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Establishing the Comet assay to determine the effects of different perturbations on DNA repair capacity

By

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Table of Contents

ACKNOWLEDGEMENTS	i
ABSTRACT	ii
OPSOMMING	iii
KEY WORDS.....	v
LIST OF SYMBOLS	vi
LIST OF ABBREVIATIONS	vii
LIST OF FIGURES.....	xii
LIST OF TABLES.....	xiii
CHAPTER 1	1
INTRODUCTION	1
CHAPTER 2	4
LITERATURE REVIEW	4
2.1. DNA damage.....	4
2.2. DNA repair mechanisms.....	5
2.3. Different aspects influencing DNA repair pertaining to this study.....	15
2.4. Comet assay	20
2.5. Aims and approach.....	22
2.6. Executed protocol	23
CHAPTER 3	25
PUBLISHED WORK.....	25
CHAPTER 4	31
MATERIALS AND METHODS.....	31
4.1. Introduction	31
4.2. Comet assay	31

4.2.10. DNA repair capacity	41
4.3. Gene expression	42
CHAPTER 5	45
RESULTS & DISCUSSION	45
5.1. Comet assay results.....	45
5.2. Gene expression results.....	61
CHAPTER 6	65
SUMMARY & CONCLUSION.....	65
REFERENCES.....	72
APPENDIX A:.....	79
PROTOCOLS FOR BUFFERS AND REAGENTS.....	79
APPENDIX B:.....	82
LIST OF SUPPLIERS AND CATALOGUE NUMBERS OF MATERIALS NOT OBTAINED FROM SIGMA ALDRICH.....	82
APPENDIX C:.....	83
PRIMER AND PROBE SEQUENCES.....	83
18S rRNA:.....	83
APPENDIX D:.....	84
RAW DATA OBTAINED (COMET ASSAY)	84

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ABSTRACT

Single cell gel electrophoresis (SCGE), more commonly known as the Comet assay, is an uncomplicated, affordable and versatile method for investigating DNA damage and repair. Existing comet-assay based methods were modified and applied in this study in order to examine the effects of different perturbations on the DNA repair capacity of different samples.

Mitochondrial functioning has a vast effect on overall cell physiology and does not simply involve the production of energy in the form of ATP that sustains common biological processes, but is also associated with important cellular occurrences such as apoptosis and ROS production. It is suggested that a change in mitochondrial function may lead to extensive ROS production which may negatively affect macromolecules, including proteins involved in DNA repair pathways, and impaired energy formation which in turn may hamper the proper occurrence of energy driven processes. Complex I and –III knock-down systems established in 143B cells are used to investigate the effect that perturbations of the energy metabolism may have on DNA repair capacity.

Metallothioneins (MTs) are known to play an imperative role in trace element homeostasis and detoxification of metals and are effective ROS scavengers. The pro-oxidant environment that heavy metal imbalance causes may result in mutagenesis and transformation through DNA damage. It is suggested that an imbalance in the metal homeostasis caused by MT knock-out may create an environment favourable for DNA damage formation and at the same time impair DNA repair pathways. Because of the multi-functionality and involvement of metallothioneins in such a wide variety of biological processes, it was considered interesting and essential to extend the investigation on the effect of the absence of metallothioneins on DNA repair. A metallothionein I and –II knock-out mouse model is employed to determine the effect of MT knock-out on DNA repair capacity.

It was clear from the results obtained that transfection of cells, as used to investigate a perturbation in the energy metabolism in 143B cells, has an impairing effect on DRC. It was also confirmed that metallothioneins play an important and diverse role in cell biology since the absence thereof inhibits both BER and NER.

OPSOMMING

Vestiging van die Komeet analise om die effekte van verskillende ingrepe op DNS-herstel te bepaal

Enkel sel jel elektroforese, beter bekend as die komeet analise, is 'n eenvoudige, bekostigbare en veelsydige metode om DNS skade en -herstel te ondersoek. Bestaande komeet analise gebaseerde metodes is in hierdie studie aangepas sodat die effekte van verskillende ingrepe op die DNS-herstel vermoë in verskillende eksemplare bestudeer kon word.

Mitochondriale funksionering het 'n groot effek op algehele sel fisiologie en behels nie net energie produksie in die vorm van ATP, wat algemene biologiese prosesse onderhou nie, maar word ook geassosieer met belangrike sellulêre gebeurtenisse soos apoptose en die produksie van vry radikale. 'n Voorstelling is gemaak dat 'n verandering in mitochondriale funksie mag lei tot die uitermatige hoë produksie van reaktiewe suurstof spesies wat makromolekules negatief mag beïnvloed. Hierdie makromolekules sluit proteïene in wat betrokke is by DNS-herstel weë, sowel as by verswakte energie produksie wat om sy beurt die verloop van energie-afhanklike prosesse mag inhibeer. Geen-inhibering van kompleks I en III van die elektron transport ketting wat in 143B selle gevestig is, is gebruik om die effek te ondersoek wat ingrepe in die energie metabolisme op DNS-herstel mag hê.

Metallotioniene (MTs) is bekend vir hul onontbeerlike rol in spoorelement homeostase en die detoksifisering van metale. Metallotioniene is ook effektiewe ROS verwyderaars (antioksidant eienskappe). Die pro-oksidant omgewing wat deur 'n swaarmetaal wanbalans veroorsaak word kan tot mutagenese en transformering lei deurdat erge DNS-skade aangerig word. Daar word voorgestel dat 'n wanbalans in die metaal homeostase wat deur die uitklop van MT-gene veroorsaak word, 'n omgewing skep wat DNS-skade begunstig en terselfde tyd DNS-herstel weë benadeel. Weens die multifunksionaliteit en betrokkenheid van metallotioniene by so 'n wye verskeidenheid biologiese prosesse, was dit noodwendig om die ondersoek uit te brei, deur die effek van die afwesigheid van metallotioniene op DNS herstel te bestudeer. 'n Metallotionien I en -II uitklop muis model is hiervoor gebruik.

Vanuit die resultate wat verkry is, was dit duidelik dat transfeksie van selle, soos wat dit gebruik is om ingrepe in die energie metabolisme van 143B selle tot stand te bring, 'n nadelige effek het op die DNS-herstel vermoë van die betrokke selle. Verder is aangetoon dat metallotioniene 'n belangrike en diverse rol in selbiologie speel, aangesien die afwesigheid daarvan beide uitsny DNS-herstelweë (BER en NER) se werking inhibeer.

KEY WORDS

Comet assay

DNA repair capacity

Base excision repair (BER)

Nucleotide excision repair (NER)

Electron transport chain

Metallothionein

LIST OF SYMBOLS

μ	Micro
%	Percent/ percentage
° C	Degrees Celsius
®	Registered trademark
™	Trademark

LIST OF ABBREVIATIONS

(In alphabetical order)

A	Adenine
AP sites	Abasic sites
APTD	Average percentage of tail DNA
ATP	Adenosine tri-phosphate
B[a]P	Benzo[a]pyrene
BER	Base excision repair
BPDE	Benzopyrene diol epoxide
BSA	Bovine serum albumin
BUT	Butenolide
C	Cytosine
Cd	Cadmium
cDNA	Complementary DNA
CI	Complex one
CIII	Complex III
CO ₂	Carbon dioxide
Cu	Copper
ddH ₂ O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo-nucleic acid
DNS	Deoksieribo-nukleïensuur

DRC	DNA repair capacity
DSB	Double strand break
DTT	1,4 - Dithiothreitol
E. g.	Exempli gratia (for example)
EDTA	Ethylenediamine tetra acetic acid
ERCC1	Excision repair cross complimenting
<i>Et al.</i>	Et alii (and others)
ETC	Electron transport chain
EtOH	Ethanol
EV	Empty vector
FBS	Fetal bovine serum
Fig.	Figure
g	Gram
G	Guanine
GFP	Green fluorescent protein
GGR	Global genomic repair
H ₂ O ₂	Hydrogen peroxide
HD	Heavily damaged
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HMPA	High melting point agarose
hOGG1	Human oxo-guanine glycosylase
HPLC	High performance liquid chromatography
HPLC	High performance liquid chromatography
HT1	Hereditary/Hepatorenal tyrosinemia type 1

i.e.	id est (that is)
KBrO ₃	Potassium bromate
KCl	Potassium chloride
kd	Knock down
LMPA	Low melting point agarose
M	Molar
mA	Miliampere
ml	Milliliter
mM	Millimolar
MMR	Mismatch repair
mRNA	Messenger ribonucleic-acid
MT	Metallothionein
MW	Molecular weight
N	Nitrogen
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (reducing agent)
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexane-dione
OH	Hydroxyl radical
OXPHOS	Oxidative phosphorylation system
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PE	Protein extract
pH	Potential of hydrogen
pHPPA	para-Hydroxy phenylpyruvic acid
rcf	Relative centrifugal force
RNA	Ribose nucleic-acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPM	Revolutions per minute
rRNA	ribosomal ribonucleic-acid
SA	Succinylacetone
SCGE	Single cell gel electrophoresis
sh-RNA	Small hairpin ribonucleic-acid
siRNA	Small interfering ribonucleic-acid
SSB	Single strand break
T	Thiamine
TCA	Tricarboxylic acid
TCR	Transcription coupled repair
TEMED	Tetramethylethylenediamine
TrisHCl	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
URKS	Unrelated knock down system
UV	Ultra violet
XPG & XPF	The genes encoding some of proteins involved in NER in were first identified in studies related to the human DNA repair disease, <i>Xeroderma pigmentosum</i> (XP), which suggested that mutations in

one or more of 7 genes (*XPA-XPG*) could contribute to the formation of the disease.

Zn

Zink

LIST OF FIGURES

Figure 2.1. The mechanism of BER.

Figure 2.2. Formation of 8-hydroxyguanine.

Figure 2.3. The mechanism of NER, global genomic repair.

Figure 2.4. Bioactivation of benzo[a]pyrene to benzopyrene diol epoxide.

Figure 2.5. Determining DRC with the Comet assay.

Figure 4.1. A simplified drawing of a frosted microscope slide.

Figure 5.1. Repair of control substrate DNA.

Figure 5.2. Repair of H₂O₂ exposed DNA.

Figure 5.3. Repair capacity of 143B cell protein extracts.

Figure 5.4. Repair of control substrate DNA.

Figure 5.5. Repair of H₂O₂ substrate DNA.

Figure 5.6. Repair capacity of mouse liver tissue protein extracts.

Figure 5.7. Repair capacity of 143B cell protein extracts.

Figure 5.8. Repair capacity of mouse liver tissue protein extracts.

Figure 5.9. Gene expression: hOGG1.

Figure 5.10. Gene expression: ERCC1.

LIST OF TABLES

Table 4.1. *Comet classes.*

CHAPTER 1

INTRODUCTION

The Comet assay has recently been used in a study performed in our laboratory to investigate the effect of disease related metabolites on DNA repair (Van Dyk *et al.*, 2010). A paper hereon was published (included in chapter 3) and the method proved to be promising for future investigations. A need have long since been recognised for a simple, yet reliable assay to determine not only the DRC of different tissue or sample types but also the effects of different perturbations or external factors on DRC. The assay described for the purpose of this dissertation was thus developed to investigate BER and NER and the effects of different perturbations thereon.

There is some evidence in the literature that the energy metabolism and heavy metal homeostasis respectively, are inherently linked to DNA repair. The use of cell culture and mouse models to investigate these phenomena is thus very relevant and useful. Several studies have shown that DNA repair plays an extremely important role in ageing and the development of various age-related diseases such as Alzheimer's disease and cancer. In this study, complex I and –III knock-down systems of the electron transport chain in 143B cells are used to investigate the effects of perturbations in the energy metabolism on DRC. Furthermore, recent studies concluded that an imbalance in the heavy metal metabolism such as when the homeostasis maintained by metallothioneins are disrupted, have a direct impairing effect on the initiating steps of excision repair pathways. A metallothionein I and –II knock-out mouse model is used to examine the effects of a perturbation in the metal metabolism on DRC, specifically in mouse liver tissue of MT knock-out mice. This study is therefore not only an expansion of an existing method- but also addresses important, still to be elucidated research questions.

This dissertation commences with a literature review in chapter 2 which gives a broad background of DNA damage and repair, the method established in this study and the perturbations under investigation. In chapter 3, a published paper is included together with the work allocation thereof. The letter of consent signed by the co-authors, to include above mentioned article in this dissertation is provided at the end of chapter 1.

The paper is given prior to chapter 4, containing the materials and methods because the method under assessment was used to produce the findings published in the included paper. The current study greatly relies on the published work and the researcher wishes to establish this alkaline Comet assay with its modifications for use in other more extensive applications, such as to determine the effect of various perturbations on the DNA repair capacity of a tissue or cell culture sample. The article was a follow up on previous work also published in the same journal (Van Dyk & Pretorius, 2005), and was accepted and available online only 8 days after it was submitted for review. In the fourth chapter, elaborated methods for the execution of the study are described and the materials used are noted. Chapter 5 gives a systematic layout of the results obtained and the figures presented are discussed accordingly. The literature and findings are summarised and brought to a close in chapter 6. A list of references is included at the end of this dissertation followed by additional supportive information pertaining to the execution of the study in appendices.



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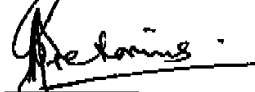
To whom it may concern,

We as co-authors of the paper: "Hereditary tyrosinemia type 1 metabolites impair DNA excision repair pathways" published in "Biochemical and Biophysical Research Communications 401: 32–36 (2010), hereby give our consent that the paper may be included in the dissertation entitled "Establishing the Comet assay to determine the effects of different perturbations on DNA repair capacity." The above mentioned dissertation is submitted by Anzaan Steenkamp in partial fulfilment of the requirements for the degree Magister Scientiae in Biochemistry at the Potchefstroom Campus of the North-West University.

Regards,
Co-authors:


E. van Dyk


G. Koekemoer


P.J. Pretorius

CHAPTER 2

LITERATURE REVIEW

2.1. DNA damage

2.1.1. Introduction

The genome is constantly confronted with factors challenging its integrity (Krokan *et al.*, 1997). These include xenobiotics, environmental stress and endogenous reactive oxygen species (ROS) (Dinant *et al.*, 2008; Zhang *et al.*, 2009). The state within a cell which is characterised by an overproduction of ROS and/or a decrease in the availability of antioxidants is known as *oxidative stress* (Franco *et al.*, 2008).

DNA damage may result in various responses such as inhibition of transcription or replication, cell cycle progression impairment, transcriptional mutagenesis, senescence and even cell death (Xu *et al.*, 2008). It is known that free radicals (ROS) are produced by the mitochondria during respiration and are consequently present in all body tissues (Mitra *et al.*, 2002). Other sources of ROS include the P450 metabolism, peroxisomes and the activation of inflammatory cells (Franco *et al.*, 2008). The production of ROS during respiration can be ascribed to so called “electron leakage”, which refers to a situation where a bottle-neck effect is created and electrons accumulate in the electron transport chain. These electrons are then transferred to oxygen molecules to form superoxide anions (Finley & Haigis, 2009). The superoxide anions are then converted to other ROS such as the enzymatic formation of hydrogen peroxide (Franco *et al.*, 2008; Finley & Haigis, 2009). ROS is known to have the ability to damage biological macromolecules such as DNA and proteins by means of oxidation *in vivo* and *in vitro* (Collins & Horvátová, 2001; Franco *et al.*, 2008). It can however, be used in biological studies to induce base lesions and single strand breaks (SSBs) (Taverna *et al.*, 2003; Mitra *et al.*, 2002).

Environmental pollutants and potential carcinogens such as polycyclic aromatic hydrocarbons (PAHs) are found in numerous products such as tobacco smoke and food, and are often found in ambient air (Cebulska-Wasilewska *et al.*, 2007; Yan *et al.*, 2010). The majority of the population is thus more or less constantly exposed to PAHs in varying concentrations depending on lifestyle and location (Takaishi *et al.*, 2009). PAHs were the first chemically identified carcinogens, they are composed of

multiple fused benzene rings and many PAHs are known to be human mutagens and carcinogens (Perera *et al.*, 2005; Bast & Hong, 2009). Nearly all kinds of genomic injury sustained by exposure to environmental pollutants can serve as potential triggers of mutagenesis or carcinogenesis (Cebulska-Wasilewska *et al.*, 2007). They are notoriously considered chemically inert but undergo biological activation to diol-epoxides which are carcinogenic and are able to bind covalently with DNA and other macromolecules (Xiong *et al.*, 2007; Bast & Hong, 2009). Binding to DNA molecules brings about distortion of the DNA structure and may cause mutations by potentially causing errors during DNA replication (Park *et al.*, 2006).

Reagents that fall in these two categories, ROS and PAHs, will be used in this study to induce damage to DNA in order to investigate the repair of the specific lesions performed by certain proteins involved in the excision repair pathways. Hydrogen peroxide and benzo[a]pyrene will be discussed in section 2.2.2.2 and 2.2.3.2 respectively.

2.2. DNA repair mechanisms

2.2.1. Introduction

As mentioned previously, DNA is not the inherently stable molecule it was once made out to be, but is constantly challenged by potentially damaging endogenous and exogenous molecules and factors (Krokan *et al.*, 1997; Dinant *et al.*, 2008). DNA repair pathways exist to prevent the gradual build up of DNA damage during ageing and illness (Mullenders *et al.*, 2009), and to protect the DNA from the mutagenic properties of various carcinogens (Xiong *et al.*, 2007). When DNA is damaged, a cascade of actions are induced within a cell, one of the most important is the arrest of the cell cycle allowing the damage to be repaired before the next round of replication takes place.

DNA repair mechanisms only make out a part of the processes that work together to maintain genome integrity (Krokan *et al.*, 1997). Because of the wide variety of damage resulting from the different kinds of damage inducing molecules (ROS, environmental toxins etc.), multiple DNA repair pathways exist (Zhang *et al.*, 2009). The existence of DNA repair mechanisms is crucial for the simple reason that unrepaired DNA damage leads to cell cycle arrest and apoptosis. The constant

surveillance and correction of DNA is also important, for the build up of misrepairs may cause chromosomes to be unstable or lead to carcinogenesis (Zhang *et al.*, 2009).

Excision repair pathways play a very important part in counteracting and rectifying DNA damage, and include base excision repair and nucleotide excision repair (Xu *et al.*, 2008). Excision repair pathways (e.g. BER, NER and MMR) operate in similar ways but differ distinctively in the points of onset. In particular, the nature of the enzymatic reactions vary and the enzymes involved in the initiating steps of these repair pathways tend to be very substrate specific since they normally act to recognise the damage to be repaired (Bast & Hong, 2009). BER and NER both depend on the chronological recruitment and commencement of a range of proteins at a site where DNA damage is present, this process occurs at timely manner for both BER and NER (Almeida & Sobol, 2007). Oxidative DNA damage is known to slow down cellular processes such as transcription and replication, whilst damage caused by PAHs (DNA adducts) may inhibit these processes to a much greater extent. It can therefore be deduced that NER is required to carry out DNA repair before replication and repair carried out by the BER pathway is required throughout the cell cycle to maintain the genomic integrity of every cell. It is also acknowledged that BER and NER are the principal excision repair pathways in postmitotic cells, which do not undergo cell division (Mitra *et al.*, 2002).

It has recently been noted that DNA repair pathways generally associated with SSBs are extensively involved in DSB repair. It is thus important to acknowledge that the different DNA repair mechanisms are not completely separated, although different DNA repair mechanisms can be distinguished, but are well intertwined (Zhang *et al.*, 2009). This statement is supported by the fact that inactivation of characteristic NER proteins impairs the repair of BER-specific substrates, indicating that the repair pathways are not completely compartmentalised but are greatly intertwined (Sato *et al.*, 1993).

2.2.2. Base excision repair

The inevitable production of ROS during normal cell respiration necessitates a repair mechanism able to reverse the damage enforced on the DNA by these molecules. BER acts to repair one nucleotide lesions originating from base damage and is said to be responsible for the removal of most alkylation damage (Xu *et al.*, 2008). There are several variations within BER considering that every incorrect base is repaired differently and that a different enzyme catalyses each reaction (Christmann *et al.*, 2003). The BER pathway is primarily responsible for the repair of oxidative DNA damage, except for DSBs (Mitra *et al.*, 2002). For the *in vitro* BER assay hydrogen peroxide will thus be used in this study to induce oxidative damage to DNA which is recognized and repaired by the BER pathway. According to Christmann *et al.* (2003) BER takes place in five distinguishable steps:

1. *Recognition, base removal and incision*
2. *Nucleotide insertion*
3. *Decision between short- and long-patch repair*
4. *Strand displacement and DNA repair synthesis*
5. *Ligation*

The very first step in BER acts to initially recognize the damaged base for eventual removal, as illustrated in figure 2.2. The enzymes involved in this recognition and removal step are termed glycosylases (Mitra *et al.*, 2002; Christman *et al.*, 2003). An abasic (AP) site results from the action of the DNA glycosylase (Mitra *et al.*, 2002). Some DNA glycosylase enzymes have bifunctional activity as it recognizes the damaged base, creates an AP site and has inherent AP-lyase activity which incises the deoxyribosephosphate backbone of the DNA (Xu *et al.*, 2008). hOGG1 is a type II glycosylase enzyme that removes the damaged guanine base and cleaves the AP site to form a single strand break (Christman *et al.*, 2003). Apurinic (AP) sites and SSBs are generated as transitional repair products during BER and necessitate end processing. The resulting SSB created by a glycosylase with AP-lyase activity are processed to create a 3'OH and 5' phosphate in order for nucleotide insertion to take place by the suitable DNA polymerase. The repair action

is completed when repair synthesis has replaced the excised base and ligation has been performed to seal the phosphodiester backbone of the DNA molecule (Xu *et al.*, 2008).

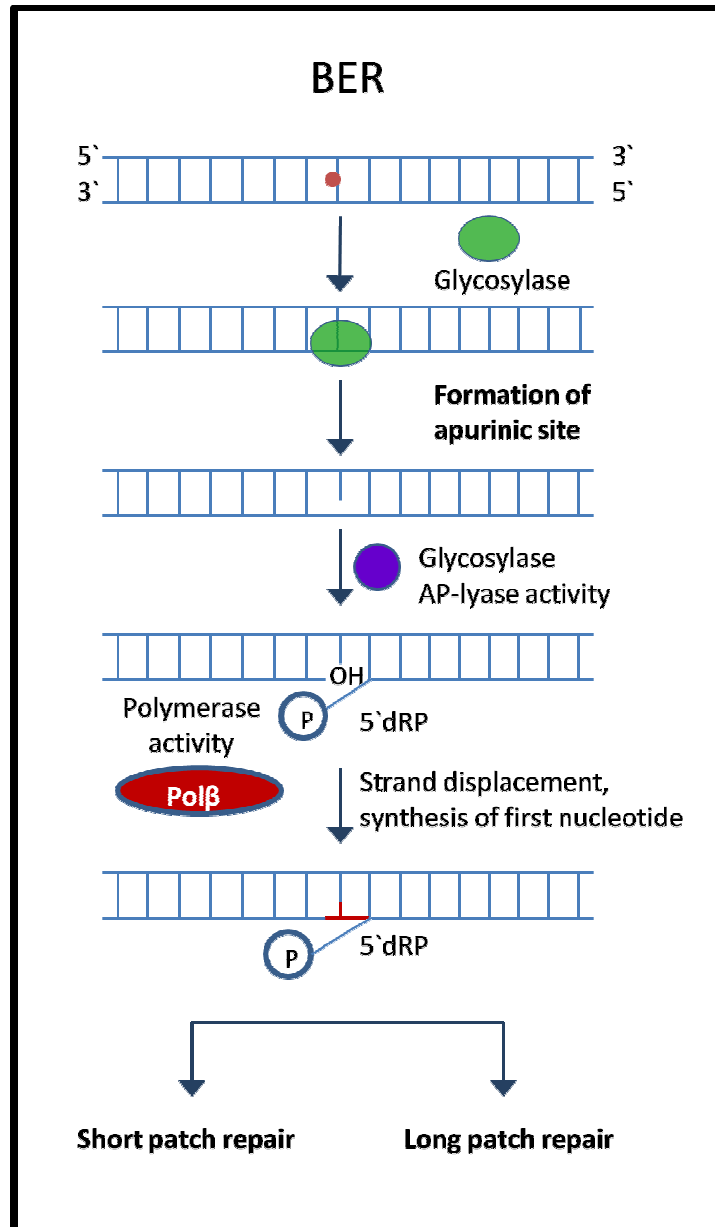


Figure 2.1. The mechanism of BER. A specific DNA glycosylase enzyme, *hOGG1*, removes the oxidized base by hydrolyzation of the N-glycosidic bond that results in an AP site. This AP site is attended to by APE and further processed via short- or long patch repair. Figure adapted from Christmann *et al.*, 2003.

2.2.2.1. *hOGG1* (EC 3.2.2)

hOGG1 (Human 8-Oxo-Guanine Glycosylase 1) is a type II glycosylase enzyme that specifically catalyses the removal of 8-oxo-guanine bases only when it is paired with C and is bifunctional because it also has AP-lyase activity (Christmann *et al.*, 2003;

Xu *et al.*, 2008). hOGG1 has a very weak affinity for the repair of 8-oxo-G when it is paired with A, these are, however, repaired by a second OGG enzyme termed OGG2 (Mitra *et al.*, 2002). hOGG1 consists of 345 amino acids and is very substrate specific (Smith *et al.*, 2006).

DNA glycosylase enzymes recognize and commence the repair of numerous DNA lesions which may have been caused by deamination, alkylation or oxidation. Bifunctional DNA glycosylase enzymes generally remove oxidized bases (Krokan *et al.*, 1997; Xu *et al.*, 2008). These lesions commonly cause bases to appear deviant and often distort the structure of the DNA double helix, in this way it can be detected by DNA glycosylases. hOGG hydrolyses the *N*-glycosidic bond between the aberrant base and the affected ribose entity, in this way removing the base and creating the AP site after which its AP-lyase activity allows it to form a SSB which brings about a one nucleotide gap (Krokan *et al.*, 1997; Xu *et al.*, 2008). Oxidised guanine bases can be further oxidized to form more intricate lesions, e.g. spiroiminodihydantoin or cyanuric acid, which are processed by different enzymes. To prevent these more complex lesions from forming, the proper working of the BER pathway is of vital importance in order that primarily oxidised bases can be removed before they are further oxidised (Xu *et al.*, 2008). In a study by Radak *et al.* (2005), it was shown that hOGG1 activity differs among red and white muscle fibers which suggest differentiation in the regulation of BER proteins. Chaudhry (2007) revealed that the activity of hOGG1 shows no dependence on the different phases of the cell cycle, whilst other enzymes demonstrate higher activity in the G1 and G2 phase.

2.2.2.2. Hydrogen peroxide

In order to assess the BER capacity of a specific tissue or sample, a substrate needs to be created. The substrate DNA must be specifically damaged so that the assay is specific for the investigation of the base excision repair pathway. As is evident from the above discussion of BER and the specificity of hOGG1, a DNA substrate containing oxidative damage in the form of 8-oxo-G is specifically recognised by the enzyme under investigation. With the intention to create such a substrate, hydrogen peroxide (H₂O₂) will be used to induce damage to the DNA.

Hydrogen peroxide is a well known and powerful oxidising agent and is even considered to be a very reactive species (Mitra *et al.*, 2002). The involvement of ROS in 8-oxo-G (guanine) formation cannot be ignored and the possibility exists to induce 8-oxo-G sites by hydroxylation of G bases (Riemscheider *et al.* 2002). Hydrogen peroxide is believed to be the most essential oxidising agent active *in vivo* in damaging DNA and other macromolecules (Mitra *et al.*, 2002). Smith *et al.* (2006) state that the method of DNA oxidation with potassium bromate (KBrO₃) is not clear but it has been proven that treatment of DNA with KBrO₃ increases the occurrence of 8-oxo-G. Gamma irradiation causes DNA damage through radiolysis of water to produce ROS (e.g. hydroxyl radical), or by directly causing double strand breaks in the DNA. Hydroxyl radicals attack electron dense sites on the DNA and this commonly leads to the formation of 8-oxo-G when interaction with guanine takes place at the C8 position (Smith *et al.*, 2006).

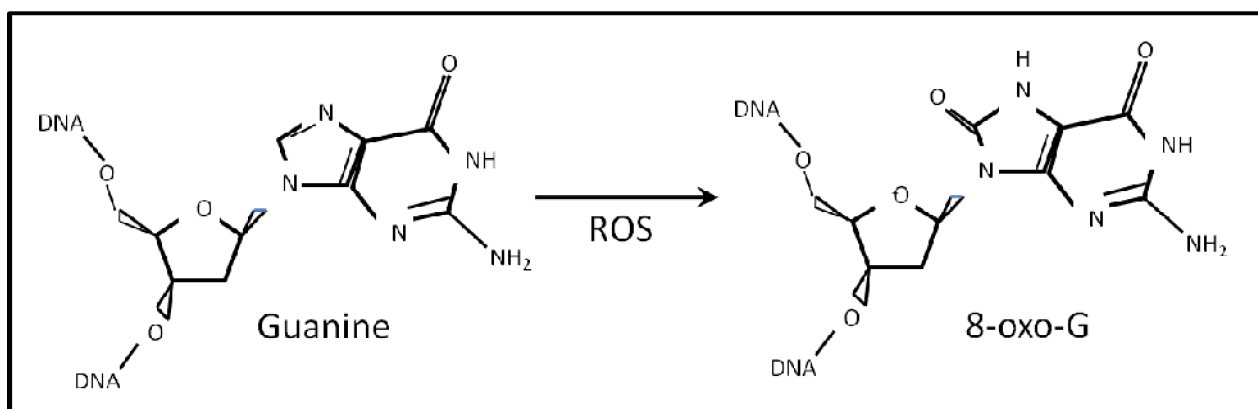


Figure 2.2. Formation of 8-hydroxyguanine. 8-oxo-G forms as a result of oxidative damage by ROS such as hydrogen peroxide. Figure adapted from Hsu *et al.*, 2004.

2.2.3. Nucleotide excision repair

Distortions in the DNA structure caused by large adducts such as those caused by PAHs need to be removed from the DNA to prevent cancer formation (Zhang *et al.*, 2009). Xeroderma pigmentosum (XP) is a rare autosomal recessive skin disorder that occurs as a result of poor NER (Nucleotide excision repair) functioning (Mullenders *et al.*, 2001; Bast & Hong, 2009; Messaoud *et al.*, 2010).

NER primarily repairs large DNA adducts caused by exposure to UV-light and chemicals such as aflatoxine and benzo[a]pyrene (Fig. 2.3) (Mitra *et al.*, 2002; Mullenders *et al.*, 2008; Zhang *et al.*, 2009; Yan *et al.*, 2010). An oligonucleotide containing the DNA adduct is removed and the intact strand of DNA is used to

synthesize a new fragment of DNA to replace the removed oligonucleotide (Krokan *et al.*, 1997). NER is considered to be the most complex excision repair mechanism since it involves the protein products of more than thirty genes (Krokan *et al.*, 1997).

There are two different pathways that constitute NER as a whole, namely global genomic repair (GGR) and transcription-coupled repair (TCR) (Christmann *et al.*, 2003; Zhan *et al.*, 2009). The NER enzyme under investigation in this study is involved in GGR and is termed excision repair cross complementing or ERRC1. As the name suggests, GGR acts to repair lesions to the DNA across the entire genome (Mullenders *et al.*, 2001). GGR proceeds in the following four steps (Bast & Hong, 2009; Zhang *et al.*, 2009):

1. *DNA damage recognition*
2. *DNA unwinding*
3. *Excision of the DNA lesion*
4. *Repair synthesis*

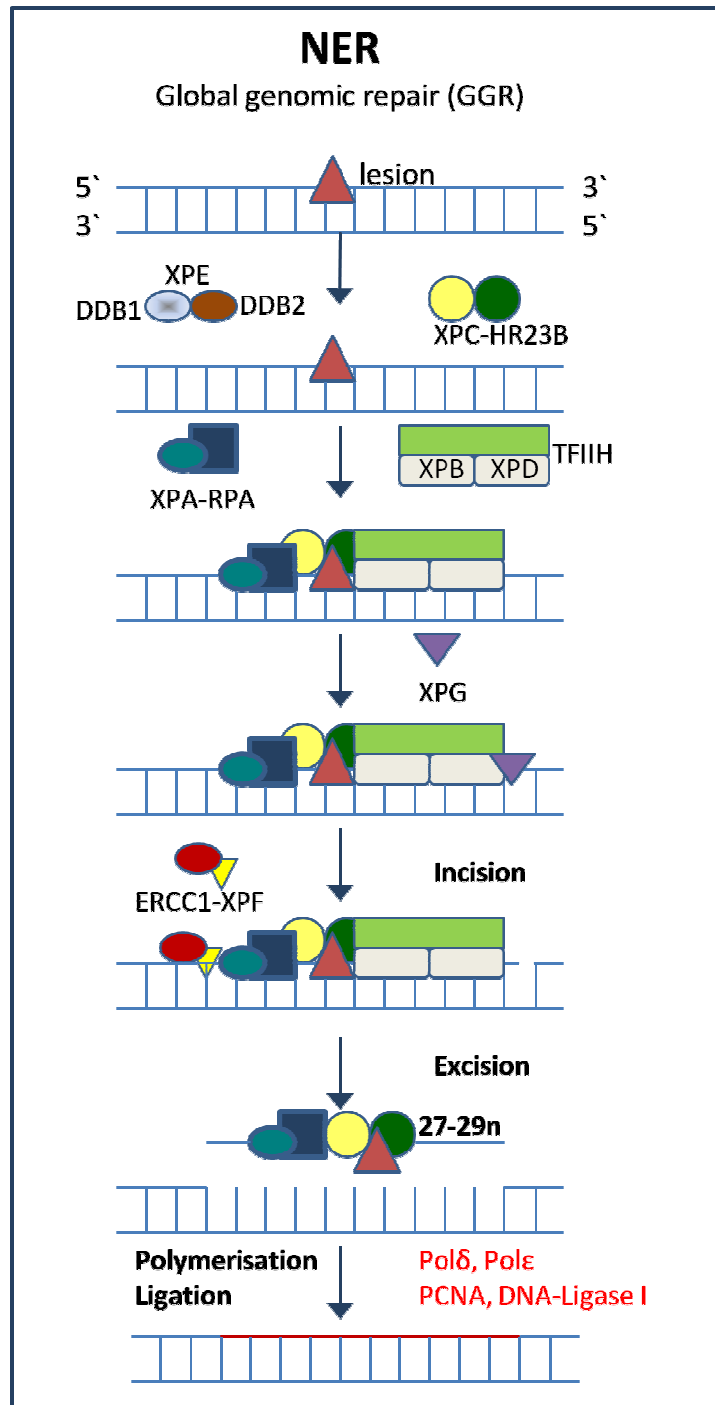


Figure 2.3. The mechanism of NER, global genomic repair. GGR acts to recognise lesions, unwind DNA and remove bulky lesions via ERCC1, resynthesis and ligation of repaired DNA takes place afterwards. Figure adapted from Christmann *et al.*, 2003.

ERCC1 acts in an XPF-ERCC1 complex to recognise and excise the lesion and it performs 5' -incision (Mullenders *et al.*, 2001), 3' -incision is performed by XPG (Christmann *et al.*, 2003), see figure 2.5. The removal or excision of the lesion is performed by the action of enzymes containing endonuclease activity (Bast & Hong, 2009). The XPF- or XPG/ERCC1 complex is engaged to cleave one strand of the

DNA at positions 3' and 5' to the lesion. This step includes the synthesis of a new oligonucleotide consisting of nearly 30 nucleotides, depending on the type of damage that is removed, to repair the gap created by excision of the lesion (Zhang *et al.*, 2009). The new oligonucleotide is constructed by 5' to 3' polymerisation followed by ligation of the free ends (Bast & Hong, 2009).

Benzo[a]pyrene will be used in this study to induce specific damage to the DNA of interest in order to investigate the effect of the different perturbations on a specific protein involved in the initiating step of NER, namely ERCC1.

2.2.3.1. ERCC1

Excision repair cross complementing 1 (ERCC1) acts in a complex with XPF to recognise damaged DNA and has structure-specific endonuclease activity (Sargent *et al.*, 2000; Enzlin & Schärer, 2002; Christmann *et al.*, 2003). The XPF-ERCC1 enzyme complex is involved in NER, interstrand crosslink repair and homologous recombination (Enzlin & Schärer, 2002). As mentioned in section 2.2.3, the dimeric ERCC1/XPF endonuclease performs 5' incision whilst XPG endonuclease is responsible for performing 3' incision (Sargent *et al.*, 2000). The 3' incision precedes the 5' incision performed by the heterodimeric endonuclease (Enzlin & Schärer, 2002). These events lead to the excision of an oligonucleotide consisting of 24 – 32 bases, and the intact strand is used to synthesize a new oligonucleotide by polymerisation, as mentioned earlier (Tsodikov *et al.*, 2005; Bast & Hong, 2009).

ERCC1/XPF requires the structural presence of XPG but is not affected by its activity (Enzlin & Schärer, 2002). According to Enzlin & Schärer (2002), the two proteins that form the dimer are very unstable when not bound together or in the absence of each other and are thus an obligate heterodimer (Tsodikov *et al.* 2005). The C-terminal domains of the two proteins interact to form the active heterodimer. Both the C-termini of XPF and ERCC1 include two helix-hairpin-helix structures (Tsodikov *et al.*, 2005). These motifs are often found in proteins that act to bind irregularly shaped DNA structures (Enzlin & Schärer, 2002; Tsodikov *et al.*, 2005).

2.2.3.2. Benzo[a]pyrene

B[a]P (Benzo[a]pyrene) is a PAH that causes damage to DNA by distorting the DNA structure when binding to it, and is also the most studied PAH (Bast & Hong, 2009; Yan *et al.*, 2010). Benzo[a]pyrene needs to be bioactivated to form benzo[a]pyrene-

7,8-diol 9,10-epoxide (BPDE), which is ultimately carcinogenic (Park *et al.*, 2006). B[a]P exerts three types of toxicity namely mutagenicity, carcinogenicity, and developmental toxicity and is therefore considered a representative PAH in biological studies (Perera *et al.*, 2005; Cebulska-Wasilewska *et al.*, 2007; Banni *et al.*, 2009). Benzo[a]pyrene has been used in cancer research on cell cultures from as early as 1977 (Yang *et al.*, 1977) which indicates the usefulness and the extent of its applications.

