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A study of the early DNA methylation events in oxidative stressed cultured mammalian cells

By

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“There is only one way to become a butterfly. And that is to grow your own wings. You can be a caterpillar with a hand glider for as long as you want but you’d only be kidding yourself. And there is only one way to grow your own wings and that is through the cocoon. But it’s dark and lonely in there it takes courage” Q’zoo

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List of abbreviations

ALS	Alkaline labile sites
AMV	Avian Myeloblastosis Virus
AP	Apurinic/Apyrimidimic
APTD	Average percentage tail DNA
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BER	Base excision repair
β-ME	β-mercaptoethanol
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CEA	Cytosine extension assay
CH_3	Methyl
CO_2	Carbon dioxide
Ct	Cycle threshold
Cu	Copper
C5	Carbon-5
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
dpm	Disintegrations per minute
DRC	DNA repair capacity
dRP	Deoxyribosephosphate
DSBs	Double strand breaks
DTT	1,4 –Dithiothreitol
ESCODD	European standards committee on oxidative DNA damage
EndoIII	Endonuclease III
EDTA	Ethylenediaminetetraacetic acid
Et al.	Et alii (and others)
Fe	Iron
Fpg	Formamido pyrimidine glycosylase

FBS	Foetal bovine serum
gDNA	Genomic DNA
GSH	Glutathione
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HM	Hypermethylated
HMPA	High melting point agarose
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
*h*OGG1	Human oxo-guanine glycosylase
i.e.	id est (that is)
IM	Intermediately methylated
KCl	Potassium chloride
KOH	Potassium hydroxide
LMPA	Low melting point agarose
M_d	Methylation dependent digest
miRNA	microRNA
M_o	Mock digest
mRNA	Messenger RNA
M_s	Methylation sensitive digest
M_{sd}	Double digest
MSP	Methylation sensitive PCR
MTT	3-(4-5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NTC	Non-template control
OEHHA	Office of Environmental Health Hazard Assessment
OGG1	8-Oxoguanine glycosylase
OH	Hydroxyl
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Protein extract
pH	Potential of hydrogen
R	Percentage DNA refractory to enzyme digestion
QC	Quality control
RNA	Ribonucleic acid
ROS	Reactive oxygen species

rpm	Revolutions per minute
RQ	Relative quantity
SAH	S-adenosyl methionine
SAM	S-adenosyl homocysteine
SCGE	Single cell gel electrophoresis
SOD	Superoxide dismutase
SSBs	Single strand breaks
TAE	Tris-acetate-EDTA
TrisHCl	2-Amino-2-(hydroxymethyl)l-3-propandiol-hydrochloride
UM	Unmethylated
W	Analytical window
[^{3}H]dCTP	Radiolabeled deoxycytidine triphosphate
8-oxoG	8-Oxoguanine
8-hydroxyG	8-Hydroxyguanine

List of symbols

α	Alpha
β	Beta
g	gram
g	G-force
L	Litre
M	Molar
mA	Milliampere
mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
mm^2	Square millimetre
ng/ml	Nanogram per millilitre
nm	Nanometre
V	Volt
µ	Micro
µg/ml	Microgram per millilitre
µM	Micromolar
%	percentage
°C	Degrees Celsius

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Abstract

Cells are continuously exposed to reactive oxygen species (ROS) causing oxidative stress. Cells can withstand and counteract ROS using defence mechanisms which range from free radical scavengers, antioxidant enzymes and DNA repair systems. Prolonged exposure of cells to oxidant species leads to the accumulation of genetic as well as epigenetic alterations. Exposure of cells to the non-radical hydrogen peroxide (H_2O_2) leads to the generation of hydroxyl radicals ($^{\bullet}OH$) by Fenton reactions when H_2O_2 reacts with a metal iron in the vicinity of DNA. These $^{\bullet}OH$ are very reactive and attack DNA giving rise to lesions such as single stand breaks and base modifications, which could influence DNA methylation.

DNA methylation is the post synthetic addition of methyl groups to the carbon 5 position of cytosine when cytosines are in the CpG dinucleotide context and is involved in gene expression. DNA methylation is considered to be very stable. Aberrant DNA methylation influences cancer related gene expression and genomic stability. The aim of this study was to investigate early changes in the global DNA -and gene specific methylation patterns of cultured mammalian cells when cells were exposed to H_2O_2. The term *early* refers to how soon following exposure to H_2O_2 over a six hour period changes in the DNA methylation pattern can be observed when exposing cells to H_2O_2 concentrations that causes oxidative DNA damage.

Changes in the *h*OGG1 promoter methylation status and gene expression were evaluated as this gene plays a crucial role in the initiation of the base excision repair pathway for the repair of oxidative DNA damage caused by H_2O_2 exposure. Results obtained with the alkaline comet assay showed that H_2O_2 exposure led to oxidative DNA damage and decreased DNA repair capacity when cells were exposed to H_2O_2 in fully supplemented medium (DMEM + 10% FBS). A change in the global DNA methylation pattern was evaluated with the cytosine extension assay and an enzyme based methylation sensitive PCR was used to evaluate the change in the promoter methylation status of *h*OGG1. Changes in the global DNA- and gene (promoter) specific methylation patterns could be observed where; a degree of global DNA hypomethylation and hypermethylation of the *h*OGG1 promoter could be observed within the six hour period of exposure to a concentration of H_2O_2 that was also associated with a high level of oxidative DNA damage. Finally, a decrease in the expression of the *h*OGG1 gene was also observed following exposure to this concentration of H_2O_2 within the six hour exposure period. These findings suggests that oxidative DNA damage influences DNA methylation (both globally and gene

specific) and that the expression of the *h*OGG1 gene is possibly influenced by promoter hypermethylation which is associated with oxidative DNA damage. Results were generated in human osteosarcoma (143B) cells. This cell line was used in order to investigate the effect of oxidative stress on the global DNA methylation pattern as well as the promoter methylation- and expression of the *h*OGG1 gene in wild type 143B cells (uncompromised complex III). Previous studies reported that in 143B cells in which complex III of the respiratory chain was compromised, by a knockdown system, deviations in the global DNA methylation pattern as well as the promoter methylation and expression of genes involved in DNA repair pathways could be observed.

Opsomming

Selle word voortdurend blootgestel aan reaktiewe suurstofspesies (ROS) wat oksidatiewe stres veroorsaak. Selle kan ROS weerstaan en teenwerk deur gebruik te maak van verdedigingsmeganismes wat wissel van vrye radikaal- opruimers, anti-oksidant ensieme en DNA-herstelmeganismes. Langdurige blootstelling van selle aan oksidant spesies kan dan lei tot die ontstaan en opeenhoping van genetiese sowel as epigenetiese veranderinge. Blootstelling van selle aan die nie-radikaal waterstofperoksied (H_2O_2) lei tot die vorming van hidroksielradikale ($^{\cdot}OH$) deur Fentonreaksies wanneer H_2O_2 met 'n yster in die omgewing van DNA reageer. Hierdie $^{\cdot}OH$ is baie reaktief en val DNA aan wat aanleiding gee tot letsels soos enkelstring breuke en basiswysigings, wat DNA-metilering kan beïnvloed.

DNA-metilering is die post-transkriptionele toevoeging van metielgroepe aan die koolstof 5 posisie van sitosien. Hierdie proses vind gewoonlik in sogenaamde CpG-eilande plaas en speel ‘n belangrike rol in geenuitdrukking. DNA-metilering word as baie stabiel beskou en is onontbeerlik vir die normale funksionering van ‘n organisme. Abnormale DNA-metilering beïnvloed kankerverwante geenuitdrukking en genomiese stabiliteit. Die doel van hierdie studie was om die vroëe veranderinge in die globale DNA en geen-spesifieke metilering patrone van gekweekte soogdierselle wat plaasvind as gevolg van die blootstelling aan H_2O_2 te ondersoek. Die term *vroëe* verwys hier na hoe vinnig binne ‘n ses uur periode van blootstelling van selle aan H_2O_2 konsentrasies wat oksidatiewe DNA skade induseer, daar verandinge in die DNA metilerings patroon waargeneem kan word.

Veranderinge in die metileringstatus en geenuitdrukking van die *hOGG1*- promoter is geëvalueer omdat hierdie geen 'n deurslaggewende rol speel in die inisiëring van basis uitsnydingsherstel vir die herstel van oksidatiewe DNA-skade wat veroorsaak word deur H_2O_2-blootstelling. Resultate wat verkry is met die alkaliese komeet analise het getoon dat die H_2O_2-blootstelling lei tot oksidatiewe DNA-skade en ‘n afname in DNA-herstelkapasiteit wanneer die selle blootgestel is aan H_2O_2 in medium wat volledig aangevul is (DMEM + 10% FBS). Veranderinge in die globale DNA metilering patroon is geëvalueer met die sitosien inbouïngs-analise (CEA) en 'n ensiem gebaseerde metilasie sensitiewe polimerase kettingreaksie (PCR) is gebruik om die verandering in die promoter metileringstatus van *h*OGG1 te evalueer. Veranderinge in die globale- en geen (promote/voorganger) spesifieke metileringpatrone kon waargeneem word waar globale hipo- en hipermetileting van die *h*OGG1-promoter waargeneem kon word binne die ses uur periode van blootstelling aan ‘n konsentrasie van H_2O_2 wat hoë vlakke van oksidatiewe skade geinduseer het. Ten slotte, kon 'n afname in die uitdrukking van die *h*OGG1 geen ook waargeneem word in die ses uur

periode van blootstelling aan die konsentrasie van H_2O_2. Hierdie bevindinge dui daarop dat die oksidatiewe DNA-skade DNA-metilering (beide globaal en geen-spesifieke) beïnvloed en dat die uitdrukking van die *h*OGG1-geen moontlik beïnvloed word deur hipermetilering van sy promoter wat gepaard gaan met DNA-skade as gevolg van blootstelling aan H_2O_2. Menslike osteosarkoma (143B) selle is gebruik in die studie. Die sellyn is gekies sodat die effek van oksidatiewe stres op die globale DNA metilerings patroon sowel as die promoter metilering patroon en die uitdrukking van die *h*OGG1 geen in wilde tipe 143B selle ondersoek kon word. Vorige studies het gevind dat geen-inhibering van kompleks III van die elektron transport ketting in 143B selle fluktuasies in die globale DNA metilerings patroon sowel as die promoter metilering en uitrukking van gene wat betrokke is by DNA skade herstel veroorsaak.

Key words

Oxidative stress; Hydrogen peroxide (H_2O_2); Mammalian cells; Human osteosarcoma (143B) cells, DNA methylation; Hypermethylation; Hypomethylation; Oxidative DNA damage; DNA repair capacity; Gene expression; Comet assay

CHAPTER 1
Introduction

1

"Epigenetics is proving we have some responsibility for the integrity of our genome. Before, genes predetermined outcomes. Now everything we do (everything we eat or smoke) can affect our gene expression and that of future generations. Epigenetics introduces the concept of free will into our idea of genetics" – R. Jirtle (Watters, 2006)

This statement made by Randy Jirtle emphasizes the well-known fact that the mammalian genome is regulated on a genomic and an epigenomic level and that we are not just a genetic blend of our parents. DNA methylation is an epigenetic mechanism and refers to the methylation of cytosines, which are located in the CpG dinucleotide context, throughout the genome (Espada and Esteller, 2010). In mammalians this process has important regulatory functions especially through the regulation of gene expression. DNA methylation can be affected by multiple factors such as environmental- and nutritional factors (Ingrosso and Perna, 2009; Rager *et al.,* 2011) which may alter the epigenotype and thereby influence the phenotype (Feil, 2006; Hitchler and Domann, 2009). There is also sufficient evidence in the literature that indicates that DNA methylation can be influenced by oxidative stress. While the change in the DNA methylation pattern of cells as a consequence of exposure to oxidative stress has been extensively studied, most studies focus on changes on a global- or gene (promoter) specific level and not both. This study aimed to examine early changes in the global- and promoter specific methylation pattern of oxidative stressed cultured mammalian cells by measuring the effect of H_2O_2 exposure over a time frame of six hours.

Furthermore, hydrogen peroxide (H_2O_2) has been used in many studies to induce oxidative stress and consequently oxidative DNA damage and was therefore chosen as exogenous source of oxidative stress for this study. The comet assay is a well-established method in our laboratory and two modifications of the basic alkaline comet assay were used in this study in order to i) measure the level of oxidative DNA damage and ii) assess the DNA repair capacity (Van Dyk *et al.,* 2010). The cytosine extension assay (CEA) has been previously applied in our laboratory to assess the global DNA methylation levels of cultured cells (Wentzel *et al.,* 2010) and was therefore a suitable method to investigate the change in the global DNA methylation pattern of the oxidative stress cultured cells. An enzyme based PCR method was used to investigate the promoter specific methylation change of a single gene (*h*OGG1) instead of making use of the methylation-specific PCR (MSP), which is dependent

on the bisulfite-mediated conversion of cytosine to uracil, in order avoid the loss and degradation of DNA during bisulfite conversion. The effect of H_2O_2 exposure on the *h*OGG1 promoter methylation status was investigated because of this genes role in the base excision repair (BER) pathway through which DNA damage caused by H_2O_2 exposure is predominantly repaired.

This dissertation commences with a review of the relevant literature on DNA methylation, oxidative stress and methods used to measure oxidative DNA damage and changes in global and gene specific DNA methylation patterns in Chapter 2. Also, the problem identification, project aim and experimental approach are given at the end of the literature review in Chapter 2. A detailed description of the methodology used in the execution of this study is given in Chapter 3. These methods include the methods used to measure cell viability (trypan blue and MTT assay), comet assay based methods, the CEA, the enzyme based methylation sensitive PCR and the gene expression assay for *h*OGG1. The results as well as an in-depth discussion of these results based on the literature are given in chapter 4. The literature and findings are summarised and brought to a close in Chapter 5, with a diagram summarising the effect of H_2O_2 exposure of cultured cells on the DNA integrity (DNA damage and repair), changes in the DNA methylation pattern and the expression of *h*OGG1. A possible link between oxidative stress induced through H_2O_2 exposure, DNA integrity, changes in the DNA methylation pattern and the expression of *h*OGG1 is also indicated in the figure.

CHAPTER 2
Literature review

2

2.1. Introduction

The genotype of a cell is connected to environmental influences by the epigenome, which determines the inheritable gene transcription pattern and therefore the phenotype of cells (Zhu and Yab, 2009, Tost, 2010). *Epi* is a Greek prefix meaning “over, on top of or above” therefore, epi (*over, above*) -genetics refers to non-genetic causes of a phenotype. There are many definitions for epigenetics. Conrad Waddington (1942) described it as gene-environment interactions that ultimately lead to a particular phenotype, which also depicts the permanent changes in gene activation and deactivation required for cellular differentiation. Epigenetics also refers to changes in gene expression which occur through modifications of the chromatin and the DNA molecule itself, rather than through any alterations of the nucleotide sequence (Franklin *et al.,* 2010). Deng and Blobel (2010) states that an epigenetic trait is defined as a stably heritable phenotype which is the result of changes in a chromosome without alterations in the DNA sequence. In the definition given by Deng and Blobel (2010) the term heritable not only refers to transgenerational inheritance or inheritance from mother to daughter cell through meiosis or mitosis, but it also refers to events that do not require cell division such as the memory of recent transcriptional activity. This can be seen in the fact that differentiated cells can also change their gene expression and local chromatin state in response to external stimuli (Roloff and Tuber, 2005).

For the purpose of this study epigenetics will be defined as the stable, reversible alteration (Espada and Esteller, 2010) of a gene’s transcriptional activity leading to changes in gene expression without directly affecting the primary DNA nucleotide sequence (Franco *et al.*, 2008; Wild and Flanagan, 2010). The epigenetic code is tissue, cell and gene specific (Cerda and Weitzman, 1997) and can change over time, while the genetic code is the same for each cell in an individual. Changes in the epigenetic code may occur in response to environmental changes on a physiological level or may be associated with pathological conditions like oncogenic transformation (Feil, 2006; Hitchler and Domann, 2009). The main two categories for epigenetic mechanisms are: DNA methylation of CpG dinucleotides and covalent modifications of histone tails (Roloff and Tuber*,* 2005; Bernstein *et al.,* 2007; Bird *et al.,* 2007; LeBaron *et al.,* 2010), these mechanisms are key players in the regulation of the transcription machinery. In mammalian cells DNA methylation occurs at the cytosine (C)

residue of the CpG dinucleotide pair (Turker and Bestor, 1997) following each cycle of DNA replication and involves the addition of a methyl (CH_3) group at the carbon-5 (C5) position of cytosine through the action of DNA methyltransferases (DNMTs). DNA methylation is a reversible epigenetic modification, which is important for the regulation of genomic stability and cellular plasticity (Bernstein *et al.,* 2007; Wu and Zhang, 2010). The second mechanism, histone modifications, occurs through DNA that winds around histone proteins for compaction and gene regulation. Histones are also subjected to post-transcriptional modifications which include acetylation, methylation, phosphorylation and ubiquitylation occurring within the amino-terminal histone tails. These post-transcriptional modifications can then either directly change the chromatin structure, to which DNA methylation is highly related (Cedar and Bergman, 2009), or it can allow the binding of specific transcription factors (Rosenfeld *et al.,* 2009). Histone modifications play an important role in DNA replication and transcriptional regulation (Portela and Esteller, 2010). Another epigenetic mechanism is non-coding RNA molecules, which is referred to as microRNAs (miRNA) (Aguilera *et al.,* 2010; Bavan *et al.,* 2011). These miRNAs are post transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs) resulting in transcriptional repression and gene silencing (Bartel, 2009). miRNAs play a role in the regulation of inflammation, apoptosis and wound healing (Bavan *et al.,* 2011).

The focus of this study is on DNA methylation as an epigenetic mechanism and the early events that take place after exposure of cultured cells to oxidative stress. Oxidative DNA damage can be measured by using the single cell gel electrophoresis which is more commonly referred to as the comet assay (Collins, 2004). The measured oxidative DNA damage can be used as a measure of oxidative stress (Collins, 2009). Oxidative stress also influences DNA methylation (Cerda and Weitzman, 1997; Franco *et al.,* 2008) which in turn influences gene expression.

2.2. DNA methylation as an epigenetic mechanism

DNA methylation refers to the post synthetic addition of methyl groups to specific sites on DNA molecules. This reaction, which occurs after every cycle of DNA replication, is catalysed by DNMTs (see figure 2.1). In mammalian cells the C5 position of cytosine is methylated when cytosines are in the CpG dinucleotide context (French *et al.,* 2009, Espada and Esteller, 2010).

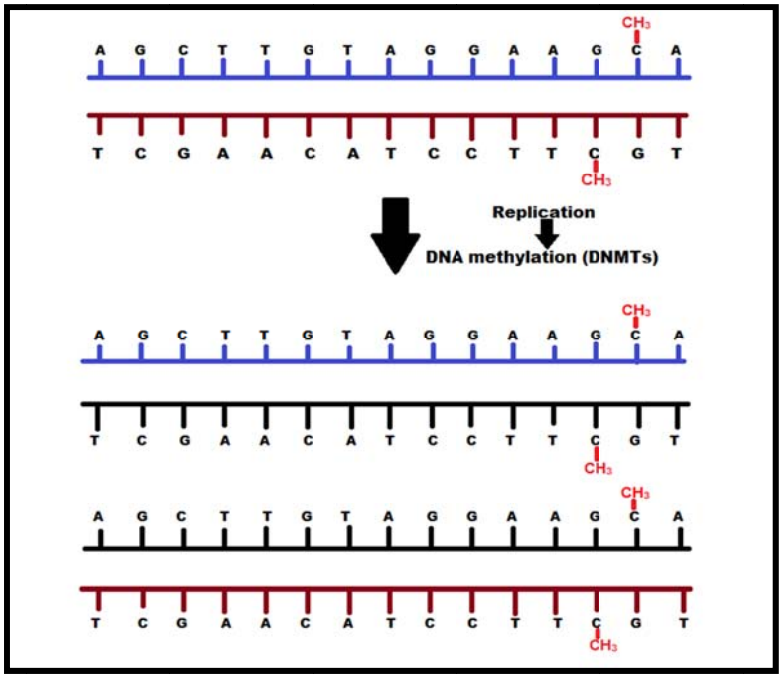

Figure 2.1 The DNA methylation process. DNA methylation occurs in cytosines in the CpG dinucleotide context, this proses occurs after every cycle of replication and is catalysed by DNA methylatransferases (DNMTs) (Adapted from Espada and Esteller, 2010).

The majority of CpGs in the genome are methylated, with the exception of CpG-islands which tend to remain hypomethylated in adult cells except on the inactivated X chromosome (French *et al.,* 2009; Espada and Esteller, 2010). CpG islands can be divined as genomic regions that are atleast 200-base pair long with 50% or higher guanine and cytosine content and 60 % or higher observed CpG ratio (Fazzari and Greally, 2004). These CpG islands are associated with the 5' ends of genes, span the promoter and the first exon of about 40% of genes in the human genome (Espada and Esteller, 2010) and are found in many housekeeping genes (French *et al.,* 2009; Portela and Esteller, 2010). When these CpG-rich sequences in the promoter regions of genes are hypermethylated it causes gene silencing (Franco *et al.,* 2008; Hitchler and Domann, 2009). It is hypothesised that promoter methylation suppresses transcription through the recruitment of methyl-CpG binding proteins which bind to 5-methyl cytosine (Choudhuri *et al.,* 2010). These methyl-CpG binding proteins then recruit histone deacetylases, histone transferases or heterochromatin proteins which then alter the chromatin structure in ways that block the access of transcription factor complexes to DNA which causes the suppressed transcription (Ziech *et al.,* 2011). For instance the deacetylation of histones results in a more condensed chromatin conformation and transcription silencing (Choudhuri *et al.,* 2010). The silencing of genes through promoter methylation can be detrimental to cells, which is evident in carcinogenesis where promoter methylation of tumor suppressor genes occurs (Brooks *et al.,* 2010).

DNA methylation is involved in gene expression, and also plays a role in a variety of epigenetic mechanisms such as X- inactivation (silencing of an X-chromosome in females) and genomic imprinting (inactivation of one parental allele) (French *et al.,* 2009, Franklin *et al.,* 2010). DNA methylation is considered as one of the most stable and permanent mechanism of epigenetic gene inactivation (Lahtz and Pfeifer, 2011) and is also relevant in the maintenance of cellular viability because it contributes to the silencing of transposons and other parasitic elements such as endogenous retroviruses (Espada and Esteller, 2010). Although DNA methylation is important for development, the presence abnormal quantities of 5-methylcytosine makes the genome unstable (Robertson and Jones, 1997). One reason for this is because the deamination of cytosine gives rise to uracil (a pyrimidine base associated with RNA) which is recognised as foreign within the DNA strand and replaced by the base excision repair (BER) pathway. While the deamination of 5-mehylcytosine gives rise to thymine (a pyrimidine base associated with DNA) which, although repaired by the BER, is not necessarily effectively recognised as foreign within the DNA strand (see figure 2.2) (Friedberg *et al.,* 2006). Therefore, the deamination of 5-methylcytosine to thymine is prone to mutations upon replication (Cooke *et al.,* 2003; Fazzari and Greally, 2004).

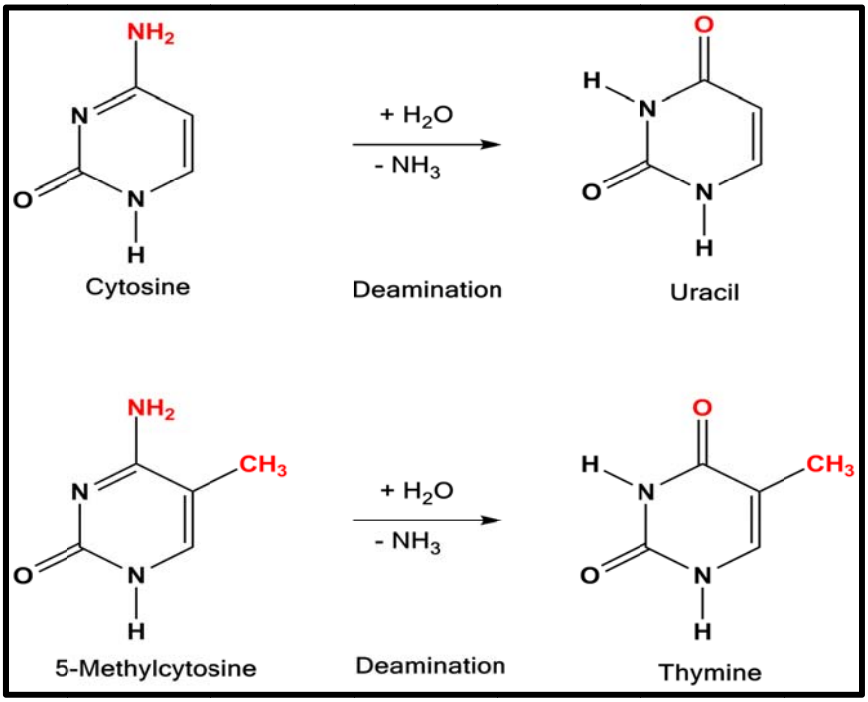

Figure 2.2: Deamination of cytosine and 5-methylcytosine. (i) The deamination of cytosine gives rise to uracil which is recognised and removed by the base excision repair pathway. (ii) The deamination of 5-methylcytosine, giving rise to thymine, is prone to mutations. (Adapted from Friedberg *et al.,* 2006).

2.2.1. The mechanism by which DNA methylation occurs

DNA methylation occurs through the addition of a methyl group to the C5 position of cytosine residues at CpG dinucleotides. S-adenosyl methionine (SAM) serves as a methyl donor for DNA methylation (Lu, 2000; Garrett and Grisham, 2005). The methyl group from SAM is transferred to the 5' position of the pyrimidine ring of cytosine, through the action of DNMTs converting SAM to S-adenosyl homocysteine (SAH), (Figure 2.3) (Hitchler and Domann, 2009; Fernandez *et al.,* 2010). SAM is a coenzyme which is required for cell growth and it is involved in the biosynthesis of essential metabolites such as polyamines, hormones and neurotransmitters which include dopamine and serotonin (Alan and Miller, 2008; Espada and Esteller, 2010). SAM is also involved in transsulfuration reactions by which SAM is converted to cysteine through a series of enzymatic steps. This formed cysteine is a precursor for the cellular antioxidant glutathione (GSH) (Lu, 2000).

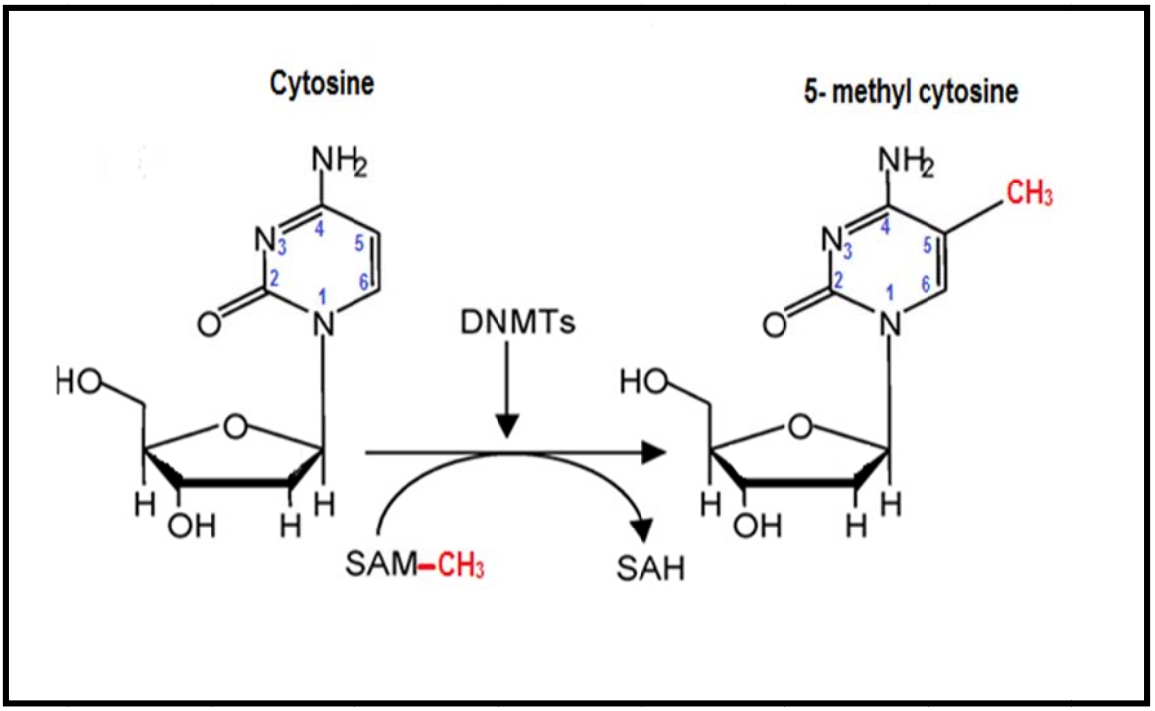

Figure 2.3: The mechanism by which DNA methylation occurs. The addition of methyl groups to the 5'-position of the pyrimidine ring of cytosine is catalysed by DNA methyltransferases (DNMTs). DNMTs use S-adenosyl methionine (SAM) as source of methyl groups, SAM is converted to S-adenosyl homocysteine (SAH) (Adapted from Espada and Esteller, 2010).

2.2.2. Types of DNA methylation

There are two known types of normal DNA methylation processes in mammalian eukaryotic cells which are: *de novo* methylation and maintenance methylation as shown in figure 2.4 (Portela and Esteller, 2010).

