DIVERSITY IN THE APPLICATIONS OF THE SINGLE CELL GEL ELECTROPHORESIS (COMET) ASSAY

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TABLE OF CONTENTS

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER FOUR

RESULTS AND DISCUSSION

CHAPTER FIVE

CHAPTER SIX

CONCLUSIONS

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OPSOMMING

Die ontwikkeling van die enkelsel gel-elektroforese tegniek (Komeetanalise) is a kragtige metode vir die meting van DNS-skade en -herstel en het gelei tot 'n groot deurbraak vir die bepaling van die impak wat interne en eksterne faktore het op deoksiribonukleïenesuur (DNS) skade. In hierdie verhandeling word die vestiging en 'n verskeidenheid toepassings van die Komeetanalise in ons laboratorium beskryf. In hierdie toepassings is ingesluit die monitering van die effek van oefening op DNA-beskadiging en -herstel ten einde die optimale oefenprogram vir middeljarige mans vas te stel; tydens die bestudering van DNA-herstel in die geval van 'n borskanker pasiënt, het ons aangetoon dat ten einde die basislyn DNA-skade te kon aantoon, dit nodig was om 'n spesifieke ingreep op die DNA toe te pas; die anti-oksidant aktiwiteit van 'n plantekstrak is ook met behulp van die Komeetanalise bestudeer; 'n industriële toepassing van die Komeetanalise was die monitering van DNA-skade en -herstel in die limfosiete van werkers wat aan chemikalieë blootgestel is. Die Komeetanalise is ook meer informatief gemaak deur die insluiting van die vertering van DNA op die mikroskoopplaatjies met die teikenspesifieke ensieme *Fpg* (formamidopirimidien DNA-glikosilase) en *Endo* III (endonuklease III). Vanuit hierdie toepassings kan ons met sekerheid stel dat die Komeetanalise herhaalbaar in sy diversiteit in ons laboratorium gevestig is.

Die mees betekenisvolle bydrae van hierdie verhandeling is die modifikasie van die Komeetanalise om die metileringsvlakke van DNA vas te stel. Hierin is die diversitieit in die sensitiwiteit teenoor die metileringstatus van hul gemeenskaplike teikengebied, van die twee endonukleases *Hpa II* en *Msp I* benut. Veranderinge in die metileringsvlakke van DNA wat met spesifieke chemikalieë aangebring is, is oortuigend aangetoon. In hierdie toepassing is ook aanduidings gekry van selspesifisiteit ten opsigte van DNA-metilering. Ons is oortuig dat hierdie modifikasie van die Komeetanalise heel uniek is en dat 'n nuwe veld van toepassings geopen is.

ABSTRACT

The development of the single cell gel electrophoresis assay (Comet assay) as a powerful method for measuring DNA strand breakage and repair, has lead to a broader understanding of the impact of certain internal and external factors on DNA damage. This study describes the establishment of the Comet assay in our laboratory and its application in a diversity of studies. These studies include the monitoring of the effect of exercise on DNA damage and repair with the purpose of establishing the optimal exercise program for middle aged men; in the analysis of DNA repair in a breast cancer patient after chemotherapy, we demonstrated that to get a really informative picture of baseline DNA damage, it is necessary to invoke some type of insult to the DNA; we also studied the antioxidant activity of plant extract and finally an industrial application of the Comet assay where we studied DNA damage and repair in the lymphocytes of people exposed to a variety of chemicals. The Comet assay was made even more informative by incorporating additional steps by digesting the DNA on the microscope slides with enzymes that recognize particular kinds of damage to the nucleic acid (formamidopyrimidine DNA glycosylase (*Fpg*) and Endonuclease III (*Endo* III). From these applications we can safely conclude that we have established the Comet assay as reproducible assay with proven diversity in its applications.

The most significant contribution of this study is the quite novel modification of the Comet assay to monitor the methylation status of DNA. In this we applied the restriction endonuclease enzymes, *Hpa* II and *Msp* I, which have different sensitivities to methylation of their common target site. Changes in the methylation levels of DNA after treatment of animals and isolated cells with chemicals with known effects on DNA, were convincingly demonstrated and indications of cell specificity in DNA methylation were observed. We believe that this modification opens a new field of applications for the Comet assay.

LIST OF ABBREVIATIONS

A

A	Adenine
AAF	2-Aminofluorene or *N*-acetyl-2-aminofluorene
ADP	Adenosine diphosphate
ALS	Alkyline lable sites
ANOVA	Analysis of variance
AP	Altered purines
APE1	Apurinic/apyrimidinic endonuclease
Artemis	Mammalian gene name
ATLD	Ataxia telangiectasia-like disorder
ATM	Ataxia-telangiectasia-mutated Protein
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase

B

BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	Base excision repair
BSA	Bovine serum albumin

C

C	Cytosine
Ca^{++}	calcium ion
CASP	Comet assay software program
Cisplatin	cis-Diammineplatimum (ii) dicholoride
CPD	cyclobutane pyrimidine dimmers
CpG	Sequences of DNA where cytosine lies next to guanine
CSA	Encodes a protein with multiple WD-40 repeats)
CSB	Encodes a DNA-dependent ATPase of the SNF2 family

D

5'dRP	5'-deoxyribose-5-phosphate
DDB 1	*Homo sapiens* gene (1127kDa): damage-specific DNA binding protein 1
DDB 2	*Homo sapiens* gene (248kDa): damage-specific DNA binding protein 2
ddH_2O	double distilled water
dG	
DMSO	dimethylsulfoxide
DNA	Deoxyribonuclecleic acid
DNS	Deoksiribonukle
DNS	Deoksiribonukleïensuur
DSBs	Double strand breaks

E

EDTA	ethylenediaminetetra-acetic acid
EMS	Ethylmethanesulfonate
Endo III	Endonuclease III
ERCC1	Exchange repair cross complementing
Exol	5'-3' Exonuclease that interacts with MutS and MutL homologs and has been implicated in the excision step of DNA mismatch repair
e.g.	exempli gratia (for example)

F

FEN1	Flap endonuclease
Fpg	formamidopyrimidine DNA glycosylase

G

g	9.8 meter/second^2
g	gram

G	Guanine
G_0	Resting phase
G1 phase	Growth and preparation of the chromosomes for replication
G2 phase	Preparation for mitosis
GGR	Global genomic repair

H

H_2O	water
H_2O_2	hydrogen peroxide
HBSS	Hanks balance salt solution
HCl	hydrochloric acid
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMPA	high melting point agarose
HNPCC	Hereditary colon cancer syndrome
Hpa II	Restriction enzyme isolated from *Haemophilus parainfluenzae* II
HR	Homologous recombination
HR23B	Human protein cooperates with XPC contains ubiquitin domain; interacts with proteasome and XPC
HRR	Homologous recombination repair

K

KCl	potassium chloride
Kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
km	kilometer
Ku 70	thyroid autoantigen 70kD
Ku 80	thyroid autoantigen 80kD

L

LMPA	low melting point agarose

LIG	ligase

M

M	Molar
mA	milli Ampere
mg	milligram
Mg^{++}	magnesium ion
$MgCl_2$	magnesium chloride
ml	milliliter
MLH1	A gene that plays a role in squamous cell carcinoma of larynx development
mM	milli Molar
MMC	Mitomycin C
MMR	Mismatch repair
MNNG	N-Methyl-N -nitro-N-nitrosoguanidine
MNU	1-Methyl-1-nitrosourea
MPG	N-Methylpurine-DNA glycosylase
MRE II	Double strand break repair protein
Msp I	Restriction enzymes isolated from *Moraxella sp.*
Mut Lα	A protein in the methyl-directed mismatch repair pathway, which functions to ensure the fidelity of the daughter DNA strand during replication
Mut Sα	A protein in the methyl-directed mismatch repair pathway, which functions to ensure the fidelity of the daughter DNA strand during replication

N

Na_2HPO_4	di-sodium hydrogen orthophosphate anhydrous
NaCl	sodium chloride
NaOH	sodium hydroxide
NBS I	DNA repair protein essential for homologous recombination repair
NER	Nucleotide excision repair

NH_3	ammonia
NHEJ	Non-homologous enjoining
nM	nano Molar

O

O	oxygen
3' OH	3'-hydroxyl
8 - oxoG	8-oxoguanine
OGG1	Oxoguanine glycosylase

P

P	phosphate
PARP	1 – poly (ADP-ribose) olymerase-1
PBS	phosphate buffer solution
PCNA	Proliferating cell nuclear antigen
pH	The pH of a solution is defined as the negative log of the hydrogen ion concentration
PhIP	2-Amino-1-methyl-6-phenyl-imidazo [4,5-b] pyridine
PKcs	Protein kinase
PMS2	Postmeiotic segregation increased 2 (S. cerevisiae)
Pol ε	DNA polymerase ε
Pol δ	DNA polymerase δ
Pol β	DNA polymerase β
PPS	Photoproducts

R

r	Correlation
Rad 50	A protein which is essential for the repair of damaged DNA during homologous repair
Rad 51	A protein which is essential for the repair of damaged DNA during

	homologous repair
Rad 52	A protein which is essential for the repair of damaged DNA during homologous repair
Rc	Repair capacity
RF-c	Replication factor C
RNA PII	RNA polymerase II
RPA	Replication protein A
ROS	Reactive oxygen species

S

S phase	Synthesis of DNA and centrosomes
SCGE	Single cell gel electrophoresis
SD	Standard deviation
SSBs	Single strand breaks
Sss I	Restriction enzyme isolated from *Spiroplasma sp.*

T

T	Thiamine
TCR	Transcription-coupled repair
TFIH	Transcription factor active in nucleotide repair
TFIIS	Transcription factor active in nucleotide repair
Tris	2-Amino-2-(hyroxylmethyl)-1.3-propandiol
TTD	Trichothiodystrophy

U

UV	Ultraviolet

V

V	Volt
V(D)J	Variable diversity joining

W

WBS	White blood cells

X

XP	Xeroderma pigmentosum
XPA	Human protein that binds and stabilizes open complex; checks for damage
XPB	Human protein: 3' to 5' helicase
XPC	Human protein that works with HR23B; binds damaged DNA; recruits other NER proteins
XPD	Human protein: 5' to 3' helicase
XPF	Human protein which is part of endonuclease (5' incision)
XPG	Human protein: Endonuclease (3' incision); stabilizes full open complex
XRCC	X-ray-repair-cross-complementing

SYMBOLS

°C	Degrees Celsius
%	Percentage
ε	Epsilon
δ	Delta
β	Beta
→	converted to
α	Alpha
µl	micro liter

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

The primary structure of deoxyribonucleic acid (DNA) is dynamic and subject to constant, although subtle, changes. Errors are induced spontaneously, especially during DNA replication and recombination (Lindahl, 1993) and these changes include mismatches (Modrich, 1997), deamination and loss of bases (Lindahl 1979; Loeb and Preston 1986; Lindahl 1993), incorporation of uracil into DNA and oxidative damage to DNA (Lindahl and Nyberg, 1974; Ames, 1989). Environmental agents also induce various kinds of DNA damage. These can be classified as physical, such as radiation and chemical agents (Singer and Kusmierek, 1982). Regardless of the cause of DNA damage, its repair is vital for safeguarding the genetic information and ultimately the survival of an organism (Lindahl, 1982). Unrepaired or incorrectly repaired lesions may lead to mutagenesis and eventually carcinogenesis (Lindahl, 1993). Accurate DNA repair is important and failure of DNA repair may lead to disruption of normal homeostasis and malignant diseases. (Sawyers *et al.*, 1991; Hoffbrand and Pettit, 1995).

Different techniques for detecting DNA damage, as opposed to the biological effects (e.g. mutations and structural chromosomal aberrations) that result from DNA damage, have been used to identify substances with genotoxic activity. Some years ago the alkaline single-cell gel electrophoresis (SCGE) assay was introduced as a novel approach for detecting DNA lesions. The development of this assay was done by Östling and Johanson (1984). The method involves the application of an electrical current to cells that results in a length dependent movement of DNA fragments. Since the DNA damage induced by toxic agents is often tissue and cell-specific, SCGE is very useful because it can detect DNA lesions in individual cells obtained under a variety of experimental conditions. This assay has a variety of applications, for instance testing chemicals for genotoxicity, monitoring environmental

contamination with genotoxins, human biomonitoring and fundamental research in DNA damage and repair (Collins, 2004).

The sensitivity and specificity of this method is of great importance in the application of the comet assay and to exploit the special features of this assay a number of analyses were performed on various groups of volunteers (e.g. a cancer patient, occupational workers, antioxidant treatment, and effect of exercise on DNA).

Although the comet assay is essentially a method for measuring DNA single and double strand breaks, the introduction of lesion-specific endunucleases allows the detection of, for example, oxidized bases and alkylation damage. To attain the objective of this study, cell suspensions from various tissues were treated with different substances to evaluate the extent of DNA damage, by inducing DNA damage with hydrogen peroxide (H_2O_2). DNA repair enzyme treatment was used to recognize particular kinds of lesions and to create breaks in the DNA. Rats were dosed with Paracetamol to evaluate *in vivo* DNA damage in different tissues. Cells from each of the tissues were incubated with different toxins, each toxin caused a specific type of DNA damage, for example alkylation (Ethyl-methane sulfonate (EMS)), DNA cross-linking agent (Mitomycin C (MMC)) and oxidation (Paracetamol). A comparison was made between the different tissues as to their response to the DNA damage.

2. STRUCTURE OF THIS DISSERTATION

Following this introduction, chapter two provides background information of the importance to maintain genetic stability and the origin of DNA damage. A short overview of the importance and functions of the different DNA repair mechanisms are given followed by the consequences of diseases caused by defective DNA repair along with the prevention and treatment of DNA damage. The focus of this dissertation was to monitor the DNA damage with the Comet assay. Furthermore, the diversity in applications of the comet assay, were broaden by incorporating

lesion-specific enzymes to measure the DNA methylation status. Lastly, the aims of, and approach to this study are given.

Chapter three consists of the materials and methods used in this study and in chapter four the results and discussion. A manuscript for publication is included as chapter five. The conclusions regarding this study are given in chapter six.

CHAPTER TWO
LITERATURE REVIEW

2.1 THE ESSENCE OF GENOMIC STABILITY

An increased tendency of the genome to acquire mutations, when various processes involved in maintaining and replicating the genome are dysfunctional, is known as genomic instability. Because there are many factors that contribute to genomic instability and since genomic stability is essential for normal cellular function, highly specific pathways have evolved to repair DNA damage and prevent genomic instability. It is important to understand the nature and causes of genomic instability, the mechanisms of DNA repair and the consequences of failure to repair damaged DNA. A dramatic increase in the level of genomic instability can occur during normal cell development and aging, genomic instability is also associated with genetic diseases and cancer (Früwald and Plass, 2002).

2.1.1 ORIGIN OF DNA DAMAGE

The origin of DNA damage falls into three broad categories. The first is the *natural endogenous cellular processes* which are involved in the overall metabolism of a organism. In addition to energy, these processes produce toxic by-products called reactive oxygen species (ROS). They belong to the different classes of free radicals, which can damage DNA as well as cellular proteins and lipids (Holmes *et al.*, 1992). Next are *external causes*, for example ultraviolet (UV) light which have been recognised as a cause of DNA damage for many years (Nocentini, 1976). Toxins like benzo[*a*] pyrene (Leffler *et al.*, 1977), medications like those used in chemotherapy (de Boer and Hoeijmakers, 2000) and cigarette smoke, all leads to DNA damage. Spontaneous and inherited *gene mutations* are the third category of DNA damage. The building blocks of genes are the nucleotides; it is arranged in a specific order in each gene. If, in the course of cell reproduction, one nucleotide is substituted for another, accidentally deleted or an extra nucleotide is added, it is called a spontaneous single base mutation (Maki, 2002). If a mutation such as this one

occurs in a germ cell (egg or sperm), the mutation can be inherited by the next generation (Boerritger *et al.*, 1992).

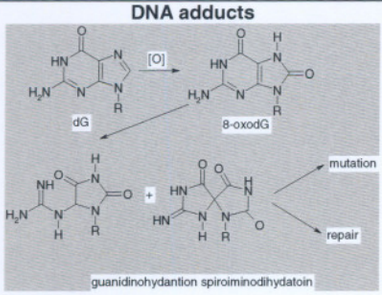
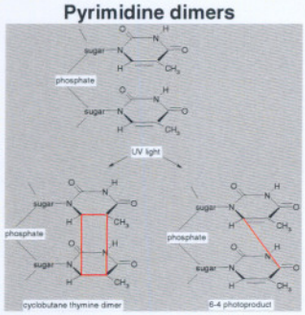
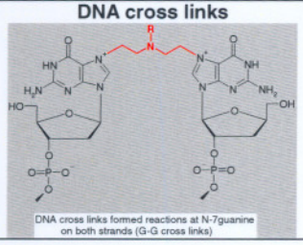
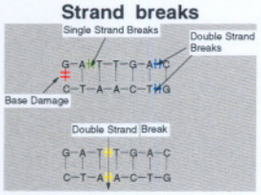
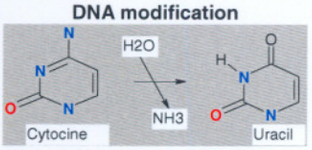
2.1.2 DIFFERENT TYPES OF DNA DAMAGE

The ability to recognize and respond to DNA damage is essential for the survival of a cell: DNA damage causes cell death, tissue degeneration, ageing and cancer (Zhou and Elledge, 2000). Actions of many agents, including UV-light, ionizing radiation, products of oxidative metabolism chemicals, can cause the DNA to sustain different types of damage: base modification, DNA adducts intra- /or inter-strand cross links and double strand breaks. Double-strand breaks (DSBs) are one of the most dangerous types of DNA damage along with inter-strand cross-links, in comparison with the other types of DNA damage. This is caused by ionizing radiation or certain chemicals such as bleomycin, and occur normally during the process of DNA replication, meiotic exchange, and V(D)J (V variable, D diversity, J joining) recombination (Lees-Miller *et al.*, 2004).

Specific chemical agents are known to alkylate or cross-link DNA bases, which can produce bulky adducts on DNA bases or break the DNA phosphate-sugar backbones (Roth and Gellert, 2000). Table 2.1 summarises the different types of DNA damages and their effects on the DNA along with a short discussion of each type of DNA damage.

DNA has a special need for metabolic stability to maintain homeostasis. If metabolic stability is not maintained, it may lead to various types of DNA damages as mentioned above. The chemical stability of DNA is maintained in two ways: by a replication process of very high accuracy that prevents most errors from occurring in the first place along with the mechanisms for repairing genetic information when DNA suffers damage (Mathews and Van Holde, 1996).

Table 2.1 Examples of DNA damage

DNA adducts  	DNA adducts are covalent adducts between chemical mutagens compounds and the DNA. Such couplings activate DNA repair processes and, unless repaired prior to DNA replication, may lead to nucleotide substitutions, deletions, and chromosome rearrangements. It is also implicated in many types of human cancesr especially where persistent oxidative stress leads to malignancy by increasing mutations and genomic instability on DNA level (Scicchitano *et al.*, 2003).
Pyrimidine dimers  	Dimers are found in the DNA chains damaged by UV irradiation. They consist of two adjacent pyrimidine nucleotides, usually thymine nucleotides, in which the pyrimidine residues are covalently joined by a cyclobutane ring. These dimers stop the DNA replication process (Noah *et. al.*, 2000).
DNA cross links  	DNA cross-links are covalent linkages that can be formed between bases on the same DNA strand ("intra-strand") or on the opposite strand ("inter-strand"). Several chemotherapeutic drugs are used against these cancer cross link DNA (Dronkert and Kanaar, 2001).
Strand breaks  	The critical target of radiation is DNA. Ionizing radiation can cause deletions, substitutions, or actual breaks in the DNA chains. DNA strand breaks, if not repaired correctly, cause abnormalities in chromosomes that may result in the cells inability to reproduce (Olive, 1998).
DNA modification  	DNA modification is the enzymatic modification of the chemical structure of specific sites in DNA. A variety of chemical changes are made to a DNA molecules just after it has been replicated e.g. DNA methylation (Wilson and Jones, 1983).

O: Oxygen; **dG**: Guanine ; **8-oxodG:** 8-oxoguanine; **UV:** Ultraviolet; **DNA:** deoxyribonucleic acid; **H_2O:** water; **NH_3:** ammonia

2.1.3 FUNCTION AND IMPORTANCE OF DNA REPAIR MECHANISMS

Cells must have the ability to repair errors in their DNA to be able to maintain genetic stability and ultimately for survival. If the DNA of dividing cells are damaged, the DNA can not be properly copied, and the cells can not divide. Instead, they can age and then die (Ozawa, 1997). In both dividing and non-dividing cells, "healthy" intact DNA is vital to its everyday functioning. The code in DNA is read by special enzymes and 'translated' into the proteins that carry out cellular and other bodily processes. Consequently, even small DNA errors can have serious intra- as well extra cellular effects. A single unrecognised and uncorrected DNA error can disable a critically needed protein, and over time, result in disease or even death. DNA repair processes act by locating the damage in the DNA and correcting it before too much of the damage is reproduced and accumulated. Some researchers hold the opinion that without DNA repair processes human cells would sustain enough damage to become useless within one year (Ozawa, 1997).

2.1.4 DIFFERENT TYPES OF DNA REPAIR MECHANISMS

The genome of living cells experience a variety of damages that threatens their genomic integrity. Cells utilise several distinct, though overlapping to a certain extend, DNA repair pathways to cope with the various kinds of structural DNA damage and replication errors (Mohrenweiser *et al.*, 2003). The significance of DNA repair is evident by the highly conserved nature of these repair pathways in evolution, and defects in any one of these major DNA repair pathways are often interconnected with other cellular processes such as cell cycle control, DNA replication, transcription, apoptosis and even immunological responses. The four major DNA repair pathways operating in eukaryotes are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), recombinational repair including homologous recombination (HR) and non-homologous end-joining (NHEJ), repair (Krokan *et al.*, 2004).

2.1.4.1 BASE EXCISION REPAIR (BER)

DNA is prone to spontaneous hydrolytic decomposition in the cell (Lindahl, 1993). In addition, ROS formed endogenously during normal cellular respiration and alkylating agents that are present naturally *in vivo*, can react with DNA to form various mutated DNA products. BER has evolved to handle these mutagenic and cytotoxic hydrolytic, oxidative and alkylation damages, which can lead to DNA lesions (Scharer and Jiricny, 2001).

The initial step in BER, the mechanism of which is shown in Fig. 2.1, is carried out by specific DNA glycosylases, which recognises and removes damaged or incorrect (e.g. uracil) bases by hydrolysing the *N*-glycosidic bond (for review see Scharer and Jiricny, 2001). During short-patch BER, 5'dRP is displaced by DNA polymerase β (Polβ), which inserts a single nucleotide (Wiebauer and Jiricny, 1990; Sobol *et al.*, 1996). Polβ is also involved in long-patch BER (Klugland and Lindahl, 1997; Dianov *et al.*, 2001), inserting the first nucleotide at reduced AP sites (Podlutsky *et al.*, 2001).

The critical step in the decision between short- and long-patch BER is the removal of 5'dRP upon the insertion of the first nucleotide. Upon dissociation of Polβ from damaged DNA, further processing occurs by proliferating cell nuclear antigen (PCNA) -dependent long-patch repair (Frosina *et al.*, 1996 and Matsumoto *et al.*, 1999). For example, the removal of 8-oxoguanine occurs mainly via short-patch BER; only 25% of lesions are repaired via the long-patch repair pathway (Dianov *et al.*, 1999). In contrast to short-patch repair, in which single base insertion by Polβ the DNA backbone is directly sealed, several additional steps occur during long-patch repair. After dissociation of Polβ and strand displacement, further DNA synthesis is accomplished by Polε or Polδ together with PCNA and Replication factor C (RF-C) (Stucki *et al.*, 1998), resulting in longer repair patches of up to 10 nucleotides. The ligation step is preformed by DNA ligases I and III (for review see Tomkinson *et al.*, 2001).

Ligase I interact with PCNA and Polβ participate mainly in long-patch BER (Prasad *et al.*, 1996, Srivastava *et al.*, 1998). DNA ligase III interacts with X-ray-repair-cross-complementing (XRCC1), Polβ PARP-1 [poly(ADP-ribose)olymerase-1] and is involved only in short-patch BER (Kubota *et al.*, 1996).

2.4.1.2 NUCLEOTIDE EXCISION REPAIR (NER)

Bulky DNA adducts, such as UV-light-induced photo-lesions [(6-4) photoproducts (6-4PPS)] and cyclobutane pyrimidine dimmers (CPD's)], intra-strand cross-links and large chemical adducts are generated from exposure to aflatoxine, benzo[*a*]pyrene and other genotoxic agents which can be repaired by NER (for review see Friedberg, 2001; Hanawalt, 2001; Mullenders and Berneburg, 2001). NER consists of two sub pathways: global genome repair (GGR) and transcription-coupled repair (TCR) (Fig. 2.2) During GGR, recognition of the DNA lesions occurs by XPC-HR23B, RPA-XPA or DDB1-DDB2. DNA unwinding is performed by the transcription factor TFIH, and excision of these lesions by XPF and dXPF-ERCC1. Finally, re-synthesis occurs by Polδ or Polε and ligation by DNA ligase I. During TCR the induction of these lesions results in blockage of RNA polymerase II (RNAPII). This leads to assembly of CSA, CSB or TFIIS at the site of the lesion, by which RNAPII is removed from the DNA or displaced from the lesion, making it accessible for the exonucleases XPF-ERCC1 and XPG to cleave the lesions in the DNA strand. Re-synthesis again occurs by Polδ or Polε and ligation by DNA ligase I (Christmann *et al.*, 2003).