It was previously demonstrated in Jurkat T-cells that exposure to B[a]p caused the up-regulation of proteins concerned with apoptosis and tumour suppression whilst proteins involved in the energy metabolism, cell structure and DNA synthesis were down-regulated (Oh *et al.*, 2004). This is an indication that induction of DNA repair, especially NER and the proteins involved may result in the suppression of the formation of proteins involved in, among others, the energy metabolism. This provides a hypothetical underlying link or connection between DNA repair and the energy metabolism in human cells.

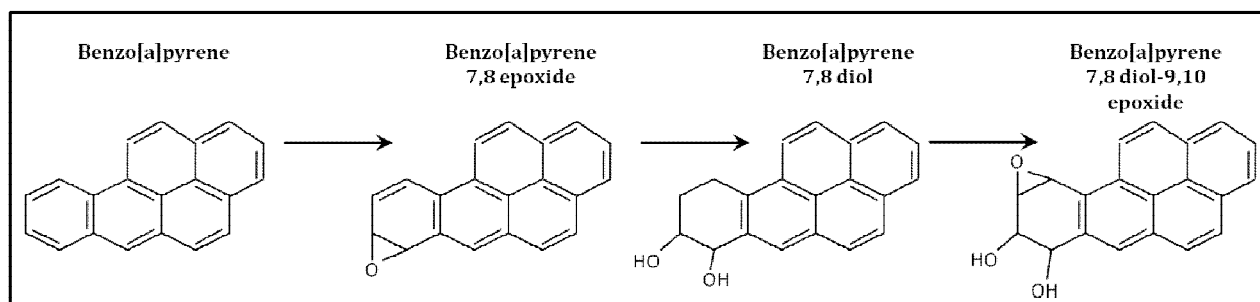


Figure 2.4. Bioactivation of benzo[a]pyrene to benzopyrene diol epoxide. *Figure adapted from Serpi, R (2003)*

Benzopyrene diol epoxide opens spontaneously at the carbon-10 position, leaving a very reactive carbonium ion that can bind to guanine bases in a covalent fashion and distorts the DNA in the process. An aromatic adduct is produced that resides within the minor groove of the DNA double helix (Bast & Hong, 2009). This distortion brings about perturbation of the DNA double helix and induces mutations that commonly cause cancer when the normal cell cycle is disrupted (Yang *et al.*, 1977). B[a]P can also produce quinone derivatives that in turn easily produce ROS, thus it is not uncommon for oxidative DNA damage to accompany the distortion brought about by the binding of BPDE to the DNA molecule (Penning *et al.*, 1996).

2.3. Different aspects influencing DNA repair pertaining to this study

2.3.1. Introduction

The alkaline Comet assay is extensively used to assess DNA damage caused by various exogenous sources (Fracasso *et al.* 2006). The method was first developed by Ostling and Johanson (1984) and its applications have since greatly expanded. More recently this assay's uses have been extended to the quantification of DNA repair capacity as described by, amongst others, Collins *et al.* (2001).

Comet assay (under alkaline conditions) was applied, as well as other established methods to quantify the DNA repair capacity of cultured 143 B cells of which the energy metabolism is altered as well as that of mouse liver tissue with perturbations in the metal metabolism.

2.3.2. Perturbations of the energy metabolism

Mitochondria are membrane surrounded organelles within the majority of eukaryotic cells that serve as the primary energy centres which provide the cell with ATP to perform various tasks. Energy in the form of ATP is produced through processes such as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Coffman, 2009). The concept of a respiratory chain and its chemiosmotic consequences was originally described by David Keilin as early as the 1920s (Mitchell, 1978). Peter Mitchell was awarded the Nobel Prize in Chemistry in 1978 *"for his contribution to the understanding of biological energy transfer through the formulation of the chemiosmotic theory"* (Nobelprize.org, 1978).

Oxidative phosphorylation is described as the production of ATP (and NADH and FADH₂ as by-products) from the main energy sources- carbohydrates, amino acids and fatty acids. ATP production is primarily driven by complex I to IV which is collectively called the electron transport chain (ETC) (Lindeque *et al.*, 2010). Complex I and III are generally regarded as the main sources of superoxide radicals originating from the ETC (Coffmann, 2009). The actual production of ATP is however performed by complex V, otherwise known as ATP synthase. Complexes I to V are located in the inner mitochondrial membrane (Lindeque *et al.*, 2010)

Mitochondrial functioning thus has an enormous effect on overall cell physiology. This functioning does not merely involve production of energy rich molecules (ATP)

for common bodily functions, for instance muscle movement and most importantly for the purpose of this study, energy to drive processes such as DNA repair, it is also associated with important cellular occurrences such as apoptosis, necrosis, regulation of calcium dynamics and the production of reactive oxygen species (ROS), (Finley & Haygis, 2009).

It is commonly perceived that ROS leads to DNA damage and lipid peroxidation that can negatively influence normal cell functioning and bring about various pathological conditions (Finley & Haigis, 2009). ROS, and more specifically hydrogen peroxide, is extensively described in section 2.1.1 and 2.2.2.2. The possibility exists that a change in mitochondrial function may lead to extensive ROS production which may negatively affect macromolecules, including proteins involved in DNA repair pathways, and impaired energy formation which in turn may inhibit the proper occurrence of energy dependant processes (Levy & Deutschman, 2007).

In a study performed in our laboratory by Kok, D (2010) on the 143B cell models with CI and CIII knock-downs, it was observed that transfection alone altered global and gene specific methylation. Kok indicated that changes in DNA methylation in the two knock-down systems differed suggesting that the amount of ROS produced in such a system may cause different methylation patterns and also determines the extent of DNA methylation. Changes in DNA methylation of specific genes are frequently used as diagnostic indicators, random alterations in gene-specific methylation in cell cultures may lead to false identification of biomarkers and can cause considerable misuse in research time and resources. Considering the immense effects that transfection with an empty vector, let alone a knock-down system pertaining to the energy metabolism, has on the methylation of 143B cells, it was deemed necessary to investigate the effects of these perturbations on the DRC of these cells.

2.3.2.1. Complex 1 knock-down

In this study two separate knock-down models will be used to study the effect of intended perturbations in the energy metabolism on DNA repair pathways. These models are established in 143B cells. Both involve the use of an RNAi mechanism for inactivation of a subunit which is crucial for the proper functioning of the complex to which it belongs.

The Clontec Inc. RNAi-Ready pSIREN-RetroQ-TetP self-inactivating retroviral expression vector was used to create the various knock-downs in 143B cells. The vector is commonly used to express double stranded short hairpin RNA (shRNA), to screen for functional shRNA oligonucleotides and in gene silencing applications by using viral mechanism of delivery (PR611410; 2006).

The first model uses sh-RNA to inactivate subunit NDUFS3 (Fe-S protein 3) of complex I (NADH:ubiquinone oxidoreductase; E.C. 1.6.5.3) of the electron transport chain (ETC). Complex I serves as the initial electron accepting complex of the oxidative phosphorylation system (OXPHOS). Leigh syndrome is probably the most common clinical feature of a deficiency of this complex, although it can be found in defects of complexes II-V as well (Finsterer, 2008).

2.3.2.2. Complex 3 knock-down

The second model uses the same type of mechanism for disabling Complex III (ubiquinol cytochrome C; E.C. 1.10.2.2) of the ETC. In this case, subunit UQCRC1 (Rieske Iron-Sulphur protein) of complex III of the ETC is inactivated. This complex catalyses the transfer of electrons from complexes I and III to cytochrome C. Deficiency of complex III has been associated with encephalopathy, liver failure and early death syndrome, to name but a few symptoms (Moslemi & Darin, 2007). The use of these models is based on the fact that increased production of ROS is commonly associated with defects in OXPHOS enzymes, especially those involving complexes I and III (Reinecke, *et al*, 2009). ROS have been shown to influence various epigenetic factors. Its effects on DNA methylation may be direct through a number of chemical mechanisms, or indirect through redox changes that can control the activity of various enzymes and other proteins (Franco, *et al*, 2008).

2.3.3. Perturbations in the metabolism of metals

Metallothioneins are tiny metal-binding proteins that were first discovered by Margoshes *et al.* (1957). These proteins have a characteristically low molecular weight, high cysteine content and contain no aromatic residues (Masters *et al.*, 1994). Regardless of the long period of time that metallothioneins have been a subject of numerous studies, the primary biological function thereof remains largely unknown (Lindeque *et al*, 2010). These tiny proteins have diverse functionality and are involved in numerous cellular processes as various factors and chemicals

induce their expression. Metallothioneins therefore have been referred to as *multi-stress proteins* by Andrews (2000), and Theocharis *et al.* (2003).

Metallothioneins are known to play an important role in trace element homeostasis and detoxification of metals and are effective ROS scavengers (Vašak, 2005; Chiaverini & De Ley, 2010). Some metals such as cadmium (Cd) are known carcinogens due to their ability to accumulate in target organs in humans and its ability to cause apoptosis, and to interfere with vital cellular processes that cooperate to control the transcription of various target genes (Liu & Kadiiska, 2009; Thevanod, 2009; Candéias *et al.*, 2010). Cadmium specifically has the capability of replacing zinc (Zn) and copper (Cu) from metallothioneins and Zn-finger domains present in various enzymes, some of which include proteins involved in DNA repair pathways (Asmuss *et al.*, 2000; Hartwig *et al.*, 2002). What's more, Cd also binds glutathione- the largest and most effective antioxidant. Heavy metal imbalance thus occurs and the cell is exposed to elevated levels of redox metals whilst antioxidant defences are depleted (Liu & Kadiiska, 2009). In the absence of two very important isoforms of metallothioneins it is thus possible for such an imbalance to occur within the cell leading to these undesired conditions. The pro-oxidant environment that heavy metal imbalance causes may result in mutagenesis and transformation through disproportionate DNA damage (Bertin & Averbeck, 2006). As suggested throughout sections 2.1 and 2.2, a constant build-up of unrepaired DNA damage can ultimately lead to sequence modifications and genetic rearrangements. Various DNA repair mechanisms exist however, to maintain genomic integrity. Hoeijmakers (2001) gives the explanation that numerous, partially overlapping intricate processes exist in harmony to identify and repair DNA damage, which would otherwise lead to serious consequences including cancer formation. It has been shown that elevated Cd levels directly affect the repair activity of proteins under investigation in this study, namely XPA and XPC (involved in the damage recognition in NER) as well as hOGG1 (Candéias *et al.*, 2010). It can thus be suggested that an imbalance or disturbance in the metal homeostasis caused by MT knock-out may create an environment favourable for DNA damage formation and at the same time impair DNA repair pathways.

In a study performed by Takashi *et al.* in 2009, it was shown that MT-I/II knock-out mice were more vulnerable to chromosomal abnormality or DNA damage than wild

type mice upon treatment with various dosage concentrations of B[a]P. Another study also showed that MT knock-out mice are more susceptible to oxidative DNA damage than wild type mice. The mice were treated with butenolide (BUT), a *Fusarium* mycotoxin, and DNA damage in cardiomyocytes was evaluated by means of the alkaline Comet assay (Yang *et al.*, 2010). There is thus evidence that metallothioneins play some role in the protection of DNA against various types of DNA damage. Because of the multi-functionality and biological involvement in such a wide variety of biological processes, it was considered interesting to extend the investigation on the effect of the absence of metallothioneins on DNA repair.

2.3.3.1. Metallothionein knock-out mice

Targeted disruption of metallothionein I and II genes was first performed by Masters *et al.* (1994). The MT- I & II knock-out mouse model used in this study was developed accordingly in the laboratory of Dr. Richard Palmiter (University of Washington) and Dr. Ralph Brinster (University of Pennsylvania). Disruption of Mt1 and Mt2 genes was performed simultaneously by inserting an in-frame stop codon into these genes' exons. While altered alleles are transcribed, they are not translated (Masters *et al.*, 1994). In brief, oligonucleotides were ligated into specific restriction sites of a plasmid that contains the sequences for both metallothionein I and II to disrupt the genes and prevent the expression thereof. The disruption construct was linearised and electroporated into AB1 embryonic stem cells. Stably transfected cells were selected and clones were screened by means of PCR. Positive clones were injected into C57BL/6 blastocytes and transplanted into pseudo-pregnant female mice. Heterozygous males and females were allowed to mate in order to give rise to mice that are homozygous for both disrupted metallothionein genes. This new generation of mice is thus unable to express either metallothionein I or II. Henceforth, the mice were only mated with the 129S7/SvEvBrd-Mt1^{tm1Bri} Mt2^{tm1Bri}/J strain of mice to preserve the genetic conditions. Dr. Palmiter pointed out that, contrary to what is published in the paper by Masters *et al.*, (1994) the oligo that is inserted into exon 1 of the Mt1 gene is reversed (it still has the KpnI site and in-frame stop codon) and the additional oligo insertion is not present. He states that these errors do not affect the phenotype but impacts PCR genotyping strategies.

Mice homozygous for the *Mt1^{tm1Bri} Mt2^{tm1Bri}* mutation are viable and fertile. According to available information on the JAX Mice Database these animals show an increased sensitivity to hepatic poisoning by cadmium. Liver tissue samples of metallothionein knock out, as well as liver tissue samples from wild type mice were used in this investigation. The MT knock-out mouse model was used by a collaborative research group in the department and henceforth characterised, as described in section 4.2.4.2.

2.4. Comet assay

2.4.1. Introduction

The assay to be used in this investigation, namely single cell gel electrophoresis (SCGE), to assess DNA damage and repair plays a central role in the study. The following description of the assay will prove that it can not only be applied to assess DNA damage but also DNA repair. More specifically, some variations of SCGE have been developed to study the initial steps of DNA excision repair pathways, BER and NER. We aspire to use this method to study the effect of the above mentioned perturbations on DNA repair.

According to Giavão *et al.* (2009) *in vitro* DNA repair assays can be divided into three distinct types. All three entails preparing substrate DNA with very specific lesions and treating it with a cell extract. The first involves the use of plasmid DNA and the repair activity is detected as strand nicking quantified by measuring the migration rate in an agarose gel during electrophoresis, or by the integration of a radioactive precursor into the DNA. The second method uses oligonucleotides that are constructed to contain a specifically damaged base and is end-labelled with a detectable tag. Active proteins cause the label to be released and this can be measured and used as an indication of repair activity. Nucleoids are used as a substrate for the proteins in the cell extract in the third method. The DNA strand breaks introduced by the excision repair enzymes present in the protein extract is measured by employing the Comet assay.

The third method, SCGE, is more commonly known as the Comet assay (Plazar *et al.*, 2007). The name is derived from the comet-like appearance of damaged nucleoids after electrophoresis has been performed. The principle of the Comet assay was originally described by Ostling and Johanson (1984), it entails that

broken strands of DNA are stretched out via electrophoresis to give rise to the comets – hence the name (Plazar *et al.*, 2007). The assay entails embedding a number of single cells in a thin layer of high melting point agarose (HMPA) and lysing these cells to remove the cell debris and expose the nucleoid as a whole, without the risk of damaging the DNA when isolating and handling it (Collins *et al.*, 2001). For the purpose of this study there will be referred to bare DNA molecules, or DNA molecules that are free of a nuclear membrane and proteins, as nucleoids. These nucleoids are submitted to electrophoresis and fluorescent staining is performed in order for the DNA to be visible when examining it under a fluorescent microscope (Nossoni, 2008). Imaging software is then used to score the resulting comets and to determine the amount of DNA present in the head and tail respectively. The Comet IV software is used by us to analyse the extent of DNA damage present for this specific study. The head of the comet-image is composed of intact, undamaged DNA whilst the tail is formed by broken strands of DNA that is stretched out by electrophoresis. Several modifications of the Comet assay exist up to date, the alkaline Comet assay will be used in this study, for its wide possibility range (Collins, 2007). This assay is used to detect single- and double strand breaks, alkali labile sites, oxidative damage to bases and DNA cross-linking to other fragments of DNA or proteins (Singh *et al.*, 1988). It is also possible to detect AP-sites as well as excision repair sites with the Comet assay when it is performed under alkaline conditions (Collins, 2007).

2.4.2. Principle

From the above summary of the Comet assay, the method appeals greatly for the use in this study as it can measure AP-sites, and single- and double strand breaks in DNA. AP-sites and strand breakage may be present at low levels in the cultured cells, arise as a result of the treatment of cells with the damage causing reagents, H₂O₂ and benzo[a]pyrene respectively, or alternatively as a result of enzyme activity (Giavão *et al.* 2009). As explained earlier, the two enzymes under investigation in this experiment are involved in excision repair pathways, act to remove damaged bases or nucleotides from the DNA molecule and therefore may result in increased strand breakage. It is important to keep in mind that increased strand breakage leads to an increase in the percentage of tail DNA and also indicates enzyme activity. Protein treatment resulting in a great difference in average percentage tail

DNA (compared to background levels) thus indicates maximal protein activity, whereas smaller differences in tail DNA before and after protein treatment point towards inhibition of the excision repair proteins under investigation.

2.4.3. Applications

The technique is generally described as simple and uncomplicated and is often engaged to determine DNA repair *in vitro* (USBweb, 2009). According to Collins (2007), single cell gel electrophoresis is less precise than HPLC for investigation of oxidative damage of DNA (e.g. 8-oxoG), however it is more truthful for the measurement of background levels of damage, for the simple reason that less handling of the exposed DNA is required in the Comet assay. This assay is also quick to perform and no potentially damaging reagents are used during the procedure other than the damage inducing reagents under investigation (Plazar, 2007). One complication to the Comet assay is that the extent of damage is dependent on internal calibration as no internal standard have been developed up to date (Collins, 2007). For this very reason it is crucial to determine the background levels of DNA damage in every single experiment.

The Comet assay was employed by researchers in our laboratory to investigate DNA damage and repair in mammalian cells exposed to pHPPA, a characteristic hereditary tyrosinemia type 1 (HT1) metabolite (Van Dyk & Pretorius, 2005). An extension of this method and its applications with some modifications is thus used in this study. A Comet assay-based method to study BER activity was first developed by Collins *et al.* in 2001, where irradiation was used to induce 8-oxo-G to the substrate DNA. This assay was later modified by using hydrogen peroxide to induce oxidative damage to the substrate DNA that is recognized by enzymes involved in BER as well. In 2006 Langie *et al.* modified the BER assay by using BPDE to damage the substrate DNA, thereby creating bulky adducts to the DNA that is repaired via NER. In this study we aim to investigate the influence of various perturbations on both BER and NER capacity by employing modified versions of these two methods specified by Collins *et al.* (2001) and Langie *et al.* (2006) respectively.

2.5. Aims and approach

Aim:

The aim of this study is to investigate DNA damage and repair in various tissue or cell culture samples to primarily assess the effectiveness of the alkaline Comet assay in concurrence with other well established methods to examine DNA repair capacity.

Approach:

Tissue- and cell culture samples with certain perturbations which may have an influence on the DNA repair capacity of the particular tissue or cell line were used in this study to prepare protein extracts, of which the DNA repair capacity was investigated.

These protein extracts, prepared from the different cell culture or tissue samples, were used to treat substrate DNA with induced damage to assess the ability of a particular protein extract to repair damage of a certain type and to a certain degree on the DNA. This investigation was primarily conducted by means of the alkaline Comet assay.

Furthermore, real time gene expression of hOGG1 and ERCC1 – the two selected enzymes from the BER and NER pathways respectively – was performed on the 143B cell variants to determine whether the effects of the different perturbations causes a change in the expression of either of these genes encoding for the two enzymes. The gene expression results give an indication whether or not a change in DRC of the cultured cells can be ascribed to altered gene expression, protein modification or other unknown factors caused by the perturbations of the energy metabolism or transfection in general.

2.6. Executed protocol

A broad overview of the research plan followed is outlined in the diagram below (fig. 2.5).

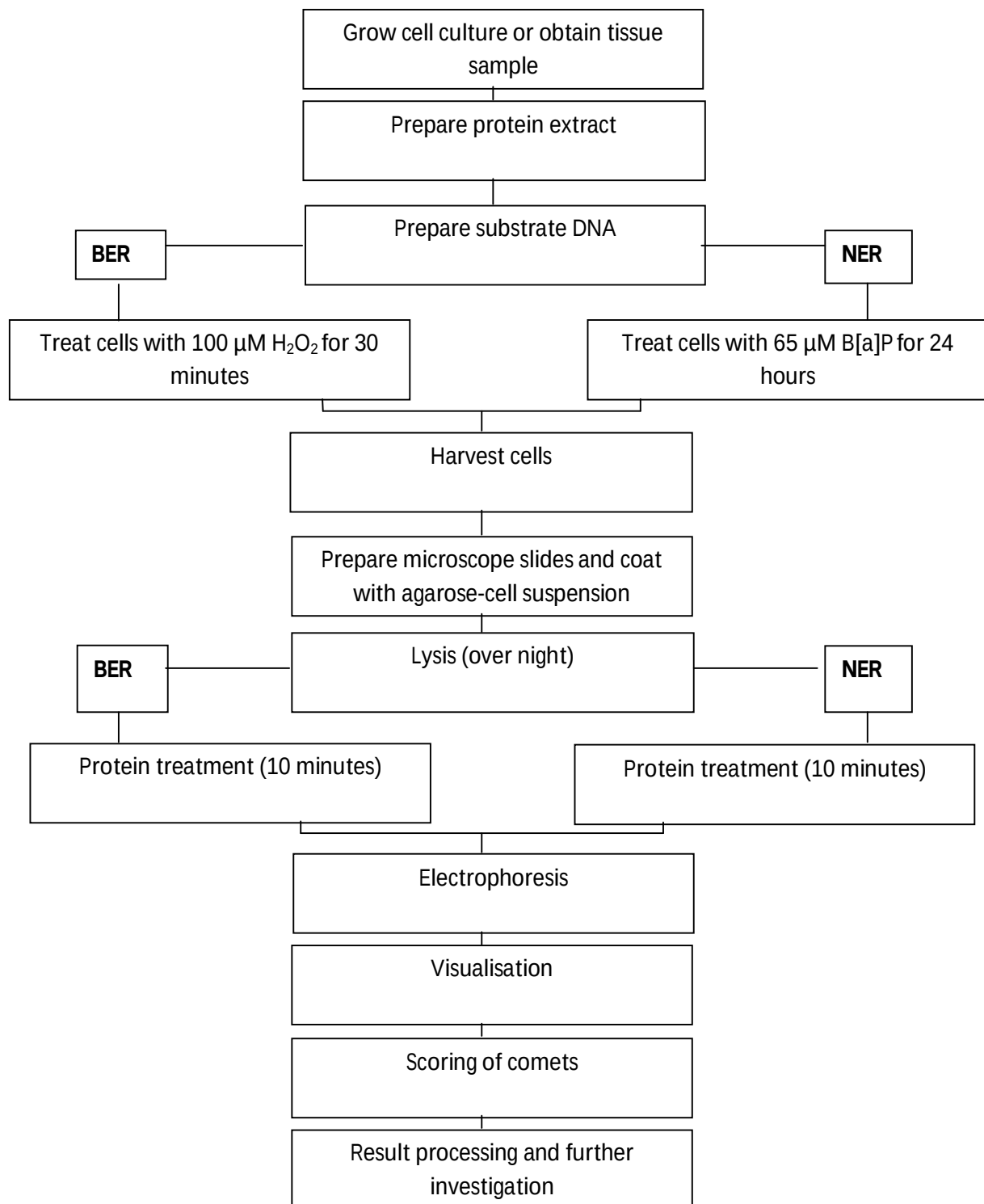


Figure 2.5. *Determining DRC with the Comet assay.* Summarised overview of the planned protocol for the completion of the Comet assay that forms a central part of this study.

CHAPTER 3

PUBLISHED WORK

The method described in this dissertation was used with some modifications to perform the experiments leading up to the findings published in the included paper. The method is thus established but in this study the researcher aims to apply it differently and plan to enhance its credibility for future use, as well as to uncover the effects of various perturbations on the DRC of a range of samples.

The study was a follow up on previous work completed in our laboratory concerning the effects of characteristic aspects of Hereditary tyrosinemia type 1 (HT1) on various cellular processes which lead to the belief that typical HT1 metabolites may affect DNA repair mechanisms, explaining the high incidence of hepatocarcinoma in HT1 patients. I, the researcher, executed most experiments recorded in the published paper, except the BER experiment where MMS was used to induce DNA damage- the latter was performed by the first author; E. van Dyk. The paper was written by Miss E. van Dyk and me, whilst statistical analysis was performed by Dr. G. Koekemoer. The study was performed and directed under supervision of Prof. P.J. Pretorius who also assisted greatly with the structure and editing of the final accepted manuscript. The consent of all co-authors was obtained to include the paper in this dissertation (see p.3).

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Hereditary tyrosinemia type 1 metabolites impair DNA excision repair pathways

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ABSTRACT

Hereditary tyrosinemia type 1 is an autosomal recessive metabolic disorder, which is caused by a defective fumarylacetoacetate hydrolase enzyme, and consequently metabolites such as succinylacetone and *p*-hydroxyphenylpyruvate accumulate. We used a modified comet assay to determine the effect of these metabolites on base- and nucleotide excision repair pathways. Our results indicate that the metabolites affected the repair mechanisms differently, since the metabolites had a bigger detrimental effect on BER than on NER.

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1. Introduction

Hereditary tyrosinemia (HT1) is an autosomal recessive metabolic disorder (OMIM 276700) caused by a defective fumarylacetoacetate hydrolase enzyme (E.C.3.7.1.2) in the tyrosine catabolism [1,2]. Intermediate metabolites such as fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and to a lesser extent *p*-hydroxyphenylpyruvate (pHPPA), *p*-hydroxyphenylacetate (pHPAA) and *p*-hydroxyphenyllactate (pHPLA) accumulate intracellularly. Characteristic of HT1 is hepatocarcinoma (HCC) and somatic mosaicism [1,3].

The molecular mechanism of HCC and somatic mosaicism is largely unknown [4]. The accumulating metabolites most probably create an internal chemical milieu instigating the molecular events underlying the HCC and somatic mosaicism. In addition to the observation that the HT1 metabolite succinylacetone (SA) inhibits DNA ligase activity [5] and the suggestion that the accumulating metabolites most likely act as alkylating agents and/or disrupt sulfhydryl metabolism [6,7], we found that the DNA repair activity in liver cells is impaired by pHPPA [8].

The mechanism and extent by which DNA repair is affected by these metabolites is still unclear. We have used a modified comet assay [9,10] to assess the effect of HT1 metabolites on the first steps of the base- and nucleotide excision repair mechanisms,

BER and NER. The primary substrate for BER is oxidized and alkylated DNA bases [11–13] and for NER large DNA adducts, caused by exposure to UV-light and chemicals such as benzo[a]pyrene [12]. For use in the modified comet assay these lesions were prepared by treating HepG2 cells with hydrogen peroxide (H₂O₂), methyl methanesulfonate (MMS) and benzo[a]pyrene (B[a]P) respectively. Repair of these lesions was investigated with protein extracts (PEs) prepared from cultured HepG2 cells exposed to the HT1 metabolites, SA and pHPPA.

2. Materials and methods

All reagents were obtained from Sigma (Johannesburg, South Africa), unless otherwise indicated.

2.1. HepG2 cells

HepG2 cells were cultured in a CO₂ incubator at 37 °C and 5% CO₂ in growth medium (Dulbecco's Modified Eagles Medium; Thermo Scientific) supplemented with 10% foetal bovine serum (FBS) (BioWhittaker), 1× Penicillin/Streptomycin (100 U Pen./ml; 100 µg Strep./ml; BioWhittaker) and 1× MEM non-essential amino acids (0.1 mM; BioWhittaker). They were harvested as follows: the cells were washed with 1× phosphate buffered saline (PBS), incubated with 1% trypsin (BioWhittaker) for 5 min, followed by the addition of 2 ml growth medium. The cells were then counted using trypan blue by mixing 25 µl trypan blue, 15 µl PBS and 10 µl cell suspension. Of this mixture 10 µl was added to the counting chamber of a haematocytometer and nine squares were counted. The volume of the cell suspension was adjusted with growth medium to 2.5 × 10⁴ cells per 50 µl. To allow recuperation

Abbreviations: HMPA, high melting point agarose; LMPA, low melting point agarose; PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI PE, heat inactivated protein extract; Stdev, standard deviation; ND, not done.

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of the cells after the harvesting procedure they were incubated at 37 °C and 300 rpm for 2 h.

2.2. Preparation of substrate DNA

Recuperated HepG2 cells were treated with H₂O₂ to serve as substrate for BER of oxidized DNA bases. Cells were divided into two aliquots of 2×10^5 cells in 400 μ l cell growth medium. The one aliquot were left untreated to serve as control and the other was exposed to hydrogen peroxide at a final concentration of 80 μ M. Both cell suspensions were incubated for 1 h at 37 °C and 300 rpm. Meanwhile, frosted microscope slides were coated with 300 μ l 1% high melting point agarose gel (HMPA). The slides were placed on a cool surface to allow it to set and remain cool until the sample was added. Fifty microliters of the untreated, or hydrogen peroxide-treated cell suspension were added to 130 μ l 0.5% low melting point agarose (LMPA) and mixed briefly by pipetting. The cell-LMPA mixture (180 μ l) was placed on one half of the pre-coated (HMPA) frosted microscope slide, and evenly distributed, taking care not to damage the thin layer of HMPA. For each of the untreated and H₂O₂ exposed cells, six microscope slides were coated. The LMPA-coated slides were allowed to solidify and were then placed overnight at 4 °C in cold lyses buffer (5 M NaCl, 0.4 M EDTA, 1% Triton X-100, 10% DMSO).

In parallel, recuperated HepG2 cells were treated with MMS to serve as substrate for BER of alkylated DNA bases. Twelve frosted microscope slides, previously coated with 300 μ l of 1% HMPA, were each coated with 2.5×10^4 cells. This was done by diluting the recuperated cells to 2.5×10^4 cells per 50 μ l cell growth medium. Fifty microliters of this diluted cell suspension were added to 130 μ l 0.5% LMPA and mixed briefly by pipetting the mixture. The cell mixture (180 μ l) was then evenly distributed on one half of the pre-coated (HMPA) frosted microscope slide. Thereafter the microscope slides were submerged in lyses buffer and left overnight at 4 °C. After lyses, the slides were washed for 15 min in PBS and then submerged in 20 μ M MMS for 30 min at 4 °C. The microscope slides were then immersed in buffer B (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml BSA, pH 7.8) for 15 min. These microscope slides were directly treated with protein extract.

To serve as substrate for NER, cells were treated with benzo[a]pyrene as described by Plazar et al. [14]. Briefly, HepG2 cells were seeded in a six-well plate at a density of 2×10^5 per well one day prior to benzo[a]pyrene treatment. Two wells were seeded with cells, one as vehicle control and the other to be exposed to benzo[a]pyrene. After 24 h the cells were exposed to a final concentration of 75 μ M benzo[a]pyrene, dissolved in DMSO, and were incubated for 24 h at 37 °C and 5% CO₂. The cells were then harvested and counted. The cell suspensions were diluted to 2.5×10^4 cells per 50 μ l growth medium of which 50 μ l was added to 130 μ l 0.5% LMPA and mixed briefly. The cell mixture (180 μ l) was evenly distributed on the frosted microscope slide pre-coated with HMPA and six microscope slides were coated for each of the different cell suspensions. These were left to solidify and were then placed overnight at 4 °C in cold lyses buffer.

2.3. Preparation of protein extracts

Three different protein extracts were prepared. Control protein extract was prepared from HepG2 cells cultured in standard growth medium. HepG2 cells were separately treated with either 50 μ M SA or 100 μ M pHPA in growth medium for 48 h at 37 °C and 5% CO₂ to prepare metabolite treated protein extracts. These cells were harvested, counted and divided into aliquots of 5×10^6 cells. The aliquots were either used directly for protein extraction or frozen (–80 °C) as pellets until used. The method to prepare the respective protein extracts were described by Collins

et al. [9] and Langie et al. [10]. In brief, each aliquot of 5×10^6 cells were resuspended in 50 μ l buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 7.8), and snap frozen in liquid nitrogen. The frozen cell pellets were then left at room temperature to thaw. Whilst the cells were thawing, a 1% Triton X-100 in buffer A solution was prepared and 15 μ l of this mixture were added per 50 μ l of the thawed cells. The cell suspension were briefly vortexed and left on ice for 10 min. The cell suspensions were then centrifuged at 14,000g at 4 °C for 5 min and the supernatant transferred to a clean tube. The protein concentration was determined with the bicinchoninic acid assay according to specifications by the manufacturer (Thermo Scientific) and was adjusted to 1 mg/ml with 0.23% Triton X-100 in buffer A. After dilution the protein extracts were aliquoted and frozen at –80 °C until use.

2.4. DNA repair assay

The microscope slides that were coated with substrate cells were removed from the lyses solution and washed for 15 min with PBS followed by a washing step with buffer B for 15 min at room temperature. A working protein extract mixture was prepared for each of the different protein extracts. The working protein extract mixture included: 50 μ l protein extract, 200 μ l buffer B and 10 μ l of a 65 μ M ATP solution. A 50 μ l aliquot of the working protein extract was evenly spread on the frosted microscope slide coated with substrate DNA imbedded in agarose. The microscope slides were then incubated for 10 min at 37 °C in a humidified atmosphere, whereafter they were placed in electrophoresis buffer (0.6 M NaOH, 50 mM EDTA) for 30 min at 4 °C. Electrophoresis was then done at 4 °C for 40 min at 30 V (200 mA). Following this, the slides were submerged for 15 min in the neutralizing buffer (0.5 M Tris-HCl, pH 7.5). The nucleoids were then stained in a 12.7 μ M ethidium bromide solution for 30 min. Slides were finally washed for 5 min in double distilled water. An Olympus 1 $\times$ 70 fluorescence microscope (200 $\times$) was used to assess the slides. The Comet IV® software was used to capture the images and to determine the percentage of DNA in the comet head and tail of a minimum of 35 randomly selected comets.

2.5. Statistical methods

Descriptive statistics were calculated and the data presented graphically using boxplots. Since the distributional assumptions of analysis of variance (ANOVA) are violated (Fig. 1), we used the two-way heteroscedastic rank-based test, Brunner–Dette–Munk (BDM) [15] to investigate whether the control cases behave differently to protein treatment when compared to the exposed group (i.e. the existence of interaction). In case of such an interaction, the various protein treatments were compared separately for the controls and the exposed groups by using the one-way non-parametric rank-based permutation test. For post-hoc analysis, all groups were compared to control protein treatment using the modified method of Dunn [16] as described in [17].

Note that the Kruskal–Wallis non-parametric test assumes that groups have the same distributional shape, therefore all group variances should be similar. This is not true for the current study (Fig. 1). As an alternative, we used BDM [15], which is a heteroscedastic version of the Kruskal–Wallis test that is robust against non-normality and heteroscedasticity. All statistical analyses were performed using R and SPSS.

3. Results

The effect of the different accumulating HT1 metabolites on the proteins involved in the initial steps of BER and NER was determined by exposing HepG2 cells to either 50 μ M SA or 100 μ M

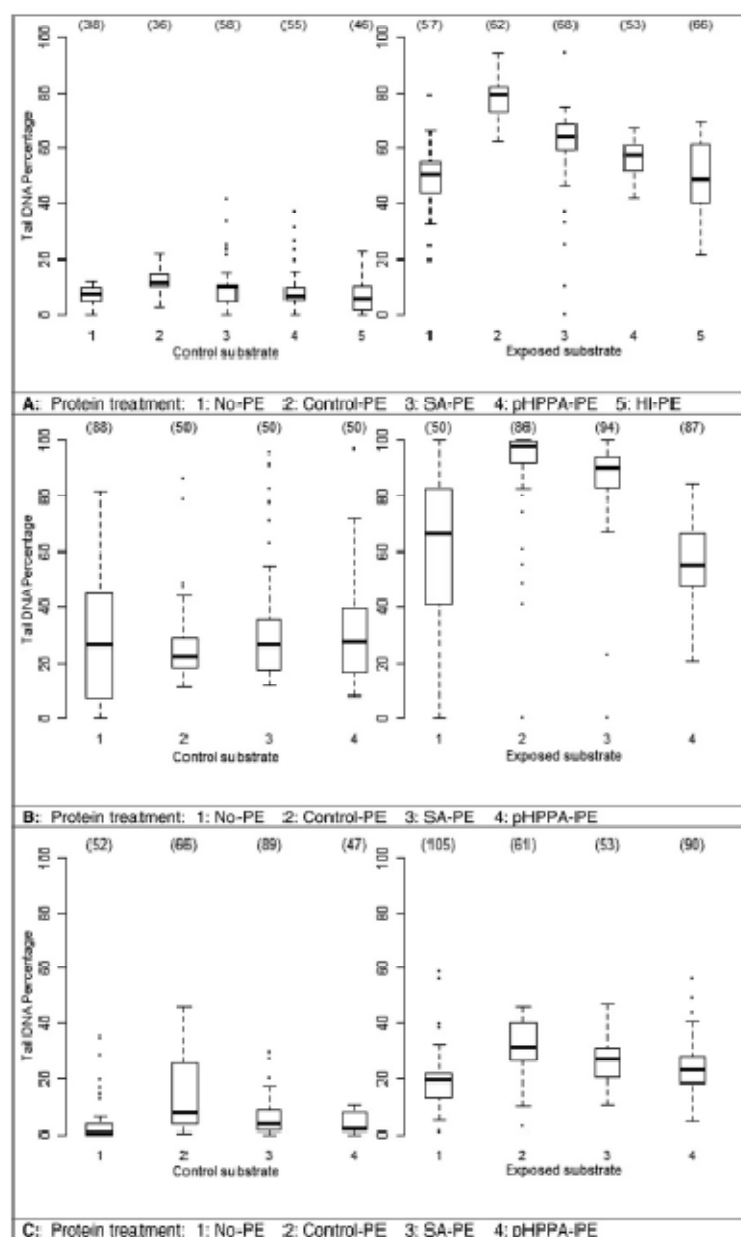


Fig. 1. Graphical representation of tail DNA percentages after different protein treatments. (A) BER (H_2O_2). (B) BER (MMS). (C) NER (H_2O_2). The number of comets that were scored for each sample is given in brackets. PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI-PE, heat inactivated protein extract.

pHPPA for 48 h. Protein extracts from these and untreated HepG2 cells were then applied to the appropriate agarose imbedded substrate DNA nucleoids, and the tail DNA percentages were determined. The extent of the increase in the percentage tail DNA measures the effectiveness of the proteins involved in the initial steps of BER and NER in their ability to recognize and excise the induced DNA lesions [9,10,18]. Control protein extract was assumed to have optimal repair capacity.