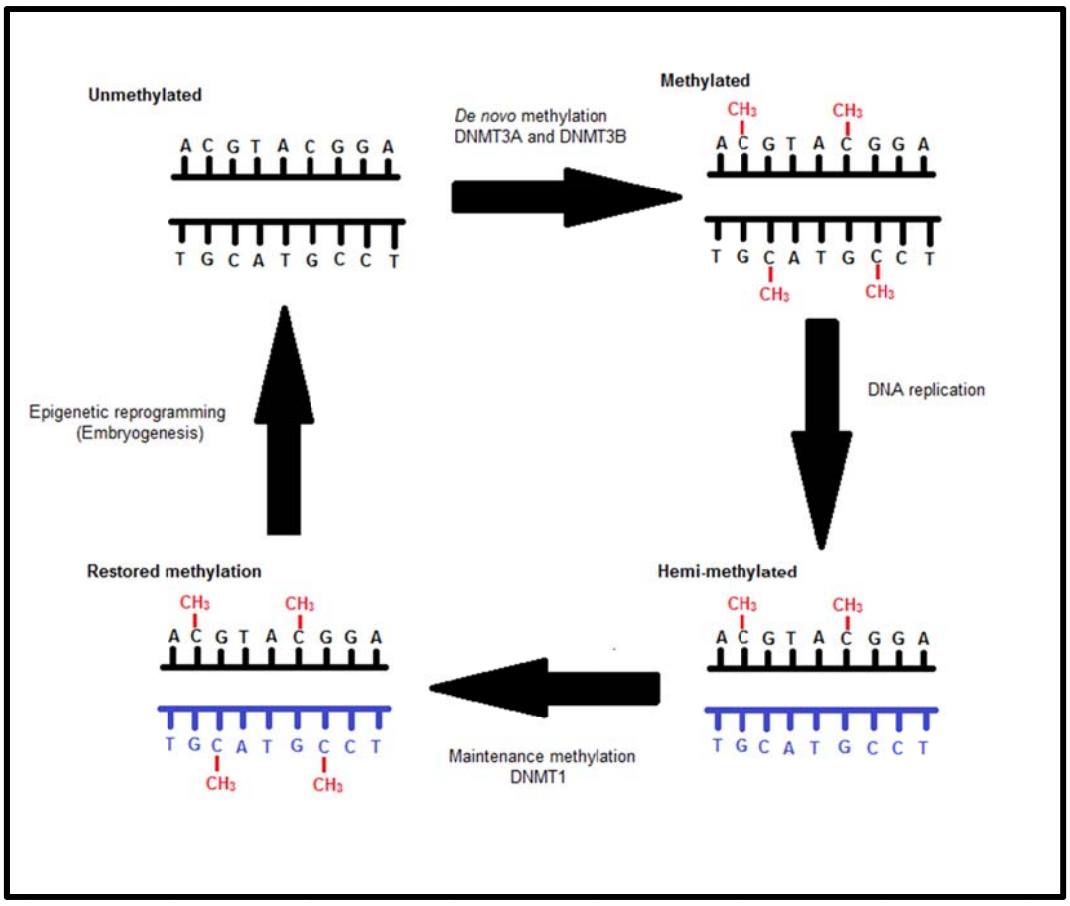

Figure 2.4: Types of DNA methylation. Two types of DNA methylation processes occur in mammalian cells; (i) *de novo* methylation which occurs through the activity of DNMT3A and DNMT3B and (ii) maintenance methylation which occurs through the activity of DNMT1. For details see text. (Adapted from Issa 2004).

De novo methylation is involved in rearranging the methylation pattern during embryogenesis as well as the differentiation process in adult cells, by methylating unmethylated DNA and thereby establishing the methylation patterns in a cell. DNMT3A and DNMT3B are responsible for *de novo* methylation. DNMT1 is responsible for maintenance methylation which is necessary in order to maintain the methylation pattern once it has been established. DNMT1 recognizes hemi-methylated DNA and methylates the single non-methylated strand following DNA replication (Jaenisch and Bird, 2003; Hamby *et al.,* 2008; Portela and Esteller, 2010).

2.3. Oxidative stress and the effect thereof on DNA and DNA methylation

Oxidative stress is characterized by excessive production of reactive oxygen species (ROS) in cells to such an extent that oxygen radicals exceed the antioxidant capacity of the cell (Ziech *et al.,* 2011). These high levels of oxidative stress are involved in the pathophysiology of human diseases such as cancer (Klaunig *et al.,* 1998; Kryston *et al.,* 2011). There are both endogenous and exogenous sources of ROS (Franco *et al.*, 2008; Kryston *et al.*, 2011). Cells withstand and counteract these sources of ROS through defence mechanisms which range from free radical scavengers such as glutathione (GSH), vitamins C and E, antioxidant enzymes such as catalase, superoxide dismutase, peroxidises and also through DNA repair mechanisms (Kryston *et al.,* 2011). Endogenous oxidative stress occurs as a result of normal cellular metabolism and oxidative phosphorylation, as well as P450 metabolism, peroxisomes and activation of inflammatory cells, and can lead to the oxidation of lipids and proteins as well as DNA damage (Cooke *et al.,* 2003; Kryston *et al.,* 2011). Exogenous sources of ROS can impact the overall oxidative status of a cell (Klaunig *et al.,* 1998) and are induced through radiation, ozone, hyperoxia, xenobiotics and chlorinated compounds, which have all been documented to cause ROS induced damage to cellular macromolecules such as DNA, RNA, lipids and proteins *in vitro* as well as *in vivo* (Franco *et al.*, 2008). ROS may play a central role in signal transduction systems as high levels of oxidative stress alters the signal pathways through oxidative damage of cell membranes, activation of transcription factors and changes in enzyme activity (Klaunig *et al.*, 1998). DNA methylation is influenced by both endogenous and exogenous oxidative stress (Cerda and Weitzman, 1997; Franco *et al.,* 2008; Cyr and Domann, 2011).

2.3.1. ROS causing oxidative DNA damage

Oxidative damage to DNA can be used as an index of oxidative stress (Collins, 2009) and can be induced through ROS such as the hydroxyl radical ($^{\bullet}OH$), the superoxide anion ($^{\cdot}O_2^-$) and the non-radical hydrogen peroxide (H_2O_2), which can be formed as byproducts of cell metabolism or occur through exogenous sources (Cerda and Weitzman, 1997; Boiteux and Radicella, 2000). Superoxide is produced as byproduct of oxygen reduction in the electron transport chain (Turrens*,* 2003; Friedberg *et al.,* 2006). The superoxide dismutase (SOD) enzyme converts superoxide to hydrogen peroxide. Hydrogen peroxide can also be produced by peroxisomes and as part of the innate immunity by neutrophils and macrophages (Guyton and Hall, 2006). Hydrogen peroxide has been used in studies to induce oxidative DNA damage (Gichner, 2003; Slameňová *et al.,* 2011; Ramos-Espinosa *et al.,* 2012) The hydroxyl radical is the most reactive of the primary ROS causing DNA

damage and can be generated from hydrogen peroxide through the reaction of hydrogen peroxide with a metal iron in the vicinity of DNA by the Fenton reaction (Friedberg *et al.,* 2006).

2.3.1.1. Consequence of oxidative DNA damage

Hydroxyl radicals attack DNA and give rise to lesions such as base modifications, apurinic/apyrimidinic (AP) sites, deletions, single strand breaks (SSBs), double strand breaks (DSBs), mutations and chromosomal rearrangements (Cerda and Weitzman, 1997, Kryston *et al.,* 2011; Freitas and De Magalhaes, 2011). 8-OxoG (figure 2.5.) is one of the most common products of oxygen radical injury in DNA (Audebert *et al.,* 2002) and can be used as a biomarker for DNA oxidation (Kim *et al.,* 2004; Malayappan *et al.,* 2007). The 8-oxoG base is also potentially mutagenic, through an alteration in the base pairing properties as it tends to pair with adenine (Collins, 2009). These 8-oxoG lesions serve as substrate for DNA repair systems. In mammalians the main pathway through which this lesion is repaired is through base excision repair (BER) (Audebert *et al.,* 2002; Friedberg *et al.,* 2006).

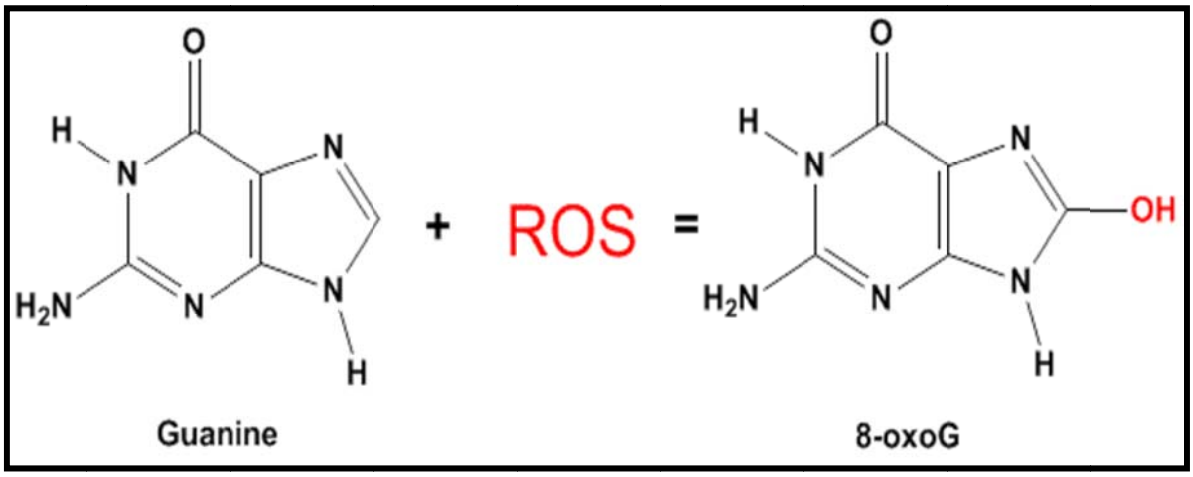

Figure 2.5: 8-Oxoguanine (8-oxoG), one of the most abundant oxidative base modifications found in mammalian DNA.

2.3.1.2. Base excision repair (BER)

The BER pathway can be divided into the short- and long patch pathways. One nucleotide is replaced by the short patch pathway, whereas 2 - 10 nucleotides are synthesised by the long patch pathway. The 8-oxoG lesion is preferentially removed by the short patch pathway (Boiteux and Radicella, 2000), and 80 - 90% of all BER occur through the short patch pathway (Sedelnikova *et al.,* 2010). BER is initiated by DNA glycosylases which cleave the

N-glycosylic bond between a modified nucleotide base and ribose, leaving the ribose phosphate chain of DNA intact and resulting in an AP site. AP endonucleases (lyase) remove the 3'-deoxyribose moiety generating single strand break with a 3'-hydroxyl adjacent to a 5' deoxyribosephosphate (dRP), nucleotide insertion then takes place by the suitable DNA polymerase. Finally, the phosphodiester backbone of the DNA molecule is sealed by ligation performed by DNA ligase (Garreth and Grisham, 2005; Xu *et al.,* 2008). (figure 2.6)

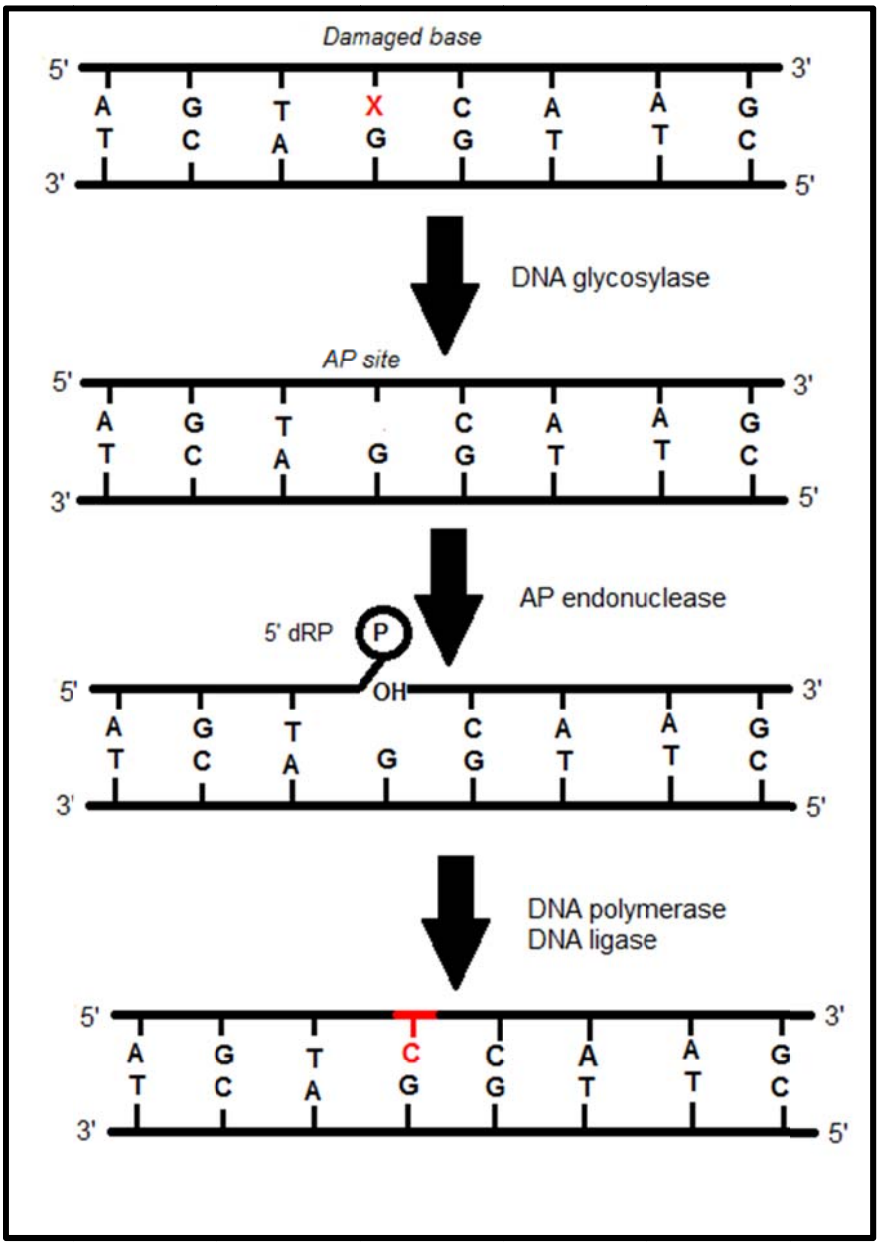

Figure 2.6: A simplified mechanism of the base excision repair (BER) pathway. An AP (apurinic/apyrimidinic) site is created by the excision of the damaged base (X) from the sugar phosphate backbone by DNA glycosylase. The DNA strand is then severed by an AP endonuclease (lyase) creating a SSB. Finally, the gap is repaired by DNA polymerase and DNA ligase (Adapted from Garrett and Grisham, 2005).

The 8-oxoguanine DNA glycosylase (OGG1) gene is involved in the BER pathway. This gene encodes the enzymes responsible for the excision of 8-oxoG (Friedberg *et al.,* 2006). Two isoforms of the Ogg1 protein (α-hOgg1 nuclear and β-hOgg1 mitochondrial) are encoded by the human OGG1 (*h*OGG1) gene. These proteins serve as DNA glycosylases for the initiation of BER in order to preferentially repair the 8-oxoG lesion in non-transcribed DNA (Audebert *et al.,* 2002; Araneda *et al.,* 2005) and also have AP lyase activity (Xu *et al.,* 2008). The promoter region of the OGG1 gene consists of a CpG island that can be silenced by DNA methylation (Dhénaut *et al.,* 2000; Araneda *et al.,* 2005). Promoter methylation of this gene is associated with different cancer types (Lahtz and Pfeifer, 2011).

2.3.2. The effect of oxidative stress on DNA methylation

ROS not only cause genetic changes such as mutations, but may also lead to epigenetic alterations by affecting DNA methylation (Franco *et al.,* 2008). The availability of SAM is linked to the redox status of cells. As mentioned in section 2.2.1. SAM is converted to cysteine which is a precursor for the cellular antioxidant glutathione; SAM also serves as a methyl donor for DNA methylation. Oxidative stress could therefore influence DNA methylation by the utilization of glutathione and thereby SAM which then affects DNA methylation. Oxidative stress also leads to the formation of single strand DNA breaks. These DNA single strand breaks contribute to a change in DNA methylation patterns because it signals *de novo* methylation (Franco *et al.,* 2008). To date there is, however, controversy over the occurrence of *de novo* methylation in cultured mammalian cells. It was first suggested that this process does not occur in cultured mammalian cells by Cerda and Weitzman (1997), but Kawasaki and Taira (2004) found that *de novo* methylation can be triggered by short interfering RNAs in cultured mammalian cells of human origin, these results were, however, unsupported. Also, of the four DNA bases, guanine has the lowest oxidation potential and is attacked preferentially upon oxidative DNA damage, because of its electron rich purine structure which allows it to react easily with oxygen radicals (Kim *et al.,* 2004). Therefore, the methylation of adjacent cytosines can be altered through the replacement of the guanine with an oxygen radical adduct such as 8-oxoG or 8-hydroxyguanine (8-hydroxyG) (Cerda and Weitzman, 1997; Hitchler and Domann, 2009).

The 8-oxoG base can directly inhibit DNMTs and thereby possibly induce demethylation of DNA (Hitchler and Domann, 2009). Cerda and Weitzman (1997) investigated the mechanisms of oxidant induced alterations in DNA methylation which might occur at specific CpG sites. They constructed a series of deoxy oligomers which contained the oxygen radical adduct 8-hydroxyG which was substituted on each guanine of the HpaII methylase

recognition site, 5'-CCGG-3' and annealed these oligonucleotides with unmethylated or methylated complementary strands. The incorporation of tritiated methyl groups was quantified and used to measure DNA methylation, making use of S-adenosyl-L-[*methyl*-^{3}H]methionine as substrate and the prokaryotic *Hpa*II DNA methylase. It was found that the substitution of the guanines with 8-hydroxyG inhibited DNA methylation of adjacent cytosines as well as the binding to the methyltransferases. They concluded that oxidative damage in nascent DNA strands and not parental strands inhibits DNA methylation.

2.4. Other factors that affect DNA methylation

The establishment and somatic maintenance of DNA methylation patterns are affected by environmental, toxicological (Aguilera *et al.,* 2010) and nutritional factors as well as cellular aging (Calvanese *et al.,* 2009) and circadian rhythm (Gallou-Kabanie *et al.,* 2007), which may alter the epigenotype and thereby influence the phenotype (Feil*,* 2006; Hitchler and Domann*,* 2009).

2.4.1. Environmental effects

The fruit fly strain *Drosophila melanogaster* provides proof that the characteristics of an organism can be affected by environmental conditions. These flies normally have white eyes. However, it was found that these flies develop red eyes when the surrounding temperature of the embryos that normally develop at 25 °C was raised to 37 °C. The offspring from these flies were found to be partly red eyed over several generations while the DNA sequence for the gene responsible for the eye colour was proven to remain the same for white-eyed parents and red-eyed offspring (Zurich, 2009). Epigenetics could therefore account for the change in eye colour, as it examines the inheritance of characteristics that do not occur as a consequence of changes in the DNA sequence. Environmental factors can also influence the epigenotype of higher organisms. Rager *et al.* (2011) exposed human lung epithelial cells to gaseous formaldehyde, a known toxic air contaminant [Office of Environmental Health Hazard Assessment (OEHHA) 2001] and found that formaldehyde exposure alters the miRNA expression profiles of human lung cells.

2.4.2. Nutritional effects

As mentioned in section 2.2.1. the enzymes (DNMTs) which are responsible for DNA methylation are dependent on the co-factor SAM. The availability of SAM is linked metabolism as can be seen in the active methyl cycle where folate is converted to N^{5}-

methyltetrahydrofolate, and then supplies a methyl group to convert homocysteine to methionine which is then converted to the universal methyl donor SAM as shown in figure 2.7. (Hitchler and Domann, 2009).

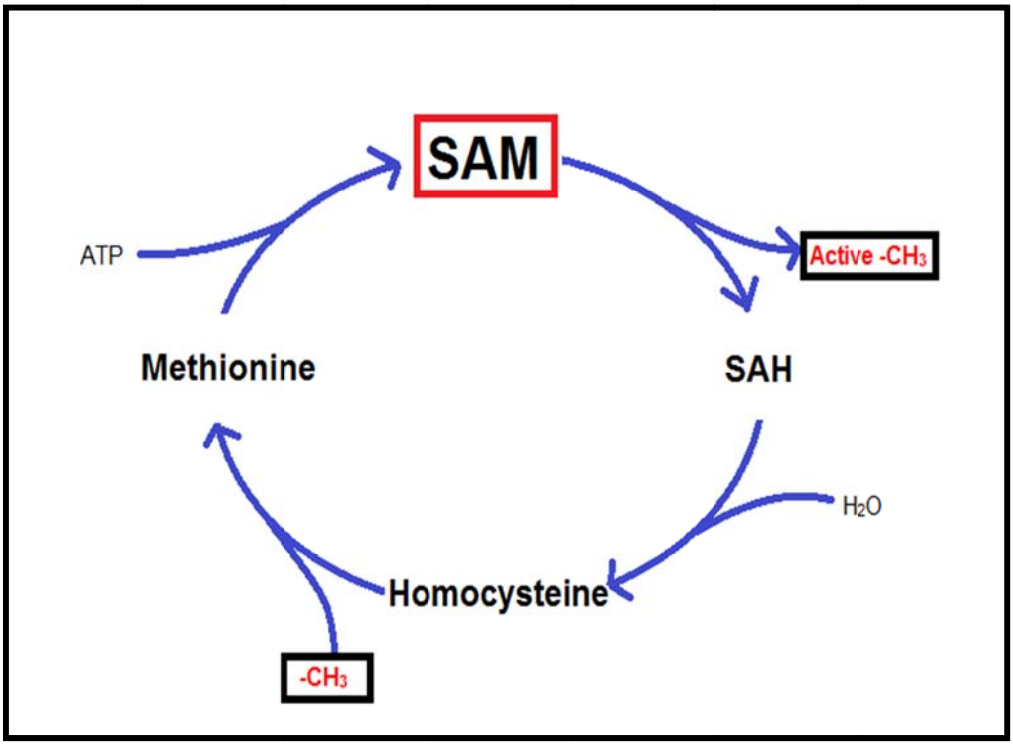

Figure 2.7: The activated methyl cycle. In the activated methyl cycle homocysteine is converted to methionine by a donated methyl group. Methionine is then converted to the universal methyl donor S-adenosyl methionine (SAM), which is converted to S-adenosyl homocysteine (SAH) by the removal of a methyl group. (Adapted from Ingrosso and Perna, 2009)

The effect of diet on DNA methylation was demonstrated in studies done with Agouti mice. Pregnant female mice were fed a diet supplemented with folic acid, Vitamin B12, choline and betaine which enhance the metabolism of SAM. It was found that these mice that are usually yellow, fat and prone to cancer and diabetes gave birth to offspring that were mainly brown, slim and healthy. These healthy offspring then also gave birth to offspring which were healthy, even though they were not fed the same diet. The change in phenotype is related to an associated increase in DNA methylation at the A^{vy} locus (Feil, 2006). Also in humans studies done on patients with hyper-homocysteinaemia showed that diet affects the methylation status of DNA. Patients with this disorder have increased levels of the inhibitor of SAM-dependent methyltransferases; S-adenosylhomocysteine (Ingrosso and Perna, 2009). In studies done by Ingrosso *et al.* (2003) it was found that patients had reduced levels of total DNA methylation compared to controls, and that supplementation with folate restored the methylation levels. Furthermore, mutagenesis can also occur because of dietary

insufficiencies of methyl donors, for example a folate deficiency could lead to abasic sites, DNA breaks and deletions. Also as a consequence of low SAM levels there is an increased tendency towards cytosine deamination and cytosine to thymine mutations instead of DNA methylation (LeBaron *et al.*, 2010).

2.5. DNA methylation in pathogenesis

As mentioned in section 2.2 in normal (healthy) cells the CpG islands that are located in the promoter or 5' end of genes are unmethylated. Abnormal i.e. increased methylation of these CpG islands leads to the repression of the transcription of associated genes (Esteller, 2007). DNA methylation is considered to be very stable and provides heritable long term silencing of genes. Aberrant DNA methylation has been associated with cancer (Wilson *et al.,* 2006; Zhu and Yab, 2009) and also other diseases such as Alzheimer's and cardiovascular diseases (Cyr and Domann, 2011). Also, as methylated cytosines exhibit an increased ability to deaminate which is catalysed when levels of SAM is low or non-existent, resulting in a high mutation rate of cytosine to thymine transitions (Neihrs, 2009; Wu and Zhang, 2010). Abnormal quantities of methylated cytosines could therefore lead to the formation of mutations which could in turn account for the involvement of DNA methylation in the carcinogenic process. During carcinogenesis oxidative injury, caused by elevated levels of ROS, could lead to the replacement of guanine with the oxygen radical adducts (section 2.3.2) , which alters the methylation of adjacent cytosines and which could then account for the aberrant DNA methylation patterns found in carcinogenesis. The role of aberrant methylation in the carcinogenesis process and whether it is leads to carcinogenesis or is a consequence of carcinogenesis and related processes such as elevated levels of ROS is still under debate.

2.6. Parameters of adverse DNA methylation

Aberrant DNA methylation patterns found in cancer is the most studied DNA methylation associated change. DNA methylation can be used as a biomarker for cancer development with most methods that are used for DNA methylation analysis making use of the ratio between methylated and unmethylated CpGs (Pogribny and Rusyn, 2012). As indicated in figure 2.8 normal (healthy) cells exhibit genome wide hypermethylation and hypomethylation at promoter CpG islands (Tost and Gut*,* 2010). In cancer cells DNA hypermethylation of the normally unmethylated CpG islands containing promoters occur, accompanied by genome wide hypomethylation (Shvachko, 2009; Esteller, 2007; Pogribny and Rusyn*,* 2012).

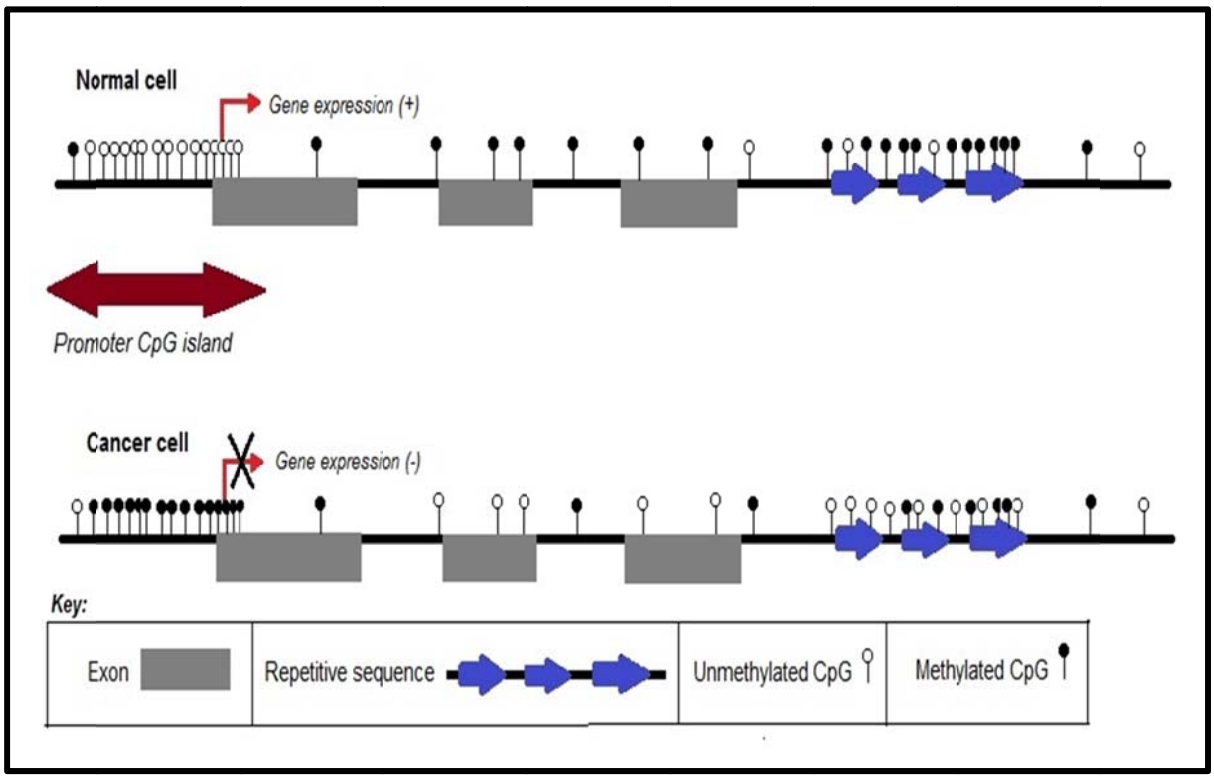

Figure 2.8: Indication of the distribution of DNA methylation in normal and cancer cells. In normal cells the promoter CpG islands normally remain unmethylated while repetitive elements and CpG dinucleotides that are spread throughout the genome are generally methylated. In contrast; in cancer cells a global loss of methylation (i.e. hypomethylation) is observed while some promoter CpG islands become methylated (Adapted from Tost and Gut, 2010).

2.6.1. DNA hypermethylation

DNA hypermethylation occurs when the methylation of the DNA domains that are normally unmethylated increases. Less than 3 % of CpGs in gene promoters are methylated in normal cells (Pogribny and Rusyn, 2012) in carcinogenesis, however, these CpGs are found to become predominantly methylated which is accompanied by transcriptional silencing of associated genes (Esteller, 2007; Tost, 2010).

2.6.2. DNA hypomethylation

DNA hypomethylation can be defined as a condition where there is a decrease in the number of methylated cytosine bases in comparison with the "normal" methylation level. (Pogribny and Rusyn, 2012). Genome wide hypomethylation can be observed when the overall genomic methylcytosine (in comparison to total cytosine) is decreased from approximately 4 % in normal tissue to 2 -3 % in cancerous tissue (Wild and Flanagan, 2010). Cancer cells have 20-60 % less 5-methylcytosine in comparison with normal cells. The

hypomethylation of the repetitive DNA sequences accounting for 20 – 30 % of the human genome is mainly responsible for this hypomethylation (Esteller, 2003). Consequences of hypomethylation include: chromosomal and genomic instability (Shvachko, 2009) through the activation and transposition of repetitive DNA elements (Wilson *et al.,* 2006; Pogribny and Rusyn, 2012), loss of imprinting and the activation of tumor promoting genes that are "normally" silenced (Wilson *et al.*, 2006).

2.7. Methods used for the measurement of DNA oxidation and changes in DNA methylation

2.7.1. Measurement of oxidative DNA damage

Oxidative DNA damage can be measured by implementing the use of bacterial repair endonucleases endonuclease III (Endo III) and formamido pyrimidine glycosylase (Fpg) (Collins, 2004; Andersson and Hellman, 2005; Collins, 2009) in conjunction with the comet assay. The comet assay which is more commonly referred to as the single cell gel electrophoresis (SCGE) is used to quantify DNA damage and allows the detection of DNA damage at levels of single cells. It has been established as a simple, cheap, rapid, flexible and sensitive method that can be used to detect single (alkaline comet assay) and double strand (neutral comet assay) breaks in DNA (Nossoni, 2008).

