspl treatment were more diffuse than following Hpall treatment, indicating that the latter contains more DNA. Also, the tails of the comets following Mspl treatment were much longer than in the case of Hpall treatment. This illustrates the differential digestibility of DNA by these restriction enzymes and reflects the level of methylation of their common target site.

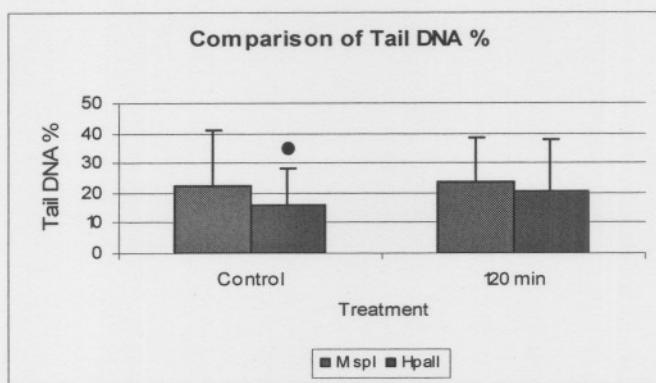


Figure 4.24. Extent of DNA damage after incubation for 120 minutes at 37°C. •($p < 0.05$) versus Mspl

Mspl cleaves CCGG sites regardless of the methylation status of CpG, whereas Hpall only cleaves the CCGG sites when no methylation is present. The difference in the % DNA in the tail between Mspl and Hpall in the control (figure 4.24) represents the difference in methylation levels of the target sites of these enzymes. Incubation of the lymphocytes at 37°C for 120 minutes in the absence of factors that could cause damage caused some demethylation since there was no significant difference in the % DNA in the tail between Mspl and Hpall. The small elevation in the amount of tail DNA following treatment with Hpall may seem insignificant. It is, however, not the case, because the difference in the values changed from being statistically significant in the control to not statistically significant after 120 min of incubation ($p < 0.05$). The significance of this result may be better illustrated by looking at the ratio between the respective amounts of tail DNA after treatment with each of the enzymes. Since Mspl will cleave the

CpG sites regardless of methylation, this will be a constant value. However, Hpall only cleaves unmethylated target sites, and an increase in the tail DNA % following treatment with Hpall will indicate a decrease in the number of methylated sites. If the value of the tail DNA % after treatment with Mspl is divided by the value of the tail DNA % after treatment with Hpall a ratio can be determined. A ratio of 1 would represent complete demethylation. In the results given in figure 4.24 the ratios are 1.4 for the control cells and 1.1 after 120 minutes of incubation. This indicates that some demethylation of the DNA occurred during the 120 minutes of incubation of the cells.

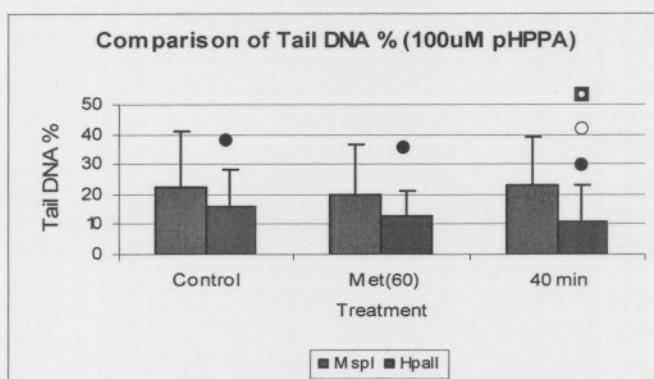


Figure 4.25. The effect of pHPPA on the methylation level of the target site of Mspl and Hpall.
 ●(p<0.05) versus Mspl; ○(p<0.05) versus Met(60); ■(p<0.05) versus figure 4.24 control

After exposure of the cells to pHPPA, this ratio between Mspl and Hpall increased from 1.4 in the control to 1.6 directly after exposure and to 2.0 after 40 minutes of repair time (figure 4.25). This increase indicates a slow increase in methylation of the enzymes with time. Thus, exposure of the lymphocytes to pHPPA, even for this relative short period, resulted in a very low level of DNA methylation, even after the metabolite has been removed. This is even more pronounced when the difference between the ratios after normal incubation and exposure to the metabolite is taken into account. Incubation of the lymphocytes without the metabolite caused a decrease in methylation. Finally exposure of the cells resulted in a small (but significant) elevation in the level of DNA methylation (figure 4.24).

In summary, pHPPA caused, amongst others, damage to DNA bases (purines > pyrimidines). It seems that the specific type of damage cannot be repaired properly since the level of repair was low. In addition, following additional oxidative stress, the DNA becomes irreparable. There could be some reasons. The first may be that the DNA damage is too extensive to repair. The second may be that the pHPPA has an inhibitory effect on the repair mechanisms of the cell. It is likely for DNA lesions to be shielded from repair enzymes by proteins that are not involved in the repair process (Rink *et al*, 1996). pHPPA can act as a reversible and competitive inhibitor of the transketolase enzyme, which is essential for the generation of ribose-5-phosphate to produce nucleic acids (Solovjeva *et al*, 1999). If the same happens to one or more of the DNA repair proteins, the lesions in the pHPPA exposed DNA may be shielded from repair. In addition to this, oxidative damage pHPPA also seem to cause slight methylation of the CpG sites in the CCGG sequences of DNA. The question now arises if it is possible for a molecule of this type to cause both oxidative damage to and methylation of DNA? Are the effects observed after treatment with Fpg truly oxidative damage, or are the observed lesions due to methylation of DNA. Fpg may also indicate alkylation damage (Speit *et al*, 2004).

Our results up to now indicated that the selected metabolites caused DNA damage in lymphocytes. Since HT1 and AIP predominantly occur in the liver (Baggott & Dennis, 1995; Mitchell *et al*, 2001), it was necessary to determine what the effect of the metabolites would be on the DNA in isolated hepatocytes.

4.3 DNA DAMAGE AND REPAIR IN HEPATOCYTES

4.3.1 DNA damage and repair after exposure of isolated hepatocytes to different metabolites

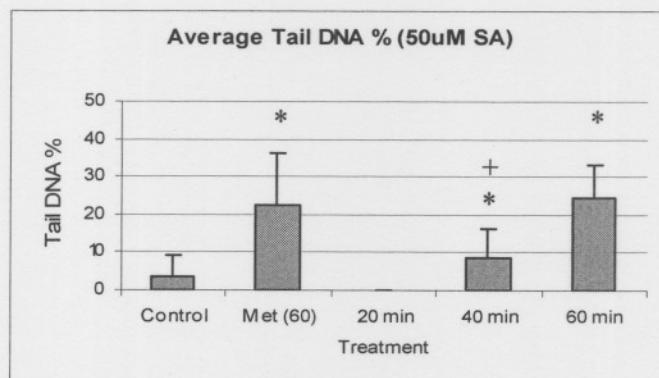
The same set of experiments that were done with the lymphocytes was repeated with isolated hepatocytes. Hepatocytes were harvested using two approaches. In the first, collagenase was used while perfusing isolated livers. Since this method easily fails and due to the observation that the collagenase in itself may cause DNA damage (Cesarone *et al*, 1984), we used another more robust method. It is called the mincing solution (Tice & Vasquez, 1999).