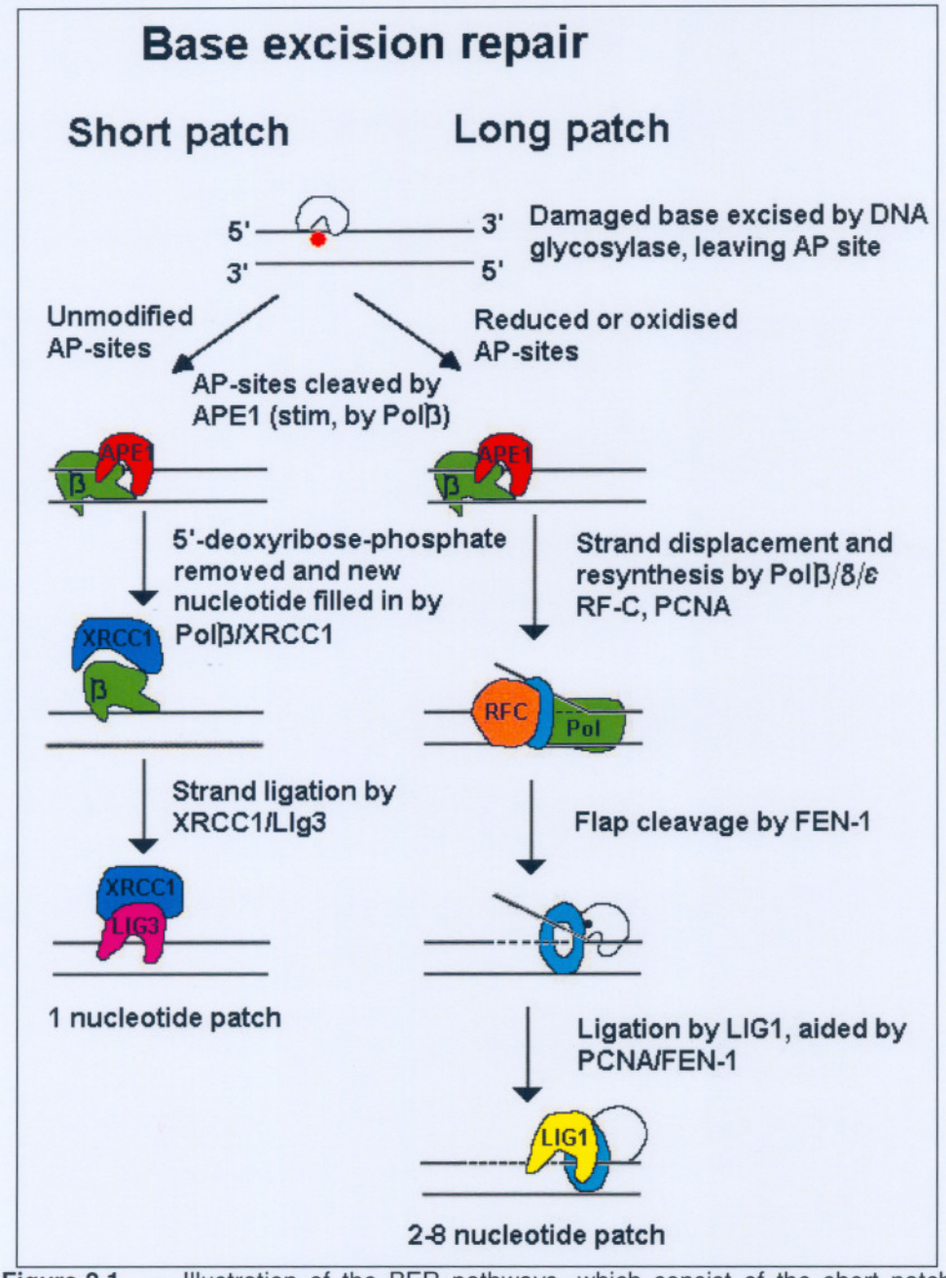


Figure 2.1 Illustration of the BER pathways, which consist of the short patch and long patch repair pathway. Only the most significant factors are shown (from Krokan *et al.*, 2004).

A: Adenine; **AP:** Altered purines; **APE1:** Apurinic/apyrimidinic endonuclease1; **BER:** Base excision repair; **FEN1:** Flap endonuclease1; **LIG3:** Ligase3; **PCNA:** Proliferating cell nuclear antigen; **Pol:** DNA polymerase; **RFc:** Replication factor C; **XRCC1:** X-ray-repair-cross-complementing; **β:** Beta; **δ:** Delta; **ε:** Epsilon

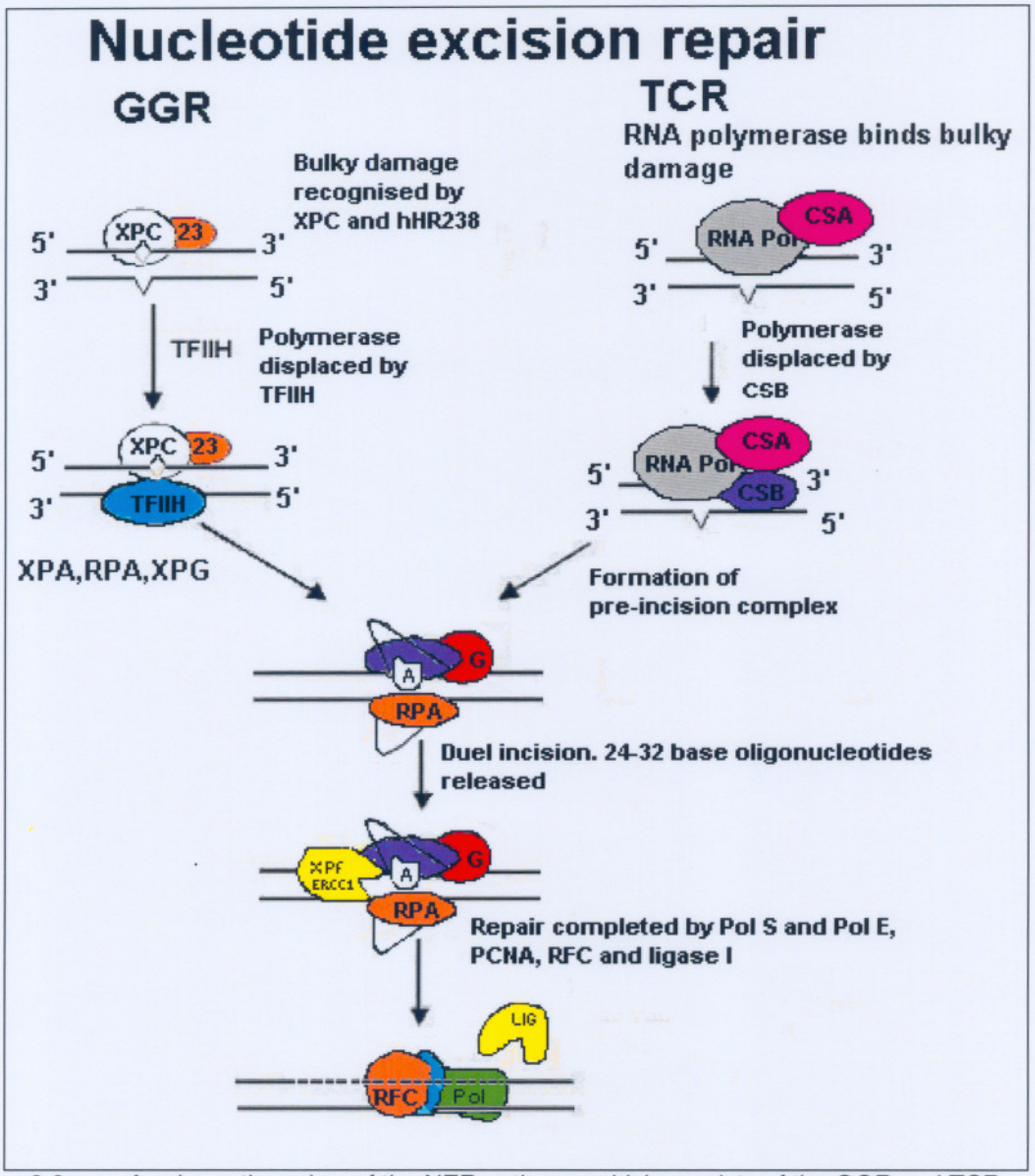


Figure 2.2 A schematic review of the NER pathway, which consists of the GGR and TCR pathway. Only the important factors are shown (from Krokan *et al.,* 2004).

A: Adenine; **AP:** Altered purines; **G:** Guanine; **CSA:** Encodes a protein with multiple WD-40 repeats; **CSB:** Encodes a DNA-dependent ATPase of the SNF2 family; **LIG:** Ligase; **PCNA:** Proliferating cell nuclear antigen; **Pol:** DNA polymerase; **RFc:** Replication factor C; **GGR:** Global genomic repair; **NER:** nucleotide excision repair; **TCR:** Transcripsion-coupled repair; **TFIIH:** Transcripsion factor active in nucleotide repair; **XPA:** Human protein that binds and stabilizes open complex, check for DNA damage; **XPC:** Human protein that works with HR23B, binds damaged DNA and recruits other NER proteins; **XPG:** Human protein: Endonuclease (3'incision), stabilizes full open complex; **RPA:** Replication protein A; **RNA pol:** RNA polymerase I **hHR238:** Human protein cooperates with XPC ubiquitin domain, interacts with proteasome and XPC; **δ:** Delta; **ε:** Epsilon

2.1.4.3 MISMATCH REPAIR (MMR)

The MMR system is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, methylation and replication errors (Modrich and Lahue, 1996; Umar and Kunkel, 1996). The main targets of MMR are base mismatches such as G/T (arising from deamination of 5-methylcytosine), G/G, A/C and C/C (Fang and Modrich, 1993). MMR not only binds to spontaneously occurring base mismatches but also to various chemically induced DNA lesions such as alkylation-induced O^6-methylguanine paired with cytosine or thymime (Duckett *et al.,* 1996), 1,2-intrastrand (CpG) cross links generated by Cisplatin (Mellon *et al.,* 1996; Yamada *et al.,* 1997), UV-induced photoproducts (Wang *et al.,* 2001), purine adducts of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxides (Wu *et al.*, 1999), 2-aminofluorene or *N*-acetyl-2-aminofluorene, and 8-oxoG (Colussi *et al.,* 2002). The importance of MMR in maintaining genomic stability and reducing the mutation load is clearly illustrated by MMR deficiency syndromes such as Hereditary colon cancer syndrome (HNPCC) (Hsieh, 2001; Lynch *et al.,* 1993). The first step by which MMR proceeds (Fig. 2.3) is the recognition of DNA lesions by MutSα (MSH2-MSH6). According to the molecular switch model, binding of MutSα-ADP triggers ADP→ATP transition, stimulating the intrinsic ATPase activity, and inducing the formation of a hydrolysis-independent sliding clamp, followed by binding of the MutLα complex (MLH1-PMS2). According to the hydrolysis-driven translocation model, ATP hydrolysis induces translocation of MutSα along the DNA. After formation of a complex composed of MutSα, excision is performed by 5'-3' Exonuclease (ExoI) and repair synthesis by Polδ (Krokan *et al.,* 2004).

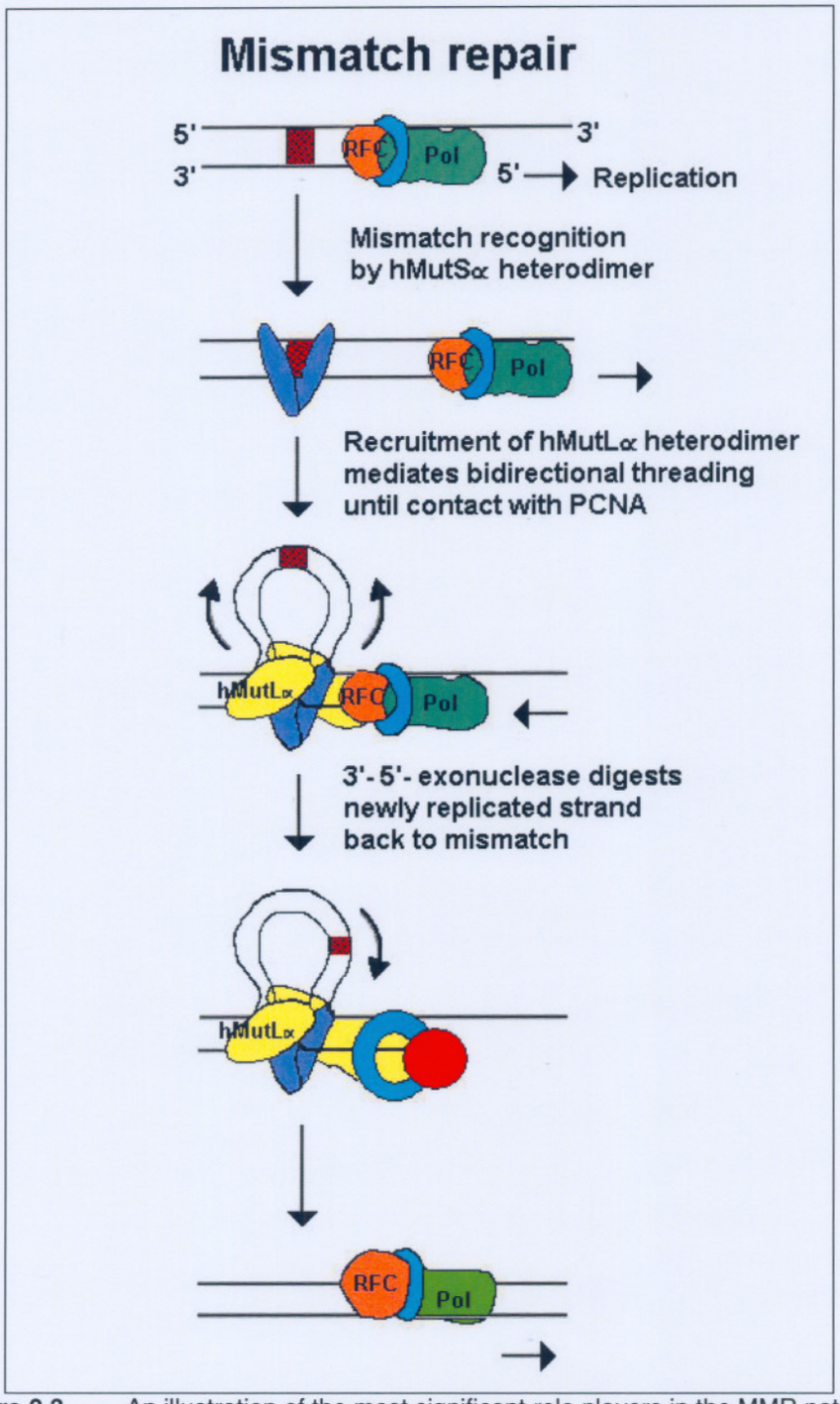


Figure 2.3 An illustration of the most significant role players in the MMR pathway (from Krokan *et al.*, 2004).

MMR: Mismatch repair; **RFC:** Replication factor C; **Pol:** DNA polymerise; **hMutL:** A protein in the methyl-directed mismatch repair pathway, which functions to ensure the fidelity of the daughter DNA strand during replication; **α:** alpha

2.1.4.4 DNA DOUBLE-STRAND BREAK (DSB) REPAIR

DSBs are highly potent inducers of genotoxic effects (chromosomal breaks and exchanges) and cell death (Dikomey *et al.,* 1998; Pfeiffer *et al.,* 2000; Lips and Kaina, 2001). In higher eukaryotes, a single non-repaired DSB inactivating an essential gene can be sufficient for inducing cell death via apoptosis (Rich *et al.,* 2000). There are two main pathways for DSB repair, homologous recombination (HR) and non-homologous end-joining (NHEJ), which are error-free and error-prone, respectively (Fig. 2.4). The use of NHEJ and HR also depends on the phase of the cell cycle. NHEJ requires little or no sequence homology and involves direct DNA end-joining (Mohrenweiser *et al.,* 2003) and occurs mainly in G0/G1. Whereas HR occurs during the late S and G2 phases of cell division (Takata *et al.,* 1998; Jonhanson and Jasin, 2000) and relies on a extensive nucleotide sequence complementary between the intact chromatid and the damaged chromatid (or homologous region) as the basis for strand exchange repair (Mohrenweiser *et al.,* 2003).

2.1.4.5 HOMOLOGOUS RECOMBINATION REPAIR (HRR)

In HRR, a homologous sequence forms a template for accurate genetic exchange, with the identical sister chromatid being preferred over homology on another chromosome (Baumann and West, 1998; Tompson and Schild, 2001; Sonoda *et al.,* 2001). HR starts with nucleolytic removal of the DSB in the 5'→ 3' direction by the MRE11-Rad50-NBS1 complex, forming a 3' single-stranded DNA fragment to which Rad52 binds. Rad52 interacts with Rad51, provoking a DNA strand exchange with the undamaged, homologous DNA molecule. Assembly of the Rad51 nucleoprotein filament is facilitated by different Rad51 prologues (such as Rad51B, Rad51C and Rad51D, XRCC2 and XRCC3). After DNA synthesis, ligation and branch migration, the resulting structure is resolved. The inhibition/mutations of HRR genes are associated with a cancer prone ataxia telangiectasia-like disorder (ATLD), characterized by neural degeneration, immunodeficiency, sterility and mild radiation sensitivity (Tompson and Schild, 2002).

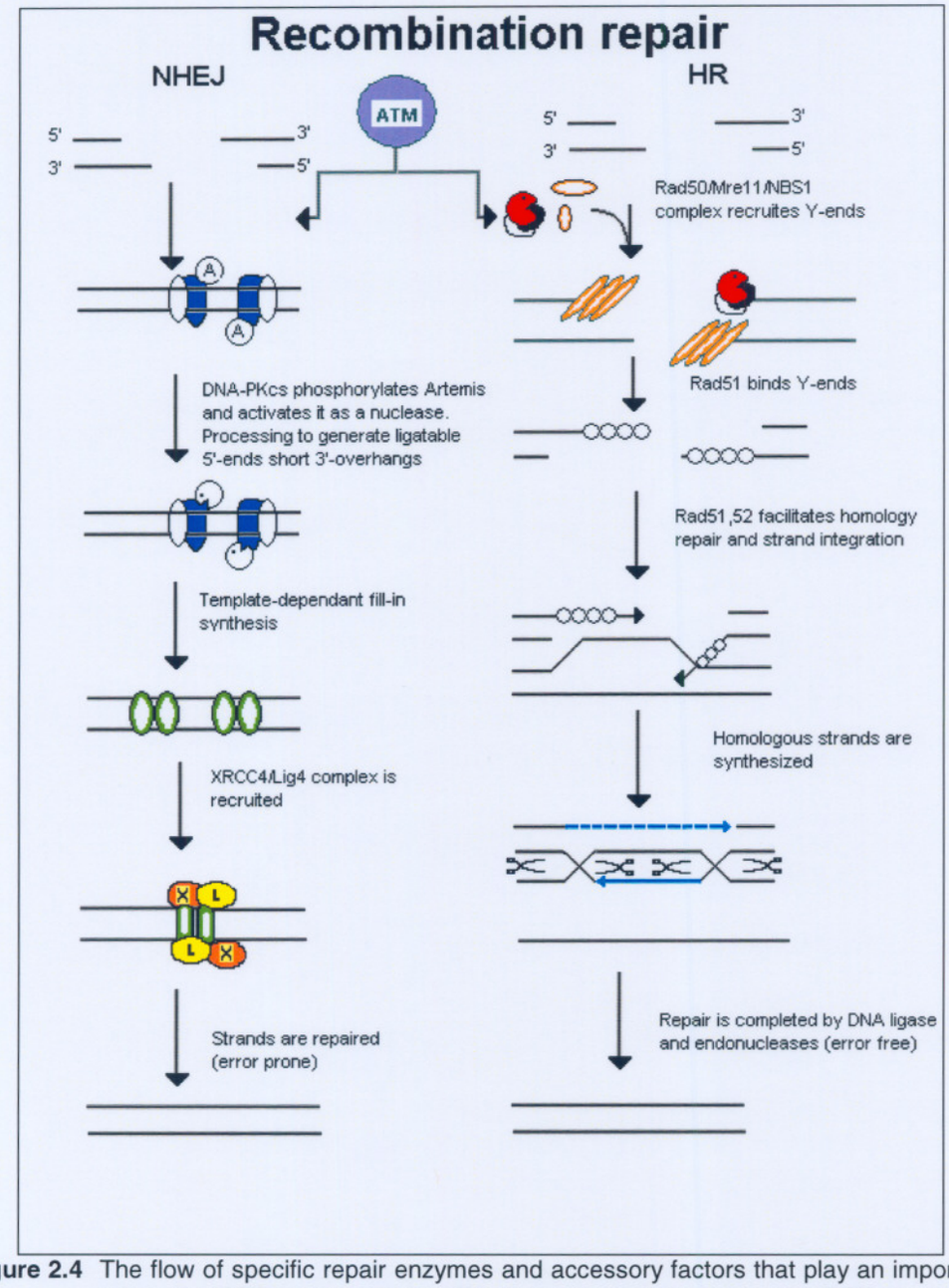


Figure 2.4 The flow of specific repair enzymes and accessory factors that play an important role in the DSB repair pathway. There are two main pathways for DSB repair, HR and NHEJ, which are error-free error (from Krokan *et al.,* 2004)

NHEJ: Non-homologous endjoining; **HR:** Homologous recombination **A:** Adenine, **ATM:** Ataxia-telangiectasia-mutated protein; **MreII:** Double-strand break repair protein; **NBSI:** DNA repair protein essential for homologous recombination repair; **Rad50/51/52:** Proteins essential for the repair of damaged DNA during homologous recombination; **X/L:** XRCC4 /LIG4 complex; **XRCC4:** X-ray-repair-cross-complementing, **LIG4:** Ligase 4

2.1.4.6 NON-HOMOLOGOUS END JOINING (NHEJ)

In NHEJ, the ends of a DSB are often modified by the addition or deletion of nucleotides then ligated to restore the covalent continuity of the broken chromosome (Mohrenweiser *et al.*, 2003). The mechanism of NHEJ begins with the recognition of binding to damaged DNA, this occurs by the Ku70-Ku80 complex. Thereafter, the Ku heterodimer binds to DNA-PKcs, forming the DNA-PK holo-enzyme. DNA-PK activates XRCC4-ligase IV, which links the broken DNA ends together. Before re-ligation by XRCC4-ligase IV, the DNA ends are processed by the MRE11-Rad50-NBS1 complex, presumably involving FEN1 and Artemis (reviewed in Doherty and Jackson, 2001).

A summary of all the important DNA repair processes are organised into five pathways (Table 2.2.) with subdivisions occurring in several of these pathways along with the exposures for which they provide protection.

Table 2.2 DNA repair pathways and the exposures for which they provide protection

DNA repair pathway	Prototypic exposure
Mismatch repair (MMR)	Naturally-occurring replication errors or recombination intermediates, as well as DNA adducts that result from treatment with certain cancer chemotherapy agents. These include: the DNA adducts of methylating agents such as MNNG and MNU
Base excision repair (BER)	Many dietary and environmental agents induce a pro-oxidant state, including heavy metals, organic and particulate components of diesel exhaust particles, certain pesticides, cigarette smoke, ionizing radiation. Cellular metabolism generates ROS that damage DNA.. Some alkylation base damage.
Nucleotide excision repair (NER)	UV-light; cigarette smoke; dietary contaminants (e.g. aflatoxin, PhIP); polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene); the aromatic amine AAF; a subset of NER genes function in the repair of DNA cross-links, such as those produced by Cisplantin.
Double strand break repair (DSB) Homologous recombinational (HR) Non-homologous end joining (NHJR)	 Ionizing radiation; cross linking agents (N,N'-bis-chloroethyl-N-nitrosourea, melphalan, mitomycin C, Cisplantin); faulty replication Similar to HRR

MNNG: N-methyl-N-nitro-N-nitrosoguanidine; **MNU:** 1-methyl-1-nitrosourea; **ROS:** Reactive oxygen species; **DNA:** Deoxyribonuclecleic acid; **UV:** Ultraviolet; **PhIP:** 2-Amino-1-methyl-6-phenyl-imidazo [4,5-b] pyridine; **AAF:** 2-Aminofluorene (Monhrenweiser *et al.*, 2003)

2.2 DISEASES CAUSED BY DEFECTIVE DNA REPAIR

The survival of an organism requires that the genetic inheritance is accurately maintained (Lindahl 1982; Lindahl and Wood, 1999). Failure in the DNA repair mechanisms leads to mutagenesis and eventually carcinogenesis (Lindahl, 1993; Monhrenweiser *et al.*, 2003; Blasiak *et al.*, 2004).

Several diseases characterised by defective DNA repair mechanisms have been identified. The most famous of these diseases are Xeroderma pigmentosum (XP), in which NER function is incomplete (de Boer and Hoeijmakers, 2000). XP is clinically characterised by the early onset of severe photosensitivity of the exposed skin areas, a high incidence of skin cancers, and to a lesser extent, to leukaemia (Fujiwara *et al.*, 1987; Kraemer *et al.*, 1994; van Steeg and Kraemer, 1999; Cleaver and Crowley, 2002). Another disease is Cockayne's syndrome. Patients with this disease have arrested growth and development, which results in a dwarfed appearance (Fujimoto *et al.*, 1969). These patients are sensitive to sunlight and they may have defective repair of UV irradiation-induced damage to DNA (Marshall *et al.*, 1980; Deschavanne *et al.*, 1984). In about half of the patients with Trichothiodystrophy (TTD) a defective NER of UV-induced DNA damage have been reported and so these patients suffer from photosensitivity (Stefanini *et al.*, 1986). Some cells from patients with Fanconi's anemia have also been reported to be defective in the repair of DNA inter-strand cross links (Moustacchi *et al.*, 1987). The DNA damage that can contribute to chronic diseases are the result from both external toxins and natural processes.

In each case where DNA damage is induced externally or occurring naturally, the outcome is an inactive DNA repair mechanism, which leads to a specific disease. The effect of unrepaired DNA damage may lead to mild or serious chronic diseases, even cancer. Thus any form of prevention or treatment for repairing the DNA damage, may aid in curing diseases that arise from defective DNA repair.

2.3 PREVENTION AND TREATMENT OF DNA DAMAGE

Understanding the role that DNA damage and its repair, or lack of repair, play in diseases are the first step to preventing or treating these types of diseases. If our DNA repair systems are in fact the 'guardians of the genome,' (Roth and Gellert, 2000), can we strengthen those guardians?

Nutrition experts have long recommended that people eat plenty of fruits and vegetables, in largely because they are such good sources of limiting DNA damage. As with any disease, one would do better to prevent DNA damage than to treat it after it has already occurred. The most valuable action to take in preventing such genetic disruption is to avoid toxins. Since most DNA damage occurs as the result of oxidative damage, scientists have looked to administering antioxidants to try to prevent DNA damage associated diseases (Fabiani *et al.,* 2001). Antioxidants are substances that protect the body against disease by countering the harmful effects of the highly-reactive forms of oxygen that accumulate in the body as by products of normal metabolic process as well as from external sources. If these reactants are left unchecked, they can damage DNA. Action of free radicals results in damage to DNA, cause mutations, damage proteins, damage lipids, and damage carbohydrates, alter function of proteins, lipids as well as carbohydrates, and create more free radicals. In a study involving dosing laboratory rats with N-acetylcysteine, which is converted by the body to glutathione, a strong antioxidant, resulted in the rats developing fewer DNA adducts (large, disruptive molecules that mess up DNA) (De Flora *et al.,* 1996).

The levels of DNA adducts in human leukocytes have been found to vary with a number of lifestyles, environmental and chemical-exposure factors. Increased dietary intake of antioxidants and essential metals, especially zinc, are known to provide protection against DNA damage (Knet *et al.*, 1991; Milner *et al.,* 2002; Knier, 2003). From this it can be concluded that there might be a possible manner of protection against DNA damage, through a better lifestyle and possible use of antioxidants.

2.4. MONITORING OF DNA DAMAGE WITH THE COMET ASSAY

In the literature it is clear that each type of DNA damage leads to the activation of a different type of DNA repair mechanisms (Monhrenweiser *et al.*, 2003). Therefore it might be expected that the induction of a specific type of DNA damage through a specific chemical reagent, for example DNA alkylation by carmustin, could lead to the activation of a specific type of DNA repair mechanism, i.e. DNA excision repair (Wozniak *et al.*, 2004). A summary of different types of chemical reagents with a specific effect on the DNA are given in chapter three Table 3.2.

Enzymes can also aid in distinguishing between the different types of DNA damage and their repair mechanisms. For example formamidopyrimidine DNA glycosylase (*Fpg*) is used to detect the major purine oxidation product, 8-oxoG, as well as other altered purines (Collins *et al.*, 1997). These enzyme-sensitive sites are converted to additional DNA breaks, which increase DNA damage. Similar enzymes like *Fpg*, with different action towards different types of DNA damage are listed in Table 4.1. (Speit *et al.*, 2004).