The comet assay, modified through the use of Fpg and Endo III, can be used for the measurement of oxidative DNA damage based on the principle that these endonucleases have appropriate specificities for oxidised bases induced through exposure to H_2O_2 and that they convert oxidised damage to single strand breaks (Andersson and Hellman, 2005). Endo III acts as a glycosylase and recognises and removes oxidised pyrimidines in DNA. The removal of the oxidised bases creates an AP site, and an associated AP-endonuclease activity then creates a break in the DNA (Collins, 2009). Fpg recognises altered purines such as imidazole-ring-opened purines or formamidopyrimidines which occur during the spontaneous breakdown of damaged purines Fpg nicks the DNA backbone at the base-free site by β-elimination through its lyase activity (Andersson and Hellman, 2005). In cellular DNA the major substrate for Fpg is the purine oxidation product 8-oxoG (Speit *et al.,* 2004; Andersson and Hellman, 2005; Collins, 2009). When comparing the use of the enzyme modified comet assay to chromatographic methods to establish the background level of base oxidation in human lymphocyte DNA based on the measurement of 8-oxoG (European standards committee on oxidative DNA damage (ESCODD) 2005), it was concluded that although the chromatographic methods like high performance liquid chromatography (HPLC)

are more precise, the comet assay is more accurate when measuring background levels of damage. The reason for this is that it is less susceptible to artefacts of oxidation during sample preparation (Collins, 2009).

2.7.2. Measurement of DNA methylation

2.7.2.1. Bisulfite treatment based methods

Sodium bisulfite treatment of DNA creates methylation-dependent sequence differences in genomic DNA. The bisulfite treatment modifies all unmethylated cytosines to uracil residues and following amplification uracil residues appear as thymines (Yang *et al.,* 2007). There are multiple methods that can be used in conjunction with bisulfite treatment of DNA in order to study DNA methylation changes (Liu and Maekawe, 2003; Tost and Gut, 2010). One such a method is bisulfite genomic sequencing. However, large quantities of DNA are necessary for conventional bisulfite sequencing to compensate for degradation and loss during bisulfite conversion. Even with modifications to the conventional method in which smaller amounts of DNA are required (Xu *et al.,* 2012) this technique is technically difficult, labor intensive and expensive which makes it unsuitable for the screening of large numbers of samples (Liu and Maekawe, 2003). Also, when studying the effect of oxidative stress on DNA methylation the bisulfite conversion method could prove to be problematic as methylated cytosine tend to spontaneously deaminate to thymine under conditions where SAM levels fluctuate (Neihrs, 2009; Wu and Zhang, 2010) which could lead to an underestimation of the amount of methylated cytosines.

Gene methylation analysis can also be performed by making use of methylation-specific PCR (MSP). This method makes use of bisulfite-mediated conversion of cytosine to uracil, followed by PCR making use of primers that are designed to distinguish methylated DNA from unmethylated DNA. One of the major drawbacks for using this technique is that it also facilitates amplification of any partially unconverted or unconverted sequence in bisulfite-treated DNA, resulting in an overestimation of DNA methylation (Sasaki *et al.,* 2003). It also tends to be more of a qualitative than a quantitatively accurate method, with strict PCR conditions such as annealing temperatures that needs to be taken into account (Liu and Maekawe, 2003; Tost and Gut, 2010). Another method that can be used for gene specific DNA methylation analysis is fluorescence-based real-time quantitative PCR analysis following sodium bisulfite treatment of DNA. This method allows the rapid screening of hundreds to thousands of samples however; it requires the use of expensive hybridization probes (Tost and Gut, 2010).

2.7.2.2. Enzyme based methods

Individual CpG positions can be analysed by making use of digestion with methylation-sensitive enzymes followed by PCR. Amplification products are produced by PCR only when digestion is inhibited by methylation (Liu and Maekawe, 2003). This method can be used to measure the methylation status of the promoter regions of genes when using predesigned primers for the gene of interest. One of the advantages of using this method is that it requires less DNA and no bisulfite conversion is needed, which makes the method simple and fast in such a manner that a large number of samples can be processed in a single day. Quantification is also improved by making use of quantitative real-time PCR with together with the use the intercalating dye *SYBR Green* (Tost and Gut, 2010). Disadvantages of the method is that it can only detect CpG methylation in methylation-sensitive restriction sites, and that false-positive results may occur if there is not complete enzymatic digestion of DNA (Liu and Maekawe, 2003). In order to minimalize the occurrence of false positive results methylation-dependent enzymes can be used in conjunction with the methylation-sensitive enzymes. The modification to this method makes it more attractive to use then bisulfite treatment based methods.

Global changes in DNA methylation patterns can be detected by making use of the cytosine-extension assay (CEA) (Pogribny *et al.,* 1999) or the comet assay modified through the use of methylation-sensitive and –dependent restriction enzymes (Wentzel *et al.,* 2010). Both of these methods utilize the difference in the methylation sensitivity of the isoschizomeric restriction endonuclease HpaII and MspI, who both recognize the same tetranucleotide sequence 5'-CCGG-3'. However, HpaII is a methylation sensitive enzyme, i.e. its function is blocked by methylated cytosines in its recognition sequence (Tost and Gut, 2010), and it is therefore inactive if any of the two cytosines are methylated. MspI on the other hand is inactive if the external cytosine is methylated (5'-^{m}CCGG-3') but is active when the internal cytosine is methylated (5'-C^{m}CGG-3') (Wentzel *et al.,* 2010; Tost and Gut, 2010). Enzyme digestion causes a 5' guanine overhang, in the CEA single nucleotide extension with radiolabeled deoxycytidine triphosphate [^{3}H]dCTP takes place.

The advantages of using the CEA for detection of abnormal methylation patterns in global DNA is that; radiolabeled incorporation is independent of the integrity of the DNA, no PCR amplification or DNA methylase reactions are required for DNA methylation detection and very low amounts of DNA (ng) are required for the assay (Pogribny *et al.,* 1999). The comet assay based method offers the advantage that it does not make use of any radiolabeled reagents, incomplete digestion might however occur as the method quantifies the integrity of

individual nucleoids and there is a possibility that not all of the restriction sites on the nucleotide may be accessible to the restriction enzymes. The CEA offers the advantage that it measures the DNA methylation of a pooled DNA sample which is in solution (Wentzel *et al.,* 2010).

2.8. Study motivation

Oxidative stress leads to lipid- and protein oxidation and also oxidative DNA damage. The oxidative DNA damage induced by high levels of ROS could lead to carcinogenesis and has a possible role in the pathogenesis of aging. Mammalian cells possess antioxidant- and specific DNA repair mechanisms which protect them against the damage induced by the oxidants to which they are exposed on a daily basis. However, prolonged exposure of cells to these oxidant species could result in the saturation of these defences. While it is well known that the genetic profile of a cell can change because of oxidative damage to DNA it should be kept in mind that the exposure of cells to oxidative stress could also lead to changes in the epigenetic profile of a cell. On an epigenetic level the DNA methylation pattern of a cell can be influenced by oxidative stress which leads to fluctuations in SAM levels and damage to DNA such as base modifications and single strand breaks in DNA. DNA methylation is considered to be very stable. Aberrant DNA methylation influences gene transcription and genomic stability which could lead to pathogenesis in cells.

Epigenetic changes such as changes in the DNA methylation pattern of a cell are considered to be reversible, whereas genetic changes such as mutations are irreversible. Therefore, detecting changes in the DNA methylation pattern of cells that are associated with oxidative damage, early on, could prove to be beneficial in disease prevention.

2.9. Project aim

The aim of this study was to examine the early DNA methylation events that occur in oxidative stressed cultured mammalian cells by examining the effect of H_2O_2 exposure on the global DNA- and promoter specific methylation pattern of 143B cells over a period of six hours. Changes in the *h*OGG1 promoter methylation status and gene expression were evaluated as this gene plays a crucial role in the initiation of the base excision repair (BER) pathway for the repair of oxidative DNA damage

2.10. Experimental approach and methodology

The approach that was followed is schematically represented in figure 2.9 and can be divided into three parts:

1) Making use of the comet assay (single cell gel electrophoresis (SCGE)) to investigate the DNA damage and repair related to the BER in 143B cells following H_2O_2 exposure,
2) Investigating the effect of H_2O_2 on the DNA methylation status of 143B cells, both globally and promoter specific,
3) Evaluating the effect of H_2O_2 exposure on the expression of the *h*OGG1 gene in 143B cells.

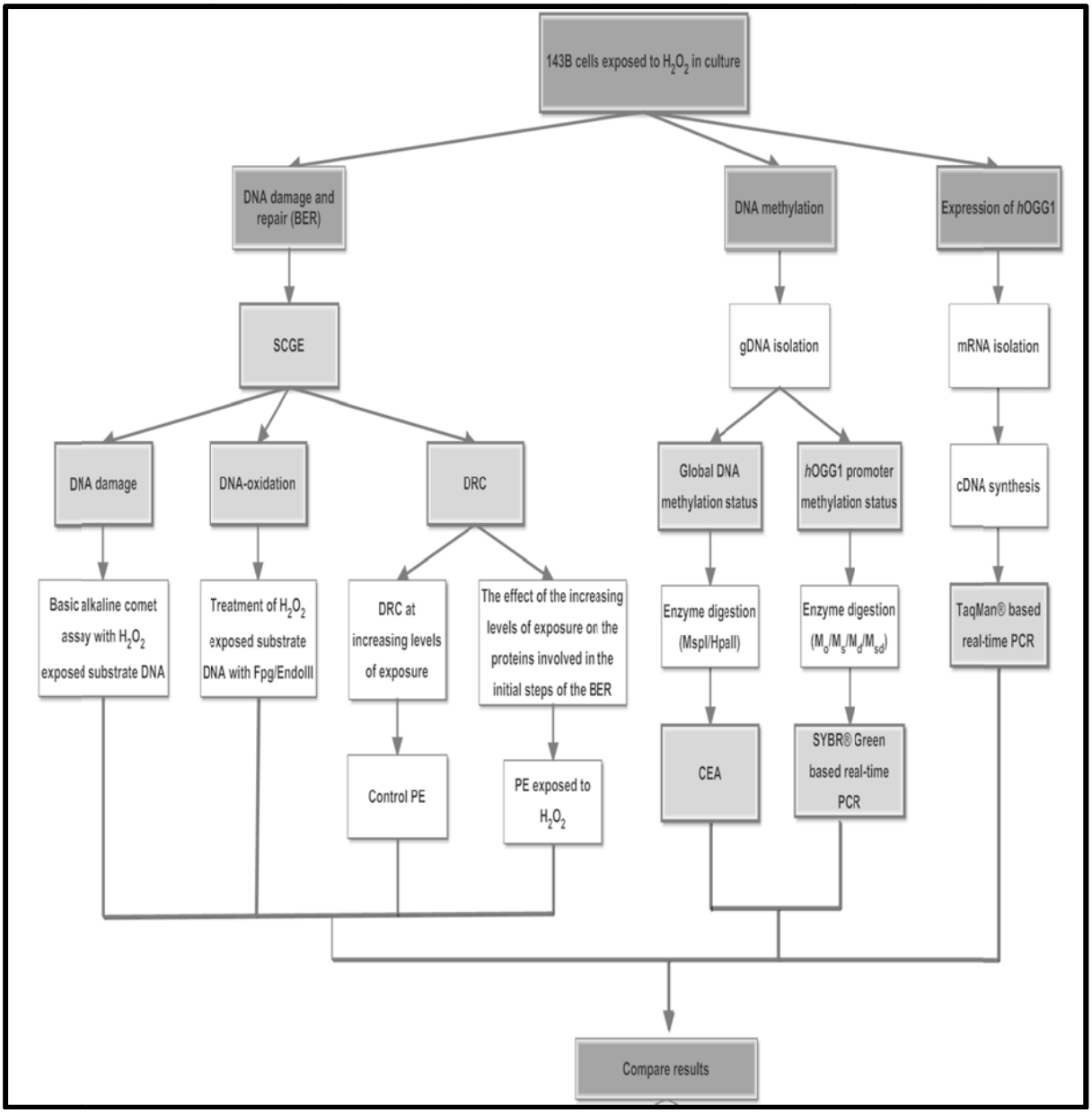

Figure 2.9: Experimental approach. 143B cells were subjected to H_2O_2 in culture. The comet assay (SCGE) was used to (i) evaluate the DNA damage, (ii) evaluate if the damage was caused by DNA oxidation and (iii) to investigate the DNA repair capacity. Change in the DNA methylation pattern caused by exposure of the cells to H_2O_2 was investigated and consisted of the evaluation of the change in (i) the global DNA methylation status and (ii) the *h*OGG1 gene promoter methylation status. Finally, the expression of the *h*OGG1 gene following exposure of the cells to H_2O_2 was investigated. Abbreviations; BER: Base excision repair; SCGE: single cell gel electrophoresis (comet assay); DRC: DNA repair capacity; PE: Protein extract; M_o: Mock digest; M_s: Methylation sensitive digest; M_d: Methylation dependent digest; M_{sd}: Double digest; CEA: Cytosine extension assay

2.11. Main objectives

The main objectives of this study were to demonstrate that:

i. The exposure of 143B cells to H_2O_2 in fully supplemented medium (DMEM + 10 % FBS) causes damage because of DNA-oxidation occurring in the cells.

ii. The activity of the proteins involved in the initial steps of the BER pathway and also the DNA repair capacity (DRC) of the 143B cells, are affected by the exposure to H_2O_2.

iii. The global DNA methylation pattern of the 143B cells is affected by the exposure of the cells to H_2O_2.

iv. The exposure of the 143B cells to H_2O_2 affects the *h*OGG1 promoter methylation status of the cells.

v. The expression of the *h*OGG1 gene is affected by the exposure to H_2O_2

vi. There is a possible relationship between the affect that H_2O_2 exposure has on the occurrence of oxidative DNA damage, the proteins involved in the initial steps of the BER/the DRC, the change in the global- and *h*OGG1 promoter methylation status and the expression of the *h*OGG1 gene.

CHAPTER 3
Materials and methods

3

3.1. Introduction

The human osteosarcoma (143B) cell line was used for this study. This cell line was used in order to investigate the effect of oxidative stress on the global DNA methylation pattern as well as the promoter methylation pattern and expression of the *h*OGG1 gene involved in the BER repair pathway in wild type 143B cells (uncompromised complex III) .Previous studies reported that in 143B cells in which complex III of the respiratory chain was compromised, induced by the stable knockdown of the Rieske protein (Levanents *et al.,* 2011), deviations in the global DNA methylation pattern as well as the promoter methylation and expression of genes involved in DNA repair pathways could be observed (Levanents *et al.,* 2011; Du Toit *et al.,* 2013). Hydrogen peroxide (H_2O_2) was used as oxidative agent and cells were exposed in culture using supplemented medium (Dulbecco's Modified Eagles Medium (DMEM) + 10 % Foetal bovine serum (FBS)). FBS supplies nutrients, growth and attachment factors and therefore enhances the survival of cells in culture. All experiments were at least done in duplicate. All of the reagents that were used were of the finest quality and were purchased from Sigma-Aldrich, unless stated otherwise. A list of the suppliers and catalogue numbers of the materials used in this study are given in appendix E.

3.2. Cell culturing conditions

Cells were cultured in a CO_2 incubator under standard conditions which consist of 5 % CO_2 and 95 % humidity at 37°C in DMEM/High-glucose medium containing 4500 mg/L glucose and 1mM sodium pyruvate (Hyclone medium. Thermo Scientific) supplemented with 10 % FBS (Lonza), 1 % penicillin/streptomycin (Lonza), 4 mM L-glutamine (Lonza) and 1 % non-essential amino acids (Lonza).

3.2.1. Exposure of cells to hydrogen peroxide (H_2O_2)

Cells were cultured in 6 well plates (Nunc) and exposed to hydrogen peroxide (H_2O_2) in culture. Cells were seeded at 2 x 10^5 cells per well and allowed to grow for 48 hours in order to reach a confluence of approximately 80 %. A stock solution of 40 mM H_2O_2 in 1 ml of phosphate buffered saline (PBS) was freshly prepared for each experiment and was diluted

to concentrations of 50 µM, 150 µM, 250 µM and 500 µM in 2 ml of culture medium (DMEM supplemented with 10 % FBS) immediately before use. The MTT assay, as described in section 3.3., was used to determine the cytotoxicity of the peroxide at these concentrations. Cells not treated with H_2O_2 were used as control. Cells were exposed to H_2O_2 for one hour prior to harvesting, as described in section 3.2.2.

3.2.2. Harvesting of cells

Cells were harvested by making use of trypsin. Medium was removed, cells were washed twice with PBS in order to remove all of the serum and medium, followed by the addition of 1 ml of the 1 x Trypsin/EDTA solution to each well and the plate was then incubated at 37°C for five minutes, where after the excess trypsin was removed and the plate was incubated at 37°C for 10 minutes to allow the cells to detach from the well surface. The release of the cells from the well surface was confirmed by microscope visualisation (100 x objective). After trypsin treatment, 2 ml of fresh growth medium was added to each well to stop the trypsin reaction. Cells were then counted and the viability of the cells were determined by using the trypan blue (dye exclusion) method (section 3.2.2.1), where after cells were diluted to 2.5 x 10^4 cells per 50 µl of medium. The trypsin-EDTA method is the most common method used to harvest adherent cells for routine serial passage because other methods, such as the use of enzyme-free dissociation buffer, causes problems with the reattachment of cells (Heng *et al.,* 2009). The use of trypsin to harvest cells for comet assay applications is also favourable as it produces intact single cells. However, trypsin is a serine protease that hydrolyses proteins, which could have a negative influence on the cells cellular integrity. Previous studies have shown that the detachment of adherent cells with trypsin-EDTA may lead alterations and damage of cell populations (Collett *et al.,* 2007) and that prolonged exposure of cells to trypsin-EDTA effects cell membrane integrity and cell viability (Sutradhar *et al.,* 2010). In this study cells were only exposed to trypsin-EDTA for a short period of time and when performing the trypan blue assay on harvested cells these cells were found to be viable (i.e. 95 % cell viability). Previous studies do however; suggest a recuperation time following the harvesting procedure prior to performing alkaline comet assay based methods (Van Dyk *et al.,* 2010; Wentzel *et al.,* 2010). Following trypsin treatment of the cells, cells were aliquoted into 400 µl aliquots and incubated at 37 °C and 200 rpm for one hour in an orbital shaker, to allow recuperation of cells which was found to be sufficient for this study.

3.2.2.1. Counting of cells

A hemocytometer and trypan blue was used to count the cells and also to determine the viability of the cells following the harvesting process. For this purpose 10 µl of the cell suspension was added to 25 µl trypan blue and topped up to a volume of 50 µl with PBS, where after 10 µl of the trypan blue/cell mixture was transferred to a hemocytometer. The cells were counted using a light microscope with the 10 X objective. The cells in the large, central gridded square and the four large corner squares (1 mm^2) were counted. A layout of the grid is given in figure 3.2. Duplicate samples were prepared and the counts averaged.

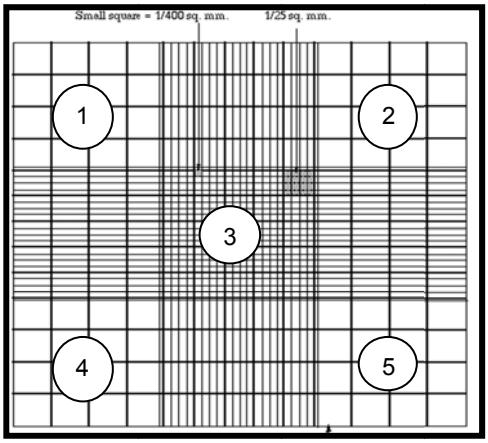

Figure 3.1: Layout of the hemocytometer grid visualised under the 10 X objective

The number of cells per µl was determined by using the following equation:

$$\text{Cells per µl} = \frac{\text{Average number of cells} \times 5 \times 10^4}{1000} \qquad \textit{Equation 3.1}$$

Where,
Average number of cells = cell count 1 + cell count 2 ÷ 2 ÷ 5
The dilution factor is 5. The number of cells counted was multiplied by 10^4 to estimate the number of cells per mL and divided by a 1000 to estimate the number of cells per µl.

The cell viability was determined by using the following equation:

$$\%\ \text{viable cell} = [1.00 - (\text{Number of blue cells} \div \text{Number of total cells})] \times 100 \qquad \textit{Equation 3.2.}$$

In order for cell to be considered viable the percentage cell viability needed to be > 95 %, which was found to be the case in all experiments performed (results not shown).

3.3. Cell viability after exposure to H_2O_2

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell viability after exposure of cells to H_2O_2. The main advantages of using the MTT assay, instead of the trypan blue method which measures cell membrane integrity, is that it is more accurate and less labour intensive (Freimoser *et al.,* 1999) also, the MTT assay measures metabolic activity instead of just membrane integrity (Sylvester 2011)

3.3.1. Principle of the method

The MTT assay is a colorimetric assay that is used to measure the cell viability and proliferation. The yellow tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by metabolically active mitochondrial dehydrogenase enzymes to non-water-soluble purple formazan crystals within the cell. This formed formazan crystals can be dissolved with detergents such as dimethylsulfoxid (DMSO). The amount of the of the formazan product can then be spectrophotometrically determined and serves as an estimate of the number of mitochondria and therefore the number of living cells (Freimoser *et al.,* 1999; Sylvester 2011).

3.3.2. Procedure

Cells were seeded at 2.5 x 10^4 cells per well in a 96-well plate. Following a 24 hour growth period cells were exposed to 0 µM, 50 µM, 150 µM, 250 µM, 500 µM, 1000 µM, 1500 µM, 2000 µM, 2500 µM, 3000 µM and 3500 µM H_2O_2 by diluting a 40 mM stock solution of H_2O_2 in 200 µl medium (DMEM supplemented with 10 % FBS) for one hour, and 150 µM and 500µM H_2O_2 for one - and six hours. Following the exposure of the cells to H_2O_2 cells were washed twice with PBS in order to remove the serum and the medium. A stock solution (5 mg/ml) of MTT was prepared by diluting MTT in PBS. Then, 20 µl of the stock solution was added to each well and medium was added to a final volume of 200 µl. DMSO (Merck) was used as a blank. Plates were then incubated at 37°C for five hours in order for the formazan crystals to form. After the incubation period, the medium was removed, 200 µl of DMSO was added to each well and the plate was incubated at 37°C (5 % CO_2) for one hour in order to dissolve the crystals. The optical density was then measured in a BioTEK plate reader

(Synergy HT and Gen5.1.1 software) at a wavelength of 560 nm. Background absorption was measured at 630 nm.

Cell viability was expressed as a percentage relative to the untreated control by using the following equation:

%cell viability = (∆*Absorbance* - ∆*Blank*)/(∆*Control* - ∆*Blank*) x 100 **Equation 3.2.**

Where,

∆Absorbance = Absorbance at 630 nm – Absorbance at 560 nm

∆Blank = Mean blank at 630 nm – Mean blank at 560 nm

∆Control = Control absorbance at 630 nm – Control absorbance at 560 nm

3.4. Assessment of the effects of oxidative stress on DNA using comet assay based methods

3.4.1. Principle of the comet assay

The comet assay consists of six basic steps: (i) lysis, (ii) electrophoresis, (iii) neutralisation, (iv) staining, (v) visualisation and (vi) scoring. The use of an alkali buffer allows the detection of alkali labile sites (ALS) as single strand breaks. High-molarity sodium chloride is used for the lysis step in order to remove membranes, cytoplasm, nucleoplasm and histones leaving behind only the nucleoid which consists of supercoiled DNA (Collins, 2004). Alkaline electrophoresis conditions are used to unwind and denature DNA after which the slides are placed in a neutralisation buffer (TrisHCl) in order to neutralize the alkaline electrophoresis buffer and to prevent unwanted DNA damage. Slides are stained by using the fluorescent agent, ethidium bromide which is an intercalating dye that binds more efficiently to double-stranded DNA than single stranded DNA (Collins, 2004). Finally, slides are scored by making use of a fluorescent microscope. Based on the tail intensity (percentage of DNA in tail), comets can be grouped into classes ranging from class 0 with the least damage to class 4 with the highest degree of damage (da Silva *et al.,* 2000) (figure 3.2)

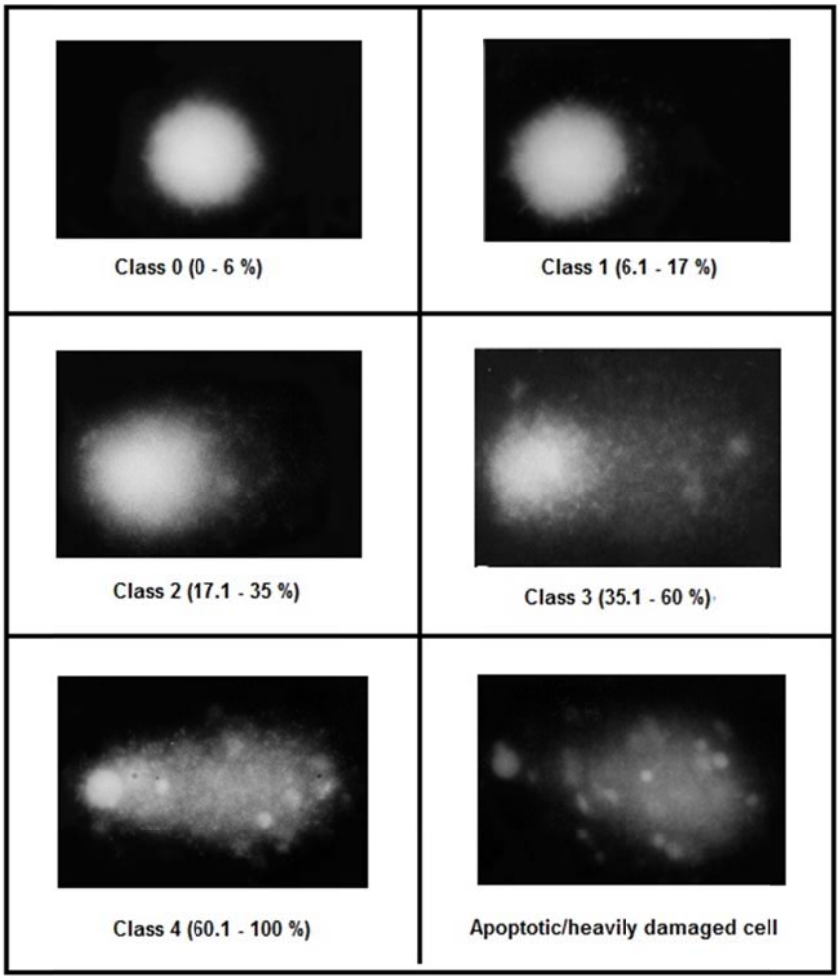

Figure 3.2: Comet classes. Class zero comets represents cells with minimall damage, class four represents cells with the most damage. Apoptotic cells are cells that are so heavily damaged that no distinction can be made between the head and the tail (Adapted from da Silva *et al.,* 2000).

3.4.2. The alkaline comet assay

The alkaline comet assay (pH > 13), which can be used to detect single strand breaks in DNA, was used (Collins *et al.,* 2008, Collins, 2009). 143B cells were exposed to H_2O_2 (section 3.2.1) and harvested (section 3.2.2) and processed further with the conventional alkaline comet assay as follows;

3.4.2.1. Preparation of slides and nucleoids

Frosted microscope slides were coated with 300 µl 1 % high melting point agarose (HMPA, in 0.1 M EDTA) and left to dry at room temperature. Following one hour incubation in the orbital shaker the 50 µl of the cell suspension was removed and placed in 150 µl of 0.5 %

low melting point agarose (LMPA, in 0.1 M EDTA). Then, 130 µl of this mixture was transferred to the HMPA pre-coated frosted microscope slide and allowed to set on ice. Slides were then lysed overnight (16-to 48 hours) in 2.5 M lysis buffer (2.5 M NaCl, 0.4 M EDTA, 1 % Triton X-100 and 10% DMSO) at 4 ºC.

3.4.2.2. Processing of slides

Following lysis, alkaline treatment was performed by immersing slides in electrophoresis buffer (5 mol/l sodium hydroxide (NaOH) and 0.4 mol/l EDTA) for 30 minutes in order to denature DNA, this was followed by 40 minutes of electrophoresis at 40 V and 80 mA at 4◦C. Slides were then neutralised in neutralising buffer (TrisHCl pH7.5) for 15 minutes, stained in ethidium bromide (12.5 µM) for one hour and then washed with water for five minutes. These slides were then visualised with the Olympus 1x70 fluorescence microscope (200 x magnification) and comets were scored using the Comet Assay IV (Perceptive Instruments Ltd.) software program. A minimum of 50 comets were randomly scored per slide and all experiments were performed in duplicate. The percentage tail DNA was used to determine DNA damage and to group comets into classes.

3.4.3. Modifications to the alkaline comet assay

The H_2O_2 exposed cells were also subjected to two modified forms of the conventional alkaline comet assay: (i) the modified comet assay to detect oxidized bases, (ii) the modified comet assay to assess DNA repair capacity *in vitro.*

3.4.3.1. Measuring oxidative DNA damage

Oxidized bases can be detected by making use of the repair endonucleases formamidopyrimidine DNA glycosylase (Fpg) and Endonuclease III (Endo III). Nucleoids embedded into agarose on frosted glass slides were treated with Endo III and Fpg (New England Biolabs) in order to detect oxidised bases following H_2O_2 exposure of cells (Collins, 2004). The source of both of these enzymes is an *E.coli* strain that carries the cloned *nth (endo III)* and *fpg* genes respectively. Both these enzymes act as an *N*-glycosylase and an AP-lyase. The *N*-glycosylase activity of Endo III releases damaged pyrimidines and that of Fpg releases damaged purines from double stranded DNA generating apyrimidinic and apurinic (AP) sites respectively, a break in the DNA is then created by an associated AP-endonuclease activity causing an increase in the percentage tail DNA which can be measured by making use of the comet assay (Collins, 2009).

3.4.3.1.1. Preparation of nucleoids from exposed cells

143B cells were exposed to H_2O_2 for one hour with the concentrations mentioned in section 3.2.1. Cells were harvested as described in section 3.2.2. After exposure of the cells to H_2O_2 the cells were processed as described in section 3.4.2.1, and the slides were lysed overnight in 2.5 M lysis buffer (2.5 M NaCl, 0.4 M EDTA, 1% Triton X-100, and 10% DMSO) at 4◦C.