4.3.1.1 Succinylacetone

The hepatocytes harvested by (A) perfusion and (B) mincing solution were exposed to 50 μ M SA for 60 minutes at 37°C and DNA repair was followed for 60 minutes.

SA caused markedly more DNA damage in hepatocytes (figure 4.26) than in lymphocytes (figure 4.2).

A:



B:

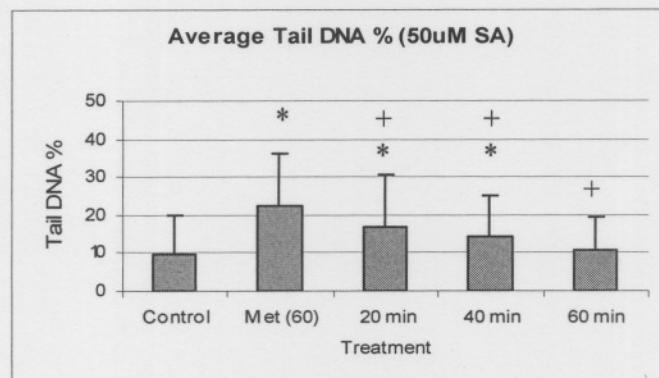
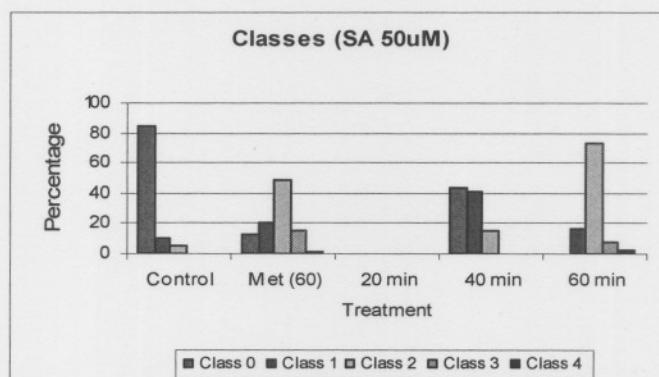


Figure 4.26. DNA damage and repair after exposure of hepatocytes to 50 μ M SA for 60 minutes at 37°C. Hepatocytes were obtained by (A) perfusion and (B) mincing solution. *($p<0.05$) versus control; + ($p<0.05$) versus Met(60)

It is also evident that DNA repair followed the same trend during the first 40 minutes in A and B. Thereafter DNA damage increased in A but decreased in B. The final calculated repair capacity in robustly harvested cells was 0.51. A possible explanation may be the different methods of cell harvesting. The harvesting of hepatocytes by perfusion involves collagenase treatment, whereas with the mincing solution no enzymes are used. The perfusion process may exhaust the energy reserves or may cause irreparable damage to the capacity of DNA repair by the cells. The reason for the apparent advantages when the mincing solution was used is unknown, except that it is a very mild treatment of the tissue to harvest viable cells.

A:



B:

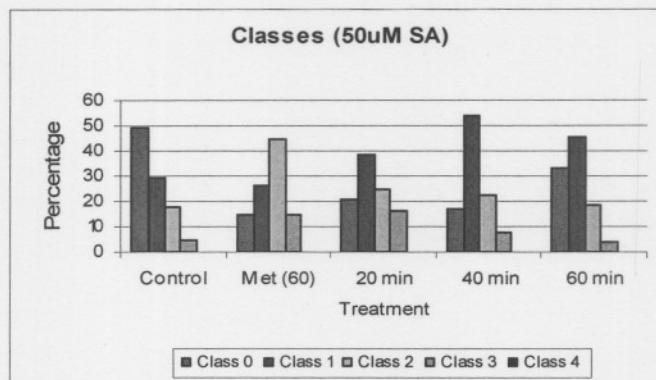


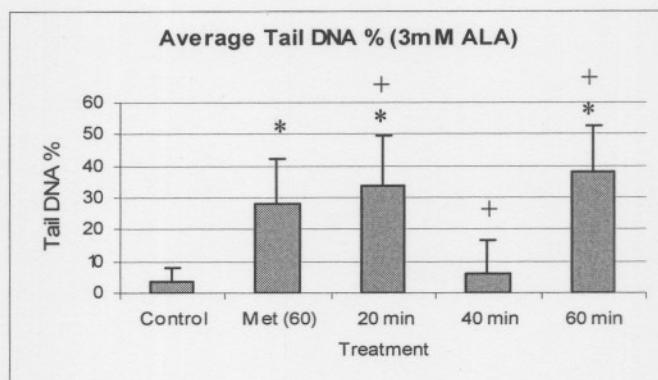
Figure 4.27. The distribution of comets after exposure of hepatocytes to 50 μ M SA at 37°C for 60 minutes. Cells harvested by (A) perfusion and (B) mincing solution.

The distribution of the comets (figure 4.27) supports the higher extent of DNA damage in hepatocytes than in lymphocytes, since there were more comets in class 2 than in lymphocytes (figure 4.3). The difference in reaction to the metabolite in the different cell preparations is also demonstrated in figure 4.27. Thus, after 60 minutes repair time, there were more comets in classes 0 and 1 in B than in A. It therefore appears that DNA damage was "delayed" in the hepatocytes prepared by the collagenase perfusion technique. This may support the notion that these cells cannot maintain DNA repair because of energy depletion.

4.3.1.2 Δ -Aminolevulinic acid

The hepatocytes harvested by perfusion (A) or mincing solution (B) were exposed to 3mM ALA for 60 minutes at 37°C and DNA repair was followed for 60 minutes at the same temperature.

A:



B:

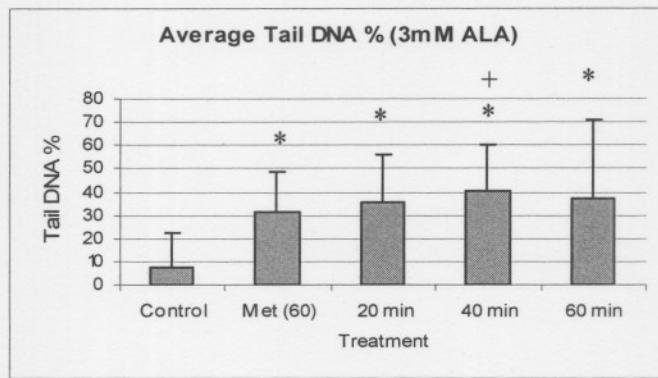
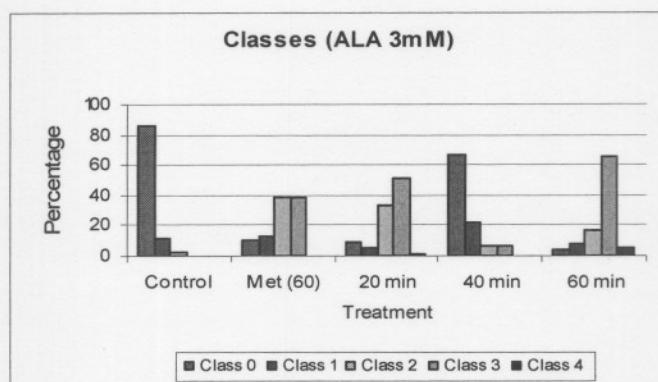


Figure 4.28. DNA damage and repair after exposure of hepatocytes to 3mM ALA for 60 minutes at 37°C. Hepatocytes were obtained by perfusion (A) and mincing solution (B). *($p<0.05$) versus control; + ($p<0.05$) versus Met(60)

ALA caused damage to DNA (figure 4.28) similar to that in lymphocytes (figure 4.4). However, in hepatocytes the extent of DNA damage was almost twice as much as in lymphocytes. In contrast to the DNA repair following exposure to SA (figure 4.26), ALA tends to increase DNA damage over time (figure 4.28). In contrast SA, the repair capacity was similar in the hepatocytes harvested by the different methods. This observation is supported by the distribution of the comets in the different classes, since more of comets were in classes 3 and 4 after exposure of the hepatocytes to ALA.