Methylation of biomolecules is the addition of methyl group to an appropriate substrate (Hubacek, 1992). When used in the context of epigenetics, DNA methylation can refer to the addition of a methyl group to a cytosine residue of DNA to convert it to 5-methylcytosine or the addition of a methyl group or groups to arginine or lysine amino acids in a protein. Methylation of DNA occurs at any CpG sites, which are sequences of DNA where cytosine lies next to guanine. The process of methylation is mediated by an enzyme known as DNA methyltransferase (Doerfler, 1983). CpG sites are quite rare in a eukaryotic genome except in regions near the promoter of a eukaryotic gene. These regions are known as CpG islands, the state of methylation of these CpG sites is critical for gene activity/expression (Robertson and Jones, 2000).

The pattern of methylation has recently become an important topic for research (Cottrell, 2004). Studies have found that in normal tissue, methylation of a gene is

mainly localized to the coding region, which is CpG poor. In contrast, the promoter region of the gene is unmethylated, despite a high density of CpG islands in the region (Bird, 1980). DNA methylation is also essential for proper embryonic development; but its presence can add additional burdens to the genome. Normal methylation patterns are frequently disrupted in tumor cells with global hypomethylation accompanying region-specific hypermethylation (Jones and Laird, 1999). During these hypermethylation events, within the promoter of a tumor suppressor gene they will silence the gene and provide the cell with a growth advantage in a manner akin to deletions or mutations. Interestingly, in cancer cells, methylation is very high even in the promoter region, raising interest in the role of methylation in the induction of cancerous properties.

Furthermore, the pattern of methylation has been shown to be a reliable marker of cancerous tissue, with a heavily methylated gene found in 90% or more patients with prostate cancer (Cottrell, 2004). Detection of certain methylation events can be used for early detection of tumors, and analysis of patterns of methylation across the genome might provide more information to the disease subtype, aggressiveness, and treatment response (Cottrell, 2004). DNA methylation research has entered the clinical field and as this research matures, methylation-based assays will make a major contribution to the field of molecular diagnostics, providing tools to fill unmet needs in current diagnostic and treatment plans for many types of cancer.

Although there are many different methods for the detection of DNA methylation (for more information concerning the different markers for monitoring DNA methylation see Havliš and Trbušek, 2002) the question now arising from this research project is: Can the DNA methylation status of single cells be monitored by the comet assay?

2.4.1. DIVERSITY IN APPLICATIONS OF THE COMET ASSAY

A broader understanding of the impact of certain internal and external factors on DNA damage and a cell's repair capacity can be obtained from the development the SCGE (comet assay) as a powerful method for measuring DNA strand breakage and

DNA repair. In this study, the main objective was to exploit the diversity in applications of the comet assay and therefore a number of experiments needed to be done to obtain the wanted results. The reproducibility of the comet assay is essential to standardize and validate this method. To achieve this, a healthy male volunteer's blood sample was analyzed once a week over a two week period. A cancer patient's repair capacity was measured during chemotherapy while a breast cancer patient's repair capacity was measured after chemotherapy. The effect of different intensities (60–90%) of exercise on DNA strand breaks were monitored in healthy male athletes (between 40-45 years). In Table 2.3 a summary of the diversity in applications of the comet assay are listed.

2.4.1 THE INCORPORATION OF ENDONUCLEASES IN THE COMET ASSAY

The comet assay was made more informative by incorporating additional steps by digesting the DNA on the microscope slides with enzymes that recognize particular kinds of damage to the nucleic acid. Endonuclease III (*Endo* III) were used to detect oxidized pyrimidines and formamidopyrimidine DNA glycosylase (*Fpg*) to detect the major purine oxidation product 8-oxoguanine as well as other altered purines (Collins and Dušinská, 1997).

2.4.3 MONITORING OF DNA METHYLATION LEVELS

The isoschizomers *Hpa* II and *Msp* I are used as DNA cleavage enzymes. Both *Hpa* II and *Msp* I recognise the same tetranucleotide sequence (5'-CCGG-3'), but display different sensitivities towards DNA methylation (Jenkins *et al.,* 2002). *Hpa* II is inactive when any of the two cytosine's are fully methylated, but cuts the hemi-methylated 5'-CCGG-3' at a lower rate compared to the unmethylated sequences whereas *Msp* I cuts 5'-C^{5m}CGG-3', but not 5'^{5m}-CCGG-3' (Mingliang *et al.,* 2000). In each case, the enzyme-sensitive sites are converted to additional DNA breaks, which increase the tail intensity of the comets. Applying this digestive step during the comet assay may enable us to measure the levels of methylation in individual cells. In this way the applicability of the comet assay can be extended even further.

Table 2.3 Diversity in applications of the comet assay

Applications	Example	Functions	Expectations
Cell types	-White blood cells (WBC) -Liver cells -Brain cells -Hemolymph -Cell cultures -Basically any form of single cell	Isolation of various cell types to perform comet analysis	Determine the different reactions of each cell type with each other through various chemical treatments.
Intervention	H_2O_2 treatment	Monitoring the DNA repair capacity	Enhancement of DNA repair capacity through different treatments e.g. antioxidants.
Bio monitoring (Humans)	-Exercise -Antioxidant treatment -DNA repair of a Cancer patient -Occupational exposures	Monitoring the effect of DNA damage caused by the various types of damage: natural endogenous cellular processes, environmental causes and gene mutations	Broadening the diversity in applications with the comet assay
Restriction enzymes	*Fpg* and *Endo* III *Hpa* II and *Msp* I	Detect oxidative damage Recognise the 5'-CCGG-3' sequence, with different sensitivity to DNA methylation	Enhancing sensitivity and specificity of the comet assay.

2.5 AIMS AND APPROACH OF THIS STUDY

The main aims of this study are to:

1. Establish the comet assay in our laboratory
2. Investigate the feasibility of using the comet assay in measuring DNA methylation levels in single cells.

The following approach was formulated for this study:

1. Applying proven published methods, with minor adjustments to suit local laboratory conditions, to set up the comet assay. Our premise is not to deviate significantly from the published methods.
2. Establishing the comet assay for measuring DNA damage and repair in single cells prepared from different tissues.
3. Use of methylation sensitive restriction enzymes to measure DNA methylation levels in single cells.

CHAPTER THREE

MATERIALS AND METHODS

ETHICAL APPROVAL

Ethical approval was obtained under the project title 'Monitoring of different DNA repair mechanisms' with approval number 04D10.

3.1 SINGLE-CELL GEL ELECTROPHORESIS / COMET ASSAY

Different techniques for detecting DNA damage have been used to identify substances with genotoxic activity. Some years ago the alkaline SCGE assay was introduced as a novel approach for detecting DNA lesions (Östling and Johanson, 1984). This method involves the application of an electrical current to cells that results in the differential transport of DNA fragments of various lengths. The image of DNA migration obtained resembles a comet with a head and a tail, hence the term comet assay (Klaude *et al.*, 1996; Singh and Stephens, 1996). Since the DNA damage induced by toxic agents is often tissue- and cell-specific, SCGE is very useful because it can detect DNA lesions in individual cells obtained under a variety of experimental conditions. In addition this technique can also be used to evaluate DNA repair (Tice, 1995; Collins, 2004).

A significant advantage of the SCGE assay is its potential applicability to any eukaryotic organism and cell type. Since the assay is also relatively inexpensive and gives results within a few hours, it is appropriate for environmental monitoring. In addition to human peripheral blood lymphocytes exposed to different agents, both *in vitro* and *in vivo* (Collins *et al.*, 1997), other cell types and organisms have also been investigates using this assay (Petras *et al.*, 1995; Tice, 1995; Verschaeve and Gilles, 1995; Sasaki *et al.*, 1997). In particular native animals, especially small mammalian species, living in or close to pollutants zones, have been used to detect hazardous pollution (Nascinbeni *et al.*, 1991; Fairbairn *et al.*, 1995; Petras *et al.*, 1995; Tice, 1995; Baker *et al.*, 1996; Salagovic *et al.*, 1996; Ralph *et al.*, 1997).

Since Singh *et al.* (1988) published their description of the SCGE assay there has been rapid growth in the use of the comet assay. The small variations in the different steps of the technique reported by different authors have been reviewed (McKelvey-Martin *et al.*, 1993; Fairbairn *et al.*, 1995; Collins, 2004; Faust *et al.*, 2004).

3.1.1 METHODOLOGY

The alkaline comet assay (pH>13) as introduced by Singh *et al.* (1988), is in its basic form the most frequently used version of the comet assay and detects DNA SSB, ALS and DNA cross-linking in individual cells. All methodological steps associated with the comet assay are equally important for obtaining reproducible and reliable results. Once a suspension of cells is obtained, the basic steps of the assay include (1) preparation of microscope slides layered with cells in agarose, (2) lysis of cells to liberate DNA, (3) exposure to alkali (pH>13) to obtain single-stranded DNA and to express ALS as SSB, (4) electrophoresis under alkaline (pH>13) conditions, (5) neutralisation of alkali, (6) DNA staining and comet visualisation and (7) comet scoring along with statistical processing of results.

3.1.2 MATERIALS

Dimethylsulfoxide (DMSO), potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH) and Triton X-100® were all obtained from Merck. Ethylenediaminetetra-acetic acid disodium salt (EDTA), Histopaque®, Hanks Balanced Salt Solution (Ca^{++}, Mg^{++} free) (HBSS) and Tris-HCl were all obtained from Sigma. Ethidium bromide was obtained from Roche. Potassium dihydrogen orthophosphate (KH_2PO_4) and di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) were all obtained from SAARCHEM. High melting point agarose (HMPA) was obtained from Separations. Low melting point agarose (LMPA) was obtained from Roche. Trypan blue stain was obtained from Bio*Whittaker.

3.1.3 SLIDE PREPARATION

Glass microscope slides were frosted to form two windows as illustrated in Fig. 3.1. The slides were permanently label and were re-used, after been washed in hot water and rinsed in distilled water.

Figure 3.1 Frosted microscope plate.

The preparation of the slides is based on uniform gels sufficiently stable to survive through to data collection, as well as to ensure easily visualised comets with minimal background noise. In this procedure the frosted slides were covered with 300µl high melting point agarose (1% HMPA) and left to solidify. Cells (50µl) were suspended in 150µl low melting point agarose (0.5% LMPA) and spread directly and evenly on the first agarose layer on a microscope slide. The number of cells (1 x 10^4) in the agarose as well as the concentration of agarose (0.5%) is important parameters for ensuring a successful analysis.

3.1.4 BLOOD AND TISSUE PREPARATION

Blood (2-3ml) were obtained from rats in a heparin tube and kept chilled (not directly on ice). The blood was carefully layered on top of 2ml Histopaque® and to obtain the lymphocytes, the tube was centrifuged for 30 minutes at 550 x g at room temperature. The first plasma layer was then removed with a Pasteur pipette and discarded. The buffy coat (containing the lymphocytes) was transferred to an Eppendorf tube and washed with 400µl phosphate buffer saline (PBS) and then centrifuged for 10 minutes at 550-x g at 4°C. The washing step was repeated with the same volume PBS afterwards the resulting pellet was resuspended in 500µl PBS and kept chilled until assay.

Brain and liver tissues (approximately 2-3mg) were collected from rats and placed into a Falcon tube containing 3ml mincing solution: HBSS (Ca^{++}, Mg^{++} free) with 20mM EDTA and 10% DMSO, and kept directly on ice for 30 minutes. The tissue was cut up in to smaller fractions and resuspended in 2ml of fresh chilled mincing solution and kept on ice for a further 30 minutes. This cell suspension is now suitable for using in the assay (Tice and Vasques, 1999).

Cells were counted and viability assessed with the trypan blue stain method and a haemocytometer. The comet assay was only performed on cells when viability exceeded 90%, and approximately 10,000 cells per microscope slide were used for analysis.

3.1.5 LYSIS

After the agarose gel has solidified, the slides were placed for at least 12 hours (overnight) in a lysis solution, consisting of high salts and detergents (5M NaCl, 0.4M EDTA, 10% DMSO, 1% Triton X-100). The lysing solution was chilled to 4°C prior to use, primarily to maintain the stability of the agarose gel (Singh *et al.,* 1988).

3.1.6 ALKALI (pH>13) UNWINDING AND ELECTROPHORESIS

Prior to electrophoresis the slides were incubated for 30 minutes at 4°C in alkaline (pH>13) electrophoresis buffer to unwind and produce single-stranded DNA lesions (0.6M NaOH, 0.05M EDTA). This will enable the detection of both double and single strand breaks.

After alkali unwinding, the unwounded DNA in the gel was electrophoresed under alkaline conditions to produce comets. The alkaline buffer used during electrophoresis was the same pH>13 buffer used during alkali unwinding. The electrophoretic conditions were 30V and 300mA, with the DNA being electrophoresed for 40 minutes at 4°C (Singh and Stephens, 1996; Yendle *et al.,* 1997).

3.1.7 NEUTRALISATION, DNA STAINING AND COMET VISUALISATION

After electrophoresis the alkali in the gels was neutralized by soaking the slides in a suitable buffer, i.e. Tris-HCl (pH 7.5) for 15 minutes at 4°C. To visualize the comets the slides were soaked in ethidium bromide (10μM), a fluorescent dye, for 15 minutes at 4°C. The slides were then washed in distilled water to remove unbound stain. The slides were examined at 200 times magnification on a Olympus XI70 inverted system microscope (Olympus Optical Co. (U.K.) LTD., London) attached to a Color View 12 (7087355) video camera equipped with a UV filter block consisting of an excitation filter (359nm) and barrier filter (461nm) and connected to a personal computer based image analysis system (analySIS® v.3, Lakewood, USA). Fifty images were randomly selected and the comet tail DNA % was measured using CASP® (v.1.2.2.) image analysis software.

3.1.8 COMET SCORING

The method used for quantifying the DNA migration with this assay, was performed by using the CASP® imaging system software program. This software is freely available from the following web address: http://www.casp.sourceforge.net. Comets were classified as damaged (Tice *et al.*, 2000) and also into four classes (See p.31).

3.1.9 PROCESSING OF THE DATA

The data obtained from each cell was collected by the CASP image analysis program, which measures the migration of the DNA in the tail. The most commonly used measurement in comet studies is the length of DNA migration. Migration length is generally believed to be related directly to fragment size and would be expected to be proportional to the level of DNA strand breaks, and inversely proportional to the extent of DNA cross-linking. The use of computerised image analysis systems to collect comet data, the measurement based on the percentage of migrated DNA along with the tail moment (a measurement of the tail length *x* a measure of DNA in the tail), introduced by Olive *et al.*, 1990, was applied in this study for processing of the data. Fig. 3.2 illustrates the method by which the computerised image analysis system operates.

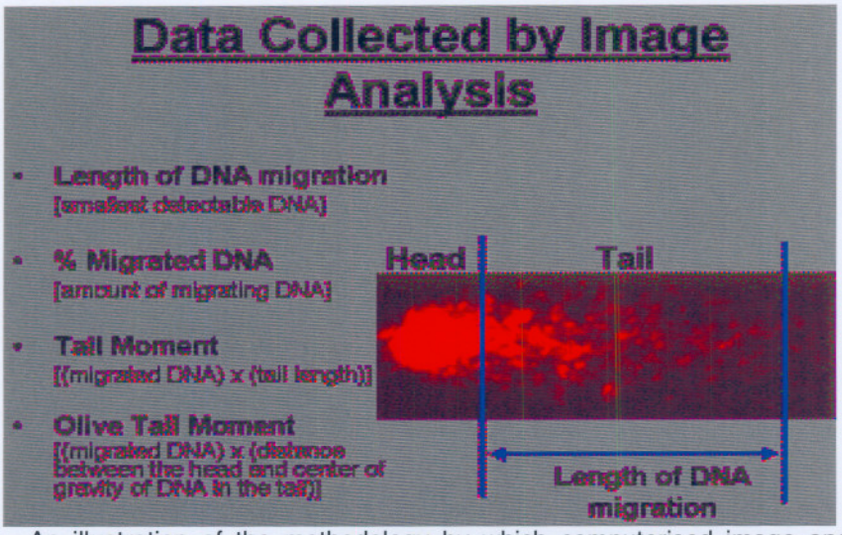


Figure 3.2 An illustration of the methodology by which computerised image analysis system programs operate for the measurement of DNA damage. Image from Bharti and Kumar, 2004.

Descriptive statistical analysis was carried out to study the significant differences after exposures (Paracetamol, EMS and MMC) were applied to the cells compared to controls. Statistical analyses were done using the Statistica 6.1 software (Copyright© StatSoft, Inc. 1984-2003). To check if there were significant differences ($p < 0.05$) in the means of untreated and treated groups, the one-way ANOVA (analysis of variance) test was employed. A minimum of 50 cells per sample was analyzed with the CASP software program and the resulting data (as a Microsoft Excel spreadsheet) were transferred to Statistica. The post hoc Duncan's test (for multiple comparisons) was then used to determine the significant differences between group means in the analysis of the variance setting. A column type graph showing the means and standard deviations (±SD) of the results was used to display the data.

According to the percentage DNA damage in the tail, the extent of DNA damage was grouped into different classes as illustrated in Fig. 3.2. The extent of DNA damage was classified as the percentage DNA in the tail, in the following way: class 0 <6%;

class 1: 6.1-17%; class 2: 17.1-35%; class 3: 34.1-60%; class 4: > 60% (Giovannelli *et al.*, 2002). Class 0 (no damage) to class 4 (highly damaged), based on the relative proportion of the DNA in the tail (Gedik *et al.*, 1992; Anderson *et al.*, 1994; Kobayashi *et al.*, 1995). By assigning a numerical value to each class, the average extent of DNA migration among cells within a sample can be calculated.

To show the shift in the degree of damage from one class to another for each of the different chemical treatments, correlation statistics were used. Correlation is a measure of the relation between two or more variables. The measurement scales used should be at least interval scales. Correlation coefficients can range from -1.00 to +1.00. The value of -1.00 represents a perfect negative correlation while a value of +1.00 represents a perfect positive correlation. A value of 0.00 represents a lack of correlation. Fig. 3.3 shows three examples of the different correlation values (a - negative correlation, b - zero correlation, c - positive correlation).

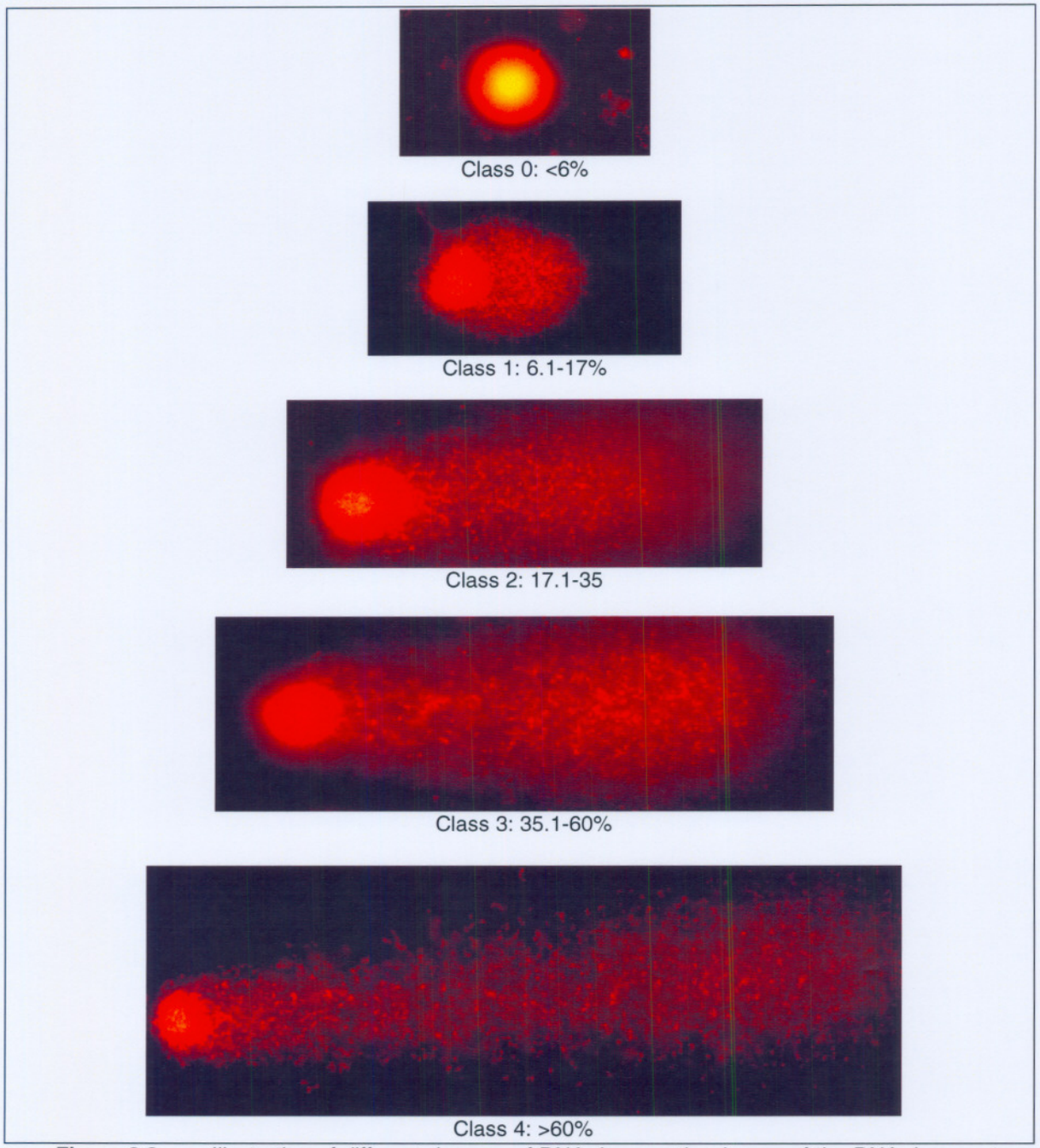
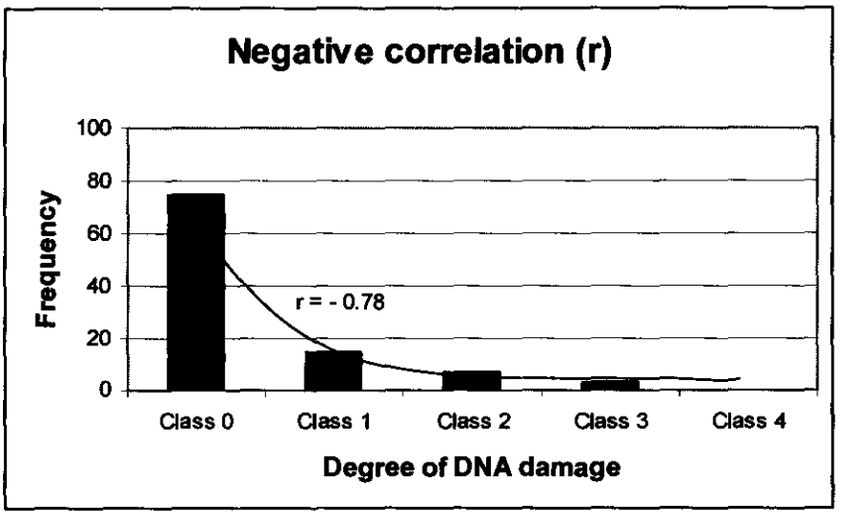
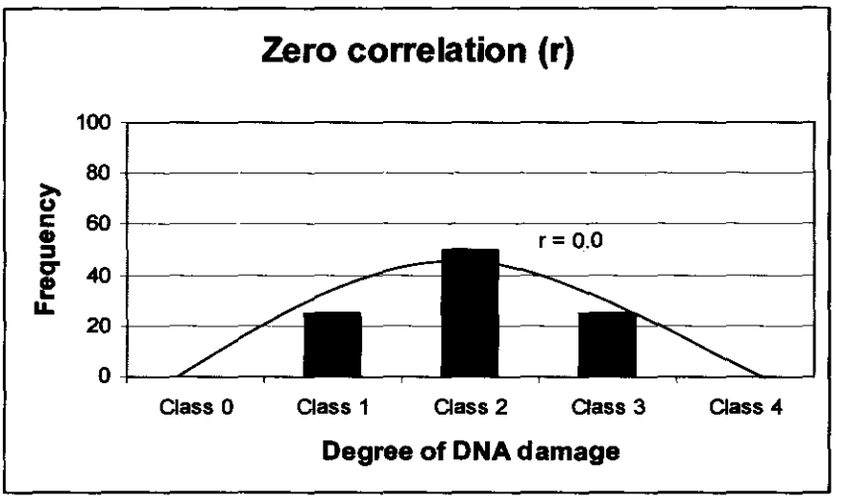


Figure 3.3 Illustration of different degrees of DNA damage the degree of the DNA damage to each cell was classified into four classes, class: 0<6%; class 1: 6.1-17%; class 2: 17.1-35%; class 3: 35.1-60% and class 4: >60% (Giovannelli *et al.,* 2002).

[a]



[b]



[c]

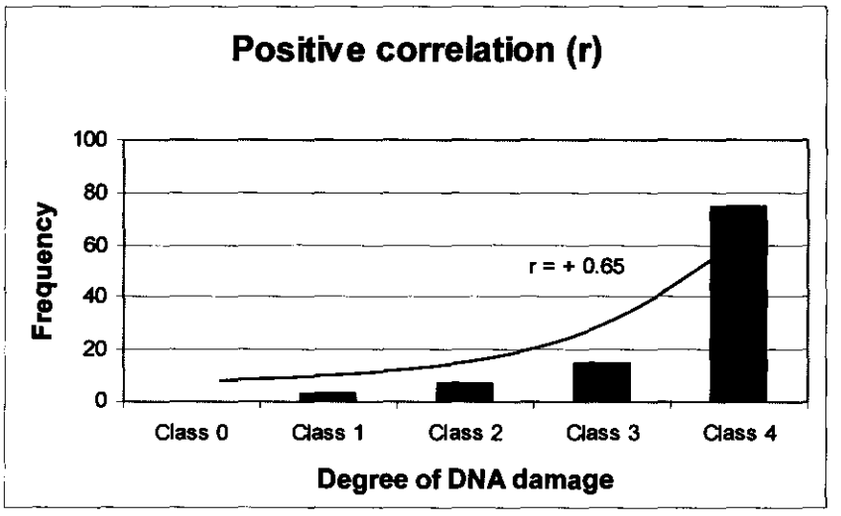


Figure 3.4 Examples of the different correlation values (a - negative correlation, b - zero correlation, c - positive correlation).

3.2 DIFFERENT APPLICATIONS OF THE SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

The applications of the comet assay were demonstrated as follows: a healthy male volunteer's blood sample was analyzed once a week over a two week period and the results compared to check reproducibility. A *breast cancer patient's* repair capacity was measured after chemotherapy. The effect of different intensities of *exercise* on DNA strand breaks were monitored in healthy male athletes (between 40-45 years), the results present represent only that of one individual. In vitro evaluation of the mutagenicity, cytotoxicity and genotoxicity of *Rosa roxburghii* in rat primary hepatocytes and its protection against tert-butyl hydroperoxide-induced oxidative stress were also assessed by applying the comet assay (Janse van Rensburg et al., 2004). The results from this study can be obtained from the reference mentioned. The effect of an apparently hazardous working environment on DNA integrity was studied with the comet assay. This was only a pilot study in which six males working in a chemical plant were involved. Blood samples were taken at six in the morning before (pre-shift) and after (post-shift) a seven hour working shift and six healthy men in the Potchefstroom area were used as controls. The flow diagram in Fig. 3.6 illustrates the experimental procedures followed during the different applications of the comet assay. All the experiments were done in duplicate.