3.4.3.1.2. Enzyme digestion of nucleoids in order to detect oxidized bases

Enzyme digestion of agarose-embedded nucleoids on glass slides was performed according to the protocol as described by Huyshamen, 2005 and Van Dyk, 2005, with a few minor modifications. Briefly, 1x enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, adjusted to pH 8.0 with KOH) was prepared. The slides containing the nucleoids were removed from the lysis buffer and washed for 15 minutes with PBS, where after it was washed for 15 minutes in 1x enzyme reaction buffer. Slides were removed from the wash buffer and the excess liquid was removed after which 50 µl of the enzyme solution ($1:10^3$dilution for Fpg and $1:10^2$ dilution of Endo III in 1 ml 1x enzyme reaction buffer) or buffer alone was placed onto the gel surface and covered with a cover slip. Slides were then placed in a pre-heated moist container and incubated at 37˚C for 30 minutes. Following incubation the cover slips were removed and the slides were washed for 5 minutes in PBS (4˚C). The slides were then further processed according to the conventional alkaline comet assay as described in section 3.4.2.2

3.4.3.2. Assessment of the DNA repair capacity

The comet assay was modified through the use of cell (protein) extracts to assess the repair capacity *in vitro* (Collins *et al.,* 2001; Langie *et al.,* 2006; Gaivão *et al.,* 2009; van Dyk *et al*., 2010). The protein extracts perform the initial steps of DNA excision repair, i.e. damage recognition and incision, there is an increase in the tail DNA as the proteins attempt to excise the damaged bases (i.e. bases with lesions). The DNA repair capacity (DRC) is defined as the ability of the proteins under investigation to perform DNA excision repair. The cell extract is made by suspending cells at high density in buffer, followed by snap-freezing of the cell suspension in liquid nitrogen and thawing in order to disrupt the structure of the cell. Detergent is then added in order to complete the release of soluble components, finally the cell debris are removed by centrifugation.

3.4.3.2.1. Preparation of protein extracts

Protein extracts were prepared from cultured 143B cells and used on substrate DNA from the same cell line in order to evaluate the DRC of the particular cell line at increasing concentrations of H_2O_2. Also, the effect of the exposure to H_2O_2 on the activity of the proteins involved in the initial steps of the base excision repair (BER) pathway was measured. In order to firstly evaluate the DRC at increasing concentrations of H_2O_2 the substrate (cells) was exposed to the increasing concentrations of H_2O_2 as described in section 3.2.1 and the protein extracts were prepared from untreated (control) cells. The effect of H_2O_2 on the activity of the proteins involved in the initial steps of the BER (i.e. the effect of the exposure to H_2O_2 on the DRC) was evaluated by exposing the substrate to 100 µM H_2O_2 in order to induce initial damage and the protein extracts were prepared from cells exposed to the increasing concentrations of H_2O_2 as described in section 3.2.1. Control substrate DNA was also exposed to the protein extracts prepared from cells exposed to increasing concentrations of H_2O_2.

Protein extracts were prepared by using the method as described by Collins *et al.* (2001), Langie *et al.* (2006) and van Dyk *et al.* (2010). Briefly, cells were harvested by using the method described in section 3.2.3.The resulting cell suspension was the centrifuged for 3 minutes at 1200 *g*, the medium was removed and the supernatant was washed twice with extraction buffer A (45 mM HEPES, 0.4M KCl, 1 mM EDTA, 0.125 mM dithiothreitol (DTT), 100% glycerol, and adjusted to pH 7.8 using KOH). Cells were then counted using the trypan blue (dye exclusion) method (section 3.2.2.1) and diluted to a final concentration of 5 x 10^6 cells/ml. The cell suspension was then centrifuged for 3 minutes at 1200 *g* and the resulting pellet was resuspended in 50 µl of buffer A per 5 x 10^6 cells. The aliquots were then snap frozen in liquid nitrogen and allowed to thaw on ice. Then, 15 µl of 1% Triton X-100 in buffer A was added to each of the 50 µl aliquots and the resulting solution was vortexed and incubated on ice for 10 minutes. After 10 minutes the solution was centrifuged at 13 000 *g* for 5 minutes at 4◦C in order to remove all of the cell debris. The supernatant was transferred to a new tube and the protein concentration was determined by using the Pierce® BCA Protein Assay Kit (Thermo Scientific) which uses bovine serum albumin as standard (Section 3.4.3.2.2). The protein extracts were then diluted with 0.23%Triton X-100 in buffer A to a concentration of 1mg/ml and stored at -80◦C until use in the repair assay.

3.4.3.2.2. Determination of the protein concentration

The Pierce® BCA Assay Kit (Thermo Scientific) is a bicinchoninic acid (BCA) based method. The principle of the method is based on the fact that under alkaline conditions the peptide bonds found in proteins react with the Cu^{2+} to produce Cu^{+} (the biuret reaction). The cuprous cation (Cu^{+}) reacts with BCA to form a purple compound which exhibits a strong absorbance at 562 nm, which is linear with increasing protein concentration. Therefore, the more proteins in the mixture, the more intense the colour will be and the higher the absorbance at 562 nm thereby allowing the quantification of proteins (Smith *et al.,* 1985; Wilson & Walker, 2005). For each reaction 200 µl of a 50:1 mixture of BCA solution and copper(II) sulphate solution was added to 2 µl sample adjusted to a final volume of 20 µl with PBS, in a 96-well plate. Bovine serum albumin (BSA) was used to create a standard curve with the following concentrations: 0 µg/µl, 4 µg/µl, 8 µg/µl, 12 µg/µl, 16 µg/µl and 20 µg/µl. The resulting mixtures were then incubated at 37°C for 30 min before reading the plate using a BioTEK plate reader (Synergy HT and Gen5.1.1 software).

3.4.3.2.3. Preparation of nucleoids from exposed cells

Cells were exposed to H_2O_2 for one hour with the concentrations mentioned in section 3.2.1. Cells were harvested as described in section 3.2.2. After exposure of the cells to H_2O_2 the cells were processed as described in section 3.4.2.1, and the slides were lysed overnight in 2.5 M lysis buffer (2.5 M NaCl, 0.4 M EDTA, 1% Triton X-100, and 10% DMSO) at 4◦C

3.4.3.2.4. Repair assay

Diluted protein extracts were thawed on ice prior to the repair assay and 200 µl of reaction buffer B (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml BSA and adjusted to pH 7.8 with KOH) was added. Langie *et al.* (2006) found that stored protein extracts had a reduced ability to incise damaged DNA when compared to freshly isolated extracts and that the addition of ATP to the protein extract causes it to regain its activity completely. ATP is required for the incision step, therefore, 10 µl of ATP (65 µM) was also added to the reaction mixture. This mixture was kept on ice until use. Slides were removed from the lysis buffer and washed for 15 minutes in PBS at 4◦C, after which it was washed in buffer B for 15 minutes at 4◦C. To assess the *ex vivo* repair capacity, 50 µl of the reaction mixture was added to each slide containing the exposed nucleoids. The slides were then covered with a cover slip, placed in a humidified container and incubated at 37◦C for 10 minutes. Following incubation, the slides were placed on ice in order to stop the enzyme reaction. Cover slips

were removed, after which alkaline treatment with electrophoresis buffer, electrophoresis, neutralization, staining and quantitation were performed as described in section 3.4.2.2. The DNA repair capacity (DRC) was calculated by using the following equation:

DRC = 1 – (Average%tailDNA:NoPE÷ Average%tailDNA:PE) **Equation 3.4.**

**PE: Protein extract*

3.5. Measuring the global DNA methylation status

After exposure of cells to 150 and 500 µM H_2O_2 (section 3.2.1) for one, three, four, five and six hours, cells were harvested (section 3.2.2) and genomic DNA (gDNA) was isolated, the cytosine extension assay (CEA) was then performed on the isolated gDNA in order to investigate the global DNA methylation status following H_2O_2 exposure.

3.5.1. Genomic DNA isolation

The Flexigene kit (Qiagen, Southern Cross Biotechnology (PTY) LDT) was used as recommended by the supplier to isolate gDNA.

3.5.1.1. Principle of the Flexigene kit

Cells are lysed with the addition of lysis buffer. Denaturation buffer containing a chaotropic salt and QIAGEN protease is added in order to disrupt mitochondrial membranes and remove proteins. DNA is precipitated by the addition of isopropanol and washed with 70 % ethanol, where after it is dried and solubilized in hydration buffer containing 10 mM Tris-HCl (pH 8.5) (QIAGEN Handbook 05/2010).

3.5.1.2. Protocol used for the Flexigene kit

The manufactures protocol for using 1-2 x 10^6 cultured cells was strictly followed. Briefly, following exposure to hydrogen peroxide as described in section 3.2.1 cells were harvested as described in section 3.2.2. Cells were then pelleted by centrifugation for 5 minutes at 300 *g* and the supernatant was removed. These cells were then disrupted by the addition of 300 µl Buffer FG1. After which 300 µl Buffer FG2, containing 3 µl QIAGEN protease, was then added to the sample followed by incubation at 65°C for 10 minutes. Following the incubation 600 µl 100% isopropanol was added to the sample and mixed thoroughly by inversion of the tube until the DNA precipitate became visible as a clump or threads. The resulting DNA was

pelleted by centrifugation at 10, 000 *g* for 3 minutes after which the supernatant was removed. The DNA was washed by adding 600 µl of 70 % ethanol and the sample was vortexed for 5 seconds. The DNA was pellet by centrifugation at 10, 000 *g* for 3 minutes, followed by the removal of the supernatant. The pellet was then air-dried until all the liquid had evaporated (at least 5 minutes). Finally, 200 µl Buffer FG3 was added and the sample was incubated at 65°C until the pellet was completely dissolved (at least 30 minutes). The isolated DNA in buffer (Buffer FG3) was then stored at 4 °C until it was used in the PCR reaction. The quantity (ng/µl) and quality (A_{260}/A_{280} = 1.8 – 2.0) of the isolated DNA was spectrophotometrically determined by making use of a NanoDrop® (ND-100 Spectrophotometer)

3.5.2. Principle of the cytosine extension assay (CEA)

The CEA is a methylation sensitive restriction enzyme based method used for genome wide methylation analysis (Unterberger *et al.*, 2012). In this study the CG methylation sensitive enzyme HpaII and the CG methylation insensitive enzyme MspI were used. These enzymes are isoschizomeric enzymes that recognise the same tetranucleotide sequence (5'-CCGG-3') but differ in sensitivity towards DNA methylation. HpaII does not cleave DNA if there is a 5-methyl group present at the internal C residue of the recognition site; MspI cleaves DNA irrespective of the presence of a methyl group at this position, but does not cleave DNA if there is a 5-methyl group present at the external C residue of the recognition site (Pogribny *et al.,* 1999; Wentzel *et al.,* 2010). Both of these enzymes leave a 5' guanine overhang after DNA cleavage, which is then followed by single nucleotide extension with radiolabeled deoxycytidine triphosphate ([^{3}H]dCTP) (Pogribny *et al.,* 1999; Wentzel *et al.,* 2010; Unterberger *et al.*, 2012). Cleavage with HpaII reveals unmethylated sites and the parallel reaction with MspI serves as a measure of the total number of possible cut sites, and also the efficiency of enzyme digestion (Pogribny *et al*, 1999, Unterberger *et al.,* 2012).

3.5.2.1. Procedure for the CEA

The CEA as described by Wentzel *et al.* (2010) was performed in order to evaluate the global DNA methylation status. Briefly, a 1 x enzyme digestion reaction consisting of 500 ng DNA, 3 µl 10 x buffer Tango and 15 U MspI or HpaII (Fermentas) was made and topped up to a volume of 20 µl with DNase free water. The resulting reaction mixture was briefly centrifuged and placed on a heating block at 37°C for 2 hours in order for the enzyme digestion to take place. It was found that better results were obtained when an additional 10

U MspI or HpaII was added to the respective reaction mixtures after the initial 2 hours incubation period and it was again incubated at 37°C for 2 hours. The incubation period was followed by a 20 minute heat inactivation period at 65°C. The enzyme digests were stored at 4°C, meanwhile a 1x CEA reaction mixture consisting of 5 µl 5x Taq buffer, 1 µl 25 mM $MgCl_2$, 5 U GoTaq polymerase (Promega) and 0.1 µl [^{3}H]dCTP ,topped up to a volume of 15 µl with DNase free water, was made. Then, 5 µl of the respective enzyme digests were diluted in 5 µl DNase free water, 15 µl of the CEA reaction mixture was added to the diluted enzyme digest and it was incubated at 56 °C for 1 hour for cytosine incorporation. Following the incubation period samples were placed on ice and 200 µl of PBS was added to each sample in order to stop the incorporation reaction. The samples were then transferred to Whatman DE-81 ion exchange filters and washed three times with PBS. Filters were air dried at room temperature overnight. Scintillation counting in 9 ml Ultima Gold™ XR (Perkin Elmer®) was performed in a liquid scintillation analyzer (Perkin Elmer® and Quantasmart™ version 3.00.5 Tri-Carb™ LSC software). Background counts were automatically subtracted from enzyme-treated samples. All samples were counted twice until sigma = 2%. Values were expressed as disintegrations per minute (dpm).

The global CpG methylation was determined by using the following equation:

Percentage CpG methylation = 100 – Hpa/Msp x 100 **Equation 3.5**

3.6. Assessment of the *h*OGG1 promoter methylation status

After exposure of cells to 150 and 500 µM H_2O_2 (section 3.2.1) for one, three, four, five and six hours, cells were harvested (section 3.2.2) and genomic DNA was isolated (section 3.5.1). Restriction enzyme digestion followed by SYBR® Green-based real-time PCR (qPCR) detection was used in order to analyse the *h*OGG1 promoter methylation status (CpG island location Chr3:9766123-9767011) after exposure to H_2O_2.

3.6.1.1. Principle of the method

The EpiTect® Methyl DNA restriction kit (Qiagen) which makes use of two enzymes ; a methylation-sensitive enzyme (Enzyme A) and a methylation-dependent enzyme (Enzyme B), was used to digest genomic DNA in preparation for real-time PCR analysis of a known and predicted CpG island in the promoter of the gene of interest (*h*OGG1). The principle of the method is based on the detection of the remaining input DNA after cleavage of the DNA

with these two enzymes. The different methylated DNA fractions are detected by performing four digests. The mock digest product (M_o) contains all of the input genomic DNA and no enzyme. The product of the methylation-sensitive digest (M_s) contains the hypermethylated DNA fraction as cleavage of the DNA with the methylation-sensitive enzyme digests unmethylated and partially methylated DNA. The product of the methylation–dependent digest (M_d) contains the unmethylated DNA fraction as cleavage of the DNA with the methylation-dependent enzyme preferentially digests methylated DNA. Finally, the product of the double digest (M_{sd}), in which both enzymes are used, measures the background as well as the success of the both enzymatic digestions and the fraction of DNA refractory to enzyme digestion (EpiTect® Methyl qPCR Assay Handbook 11/2011).

3.6.1.2. Procedure

The manufactures protocol was strictly followed. Briefly, 5 x digestion buffer was thawed on ice and vortexed. Genomic DNA was diluted by using 1 µg of Genomic DNA, adding 26 µl of 5 x digestion buffer and adjusting the volume to 120 µl with DNase free (PCR grade) water. Finally, the dilution mixture was vortexed and briefly centrifuged. The setup of the digestion mixtures are given in table 3.1:

Table 3.1: Set up for enzyme digestion reactions

COMPONENT	TUBE			
	Mo	Ms	Md	Msd
Dilute genomic DNA (µl)	28	28	28	28
ddH_2O (DNase-free) (µl)	2	1	1	---
Enzyme A* (µl)	---	1	---	1
Enzyme B (µl)**	---	---	1	1
Total (µl)	30	30	**30**	**30**

***Enzyme A: Methylation sensitive enzyme; Enzyme B: Methylation dependent enzyme*

The digestion mixtures were then briefly vortexed, centrifuged, and then incubated for 16 hours at 37 °C in a thermocycler (Biometra). Following the 16 hour incubation period enzymes were inactivated at 65 °C for 20 minutes. The DNA digests were stored at -20°C until they were used in the PCR reactions.

3.6.2.1. Principle of the qPCR assay

Following the digestion of the DNA (as described in section 3.6.1.2.) the remaining DNA in each individual enzyme reaction is quantified by real-time PCR. The EpiTect® Methyl real-time PCR (qPCR) assay, which consists of a mix of two primers that enable the analysis of the promoter methylation status of the gene (*h*OGG1) of interest, was used. Sequence information about the primers are not available but the assay reference number is supplied in Appendix A. SYBR® Green-based, quantitative, real-time PCR is used to determine the relative amounts of methylated, unmethylated and intermediary methylated DNA by comparing the amount of DNA in each digest with that of the mock (no enzyme added) digest (EpiTect® Methyl qPCR Assay Handbook 11/2011).

3.6.2.2. Procedure for qPCR

The 7500 Real-Time PCR instrument from Applied Biosystems™ together with the EpiTect® Methyl qPCR assay (Qiagen) for *h*OGG1 and the KAPA SYBR® FAST Master Mix (2x) ABI Prism™ was used. A 1x PCR mix containing 12.5 µl 2x master mix, 10.5 µl DNase free water and 1 µl primer assay was made. For each digest 24 µl of this mix was then added to 1 µl of the enzyme digests. All reactions were performed in duplicate and a no template control (NTC), containing all the reagents of the reaction mixture but no DNA (enzyme digest), was included in each run. Air bubbles were removed and the mixture was centrifuged for 1 minute at 500 *g*.The PCR run was configured as follows: 95°C for 10 minutes; followed by 40 cycles of (97°C for 15 seconds; 72°C for 60 seconds), the cycling program was followed by the default melting curve for the 7500 Real-time PCR instrument. The 10 minute step at 95°C caused the activation of the HotStart DNA polymerase and the 60 second cycle at 72°C was necessary for the detection and recording of the SYBR® Green fluorescence at the end of the 72°C annealing, extension step of each cycle. The cycle threshold (Ct) was chosen manually at 0.02 and was set to the log linear range of the amplification plot and kept constant throughout the investigation.The baseline was set by using a linear view of the amplification plots; the instrument was then set to use readings from cycle 2 through to the cycle just before the earliest visible amplification which was found to be cycle 15. The baseline was also kept constant throughout the investigation. Data analysis was performed by making use of the raw Threshold Cycle (C_t) values and the Methylation data analysis single assays Excel-based data analysis template which was downloaded from www.sabiosciences.com/dna_methylation_data_analysis.php

3.6.2.3. Conformation of the presence of the PCR product

The presence of the PCR product (259 bp) was confirmed by running the PCR product for each digest on a 2 % agarose gel. The 2 % agarose gel was prepared by diluting 2 g of low EEO molecular grade agarose (WhiteSci) in 100 ml 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA. pH 8) and adding 10 µl of a 10 mg/ml ethidium bromide solution. The samples were loaded on to the 1.5 mm lanes of the gel by mixing 3 µl of the PCR product with 2 µl of 5 x loading gel (Gel Pilot, Qiagen). A 50 bp DNA ladder (Fermentas) was used. Electrophoresis took place at 100 mA, 80 V for 90 minutes.

3.7. Expression of *h*OGG1

Cells were exposed to 500 µM H_2O_2 (section 3.2.1) for three, four, five and six. The expression of *h*OGG1 was then investigated through mRNA isolation following exposure to H_2O_2, cDNA synthesis and real-time PCR.

3.7.1. mRNA isolation

RNA was isolated from cells by making use of a NucleoSpin® RNAII kit (Macherey Nagel).

3.7.1.1. Principle of the NucleoSpin® RNAII kit

Cells are lysed by the addition of lysis buffer which contain large amounts of chaotropic ions and immediately and temporary inactivates RNases (O'Leary 1999). It also creates appropriate binding conditions which favors the adsorption of RNA to the silica membrane (filters). β-mercaptoethanol (β-ME) is also added to the lysis buffer. β-ME is a reducing agent which irreversibly denatures RNases by reducing disulfide bonds and destroying the native conformation required for enzyme function (Nelson *et al.,* 2005). An rDNase removes contaminating DNA which could also bind to the silica membrane. Salts, metabolites and macromolecular cellular components are removed by simple washing steps with the appropriate buffers. Pure RNA is eluted under low ionic strength conditions with RNase free water (Macherey-Nagel, 03/2011, Rev 13)

3.7.1.2. Protocol used for the NucleoSpin® RNAII kit

The manufactures instructions were used and a few modifications were made in order to obtain optimum results. Cells were lysed by adding 350 µl RA1 (lysis) buffer directly to each

well, which was mixed by pipetting and then transferred 2 ml eppendorf tubes, after which 3.5 µl β-ME was added to each sample and mixed briefly by vortexing. The mixture was then transferred to a purple ring filter column in collection tubes and centrifuged for 1 minute at 11 000 *g*. The column was discarded and 70 % ethanol was added to the filtrate in the collection tubes, mixed by pipetting, transferred to a blue ring filter column in a new collection tube and centrifuged for 30 seconds at 11 000 *g*. The filtrate was discarded and the column placed in a new collection tube. Then, 350 µl membrane desalting buffer (MBD) was added to each filter and the collection tube and column was centrifuged for 1 minute at 11 000 *g*, the filtrate was discarded and the tubes were centrifuged again for 30 seconds at 11 000 *g*. A 1x mixture containing 10 µl rDNase and 90 µl R_xn buffer was made and 95 µl of this mixture was added to each filter and incubated at room temperature for 15 minutes. Following the incubation period 200 µl RA2 (wash) buffer was added to each filter and centrifuged for 30 seconds at 11 000 *g*. The filtrate was discarded and the column was placed in a new collection tube and 600 µl RA3 (wash) buffer was added to each filter. Centrifugation was performed at 11 000 *g* for 30 seconds. The filtrate was discarded and the column placed in microcentrifuge tubes for mRNA elution by the addition of 40 µl RNase free water to each filter, which was then incubated for 1 minute at room temperature. Samples were then centrifuged for 1 minute at 11 000 *g*, the filtrate was placed on the filter again and centrifuged again for 1 minute at 11 000 *g*. The quantity (ng/µl) and quality (A_{260}/A_{280} = >2.00) was determined with a NanoDrop® (ND-100 Spectrophotometer) and the isolated mRNA was then stored at –20 °C.

3.7.2. cDNA synthesis

cDNA synthesis was performed from the isolated mRNA (section 3.7.1) by making use of a AMV reverse transcriptase (SBS Genetech Co.,Ltd.)

3.7.2.1 Background

An oligo-dT primer is used to prime all mRNAs simultaneously if the mRNA has a 3'-poly(A) tail. cDNA is synthesized from the mRNA by making use of the AMV reverse transcriptase (RNA-dependent DNA polymerase).

3.7.2.2. Procedure

A 1x reaction mixture consisting of; 1 µl of 10 µM oligo-dT primer (Oligo dT,18mer; IDT), 1 µl of 10 µM 18s rRNA reverse primer (sequence information given in Appendix A), 1.5 µg RNA (maximum 8 µl), was made and the volume was adjusted to 10 µl with PCR grade water. The reaction mixture was incubated at 65°C for 2 minutes so that the primers could anneal to the RNA templates. Following the 2 minute incubation period, samples were put on ice for 5 minutes. Meanwhile, a 1x mixture containing 2 µl 5 x AMV RT buffer, 3 µl HPLC grade water, 2 µl dNTP mix (Novagen, 10 mM of each dNTP), 0.5 µl (40 U/µl) RNasin® (ribonuclease inhibitor from Promega) and 0.5 µl AMV reverse transcriptase (25 U/µl, SBS Genetech Co.,Ltd), was made and 10 µl of this mixture was then added to the samples that were placed on ice. The samples were placed in a thermocycler (Biometra) and incubated overnight (approximately 16 hours) at 45°C. Following the incubation period, the samples were kept at 94°C for 5 minutes in order to stop the reaction. The synthesized cDNA was stored at 4°C. Prior to being used in the real-time PCR gene expression assay, cDNA was diluted 1:10 with PCR grade water.

3.7.3. Real-time PCR for gene expression

The gene expression profile of hOGG1 was determined by real-time PCR with the 7500 Real-Time PCR instrument from Applied Biosystems™ by making use of TaqMan® gene expression assays (Applied Biosystems™). These TaqMan® gene expression assays for the gene of interest (*h*OGG1) and the reference gene (*18S* rRNA) contains the forward and reverse primers for the respective genes as well as the FAM™ TaqMan® MGB probe. Sequence information about the primers and probes are not available but the assay reference numbers are supplied in Appendix A:

3.7.3.1. Procedure

The TaqMan® gene expression assays for *h*OGG1 (Applied Biosystems™) was performed with minor adjustments to the manufactures original protocol in order to obtain optimum results. Single plex reactions were used as both the gene of interest (*h*OGG1) and the reference gene (*18S* rRNA) contain the FAM™ TaqMan® MGB probe. All reactions were performed in triplicate and a no template control (NTC), containing all the reagents of the reaction mixture but no DNA, was included in each run. A 1x reaction mixture consisting of

10 µl 2.5x TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 1 µl *h*OGG1 TaqMan® gene expression mix or 1 µl 18s rRNA TaqMan® gene expression mix and 5µl PCR grade water, was made. For each reaction 4 µl of the respective diluted cDNA was then added to the reaction mixture. Air bubbles were removed and the mixture was centrifuged for 1 minute at 500 *g*. The PCR program was set to 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence reading took place at the end of the 60°C annealing, extension step of each cycle. The 7500 System SDS Software version 2.05 (Applied Biosystems, 7500 Real Time PCR System) was used to analyze the obtained results. A relative quantification study was performed when the run was complete, gene expression results were analyzed using a control sample as a calibrator. The relative quantities of the targets were normalized against the relative quantities of the endogenous control, 18s rRNA. The cycle threshold (Ct) was chosen manually at 0.025 and was set to the log linear range of the amplification plot and kept constant throughout the investigation.

3.8. Statistical analysis

The statistical significance of changes observed after the treatment of cells with H_2O_2 relative to the control group was determined by making use of a unpaired student's t-Test, with a two tailed distribution where $p<0.1$ was considered as indicating statistical significance. The statistical analysis was only used to support the obtained results as statistical significance or the lack thereof does not necessarily imply biological relevance or a lack thereof (Martínez-Abraín, 2008; EFSA, 2011).

CHAPTER 4
Results and discussion

4

4.1. Introduction

Cells of the mammalian body are exposed to both exogenous and endogenous sources of ROS, e. g. endogenously produced H_2O_2 (Kryston *et al.,* 2011). The latter can occur during the reduction of oxygen to water during mitochondrial respiration when the electron transport chain leaks electrons to oxygen resulting in superoxide (O_2^-) which can be converted to H_2O_2 (Turrens*,* 2003; Friedberg *et al.,*2006). The formed H_2O_2 can then react with DNA and cause DNA damage. The oxidation of guanine bases by H_2O_2 produces 8-oxoguanine (8-oxoG), which is potentially mutagenic (Malayappan *et al.,* 2007). This lesion, irrespective of the source of the ROS, is commonly recognised and removed by enzymes involved in the base excision repair (BER) pathway (Audebert *et al.,* 2002; Collins, 2009), however, continued exposure of cells to H_2O_2 could lead to the saturation of this and other defences. The replacement of guanine with 8-oxoG can also influence the DNA methylation pattern (Hitchler and Domann*,* 2009).

Changes in the global DNA methylation pattern affects the genomic stability of cells (Shvachko, 2009), whereas the change in the promoter methylation status of specific genes leads to changes in gene expression (up or down regulated) (Hitchler and Domann*,* 2009), both of which could be detrimental to cells and contributes to aging and cancer (Issa, 1999; Liu *et al.,* 2011). However, exposure of cells to H_2O_2 and other ROS could also prove to be beneficial to cells (Sedelnikova *et al.,* 2010), for instance, the production of H_2O_2 by macrophages and neutrophils play a role in the innate immunity, killing bacteria and other pathogens (Guyton and Hall, 2006).

In this study oxidative stress was induced by exposing cultured cells to H_2O_2. Oxidative damage to DNA can be used as an index of oxidative stress (Collins, 2009). The effect of H_2O_2 exposure on the DNA integrity was firstly investigated by making use of various forms of the comet assay to investigate the DNA damage and repair. This was done in order to determine which concentrations of H_2O_2 to use for the DNA methylation studies and also to make sure that the observed changes in the DNA methylation pattern did in fact occur because of changes in the integrity of the DNA caused by oxidative stress. Subsequently the effect of H_2O_2 exposure on the DNA methylation pattern (globally and gene specific with

regard to the promoter methylation status of *h*OGG1) and the expression of the *h*OGG1 gene which is involved in the BER pathway was then evaluated. These results are presented and discussed in this chapter

All the results were processed by making use of Microsoft Office Excel 2007® and plotted by making use of GraphPad Prism® (Version 5.03). All the experiments were performed at least in duplicate. The error bars in figures represent the standard deviation between the averages of duplicate repeats, unless stated otherwise.

4.2. Cell viability following H_2O_2 exposure

Cell viability was measured in order to assure that the levels of H_2O_2 used in this study were not cytotoxic and that the results are representative of healthy but stressed living cells. In order to establish the maximum level of exposure that was not harmful to cells, cells were exposed to freshly prepared H_2O_2 in culture. For *in vitro* studies guidelines suggest that doses that decrease the viability of cells by more than 30 % when compared to control groups should not be used, as it is an indicator of cytotoxicity (Tice *et al.,* 2000).

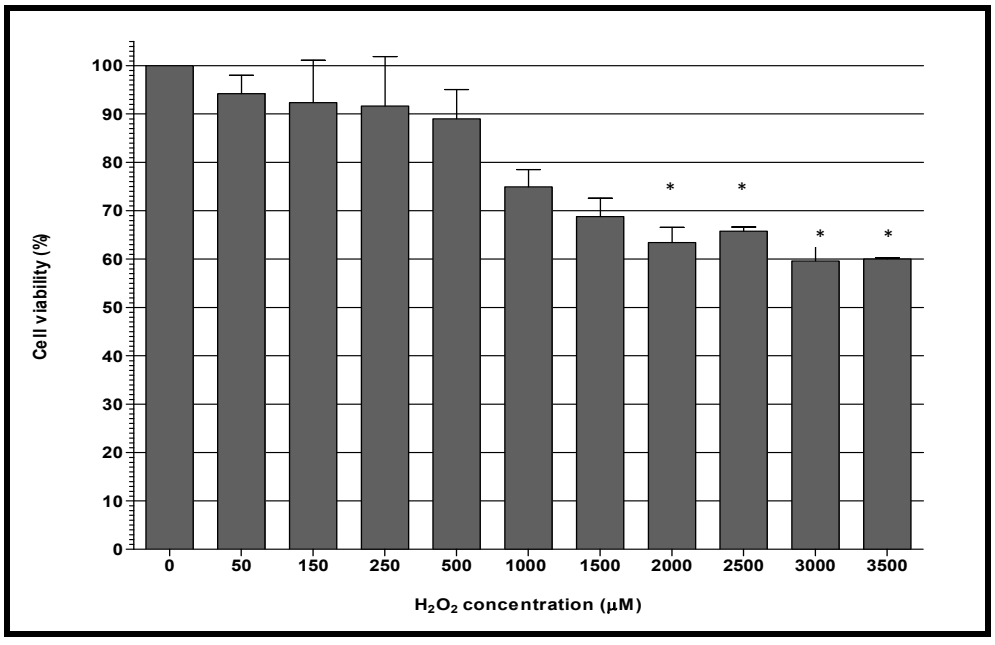

Figure 4.1: The effect of H_2O_2 exposure on the viability of 143B cells in culture. The MTT assay was used in order to determine the viability of the cells following one hour of exposure of the cells to increasing amounts of H_2O_2 in fully supplemented medium (DMEM + 10 % FBS). The triplicate repeats from two separate experiments were averaged and plotted as bars. The error bars represents the standard deviation between the averages of the two separate experiments. Cell viability was

expressed as a percentage relative to the untreated control (0 μM H_2O_2). (*p<0.1 relative to the untreated control (0 μM)).