The tail DNA percentages for the three experiments are presented on the various factor levels, i.e. control vs. exposed groups and the different protein treatment levels, in Fig. 1 and Table 1.

In all the experiments, the control groups have lower tail DNA percentages (TDP) on expectation. For the BER experiments, the control groups reacted differently to the treatment when compared to the exposed groups. This interaction was statistically significant at a 5% level. No statistical interaction was observed for the

Table 1

Mean and median tail DNA percentages after treatment of nucleoids with protein extract. Standard deviations of tail DNA percentages are given in brackets. BER, base excision repair; NER, nucleotide excision repair; H₂O₂, hydrogen peroxide; MMS, methyl methanesulfonate; B[a]P, benzo[a]pyrene; PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI PE, heat inactivated protein extract; Stdev, standard deviation; ND, not done.

		BER (H ₂ O ₂)		BER (MMS)		NER (B[a]P)	
		Control substrate	Exposed substrate	Control substrate	Exposed substrate	Control substrate	Exposed substrate
-PE	Median	7.32	50.72	26.21	66.37	0.29	19.17
	Mean	6.85	50.10	26.85	60.95	3.90	19.10
	Stdev	(3.40)	(9.87)	(21.76)	(29.84)	(7.50)	(8.40)
+CPE	Median	11.76	79.20	22.60	97.90	7.75	32.03
	Mean	12.34	77.89	26.29	88.55	13.43	31.51
	Stdev	(3.90)	(7.07)	(14.72)	(24.65)	(13.74)	(9.39)
+SA-PE	Median	10.01	64.04	26.41	90.36	3.49	27.12
	Mean	9.77	61.33	32.70	87.12	5.76	26.41
	Stdev	(7.68)	(14.09)	(22.36)	(13.74)	(6.27)	(8.75)
+pHPPA-PE	Median	6.69	57.29	27.09	55.60	2.04	23.03
	Mean	8.59	56.14	32.94	56.65	4.09	24.20
	Stdev	(7.15)	(6.57)	(22.34)	(14.10)	(3.54)	(8.75)
+HI PE	Median	5.73	49.03	ND	ND	ND	ND
	Mean	6.78	49.67				
	Stdev	(5.37)	(12.00)				

NER (B[a]P) experiment using the two-way rank-based permutation test.

Subsequently, the effect of protein treatment was separately investigated for the controls and exposed groups. For all the experiments, except for the control group of the BER(MMS) experiment, a statistical significant protein treatment effect was observed (one-way BDM).

For the BER(H₂O₂) experiment we used heat inactivated protein extract, to assess whether the increase in TDP after the addition of PE is truly due to the addition of PE. The TDP for untreated exposed substrate nucleoids and heat inactivated PE treated substrate nucleoids should therefore be similar. The Dunn [16] post-hoc test comparing all the possible group comparisons was performed, which revealed that the TDP of untreated exposed substrate nucleoids and heat inactivated PE treated substrate nucleoids does not differ significantly. It is therefore clear that the addition of PE to H₂O₂-substrate nucleoids caused a change in the TDP, which is an indication of the ability of the proteins involved to recognize and excise the oxidized DNA lesions induced by H₂O₂.

Non-parametric post-hoc analysis comparing the various protein treatments to the control protein treatment (i.e. protein extract with optimal repair) revealed that all treatments were significantly different from the control protein treatment.

In specific, there was a significant increase in the TDP of H₂O₂ exposed nucleoids after treatment with control-PE, SA-PE and pHPPA-PE. The post-hoc analysis also indicated a significant decrease in the TDP after the treatment of H₂O₂ exposed nucleoids with protein extracts that were exposed to SA or pHPPA when compared to treatment with control-PE.

Also, a significant increase was measured between the TDP of MMS-substrate nucleoids not treated with PE and the control-PE treated MMS-substrate nucleoids (Fig. 1 and Table 1). Compared to the TDP of the MMS-substrate nucleoids treated with control-PE, the TDP of MMS-substrate nucleoids treated with SA-PE or pHPPA-PE decreased significantly. Furthermore, the TDP of MMS-substrate nucleoids not treated with PE and the nucleoids treated with pHPPA-PE did not differ significantly [16].

A significant increase was measured in the TDP of B[a]P exposed nucleoids after treatment with control-PE. On the other hand, a significant decrease was measured in the TDP of SA-PE and pHPPA-PE treated B[a]P exposed nucleoids, when compared to treatment with control-PE.

4. Discussion

The preservation of genomic integrity is particularly important for organism survival [11,13]. Different DNA repair mechanisms exist for this purpose, including direct reversal, mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) [12,13]. Of these repair mechanisms, BER and NER play an integral role in repairing single strand lesions. The fact that excision repair pathways operate in very similar ways but differ quite distinctively in the points of onset [11] allows investigation of the effect of genotoxic agents, such as HT1 metabolites, on both BER and NER in cultured cells.

The primary substrate of the BER pathway is oxidized and alkylated DNA bases [11–13]. In this study, hydrogen peroxide was used to induce DNA oxidation, which entails the formation of 8-oxo-G [19]. The very first step in BER is the hydrolysis of the N-glycosidic bond between the two linked bases (8-oxo-G paired with C) in order to remove the damaged base [13]. Human 8-oxo-guanine glycosylase 1 (hOGG1) is a type II glycosylase enzyme that specifically catalyses the removal of 8-oxo-guanine bases and cleaves the AP site to form a single strand break [20].

The decrease in TDP of H₂O₂-substrate nucleoids treated with SA-PE and pHPPA-PE compared to control-PE treated substrate nucleoids suggests that the base excision repair pathway were impaired by exposure of HepG2 cells to SA and even more so after exposure to pHPPA. It is therefore likely that these metabolites affect the capacity of hOGG1 to excise the 8-oxo-G DNA lesions. However, 8-oxo-G is not exclusively removed by hOGG1. There is a back-up mechanism that appears to require the CSB gene product, but whether this gene product is necessary for direct participation in the BER pathway or for the expression of DNA glycosylases, is unclear [21]. The very low level of initiation of the removal of 8-oxo-G by the pHPPA exposed protein extract, suggests that exposure to pHPPA might also affect this back-up pathway. The precise nature of these perturbing effects of SA and pHPPA, however, still needs to be clarified.

MMS is an extensively used [22,23] SN2-type DNA alkylating agent that methylates the ring nitrogens of purines, to generate 7-methylguanine and 3-methyladenine, which causes base mispairing and replication blocks [21,23]. MPG or N-methylpurine DNA glycosylase is a type I DNA glycosylase, involved in the repair

of these alkylated DNA bases [13,21], by removing the modified base thus creating an AP site. The incision of the phosphodiester bond of the AP site is done by AP endonuclease (APE1), which interacts with and is stimulated by XRCC1 [13].

Our results showed that exposure of HepG2 cells to SA and pHPPA caused a decrease in the efficiency of PE from these cells to recognize and incise DNA lesions when compared to PE from unexposed HepG2 cells. The inter-dependant nature of the BER initiating proteins (MPG, APE1 and XRCC1), however, makes it difficult to conclude which of these proteins are the most adversely affected by SA and pHPPA.

NER primarily acts to repair large DNA adducts caused by exposure to chemicals such as benzo[a]pyrene (B[a]P) [12]. B[a]P is a polycyclic aromatic hydrocarbon that causes DNA damage by distorting the DNA structure when binding to it [10]. It needs to be bioactivated to form benzo[a]pyrene diol epoxide (BPDE), which is carcinogenic. B[a]P can be used to induce specific damage to the DNA in order to investigate transcription coupled NER (TC-NER). Langie and colleagues demonstrated a significant correlation between the removal of BPDE-adducts and the DNA repair capacity of cells, and suggested that increased tail moments and percentage fluorescence in the comet tail are indicative of the NER capacity of the protein extracts [10]. The modified comet assay therefore measures the effect on ERCC1 (excision repair cross complementing) and associated proteins involved in specifically the initiating steps of NER. ERCC1 acts in an XPF-ERCC1 complex to excise the lesion and perform the 5'-incision, the 3'-incision is performed by XPG [13].

Our results have shown that the repair ability of protein extracts, prepared from cells exposed to either SA or pHPPA, was reduced. SA and pHPPA therefore affects the initiating proteins of NER, but do not debilitate them. Similar to the repair of alkylating DNA damage, the NER initiating proteins work in an integrated fashion, thereby making it difficult to distinguish which of the proteins are affected. It remains to be seen if exposure to SA and/or pHPPA has an effect on the expression level of these proteins or if it is a structural interaction impairing its function.

5. Conclusion

In conclusion, we have shown that some of the metabolites associated with HT1, namely SA and pHPPA have an impairing effect on both BER and NER. Our results indicated that exposure to these metabolites affected the repair mechanisms differently, since the metabolites had a bigger detrimental effect on the initiating proteins of BER than on those of NER. The harmful effect of the metabolites might therefore contribute to the development of HCC in HT1 patients. We furthermore observed that exposure to SA did not have as big an impact on repair proteins than exposure to pHPPA. This observation also confirms our previous suggestion that exposure to pHPPA has an effect on DNA repair [8]. Although, further investigation is still needed to define the mechanism by which SA and pHPPA affect the proteins initiating DNA repair, our results nonetheless sets the base for investigation into the effect of the HT1 accumulating metabolites, SA and pHPPA, on DNA repair.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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CHAPTER 4

MATERIALS AND METHODS

4.1. Introduction

All experiments were carried out in duplicate. All the reagents used for the performance of the experiments were of the finest quality available and were purchased from Sigma-Aldrich, unless stated otherwise. Appendix A contains instructions and recipes for the preparation of buffers, agarose, etc.

All procedures concerning the culturing of the cells were performed aseptically in the cell culture laboratory of the Division of Biochemistry of the North-West University, Potchefstroom Campus under supervision of Dr. Oksana Levanets.

4.2. Comet assay

4.2.1. Preparation of microscope slides

A frosted microscope slide (fig. 4.1) was coated with 300 μ l of 1 % high melting point agarose gel. The slide was then placed on a cool surface to set and kept cool until the sample was added.

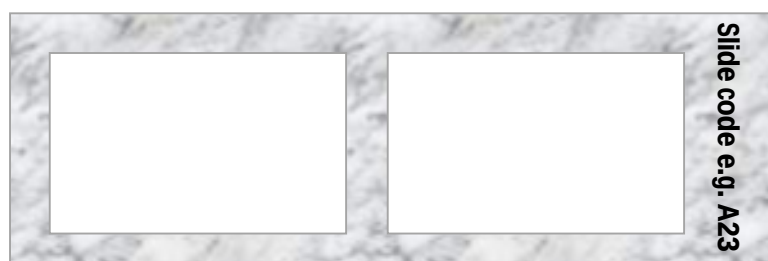


Figure 4.1. A simplified drawing of a frosted microscope slide. *The frosted frame of the microscope slide ensures that the HMPA does not slide off, but sticks to the slide. Two non-frosted windows provide separate compartments for the cells to be mounted on.*

4.2.2. Culturing of cells

143B cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C, 5% CO₂. This mixture of DMEM and FBS will be referred to as media, or growth media for the purpose of this study. Cells to be treated with H₂O₂ or that were left untreated for the preparation of the protein

extracts were cultured in 75 cm² flasks obtained from NUNC. Cells to be treated with B[a]P were cultured in 6-well plates obtained from the same company.

4.2.3. Harvesting of cultured cells

The cell-culturing flask was carefully removed from the 37°C incubator and the cells were inspected under the microscope before treatment with the damage causing reagent. (The procedure for the treatment of the cells with the damage inducing reagents, H₂O₂ or B[a]P will be elaborated upon in section 4.2.4.) Following treatment of the cells with either of the damage causing reagents, growth media was discarded and the cells were washed with PBS after which the cells were trypsinated for one minute. After trypsination, approximately 3 ml new growth media was added to stop the trypsin reaction. The cells were then counted with the trypan blue method, briefly 10 µl of cell suspension was combined with 15 µl of PBS and 25 µl of trypan blue staining solution. Thereafter, 10 µl of this mixture was placed on each window of the haemocytometer and the cells were counted under a light microscope. 25 000 cells in 50 µl of growth media were used to coat each window of a slide, the volume of the cell suspension was adjusted with growth medium according to the amount of slides needed for a particular experiment. The resulting cell suspension was used as described in section 4.2.5.

4.2.4. Preparation of protein extracts

Different protein extracts were prepared for the purpose of this study in order to investigate the effects of the different perturbations on DRC.

4.2.4.1. Complex I and III knock-downs in 143B cells

Each of the cell cultures with different perturbations were used to prepare the protein extract in order to investigate the effect of that perturbation on the DNA repair capacity of that cell line or culture. 143B cells (five variants) were cultured as described in section 4.2.2.

The five variants included the following:

- 143B control cell line

- 143B control cell line with empty retroviral vector (pSIREN-RetroQ TetP)
- 143B control cell line with an unrelated knockdown system (anti-eGFP sh-RNA in the same vector)
- 143B cell line with Complex I knockdown
 - NADH-ubiquinone oxidoreductase
 - Subunit NDUFS 3
- 143B cell line with Complex III knockdown
 - Ubiquinol cytochrome C reductase
 - Subunit UGCRFS1

This model with different perturbations in the energy metabolism, brought about by means of transfection, was previously used in a study investigating the effects of the perturbation on global and gene-specific DNA methylation. This model was characterised as in described in Kok (2010), p. 19-22, and used accordingly.

The five variants were kept separate and five different protein extracts were thus prepared. Protein extracts prepared from untransfected/unaltered 143B cells was used as a control or reference. 143B cells containing an empty vector was used to prepare a protein extract to measure the effect of the transfection alone on the DRC of 143B cells. A protein extract prepared from 143B cells with an unrelated knock-down system (GFP) was used to determine the effect of a knock-down system on the DRC of these cells. Furthermore, 143B cells of which complex I and III are respectively knocked-down were used to prepare protein extracts to investigate the effect of perturbations in the energy metabolism on the DRC of 143B cells. These cells were also harvested using the method described previously and was either used directly for protein extraction or frozen in pellets until protein extraction was performed.

The method described by Langie *et al.* (2006) was followed for protein extraction from 143B cells. In brief, the required amount of 143B cell pellets were thawed (if frozen), 5×10^6 cells produce enough protein extract to coat several microscope slides.

These cells were resuspended in 50 µl of Buffer A (50 µl per 5×10^6 cells), and snap frozen in liquid nitrogen to halt all cell processes. The microcentrifuge tubes were taken out of the liquid nitrogen with pincers and left to thaw on ice. In the meantime 10 µl of Triton X-100 were added to 990 µl of buffer A. Fifteen micro litres of this mixture was added per 50 µl of protein extract needed. These tubes were vortexed briefly and incubated for ten minutes on ice for the lysis of the cells to take place. After incubation, the tubes were centrifuged at 14 000 G (11 000 rpm) at 4°C for five minutes. The supernatant was removed from each tube and added to a new, clean tube. Protein concentration was determined by employing the bichinonic acid method and a plate reader, after which the concentration was adjusted to 1mg/ml with 0.23% Triton X-100 in buffer A. The protein extracts were stored on ice until use or frozen in aliquots at -80°C until needed for the Comet assay.

4.2.4.2. Metallothionein knock-out mice

Prof. Juan Hidalgo from the Autonomous University of Barcelona, Spain provided 10 breeding pairs of mice that have a single allele disruption in the MT-I & II gene locus (heterozygote, MT+/-), as originally described by Masters et al. (1994). For this, a permit was obtained from the Department of Agriculture South Africa. These mice were kept and used for breeding at the pathogen free unit of the Animal Research Centre at the North West University, Potchefstroom Campus. Heterozygotes (MT-/+) were used during breeding to produce MT-knock-out (MT-/-), wild-type (MT+/+) or heterozygote mice. Ethical approval for the performance of the study was obtained from the Ethical Committee of the North-West University under the title: *Investigation of the effect of metallothionein expression on the pathophysiology of disorders associated with deficient mitochondrial bioenergetics in mice*. Ethics committee of the NWU approval number: 06D07.

Animals used in this study were fed a standard laboratory diet (Rainbow Farms (Pty) Ltd) and had free access to food and water and were kept under the following conditions: temperature: 21°C ± 1 °C; relative humidity: 55 ± 5 %; lighting: 350 lux, 1 meter off the ground; nycthemeral cycle: 12h/12h; spectrum: fluorescent “daylight” tubes that supply full spectrum white light; air exchanges per hour: 16 – 18 times the volume (fresh uncirculated air); air pressure: positive in animal rooms. Airtight doors

maintain positive air-pressure barriers between departments; air quality: HEPA (high efficiency filters) were used.

The liver tissues of each of the four groups of mice were used to prepare the protein extracts for this part of the study. Firstly, wild type mice were treated with PBS to serve as a vehicle control, and rotenone (30mg/ kg/ 2 days) suspended in PBS. Rotenone is a complex I inhibitor and its blocking effects in the electron transport chain was first observed in 1960 by Lindahl and Öberg. The treatment was administered subcutaneously every second day for 21 days. The metallothionein knock-out mice were also injected with the same substances at the determined time intervals. Mice (66) were randomly divided into 11 groups (6 mice per group), so that all groups contained mice of both genotype groups on both treatments, to minimise the effect of day-to-day variation. Mice of different treatment groups were kept in separate cages to eliminate cross contamination.

Mice were identified and blood was collected under halothane anaesthesia. The mice were then killed by severing the diaphragm, and various other tissues (liver, heart, skeletal muscle and brain) were collected post mortem. As this study forms part of a bigger ongoing study performed by research colleagues in our division, only a small amount of liver tissue from each animal was provided for this specific investigation. After sacrifice, a section of the liver tissue of each mouse was wrapped in foil, put in a micro-centrifuge tube and snap frozen in liquid nitrogen after which it was stored at -80°C until further use.

The MT knock-out mouse model was characterised by research colleagues. Neither genotype nor treatment group had statistically significant influences on ROS levels. Complex I activity was reduced by rotenone mostly in the muscle and heart tissue, however a statistically meaningful difference was seen in complex I activity in the liver tissue between the wild type and knock-out rotenone treated group. The wild type rotenone treated group had lower complex I activity compared to the knock-out group. None of the other groups had significantly differing complex I activity in the liver tissue.

Approximately 30mg – 50 mg of liver tissue is needed to produce sufficient protein extract to perform several assays. The tissue was removed from the freezer and

weighed accordingly. The needed amount of liver tissue was put into a new micro-centrifuge tube and left in liquid nitrogen. The tube was taken out of the liquid nitrogen with pincers and held whilst grinding the frozen tissue in the tube with a special pestle which fits tightly in a 1.5 ml micro-centrifuge tube. The ground tissue was left in the tube and frozen in liquid nitrogen again. The pestle was thoroughly cleaned before the next sample was ground.

The protein extract was prepared from 30-50 mg of ground tissue in the same manner as described in the previous section with minor modifications. One hundred micro litres of buffer A was added to approximately 50 mg of ground tissue. The mixture was snap frozen in liquid nitrogen and removed after which it was left to thaw. The contents were mixed and 15 µl of a 1% Triton X-100 in buffer A mixture was added per 50 µl of protein extract. The suspension was vortexed and incubated on ice for 10 minutes. The tubes were centrifuged at 14 000 G (11 000 rpm) for 5 minutes at 4°C. The supernatant was transferred to a clean tube and the protein concentration was determined by using the bichinonic acid assay. Protein concentration was adjusted to 1mg/ml with 0.23% Triton X-100 in buffer A and kept on ice until use or frozen in aliquots at -80 °C.

4.2.5. Treatment of cells with damage causing reagents, H₂O₂ or B[a]P

4.2.5.1. H₂O₂ treatment

Control 143B cells were treated with hydrogen peroxide to serve as the substrate DNA in the BER experiment. A 40 mM hydrogen peroxide stock solution was prepared by diluting 6 % hydrogen peroxide with PBS. Approximately 200 000 cells were sown per well of a 6-well plate on the previous day, or allowed to adhere to the culturing plate a few hours, before treatment would commence. The growth medium was removed from cultured cells and 5 µl of 40 mM hydrogen peroxide stock solution was added together with 1995 µl of new growth medium. This ensures treatment of the cells with 100 µM of H₂O₂. The cells were incubated with H₂O₂ for 30 minutes after which they were harvested as described in section 4.2.3.

4.2.5.2. B[a]P treatment

For the NER experiment, benzo[a]pyrene was used to induce specific damage to the substrate DNA. A different procedure had to be followed to treat cells with

benzo[a]pyrene due to the insolubility of the substance in an aqueous solution. The basic treatment was performed as described by Plazar *et al.* (2007). It was decided that cells were to be treated with 65 μ M of B[a]P to induce damage to the DNA. This decision was based on results found in a previous study performed in 2009 (Steenkamp, 2009). B[a]P concentrations that are too low does not damage the cell to a great enough extent to investigate repair capacity and when the concentration becomes too high, it fails to damage the DNA to a noticeably high extent due to the fact that B[a]P is insoluble in an aqueous solution such as growth medium. A 0.1 M stock solution was prepared by dissolving 0.126 g of benzo[a]pyrene in 5 ml of DMSO. The solution was filtered using a micro-filter attached to a syringe. A second stock solution was prepared from the original stock solution to ensure that the end concentration of DMSO in the growth media was 0.2%. It is important that the concentration of DMSO stay constant in order for the effect of DMSO to be taken into account by performing a vehicle control study in parallel with the benzo[a]pyrene treatment. The second stock solution was prepared as follows:

- Stock 2 (32.5 mM): 325 μ l of 0.1 M B(a)P + 675 μ l DMSO

Cells were sown two days prior to benzo[a]pyrene treatment, and the old growth medium was removed just before adding fresh medium and benzo[a]pyrene. Four micro litres of the second stock solution was added to 1996 μ l of growth medium after cells were sown in a six well plate. By doing this, 0.2% DMSO was present in every well of the plate with growth medium (except control). With each experiment, control and vehicle control experiments were performed. For the control experiment, cells were incubated with 2 ml of growth medium and no benzo[a]pyrene or pure DMSO was added, the vehicle control experiment was performed by adding 4 μ l of DMSO (without benzo[a]pyrene) together with 1996 μ l of growth medium to approximately 200 000 143B cells. When 4 μ l of the second stock solution was added to the cells together with 1996 μ l of medium, the final benzo[a]pyrene concentration was as follows:

- 4 μ l of stock 2 + 1996 μ l of growth medium: 65 μ M of benzo[a]pyrene

The plate was incubated at 37°C for 24 hours, after which the cells were harvested in the same manner as described before 4.2.3. These cells were then directly used in the preparation of the substrate DNA.

4.2.6. Preparation of substrate DNA

Fifty micro litres of the hydrogen peroxide- or benzo[a]pyrene treated cells in suspension (5×10^6 cells per ml) were added to 130 μ l of 0.5 % LMPA and mixed briefly by pipetting. It is important to allow the LMPA to cool down after preparation, before adding the cell suspension, as the heat may cause additional damage to the DNA that will interfere with the results obtained. The cell mixture (180 μ l) was placed on one window of the pre-coated (HMPA) frosted microscope slide, and evenly distributed with the pipette tip held at a flat angle, taking care not to damage the thin layer of HMPA. These were left to cool in order for the agarose-cell mixture to set before it was placed in cold lysing buffer overnight at 4°C. The lysis step removed cell debris and exposed the nucleoid or intact DNA molecule.

4.2.7. Protein treatment of nucleoids

The nucleoids embedded in the agarose on the microscope slides that were to be treated with protein extract were taken out of the lysis solution and were washed for 15 minutes with PBS after which a washing step with buffer B for 15 minutes was performed. A protein extract mixture was prepared for each of the different protein extracts. The mixture includes the following: 50 μ l protein extract, 200 μ l buffer B and 10 μ l of ATP (65 μ M). According to experiments performed by Langie *et al.* (2006) the protein extract regains its activity completely when ATP is added. Adding ATP to the protein extracts prepared from 143B cells that have an alteration in their energy metabolism, ensures that the results are not influenced by the possibility that insufficient ATP is present to perform DNA repair. If a change in DRC is observed in the protein extracts prepared from these cells, it is thus clear that lack of sufficient energy is not to blame. Of this mixture 50 μ l were added to each window of a microscope slide coated with substrate DNA. Microscope slides were then incubated at 37°C for 10 minutes when treating the nucleoids of hydrogen peroxide damaged cells with protein extract as well as when treating the nucleoids of B[a]P damaged cells with protein extract.

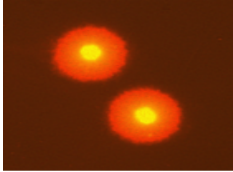
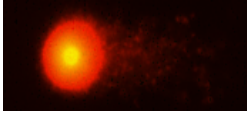
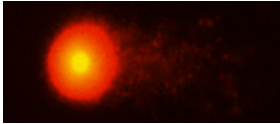
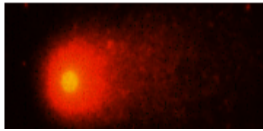
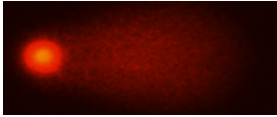
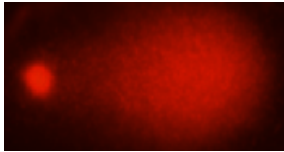
4.2.8. Conducting final steps of the Comet assay

Directly after protein treatment, the slides were placed in cold electrophoresis buffer for 30 minutes to allow the DNA to unwind and to stop protein activity. Electrophoresis was then performed at 4°C for 40 minutes at 30 V (200 mA). After electrophoresis the slides were put in neutralizing buffer (TrisHCl) for 15 minutes after which staining was performed by putting the slides in a 12.5 µM ethidium bromide solution for 30 minutes. Slides were washed for 5 minutes in double distilled water before scoring the comets.

4.2.9. Scoring of comets and processing results

Slides were studied with the Olympus 1x70 fluorescence microscope (200 x magnification) and pictures of the comets were captured with the Comet IV software. The Comet IV software was also used to score the comets, the software determines the amounts of DNA present in the head and tail of a comet, respectively. Image analysis software can generally be used to determine a range of variables including tail length, -moment, % tail DNA, and % DNA in the head of the comet. In this study, the % tail DNA was primarily used for its advantage over studies using tail length and – moment. Tail length and – moment are reliable variables within a study but is not comparable across different studies (Lovell & Omori, 2008). The percentage of tail DNA can be used to determine DNA damage and repair as tail DNA correlates with the amount of DNA damage. The data obtained was further processed in Microsoft Excel®. The average percentage tail DNA (APTD) for each group of comets was calculated, together with the standard deviation for each group. The extent to which DNA is damaged was previously used by Giovanelli *et al.* (2002) to group the comets into classes (table 4.1). These classes range from class zero to class four, with comets in class zero having minimum DNA damage and comets in class four being more extensively damaged. A fifth class was distinguished that contains comets that are heavily damaged (HD). A good representation of the data distribution can be obtained by sorting the comet data into comet classes and presenting it graphically.

Table 4.1. Comet classes. (Adapted from Giovanelli et al. (2002))

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

The table summarizes the different classes that are determined by the extent of DNA damage. Class zero contains comets that are minimally damaged whilst comets in class four are more extensively damaged. When practically no distinction can be made between the head and the tail of the comet, it is considered to be heavily damaged.

4.2.10. DNA repair capacity

4.2.10.1. Introduction

DNA repair capacity (DRC), for the purpose of this study, is an indication of the ability of the proteins under investigation to perform DNA excision repair. DRC is expressed as a value between zero and one. A DNA repair capacity value that approaches one has the ideal repair ability, however a good value (as expected from control protein extract) is normally between 0.3 and 0.5. Different protein extracts will be prepared for the purpose of this study as will be described in section 4.2.4. Briefly, the protein extract prepared from control 143B cells is referred to as control protein extract, furthermore protein extracts are also prepared from 143B cells transformed with an empty retroviral vector, the same cells transformed with a retroviral vector with an unrelated knock-down system (green fluorescent protein or GFP), 143B cells of which complex I of the ETC is knocked down and 143B cells of which complex III is knocked down. In the investigation of the effects of perturbations in the metal metabolism four groups are distinguished. The first is a metallothionein knockdown mouse model treated with the vehicle control, PBS, the second is the metallothionein knock-down mouse model treated with rotenone. Wild type mice were also treated with PBS (vehicle control) or rotenone to form the third and fourth group. Protein extracts are prepared from the mice livers in each group.

The repair capacity was calculated by using the following formula (Bhaba, 2008):

$$\text{DNA repair capacity} = 1 - \left(\frac{\text{Average \% tail DNA: No PE}}{\text{Average \% tail DNA: Control PE}} \right)$$

This equation will be used to determine DNA repair capacity for the different protein extracts in all experiments, where *Average % tail DNA: Control PE* is substituted with the average tail DNA measured in each case for the different protein extracts from each experiment investigating the effects of perturbations in the energy metabolism and metal metabolism on DNA repair pathways, respectively. The DNA repair capacity values can then be used to compare the activity of the different protein extracts, it will provide an indication of the impairing effects of the different perturbations under investigation on the proteins involved in the examined DNA repair mechanisms.

4.2.10.2. Statistical analysis

It was attempted that at least 50 comets were scored per sample, making sure that the entire window of the microscope slide was covered, but avoiding the very edges of the window where it reaches the frosting (Collins, 2004). Class distribution plots were constructed in Microsoft Excel ® to show the distribution of the data.

4.3. Gene expression

4.3.1. mRNA isolation

For the experiment concerning the 143B cells with perturbations in the energy metabolism, cells were cultured and harvested as previously described (section 4.2.3). Cells were pelleted by means of brief centrifugation and the medium was discarded to leave the dry cell pellets for RNA isolation. An RNA isolation kit (NucleoSpin® RNAII obtained from Machery Nagel) was used to isolate the mRNA from the 143B cells. The manufacturer's instructions were mainly followed, however, a few modifications were made to obtain optimum results.

To each cell pellet, 350 µl of RA1 buffer was added. The cells were mixed with the buffer by means of pipetting, after which 3.5 µl β-mercaptoethanol was added to each sample and was mixed briefly by vortexing. The mixtures were transferred to purple ring filter tubes in collection tubes and centrifuged for one minute at 11 000 G. The columns were discarded after centrifugation and the rest of the protocol followed was performed with the filtrate. The filtrate was mixed with 350 µl of 70% ethanol in the collection tube and transferred to a blue filter column in a new collection tube and centrifuged for 30 seconds at 11 000 G, the old collection tube was discarded. The filtrate was discarded and 350 µl of MDB was added to each column and centrifuged at 11 000 G for 1 minute after which the flow-through was discarded and the tubes were centrifuged again for 30 seconds at the same speed. The following mixture was prepared in a microcentrifuge tube for each sample: 10 µl rDNase, 90 µl reaction buffer. For each sample, 95 µl of this mixture was added on the column and incubated at room temperature for 15 minutes. After incubation, 200 µl RA2 buffer was added to each sample on the column and centrifuged for 30 seconds at 11 000 G. The filtrate was discarded and the column was placed in a new collection tube. Six hundred µl RA3 buffer was added to each column. Centrifugation was performed at 11 000 G for 2 minutes. The filtrate was once again

discarded and the columns were placed in microcentrifuge tubes for elution of the mRNA. Each column was incubated for 1 minute at room temperature with 40 µl of RNase free water. The samples were centrifuged for 1 minute at 11 000 G, the filtrate was placed on the column again and centrifuged again for 1 minute at 11 000 G. RNA concentration was determined with a NanoDrop® (ND-100 Spectrophotometer) and used on the same day for cDNA synthesis. The rest of the isolated mRNA was stored at – 80 for future use.

4.3.2. cDNA synthesis

The MMLV high performance reverse transcriptase cDNA synthesis kit (Epicentre® Biotechnologies) was used to synthesise cDNA from the isolated RNA (section 4.3.1). For each sample a reaction mixture was put together in a 250 µl PCR tube. Each reaction contains the following: 1 µl of oligo dT primers, 1 µl of 18s rRNA reverse primer (Eurofins, 100 pmol/µl) 1.5 µg of RNA from the specific sample (maximum volume 8 µl), and the volume of each reaction was adjusted to 10 µl with HPLC grade water. The reaction mixtures were incubated at 65°C for 2 minutes to allow the primers to anneal to the RNA templates. After the 2 minute incubation, all samples were chilled on ice for 5 minutes after which the following reagents were added to each: 2 µl of 10 x MMLV reaction buffer, 2 µl of 0.1 M DTT, 3 µl of HPLC grade water, 2 µl of dNTP mix (New England Biolabs®, 10 mM of each dNTP), 0.5 µl (40 U) of RNasin® (a ribonuclease inhibitor from Promega Incorporation), and 0.5 µl MMLV high performance reverse transcriptase (Epicentre® Biotechnologies). The tubes were placed in a thermocycler and incubated at 37°C overnight. Tubes were kept at 85°C for 5 minutes after overnight incubation to stop the reaction. Tubes were removed from the thermocycler and stored at 4°C. For use in a real-time PCR gene expression assay, cDNA was diluted (1:250) by adding 4 µl of synthesised cDNA to 996 µl of HPLC grade water.

4.3.3. Real time PCR

TaqMan ® gene expression assays for hOGG1 and ERCC1 were obtained from Applied Biosystems™ and assays were performed according to the manufacturer's instructions. A reaction mixture was assembled in duplicate for each sample and a no template control was included for each run. The no template control contained all reagents included in the reaction mixture but no DNA was added and the total volume was adjusted to 20 µl with HPLC grade water. All preparations were

performed aseptically and reaction mixtures were compiled in a RNA-free flow hood, cDNA was added to samples in a different preparation area to minimise the possibility of contamination. Each reaction contained the following: 10 µl TaqMan® real-time master mix, 1 µl of hOGG1 or ERCC1 TaqMan® gene expression assay mix (containing forward and reverse primers for the gene of interest as well as FAM™ TaqMan® MGB probes), 1 µl of 18s rRNA TaqMan® gene expression assay mix, and 6 µl of HPLC water. Two µl of diluted cDNA from the appropriate sample was added to the corresponding tube. The liquid in the tubes were collected at the bottom of each tube by means of centrifugation, making sure that no air bubbles are trapped in the liquid that could influence real-time PCR detection. A relative quantification was performed and a specifically regulated thermal program was run on the machine, the temperature was increased to 95°C for 10 minutes after which 40 repetitions of the following cycle was carried out: 95°C for 15 seconds followed by 1 minute at 60°C. The 7500 System SDS Software was used to operate the real time PCR system (Applied Biosystems, 7500 Real Time PCR System) and analyse the results obtained. When the run was complete, a relative quantification study was performed and the gene expression results were analysed using a control sample as a calibrator. The cycle threshold (Ct) values for both runs were chosen manually at 0.15. The Ct value was set to the log linear range of the amplification plot and kept constant throughout the investigation. The control sample was used as a calibrator. The relative quantities of the targets were normalised against the relative quantities of the endogenous control, 18s rRNA.

CHAPTER 5

RESULTS & DISCUSSION

5.1. Comet assay results

The Comet assay was performed to eventually give an indication of the DNA repair capacity of each protein extract that was prepared from different tissue or cell culture samples. In this section the data obtained with the Comet assay IV scoring software is presented visually. The results processed in Microsoft Excel ® representing the DRC of each protein extract are also presented visually for each experiment performed. All results obtained pertaining to the BER investigations are discussed first followed by the results obtained in the NER experiments.

5.1.2. BER

In order to investigate the BER capacity in the various tissue- and cell culture samples, substrate DNA was prepared by treating control 143B cells with 100 μ M of H₂O₂. Hydrogen peroxide oxidises guanine bases to produce 8-oxo-G, commonly recognised and removed by enzymes involved in the BER pathway. Control- and H₂O₂ substrate DNA was treated with each protein extract for 10 minutes. The protein mixture was supplemented with 2.5 mM ATP to ensure sufficient energy is available for the repair process to be carried out (see section 4.2.7). The experiment is designed to examine the initial steps of the excision repair pathways. An observed increase in the average percentage of tail DNA upon protein treatment indicates protein activity. The extent to which protein treatment increases the APTD was used to quantify the DRC of each individual protein extract.

In both the BER and NER investigations the results for the perturbations in the energy metabolism are presented first, followed by the results obtained from the experiments pertaining to the role of metallothioneins on DRC. A graph is shown that explicate the APTD of the groups investigated. This graph is supported by a *class distribution* graph in each case. A class distribution graph explains the distribution of the data for statistical relevance. Above mentioned graphs are presented as components A and B of a particular figure and are given for the control substrate DNA treated with various protein extracts as well as for the H₂O₂ (in the case of the BER investigation) exposed or B[a]P (in the case of the NER

investigation) exposed substrate DNA treated with the different protein extracts. A graph is also presented for each experiment that shows the DRC of each protein extract as an arbitrary unit. The DRC was calculated for control and H₂O₂ or B[a]P exposed substrate DNA in order to observe whether or not control and exposed substrate DNA groups respond in a similar manner to protein treatment. The DRC graphs are given as components A and B where the graph termed A shows the DRC calculated from the control substrate DNA upon protein treatment and the graph named B illustrates the DRC as calculated from the APTDs that were obtained from the exposed substrate DNA treated with the various protein extracts. Raw data for all BER experiments are included in appendix D.