A:



B:

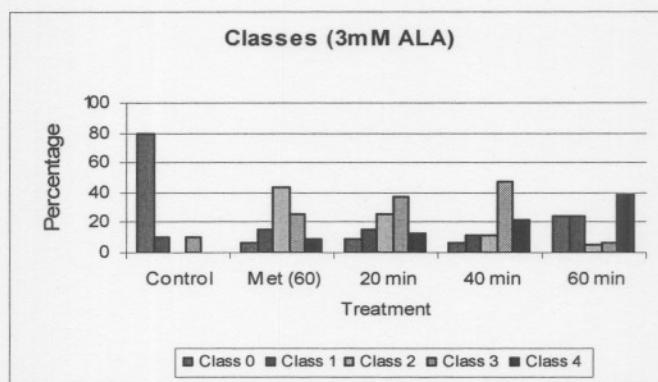


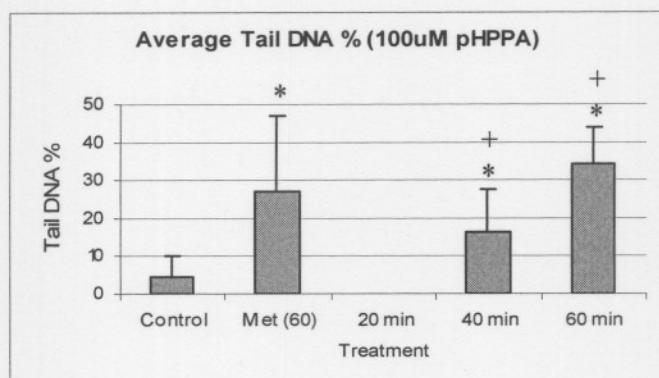
Figure 4.29. The distribution of comets after exposure of hepatocytes to 3mM ALA at 37°C for 60 minutes.

The distribution of the comets showed a steady increase in DNA damage rather than repair (figure 4.29), i.e. more comets were in classes 3 and 4 during the repair time. The different methods of harvesting hepatocytes had no effect on DNA repair (figure 4.29). Increased DNA damage, i.e. most comets in classes 3 and 4, were observed.

4.3.1.3 pHPPA

Following the exposure of the isolated hepatocytes to pHPPA, DNA repair was allowed to take place for 60 minutes.

A:



B:

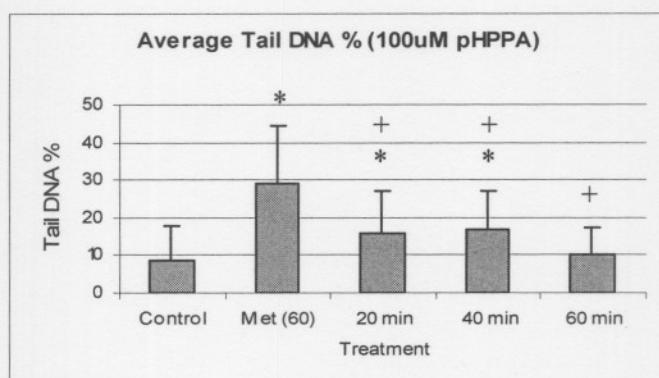
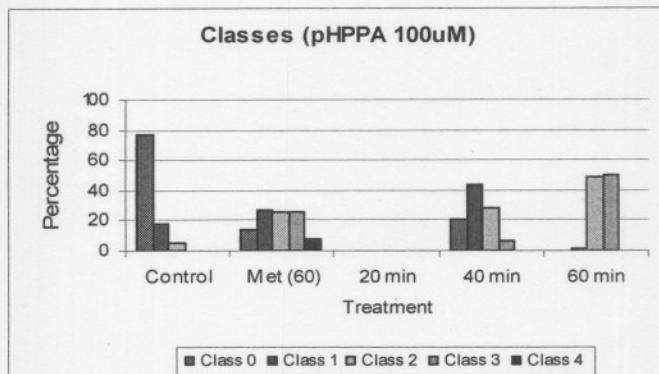


Figure 4.30. DNA damage and repair after exposure of hepatocytes to 100 μ M pHPPA for 60 minutes at 37°C. Hepatocytes were obtained by perfusion (A) and mincing solution (B). *($p<0.05$) versus control; + ($p<0.05$) versus Met(60)

Exposure of the isolated hepatocytes to 100 μ M pHPPA resulted in an increase in DNA damage, when compared to SA and ALA. However, the extent of damage was less in hepatocytes than in lymphocytes. Again the difference in results between the different harvesting methods with relation to DNA repair was observed (figure 4.30). In the case of the perfusion prepared cells no apparent repair of the DNA took place. The cells harvested by mincing solution showed some repair.

A:



B:

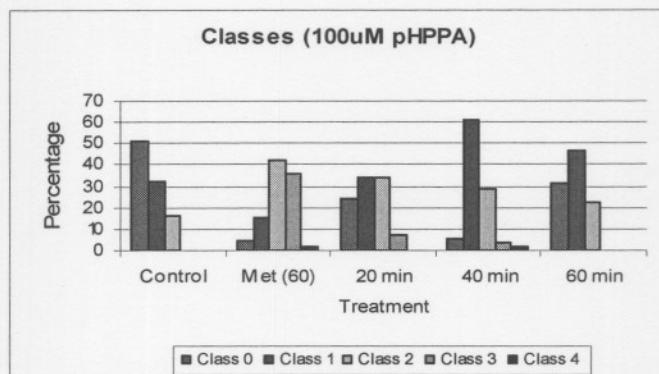


Figure 4.31. Distribution of comets after exposure of hepatocytes to 100 μ M pHPPA for 60 minutes at 37°C. After exposure the DNA repair was measured for 60 minutes. Hepatocytes were harvested by (A) perfusion and (B) mincing solution.

This is supported by the distribution of the comets in different classes (figure 4.31). The increase in DNA damage directly after exposure to pHPPA is due to the fact that more comets were present in classes 2 - 4. In both A and B there was a shift in the distribution of comets from classes 2 - 4 to classes 0 - 1 after 40 minutes repair. The marked difference after 60 minutes repair time was confirmed by this observation that most of the comets in A was in class 2 - 3, whereas in the case of B the pattern resembled that of the control. It is obvious that the two methods of harvesting hepatocytes clearly gave different results.

In summary, that exposure to either SA or ALA caused more DNA damage in hepatocytes than in lymphocytes. In contrast, exposure of hepatocytes to pHPPA caused less DNA damage. The harvesting methods also gave different results with relation to DNA repair capacity following exposure to SA and pHPPA.