The MTT assay was used to evaluate the cell viability of 143B cells following the exposure to H_2O_2. The cell viability was expressed as a percentage relative to the untreated control. For this study only H_2O_2 concentrations that gave a viability of above 80 % were chosen and used in order to assure that the cells remain viable. The results in figure 4.1 show that the cell viability was higher than 80 % at concentrations ranging from 50 μM to 500 μM, and declined (< 80 %) when cells were exposed to concentrations of 1000 μM and higher. In addition, it seems as if the cell viability was more drastically affected at peroxide concentrations beyond 500 μM. Based on these observations, cells were exposed to concentrations of H_2O_2 ranging from 50 to 500 μM for the remainder of the experiments. Also, although the minimum level for viable cells is 70 % the results in figure 4.1 supports the choice of using a cell viability percentage of higher than 80 % as an indication of viable cells. The reason for this is because initially a smaller decrease in the slope of the graph was observed between 50 μM and 500 μM of H_2O_2, however, as the concentration of H_2O_2 was increased to beyond 500 μM and the cell viability decreased to below 80 % a notable decrease in the slope of the graph could be observed.

4.3. Measuring DNA damage with the alkaline comet assay.

The alkaline comet assay in its most basic form can be used to detect DNA strand breaks and alkali-labile sites, which include apurinic and apyrimidinic (AP) sites or baseless sugars which appear as breaks (Collins *et al.,*2008; Collins, 2009). Following the exposure of the 143B cells to increasing concentrations of H_2O_2 in culture, cells were investigated with the basic alkaline comet assay in order to evaluate the DNA damage caused by H_2O_2 exposure. The APTD represents the percentage of DNA found in the tail of the comets and served as an indicator of the amount of DNA damage. An increase in the APTD serves as an increase in the amount of DNA damage and these results were confirmed by evaluating the class distribution of the resulting comets (figure 4.2) which gives an indication of the distribution of the data. As mentioned in section 3.4.1 and illustrated in figure 3.2, based on the tail intensity (percentage of DNA in tail), comets can be grouped into five classes ranging from class 0 with the least damage (i.e. least amount of DNA in the comet tail) through to class 4 with the highest degree of damage (i.e. highest amount of DNA in the comet tail) (da Silva *et al.,* 2000). The different classes of comets are represented in the class distribution graph, which are included in all the comet assay based results.

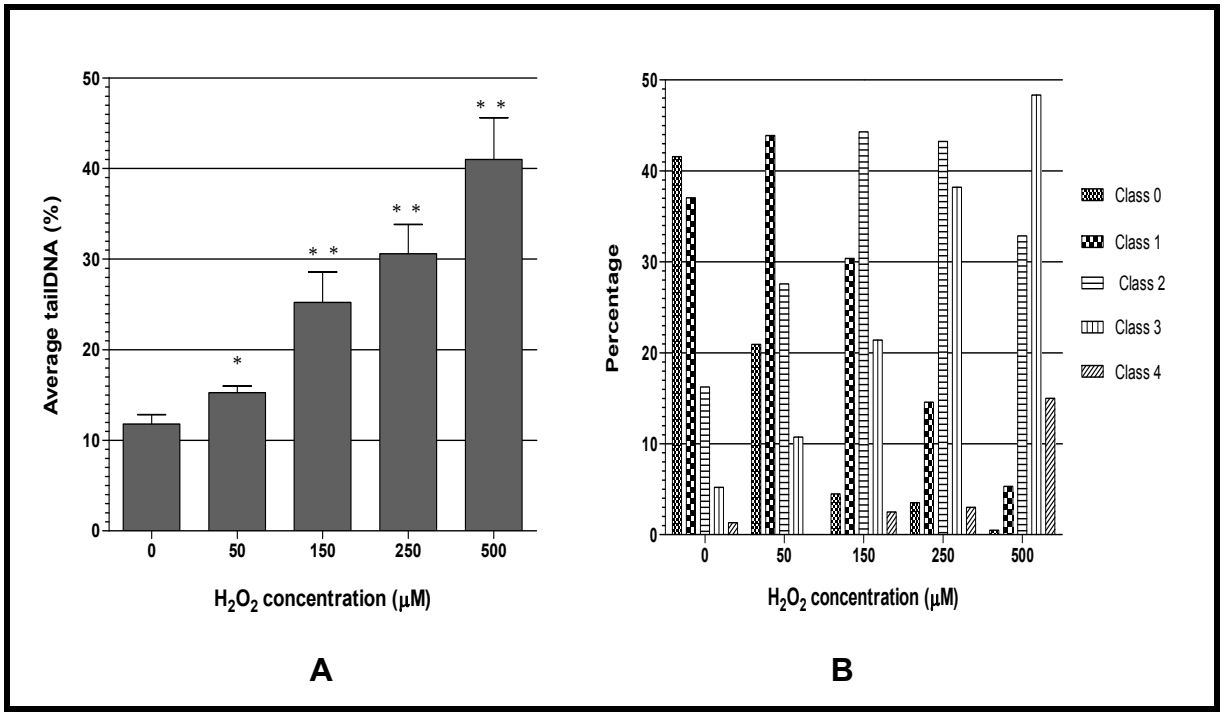

Figure 4.2: DNA damage in cultured 143B cells following H_2O_2 exposure. The basic alkaline comet assay was used to measure the DNA damage following exposure of cells to increasing amounts of H_2O_2 for one hour. (A) APTD after exposure to increasing concentrations of H_2O_2 and (B) Class distribution of cells exposed to H_2O_2. (Where: Class 0: 0- 6% tail DNA; Class 1: 6.1 – 17 % tail DNA; Class 2: 17.1 – 35 % tail DNA; Class 3: 35.1 – 60 % tail DNA and Class 4: 60.1 – 100 % tail DNA). Cells were exposed to H_2O_2 for one hour in fully supplemented medium (DMEM + 10% FBS) at 37 ºC. Results represent the average of two separate experiments consisting duplicate repeats respectively, where 50 comets were scored per individual repeat. The error bars represent the standard deviation between the averages of the two separate experiments. (*$p<0.1$ and **$p<0.05$ relative to the untreated (0 µM) control). The class distribution graph represents the combined class distribution for the two separate experiments.

It is evident from these results (figure 4.2. A) that the APTD increased as the concentration of H_2O_2 increased. Also, when comparing these results to the cell viability results (figure 4.1) it is clear that increase in the APTD represents healthy cells damaged through the exposure to H_2O_2. The reason for this is because the cell viability seemed to remain stable and represented healthy cells when using concentrations of H_2O_2 ranging from 50 µM to 500 µM, however, a notable increase in the APTD could be observed between 50 µM and 500 µM. Cells not exposed to H_2O_2 served as an estimate of the baseline DNA damage in the cells. Even though these cells were not exposed to the DNA oxidative agent, there was still a relatively small degree of damage (12 %). This can be ascribed to the fact that cells also experience a degree of endogenous oxidative damage because of normal cellular

metabolism and oxidative phosphorylation as described in section 2.3. Some variation in the baseline DNA damage occurred throughout this study (as indicated in the appropriate figures) therefore a control group was included in every experiment performed. The class distribution graph (figure 4.2 B) indicates that the comets from the control group (0 µM H_2O_2) were predominantly found in class 0 and 1 (0 – 6 % and 6.1 -17 % tail DNA respectively) which indicated the least damage. As the concentration of H_2O_2 increased, the class distribution moved more toward the higher damaged groups, until ultimately at 500 µM H_2O_2 class 3 (35.1 – 60 % tail DNA) was dominant and the percentage of class 4 (60.1 – 100 % tail DNA) comets also increased, which served as indicator that the degree of DNA damage increased with an increase in H_2O_2 concentration.

4.3.1. Measurement of oxidative DNA damage after exposure to H_2O_2 in culture

The results obtained up until now with the basic alkaline comet assay (figure 4.2) are not necessarily an indication of oxidative damage to DNA. There are two reasons for this:

(i) The observed increase in tail DNA following peroxide exposure of the cells, can also be a result of intermediates in cellular DNA repair (Cipollini *et al.,* 2006; Nossoni, 2008)

(ii) Cells were exposed to H_2O_2 in culture in fully supplemented medium (DMEM + 10 % FBS), FBS protects cells from apoptosis and oxidative stress (Francis, 2010). The serum was not removed because growing the cells in the absence of serum will have a wide range of effects on the metabolism of the cells; the presence of the serum was deemed to be more “natural” conditions for the cells.

Therefore, the basic alkaline comet assay was modified by including the use of the oxidative DNA damage lesion-specific enzymes: endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (Fpg) (figure 4.3). Both of these enzymes detect oxidized bases in cells treated with H_2O_2 (Collins, 2004) and the use of these enzymes make the comet assay more sensitive en specific for the detection of oxidative DNA damage(Collins *et al.,* 2008.; Collins, 2009; Nossoni, 2008). This method was previously applied in our laboratory to measure oxidative damage inflicted on DNA (Huysamen, 2005; Van Dyk, 2005).

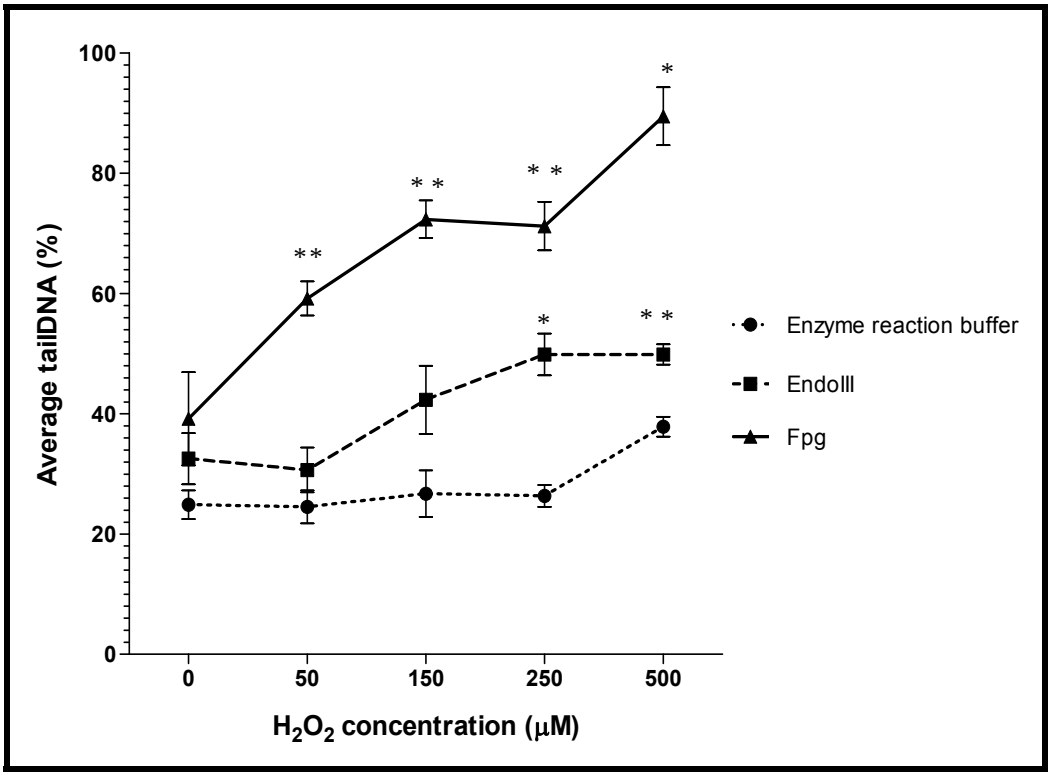

Figure 4.3: The detection of oxidised bases in peroxide exposed 143B cells. The comet assay was modified by the use of formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III) following exposure to H_2O_2, in culture (DMEM supplemented with 10 % FBS) for one hour at 37ºC. Results represent the averages of two separate experiments, where 50 comets were scored in each individual experiment. The error bars represent the standard deviation between the averages of the two separate experiments. (*p<0.1 and **p<0.05 relative the enzyme reaction buffer group)

These results (figure 4.3) indicate that when the treatment of the H_2O_2 exposed DNA with enzyme reaction buffer (non-enzyme treated group) was compared to those treated with restriction enzymes, Endo III and Fpg respectively, a higher increase in the APTD was observed in the latter. These enzymes recognise oxidised pyrimidines (Endo III) and purines (Fpg) and convert them to additional DNA breaks that increase the tail intensity of the comets (Collins *et al.,* 2008). This increase in the APTD therefore represents oxidative damage DNA (Andersson and Hellman, 2005; Collins, 2009) and increased with increasing amounts of H_2O_2. When comparing the results from the enzyme reaction group to the results in figure 4.2.A the APTD seems to remain constant in response to H_2O_2 up to the point where cells were treated with 250 μM H_2O_2, whereas a linear increase in the APTD could be observed in figure 4.2.A. One of the short comings of the comet assay is that inter- and intra-experimental variability occurs (Collins, 2004; Nossoni, 2008) this variability can explain the difference in the results even though similar experiments were performed.

However, when comparing the APTD of the Endo III and Fpg group to that of the enzyme reaction group in figure 4.3 these results do indicate how the use of these enzymes makes the comet assay more sensitive and specific for the detection of oxidative DNA damage because:

- A linear increase in the APTD with increasing amounts of H_2O_2 could be observed however, at lower amounts of H_2O_2 this increase is less apparent for non-enzyme treated group in comparison to Endo III and Fpg treated groups.
- An increase in the APTD could be observed for the control DNA (0 µM H_2O_2) between the non-enzyme treated groups and the enzyme treated groups (24 % (no enzyme) to 32 % (Endo III) to 39 % (Fpg)). The significance of the inclusion of these enzymes in the comet assay is emphasized by the revealing of specific DNA-lesions in even the control DNA.
- Furthermore, the APTD of the non-enzyme treated group increased from 26 % at 250 µM H_2O_2 to 37 % after exposure to 500 µM H_2O_2. The treatment of DNA exposed to 500 µM of H_2O_2 with the restriction enzymes indicated that there was an increase in the APTD from 37 % (non-enzyme treated group) to 49 % (Endo III) and to 89 % (Fpg). Relative to the non-enzyme treated group these increases in the APTD observed with the enzyme treated groups had statistical significance ($p<0.1$) and biologically it serves as indication of increased oxidative DNA damage caused by the exposure of the cells to H_2O_2.

Finally, the increase of the APTD was significantly more with Fpg compared to the Endo III treated DNA. This could be ascribed to the fact that Fpg not only measures oxidised purines but also ring-opened purines or formamidopyrimidines (Fapy) (Collins *et al.,* 2008). Fpg also enhances the DNA damaging effect of alkylating agents and it should therefore be considered that Fpg sensitive sites represent more than just DNA bases damaged by oxidation (Speit *et al.,* 2004). However, for this study, cells were exposed to H_2O_2 which oxidises guanine to 8-oxoG, which is a major substrate for Fpg (Andersson and Hellman, 2005) and the increasing APTD observed in figure 4.3 could therefore be ascribed as a result of increasing oxidative DNA damage with increasing concentrations of H_2O_2.

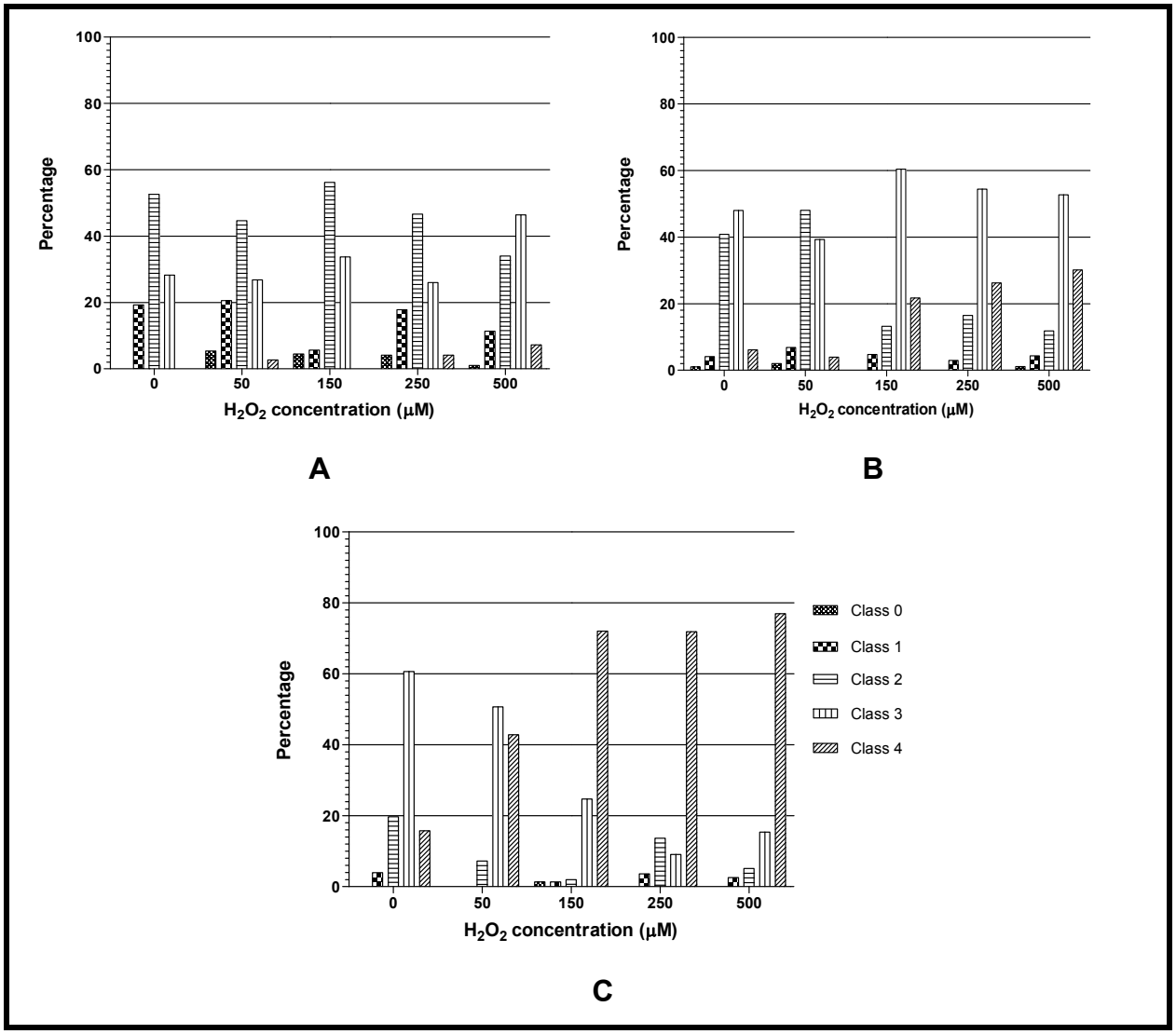

Figure 4.4: The class distribution of the comets after H_2O_2 exposure. Substrate DNA treated with (A) non-enzyme treated group (enzyme reaction buffer)), (B) Endo III and (C) Fpg. (Where: Class 0: 0- 6% tail DNA; Class 1: 6.1 – 17 % tail DNA; Class 2: 17.1 – 35 % tail DNA; Class 3: 35.1 – 60 % tail DNA and Class 4: 60.1 – 100 % tail DNA). Results represent the combined class distribution for two separate experiments, where 50 comets were scored in each individual experiment.

The class distribution graph (figure 4.4) supports the previous results (figure 4.3) that showed an increase in the amount of oxidative DNA damage following exposure of cells to increasing amounts of H_2O_2, that was detected when using the restrictions enzymes in conjunction with the comet assay (non-enzyme treated group < Endo III < Fpg). The class distribution graph for the non-enzyme treated group (figure 4.4 A) indicates that comets were found in lower damaged classes in comparison to Endo III and Fpg treated groups. Also, when comparing the class distribution graph from the Endo III treated group (figure 4.4 B) to that of the Fpg treated group (figure 4.4 C) it indicates that comets were found in lower

damaged classes for Endo III treated group in comparison to the Fpg treated group. The comets for the Fpg treated group was overall found be in the highest damaged groups.

In summary, treatment of DNA from 143B cells with these oxidative DNA damage lesion specific enzymes revealed that there was an increase in the level of oxidative DNA damage caused with increasing concentrations of H_2O_2 even though the cells were exposed to H_2O_2 in medium supplemented with 10 % FBS. The next step in this investigation was to establish whether exposure of the cells to H_2O_2 also affected their DNA repair capacity and is described in the next section.

4.3.2. Measurement of the DNA repair capacity (DRC) of 143B cells following exposure to H_2O_2

The activity of the BER following the exposure to H_2O_2 was investigated by making use of a modified form of the alkaline comet assay that makes use of protein extracts prepared from the cultured cells. The initial steps of the BER pathway (i.e. recognition and incision) are investigated through the use of this method. Previous studies were performed in our laboratory in order to investigate if an increase in APTD following the treatment of substrate DNA with protein extract is truly due to the addition of the protein extract (Van Dyk *et al.,* 2010). In this investigation control substrate DNA was treated with control- and heat inactivated protein extracts. Results showed that the heat inactivated protein extract did not cause an increase in the APTD, while treatment with control protein extract did lead to an increase in APTD. The same method that was used by Van Dyk *et al.* (2010) was used in this study. Therefore, in this study an increase in the APTD upon treatment of the substrate DNA with the respective protein extract was used to indicate protein activity. The DRC was quantified by the extent to which treatment with the protein extract increased the APTD compared to the untreated group. The higher the increase APTD following treatment with the protein extract the better the repair activity (DRC) of the cells.

4.3.2.1. DRC at increasing levels of exposure to H_2O_2

The *in vitro* DNA repair assay measures the ability of the repair enzymes in a cell extract to detect and make single strand breaks at the site of specific lesions in a DNA substrate (Cipollini *et al.,* 2006; Gaivão *et al.,* 2009). For this study H_2O_2 was used which creates a specific lesion such as 8-oxoG that is repaired by the BER. The effect of the increasing levels of H_2O_2 exposure on the DRC of 143B cells was investigated by making use of a

control protein extract, i.e. protein extract prepared from cells that were not exposed to H_2O_2. The substrate DNA was prepared from cells that were exposed to H_2O_2 and a control group was included. Active excision repair proteins increases the APTD of substrate DNA as it attempts to excise the lesions that are present on the DNA. Therefore, the control protein extract used for this part of the study should theoretically have the optimal repair capacity as there are no perturbations present that could influence the capacity of the DNA repair enzymes to excise the damaged bases.

An increase in the APTD between the substrate DNA not treated with protein extract and the substrate DNA treated with protein extract (figure 4.5) indicates that there was excision enzyme activity. From the results it can be seen that there was an increase in the APTD of the H_2O_2 exposed substrate DNA (not treated with protein extract or - PE) with an increase in the H_2O_2 concentration. This increase is expected as the APTD of the H_2O_2 exposed substrate DNA in the absence of protein extract treatment measures the amount of DNA damage. The amount of DNA damage increases with increased amounts of H_2O_2 to which cells were exposed. The increase the APTD that is observed following protein extract treatment (+ PE) serves as indication of active excision activity of the proteins involved in the initial steps of the BER and therefore the repair activity of the cells. At first glance it seems that the repair activity is not affected by the increase in the amount of H_2O_2 used. However, when comparing the APTD measured when exposing substrate DNA to increasing amounts of H_2O_2 (- PE) to the APTD measured when treating the substrate DNA with protein extract (+ PE) the increase in the APTD between the – PE and the + PE group is smaller as the amount of H_2O_2 used increases, which serves as an indication that the excision activity of the proteins under investigation is affected when exposing substrate DNA to increasing amounts of H_2O_2.

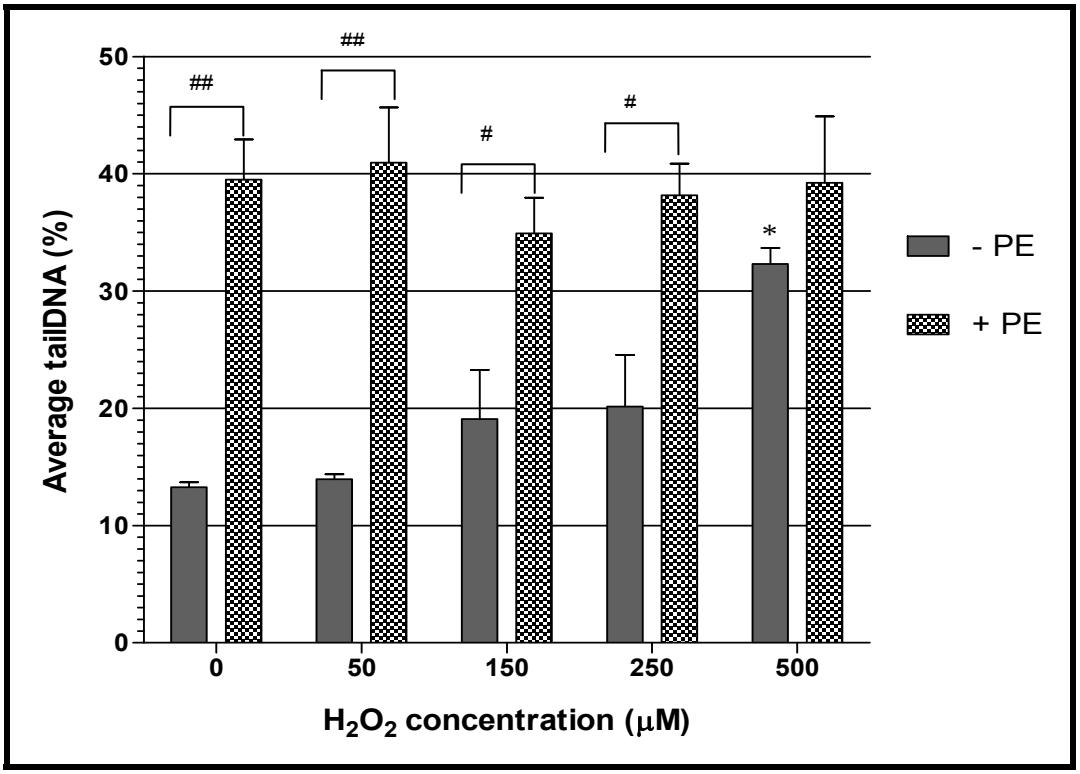

Figure 4.5: The repair of H_2O_2 exposed substrate DNA. A modified form of the alkaline comet assay which uses protein extract (PE) to perform *in vitro* DNA repair was used, the APTD without treatment with control protein extract (- PE) and following treatment with control protein extract (+ PE) when cells were exposed to increasing amounts of H_2O_2 in culture (DMEM supplemented with 10 % FBS) at 37ºC for one hour are given. The - PE group represents the damage induced by H_2O_2, whereas the + PE group represents the repair activity of the control PE when applying the control PE to substrate DNA exposed to increasing amounts of H_2O_2 for one hour. Results represent the average of two separate experiments consisting duplicate repeats respectively, where 50 comets were scored per individual repeat. The error bars represent the standard deviation between the averages of the two separate experiments. (*$p<0.05$ relative to the untreated (0 μM) control also, ##$p<0.05$ and #$p<0.1$ – PE relative to + PE)

The class distribution results (figure 4.6 B) supports the previous notion that the slight decrease (on average) observed in the APTD of +PE does not seem to be of any importance when not comparing it to the increase in the APTD of –PE. The class distribution of the H_2O_2 exposed substrate DNA not treated with protein extract (- PE) **(**figure 4.6 A) shows a notable increase in the number of comets present in classes representing a higher degree of DNA damage (class 2, 3 and 4) at 500 μM H_2O_2 from comets found predominantly in the classes representing a low degree of damage (class 0 and 1) when the cells were exposed to 0 μM H_2O_2. This change in the class distribution was not as apparent in the H_2O_2 exposed substrate DNA treated with the control protein extract (+ PE) (figure 4.6. B).

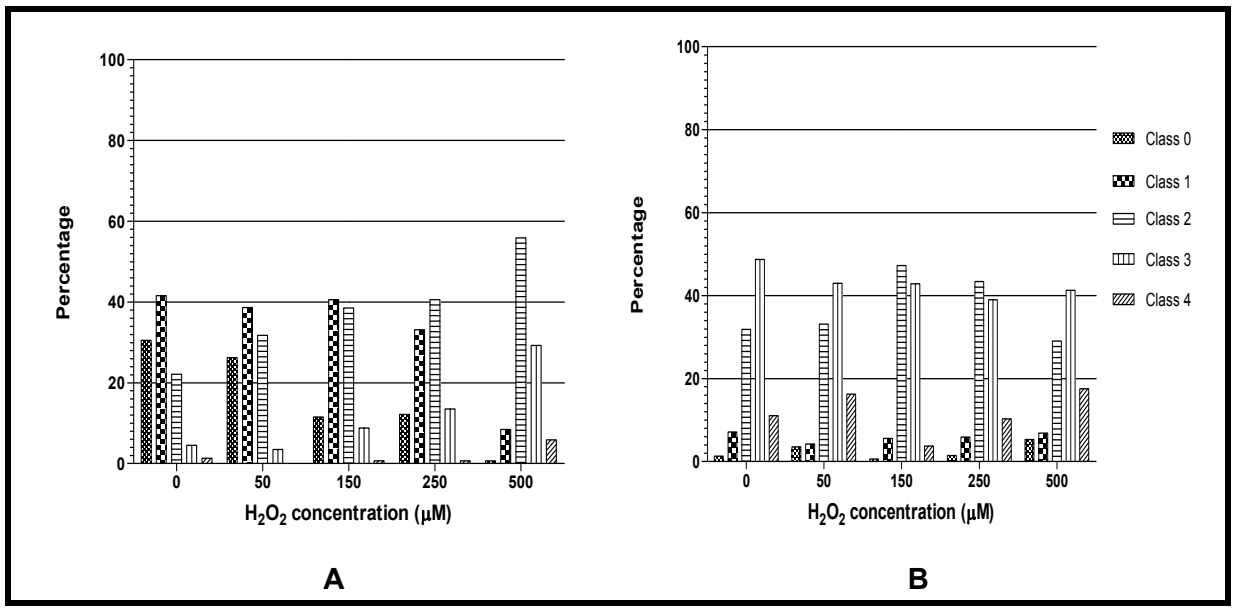

Figure 4.6: The class distribution of the comets from the H_2O_2 exposed substrate DNA. (A) Without protein extract and (B) following treatment with control protein extract. (Where: Class 0: 0-6% tail DNA; Class 1: 6.1 – 17 % tail DNA; Class 2: 17.1 – 35 % tail DNA; Class 3: 35.1 – 60 % tail DNA and Class 4: 60.1 – 100 % tail DNA). Results represent the combined class distribution for two separate experiments consisting of duplicate repeats respectively, where 50 comets were scored in each individual experiment.