3.3 MONITORING DNA LESIONS WITH THE COMET ASSAY IN DIFFERENT TISSUES OF THE RAT

Various treatments to different cell types (white blood cells, brain and liver cells) from rats were treated with a variety of substances to evaluate the extend of DNA strand breaks and in some cases also the repair capacity of the isolated cell suspensions. The first of these experiments was the dosing of rats with 300mg/kg Paracetamol to evaluate *in vivo* DNA damage and the repair capacity in each tissue, compared with an untreated group of rats. Secondly DNA repair enzymes were used to recognize a particular kind of lesion that creates breaks in the DNA strand. Lastly, cells from each of the tissues were incubated with two different toxins, each causing a specific type of DNA damage for example alkylation, methylation and oxidation.

The flow diagram in Fig. 3.7. gives a summary of the experimental procedures used to monitor different DNA lesions in various tissues of the rat.

3.3.1 MONITORING SPECIFIC DNA LESIONS

Measuring DNA strand breaks gives to a certain extend limited information. Breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined. It may in fact be apurinic/apyrimidinic sites (i.e., AP sites or baseless sugars), which are alkali labile and therefore appear as breaks. Or it may be intermediates in cellular repair, because both nucleotide and base excision-repair processes cut out and repair damage (Collins, 2004).

To make the comet assay more specific, sensitive and more informative, an additional step of digesting the DNA on the microscope slides with an enzyme that recognizes a particular kind of damage and creates a break. *Endo* III was used to detect oxidized pyrimidines (McKelvey-Martin *et al.*, 1993) and *Fpg* to detect the major purine oxidation product 8-oxoG as well as other altered purines (Collins *et al.*, 1997). In each case, the enzyme-sensitive sites are converted to additional DNA breaks, which increase tail intensity. Applying this activity to different tissues may shed some light on differential damage and repair in different tissues.

3.3.1.1 METHODOLOGY FOR *Fpg* AND *Endo* III

The comet assay as described in Section 3.1, was modified for the detection of specific types of damage with the use of specific enzymes. These enzymes identify specific DNA base modifications that are converted to strand breaks. These strand breaks are then detected by the comet assay at pH>13 and by comparing the DNA migration in enzyme treated and buffer treated slides (Fig. 3.3), interpretations regarding the degree of specific types of damage to DNA can then be made.

3.3.1.2 MATERIALS

Enzymes (*Endo* III and *Fpg*) and their buffers were obtained form New England BioLabs and other chemicals from either Merck or Sigma.

Table 3.1 Description of *Endo* III and *Fpg* enzymes

Enzyme	Mechanism	Supplier
Endo III	The N-glycosylase activity releases damaged pyrimidines from double-stranded DNA, generating an AP site. The AP-lyase activity of the enzyme cleaves 3' to the AP site leaving a 5' phosphate and a 3' ring opened sugar. Some of the damaged bases recognized by *Endo* III include urea, 5,6 dihydroxythymine, thymine glycol, 5-hydroxy-5 methylhydanton, uracil glycol, 6-hydroxy-5, 6-dihdrothimine and methyltartronylurea (Dizdaroglu *et al.*, 1993; Hatahet *et al.*, 1994).	BioLabs
Fpg	The N-glycosylase activity releases damaged purines from double stranded DNA, generating an AP site. The AP-lyase activity cleaves both 3' and 5' to the AP site thereby removing the AP site and leaving a 1 base gap. Some of the damaged bases recognized and removed by *Fpg* include 7,8-dihydro-8-oxoguanine (8-oxoG), 8-oxoadenine, fapy-guanine, methyl-fapy-guanine, fapy-adenine, aflatoxinB_1-fapy-guanine, 5-hydroxy-cytosine and 5-hydroxy-uracil (Tchou *et al.*, 1994; Hatahet *et al.*, 1994).	BioLabs

3.3.1.3 TREATMENT WITH ENZYMES *Fpg* AND *Endo* III

Slides were equilibrated in enzyme reaction buffer containing HEPES [40mM], KCl [0.1M]; EDTA [0.5M]; BSA [0.2mg/ml] at pH 8 for 15 minutes. The slides were removed from the buffer and excess liquid was dabbed off. A volume of 50µl enzyme solution (for *Fpg* and *Endo* III) consisting of 0.25µl enzyme, 5µl enzyme buffer and 44.75µl ddH_2O was placed onto gel surface. Slides were put in a moist box (plastic container lined with damp paper towels) and incubated at 37°C for 30 minutes. After incubation with enzyme, slides were placed in an electrophoresis tank which contained a pre-chilled electrophoresis solution for 30 minutes. The remaining procedure remains exactly the same as described in Sections 3.1.7 – 3.1.12. For each experiment there was a control, a no enzyme treated and an enzyme treated slide (Protocol according to Collins, 2004).

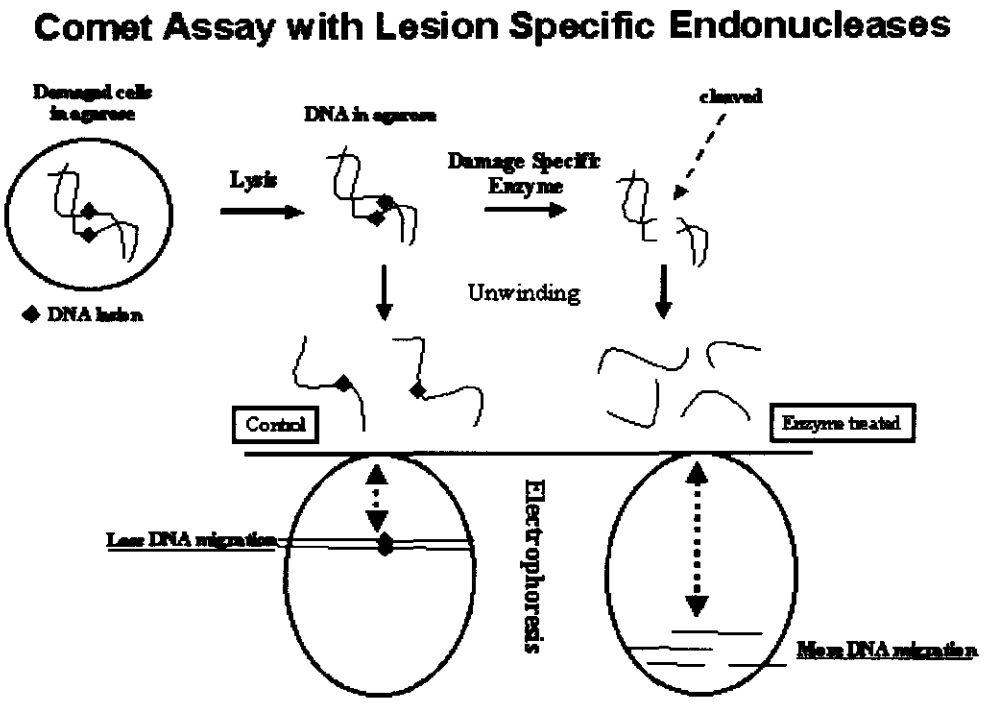


Figure 3.5 Comet assay performed with specific restriction enzyme. The figure indicates damaged cells imbedded in agarose gel after lysis only DNA remains, which was treated with and without (which represents the control) the specific restriction enzyme. Image from Bharti and Kumar, 2004.

3.3.2 MONITORING DNA METHYLATION LEVELS

The role of DNA methylation in eukaryotic cells is not fully understood yet, but it is clear that methylation has two major functions: gene expression control and protection of the host organism against expression of undesired sequences (Hubacek, 1992). Methylation regulates vital cellular processes, thus any inappropriate methylation/ demethylation could cause fatal or lethal consequences, e.g. cancer (Havliš and Trbušek, 2002). The major problem with methylation occurs when CpG sites within a promoter CpG island are predominantly methylated, the chromatin is normally condensed in that region and there is little or no gene expression of the associated gene (Magdinier *et al.,* 2002). When there is such an aberrant methylation of tumour suppressor genes, it can lead to abnormal cellular growth. The isoschizomers *Hpa* II and *Msp* I are used as DNA cleavage enzymes. Both *Hpa* II and *Msp* I recognise the same tetranucleotide sequence (5'-CCGG-3') but display differential sensitivity to DNA methylation (Jenkins *et al.,* 2002). *Hpa* II is inactive when any of the two cytosine's is fully methylated, but cuts the hemi-

methylated 5'-CCGG-3' at a lower rate compared to the unmethylated sequence, whereas, *Msp* I cuts although slowly 5'-C^{5m}CGG-3', but not 5'-^{5m}CCGG-3' (Mingliang *et al.,* 2002). To monitor this type of methylation status the restriction endonucleases *Hpa* II and *Msp* I could be used with the comet assay, because the difference in DNA strand breaks reflects the difference in DNA methylation levels.

The development of the comet assay with the use of these enzymes can contribute in understanding the role of DNA methylation in cancer and other methylated DNA related diseases.

3.3.2.1 METHODOLOGY FOR *Hpa* II AND *Msp* I

The comet assay, as described in Section 3.1, was modified for the detection of specific types of damage with the use of specific enzymes. These enzymes identify specific DNA base modifications that are converted to strand breaks. These strand breaks are then detected by the comet assay at pH>13 and by comparing the DNA migration in enzyme treated and buffer treated slides, interpretations regarding the degree of specific types of damage to DNA can then be made.

3.3.2.2 MATERIALS

Enzymes (*Hpa* III and *Msp I*) and their buffers were obtained form New England BioLabs and other chemicals from either Merck or Sigma.

Table 3.2 Description of the *Hpa* II and *Msp* I enzymes

Enzyme	Mechanism	Supplier
CpG Methylase (*Sss* I): ^{CH_3} 5'...CG...3' 3'...GC...5' $_{CH_3}$	The CpG Methylase, *Sss* I, methylates all cytosine residues (C^5) within the double-stranded dinucleotide recognition sequence 5'...CG...3' (Nur *et al.*, 1985)	BioLabs
Hpa II and *Msp* I: 5'...CCGG...3' 3'...CCGG...5'	*Hpa* II is an isoschizomer of *Msp* I. When the external C in the sequence CCGG is methylated, *Msp* I and *Hpa* II cannot cleave. However, unlike *Hpa* II, *Msp* I can cleave the sequence when the internal C residue is methylated (Nur *et al.*, 1985)	BioLabs

3.3.2.3 TREATMENT WITH ENZYMES *Hpa* II AND *Msp* I

Slides were equilibrated in a buffer containing Tris-HCl [10mM]; $MgCl_2$ [10mM]; NaCl [50mM] at pH 7.5 for 15 minutes. The slides were removed from the buffer and excess liquid was dabbed off. A volume of 50µl enzyme solution (for *Hpa* II and *Msp* I) consisting of 0.25µl enzyme, 5µl enzyme buffer and 44.75µl ddH_2O was placed onto gel surface. Slides were placed in a moist box (plastic container lined with damp paper towels) and incubate at 37°C for 60 minutes. After incubation with enzyme, slides were placed in an electrophoresis tank that contained a pre-chilled electrophoresis solution for 30 minutes. The remaining procedure was as described in Sections 3.1.7 – 3.1.12.

3.3.3 METHYLATION POSITIVE CONTROL

Paracetamol is a widely used analgesic and antipyretic. At high doses it exerts toxic effects primarily through the toxic metabolite *N*-acetyl-*p*-benzoquinone imine in the liver and lymphocytes (Lertratanangkoon *et al*, 1997). The primary immediate result is the depletion of glutathione and the covalent modification of proteins. There is no indication that Paracetamol causes primary damage to the brain, but it has a highly targeted action in the brain, blocking an enzyme involved in the transmission of pain

(Flower and Vane, 1972). The oral dosing of rats with 300mg/kg Paracetamol for a time period of three days will assist the evaluation of different types of DNA damage and DNA repair capacity that might exit *in vivo* using comet assay.

3.3.3.1 METHODOLOGY

Three male Sprague Dawley rats with an average body weight between 230-260g were orally dosed with 300mg/kg Paracetamol daily for three days. All the rats had free access to a commercial pellet diet and water under standard laboratory conditions. On the fourth day they were killed by decapitation together with three untreated rats; 2-3ml blood was collected and the brain and liver were removed. The blood and tissues collected were then treated as described in Section 3.3.1.3, 3.3.2 and 3.3.3.

3.3.3.2 MATERIALS

Paracetamol was obtained from Sigma.

3.3.3.3 HYDROGEN PEROXIDE TREATMENT (H_2O_2)

H_2O_2 is one of the most powerful oxidizers known. Through catalysis, it can be converted into hydroxyl radicals that cause oxidative DNA damage (Halliwell *et al.*, 2002). By treating lymphocytes, liver and brain cells with a known concentration of hydrogen peroxide, a specific degree of DNA damage is obtained. The degree of DNA damage is then used to compare the DNA repair capacity in the different types of cells.

3.3.3.4 METHODOLOGY

After cells were obtained as previously described a control sample was removed. The remaining cells were treated with 60µM H_2O_2 for 20 minutes at 37°C. The cells were washed twice with 400µl PBS to remove all the H_2O_2 and were then resuspended in 450µl Hams F10. The cells were then incubated for another 20 minutes and 40 minutes to monitor DNA repair capacity.

3.3.3.5 MATERIALS

Hams F10 and hydrogen peroxide (H_2O_2) were obtained from Merck.

3.3.4 INDUCTION OF VARIOUS DNA LESIONS WITH CHEMICAL COMPOUNDS

As discussed in Section 2.8 the following chemical reagents listed in Table 3.3 were used to induce specific types of DNA damage.

Table 3.3 Summary of chemical reagents and their effect on DNA

Chemical reagent	Function	Type of DNA damage	Activated DNA repair mechanism
EMS (Ethylmethanesulfonate) **$C_3H_8O_3S$**	EMS readily alkylates nucleophiles such as hydroxyl, amino and sulfhydryl groups in biological materials (e.g., proteins) (Hrushesky *et al.*, 1972).	EMS is a mutagen that reacts with bases and adds methyl or ethyl groups (Montelon, 1998).	Depending on the affected base may then degrade to yield a baseless site, which is mutagenic and recombinogenic, or mispair to result in mutations upon DNA replication (Montelon, 1998).
MMC (Mitomycin C) **$C_{15}H_{18}N_4O_5$**	Cross-linking agent that induce DNA strand breaks (McKenna *et al.*, 2003).	MMC a known DNA cross linker as well as an oxidating DNA damaging agent (McKenna *et al.*, 2003).	Inhibitor of DNA synthesis, nuclear division and cancer cells (McKenna *et al.*, 2003).

3.3.4.1 METHODOLOGY

Lymphocytes were used to determine the best optimum concentration for causing DNA damage for each of the different chemical reagents by the method described is Section 3.1.1. The only modification was the incubation of the lymphocytes with the chemicals at 37°C for 60 minutes. Once the optimum concentration for each of the chemical reagents was determined, lymphocytes, liver and brain cells were treated with the toxins at that specific concentration as well as enzyme treatments (Section .2.2 and 3.2.3).

3.3.4.2 MATERIALS

Ethanol, NaCl and KCl were obtained from Merck. EMS and MMC were all obtained from Sigma. KH_2PO_4 and Na_2HPO_4 were all obtained from Roche.

3.3.5 FLOW DIAGRAM SUMMARIZING EXPERIMENTAL PROTOCOL

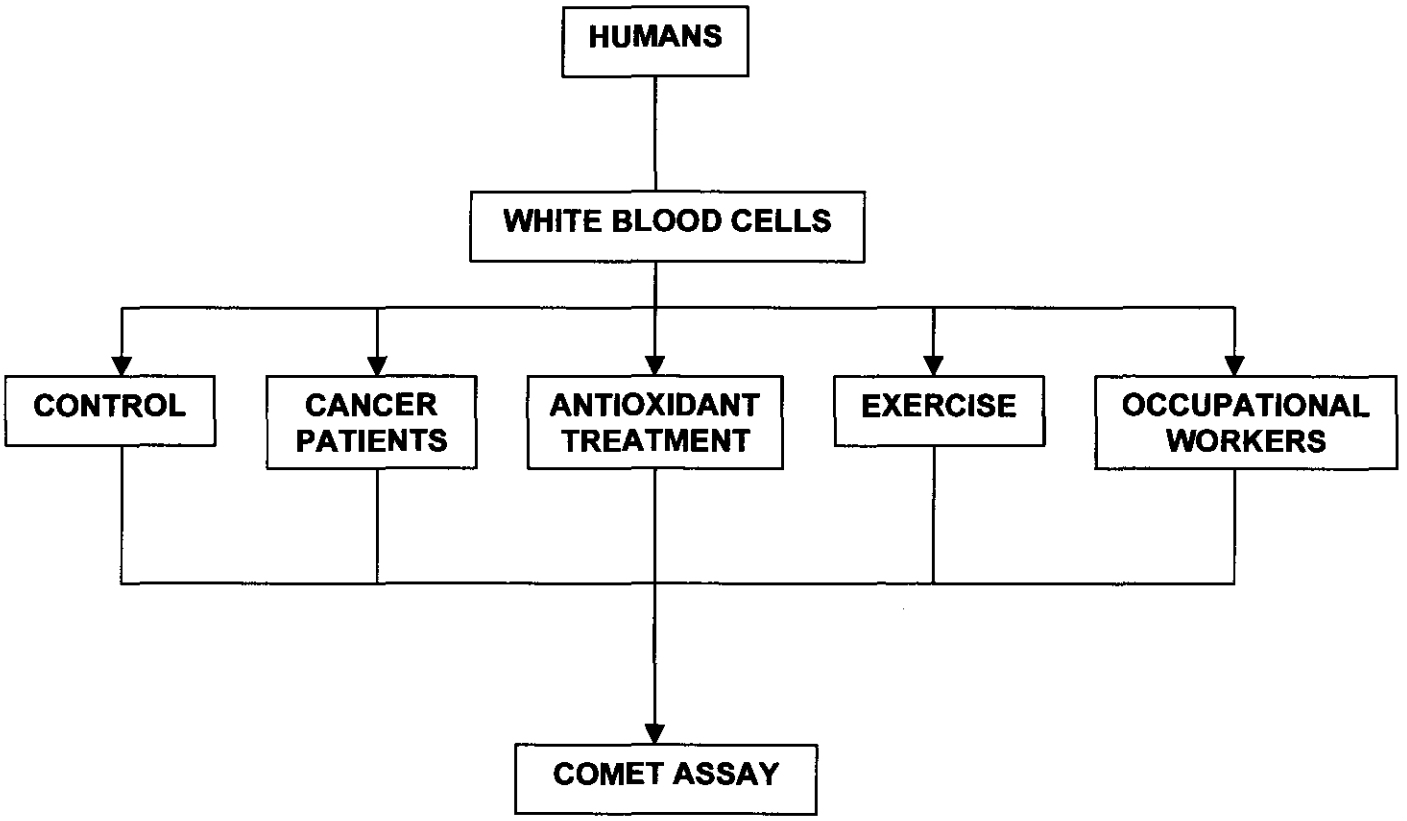


Figure 3.6 Flow diagram summarizing the experimental procedures followed.

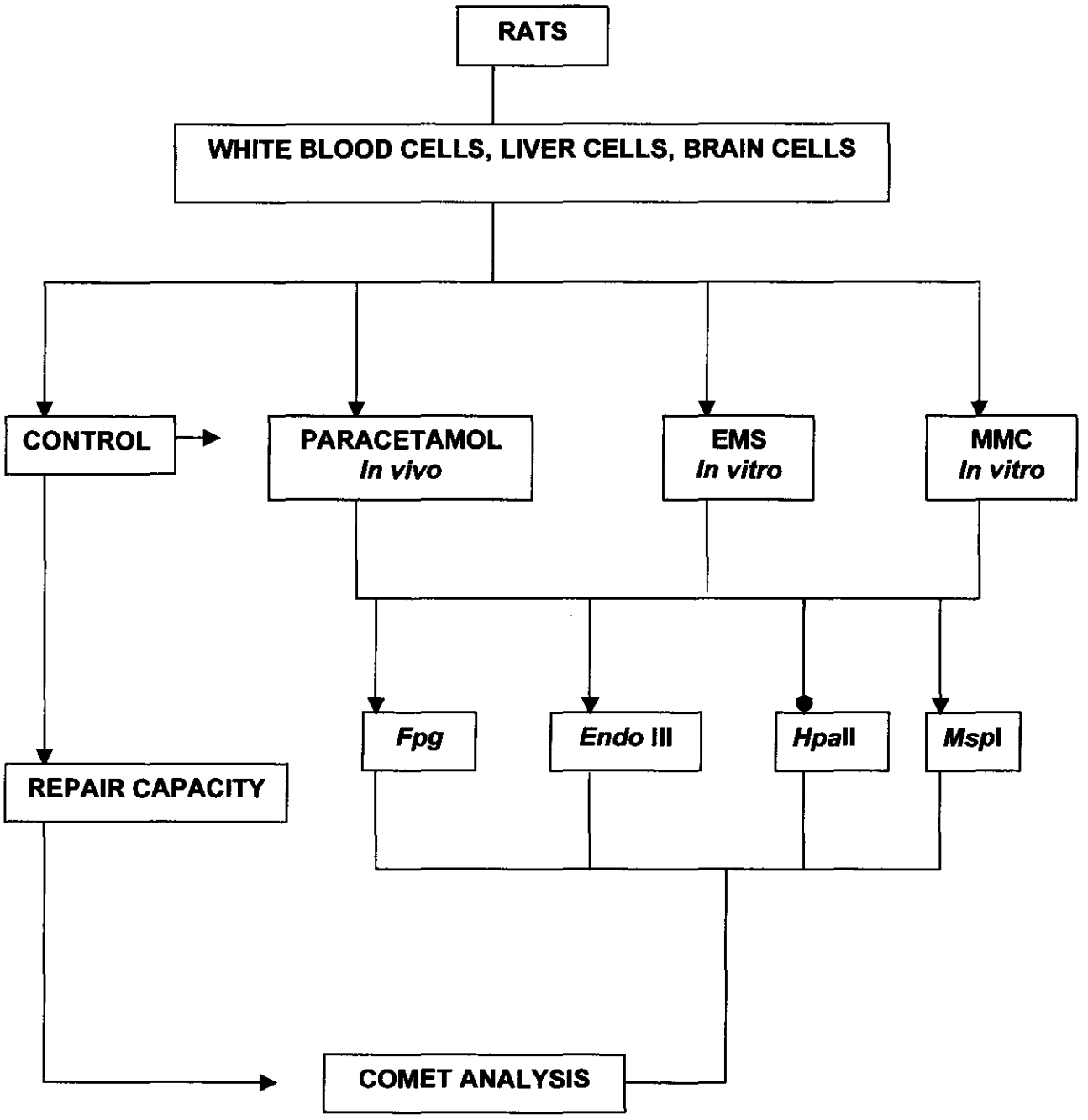


Figure 3.7 Flow diagram summarizing the experimental procedures followed to monitor different DNA lesions in various tissues of the rat.

EMS: Ethylmethanesulfonate; **MMC:** Mitomycin C; ***Fpg*:** Formamidopyrimidine DNA glycosylase; ***Endo*III:** Endonuclease III; ***Hpa*II:** Restriction enzyme isolated from *Haemophilus parainflueanzae* II; ***Msp*I:** Restriction enzyme isolated from *Moraxella sp.*

CHAPTER FOUR

RESULTS AND DISCUSSION

The results on the diversity in applications of the single cell gel electrophoresis (comet assay) have been divided into four different sections. In the first section a discussion of the establishment of the comet assay is given along with all the experimental adjustments to establish this technique in our laboratory. Isolation of cells from different tissues is given in section two, together with the experimental procedures and results obtained. The diverse applications of the comet assays are listed in the third section. The fourth section contains the monitoring of the DNA methylation status of DNA with the comet assay. The use of the comet assay for monitoring the DNA methylation status of single cells is to the best of our knowledge a very novel application.

4.1 ESTABLISHING THE COMET ANALYSIS

Recent reviews on the comet assay for measuring DNA damage and repair (Singh *et al.*, 1988; Tice, 1995 and Collins, 2004) were used as a guideline for the use of the comet assay in our laboratory. A few practical adjustments were made in the described methods to establish the comet assay in our laboratory.

Initially whole blood was used to perform the comet assay. The blood was only washed with PBS to remove the plasma. Although the lyses buffer removed most the cell membranes and other proteins, there was still a lot of background in the pictures obtained (see Fig. 4.1 [a]). This tended to complicate the data analysis and the interpretation of the results. Whole blood samples can still be used for the comet assay, although it would be more reliable to add an extra washing step in the procedure to make the data analysis less complicated. As this was a limitation for the analyses, a method for isolating only the white blood cells with Histopaque® was investigated (see Section 3.1.4 for the methodology). It proved to be successful as there was no background in the pictures (see Fig. 4.1 [b]).

This isolation technique facilitated the interpretation of the data analysis and the validation of the results obtained.

When using blood samples, care must be taken to keep the samples cold but not directly on the ice. This prevents local freezing and unnecessary damage to the DNA that could influence the results. It is often useful to store cells collected during a study, so that it can be analyzed on a later date. However, it is not appropriate in the comet assay to keep fresh blood samples for longer than 24 hours before analysis, as this may cause cells to lyse and cause additional damage to the DNA and interference with the DNA's integrity. Lymphocytes in a medium of PBS without serum but with 10% DMSO can be frozen and then stored at -70°C, or it can be stored in liquid nitrogen for months or even years (Collins, 2004).

Another practical aspect to consider is the amount of LMPA in which the cells are suspended. Initially 50µl of cells were suspended in 300µl of LMPA. This made the visualization of the comet assay very difficult, because there were too many different layers of cells in the gel and this hindered the focusing of the microscope. The use of only 150µl of LMPA along with 50µl cells, made it easier for visualizing and focusing of the microscope. After all these practical adjustments were made, the comet assay was performed with ease and very reproducible in the laboratory and reliable results were obtained.

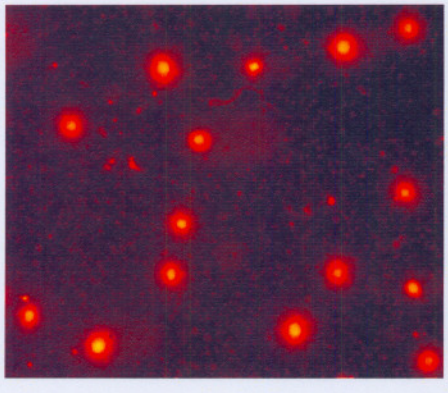
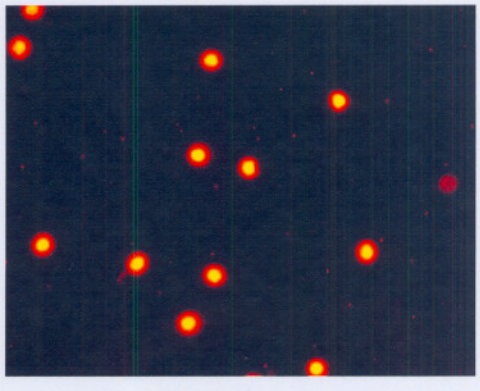
[a]  [b] 

Figure 4.1 A comparison between whole blood cell samples [a] and isolated white blood cell samples [b] after comet assay was performed on control subjects.

4.2 ISOLATION OF CELLS FROM DIFFERENT TISSUES

Many different methods for isolating cells from different tissues have been published (http://www.tissuedissociation.com/techniques.html), but not all the methods are suitable for performing the comet assay. The reason for this is that some of the methods induce DNA damage during cell isolation resulting to false positive results. Many pilot studies were performed to isolate cells for use in the comet assay, before a final way for isolating cells with undamaged DNA were established.