5.1.2.1. Perturbations of the energy metabolism

The substrate DNA for all experiments reported in section 5.1.2. was prepared from control 143B cells or 143B cells treated with 100 μ M of H₂O₂ as described in section 4.2.5. and 4.2.6. Throughout chapter 4 various abbreviations were used. These abbreviations include:

PE	protein extract	CI kd	complex I knock-down
EV	empty vector	CIII kd	complex III knock-down
URKS	unrelated knock-down system		

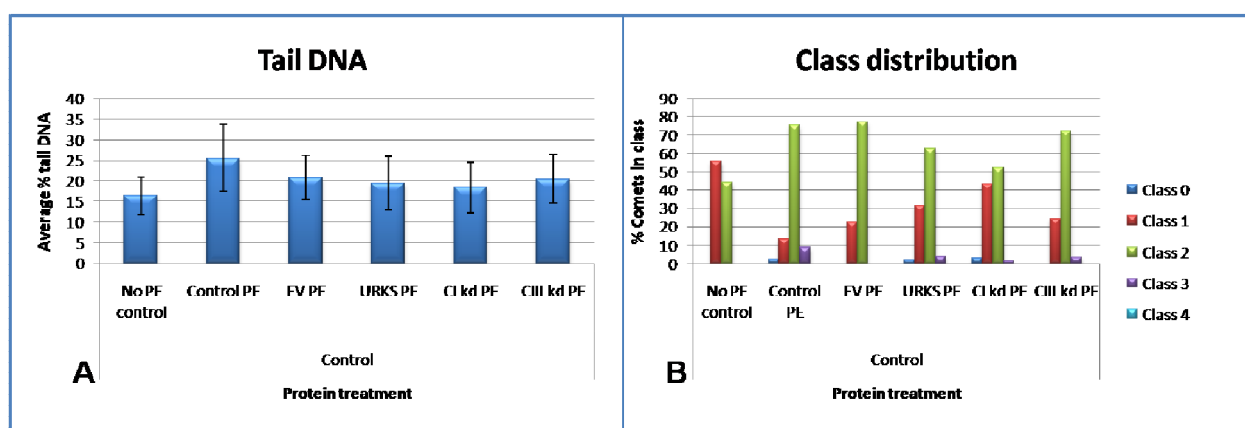


Figure 5.1. Repair of control substrate DNA. A. Graphical representation of the APTD of control substrate DNA upon treatment with various protein extracts prepared from a range of 143B cell cultures. B. Class distribution graph of control substrate DNA after protein treatment

The average percentages of tail DNA varies evidently between the groups of control substrate DNA treated with different protein extracts as can be seen in figure 5.1.A. On first glance, when taking into account the standard deviation of each group, there seems to be little difference between the treatment groups but upon further

processing of the results the variation between the different groups becomes more apparent. The substrate DNA for the control experiment was left untreated to investigate the baseline DNA damage. The baseline DNA damage proved to be approximately 16%. The integrity of the genome is constantly endangered by various sources as explained in section 2.1.1. Even under controlled conditions such as those that the cell cultures are submitted to, some variation in baseline DNA damage occurs between batches of cells. For this reason a control is included for every experiment as suggested by Lovell & Omori (2008). As explained earlier, active excision repair proteins increase the APTD of the substrate as it attempts to excise lesions present on the DNA. Theoretically, protein extract prepared from the 143B control cell line should have optimal repair capacity as no perturbations are present that could potentially influence or change the capacity of the DNA repair enzymes to excise damaged bases. Treatment with the control protein extract caused the APTD to increase to about 25%. The APTD of control substrate DNA treated with protein extracts prepared from 143B cells containing an empty retroviral vector increased to a little above 20%. Protein extract prepared from 143B cells expressing the GFP (URKS) increased the APTD of control substrate DNA to nearly 20% upon treatment. Control substrate DNA treated with protein extract prepared from complex I knock-down 143B cells caused the APTD of control substrate DNA to increase to approximately 18%. Protein extract prepared from complex III knock-down 143B cells caused the APTD of control substrate DNA to increase to roughly 20%. The data obtained in the control BER experiment showed low APTD values with a relatively high standard deviation. The reason for this may be that no specific damage was intentionally induced in the DNA and inevitably various kinds of background DNA damage were present. It is possible that the damage on the DNA may have been repaired by various DNA repair proteins belonging to any of various repair pathways. Repair of different kinds of DNA damage occur at different rates and require diverse factors and energy to take place. The latter gives a possible explanation as to the high standard deviation occurring in the data.

Figure 5.1.B illustrates the distribution of the data shown in figure 5.1.A. The comets scored for each experiment was grouped into different classes according to the extent of DNA damage measured by the percentage of DNA in the tail of the comet. These classes were established by Giovanelli *et al.* (2002) and were described in

section 4.2.9 and the different classes are given in table 4.1. The class distribution for each case reflects the APTD of that group and illustrates the distribution of the data obtained. Control substrate DNA not treated with protein extract produced comets primarily in class 1 reflecting that the majority of the comets have between 6.1 and 17% of DNA in the tail. Approximately 40% of the comets in this group have 17.1 -35% of DNA in the comet tails, these comets are thus in class 2. The control substrate DNA treated with various protein extracts produced comets mainly in class 2 for all experimental groups. In addition to the majority of comets in class 2, treatment with control protein extract generated minimal comets in classes 1, 3 and 0 – in order of abundance. Treatment with EV PE caused around 80% of the resulting comets to be in class 2 and approximately 20% of the comets fall in class 1. Besides the majority of the comets being in class 2 in the experiment concerning control substrate DNA treated with URKS PE, a large amount of comets were also in class 1, and less comets appeared in classes 3 and 0. Treatment of control substrate DNA with CI kd PE produced nearly as much comets in class 1 as there are in class 2, a few comets also occur in classes 0 and 3. Control substrate DNA treated with CIII kd PE caused comets to fall primarily in class 2 as previously mentioned, nearly 20% of comets in this group were also in class 1 and very few comets were observed in class 3.

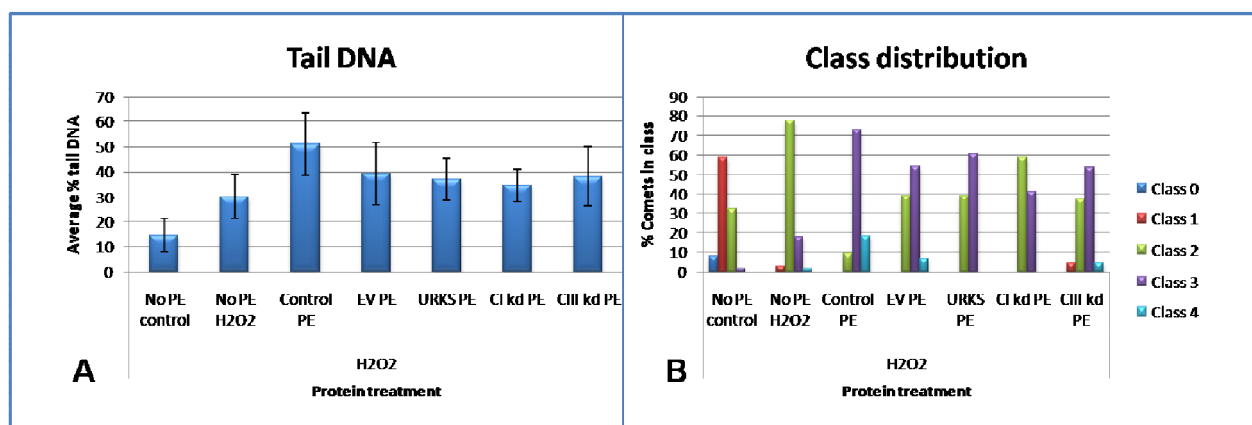


Figure 5.2. Repair of H_2O_2 exposed DNA. A. Graphical representation of the APTD of H_2O_2 substrate DNA upon treatment with various protein extracts prepared from a range of 143B cell cultures. B. Class distribution graph of H_2O_2 substrate DNA after protein treatment

The APTDs of H_2O_2 damaged substrate DNA treated with various protein extracts prepared from 143B cells with different perturbations are shown in figure 5.2.A. Cells treated with 100 μM of hydrogen peroxide yielded nucleoids with roughly 30% of APTD. This confirms the well known fact that H_2O_2 causes oxidation and damage

to DNA that can be observed as strand breaks (Benhusein *et al*, 2010). Upon treatment with control PE the APTD increases to about 50%. This large increase is an indication of the control PE's ability to optimally repair DNA damage induced by H₂O₂. EV PE caused the APTD of H₂O₂ damaged substrate DNA to increase to an estimated value of 40%. Hydrogen peroxide exposed substrate DNA treated with URKS PE leads to an APTD of nearly 40%. CI kd PE causes the APTD of the substrate DNA to increase to approximately 35% and CIII kd PE causes an APTD of nearly 40%, a value just between that of which is caused by EV PE and URKS PE.

The class distribution of the data obtained in this experiment greatly reflects the APTDs of each group and shows that the data is well distributed. The class distribution graph (figure 5.2.B) also confirms the lower standard deviation on the data obtained in this experiment compared to the control BER experiment in figure 4.1. Comets obtained from control substrate DNA not treated with protein extract proved mainly in class 1 and approximately 30% of the comets were in class 2. Only a few comets in this group were found in class 0 and a small amount of comets proved to be in class 3. Substrate DNA prepared from 143B cells treated with H₂O₂ produced comets primarily in class 2. Nearly 20% of the comets were in class 3 and very few proved to be in classes 1 and 4. Hydrogen peroxide substrate DNA treated with control PE produced comets largely in class 3, and less comets in classes 4 and 2 – in descending order. The large number of comets in class 3 mirrors the high APTD as seen in figure 5.2.A, as control PE have optimal DNA repair capacity and is therefore able to excise a large amount of oxidative damage, leading to a large increase in APTD. Treatment of H₂O₂ substrate DNA with EV PE generated comets that were mostly in class 3, as does treatment with URKS- and CIII kd PE. Furthermore, treatment with EV PE also produced a large quantity of comets in class two and very few comets in class 4. In addition to the large number of comets in class three caused by treatment of H₂O₂ substrate DNA with URKS PE, approximately 40% of the comets in this group were in class 2. Upon treatment of H₂O₂ substrate DNA with CI kd PE, nearly 60% of the comets were in class 2. The rest of the comets in this group proved to be in class 3. Treatment of H₂O₂ substrate DNA with CIII kd PE produced comets mainly in class 3 and nearly 40% of the comets were in class 2. A few comets in this group were also found in classes 2 and 4.

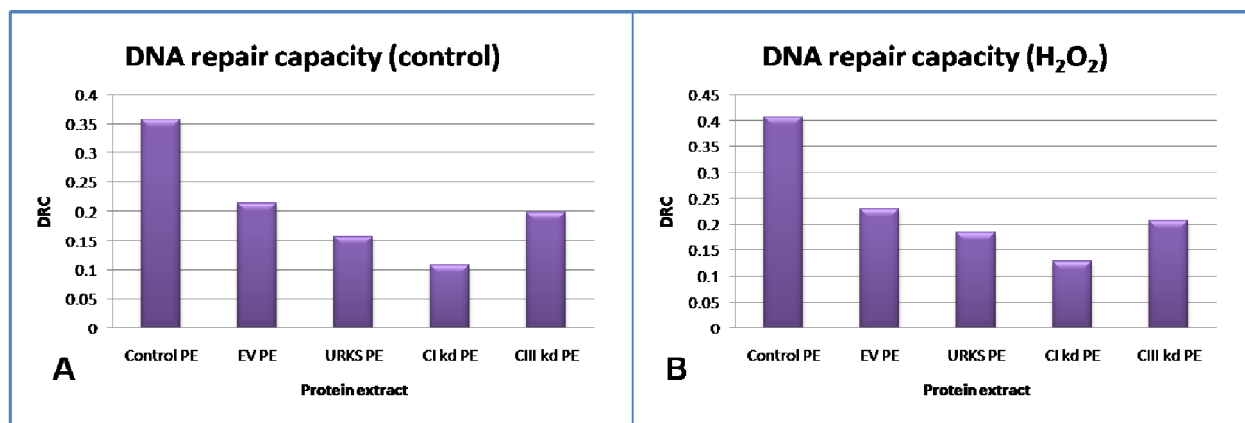


Figure 5.3. Repair capacity of 143B cell protein extracts. *Protein extracts acting on A, control substrate DNA and B, on H₂O₂ substrate DNA.*

As seen in figure 5.3.A and -5.3.B, the DRC for the various protein extracts calculated from the data shown in figures 5.1.A and –B, and 5.2.A and –B, have the same tendency. The DRC values graphically presented in this chapter were calculated as described in section 4.2.10.1. As suggested earlier, control PE should theoretically have optimal DRC. This theory is proved and illustrated in figure 5.3. In both the control and H₂O₂ experiment, the control PE proved to have the highest DRC value. The EV PE had a DRC value of nearly half of that of control PE, indicating that transfection with an empty retroviral vector inhibits the BER capacity of 143B cells. The DRC of the URKS PE showed to be even lower in both cases, suggesting that the expression of a protein further reduces the BER capacity of the cells. Metabolic channelling is the phenomenon where a cell or organism applies its energy to perform a specific process and this may lead to the cell or organism to neglect other vital processes. Expressing an extra, foreign protein may put severe metabolic stress on a cell and other energy- or resource dependant processes may suffer as fewer resources are available for the cell's normal biological functioning. These processes include replication, transcription, translation, DNA repair and many others. The DRC calculated for CI kd PE proved to be even lower than that of URKS PE. Whether or not the complex I knock-down is the cause of the decrease in DRC is yet to be uncovered. The protein extract prepared from 143B cells of which complex III is knocked-down has a DRC value lower than that of control- and EV PE but faintly higher than the DRC values of URKS- and CI kd PE. DRC values presented in figure 5.3.A and 5.3.B are calculated from one BER experiment performed in duplicate. The experiment was however repeated (results not shown). Although the results were similar and showed comparable effects, some variation

was observed in the DRC values for the various protein extracts. A uniform reduction of DRC values was observed in all protein extracts transfected with the retroviral vector (including EV, URKS, CI kd and CIII kd) compared to the control protein extract prepared from normally cultured 143B cells. The decrease in DRC for the complex I and III knock-down models cannot be ascribed to the perturbation in the energy metabolism with certainty because of the limited characterisation of the 143B cell model used and the limited information available pertaining to the knock-down. However, the DRC information together with previous work performed in our laboratory (Kok, 2010) suggest that mere transfection of cells have much larger impact on various biological functions of the cell than previously acknowledged.

5.1.2.2. Perturbations in the metal metabolism

The experiments for which the results are shown in this section (5.1.2.2) were performed by treating substrate DNA prepared from 143B cells (control and treated with hydrogen peroxide) with different protein extracts prepared from the liver tissue of control and MT knock-out mice. A protein extract was prepared from a small amount of liver tissue from one animal in a designated group. Five protein extracts (from individual mice) were prepared for each group and the experiments were repeated for each of the protein extracts prepared. Figure 5.4.A and 5.4.B give the results obtained after treatment of substrate DNA with protein extract prepared from a single animal's liver tissue. Results obtained for all tissue tested (animal identity is known and documented) are included in appendix E. All results were, however, considered and used to calculate the average DRC of each type of tissue as presented in figures 5.6.A and -B, and 5.12.A and -B.

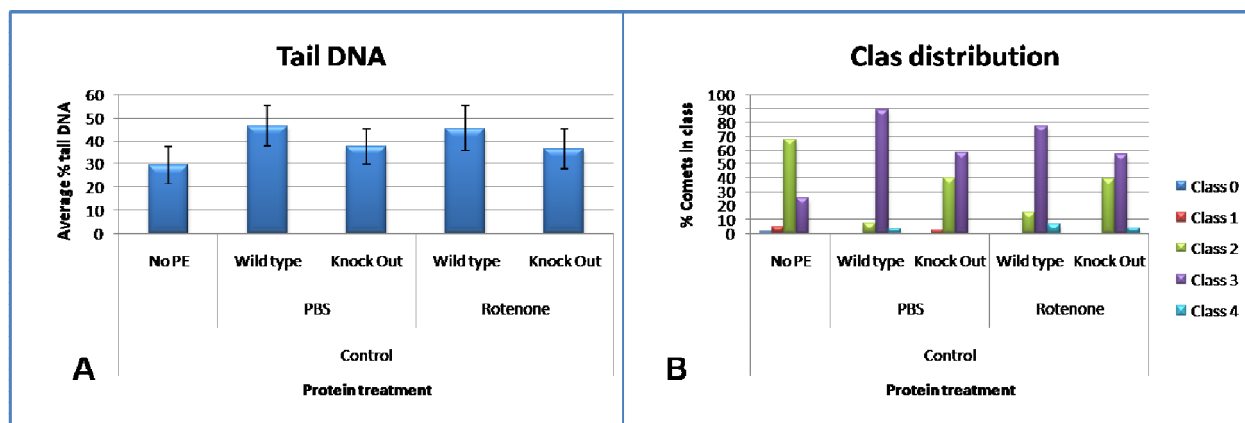


Figure 5.4. Repair of control substrate DNA. A. Graphical representation of the APTD of control substrate DNA upon treatment with various protein extracts prepared from mouse liver tissue. B. Class distribution graph of control substrate DNA after protein treatment

The APTDs of control substrate DNA treated with protein extracts prepared from mouse liver tissues described in section 2.3.3. are shown in figure 5.4.A. Control substrate DNA left untreated yielded comets with an APTD of nearly 30%. It is mentioned by Lovell & Omori (2008) that negative control cells may have between 10- and 20% of DNA in the tail. The high APTD of the negative control group can be ascribed to numerous factors. These include the fact that cell culture conditions, although ideally designed and maintained, may cause variation in cell metabolism and biological functioning as explained in section 5.1.2.1. When the cells are applied to the pre-coated microscope slide, the LMPA needs to be cooled before the cell suspension is added and the mixture is accordingly applied to the slide as described in section 4.2.6. If however, ample time is not allowed for the LMPA to cool down before adding the cell suspension, the high temperature may lead to increased DNA strand breaks. Even though the control group has a relatively high APTD, a control group is included for every single experiment as suggested by Lovell & Omori (2008) and the experimental results and processing thereof will accordingly not be greatly affected as an “internal standard” is included for each experiment. Control substrate DNA treated with protein extract prepared from wild type mouse liver tissue in the vehicle control (PBS treated) group produced comets with an APTD of approximately 48% that theoretically should have optimal repair capacity. The protein extract prepared from the metallothionein knock-out liver tissue caused the APTD of control substrate DNA to increase to roughly 39% upon treatment. Control substrate DNA treated with protein extract prepared from wild type mouse liver tissue in the rotenone treated group showed little difference

compared to the same tissue in the vehicle control group. The APTD of the comets in this group were slightly more than 45%. Protein extract prepared from the MT knock-out mouse liver tissue in the rotenone treated group, applied to control substrate DNA, yielded comets with a, APTD of roughly 38%.

The class distribution of the data presented in figure 5.4.A is shown in figure 5.4.B. Comets yielded from the control substrate DNA left untreated were mainly in class 2, some comets were also found in class 3. Negligible quantities of comets were in classes 1 and 0. Control substrate DNA treated with protein extracts prepared from wild type mouse liver tissue (vehicle control group) produced comets primarily in class 3, as well as a small amount of comets in classes 2 and 4, in descending order of abundance. Treatment of control substrate DNA with MT knock-out PE in the group treated with PBS (vehicle control) gave rise to comets of which nearly 60% are in class 3, approximately 40% of the comets are in class 2 and a few comets were in class 1. Wild type PE in the rotenone group applied to control substrate DNA caused comets to be mainly in class 3, whilst approximately 14% were in class 2 and less than 10% of the comets were in class 4. Control substrate DNA treated with protein extract prepared from MT- knock-out mouse liver tissue in the rotenone treated group produced comets of which roughly 60% were in class 3, nearly 40% were in class 2 and less than 5% of the comets were in class 4.

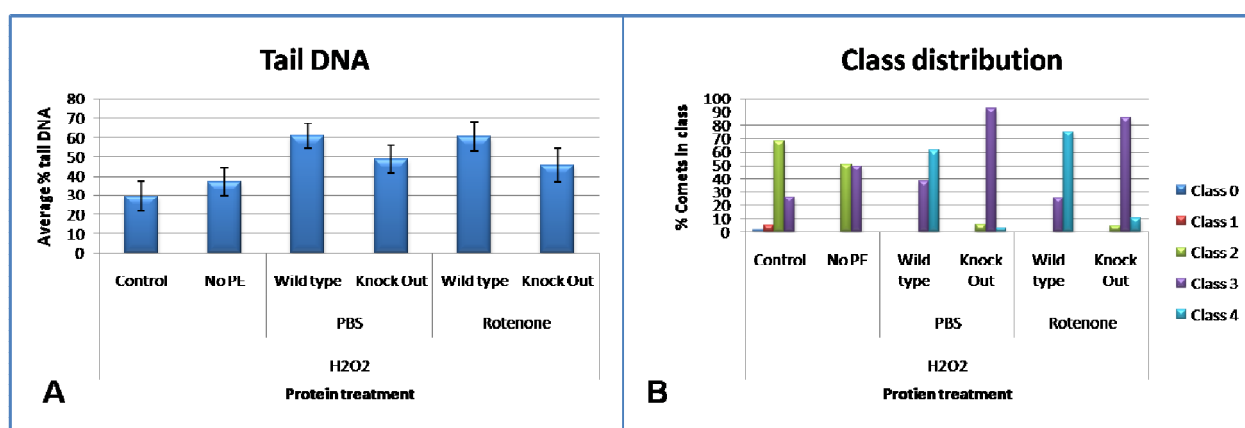


Figure 5.5. Repair of H_2O_2 substrate DNA. A. Graphical representation of the APTD of H_2O_2 substrate DNA upon treatment with various protein extracts prepared from mouse liver tissue. B. Class distribution graph of H_2O_2 substrate DNA after protein treatment.

In figure 5.5.A and 5.5.B, the APTDs of hydrogen peroxide treated substrate DNA incubated with various protein extracts prepared from mouse liver tissues are shown. Control substrate DNA not treated with protein extract once again showed

comets with an APTD of about 30% as explained in the discussion of figure 5.4.A. Substrate DNA prepared from 143B cells treated with hydrogen peroxide and left untreated had an APTD of nearly 40%. Hydrogen peroxide treated substrate DNA treated with wild type PE (vehicle control group) yielded comets with an APTD of just over 60%. The large increase in APTD upon protein treatment indicates efficient excision repair capacity of the protein extract prepared from mouse liver tissue in the control group. Treatment of H₂O₂ substrate DNA with knock-out PE in the vehicle control group caused comets to have an APTD of nearly 50%. The increase in APTD upon treatment of H₂O₂ substrate DNA with wild type PE was larger than the increase that occurred upon treatment with MT-knock-out PE. Hydrogen peroxide substrate DNA treated with protein extract prepared from wild type mouse liver tissue in the rotenone treated group gave rise to comets with an APTD of 60%, which does not differ greatly from the comets that resulted from treatment with wild type PE in the PBS administered group. Treatment of H₂O₂ substrate DNA with metallothionein knock-out PE in the rotenone group produced comets that have an APTD of approximately 46%. As explained previously, a larger increase in APTD after protein treatment indicates better repair capacity as the excision repair proteins present in the protein extract acts to remove the damage present on the DNA by excising damaged bases (specifically 8-oxo-G) and in doing so causes single strand breaks or AP sites. A larger increase in APTD upon protein treatment thus indicates high DNA excision repair capacity.

The class distribution of the comets in support of figure 5.5.A is given in figure 5.5.B. Class distribution for the control group of substrate DNA, left untreated was discussed previously in the explanation of figure 5.4.B. Substrate DNA prepared from H₂O₂ treated 143B cells and not treated with protein extract was used to establish the amount of DNA damage caused by the exposure to H₂O₂ alone, these comets were equally abundant in classes 2 and 3. Hydrogen peroxide exposed substrate DNA treated with wild type (PBS) PE produced comets primarily in class 4 and approximately 40% comets in class 3. Treatment of H₂O₂ substrate DNA with MT-knock-out PE (vehicle control group) produced comets that are mainly class 3, a very small amount of comets were also found in classes 2 and 4 – in descending order of abundance. Comets generated by treatment of H₂O₂ substrate DNA with wild type (rotenone) PE were mostly in class 4, although approximately 25% of the

comets were in class 3. Hydrogen peroxide substrate DNA treated with metallothionein knock-out PE prepared from mouse liver tissue in the rotenone treated group, generated comets of which approximately 87% were in class 3, roughly 10% were in class 4 and very few comets were found in class 2.

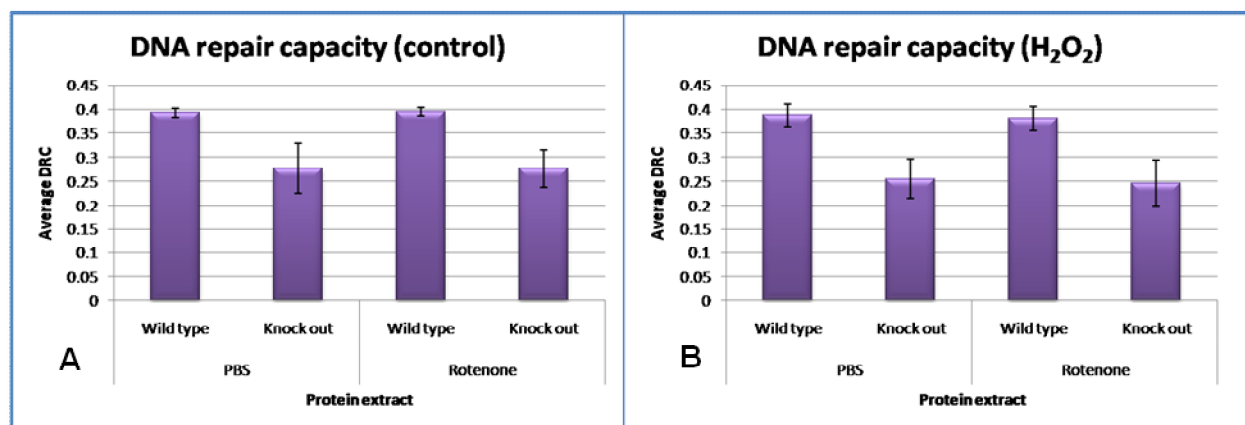


Figure 5.6. Repair capacity of mouse liver tissue protein extracts. *Protein extracts acting on A, control substrate DNA and B, on H₂O₂ substrate DNA*

Figures 5.6.A and 5.6.B show that the DRC calculated for different protein extracts with perturbations in the metal homeostasis have the same trend when acting on control and H₂O₂ substrate DNA. Once again, protein extracts prepared from the liver tissue of wild type mice showed optimal DRC with an approximate value of 0.4 which is considered a satisfactory DRC for a control group. Rotenone treatment in comparison with the vehicle control treatment (PBS) seems to have little or no detectable effect on the DRC of the various protein extracts in wild type and knock-out groups. MT knock-out protein extract acting on control substrate DNA have an estimated DRC value of 0.275. This value is considerably lower than that of the wild type protein extract indicating that MT knock-out impairs DNA repair in some way. The same applies for the knock-out protein extracts acting on H₂O₂ substrate DNA. The wild type groups show DRC values approaching 0.4, indicating optimal repair capacity of H₂O₂ induced DNA damage. MT knock-out protein extracts have a calculated DRC of roughly 0.25 for both the vehicle control and rotenone treatment groups. The protein extracts prepared from MT knock-out mouse liver tissue (in PBS and rotenone treated groups) thus appear to have definite impaired DNA repair capability of oxidative damage. In this case it can be deduced that the perturbation in the metal homeostasis has an effect on the proteins involved in the initial repair stages of BER that specifically act to repair lesions such as 8-oxo-G caused by H-

$_2\text{O}_2$. A similar observation was made by Candéias *et al.* (2010), who analysed the effects of cadmium exposure, which is also caused by the absence of metallothionein in the mouse model used in this study, on the recognition and excision stages BER and NER. It was found in the study by Candéias *et al.* (2010) that both repair pathways are inhibited by the presence of Cd but have different sensitivities to exposure concentrations. They have shown that Cd is a very powerful DNA repair inhibitor. These findings are clearly supported by the DRC calculations from the data obtained in this study. Since Yang *et al.*, (2010) also observed that MT knock-out mice are more susceptible to oxidative DNA damage it may be presumed that the protective role of metallothioneins against oxidative stress or heavy metal imbalance and the effects thereof was underestimated in the past. The vast effect of the absence of metallothioneins on the DRC of mouse liver tissue suggests that the lack of anti-oxidant defences may cause improper functioning of DNA repair proteins as described in section 2.3.3., however the effects are not restricted to proteins involved in repair pathways. Other possibilities include the imbalance of the metal metabolism and lack of metal detoxification in the absence of metallothioneins which may lead to apoptosis of cells, cellular adaptation – causing metabolic channelling once again, glutathione depletion and also extreme DNA damage. Perhaps a combination of all the above speculations is also possible.

5.1.3. NER

The experiments performed in the investigation concerning the NER capacity of different tissue- and cell culture samples was performed in a similar manner to that of the BER investigation. However, 143B cells were treated with 65 μM of B[a]P for 24 hours to serve as the substrate DNA for the NER related DNA repair enzymes present in the protein extracts to act on. B[a]P is bio-activated to BPDE and binds covalently to DNA molecules to distort the structure thereof and is ultimately carcinogenic (Park *et al.*, 2006). Bulky adducts to DNA such as those caused by BPDE are generally recognised and repaired by enzymes associated with NER. Protein treatment was performed as described in section 4.2.7. The same principle applies for the NER study as was explained for the BER experiments; an observed increase in the average percentage of tail DNA upon protein treatment indicates

excision repair activity. The DRC applying to the NER capability of each protein extract was calculated as depicted in section 4.2.10.1.

The APTD graphs and raw data obtained for all NER experiments are included in appendix D. All results obtained were considered to calculate the DRC for each protein extract. Again, as for the BER experiments, ostensibly the results for the NER experiments seem futile when considering the considerably high standard deviation, but when the data is further processed the effects of the perturbations on the DRC of the various tissues are evident. Graphs in this section are similar in layout as those included in section 5.1.2. for the BER experiment and thus, only essential findings are discussed in section 5.1.3. DRC graphs are presented in this section for the protein extracts prepared from cell cultures with perturbations in the energy metabolism as well as for protein extracts prepared from wild type and MT knock-out mice in the two treatment groups (see section 2.3.3).

5.1.3.1. Perturbations in the energy metabolism

The substrate DNA for all experiments reported in section 5.1.3. were prepared from control 143B cells or 143B cells treated with 65 μ M of B[a]P as described in section 4.2.5. and 4.2.6.

Baseline DNA damage for all experiments discussed in this section were low, as expected. The APTD in general, even upon protein treatment, was lower than that observed in the BER investigation. The vehicle control reagent on its own had nearly a negligible effect on the occurrence of DNA damage as it caused very little increase in APTD of 143B cells after treatment thereof at 0.2% for 24 hours. Treatment of the vehicle control substrate DNA with various protein extracts (EV-, URKS-, CI kd- and CIII kd PE) showed a small increase in APTD to various extents indicating active excision repair taking place. Class distribution supported the APTD noted for each treatment group but in some cases reflected somewhat larger standard deviation and thus larger distribution of the data, compared to that calculated and detected in the BER experiment.

B[a]P treatment resulted in a notable increase in APTD of the substrate DNA. As expected, treatment with control protein extract caused the largest increase in APTD indicating optimal repair capacity. EV-, URKS-, CI kd- and CIII kd PE also caused the APTD to increase upon treatment of B[a]P substrate DNA with each of

the mentioned PEs. Class distribution once again portrayed the APTD data obtained well, and the considerably high standard deviation as well as data distribution was reflected in the class distribution records. As noted in section 2.2.3.2., some PAHs such as B[a]P have the ability to produce ROS indirectly through the formation of quinone derivatives and thus may lead to oxidative DNA damage in addition to adduct formation. Damage and repair of different DNA lesions are timely processes and as expected do not occur at the same rate for various kinds of damage or the removal thereof. Since B[a]P, amongst other PAHs, have the aptitude to form adducts to DNA that is typically repaired by NER, as well as the ability to indirectly cause oxidative damage, usually recognised and repaired by proteins in the BER pathway, it may cause DNA damage that require a range of proteins from BER and NER to excise the induced damage. The repair of a variety of DNA damage does not take place at precisely the same tempo, as explained. The high standard deviation may thus be ascribed to the fact that different DNA aberrations are in different phases of repair at the time when protein activity is stopped (after 10 minute incubation with protein extract) by placing the slides in cold electrophoresis buffer as reported in section 4.2.7.

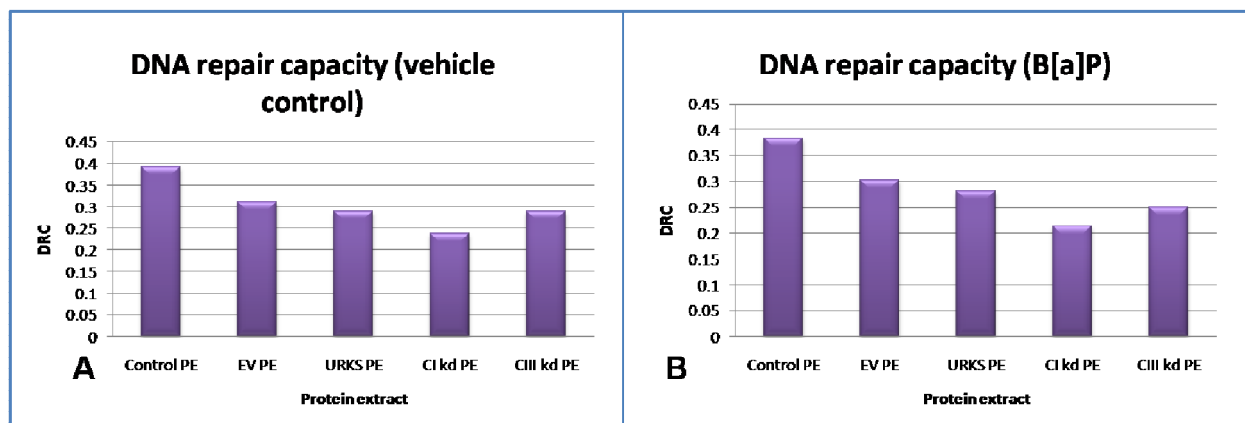


Figure 5.7. Repair capacity of 143B cell protein extracts. *Protein extracts acting on A, vehicle control substrate DNA and B, B[a]P substrate DNA*

From figures 5.7.A and 5.7.B it is evident that the DRC calculated for the different protein extracts prepared from 143B cells with perturbations in the energy metabolism revealed a similar trend when acting on vehicle control substrate DNA than when acting on B[a]P treated substrate DNA. A similar trend was also observed for the same group of protein extracts in the BER investigation. Both in the

vehicle control and B[a]P study the control PE showed the highest DRC value of nearly or close to 0.4, representing a good value and optimal DRC for control 143B cells. EV PE showed a lower DRC value around 0.3 in both cases which clearly shows that the DRC is inhibited to a small extent by transfection with the empty retroviral vector. The DRC values calculated for URKS PE were even lower than that of EV PE, as was also the case in the BER findings shown in figures 5.3.A and 5.3.B. This once again proposes that the expression of a protein puts stress on the cell's metabolism and causing other processes, including DNA repair, to suffer there under. CI PE has the smallest DRC value at approximately 0.24 for the vehicle control experiment and just above 0.2 for the experiment where B[a]P was used to induce damage to the substrate DNA. A vaguely higher DRC was noted for CIII kd PE in both cases; nearly 0.3 for the vehicle control study and roughly 0.25 for the B[a]P study. Yet again the results obtained from repeat experiments (not shown) cannot be used to confirm for certain whether or not the inhibition is due to the complex I or –III knock down. It can however be inferred that the transfection with the retroviral vector used in this model has an enormous effect on DNA repair, as observed in this study, and other cellular functions and phenomena such as global and gene specific DNA methylation patterns (Kok, 2010).

5.1.3.2. Perturbations in the metal metabolism

The experiments for which the results are discussed in this section were performed by treating control and B[a]P substrate DNA, from 143B cells, with protein extracts prepared from mouse liver tissue of animals with different genotype and in different treatment groups as described in section 2.3.3. Once again, as for the experiment pertaining to the BER study in the corresponding section (perturbations in the metal metabolism), protein extracts were prepared from the liver tissue of 5 individual animals in each group. The results discussed in this section represent an overview of all experiments performed (with 5 protein extracts representing each treatment group), and all experiments performed were taken into account to calculate the average DRC of each tissue type as presented in figures 5.8.A and –B.

As in the NER experiment concerning the investigation of the effect of perturbations in the energy metabolism on DRC, B[a]P caused a prominent increase in APTD compared to vehicle control substrate DNA. As anticipated, the protein extracts prepared from wild type mouse liver tissue caused APTD of vehicle control- and

B[a]P substrate DNA to increase with the highest percentage value, indicating good excision repair capacity. Protein extracts prepared from MT knock-out mouse liver tissue caused an increase in the APTD of control- and B[a]P substrate DNA upon treatment as well, but to a lesser extent than wild type protein extract. There seemed to be little notable difference between the PBS and rotenone treatment groups, differences observed can be considered coincidental and can be neglected due to the standard deviation observed. The class distribution of the APTDs in this experiment depicts the data well, although once again the large standard deviation calculated was evident in the class distribution. The reason for the latter is suggested to be the same as described in section 5.1.3.1.