On the other hand, exposure to ALA gave similar results for the different methods, i.e. increased damage.

4.3.2 DNA damage and repair after exposure of the hepatocytes to the metabolites and to additional oxidative stress

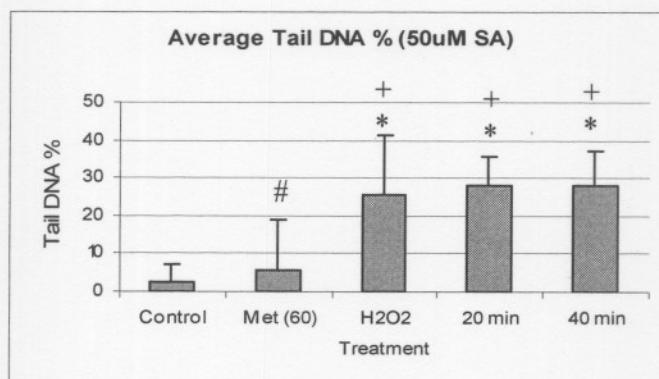
We also determined the effect exposure to the metabolites followed by oxidative stress to the DNA repair capacity of the hepatocytes.

4.3.2.1 Succinylacetone

Hepatocytes harvested by perfusion (A) and with the mincing solution (B) were exposed to 50 μ M SA for 60 minutes at 37°C whereafter the metabolite was removed and the effect of H₂O₂ was studied.

Exposure of the SA-treated cells to H₂O₂ increased DNA damage and increased DNA damage was apparent during the repair time after exposure to H₂O₂ (figure 4.32). There was also a difference in results between the different harvesting methods. DNA damage increased after the additional oxidative stress in hepatocytes harvested by perfusion (A) and decreased in hepatocytes harvested with the mincing solution (B), similar to the results when no H₂O₂ was used (figure 4.26). However,

A:



B:

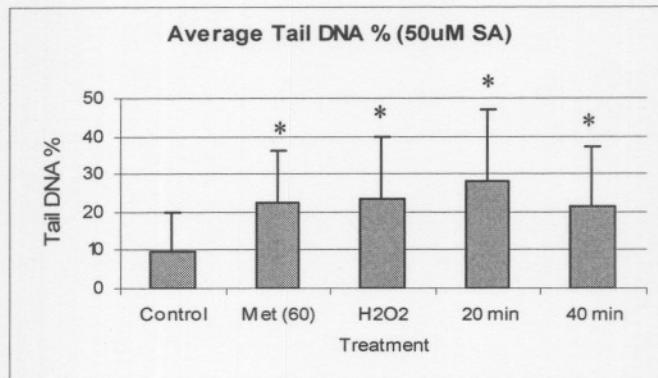
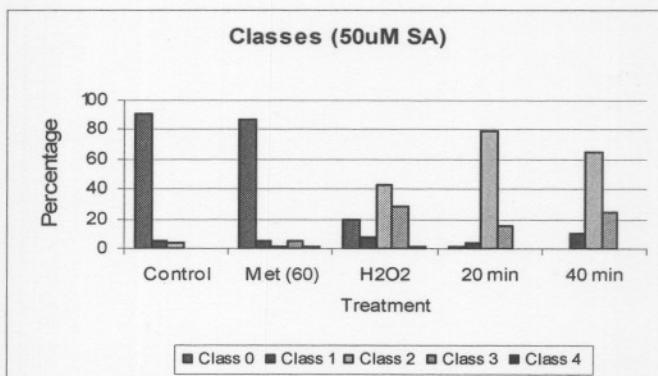


Figure 4.32. DNA damage and repair in SA-treated hepatocytes after exposure to H₂O₂. *($p<0.05$) corresponding to the control; + ($p<0.05$) versus Met(60); #($p<0.05$) versus H₂O₂

oxidative stress apparently had an effect on the DNA repair capacity of the hepatocytes harvested with the mincing solution since the repair capacity was 0.04 as compared to 0.51 (figure 4.26).

The distribution of comets is better indication of the difference in reaction to additional oxidative stress between the two harvesting methods (figure 4.33). Harvesting by perfusion apparently caused a higher degree of DNA damage since more comets were present in classes 2 and 3 over time.

A:



B:

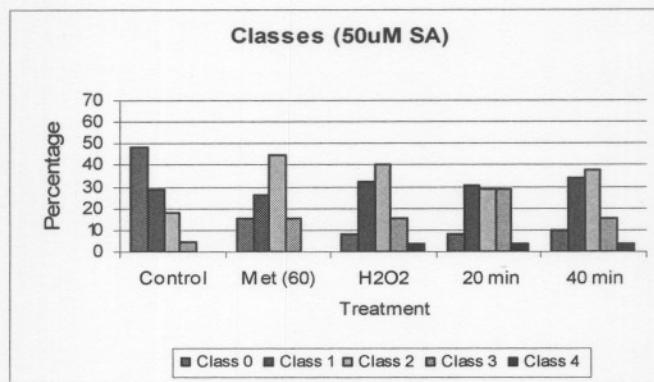


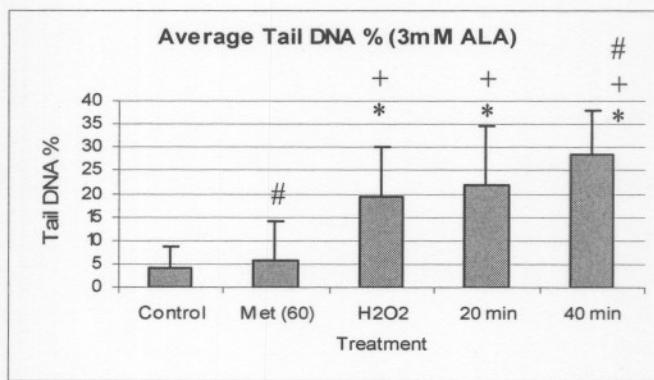
Figure 4.33. Distribution of comets after exposure of SA-treated hepatocytes to H₂O₂.

The effect on hepatocytes harvested with the mincing solution had a smaller effect. Comet distribution shifted from classes 2 and 3 after 20 minutes repair time to classes 1 and 2 after 40 minutes repair time. DNA repair therefore took place. DNA repair in figure 4.33 B was, however, slower than in figure 4.27 B, which indicates that the additional oxidative damage did have an effect on the DNA repair ability of the SA-treated cells.

4.3.2.2 Δ-Aminolevulinic acid

The effect on the DNA repair capacity of ALA-treated hepatocytes was determined after the hepatocytes were exposed to H₂O₂.