Collagenase catalyses the hydrolysis of collagen and gelatine and is used for the disintegration of tissues and for the isolation of single cells (Balconi and Dejana, 1986). Trial runs were performed with the *collagenase* enzyme to isolate cells from liver, heart and skeletal muscle. The results showed that the amount of cells that were isolated was not enough to successfully carry out the comet assay (see Figure 4.2). The enzyme also caused unnecessary DNA damage. This might have been due to the possible contamination of the *collagenase* with DNAase(s). Therefore, the application of *collagenase* was not suitable in our hands for the isolation of single cells to perform the comet assay.

In collaboration with the Division of Physiology on our campus the isolation of single cells from heart muscle with the Langendorff apparatus was investigated. The hearts were obtained from ether-anaesthetized animals and were perfused on a modified Langendorff apparatus with Ca^{2+}-free Hank's solution, 3% fetal calf serum, 95% O_2, 5% CO_2, at pH 7.4 for 12 minutes. This was followed by the same medium with 0.1% *collagenase* for 12 minutes and finally with enzyme-free medium containing EDTA for 12 minutes (Bishop and Drummond, 1979). This is, however, a time consuming procedure and the results obtained (see Fig. 4.3) were not satisfactory. The amount of single cells isolated was too small and too much DNA damage was induced during this isolation procedure.

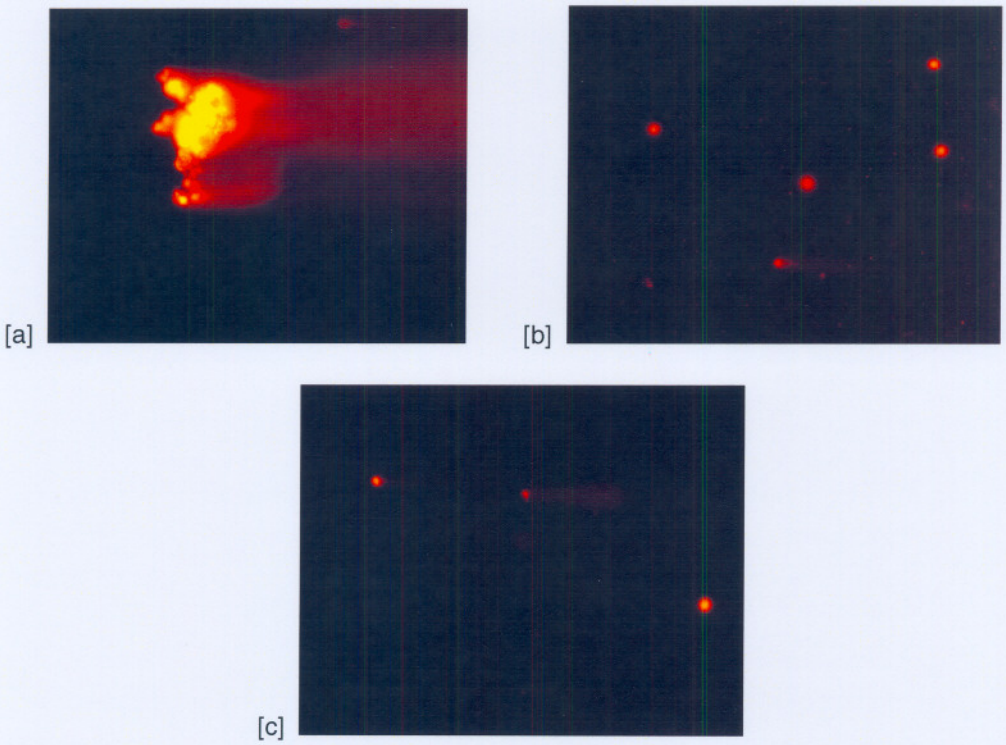


Figure 4.2 Comets after isolation of cells from different tissues [a] heart muscle [b] liver cells [c] skeletal muscle with *collagenase.*

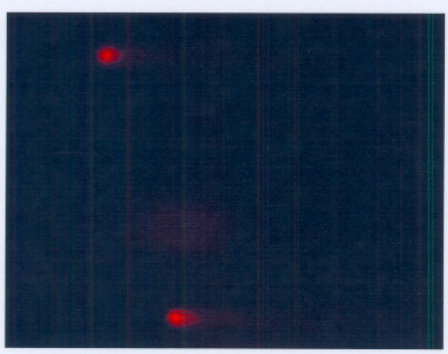


Figure 4.3 Comets after the isolation of heart cells with the Langendorff procedure.

Another method investigated was the manual homogenising of tissues. The tissue was minced into small pieces with a scalpel. The amount of single cells yielded from this method was not enough to perform the comet assay. There was still too much DNA damage induced to the cells during this procedure (see Fig. 4.4). The reproducibility of this method was also problematic. A Potter-Elvehjem homogeniser was tried as an alternative method for the isolation of single cells from tissues. The amount of cells obtained with this procedure was suitable for performing the comet assay, but in the majority of the cells the DNA was damaged (see Fig. 4.4).

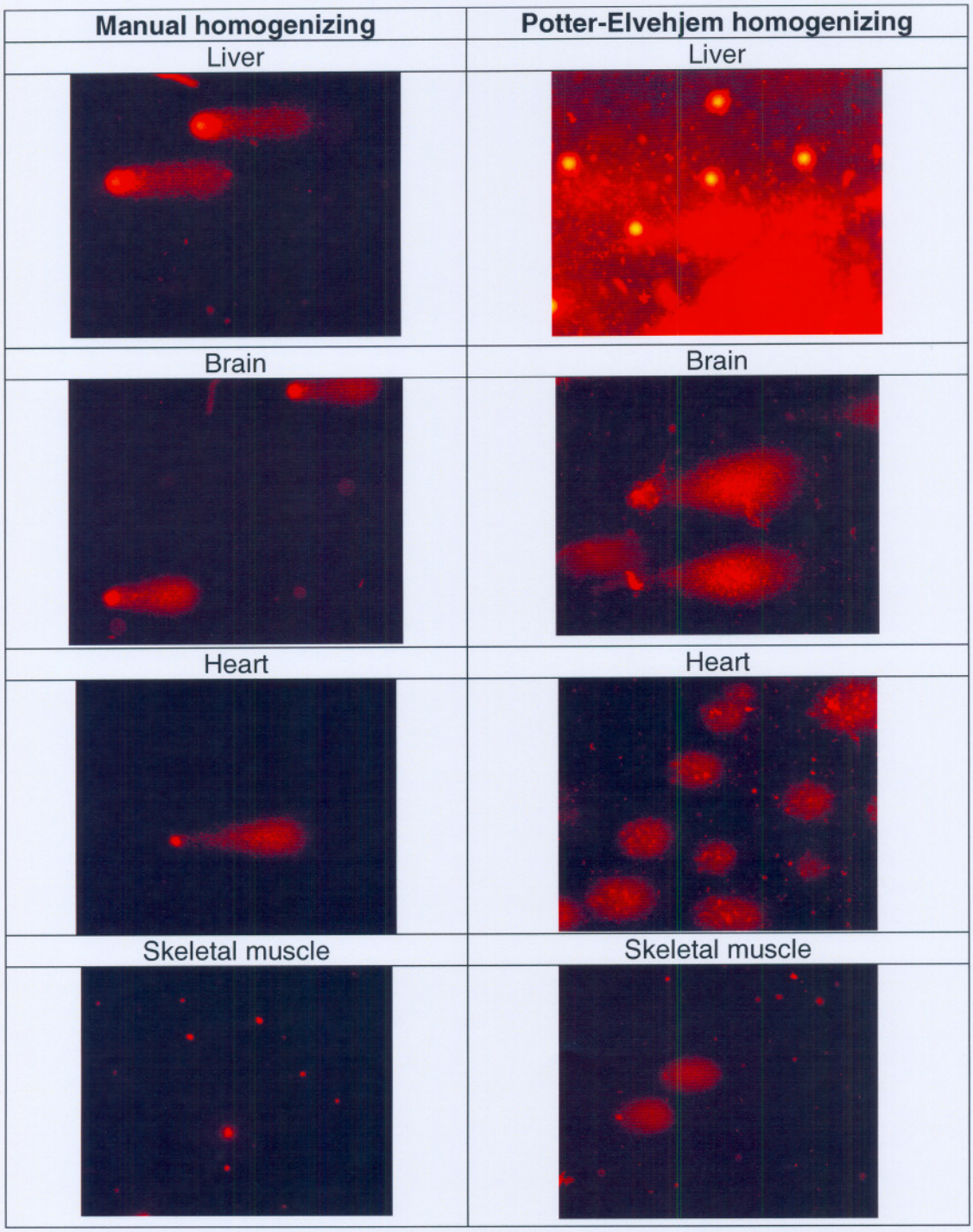


Figure 4.4 Comet obtained with different homogenization methods for cell isolation.

Tice and Vasques (1999) published a protocol for the isolation of single cells from tissues using a mincing solution (Section 3.1.4). Results obtained by using this method proved to be the superior way for isolating single cells from all of the

different tissues that were used in this study (see Fig. 4.5). Sufficient amounts of cells were obtained and the DNA was intact to perform the comet assay. Because of the great success and efficiency with the use of the mincing solution, the isolation of cells from various tissues for this study was done using this method.

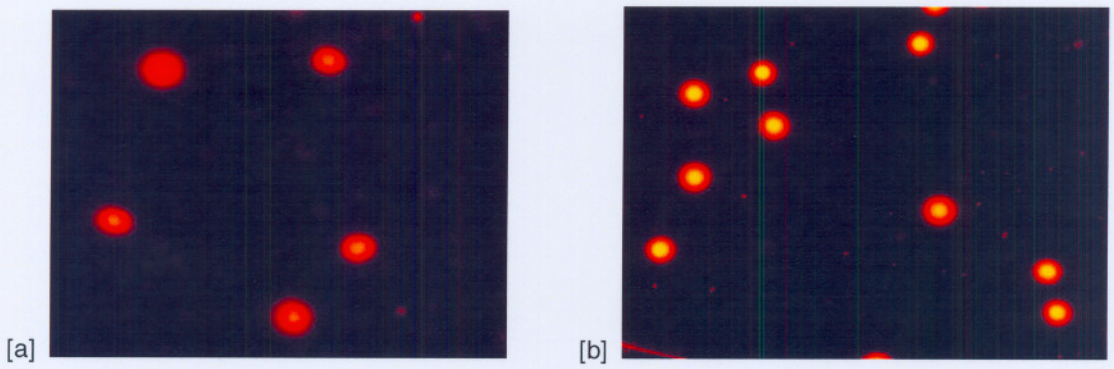


Figure 4.5 Isolation of cells from different tissues [a] brain cells and [b] liver cells in a mincing solution.

The comet assay has already proved to have a broad applicability for the measurement of DNA integrity in different cell types. A group effort was made between the Divisions of Biochemistry and Zoology to attempt the analysis of fresh water crab hemolymph with the use of the comet assay. The results are shown in Fig. 4.6. Cells were suspended in two different solutions (HAMS and PBS) or applied directly. It is apparent from the results that single cells can be obtained from the hemolymph of freshwater crabs. The isolation of the cells from hemolymph was only a pilot study to investigate if it was possible to use the comet assay to detect DNA damage in aquatic animals. The main aim of this pilot study was to validate the comet assay for application on marine animals (Abalone) exposed to pollution. Although there are still some shortcomings in the isolation procedure, e.g. the type of syringe used to obtain the hemolymph and ensuring that the control animals were not exposed to any form of DNA damaging factors that might influence the results. The use of the comet assay to detect DNA damage in freshwater crabs has shown to be possible in our laboratory. There have been some recent studies to link DNA strand breaks in aquatic animals to pollution (Lee and Steinert, 2003). Further

investigation is required before the comet assay can be used as a standard method of pollution in aquatic environments.

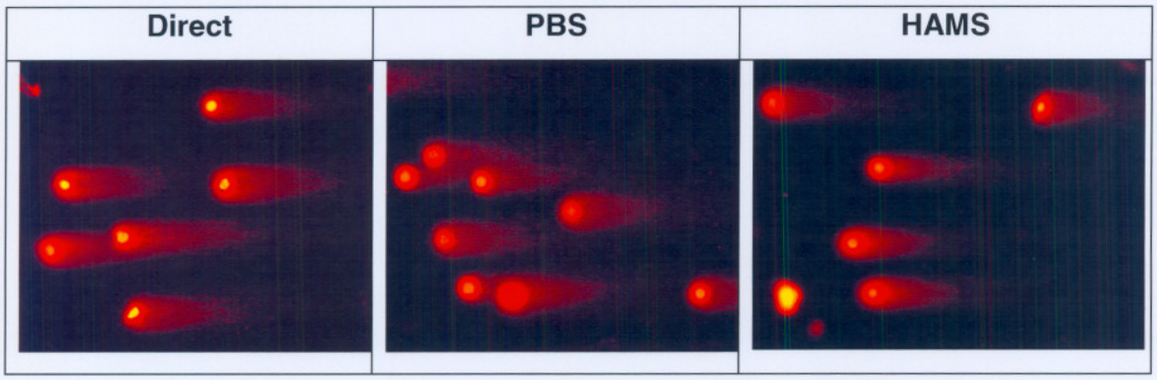


Figure 4.6 Use of the comet assay to measure DNA damage in hemolymph.

4.3 OTHER APPLICATIONS

In this section a diversity of applications of the comet assay as performed in our laboratory is described. The reproducibility of the comet assay is essential to standardize and validate this method. To achieve this, a healthy male volunteer's lymphocytes were analyzed once a week over a two-week period. A breast cancer patient's repair capacity was measured after chemotherapy. The effects of different intensities (60% and 90%) of exercise on DNA strand breaks were monitored in healthy male athletes, (between 40-45 years). The result of one athlete is given. Rat primary hepatocytes were isolated to evaluate the geotaxis effect of *Rosa roxburghii* fruit extract (antioxidant) (for results see Janse van Rensburg *et al.*, 2004). The comet assay was also used to determine the genotoxic effect of the working environment in a chemical plant.

4.3.1. REPRODUCIBILITY OF THE COMET ASSAY

To standardize the comet assay the reproducibility of this assay was monitored. The lymphocytes from a healthy male volunteer were analyzed once a week over a two-week period. In Fig. 4.7 the results of the first week showed a low level of DNA strand breaks in the control and a high level of DNA strand breaks after H_2O_2 treatment. DNA repair took place during the 20 and 40 minutes allowed for repair.

The results obtained were as expected as the subject was not exposed to any DNA damaging agents before the sample was collected. H_2O_2 was applied to the cells to induce damage to the DNA and to monitor the repair capacity. The results of the second week had no statistical difference with the first week's results (Fig. 4.7).

We used the following formula (Equation 4.1) to calculate the DNA repair capacity of an individual.

Equation 4.1. Repair capacity formula

$$Rc = 1 - [\text{average tail DNA } (\%)_{40\text{minutes}} / \text{average tail DNA } (\%)_{H2O2\text{ treatment}}]$$

Rc = repair capacity

A value closer to 1 is an indication of a good repair capacity, while a value closer to 0 is an indication of poor repair capacity. This values serves as an indicator to monitor possible treatments. Every subject serves as its own control.

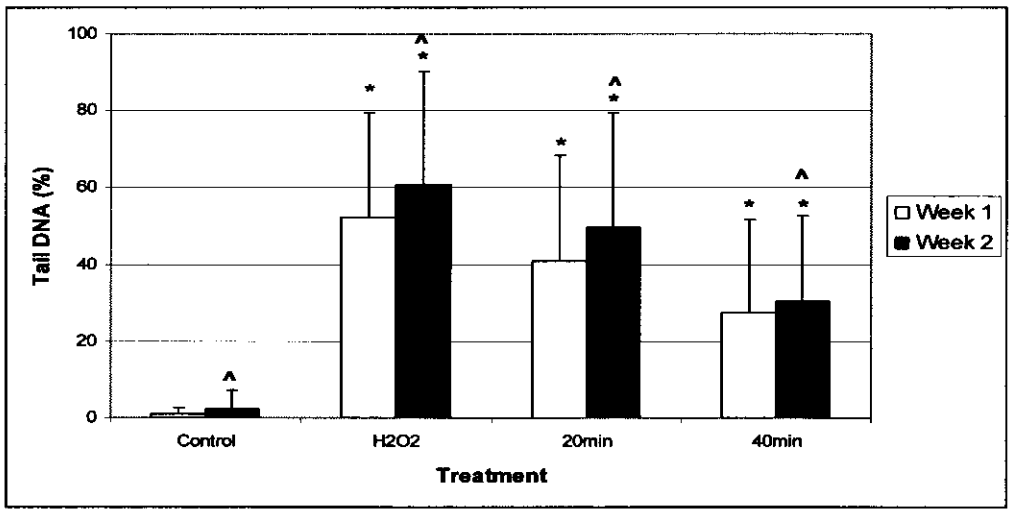


Figure 4.7 Reproducibility of the comet assay. A healthy male volunteer's blood sample [▫] first week and [▪] second week (*p<0.05, ^p>0.05).

The repair capacity was virtually the same over the experimental period, the Rc value for week 1 was 0.673 and for week 2 it was 0.639. It seems safe to conclude that in our hands the comet assay is adequately reproducible.

4.3.2 EVALUATING THE REPAIR CAPACITY AFTER CHEMOTHERAPY

Fig. 4.8 shows the repair capacity in the case of a breast cancer patient soon after chemotherapy. After H_2O_2 treatment the extent of DNA strand breaks increased to 50% compared to the control (0.5%). This is because H_2O_2 is a known oxidizing agent. When comparing the time allowed for repair to take place in the DNA, there is no significant difference between the values at 20 minutes (50% tail DNA) and 40 minutes (49% tail DNA). The breast cancer patient's repair capacity after chemotherapy shows an Rc value of 0.059. There is no significant change in the repair capacity although the baseline level of DNA damage (control, Fig. 4.8) is very low.

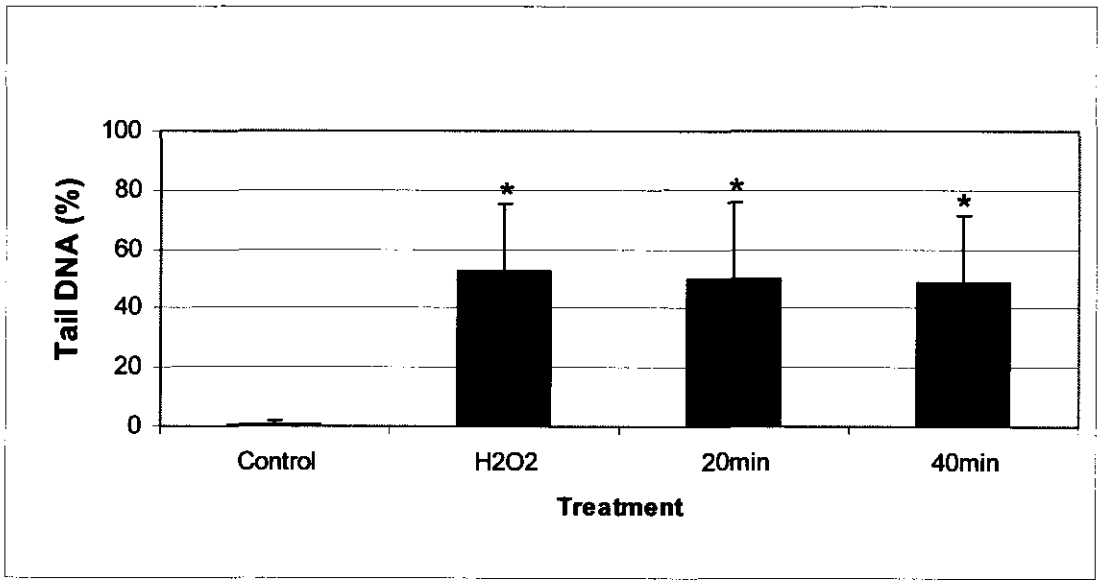


Figure 4.8 DNA damage and repair by lymphocytes of a breast cancer patient (*p<0.05).

A word of caution is necessary: in using the comet assay to measure baseline DNA damage in cancer patients, it seems to be necessary to invoke some kind of damage to the DNA and measure the repair capacity to really get an informative comet assay. A similar caution was recently raised by Wang, *et al* (2005) where they suggest intervention like *Fpg* digestion to obtain more informative baseline DNA damage values. The lack of repair that is taking place in this patient's white blood cells could be contributed by the effect of the chemotherapy on the repair capacity machinery.

4.3.3 THE EFFECT OF EXERCISE ON DNA STRAND BREAKS

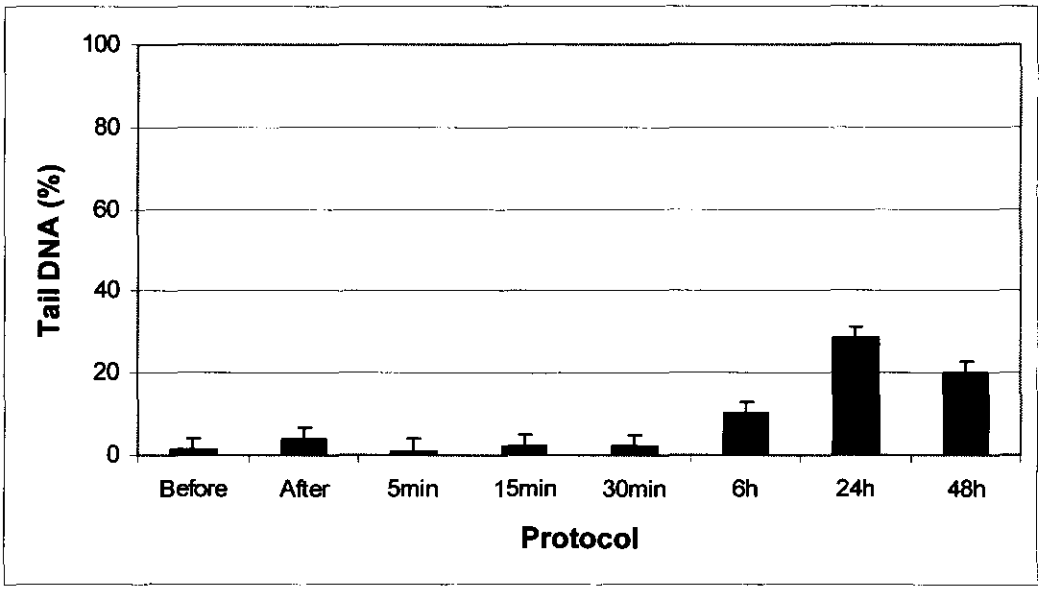
According to collaborators of the Institute for Biokinetics on our campus it is problematic to determine the optimal exercise regimens that must be prescribed to middle aged to elderly people. We suggested using the comet assay in helping to solve this dilemma by measuring DNA damage and repair during a series of exercise programmes, ranging from 60% of the maximum capacity to 90%. In Fig. 4.9 the results are given of a healthy male of 55 years old and only the results of the 60% and 90% of his maximum exercise capacity are given to illustrate the applicability of the comet assay to this discipline. A blood sample was taken immediately before starting the exercise bout and then at various intervals thereafter.

From these results it is clear that an increase in the intensity of exercise results in an increase in DNA damage as measured in white blood cells and also that repair takes longer after the higher intensity exercise. An interesting feature of these results is an apparent biphasic course of the repair process. Although this study is still in progress it looks promising in that it may help to determine the optimal exercise intensity of an individual on a more scientific basis.

4.3.4 MONITOR THE EFFECT OF CHEMICAL EXPOSURE

The comet assay was also used to determine the genotoxic effect of the working environment in a chemical plant. In the case of a control group, which consisted of six healthy males, the average baseline tail DNA % was 5% (Fig. 4.10, [a]). This low level of DNA strand breaks is characteristic of people not exposed to a hazardous chemical environment (Faust *et al.,* 2004). After H_2O_2 treatment of the isolated lymphocytes, the tail DNA % increased to 60%, which is to be expected. In the time allowed for repair, the percentage damaged DNA decreased to 23% after 40

[a]



[b]

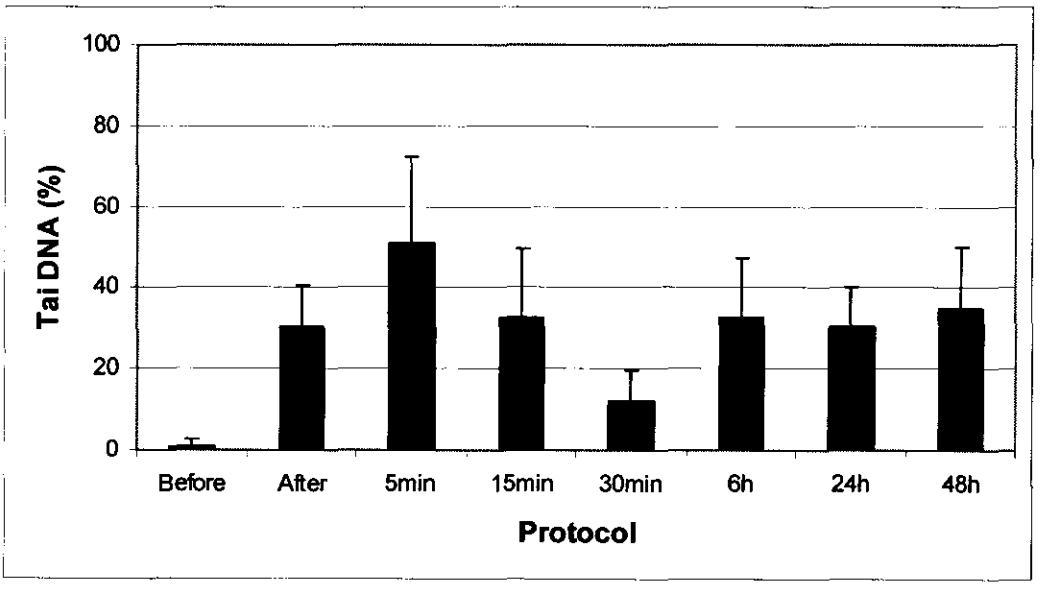


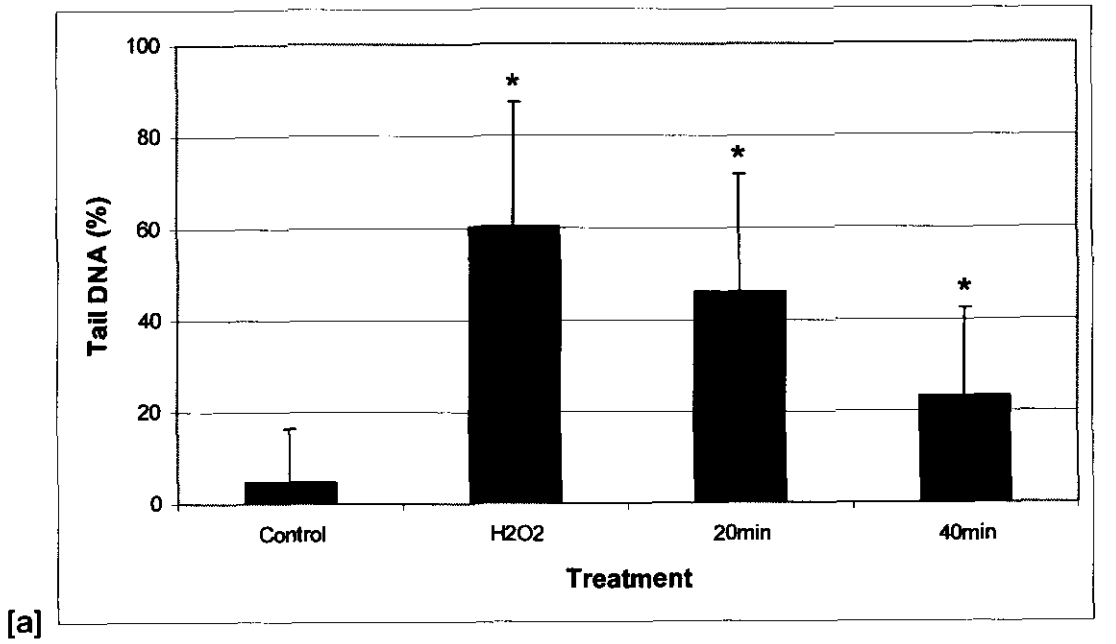
Figure 4.9 An illustration of the effect of different intensities of exercise in a healthy male on DNA damage and repair: [a] 60 % and [b] 90% intensity of exercise.

minutes. Expressed as the repair capacity, calculated from the mean values presented in this figure, a number of 0.617 is obtained which is in the normal to higher range. In the case of the chemical plant workers, the pre-shift baseline tail DNA % is 22% and for the post-shift it is 27% (Fig. 4.10, [b]). These values differ significantly from those of the control subjects and may be ascribed to a long-term exposure of these people to hazardous chemicals and also to too short repair times between shifts. The nutritional habits of these subjects are currently under investigation. The pre-shift value of the repair capacity observed for the chemical plant workers was as low as 0.288 and the post-shift was even slightly lower at 0.207.