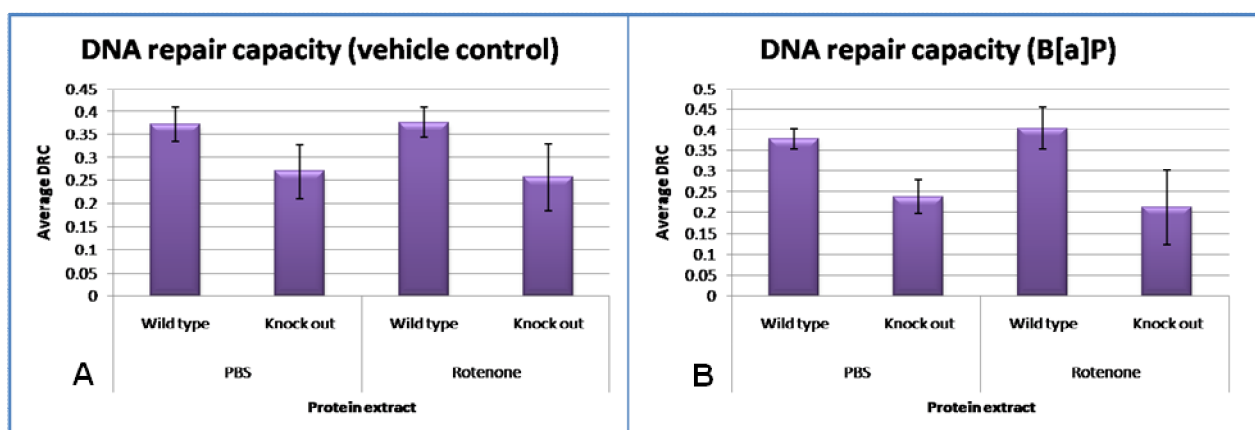


Figure 5.8. Repair capacity of mouse liver tissue protein extracts. Protein extracts acting on A, vehicle control substrate DNA and B, on B[a]P substrate DNA

The results obtained by treating vehicle control- and B[a]P substrate DNA with protein extracts prepared from wild type and MT knock-out mouse liver tissue (in two treatment groups), were used to calculate the DRC (concerning NER) for each tissue type under investigation in this part of the study (shown in figures 5.8.A and – B). DRC values for the different protein extracts have a similar trend when acting on vehicle control substrate DNA and B[a]P substrate DNA. A comparable tendency was also observed for the same group of protein extracts in the BER analysis. The wild type protein extracts showed optimal DRC in the vehicle control and B[a]P investigation, and as mentioned previously, no noteworthy difference between the PBS and rotenone treatment groups could be observed within this particular study. An average DRC value of approximately 0.375 were calculated for the protein extract prepared from wild type mouse liver tissue acting on the vehicle control substrate DNA and an average value approaching 0.4 were calculated for the same

protein extract acting on B[a]P substrate DNA. From these values, it is apparent that wild type mice have optimal NER capacity. Considerably lower DRC values were calculated for MT knock-out protein extracts in both treatment groups (PBS and rotenone). Protein extract prepared from MT knock-out mouse liver tissue had an average DRC value of approximately 0.25 – for both PBS and rotenone administered treatment groups in the vehicle control experiment. The same protein extracts showed average DRC values between 0.2 and 0.25 when acting on B[a]P substrate DNA. From figures 5.8.A and –B the impairing effect of the absence of metallothioneins on the NER capacity of mouse liver tissue is unmistakable. Candéias *et al.* (2010) observed that the recognition and excision steps of both BER and NER were directly affected by the absence of metallothioneins in a mouse model. Since PAHs such as B[a]P have the ability to cause oxidative damage to DNA indirectly (see section 2.2.3.2) as well as to distort the DNA structure, one inherently measures BER capacity together with NER capacity when performing an NER assay. Metallothionein knock-out clearly has an impairing effect on BER capacity from what is known in the literature described in the corresponding section and from the results obtained in this investigation pertaining to the BER capacity of various tissue and cell culture samples. One would assume therefore that this would also be reflected when an NER assay is performed, which was plainly the case. The findings in this section are supported by the study performed by Candéias *et al.* (2010), where an imbalance of the metal metabolism inhibited the recognition and excision stages of NER, precisely these steps are also investigated with this specially designed Comet assay.

5.2. Gene expression results

Quantitative real time PCR was performed to determine whether perturbations in the energy metabolism brought about by means of transfection, which clearly affects DRC of these samples, causes altered gene expression levels. The genes under investigation are two representative genes encoding for proteins involved in the initial stages of BER and NER, namely hOGG1 and ERCC1. Gene expression analysis was performed in support of the results obtained in section 5.1, and should be regarded as such. The data obtained by performing quantitative real time PCR on the 7500 System SDS Software are presented visually in this section and

discussed accordingly. Primers available for gene expression analysis were hOGG1 and ERCC1 which are human genes, although these genes and the corresponding proteins are similar in rodents (mice used in MT study), the primers could only be applied for analysis of the five 143B cell variants. The 18s rRNA gene was used for hOGG1 and ERCC1 expression normalisation. The control sample was used as a calibrator and thus the expression levels of the two genes of interest were set to 1, but the gene expression levels were plotted as $\log_{10}$ values (the $\log_{10}$ of 1 equals 0), the expression levels of the calibrator or control sample appear as 0 on the graph. No bars appear for the endogenous control (18s rRNA) as the relative quantities of the targets were normalised against the relative quantities of the endogenous control, and the expression level thereof is 0.

In this section (5.2) abbreviations are used as described in section 5.1, in addition- no template control is abbreviated as NTC.

5.2.1. hOGG1

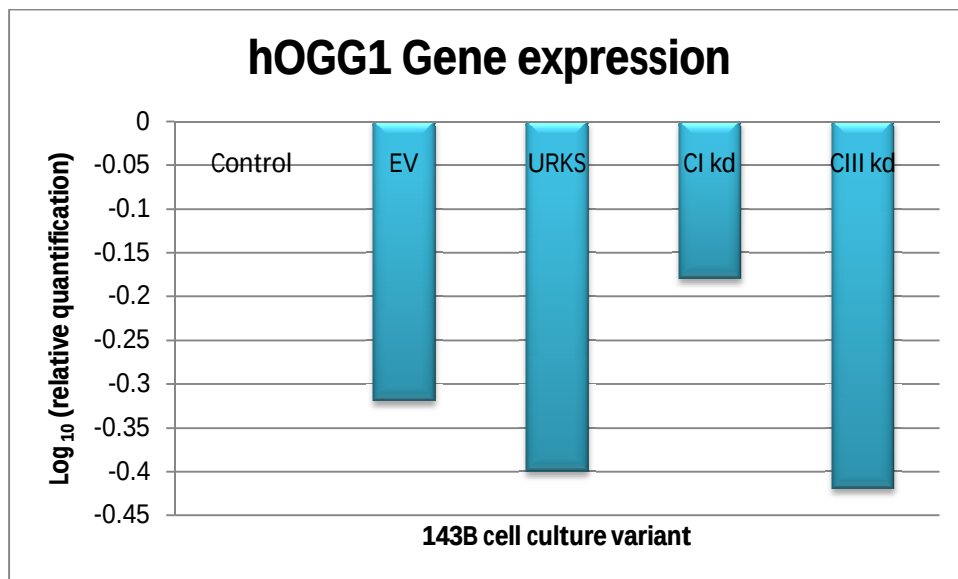


Figure 5.9. Gene expression: hOGG1. The hOGG1 gene expression of each sample group is given relative to that of the expression of hOGG1 in the control 143B cell line.

From figure 5.9 it is evident that hOGG1 gene expression is lower in all perturbed cell lines compared to the control 143B cell line. 143B cells transfected with an empty vector shows a large down regulation of the hOGG1 gene with a value of -0.32. The expression of the same gene in the URKS established in 143B cells is even lower at -0.4. The relative gene expression value of hOGG1 in the CI knock-down system is -0.18 indicating that this knock-down inhibits the expression of

hOGG1 to a lesser extent than both EV and URKS. The CIII knock-down system down regulates hOGG1 gene expression by the greatest relative gene expression value, -0.42. The results obtained suggest that the perturbations established in 143B cells by means of transfection directly affect the gene expression of hOGG1, a glycosylase enzyme associated with the recognition and early repair of oxidised base in the BER pathway. However, the extent to which hOGG1 gene expression is down regulated in each case does not portray in parallel the extent to which DRC is impaired in each case, as described in section 5.1.2.1. This suggests that transfection and the perturbations in the energy metabolism brought about impair BER capacity on at least two levels, i. e. gene expression and protein function.

As mentioned earlier, DNA repair proceeds as an intricate process involving a cascade of enzymes acting to repair a specific lesion. hOGG1 was investigated in this study as a representative protein of the initial stages of the BER pathway but cannot be held solely responsible for the repair of 8-oxo-G base lesions. The expression of the gene encoding for this protein is, however, down regulated by transfecting 143B cells with (even) an empty retroviral vector, and even more so when a foreign protein is expressed within the cell, as well as when knock-downs of complex I and III are established in these cells. The fact that DRC is impaired and hOGG1 gene expression is down regulated in all four investigated 143B cell groups propose that transfection of cells with a vector has an enormous harmful effect on the cells and certainly may lead to altered cell biology.

5.2.2. ERCC1

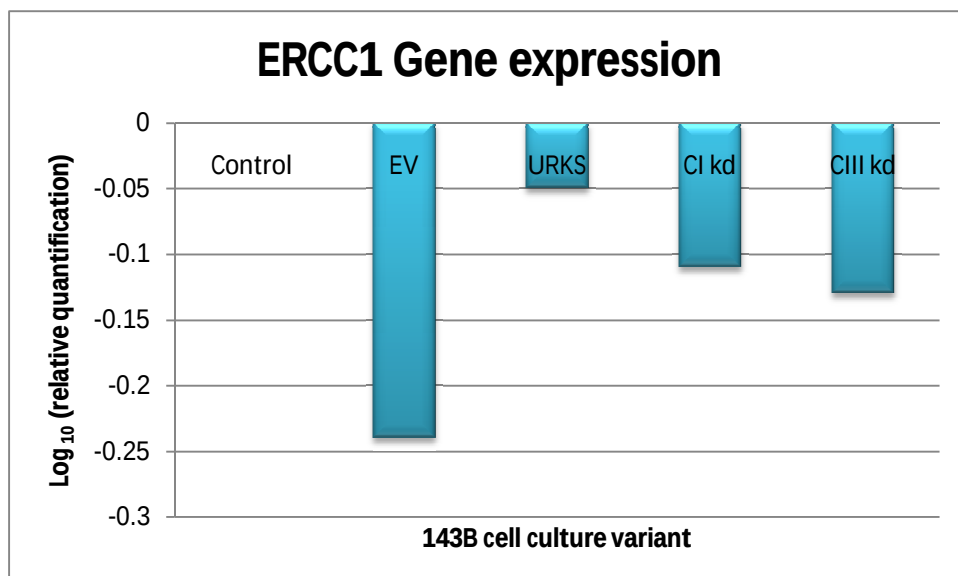


Figure 5.10. Gene expression: *ERCC1*. The *ERCC1* gene expression of each sample group is given relative to that of the expression of *ERCC1* in the control 143B cell line.

The gene expression values of the four variants of 143B cells examined can be seen in figure 5.10. Once again, as is the case with hOGG1 gene expression, *ERCC1* gene expression is down regulated in all four variants compared to the control 143B cell line. *ERCC1* gene expression is greatly down regulated (relative value of -0.24) by transfection with an empty retroviral vector. 143B cells expressing the green fluorescent protein (URKS) show very little down regulation of *ERCC1* with a relative expression value of -0.05. The CI knock-down system down regulates *ERCC1* gene expression more than URKS but not as great as EV, with a relative gene expression value of -0.11. A value of -0.13 was obtained for the 143B cells which have a CIII knock-down, suggesting that *ERCC1* is down regulated slightly. The down regulation of *ERCC1* is not as prominent as those seen in figure 5.9 for hOGG1 in the same variants of 143B cells. Again, the gene expression results obtained for *ERCC1*, the enzyme under investigation in the NER study, does not depict the extent to which NER capacity is impaired in section 5.1.3.1. NER is an interdependent and complex method of DNA lesion repair as describe in section 2.2.3. The impaired DRC caused by transfection of 143B cells cannot exclusively be ascribed to the fact that *ERCC1* gene expression is down regulated because the down regulation is very small in some cases (e.g. URKS). The results showed in figure 5.10 suggest that transfection impairs DRC by causing down regulation of *ERCC1*, a gene encoding for one of the proteins acting in a complex to recognise and excise bulky adducts to the DNA. This is not, however, the only way in which DRC is hampered for the extent of impairment is greater than can be ascribed to down regulation of *ERCC1*. The mechanism in which transfection interferes with the cell's DRC is still unknown.

CHAPTER 6

SUMMARY & CONCLUSION

The effect of different perturbations on DNA repair capacity was determined by employing the single cell gel electrophoresis-, or Comet assay. The Comet assay is known to be a simple, reliable and uncomplicated qualitative method to investigate DNA damage and repair (USBweb, 2009). The method is also acknowledged for its ability to detect AP-sites, SSBs as well as DSBs, and is therefore suitable for the purpose of this study. Existing methods were previously modified to suit the specific needs of this study and the method was optimised for use in our laboratory (Van Dyk *et al.*, 2010). The methods used to study BER and NER, the two excision repair pathways under investigation, differ and was adapted from that described by Collins *et al.* (2001) and Langie *et al.* (2006) respectively. The B[a]P treatment method of Plazar *et al.* (2007) was, however, used in conjunction with the NER investigation method of Langie *et al.* (2006).

It is acknowledged that free radicals (ROS) are produced by the mitochondria through respiration and are accordingly present in all body tissues (Mitra *et al.*, 2002). Other sources of ROS include the P450 metabolism, peroxisomes and the activation of inflammatory cells (Franco *et al.*, 2008). Mitochondrial functioning has an enormous effect on overall cell physiology and does not merely involve the production of ATP for general cellular functions. ATP serves to drive energy dependent processes such as DNA repair, which is also associated with imperative cellular occurrences such as apoptosis, necrosis, regulation of calcium dynamics and the production of reactive oxygen species (ROS). It is commonly understood that ROS leads to DNA damage and lipid peroxidation that can negatively influence normal cell functioning and bring about various pathological conditions (Finley & Haigis, 2009). The possibility exists that a change in mitochondrial function (as in the case of 143B cell variants used in this study) may lead to overproduction of ROS which may negatively affect macromolecules, including proteins involved in DNA repair pathways, and impaired energy production which in turn may restrain the proper occurrence of energy dependant processes (Levy & Deutschman, 2007).

For the purpose of this study, two separate knock-down systems, established in 143B cells, were used to examine the effect of perturbations in the energy

metabolism on DNA repair pathways. The first is an sh-RNA induced inactivation of subunit NDUFS3 (Fe-S protein 3) of complex I (NADH:ubiquinone oxidoreductase; E.C. 1.6.5.3) of the electron transport chain (ETC). Complex I serves as the primary electron accepting complex of the oxidative phosphorylation system (OXPHOS). Leigh syndrome is possibly the most familiar clinical trait of a deficiency of this complex (Finsterer, 2008). The second model uses the same of mechanism for disabling Complex III (ubiquinol cytochrome C; E.C. 1.10.2.2) of the ETC. In this case, subunit UQCRC1 (Rieske Iron-Sulphur protein) of complex III of the ETC is inactivated. Complex III catalyses the transfer of electrons from complexes I and II to cytochrome C. Deficiency of complex III has been linked with encephalopathy, liver failure and early death syndrome (Moslemi & Darin, 2007). These knock-down systems, together with several controls, were used in this study.

The second model investigated in this study, involved a perturbation in the homeostasis of the metal metabolism. Metallothioneins are small metal-binding proteins with characteristically low molecular weight, high cysteine content and contain no aromatic residues (Margoshes *et al.* 1957; Masters *et al.*, 1994). Metallothioneins have diverse functionality and are involved in numerous cellular processes as various factors and chemicals induce their expression (Theocharis *et al.* 2003). Metallothioneins are known to play a central role in trace element homeostasis and detoxification of metals and are effective ROS scavengers (Vařak, 2005; Chiaverini & De Ley, 2010). Heavy metal imbalance can occur in the absence or malfunctioning of metallothioneins and the cell is consequently exposed to elevated levels of redox metals (Liu & Kadiiska, 2009). The pro-oxidant milieu that a heavy metal imbalance causes may result in mutagenesis through unrestrained DNA damage (Bertin & Averbeck, 2006). Several, partially overlapping elaborate processes exist in agreement to identify and repair DNA damage, which would otherwise lead to serious possible consequences including cancer development (Hoeijmakers 2001). It was thus suggested that an imbalance or disturbance in the metal homeostasis caused by MT knock-out may create an environment favourable for DNA damage formation and at the same time impair DNA repair pathways. Previous studies performed concluded that MT knock-out mice were more susceptible to oxidative DNA damage, (Yang *et al.*, 2010), and chromosomal aberrations upon treatment with B[a]P (Takashi *et al.* in 2009). Targeted disruption

of metallothionein I and II genes was first performed by Masters *et al.* (1994), this model was used in the current study.

The genome is continuously confronted with factors challenging its integrity (Krokan *et al.*, 1997). These include xenobiotics, environmental stress and endogenous reactive oxygen species (ROS) (Dinant *et al.*, 2008; Zhang *et al.*, 2009). Excision repair pathways play an imperative role in counteracting and rectifying DNA damage, and include base excision repair and nucleotide excision repair (Xu *et al.*, 2008). DNA repair pathways exist to prevent the gradual build up of DNA damage during ageing and poor health (Mullenders *et al.*, 2009), and to guard DNA from the mutagenic properties of various carcinogens (Xiong *et al.*, 2007). Excision repair pathways, BER and NER, operate in comparable ways but differ distinctively in the points of onset and are both substrate specific. hOGG1, involved in the BER pathway, is classified as a type II glycosylase enzyme that catalyses the removal 8-oxo-guanine bases paired with cytosine. The enzyme under investigation in the NER pathway acts in an XPF-ERCC1 complex to recognise bulky adducts to the DNA and to perform 5' incision (Christmann *et al.*, 2003). The initial stages of excision repair pathways that are monitored in this particular study, give rise to strand breaks or AP sites, the latter form SSBs under alkaline conditions.

DNA repair capacity (DRC), for the purpose of this investigation, is an indication of the ability of the proteins under investigation to carry out DNA excision repair. DRC is expressed as a value between zero and one. A DRC value that approaches one has ideal repair ability, however a good value (as expected from control protein extract) is normally between 0.3 and 0.5. It should always be kept in mind that active excision repair proteins increase the APTD of the substrate DNA as it attempts to excise lesions present on the DNA.

Certain factors had to be taken into account before the experiments could be performed in order to assess the effects of the various perturbations on DRC. Most of these issues were brought to light and resolved in the study leading up to the findings published in the included paper in chapter 3. A proof of principle investigation was performed by treating substrate DNA with control- and heat inactivated protein extracts. Heat inactivated protein extract did not cause an increase to the APTD of the substrate DNA proving that the increase in APTD is

indeed a result of protein treatment, and the data can be used to calculate the excision repair ability of various protein extracts. It was also determined that cells had to be incubated at 37°C with the damage causing reagent and the treatment concentrations of these reagents were determined.

A uniform decrease of DRC values was detected in all protein extracts prepared from transfected 143B cells (including EV, URKS, CI kd and CIII kd) compared to the control protein extract prepared from normally cultured 143B cells in both the BER and the NER investigations. From the results it appears that BER is affected to a greater extent than NER when comparing the DRC values as presented in figures 5.3 and 5.7. However, the reduction in DRC for the complex I and III knock-down models cannot be ascribed to the perturbation in the energy metabolism. Gene expression analysis showed that hOGG1 and ERCC1 gene expression is down regulated in the four different 143B cell models compared to control 143B cells, but the mechanism in which the transfection brings about impairment of DRC is still unknown. These findings together with previous observations (Kok, 2010) suggest that mere transfection of cells have a much larger impact on diverse biological functions of the cell than previously recognised.

A number of PAHs including B[a]P have the capacity to produce ROS indirectly through the formation of quinone derivatives and may thus lead to oxidative DNA damage in addition to adduct formation. The repair of different DNA lesions are timely processes and do not occur at the same rate for various kinds of damage. Since B[a]P, amongst other PAHs, have the aptitude to form adducts to DNA that is typically repaired by NER, as well as the ability to indirectly cause oxidative damage, usually recognised and repaired by proteins in the BER pathway, it may cause DNA damage that require a range of proteins from BER- and NER pathways to excise the induced damage. The repair of a variety of DNA damage does not take place at precisely the same tempo, and the high standard deviation observed in all NER experiments may thus be ascribed to the fact that diverse DNA abnormalities are in different phases of repair at the time when protein treatment is ended.

Definite impairment of both excision repair pathways was observed in protein extracts prepared from MT knock-out mouse liver tissue compared to wild type mouse liver tissue. Rotenone treatment in comparison with the vehicle control treatment (PBS) seems to have little or no apparent effect on the DRC of the

various protein extracts in wild type and knock-out groups as observed in the current study. No significant change or elevation in ROS levels was observed in the different genotype or treatment groups. Furthermore, rotenone treatment caused inhibited complex I activity mostly in the muscle and heart tissue. In the liver tissue, rotenone treated wild type tissue had lower complex I activity than rotenone treated knock-out tissue. Rotenone may thus affect the energy metabolism in wild type or MT knock-out mice to some extent in the liver but is not reflected in the DRC values obtained during the course of this study. Perturbations in the metal homeostasis have a distinct impairing effect on the proteins involved in the initiating steps of BER as well as on the proteins engaged in the early stages of NER. An analogous observation was made by Candéias *et al.* (2010), who analysed the effects of cadmium exposure on the recognition and excision stages of BER and NER. It was found in the study by Candéias *et al.* (2010) that both repair pathways are inhibited by the presence of Cd, and the lack of metallothioneins to maintain proper and healthy metal homeostasis. Their findings are undoubtedly supported by the DRC calculations from the data obtained in this study. Since Yang *et al.* (2010) also made the observation that MT knock-out mice are more predisposed to oxidative DNA damage, one may assume that the protective role of metallothioneins was undervalued in the past.

The vast effect of the deficiency of metallothioneins on the DRC of mouse liver tissue proposes that excessive damage to macromolecules due to the lack of antioxidant defences provided by metallothioneins may cause mal-functioning of DNA repair proteins. The effects, however, are not restricted to proteins involved in repair pathways. Other possibilities include that the imbalance of metal homeostasis and lack of heavy metal detoxification in the absence of metallothioneins may lead to apoptosis, cellular adaptation – causing metabolic channelling, glutathione depletion and above all - extreme DNA damage. Perhaps a combination of all the above speculations is also probable.

Plazar *et al.* (2007) questioned the reproducibility of their method when using human cells. This potential predicament applies to this study as well and it was accordingly crucial to perform all experiments in duplicate and that a control experiment was conducted in parallel with every trial to serve as calibration for every study individually, as background DNA damage is known to vary between

batches of cells. Another possible shortcoming of this method is that the assay was developed to monitor or investigate a single step in the respective excision repair pathways, BER and NER, however it is inevitable that a cascade of enzymes are involved in the repair process. This method does not provide a holistic representation of the effects of the different perturbations on the total repair processes, but merely the effect thereof on enzymes involved in the initiating steps of each pathway. The method is not quantitative but provides the researcher with a good general idea of the effects of various perturbations on DNA repair capacity.

The shortcomings of the method are minor and mostly lead to new questions to be answered and research possibilities in the field. The fact that transfection has diverse, previously overlooked effects of the normal biological functions of cells are of great concern. This is alarming, for transfection is extensively used in common biological research experiments. Furthermore, there is still room for the clarification of the mechanism by which metallothioneins protect macromolecules such as DNA and proteins against damage of various origins, besides its anti-oxidant properties and metal binding capability. Further investigation will surely lead to the discovery of metallothioneins' extended functions, as the primary biological purpose of these multi-stress proteins is yet to be determined. Despite some negative aspects and new questions arising, the aim of the study was achieved.

The approach that was followed proved to be successful to determine the effects of different perturbations on DNA excision repair capacity. The changes in DRC that was observed in this study could not be attributed to a perturbation in the energy metabolism (where the 143B cell model is used) but was instead ascribed to the effect of transfection of cells. Although MT knock-out did not cause significant changes in ROS levels or complex I activity in the tissue under investigation, impaired DRC was observed in the MT knock-out liver tissue. This mechanism of impairment is still to be clarified in future studies and is not ascribed to altered energy metabolism. Now more than ever the Comet assay is thought of as a versatile and promising quantitative assay to measure DNA damage and repair. The comet assay can thus be used to investigate DRC in various tissue or cell culture samples with diverse perturbations. The aims of this study were successfully achieved as is emphasized by the history of the published paper- the paper was

received by the journal on the 31st of August 2010 and accepted and available online by the 7th of September 2010. The Comet assay was applied to establish what the effects of certain perturbations were on different types of samples, and the perturbations were unrelated – contributing to the versatility and adaptability of this assay.

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APPENDIX A:

PROTOCOLS FOR BUFFERS AND REAGENTS

Buffer A

Hepes	0.7153 g
KCl [4 mM]	6.67 ml
EDTA	0.0195 g
DTT [0.125 M]	53.4 µl
Glycerol [100 %]	6.67 ml

Add 60 ml ddH₂O and adjust pH to 7.8 with KOH, store at room temperature. Note that DTT becomes unstable at room temperature, for this reason it is important to return it to 4°C directly after use.

To prepare KCl [4 mM]:
(MW = 74.55 g/mol)

KCl	0.1419 g
ddH ₂ O	500 ml

To prepare DTT [0.125 M]:
(MW = 154.25 g/mol)

DTT	0.01446 g
ddH ₂ O	750 µl

Buffer B (Enzyme buffer)

Hepes	2.145 g
EDTA	0.015 g
Glycerol [100 %]	4 ml
BSA [98 %]	0.060 g

Add 170 – 180 ml ddH₂O and adjust the pH to 7.8 with KOH. Adjust the volume to 200 ml.

Electrophoresis buffer

NaOH [5 M]	60 ml
EDTA [0,4 M]	2.4 ml

Fill up to one liter with ddH₂O.

Ethidium bromide (Staining solution) (12.5 µM)

Ethidium bromide	0.005 g
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Dissolve in one litre ddH₂O and store at 4°C for at least one hour before use. Note that ethidium bromide is carcinogenic and care should be taken when handling it. Cover the container with foil as ethidium bromide is sensitive to light.

High melting point agarose (1 %)

HMPA	1 g
EDTA [0.1M]	100 ml

Dissolve the HMPA in the EDTA in the microwave. Observe the flask and take out when the mixture boils, swirl to mix and bring to the boil again. Repeat until the agarose is completely dissolved in the liquid. Prepare in small volumes that are sufficient only for the specific experiment. (E.g. 0.1 g HMPA in 10 ml EDTA)

Low melting point agarose (0,5 %)

LMPA	0.5 g
EDTA [0.1 M]	100 ml

Dissolve the LMPA in the EDTA in the microwave. Observe the flask and take out when the mixture boils, swirl to mix and bring to the boil again. Repeat until the agarose is completely dissolved in the liquid. Prepare in small volumes that are sufficient only for the specific experiment. (E.g. 0.01 g LMPA in 2 ml EDTA). Place in warm water or 37 °C water bath.

Lysis buffer

NaCl [5 M]	500 ml
EDTA [0.4 M]	250 ml
Triton X-100	10 ml
DMSO [10 %]	100 ml
ddH ₂ O	140 ml

To prepare NaCl [5 M]:
(MW = 58.44 g/mol)

NaCl	292.2 g
ddH ₂ O	1000 ml

Adjust the pH of 500 ml ddH₂O to between 7 and 8 with approximately 90 ml NaOH (this step is very important, otherwise the EDTA will not dissolve in the water). Adjust the final volume to one liter after the EDTA has dissolved.

To prepare the EDTA [0.4 M]:

(MW = 372.24 g/mol)

EDTA	148.89 g
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Use a magnetic stirrer to dissolve the salt in the water. Note that NaCl forms brown foam and should be filtered before use. Use a syringe to measure off the Triton X-100.

To prepare NaOH [5 M]:

(MW = 40 g/mol)

NaOH pellets	100 g
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Dissolve the NaOH pellets in 350 ml ddH₂O. Note that extra care should be taken as this is an exothermic reaction and the mixture heats up. Place the container with solution in ice water to cool and adjust the volume to 500 ml with ddH₂O when the solution has cooled.

Neutralizing buffer/Tris HCl

Tris HCl	63.04 g
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ddH ₂ O	500 ml
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Dissolve the Tris HCl in the water and cool (4°C) for at least one hour. Adjust the pH to 7 with NaOH [0.6 M]. Approximately 65 – 70 ml of NaOH is needed.

Phosphate buffered saline (PBS)

PBS tablet	1
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ddH ₂ O	200 ml
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Dissolve one tablet in 200 ml of ddH₂O.

APPENDIX B:**LIST OF SUPPLIERS AND CATALOGUE NUMBERS OF MATERIALS
NOT OBTAINED FROM SIGMA ALDRICH**

Reagent	Company	Catalogue number
18s rRNA reverse primer	Eurofins	010755687
75 cm ² flasks	NUNC	
BCA TM Protein assay kit	Thermo Scientific	23227
DMEM	Thermo HyClone	SH 30243 FS
dNTP mix	New England Biolabs	N0447S
FBS	BioWhittaker ® Sera	DE14-801F1
MMLV High performance reverse transcriptase	Epicentre Biotechnologies	RT80110K
NucleoSpin ®RNA II	Machery-Nagel	740 955.50
Oligo dT	QIAGEN	79237
Penicillin/Streptomycin	Lonza Walkerville, Inc	17-160E
TaqMan® gene expression assays	Applied Biosystems	Assay IDs: hOGG1: Hs00213454_m1 ERCC1: Hs01012158_m1
18s rRNA TaqMan® gene expression assay	Applied Biosystems	4319413

APPENDIX C:

PRIMER AND PROBE SEQUENCES

cDNA synthesis:

18s rRNA reverse primer:

5' CGGACATCTAAGGGCATCAC 3'

Gene specific primers used for real time PCR (gene expression):

Primer and probe sequences of hOGG1-, ERCC1- and 18S rRNA gene expression assays are not disclosed by the manufacturer, assays are however standardised.

Assay IDs:

hOGG1: Hs00213454_m1

ERCC1: Hs01012158_m1

18S rRNA: Hs99999901_s1

APPENDIX D:

RAW DATA OBTAINED (COMET ASSAY)

BER

Perturbations in the energy metabolism

	% Tail DNA (Control- BER experiment)					
	No PE control	Control PE	EV PE	URKS PE	CI kd PE	CIII kd PE
	9.546852025	33.85975	23.70406	23.58883	17.11363	24.79773
	23.63790725	3.851995	21.60909	14.40565	19.50066	20.01946
	21.21390113	22.71382	15.86261	12.70942	13.03871	19.67198
	28.63692434	29.41083	18.99297	21.24027	17.16356	12.16294
	13.04404145	27.71612	15.75298	24.03601	25.60651	23.00817
	16.83308869	31.12797	25.66097	13.66732	22.53516	17.82382
	23.80533344	24.63785	23.84723	4.909706	11.71844	24.05189
	16.22488912	36.93438	22.05451	9.834758	3.509509	19.93784
	15.59655875	20.19788	10.16944	21.10055	10.02972	38.34324
	19.80520585	29.21017	15.65257	23.2041	23.73791	20.32152
	18.42203232	10.74294	19.80419	10.90926	30.78363	32.45777
	18.05693747	29.26792	12.33407	15.8113	10.96978	23.96156
	16.56415344	29.82061	19.18294	27.06255	14.80337	19.36377
	25.34753932	11.03369	25.6858	22.49092	22.70226	20.12172
	20.5313377	34.30446	20.25385	36.17187	12.95655	18.4513
	16.09552691	19.58832	18.47814	15.16959	11.93992	20.34183
	15.06024926	21.7467	15.40915	18.86655	37.53825	27.06313
	15.14651597	20.5814	17.9813	12.12339	19.45946	38.99617
	13.35788016	25.6185	33.23567	21.20176	22.33269	21.00227
	20.62641783	32.36489	32.51536	17.3525	30.19901	24.51724
	13.46588929	25.71429	16.76559	17.11088	15.89592	14.52691
	17.2280428	12.98004	20.54773	21.68332	8.357657	23.07247
	29.76508121	33.05905	33.8714	21.47833	15.46927	14.06483
	10.68112133	11.6379	20.82085	15.39195	22.58605	15.07347
	19.63323782	31.65173	21.10561	22.89118	16.90084	23.31619
	17.37420582	23.4976	23.64521	27.82147	23.69412	19.97732
	10.73547014	19.28512	17.25	13.18263	10.21629	10.9688
	18.11016312	14.78481	19.67341	19.72	14.83094	33.06886
	13.63750479	36.14231	13.4702	19.47816	19.07317	16.65458
	13.46055811	39.67733	14.97127	16.58212	23.37105	22.31072
	14.01319925	32.45115	20.55627	20.37402	13.85058	19.36784
	17.18123209	28.03266	23.27775	28.17941	24.47269	23.54248
	22.06553777	30.0595	25.05777	16.69049	19.74489	18.60656
	14.12965664	32.2761	21.00885	18.16038	20.86792	12.06075
	7.480100951	27.03319	20.3665	19.6973	24.69968	21.27741
	13.69773556	20.22579	23.91254	16.60083	16.53708	10.80871

	18.31064563	13.23244	22.59504	10.95985	29.63691	18.04313
	13.44423481	37.95777	10.80778	18.70972	16.32232	19.61294
	9.109174019	28.07538	25.49697	18.76634	29.41403	15.58407
	19.20219397	29.41802	18.42094	23.14458	18.95864	19.6547
	18.18701023	26.56094	24.52174	18.89474	18.15038	26.94518
	13.67189539	19.82322	23.98564	12.26239	16.81612	21.20898
	21.08271901	30.97446	23.29949	22.70094	16.25909	18.06492
	15.19641767	20.75823	18.81466	21.29537	20.329	22.54988
	12.50521812	29.58506	27.8338	25.56156	21.97962	10.8847
	10.22377304		24.44788	20.80764	20.94272	18.0344
	19.43710075		22.09935	14.29988	22.27494	19.38925
	16.75005249		22.58586	20.07903	12.88733	16.27744
	11.4572504		14.3667	18.05801	15.64623	23.2364
	19.89314618		23.78018	21.36419	5.938007	24.64887
	16.61684783		22.14442	46.02021	14.86392	22.84438
	20.32197366		10.23094		24.51918	14.00239
	20.10926903		30.818		18.29515	17.36543
	20.26597623		19.66982		22.46009	12.99652
	7.623865356		12.01708		19.0433	26.08077
	18.1077205		29.91631		21.94609	18.27296
	13.5507689		23.85105		26.08034	14.48234
	12.16010485		19.88032		15.3097	21.99431
	15.38706872		20.68705		10.33654	
	15.16090584		19.68871		15.29404	
	13.87814098		11.45263		14.62377	
	12.35647326		26.56945		13.63193	
	17.72956303				15.57428	
	6.145385737				19.45284	
	18.22841155				10.61194	
	16.4906238					
Average	16.40630243	25.54721	20.87854	19.48673	18.397	20.47047
Standard deviation	4.626917357	8.155645	5.418741	6.579607	6.246981	5.891601
DNA repair capacity:		0.357804	0.214203	0.158078	0.108208	0.198538
Count	66	45	62	51	65	58
Number of comets in class:						
0-6%	0	1	0	1	2	0
6.1-17%	37	6	14	16	28	14
17.1-35%	29	34	48	32	34	42
35.1-60%	0	4	0	2	1	2
>60%	0	0	0	0	0	0

Percentage comets in class:						
Class 0	0	2.222222	0	1.960784	3.076923	0
Class 1	56.06060606	13.33333	22.58065	31.37255	43.07692	24.13793
Class 2	43.93939394	75.55556	77.41935	62.7451	52.30769	72.41379
Class 3	0	8.888889	0	3.921569	1.538462	3.448276
Class 4	0	0	0	0	0	0

	% Tail DNA (H ₂ O ₂ - BER experiment)						
	No PE control	No PE H2O2	Control PE	EV PE	URKS PE	CI kd PE	CIII kd PE
	7.57085	37.62289	62.44049	49.29923	38.43307	29.54198	46.68535
	20.75472	16.65348	47.34104	38.16121	45.5042	30.78195	28.45471
	25.34349	52.80702	45.0702	25.80982	24.32909	32.55383	24.3825
	14.73048	39.10804	88.509	25.66775	30.06312	37.36086	47.61884
	7.98154	21.59568	69.48399	42.31507	49.85532	32.331	37.98507
	19.28038	28.59631	72.9106	36.34868	28.25186	38.17372	67.82348
	10.91901	39.77674	56.07526	37.2286	45.97542	45.76441	30.6551
	6.98895	23.75427	61.40684	60.96715	42.66333	33.49326	40.02658
	4.450471	37.78758	44.34587	49.80057	47.76396	21.23592	34.7806
	6.087317	32.36406	61.44315	63.09902	46.7427	40.00786	49.27998
	10.16406	26.70084	44.52793	40.88246	49.41778	29.60132	39.32675
	14.78261	29.08965	42.23642	32.65272	32.09652	39.61141	11.47595
	13.41425	20.63257	40.79127	29.48944	32.57456	34.161	27.44541
	23.48984	26.13215	57.59036	38.04593	44.96496	30.32237	28.11635
	13.2219	33.68049	53.91511	44.89745	41.61088	29.46903	42.66116
	6.842495	32.96467	59.14316	35.46758	18.82098	34.87061	35.22589
	18.32948	31.94573	43.4058	29.93579	47.8273	42.54578	44.95403
	14.64958	23.98883	53.58156	40.05807	57.4423	34.30714	40.89485
	3.661673	30.37309	40.34091	53.60098	27.56637	32.68144	27.44541
	10.65674	21.54008	56.49402	46.42804	34.53463	29.09216	28.11635
	11.95051	28.28283	31.96474	42.86419	33.54544	37.03333	44.95403
	7.215244	24.85186	40.65395	43.74294	30.11793	31.18027	40.89485
	16.67202	33.36314	40.45952	30.01772	20.53881	32.42236	45.22589
	15.78873	34.15256	32.17666	31.73632	37.60556	37.9646	24.60775
	12.80649	24.09309	34.59459	88.81873	53.61021	25.03791	21.58732
	17.54567	22.84835	54.19503	32.15054	48.37312	50.85158	46.79049
	10.22853	21.22045	41.3569	40.62305	37.84696	37.61194	36.0501
	15.66781	55.07425	48.36722	49.53812	36.10599	33.63041	32.22418
	4.569705	25.79302	57.38174	32.4007	45.5014	27.39018	54.56477
	17.08543	30.43847	40.14405	49.55236	26.87745	43.51162	21.74956
	22.39127	25.47031	59.82717	43.24362	38.80047	47.71071	33.02671
	17.90386	28.3671	47.49106	23.76224	24.33862	25.29735	30.13849
	16.92579	34.24591	52.37654	82.81107	28.04412	28.3259	49.51673
	15.62903	27.51331		36.29605	27.04472	38.52588	36.24256
	15.50486	33.26881		34.11486	43.39355	34.30079	44.38986