A:



B:

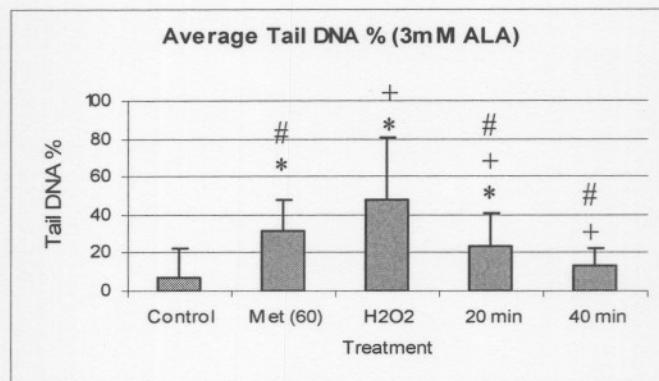
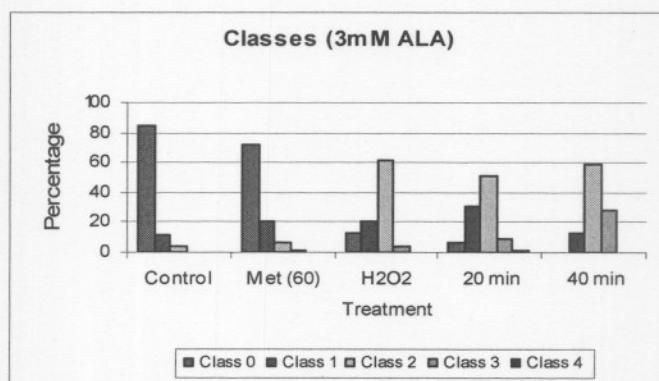


Figure 4.34. DNA damage and repair in ALA-treated hepatocytes after treatment with H₂O₂. Hepatocytes were obtained by perfusion (A) and mincing solution (B). *(p<0.05) versus control; + (p<0.05) versus Met(60); #(p<0.05) versus H₂O₂

ALA caused DNA damage in hepatocytes (figure 4.34). After exposure of the ALA-treated cells to oxidative stress, the percentage of tail DNA increased, indicating increased DNA damage. DNA damage increased markedly in the hepatocytes harvested by perfusion after the oxidative stress was removed. No repair took place in the 40 minutes that was allowed for DNA repair. In contrast repair took place in the cells harvested by the mincing solution (figure 4.34, B). This observation is clearly supported by the results of the distribution of the comets (figure 4.35).

A:



B:

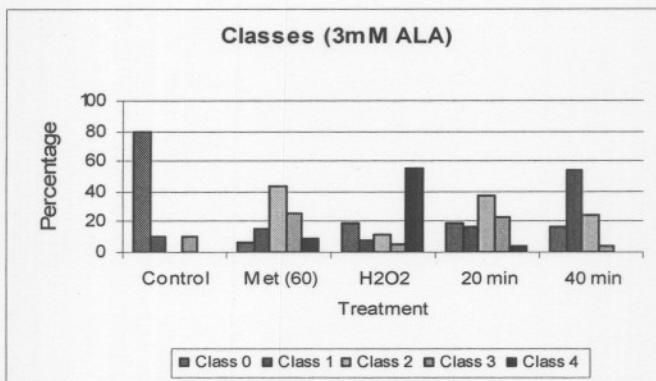


Figure 4.35. The distribution of comets after exposure of hepatocytes to 3mM ALA for 60 minutes at 37°C. DNA repair was measured for 40 minutes in two 20 minute intervals, after cells were placed under oxidative stress.

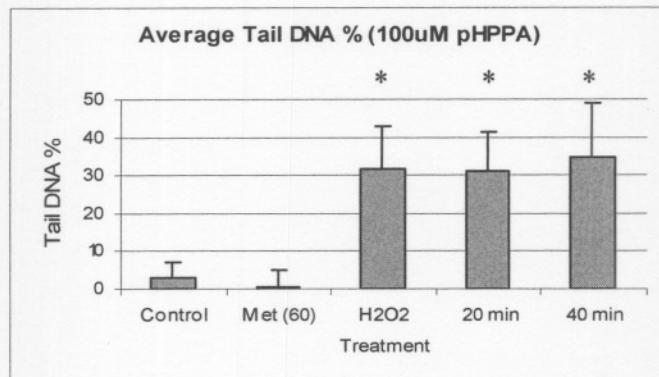
The difference in distribution of the comets (figure 4.35) again indicates the difference in the results obtained in the cells harvested by the different methods. ALA caused more damage to DNA in hepatocytes harvested by the mincing solution, since there were more comets in classes 2 - 4 in B (met60) than in A. DNA repair in the hepatocytes harvested by mincing solution was also improved (figure 4.35).

4.3.2.3 pHPPA

Hepatocytes were exposed to 100µM pHPPA for 60 minutes at 37°C. The effect of oxidative stress on the DNA repair capacity of the hepatocytes were then determined.

pHPPA caused DNA damage in isolated hepatocytes (figure 4.36).

A:



B:

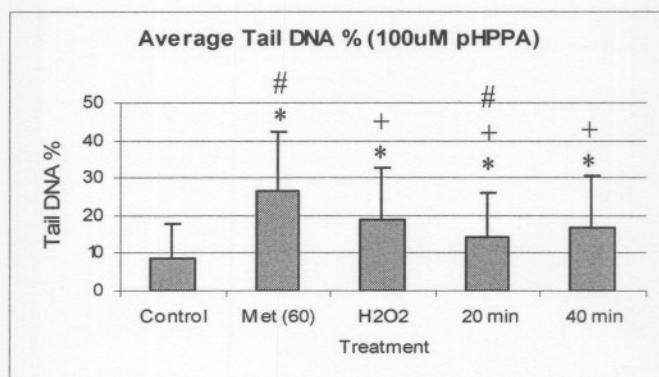
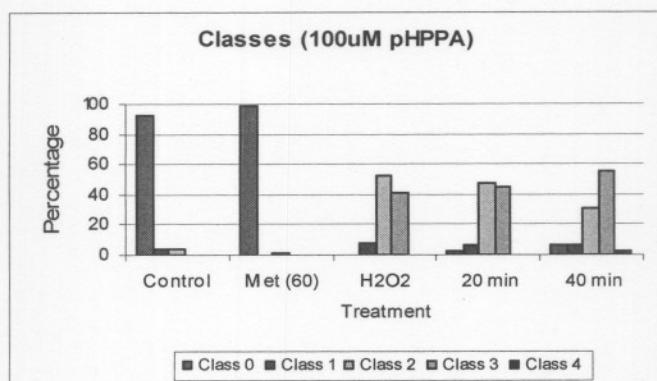


Figure 4.36. DNA damage after exposure of hepatocytes to 100 μ M pHPPA for 60 minutes at 37°C. Hepatocytes were obtained by perfusion (A) and mincing solution (B). *($p<0.05$) versus control; + ($p<0.05$) versus Met(60); #($p<0.05$) versus H₂O₂.

As in previous cases the exposure of hepatocytes to oxidative stress caused DNA damage to increase during the time allowed for repair (figure 4.30, A). Again, repair of the damaged DNA differed between the two methods of harvesting hepatocytes. The cells harvested by collagenase perfusion had impaired DNA repair capacity whereas the hepatocytes harvested with mincing solution had an increased repair capacity of 0.37.