The DNA repair capacity (DRC) of 143B cells exposed to H_2O_2 was calculated as described in section 3.4.3.2.4 (equation 3.4). The optimal DRC, which was represented by the control substrate DNA treated with the control protein extract, was found to be approximately 0.67 arbitrary units for the 143B cells as shown in figure 4.7. The DRC 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 DRC value that approaches one has the ideal repair ability.

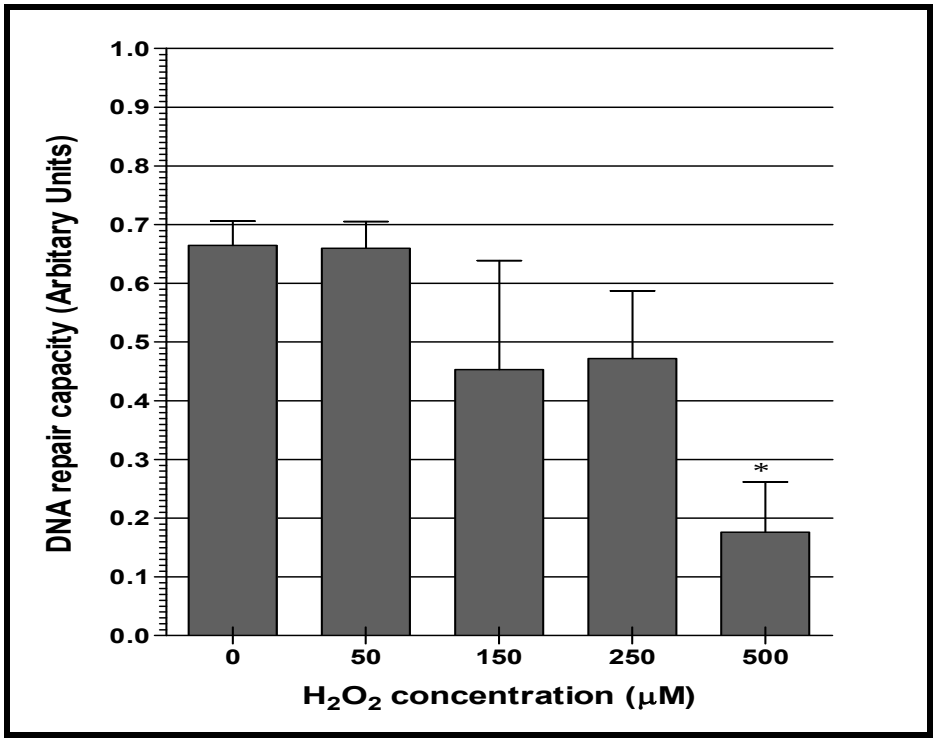

Figure 4.7: The DNA repair capacity of 143B cells following exposure to H_2O_2. The DRC of control protein extract when substrate DNA was exposed to increasing amounts of H_2O_2 for one hour is given. Results represent the average of two separate experiments consisting duplicate repeats respectively, where 50 comets were scored per individual repeat. The error bars represents the standard deviation between the averages of the two separate experiments. (*$p<0.05$ relative to the untreated (0 μM) control).

The results revealed a decrease in the DRC of 143B cells with an increase in the H_2O_2 concentration. This could indicate that as the amount of damage that is induced increases the repair activity of the repair enzymes under investigation gets overwhelmed (defences are saturated) to such a point that the DRC decreases.

4.3.2.2. The effect of increasing levels of H_2O_2 on the DRC

The *in vitro* DNA repair assay was also used in order to measure the effect of oxidation on the repair enzymes involved in the initial steps of the BER. Exposure to ROS affects signal transduction systems as high levels of oxidative stress alters the signal pathways through oxidative damage of cell membranes, activation of transcription factors and changes in enzyme activity (Klaunig *et al.*, 1998). In order to evaluate the effect of H_2O_2 exposure on the repair enzymes protein extracts were prepared from 143B cells exposed to H_2O_2 in culture. Both the control substrate DNA and the H_2O_2 exposed substrate DNA were treated with these protein extracts, and the DRC was calculated in order to see if the control- and H_2O_2

exposed substrate DNA had a similar response to the protein treatment. The H_2O_2 exposed substrate DNA was derived from 143B cells exposed to 100 µM H_2O_2. Protein extracts included a control protein extract and protein extracts prepared from 143B cells exposed to H_2O_2 (50, 150, 250 and 500 µM). As discussed in section 4.4.2.1. theoretically the control protein extract should have the optimal repair capacity and the introduction of perturbations such as ROS should have a negative influence on the repair capacity. Control substrate DNA that was not treated with protein extract was also included in order to measure the optimal DNA repair activity in the protein extract.

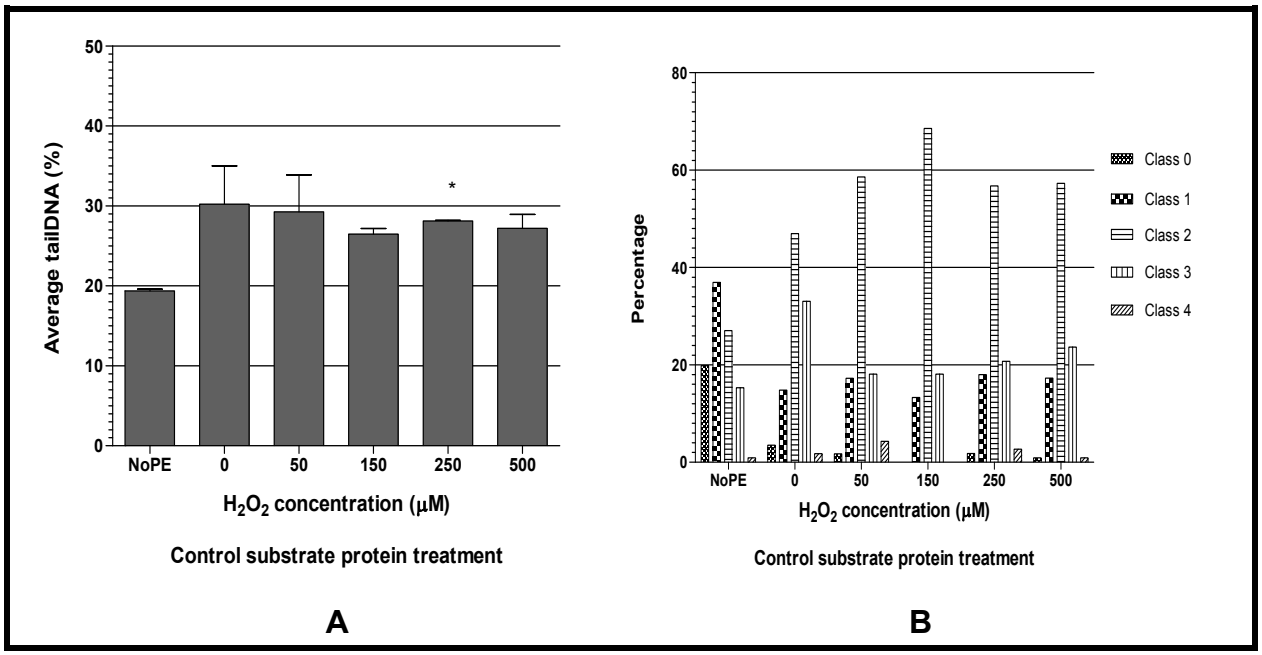

Figure 4.8: The effect of oxidation on the repair of control substrate DNA. A modified form of the alkaline comet assay which uses protein extract (PE) to perform *in vitro* DNA repair was used. The results for the control substrate DNA (i.e. not exposed to H_2O_2) treated with PE prepared from cells exposed to increasing amounts of H_2O_2 (50 – 500 µM) for one hour are given. A group that was not treated with PE (NoPE) and a control PE (0 µM H_2O_2) was also included. The repair activity is quantified by the degree of the increase in the APTD observed between the substrate DNA not treated with PE and the substrate DNA treated with PE. (A) The APTD of control substrate DNA upon treatment with protein extracts prepared from 143B cells exposed to increasing amounts of H_2O_2 for one hour; (B) The class distribution of the control DNA substrate after protein treatment. (Where: NoPE; No protein treatment; Class 0: 0- 6% tail DNA; Class 1: 6.1 – 17 % tail DNA; Class 2: 17.1 – 35 % tail DNA; Class 3: 35.1 – 60 % tail DNA and Class 4: 60.1 – 100 % tail DNA). Results represent the average of two separate experiments, where 50 comets were scored per individual experiment. The error bars represent the standard deviation between the averages of the two separate experiments. (*$p<0.05$ relative to NoPE). The class distribution graph represents the combined class distribution for the two separate experiments.

In figure 4.8 A it can be seen that there was an increase in the APTD between the control substrate DNA not treated with protein extract and the control substrate DNA treated with control protein extract. This increase in the APTD indicated that there was DNA repair enzyme activity. When comparing the increase in APTD following treatment with control protein extract to the increase in APTD following treatment with protein extract prepared from cells exposed to H_2O_2 a slight decrease in the APTD could be observed. At first glance it seems that this decrease in the APTD occurs with an increase in the H_2O_2 concentration. However, when taking the class distribution (figure 4.8 B) into account this observed decrease did not seem to be of significance, as there seemed to be no notable change in the class distribution between the control substrate DNA treated with the protein extracts prepared from cells exposed to H_2O_2. The only notable change in the class distribution could be observed between the control substrate DNA that did not undergo protein treatment, the control substrate DNA treated with the control protein extract and the control substrate DNA treated with protein extract prepared from cells exposed to H_2O_2. This difference in class distribution did not depend on the concentration of H_2O_2 used.

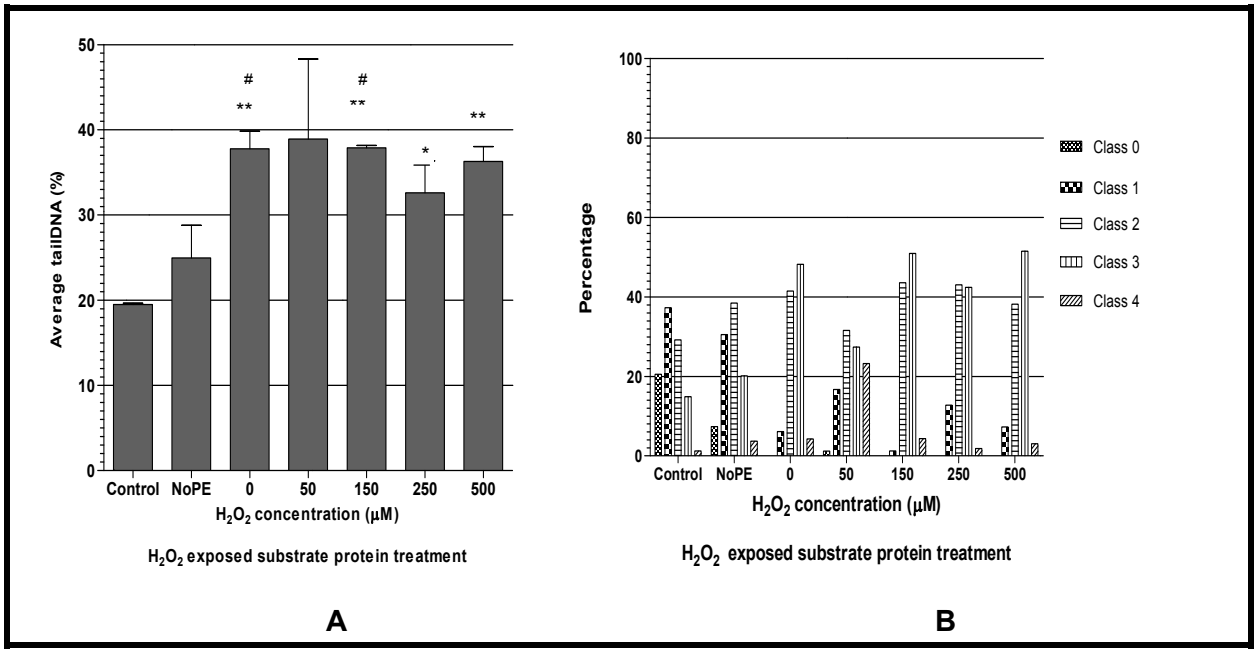

Figure 4.9: The effect of oxidation on the repair of H_2O_2 exposed DNA. A modified form of the alkaline comet assay which uses protein extract (PE) to measure *in vitro* repair activity was used. The results for the H_2O_2 exposed substrate DNA (exposed to 100 µM of H_2O_2) treated with PE prepared from cells exposed to increasing amounts of H_2O_2 (50 – 500 µM) for one hour are given. A control group (not exposed to H_2O_2 and not treated with PE) as well as a group that was not treated with PE (NoPE) and a control PE (0 µM H_2O_2) was also included. The repair activity is quantified by the degree of the increase in the APTD observed between the substrate DNA not treated with PE and the

substrate DNA treated with PE. (A) The APTD of H_2O_2 exposed substrate DNA upon treatment with protein extracts prepared from 143B cells exposed to increasing amounts of H_2O_2 for one hour, (B) The class distribution of H_2O_2 exposed DNA substrate after protein treatment. (Where: NoPE; No protein treatment; Class 0: 0- 6% tail DNA; Class 1: 6.1 – 17 % tail DNA; Class 2: 17.1 – 35 % tail DNA; Class 3: 35.1 – 60 % tail DNA and Class 4: 60.1 – 100 % tail DNA). Results represent the average of two separate experiments, where 50 comets were scored per individual experiment. The error bars represent the standard deviation between the averages of the two separate experiments. (**$p<0.05$ and *$p<0.1$ relative to control; #$p<0.1$ relative to NoPE). The class distribution graph represents the combined class distribution for the two separate experiments.

In figure 4.9 the APTD and the class distribution of the H_2O_2 exposed substrate DNA following protein treatment is given. A control substrate DNA group was used as an indicator of the background level of DNA damage prior to the H_2O_2 treatment and as mentioned earlier some variation in the baseline DNA damage was observed from 12 % in figure 4.2. A to 19 % in figure 4.9 A which lead to the inclusion of a control group for every experiment. An increase in the APTD (figure 4.9 A) between the H_2O_2 exposed substrate DNA without protein treatment and the H_2O_2 exposed substrate DNA with protein treatment represents increased excision enzyme activity. When comparing the increase in APTD following the treatment with control protein extract to the increase in APTD following the treatment with protein extract prepared from cells exposed to H_2O_2 (figure 4.9 A) no notable change in the APTD was evident, except at 250 µM H_2O_2, where there was a slight decrease in the APTD. When taking the class distribution of the comets (figure 4.9 B) into account, this decrease in the APTD did not seem to be of significance, as there seemed to be no notable change in the class distribution between the H_2O_2 exposed substrate DNA treated with the control protein extract and the H_2O_2 exposed substrate DNA treated with the protein extracts prepared from the cells exposed to 250 µM H_2O_2. The only real notable change in the class distribution could be observed between the H_2O_2 exposed substrate DNA that did not undergo protein treatment and the H_2O_2 exposed substrate DNA treated with the protein extracts with more comets found in the higher damaged classes (class 2, 3 and 4) following protein treatment in comparison to the control group and the group not treated with protein extract. This difference in the class distribution did not depend on the concentration of H_2O_2 used.

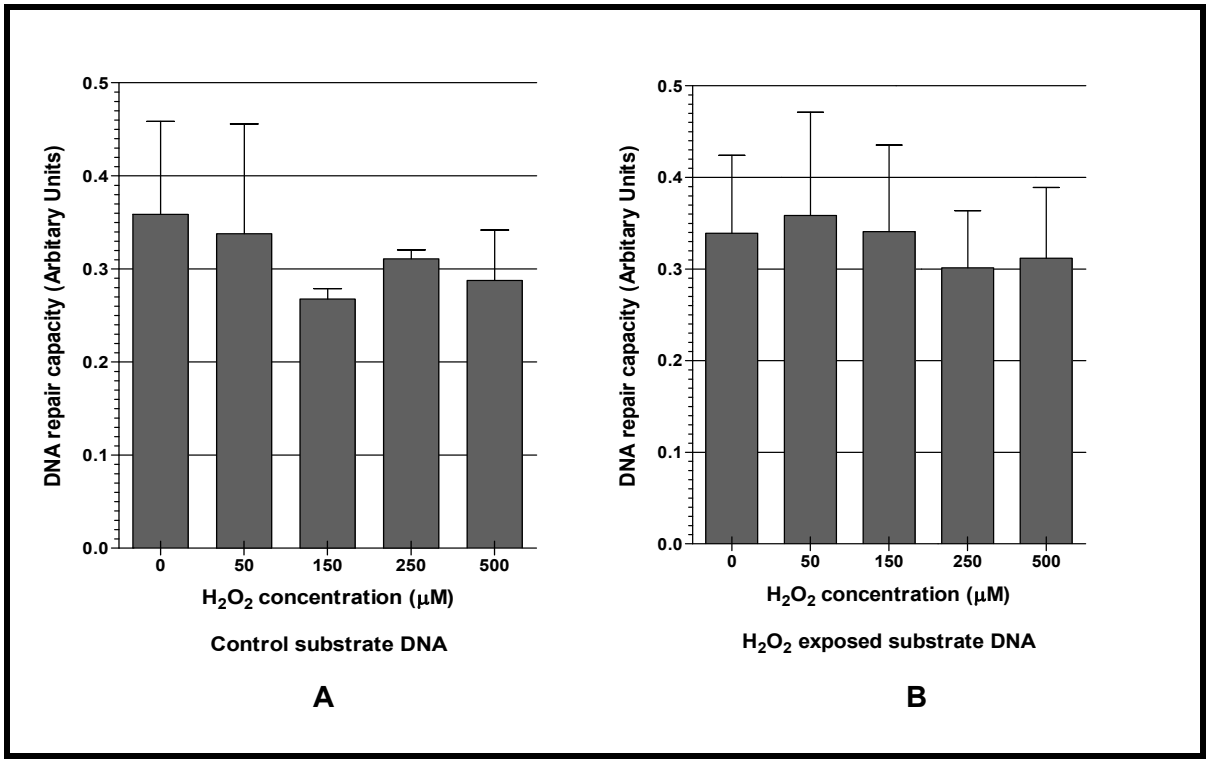

Figure 4.10: The effect of H_2O_2 exposure on the DNA repair capacity of 143B cells. Substrate DNA was treated with protein extracts prepared from 143B cells exposed to increasing amounts of H_2O_2 for one hour. (A) The DRC of control substrate DNA and (B) The DRC of H_2O_2 exposed DNA substrate. Results represent the average of two separate experiments, where 50 comets were scored per individual experiment. The error bars represent the standard deviation between the averages of the two separate experiments.

Using these results, the DRC of the control substrate DNA and the H_2O_2 exposed substrate DNA was calculated as described in section 3.4.3.2.4 (equation 3.4) and are represented in figure 4.10. The significance of the inclusion of these protein extracts in the comet assay is emphasized by the (slight) decrease in the DRC observed between the treatment of the control substrate DNA with control protein extract (0 μM H_2O_2) and protein extracts prepared from cells exposed to H_2O_2 ($p > 0.1$) (figure 4.10 A). However, the results for the H_2O_2 exposed (damaged) substrate DNA did not reveal any noteworthy change in the DRC when using protein extracts prepared from cells exposed to increasing amounts of H_2O_2 (figure 4.10 B). A decrease in the DRC would have been expected as the exposure of the cells to H_2O_2 would induce more damage to DNA, in comparison to the control group, that would provide the excision repair enzymes with the more target (damaged) substrate and evoke more enzyme activity however, it would have been expected that the oxidation caused by the exposure to H_2O_2 would have decreased the excision activity of the enzymes. This was not found to be true.

Also, the treatment of the control substrate DNA with the control protein extract (figure 4.10.A) should serve as an indicator of the optimal DRC of 143B cells. However, in section 4.3.2.1 the optimal DRC of 143B cells was found to be approximately 0.67 arbitrary units and the results in figure 4.10 A shows an optimal DRC of approximately 0.36 arbitrary units. One of the short comings of the comet assay is that inter- and intra-experimental variability occurs (Collins, 2004; Nossoni, 2008), this can be observed when comparing the APTD of the control in figure 4.5 (approximately 13 %) to that in figure 4.8 (approximately 19%) and figure 4.9 (approximately 20 %). This variability can be used to explain the difference in the observed optimal DRC between these experiments.

In summary: The results given in this section illustrate the effect of H_2O_2 on the DNA damage and repair of cultured eukaryotic cells. The occurrence of false positive results was excluded by only using concentrations that were not cytotoxic and hence did not affect cell viability. Also, following the harvesting process with trypsin, cells were allowed to repair for one hour in order to minimalize the damaging effect that this serine protease could have on the cellular integrity. Results obtained with the basic alkaline comet assay revealed a direct relationship with the increase in the level of DNA damage and the increase the amount of H_2O_2 the cells were exposure to. The use of the oxidative DNA damage lesion-specific enzymes Endo III and Fpg made it possible to conclude that the bulk of the increase in DNA damage occurred because of an increase in DNA oxidation. Finally, it was found that the DRC of 143B cells decreased with an increase in the level of exposure to H_2O_2, possibly because the exposure to increasing levels of H_2O_2 (ROS) leads increased amounts of DNA damage which in turn leads to decreased activity of the repair enzymes involved in the initial steps of the BER.

The damage caused by DNA oxidation has the potential to lead to mutations if not repaired by the appropriate DNA repair machinery (Cooke *et al.,* 2003; Kryston *et al.,* 2011), thereby negatively influencing the integrity of the genome of an organism. In addition, the literature alludes to a link between oxidative stress conditions and deviations in DNA methylation in mammalian cells (Cerda and Weitzman, 1997; Cyr and Domann, 2011). The focus of the next part of this study is, therefore, whether the observed damage to DNA by peroxide treatment is accompanied by deviations in the DNA methylation pattern of the 143B cells, both globally and promoter specific.

4.4 Measurement of the global CpG methylation status

The CEA (cytosine extension assay) offers the opportunity to assess changes in the DNA methylation level in global DNA and within CpG islands as was first described by Pogribny *et al.* (1999) and applied in our laboratory (Wentzel *et al.,* 2010). The effect of the exposure of 143B cells (in culture) to H_2O_2 on the global DNA methylation pattern of the cells was investigated with the CEA assay. For this study the methylation sensitive restriction enzyme HpaII, which has relatively frequent recognition sites that occur randomly throughout the genome, was used. The results therefore represent the genome wide changes in the global CpG methylation level of 143B cells that occur outside of CpG islands.

Prior to performing the CEA the MTT-assay was used to determine the viability of the cells following exposure of the cells to 150 and 500 µM H_2O_2 for six hours. The same parameters as described in section 4.2 were used to evaluate the viability of the cells. The results in figure 4.11 show that after six hours of exposure cells are viable at both concentrations (i.e. % cell viability > 80 %). However, in comparison with results in figure 4.1 a decrease in the cell viability could be observed following this prolonged period of exposure. This observed decrease in the cell viability percentage between one hour and six hours of exposure did however not prove to be of statistical significance ($p>0.1$) which supports the use of these conditions (concentrations over a period of six hours) in order to evaluate changes in the DNA methylation pattern while results still represent healthy but stressed cells.

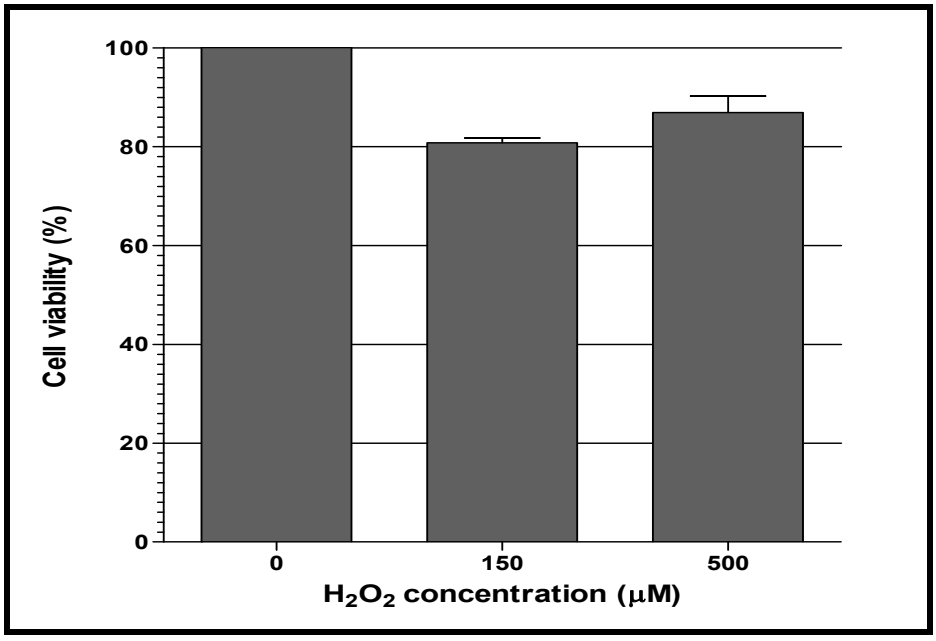

Figure 4.11: The effect of H_2O_2 exposure on the cell viability of 143B cells in culture. The MTT assay was used in order to determine the viability of the cells following six hour of exposure of the cells to 150 and 500 μM of H_2O_2 in fully supplemented medium (DMEM + 10 % FBS). The triplicate repeats from two separate experiments were averaged and plotted as bars. The error bars represents the standard deviation between the averages of the two separate experiments. Cell viability was expressed as a percentage relative to the untreated control (0 μM H_2O_2). (P>0.1 relative to the control)..

Normally cells exhibit genome wide hypermethylation of CpGs outside of CpG islands (Tost *et al.,* 2010). Hypomethylation of these CpGs leads to chromosomal and genomic instability (Shvachko, 2009). Although a cancer cell line was used for this study and a decrease in genome wide DNA methylation is associated with cancer cells in comparison to non-cancerous cells (Shvachko, 2009; Tost and Gut, 2010; Pogribny and Rusyn, 2012), DNA hypomethylation is defined as a decrease in the number of the methylated cytosine bases in comparison with the "normal" methylation level (Pogribny and Rusyn, 2012). For this study a decrease in the percentage of the global CpG methylation of the exposed cells in comparison to that of the control group was used as an indicator of possible DNA hypomethylation. Therefore, the results obtained with the control group was used as an indicator of the "normal" global CpG methylation level of the 143B cells and the change in the global CpG methylation pattern was evaluated relative to that of the control group

Cells were exposed to 150 and 500 μM H_2O_2 for one, three and six hours in order to investigate a possible threshold effect of DNA oxidation on DNA methylation. The DNA was

then isolated from the cells and the CEA was performed. These results are given in figure 4.12.

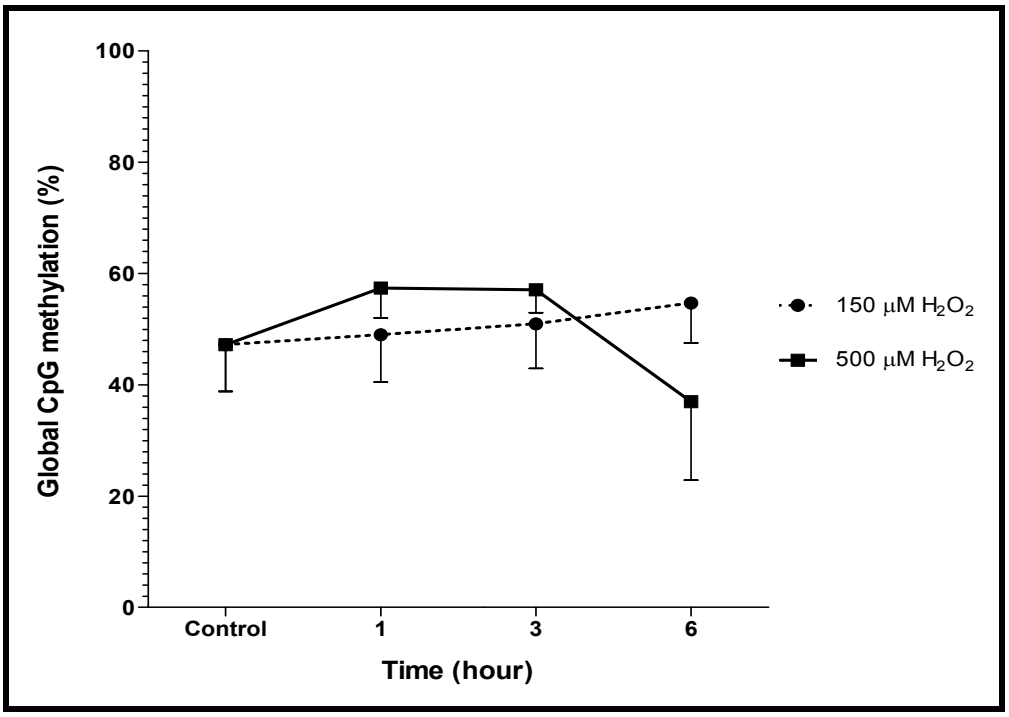

Figure 4.12: The effect of H_2O_2 on the global CpG methylation status of 143B cells. The figure represents the results obtained with the cytosine extension assay (CEA) when the effect of H_2O_2 on the global CpG methylation status at one, three and six hours was investigated following the exposure to 150- and 500 μM of H_2O_2. The change in the global CpG methylation pattern was evaluated relative to the control group (untreated cells). The results represent the average of three separate experiments performed in duplicate. The error bars represent the standard deviations between the averages of the separate experiments.