	14.54952	37.66821		33.53506	40.65764	37.82588	45.61948
	3.042228	34.6653		29.39127	36.5065	41.31445	44.45265
	37.67615	25.52674		32.28408	25.52888	35.91398	44.98554
	8.394282	33.78774		31.61342	33.16411	35.50107	28.66242
	15.11159	24.61191		44.3265	38.50211	30.24393	47.25958
	25.51531	22.03739		26.01433	30.37278	26.83141	70.16291
	7.965398	22.0944		25.22506	35.00541	32.38095	41.32398
	17.94952	37.10934		27.55035	33.09828	32.03114	49.89446
	10.84568	30.15983		37.89463	48.77724	42.29882	62.80118
	18.53029	26.05488		42.09837	24.21855	22.33238	46.07002
	18.37904	27.93232		42.38265	31.03093	46.27325	11.60123
	22.61301	38.62834		47.25426	38.27423		40.07789
	11.06269	22.34031		26.82185	44.26508		34.00113
	24.56544	24.91191		31.32201	35.07913		28.02109
	15.36649	11.85361		29.47454	35.59211		31.23881
	17.93504	38.81321		31.48003	42.99587		30.10575
	27.17232	30.11078		36.23886	32.6048		47.24339
	9.039753	20.78624		36.05563	33.23516		47.92866
	15.16489	23.27229		41.8422	38.10567		43.86952
	1.983189	64.52682		41.36123	35.11646		46.3455
	6.349511	25.53962		20.84925	32.27267		43.43106
	16.14674	30.91385		38.85144	41.4892		34.90377
	17.36405	36.46623		37.98799	36.53473		31.70556
	19.61573	29.91097		42.24705	37.75598		32.60045
	12.78229	55.97733			41.90154		19.47971
	12.31793	25.22845			39.59219		53.16892
	18.2725	33.48443					41.41149
	14.30434	29.74017					34.46691
		25.17747					11.77208
		27.49506					43.59553
		28.04924					30.258
		21.72353					39.52604
Average	14.46381	30.18948	50.97097	39.25305	37.04688	34.60107	38.10899
Standard deviation	6.593368	9.034675	12.25959	12.39761	8.325181	6.556887	11.74232
DNA repair capacity:			0.407712	0.230901	0.185101	0.127498	0.207812
Count	63	67	33	59	61	46	67
Number of comets in class:							
0-6%	5	0	0	0	0	0	0
6.1-17%	37	2	0	0	0	0	3
17.1-35%	20	52	3	23	24	27	25
35.1-60%	1	12	24	32	37	19	36

>60%	0	1	6	4	0	0	3
Percentage comets in class:							
Class 0	7.936508	0	0	0	0	0	0
Class 1	58.73016	2.985075	0	0	0	0	4.477612
Class 2	31.74603	77.61194	9.090909	38.98305	39.34426	58.69565	37.31343
Class 3	1.587302	17.91045	72.72727	54.23729	60.65574	41.30435	53.73134
Class 4	0	1.492537	18.18182	6.779661	0	0	4.477612

Perturbations in the metal metabolism

	% Tail DNA (Control- BER experiment)				
	PBS		Rotenone		
	No PE	Wild type	Knock Out	Wild type	Knock Out
	9.546852	7.113625291	13.85974688	5.797725782	3.588828549
	3.637907	9.500661225	3.851994894	14.01945904	4.405649618
	2.213901	5.038705481	12.71381579	9.671983599	13.7094157
	8.636924	7.163561077	9.41083489	12.1629427	4.240274424
	3.044041	15.6065121	7.7161174	5.008168028	4.036006727
	6.833089	12.53515791	13.12796724	7.823819723	4.667315175
	2.805333	11.71844388	4.637846905	14.05188926	3.909706241
	6.224889	13.5095093	6.934382116	10.93784365	19.83475783
	5.596559	20.02972481	5.197879859	8.343237042	4.100554236
	9.805206	13.7379081	9.210174029	10.32152231	3.204104903
	8.422032	20.78362865	10.74294205	12.45777148	10.9092596
	8.056937	20.96978274	9.267917411	15.96155602	5.811296115
	6.564153	14.80336967	9.820608182	9.363774841	17.06254949
	5.347539	12.70226007	11.0336895	10.12171561	1.490923184
	3.531338	12.95654513	4.304463888	8.451296993	3.171866448
	6.095527	21.93992018	9.588316301	10.34183117	5.169593118
	5.060249	17.53824757	13.74670037	7.063131448	18.86655261
	5.146516	9.459459459	4.581401098	8.996173756	12.12338594
	3.35788	12.33268671	15.61850312	51.00226599	4.201755099
	4.626418	10.19900646	4.364894714	4.517241379	17.35250399
	3.465889	15.89591554	5.714285714	4.526911628	7.110883771
	7.228043	8.357656514	12.98003936	13.07247488	11.68331507
	2.765081	5.469273743	13.05904935	4.064825109	6.478334005
	20.68112	2.586051308	11.63790493	5.07346586	5.391949153
	9.633238	6.900842099	13.65172855	13.31619025	13.89118441
	7.374206	4.694120745	13.49760438	9.977322961	7.821473359
	0.73547	10.21629066	9.285120832	10.9688045	14.18263045
	2.110163	4.830940837	14.78480856	4.068860809	9.719999181
	3.637505	9.073167988	6.14231158	6.654583118	9.478162223
	3.460558	5.37104846	9.677332375	5.310724976	6.582124906
	4.013199	13.85057983	3.451154529	9.367840475	4.374020519
	7.181232	4.472685817	8.032660267	5.542483899	14.17940718

	22.06554	9.744890165	4.059497248	8.606557377	6.690488795
	4.129657	5.867921982	4.27610094	12.06074936	8.160379574
	7.480101	4.699675627	7.033186242	4.277414245	19.69729671
	3.697736	6.537080599	13.22579474	4.808710699	6.600827494
	8.310646	9.636910732	⁴ .232400668	8.043126082	14.95985233
	3.444235	6.32231981	7.957774532	9.612944302	8.709718588
	9.109174	9.414034046	8.075383129	6.584066471	8.766342165
	9.202194	8.958642023	9.418022528	9.654695336	13.14458178
	8.18701	8.150378045	16.56094467	6.94518192	18.89473684
	4.671895	6.816116328	9.823218606	5.208983609	4.262391869
	1.082719	6.259085079	11.97446393	18.0649192	12.70093865
	5.196418	10.32900244	10.75822604	15.54987531	11.29536737
	3.505218	11.97961799	9.585062241	10.88469906	2.561564386
	3.223773	10.94272435		8.0344035	10.80764408
	9.437101	2.274936217		9.389252949	12.29987746
	2.750052	4.887329689		6.277438898	10.07902736
	1.45725	5.646230649		5.236398543	18.058009
	9.893146	5.938007305		5.64886805	11.36419001
	3.616848	5.863921842		12.844377	16.020207
	0.321974	4.51918391		14.00239017	
	20.10927	8.295153215		7.365432199	
	20.26598	2.460087083		12.99652021	
	7.623865	9.043297076		16.08076772	
	8.107721	11.94609471		8.272961074	
	3.550769	6.080338826		14.48233562	
	2.160105	13.30970102		11.99431129	
	5.387069	10.33654038			
	5.160906	5.294043848			
	3.878141	4.623767198			
	2.356473	23.63193046			
	7.729563	5.574276889			
	6.145386	19.45284012			
	8.228412	10.61193545			
	6.490624				
	3.406657				
	5.391456				
	8.559869				
	4.806067				
	8.687169				
	20.76454				
	3.140477				
	1.317258				
	4.331093				
	21.88668				
	3.974383				
	6.532119				
	3.431293				
	12.93058				

	5.477629				
	3.937194				
Average	6.504761	9.797004684	9.417997089	10.02219342	9.565161268
Standard deviation	4.815638	5.066096313	3.599287907	6.526013082	5.208056627
Median	5.271978	9.073167988	9.501542384	9.365807658	8.766342165
DNA repair capacity:		0.336045912	0.309326468	0.350964301	0.319952782
Count	82	65	44	58	51
Number of comets in class:					
0-6%	46	19	10	14	17
6.1-17%	30	39	34	42	27
17.1-35%	6	7	0	1	7
35.1-60%	0	0	0	1	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	56.09756	29.23076923	22.72727273	24.13793103	33.33333333
Class 1	36.58537	60	77.27272727	72.4137931	52.94117647
Class 2	7.317073	10.76923077	0	1.724137931	13.7254902
Class 3	0	0	0	1.724137931	0
Class 4	0	0	0	0	0

	% Tail DNA (H ₂ O ₂ - BER experiment)					
			PBS		Rotenone	
	Control	No PE	Wild type	Knock Out	Wild type	Knock Out
	9.546852	40.42664	65.96765198	49.35462409	62.08088196	42.9937477
	3.637907	28.59289	51.24673354	51.6602274	67.81984334	22.1719457
	2.213901	22.83227	55.3366181	46.05217391	60.20123424	46.6599009
	8.636924	35.12328	65.76203209	42.48932948	63.51143257	65.91081027
	3.044041	25.05698	62.83053136	53.46695682	64.3902439	41.87785388
	6.833089	32.90155	53.09532217	49.52088879	60.65165877	44.90344034
	2.805333	31.55161	54.17149754	46.40953997	66.21112158	39.79553516
	6.224889	32.39362	62.4354712	23.93918349	38.67957046	40.18340828
	5.596559	22.85746	76.61858805	52.45847857	60.51088375	47.42876998
	9.805206	30.80645	57.22583153	50.20633676	60.43562439	38.138833
	8.422032	38.16327	48.0894101	39.97757357	65.97431529	36.39262787
	8.056937	36.42174	66.97654636	51.19918618	60.92415871	39.37047882
	6.564153	33.96541	60.30249175	46.32120796	51.80857388	40.74332232
	5.347539	33.24328	65.03521713	54.48050594	68.12382739	51.07089312

	3.531338	30.5618	65.27861597	48.25542878	60.20127768	53.88373531
	6.095527	37.45241	64.62352305	48.12644057	69.56172029	42.43963425
	5.060249	40.96052	50.41116006	51.15479115	62.51228501	42.49180021
	5.146516	48.45171	70.4259455	52.27272727	60.86836087	43.59618282
	3.35788	43.33333	56.81479731	48.06117105	52.18673219	46.70463174
	4.626418	33.00643	55.5163728	53.85050963	60.99933378	42.71091898
	3.465889	51.00386	60.11135398	49.83431269	68.11363773	40.8763977
	7.228043	38.52441	64.45672192	55.79042294	60.63897764	49.72630174
	2.765081	35.34091	61.80072029	48.61151151	51.44269046	55.32849503
	20.68112	29.09138	63.41511286	51.64293538	63.66538374	37.57122619
	9.633238	34.27461	59.36603457	58.7431694	51.83817062	41.28546683
	7.374206	34.47417	64.11905868	54.92845537	65.65488565	41.36291468
	0.73547	31.96142	54.05574243	64.79099678	62.04996247	45.03467763
	2.110163	36.21747	64.90325237	49.93324699	63.80909173	38.17427051
	3.637505	34.68736	55.74117647	50.87360087	66.90293322	31.71422659
	3.460558	33.87398	61.98795571	51.20183811	64.19421488	38.75078608
	4.013199	33.16063	54.3750839	55.47434734	67.21647806	43.69797235
	7.181232	36.64583	53.49340369	35.4820457	68.81670534	53.76110812
	22.06554	36.42161	60.26525199	41.61107568	68.22652486	39.59243032
	4.129657	50.84064	69.73760933	47.26491134	63.89270705	45.85509265
	7.480101	37.62378	64.60363232	57.29064294	44.70387841	68.30679357
	3.697736	30.04837	63.40065018	47.52071383	61.06674043	67.36525229
	8.310646	36.22133	60.07421614	34.25373425	48.12306961	45.12524686
	3.444235	33.43939	73.45238095	45.87961985	58.61915588	61.24626547
	9.109174	38.49203	53.39661017	41.01542042	55.10326358	62.02788261
	9.202194	34.86433		45.50905744	52.97836129	56.3252149
	8.18701	36.18633			60.25747354	50.29276453
	4.671895	32.8244			68.5825957	48.17614558
	1.082719	43.38453			38.20035993	63.5053195
	5.196418	38.09204			68.05844907	66.62827949
	3.505218	45.71317				45.14491588
	3.223773	38.91684				64.67463806
	9.437101	34.96591				49.2628391
	2.750052	32.60346				45.25973552
	1.45725	34.22922				47.39580117
	9.893146	44.10913				
	3.616848	33.69151				
	0.321974	33.96854				
	20.10927	46.16877				
	20.26598	33.94571				
	7.623865	35.97845				
	8.107721	58.25849				
	3.550769	58.05189				
	2.160105	39.16176				
	5.387069	37.81994				
	5.160906	35.63506				
	3.878141	39.77185				
	2.356473	33.94202				

	7.729563	38.70134				
	6.145386	30.05243				
	8.228412	34.255				
	6.490624	30.58998				
	3.406657	31.60324				
	5.391456	32.93196				
	8.559869	53.54755				
	4.806067	30.15588				
	8.687169	58.50778				
	20.76454					
	3.140477					
	1.317258					
	4.331093					
	21.88668					
	3.974383					
	6.532119					
	3.431293					
	12.93058					
	5.477629					
	3.937194					
Average	6.504761	36.80386	60.79282886	48.67273351	60.45019979	47.20279452
Standard deviation	4.815638	7.348825	6.444213656	7.135753646	7.66414274	9.881451197
Median	5.271978	34.96591	61.80072029	49.67760074	61.55835145	45.12524686
DNA repair capacity:			0.394601875	0.243850469	0.391170505	0.220303268
Count	82	71	39	40	44	49
Number of comets in class:						
0-6%	46	0	0	0	0	0
6.1-17%	30	0	0	0	0	0
17.1-35%	6	36	0	2	0	2
35.1-60%	0	35	15	37	11	39
>60%	0	0	24	1	33	8
Percentage comets in class:						
Class 0	56.09756	0	0	0	0	0
Class 1	36.58537	0	0	0	0	0
Class 2	7.317073	50.70423	0	5	0	4.081632653
Class 3	0	49.29577	38.46153846	92.5	25	79.59183673
Class 4	0	0	61.53846154	2.5	75	16.32653061

Count	38	34	38	26	33
Number of comets in class:					
0-6%	13	0	2	1	4
6.1-17%	18	22	25	13	22
17.1-35%	7	12	11	12	7
35.1-60%	0	0	0	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	34.21053	0	5.263158	3.846154	12.12121
Class 1	47.36842	64.70588	65.78947	50	66.66667
Class 2	18.42105	35.29412	28.94737	46.15385	21.21212
Class 3	0	0	0	0	0
Class 4	0	0	0	0	0

	% Tail DNA (H ₂ O ₂ - BER experiment)					
			PBS		Rotenone	
	Control	No PE	Wild type	Knock Out	Wild type	Knock Out
	7.792102	39.8434	73.66364	34.56533	79.34066	61.82134
	4.88756	55.79755	70.6568	53.21579	62.13035	58.68737
	11.35669	51.35177	73.94287	64.78293	92.50299	54.14475
	1.004801	52.67137	61.68224	56.50244	63.02352	61.09512
	6.126296	46.10169	79.00574	56.56364	74.50756	62.62016
	1.063908	61.53345	73.61553	57.52732	76.68524	60
	13.27992	33.1	59.99836	45.01397	63.07771	60.08929
	1.262334	54.01265	67.62471	62.41245	62.03704	58.87965
	20.08999	56.81984	83.38191	58.72462	59.45544	64.34178
	17.43484	47.98201	56.22257	55.19896	58.41149	57.94673
	3.296703	31.6704	84.00757	60.0198	67.85004	58.58728
	20.35311	39.81413	81.92568	65.18547	54.22265	60.94994
	6.592083	40.29797	42.33404	51.34418	72.18692	53.97484
	1.270908	37.92728	82.85354	68.15661	60.61464	52.10526
	6.51711	17.76288	64.51418	58.42275	61.44578	68.09325
	5.508384	38.8618	67.07096	69.6118	65.11813	59.71831
	7.366412	56.13961	81.4122	50.373	61.94046	59.79722
	12.02338	26.03314	57.25154	56.85906	62.77723	53.59586
	12.18086	58.59699	38.77134	65.53395	59.29271	35.36576
	7.512747	54.29185	57.17839	46.96864	67.80842	54.46821
	8.807952	20.51747	51.76108	58.18413	61.82134	51.86776
	2.773678	53.48299	63.86214	60.16883		53.90845
	22.11104	51.61421	44.76955	50.72008		57.50776
	15.69544	24.54074	62.18302	61.59098		64.9141
	30.23857	40.04232	84.89103	60.29746		69.84372
	8.499507	48.75596	86.74389	68.86499		55.4144
	11.98494	55.02368		52.86249		68.93835

	17.11553	53.89041				61.17272
	16.59259	32.68923				
	5.739207	40.35225				
	5.504778	44.55809				
	9.555215	43.4341				
	21.30047	40.42932				
	4.434033					
	11.92715					
	4.790454					
	9.976643					
	3.015531					
Average	9.920602	43.93759	67.35863	57.39525	66.01192	58.56605
Standard deviation	6.90926	11.47144	13.85086	7.858525	8.771942	6.645937
Median	8.145805	44.55809	67.34783	58.18413	62.77723	59.29898
DNA repair capacity:			0.347707	0.234473	0.334399	0.249777
Count	38	33	26	27	21	28
Number of comets in class:	13	0	0	0	0	0
0-6%	18	0	0	0	0	0
6.1-17%	7	7	0	1	0	0
17.1-35%	0	25	8	15	4	17
35.1-60%	0	1	18	11	17	11
>60%						
	34.21053	0	0	0	0	0
Percentage comets in class:	47.36842	0	0	0	0	0
Class 0	18.42105	21.21212	0	3.703704	0	0
Class 1	0	75.75758	30.76923	55.55556	19.04762	60.71429
Class 2	0	3.030303	69.23077	40.74074	80.95238	39.28571
Class 3						
Class 4						

	% Tail DNA (Control- BER experiment)				
	PBS			Rotenone	
	No PE	Wild type	Knock Out	Wild type	Knock Out
	2.143785	17.16964	10.0111	17.71741	10.29891
	10.57981	18.00694	15.76826	13.79346	12.07102
	10.25718	12.78025	11.82759	18.7361	11.45318
	11.08853	26.27761	10.09376	10.14231	20.60137
	8.982892	21.98969	10.02932	11.4016	21.02242
	10.70838	12.31827	11.42562	15.068	10.42703
	7.362604	19.20328	20.46875	9.693221	14.50692
	6.95044	12.46252	11.97364	9.301334	12.89331
	2.300138	12.44032	15.64939	28.6733	15.1275

	9.010587	13.43494	21.25248	8.533442	10.26584
	10.95867	17.60425	10.44605	16.13282	14.12805
	9.185989	13.24586	10.22127	14.37863	10.17022
	6.438029	16.83301	10.05145	23.13101	20.12174
	10.39368	29.18993	18.46647	23.49438	25.5064
	10.66584	11.47035	10.60845	21.72371	10.22787
	14.76488	23.84328	12.85191	14.65178	16.5757
	23.70629	14.50958	10.24746	16.85511	30.03666
	2.509273	17.0807	10.02222	19.05258	11.1353
	0.658612	19.89814	11.52416	17.10156	5.843228
	15.08845	18.96806	10.03128	13.06174	12.29527
	8.013764	13.64834	10.4628	15.8848	14.43625
	0.32475	18.42683	10.01801	9.233322	10.28553
	0.033542	17.92576	10.02656	12.27362	10.64875
	1.664018	17.38375	10.14868	19.51603	10.55721
	4.898906	15.87159	10.21661	17.27709	10.4299
	7.234915	19.25315	10.5828	18.43692	7.278581
	7.285559	19.39288	11.05222	10.21431	17.23551
	41.46471	15.52763	10.15577	9.855025	10.08599
	24.77419	11.45761	10.90707	17.87593	11.22124
	33.66362	19.45729	10.03298	17.19454	20.24592
	10.17605	16.51987	11.487	9.866766	23.54644
	4.705816	19.85428	11.09485	7.090715	12.18851
	10.29687	9.286653	0	27.79473	0
	10.03239	15.81953	11.08349	18.4998	10.66365
	4.257737	14.01071	11.37565	17.04112	21.87254
	2.805445	19.23105	14.67639	29.36126	12.69398
	11.59051	15.97961	15.78463	16.38402	23.37027
	12.38935	9.519904	14.54612	19.21247	17.44409
	0	18.33938	10.62342	12.60191	10.60187
	10.0063	17.90681	10.28536	17.38468	13.75529
	0	9.91328	17.55649	16.3702	2.913577
	10.05004	9.505566	13.64939	8.955664	10.0581
	0	9.839642	19.42494	17.94387	10.79219
	9.812851	12.54947	61.72589	11.399	20.7365
	21.45356	19.60812	18.37868	17.03497	4.989084
	23.04526	8.468658	20.36148	15.5432	
	10.83787	12.15399	17.60976	17.1974	
	6.86901	19.16725	10.30358	14.30859	
	9.069671	12.98738	11.4142	19.0754	
	3.860809		11.83826	13.38353	
	3.523743		7.523542		
	4.067877		18.50585		
	12.40862		10.92431		
	10.46196		13.65623		
	10.13148		10.66923		
	10.00524		10.80122		
	10.04016		13.25336		

	10.29468		10.4059		
	11.63786		20.01896		
			12.96551		
			18.20697		
			7.746177		
			12.58147		
			10.57798		
			14.14558		
			50.31678		
			10.08877		
			10.0326		
			11.37435		
			10.42907		
Average	9.609206	16.07618	13.62882	15.93709	13.61686
Standard deviation	7.723959	4.418781	8.227611	5.060577	6.028767
Median	10.00524	16.51987	11.08917	16.37711	12.07102
DNA repair capacity:		0.40227	0.294935	0.397054	0.294316
Count	59	49	70	50	45
Number of comets in class:					
0-6%	17	0	1	0	4
6.1-17%	36	26	56	27	29
17.1-35%	5	23	11	23	12
35.1-60%	1	0	1	0	0
>60%	0	0	1	0	0
Percentage comets in class:					
Class 0	28.81356	0	1.428571	0	8.888889
Class 1	61.01695	53.06122	80	54	64.44444
Class 2	8.474576	46.93878	15.71429	46	26.66667
Class 3	1.694915	0	1.428571	0	0
Class 4	0	0	1.428571	0	0

	% Tail DNA (H ₂ O ₂ - BER experiment)					
			PBS		Rotenone	
	Control	No PE	Wild type	Knock Out	Wild type	Knock Out
	2.143785	50.1687	69.55983	52.5987	75.66046	64.05746
	10.57981	34.34362	61.47825	64.15066	75.34134	56.19021
	10.25718	48.92524	76.86021	52.14732	80.8361	75.49815
	11.08853	54.9396	75.8093	60.21502	80.12726	60.58263
	8.982892	31.28931	93.6873	67.28857	60.10152	54.4006
	10.70838	44.82991	73.83818	65.04598	79.6891	63.47961
	7.362604	52.96403	95.06657	50.67715	88.06516	62.61487

	6.95044	52.43093	63.23909	54.16022	63.8783	60.22719
	2.300138	40.35131	71.21212	60.20389	61.82352	76.46946
	9.010587	38.23576	60.87645	58.0327	64.83778	61.68723
	10.95867	43.13548	67.52557	53.79665	80.06465	50.27206
	9.185989	43.35623	72.41026	57.41901	73.38609	70.11973
	6.438029	55.60886	74.5543	60.87242	70.32677	60.36054
	10.39368	52.62255	66.22383	52.6989	70.77374	50.78093
	10.66584	49.89936	73.30016	62.8804	90.474	67.90598
	14.76488	36.50126	85.51944	61.24876	85.17112	56.81761
	23.70629	40.09501	76.4761	6.812843	70.39516	59.03882
	0	37.28119	62.71017	62.94906	80.30697	60.28226
	2.509273	38.92354	74.04902	60.31837	73.99129	70.59419
	0.658612	52.63081	71.47204	54.28248	78.54872	65.33899
	0	51.98433	61.99454	61.89752	71.12393	62.40738
	15.08845	38.32947	74.06943	57.08622	70.10252	71.04279
	8.013764	40.10259	67.87649	52.11435	88.58986	50.28211
	0.32475	54.31434	76.13643	63.00497	80.71498	53.22478
	0.033542	30.83218	66.25	61.58742	71.43567	60.54038
	1.664018	53.03783	90.13144	50.60157	81.43455	66.27217
	4.898906	46.21443	71.7431	64.50288	64.48894	60.59599
	7.234915	31.51057	64.50764	59.37178	76.52729	64.28648
	7.285559	34.52987	75.01991	54.05733	71.77381	62.40896
	41.46471	40.1424	68.03887	67.47304	73.28837	50.23866
	24.77419	10.0021	65.13221	36.76357	72.95599	60.06143
	33.66362	52.74907	66.15737	52.2935	75.29855	62.59381
	10.17605	50.31508	76.02035	63.16364	73.49937	51.29799
	4.705816	52.89035	75.46469	59.89578	68.50024	51.06043
	10.29687	47.2125	85.03725	64.3807	74.10162	54.74395
	10.03239	35.46017	76.15991	67.27356	68.35753	67.03019
	4.257737	35.37233	70.67532	65.06381	79.24053	60.39898
	2.805445	54.08507	70.0621	54.1724	69.43996	61.26277
	11.59051	53.68436	69.47559	59.74026	71.4064	55.82999
	12.38935	31.94541	96.0459	60.96944	70.66048	50.32469
	0	54.61368	91.33769	56.30053	69.22266	60.13937
	10.0063	53.55683	67.47967	62.09794	72.61716	62.50941
	0	24.83675	97.15837	55.86994	71.27328	52.75067
	10.05004	33.11877	86.03844	51.12316	71.21163	64.69205
	0	37.02636	66.68535	60.42071	67.52161	52.54136
	9.812851	51.92661	68.50292	59.12735	71.67468	51.74219
	21.45356	55.99873	87.02211	54.55235	68.86513	51.53373
	23.04526	66.44785	86.69106	50.06159	75.54899	68.67347
	10.83787	64.53495	96.22347	63.18308	69.11027	54.85334
	6.86901	59.50854	69.11552	67.32086	76.49615	52.20099
	9.069671	47.17949	73.16786	58.9461	70.24782	50.66337
	3.860809	34.34467	66.78314	65.25359	70.37997	53.16284
	3.523743	31.33154	69.64956	52.90176	75.20776	51.8371
	4.067877	41.29929	67.64098		74.04993	64.58435
	12.40862	53.76092	87.1392		76.18163	64.75324

	10.46196	32.60373			67.21912	61.13184
	10.13148	42.63889				65.66599
	10.00524	53.9548				60.38498
	10.04016	54.48849				53.62704
	10.29468	46.6787				54.64144
	11.63786	50.19363				50.03963
		51.94338				50.25376
		56.94785				57.81075
		34.8023				50.88489
		34.62838				62.40578
		36.24239				61.19486
		52.54218				60.53032
						51.33436
Average	9.29415	44.78204	74.77277	57.55418	73.63513	59.10538
Standard deviation	7.787675	10.33126	9.949984	9.213078	6.284983	6.656146
Median	9.812851	46.6787	72.41026	59.74026	72.78658	60.3214
DNA repair capacity:			0.401092	0.221915	0.391839	0.242336
Count	61	67	55	53	56	68
Number of comets in class:						
0-6%	19	0	0	0	0	0
6.1-17%	36	1	0	1	0	0
17.1-35%	5	13	0	0	0	0
35.1-60%	1	51	0	27	0	30
>60%	0	2	55	25	56	38
Percentage comets in class:						
Class 0	31.14754	0	0	0	0	0
Class 1	59.01639	1.492537	0	1.886792	0	0
Class 2	8.196721	19.40299	0	0	0	0
Class 3	1.639344	76.1194	0	50.9434	0	44.11765
Class 4	0	2.985075	100	47.16981	100	55.88235

	% Tail DNA (Control- BER experiment)				
		PBS		Rotenone	
	No PE	Wild type	Knock Out	Wild type	Knock Out
	2.684868	8.492454	4.147308	10.00236	11.72331449
	7.244614	11.89948	6.742177	1.74801	12.79917544
	10.12814	15.40033	1.446167	1.216427	12.02578269

	10.28505	9.004355	0.815503	15.41695	10.36421782
	8.206408	4.148647	4.035853	8.273682	10.03413261
	4.313053	10.84309	5.426337	10.03136	7.496117549
	1.910314	10.23728	3.569912	8.861265	10.17246506
	6.01305	8.212931	1.100445	10.87794	7.243914104
	17.29133	11.72858	16.34558	10.76574	6.774911587
	3.164711	10.46993	3.259098	10.55323	8.448703323
	5.628014	1.049029	4.485889	12.2225	7.660085811
	0.2415	14.87088	0.787926	10.57555	9.058007699
	12.48212	5.498961	0.234361	7.119549	4.315109259
	7.020397	12.86234	6.158584	10.50146	11.08593534
	11.81873	4.224392	3.482587	17.92678	6.349157827
	8.416998	11.44487	0.733432	11.99319	6.134459205
	6.955688	10.68084	0.883126	11.46872	6.78204587
	0.752528	12.69662	0.504197	11.56009	11.22644613
	4.466966	14.99842	0.474449	7.732694	2.559271471
	3.095869	19.34524	7.705965	20.85266	6.815545994
	12.58882	10.31452	5.024241	3.771619	6.509746089
	8.501833	5.822233	0.361464	10.80154	4.116374799
	9.845967	8.490718	35.15951	1.840691	10.05924463
	5.127436	12.53511	8.327794	16.72977	3.230272266
	9.358623	12.48764	1.103706	1.79938	5.840120017
	9.17494	15.50413	9.809983	12.61763	10.58822229
	2.610185	7.50263	0.799411	4.649605	12.94632832
	10.30355	9.734872	25.6538	15.29478	1.918092276
	5.587988	11.79115	1.652573	5.792198	11.62648965
	2.79189	4.859236	0.275107	6.356694	5.011808467
	3.292826	11.1595	7.89749	12.00592	5.24955009
	3.505446	15.7377	7.422073	10.39026	4.243373104
	1.666332	10.59608	4.154878	1.18385	1.170598372
	0.138161	12.92796	16.40457	11.59884	6.02210531
	1.106633	15.085	20.23858	10.59307	3.058103976
	4.167565	11.81307	13.87294	2.06071	11.08928138
	5.91135	1.18094	40.68259	7.155293	5.914976932
	2.202474	7.948718	0.110445	6.356128	12.38195616
	3.776714	10.91632	6.41225	10.9818	5.133182065
	1.348121	7.24633	0.909412	32.939	13.60084477
	6.701025	11.86957	1.357576	14.34277	4.982019778
	6.582328	10.04806	13.11687	10.12315	5.555071833
	11.58989	11.16978	34.98692	6.812287	11.0552636
	7.975663	9.327106	7.797623	11.08096	11.33733704
	7.227718	10.88683	5.321742	15.81263	3.922622246
		14.91909	9.4855	8.8027	6.511684741
		10.17229	11.8023	6.40804	16.88010393
		10.54275	18.13847	27.94956	12.85837685
		10.0108	7.079379	7.80057	13.83739967
		7.856632	1.081023	10.09394	7.801063256
		16.04656		5.173552	15.17384205

		7.255363		2.416135	4.334038055
		10.04471		9.231762	9.593070763
		9.373571			7.023399304
		11.87436			12.70024051
		2.699317			2.565753628
		12.76394			11.75756546
		10.6382			14.00315418
		5.079299			
		7.681948			
		15.77429			
		13.81765			
		3.985123			
		9.311446			
		9.617677			
		13.41794			
		10.70573			
		3.918772			
Average	6.11564	10.18487	7.775583	10.01259	8.287439261
Standard deviation	3.879311	3.730426	9.477651	6.020534	3.765961572
Median	5.91135	10.56942	4.755065	10.12315	7.57810168
DNA repair capacity:		0.399537	0.213481	0.389205	0.262059087
Count	45	68	50	53	58
Number of comets in class:					
0-6%	23	11	28	11	18
6.1-17%	21	56	16	38	40
17.1-35%	1	1	4	4	0
35.1-60%	0	0	2	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	51.11111	16.17647	56	20.75472	31.03448276
Class 1	46.66667	82.35294	32	71.69811	68.96551724
Class 2	2.222222	1.470588	8	7.54717	0
Class 3	0	0	4	0	0
Class 4	0	0	0	0	0

	% Tail DNA (H ₂ O ₂ - BER experiment)			
			PBS	Rotenone

	Control	No PE	Wild type	Knock Out	Wild type	Knock Out
	2.684868	35.44632	56.89655	46.82619	64.06836	59.70508
	7.244614	31.57244	64.75047	69.68671	68.41244	40.21541
	10.12814	44.54472	59.43125	61.91017	50.65659	50.0334
	10.28505	33.82052	67.19277	61.51357	79.87365	50.55887
	8.206408	32.10685	52.96641	40.50541	60.72254	63.84398
	4.313053	42.36791	50.25795	69.40189	64.45263	50.63936
	1.910314	33.4579	71.00384	61.99051	59.39986	50.4394
	6.01305	43.72754	55.41825	51.0216	55.33162	52.21611
	17.29133	21.50582	54.27536	51.97967	44.71542	54.04949
	3.164711	33.23935	61.92441	44.12917	52.09516	55.73354
	5.628014	31.538	51.39797	48.94247	57.26921	50.00845
	0.2415	35.55233	63.67406	40.84036	41.76781	63.65495
	12.48212	38.22852	65.66894	56.27566	61.73193	60.29179
	7.020397	33.50302	53.2556	42.05635	51.94128	40.56272
	11.81873	39.88992	60.09897	40.93294	60.962	53.03087
	8.416998	30.27897	66.93041	53.43819	61.77367	52.98172
	6.955688	36.24522	51.14504	50.24218	60.52926	50.986
	0.752528	34.50999	56.58449	54.80129	60.23057	52.07921
	4.466966	27.88256	53.84428	49.12721	61.20302	61.49662
	3.095869	31.72608	50.77026	48.041	50.51388	60.72508
	12.58882	34.28601	51.47259	53.73566	50.13454	40.05021
	8.501833	41.08245	60.24279	51.26396	64.67932	49.24322
	9.845967	35.98753	61.43782	56.59747	61.79422	55.55533
	5.127436	33.14905	63.12574	60.83291	64.87992	44.34021
	9.358623	38.35491	51.48322	47.58105	52.92696	53.85536
	9.17494	46.70877	50.30869	33.49387	58.62634	47.16113
	2.610185	31.5339	51.0548	47.09893	53.48861	54.75098
	10.30355	40.22938	51.78623	57.68159	63.33518	53.66064
	5.587988	36.08511	53.19942	47.05161	70.82813	47.72703
	2.79189	29.58864	68.39905	59.96584	60.1526	60.81004
	3.292826	38.21832	62.62434		50.79394	54.46508
	3.505446	31.0615	51.37945		43.03568	50.98386
	1.666332	39.12551	52.75175		63.18665	55.46407
	0.138161	32.10094	53.94884		55.67891	35.4343
	1.106633	30.31599	52.84015		55.32829	46.47147
	4.167565	42.85302	63.55014		51.23472	52.30349
	5.91135	35.56312	62.41889		51.80004	52.06632
	2.202474	25.71075	51.99604		43.45823	55.69424
	3.776714	35.0678	50.48755		57.4219	58.18049
	1.348121	36.92293	54.19466		64.36265	49.19729
	6.701025	31.05599	62.75394		52.8955	40.7485
	6.582328	39.01946	52.64317		55.35815	56.60702
	11.58989	26.4751	63.90914		55.92669	57.38544
	7.975663	33.32831	50.24939		51.34699	53.67628
	7.227718	42.44451	51.54488		57.40991	53.63979
		48.16968	66.80283			42.48638
		35.97665				41.25685

		32.48273				53.56293
		32.18789				51.54136
		34.01045				53.96789
		26.90595				50.65397
Average	6.11564	35.04208	57.26289	51.96551	57.283	51.88614
Standard deviation	3.879311	5.418657	6.18669	8.601031	7.515704	6.340773
Median	5.91135	34.28601	54.23501	51.14278	57.40991	52.30349
DNA repair capacity:			0.388049	0.325667	0.388264	0.324635
Count	45	51	46	30	45	51
Number of comets in class:						
0-6%	23	0	0	0	0	0
6.1-17%	21	0	0	0	0	0
17.1-35%	1	27	0	1	0	0
35.1-60%	0	24	28	23	26	45
>60%	0	0	18	6	19	6
Percentage comets in class:						
Class 0	51.11111	0	0	0	0	0
Class 1	46.66667	0	0	0	0	0
Class 2	2.222222	52.94118	0	3.333333	0	0
Class 3	0	47.05882	60.86957	76.66667	57.77778	88.23529
Class 4	0	0	39.13043	20	42.22222	11.76471

	% Tail DNA (Control- BER experiment)				
		PBS		Rotenone	
	No PE	Wild type	Knock Out	Wild type	Knock Out
	8.576579	10.04023	1.028999	10.55628	2.282555
	1.282214	14.20949	7.024955	12.78997	0.148417
	7.855184	11.14228	10.25554	11.39347	10.1171
	4.604483	13.57136	11.14676	19.98721	10.07988
	16.95914	11.27492	10.15515	4.095167	1.792677
	11.58898	10.13346	10.00927	8.176305	1.077188
	22.37381	10.41877	9.141017	14.75082	1.09108
	10.23192	14.62879	11.27963	9.305595	10.07332
	10.294	0.013288	17.26755	21.7705	1.682288
	10.97814	3.471898	10.02656	16.55686	1.299713
	10.75601	10.84807	12.1629	18.03724	10.1332