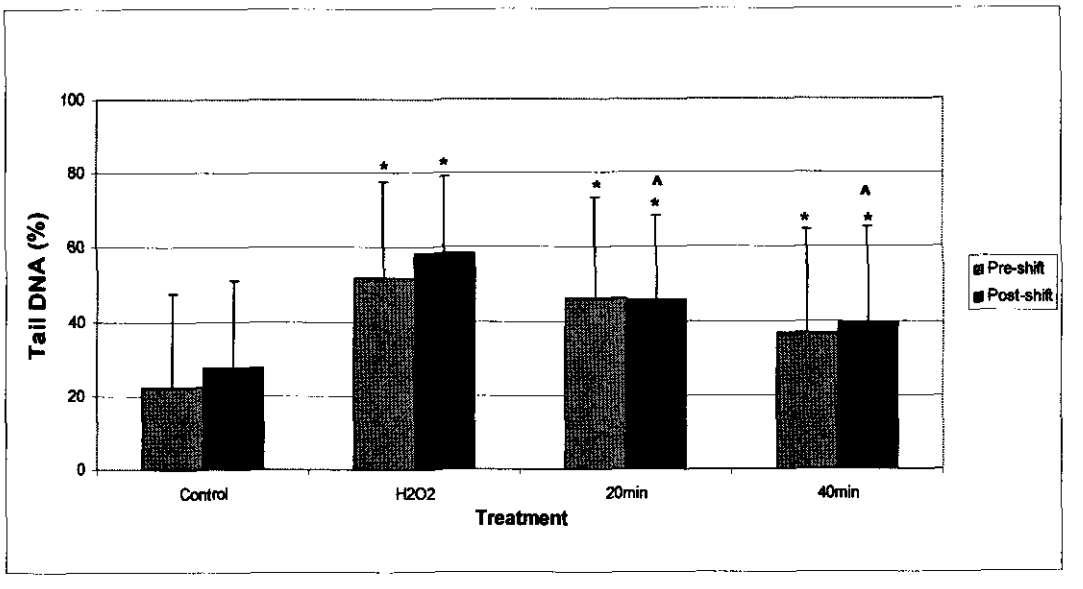
Although this was a pilot study with small numbers of people involved, it emphasizes the value of the comet assay and we as sure that it can be of great value in monitoring any intervention, e.g. dietary supplements.

4.4 COMET ASSAY SUCCESSFULLY ESTABLISHED

The establishment of the comet assay in our laboratory was successfully done using previous reviews of the comet assay (Singh *et al.,* 1988; Tice, 1995 and Collins, 2004) as basic guidelines to set up this method. Minor adjustments were made to ensure the reproducibility of the comet assay in our hands. The establishment of the comet assay allowed us to use this technique to broaden our knowledge on DNA damage and repair in the various applications of this technique.



[a]



[b]

Figure 4.10 DNA damage and repair in chemical plant workers. [a] control subjects [b] people working in a chemical plant (*p<0.05; ^p>0.05).

During setting up of the comet assay, all the apparatus (e.g. electrophoresis chamber) were kept clean and it was ensured that the buffers were not contaminated, for this may adversely influence the results. The slides must be carefully placed in each of the buffers to prevent the gels dislodging from the slides.

It should be noted that when electrophoresis is performed that the slides were evenly distributed in the buffer chamber to allow for a uniform current flow. Consistency was maintained during the performance of the comet assay, e.g. time of lyses, unwinding, neutralization and staining of the DNA on the slides.

The comet assay has become a popular technique on campus and we became involved in a variety of projects from different divisions of the University e.g. Physiology, Biokinetics and Zoology. This is a very strong indication of the versatility and diversity of the comet assay.

A most striking aspect of the results obtained is a very high level of irreparable DNA in the breast cancer patient after chemotherapy (see Fig. 4.8). The lymphocytes from the patient had no ability to repair DNA damage induced by hydrogen peroxide. Another study was published on monitoring breast cancer patients by means of the comet assay (Blasiak *et al.*, 2004).

With an increase in intensity of exercise the DNA damage also increased (see Fig. 4.9). In another paper regarding the effect of endurance exercise on DNA damage (Mastaloudis *et al.*, 2004) using the comet assay the results also showed that with an increase in the intensity of exercise increased the amount of DNA damage.

Exposure to hazardous chemicals is a high risk in most of the human populations (Faust *et al.*, 2004). The comet assay is a promising method for human biomonitoring studies to evaluate the extent of DNA damage with exposure to hazardous chemicals in the working environment. The results obtained (see Fig. 4.10) showed that this method is sufficiently sensitive to detect low levels of DNA damage in human lymphocytes.

4.5 THE MONITORING OF THE METHYLATION STATUS OF DNA WITH THE COMET ASSAY

The methylation status of the DNA was determined by using the methylation enzymes (isoschizomers) *Hpa* II and *Msp* I. These enzymes recognize the sequence

CCGG depending of which cytosine residue is methylated (see Section 3.3.2) and were used in conjunction with the lesion specific enzymes *Fpg* and *Endo* III. This was done to differentiate between oxidative damage to the DNA and changes in the methylation levels of the DNA.

4.5.1 DNA METHYLATION LEVELS IN DIFFERENT CELL PREPARATIONS

Cells (WBC, brain- and liver cells) were isolated from rats (see Section 3.1.4) that were not exposed to any harmful chemicals or environments. The mean of the tail DNA (%) was determined from the total group (four rats). The comet assay was performed on the isolated cells as described in Section 3.1.1. The repair capacity was measured as well as the effect of different enzyme *(Fpg, Endo* III, *Hpa* II and *Msp* I) treatments as described in Section 3.2.2 and 3.2.3. The results are listed in Tables 4.1 - 4.2 and Fig. 4.11 - 4.12.

Table 4.1 Tail DNA (%) in various cell types from control animals

Tail DNA (%)	WBC	brain cells	liver cells	T-test between the different tissues
Control	12 (±13)	3 (±4)	7 (±8)	* # ×
H_2O_2	52 (±26)	32 (±16)	36 (±23)	* # ×
20 minutes	40 (±16)	20 (±11)	28 (±16)	* # ×
40 minutes	38 (±13)	15 (±14)	26 (±10)	* # ×
Fpg	19 (±19)	9 (±9)	31 (±33)	* # ×
***Endo* III**	3 (±8)	8 (±11)	19 (±27)	* # ×
***Hpa* II**	10 (±9)	16 (±11)	24 (±15)	* # ×
***Msp* I**	24 (±12)	16 (±14)	27 (±15)	* ×

* $p<0.05$ (Significant difference between WBC and Brain cells), # $p<0.05$ (Significant difference between WBC and Liver cells), × $p<0.05$ (Significant difference between Brain- and Liver cells)

The extent of DNA strand breaks in isolated WBC, brain and liver cells from control animals was measured with the comet assay (Table 4.1 and Fig. 4.11).

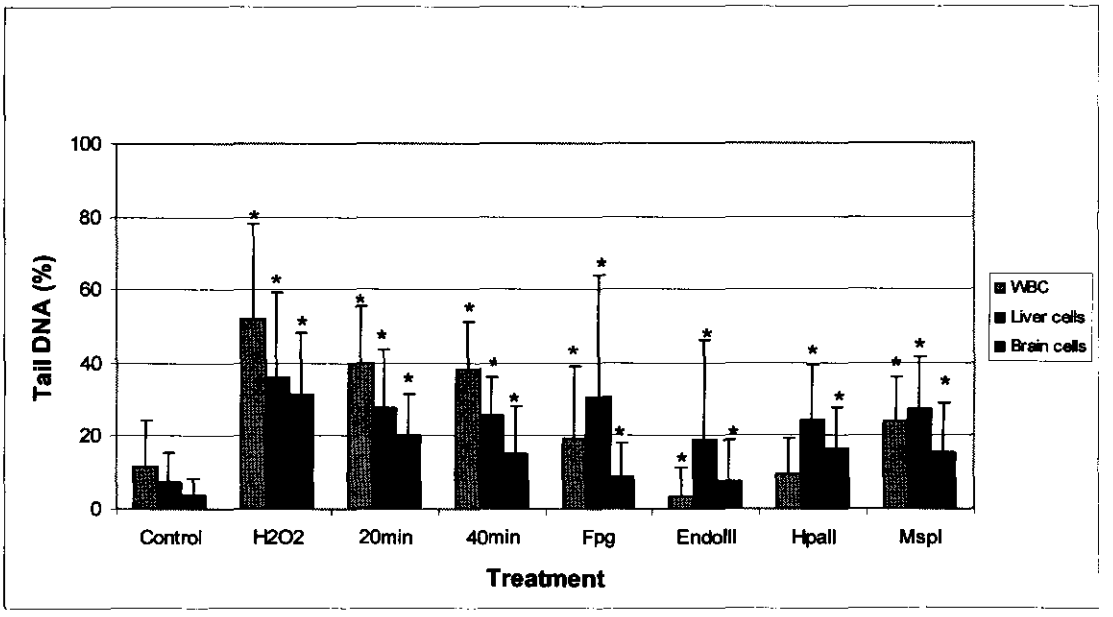


Figure 4.11 DNA damage and repair in single cells isolated from various tissues control animals (*p<0.05).

The tail DNA of all the control cells is below 10%, which is to be expected because nor the animals or the cells were exposed to any DNA damaging agents. An increase of at least 32% tail DNA in all three cells types is observed after the *in vitro* treatment with H_2O_2 of the isolated cells. The WBC shows more DNA damage in comparison with the brain and liver cells and there is a significant difference between each cell types tail DNA (%). The reason for this could be attributed to the relative short life span of lymphocytes (1 day) after leaving the bone marrow in comparison with the life span of liver cells (200 days) (http://www.geocites.com/medinotes/his_liver.html) and brain cells (30-50 years) (http://www.madsci.org.html).

The 20 and 40 minutes (Fig. 4.11) are the time periods allowed for DNA repair after the H_2O_2 was removed. In all three cell types DNA repair is taking place since significant decrease in tail DNA (%) is apparent in Fig. 4.12. In all the three cell types repair does take place, but the repair capacity of each cell type differs (WBC:0.269, liver:0.277 and brain:0.525) The rate of DNA repair differs from each other maybe because of the different functions of each cell type, for example lymphocytes provides protection against infection and disease and may be damaged in this

process; the liver cells filter harmful substances from the blood (such as alcohol and complex chemicals) and the brain is the control centre for virtually every vital activity necessary to survive (Guyton and Hall, 1996). The brain cells have a higher mitochondrial content to produce more energy to detoxify the cells from harmful compound to repair quickly to maintain the major functions of the body. These exposures may hinder the repair processes in lymphocytes and liver cells to a larger extent than in brain cells.

To measure the level of DNA oxidation, the DNA on the slides was digested with *Fpg* and *Endo* III. There is a slight increase in tail DNA (%) after *Fpg* (detects oxidized purines) and *Endo* III treatment (detects oxidized pyrimidines), especially in the liver cells since more substrates are available in the DNA for these enzymes. The liver is the detoxification organ of the body (Guyton and Hall, 1996) and is exposed to more harmful substances (natural/manufactured) and this may result in a higher susceptibility to oxidative stress. The brain has a very fast metabolic rate to remove all the harmful substances (Guyton and Hall, 1996). There is a significant difference between each cell type reaction towards these lesion specific enzymes.

To measure the level of DNA methylation, the DNA on the slides was digested with *Hpa* II and *Msp* I respectively. Comparing *Hpa* II treatment with the baseline values of tail DNA (%) it is evident that only in the liver and brain cells more restriction sites are present. However, for *Msp* I even more restriction sites are present in all three cell types comparing to the baseline values. In the case of only the white blood cells there was an increase in the tail DNA (%) in comparison with *Hpa* II treatment. Both *Msp* Hpa II and I are methylation-sensitive enzymes that cut between cytosine residues at 5'-CCGG-3' site. *Msp* I will not cut if the external cytosine is methylated, and *Hpa* II will not cut if the internal cytosine is methylated. Both *Msp* I and *Hpa* II will cut if the site is unmethylated (Mann and Smith, 1977).

In the different cell types there are 10% (WBC), 16% (brain) and 24% (liver) tail DNA (%) *Hpa* II treatment, and 24% (WBC), 16% (brain) and 27% (liver) with *Msp* I treatment.

The significance of differences in tail DNA (%) between tissues was investigated in Table 4.2. Four statistical tests were applied to analyse the data: one nonparametric (Kurskal-Wallis tests) and three one-way ANOVA (Bonferroni, Duncan's and Unequal N HSD) tests. The incorporation of the results of these statistical tests was used to validate and determine the significance of the data that has been generated.

Most of the different treatments listed in Table 4.2 differ statistically from one another with all four statistical tests. From this data the following becomes evident regarding the DNA methylation levels. The *Hpa* II and *Msp* I enzyme treatment differs slightly but significantly from each other and from the baseline values.

Table 4.2 Statistical processing of the results obtained from the control animals

Kruskal-Wallis Nonparametric (*)/Bonferroni-test (#)/Duncans-test (^)/Unequal N HSD-test (×)																							
	Control			H_2O_2			*20 minutes*			*40 minutes*			*Fpg*			*Endo* III			*Hpa* II			*Msp* I		
	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain
Control				* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^ ×	* # ^ ×	* ^ ×	^	* # ^ ×	^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
H_2O_2	* # ^ ×	* # ^ ×	* # ^ ×				# ^ ×	* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^ ×	* ^	* ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
20 minutes	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	# ^ ×				* ^		* ^	* ^ ×		* ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	^		^	* # ^		* # ^
40 minutes	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^		* ^				* ^ ×	^	* ^ ×	* # ^ ×	* ^	* # ^ ×						
Fpg	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^	* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×		* # ^ ×					# ^ ×		* # ^ ×	^	* # ^ ×	* # ^ ×		* # ^ ×
Endo III	^	* # ^ ×	^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^	* # ^ ×		# ^ ×					* # ^ ×	* ^	* # ^ ×	* # ^ ×	* # ^	* # ^ ×
Hpa II	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^ ×	* # ^ ×	^		^				* # ^ ×	^	* # ^ ×	* # ^ ×	*^	* # ^ ×						
Msp I	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^		* # ^				* # ^ ×		* # ^ ×	* # ^ ×	* # ^	* # ^ ×						

(p<0.05 * # ^ ×)

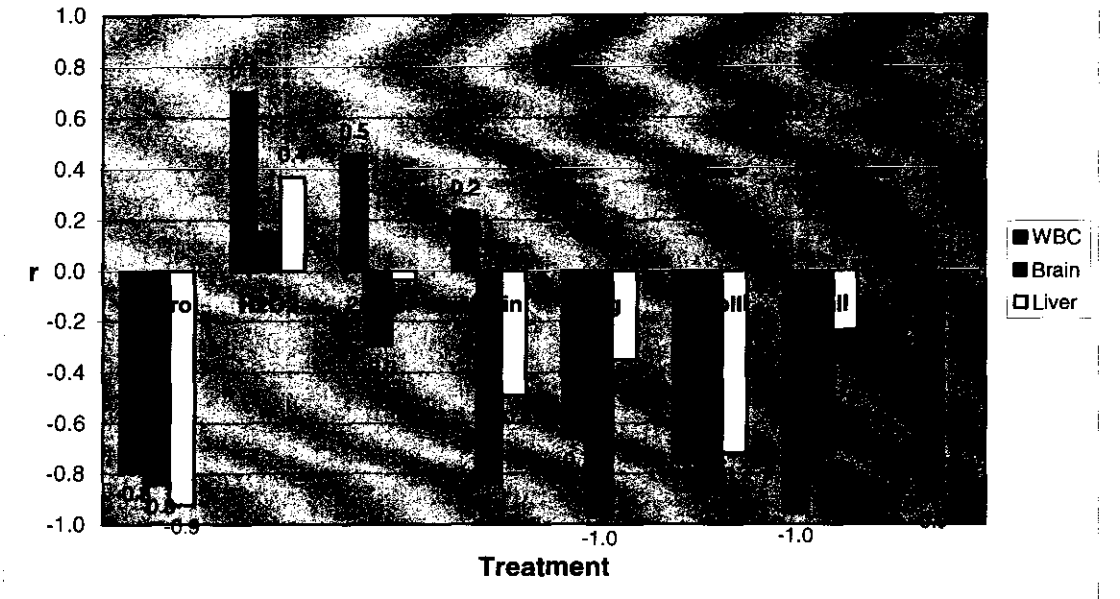


Figure 4.12 Shift in the degree of DNA damage. The Spearman correlation (r) was used to demonstrate the shift between the different classes of DNA damage.

Fig. 4.12 illustrates a shift in the degree of DNA damage [from: -1 (Class 0) to 0 (Class 1-2) to +1(Class 3-4)] with each of the various treatments of the different cell types. The use of the Spearman correlation for analysing the data was performed in order to improve and refine the interpretation of the data generated with the comet assay.

In the control group all the different cell types (WBC, brain and liver) are situated in the -1 region. In this region the extent of DNA damage falls into Class 0 (0-6% DNA damage). This represents baseline DNA damage. A complete shift takes place with H_2O_2 treatment to the +1 region. This extent of DNA damage falls into Class 3-4 (35-100% DNA damage). This shows that DNA damage has occurred with H_2O_2 treatment, which is an oxidizing agent. The WBC was more sensitive to the H_2O_2 treatment in comparison to the liver and brain cells. In the 20 minutes and 40 minutes repair time the values move back towards a more negative region (approximately Class 1-2: 17-35% DNA damage). This indicates that repair has taken place within in the cells.

Fpg treatment shows a value closer to -1 (Class 0) in the WBC and brain cells compared to the liver cells -0.3 (Class 2). This indicates that the liver is more prone to oxidative stress than the other organs. *Endo* III treatment has a value closer to -1 for all the cell types, an indication of virtually no degree of DNA damage. The DNA of the three cell types, after treatment with *Fpg* and *Endo* III, fall into Class 0-1 shows that there was no oxidised substrates for these enzymes to cut. This is an indication that there was no significant oxidative stress in the control animals.

The WBC and brain cells show a strong shift to the -1 (Class 0-1) value with *Hpa* II treatment, where as the liver cells have a shift to the -0.2 (Class 2-3) value. This indicates that the WBC and brain cells have a higher level of methylated DNA sites than that of the liver cells. As the degree of DNA damage falls into the Class 0-1 region, after *Hpa* II treatment, it is an indication that there is a high level of methylated sites on the DNA. Because the methyl group on the cytosine base in the CCGG sequence hinders *Hpa* II from cutting the DNA (*Hpa* II is methylation sensitive). The amount of methylated DNA sites in the liver cells were much less for it was in the Class 2-3 region. The tail DNA (%) increased in the WBC and liver cells after *Msp* I treatment and the correlation shift to 0 (Class 2-3). The brain cells were more towards the -1 region. Methylated DNA sites increase the degree of DNA damage after *Msp* I treatment, because *Msp* I is not methylation sensitive and cuts the methylated cytosine base of the CCGG restriction site. The WBC and liver cells have a Class 2-3 degree of DNA damage after *Msp* I treatment, indicating a higher level of DNA damage than that of the brain cells (Class 0-1).

In conclusion, from these results it can be concluded that the restriction enzymes *Hpa* II and *Msp* I can be used in the comet assay to measure the global methylation levels of DNA in single cells. In this case and in the following work, the use of *Fpg* and *Endo*III shows that DNA methylation can be demonstrated with the comet assay together with other types of DNA damage.

To study whether changes in the methylation levels of single cells could be demonstrated with the comet assay, animals and cells were treated with various chemicals affecting the methylation levels of DNA. These results are reported in the following section.

4.5.2 TREATMENTS AFFECTING THE DNA METHYLATION LEVELS

Various cell types (WBC, liver- and brain) were exposed to different chemical treatments to monitor the effect it might have on the DNA methylation. Using restriction enzymes Hpa II and Msp I monitored the methylation status of the DNA.

4.5.2.1 PARACETAMOL TREATED ANIMALS

The following results were obtained from rats treated with Paracetamol (300mg/kg) daily over a three day period. Cells (WBC, brain- and liver) were isolated from the rats and the comet assay was performed on the cells as described in Section 3.2.1. The repair capacity was measured as well as the effect of different enzyme (*Fpg*, *Endo* III, *Hpa* II and *Msp* I) treatments as described in Section 3.2.2 and 3.2.3. The results are listed in Table 4.3 - 4.4 and Fig. 4.13 - 4.14.

Table 4.3 Tail DNA (%) after Paracetamol treatment

Tail DNA (%)	WBC	brain cells	liver cells	T-test between the different tissues
Control	23 (±11)	5 (±8)	17 (±10)	* # ×
H_2O_2	46 (±23)	24 (±14)	41 (±25)	* # ×
20 minutes	32 (±13)	22 (±14)	28 (±22)	* # ×
40 minutes	29 (±15)	8 (±11)	23 (±8)	* # ×
Fpg	44 (±15)	18 (±6)	31 (±21)	* # ×
***Endo* III**	37 (±16)	5 (±7)	23 (±17)	* # ×
***Hpa* II**	18 (±13)	12 (±18)	5 (±11)	* # ×
***Msp* I**	30 (±12)	44 (±25)	37 (±27)	* # ×

* $p<0.05$ (Significant difference between WBC and Brain cells), # $p<0.05$ (Significant difference between WBC and Liver cells), × $p<0.05$ (Significant difference between Brain- and Liver cells)

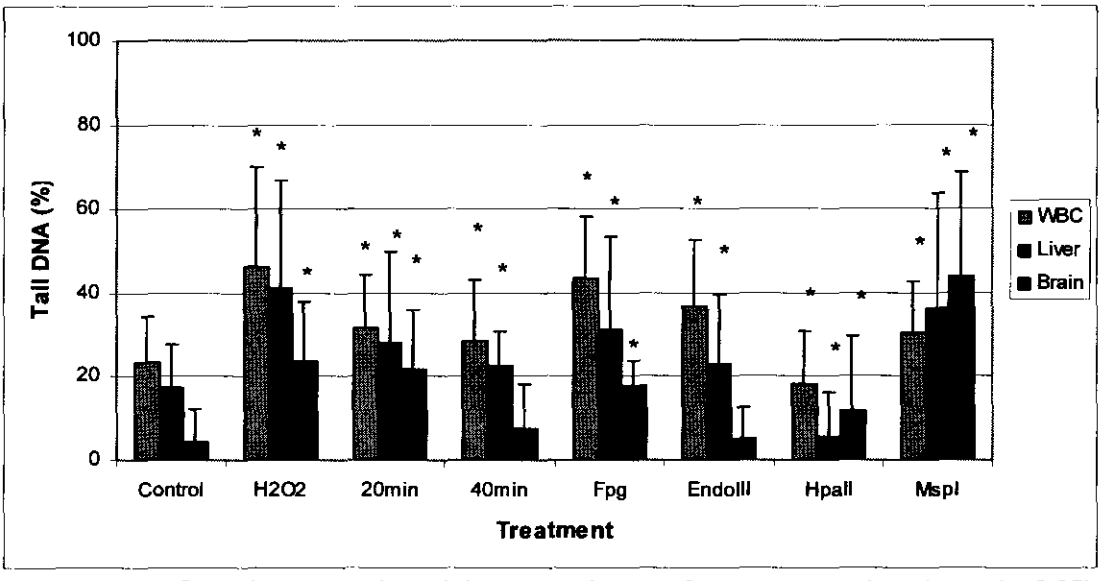


Figure 4.13 DNA damage and repair in various tissues after Paracetamol treatment ($p<0.05$).

The WBC and liver cells in the control group, after *in vivo* treatment with Paracetamol (Table 4.3 and Fig. 4.13), had 23% and 17% tail DNA (%) in comparison with the brain (5%). The increase in the DNA damage indicates that Paracetamol had a slight toxic effect on the WBC and liver cells in comparison with the untreated rats (Fig. 4.11). The WBC and liver cells help with the filtering of blood (Guyton and Hall, 1996) and will therefore be more prone to the damaging effect of Paracetamol. The brain has a faster metabolic rate compared to WBC and liver cells, because of its high mitochondrion concentration, leading to a slow effect of Paracetamol and its metabolites with no immediate influence on the cells (Hinson, 1980).

Exposing the cells to H_2O_2 markedly increased the tail DNA in WBC (46%) and liver cells (41%), but to a lesser extent in the brain (22%). In the entire group of cells the amount of DNA lesions nearly doubled after H_2O_2 treatment. The repair rate of all the cells was approximately the same as for the untreated group of rats (Fig. 4.11). After the 20th minute repair time the DNA damage for each cell type was as follows: WBC (32%), liver cells (28%) and brain cells (24%) and in the 40th minute: WBC (29%), liver cells (23%) and brain cells (8%). The Paracetamol group did show a slightly better repair capacity that the untreated group: WBC-

0.369, liver cells-0.439 and brain cells-0.666. The reason for this could be because Parcetamol has antioxidant characteristics in low non-toxic doses (Black, 1984).

In all three cell types tail DNA (%) increased (WBC-44%; brain-18% and liver 31%) with *Fpg* treatment, which recognises oxidative DNA base damage, in particular 8-OH guanine (Albertini *et al.*, 2000 and Tice *et al.*, 2000). Only the WBC and liver cells showed an effect towards *Endo* III treatment which detects oxidized pyrimidines, WBC with 37% and liver cells with 23% tail DNA. Brain cells showed no effect after the *Endo* III treatment since there was only 5% tail DNA. The difference between the effects in the absence and presence of *Fpg* and *Endo* III were significant. The use of these two lesion-specific enzymes is a useful modification of the comet assay because it increases its sensitivity and its specificity. *Fpg* and *Endo* III are widely used for the detection of various forms of DNA damage (Speit *et al.*, 2004).

In the results obtained after *Hpa* II and *Msp* I treatment, a strong indication towards increased methylation levels after treatment with Paracetamol can be observed. Treatment with *Hpa* II showed the following: WBC-18%; brain cells-12% and liver cells- 5% tail DNA. This low level of tail DNA (%) is an indication of the presence of methylated DNA bases because when the cytosine base of the CCGG sequence is methylated, it prevents *Hpa* II from cutting the DNA. The *Msp* I treatment showed elevated levels of DNA strand breaks in all the cell types (WBC-30%, brain cells-44% and liver cells -37%) indicating that there were many restriction sites for *Msp* I as *Msp* I is not methylation sensitive. Therefore the results in Figure 4.14 show that administration of Paracetamol to the rats increased the methylation levels of DNA in the cells and that this phenomenon can be illustrated with the comet assay.