The results in Figure 4.12 show that the "normal" global CpG methylation level of the 143B cells is approximately 47 %. Exposure of the cells to 150 μM H_2O_2 did not affect the global CpG methylation status of the cells. Exposure of the cells to 500 μM H_2O_2 initially had no negative effect on the global CpG methylation status, however, after six hours of exposure there was a decrease in the global CpG methylation level from 47 % ("normal" level) to 36 %. Because the decrease in the global CpG methylation levels was observed between three and six hours, the effect H_2O_2 exposure between these two times was further investigated. Consequently, the effect of H_2O_2 exposure on the global CpG methylation status of the cells at four and five hours of exposure to 500 μM H_2O_2 was also investigated with the CEA and these results are given in figure 4.13.

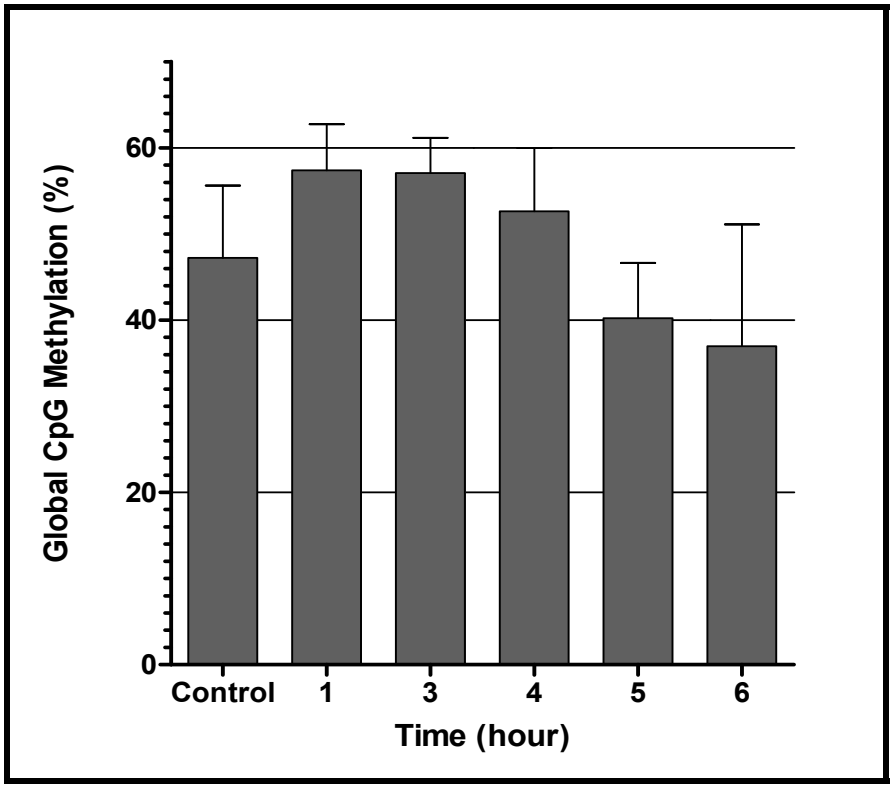

Figure 4.13: The effect of H_2O_2 on the global CpG methylation status of 143B cells. The figure represents the results with the cytosine extension assay (CEA) when the effect of the exposure to 500 µM H_2O_2 on the global CpG methylation status between three and six hours was further investigated. The change in the global CpG methylation pattern was evaluated relative to the control group (untreated cells). The results represent the average of three separate experiments performed in duplicate. The error bars represent the standard deviations between the averages of the three separate experiments.

The results in figure 4.13 show that the level of global CpG methylation, after reaching a peak after one to three hours, starts to continuously decrease after three hours of exposure to 500 µM H_2O_2. After five and six hours of exposure the level of global CpG methylation is lower than that of the control. When performing a two tailed students T-test this decrease did not prove to be of statistical significance ($p>0.1$ relative to the control). However, in cancer cells the repetitive sequences and the CpG dinucleotides found throughout the genome are hypomethylated (figure 2.8; section 2.6) therefore the effect of H_2O_2 exposure (and DNA demethylating agents) on the global CpG methylation pattern is not expected to be dramatic (Ou *et al.,* 2006). This decrease might however, be of biological relevance (EFSA, 2011) as it correlates with high levels oxidative DNA damage detected following exposure of cells to 500 µM H_2O_2 as shown in section 4.3.1. (figure 4.3). Oxidative DNA damage has been linked to DNA hypomethylation in other studies (Tunc and Tremellen, 2009; Hitchler and

Domann, 2009; Bhusari *et al.,* 2010). Therefore, exposure of the cells to the higher of the two concentrations (150 µM vs. 500 µM) possibly led to a degree of global DNA hypomethylation in the 143B cells, and the possible threshold value of exposure to this high level of DNA oxidation is roughly four hours.

In summary: The results given in this section illustrate the effect of H_2O_2 exposure on the global CpG methylation status of 143B cells. The change that was observed in the global DNA methylation level of the cells from 47 % ("normal" level) to 36 % following the exposure to 500 µM of H_2O_2 for six hours is supported by the results in section 4.3.1 which showed that the exposure of the cells to a high level of H_2O_2 (500 µM) caused an increase in the level of oxidative DNA damage measured with the oxidative DNA damage lesion specific enzymes EndoIII and Fpg. Fpg is specific for the oxidised guanine base 8-oxoG (Friedberg *et al.,* 2005), which results from the oxidation of DNA by H_2O_2 exposure, the presence of 8-oxoG next to a methylated cytosine can directly inhibit DNMTs and thereby induce demethylation of the DNA (Hitchler and Domann, 2009; Bhusari *et al.,* 2010) which would lead to a decrease in the amount of methylated cytosines found throughout the genome and possibly lead to the observed genome wide hypomethylation.

For the next part of the study, the effect of the exposure to H_2O_2 on the promoter methylation status of the *h*OGG1 gene, involved in the BER, was investigated. The *h*OGG1 gene encodes for the OGG1 protein which is responsible for the removal of oxidized and ring-opened purines which include 8-oxoG and FapyG (Friedberg *et al.,* 2005). The same DNA that was isolated from 143B cells exposed to 500 µM of H_2O_2 and used in the CEA was used for the next part of the study in order to compare the effect of the observed oxidative DNA damage on the global- (section 4.4) and promoter specific methylation status of the cells. The results for the effect of the exposure to 150 µm of H_2O_2 on the promoter methylation status of the *h*OGG1 gene in the 143B cells did not show any noteworthy change in the promoter methylation status and are given in Appendix B.

4.5. Evaluation of the promoter methylation status of the *h*OGG1 gene

The DNA methylation status of the CpG islands (location Chr3:9766123-9767011) of *h*OGG1 after exposure to H_2O_2 was investigated through restriction enzyme digestion followed by real-time PCR as described in section 3.6. Predesigned primers make it possible to detect the methylation status of the promoter region of the gene of interest (i.e. *h*OGG1). The majority of all human genes promoters contain CpG islands. The majority of the CpGs in

the genome are methylated, with the exception of CpG-islands which tend to remain unmethylated (Franco *et al.,* 2008; Espada and Esteller, 2010). A change in the methylation status of CpG islands found in the regulatory regions of genes (such as the promoter region) correlates with the transcriptional state of the cells. Hypermethylation of the CpG islands found in the promoter regions of genes represses transcription (Tost, 2010) while hypomethylation is associated with gene transcriptional activity (Tunc and Tremellen*,* 2009).

Before the results obtained for this section was considered the QC report, which is automatically generated by the EpiTect® Methyl PCR Array Excel-based data analysis template, was taken into account. The QC report for each run is given in appendix C. The template automatically calculates and displays the analytical window (W) and the percentage of DNA refractory* (R) to the restriction enzyme digestion. In order for the enzyme digestion to be efficient the following parameters are automatically set by the template:

1. The analytical window (W) of the assay, which is represented by the difference in the C_T values between the double and the mock digest, had to be greater than 2 (i.e. $C_T[M_{sd} - M_o] > 2$). When W is > 2, the results can be considered as reliable and meaningful as it means that more than 75 % of all DNA molecules in the sample are digested.
2. The analysis is reported as a “Failure” if the analytical window is less than two (W < 2) as this would mean that the refractory DNA percentage is greater than 25 % (R > 25 %)

*DNA refractory (R) to the restriction digestion = DNA that is not digested

The W was > 2 and the R was < 25 % for all of the PCR results obtained and used in this part of the study as the QC results in Appendix C shows.

Also, because restriction based PCR methods are prone to false positive results, the PCR products from each real-time PCR reaction was run on a 2 % agarose electrophoresis gel in order to confirm the presence of the 259 bp PCR product. Regardless of the drawbacks to this method, it was chosen because it offers the advantage that no bisulfite conversion of the DNA is necessary and hence smaller amounts of DNA can be used. The results for the control, three-, five- and six hours are given in figure 4.14 and indicate that the 259 bp product was indeed present. The PCR products for one and four hours were also evaluated and gave similar results as shown in appendix D.

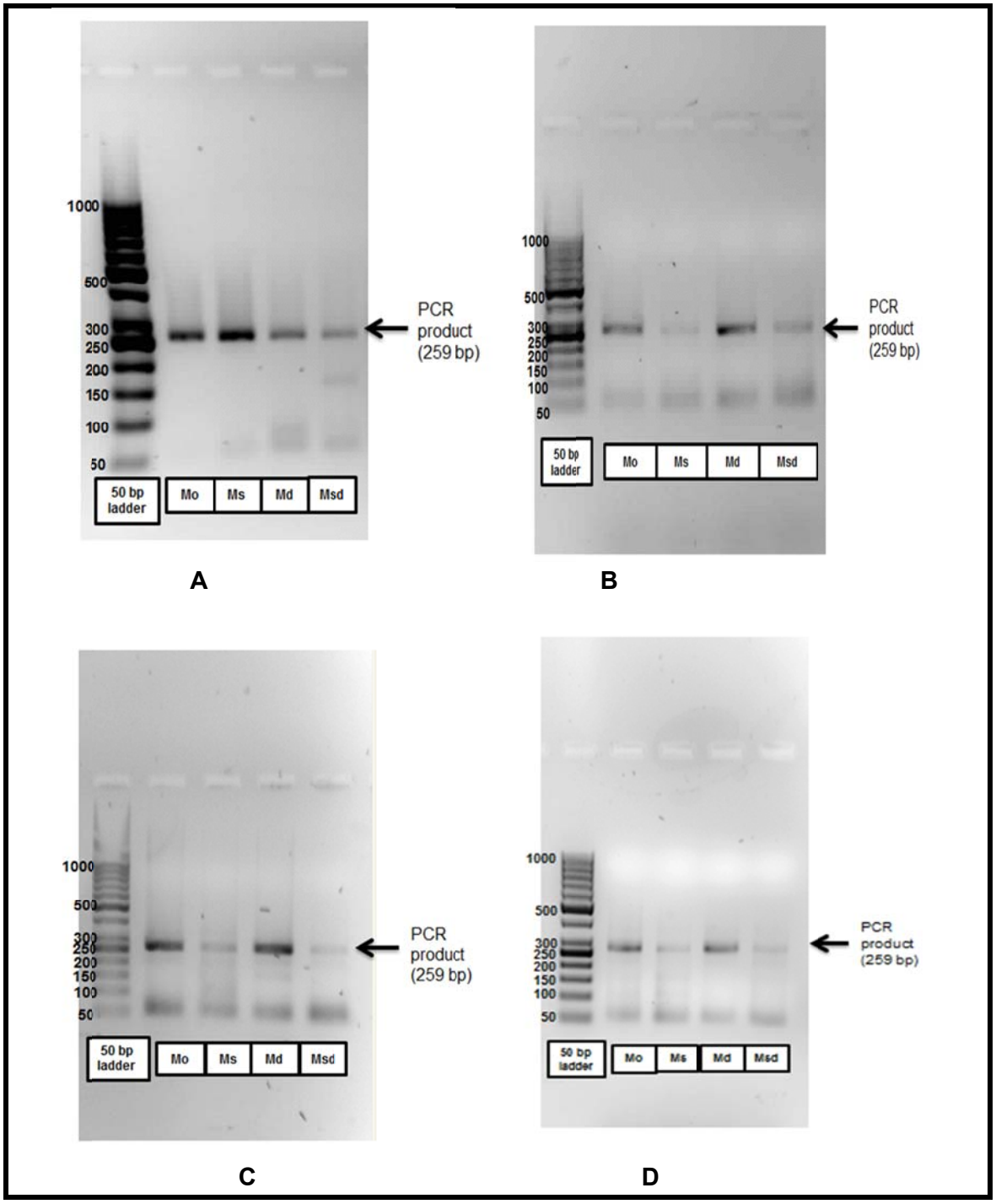

Figure 4.14: Gel electrophoresis confirmation of the PCR product. The PCR products obtained with the real-time PCR were run on a 2 % agarose gel in order to confirm if the 259 bp product was present and hence that amplification was successful. Each gel represents the different enzyme digest for samples exposed to 500 µM for different time periods respectively (A) Controll, (B) three hours, (C) five hours and (D) six hours. A 50 bp DNA ladder was used. (Mo: Mock digest; Ms: Methylation sensitive digest; Md: Methylation dependent digest and Msd: Double digest)

The results in figure 4.15 shows the fraction of the DNA that is hypermethylated (HM), unmethylated (UM) and intermediately methylated (IM) following the exposure to H_2O_2. The control group shows an initial level of hypermethylation (approximately 2 %) which could be due to the fact that a cancer cell line was used, since cancer cells have irregular methylation patterns (Kryston *et al.,* 2011). A change in the methylation pattern could be observed after three hours of exposure of the cells to peroxide. However, according to the manufacture's guidelines for data analysis and interpretation, the minimum level of hypermethylation (HM) considered to be positive (i.e. of biological relevance) can be set at 10 to 20 %. A sample can be considered to have undergone hypomethylation when the percentage of UM fraction is < 10 % and if the IM fraction of the DNA is > 60 % it may be considered as having biological significance (SABiosciences User Manual Version 2.1, 3/10/2010).

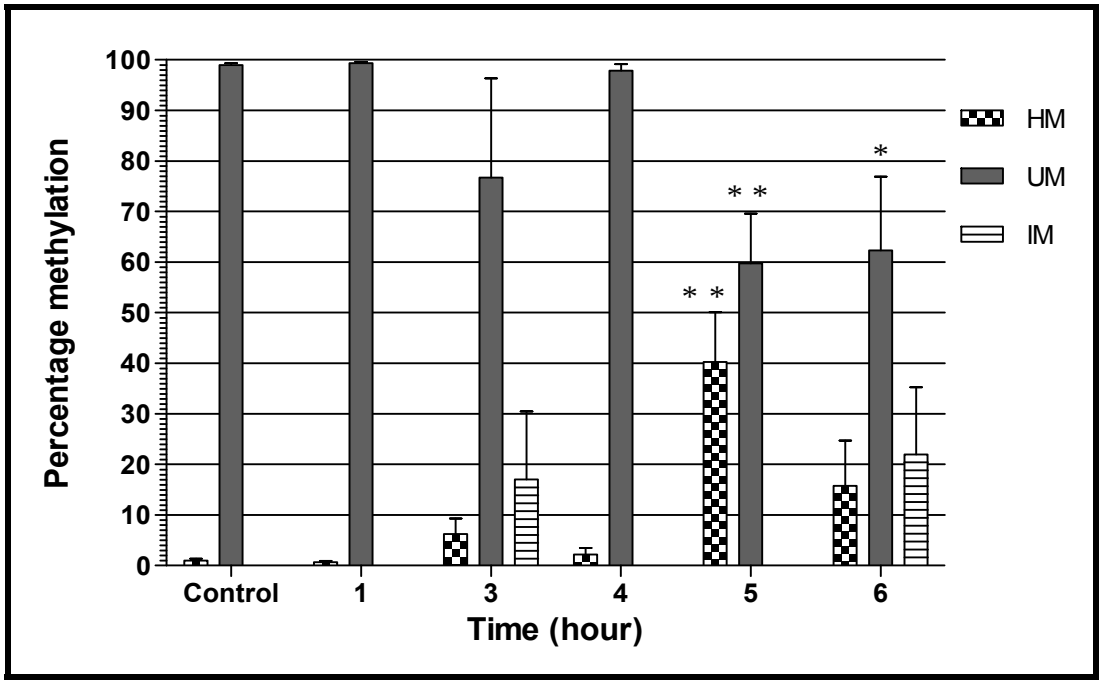

Figure 4.15: The effect the exposure to H_2O_2 on the *h*OGG1 promoter methylation status. The effect of the exposure of the 143B cells to 500 µM H_2O_2 on the promoter methylation status of the *h*OGG1 gene was investigated over a period of six hours by making use of an enzyme restriction based real-time PCR. Results represent the average of two separate experiments performed in duplicate; the error bars represent the standard deviation between the averages of the two separate experiments. (Where: HM: Hypermethylated; UM: Unmethylated; IM: Intermediately methylated). (*p<0.1 and **p<0.05 relative to the control)

When taking the guidelines into consideration, the only biological relevant changes in the methylation status of the promoter region of *h*OGG1 (figure 4.15) occurred after five and six hours of exposure to 500 µM of H_2O_2 (i.e. HM > 10 %). The percentage of the HM fragment

is approximately 40 % after five hours of exposure and approximately 16 % after six hours of exposure. However, only the observed hypermethylation after five hours of exposure (40 % HM) is supported by statistical analysis ($p<0.05$ relative to the control). There is a spike in the percentage HM DNA at three hours from the initial level (2 %) observed in the control group to approximately 7 %. However, this change is not considered to be of biological relevance (HM < 10 %).

Hypermethylation of the promoter region of genes is associated with repressed gene expression (Tost, 2010). In order to further investigate if the observed hypermethylation was of biological relevance the expression of the *h*OGG1 gene under the same conditions was investigated.

4.6. Evaluation of the effect of H_2O_2 exposure on the expression of *h*OGG1

The results in figure 4.16 show that the expression of the *h*OGG1 gene was influenced by DNA oxidation caused by the exposure of the cells to H_2O_2. Initially an increase in the level of expression could be observed at three hours of exposure which might have occurred as the cells try to repair the damage caused by the DNA oxidation. However, the expression starts to decrease at four hours; this could be a consequence of the saturation of the defence that is provided by the BER.

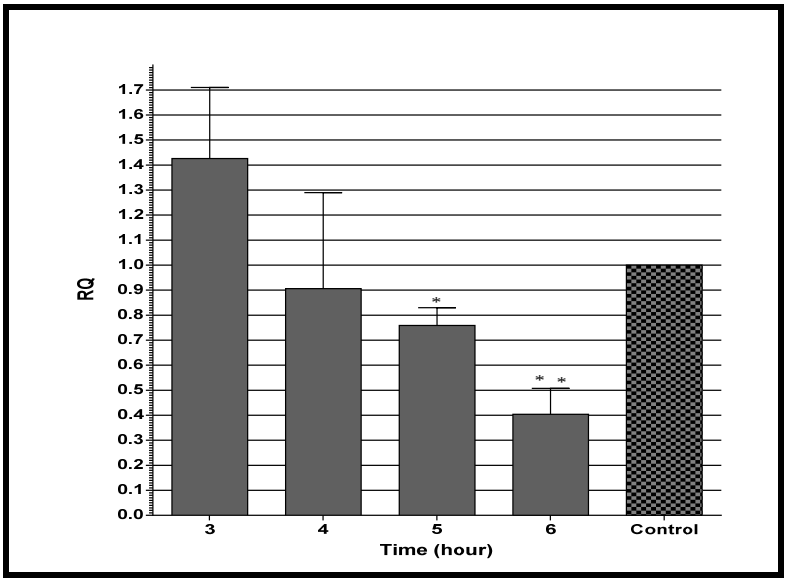

Figure 4.16: Expression of the *h*OGG1 gene. The results represent the effect of the exposure of the cells to 500 µM on the expression of *h*OGG1, over a period of six hours. Results represent the mean of two biological repeats consisting of triplicate repeats respectively. Expression levels were

normalized to 18S rRNA. Error bars represent the standard deviation between the average of the two biological repeats (Where RQ: Relative quantity) (*p<0.1 and **p<0.05 relative to the control group)

When comparing the results of the effect of DNA oxidation on the expression of the *h*OGG1 gene (figure 4.16) to the percentage hypermethylation measured in the *h*OGG1 promoter (figure 4.17) it seems that there is a relationship between the methylation status of *h*OGG1 promoter and the expression of the *h*OGG1 gene.

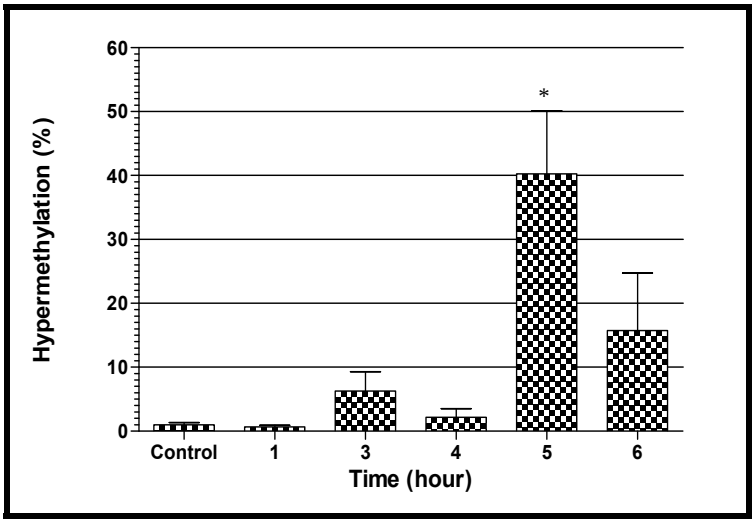

Figure 4.17: Percentage hypermethylation measured in the *h*OGG1 promoter. The effect of the exposure to 500 µM H_2O_2 on the methylation status of the *h*OGG1 promoter was investigated over a period of six hours by making use of an enzyme restriction based real-time PCR. Results represent the average of two separate experiments performed in duplicate; the error bars represent the standard deviation between the averages of the two separate experiments (*p<0.05 relative to the control group).

An investigation into the characterization of the *h*OGG1 promoter by Dhénaut *et al.,* (2000) revealed that the *h*OGG1 gene has characteristics of a housekeeping gene; with a G and C rich promoter region and two CpG islands and Alu repeats within the promoter and the 5' sequence of the transcription region. Promoter methylation has been associated with the *h*OGG1 gene (Guan *et al.,* 2008; Lahtz and Pfeifer, 2011). The observed hypermethylation of the promoter CpG islands could be caused by *de novo* methylation because oxidative stress leads to the formation of single strand breaks and these DNA single strand breaks signals *de novo* methylation (Franco *et al.,* 2008). However, a continuous decrease in the expression of the *h*OGG1 gene (figure 4.16) could be observed while a continuous increase in the hypermethylation of the *h*OGG1 promoter (figure 4.17) was not observed. When SA< levels are low 5-methylcytosine is converted to thymine by spontaneous deamination (Wu

and Zhang, 2010; LeBaron *et al.*, 2010) which could possibly be responsible for the decrease in the degree of hypermethylation of the *h*OGG1 promoter between five- and six hours of exposure to H_2O_2. The deamination of 5-methylcytosine to thymine (Friedberg *et al.,* 2006) could cause an underrepresentation of methylated cytosines which then causes a decrease in the degree of hypermethylation measured although a degree of hypermethylation of the *h*OGG1 promoter still occurred after six hours of exposure to H_2O_2.

Also, at four hours of exposure to H_2O_2 a decrease in the *h*OGG1 expression could be observed although an increase in the hypermethylation of the *h*OGG1 promoter could not be observed. Could a decrease in the expression of the *h*OGG1 gene lead to the observed gain of DNA methylation of the *h*OGG1 promoter? (Feil, 2006) Li *et al.* (2009) found that the exposure of osteoblast cells to H_2O_2 induces G2 cell cycle arrest and inhibits cell proliferation without affecting cell viability. In the literature it is stated that substances that may have an impact on the cell cycle and on cellular proliferation could alter gene expression and chromatin (Feil, 2006). Could exposure of the 143B cells which is an osteosarcoma cell line to H_2O_2 also affect the cell cycle and cellular proliferation?

The results in figures 4.16 and 4.17 do however show that the expression of the *h*OGG1 gene decreased when the *h*OGG1 promoter was hypermethylated after five hours of exposure to H_2O_2. Therefore it can be concluded that the hypermethylation of the *h*OGG1 promoter possibly causes decreased expression of the *h*OGG*1* gene.

Final summary

Exposure of 143B cells to H_2O_2 in culture lead to oxidative DNA damage that increased as the amount of H_2O_2 was used increased. A decrease in the DRC, which could have been caused by a decrease in the activity of the repair enzymes involved in the initial steps of the BER, could be observed following exposure of cells to increasing amounts of H_2O_2. Finally, a decrease in the global DNA methylation pattern as well as an increase in the promoter specific methylation pattern of the *h*OGG1 gene could be observed that was accompanied by a decrease in the expression of the *h*OGG1 gene. A link between oxidative DNA damage and DNA hypomethylation has been shown to exist (Tunc and Tremellen*,* 2009) therefore, the observed decrease in the global CpG methylation could possibly be a consequence of the observed oxidative DNA damage following H_2O_2 exposure. The observed hypermethylation of the promoter of the *h*OGG1 gene can also possibly be a consequence of oxidative stress, induced by H_2O_2 exposure, leading to the formation of single strand

breaks in DNA that triggers *de novo* methylation (Franco *et al.,* 2008) of the CpG islands located in the promoter region of the *h*OGG1 gene (Dhénaut *et al.,* 2000) leading to a gain of methylated cytosines. Hypermethylation of CpG islands located in promoter areas of genes are associated with suppressed gene expression (Tost, 2010). A decrease in the expression of the *h*OGG1 gene was observed at time intervals were hypermethylation of the *h*OGG1 promoter was also observed.

CHAPTER 5
Summary and Conclusion

5

Cells of the mammalian body are continuously exposed to ROS, both endogenously and exogenously. Oxidative stress is characterized by excessive production of or exposure to ROS to such an extent that oxygen radicals exceed the antioxidant capacity of the cell (Ziech *et al.,* 2011). Cells withstand and counteract the effects of ROS through defence mechanisms such as DNA repair mechanisms (Kryston *et al.,* 2010), which are important for the maintenance of genomic integrity and - stability. ROS induces damage to cellular macromolecules such as DNA, RNA, lipids and proteins *in vitro* as well as *in vivo* (Franco *et al.*, 2008). One of the main repair pathways through which oxidative DNA damage in mammalian cells is repaired is the BER pathway (Boiteux and Radicella, 2000; Audebert *et al.,* 2002). There is also ample evidence in the literature which shows that DNA methylation is affected by oxidative stress (Cerda and Weitzman, 1997; Franco *et al.,* 2008; Hitchler and Domann, 2009; Cyr and Domann, 2011). While DNA methylation is an important epigenetic mechanism which regulates gene expression, the presence of abnormal quantities of methylated cytosines causes genomic instability. Furthermore, the deamination of 5-methylcytosine gives rise to thymine (Friedberg *et al.,* 2006) which is prone to mutations upon replication (Cooke *et al.,* 2003; Fazzari and Greally, 2004). The thymine-guanine mismatch is repaired by the BER (Neihrs, 2009; Friedberg *et al.,* 2006; Wu and Zhang, 2010). This indicates that there is also an important link between DNA methylation and DNA repair

Continuous exposure of cells to oxidative stress could lead to the saturation of defences such as BER, which could lead to changes in the genetic as well the epigenetic profile of a cell. The aim of this study was to examine the early DNA methylation events that occur in oxidative stressed cultured mammalian cells by examining the effect of H_2O_2 exposure on the global- and promoter specific methylation pattern of 143B cells. The term *early* refers to how soon following exposure to H_2O_2 over a six hour period changes in the methylation pattern can be observed when exposing cells to H_2O_2 concentrations that causes oxidative DNA damage. Changes in the *h*OGG1 promoter methylation status were evaluated as this gene plays a crucial role in the initiation of the BER for the repair of oxidative DNA damage

One of the main objectives of this study was to demonstrate that the exposure of cells to H_2O_2 in fully supplemented medium (DMEM + 10 % FBS) causes DNA damage because of

DNA-oxidation occurring in the cells, even at low concentrations of H_2O_2, when cells were exposed to H_2O_2 for one hour. Normally cells are exposed to oxidative agents in the absence of FBS because FBS can protect cells from oxidative stress (Francis, 2010) however, for this study serum was used because the presence of serum was deemed to reflect more "natural" conditions for the cells. The results obtained with the basic alkaline comet assay (section 4.3, figure 4.2.) indicated that there was an increase in the DNA damage measured with an increase in the amount of H_2O_2 that the cells were exposed to. However, the increase in the measured damaged was not necessarily an indication of oxidative damage to DNA caused by H_2O_2 exposure, and could have resulted because of intermediates in cellular DNA repair which led to an increase in the tail DNA following peroxide exposure (Cipollini *et al.,* 2008; Nossoni, 2008). The use of the oxidative DNA damage lesion-specific enzymes, Endo III and Fpg, in conjunction with the comet assay indicated that there was however an increase in the level of oxidative DNA damage with an increase in the amount of H_2O_2 used even though cells were exposed to H_2O_2 in cell culture medium containing 10 % FBS (section 4.3.1, figure 4.3).

Also, guanine has the lowest oxidation potential of the four DNA bases and is attacked preferentially upon oxidative DNA damage, because of its electron rich purine structure which allows it to react easily with oxygen radicals (Kim *et al.,* 2004). One of the major substrates for the Fpg enzyme is the 8-oxoG base (Andersson and Hellman, 2005) which can result from exposure of cells to H_2O_2. The results indicated that there was a substantial increase in the tail DNA and therefore the amount of oxidative DNA damage measured when using Fpg in conjunction with the comet assay (non-enzyme treated group< Endo III < Fpg) even at the lowest (50 μM) of the concentrations of H_2O_2 used. This could indicate that there was a substantial increase in the level of 8-oxoG with an increase in the amount of H_2O_2 that the cells were exposed to. Also, the measured oxidative DNA damage can be used as a measure of oxidative stress (Collins, 2009) therefore cells experienced oxidative stress through the exposure to H_2O_2.