	10.66532	10.09517	12.28868	11.31553	7.065934
	0.008798	10.04567	1.689348	3.684179	11.01124
	4.619698	13.07346	10.1571	10.11798	10.20007
	10.64204	13.38143	12.87362	11.24017	6.773137
	18.45985	1.182944	9.659192	7.982028	12.81286
	10.48251	10.29712	2.913395	11.33059	6.835273
	10.56642	11.88159	3.950216	5.376913	13.19686
	1.411642	10.04862	12.48627	10.9455	32.22486
	5.440291	18.78412	12.54209	15.19743	16.93656
	0.532993	5.348832	7.992781	4.139628	7.948483
	0.148235	7.474355	15.20101	6.05647	0.05814
	15.22213	12.33231	8.815702	10.95476	4.434162
	0.012374	12.2311	21.72995	10.57717	2.487438
	1.589349	11.02029	6.665835	13.25173	1.349114
	0.021225	14.4869	11.09433	11.02663	2.788172
	1.27265	10.76297	15.01965	4.315768	17.60318
	1.886555	10.15667	7.564474	11.65037	2.470291
	4.081937	10.88238	24.01177	10.09379	12.3848
	1.996904	13.58389	10.14111	5.522721	11.77735
	10.51221	12.43299	10.86581	10.31919	12.06742
	1.724943	12.87258	10.62958	7.387077	17.09218
	1.063328	0.016124	12.70613	9.976193	20.0161
	8.207569	13.98234	1.947863	9.265552	4.841899
	4.059373	2.196281	16.80426	11.70929	16.50343
	8.461454	22.58261	5.368785	4.52536	4.592876
	1.22842	20.42075	13.60428	5.633543	49.99491
	10.04133	13.36572	12.81443	8.279843	10.98879
	0.520913	20.31534	5.650287	12.36503	12.80946
	10.42871	18.79076	11.20415	16.05125	0.009752
	10.15192	7.140005	3.80422	21.87846	9.324561
	11.58186	17.81522	10.0192	3.941702	11.84662
	5.517361	10.18324	2.90773	42.75453	6.017905
	10.17873	4.650457	25.89182	11.65252	7.336991
	1.711151	10.063	22.95366	10.04956	51.67804
	1.813768	14.86694	4.541815	12.676	2.159537
	1.479939	1.002739	15.13522	8.514778	10.37619
		21.66755	10.55909	15.66589	5.551476
		16.01328	11.07945	7.450178	0.21931
		20.07836	10.84386		
		5.387501	2.535673		
		4.084682	10.84388		
		2.597095	15.42297		
		11.09916	5.471881		
		1.478397	1.178604		
		10.34902	2.952904		
		10.20805			
		12.0541			
		9.214168			

		9.821749			
		5.611162			
Average	6.664774	10.80629	10.25058	11.27132	9.868628
Standard deviation	5.523585	5.400358	5.596531	6.390223	10.66559
Median	5.517361	10.84807	10.40732	10.57717	7.948483
DNA repair capacity:		0.383251	0.349815	0.408696	0.32465
Count	47	61	56	49	49
Number of comets in class:					
0-6%	24	13	14	9	19
6.1-17%	21	40	37	35	24
17.1-35%	2	8	5	4	4
35.1-60%	0	0	0	1	2
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	51.06383	21.31148	25	18.36735	38.77551
Class 1	44.68085	65.57377	66.07143	71.42857	48.97959
Class 2	4.255319	13.11475	8.928571	8.163265	8.163265
Class 3	0	0	0	2.040816	4.081633
Class 4	0	0	0	0	0

NER

Perturbations in the energy metabolism

	% Tail DNA (Vehicle control- NER experiment)					
	No PE	Control PE	EV PE	URKS PE	CI kd PE	CIII kd PE
	6.160557	3.443761	0.556691	3.012704	8.737585	5.462543
	0	5.825804	6.897519	11.94481	1.963056	4.0841
	3.707655	3.222982	8.9778	8.527654	1.747784	3.34252
	4.696439	4.627888	19.57154	9.483651	5.890013	1.510245
	0	5.720861	5.30756	4.156212	1.115911	2.878252
	1.696962	10.1324	6.839501	12.76452	13.90282	8.664423
	0.040768	6.452442	7.609605	6.999771	3.696715	13.58913
	8.872919	9.961349	2.733915	3.78493	0.806481	19.04424
	2.024117	2.649488	4.768097	2.767974	4.119762	7.613041
	1.36688	6.906583	4.42174	3.345405	19.00913	2.719008

	1.56075	17.44026	3.726375	7.781649	3.963448	7.555895
	1.027879	10.49974	3.501837	3.244755	4.790669	10.89338
	6.108222	17.87764	8.333333	14.50083	4.463828	4.434409
	1.732663	8.054718	19.47969	3.369769	4.065827	5.086972
	0	7.801375	2.925532	3.939619	3.947539	0
	0	8.619646	10.28414	10.42093	1.151777	0.248267
	0.617739	3.281991	16.41023	8.198753	2.461861	20.23614
	0.63643	1.787225	3.039449	6.562657	2.017045	8.800077
	4.50368	9.322798	3.687025	5.633157	5.71794	9.710988
	6.027947	8.087071	4.55232	35.8147	4.029612	2.161626
	2.750207	2.878598	11.14746	3.394168	9.661983	13.78785
	4.09249	8.458277	9.769654	12.42015	8.88712	2.892648
	2.255795	1.134936	4.23203	3.621714	5.61753	10.87037
	12.32343	13.4736	0	7.161883	3.116627	0.351865
	3.502658	19.2354	18.00816	12.55932	0.847021	10.11734
	1.927461	17.20611	17.3529	3.74169	1.116091	3.324035
	4.877947	5.406245	6.348921	4.789731	1.778963	3.401833
	0.019892	5.87184	18.84799	0.819308	22.04577	10.03141
	13.04252	1.273269	0.337903	1.002532	3.201491	4.171967
	0	19.14983	1.555556	0.154766	3.10081	2.871142
	54.52036	8.675066	2.673743	5.125972	16.70356	4.926108
	3.253682	17.12159	3.789894	0.032526	5.289842	12.01203
	10.80909	10.56273	2.556268	9.977827	1.926174	5.985579
	9.448923	2.300421	5.979646	1.669116	0	3.183244
	0.019428	3.352451	3.628954	0.699028	0.702528	0.714043
	1.478451	4.831471	1.926881	17.16103	19.53772	5.402208
	1.932421	1.036693	5.26365	5.710869	11.72255	11.11219
	3.7203	1.537568	10.70194	0	2.403586	3.936753
	5.839658	3.26288	5.979868	0.396837	13.95912	2.01709
	0	9.781808	4.848373	3.127709	6.116782	6.465831
	0.198229	0.121045	11.78595	4.682904	5.484165	2.246143
	1.49426	14.96851	8.216688	7.618598	8.704397	1.145828
	0.583765	5.197494	4.385506	0.480169	2.681187	5.573697
	1.69216	12.02115	4.055297	15.1311	2.178384	10.39741
	0	0.309158	26.79629	6.326179	9.096697	6.633932
	5.740097	6.981746	5.540821	6.386054	1.381228	3.209158
	9.9536	7.398588	4.030751	4.954833	1.582443	2.466353
	5.647492	6.114467	6.945607	0	19.96269	10.09103
	7.665672	0	2.2302	37.9349	2.492281	4.410267
	5.122373	0.107842	3.945704	6.61818	4.032937	10.01951
		10.52632	0	3.171944	9.872697	10.40385
		13.03783	10.89825	2.099922	5.823327	
			2.624192	1.965468	4.821988	
			14.71258	3.166388	3.937228	
			3.571818	23.69429	11.87784	
			0.84868	6.535583	0.01789	
			2.192873	9.101145	4.183032	

			10.51126	0.405324	1.921213	
			6.780414	3.284962	10.7801	
			0.034529	0	7.855358	
			5.184425	3.234374		
			5.758398	16.05013		
			4.07933	1.949323		
			4.250426	4.088704		
			3.652478	9.552892		
			5.746953	2.809902		
			0.553896	1.358295		
				19.85873		
				1.306812		
				0.046414		
				1.090856		
				5.53391		
				8.993183		
				0.743232		
				6.149767		
				0.51815		
				8.219304		
				3.357371		
				0.188127		
				7.435427		
Average	4.493879	7.404826	6.535926	6.323293	5.900353	6.317803
Standard deviation	8.006859	5.360366	5.520927	6.942016	5.362454	4.605596
DNA repair capacity:		0.393115	0.312434	0.289313	0.238371	0.288696
Count	50	52	67	80	60	51
Number of comets in class:						
0-6%	39	24	43	48	42	30
6.1-17%	10	22	18	27	14	19
17.1-35%	0	6	6	3	4	2
35.1-60%	1	0	0	2	0	0
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	78	46.15385	64.1791	60	70	58.82353
Class 1	20	42.30769	26.86567	33.75	23.33333	37.2549
Class 2	0	11.53846	8.955224	3.75	6.666667	3.921569
Class 3	2	0	0	2.5	0	0

Class 4	0	0	0	0	0	0
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	% Tail DNA (B[a]P- NER experiment)							
	Control	VC	No PE	Control PE	EV PE	URKS PE	CI kd PE	CIII kd PE
	0	6.160557	13.46796	21.66614	31.1661	6.593886	4.634327	24.63026
	2.541637	0	6.350325	13.37506	20.33409	10.19077	24.05763	10.93183
	0.699891	3.707655	3.96316	23.43539	17.63394	8.260601	9.105032	6.343654
	2.47809	4.696439	9.648958	14.69725	14.4048	5.626197	11.85409	26.60617
	4.172567	0	13.50232	19.53637	14.31673	39.82235	30.71221	16.42015
	5.687548	1.696962	10.79048	14.65737	11.25711	4.090072	12.9012	6.990794
	1.342903	0.040768	9.203396	19.05961	13.74055	14.23156	22.58739	10.53499
	1.565092	8.872919	21.84308	20.77973	37.49625	17.91556	6.784671	20.61408
	1.570241	2.024117	0	23.14756	6.533187	3.809372	12.5936	15.0093
	1.052062	1.36688	10.10727	12.82501	23.21429	20.71153	22.95172	3.715433
	4.599154	1.56075	8.107711	16.85175	11.06391	12.6998	19.06609	4.327451
	2.489098	1.027879	9.597717	12.4321	21.01651	14.20519	12.47117	16.26788
	2.279647	6.108222	11.22157	18.64564	13.72491	10.43232	12.53093	24.69884
	0	1.732663	7.083619	19.76615	17.26119	10.44221	2.682531	8.805706
	0.09892	0	5.879847	29.76291	29.9471	27.20662	5.007494	34.28452
	25.55972	0	6.554957	26.89262	21.81691	11.1444	0.189542	13.82464
	0.411962	0.617739	12.21335	17.52657	16.48996	17.24252	28.83466	4.868463
	1.458907	0.63643	1.61139	13.19842	14.29407	16.6015	34.08626	10.25282
	0	4.50368	11.28821	17.2115	17.75639	13.38437	7.086174	15.46019
	22.84011	6.027947	2.542345	27.51242	12.95486	22.43361	13.46477	3.369282
	7.158313	2.750207	17.19991	30.17621	26.39311	9.604706	13.73626	16.10174
	16.5561	4.09249	3.128435	15.8987	22.92368	16.39948	12.85268	8.0228
	4.389071	2.255795	7.06502	15.14277	21.6167	8.45784	2.265485	28.76701
	2.527864	12.32343	14.5972	16.99876	13.25423	17.86988	10.51309	37.43499
	5.115438	3.502658	11.91027	13.23746	15.53113	15.31292	4.355593	12.79682
	0	1.927461	3.389582	13.66491	11.57557	31.90022	17.61422	25.18098
	1.13761	4.877947	10.81337	18.59129	13.69994	10.1563	10.7033	22.27107
	0	0.019892	12.19828	24.92995	15.73058	12.44377	14.26348	2.928203
	1.096792	13.04252	9.431984	14.7594	0.110279	21.50123	20.84184	13.54565
	0.016743	0	17.50075	17.31447	20.25416	26.74549	18.99521	17.04829
	0.246139	54.52036	10.44966	21.49589	14.43676	26.53816	11.29039	14.79982
	6.382979	3.253682	10.94175	18.26079	15.28857	8.535982	14.79991	25.49668
	0.422164	10.80909	2.327313	18.80894	12.66971	23.35709	22.19742	7.214467
	2.962454	9.448923	10.79132	10.18435	3.321784	4.060816	28.55845	1.243037
	5.091913	0.019428	10.18148	23.29084	27.68558	9.872272	8.266137	15.62886
	4.835703	1.478451	14.2871	14.17434	10.34185	18.9025	11.77894	31.87707
	2.210756	1.932421	18.34129	14.75151	10.50121	8.650243	7.575269	23.58067
	0.049829	3.7203	7.499653	27.18375	10.8137	40.53321	22.55152	7.786763
	0.012428	5.839658	25.29965	7.735011	15.43892	11.03698	9.654195	7.733985
	12.58687	0	11.93597	16.14895	8.328509	31.14203	21.79146	3.861781
	14.48651	0.198229	17.47861	28.87609	6.72453	4.775828	6.309562	2.68388
	0.063955	1.49426	11.11012	16.43644	12.43167	28.33099	28.68293	30.6619

	5.216057	0.583765	10.39918	14.17214	17.77247	10.10751	11.56739	23.47051
	1.205012	1.69216	12.89092	20.96596	13.88903	13.47674	7.2892	18.86965
	3.039198	0	15.1353	12.52847	8.027823	25.90803	17.65118	2.78361
	1.778773	5.740097	14.21461	19.74086	16.39487	26.25574	19.84938	10.43933
	7.630159	9.9536	7.310815	10.43883	15.77683	10.42721	24.63729	8.14001
	0	5.647492	11.98164	20.11897	18.16522	18.16522	11.69938	1.720631
	13.60032	7.665672	10.08373	12.19883	17.31336	37.31336	4.176334	18.15064
	2.722418	5.122373	21.2337	20.65011	31.61543	31.79896	12.88344	19.4059
			13.14195		11.74552	26.51	12.41077	18.6426
			10.51217		21.11962	19.76823	12.1935	24.63177
			38.8892		10.47424	10.47424	9.507477	10.52794
			10.11891		20.75942	32.29342	19.79935	20.37335
			13.53182		10.15148	11.1535	11.13172	
			7.605802		26.51	21.31732		
			11.82626		10.78143	9.58368		
			6.772169		14.26618	2.471226		
			13.70819		16.20436	3.692435		
			11.18149		10.47453	9.28422		
			13.44605		18.49937	9.507371		
						12.99621		
						32.15377		
						2.515723		
						0.498872		
						4.368594		
						17.60761		
						11.16486		
						19.01204		
						2.943466		
						14.11351		
						10.64077		
Average	4.067782	4.493879	11.25968	18.23709	16.15469	15.70411	14.32719	15.03294
Standard deviation	5.708444	8.006859	6.021929	5.303675	6.912465	9.623011	7.838223	9.225803
DNA repair capacity:				0.382595	0.303009	0.283011	0.214104	0.251
Count	50	50	61	50	61	72	55	54
Number of comets in class:								
0-6%	41	39	8	0	2	11	7	10
6.1-17%	7	10	45	23	36	33	29	23
17.1-35%	2	0	7	27	22	25	19	20
35.1-60%	0	1	1	0	1	3	0	1

>60%	0	0	0	0	0	0	0	0
Percentage comets in class:								
Class 0	82	78	13.11475	0	3.278689	15.27778	12.72727	18.51852
Class 1	14	20	73.77049	46	59.01639	45.83333	52.72727	42.59259
Class 2	4	0	11.47541	54	36.06557	34.72222	34.54545	37.03704
Class 3	0	2	1.639344	0	1.639344	4.166667	0	1.851852
Class 4	0	0	0	0	0	0	0	0

Perturbations in the metal metabolism

	% Tail DNA (Vehicle control- NER experiment)				
	PBS			Rotenone	
	No PE	Wild type	Knock out	Wild type	Knock out
	0	1.799815	2.527093	10.81736	11.14489
	2.541637	5.842704	12.03381	3.825952	3.044063
	0.699891	4.477649	6.775206	1.078221	2.497668
	2.47809	9.316315	2.652819	2.913466	5.59905
	4.172567	0.433235	0.730787	6.886692	1.877998
	5.687548	9.231868	0	6.298461	7.055351
	1.342903	9.522987	2.508598	10.46814	8.716981
	1.565092	6.578472	2.963757	6.346328	4.284319
	1.570241	4.720131	0.562092	8.506284	0.42796
	1.052062	7.102172	1.885691	8.115976	11.75948
	4.599154	10.97045	0.07917	0	2.029301
	2.489098	12.91913	0.069235	3.989267	3.989267
	2.279647	5.37508	1.997738	9.034571	20
	0	6.250217	4.897603	10.02279	5.861214
	0.09892	0	6.599943	8.070866	8.750744
	25.55972	7.657799	0.336964	17.30964	7.511913
	0.411962	11.89792	9.6864	4.324539	7.874502
	1.458907	0	0	4.589878	1.135329
	0	12.86331	11.51137	18.93546	3.386254
	22.84011	11.94177	1.422914	10.44761	2.879035
	7.158313	0	15.54523	8.691213	10.65455
	16.5561	11.83892	12.00529	3.062697	10.46844
	4.389071	1.470588	12.89181	10.89704	2.049033
	2.527864	7.094581	7.432878	3.676169	0.130012
	5.115438	9.698722	20.02123	19.99905	11.4605
	0	8.037349	5.911358	17.8774	4.004621
	1.13761	3.894023	0.927381	3.328525	2.792282
	0	9.959966	9.210378	5.77717	6.6149
	1.096792	1.794881	2.67438	6.088237	0.012614
	0.016743	4.821808	3.148945	1.590668	2.137941

	0.246139	9.601707	6.700121	1.071299	8.468166
	6.382979	3.353948	3.607125	10.24912	2.478052
	0.422164	15.74964	10.42725	0	15.93346
	2.962454	8.802481	11.29747	1.409572	6.520336
	5.091913	7.86104	10.01382	5.275137	5.159156
	4.835703	7.380194	8.107477	1.25446	7.037416
	2.210756	11.14878	2.496241	5.018308	12.59308
	0.049829	11.57697	1.391495	10.23073	4.017306
	0.012428	12.97538	7.730727	1.762794	0.074018
	12.58687	10.15781	2.415866	6.781898	3.593563
	14.48651	9.162133	3.917622	7.837822	4.336867
	0.063955	13.54998	13.36326	4.305926	0.434551
	5.216057	1.978888	7.20208	9.532925	2.079938
	1.205012	0	3.24553	3.420513	1.639469
	3.039198	4.32999	4.339413	6.376117	8.370581
	1.778773	4.532827	6.070826	3.554344	6.33256
	7.630159	3.632091	0.524957	9.270116	2.214205
	0	7.681062	8.403255	1.705619	2.00379
	13.60032	11.07949	0.635463	2.539048	4.031511
	2.722418	1.462882	0.964092	5.328729	0.177567
		5.898707	0.083348	2.037933	
		1.70555	3.013638	5.447204	
		9.22669	2.532401	9.498934	
		14.55135	11.74985	2.322301	
		2.813391	10.0552	6.943988	
		0.101186	6.829337	2.286396	
		5.768441	0.282047	8.591213	
		7.232711	2.320045	4.441939	
		1.844466	5.014766	3.823572	
			7.02732	2.845194	
			3.483112	6.442707	
			3.149031	23.32599	
			21.32111	7.127895	
			1.128244		
			10.76119		
			1.714762		
Average	4.067782	6.824943	5.489842	6.587769	5.352916
Standard deviation	5.708444	4.271369	4.924862	4.894057	4.34523
Median	2.245202	7.102172	3.545118	5.77717	4.024408
DNA repair capacity:		0.403983	0.259035	0.382525	0.240081
Count	50	59	66	63	50
Number of					

comets in class:					
0-6%	41	26	39	32	31
6.1-17%	7	33	25	26	18
17.1-35%	2	0	2	5	1
35.1-60%	0	0	0	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	82	44.0678	59.09091	50.79365	62
Class 1	14	55.9322	37.87879	41.26984	36
Class 2	4	0	3.030303	7.936508	2
Class 3	0	0	0	0	0
Class 4	0	0	0	0	0

	% Tail DNA (B[a]P- NER experiment)					
			PBS		Rotenone	
	Vehicle'Control	No PE	Wild type	Knock out	Wild type	Knock out
	0	3.467961	22.863	10.71532	44.07567	12.04729
	2.541637	6.350325	10.90154	12.1888	35.43183	17.18017
	0.699891	23.96316	13.53916	12.93508	21.39135	11.44778
	2.47809	9.648958	18.98504	11.78247	12.46225	15.20625
	4.172567	43.50232	20.48028	17.12323	8.139607	14.62525
	5.687548	10.79048	13.48641	14.6057	23.03679	14.12259
	1.342903	9.203396	13.98601	13.52774	10.69148	14.70614
	1.565092	1.843077	18.09178	12.1977	23.87537	6.964503
	1.570241	0	14.84974	15.76087	48.69887	10.09553
	1.052062	10.10727	22.43054	18.15148	39.37342	11.30906
	4.599154	28.10771	20.44101	21.1062	12.33236	4.130603
	2.489098	9.597717	16.04855	9.257428	15.28677	6.803961
	2.279647	11.22157	22.33572	15.19268	13.67807	0.027167
	0	47.08362	16.14929	14.42159	16.17392	12.80788
	0.09892	15.87985	16.56407	9.356037	10.12605	15.38531
	25.55972	6.554957	18.28076	17.74218	17.13922	8.677289
	0.411962	2.213348	17.40453	13.33702	9.099819	10.29335
	1.458907	1.61139	23.11669	10.21896	22.28532	9.19175
	0	1.28821	20.53392	22.13717	8.202578	17.46892
	22.84011	2.542345	19.78044	6.218052	12.37329	14.58333
	7.158313	17.19991	13.17694	5.620669	25.49954	10.12508
	16.5561	3.128435	23.11236	13.80027	20.29084	11.32842
	4.389071	7.06502	22.23067	22.18573	8.41247	11.55987
	2.527864	14.5972	25.3676	13.56976	12.85399	4.371244
	5.115438	11.91027	23.3787	11.50873	22.97692	13.8574
	0	3.389582	10.73147	9.747366	7.098856	5.916962
	1.13761	0.813366	13.80313	8.084814	7.670435	17.97886

	0	2.198278	11.03535	12.54221	10.58893	13.06977
	1.096792	9.431984	19.77469	11.48176	23.23644	5.541953
	0.016743	7.50075	23.23915	9.103685	5.161947	7.400787
	0.246139	0.449662	11.0887	20.15919	4.456293	27.44329
	6.382979	0.941748	23.96787	16.06993	24.33854	17.7429
	0.422164	2.327313	20.74922	10.27653	13.307	11.43168
	2.962454	0.791325	26.46396	3.847138	12.15945	13.13355
	5.091913	0.181477	29.10921	10.19413	6.299227	7.514797
	4.835703	14.2871	28.43841	34.14708	11.31806	7.704712
	2.210756	18.34129	23.59506	17.35738	10.93201	13.82472
	0.049829	7.499653	19.56333	20.19832	14.21531	8.459999
	0.012428	5.299648	26.84893	11.53356	16.17573	11.59482
	12.58687	1.935974	23.85797	16.84538	17.41272	6.633246
	14.48651	17.47861	16.13622	16.25692	22.18944	14.29408
	0.063955	21.11012	7.486228	16.54455	11.41951	29.30448
	5.216057	0.399181	21.17964	7.674965	15.69305	15.31816
	1.205012	2.890918	12.39673	10.67815	6.542526	5.741
	3.039198	25.1353	16.4098	12.58748	17.28999	18.99642
	1.778773	41.21461	11.22824	14.6212	10.04292	1.497714
	7.630159	7.310815	20.95494	12.86997	20.14073	19.64495
	0	1.981641	20.15795	8.246166	15.51889	15.67886
	13.60032	10.08373	17.91379	15.83469	9.133682	12.23039
	2.722418	21.2337	20.61509	14.36982	10.3203	16.56618
		23.14195	31.7327	5.448044	23.58815	11.61119
		10.51217	22.63911	10.4405	14.157	10.13686
		38.8892	24.08526	12.4762	10.63655	23.7711
		0.118906	18.07813	13.97157	17.40099	17.90462
		23.53182	19.03591	8.518135	16.39735	9.945525
		7.605802	23.77739	13.25947	30.21702	8.688831
		1.826263	14.57198	31.57895	15.73994	14.9711
		16.77217	11.19456	20.57987	18.70153	24.4415
		3.708188	14.61206		16.11202	12.52955
		11.18149	13.68876		11.42351	12.35653
		3.446055	16.5169		13.55302	0
			19.74618		15.88369	7.626539
			16.33729		18.90557	8.512156
			18.12561		23.3071	24.42994
			4.736242		13.28317	13.20659
			2.977403		15.15468	27.12571
			17.33239		25.28173	16.06076
			18.62439		23.79406	11.85562
			17.06903		24.70524	26.79607
			18.41633		28.16456	14.1219
						10.41696
						10.93187
						15.0046
						13.27056
						30.41543

						14.17768
Average	4.067782	10.88263	18.39396	13.83114	17.04252	13.0696
Standard deviation	5.708444	11.28716	5.481529	5.541825	8.648132	6.305467
Median	2.245202	7.50075	18.52036	13.09728	15.60597	12.44304
DNA repair capacity:			0.408359	0.213179	0.361443	0.167333
Count	50	61	70	58	70	76
Number of comets in class:						
0-6%	41	25	2	3	2	8
6.1-17%	7	22	24	43	40	53
17.1-35%	2	10	44	12	24	15
35.1-60%	0	4	0	0	4	0
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	82	40.98361	2.857143	5.172414	2.857143	10.52632
Class 1	14	36.06557	34.28571	74.13793	57.14286	69.73684
Class 2	4	16.39344	62.85714	20.68966	34.28571	19.73684
Class 3	0	6.557377	0	0	5.714286	0
Class 4	0	0	0	0	0	0

	% Tail DNA (Vehicle control- NER experiment)				
	PBS			Rotenone	
	No PE	Wild type	Knock out	Wild type	Knock out
	4.016572	6.162188	12.43656	7.665203	13.76812
	0.369421	17.09503	12.89713	5.901653	7.107231
	13.03848	5.875865	6.127886	6.498762	9.826418
	19.14209	11.64146	3.111111	8.032026	10.70036
	5.878922	11.42885	5.700173	12.95196	16.7079
	3.013029	9.57255	2.783231	11.02915	10.02711
	2.346965	10.42423	10.31441	1.243781	0.445851
	3.608409	3.031869	9.308511	11.0663	9.278432
	15.93692	7.094952	11.40885	2.548277	7.043355
	5.91898	7.785161	3.2459	2.435131	26.90719
	4.436235	21.78453	9.269972	27.10478	10.79425
	9.093768	9.949555	2.828226	6.294086	2.796611
	2.836671	3.525611	20.33464	8.802737	2.230534
	8.219953	1.082674	11.34427	4.066166	6.782977
	4.567005	6.547619	1.152798	26.50205	7.445162

	2.09763	12.3885	4.076323	10.51891	15.55744
	6.835771	2.444728	5.828011	12.27026	15.00768
	3.333333	5.006544	6.607113	2.114082	18.56192
	16.64422	9.309199	25.70555	3.930608	5.447562
	14.32335	7.391046	7.785578	17.89731	3.440685
	6.413973	1.561552	5.021119	1.569943	7.811723
	4.305222	6.948529	11.61871	11.67603	13.64315
	11.12047	4.196739	5.507452	19.13075	21.77941
	6.895288	11.27142	16.6261	1.676529	4.497952
	2.911911	9.359112	9.886792	19.01906	9.787961
	0.242326	8.683128	9.62984	3.338262	3.518509
	5.164603	7.239307	4.961624	12.53397	7.326786
	6.545855	21.61248	13.30137	12.75171	2.378416
	7.755312	6.776297	4.943457	9.387352	7.900005
	3.44728	12.33091	10.21073	14.68262	11.01977
	3.445143	12.67848	2.609629	1.704022	16.34646
	1.315789	11.82846	8.609497	13.34746	16.13228
	2.766667	2.940102	14.77281	0.589302	0.256978
	5.797458	11.84898	5.787896	11.44014	5.225931
	8.460358	1.308489	8.347726	6.399705	2.863849
	4.700599	3.456443	7.014234	2.835794	19.27617
	7.225659	12.71197	2.343159	1.09846	2.919409
	4.648215	10.4993	16.41083	10.6437	2.397195
	2.573933	6.568803	6.298584	21.07618	2.798619
	6.490125	2.178761	6.733216	0.374043	9.648177
	3.237329	11.58842	8.599976	2.632794	2.511392
	3.68254	17.78726	14.18178	11.16123	1.558485
	2.427857	7.792506	5.890288	5.078554	23.29612
	6.392295	20.36209	8.981818	12.93167	25.2503
	6.638073	1.335445	8.365484	2.900443	2.632675
	5.149893	5.727452	6.30598	3.202468	0.770618
	5.241314	8.539823	3.558079	10.01633	1.778783
	5.394411	16.39031	4.224164		3.444245
	3.145736	12.75045	3.29438		18.04066
	6.264102	1.154571	3.812257		2.208713
	8.457673		4.346211		0.041802
	7.775401		0.828729		15.45466
	5.241706				7.21536
					12.70687
					1.461791
					10.9805
					3.391859
Average	5.979854	8.779395	7.986349	8.767484	8.774568
Standard deviation	3.948158	5.303592	4.957367	6.680506	6.888906
Median	5.241314	8.166165	6.670164	8.032026	7.326786

DNA repair capacity:		0.318876	0.251241	0.317951	0.318502
Count	53	50	52	47	57
Number of comets in class:					
0-6%	32	15	22	19	24
6.1-17%	20	30	28	22	26
17.1-35%	1	5	2	6	7
35.1-60%	0	0	0	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	60.37736	30	42.30769	40.42553	42.10526
Class 1	37.73585	60	53.84615	46.80851	45.61404
Class 2	1.886792	10	3.846154	12.76596	12.2807
Class 3	0	0	0	0	0
Class 4	0	0	0	0	0

	% Tail DNA (B[a]P- NER experiment)					
			PBS		Rotenone	
	Vehicle Control	No PE	Wild type	Knock out	Wild type	Knock out
	4.016572	11.53849	19.06307	23.64085	18.34145	20.32926
	0.369421	14.71559	19.04933	12.34842	22.54456	17.74437
	13.03848	19.90251	20.00123	23.23333	22.31789	20.92646
	19.14209	17.01225	33.73394	15.19945	14.46388	20.16265
	5.878922	10.08579	11.28891	11.79219	15.28893	21.44964
	3.013029	7.439024	9.177899	14.06844	23.2348	27.9628
	2.346965	19.27979	29.77029	34.55633	15.84109	12.03361
	3.608409	7.635998	46.0815	37.16628	13.35896	23.83673
	15.93692	14.37368	16.89129	24.4881	19.48979	10.20208
	5.91898	11.94183	13.93743	16.56039	20.5061	17.58727
	4.436235	8.921857	20.67634	11.66709	25.24764	16.47598
	9.093768	15.75901	20.12427	14.07518	26.37571	11.17607
	2.836671	7.238752	2.153478	13.56892	19.10591	12.40432
	8.219953	14.59982	9.098511	18.65219	5.459125	17.73664
	4.567005	16.97038	14.70022	13.23993	22.49919	35.31142
	2.09763	7.156195	10.72399	10.48998	21.96632	27.02936
	6.835771	18.41914	20.16935	6.742156	27.14435	38.75265
	3.333333	8.180961	10.77192	5.652588	10.70224	14.39063
	16.64422	10.27963	11.61023	35.25427	24.03017	33.91379
	14.32335	8.099807	22.5641	7.191465	18.34193	10.94658
	6.413973	12.404	12.87237	16.57777	12.57712	18.87633
	4.305222	17.7375	27.76991	13.43158	23.1018	4.27034

	11.12047	19.0955	16.94759	34.18566	21.0616	12.83186
	6.895288	11.41082	12.84472	4.554274	16.83767	6.437768
	2.911911	16.39957	38.18291	9.952294	19.16964	6.95751
	0.242326	13.78977	7.752354	11.28994	16.53907	14.10005
	5.164603	12.83137	25.52165	5.51577	21.22757	36.01512
	6.545855	16.5155	29.60248	36.18668	22.40467	18.45249
	7.755312	15.88056	21.09355	29.6589	7.760734	16.71264
	3.44728	9.335009	26.91857	8.469945	18.7609	7.724008
	3.445143	8.093449	17.56577	13.05699	25.04945	12.85483
	1.315789	11.06546	38.9231	14.66004	23.99747	15.94918
	2.766667	15.90768	27.70695	4.67485	29.9422	17.46281
	5.797458	11.80467	9.840917	16.92592	22.23052	11.04453
	8.460358	8.607285	20.80472	43.55727	36.18281	23.60467
	4.700599	14.63317	20.71183	16.21769	13.74177	20.29313
	7.225659	16.13302	34.19496	5.870202	23.23152	13.084
	4.648215	3.728448	24.44762	37.02885	27.54383	22.56328
	2.573933	20.01671	31.27513	10.1193	17.8341	7.466563
	6.490125	14.33772	20.78478	25.98024	16.43476	16.31056
	3.237329	17.32145	10.58394	21.94229	21.25705	3.182105
	3.68254	14.08006	20.60722	14.07277	17.55448	
	2.427857	12.28748	21.31509	8.726735	16.02638	
	6.392295	15.64601	19.37003	6.277945	26.02836	
	6.638073	15.21377	27.88899	29.00595	38.4538	
	5.149893	13.96249	11.87185	5.774818	4.615826	
	5.241314	16.12777	26.78519	8.944894	12.43954	
	5.394411	12.11125	20.1545	17.41908	28.45832	
	3.145736	17.82598	26.68091	18.49037	18.7336	
	6.264102	16.86259	18.38629	18.22745	29.84536	
	8.457673	12.71581		13.88991	25.50536	
	7.775401	14.43606				
	5.241706	17.24043				
Average	5.979854	13.49262	20.61986	17.0642	20.40799	17.47722
Standard deviation	3.948158	3.867592	8.937461	10.06107	6.709763	8.471587
Median	5.241314	14.33772	20.16192	14.07277	21.0616	16.71264
DNA repair capacity:			0.345649	0.209302	0.338856	0.227988
Count	53	53	50	51	51	41
Number of comets in class:						
0-6%	32	1	1	6	2	2
6.1-17%	20	42	16	27	13	19
17.1-35%	1	10	30	13	34	17

35.1-60%	0	0	3	5	2	3
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	60.37736	1.886792	2	11.76471	3.921569	4.878049
Class 1	37.73585	79.24528	32	52.94118	25.4902	46.34146
Class 2	1.886792	18.86792	60	25.4902	66.66667	41.46341
Class 3	0	0	6	9.803922	3.921569	7.317073
Class 4	0	0	0	0	0	0

	% Tail DNA (Vehicle control- NER experiment)				
		PBS		Rotenone	
	No PE	Wild type	Knock out	Wild type	Knock out
	6.160557	8.845552	6.431175	3.665437	0.089526
	0	15.20974	9.83779	8.27911	3.07771
	3.707655	2.997693	7.506196	6.121906	3.118558
	4.696439	10.36036	3.92294	7.213439	0.184659
	0	1.077516	1.664802	6.209694	18.2155
	1.696962	9.436601	4.679099	4.634197	17.81549
	10.04077	11.53173	8.676225	6.47528	10.65529
	18.87292	11.97627	2.774139	7.768097	8.876997
	2.024117	2.948222	4.80266	5.018656	0.575941
	1.36688	1.50278	17.48615	3.868876	6.746427
	1.56075	9.463511	32.79072	2.272256	7.210414
	1.027879	8.922108	9.398992	6.071467	10.73086
	16.10822	3.971834	4.15255	2.520619	5.220506
	1.732663	5.132523	9.832564	13.61764	7.461677
	0	20.5954	2.186705	16.84278	2.050621
	0	3.909727	5.12575	28.29744	4.403748
	0.617739	7.858636	5.890473	5.252752	4.058943
	0.63643	8.487592	3.819748	16.45933	2.866044
	4.50368	4.390434	5.760244	9.590856	1.822563
	6.027947	12.45604	3.68502	28.34804	1.862985
	2.750207	10.73197	3.393303	6.841603	2.467268
	4.09249	11.85905	5.097809	3.357414	1.465453
	2.255795	4.063218	7.31312	6.844559	3.697682
	2.323427	16.88182	6.527344	8.148577	0.116681
	3.502658	7.493187	9.351436	6.548716	8.162834
	1.927461	7.966047	4.343828	4.625259	4.713847
	14.87795	2.13708	8.387833	5.173091	4.8148
	0.019892	14.44738	4.95868	3.639299	0.1441
	13.04252	5.098973	4.848363	1.832394	9.534623
	0	4.005842	7.770779	4.328633	7.66797
	5.520362	15.06708	7.182438	7.033975	9.372453
	3.253682	14.06336	1.379143	1.547991	0.533008

	10.80909	2.390126	5.741943	8.565471	6.649433
	9.448923	8.430541	4.029395	6.872863	10.54557
	0.019428	6.699862	4.556826	5.88386	10.70186
	1.478451	9.916575	5.530399	4.720939	13.36791
	1.932421	7.976528	4.332772	7.304567	9.047541
	3.7203	1.980995	7.784383	3.967582	8.202866
	5.839658	6.578548	3.749174	8.536209	6.894785
	0	2.120026	3.937463	8.069249	6.008965
	10.19823	13.65384	2.627471	8.014196	13.67524
	1.49426	9.31845	9.126892		8.318684
	0.583765	16.8954	3.883895		1.670928
	1.69216	1.966136	1.207574		0
	0	6.795274	7.13147		8.035007
	5.740097	2.084378	7.511063		5.742772
	9.9536	2.499005	23.19172		7.72505
	15.64749	5.406946	11.06742		8.334617
	7.665672	1.842752	20.5324		5.054152
	5.122373	9.170838	14.43743		3.305613
		3.182041	13.59908		1.168083
		7.20836	12.37635		10.76712
		7.831958	3.118347		9.970337
		9.605926	5.144988		6.894631
		9.208663	7.345007		3.961581
		0.004474	6.817099		12.08933
		6.400896	3.91776		11.37531
		2.579734	9.126071		8.684557
		8.938443	9.224871		12.88818
		8.108241	4.975037		7.86749
		11.3601	9.829639		8.172573
			3.892652		15.34353
			2.095239		12.5364
			3.102153		6.02249
			3.235188		9.137921
			8.772988		
			9.475796		
Average	4.513879	7.623678	7.125462	7.570349	6.767626
Standard deviation	4.863063	4.597793	5.253463	5.77705	4.416217
Median	2.536817	7.858636	5.741943	6.47528	6.894785
DNA repair capacity:		0.407913	0.366514	0.403742	0.333019
Count	50	61	67	41	65
Number of comets in					

class:					
0-6%	37	23	36	17	27
6.1-17%	12	37	27	22	36
17.1-35%	1	1	4	2	2
35.1-60%	0	0	0	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	74	37.70492	53.73134	41.46341	41.53846
Class 1	24	60.65574	40.29851	53.65854	55.38462
Class 2	2	1.639344	5.970149	4.878049	3.076923
Class 3	0	0	0	0	0
Class 4	0	0	0	0	0