A:



B:

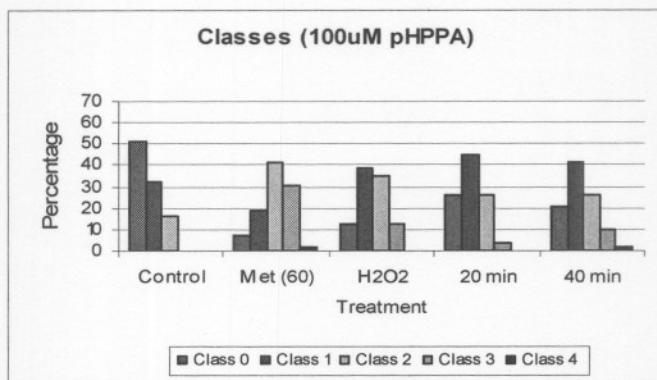


Figure 4.37. Distribution of the comets after exposure of pHPPA-treated hepatocytes H₂O₂ for 20 minutes at 37°C. After exposure the DNA repair was measured for 40 minutes after exposure.

The increased DNA damage following oxidative stress in the hepatocytes harvested by perfusion and the decreased DNA damage in hepatocytes harvested with the mincing solution is clearly visible in the results given in figure 4.37. The increased damage in A is apparent from the increase in the percentage of comets in class 3, which indicates DNA damage of 60%. In B, the number of comets in class 3 decreased and increased in class 0. This shows that DNA repair took place in mincing solution harvested hepatocytes.

In summary exposure of hepatocytes to oxidative stress following exposure to the metabolites (SA; ALA; pHPPA) increased in the extent of DNA damage. The repair capacity of cells following exposure to the metabolites differed with the methods of harvesting. Those cells harvested by mincing solution showed continuous DNA repair in the time allowed. This was not so for hepatocytes harvested by perfusion. The lower repair capacities following exposure to SA

and pHPPA and H₂O₂ indicate that the damage caused by these metabolites were persistent.

The results in both isolated lymphocytes and hepatocytes are summarised in Table 4.1.

Table 4.1. DNA damage and repair to lymphocytes and hepatocytes after exposure to the metabolites alone and together with H₂O₂.

		Damage	CRC	H ₂ O ₂ Damage	CRC
SA	Lymphocytes	+	0.33	+	0.3
	Hepatocytes (P)	++	N.A.	+++	N.A.
	Hepatocytes (MS)	++	0.51	++	0.04
ALA	Lymphocytes	+	0.36	+	0.18
	Hepatocytes (P)	+++	N.A.	++++	N.A.
	Hepatocytes (MS)	+++	N.A.	++++	0.7
pHPPA	Lymphocytes	+++	0.622	+++	N.A.
	Hepatocytes (P)	++	N.A.	+++	N.A.
	Hepatocytes (MS)	++	0.65	++	0.37

ALA = δ-Aminolevulinic acid; CRC = Calculated repair capacity; MS = Mincing Solution; P = Perfusion; pHPPA = p-Hydroxyphenylpyruvic acid; SA = Succinylacetone. ++: Reference point of damage, mild damage; +: minute damage; +++: moderate damage; ++++: severe damage; N.A.: not available

Exposure of the lymphocytes and hepatocytes to the respective metabolites caused DNA damage of various magnitudes (Table 4.1). The amount of DNA damage in the isolated lymphocytes was similar to that of hepatocytes harvested with the mincing solution. Hepatocytes harvested by perfusion had more severe DNA damage. It is also apparent that damage caused by SA was slightly more extensive in hepatocytes than in lymphocytes, following exposure to H₂O₂. DNA damage slightly increased. The relatively low calculated repair capacity values indicate that the DNA damage caused by SA inhibited the ability of cells to repair damaged DNA. The repair capacities also indicated that the damage to lymphocytes was not as persistent as the damage to the isolated hepatocytes. There was almost no difference in the calculated repair capacities in lymphocytes, while the repair capacity in hepatocytes was much lower. Similar to SA, ALA also caused DNA damage and after the addition of H₂O₂ the damage

increased slightly. Again the hepatocytes were more sensitive than the isolated lymphocytes. The calculated repair capacities confirm this. Exposure of the cells to pHPPA also caused DNA damage. It appeared that the lymphocytes were more prone to damage than the hepatocytes. At first it seems as if the damage caused by pHPPA had no effect on DNA repair capability of the cell as it was relatively high in both cell types. Additional use of H₂O₂ apparently caused no additional DNA damage. The marked increase in DNA damage in isolated lymphocytes and the much lower repair capacity of the hepatocytes, however, suggest that specific damage caused by pHPPA may have had an effect on the repair mechanisms of the cell (see 4.2.3.4). The damage to DNA caused by pHPPA thus seems to be more persistent than was suggested by Mitchell *et al*, 2001.

CHAPTER 5

SUMMARY AND CONCLUSION

Hereditary Tyrosinemia type 1 (HT1) is an autosomal recessive disorder (Prieto-Alamo & Laval, 1998) caused by a deficiency of the fumarylacetoacetate hydrolase (FAH) enzyme (Jorquera & Tanguay, 1999), the last enzyme in the tyrosine catabolic pathway. The deficiency is caused by any of 42 mutations on chromosome 15q23-q25. It leads to severe symptoms e.g. a mosaic pattern of liver cirrhosis (Mitchell *et al*, 2001) and ultimately to hepatocellular carcinoma. Under pathological conditions accumulating metabolites such as succinylacetone (SA), fumarylacetoacetate (FAA), maleylacetoacetate (MAA) and to a lesser extent also p-hydroxyphenylpyruvic acid (pHPPA) are responsible for the clinical symptoms and features of HT1 (Mitchell *et al*, 2001). FAA and MAA both act as alkylating agents, but has never been isolated, either as circulating or excreted metabolites (Mitchell *et al*, 2001). SA reacts with amino acids, especially lysine, via Schiff-base formation to form stable adducts. This may consequently inhibit enzymes like DNA-ligase which can lead to mutations and eventually develop into cancer (Prieto-Alamo & Laval, 1998). In addition to inhibition of DNA-ligase, SA also inhibits δ-aminolevulinic acid dehydratase resulting in the accumulation of δ-aminolevulinic acid (Deepali *et al*, 2004).

The overproduction and accumulation of δ-aminolevulinic acid (ALA) is one of the main characteristics of acute intermittent porphyria (AIP) (Baggott *et al*, 1995). AIP is an autosomal dominant disorder of the porphobilinogen deaminase enzyme (Kappas *et al*, 2001) and can be due to any of 237 possible mutations on chromosome 11q23. Its clinical features include severe abdominal pain, constipation and urinary retention (James & Hift, 2000). Since ALA and porphobilinogen is elevated, it has previously been linked to the increased

production of reactive oxygen species, which can subsequently cause single-strand DNA breaks (Douki *et al*, 1998).

Although the genetic deficiencies of HT1 that are responsible for this disorder are well established, the mechanisms through which the hepatic and renal symptoms arise are still unknown (Mitchell *et al*, 2001). The hypothesis was that the early metabolites of tyrosine catabolism such as pHPPA are unlikely to cause any hepatorenal symptoms, since they are also present in disorders without any hepatorenal symptoms. It seemed likely that the final metabolites of tyrosine catabolism, SA, is toxic and may act as alkylating agents (Mitchell *et al*, 2001) that may cause the underlying biochemical and clinical symptoms. In order to better understand these processes, especially those that result in the formation of hepatocarcinoma, it became necessary to determine the effect the metabolites characteristic of HT1 on the integrity of DNA.