The same statistical tests were used in Table 4.4 as was used previously. For this study the most significant difference we are interested in are for the *Hpa* II and *Msp* I. From this data the following becomes evident regarding the DNA

methylation levels: The relative large difference in tail DNA (%) after *Msp* I treatment relative to *Hpa* II treatment is indicative of the higher level of DNA methylation in especially the liver and brain cells. This phenomenon is more pronounced when the results depicted in Fig. 4.13 is compared to that of the untreated animals (Fig. 4.11).

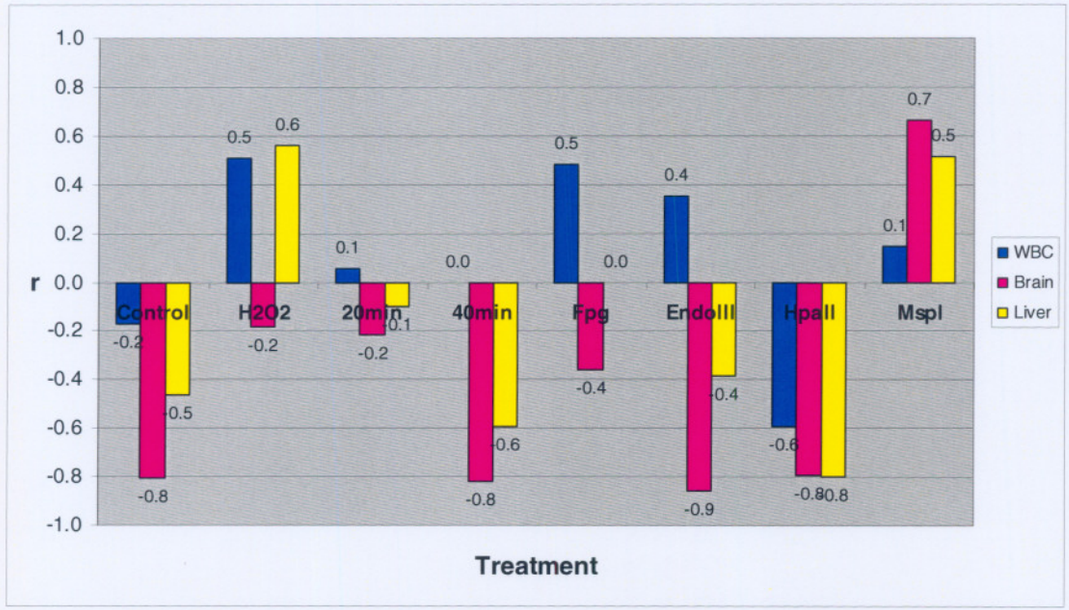


Figure 4.14 Shift in the degree of DNA damage of Paracetamol treated rats. The Spearman correlation (r) was used to demonstrate the shift between the different classes of DNA damage.

Fig. 4.14 illustrates the shift in the degree of DNA damage [from: -1 (Class 0) to 0 (Class 1-2) to +1 (Class 3-4)] with each of the various treatments of the different cell types after the rats were dosed with Paracetamol. Each of the cell types responded differently on the Paracetamol treatment when compared with each other. In the control group the WBC were more prone to the effect of Paracetamol treatment for it had a value of -0.2 (Class 2-3: 17-60% tail DNA). The liver cells showed a lower value of -0.5 (Class 1-2: 6-17% tail DNA) and the brain cells displayed almost no levels of DNA damage for it had a value of -0.8 (Class 0: 1-6% tail DNA).

Table 4.4 Statistical processing of the results obtained from the Paracetamol treated animals

Kruskal-Wallis Nonparametric (*)/Bonferroni-test (#)/Duncans-test (^)/Unequal N HSD-test (×)																								
	Control			H_2O_2			*20 minutes*			*40 minutes*			*Fpg*			*Endo* III			*Hpa* II			*Msp* I		
	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain
Control				* # ^ ×	^	* # ^ ×	^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×		* # ^	* # ^ ×	* ^ # ×	* # ^ ×	* # ^ ×		# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
H_2O_2	* # ^ ×	# ^	* # ^ ×				* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×		# ^		* # ^ ×	# ^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	# ^ ×	# ^ ×
20 minutes	^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×							* # ^ ×	* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×			# ^ ×
40 minutes	* # ^ ×	# ^ ×		* # ^ ×	^	* # ^ ×		* # ^ ×	* # ^ ×				* # ^ ×		* #	^			* # ^ ×	* # ^ ×			# ^ ×	* # ^ ×
Fpg	* # ^ ×	* # ^ ×	* # ^ ×		# ^ ×		* # ^ ×	* # ^ ×		* # ^ ×		* #				* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	#	* # ^ ×	^	* # ^ ×
Endo III	* # ^ ×	^		* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	* ^ ×	^	* ^		* # ^ ×	* # ^ ×	* # ^ ×		* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	#	* # ^ ×	* # ^ ×	* # ^ ×
Hpa II	# ^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×			* # ^ ×	* # ^ ×	#	* # ^ ×	* # ^ ×	#				* # ^ ×	* # ^ ×	* # ^ ×
Msp I	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	# ^ ×	# ^ ×	* # ^		# ^ ×			* # ^ ×	* # ^ ×	^	* # ^ ×	* # ^ ×	^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×			

(p<0.05 * # ^ ×)

For all three the cell types, the DNA strand breaks increased with H_2O_2 treatment, WBC: +0.5 (Class 4), brain: -0.2 (Class 2-3) and liver: +0.6 (Class 4). This increase in DNA strand breaks is due to the oxidizing effect of H_2O_2 on the DNA. The degree of DNA damage decreased significantly over the 20 and 40 minutes set out for repair, especially in the liver (-0.1 and -0.6) and brain (-0.2 and -0.8) cells compared to the WBC (+0.1 and 0).

Treatment with *Fpg* increased the degree of DNA damage in all three cells types, WBC +0.5 (Class 4: 60-100% tail DNA), brain cells -0.4 (Class 1-2: 17-35% tail DNA) and liver cells 0 (Class 2-3:17-60% tail DNA). The increasing degree of DNA damage with *Fpg*, is because of high levels of oxidized purines sites on the DNA. *Endo* III treatment showed a slight elevation in the degree of DNA damage in the WBC (+0.4: Class 3) and liver cells (-0.4: Class 2) but no significant increase in DNA damage was detected in the brain cells (-0.9: Class 0). The treatment of cells with *Endo* III helped with the detection of oxidized pyrimidines, therefore in the WBC and liver cells the level of oxidized pyrimidines increased with Paracetamol treatment, but no significant increase was detected in the brain cells.

After the DNA on the slides were treated with *Hpa* II the WBC shifted to -0.6 (Class 2: 17-35% DNA damage) while the brain and liver cells were at -0.8 (Class 0: 0-6% DNA damage). All the cell types have a negative value indicating that the degree of DNA damage after *Hpa* II treatment was not very significant. This low level of DNA damage after *Hpa* II treatment indicates towards the presence of methylated DNA bases on the DNA string, especially if it is compared to the *Msp* I results. Treatment with *Msp* I displayed high levels of DNA damage in the WBC +0.7 (Class 4: 60-100% tail DNA), liver cells +0.5 (Class 3: 35-60% tail DNA) and brain cells +0.1 (Class 2-3: 17-60% tail DNA). As *Msp* I is not methylation sensitive as *Hpa* II is it will therefore cut the DNA when it is methylated.

As mentioned before, the use of these two restriction enzymes is a useful modification of the comet assay because it increases both its sensitivity and its specificity towards the detection of methylated DNA bases after an *in vivo* insult to the DNA in various cell types.

This result is rather surprising since it was shown that Paracetamol intoxication causes depletion of glutathion and eventually of the erosion of methylating capacity of a cell (Lertratanangkoon *et al*, 1997; James, *et al*, 2003). In this case Parcetamol apparently acted as an antioxidant (Black, 1984) and the observed increased methylation of DNA may be linked to this phenomenon but we have no proof for this speculation.

4.5.2.2 EMS TREATED CELLS

The following results were obtained from a group of four rats and exposure of their isolated WBC, brain- and liver cells to EMS, a DNA alkylating agent (Monleton, 1998). The comet assay was performed on the cells as described in Section 3.2.4 along with the various enzyme treatments as described in Sections 3.2.4 and 3.2.3. The results are listed in Table 4.5 - 4.6 and Fig. 4.15 - 4.16.

Table 4.5 Tail DNA (%) after exposure to EMS

Tail DNA (%)	WBC	brain cells	liver cells	T-test between the different tissues
Control	8 (±6)	4 (±3)	4 (±3)	* #
EMS	25 (±14)	15 (±10)	38 (±22)	* # ×
EMS+*Fpg*	9 (±4)	10 (±7)	12 (±10)	# ×
EMS+*Endo* III	9 (±3)	7 (±4)	10 (±6)	* # ×
EMS+*Hpa* II	22 (±18)	9 (±9)	11 (±7)	* # ×
EMS+*Msp* I	23 (±21)	32 (±18)	19 (±15)	* ×

* $p<0.05$ (Significant difference between WBC and Brain cells), # $p<0.05$ (Significant difference between WBC and Liver cells), × $p<0.05$ (Significant difference between Brain- and Liver cells)

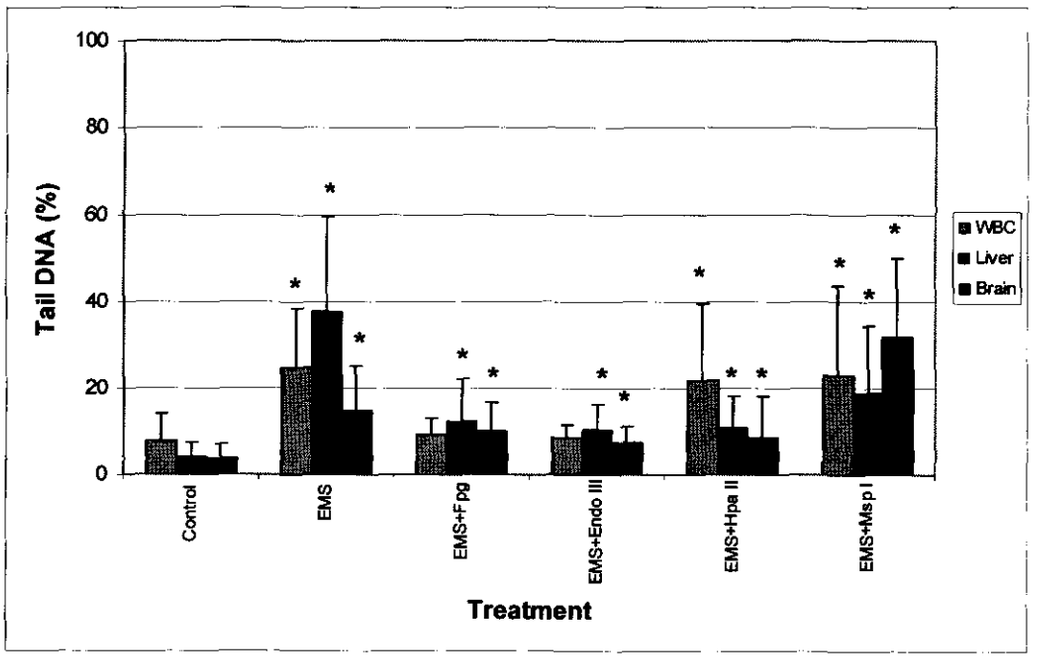


Figure 4.15 The effect of *in vitro* EMS treatment on the tail DNA (%) of the various cell types (*p<0.05).

As expected, there was little baseline damage (less than 10%) in all the control groups (Table 4.5 and Fig. 4.15). After 60 minute incubation with EMS there was a significant increase in DNA strand breaks in all the cell types (WBC-25%, brain-15% and liver-38%). This is because EMS is a well-known alkylating agent that reacts with bases and adds mainly ethyl but also methyl groups to DNA (Montelon, 1998). The detection of DNA damage through the application of *Fpg* and *Endo* III ranged between 7-12% for all the different cell types, which is not a significant difference from the control. These results show that there was no high level of oxidative stress induced by EMS.

In comparison with *Hpa* II treatment, *Msp* I treatment increased the tail DNA (%) in all the cell types investigated. This result illustrates that EMS did indeed add methyl residues to the DNA an observation that corresponds with the results presented in Table 4.1 and Fig. 4.11 and also to observations described in the literature (Lawely, 1974).

The same statistical tests were applied in Table 4.6 as was used previously. Comparing with the results given in Fig. 4.12, the most interesting information for us was the differences between the *Hpa* II and *Msp* I treatments of the DNA: the *Hpa* II and *Msp* I enzyme treatment differs significantly from each other and from the baseline values. Again, in the liver and brain cells the level of DNA methylation was higher relative to the lymphocytes.

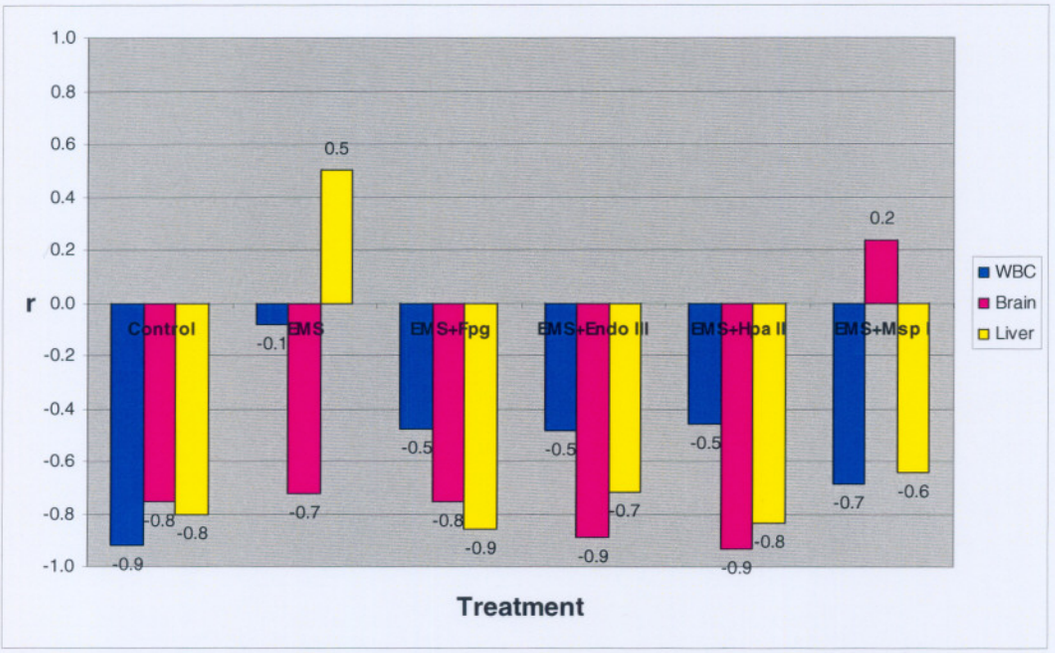


Figure 4.16 Shift in the degree of DNA damage of EMS treated rats. The Spearman correlation (r) was used to demonstrate the shift between the different classes of DNA damage.

Fig. 4.16 illustrates the shift in the degree of DNA damage [from: -1 (Class 0) to 0 (Class 1-2) to +1 (Class 3-4)]. In the control group, all the different cell types (WBC, brain and liver) are situated in the -1 region. In this region the extent of DNA damage falls into Class 0 (0-6% tail). A complete shift takes place with the EMS treatment to the more +1 region for the liver cells (+0.5). This extent of DNA damage falls into Class 3-4 (35-100% tail DNA). The WBC shifted to the 0 region (-0.1), which represents Class 2-3 (17-60% tail DNA), while the brain cells showed no significant shift after EMS treatment. This indicates that DNA damage was induced during EMS incubation. The WBC and liver cells were more sensitive to the effect of EMS in comparison to the brain cells.

Table 4.6 Statistical processing of the results obtained from the EMS treated group

Kruskal-Wallis Nonparametric (*)/Bonferroni-test (#)/Duncans-test (^)/Unequal N HSD-test (×)																		
	Control			*EMS*			*Fpg*			*Endo* III			*Hpa* II			*Msp* I		
	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain
Control				* # ^ ×	* # ^ ×	* # ^ ×		* # ^ ×	# ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
EMS	* # ^ ×	* # ^ ×	* # ^ ×				* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
Fpg		* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×						* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	# ^ ×	* # ^ ×
Endo III		* # ^ ×	* # ^ ×	* # ^ ×	* # ^×	* ^							* # ^ ×			* # ^ ×	# ^ ×	* # ^ ×
Hpa II	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×			* # ^ ×						* # ^ ×	* # ^ ×	* # ^ ×
Msp I	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×			

(p<0.05 * # ^)

Fpg treatment shows a closer value to -1 (Class 0) in the brain and liver cells compared to the WBC cells -0.5 (Class 2). *Endo* III treatment showed similar results as that of *Fpg*. These results can be interpreted that EMS does not cause high levels of strand breaks in the different cells types.

The results given in Fig. 4.16 must be interpreted in conjunction with that of the controls presented in Fig. 4.12. The brain (-0.9) and liver (-0.8) cells had no significant shift in DNA damage with *Hpa* II treatment. The extent of DNA damage in both cells types were between 0-6% (Class 0), whereas the WBC cells had a shift to -0.5 (Class 2-3: 17-60% tail DNA). This indicates that the brain and liver cells had less DNA damage hence lower methylation levels than that of the WBC cells indicated by *Hpa* II treatment. *Msp* I treatment shifted the extent of DNA damage in the case of the brain cells to a value of +0.2 (Class 3-4: 35-100% tail DNA), liver cells shifted to -0.6 (Class 2-3: 17-60% tail DNA) and the WBC had a value of -0.7 (Class 1-2: 6-35% tail DNA). Brain cells seem to be more prone to DNA methylation in comparison with the liver and WBC, which showed a lower level of methylation induced by EMS. However, it seems that the WBC and liver cells are more prone to DNA damage than the brain cells.

4.5.2. MMC TREATED CELLS

The following results were obtained from exposing WBC, brain- and liver cells to MMC. The comet assay was performed on the cells as described in Section 3.2.4 along with the various enzyme treatments as described in Sections 3.2.2 and 3.2.3. The results are listed in Table 4.7 - 4.8 and Fig. 4.17 - 4.18. As in the previous section on EMS treatment of the isolated cells, these results must be looked at in relation to those given in Fig. 4.11 and 4.12 for the control cells.

Table 4.7 Tail DNA (%) after MMC treatment

Tail DNA (%)	WBC	brain cells	liver cells	T-test between the different tissues
Control	6 (±8)	6 (±7)	8 (±7)	
MMC	28 (±14)	18 (±10)	10 (±14)	* #
MMC+*Fpg*	40 (±17)	26 (±15)	15 (±15)	# ×
MMC+*Endo* III	14 (±19)	29 (±14)	14 (±16	# ×
MMC+*Hpa* II	32 (±13)	6 (±10)	10 (±8)	* # ×
MMC+*Msp* I	42 (±15)	12 (±10)	10 (±13)	* #

p<0.05 (Significant difference between WBC and Brain cells), # p<0.05 (Significant difference between WBC and Liver cells), × p<0.05 (Significant difference between Brain- and Liver cells)

As seen In Table 4.7 (graphical presentation of the results in Fig. 4.17), the control group of cells did not show high levels of tail DNA (WBC-6%, brain-6%and liver-8%) for they were not subjected to any harmful substances before isolation.

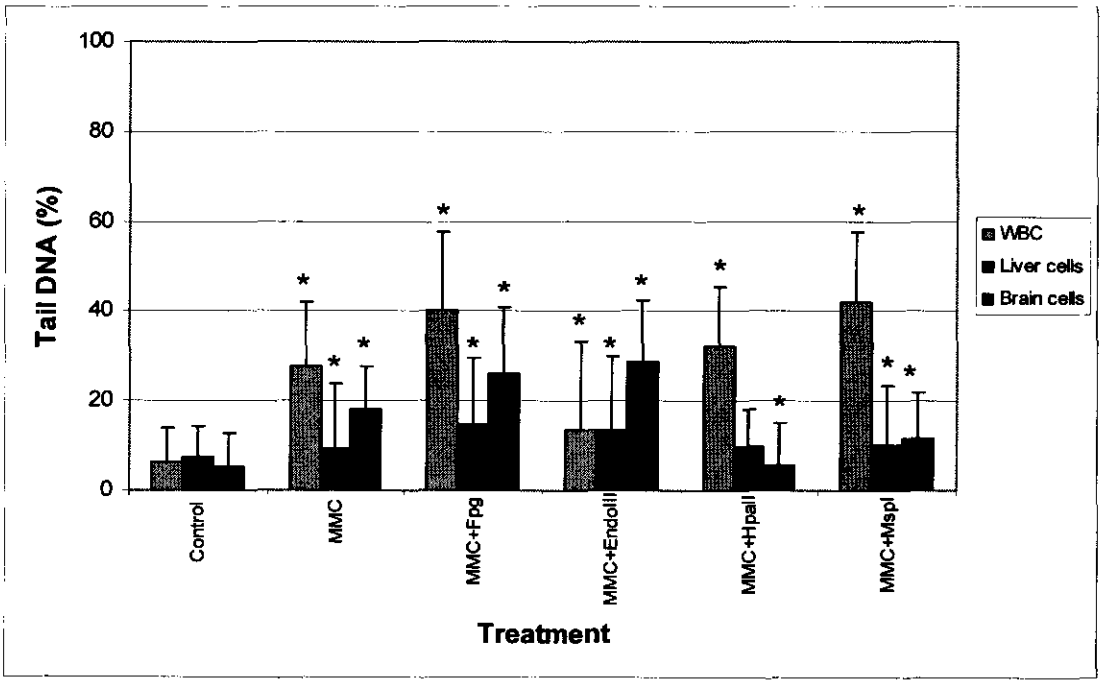


Figure 4.17 The *in vitro* effect of MMC treatment on the tail DNA (%) of various cell types (*p<0.05).

After incubation with MMC the level of DNA strand breaks increased in the WBC to 28%, brain cells to 18% and only a slight increase in the liver cells to 10%. This increase in DNA strand breaks is because MMC is a known DNA cross-linker as well as an oxidizing agent of DNA (McKenna *et al.,* 2003). The WBC and brain cells responded more to the MMC treatment in comparison to the liver.

The results obtained with *Fpg* and *Endo* III treatment strongly suggest an oxidative damaging effect of MMC on the DNA. There is a significant increase in tail DNA (%) in the WBC (*Fpg*- 40% and *Endo* III-14%), brain cells (*Fpg*- 26% and *Endo* III-29%) and the liver cells (*Fpg*- 15% and *Endo* III-14%) in comparison with the control group and after MMC incubation. The results show that these lesion specific enzymes enhance the sensitivity of the comet assay for the detection of the effect of oxidative stress on DNA in the different cell types.

As mentioned before in Section 3.3.2, *Hpa* II and *Msp* I are indicators of methylated DNA bases, therefore if any methylation of the DNA sites had taken place, *Msp* I would cut the DNA at the methylated bases and will result in an increased DNA damage which will be reflected in an increase in tail DNA (%). Whereas *Hpa* II will be blocked by methylated bases and prevent DNA strand breaks. In the brain and liver cells, no significant methylation had taken place after incubation with MMC and digestion of DNA with *Hpa* II and *Msp* I, since the extent of DNA damage for these two cell types was between 6-10% for *Hpa* II and 10-12% for *Msp* I. Without an increase in DNA strand breaks with *Msp* I and a decrease in DNA strand breaks with *Hpa* II, no methylation had taken place. Interestingly, the amount of DNA strand breaks increased significantly in the WBC to 42% after *Msp* I treatment and to 32% after *Hpa* II treatment. This strongly indicates that methylation has taken place in the WBC after the MMC incubation period. WBC therefore, is more prone to DNA methylation than brain and liver cells after treatment with MMC.

The same statistical tests were used in Table 4.8 as was used previously. We have the greatest interest in the differences between the results obtained with *Hpa* II and *Msp* I. From this data, the following becomes evident regarding the DNA methylation levels. Contrary to the effect of the previously described interventions, only in the WBC a slight increase in DNA methylation was observed in this case.

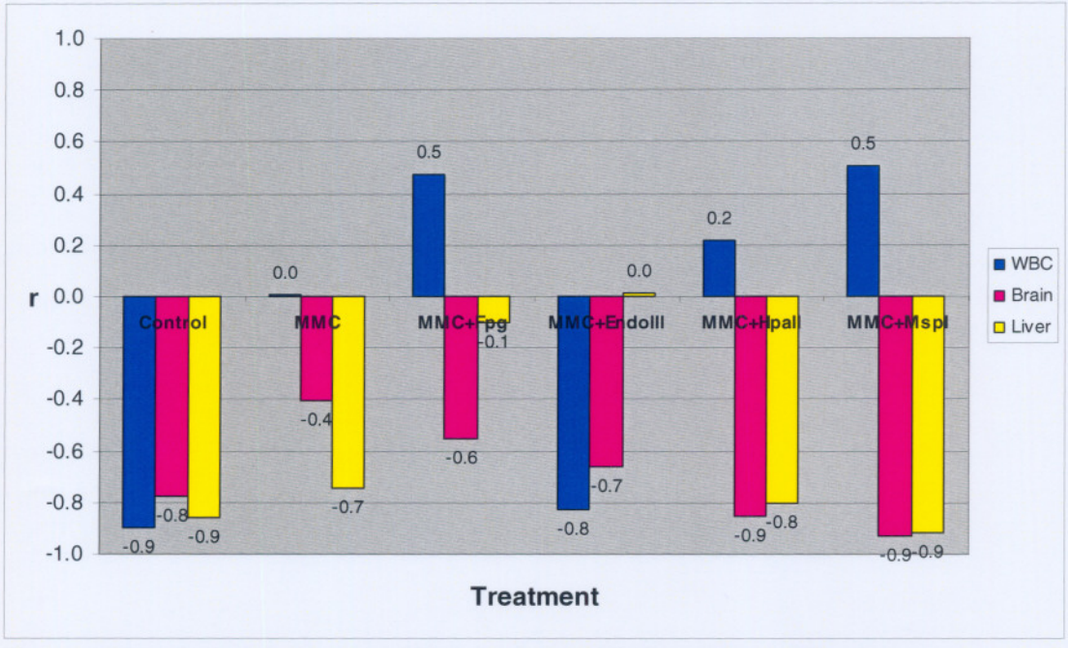


Figure 4.18 Shift in the degree of DNA damage of MMC treated rats. The Spearman correlation (r) was used to demonstrate the shift between the different classes of DNA damage.

Fig. 4.18 is an illustration of the shift in the degree of DNA damage [from: -1 (Class 0) to 0 (Class 1-2) to +1 (Class 3-4)] with each of the various treatments of the different cell types after they were isolated and incubated with MMC. In the control group, all the cells types had a value near the -1 range (Class 0:1-6% tail DNA). Each of the cell types responded differently with the MMC treatment when compared to each other. In all three the cell types, the DNA strand breaks increased with MMC treatment, except for the liver cells. The WBC showed a value of 0 (Class 2-3: 17-60% tail DNA) and the brain -0.4 (Class 1-2: 6-35% tail DNA) and the liver -0.7 (Class 1: 6-17% tail DNA).

