DNA DAMAGE AND REPAIR DETECTED BY THE COMET ASSAY IN LYMPHOCYTES OF AFRICAN PETROL ATTENDANTS: A PILOT STUDY.

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Hons. B.Sc.

Mini-dissertation submitted in partial fulfilment of the requirements for the degree Master of Science in Occupational Hygiene at the North-West University.

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"Now to Him who is able to do exceedingly abundantly above all that we ask or think, according to the power that works in us, to Him *be* glory..."

Eph. 3:20-21 (NKJV)

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Preface

This mini-dissertation was written in article format. The empirical work consists of a pilot study on the effects of exposure of petrol attendants to petrol vapours. This study determined the exposure levels of petrol VOCs (volatile organic compounds) to which petrol attendants are exposed during an 8 hour working shift. The genotoxicity of petrol VOCs was also investigated. The comet assay was used to determine the baseline DNA damage in lymphocytes of petrol attendants and a group of matched controls. The effect on DNA repair capacity was also determined with the additional exposure of the cells to H_2O_2 (Collins, 2004). This study has been approved by the North-West University Ethics Committee; Ethical approval number: 06M11. Every subject gave their informed written consent prior to the commencement of the study.

In Chapter 1 a review is given of the relevant literature on the background and characteristics of petrol and its constituents. The health effects and toxicological influences of some of these constituents are also discussed in this review. This chapter also describes the different types of DNA damage and repair as well as several factors that may influence their levels. Chapter 2 consists of a manuscript written as an article in accordance with the format required by the journal to which it will be submitted for publication. Tables and figures will be included as part of the text and not at the end of the article to ensure that the article is presented in a readable and understandable format. Due to the limited word count of the article (< 5 000 words), a brief description of the methods will be given in the article and the detailed methods will be given in the annexure at the end of the mini-dissertation. The article is entitled *"DNA damage and repair detected by the comet assay in lymphocytes of African petrol attendants"*, and will be submitted to the Annals of Occupational Hygiene for peer reviewing and publication. Chapter 3 provides a final summary and conclusion, as well as recommendations for further studies.

The references used in Chapter 1 and the preface are provided according to the mandatory style stipulated by the North-West University at the end of Chapter 1. The

relevant references of Chapter 2 are provided at the end of the chapter according to the author's instructions of the Annals of Occupational Hygiene.

Author's contribution

The study reported in this dissertation was planned and executed by a team of researchers. The contribution of each of the researchers is depicted in Table 1.

Table 1. Research team.

NAME	CONTRIBUTION
Ms. G. S. Colane	Responsible for: ▪ Literature searches, interpretation of data and writing of the article; ▪ Recruiting subjects; ▪ Sampling of personal exposure data; ▪ Comet assay experimentation.
Mr. P. J. Laubscher	▪ Supervisor ▪ Assisted with designing and planning of the study, approval of protocol, interpretation of the results and documentation of the study.
Mr. J. L. Du Plessis & Prof. P. J. Pretorius	▪ Co-supervisor ▪ Assisted with the approval of the protocol, interpretation of the results, reviewing of the dissertation and documentation of the study; ▪ Giving guidance with scientific aspects of the study.
Ms. E. van Deventer	▪ Assisted with the personal exposure measurements.
Ms. E. van Dyk & Mrs. R. Preston	▪ Guidance on execution of the comet assay technique; ▪ Assisted with interpretation of DNA damage and repair data.

NAME	CONTRIBUTION
Sr. M. C. Lessing & Mrs. C. Fourie	▪ Assisted with the collection of blood samples.
Prof. F. Steyn	▪ Assisted with statistical analysis of data.
Prof. F. van der Westhuizen	▪ Assisted with the testing of oxidative and antioxidant status.
Dr. L. Du Plessis	▪ Assisted with the interpretation of oxidative and antioxidant status data.

The following is a statement from the co-authors that confirms each individual's role in the study:

I declare that I have approved the above mentioned article and that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that it may be published as part of Goitsemang Colane's M.Sc (Occupational Hygiene) dissertation.

Mr. P. J. Laubscher
(Supervisor)

Mr. J. L. Du Plessis
(Co-supervisor)

Prof. P. J. Pretorius
(Co-supervisor)

Ms. E. van Deventer

Ms. E. van Dyk

Dr. L. Du Plessis

List of Abbreviations

A	- Adenosine
AAPH	- Azobis (2-amidnopropane) dihydrochloride
ACGIH	- American Conference of Governmental Industrial Hygiene
ANCOVA	- Analysis of covariance
ATSDR	- Agency for Toxic Substances and Disease Registry
BER	- Base excision repair
BSA	- Bovine serum albumin
BTX	- Benzene, Toluene, Xylene
C	- Cytosine
CA	- Chromosome aberrations
CARR U	- Carratelli Units
CCOHS	- Canadian Centre for Occupational Health & Safety
C-H bonds	- Carbon-Hydrogen bonds
CL	- Control limit
CNS	- Central nervous system
CO	- Carbon monoxide
Cu^{2+}	- Copper
ddH_2O	- Double distilled water
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
d-ROMs	- Diacron reactive oxygen metabolite
DSBs	- Double-strand breaks
EDTA	- Ethylenediaminetetraacetic acid
Endo III	- Endonuclease III

List of Abbreviations (continues...)

ETBE	- Ethyl tertiary butyl ether
Fe^{2+}	- Ferrous iron
Fe^{3+}	- Ferric iron
$FeCl_3$	- Ferric chloride
$FeSO_4$	- Ferric sulphate
FPG	- Formamidopyrimidine DNA glycosylase
FRAP	- Ferric reducing antioxidant power assay
G	- Guanine
GC-MS	- Gas chromatography-mass spectrometry
GSH	- Gluthathione
GPx	- Glutathione peroxidase
GR	- Glutathione reductase
H^+	- Hydrogen atom
HEPES	- 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMPA	- High melting point agarose gel
HNO_2	- Nitrous acid
$HO_2^{\cdot}$	- Hydroperoxyl radical
HOCl	- Hypochlorous acid
H_2O_2	- Hydrogen peroxide
IARC	- International agency for research on cancer
KCl	- Potassium chloride
K_2HPO_4	- Dipotassium hydrogen phosphate
LMPA	- Low melting point agarose gel

List of Abbreviations (continues...)

mg/m^3	- Milligrams per cubic meter
mM	- Millimolar
MMR	- Mismatch repair
MMT	- Methylcyclopentadienyl manganese tricarbonyl
MN	- Micronuclei
Mn_3O_4	- Manganese oxide
MTBE	- Methyl tertiary-butyl ether
µl	- Microliter
µM	- Micromolar
$NaAc.3H_2O$	- Acetate buffer
NaCl	- Sodium chloride
NaH_2PO_4	- Sodium dihydrogen phosphate
NaOH	- Sodium hydroxide
NER	- Nucleotide excision repair
NHEJ	- Non-homologous end joining
NIOSH	- National Institute for Occupational Safety & Health
nM	- Nanomolar
$NO^{\cdot}$	- Nitric oxide
$NO_2^{\cdot}$	- Nitrogen dioxide
N_2O_3	- Dinitrogen trioxide
N_2O_4	- Dinitrogen tetroxide
OEL	- Occupational exposure limit
$OH^{\cdot}$	- Hydroxyl radical
OH^-	- Hydroxyl ion
$ONOO^-$	- Peroxynitrite
OSHA	- Occupational Safety and Health Administration
$O_2^{\cdot -}$	- Superoxide anion radical

List of Abbreviations (continues...)

O_3	- Ozone
$^1O_2$	- Singlet oxygen
8-oxodG	- 8-oxo-2 deoxyguanosine
PBS	- Phosphate buffered saline
PCA	- Perchloric acid
Ppb	- Parts per billion
Ppm	- Parts per billion
RC	- Repair capacity
RL	- Recommended limit
RNS	- Reactive nitrogen species
ROS	- Reactive oxygen species
$RO_2^{\cdot}$	- Peroxyl radical
$RO^{\cdot}$	- Alkoxyl radical
R-OOH	- Hydroperoxides
Rpm	- Revolutions per minute
$PbBr_2$	- Lead dibromide
$Pb(CH_2CH_3)_4$	- Tetraethyl lead
PbO	- Lead oxide
SCE	- Sister chromatid exchange
SD	- Standard deviation
SOD	- Superoxide dismutase
Ssb	- Single strand breaks
T	- Thymine
TAC	- Total antioxidant capacity
TAME	- Tertiary amyl methyl ether

List of Abbreviations (continues...)

TBA	- Tertiary butyl alcohol
TL	- Tail length
TLV	- Threshold limit value
TPTZ	- 2,4,6-Tripyridyl-1,3,5-triazine
TPTZ-Fe^{3+}	- Ferris-trypyridyltriazine complex (colourless)
TPTZ-Fe^{2+}	- Ferrous-trypyridyltriazine complex (coloured)
Tris-HCl	- Tris (hydroxymethyl) aminomethane hydrochloride
TWA	- Time weighted average
UV	- Ultra violet
VOC	- Volatile organic compounds
WHO	- World Health Organisation

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Abstract

Petrol attendants are exposed to petrol volatile organic compounds (VOCs) which may have genotoxic and carcinogenic effects. The single cell gel electrophoresis assay (comet assay) is a method highly sensitive to DNA damage induced by environmental and occupational exposure to carcinogenic and mutagenic agents. The aim of this study was to evaluate the level of exposure of petrol attendants to petrol VOCs and also to determine their effect on DNA damage and repair in lymphocytes of African petrol attendants. The exposed group consisted of 20 subjects, randomly selected from three petrol stations. A control group of 20 unexposed subjects was also chosen and matched for age and smoking habits with the exposed group. Sorbent tubes were used to assess personal exposure of petrol attendants. The comet assay was used to investigate the basal DNA damage and repair capacity in isolated lymphocytes of petrol attendants and control subjects. Blood samples were taken from the petrol attendants at the end of their 8 hour working shift and also from the control subjects. The petrol attendants were found to be exposed to levels of petrol VOCs lower than the occupational exposure limit (OEL) for constituent chemicals. A significant relationship was found between the volume of petrol sold during the shift and the average concentrations of benzene, toluene and the total VOCs measured. However, relative humidity had a negative correlation with the average concentrations of benzene, toluene, xylene and the total VOCs. Significantly higher basal DNA damage was observed with the exposed group compared to the control group. The period of exposure influenced the level of DNA damage and the calculated repair capacity. Smoking and age had a significant influence on the level of DNA damage. DNA repair capacity was delayed in smokers of both exposed and non-exposed group.

Keywords: DNA damage; DNA repair; Comet assay, Petrol attendant; Volatile organic compounds.

Opsomming

Petroljoggies word blootgestel aan vlugtige organiese verbindings in petrol wat genotoksiese en karsinogeniese effekte kan veroorsaak. Enkelsel gel-elektroforese (Komeetanalise) is 'n hoogs sensitiewe metode vir die bepaling vir die mate van DNA-skade wat veroorsaak word deur omgewings- en beroepsblootstelling aan karsinogeniese en mutageniese agense. Die doel van hierdie studie was om die mate van blootstelling van petroljoggies aan vlugtige organiese komponente van petrol, sowel as die effek daarvan op DNA beskadiging en herstel in limfosiete van swart petroljoggies te bepaal. Die proefgroep van 20 petroljoggies is ewekansig gekies uit die werknemers van drie petrolstasies. Die kontrole groep van 20 nie-blootgestelde proefpersone was gepaar met die proefgroep ten opsigte van ouderdom en rookgewoontes. Geaktiveerde koolstof adsorpsie buise is gebruik om die persoonlike blootstelling van die petroljoggies te bepaal. Die Komeetanalise is gebruik om die basale vlak van DNA-skade sowel as die herstelvermoë van die DNA in geïsoleerde limfosiete van beide die proef- en die kontrolegroep te bepaal. Bloedmonsters van die petroljoggies is aan die einde van hulle werkskof van 8 ure, en by die proefgroep op die tyd wat hulle beskikbaar was, versamel. Die blootstellingsvlakke van die petroljoggies aan vlugtige organiese verbindings was laer as die beroepsblootstellingsdrempels (TBG-BBd) van die onderskeie chemikalieë. 'n Betekenisvolle verwantskap is aangetoon tussen die volume petrol verkoop en die gemiddelde konsentrasies van benseen, tolueen en totale vlugtige organiese verbindings. Relatiewe humiditeit het 'n negatiewe korrellasie getoon met die gemiddelde konsentrasies van benseen, tolueen, xileen en totale vlugtige organiese verbindings. 'n Betekenisvolle hoër basale vlak van DNA skade is waargeneem by die proefgroep as by die kontrole groep. Die tysduur van blootstelling het ook die vlakke van DNA-skade en herstel beïnvloed. Rook en ouderdom het 'n betekenisvolle invloed op die vlak van DNA-skade gehad. Die DNA herstelkapasiteit was vertraag in beide die blootgestelde en die nie blootgestelde groep.

Sleutelwoorde: DNA-skade; DNA-herstel; Komeetanalise; Petroljoggies; Vlugtige organiese verbindings.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Various techniques are presently used to detect early biological effects of DNA damaging agents in environmental and occupational locations (Møller *et al.*, 2000). These techniques have been useful in studies of environmental toxicology, carcinogenesis, human epidemiology and aging (Singh *et al.*, 1988). Effects of environmental toxicology, cancer and aging are often tissue and cell-type specific, thus it became important to develop a technique which can detect DNA damage in individual cells. Singh *et al.* (1998) have developed a simple approach, the comet assay, for sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. The single cell gel electrophoresis (SCGE) or the comet assay is a sensitive technique for detecting double strand breaks (*dsb*), single strand breaks (*ssb*) and/or alkali-labile sites at a single cell level (Andreoli *et al.*, 1997; Collins, 2004). It is fast, sensitive, visual and inexpensive compared to conventional techniques which are laborious and time consuming (Fontaine *et al.*, 2004). The single cell approach allows for robust statistical data analysis. Another advantage is that any eukaryotic cells are agreeable to DNA damage analysis. For these reasons, the comet assay has been widely used in diverse research fields, ranging from biomonitoring and studies of DNA repair processes to genotoxicity assessments (Tice *et al.*, 2000).