Metabolites such as SA and ALA cause DNA damage (Prieto-Alamo & Laval, 1998; Di Mascio *et al*, 2000). It is, however, not clear whether this damage is repairable or if it is persistent. This may lead to mutations and ultimately to cancer. In addition, the nature of the damage to DNA is not clear. The aim of the study was to determine if the DNA damage caused by the metabolites characteristic of HT1 and AIP is reversible or persistent. This was done by using the comet assay to determine levels of DNA damage and repair in isolated lymphocytes and hepatocytes after exposure to SA, ALA and pHPPA. The comet assay was used since it measures double strand breaks (DSB's), single strand breaks (SSB's) alkali labile sites, oxidative DNA base damage, DNA-DNA/protein/DNA-drug cross linking and DNA repair (Singh *et al*, 1988; Dhawan, 2004). In view of this, it is highly suitable to study genetic toxicology (Collins, 2004).

The initial experiments where isolated lymphocytes were exposed to the respective metabolites, repair of the damaged seemed likely although to varying

degrees. The exposure of the isolated lymphocytes to SA inhibited the DNA repair capacity of the cells somewhat. This was evident by the initially slow repair and the relatively low repair capacity. A similar slight inhibitory effect on the repair capacity was seen after exposure to ALA. Exposure of isolated lymphocytes to pHPPA caused DNA damage, but damage was more pronounced, as was repair. Exposure to pHPPA appeared not to inhibit the capacity of cells to repair DNA. The DNA repair capacity was almost double following exposure to the other metabolites.

The results did, however, not indicate if the DNA damage was persistent or not. To get a better indication of the capacity of cells to repair damaged DNA, the lymphocytes were exposed to H₂O₂ after the metabolites were removed. The repair of DNA was then measured. The results showed that this approach gave a better indication of the effect of the metabolites on the capacity of the cells for DNA repair. It appeared that exposure of the lymphocytes to oxidative stress after exposure to SA had no additional effect on DNA repair capacity. This is evident from the almost unchanged repair capacity (see 4.2.2.1). Exposure of the lymphocytes to H₂O₂ following exposure to ALA, inhibited the repair capacity i.e. it was almost half that of the repair capacity after exposure to ALA alone (see 4.2.2.2). In contrast, the addition of H₂O₂ after exposure to pHPPA increased DNA damage markedly (see 4.2.2.3). This was in contrast to the results of Mitchell *et al* (2001) on the involvement of pHPPA. They concluded that pHPPA is not involved in the pathophysiology of HT1. In view of this, we attempted to identify the type of damage caused by pHPPA. This was done by using the lesion specific enzymes Fpg, EndoIII, Mspl and Hpall to add to the pHPPA treated nucleoids.

The results with Fpg and EndoIII (see 4.2.3.2 and 4.2.3.3), strongly suggested that pHPPA caused oxidative damage to both purines and pyrimidines, but with more damage to the purines i.e. there were more DNA damage after treatment with Fpg than after treatment with EndoIII. The damage caused by pHPPA was

difficult to repair since the repair capacity was low. In addition, after H₂O₂ treatment markedly increased DNA damage. This could be due to one of two possible reasons. The DNA damage caused by pHPPA and H₂O₂ may have been too extensive to repair, or, the specific damage caused by pHPPA may have had an effect on the repair mechanisms of the cell. It was possible for DNA lesions to be shielded from repair enzymes by proteins that are not involved in the repair process (Rink *et al*, 1996). pHPPA also acts as a reversible and competitive inhibitor of the transketolase enzyme, which ultimately is essential for the production of nucleic acids (Solovjeva *et al*, 1999). If the same type of inhibition applies to one or more of the DNA repair proteins, the lesions in the DNA following exposure to pHPPA may be shielded from repair. In addition the results of treatment of the nucleoids with Mspl and Hpall, suggested that pHPPA may cause methylation damage to DNA, since the *in vitro* methylation of DNA is possible (Horváthová *et al*, 1998). It remains unclear if pHPPA could cause both oxidative damage and DNA methylation, since DNA containing oxidative lesions is poor acceptors of methyl groups (Wachsman, 1997). The answer is probably the fact that Fpg is also sensitive to alkylation damage (Speit *et al*, 2003). Alkylation damage should not be excluded if the type of DNA damage cannot be ascertained. The increase in the percentage of DNA in the tail of the comets, which was observed after treatment with Fpg, may thus be due to alkylation of the DNA during pHPPA-treatment. This is speculation and should be investigated further.

Our results suggest that exposure of lymphocytes to SA inhibits DNA repair, but that some repair did take place. This also the case for ALA. At first glance, the damage to DNA caused by pHPPA appeared to be completely repairable. However, taking other results into account, the final conclusion was that the damaging effect of pHPPA on DNA was much more persistent.

These results were obtained with lymphocytes. Both the deficiencies for HT1 and AIP occur mainly in the liver (Baggott *et al*, 1995; Mitchell *et al*, 2001). Therefore, we repeated the studies on isolated hepatocytes.

The hepatocytes were harvested by collagenase perfusion and with a mincing solution. SA, ALA and pHPPA caused damage to DNA similar to that in lymphocytes. The damage was more pronounced following exposure to SA and ALA, and less pronounced in hepatocytes exposed to pHPPA. In each of the cases initial DNA repair seemed to take place. However, time of repair differed between different harvesting methods (see 4.3.1.1-3). In hepatocytes harvested with the mincing solution, continuous DNA repair was seen. In the hepatocytes harvested by perfusion there was an increase in DNA damage after 60 minutes of repair time. Similar results were seen after the metabolite-treated hepatocytes were exposed to H₂O₂. In each of these cases, the exposure of the cells to the additional oxidative stress resulted in increased DNA damage. Continuous DNA repair was seen in the hepatocytes harvested with the mincing solution. In the hepatocytes harvested by perfusion an initial period of DNA repair was followed by an increase in DNA damage. The difference in the harvesting methods may hold the key to these contrasting results. The harvesting of the hepatocytes with the mincing solution is a milder way to isolate viable cells, since no enzymes are used. In contrast, collagenase is used with the harvesting of hepatocytes by perfusion. It is likely that the collagenase activity may deplete the energy resources of cells during the incubation period. The lack of sufficient energy in turn, may have caused the delayed DNA damage.

The results therefore suggest the minced isolated hepatocytes were more sensitive to the genotoxic effect(s) of SA and ALA and less sensitive to that of pHPPA. This is supported by the fact that similar results were observed in the lymphocytes. It is also not clear that perfusion were detrimental to the cells, and that the results were not very reliable.

These observations are based on a relative short exposure time of the cells to the metabolites. It is still unclear what will happen to DNA damage and repair with longer exposure of the cells to these metabolites. In a preliminary experiment where hepatocytes prepared with the mincing solution were exposed for 180 minutes to the metabolites the following results were obtained (Table 5.1).

Longer exposure with the respective metabolites markedly increased DNA damage. It appeared that exposure of the isolated hepatocytes to SA for 180 minutes and to pHPPA for 120 minutes caused irreversible DNA damage. All of the comets were present in the heavily damaged class. This may be similar to the *in vivo* findings with HT1 liver cells.

Table 5.1. The extent of DNA damage and the distribution of the comets.