Table 4.8 Statistical processing of the results obtained from the MMC treated group

Kruskal-Wallis Nonparametric (*)/Bonferroni-test (#)/Duncans-test (^)/Unequal N HSD-test (×)																		
	Control			*MMC*			*Fpg*			*Endo* III			*Hpa* II			*Msp* I		
	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain	WBC	liver	brain
Control				* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
MMC	* # ^ ×	* # ^ ×	* # ^ ×				* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	^	# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×
Fpg	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×					* # ^ ×	^	^	* # ^ ×	* # ^ ×	# ^ ×		* # ^ ×	* # ^ ×
Endo III	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	^	* # ^ ×	^	^				* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×
Hpa II	* # ^ ×		* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	# ^ ×	* # ^ ×	* # ^ ×					* # ^ ×	* # ^ ×	* ^
Msp I	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×		* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* # ^ ×	* ^			

(p>0.05 * # ^ ×)

Treatment with *Fpg* increased the degree of DNA damage in all three cells types, WBC: +0.5 (Class 3-4: 35-100% tail DNA), brain cells: -0.6 (Class 1-2: 17-35% tail DNA) and liver cells: -0.1 (Class 2-3: 17-60% tail DNA). The increasing degree of DNA damage with *Fpg*, is because of high levels of oxidized purines in the DNA. *Endo* III treatment showed a significant elevation in the degree of tail DNA in the liver cells (0: Class 3) but no significant increase in tail DNA was detected in the WBC (-0.8: Class 0) and the brain cells (-0.7: Class 1). The treatment of cells with *Endo* III assisted with the detection of oxidized pyrimidines. Therefore, in the liver cells the level of oxidized pyrimidines increased with MMC treatment, but no significant increase was observed in the WBC and brain cells.

After treatment with *Hpa* II the WBC shifted to the +0.2 region (Class 2-3: 17-60% tail DNA), whereas the brain and liver cells stayed in the -1 region. The treatment with *Msp* I displayed high levels of DNA damage in the WBC +0.5 (Class 4: 60-100% tail DNA), but no significant damage occurred in the brain and liver cells, both were -0.9 (Class 0: 0-6% DNA damage). As *Msp* I is known to digest methylated restriction sites, this result indicates that there are relatively high levels of methylated DNA bases after MMC treatment in the WBC but not so in the brain and liver cells.

All these results show that the comet assay is capable to reveal baseline DNA methylation levels as well as *in vivo* and *in vitro* induced changes in DNA methylation.

CHAPTER FIVE

ARTICLE

Chapter five is in an article format and was written according to the instructions for authors of the scientific journal, Mutation Research. (http://authors.elsevier.com/GuideForAuthors.html.).

Application of the comet assay to detect DNA methylation levels in rat tissue

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Abstract

The development of the single cell gel electrophoresis assay (comet assay) as a powerful method for measuring DNA strand breaks and repair, has lead to a broader understanding of the impact of certain internal and external factors on DNA damage and a cell's repair capacity. Along the years the comet assay was made more informative by incorporating additional steps by digesting the DNA on the microscope slides with enzymes that recognise particular kinds of damage to nucleic acids. Endonuclease III (*Endo* III) are used to detect oxidised pyrimidines and formamidopyrimidine DNA glycosylase (*Fpg*) to detect the major purine oxidation product 8-oxoguanine as well as other altered purines.

To investigate whether the comet assay can be modified to study the DNA methylation levels in cells, the use of the isoschizomeric restriction enzymes *Hpa* II and *Msp*I were investigated. Both these enzymes recognize the sequence 5'–CCGG–3' but they have different sensitivities for methylation of the cytosine residues in the target site. Treatment of rat lymphocyte DNA on the slides with MspI resulted in a significant increase in the tail DNA of the comets in relation to the controls and to treatment with HpaII. Analysis of DNA exposed in vitro and in vivo to chemicals that change the methylation levels of DNA showed that these changes can be detected with the comet assay in a background of other types of DNA damage

We convincingly demonstrated that the Comet assay can indeed be used to study the global methylation levels of single cells. This opens a new range of applications for the comet assay.

1. Introduction

A variety of DNA lesions can be detected using the alkaline method of the single cell gel electrophoresis (Comet) assay (1). These include DNA double strand breaks (DSB), DNA single strand breaks (SSB) and alkali-labile sites (ALS). Modifications to the comet assay have allowed the use of lesion specific endonucleases to detect specific base damage as SSB's (2,3). For example, *Fpg* acts both as an *N*-glycosylase and an AP-lyase. The *N*-glycosylase activity releases damaged purines from double strand DNA generating an apurinic site (AP-site). The AP-lyase activity cleaves both 3' and 5' to the AP-site thereby removing the AP-site and leaving a 1-base gap. Some of the damaged bases recognised and removed by *Fpg* include 8-oxoguanine, 8-oxoadenine, 5-hydroxy-cytosine and 5-hydroxy-uracil. It has been shown that the *Fpg* enzyme detects alkylation damage with high sensitivity when using the comet assay since *Fpg* strongly enhanced the detection of mitomycin C (MMC) and ethylmethylsulphonate (EMS) induce DNA modifications (4). Thus, the use of repair enzymes is another way to characterise DNA damage induced by reactive oxygen species (5). *Endo* III acts both as an N-glycosylase and an AP-lyase. The N-glycosylase activity releases damaged pyrimidines from double stranded DNA, generating an AP site. The AP-lyase activity of the enzyme cleaves 3' to the AP site leaving a 5' phosphate and a 3' ring opened sugar. Some of the damaged bases recognised and removed by *Endo* III include 5, 6 dihydroxythymine and thymine glycol (4).

The role of DNA methylation in eukaryotic cells is not fully understood yet, but it is clear that methylation has two major functions: gene expression control and

protection of the host organism against expression of undesired sequences (6). Methylation regulates vital cellular processes, thus any inappropriate methylation/demethylation could have lethal consequences, e.g. cancer (7). The major problem with DNA methylation occurs when CpG sites within a promoter CpG islands are aberrantly methylated, the chromatin is normally condensed in that region and there is little or no gene expression of the associated gene (8). Such an aberrant methylation of tumour suppressor genes can lead to abnormal cellular growth. The isoschizomers *Hpa* II and *Msp* I are recognise the same tetranucleotide sequence (5'-CCGG-3') but display differential sensitivity to DNA methylation (9). *Hpa* II is inactive when any of the two cytosines is methylated, but cuts the hemi-methylated 5'-CCGG-3' at a lower rate compared to the unmethylated sequences whereas *Msp* I cuts 5'-C^{5m}CGG-3', but not 5'-^{5m}CCGG-3' (10).

The difference in sensitivity to DNA methylation was used to study whether the comet assay could be used to measure a part of the methylation status of DNA. A higher level of DNA methylation would result in a larger increase in the amount of DNA in the comet tails in the case of *Msp* I relative to *Hpa* II (11).

2. Materials and methods

2.1. Rat blood cells

Ethical approval was obtained from the local Ethics Committee of the Northwest University with number 04D10. Heparinised blood samples were obtained after decapitation of healthy Spraque-Dawley male rats (230-260g) which represented the untreated control group. Blood were also collected from male Spraque-Dawley rats treated with 300mg/kg Paracetamol once a day for three days. The animals were housed in an air-conditioned room at 25°C with a 12 hour light/dark cycle and had free access to tap water and rat chow. Peripheral blood lymphocytes were separated from freshly collected whole blood on Histopaque® gradients (Sigma-Aldrich Co., St. Louis, USA), washed twice in 1 x PBS (phosphate-buffered saline) and suspended in PBS. The various treatments were performed with the isolated cells. Cells were counted and viability assessed with the trypan blue stain method. The comet assay was only performed on cells when viability exceeded 90% and approximately 10,000 cells per microscope slide were used.

Cells were treated with hydrogen peroxide (H_2O_2) at a final concentration of 60μM for 20 minutes at 37°C to induce oxidative damage.

2.2 Chemical treatment

The isolated cells were exposed to the alkylating agent ethylmethanesulfoneate (EMS, CAS No.: 62-50-0), and the DNA cross linking agent mitomycin C (MMC, CAS No.: 50-07-7). The chemicals were diluted with PBS immediately before use and the cells were exposed for 60 minutes at 37°C. O^6-Benzylguanine (O^6BG,

CAS No.:19916-73-5) was diluted with 1% DMSO before use and incubated with the cells for 60 minutes at 37°C.

2.3. Comet assay

The Comet assay was performed under alkaline conditions according to the procedure previously described (12) with minor modifications to suit local laboratory conditions. A freshly prepared suspension of cells in 0.5% low melting point agarose (Roche Diagnostics, Basel, Switzerland) dissolved in 0.5M EDTA (Sigma-Aldrich Co., St. Louis, USA) was spread over frosted microscope slides pre-coated with 0.1% high melting point agarose (Roche Diagnostics, Basel, Switzerland). The cells were then lysed overnight at 4°C in a buffer consisting of 5M NaCl, 0.4M EDTA, 1% Triton X-100 and 10% DMSO, pH 7.5. After lysis, DNA was allowed to unwind for 30 minutes in the electrophoresis solution consisting of 0.6M sodium hydroxide, 0.05M EDTA, pH > 13. Electrophoresis was conducted at 4°C for 40 minutes at 500mA and 40 Volts. The slides were then neutralised with 0.4M TrisHCl, pH 7.5 and stained with ethidium bromide (10µg/ml).

The slides were examined at 200 x times magnification on a Olympus XI70 inverted system microscope (Olympus Optical Co. (U.K.) LTD., London) attached to a Color View 12 (7087355) video camera equipped with a UV filter block consisting of an excitation filter (359nM) and barrier filter (461nM) and connected to a personal computer based image analysis system (analySIS® v.3, Lakewood, USA). Fifty images were randomly selected and the comet tail DNA % was measured using CASP (v.1.2.2.) image analysis software.

2.4. Enzyme treatment

When the comet assay was combined with enzyme treatment, slides were washed for 10 minutes in enzyme buffer (for *Hpa* II and *Msp* I: 10mM Tris, 10mM $MgCl_2$, 50mM NaCl, pH 7.5) after cell lyses. All the enzymes were obtained from New England BioLabs. The slides were then coated with 50µl of either buffer alone or buffer containing 2.5U of either of *Hpa* II and *Msp* I and were then put in small container lined with damp paper. The container was closed with a tight fit cover and then incubated for 60 minutes at 37°C. The slides were then processed as described above for the comet assay.

2.5. Statistical analysis

Values are expressed as means (± SD) and analysed using one way ANOVA followed by Duncan's multiple range tests for multiple mean comparisons.

3. Results

3.1. Control and treated lymphocytes

When treating the DNA of the isolated lymphocytes on the slides with *Msp* I, significant more tail DNA was observed in the comets relative to the control and *Hpa* II treatment (Figure 1) . This indicates that there are restriction sites for *Hpa* II are not available to the enzyme as are the sites for *Msp* I. This difference reflects the level of methylation of the CCGG sites in the DNA of the cells examined. In the use of restriction endonucleases suppliers as a rule recommend rather rigid guidelines for their use, however, *Hpa* II and *Msp* I were active under rather robust conditions on the slides in a sealable plastic container lined with damp paper towels.

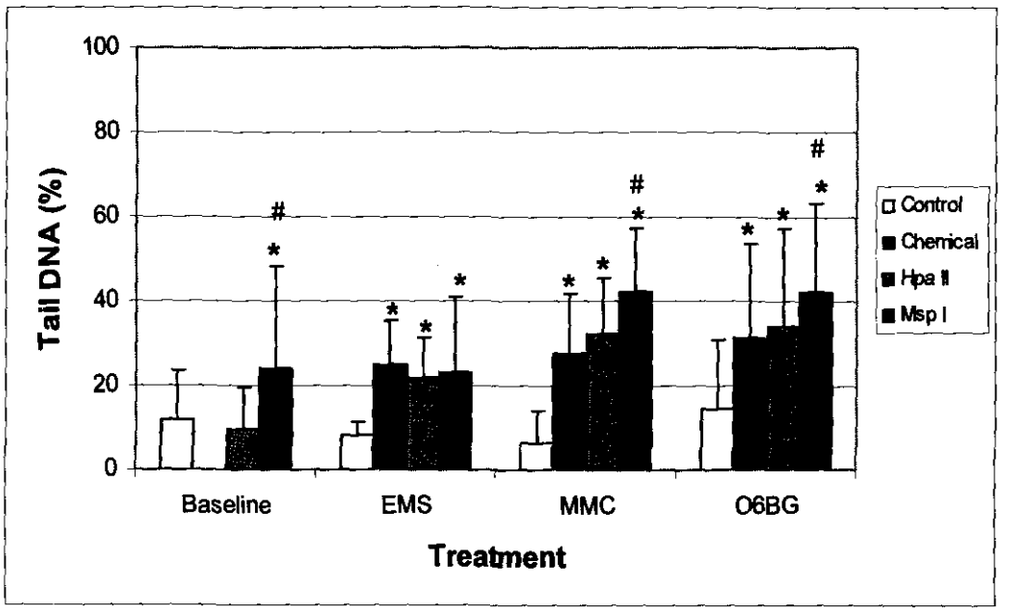

Figure 1. Restriction enzyme treatment of control and treated lymphocytes. The cells were exposed to the different chemicals as described in materials and methods before treatment with *Hpa* II and *Msp* I. (* statistically significant different from the control: $p<0.05$, # statistical significant difference between *Hpa* II and *Msp* I: $p<0.05$).

Figure 2 shows typical examples of lymphocyte DNA comets obtained after treatment of the DNA on the slides with the two restriction enzymes. Comets originating from the controls (a) have a nearly symmetrical shape with practically no tails. *Msp* I (c) treatment resulted in much smaller heads with longer and denser tail DNA in comparison with *Hpa* II treatment (b).

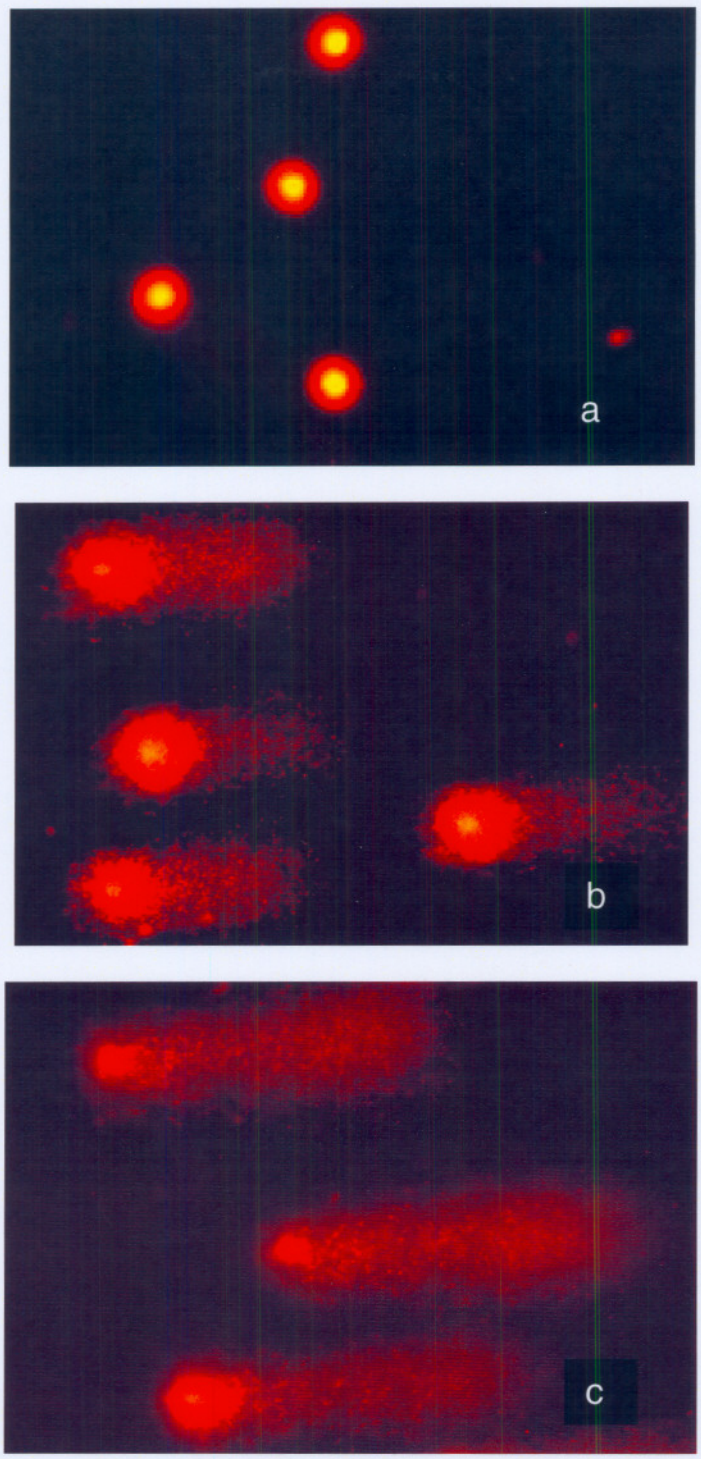

Figure2: Representative examples of the comets of the control and enzyme treated lymphocytes. (a) Control (b) Normal+*Hpa* II (c)Normal+*Msp* I.

To determine whether changes in the DNA methylation levels can be detected by the comet assay, the isolated lymphocytes were exposed to chemicals with a known effect on DNA. The results depicted in Figure 1 were obtained after the in

vitro treatment of isolated lymphocytes with chemicals having different effects on DNA. Exposing the cells to EMS, a moderate alkylating compound (13), resulted in a significant increase in the tail DNA but no further increase was observed following treatment of the DNA with either of the restriction enzymes. Exposure of the cells to MMC, a DNA cross-linker as well as an oxidising agent of the DNA compound (14), also resulted in an increase in the tail DNA but in the case of *Msp* I treatment, a significant increase relative to *Hpa* II treatment in the tail DNA was observed. Very similar results were obtained after exposure of the cells to O^6BG, an alkylating agent (15).

To study the effect of an in vivo intervention on the degree of DNA methylation, rat were administered a non-toxic dose of Paracetamol. This in vivo intervention shows a significant increase in the tail DNA after *Msp* I treatment relative to *Hpa* II treatment in the control and drug treated animals (Figure 3). However, a slight but significant increase in *Msp* I sites was caused by the in vivo drug treatment.

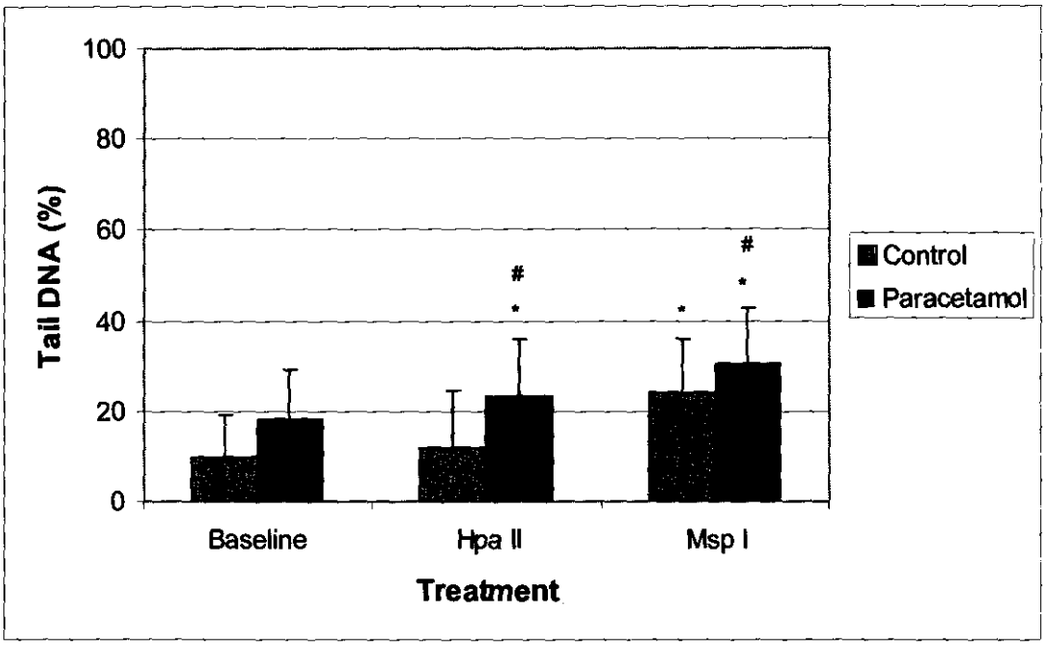

Figure 3. The effect of in vivo administration of Paracetamol. This represent the data obtained from four animals. (* statistically significant different from the control: $p<0.05$, # statistical significant difference between *Hpa* II and *Msp* I: $p<0.05$).

4. Discussion

The aim of this study was to determine whether the comet assay could be modified to detect the baseline DNA methylation levels in cells and any changes therein by making use of the isoschizomeric restriction endonucleases *Hpa* II and *Msp* I which have different sensitivities to methylation of their target site. It is generally believed that *Msp* I is less sensitive to DNA methylation and that an increase in the tail DNA relative to treatment with *Hpa* II, would to a large extent reflect the DNA methylation levels in individual cells (11). An analogous principle is applied to the use of the *Fpg* protein in the comet assay to show the presence of for instance oxidative modified purines (4).

The results obtained in this modification of the comet assay met our expectations to measure DNA methylation levels with the lesion specific enzymes such as *Hpa* II and *Msp* I. This was possible in a background of other types of DNA damage caused by the chemicals to which the cells and animals were exposed to. The use of the various chemicals illustrated that changes in the levels of DNA methylation, also in a background of DNA damage, can be demonstrated with this modification of the comet assay. This modification of the comet assay may become useful, for example when a substance with unknown mode of action is tested when DNA methylation and/or oxidation is expected to take place.

This results obtained after administration of Paracetamol to the animals was rather surprising since it was shown that Paracetamol intoxication causes depletion of glutathion and eventually of the erosion of methylating capacity of a cell (16,17). In the experiments described here Parcetamol at the concentration used, apparently acted as an antioxidant (18) and the observed increased methylation of DNA may be linked to this phenomenon, but we have no proof for this speculation.

In this study the use of specific restriction enzymes with different sensitivities to methylation of their target sites, enabled us to detect baseline DNA methylation Levels as well as changes induced in vivo and in vitro. This modification of the comet assay may have great value for the early detection of diseases and pathological conditions due to exposure to hazardous chemicals. The benefits of possible treatments can also be monitored.

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CHAPTER SIX

CONCLUSIONS

DNA damage and repair receive much attention in the research and medical societies worldwide. The reason for this is that DNA is the core of the information for all the functions in the human body. If the DNA has been damaged, repair needs to take place before replication, otherwise it might lead to the formation of mutations and disease (e.g. cancer).

The aim of this study was to establish the comet assay in our laboratory and to expand its diversity of applications by investigating the possibility to measure DNA methylation levels in single cells.

6.1. ESTABLISHING THE COMET ASSAY

A diversity of techniques already exists to detect DNA damage (Jenssen and Ramel, 1980 and Grisolia, 2002). The monitoring of DNA damage on a single cell level is now possible by applying the comet assay (Singh *et al.*, 1988). Reviews published on the comet assay (Singh *et al.*, 1988; Tice, 1995; Collins, 2004) were used as a basic guideline to establish this technique in our laboratory. After minor adjustments to these methods the comet assay was established successfully in our laboratory.

6.1.1. ISOLATION OF CELLS FROM DIFFERENT TISSUES

The successful isolation of single cells to perform the comet assay from brain and liver tissue was done by using a "mincing solution" and very mild treatment of the tissues (see Section 4.2). This isolation procedure made it possible to monitor the response, with regard to DNA damage and repair, of different cell types exposed various chemical compounds, e.g. Paracetamol, EMS and MMC.

6.1.2. DIVERSITY IN APPLICATIONS OF THE COMET ASSAY

In the study presented experiments were done to properly establish the comet assay in our laboratory. The first aim in this regard was to investigate the *reproducibility* of the assay as performed by us by analysing the lymphocytes of a healthy male volunteer once a week for two weeks. In the results obtained (Fig 4.8) it was clear that the comet assay is very reproducible in our hands when performed according to the protocol described in Chapter 3.

The results obtained from the *breast cancer patient* soon after chemotherapy show that the comet assay is able to detect the inability of DNA to repair. A note of caution is appropriate at this point: when using the comet assay to measure baseline DNA damage in cancer patients, it seems to be necessary to invoke some kind of damage to the DNA and measure the repair capacity to really get an informative comet assay. This note stems from the observation (Fig 4.9) that the baseline level of DNA damage in the cancer patient was as low as that observed in normal healthy persons but that the effect of the chemotherapy could only be observed after exposing the isolated lymphocytes to peroxide.

The affect of a antioxidant on DNA damage and repair in rat liver cells was investigated with the comet assay. We were able to demonstrate that a *Rosa roxburghii* fruit extract was not genotoxic in rat primary hepatocytes and that this extract has a beneficial effect on the integrity of DNA (Janse van Rensburg *et al.*, 2004).

A rather novel application of the comet assay was the monitoring of DNA damage and repair after prescribed *exercise* programmes executed by middle aged men. From the results obtained we concluded that strenuous exercise does induce DNA damage and that the effect of different intensities of exercise on DNA damage is an interesting phenomenon that merits further investigation. In addition, the comet assay can be used in determining the optimal exercise programme for an individual.

From the experiments we did it can be concluded that in the *occupational exposure* study the comet assay proved to be a promising tool for human bio-monitoring studies since it has been used successfully for assessing pollution-induced DNA damage and adverse effects on DNA repair. As can be seen from the literature this application of the comet assay is indeed applied to a diversity of occupational exposures (Collins *et al.*, 1997) and can be used fruitfully in South Africa.

Ever since we have established the comet assay in our laboratory, it very soon became popular and in demand on campus because of the important information it delivers regarding the integrity of a cell's genetic apparatus. The major advantages of the comet assay are that it is economical, its performance is straightforward and results can be obtained in a relative short time.

6.4. MONITORING THE METHYLATION STATUS OF DNA

The major contribution of this study was the expansion in the versatility of the comet assay by showing that the comet assay could be modified to measure the DNA methylation levels in various cell types. The use of lesion specific enzymes such as *Fpg* and *Endo* III in conjunction the restriction enzymes *Hpa* II and *Msp* I, were shown to be useful agents in the comet assay because their use show that the methylation levels of DNA could be demonstrated together with or in a background of other types of damage to DNA. This may become useful, for example, when a substance with an unknown mode of action is tested when DNA oxidation and/or methylation is expected to take place. The results also show the usefulness of the DNA damaging agents, EMS and MMC, in the comet assay. The use of these compounds allowed us to demonstrate that the comet assay is capable to indicate changes in the DNA methylation levels of single cells. In this regard an interesting observation was made: a degree of cell specificity in the DNA methylation apparatus was observed (see Sections 4.6.2.2 and 4.6.2.3). Further study is necessary to confirm this observation.

As mentioned previously, the increased methylation of DNA in especially the liver and brain cells (Section 4.6.2.1) after the administration of a non-toxic dose of

Paracetamol to rats, could not be satisfactorily explained and therefore also needs further investigation.

In this study the use of lesion-specific enzymes in the comet assay helped us to show the presence of methylated DNA bases in a background of oxidised DNA bases which usually is an novel extension of the standard comet assay. With these results in mind, we are convinced that there is a great opportunity to make use of the comet assay in combination with these restriction endonucleases, for the early detection of DNA methylating related diseases as has been shown with other methods to detect DNA methylation (Fruhwald, 2003). The benefits of appropriate interventions can also be monitored.

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