In view of the high sensitivity of the comet assay to measure DNA damage induced by environmental and occupational exposure to carcinogenic and mutagenic agents, the comet assay will be used to study the occurrence of DNA damage in peripheral lymphocytes of African petrol attendants. These workers experience exposure to levels of petrol vapours which may pose a risk of adverse effects. Petrol is a complex mixture of low-molecular mass compounds, mainly paraffins, naphthenes, olefins and aromatics, which can cause mutations and cancer (Pitarque *et al.*, 1997). Aromatic compounds of petrol are predominantly benzene, toluene and xylene (BTX) (Periago *et al.*, 1997). Benzene, from a toxicological view, is the most hazardous component and has been classified as a human carcinogen by the International Agency for Research and Cancer (IARC, 1989) and the American Conference of Governmental Industrial Hygienists (ACGIH, 2003). Although toluene and xylene are not classifiable as human carcinogens,

their exposure can lead to neurological effects such as headache, dizziness, fatigue, tremors, incoordination, anxiety, impaired short-term memory and inability to concentrate (ATSDR, 2006 & 2007).

Workers can be exposed to relatively high levels of petrol vapours in petrochemical refineries and petrol service stations, or to low levels of petrol vapours in the general population (Pitarque *et al.,* 1997). Previous studies done on exposure to VOCs for individuals with occupations associated with exposure to petrol vapour emissions, yielded evidence that workers were exposed to significantly higher levels of aromatic hydrocarbons. In Italy, Periago *et al.* (1997) found that hydrocarbons were elevated in ambient air, and also that climatic conditions can increase the risk of exposure during shifts, especially during summer (Periago & Prado, 2005). According to a study done in Thailand, service station attendants showed a more elevated exposure to benzene than any other occupation studied (Navasumriti *et al.,* 2005). However, all of these studies were conducted with different ethnic groups in different countries, but no studies on the occupational exposure to petrol vapours have been done in black African petrol attendants.

Biological effects of DNA damaging agents have been detected by the use of a number of techniques. Singh *et al.* (1988) developed a simple approach, the comet assay, for sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. Occupational exposure to lead was found to induce in vivo relevant biological effects according to a study by Fracasso *et al.* (2002). This study confirmed previous observations of toxic effects of lead on lymphocytes. The comet assay or SGCE analysis of *ssb* (single strand breaks) and alkali labile sites reported in a study by Andreoli *et al.* (1997) showed a significant excess of DNA damage in circulatory lymphocytes of petrol attendants who were occupationally exposed to low benzene levels, compared to an age-matched reference group. In other study by Fraceschettti *et al.* (2005) the comet assay was used to determine the significant correlation between the exposure of petrol attendants to low levels of benzene and DNA damage.

3. Hypothesis

It is, therefore, hypothesised that since African petrol attendants are routinely exposed to VOC levels, they may be subjected to increased oxidative DNA damage and reduced DNA repair capacity.

3. Aims and approach of the study

The general aims of this study were:

- To characterise the personal exposure of African petrol attendants to petrol VOCs,
- To analyse the occurrence of oxidative DNA damage and the level of DNA repair in peripheral lymphocytes of a group of African petrol attendants who are occupationally exposed to petrol VOCs, and
- To compare the level of oxidative DNA damage and repair in peripheral lymphocytes of a group of African petrol attendants and a paired group of control subjects not exposed to petrol.

The results will be corrected for confounding factors such as age and smoking habits.

The approach of this study was formulated as follows:

The levels of petrol VOCs to which each petrol attendant was exposed during an 8 hour working shift were determined. DNA damage and repair were also measured, using the comet assay to provide information on the possible genotoxic effects of petrol on the subjects. Oxidative and antioxidant status were also measured to determine the level of oxidative stress after exposure to petrol VOCs.

CHAPTER 2

LITERATURE REVIEW

1. Introduction

This chapter will focus on a concise review of the literature that is necessary for the understanding and interpretation of the article presented in this dissertation. It will give a background on the characteristics of petrol and some of its constituents. The health effects and toxicological influences of some of these constituents will be discussed in this review. As one of the aims of this study is to investigate the genotoxicity of petrol on petrol attendants, the different methods used will also be reviewed.

2. Characteristics of petrol

Gasoline or petrol is a generic name for petroleum fuel which is mainly used for internal combustion engines. Petroleum is a thick, dark brown or greenish coloured flammable liquid. Also known as crude oil, it is one of the most important fuel sources currently used in the modern world. Petrol is a complex mixture which does not occur naturally in the environment, but it is produced from crude oil or synthesized from gas through refining processes. The composition of petrol varies according to the type of crude oil from which it originates and the differences in processing techniques and refineries from which it is blended (Periago *et al.*, 1997). This complex mixture is made of low-molecular mass compounds mainly paraffinic, naphthenic, olefinic and aromatic with a carbon number ranging from 3-11 (Periago & Prado, 2005). Its performance is determined primarily by its volatility (tendency to boil and its vapour pressure), its quality, cleanliness and stability. It may also contain oxygenates, lead, detergents and other additives to improve its performance (Caprino & Togna, 1998).

3. VOCs in petrol and occupational exposure

The most likely way that a person might be exposed to petrol is by breathing its vapour at a service station. When petrol attendants fill a car's fuel tank, they may be exposed to vapours and different types of VOCs in petrol. If the hose from the petrol tank has a leak or the car tank overfills, the petrol attendant may be exposed to more gasoline vapours and the petrol may even spill on his skin. Some of the chemicals in petrol are expected to penetrate the skin more easily than others. Previous studies done have shown that workers in petroleum-related industries, who routinely work near VOC sources, are

exposed to highly-elevated VOC levels during their work-time. A study done by Navasumriti *et a.* (2005) showed an elevated exposure level in petrol attendants working an 8 hour shift refuelling motor vehicles with petrol, and not involved in any other responsibilities during their workday. In this study atmospheric levels of benzene at various sites in Bangkok were determined. Individual exposure levels in factory workers (73.55 ppb) and petrol attendants (121.67 ppb) were significantly higher than control workers (4.77 ppb, $p< 0.001$) who were not exposed to petrol. In another study done by Bono *et al.* (2003) in Italy, three types of urban occupational exposure to the same hydrocarbons were compared to verify the different expected levels of exposure. The three categories compared were petrol attendants, traffic officers and municipal employees. Personal exposure showed that only the petrol attendants were exposed to significantly higher levels of benzene compared to the other two professional categories, in both summer and winter (Bono *et al.,* 2003).

VOCs associated with the exposure to motor vehicle exhaust and/or petrol vapour emissions are pollutants of concern because of their toxicity (Jo & Song, 2001). An important group of aromatic compounds of petrol is composed of benzene, toluene and xylene (BTX) (Periago *et al.*, 1997). Other toxic substances present in petrol are tetraethyllead, tetramethyllead, butadiene, n-hexane and trimethylpentane. Some of these substances can be rapidly absorbed through inhalation and skin contact. Since lead-containing antiknock additives have been reduced and eliminated, more aromatics are blended into petrol for antiknock purposes, and thus benzene concentrations have increased (Roma-Torres *et al.*, 2006).

Personal exposure is a key concept in relating air pollutant concentrations to health effects. Personal exposure is essentially the average concentration of a pollutant that a person is exposed to over a given period of time. During car refuelling, an air stream saturated with petrol vapour is evacuated from the fuel tank of the car. The volume of air is exactly equal to the volume of petrol pumped. Thus, the volume of petrol sold during the shift could have an influence in the contamination of the air near the respiratory zone of each exposed subject. A study done by Periago *et al.* (1997) showed a significant

relationship between the volume of petrol sold during the shift and the ambient concentration of BTX for each worker sampled. Furthermore, since the study was done in two different seasons with different ambient temperatures, a significant difference was found between the time-weighted average (TWA) concentrations of VOCs measured with the ambient temperature.

Technical specifications for petrol have been changed and regulations put in place to reduce the content of benzene and other VOCs in petrol. The South African Occupational Health and Safety Act, 1993 (Act No. 85 of 1993) has established new regulations for hazardous chemical substances (Table 1).

Table 1. Occupational exposure limits for VOCs (OHS Act 85 of 1993).

	TWA OEL-CL [a]		TWA OEL-RL [b]	
Substance	ppm	mg/m^3	ppm	mg/m^3
Benzene	5	16		
Ethylbenzene			100	435
n-Heptane			400	1 600
n-Hexane			20	70
Pentane			600	1 800
Toluene			50	188
White spirits			100	575
Xylene			100	435

[a] CL control limit

[b] RL recommended limit

4. Health risks and toxicological influences of petrol constituents

4.1. Benzene

Benzene is normally a minority component representing one-tenth of the aromatic content of the petrol. It is derived from petroleum and is an important antiknock agent in

unleaded petrol. It is an aromatic hydrocarbon whose composition differs from other hydrocarbons with its absence of a methyl group attached to the benzene ring (Bono *et al.*, 2003). Benzene is characterised as a known carcinogen for all routes of exposure, according to several organisations such as the International Agency for Research on Cancer (IARC, 1989) and the American Conference of Governmental Industrial Hygienists (ACGIH, 2003). It is an ever-present industrial and environmental pollutant. It is present in both evaporative and combustible automobile emissions, has been detected in cigarette smoke, and is commonly used as an industrial solvent in the workplace. It is an established cause of human leukaemia that is thought to act by producing chromosomal aberrations and alterations in cell differentiation (Çelic *et al.,* 2003).

The use of benzene has been restricted, following recognition of its carcinogenic characteristics. Progressive reduction of the use of benzene, along with continuous reduction of threshold limiting values (TLVs) have ensured that exposures to high levels of benzene in the workplace no longer constitute a serious problem (Roma-Torres *et al.*, 2006). This has led to a shift in the interest of studies on the health risks to low occupational and environmental exposure levels of benzene (Franceschetti *et al.*, 2005). This industrial organic solvent has a TWA OEL-CL of 5 ppm (OSHA, 1995).

About 50% of inhaled benzene in air is absorbed into the body. The primary route of entry is through the nose to the lungs. Once in the body the chemical tends to rest in the fatty tissue. As a result of long-term exposure, benzene may exert a damaging effect on cells, thus inducing certain forms of leukaemia. Benzene is of particular interest as it is one of the few chemicals known to increase the risk of acute myeloid leukaemia significantly in occupational settings (Çelic *et al.*, 2003 & Hinwood *et al.*, 2005). The first case of leukaemia from benzene exposure was documented in 1928 (Smith & Zang, 1998). Epidemiological studies indicate a positive correlation between high levels of benzene with a higher incidence of aplastic anaemia and leukaemia (Raabe & Wong, 1996). Chronic exposure to benzene can lead to serious haematological effects and high chronic exposure leads to the onset of irreversible bone-marrow depression, characterised

by leukocytopenia, thrombocytopenia, granulocytopenia, pancytoponia, aplastic anaemia and leukaemia (Snyder, 2000).

Biological monitoring of human exposure to benzene has been performed as a component of health surveillance programmes in several countries. Several reports available suggest that exposure to benzene is a serious health problem. Even a small percentage of benzene in automobile petrol of about 3% is a serious health risk to petrol pump workers (Verma & Rana, 2001). Measurements done by Navasumrit *et al.* (2005) showed possible early biological effects of benzene exposure which are indicative of health risk in petrol attendants. Although the exposure level for these petrol attendants of 0.12 ppm was lower than the ACGIH limit of 0.5 ppm, the levels were right at the NIOSH limit of 0.1 ppm. DNA damage, determined as DNA strand breaks, was found to be elevated in petrol attendants at these exposure levels. This is in agreement with other studies in workers exposed to low levels of benzene (Andreoli *et al.*, 1997 & Franceschetti *et al.*, 2005). There has been an increased risk of lung cancer reported in service station attendants (Brandi *et al.*, 1998). Damage to both the humoral and cellular components of the immune system has been known to occur in humans following inhalation exposure. This is manifested by decreased levels of antibodies and decreased levels of leukocytes in workers. Animal data support these findings (ATSDR, 2006).

4.2. Alkylbenzenes: Toluene and Xylene

Alkylbenzenes are single ring aromatic compounds containing one (toluene) or more (xylene) saturated aliphatic side chains. Toluene occurs naturally in crude oil and is largely used as a solvent carrier in paints, thinner, adhesives, inks and pharmaceutical products. It is also used as an additive in cosmetic products as a raw material for the production of polyurethane foam (toluene diisocyanate). It is also blended together with benzene and xylene into petrol. Xylene is an aromatic hydrocarbon that exists in three isomeric forms: *ortho, meta and para.* About 92% of mixed xylenes are blended into petrol and are also being used in a variety of solvent applications (Roma-Torres *et al.*, 2006).

Toluene and xylene occur in small amounts in petrol blends and standard petrol formulations as a result of the octane process. They are mainly absorbed by inhalation and through the skin. Xylenes are more potent skin irritants than benzene or toluene. Toluene and xylene are considered not classifiable as human carcinogens by IARC and ACGIH because of inadequate evidence for carcinogenicity of both compounds in humans and experimental animals (Caprino & Togna, 1998).

The Canadian Centre for Occupational Health and Safety (CCOHS, 1997) showed that toluene exposure symptoms are related to exposure concentration. At approximately concentrations between 50 and 100 ppm, irritation of the nose, throat and respiratory tract can occur, as well as slight drowsiness, headache, fatigue and dizziness. Concentrations over 200 ppm can cause symptoms similar to drunkenness (giddiness), numbness, and mild nausea, while over 500 ppm can result in mental confusion and loss of coordination. Higher concentrations (10 000 ppm) lead to further depression of the central nervous system which can result in unconsciousness and death. The CCOHS revealed that both short-term and long-term exposure to high concentrations of xylene affect the central nervous system similar to toluene exposure. Due to these neurological effects, the occupational exposure limits were set as can be seen in Table 1.1 to prevent acute and chronic effects on the central and peripheral nervous system from exposures to toluene and xylene.

4.3. Leaded and unleaded petrol

In order to get the maximum energy from the burning fuel in modern car engines, the petrol vapour-air mixture is highly compressed before it is sparked. On the other hand some hydrocarbons tend to ignite under pressure before they are sparked. In this way the engine runs roughly, and this is referred to as "knocking". Branched-chain alkanes tend to resist this pre-ignition better than alkanes with unbranched chains. Alkanes and fuel mixtures are given octane ratings depending on their knocking tendency. 2, 2, 4-Trimethylpentane (containing 8 carbons and is an isomer of octane) has an octane rating of 100; while heptane has a rating of 0. The octane number of a petrol is the % of 2,2,4-

trimethylpentane in a mixture with heptane that has the same knocking characteristics as the petrol under investigation (Meusinger & Moros, 2001).