The second objective of the study was to demonstrate that proteins involved in the initial steps of the BER pathway and also the DNA repair capacity (DRC) of the cells, are affected by the exposure to H_2O_2. Protein extracts perform the initial steps of DNA excision repair, i.e. damage recognition and incision. The APTD of the comets increase as the active excision repair proteins try to excise (i.e. remove) damaged bases. The DRC was quantified by the extent to which protein treatment increased the APTD compared to the untreated group. The higher the increase APTD following protein treatment the better the repair activity (DRC) of

the cells were. The DRC 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 DRC value that approaches one has the ideal repair ability. The BER pathway is primarily involved in the repair of oxidative DNA damage; therefore the effect of H_2O_2 exposure on the proteins involved in the initial steps of the BER pathway was investigated. A decrease in the DRC could be observed when a control protein extract was used and the substrate DNA was exposed to increasing amounts of H_2O_2 (section 4.3.2.1, figure 4.7). The decrease in the DRC of the control protein extract with the exposure of the substrate DNA to increasing amounts of H_2O_2 could indicate that the repair activity of the proteins under investigation finally becomes saturated as the amount of damage in the substrate DNA increases.

The third objective of the study was to demonstrate that the global DNA methylation pattern of the cells is affected by the exposure of the cells to H_2O_2. Cells normally exhibit genome wide hypermethylation of CpG dinucleotides that occur outside of CpG islands (Tost *et al.,* 2010). DNA hypomethylation is defined as a decrease in the number of the methylated cytosine bases in comparison with the “normal” methylation level (Pogribny and Rusyn, 2012). For this study a decrease in the percentage of the global CpG methylation of the exposed cells in comparison to that of the control group was used as an indicator of possible DNA hypomethylation. Results indicated that the global DNA methylation pattern of the cells was affected by the exposure of the cells to H_2O_2 when exposing cells to high concentrations of H_2O_2 (section 4.4, figure 4.13). Comet assay based results only represented oxidative DNA damage results when cells were exposed to increasing amounts of H_2O_2 for a short (one hour) period of time. The highest of the H_2O_2 concentrations used (500 µM H_2O_2) caused the most damage after one hour of exposure and after prolonged exposure (five and six hours respectively) a degree of global DNA hypomethylation in comparison to the control group could be observed (from 47 % in control group to 40 % and 36 % respectively at five and six hours of exposure), possibly because of a further increase in the level of oxidative DNA damage. On the other hand, exposure of cells to a lower concentration (150 µM) of H_2O_2 for the same period of time did not affect the global DNA methylation pattern although a substantial amount of oxidative DNA damage could be measured when exposing cells to this level of H_2O_2 (section 4.3.1; figure 4.3).

The use of the methylation sensitive restriction enzyme HpaII in the CEA, led the observed DNA hypomethylation to represent genome wide changes in the global CpG methylation level of 143B cells that occur outside of CpG islands. Further studies are necessary in order to confirm if this observed change is accompanied by a change in the global CpG

methylation level within CpG islands located throughout the genome. The observed global hypomethylation can be ascribed to the high levels of 8-oxoG which is present following the exposure to H_2O_2, as was measured with the comet assay modified through the use of Fpg (section 4.3.1; figure 4.3). The 8-oxoG base can directly inhibit DNMTs and thereby possibly induce demethylation of DNA (Hitchler and Domann, 2009). Oxidative DNA damage has been linked to DNA hypomethylation in other studies (Tunc and Tremellen, 2009). The decrease in global DNA methylation pattern could therefore be of biological relevance. Results indicate that a high degree of oxidative DNA damage, which is caused by the exposure of cells to higher concentrations of H_2O_2, is necessary in order to affect the global DNA methylation pattern in the time frame of six hours.

The fourth objective of the study was to demonstrate that the exposure of the cells to H_2O_2 affects the *h*OGG1 promoter methylation status of the cells. The 8-oxoguanine DNA glycosylase (OGG1) gene is involved in the BER pathway and encodes the enzymes responsible for the excision of 8-oxoG. These enzymes serve as DNA glycosylases for the initiation of BER in order to preferentially repair the 8-oxoG lesion in non-transcribed DNA (Audebert *et al.,* 2002; Araneda *et al.,* 2005). Studies have revealed that the promoter (hyper) methylation has been associated with the *h*OGG1 gene (Guan *et al.,* 2008; Lahtz and Pfeifer, 2011). Results from this study also indicated that hypermethylation of the *h*OGG1 promoter occurred following exposure of cells to a high concentration (500 µM) of H_2O_2 over a time frame of six hours (section 4.5, figure 4.15.). The effect of the exposure of cells to lower concentration (150 µM) of H_2O_2 was also evaluated and although a substantial amount of oxidative DNA damage could be measured when exposing cells to this level of H_2O_2 (section 4.3.1; figure 4.3) no significant change in the *h*OGG1 promoter methylation status could be observed (Appendix B). These results also indicated that a high degree of oxidative DNA damage, which is caused by the exposure of the cells to higher concentrations of H_2O_2, is necessary in order to affect the *h*OGG1 promoter methylation pattern in the time frame of six hours

Hydrogen peroxide affects promoter methylation through the generation of hydroxyl radicals through Fenton reactions (Friedberg *et al.,* 2006). The hydroxyl radical is the most reactive of the primary ROS causing DNA damage by attacking DNA and giving rise to lesions such as single strand breaks (SSBs) (Kryston *et al.,* 2011). These single strand breaks in DNA can trigger *de novo* methylation (Franco *et al.,* 2008) which could explain the observed hypermethylation of the *h*OGG1 promoter after five hours of exposure to 500 µM of H_2O_2.

The fifth objective of the study was to demonstrate that the expression of the *h*OGG1 gene is affected by the exposure to H_2O_2. The effect of the exposure of the cells to 500 µM of H_2O_2 on the expression of the *h*OGG1 gene was evaluated as exposure of the cells to this level of peroxide affected both the global- DNA and *h*OGG1 promoter methylation status of the cells. A change in the expression of the *h*OGG1 gene following the exposure to H_2O_2 was observed. This change in expression correlated with the change in the promoter methylation status, where hypermethylation of the promoter was associated with a decrease in the expression (section 4.6, figure 4.16 and 4.17)

The final objective of the study was to demonstrate a possible relationship between the affect that H_2O_2 exposure has on the occurrence of oxidative DNA damage, the activity of proteins involved in the initial steps of the BER (the DRC), the change in the global- and *h*OGG1 promoter methylation status and the expression of the *h*OGG1 gene. This possible relationship is summarised in figure 5.1.

This figure shows that during the exposure of cultured cells to H_2O_2 in fully supplemented medium (DMEM + 10 % FBS), the H_2O_2 reacts with a metal iron in the vicinity of DNA to generate hydroxyl radicals ($^{\cdot}OH$) and other oxidants by the Fenton reaction (Friedberg *et al.,* 2006). The hydroxyl radicals then attack DNA and gives rise to damage such as base modifications and single strand breaks in DNA. Exposure to increased amounts of H_2O_2 leads to increased oxidative damage and especially prevalence of the modified 8-oxoG base, as well as a decrease in the DRC and a decrease in the repair activity of the proteins involved in the initial step of the BER possibly because of high degrees of damage to DNA that could overwhelm (saturate) the activity of the enzymes. The oxidative DNA damage and the presence of the 8-oxoG lesion contribute to the observed global DNA hypomethylation (Hitchler and Domann, 2009; Tunc and Tremellen*,* 2009). The formation of single strand breaks in DNA could account for the observed promoter methylation (hypermethylation) of the *h*OGG1 promoter, which in turn could account for the decrease in the expression of the *h*OGG1 gene.

The decrease in the activity of the repair enzymes possibly correlates with the decrease in the expression of the *h*OGG1 gene which encodes for the Ogg1 protein which acts as a DNA glycosylase and is involved in the initial steps of the BER namely the excision of 8-oxoG. However, this possibility needs further investigation.

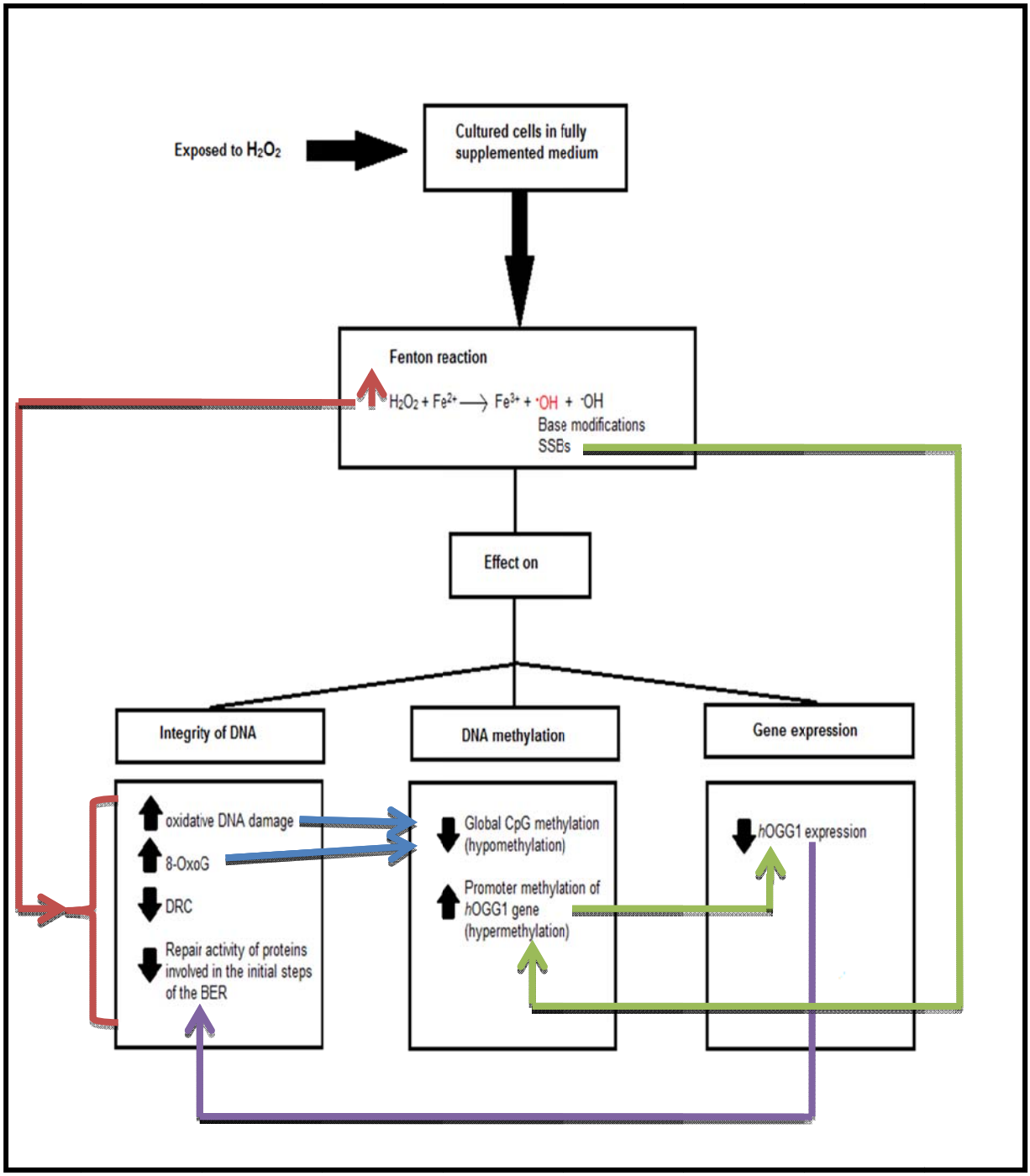

Figure 5.1 Summary of the effect of H_2O_2 exposure on cultured cells. Exposure of cells to H_2O_2 in culture, in fully supplemented medium (DMEM + 10 % FBS), leads to generation of hydroxyl radicals ($^{\cdot}OH$) through Fenton reactions in the vicinity of DNA. An increase in the amount of H_2O_2 affects the integrity of DNA, which in turn can affect the DNA methylation pattern globally. The generation of the hydroxyl radical also influences the *h*OGG1 promoter methylation pattern, which affects the expression of the *h*OGG1 gene through the formation of single strand breaks. (?) The decrease in the *h*OGG1 expression could possibly account for the decrease in the repair activity (i.e. excision activity) of proteins involved in the initial steps of the BER. (Where; SSBs: Single strand breaks and DRC: DNA repair capacity)

Final conclusion and future prospects

All of the objectives of the current study could be achieved and it could be determined that;

i. Oxidative DNA damage occurred even though cells were exposed to H_2O_2 in fully supplemented medium containing 10 % FBS
ii. The DRC and the repair activity of the proteins involved in the initial steps of the BER were affected by the exposure of the substrate DNA to H_2O_2
iii. The global DNA methylation pattern of the cells was affected by the exposure of the cells to H_2O_2.
iv. The exposure of the cells to H_2O_2 affected the *h*OGG1 promoter methylation status of the cells.
v. The expression of the *h*OGG1 gene was found to be affected by the exposure to H_2O_2
vi. A possible relationship between the affect that H_2O_2 exposure had on the occurrence of oxidative DNA damage, the activity of the proteins involved in the initial steps of the BER/the DRC, the change in the global DNA- and *h*OGG1 promoter methylation status and the expression of the *h*OGG1 gene could be determined.

Consequently the aim of the study was also achieved. Exposure of 143B cells to H_2O_2 in culture affected both the global DNA methylation pattern as well as the *h*OGG1 promoter methylation pattern by eventually leading to a degree of global DNA hypomethylation and hypermethylation of the *h*OGG1 promoter. A decrease in the expression of the *h*OGG1 gene could also be observed which was found to occur when the *h*OGG1 promoter was also hypermethylated. Furthermore, the aim of the study was to evaluate *early* DNA methylation changes in oxidative stressed cultured mammalian cells. In order to achieve this aim changes in the methylation pattern (globally- and gene specific) was measured over a period of six hours in order to evaluate if exposure to amounts of H_2O_2 that induce oxidative DNA damage has an effect on DNA methylation during this short period of time.

The amount of H_2O_2 used for this study was determined by measuring the cell viability at increasing concentrations of H_2O_2 (section 4.2; figure 4.1) and the highest concentration of H_2O_2 that still produced viable cells was used. The effect of 150 and 500 µM H_2O_2 on the DNA methylation pattern was measured. Although similar cell viability results were obtained at both these concentrations, 500 µM H_2O_2 caused more DNA damage (section 4.3; figure 4.2) and oxidative DNA damage (section 4.3.1; figure 4.3). It could therefore be concluded that results obtained by the exposure of the cells to 500 µM of H_2O_2 reflected healthy but stressed cells. Changes in the DNA methylation pattern could be observed within a six hour

time frame when exposing the cells to 500 µM but not 150 µM of H_2O_2, which possibly indicates that higher degrees of oxidative stress are necessary in order to produce an acute effect. Chronic exposure to lower concentrations of H_2O_2 could possibly have the same effect on the DNA methylation pattern.

Furthermore, although a cancer cell line was used for this study and in comparison to normal (healthy) cells they show abnormal DNA methylation patterns i.e. genome wide DNA hypomethylation and promoter hypermethylation (Kryston *et al.,* 2011), this study was designed in order to study a change in the DNA methylation patterns caused by oxidative stress and therefore results indicate a change relative to the "normal" DNA methylation pattern of the cells represented by a control group. In this study oxidative stress was induced by exposing cultured cells to H_2O_2. Oxidative damage to DNA can be used as an index of oxidative stress (Collins, 2009). The effect of H_2O_2 exposure on the DNA integrity was firstly investigated by making use of various forms of the comet assay to investigate the DNA damage and repair. This was done in order to make sure that the observed changes in the DNA methylation pattern did in fact occur because of changes in the integrity of the DNA caused by oxidative stress. It can therefore be concluded that the changes in the DNA methylation pattern of the cells occurred because of oxidative stress induced by H_2O_2 exposure.

Finally, there is still controversy over the cause and effect relationship between changed DNA methylation patterns and gene expression. As mentioned by Feil (2006) it is not known whether the gain of DNA methylation at specific promoters and CpG islands causes loss of gene expression or whether the change in the DNA methylation is a consequence of the loss of expression. Even though gain of DNA methylation (hypermethylation) of the *h*OGG1 promoter was observed at time intervals where a decrease in *h*OGG1 expression was also observed it still remains to be investigated whether this observed hypermethylation is truly responsible for the repressed gene expression as a decrease in the *h*OGG1 expression could also be observed following exposure to of cells to H_2O_2 (section 4.6; figure 4.16) even though hypermethylation of the *h*OGG1 promoter was not observed (section 4.6; figure 4.18). Could it be possible that the loss of expression of the *h*OGG1 gene is caused by an effect of H_2O_2 exposure on the cell cycle and cellular proliferation without affecting the cell viability, that then leads to hypermethylation of the *h*OGG1 promoter?(Feil, 2006; Li *et al.,* 2009). Future studies needs to address this issue.

Also, the effect of the induced oxidative stress was only studied in a single DNA repair pathway. Oxidative DNA lesions are counteracted by multiple overlapping repair processes and because this provides a fail-safe element to DNA repair (Cooke *et al*, 2003; Friedberg *et al.,* 2006) the impairment of one repair process does not necessarily affect the repair of a particular lesion. The main two repair pathways that are used for the removal of oxidized DNA base lesions are base excision repair (BER) and nucleotide excision repair (NER) (Cooke *et al.,* 2003; Freitas and De Magalhaes, 2011). In order to therefore fully grasp the effect of oxidative stress on DNA integrity and DNA methylation future studies need to address the effect of oxidative stress induced DNA damage on the promoter methylation status and expression of multiple genes from multiple repair pathways.

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Appendix A

Primer and Probe sequences

cDNA synthesis:

18S rRNA reverse primer 5' CGGACATCTAAGGGCATCAC 3'

Gene specific primers used for real-time PCR (methylation analysis and gene expression):

Primer and probe sequence of the EpiTect® Methyl qPCR Assay for *h*OGG1, *h*OGG1- and 18S rRNA gene expression assays are not disclosed by the manufacturer, assays are however standardised.

Assay ID (gene expression assay):

*h*OGG1: Hs00213454-m1

18S rRNA: Hs99999901_s1 (RefSeq: X03205.1)

Assay ID (EpiTect® Methyl qPCR Assay):

*h*OGG1: Hs380271

Appendix

B

***h*OGG1 promoter methylation status when cells were exposed to 150 µM H_2O_2.**

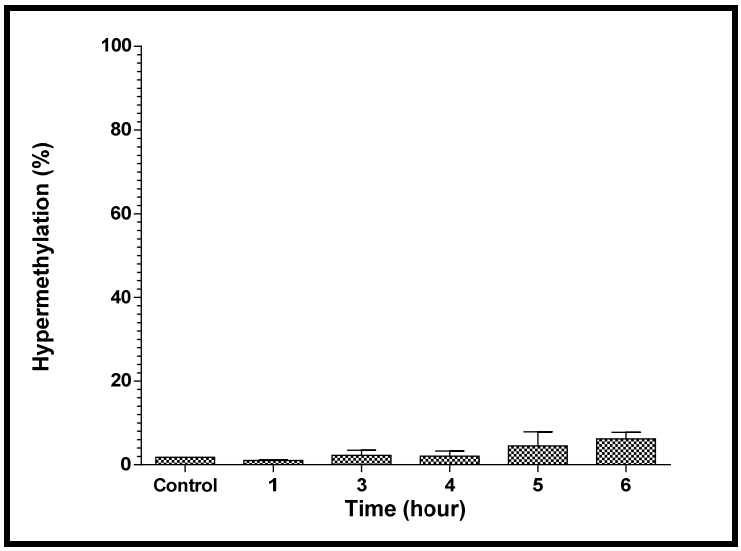

Figure B.1: Percentage hypermethylation measured in the *h*OGG1 promoter. The effect of the exposure to 150 µM H_2O_2 on the methylation status of the *h*OGG1 promoter was investigated over a period of six hours.

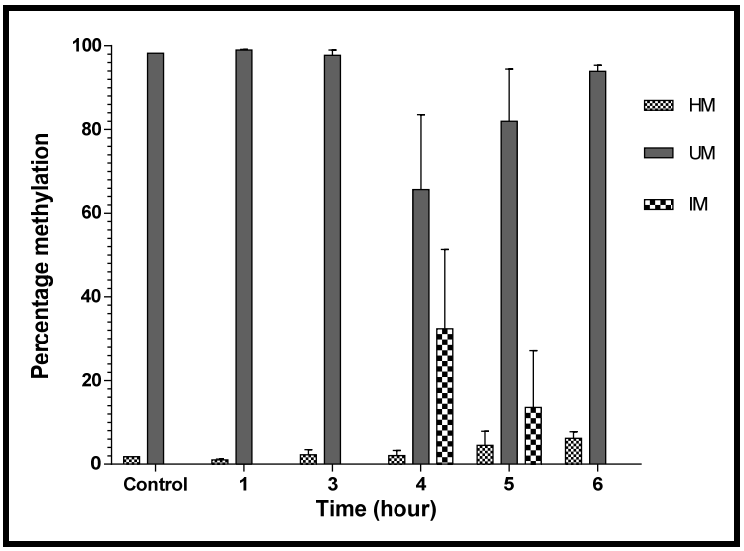

Figure B.2: The effect the exposure to H_2O_2 on the *h*OGG1 promoter methylation status. The effect of the exposure of the 143B cells to 150 µM H_2O_2 on the promoter methylation status of the *h*OGG1 gene was investigated over a period of six hours. Results represent the average of two individual experiments performed in duplicate; the standard deviation is representative of the standard deviation between the separate experiments. (Where: HM: Hypermethylated; UM: Unmethylated; IM: Intermediately methylated).

Results were not considered to be significant because:

HM <10 %,

UM > 10 % and

IM < 60 %

Results were considered to be significant when:

HM > 10 %

UM < 10 %

IM > 60 %

These considerations were in accordance with the manufacture's guidelines for data analysis and interpretation (SABiosciences User Manual Version 2.1, 3/10/2010).

Appendix C

Table C.1: Quality control (QC) report for EpiTect® Methyl PCR Assay (500 µM H_2O_2).

	Repeat 1		Repeat 2	
	W	**R**	**W**	**R**
Control 1	10.2702	0.081 %	11.6121	0.032 %
Control 2	12.3782	0.019 %	12.406	0.018 %
Control 3	6.02599	1.535 %	8.40781	0.294 %
Control 4	5.99946	1.563 %	5.54701	2.139 %
One hour 1	9.49645	0.138 %	8.33269	0.310 %
One hour 2	9.101	0.812 %	9.39144	0.149 %
One hour 3	13.4629	0.009 %	13.2239	0.010 %
Three hours 1	4.14509	5.652 %	4.33921	4.940 %
Three hours 2	1.5449	Failure	4.45094	4.572 %
Three hours 3	10.047	0.095 %	10.5191	0.068 %
Four hours 1	3.69859	7.702 %	3.53541	8.625 %
Four hours 2	7.93602	0.408 %	9.14778	0.176 %
Five hours 1	3.7264	7.555 %	3.24106	10.577 %
Five hours 2	2.60066	16.486 %	2.11723	23.049 %
Six hours 1	2.84419	13.926 %	2.5531	17.039 %
Six hours 2	12.0401	0.024 %	12.3819	0.019 %

Where; W = Analytical window and R = Percentage DNA refractory to enzyme digestion.

Gel electrophoresis confirmation of the PCR product

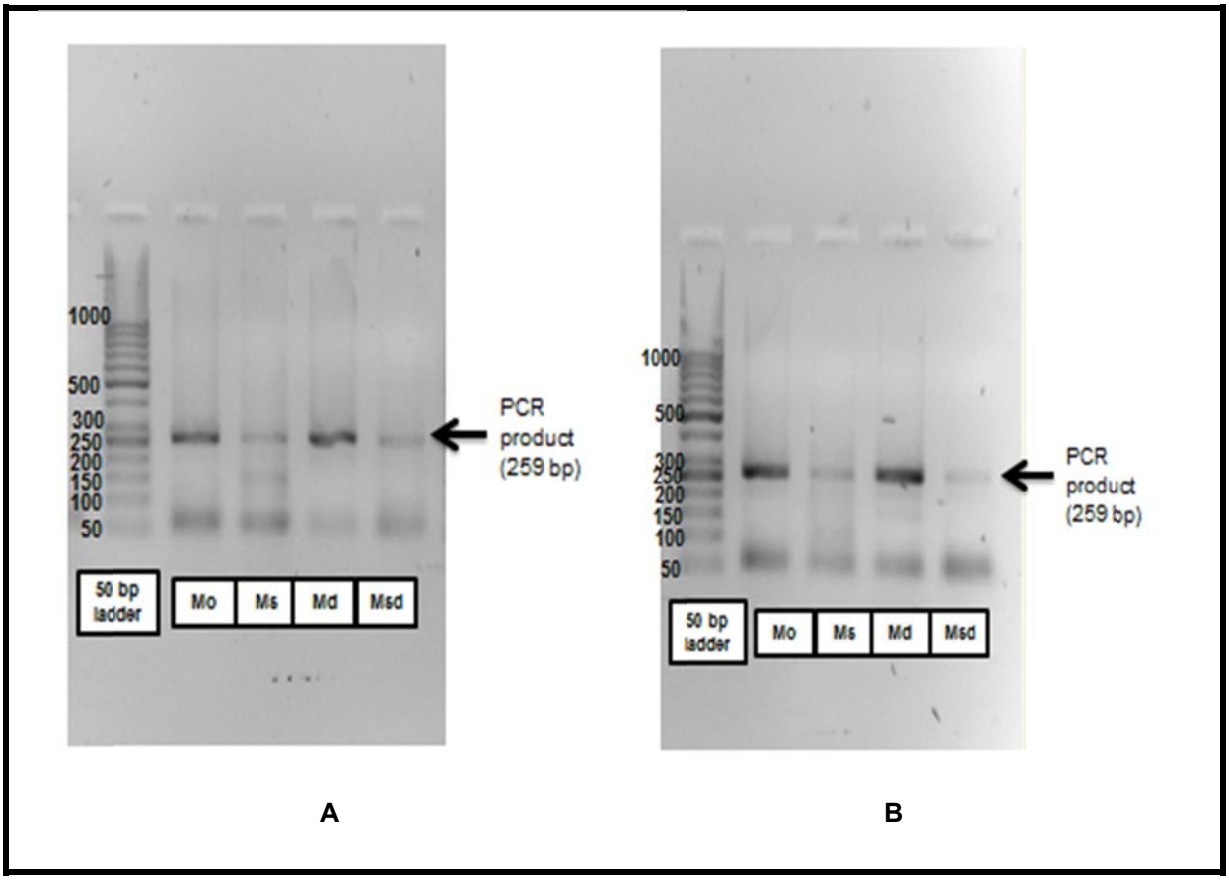

Figure D.1: Gel electrophoresis confirmation of the PCR product. The PCR products obtained with the real-time PCR were run on a 2 % agarose gel in order to confirm if the 259 bp product was present and hence that amplification was successful. Each gel represents the different enzyme digest for samples exposed to 500 µM for different time periods respectively (A) One and (B) four hours. A 50 bp DNA ladder was used. (Where: Mo: Mock digest; Ms: Methylation sensitive digest; Md: Methylation dependent digest and Msd: Double digest)

Appendix E

Table E.1. List of suppliers and catalogue numbers of materials

Reagent	Company	Catalogue number
2- Mercaptoethanol	Merck, BDH chemicals	44143
6-well plate	NUNC	140685
18S rRNA reverse primer	Eurofins	010755687
18S rRNA TaqMan® gene expression assay	Applied Biosystems	4333760T
75 cm^2 flask	NUNC	156472
96 well plate	TPP	92096
Agarose; Molecular grade low EEO	Hisppanagar, Whitehead Scientific	D-1 LE
ATP disodium salt hydrate	Sigma-Aldrich	A3377
AMV reverse transcriptase	SBS Genetech Co., Ltd.	FAM-500
BCA™ Protein assay kit	Thermo Scientific	23227
Albumine bovine (BSA)	Sigma-Aldrich	A-7906
Deoxynucleotide mix (dNTPs)	Novagen ® EMD4Biosciences	71004
DMSO (Dimethylsulfoxide)	Merck	1.02952.1000
Dulbecco's Modified Eagles Medium (DMEM; Hyclone)	Thermo Scientific	SH30243.FS
EDTA	Sigma-Aldrich	E5134

EndoIII	New England Biolabs	M0268S
EpiTect® Methyl qPCR Assay (*h*OGG1)	Qiagen	335001 MePH04523-1A
EpiTect® Methyl DNA restriction Kit	Qiagen	335451
Ethanol absolute	Merck	1.00983.2500
Ethidium Bromide	Sigma-Aldrich	E8751
FlexiGene DNA kit	Qiagen	51204
Foetal bovine serum (FBS) Gama irradiated	BioWhittaker ® Sera, Lonza	DE14-801F1
Fpg	New England Biolabs	M0240S
Generuler^{TM} 50bp DNA ladder	Fermentas	SM0372
GoTaq® DNA polymerase	Promega	M3175
[^{3}H]deoxycytidine triphosphate ([^{3}H]dCTP)	GE Healthcare	NET601A250UC
HEPES	Sigma-Aldrich	H3375-250G
HMPA	Techcomp Ltd.	9201
Hpa II	Fermentas	ER0512
Hydrogen peroxide (H_2O_2)	UNIV AR	3063820
KAPA SYBR ® FAST master mix (2x) ABI Prism^{TM}	KAPABIOSYSTEMS, Lasec	KK4604
L-Glutamine	BioWhittaker, Lonza	17-605E
LMPA	Roche	1441345
MEM non-essential amino acids	BioWhittaker, Lonza	BE13-114E

Msp I	Fermentas	ER0541
Nucleospin® RNA II kit	Macherey-Nagel	740 955
Oligo(dT) 18mer	IDT	51-0115-07
Penicillin/Streptomycin	BioWhittaker, Lonza	17-602E
Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417
Potassiumchloride (KCl)	Sigma-Aldrich	P9333
2-Propanol (Isopropanol)	Sigma-Aldrich	I9516
Sodiumchloride (NaCl)	Sigma-Aldrich	S9625
Sodiumhydroxide (NaOH)	Sigma-Aldrich	S5881
TaqMan® gene expression assay (*h*OGG1)	Applied Biosystems	4331182
TaqMan® universal PCR master mix, No AmpErase® UNG	Applied Biosystems	4324018
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich	M5655
TrisHCl	Sigma-Aldrich	T5941
Triton X-100	BDH	BB306324N
Trypan blue	Sigma-Aldrich	93595
Ultima Gold^{TM} XR	Perkin Elmer®	6013119
Water – HiPerSolv for HPLC^{TM}	Merck, BDH AnalaR®	1.15333.2500
Whatman® DE-81 ion exchange filters	Sigma	Z286591