	Control		60 min		120 min		180 min	
	SA	pHPPA	SA	pHPPA	SA	pHPPA	SA	pHPPA
Average Tail DNA	9.5±10.3	8.7±8.9	18.5±6.8	27.5±12.8	22.7±14.9			
Class 0	48.9	51.0	0.0	4.3	0.0	0.0	0.0	0.0
Class 1	28.9	32.7	47.4	4.3	75.0	0.0	0.0	0.0
Class 2	17.8	16.3	52.6	65.2	0.0	0.0	0.0	0.0
Class 3	4.4	0.0	0.0	21.7	25.0	0.0	0.0	0.0
Class 4	0.0	0.0	0.0	4.3	0.0	0.0	0.0	0.0
Heavily Damaged	0.0	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Since it can clearly advance our knowledge on DNA damage and repair we recommend that the exposure time to the metabolites be lengthened. In addition, cells must be exposed to combinations of the metabolites, to more closely resemble the *in vivo* situation where metabolites are simultaneously elevated in patients with HT1. We also observed some extent of methylation of CpG sites in the CCGG sequences of DNA. This supports the proposed mechanism that underlies the pathophysiology of HT1 (Mitchell *et al*, 2001). This does need further clarification.

The aim of this study was to determine the effect of SA, ALA and pHPPA on DNA damage and repair in human cells. We conclude that SA, ALA and pHPPA have genotoxic effects in both isolated lymphocytes and hepatocytes. The DNA damage caused by these metabolites appeared to be persistent. This, in turn can lead to the severe clinical symptoms observed in patients with HT1 and AIP. The study therefore contributed to a better understanding of the pathophysiology of Hereditary Tyrosinemia Type 1. Contrary to previous reports (Mitchell *et al*, 2001), pHPPA may have a definitive role in contributing to the clinical features of HT1. Finally, the alleged DNA damage caused by pHPPA may cause hepatocarcinogenesis, characteristic of HT1.

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APPENDIX A

LYSIS BUFFER

NaCl [5M]: 500 ml
EDTA [0.4M] (pH 7-8): 250 ml
Triton X-100: 10 ml
DMSO [10%]: 100 ml
ddH₂O: 140 ml

ELECTROPHORESIS BUFFER

NaOH [0.6M]: 500 ml
EDTA [0.05M]: 20 ml
ddH₂O: 480 ml

NEUTRALISING BUFFER

TrisHCl 63.04 g in 500 ml ddH₂O
Adjust pH to 7.5 after 1 hour in fridge with [0.6M] NaOH
Fill to 1000 ml

PHOSPHATE BUFFERED SALINE (PBS)

NaCl: 8 g
KCl: 0.2 g
Na₂HPO₄: 1.15 g
KH₂PO₄: 0.2 g
Dissolve in 1000 ml ddH₂O
Adjust pH to 7-8

STAINING BUFFER

Ethidium Bromide: 0.005 g

ddH₂O: 1000 ml

Store in fridge (4°C)

Table A.1. Supplier companies and catalogue numbers of reagents used.

REAGENT	COMPANY	CATALOGUE NUMBER
DMSO (Dimethylsulfoxide)	Sigma-Aldrich	D 5879
EndolIII	New England Biolabs	M0268S
EDTA	Sigma-Aldrich	E 5134
Ethidium Bromide	Sigma-Aldrich	E 8751
Fpg	New England Biolabs	M0240S
H ₂ O ₂	Sigma-Aldrich	H 1009
Hams F10	Addcock Ingram	12-618F
Histopaque®	Sigma-Aldrich	10771
HMPA	Techcomp Ltd.	9201
Hpall	New England Biolabs	R0171S
KH ₂ PO ₄	Merck	1.05108.0500
LMPA	Roche	1441345
Mspl	New England Biolabs	R0106S
Na ₂ HPO ₄	Merck	1.06566.0500
Potassiumchloride (KCl)	Sigma-Aldrich	P 9333
Sodiumchloride (NaCl)	Sigma-Aldrich	S 9625
Sodiumhydroxide (NaOH)	Sigma-Aldrich	S 5881
TrisHCl	Sigma-Aldrich	T 5941
Triton X-100	Sigma-Aldrich	T 8787
Trypan blue	Sigma-Aldrich	93595

ENZYME BUFFER FOR FPG AND ENDOIII

HEPES [40mM]: 2.08 g

KCl [0.1M]: 1.49 g

EDTA [0.5mM]: 0.04 g

BSA [0.2mg/ml]: 40 mg

ddH₂O: 200 ml

ENZYME BUFFER FOR MSPI AND HPAII

Obtained from New England Biolabs.

WILLIAM'S MEDIUM E (WE)

10.76 g Williams's Medium E in 1 L ddH₂O

Add 2.2 g NaHCO₃

Adjust pH to 7.2 with HCl

- (Make 2 bottles, one for preparation of other reagents and one for perfusion)

L-GLUTHAMINE 0.2M = 1.46 g in 50 ml WE

HEPES 13 g in 50 ml WE

EGTA 0.95 g in 50 ml WE

L-PROLINE 0.2M = 1.15 g in 50 ml WE

WE FOR PERFUSION

Total volume: ± 250 ml

250 ml WE

2.5 ml HEPES

Keep on ice: 140 – 150 mg Collagenase*

118 mg CaCl₂·H₂O*

* Weigh out separately and add just before perfusion

WE + 10 % FETAL BOVINE SERUM (FBS)

Total volume: **300 ml**
 1 g FBS
 3 ml HEPES
 3 ml Gluthamine
 0.3 ml Insulin*
 Fill up to 300 ml with WE

* Dilute 5X: **0.2 ml Insulin + 0.8 ml WE beforehand**

HANK'S BALANCED SALTS

9.52 g Hank's Balanced Salts (HBSS) in 1 L ddH₂O
Add 0.35 g NaHCO₃
Adjust pH to 7.2 with NaOH

HANK'S WASH

Total volume:	250 ml	500 ml
	25 ml HBSS	50 ml HBSS
	2.5 ml HEPES	5 ml HEPES
	222.5 ml ddH ₂ O	445 ml ddH ₂ O

HANK'S PERfusion

Total volume:	250 ml	500 ml
	25 ml HBSS	50 ml HBSS
	2.5 ml HEPES	5 ml HEPES
	2.5 ml EGTA	5 ml EGTA
	220 ml ddH ₂ O	440 ml ddH ₂ O

Table A.2. Supplier companies and catalogue numbers of reagents used.

REAGENT	COMPANY	CATALOGUE NUMBER
CaCl ₂ ·2H ₂ O (Calcium Chloride)	Merck	1524920
Collagen	Sigma-Aldrich	C4387
Collagenase	Sigma-Aldrich	C0130
EGTA	Sigma-Aldrich	E4378
EtOH (70%)	Merck	1000658
FBS (Fetal bovine serum)	Delta Bioproducts	14-501AI
HBSS (Hank's Balanced Salts)	Sigma-Aldrich	H2387
HCl	F.C. Scientific	
HEPES	Sigma	H0763
Insulin (Humulin)	Lily	HI0410
L-Glutamine	Sigma-Aldrich	G8540
L-Proline	Merck	7434
NaHCO ₃	F.C. Scientific	
NaOH	Saarchem	5823200
Pyruvate (Na)	Sigma-Aldrich	P2256
Williams's Medium E	Sigma-Aldrich	W4125