The Scottish Environment Protection Agency found as long ago as 1922 that if tetraethyl lead, $Pb(CH_2CH_3)_4$, was put into petrol, particles of lead and lead oxide (PbO) are formed on combustion. This then helps the petrol to burn more slowly and smoothly, preventing knocking and giving higher octane ratings. 1,2-Dibromoethane is also added to the petrol to remove the lead from the cylinder as $PbBr_2$, which is released as vapour into the environment (Cotton, 2002; WHO, 2003). Using higher-octane leaded petrol led to more powerful high-compression engines being built. But two problems resulted from this. Firstly, lead released from car exhausts is dispersed into the environment and has been linked to a number of health problems. Lead particles, once in the atmosphere, typically from vehicle emissions, can be inhaled. The second problem is that car exhausts contain environmentally unfriendly gases, such as CO and nitrogen oxides (Singh & Singh, 2006).

Leaded petrol also contains 1,2-dichloroethane, a colourless, sweet smelling liquid which evaporates easily. This compound was used in the past as a lead scavenger in leaded petrol. High levels of 1,2-dichloroethane, cause a range of adverse effects on the lungs and irritation of the eyes and respiratory system. At normal levels it is unlikely to have adverse effects (Scottish Environment Protection Agency, 2005). Typically 30 to 50% of the inhaled particles are retained in the lung and further sub-fractions absorbed either through the lung or gastrointestinal tract into the bloodstream. Once absorbed into the body a wide range of toxicological effects occur. These include effects on the blood, the nervous system, the kidneys and reproductive, cardiovascular, liver and gastrointestinal systems (ATSDR, 2004). Exposure to lead can have a broad range of health effects depending on the amount of lead present and the length of exposure. Generally, the greater the level of exposure, the greater the impact on health will be (Unionsafe, 2002).

To improve performance of fuel, combustion must be rapid. With the search for suitable antiknock agents came the development of alkyl leads (tetramethyl and tetraethyl lead) as

cost-effective octane enhancers. A study done on employees exposed for an average period of 14 years to organic compounds of lead showed that neurotoxic damage can occur from exposures to such antiknock additives. It has been known for decades that organolead compounds are potent neurotoxins on the CNS and its development. Tetraethyl lead is known to have a toxic impact on the CNS, as suggested by pathological changes in brain stem neurons and subtle cognitive and neurological deficits. Introduction of unleaded fuel brought about improvements in the gradual decrease in the lead content of petrol (Caprino & Togna, 1998).

With the increasing use of unleaded petrol, however, emissions and concentrations in air have declined steadily in recent years (Rodamilans *et al.*, 1996; Mathee *et al.*, 2006; Singh & Singh, 2006; Wang *et al.*, 2006). The emissions for benzene, toluene, the xylenes and 1,3-butadiene in vehicles using unleaded and leaded petrol respectively, are found to be significantly lower when unleaded petrol is substituted for leaded petrol (Duffy *et al.*, 1998).

4.4. MMT (methylcyclopentadienyl manganese tricarbonyl)

Certain metals, even when released into the environment in low concentrations, may exert toxicity to living organisms over a long time. One of the constituents to leaded petrol is methylcyclopentadienyl manganese tricarbonyl (MMT). This compound was introduced as an octane boosting and "anti-knock" agent, thus either replacing or reducing the lead content in petrol. When used as an octane improver in petrol, MMT leads to increased airborne levels of manganese in the form of Mn_3O_4 (Caprino & Togna, 1998). Manganese, unlike lead, is a normal and essential component of the human diet which is also considered to be an important metal to the mitochondrial oxidative processes for all living mammals, but may also be toxic at high concentrations. Both deficiency and excess of manganese have been associated with detrimental health effects. The major toxicological effects of manganese, observed after long occupational exposure, are on the lungs (manganese pneumonia) and the central nervous system (manganism) (Abbott, 2003). Excessive exposure via inhalation has also been shown to accumulate in

the brain, causing irreversible brain disease, to some extent similar to Parkinson's Disease (Röllin *et al.*, 2005).

4.5. Oxygenates

Oxygenates are used as antiknock agents in place of lead derivatives and as substitutes for high octane aromatics in fuel. They diminish exhaust emissions of carbon monoxide and hydrocarbons by permitting more efficient fuel combustion (Lee *et al.*, 2002). Oxygenates include substances such as ethanol, methanol, methyl tertiary butyl ether (MTBE), ethyl tertiary butyl ether (ETBE), tertiary butyl alcohol (TBA), and tertiary amyl methyl ether (TAME). It should be noted that exposure to ethanol in petrol should not increase the risk of toxicity in human health. Potential levels of exposure are much lower compared with those levels associated with the toxic effects observed in experimental animals. Methanol is well absorbed in humans following inhalation or ingestion. It produces a transient mild depression of the CNS with headache, vertigo and vomiting. MTBE is an aliphatic ether and it is a volatile, colourless and inflammable liquid, which has been employed as an octane enhancer in petrol. The oxygen atom in MTBE helps provide extra oxygen content for complete combustion, and gives it an octane rating of 116 (Caprino & Togna, 1998; Cotton, 2002).

5. Genotoxicity of petrol

Exposure to petrol vapours has been classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC, 1989) and the American Conference of Governmental Industrial Hygienists (ACGIH, 2003). This is mainly due to the carcinogenicity of some components such as benzene. An increased level of cytogenetic damage in peripheral blood lymphocytes of workers occupationally exposed to petroleum and petroleum derivatives has been demonstrated using different genetic end-points such as sister chromatid exchange (SCE), DNA strand breaks and micronuclei (Çelic *et al.*, 2003). Several studies have also investigated the ability of lead to act as a co-carcinogen. Such an effect seems to be, in part, due to interference with DNA repair process and thus enhancing the genotoxicity of other DNA damaging agents (i.e. UV radiation and alkylating compounds). Lead can also take part in the Fenton reaction to

generate hydroxyl radicals, singlet oxygen and other highly damaging reactive oxygen species (ROS) that are well-known to cause DNA damage. On the other hand, chemicals that produce ROS may induce genotoxic effects when the redox state of the cell shifts to a more oxidised state (oxidative stress) (Fracasso *et al.*, 2002).

5.1. DNA damage

DNA damage can be subdivided into two main categories; i.e. endogenous damage such as attack by ROS produced from normal metabolic by-products (spontaneous mutation), especially the process of deamination; and exogenous damage caused by external agents such as ultraviolet radiation, x-rays, hydrolysis and mutagenic chemicals (especially aromatic compounds that act as DNA intercalating agents) (Friedberg, 2004).

Human genetic material is constantly exposed to physical and chemical substances of both intracellular and environmental origin. These include UV radiation, X-rays and chemical reactive species. Such agents or substances of endogenous and exogenous origin may directly or indirectly cause DNA damage. This includes chemical modification of the bases which in turn disrupts the DNA molecule's regular helical structure by introduction of foreign chemical bonds or formation of adducts that do not fit the standard double helix. DNA strand breaks can result from a direct modification of DNA by chemical agents or their metabolites. It can also be from a process of DNA excision repair, replication and recombination or from apoptosis. Also, direct breakage of the DNA strand occurs when ROS interact with DNA (Møller *et al.*, 2000).

5.1.2. Types of DNA damage

DNA contains many potential reactive sites and its structure can be modified in a number of ways. Although DNA is the carrier of genetic information, it has limited chemical stability. Hydrolysis, oxidation and nonenzymatic methylation of DNA occur at significant rates *in vivo*, and are counteracted by specific DNA repair processes. The spontaneous decay of DNA is likely to be a major factor in mutagenesis, carcinogenesis and ageing (Lindahl, 1993). There are several types of DNA damage due to endogenous cellular processes, mainly oxidation, alkylation, hydrolysis and mismatching of bases.

Oxidative DNA damage is caused by agents such as singlet oxygen and peroxide radicals. It causes base and sugar-phosphate backbone damage and breakage. Oxidative DNA damage is an inevitable consequence of cellular metabolism, with a tendency for increased levels following toxic insult. Although more than 20 base lesions have been identified, only a fraction of these have received appreciable study, most notably 8-oxo-2 deoxyguanosine. 8-oxodG is promutagenic and can induce a G:C to T:A transversion at DNA replication. This lesion has been the focus of intense research interest (Cooke *et al.*, 2003).

Alkylation damage (usually methylation) includes a variety of DNA base modifications which may result in mutations and eventually lead to carcinogenesis. Living cells counteract these lesions by a set of repair enzymes which specifically recognize these alkylated bases, often producing sites of base loss (Drabløs *et al.*, 2004).

The simplest reaction that is potentially harmful to DNA is hydrolysis (Lindahl, 1993). Endogenous cellular process can cause hydrolysis of bases such as deamination, depurination and depyrimidination. Abasic sites resulting from such hydrolysis lose their genetic encoding and can thus lead to mutations during replication. Mismatch of bases also results due to DNA replication in which wrong DNA base is stitched into place in a newly forming DNA strand (Cooke *et al.*, 2003).

Covalent binding of chemicals to DNA with the formation of chemically stable products known as adducts plays a major role in the mode of action of chemical mutagens and carcinogens. These adducts range in size and complexicity from simple alkyl groups (methyl or ethyl) to bulky multi-ring residues from chemicals such as polycyclic aromatic hydrocarbons and aromatic amines (Godschalk *et al.*, 2002).

5.2. DNA repair

Any DNA damage must be repaired in order to maintain the integrity of the genomic information. The integrity of DNA is vital to cell survival and reproduction. Garrett and Grisham (2005) explained several mechanisms which recognise lesions on the DNA

strand and remove them through a number of diverse reaction sequences. These mechanisms are described below. For recent extensive reviews, see Guetens *et al.* (2002), Christmann *et al.* (2003), Cooke *et al.* (2003), Friedberg (2004) and Berwick and Veins (2005). A summary of repair mechanisms from these sources is given below.

Two fundamental types of molecular mechanisms for DNA repair can be distinguished as follows:

1. Mechanisms that excise and replace damaged regions by replication, recombination, or mismatch repair; and
2. Mechanisms that reverse damaging chemical changes in DNA, and these includes excision repair systems.

5.2.1. Mismatch repair (MMR)

The mismatch repair system corrects errors induced during DNA replication. It scans newly synthesised DNA for mispaired bases, excises the mismatched region and then replaces it by DNA polymerase-mediated local replication. It is vital in such replacements to note which base of the mismatched pair is corrected (Garrett & Grisham, 2005). Therefore, the steps by which MMR proceeds are recognition of DNA lesions, strand discrimination, as well as excision and repair synthesis.

5.2.2. Excision repair

Many damaged or modified bases are replaced via excision repair systems. The two fundamental excision repair systems are base excision and nucleotide excision.

5.2.3. Base excision repair (BER)

BER acts on single bases that have been damaged through oxidation or other chemical modifications during the normal cellular process. A damaged base is excised from the sugar-phosphate backbone by DNA glycosylase, creating an AP site. Then an apurinic/apyrimidinic endonuclease cuts the DNA strand and an excision nuclease removes the AP site and several nucleotides. DNA polymerase I and DNA ligase then

repair the gap. The information of the complementary strand is used to dictate which bases are added in re-filling the gap.

5.2.4. Nucleotide excision repair (NER)

NER recognises and repairs larger regions of damaged DNA than BER. It is the main pathway for removal of carcinogenic lesions caused by sunlight or other mutagenic agents. The NER system cuts the sugar-phosphate backbone of a DNA strand in two places, one on each side of the lesion, and removes the region. The resultant gap is then filled in using DNA polymerase and the sugar-phosphate backbone is covalently closed by DNA ligase.

5.2.5. Double-strand breaks

Double-strand breaks in which both strands in the double helix are cut are particularly hazardous to the cell because they can lead to genome rearrangement. There are also two mechanisms for the repair of double-strand breaks, namely non-homologous end joining and homologous recombination repair.

5.2.6. Non-homologous end joining (NHEJ)

In NHEJ, DNA ligase IV directly joins the two ends. To guide accurate repair, NHEJ relies on short homologous sequences called microhomologies present on the single-stranded tail of the DNA ends to be joined. If these overhangs are compatible, repair is usually accurate. NHEJ can also induce mutations during repair. Loss of damaged nucleotides at the break site can lead to deletions and joining of non-matching termini forms translocations. NHEJ is especially important before the cell has replicated its DNA, since there is no template available for repair by homologous recombination.

5.2.7. Homologous recombination

Recombination repair requires the presence of an identical or nearly identical sequence to be used as a template for repair of the break. This pathway allows a damaged chromosome to be repaired using a sister chromatid or a homologous chromosome as a template. DSBs caused by the replication machinery attempting to synthesise across a

single-strand break or unrepaired lesion cause collapse of the replication fork and are typically repaired by recombination.

5.3. Oxyradicals and DNA damage

5.3.1. Free radicals and reactive oxygen species

A free radical is any species that has one or more unpaired electrons, making it unstable and reactive. Most biological molecules have two electrons that spin in the external orbit to make it stable (Halliwelll & Gutteridge, 1984). A free radical, depending on its reduction or oxidation potential, tends to extract an electron from a nearby molecule to reach its stability, and the target molecule in turn becomes a new radical (Iorio, 2002).

All aerobic life is constantly exposed to oxidant pressure from molecular oxygen and reactive oxygen species (ROS). ROS is a collective term which includes oxygen radicals (superoxide, peroxyl, and alkoxyl) and certain non-radicals that are either oxidizing agents or easily converted into radicals (Guetens *et al.*, 2002). Reactive nitrogen species (RNS) include species derived from nitrogen (Halliwell & Gutteridge, 2000). Different types of ROS and RNS are summarised in Table 2.

Table 2. Different types of ROS and RNS molecules (Halliwell & Gutteridge, 2000).

	ROS		**RNS**	
	Name	Symbol	Name	Symbol
Radicals	Hydroxyl	$OH^{\cdot}$	Nitric oxide	$NO^{\cdot}$
	Peroxyl	$RO_2^{\cdot}$	Nitrogen dioxide	$NO_2^{\cdot}$
	Alkoxyl	$RO^{\cdot}$		
	Hydroperoxyl	$HO_2^{\cdot}$		
	Superoxide	$O_2^{\cdot -}$		
Non-radicals	Hypochlorous acid	HOCl	Nitrous acid	HNO_2
	Ozone	O_3	Dinitrogen tetroxide	N_2O_4
	Singlet oxygen	$^1O_2$	Dinitrogen trioxide	N_2O_3
	Hydrogen peroxide	H_2O_2	Peroxynitrite	$ONOO^-$

A range of cellular process, external factors and/or disease states can lead to the formation of ROS and RNS (Ferguson *et al.*, 2006). Physical, chemical and biological agents can directly induce ROS generation (Onunkwor *et al.*, 2004). Such physical agents include ionizing and UV radiation. Ozone is also a chemical agent which is able to stimulate free radical production in living organisms. While radiation and ozone directly stimulate ROS production, some chemical agents such as aromatic polycyclic hydrocarbons and several drugs can also increase free radical production by indirect mechanism, like stimulating cytochrome P_{450} (Iorio, 2002).

Not only are there several different types of ROS and RNS, but these also lead to a range of effects on different macromolecules (Table 3).

Table 3. Oxidative damage to macromolecules (Ferguson *et al.*, 2006).

Target molecule	Nature of damage
Lipid	Peroxidation
	Generation of reactive products
Protein	Cross-linking or aggregation
	Enzyme inactivation
	Fragmentation
DNA	Base modification
	Single-strand DNA breaks
	Double-strand DNA breaks

Both ROS and RNS possess carcinogenic characteristics and have been implicated in human cancer development due to the potentially mutagenic oxidised bases in DNA. However, DNA damage is better regarded as a marker of exposure to genotoxic agents than as an indicator of the likelihood that cancer will occur in an individual (Collins, 2005). The development of cancer depends on a number of factors including the extent of DNA damage, antioxidant defences, DNA repair systems, the efficiency of removal of

oxidised nucleosides before incorporation into DNA, and the cytotoxic effects of ROS in large amounts or growth promoting effects in small amounts (Guetens *et al.*, 2002).

5.3.2. Oxidative DNA damage from ROS

H_2O_2 has the ability to cross the cell membranes easily and, therefore, enter the cells. Upon entry, H_2O_2, reacts with endogenous superoxide anions ($O_2^{\cdot -}$) and transition metal ions (Fe^{2+} and Cu^{2+}) via the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$ (Halliwell & Gutteridge, 1984). This results in the formation of the highly reactive hydroxyl radical ($OH^{\cdot}$). The hydroxyl radical is the ultimate DNA-attacking agent (Guetens *et al.*, 2002). It reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H^+ atom from the methyl group of thymine and each of the C-H bonds of 2'-deoxyribose. Such additions to the C5-C6 double bond of pyrimidines leads to C5-OH and C6-OH adduct radicals and H atom abstraction from thymine results in the allyl radical (Marnett, 2000 and Cooke *et al.*, 2003). Oxidative DNA damage caused by $OH^{\cdot}$ includes single-strand breaks, double-strand breaks, alkali-labile regions and oxidized purines and pyrimidines. If DNA damage is not repaired, accumulation of modified nucleotides may result, negatively affecting the integrity of the genome (Mohrenweiser *et al.*, 2003). As a result of this, cells have defence mechanisms to repair damaged DNA.

5.3.3. Antioxidant defence system

Antioxidants are substances which target and neutralise damaging free radicals in the body. The cell has developed an efficient defence system to control the production of ROS. When an organism is exposed to ROS, antioxidant defences are included in order to prevent or limit oxidative stress. This antioxidant system is important and consists of radical scavenging antioxidants and preventative antioxidants (Halliwell & Gutteridge, 2000).

Radical scavenging antioxidants scavenge radicals by directly reacting with the radical molecule to remove it by donating an electron to the reactive species (Halliwell & Gutteridge, 2000). These radical scavenging antioxidants include either hydrophilic (e.g.

ascorbate or Vitamin C, uric acid, billirubin, albumin) or hydrophobic compounds (e.g. tocopherols or Vitamin E, carotenoids) (Iorio, 2002; Ferguson *et al.*, 2006). Vitamin C, E and carotenoids are derived from the diet and others like gluthathione (GSH), uric acid, billirubin and albumin are synthesised by the cell. These substances or molecules act as chain-breaking antioxidants. They give off an electron to the ROS and they in turn convert into a new radical. This newly formed radical is poorly reactive and unable to attack, consequently stopping the chain reaction (Guetens *et al.*, 2002).

Preventative antioxidants are those that suppress the formation of free radicals and can be divided into two groups. The first are sequestrators or chelators, i.e. transferring, lactoferrin, hemopexin and albumin. The second group is the antioxidant enzyme system consisting of superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPx) (Matés *et al.*, 1999).

5.3.4. Oxidative stress

Oxidative stress is defined as a process in which the dynamic redox balance between oxidants and antioxidants is intensely shifted towards oxidative potentials (Serafini & Rio, 2004). Oxidative stress can either result in adaptation of the cell to the new conditions or cell injury (Halliwell & Gutteridge, 2000). Mild exposure of an organism to oxidative stress often leads to an increase in synthesis of cellular antioxidant defence system. This happens in order to repair the oxidant/antioxidant balance, to protect the cell against oxidant attack and also to prepare the cell for possible stronger oxidative attack. When ROS production is exaggerated and/or the cell's ability to inactivate ROS is reduced, the cell undergoes free radical damage, despite antioxidant defence (Iorio, 2002). Irreversible cell injury causes cell death by either necrosis or apoptosis. Necrosis involves swelling and rupture of the cell and affecting adjacent cells by releasing its contents into the surrounding area. Cell death by apoptosis is regulated and does not affect surrounding cells (Cobb *et al.*, 1996; Wochna *et al.*, 2007). Oxidative stress is implicated in aging and human diseases such as cancer and certain neurodegenerative diseases (Brenneisen *et al.*, 2005).

6. Factors that influence the level of DNA damage

There are several factors that have been reported to cause variations in the level of DNA damage in healthy untreated individual cells. Many of these factors are age, diet, exercise, smoking and period of exposure. There are now several studies that have described these variations in the basal level of DNA damage among healthy individuals. Some of them are discussed below.

6.1. Age

The effect of the age of an individual in the comet assay has been assessed in statistical analyses in most of the biomonitoring studies which have apparently yielded conflicting results. In a study done by Andreoli *et al.* (1997) no correlation was found between the extent of DNA damage and the age of the subjects in both the exposed (12 petrol attendants) and control subjects (12 healthy blood donors). However, Roma-Torres *et al.* (2006) observed with the effect of age, a significant increase of chromosome aberrations (CA) and micronuclei (MN) frequencies when comparing the elder (>50 years) with the youngest group (>30 years) within controls.

Another study of 80 individuals from Greece showed that men at the age of 55-60 years had an average of 14.5 % more DNA damage than men at the age of 20-25 years (Piperakis *et al.*, 1998). Overall, the age of the individual appears to have little effect on the mean basal level of DNA damage (Møller *et al.*, 2000).

6.2. Smoking

Smoking has always been one of the first exposure circumstances to which researchers turn their attention as a source of an agent that would produce a positive effect. Biomonitoring studies have, therefore, often included both smokers and non-smokers.

A study by Andreoli *et al.* (1997) showed no correlation between the extent of DNA damage and the smoking habits of the subjects in both the exposed (12 petrol attendants) and control subjects (12 healthy blood donors). Also, no association was found between smoking and chromosome aberrations (CA), micronuclei (MN) or DNA damage (Roma-

Torres *et al.*, 2006). The comet assay carried out by Franceschetti *et al.* (2005) to determine lymphocyte DNA damage in benzene exposed and unexposed subjects showed that smoking habits did not interfere with the results because they did not find any differences in mean values of comet parameters between smokers and non-smokers.

However, in a study done by (Şardas *et al.*, 1997) on professional colourists, both the exposed and control smoker subjects showed a greater proportion of damaged DNA. SCE frequency was found to be higher in smokers than in non-smokers in studies done by Çelik *et al.* (2003) and Pitarque *et al.* (1997). Fracasso *et al.* (2006) analysed lymphocyte DNA damage in subjects who were never-smokers, active-smokers, non-smokers and also ex-smokers exposed to second-hand tobacco smoke at the workplace, in order to investigate and compare their basal DNA damage. They observed that the active smokers showed significant high levels of basal DNA damage compared to other groups.

6.3. Period of exposure

With the period/time of exposure, the levels of CA and Tail length (TL) increased in a study done by Roma-Torres *et al.* (2006). The group of workers studied was divided into two, according to the duration of exposure and taking into account that 14 individuals were exposed for less than 5 years and 32 individuals for more than 19 years. The comet assay carried out by Franceschetti *et al.* (2005) to determine lymphocyte DNA damage in benzene exposed and unexposed subjects, showed that only the exposed group displayed a significant increase relative to the control group.

6.4. Exercise

Although exercise is regarded as promoting good health and well-being, excessive exercise is associated with oxidative stress, which is reflected by higher levels of oxidative DNA damage (8-oxodG) and lipid peroxidation (Møller *et al.*, 2000). Before the effect of exercise is known in sufficient detail, information about the type and intensity of the exercise as well as the time since the last session of exercise should be obtained in biomonitoring studies (Møller *et al.*, 2000).

6.5. Diet

A number of studies have investigated the effect of nutrients, antioxidants or a combination of antioxidants on the level of DNA damage as assessed by the comet assay in humans (Møller *et al.*, 2000). A review of such studies published in 2002 concluded that a single administration of antioxidant supplements, fruits, vegetables or natural products could reduce DNA damage in the following hours (Møller & Loft, 2002). A group of petrol attendants and auto-mechanics in Nigeria, occupationally exposed to lead, were supplemented with ascorbic acid (Vitamin C) for 2 weeks in a study done by Onunkwor *et al.* (2004). The findings suggested a protective effect of ascorbic acid in lead-induced toxicity. Following a review by Møller and Loft (2004) accumulating evidence from the intervention studies suggests that the repair activity of oxidative DNA damage may be elevated in subjects following ingestion of antioxidants.

6.6. Gender

As for gender, a statistical analysis is routinely carried out to evaluate any difference between the sexes in the study population. A study done in India by Bajpayee *et al.* (2002) described a gender-related difference in the basal level of DNA damage in a healthy Indian population, with the males showing higher damage than females, although no reason for these finding was discussed. In contrast, the results of a study done in Brazil indicated that H_2O_2 induces DNA damage in human lymphocytes independently of gender (Braz *et al.*, 2007). Generally at present the effect of gender must be regarded as a matter of controversy since most studies reported that men had more DNA damage than women, and in contrast, other studies state that women had more basal DNA damage than men (Møller *et al.*, 2000). Some studies used single measurements while others used repeated measurements (Møller *et al.*, 2000).

7. Measurement of oxidative DNA damage

Biological monitoring of exposure to chemical substances has become an important strategy in the evaluation of risks to human health in order to improve conditions of occupational health and safety (Roma-Torres *et al.*, 2006).

7.1. Comet assay

The single cell gel electrophoresis, or comet assay as it is well known, provides a measurement of single or double-strand DNA breaks at the level of the single cell (Horváthová *et al.*, 1998). The technique involves the evaluation of cells kept in agarose gel (on a microscope slide), submitted to electrophoresis and dyed with ethidium bromide. Cells with damaged DNA form a comet consisting of a head (nuclear matrix) and a tail (formed by DNA fragments). The amount of DNA that has migrated is correlated with the degree of damage (Singh *et al.*, 1988). DNA damage level increases from class I to V (Giovannelli *et al.*, 2002), as indicated by the increasing tail migration (class 0: 0–6%; class I: 6.1–17%; class II: 17.1–35%; class III: 35.1–60%; class IV: 60.1–100%; and class V: heavily damaged). *See Figure 1.*

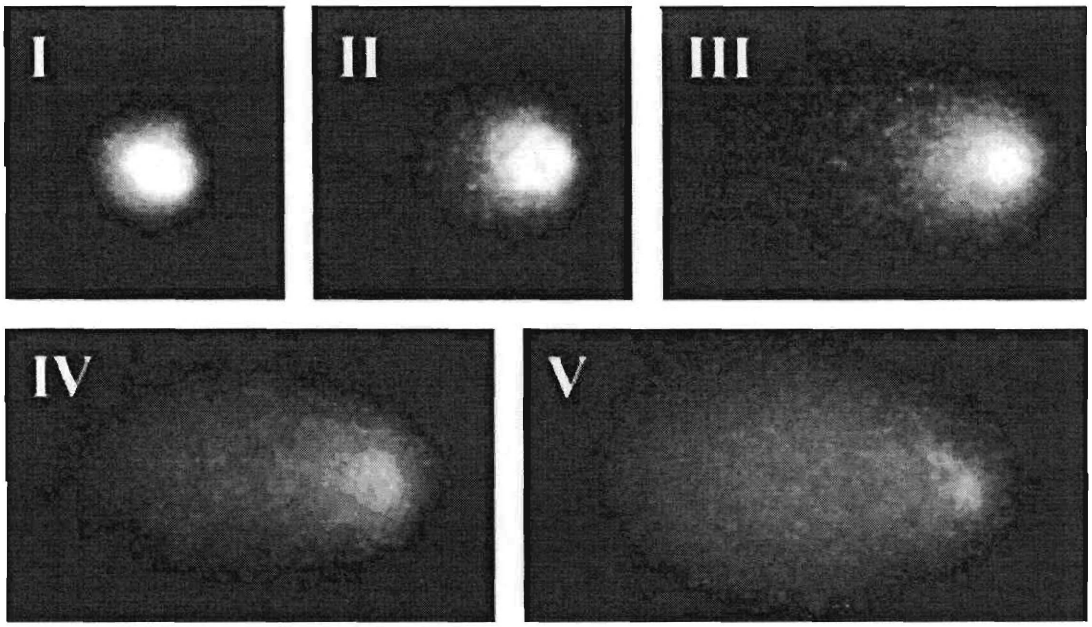

Figure. 1. Illustration of the different classes of comets, corresponding to increasing DNA damage. Each image represents one class of damage as indicated in each panel (Giovannelli *et al.*, 2002).

The comet assay is extremely versatile, rapid, sensitive, and is used extensively in Biology, Medicine and Toxicology due to its capacity and sensitivity in demonstrating DNA breaks, both single and double strands breaks and alkali-labile sites (Sardas *et al.*, 1997). The alkaline conditions cause the separation of the paired bases, enabling the detection of simple chain ruptures (Martino-Roth *et al.*, 2003).

Measuring DNA strand breaks gives limited information. Oxidatively altered bases can be detected by using an enzyme-modified version that includes a DNA digestion step using either a DNA glycosylase or an endonuclease enzyme. These enzymes recognise a particular kind of damage and create a single strand break. The oxidatively altered base may in fact be apurinic or apyrimidic sites (i.e. AP sites or baseless sugars), which are alkali labile and, therefore, appear as breaks. Oxidised purines including 8-oxodG can be detected after incubation with formamidopyrimidine DNA glycosylase (FPG), whereas oxidised pyrimidines are revealed by endonuclease III (Endo III). In each case, the enzyme-sensitive sites converted to additional DNA breaks increase tail intensity. A further variant of the technique exposes white blood cells *ex vivo* to DNA-breaking agents such as hydrogen peroxide (H_2O_2) as a measure of the way in which a dietary regimen has enhanced the innate ability of cells to repair damage (Collins, 2004; Ferguson *et al.*, 2006).

7.2. Assays for oxidative stress and antioxidant status

Analysis of oxidative stress and its relationship to antioxidant need are usually approached by two strategies. The first measures damage caused by free radicals and the second measures the antioxidant reserve or capacity.

Hydroperoxides (R-OOH) are formed as a result of lipid peroxidation and can be measured in biological fluids (Iorio, 2002). It is important to note that hydroperoxides are considered a marker of oxidative stress and should not be confused with hydrogen peroxide (H_2O_2). Because of their stability and good oxidant capacity, hydroperoxides, are detectable in biological fluids by means of the d-ROMs (diacron reactive oxygen metabolite) test. The d-ROMs test takes advantage of the property of hydroperoxides to generate hydroperoxyl and alkoxyl radical in the presence of iron ions. Hydroperoxides in serum, after reacting with a chromogenic substrate, develop a coloured derivative (pink to red). This detectable coloured complex is then quantified spectrophotometrically. The amount of hydroperoxides, which correlates directly with the detected colour intensity, is expressed as arbitrary units ("CARR U", i.e. Carratelli Units) (Iorio, 2002).

In order to determine the ability of an individual or population group to cope with oxidative stress, it is essential to measure levels of free radical scavengers. The variety of antioxidant defences in plasma makes it difficult to separate the contribution of each one. This has led to the measurement of total antioxidant capacity, rather than individual antioxidant components. For recent extensive reviews on these assays, see Cao and Prior (1999), Prior (2004), Serafini and Rio (2004), Collins (2005) and Ferguson *et al.* (2006). The FRAP (ferric reducing antioxidant power) assay measures the ability of plasma samples to reduce the colourless ferris-trypyridyltriazine complex (TPTZ-Fe^{3+}) to its ferrous coloured form (TPTZ-Fe^{2+}) (Benzie & Strain, 1999). It is a simple and highly reproducible spectrophotometric assay using ascorbic acid (Vitamin C) as a standard. Disadvantages of this assay are that it does not work at physiological conditions (pH 3.6) and does not measure the contribution of liposoluble antioxidants and thiols groups. The multivariate nature of the oxidative stress status requires the use of a combination of different assays to better evaluate the status (Cutler, 2005).

8. Summary

Petrol is a complex mixture of low-molecular mass compounds, some of which are carcinogenic, genotoxic and others neurological effects. Occupational exposure to petrol VOCs poses a health risk. In view of this occupational health risk it is important to determine the level of exposure to petrol VOCs and also evaluate the effect caused by this exposure.

Following this review, in Chapter 3, is an article investigating the genotoxic effect of petrol VOCs on lymphocytes of petrol attendants. This article comprises of a study of the level of occupational exposure of petrol attendants to petrol VOCs, as well as its effect on the oxidative DNA damage and DNA repair capacity.

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CHAPTER 3

ARTICLE

DNA damage and repair detected by the comet assay in lymphocytes of African petrol attendants: A pilot study.

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1. Abstract

Petrol attendants are exposed to petrol volatile organic compounds (VOCs) which may have genotoxic and carcinogenic effects. The single cell gel electrophoresis assay (comet assay) is a method highly sensitive to DNA damage induced by environmental and occupational exposure to carcinogenic and mutagenic agents. The aim of this study was to evaluate the level of exposure of petrol attendants to petrol VOCs and also to determine their effect on DNA damage and repair in lymphocytes of African petrol attendants. The exposed group consisted of 20 subjects, randomly selected from three petrol stations. A control group of 20 unexposed subjects was also chosen and matched for age and smoking habits with the exposed group. Sorbent tubes were used to assess personal exposure of petrol attendants. The comet assay was used to investigate the basal DNA damage and repair capacity in isolated lymphocytes of petrol attendants and control subjects. Blood samples were taken from the petrol attendants at the end of their 8 hour working shift and also from the control subjects. The petrol attendants were found to be exposed to levels of petrol VOCs lower than the occupational exposure limit (OEL) for constituent chemicals. A significant relationship was found between the volume of petrol sold during the shift and the average concentrations of benzene, toluene and the total VOCs measured. However, relative humidity had a negative correlation with the average concentrations of benzene, toluene, xylene and the total VOCs. Significantly higher basal DNA damage was observed with the exposed group compared to the control group. The period of exposure influenced the level of DNA damage and the calculated repair capacity. Smoking and age had a significant influence on the level of DNA damage. DNA repair capacity was delayed in smokers of both exposed and non-exposed group.

2. Introduction

Petrol is a complex mixture of low-molecular mass compounds, mainly paraffins, naphthenes, olefins and aromatics, which can cause mutations and cancer (Pitarque *et al.*, 1997). Aromatic compounds of petrol are predominantly benzene, toluene and xylene (BTX) (Periago *et al.*, 1997). Benzene, from a toxicological view, is the most hazardous component and has been classified as a human carcinogen by the International Agency for Research and Cancer (IARC, 1989) and the American Conference of Governmental Industrial Hygienists (ACGIH, 2003). Although toluene and xylene are not classifiable as human carcinogens, their exposure can lead to neurological effects such as headache, dizziness, fatigue, tremors, incoordination, anxiety, impaired short-term memory and inability to concentrate (ATSDR, 2006 & 2007). The South African Occupational Health and Safety Act, 1993 (Act No. 85 of 1993) established regulations for hazardous chemical substances. The time weighted average occupational exposure limits (TWA-OEL) for petrol VOCs are: benzene (16 mg/m^3), ethylbenzene (435 mg/m^3), toluene (188 mg/m^3) and xylene (435 mg/m^3).

Workers can be exposed to relatively high levels of petrol vapours in petrochemical refineries and petrol service stations, or to low levels of petrol vapours in the general population (Pitarque *et al.,* 1997). Previous studies done on exposure to VOCs for individuals with occupations associated with exposure to petrol vapour emissions, yielded evidence that workers were exposed to significantly higher levels of aromatic hydrocarbons. In Italy, Periago *et al.* (1997) found that hydrocarbons were elevated in ambient air, and also that climatic conditions can increase the risk of exposure during shifts (Periago & Prado, 2005). According to a study done in Thailand, service station attendants showed a more elevated exposure to benzene than any other occupation studied (Navasumriti *et al.,* 2005). However, all of these studies were conducted in different countries, but no studies on the occupational exposure to petrol vapours have been done in black African petrol attendants.

Biological effects of DNA damaging agents have been detected by the use of a number of techniques. Singh *et al.* (1988) developed a simple approach, the Comet assay, for sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. Occupational exposure to lead was found to induce *in vivo* relevant biological effects according to a study by Fracasso *et al.* (2002). This study confirmed previous observations of toxic effects of lead on lymphocytes. The Comet assay reported in a study by Andreoli *et al.* (1997) showed a significant excess of DNA damage in circulatory lymphocytes of petrol attendants who were occupationally exposed to low benzene levels, compared to the age-matched reference group. In other studies there is a lack of clear indication on the exposure levels at the workplace and, therefore, it is difficult to determine if the presence or absence of genetic damage can be associated with high or low levels of exposure.

This study, therefore, aims to characterise the personal exposure of petrol attendants to petrol VOCs during an 8 hour working shift, and also to analyse the occurrence of oxidative DNA damage and the level of DNA repair in peripheral lymphocytes of a group of African petrol attendants, and a paired group of controls, using the comet assay. The results will be corrected for confounding factors such as age and smoking habits.

3. Materials and Methods

3.1. Subjects investigated

A group of 20 petrol attendants (smokers and non-smokers) from three different petrol stations at different locations within Potchefstroom were recruited for this study. These petrol stations were chosen on the basis of the volume of petrol sales per month. The control group consisted of 20 healthy men recruited form non-petroleum occupants and students at the North-West University, Potchefstroom Campus. These subjects were matched for age and smoking habits with the experimental group. They had no history of occupational or recreational exposure to petrol vapours or any other suspicious genotoxicity agents. Health conditions and lifestyle factors such as smoking, alcohol

consumption, use of pharmaceutical drugs and occupational history were assessed by means of questionnaires.

All subjects were fully informed about the procedures and objectives of this study and an informed consent form was signed by each subject prior to the study. Ethical approval for this study was obtained from the Ethics Committee of the North-West University, Potchefstroom Campus. Ethical approval number: 06M11.

3.2. Sampling strategy

Sampling conditions: All the petrol attendants were working at the time when the air as well as blood samples were collected. Samples were taken on a Friday which was considered the worst-case-scenario, since it was the day of the week on which the highest volumes of petrol are usually sold. Climate conditions (temperature, humidity and wind speed), work shifts and the volume of petrol sold were measured. The climate conditions were measured with the use of the Vantage Pro Weather station.

Work study: A work study was done on the experimental group which reflected the manner in which the individual poured petrol into the car's petrol tank and whether or not the individual was involved in any other tasks during car refuelling. The work study also showed the volume of petrol each attendant poured. A table was designed which the petrol attendant filled in during his sampling period to complete the work study.

Air sampling: The sampling period was for an 8 hour working shift, after which the samples were capped, labelled, transported to the laboratory and stored at 4°C until sent for analysis at an accredited laboratory.

Blood samples: Blood samples (6 ml) were drawn into heparin tubes by a qualified nurse at the end of the shift every Friday from the petrol attendants and throughout the week from the control subjects. Each sample was capped, labelled and kept at 4°C before laboratory analysis at the end of each sampling period.

3.3. Personal exposure measurements

The NIOSH method 1501 was used to measure personal exposure of petrol attendants with a few modifications (NIOSH, 2003).

Measurements of laboratory and field blanks were taken for quality control. The sampling train was prepared prior to each monitoring and each sampling pump was pre- and post-calibrated at a flow rate of 0.125 L/min. After each sampling, the samples were sealed, kept at 4°C and sent for gas chromatography (GC-MS) analysis at an accredited laboratory (Chemtech Laboratory Services). The mass of every organic contaminant, as determined by the GC-MS analysis, was converted to time-weighted average concentration in air.

3.4. Genotoxicity studies

Lymphocyte preparation: Human peripheral blood lymphocytes were isolated from heparinized blood on Histopaque. The plasma was removed and kept frozen at -70°C for the free radical assay (dROMs) and the antioxidant assay (FRAP). The isolated cells were washed twice with PBS and then re-suspended in 1 000 µl PBS, vortexed and kept at 4°C for ±1 hour. Each sample contained approximately 1 000 cells.

Comet assay: The comet assay was performed under alkaline conditions as described in a study by Van Dyk and Pretorius (2005), with minor modifications. Briefly, 30 µl of the cell suspension was mixed with 90 µl LMPA (low melting point agarose) at 37°C, and evenly layered onto the microscope slides pre-coated with 300 µl HMPA (high melting point agarose). Slides were kept on ice to solidify and also to stop cell metabolism. The remaining cells were exposed to 40 µl H_2O_2 [600 µM] for 10 min at 37 °C. The exposed cells were washed with PBS, re-suspended in 280 µl HAMS F10 solution, and incubated for 30, 60 and 90 min at 37 °C. At each time point an aliquot of cells was spread on the slides as described above. The slides were then immersed in chilled lysis buffer at 4 °C and kept overnight. When the DNA in the agarose was treated with the lesion specific enzymes Fpg and EndoIII, the slides were first rinsed with ddH_2O and then covered with a small volume of enzyme buffer containing the respective enzyme. Incubation was done

in a damp container for 30 min at 37 °C. Thereafter, the slides were placed in a horizontal electrophoresis tank containing freshly prepared and chilled electrophoresis buffer. The slides were left for 30 min to allow DNA unwinding and electrophoresis was then performed at 30 V and 300 mA at 4 °C for 40 min. The slides were rinsed with ddH_2O and then placed in a neutralizing buffer for 15 min at 4 °C. Staining of the slides was done with ethidium bromide. Photographs of the comets were taken with an Olympus IX70 Inverted system microscope (200X magnification). For each sample a minimum of 50 comets were taken and the DNA tail intensity determined under constant sensitivity with Comet Assay IV software (Perspective Instruments) and the data were processed in Microsoft Excel. The repair capacity was calculated as $RC = 1 - \left(\frac{\%\ tail\ DNA\ 60\,\text{min}}{\%\ tail\ DNA\ H_2O_2} \right)$.

The comets were classified into different classes according to the amount of DNA tail intensity (Giovannelli *et al.*, 2002): class 0, 0-6 %; class 1, 6.1-17 %; class 2, 17.1-35 %; class 3, 35.1-60 %; and class 4, >60 %. For comets which could not be scored by the software due to having a very small head and a long tail, manual scoring was done, and an additional class for "heavily damaged" cells was created to accommodate such comets (Van Dyk & Pretorius, 2005).

3.5. Oxidative stress

Oxidative stress is a process in which the dynamic redox balance between oxidants and antioxidants is intensely shifted towards oxidative potentials (Serafini & Rio, 2004). Analysis of oxidative stress and its relationship to antioxidant need are usually approached by two strategies. The first measures damage caused by free radicals and the second measures the antioxidant reserve or capacity.

3.5.1. Oxidative status assay

The oxidative status measuring mostly hydroperoxides present in serum was measured with the aid of an assay kit (d-ROMs test) from DIACRON International. Reagents were used as supplied in the d-ROMs test kit and the test was performed as described in the protocol with a few modifications according to Davis *et al.* (2007). Absorbance changes

were measured kinetically at 560 nm in 3 min intervals for 15 min using a Bio-Tek FL 600 microplate reader. The concentration of hydroperoxides in the sample was expressed in Carratelli (CARR) units (Iorio, 2002).

3.6. Antioxidant capacity

3.6.1. Ferric reducing antioxidant power (FRAP)

Plasma samples were prepared as discussed in Section 3.4. The ability of antioxidants in the sample to reduce the colourless ferris-trypyridyltriazine complex (TPTZ-Fe^{3+}) to its ferrous coloured form (TPTZ-Fe^{2+}) was measured following the method described by Benzie and Strain (1996), with a few modifications. A standard series of 0 – 100 µM ferric sulphate ($FeSO_4$) was used for the assay. Plasma (15 µl) and ddH_2O (85µl) were added to the wells of a microtiter plate. Two hundred and fifty µl FRAP reagent, consisting of a mixture of acetate buffer (300 mM $NaAc.3H_2O$), TPTZ (10 mM), $FeCl_3$ (0.45 mg/ml) in the ratio of 10:1:1 was added to standards and samples. The plate was read after 3 min (25° C) at 593 nm in a BIO-TEK FL600 microplate reader. Linear regression of data from the standards was used to calculate the antioxidant capacity (R^2-value ± 0.99 was linear).

The oxidative stress status can be determined as follows:

$$Oxidative\ Stress\ Status\ ratio = \left(\frac{Oxidative\ status}{Antioxidant\ status} \right).$$

This ratio is calculated on the bases of in-house reference ranges from a study done at the Biochemistry laboratory at the North-West University, Potchefstroom Campus (Professor F.H. van der Westhuizen, School of Biochemistry, North-West University).

3.7. Statistical analysis

All data were transferred to Microsoft Excel and statistically analysed using the computer software package Statistica© version 7 (Statsoft Inc, Tulsa, OK, USA). Variables were tested for normality and non-normally distributed data were transformed into approximately normal distribution using logarithmic transformation. The Student *t*-test

was used to compare mean exposure levels. Spearman's correlation analysis was used to determine the correlation between the variables (level of DNA damaged, repair capacity and oxidative stress status) and the personal exposure and volume petrol sold. The ANCOVA was used to test the statistical significance of the difference between the means of variables studied within the two groups adjusted for age and smoking status. A regression analysis was used to determine the association between petrol VOCs as dependent variables and their influential factors as independent variables. Another regression analysis, assessing the effect of age, petrol exposure and smoking on the level of DNA damaged and repair capacity as well as the oxidative stress status of the exposed group was done.

4. Results and Discussion

Table 1 shows the demographic characteristics of the subjects studied (exposed and control group). Seventy percent of the workers fall in the age group 25-35 years, with only 10% in the younger and 20% in the older group. The number of smokers (55%) was higher than the non-smokers (45%) in both groups. BTX form an important group of aromatic compounds of petrol (Periago *et al.*, 1997) and were chosen as target VOCs due to the carcinogenic properties of benzene (IARC, 1989; ACGIH, 2003) and the neurological effects of toluene and xylene (ATSDR, 2006 & 2007). The results of the personal exposure monitoring are given in Table 2. Both the laboratory and field blanks were analyzed for contamination and none was identified as there were no VOCs in the blanks. The mean values and standard deviations for BTX and total VOCs as well as the influential factors are shown for the whole sampling period. The exposure levels of BTX (0.65, 0.73 and 0.31 mg/m^3 respectively) for the exposed population studied were much lower than the occupational exposure limit values. Of the three VOCs, toluene yielded the highest exposure level (0.73 mg/m^3). Even at these low levels, exposure should be monitored and controlled as these levels can also have genotoxic effects (Hakkola, 2001; Franceschetti *et al.*, 2005)

Table 1. Characteristics of the study population.

	Exposed group	Control group
Number of subjects	20	20
Age (years)		
<25	2	2
25-35	14	14
>35	4	4
Smoking habits		
Smokers	11	11
Non-smokers	9	9

Table 2. Summary of personal exposure monitoring.

Subject	Benzene (mg/m^3)	Toluene (mg/m^3)	Xylene (mg/m^3)	Total VOCs (mg/m^3)	Temp (°C)	Relative Humidity (%)	Wind Speed (m/s)	Volume Sold (*l*)
Lab & field Blanks	0	0	0	0	-	-	-	-
1	0.06	0.08	0.04	3.02	23.4	57	2.2	470.37
2	0.31	0.34	0.14	18.02	23.9	28	2	1072.03
3	0.29	0.3	0.12	19.08	24.5	52	2.1	1455.07
4	0.22	0.25	0.1	13.188	27.2	38	2.6	1042.28
5	0.21	0.29	0.19	17.47	21.9	67	1.3	1336.48
6	0.61	0.71	0.31	39.88	23.9	35	1.3	1625.58
7	0.46	0.65	0.08	27.99	21.6	68	1.4	1542.13
8	1.26	1.26	0.54	94.55	20.9	32	2.1	1425.22
9	0.55	0.68	0.39	37.1	21.2	32	2.2	1002.02
10	1.17	1.17	0.49	87.6	22.9	34	1.4	1345.18
11	0.54	0.58	0.27	39.74	23.8	27	2	1159.95
12	0.73	0.9	0.53	49.57	22.2	36	1.3	1655.06
13	0.65	0.76	0.35	44.35	24	26	2	935
14	0	0.66	0.31	34.25	23.5	36	1.2	1207.93
15	1.27	1.25	0.47	87.22	26.7	23	0.5	1334.6
16	1.85	1.73	0.5	99.61	23.9	48	2.5	1686.03
17	0.27	0.34	0.17	30.12	28.7	34	2.2	1165.86
18	0.83	0.85	0.32	75.58	28.9	25	0.6	1397.54
19	1.00	1.02	0.41	75.02	27.2	22	0.5	1203.37
20	0.62	0.69	0.37	38.11	21.2	32	2.1	1105.85
Mean*	0.65	0.73	0.31	46.57	24.08	37.60	1.68	1258.38
SD*	0.47	0.41	0.16	29.53	2.46	13.70	0.65	288.43

*Mean concentrations and standard deviation (SD).

A correlation was determined between BTX as well as the total VOCs and the varying climatic condition together with the volume petrol sold for the whole sampling period. Table 3 shows a correlation study with p-values < 0.05 and r-values $\pm$ 0.4 or larger, for significant correlations. Logarithmic transformations were made for benzene, toluene, and total VOCs due to their skewed distribution. No log transformation was necessary for xylene as it was normally distributed. A significant relationship was found between the levels of exposure to benzene, toluene and total VOCs and the volume of petrol sold and relative humidity. This can be explained by the airstream saturated with petrol vapour which was evacuated from the car's petrol tank during petrol refilling. The volume of airstream evacuated is exactly equal to the volume of petrol pumped, thus the significant influence of volume petrol sold. Xylene only showed a significant relationship with relative humidity. The volume of petrol sold showed a positive correlation with BTX and total VOCs, while relative humidity gave a negative significant correlation of -0.497, -0.453, -0.518 and -0.527 with BTX and the total VOCs respectively. This implies that when the volume of petrol sold increases, the emissions of benzene, toluene and total VOCs increases, as shown by correlation coefficients of 0.436 - 0.651. A similar statistically significant increase was observed by Periago *et al.* (1997) between levels of aromatic compounds in the air and the volume of petrol dispensed. However, increased relative humidity causes a decrease in BTX and the total VOCs concentration, and *vice versa*. Temperature and wind speed showed no correlation with exposure levels studied.

Table 3. Correlation between BTX, total VOCs and the influential factors studied.

		Volume petrol sold		Temperature		Relative humidity		Wind speed	
	N	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Benzene	20	0.634	0.004*	-0.023	0.926	-0.497	0.03*	-0.358	0.133
Toluene	20	0.651	0.002*	-0.741	0.756	-0.453	0.045*	-0.376	0.103
Xylene	20	0.436	0.055	-0.179	0.448	-0.518	0.019*	-0.286	0.221
Total	20	0.638	0.002*	0.071	0.767	-0.527	0.017*	-0.403	0.078

* Marked correlations significant at $p < 0.05$

Table 4 shows the regression analysis of the personal monitoring of the exposed group. In these regression models, with BTX and total VOCs as dependent variables and the presence of volume of petrol sold, temperature, relative humidity and wind speed used as independent variables, there was a good association with the four dependent variable $\log_{10}$ (benzene): $R^2 = 0.697$; $\log_{10}$ (toluene): $R^2 = 0.588$; xylene: $R^2 = 0.702$; $\log_{10}$ (total VOCs) $R^2 = 0.721$. Volume of petrol sold ($P = 0.0003$) and relative humidity ($P = 0.001$) showed a significant influence in the exposure levels of benzene. These two variables as well as temperature ($P = 0.112$) and wind speed ($P = 0.647$) explained the 69.7% of the variance in $\log_{10}$ (benzene). With logarithm of toluene exposure, the volume of petrol sold ($P = 0.0002$) and relative humidity ($P = 0.001$) also showed a significant influence. Together with temperature ($P = 0.051$) and wind speed ($P = 0.437$), these four variables accounted for 58.8% of the variance in $\log_{10}$ (toluene). Similarly, volume of petrol sold ($P = 0.0001$) and relative humidity ($P = 0.0003$) had a significant influence in the exposure of $\log_{10}$ (total VOCs) and together with temperature ($P = 0.268$) and wind speed ($P = 0.499$) they accounted for 72.1% of the variance in the logarithm of total VOCs exposure. However, volume of petrol sold ($P = 0.014$), relative humidity ($P = 0.001$) and temperature ($P = 0.014$) significantly influenced xylene exposure. Together with wind speed ($P = 0.511$) these three variables accounted to 70.2% of the variance in Xylene exposure. Therefore, volume petrol sold and relative humidity significantly influenced the level of exposure, as was seen in Table 3.

Table 4. Regression of $\log_{10}$ (Benzene), $\log_{10}$ (Toluene), Xylene and $\log_{10}$ (Total VOCs) as dependent variables with independent variables in the total subject group (N=20). Beta and level of significance *P* are shown.

Independent variable		**Dependent variable**			
		Log_{10} (Benzene)	Log_{10} (Toluene)	Xylene	Log_{10} (Total VOCs)
Volume petrol sold	*ß*	0.655	0.654	0.439	0.658
	P	0.0003*	0.0002*	0.014*	0.0001*
Temperature	*ß*	-0.247	-0.296	-0.457	-0.156
	P	0.112	0.051	0.014*	0.268
Relative humidity	*ß*	-0.64	-0.605	-0.712	-0.63
	P	0.001*	0.001*	0.001*	0.0003*
Wind speed	*ß*	-0.069	-0.113	-0.112	-0.095
	P	0.647	0.437	0.511	0.499

* Significant at $p < 0.05$

Table 5 shows the effect of exposure to petrol VOCs on DNA damage, repair capacity and oxidative stress status. The parameter tail intensity was used to quantify the level of DNA damage (Collins, 2004) in lymphocytes of both the control and exposed subjects. This parameter was used as it bears a linear relationship to DNA break frequency, is relatively unaffected by threshold setting and allows discrimination of DNA damage over the widest possible range (0-100% DNA in tail) (Collins, 2004). DNA damage and oxidative stress status were not normally distributed and, therefore, log transformations for both were used. For all variables under comparison, the exposed group showed a tendency of higher values in comparison with the control group. However, only the level of DNA damage showed a significant difference between these groups ($p< 0.05$). Although the difference is very small, the exposed group had a better DNA repair capacity than the control group. The comet analysis in studies by Andreoli *et al.* (1997) and Franceschetti *et al.* (2005) showed a significant degree of DNA damage in circulating lymphocytes of workers occupationally exposed to low benzene levels, compared to age-matched controls.

The reference ranges of a study done at the Biochemistry laboratory of the North-West University (Professor F.H. van der Westhuizen, School of Biochemistry, North-West University, Potchefstroom Campus) on a group of healthy individuals were 343.01 – 838.38 μM FRAP units (mean, 565.38; n = 81), while the dROMs values ranged from 238.41 to 741.21 Carr U (mean, 438.63; n = 75). Hydroperoxides (dROMs test) in the control group studied varied between 208 and 555 Carr U, while the exposed group varied between 208 and 600 Carr U. There was no significant difference between the mean values of the two groups and they were both within the given reference ranges. The FRAP values for the control subjects were within the reference ranges, with only one subject having 332 μM FRAP units. Five of the exposed subjects had values below the given reference ranges. Comparing the mean FRAP values of both groups studied, the control group had a higher value of 486.6 μM FRAP units compared to the exposed group with 393.95 μM FRAP units. It is possible that the antioxidant defences in the exposed group were overwhelmed by external factors such as environmental stressors and dietary intake. However, the compliance of the diet of subjects was not considered as

it was not reliable. The exposure to petrol VOCs compromised the antioxidant capacity, which probably lead to increased oxidative stress in the exposed group.

Table 5. Results of DNA damage, repair capacity and oxidative stress status in control and exposed groups.

	N	DNA damage (tail intensity)	Calculated Repair Capacity	Oxidative Stress Status
Control group	20	6.30 ± 3.37	0.25 ± 0.24	0.90 ± 0.25
Exposed group	20	15.06 ± 9.10*	0.32 ± 0.28	1.32 ± 0.84

Mean ± SD

* Significantly different from control group ($p<0.05$), according to independent samples t-test.

Figure 1 represents the mean DNA damage and repair values of the exposed group versus the control group (n = 20). Following exposure to petrol VOCs, basal DNA damage was measured before treatment of the cells with H_2O_2 (control), directly after H_2O_2 treatment and after incubation period of 90 min (30 min intervals). The basal DNA damage (control) was low and it increased after H_2O_2 treatment in both study groups. A significant basal DNA damage ($p < 0.05$) was seen in the exposed group. DNA repair capacity of a cell was measured by inducing oxidative DNA damage with H_2O_2 and then monitoring the rate at which the lesions repair (Collins, 2004). In Figure 1, after H_2O_2 removal, some repair took place for the subsequent 90 min incubation. This is also evident with the calculated repair capacity of 0.32 and 0.25 for the exposed and control groups respectively, as represented in Table 5.

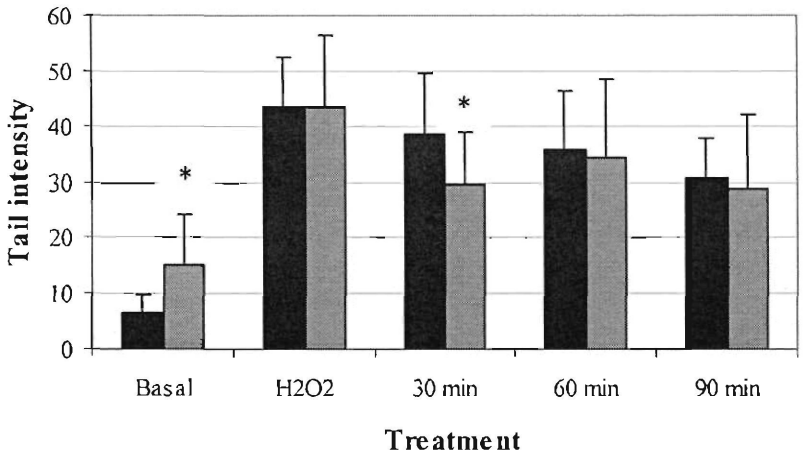
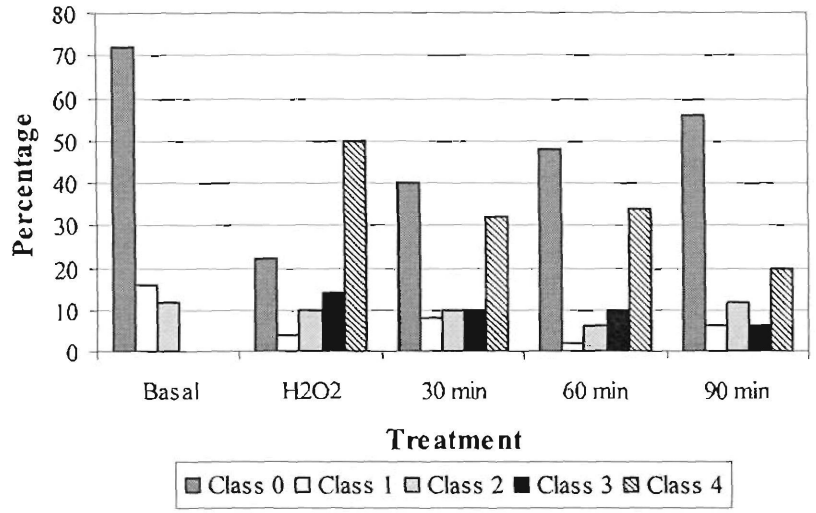


Figure 1. Mean ± SD values of DNA damage and repair in lymphocytes of control (black) and exposed (grey) subjects. Error bars show the standard deviation of the mean values. (*$P < 0.05$) Control versus exposed group.

In Figure 2, analysis of the class distribution of comets (Giovannelli *et al.*, 2002) in a subject representative of the average control (Fig. 2A) and exposed (Fig. 2B) groups was done. In the control group the majority of comets were in class 0 while for the exposed group they were distributed between classes 0 and 1. Approximately 75% of the total DNA was still intact or slightly damaged in the control group. The group exposed to petrol showed a moderate basal damage as indicated by the clustering of comets in class 1 and 2. However, it is clear from the results that oxidative stress by H_2O_2 caused more DNA damage with the formation of class 4 comets in both groups studied. Although Figure 1 showed no significant difference between the two groups after additional oxidative stress, this distribution of comets showed an increased presence of class 4 comets in the control group than in the exposed group. During the 90 min incubation period class 4 comets decreased substantially, with a concomitant increase in class 0 comets. The abrupt increase in DNA damage observed at 60 min repair time (Fig. 2B) may be due to the mechanisms of nucleotide excision repair (NER). The NER system cuts the sugar-phosphate backbone of a DNA strand in two places, one on each side of the lesion and removes the region. The resultant gap is then filled in using DNA polymerase and the sugar-phosphate backbone is covalently closed by DNA ligase (Christmann *et al.*, 2003), as can be seen by continued repair during the subsequent 30 min. This distribution of comets showed some repair since at 90 min repair time, 40% of the comets were in class 0 while class 4 comets were reduced to 14% in the exposed group. A similar pattern of comets distribution was seen at 90 min repair time in the control group (Fig. 2A) with 56% of the comets in class 0 and 20% in class 4.

A



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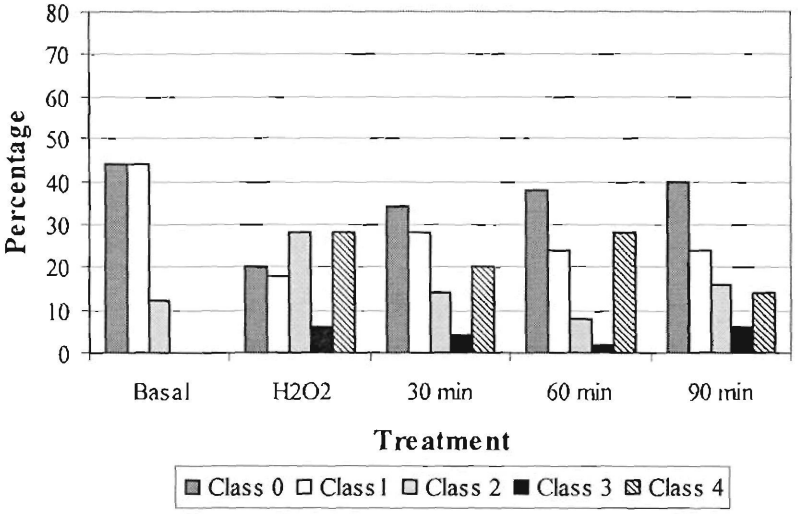


Figure 2. Distribution of comets in classes after DNA damage and 90 min repair time. Subject representative of average control group (A) and average exposed group (B).

According to the correlation study depicted in Table 6, only DNA damage and oxidative stress status showed significant correlations with the volume of petrol sold and personal exposure, respectively. No correlations were found with the calculated repair capacity.

Table 6. Spearman's correlation between variables studied and the personal exposure and volume of petrol sold.

		Personal exposure	Volume petrol sold
DNA damage	20	0.311	0.638*
Calculated Repair Capacity	20	-0.194	0.118
Oxidative Stress Status	20	0.460*	0.268

* Marked correlations are significant at $p < 0.05$

Figure 3 represents the effect of period of exposure on DNA damage and repair in lymphocytes of petrol attendants. These were also compared to the average control group. The exposed subjects were divided into four categories (<1 year; 1-3 years; 5-10 years; and >10 years) according to the period of exposure to petrol. Subjects exposed for more than 10 years showed the highest level of basal DNA damage, while those exposed for less than a year had minimal DNA damage. Additional oxidative stress caused an increase in DNA damage in all groups studied. Subjects exposed for less than a year showed a similar tendency as the control group, although the basal damage was slightly higher than the control group. The addition of H_2O_2 caused an increase in tail intensity by 30 %, however, the damage was lower than the other compared groups. A similar pattern was observed in the group exposed for 1-3 years. Both subjects exposed for 5-10 years and more had the greatest oxidative damage after treatment with H_2O_2. There was an abrupt initial repair which can be seen by the steep slope during the first 30 min after oxidative damage (H_2O_2) in the exposed groups. However, in the groups exposed for more than 1 year, this repair could not be maintained for the whole 90 min repair time given. In contrast, the slope of the control group and to some extent the group exposed for less than 1 year gradually decreased and repair was maintained for the whole repair time given. It can be deduced that DNA repair could not be maintained in the groups exposed for longer periods, because the exposure to petrol VOCs inhibited the repair of single strand breaks in the initial repair process. Therefore, due to such long periods of exposure, DNA repair was compromised. This is also observed with the mean calculated repair capacity of 0.29 in subjects exposed for more than 10 years and 0.46 in those exposed for less than a year (data not shown).

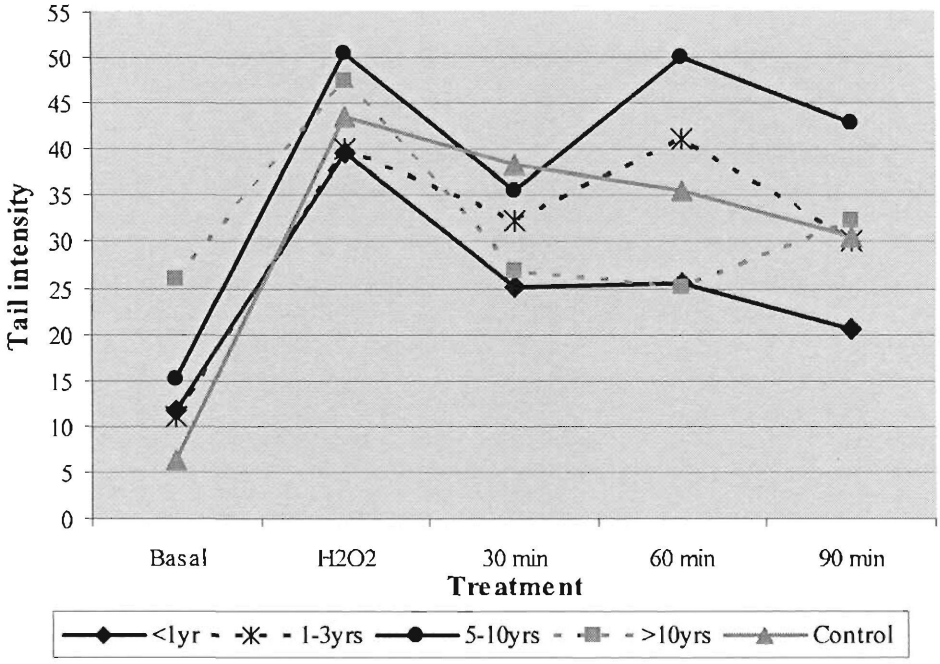


Figure 3. Effect of period of exposure on DNA damage and repair in lymphocytes of petrol attendants. The cells were exposed to 40 µl H_2O_2 at 37°C for 90 min.

The ANCOVA was used to test the statistical significance of the difference between the means of the variables studied (the level of DNA damaged, repair capacity and oxidative stress status) and the two groups, adjusted for age and smoking status in Table 7. The results as summarized in Table 7 show no statistical significance, except for the level of DNA damaged ($p<0.05$). Therefore, age and smoking only have an influence in the level of DNA damaged and no effect on repair capacity and oxidative stress status. However, in a study by Andreoli *et al.* (1997) no significant association was found between DNA damage and either age or smoking. This was suspected to be due to the narrow age range and the small number of smokers in the population.

Table 7. Comparison of the studied variables of the two groups (control and exposed), adjusted for age and smoking status).

	Adjusted mean for Control group	Adjusted mean for Exposed group	*p-value*
DNA damage	7.638	9.397	<0.001*
Calculated Repair Capacity	0.197	0.372	0.285
Oxidative Stress Status	1.048	0.855	0.727

* Significant at $p < 0.05$

Table 8 represents the regression analysis of the effect of age, petrol exposure and smoking on the level of DNA damage and repair as well as oxidative stress status of the exposed group (petrol attendants). In this regression model the level of DNA damaged, repair capacity and oxidative stress status were used as dependant variables and the age, petrol exposure and smoking status of the petrol attendants as independent variables. There was a good association with the three dependent variable (DNA damage: R^2 = 0.154; repair capacity: R^2 = 0.421; oxidative stress status: R^2 = -0.088) (data not shown). Petrol exposure (P = 0.041) showed a significant influence in the level of DNA damaged, while age (P =0.887) and smoking (P = 0.623) had no significant influence. These three variables explained the slight variance of 15.4% in the level of DNA damaged. Both age (P = 0.597) and petrol exposure (P = 0.601) had no significant influence on repair capacity, only smoking (P = 0.002) showed a significant influence. These variables accounted for 42.1% of the variance in the repair capacity. No significant influence was indicated for the oxidative stress status since the R^2 value was -0.08. Andreoli *et al.* (1997) found no correlation between the extent of DNA damage and the ages or smoking habits of the subjects. However, a study done by Fracasso *et al.* (2006) demonstrated that active and ex-smokers displayed significantly high levels of basal DNA damage associated with a decrease in DNA repair capacity compared to never-smokers. Other authors also associated cigarette smoke to higher DNA damage and slower or inhibited DNA repair kinetics (Şardas *et al.*, 1997).

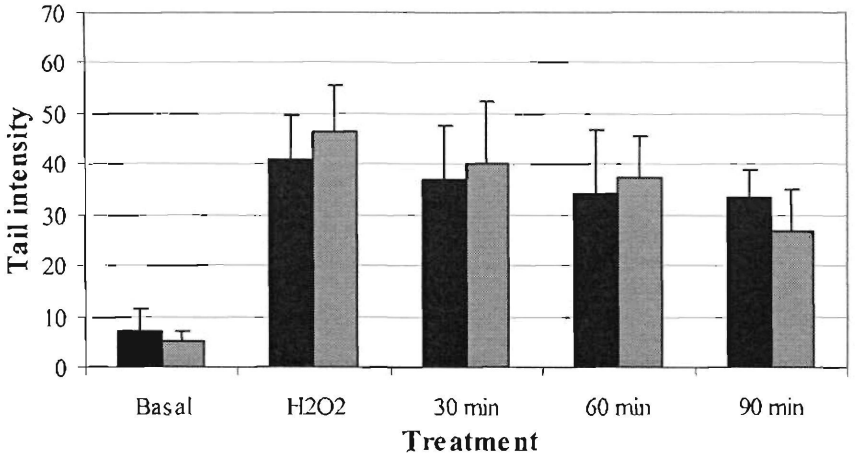
Table 8. Regression of DNA damaged, repair capacity and oxidative stress status as dependent variables with independent variables in the exposed subject group (N=20). Beta and level of significance *P* are shown.

Independent variable		**Dependent variable**		
		DNA damage	Calculated Repair Capacity	Oxidative Stress Status
Age	*ß*	-0.036	-0.112	0.102
	P	0.887	0.597	0.726
Petrol exposure	*ß*	0.555	-0.11	0.165
	P	0.041*	0.601	0.567
Smoking	*ß*	-0.107	-0.658	0.135
	P	0.623	0.002*	0.585

* Significant at $p < 0.05$

Figure 4 shows the effect of smoking on DNA damage and repair in both groups studied. There was a higher basal DNA damage in smokers compared to non-smokers in the control group (Fig. 4A). Even though basal DNA damage increased significantly ($p<0.05$) in the exposed group (Fig. 4B), no difference was seen between smokers and non-smokers. Additional oxidative stress caused further increase in DNA damage of smokers and non-smokers in both the exposed and control groups. Smokers in both the exposed and control groups showed delayed repair capacity in the 90 min repair time given. In the control group, smokers showed some DNA repair which later reached a plateau in the last 60 min repair time, while non-smokers showed a possibility of repair (although very slow), if given more time to repair. The exposed group had a more abrupt DNA repair pattern during the first 30 min. The smokers in this group had a sudden increase in tail intensity during the 60 min repair time which resembled that of oxidative damage. This increased DNA damage subsequently decreased again after further 30 min repair time. However, the non-smokers maintained a constant level of tail intensity with some decrease in the last 30 min repair time. This showed possible further DNA repair, given enough time. These findings confirm the results of a regression analysis (Table 8) where smoking had a significant influence ($p<0.05$) on the calculated DNA repair capacity.

A



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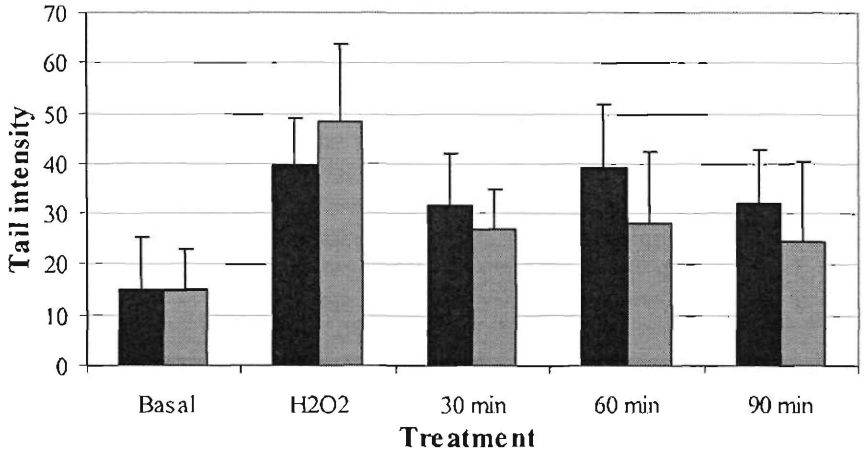

Figure 4. Effect of smoking on DNA damage and repair in lymphocytes of control subjects (A) and exposed subjects (B). Smokers (black) and non-smokers (grey).

5. Conclusions

The results of the occupational exposure of petrol attendants to petrol VOCs obtained in this study were lower than the OEL (OHS Act 83 of 1995). Nevertheless, such exposures should be carefully monitored and kept under control because of their possible neurotoxic and carcinogenic effects (IARC, 1989; ACGIH, 2003). Exposure to these low levels resulted in increased levels of DNA damage and decreased DNA repair capacity. These results are similar to those obtained in other countries (Andreoli *et al.*, 1997; Franceschetti *et al.*, 2005; Navasumrit *et al.*, 2005; Roma-Torres *et al.*, 2006). The antioxidant capacity decreased in the group exposed to petrol VOCs. This decrease in antioxidant capacity can contribute to a state of oxidative stress, which can cause oxidative damage to proteins, lipids and DNA. The exposure to petrol VOCs compromised the antioxidant capacity, which probably leads to increased oxidative stress in the exposed group. Age and smoking both had a significant effect on the level of DNA damage. Furthermore, smokers had a decreased repair capacity while non-smokers maintained a low level of DNA damage and possibility of continued repair. Another factor which influenced the level of DNA damage was the period of exposure to petrol VOCs. Subjects exposed for longer periods had the highest level of DNA damage and a compromised DNA repair process. This warrants further study of the repair process of damaged DNA. Due to the small size of the study, a further investigation involving a larger group of subjects is necessary to yield a broader spectrum of the effects of exposure to petrol VOCs. Also, other methods and confounding factors should be used in biomonitoring studies to enhance the ability of the comet assay in determining the effects of chronic exposure to low levels of petrol VOCs.

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CHAPTER 4

SUMMARY AND CONCLUSION

General summary, conclusion and recommendations

Occupational exposure to petrol VOCs may pose a genotoxic risk. Petrol is composed of many compounds, some of which may have neurotoxic effects (toluene and xylene) and others which have been confirmed as human carcinogens (benzene) by the International Agency for Research and Cancer (IARC, 1989) and the American Conference of Governmental Industrial Hygienists (ACGIH, 2003). Previous studies yielded evidence that workers with occupations associated with petrol vapour were exposed to significantly higher levels of aromatic hydrocarbons (Periago *et al.*, 1997; Hakkola *et al.*, 2001; Jong & Song, 2001; Navasumriti *et al.*, 2005; Periago & Prado, 2005). In Italy, Periago *et al.* (1997) found that hydrocarbons were elevated in ambient air and also that climatic conditions can increase the risk of exposure during shifts, especially during summer (Periago & Prado, 2005). According to a study done in Thailand, service station attendants showed a more elevated exposure to benzene than any other occupations studied (Navasumriti *et al.*, 2005). However, all of these studies were conducted with different ethnic groups in different countries, but no studies on the occupational exposure to petrol vapours have been done on black African petrol attendants.

The OHS Act 1993 (Act No. 85 of 1993) established new regulations for hazardous chemical substances in order to reduce the exposure levels of these compounds. Even at exposure levels below the OEL (occupational exposure limit) value, subjects in this study showed elevated levels of DNA damage and decreased DNA repair capacity. Exposures to low levels of benzene have been proven to increase the level of DNA damage and decrease DNA repair capacity in studies by Andreoli *et al.* (1997), Franceschetti *et al.* (2005) and Roma-torres *et al.* (2006).

Certain techniques have been developed to investigate the biological effects of DNA damaging agents. One of the important and sensitive techniques, the comet assay, was developed to detect DNA damage and repair in individual cells (Singh *et al.*, 1988). Considering the high sensitivity of the comet assay to DNA damage induced by environmental and occupational exposure to carcinogens and mutagenic agents (Andreoli

et al., 1997; Tice *et al.*, 2000), this technique was used to study the occurrence of DNA damage in lymphocytes of African petrol attendants and their age-matched controls.

The personal monitoring of petrol attendants exposed to petrol VOCs yielded exposure levels below the OEL. There was a significant difference in the level of DNA damage between the exposed group and the unexposed control group. Not only was the DNA damage higher in the exposed group, but also the repair capacity decreased as a result of this exposure. Although the exposure levels were below the OEL, chronic exposure to petrol VOCs led to highly elevated levels of DNA damage and also compromised the DNA repair process. These results are similar to those obtained in other studies done in Italy and Thailand (Andreoli *et al.*, 1997; Franceschetti *et al.*, 2005; Navasumrit *et al.*, 2005; Roma-Torres *et al.*, 2006). The two confounding factors taken into account in this study, age and smoking, significantly influenced the level of DNA damage and repair. Smokers yielded increased levels of DNA damage and less repair capacity than the non-smokers. The oxidative stress status was also higher in the exposed group than in the control group. The level of hydroperoxides in both groups was the same but there was a decrease in antioxidant capacity in the exposed group. It is possible that the antioxidant defences in these subjects were overwhelmed by the petrol exposure or other factors such as dietary intake. However, due to the non-compliant diet study of the subjects, the diet of the subjects was not reliable.

The hypothesis of this study was that African petrol attendants, as they are routinely exposed to petrol VOCs, may be subjected to increased oxidative DNA damage and reduced DNA repair capacity. The general aim of this study was to investigate the levels of exposure of petrol attendants to petrol VOCs and to determine the level of DNA damage and repair affected by such exposures. The hypothesis of this study is, therefore, accepted since even at low levels, exposure of subjects to petrol VOCs gave rise to genotoxic effects.

For further studies it is recommended that such a study should be done on a larger group of subjects to give a broader spectrum of the results. Other confounding factors which

can possibly affect the level of DNA damage and repair detected by the comet assay should be considered and used as criteria in selection of the study population. A similar study can also be done on workers in the petrol delivery and offloading operations. Other biological monitoring methods should be done to enhance the ability of the comet assay to detect the effects of low level exposures. Also the type of damage and repair process which the cells undergo should be investigated. A detailed study of the dietary intake of the subjects is necessary to determine the effects of antioxidants in oxidative DNA damage.

Annexure A

Consent

Title of the project:

.....
.....
.....

I, the undersigned ..... (full names)

read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

all the subjects used in the study (individual signed consent forms available on request)

(Signature of the subject)

Signed at .....on .....

Witnesses

1. .....

2. .....

Signed at ..... on .....

Annexure B

Demographic and lifestyle questionnaire (experimental group)

All information given in this questionnaire is confidential

(mark with an 'X')

1. Subject number:		Date:	
2. Name:			
3. Age		20 – 24	
		25 – 29	
		30 – 34	
		35 – 39	
		40 – 44	
		45 +	
4. What language do you speak? _____			
5. Where do you stay? _____			
6. How long have been in Potchefstroom? _____			
7. What is your occupation? _____			
8. How long have you been working at your current job? _____			
Work shift:		Day	
		Night	
Shift duration:		8 hrs	
		10 hrs	
		12 hrs	
9. How long have you been working with petrol? _____			
10. Previous occupation: _____			
11. Do you have other sources of income other than your job?		No	Yes
If yes, please name the source of income: _____			

12. Do you suffer from any of the following?		
Hypertension (High blood pressure)	No	Yes
Diabetes	No	Yes
Stroke	No	Yes
Heart disease	No	Yes
Cancer	No	Yes
TB	No	Yes
Sexually transmitted disease	No	Yes
Other, please specify: _____		
13. Do you take any medication?	No	Yes
If yes, please list medication: _____ _____ _____ _____ _____		
14. Do you smoke?	No	Yes

If yes, mark what you smoke and indicate the amount per day/week	Type of smoking	Amount per day / week
Cigarettes		
Cigars		
Tobacco (zoll)		
Snuff		
Pipe		

Other, please specify: _____		
How long have you been smoking (years)? _____		
If you don't smoke at the moment, were you smoking regularly before?	No	Yes
If yes, for how long were you smoking before? _____		
15. Do you use alcohol?	No	Yes

If yes, mark the type of alcohol you mainly use:		
Traditional beer (homemade)		
Tlokwe		
Beer (commercial)		
Spirits		
Wine		
16. Do you do physical exercises?	No	Yes
If yes, mark the type of exercise and specify the hours per day/per week:	Per day	Per week
Gymnasium exercises (e.g. weights)		
Training for long distance running (e.g. gym or road)		
Swimming		
Cycling		
Other, please specify: _____		
17. What did you eat for: ▪ Breakfast:_____ ▪ Lunch: _____ ▪ Supper: _____		
18. Do you have any hobbies (e.g. painting, carpentry, gardening, etc.)?	No	Yes
If yes, please specify: _____		
19. What are your experiences on working with petrol? _____ _____ _____ _____ _____ _____		

Demographic and lifestyle questionnaire (control group)

All information given in this questionnaire is confidential

(mark with an 'X')

1. Subject number:	Date:	
2. Name:		
3. Contact details:		
4. Age	20 – 24	
	25 – 29	
	30 – 34	
	35 – 39	
	40 – 44	
	45 +	
5. What is your home language? _____		
6. Where do you stay in Potchefstroom? _____		
7. How long have been in Potchefstroom? _____		
8. Where were you from, before coming to Potchefstroom? _____		
9. Are you a student or employee? _____		
10. If employee, name your current occupation. _____		
11. How long have you been working at your current job? _____		
12. Do you have other sources of income other than your job?	No	Yes
If yes, please name the source of income: _____		
13. What is your previous occupation? _____		
14. Have you ever worked with petrol before?	No	Yes
15. If yes, how long have you worked with petrol before? _____		
17. Do you suffer from any of the following?		
Hypertension (High blood pressure)	No	Yes
Diabetes	No	Yes

Stroke	No	Yes
Heart disease	No	Yes
Cancer	No	Yes
TB	No	Yes
Sexually transmitted disease	No	Yes
Other, please specify: _____		
18. Do you take any medication?	No	Yes
If yes, please list medication: _____ _____ _____ _____ _____		
19. Do you smoke?	No	Yes

If yes, mark what you smoke and indicate the amount per day/week	Type of smoking	Amount per day / week
Cigarettes		
Cigars		
Tobacco (zoll)		
Snuff		
Pipe		
Other, please specify: _____		

If you don't smoke at the moment, were you smoking regularly before?	No	Yes
If yes, for how long were you smoking before? _____		
20. Do you use alcohol?	No	Yes
If yes, mark the type of alcohol you mainly use:		
Traditional beer (homemade)		
Tlokwe		
Beer (commercial)		

Spirits		
Wine		
21. Do you do physical exercises?	No	Yes
If yes, mark the type of exercise and specify how many times per day/per week:	Per day	Per week
Gymnasium exercises (e.g. weights)		
Training for long distance running (e.g. gym or road)		
Swimming		
Cycling		
Soccer		
Other, please specify: _____		
22. Do you have any hobbies (e.g. painting, carpentry, gardening, etc.)?	No	Yes
If yes, please specify: _____		

Annexure C

Preparation of buffers

LYSIS BUFFER

NaCl[5M]:	500 rnl
EDTA [0.4M] (pH 7-8):	250 rnl
Triton X-100:	10 rnl
DMSO [l0 %]:	100 rnl
ddH_2O:	140 rnl

ELECTROPHORESIS BUFFER

NaOH [0.6M]:	500 rnl
EDTA [0.05M]:	20 rnl
ddH_2O:	480 rnl

NEUTRALISING BUFFER

TrisHCl 63.04 g in 500 ml ddH_2O

Cool to 4°C and adjust pH to 7.5 after with [5M] NaOH

Adjust volume to 1000 ml

PHOSPHATE BUFFERED SALINE (PBS)

NaCI:	8 g
KCI:	0.2 g
NaH_2PO_4:	1.15 g
K_2HPO_4:	0.2 g

Dissolve in 1000 ml ddH_2O

Adjust pH to 7-8; store at 4°C

STAINING BUFFER (NB! Carcinogenic)

Ethidiurn Bromide: 0.005 g

Dissolve in 1000 ml ddH_2O

Store in fridge (4°C)

ENZYME (Fpg and Endo III) REACTION BUFFER

40 mM HEPES: 2.08 g

0.1 M KCl: 1.49 g

0.5 M EDTA: 0.04 g

0.2 mg/ml BSA: 40 mg

Make up to 200 ml with ddH_2O