	% Tail DNA (B[a]P- NER experiment)					
			PBS		Rotenone	
	Vehicle Control	No PE	Wild type	Knock out	Wild type	Knock out
	6.160557	5.121267	22.43938	16.80104	19.70199	24.78168
	0	20.09343	40.03692	18.49853	26.52314	25.43711
	3.707655	14.18079	32.83752	17.8706	18.6201	26.63487
	4.696439	2.523428	24.71218	23.93597	21.19955	17.43983
	0	20.84836	19.73778	19.68018	16.59872	33.82954
	1.696962	13.66151	34.99964	22.96553	17.34534	17.08183
	10.04077	15.10044	17.91614	14.93311	33.14608	16.78546
	18.87292	9.87797	18.70678	16.3037	24.00674	14.68753
	2.024117	25.6125	23.70253	11.4173	43.70226	16.33404
	1.36688	13.53985	29.06323	10.94834	23.9424	21.89092
	1.56075	18.38617	28.85191	20.84823	17.20587	14.92156
	1.027879	17.47195	20.16045	12.67028	17.15544	24.28483
	16.10822	20.48177	25.76272	19.77466	31.66842	19.77983
	1.732663	28.98489	28.38079	13.43553	31.76646	15.46408
	0	17.81948	28.28623	35.96744	13.96371	2.924464
	0	22.68498	18.07188	25.47426	14.26482	26.90564
	0.617739	17.06682	18.9756	6.265092	24.44944	28.61882
	0.63643	15.14606	19.74364	16.80743	26.98005	13.18984
	4.50368	5.732183	20.92211	33.33225	14.46799	15.13825
	6.027947	8.505498	21.18736	10.35388	18.40008	17.05457
	2.750207	4.437358	20.11968	17.72403	13.55494	15.05361
	4.09249	16.91618	17.50504	12.26524	17.95894	12.30164
	2.255795	23.31824	20.59929	24.02808	13.42003	24.69449
	2.323427	5.052324	16.05151	21.29626	21.46688	15.07565
	3.502658	4.975368	5.762289	11.12613	18.40283	15.30389
	1.927461	15.49375	30.07665	10.79156	27.83323	24.24966
	14.87795	5.740168	15.6314	22.04694	35.96109	13.22103
	0.019892	4.200319	24.16832	26.45038	28.631	24.11965

	13.04252	15.35881	2.82367	29.993	20.38086	25.35607
	0	4.14996	34.46154	35.71544	32.10786	23.98667
	5.520362	16.09412	20.73603	12.79445	21.59254	33.97031
	3.253682	15.1468	13.84972	16.58636	31.60606	23.65576
	10.80909	13.83043	0.047912	17.11283	16.7354	20.47959
	9.448923	13.27092	18.38942	26.57333	38.87467	19.90615
	0.019428	10.39574	17.83665	22.52981	16.05962	28.45697
	1.478451	11.53581	17.58906	12.79284	18.12829	27.42031
	1.932421	14.34483	43.00533	21.58451	21.27744	15.00854
	3.7203	16.02008	12.40265	22.05362	14.89064	27.81423
	5.839658	6.254672	24.46598	10.30513	30.64713	25.85999
	0	37.58101		12.74564		20.83908
	10.19823	13.26264		10.2845		15.60421
	1.49426	24.43703		15.91763		12.34082
	0.583765	13.20534		28.12553		22.7651
	1.69216	22.70189		26.82352		21.83419
	0	3.435872		16.6165		26.25169
	5.740097	18.24314		14.00262		21.08534
	9.9536	17.28466				25.95774
	15.64749	9.752242				23.47389
	7.665672	19.9032				26.55477
	5.122373	12.02369				23.7065
		11.59037				16.45407
		7.35628				11.05887
		5.180549				15.60698
		11.75484				26.07274
		3.270007				22.17335
		16.76252				20.75816
		2.866679				23.18608
		16.39316				24.931
		6.297017				11.82858
		18.10514				23.6691
		13.19487				22.84186
		14.57296				21.99269
		13.38415				7.229246
		51.04008				7.3
		7.883086				19.28509
		3.553037				7.720035
		6.461756				17.68215
		2.604447				25.4952
		15.19221				17.52832
		1.425525				13.43285
		4.923585				16.21783
		9.155985				21.23052
		10.34256				19.30503
		14.64555				11.20774

Average	4.513879	13.36706	21.79531	18.83846	22.93944	19.9154
Standard deviation	4.863063	8.280077	8.850739	7.09206	7.696012	6.212233
Median	2.536817	13.462	20.59929	17.41843	21.19955	20.79862
DNA repair capacity:			0.3867	0.290438	0.417289	0.328808
Count	50	74	39	46	39	74
Number of comets in class:						
0-6%	37	17	3	0	0	1
6.1-17%	12	38	4	22	9	25
17.1-35%	1	17	30	22	27	48
35.1-60%	0	2	2	2	3	0
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	74	22.97297	7.692308	0	0	1.351351
Class 1	24	51.35135	10.25641	47.82609	23.07692	33.78378
Class 2	2	22.97297	76.92308	47.82609	69.23077	64.86486
Class 3	0	2.702703	5.128205	4.347826	7.692308	0
Class 4	0	0	0	0	0	0

	% Tail DNA (Vehicle control- NER experiment)				
		PBS		Rotenone	
	No PE	Wild type	Knock out	Wild type	Knock out
	6.20645	16.52842	15.93616	15.85628	13.07792
	17.51423	4.815003	25.73469	14.23748	11.35201
	10.56895	18.21163	11.33549	13.94253	16.03843
	15.71672	8.012042	18.26921	19.35744	19.71842
	17.0191	23.60037	27.11345	20.76082	15.02287
	10.36207	22.86422	10.43187	11.61477	14.2332
	11.16888	16.48243	16.89106	30.87076	19.70612
	4.445228	19.61299	12.14466	25.48815	20.88169
	11.93718	11.08969	3.944261	23.53491	17.45619
	9.338461	36.3915	16.39747	22.25642	11.5984
	19.53253	22.52144	34.94151	19.62573	18.70771
	12.83875	31.89867	11.09478	27.4429	9.128948
	16.33472	25.5116	17.02844	25.55018	5.096606
	10.90995	10.4634	22.66222	23.54592	14.45717
	6.718242	18.6947	15.42636	22.25642	11.49715
	11.20267	12.4994	8.608422	27.42009	3.079098
	15.97433	12.90323	6.676507	19.62573	15.24313

	5.13511	14.59446	36.5985	18.23626	34.23393
	15.4284	29.47441	15.2507	33.31552	19.48211
	14.64788	16.26514	9.688877	18.65921	24.6837
	8.168508	29.00795	16.13229	19.00326	16.8838
	13.44606	16.68976	7.888439	28.73397	18.25239
	12.97697	23.37997	16.19952	24.95592	7.817375
	12.03777	11.04807	9.334815	4.102324	4.051639
	12.91891	10.68919	34.36388	14.70969	24.57532
	10.94419	19.2387	14.77472	14.06296	7.33524
	7.509423	14.12688	14.19006	29.85857	10.82698
	13.97365	17.86203	32.8007	28.35533	16.30103
	12.74165	9.328744	14.84293	15.16862	20.60417
	13.29621	22.39318	9.288176	11.63109	13.76544
	8.530962	17.48335	11.40626	17.97217	24.75235
	13.90922	20.11296	20.72184	14.85915	18.56445
	10.76554	9.798254	5.409486	37.78618	4.801229
	11.19635	14.47205	8.069493	27.06196	16.3533
	9.920113	19.39856	11.2784	14.76131	10.9498
	11.59073	20.85315	7.838014	19.39879	40.0034
	41.45038	35.01901	14.58849	7.945421	11.33421
	4.084536	21.38966	8.784875	3.515005	16.13241
	5.982679	22.04316	14.43188	21.67633	23.21461
	6.554182	21.26185	13.59382	22.69661	18.96352
	10.55736	14.39251	16.19742	21.42051	18.71629
	19.65151	17.18922		25.57504	20.87908
	7.856798	25.54913		32.74968	14.45283
	12.31356	16.52468		16.55003	17.52407
	11.09427	31.09688		29.25508	6.772354
	10.31991	33.8128		18.44492	
	13.1578	11.17261		16.51115	
	14.58758	20.2065		7.552716	
	11.4473	35.97618		17.82866	
	10.82525			10.99404	
	13.65491			12.77496	
	11.94376			14.22681	
	11.76846				
	18.455				
Average	12.27095	19.4684	15.56854	19.918	15.96716
Standard deviation	5.418556	7.640729	8.085818	7.448276	7.264417
Median	11.67959	18.6947	14.58849	19.37812	16.13241
DNA repair capacity:		0.369699	0.211811	0.383926	0.231488
Count	54	49	41	52	45

Number of comets in class:					
0-6%	4	1	2	2	4
6.1-17%	44	19	29	17	22
17.1-35%	5	26	9	32	18
35.1-60%	1	3	1	1	1
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	7.407407	2.040816	4.878049	3.846154	8.888889
Class 1	81.48148	38.77551	70.73171	32.69231	48.88889
Class 2	9.259259	53.06122	21.95122	61.53846	40
Class 3	1.851852	6.122449	2.439024	1.923077	2.222222
Class 4	0	0	0	0	0

	% Tail DNA (B[a]P- NER experiment)					
	Vehicle Control	No PE	PBS		Rotenone	
			Wild type	Knock out	Wild type	Knock out
	6.20645	22.77301	32.57789	12.33898	21.80311	26.9202
	17.51423	11.83176	43.10124	17.89146	12.30092	7.731697
	10.56895	22.07547	29.06776	22.00794	18.55986	26.32094
	15.71672	27.36315	34.24864	30.18621	26.7737	20.41284
	17.0191	13.75806	22.48999	14.9704	14.97561	30.68787
	10.36207	19.49494	29.14928	24.17149	19.03471	18.0574
	11.16888	15.85099	34.19688	13.91553	4.81457	45.69828
	4.445228	22.97757	23.76205	19.80371	10.52345	27.37921
	11.93718	27.32833	45.39399	21.20986	23.48654	10.15339
	9.338461	13.69791	24.59345	20.31382	26.32097	23.17221
	19.53253	19.19265	25.16152	20.7135	34.90335	15.70765
	12.83875	14.62238	22.15466	43.15151	31.00166	34.95224
	16.33472	21.69378	24.38766	35.72703	26.74704	24.73573
	10.90995	18.25413	38.59469	19.1451	27.61125	23.13243
	6.718242	19.88864	29.67577	20.53097	19.55771	12.29535
	11.20267	11.91073	20.39187	19.30823	20.13866	11.74659
	15.97433	25.06149	41.14749	23.6348	22.28005	14.88274
	5.13511	5.608606	14.84459	26.71207	57.3609	11.11183
	15.4284	16.88976	20.84251	22.2347	16.02947	22.9869
	14.64788	17.82324	57.82007	20.26355	21.90083	10.13731
	8.168508	6.410943	20.47639	23.45303	21.17673	25.81164
	13.44606	15.62321	24.78689	16.98511	21.41815	30.05786
	12.97697	18.31551	25.7807	24.13835	30.19927	14.65846
	12.03777	27.1883	35.16823	16.92767	22.15458	16.55135
	12.91891	17.26463	19.71746	22.73167	21.33096	20.38697
	10.94419	24.91588	24.98646	16.4293	28.30907	16.92786

	7.509423	23.23476	20.62049	27.60131	25.80153	20.8805
	13.97365	20.73444	24.33691	34.44288	25.17422	22.64979
	12.74165	21.45954	25.46781	21.85231	25.07923	17.41975
	13.29621	24.13771	58.52649	21.68768	25.28195	4.799922
	8.530962	15.06732	38.04374	39.52046	15.69505	28.47747
	13.90922	17.02849	23.32799	13.81455	32.92321	17.64094
	10.76554	18.09955	24.58857	16.65459	22.16911	20.17033
	11.19635	15.50003	23.36186	36.41296	28.25502	13.57546
	9.920113	25.85578	25.9834	27.47777	23.80225	25.41247
	11.59073	19.12515	30.936	18.41499	14.67593	15.97734
	41.45038	19.77059	38.33454	19.78619	24.34764	14.69173
	4.084536	19.06293	21.50931	28.1155	27.87264	14.27928
	5.982679	15.93838	25.43369	34.47258	22.39794	17.70382
	6.554182	24.48331	22.0816	32.74235	12.40812	26.68638
	10.55736	20.25543	37.30195	17.65592	11.35084	31.08787
	19.65151	14.18893		13.84029	26.42059	8.794277
	7.856798			18.83997	20.35525	34.49749
	12.31356			30.25055	16.85445	22.3832
	11.09427			35.88576	15.95442	17.99885
	10.31991			16.80489	10.3458	31.90771
	13.1578			17.78726	14.44557	24.95333
	14.58758			13.33779	3.759311	25.67516
	11.4473			26.06217	17.22234	19.76952
	10.82525			12.71578	23.52049	
	13.65491			23.3277	22.42134	
	11.94376			56.56955	21.42053	
	11.76846			17.42661	27.06379	
	18.455			31.63218	31.77786	
					19.44866	
					36.46484	
					27.26515	
					24.83967	
Average	12.27095	18.85137	29.37494	23.59312	22.37117	20.81734
Standard deviation	5.418556	5.103769	9.722611	8.667961	8.258122	8.0768
Median	11.67959	19.09404	25.43369	21.44877	22.22458	20.38697
DNA repair capacity:			0.35825	0.20098	0.451484	0.094439
Count	54	42	41	54	58	49
Number of comets in class:						
0-6%	4	1	0	0	2	1
6.1-17%	44	13	1	12	12	16

17.1-35%	5	28	30	36	42	31
35.1-60%	1	0	10	6	2	1
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	7.407407	2.380952	0	0	3.448276	2.040816
Class 1	81.48148	30.95238	2.439024	22.22222	20.68966	32.65306
Class 2	9.259259	66.66667	73.17073	66.66667	72.41379	63.26531
Class 3	1.851852	0	24.39024	11.11111	3.448276	2.040816
Class 4	0	0	0	0	0	0

	% Tail DNA (Vehicle control- NER experiment)				
		PBS		Rotenone	
	No PE	Wild type	Knock out	Wild type	Knock out
	8.624397	14.49786	16.79293	12.80017	7.239857
	6.52849	21.411	6.820186	17.98774	16.53899
	10.99167	4.042387	14.87004	11.49163	8.3881
	4.336785	10.01758	5.52836	12.81585	7.867855
	2.057678	23.2283	15.8993	16.66452	8.255833
	12.90624	11.54922	15.78109	10.06249	6.095819
	6.275133	10.25478	15.10667	11.02936	8.111387
	7.007007	18.00722	6.023939	10.05394	0
	6.372347	10.01462	8.550217	14.00269	17.25315
	7.584476	10.5704	4.087798	10.11672	8.104902
	9.176422	12.82206	13.3213	12.01894	9.008524
	6.719278	16.21648	15.1716	29.64582	8.259538
	6.584156	19.0392	15.29149	9.10354	17.22391
	21.9371	10.17517	6.464891	10.61028	8.016309
	5.847137	11.47427	15.7239	13.09805	8.952923
	31.0896	17.03341	6.823297	19.22219	7.044606
	9.790584	10.04227	5.739259	12.84328	8.087091
	7.312925	12.92334	5.37118	12.71262	8.010836
	14.87855	9.011783	5.2007	14.65315	7.006153
	6.992686	17.15829	5.656394	8.913349	6.2905
	14.1704	18.13733	7.631313	9.14876	16.03802
	10.14503	15.34186	8.243802	10.0544	6.836475
	6.819633	17.43567	14.1632	13.90619	8.533179
	5.27153	10.17488	9.733093	10.05428	10.23981
	8.229639	11.32531	14.4376	15.21111	17.93259
	6.621516	19.00791	9.540649	10.0459	18.06672
	5.927746	10.29622	9.463866	15.39365	12.28926
	4.396822	7.037268	8.892278	16.68744	8.981371
	8.835314	10.01023	15.15282	16.79788	6.032795
	6.456947	12.07059	11.82128	18.81594	8.509488
	10.69269	11.96403	18.37933	17.39309	0

	7.37807	10.20813	7.665383	10.01653	7.754601
	9.644416	14.57054	15.39121	13.07513	8.094968
	11.5888	6.225636	8.607093	11.40173	7.244948
	7.84267	9.650161	14.12485	21.76158	17.89225
	6.032958	17.19828	14.47146	14.60458	19.94764
	2.76306	15.79317	14.44251		9.548853
	5.619157	9.227891	6.678019		6.695315
	5.816002		13.70995		6.49968
	7.040406		14.59495		8.819639
	7.444783		5.556949		9.001208
	2.389215		9.614355		8.556704
	7.052213		18.28604		7.982255
	5.946749		14.72128		8.733824
	6.495012		5.60166		8.660302
	7.589828		15.37943		7.524699
	12.87692		4.556236		7.723759
	11.00874		13.4865		7.975178
	6.804073		15.92201		8.437516
			13.22393		9.268153
			5.153706		8.102528
			15.1943		7.833964
			5.342141		9.40643
			14.6602		8.631686
			16.65202		7.952492
			12.17598		3.513581
			14.70566		7.125126
					18.10193
					18.88192
					18.49584
					17.96178
					16.72697
					8.696388
					18.0725
Average	8.406387	13.03065	11.25617	13.72818	9.923134
Standard deviation	4.755185	4.320953	4.346371	4.25468	4.585831
Median	7.040406	11.75663	13.22393	12.82957	8.412808
DNA repair capacity:		0.354876	0.253175	0.387655	0.15285
Count	49	38	57	36	64
Number of comets in class:					
0-6%	11	1	11	0	3

6.1-17%	36	27	44	30	50
17.1-35%	2	10	2	6	11
35.1-60%	0	0	0	0	0
>60%	0	0	0	0	0
Percentage comets in class:					
Class 0	22.44898	2.631579	19.29825	0	4.6875
Class 1	73.46939	71.05263	77.19298	83.33333	78.125
Class 2	4.081633	26.31579	3.508772	16.66667	17.1875
Class 3	0	0	0	0	0
Class 4	0	0	0	0	0

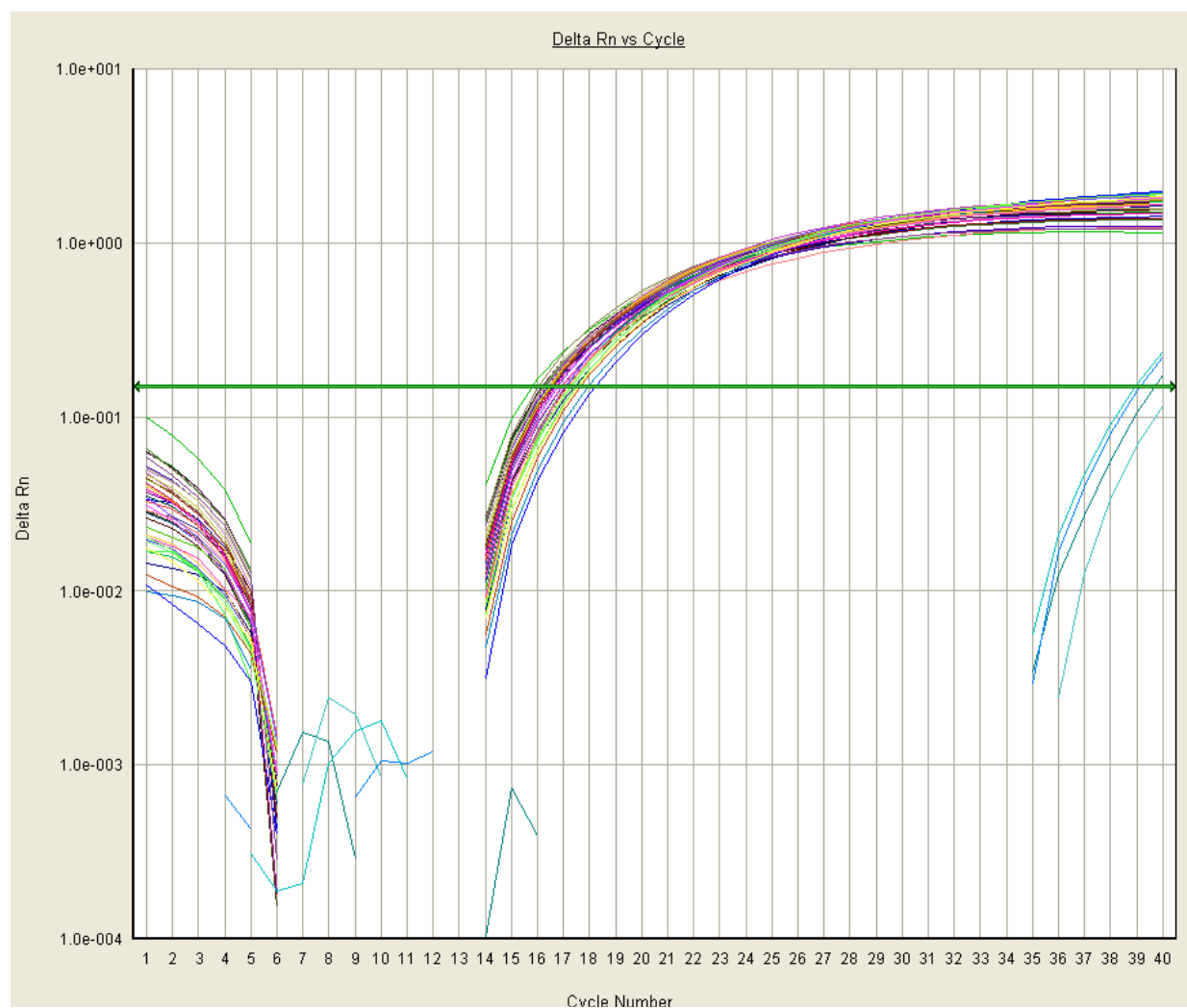
	% Tail DNA (B[a]P- NER experiment)					
			PBS		Rotenone	
	Vehicle Control	No PE	Wild type	Knock out	Wild type	Knock out
	8.624397	17.47875	28.14383	30.04279	31.80229	26.65619
	6.52849	20.22068	32.25615	21.28156	39.98592	31.35231
	10.99167	21.21919	38.62533	32.02783	33.40211	41.13286
	4.336785	19.86058	43.54959	30.09031	30.93539	27.01221
	2.057678	18.32547	37.31175	20.47284	30.02737	29.05753
	12.90624	21.71048	19.04629	31.86541	40.43594	33.02722
	6.275133	22.33652	40.96917	33.401	24.77336	21.32383
	7.007007	33.79065	35.67455	22.6021	41.32817	26.54
	6.372347	19.51173	36.11211	20.58272	38.67044	35.77412
	7.584476	24.71008	45.3512	27.31477	38.92601	21.01253
	9.176422	20.64189	16.73777	32.22671	31.44559	25.26182
	6.719278	22.71799	20.08202	24.73574	37.9228	43.2424
	6.584156	24.86977	29.56901	21.0178	36.41875	35.01703
	21.9371	17.62452	31.0964	21.26164	38.95936	2.217336
	5.847137	10.02655	35.07752	24.89828	46.7173	33.50507
	31.0896	20.00873	37.56676	23.53872	46.98838	33.85222
	9.790584	20.12573	34.36242	33.93794	16.88936	32.48749
	7.312925	29.57553	34.32265	15.05912	28.69565	26.97188
	14.87855	12.48519	39.09091	30.19293	34.37613	23.46856
	6.992686	21.82347	30.24094	36.71651	41.24078	16.47175
	14.1704	25.99725	14.64501	37.07351	28.83304	20.87765
	10.14503	20.93422	22.79505	20.02504	23.75893	2.130973
	6.819633	22.52484	39.78273	34.32563	28.37875	7.830149
	5.27153	19.30054	31.82188	35.21521	42.62249	22.55099
	8.229639	21.94063	22.29292	26.5185	30.47287	42.40829
	6.621516	23.60282	22.67213	31.12959	31.25528	22.01528
	5.927746	28.65366	34.83911	49.44612	36.25554	47.56426
	4.396822	14.40078	45.95883	38.38396	32.11556	41.72748
	8.835314	16.36973	31.27521	21.62082	49.87308	29.30611
	6.456947	12.79402	22.48791	39.18172	45.1884	24.31528

	10.69269	12.58955	38.50676	37.96749	39.1874	41.17239
	7.37807	28.21033	34.81099	28.90054	37.38765	27.23149
	9.644416	15.70753	31.95486	20.20479	38.44604	16.12249
	11.5888	23.79726	38.91412	30.36038	31.79392	12.732
	7.84267	22.56286	28.17081	32.0364	21.16373	48.66834
	6.032958	19.42205	31.72508	24.08113	34.16269	47.13984
	2.76306	25.65167	47.57953	37.68793	32.31314	17.54891
	5.619157	24.56253	21.14658	34.57822	47.50882	46.91059
	5.816002	14.69724	41.93448	20.63091	35.09879	14.27158
	7.040406	12.69435	44.15001	32.37721	29.72222	7.045945
	7.444783	17.62137	20.456	42.02484	35.32578	33.36459
	2.389215	11.93804	38.88354	30.01938	36.19249	11.99234
	7.052213	25.51793	22.92275	30.89328	31.78481	28.80131
	5.946749	11.41314	19.65021	36.53869	41.94926	9.921456
	6.495012	27.8633	30.38207	33.14505	38.6949	28.66083
	7.589828	11.66489	37.39638	31.90723	35.54107	16.32443
	12.87692	11.89072	37.24961	42.44239	37.9421	18.8579
	11.00874	20.04872	33.00372	26.15781	33.51792	21.71678
	6.804073		34.38207	14.55522	35.06245	20.9011
			39.89044	12.58888	30.2141	25.42983
			24.92116	28.11111	37.81189	39.84504
			21.83629	31.01417	49.92426	22.01019
			20.46794	37.13477	41.05379	52.89194
			32.60654	11.59939	35.23158	15.60337
			39.95611	28.23492	46.50225	33.67308
			37.5014	28.55838	47.61006	20.71148
			37.18958	16.53133		2.260682
			48.71148	20.24113		16.62953
			44.1468	10.0319		38.86703
			33.30726	13.22626		19.65177
				10.09533		28.36625
				14.26348		
				37.8933		
				12.88647		
Average	8.406387	20.07157	32.79186	27.57979	36.06854	26.41697
Standard deviation	4.755185	5.495854	8.383932	8.869657	6.975727	12.09361
Median	7.040406	20.1732	34.34253	29.45996	35.86678	26.54
DNA repair capacity:			0.38791	0.272236	0.443516	0.240202
Count	49	48	60	64	56	61
Number of comets in class:						

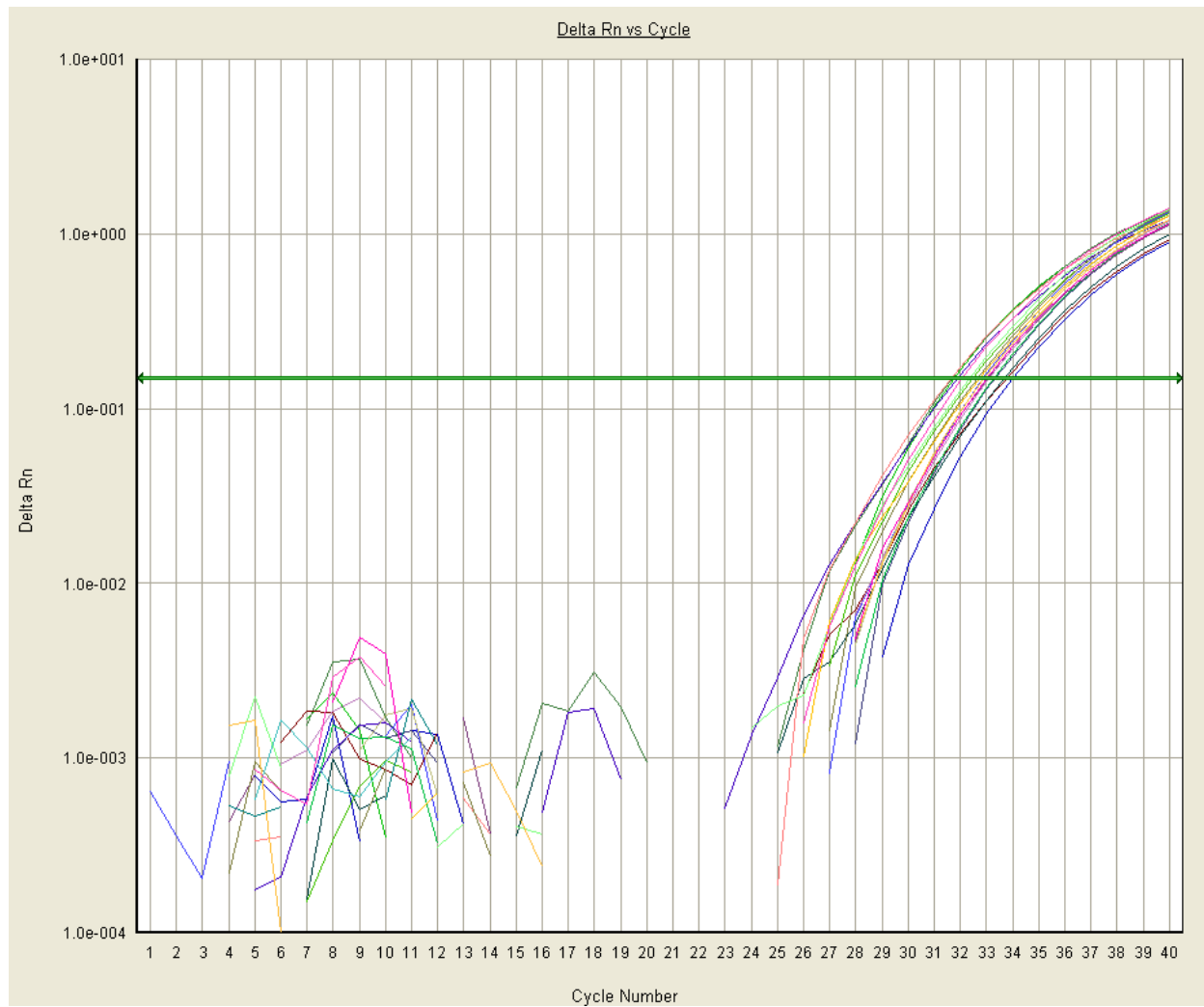
0-6%	11	0	0	0	0	3
6.1-17%	36	13	2	10	1	11
17.1-35%	2	35	32	41	22	33
35.1-60%	0	0	26	13	33	14
>60%	0	0	0	0	0	0
Percentage comets in class:						
Class 0	22.44898	0	0	0	0	4.918033
Class 1	73.46939	27.08333	3.333333	15.625	1.785714	18.03279
Class 2	4.081633	72.91667	53.33333	64.0625	39.28571	54.09836
Class 3	0	0	43.33333	20.3125	58.92857	22.95082
Class 4	0	0	0	0	0	0

RAW DATA OBTAINED (REAL TIME PCR: GENE EXPRESSION ASSAY)

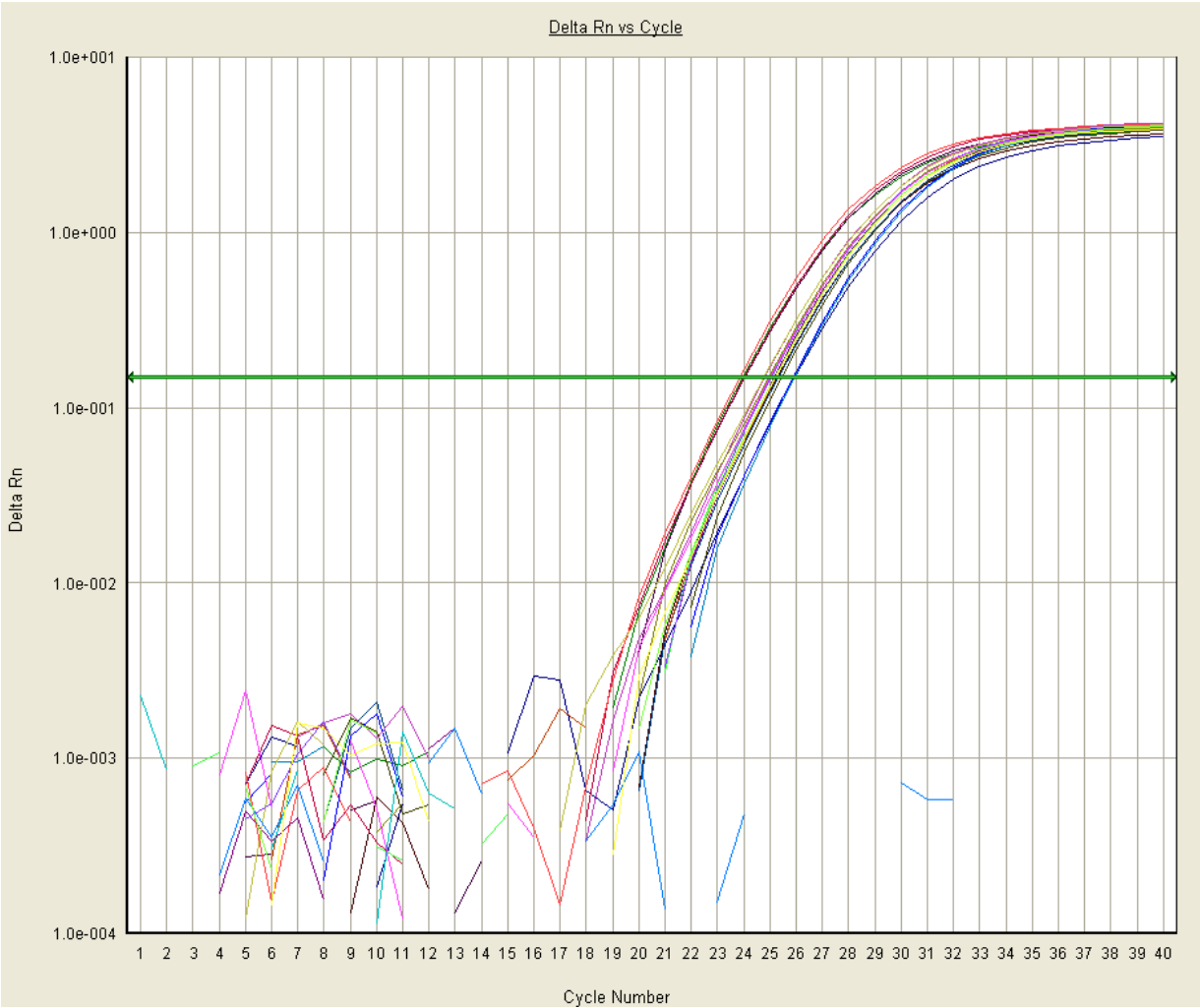
18s Amplification plot:



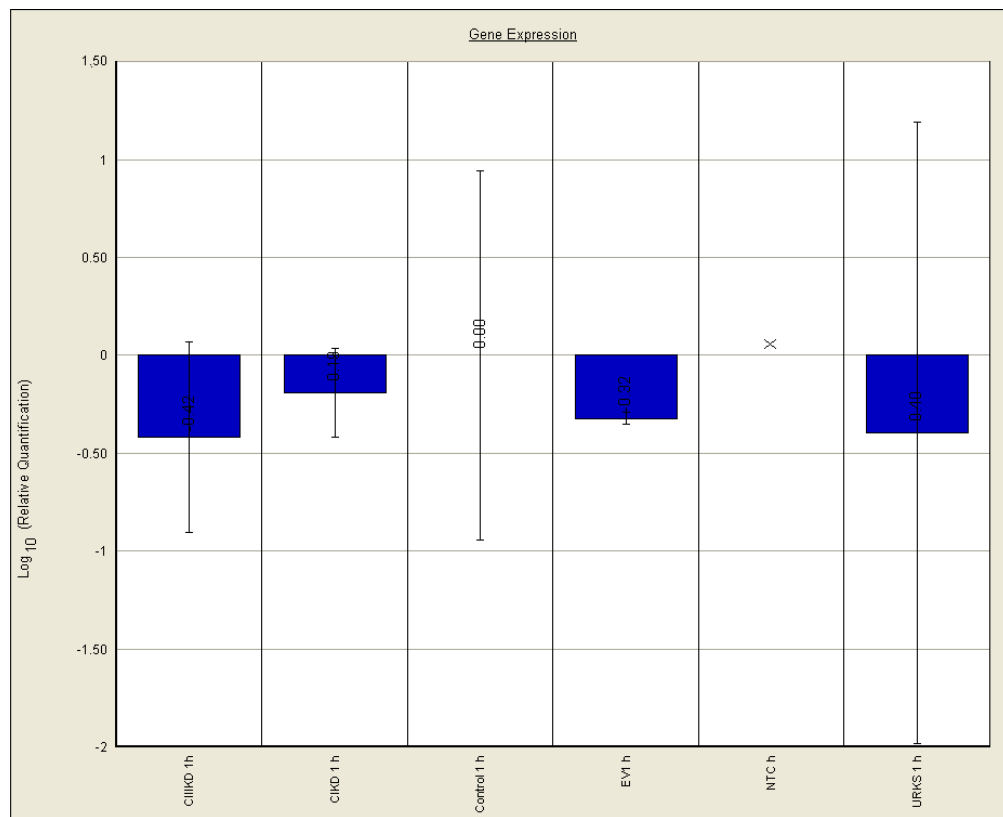
hOGG1 Amplification plot:



ERCC1 Amplification plot:



hOGG1 Gene expression plot:



ERCC1 Gene expression plot:

