Evaluation of DNA damage and DNA repair by the comet assay in workers exposed to organic solvents

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ABSTRACT

Rapid development of the chemical industry over the past two decades makes the study of the effects of chemical substances (such as benzene) on the human body essential. Benzene toxicity involves both bone marrow depression and leukemogenesis caused by damage to various classes of hematopoietic cells and a variety of hematopoietic cell functions. Studies of the relationship between the metabolism and toxicity of benzene indicate that several metabolites of benzene play significant roles in generating benzene toxicity. Benzene is metabolized, primarily in the liver, to a variety of hydroxylated and ringopened products that are transported to the bone marrow where subsequent secondary metabolisms occur.

Maintenance of the genomic integrity is of crucial importance for all organisms. Damage to the DNA that makes up human genes is constantly inflicted by a large number of agents and can have severe effects if it persists. Modification of DNA can lead to mutations, which alter the coding sequence of DNA and can lead to cancer in mammals. Other DNA lesions interfere with normal cellular transactions such as DNA replication or transcription, and are deleterious to the cell. Thus it is obvious that the stability of the genome must be under continuous surveillance. This is accomplished by DNA repair mechanisms, which remove DNA lesions in an error-free, or in some cases, error-prone way. Defects in DNA repair give rise to hypersensitivity to DNA damaging agents, accumulation of mutations in the genome, and finally to the development of cancer and various metabolic disorders.

The alkaline single cell gel electrophoresis assay (comet assay) was applied to study the occurrence of DNA damage and repair in peripheral lymphocytes of 27 experimental human subjects with occupational exposure to benzene and 9 control human subjects with no occupational exposure to benzene. Two blood samples (pre-shift and after-shift respectively) were obtained from each of the control as well as the experimental subjects. After electrophoresis, scoring was done by using the CASP software program. Two of several variables were of primary interest, i.e., Tail DNA% and Tail

Moment. Statistical analysis clearly indicated that significant differences exist between pre-shift and after-shift data for both variables. Results revealed that there are also different behaviour patterns for the experimental and control groups, pre-shift and after-shift, for both variables during all stages of the comet assay. It became evident for both Tail DNA%-data and Tail Moment-data that DNA damage was indeed present at the exposed (experimental) group of workers. For the experimental group, after-shift data exceeded pre-shift data in general. Different behaviour was noticed between the experimental and control-data during the repair process. Also, the initial pre-shift data-values of the experimental group, is much higher (about 9%) than that of the control group, which may be an indication of a carried-over effect which may exist from the previous shift, due to the exposure to benzene.

After-shift urine samples were also obtained from the experimental and control subjects and analised for urinary trans-trans-muconic acid (ttMA), a metabolite of benzene, with the aid of a high-performance liquid chromatography (HPLC/UV) detector. The exposed workers showed a markedly higher (1,8 times) ttMA mean value than the control persons, and the two-sample t-test confirmed this significant difference.

Opsomming

Die vinnige ontwikkeling van die chemiese bedryf die afgelope twee dekades, maak die studie van die uitwerking van chemiese stowwe (bv. benseen) op die menslike liggaam noodsaaklik. Benseentoksisiteit sluit beide beenmurgonderdrukking en leukemigenese in, wat veroorsaak word deur verskillende klasse van hematopoïetiese selle en 'n verskeidenheid van hematopoïetiese selfunksies. Studie van die verwantskap tussen die metabolisme en toksisiteit van benseen, dui aan dat verskeie metaboliete daarvan 'n baie belangrike rol in die ontwikkeling van toksisiteit speel. Benseen word hoofsaaklik in die lewer gemetaboliseer en afgebreek tot gehidroksileerde, oopring produkte. Hierdie produkte word vervoer na die beenmurg waar dit verdere metabolisme ondergaan.

Die instandhouding van genoomintegriteit is van kardinale belang vir alle organismes. Beskadiging van DNA word veroorsaak deur 'n groot aantal ingrepe wat nadelige gevolge inhou indien dit volgehou word. Wysiging van die DNA lei tot mutasies wat die kodering van DNA verander en lei dan tot kanker in soogdiere. Ander DNA-letsels beïnvloed normale sellulêre aktiwiteite soos bv. DNA-replikasie en DNA-transkripsie en beskadig dus die sel. Dit is daarom belangrik dat die stabiliteit van die genoom voortdurend geëvalueer moet word en word in die liggaam bewerkstellig deur DNA-herstelmeganismes, wat DNA-letsels in 'n foutlose en in sommige gevalle 'n uitgebreide, komplekse wyse, verwyder. Beskadiging van die DNA-herstelmeganismes gee aanleiding tot hipersensitiwiteit vir DNA-beskadigde middels, akkumulasie van mutasies in die genoom en uiteindelik tot die ontwikkeling van kanker en verskillende metaboliese afwykings.

Die sogenaamde komeet-analise was gebruik om die verskynsel van DNA-beskadiging en DNA-herstel in perifere limfosiete te ondersoek by 27 eksperimentele proefpersone met werksverwante blootstelling aan benseen,

en vergelyk met 9 kontrole persone wat geen werksverwante blootstelling aan benseen ondergaan het nie. Twee bloedmonsters (voor- en naskof onderskeidelik), was van elk van die voorwerpe van die kontrole sowel as die eksperimentele groepe verkry. Na elektroferese is die komete geëvalueer deur middel van die CASP sagteware-program. Slegs twee van vele veranderlikes was bestudeer, nl. "Tail DNA%" en "Tail Moment". Statistiese analise het duidelik aangetoon dat betekenisvolle verskille bestaan in die voorskof en naskof resultate vir albei van die veranderlikes. Beide "Tail DNA%-data" en "Tail Moment-data" toon beduidende DNA-beskadiging by die blootgestelde (eksperimentele) groep werkers. Naskof-data het oor die algemeen groter waardes getoon as voorskof-data. Die gemiddelde aanvangs voorskof-waardes van die eksperimentele groep is baie hoër (omtrent 9%) as die ooreenstemmende waardes verkry uit die kontrolegroep. Dié resultaat dui op 'n moontlike oordraagbare effek wat uit die vorige skof kon ontstaan as gevolg van blootstelling aan benzene. Resultate toon dat daar verskillende gedragspatrone vir die eksperimentele- en kontrolegroepe, voor- en naskof, vir albei veranderlikes bestaan tydens alle fases van die komeet-analise, veral tydens die herstelproses.

Naskof urienmonsters is verkry van die eksperimentele- sowel as die kontrolegroepe. Die urienmonsters is ontleed vir trans-trans-mukoniese suur (ttMA), 'n metaboliet van benseen, met behulp van 'n "high-performance liquid chromatography" (HPLC/UV) skandeerder. Die blootgestelde werkers toon 'n beduidende hoër (1,8 keer) gemiddelde ttMA waarde as die kontrole groep, en die twee-steekproef t-toets bevestig die beduidende verskil.

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CHAPTER 1

OVERVIEW

1.1 Introduction

The rapid development of the chemical industry the past two decades, makes the study of the effects of chemical substances on the human body essential (Schärer, 2003:2946). Some petroleum products such as gasoline and heavy fuel oils contain cancerous substances such as benzene and polycyclic aromatic hydrocarbons (PAHs). These products have a widespread use and there are many people in several occupational groups that are exposed to benzene or PAHs from petroleum products. Järvholm *et al.* (1997:686) state that refinery workers, petroleum distribution workers, workers manufacturing lubrication oils and tank cleaners fall into this category. The risk for cancer due to exposure in these industries depends on the type of product used and the dose. There are studies of refinery workers that show an increased risk of leukaemia and squamous cell skin cancer, attributed to exposure to benzene and PAHs respectively (Christie *et al.*, 1991:511; Wong *et al.*, 1989:283). There are also a few studies of distribution workers exposed to petroleum products. A British study indicated an increased risk of kidney cancer and leukaemia (Rushton, 1993:561), a Canadian study indicated an increased risk of leukaemia in tanker drivers (Schnatter *et al.*, 1993:85), and a study from the United States indicated an increased risk of acute myeloid leukaemia, although the increase was not significant (Wong *et al.*, 1993:65).

Benzene has been extensively used in industry as a volatile solvent and later as a sorting material for the synthesis of other chemicals (Andrews and Snyder, 1992:681). The level of benzene exposure has been reduced and, in some workplaces, other solvents have replaced benzene. However, industrialization leads to more emissions of benzene into the environment (Zhu *et al.*, 2001:173). It has been reported that benzene has cytotoxic (Farris *et al.*, 1997:137), hemotoxic (Andrew *et al.*, 1995:305), immunotoxic

(Hsieh *et al.*, 1991:28), and genotoxic properties (Tuo *et al.*, 1999:31; Daiker *et al.*, 2000:185). In biological systems, inhaled benzene is converted to benzene oxide, which is rearranged to form phenol, or reacts with glutathione to form premercapturic acid. Phenol is converted finally to the metabolites catechol, hydroquinone and benzoquinone. In another pathway, benzene oxide is converted to benzene glycol, which is metabolized by ring opening to muconic acid that appears in urine (Hoffmann *et al.*, 1999:44). Benzene metabolites bind covalently to cellular molecules, proteins and DNA in tissues. Andrews *et al.* (1992:690) stated that this binding is implicated in mechanisms of toxicity (inhibiting cell replication) and carcinogenicity (initiation of leukemia). Benzene is also supposed to act as a mutagen via an indirect mechanism, leading to oxidative DNA damage through the formation of hydroxyl radicals via hydrogen peroxide (Andreoli *et al.*, 1997:100).

In recent years, single cell gel electrophoresis (SCGE), referred to as 'the comet assay', has been widely used to detect strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites. This technique has been shown to be a very sensitive method and a useful tool to detect genetic damage at the individual cell level and in human biomonitoring (Kassie *et al.*, 2000:15; Meller *et al.*, 2002:1015). The DNA damaging property of benzene and its metabolites was investigated using peripheral blood mononuclear cells, using the comet assay method, and benzoquinone was found to be the most damaging compound (Fabiani *et al.*, 2001:3). Studies on the peripheral lymphocytes of human subjects occupationally exposed to low levels of benzene, utilising the comet assay method, showed a significant excess of DNA damage compared with the lymphocytes of matched unexposed controls (Andreoli *et al.*, 1997:101). The majority of lymphocytes, which are produced by the lymph nodes, spleen, thymus and bone marrow, i.e. T-lymphocytes, are long lived, having a life-span of 4 to 10 years. The remaining lymphocytes such as B-lymphocytes, representing 15% of the lymphocyte population, are short-lived, lasting only 3–4 days (Carson, 1995:917).

1.2 Present study

Persons handling petrol are exposed to benzene (Järvholm *et al.*, 1997:688). Furthermore, benzene typically represents PAHs, and therefore the metabolism and effects of benzene, as well as the metabolites resulting from exposure to benzene are of interest in this study. In view of the research results mentioned above, as well as the availability of structures to study characteristics of DNA, an investigation regarding the health of workers in the local petroleum industry is justified and possible. The main elements of the study will now be defined briefly.

1.2.1 Problem statement

The following question is investigated: Does DNA damage occur in workers at the fuel section of Sasol Synfuels at Secunda? If so, to which extent does DNA damage occur and in which way is the DNA repair influenced?

1.2.2 Aims of study

The aim of this study is to investigate empirically whether DNA damage occurs in workers of the refinery section of Sasol Synfuels at Secunda. If this is found to be present, the level of DNA damage, as well as the level of DNA repair, will be determined. The genotoxic potential of an environment where Volatile Organic Compounds (VOC's) are present, is therefore investigated.

1.2.3 Hypothesis

A working environment where workers are exposed to petroleum and other VOC's causes DNA damage and affects DNA repair.

1.3 Contents and planning

In order to derive reliable answers to the questions raised in paragraph 1.2, a thorough background study of the contributing factors regarding the effects of petroleum compounds on DNA integrity, especially benzene, is essential. In this study, we address topics of close interest to the hypotheses and aims mentioned in the previous paragraph.

In Chapter 2 a review of existing literature is given. In Chapter 3 the comet assay method is discussed, and in Chapter 4 the present study is defined and methods are described in detail. In Chapter 5 statistical methods and analysis will be explained and displayed, together with results, discussions, conclusions and recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In the early 19th century, benzene, toluene, xylenes and other alkyl benzenes were shown to be the major constituents of coal tar naphtha, a by-product of coal gas manufacture that was used as a solvent for rubber. The value of the alkyl benzenes and other aromatic hydrocarbons both as solvents and starting materials in many industrial processes was soon realised. In the 20th century, crude oil or petroleum was also shown to be a rich source of aromatics (Pitarque *et al.*, 1999:195).

According to Aw (2000:261) VOCs are an important group of air pollutants to be investigated, as they contribute to the most serious air pollution problems. Firstly, they have been demonstrated to be active in the formation of photochemical smog and ground-level ozone production. Secondly, several VOCs found in urban air are classified as carcinogenic compounds (1,3-butadiene and benzene).

PAHs are ubiquitous environmental pollutants and many of them are known as carcinogenic (IARC, 1987:233) and mutagenic compounds. The PAHs recognized as carcinogenic are mostly associated with particulate matter (Lyal *et al.*, 1988:2550). These organic compounds are produced by high-temperature reactions, such as incomplete combustion and pyrolysis of fossil fuels and other organic materials. They undergo thermal decomposition and react with a number of atmospheric chemicals producing derivatives that can be more toxic than the original compounds (Nicolaou *et al.*, 1984:103).

Carbonyls are among the major species of organic compounds involved in photochemical air pollution, since aldehydes and ketones play an important role as products of photo-oxidation of gas-phase hydrocarbons as a major

source of free radicals. Motor vehicle exhaust is the primary emission source of carbonyl compounds in urban areas (Granby *et al.*, 1997:1403).

2.2 Benzene

Benzene is a member of the aromatic compounds and it is therefore justified that research is conducted in an effort to establish possible effects of benzene on mechanisms of toxicity and cell damage (Aw, 2000:261).

According to Aw (2000:261), aromatic organic compounds constitute more than 50% of the different chemicals in common use in occupational settings worldwide. This group of chemicals consists of unsaturated cyclic compounds based on the benzene ring – benzene being the simplest homologue within the group. The term 'aromatic' originally stemmed from the pleasant odour characteristic of the earlier recognized compounds in the group (e.g. some esters used in perfumes and flavourings). However, many of the aromatic hydrocarbons today are odourless.

Benzene was first demonstrated in coal tar in 1845, and most aromatic compounds were previously isolated from the coal tar fraction of coal distillation. Destructive distillation of coal at 1000-1300°C yields coke, coal gas, coal tar and water-soluble volatile compounds, with the coal tar fraction containing over 200 different organic chemicals. The main source of aromatic compounds nowadays is petroleum (Ginger and Jeanne, 2001:639).

While there are considerable animal data on the health effects of these compounds, acute and/or chronic effects in humans have only been documented for a few. Reports of ill health in humans tend to be based on clinical cases of occupational or intentional (suicide or homicide) over-exposure, or on epidemiological studies of exposed workers. Occupational

epidemiological data related to exposure to only one compound are uncommon. Workplace exposures tend to be due to a mixture of related or unrelated compounds or within a particular process in a specific job category, or in a common industry (Aw, 2000:263).

According to Ginger and Jeanne (2001:641), benzene is a volatile, colourless, highly flammable liquid that was first discovered in 1825 by Faraday, who isolated it from a liquid condensed by compressing oil gas. In modern times, most (98%) benzene is commercially derived from petrochemical and petroleum refining industries. Benzene is a by-product of various combustion processes, such as forest fires and the burning of wood, garbage, organic wastes, and cigarettes (Hattemer-Frey *et al.*, 1990:221); it is also released to the air from crude oil seeps and volatilizes from plants (Brief *et al.*, 1980:616).

Currently, worldwide production of benzene is estimated at approximately 15 million tons (13.6×10^6 metric tons) (Fishbein, 1992:8). Production in the United States alone is increasing at a rate of 3% annually (ATSDR, 1996), approaching 6 million tons (5.4×10^6 metric tons) of benzene produced in the United States in 1990 and 14.0 billion pounds in 1993 (Fishbein, 1992:5).

Benzene is one of the world's major commodity chemicals. Its primary use (95% of production) is as an intermediate in the production of other chemicals, predominantly ethyl benzene (for styrofoam and other plastics), cumene (for various resins), and cyclohexane (for nylon and other synthetic fibres) (ATSDR, 1993:1150). Benzene is an important raw material for the manufacturing of synthetic rubbers, gums, lubricants, dyes, and pharmaceutical and agricultural chemicals; it is also found in consumer products such as glues, paints, and marking pens (Ginger and Jeanne, 2001:644).

Benzene is also a natural component of crude and refined petroleum. The mandatory decrease of lead alkyls in gasoline has led to an increase in the aromatic hydrocarbon content of gasoline to maintain high octane levels and antiknock properties. In the United States, gasoline typically contains less than 2% benzene by volume, but in other countries benzene concentrations can be as high as 5% (Wolf, 1956:387).

Occupational exposures predominantly account for human exposure to benzene. In 1987, approximately 238,000 workers employed by the rubber industry, oil refineries, chemical plants, the shoe manufacturing industry, gasoline storage facilities, and service stations were exposed to benzene (Mehlman, 1991:143), with an additional 2 to 3 million US workers potentially exposed to benzene (Fishbein, 1992:9).

As can be seen from the environmental sources listed, inhalation accounts for up to 99% of the total daily intake of benzene (ATSDR, 1996:915). Anthropogenic emissions to the air are approximately 34,000 metric tons per year, primarily because of industry-related releases to the environment (TRI, 1994:7). Levels of benzene found in various places are listed in Table 2.1. Smoking, however, is the largest anthropogenic source of benzene exposure for the general public.

The estimates of daily intake of benzene from a single cigarette vary: from 5.9 to 73.0 micrograms (μg) (Brunnemann *et al.*, 1990:1863), from 10 to 31 μg (Thomas, 1986:7), 30 μg (Fishbein, 1992:5), 40 μg (Travis *et al.*, 1990:400), 57 μg (Wallace *et al.*, 1987:272), and 90 μg (Gilbert *et al.*, 1982:110).

Passive or "second-hand" smoke is also a source of exposure. According to Hattemer-Frey *et al.* (1990:222), non-smokers who live with a smoker have

about 30% to 50% higher benzene levels in their breath than do non-smokers who do not live with a smoker.

TABLE 2.1. Levels of benzene in air.

Location of Air Sample	Level (ppb*)
Urban	4.0-160
Remote/Rural	0.16-3.5
Suburban residential-remote from traffic	1.8-4.5
Indoor	1.8
Workplace	2.1
Near chemical plant	14.0
Near refineries	9.0
Gas stations	<1.0-32

*ppb = parts per billion

(Source: ATSDR, 1996:989)

Ingestion of contaminated food items (Table 2.2) has been suggested as a potentially important pathway of human exposure to benzene (Hattemer-Frey *et al.*, 1990:228).

Benzene, however, has only a low-to-moderate bioconcentration potential in aquatic organisms (Miller *et al.*, 1995:522) and some plants (Geyer *et al.*, 1984:269); therefore, ingestion of contaminated food items probably accounts for less than 1% of the average daily intake of benzene by the general population of the United States (Hattemer-Frey *et al.*, 1990:231).

TABLE 2.2. Food containing benzene.

Food Containing Benzene (level in μg/kg* where available)		
Vegetables	**Fruits**	**Meat, Fish, and Poultry**
Dry red beans	Apple	Cooked beef (2-19)
Leek	Citrus fruit	Irradiated beef (19)
Mushroom	Cranberry and bilberry	Cooked chicken (<10)
Onion, *roasted*	Black currants	Egg, hard-boiled (500-1900)
Parsley	Guava	
Potato, *cooked peel*	Cayenne pineapple	Egg, uncooked (2100)
Soybean milk	Strawberry (trace)	Haddock fillet (100 to 200)
Trassi, *cooked*	Tomato, *hothouse*	Lamb, heated (<10)
Beverages	**Dairy products**	Mutton, heated (<10)
Cocoa	Butter (0.5)	Veal, heated (<10)
Coffee	Blue cheese	Codfish
Jamaican rum (120)	Cheddar cheese	**Nuts**
Tea	Other cheese	Filbert, *roasted*
Whiskey		Peanut, *roasted*
		Macadamia nut

*μg/kg = micrograms per kilogram

(Source: Powell *et al.*, 1986:60; Marcus, 1987:205).

2.2.1 Toxicokinetics and metabolism

Since the 1970s exposure to benzene has been shown to cause hematotoxicity and leukemia (Aksoy, 1989:194). The IARC has concluded that there is sufficient evidence to classify benzene into the group 1 human carcinogen category and exposure to benzene is most significantly associated with the development of acute myeloid leukemia (Au *et al.*, 2002:153). In addition, some reports showed that exposure to benzene is linked to the development of other types of leukemia and lymphoma. Various

mechanisms have been proposed for the induction of leukemia by benzene (Au *et al.*, 2002:154).

Absorption of benzene varies with route of exposure. In humans, respiratory uptake has been determined to vary from approximately 47% to 80% (Ginger *et al.*, 2001:642), although dermal absorption can range from 0.05% to 0.2%.

Absorption data for oral exposure in humans is not available; however, in animals, absorption rates following oral exposure to benzene were found to be from 90% to almost 100% and are vehicle-dependent.

Benzene must be metabolized to exert its toxic effects (Snyder *et al.*, 1996:1165); however, this process is complex, consisting of multiple pathways (Figure 2.1). Benzene is metabolized by cytochrome P-450-dependent multifunction oxidase enzymes (Ginger *et al.*, 2001:665), and benzene metabolism leads either to more toxic products, via pathways involving those leading to ring breakage and benzoquinone, with muconic acid and hydroquinone, respectively, as conjugates, with prephenyl and phenyl mercapturic acids and phenyl conjugates as the markers, respectively.

The liver is the major organ for the metabolism (biotransformation) of benzene. There is, however, increasing evidence that other organs such as the skin, lungs, kidney and skeletal muscle may have a significant capability for biotransformation, albeit at a lower level of activity than the liver. Detoxification does not always occur and, for chemicals, toxicity may be enhanced as a result of biotransformation (Blain, 2000:264).

Phenol, hydroquinone, and catechol are the major metabolites of benzene in mammals (Ong *et al.*, 1996:330). Hydroquinone and benzoquinone inhibit

proliferation and differentiation in lymphocytes in culture at noncytotoxic concentrations; phenol or catechol suppress lymphocyte growth or function at concentrations that result in cell death.

In addition, hydroquinone and catechol have been shown to reduce the number of spleen and bone marrow progenitor B-lymphocytes and to inhibit polyclonal plaque-forming cells (Hsieh *et al.*, 1990:320). However, these metabolites can affect each other's rate of metabolism because they are substrates for the same cytochrome P-450 enzymes (Cox, 1991:453). These interactions might help to explain the non-linear relation between administered benzene concentrations and internal doses of metabolites. Indeed, it has been reported that a higher proportion of metabolites are produced at lower exposure concentrations (Ginger *et al.*, 2001:647).

Sex differences are believed to play a role in the toxicity of benzene; however, the literature has been contradictory. A number of studies have reported that females are more susceptible in both humans (Mallory *et al.*, 1939:355; Ito, 1962a:268; Sato *et al.*, 1975:321) and animals (Hirokawa, 1955:275). In contrast, male mice (Gad-El-Karim *et al.*, 1986:464) are more susceptible to benzene-induced chromosomal damage than are female mice.

Still other studies (Yin *et al.*, 1996:1339) have found male and female humans to be equally susceptible to the effects of benzene exposure. It is important to note that the end points of these studies are variable. The health effects of benzene exposure also depend both on the species exposed (Zhu *et al.*, 1995:183) and the route of exposure (Cox,1991:453).

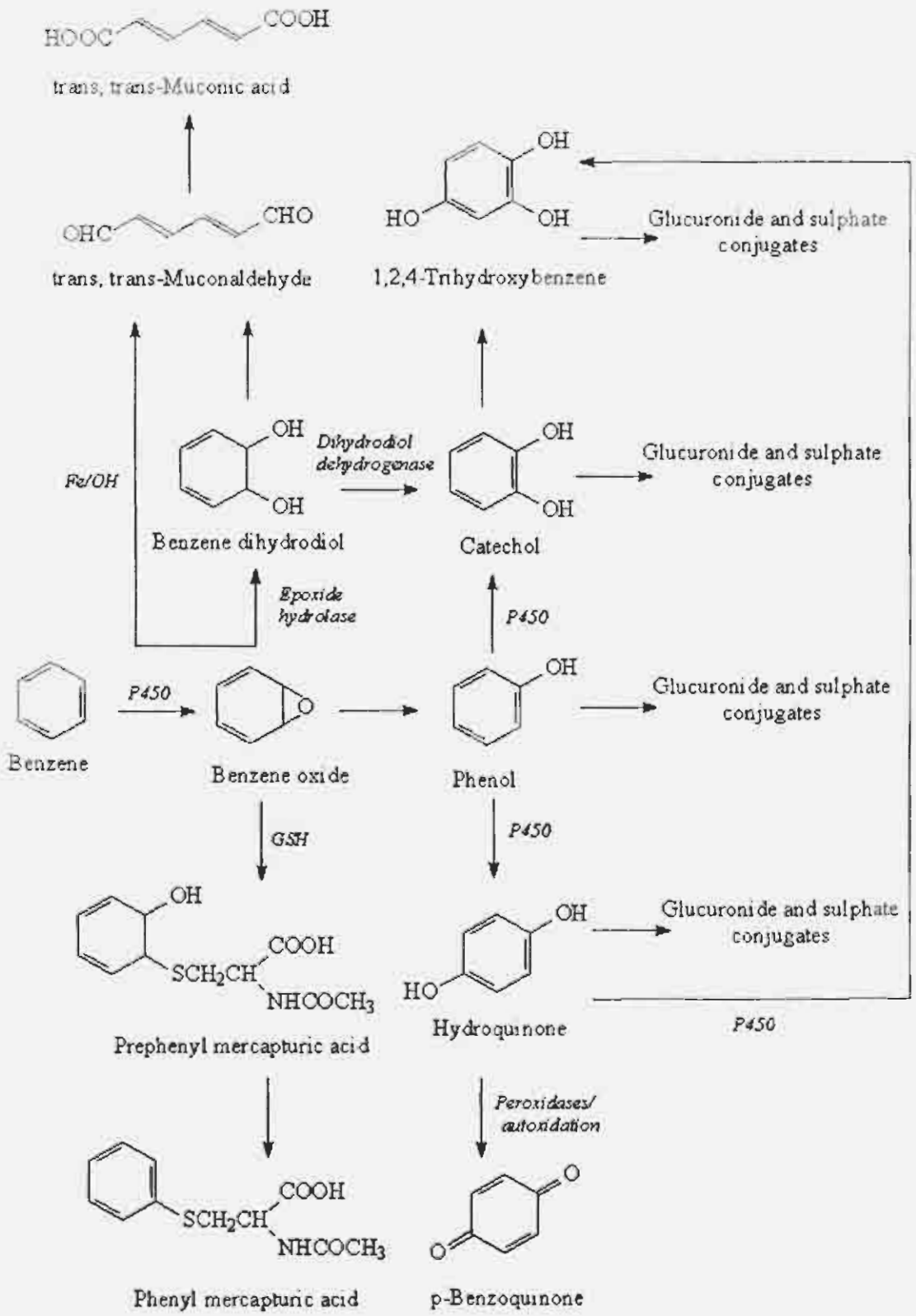

Figure 2.1. Scheme for the metabolism of benzene. (Adapted from Snyder and Hedli, 1996:1169).

All things being equal, humans tend to form lower internal doses of reactive metabolites than do animals (Reitz *et al.*, 1989:62) and the route of exposure has little or no effect on subsequent metabolism of benzene in humans (Sabourin *et al.*, 1989:441). It should be noted, however, that Goldstein (1977:69) postulated that humans might have a genetic predisposition to benzene toxicity. Age can also play a role in the metabolism of benzene, with younger animals displaying a higher rate of metabolism, and a greater susceptibility to toxic effects, than do older animals (McMurry *et al.*, 1994:4).

According to Ginger *et al.* (2001:649), following inhalation exposure, most benzene is excreted unchanged in exhaled air. Human excretion of absorbed benzene involves a biphasic urinary excretion of conjugated derivatives (sulphates and glucuronides). Animal data show a similar pattern; that is, unmetabolized benzene is excreted primarily through exhalation, but metabolized benzene is excreted mainly in the urine (Ginger *et al.*, 2001:644).

Ethanol has been shown to alter benzene metabolism. For example, ethanol has been shown to induce CYP2E1, a cytochrome P-450 enzyme responsible for benzene metabolism (Gut *et al.*, 1993:237; Medinsky *et al.*, 1994:119). Nakajima *et al.* (1985:23) found that pre-exposure of male rats to ethanol not only increased the rate of metabolism of benzene by hepatic microsomes six fold, but also significantly increased the rate of clearance of benzene from the blood. Ethanol has also been shown to enhance the toxicity of benzene in humans as well as in animals (Ginger *et al.*, 2001:651).

Other substances may also affect the metabolism of benzene. Workers exposed to a combination of benzene and toluene produce significantly lower urinary phenol (a biomarker for benzene exposure) than those exposed to either benzene or toluene alone (Inoue *et al.*, 1988:15); toluene has also been

shown to lower the toxicity of benzene in animals, or to detoxification, via pathways leading to mercapturic acid products (Hsieh *et al.*, 1990:320).

Physiologically based pharmacokinetic (PBPK) models are used to allow interdose, interspecies and interroute pharmacokinetic extrapolation as well as prediction of target tissue exposure (Spear *et al.*, 1991:641). According to Cox *et al.* (1992:401) PBPK models are useful tools to apply to correct risk assessments for nonproportional relations between administered and internal doses in test species; differences between routes of administration in terms of internal doses formed from a given amount of administered benzene; and interspecies metabolic differences in the production of external doses from administered doses. Several models are currently available (Woodruff *et al.*, 1989:254; Ginger *et al.*, 2001:668). Each varies in structure, the parameter values assigned, the data from which the values were derived and metabolic constants.

For discussions of these various models, the reader is referred to Spear *et al.* (1991:641), Cox *et al.* (1992:401), and ATSDR (1996:1019).

2.2.2 Urinary Metabolites

Urinary phenol, trans-trans-muconic acid (ttMA) and S-phenylmercapturic acid (PMA) are the three excreted metabolites that have been used most as biomarkers of benzene exposure in occupational studies. The utility of urinary phenol as a biomarker of benzene exposure is limited to inhalation exposures at air concentrations exceeding 3 mg/m^3 and has been found to be proportional to benzene concentrations at exposures as high as 620 mg/m^3 (Inoue *et al.*, 1986:692; Roush *et al.*, 1985:385; Drummond *et al.*, 1988:256). Additional sources of urinary phenol due to diets and ingestion of medicine (Waritz, 1985:257) have precluded its use as a biomarker of environmental benzene exposures.

Urinary S-phenylmercapturic acid is a metabolite of the ring hydroxylation pathway of benzene. It was found to be increased in a dose-response fashion to benzene in the urine of coke production workers, in a similar manner as urinary phenol, and to be higher in the postshift urine compared to prework urine of two workers exposed to 1.1 and 0.15 ppm benzene when no discernable differences in the urinary phenol levels were detectable (Stommel *et al.*, 1989:279). This suggested that urinary S-phenylmercapturic acid is a more sensitive biomarker of benzene exposure than urinary phenol.

Urinary S-phenylmercapturic acid appears to be a specific and sensitive biomarker of benzene, whose only apparent source is the ring hydroxylation pathway; thus it has the potential to be a useful biomarker of low-level benzene exposures (Boogaard *et al.*, 1995:611). However, it is excreted in very low quantities (μg/g creatinine) and requires a complex analytical method to be analyzed.

Urinary trans-trans-muconic acid has been used as a biomarker of sub-mg/m^3 exposures (Witz *et al.*, 1990:613; Bechtold *et al.*, 1991:473; Ducos *et al.*, 1992:309), the upper range of environmental benzene exposure. But other sources of this urinary metabolite have been identified, such as metabolism of sorbic acid (a food additive), which makes it a nonspecific biomarker of low-level environmental benzene exposure (Ducos *et al.*, 1990:529). However, according to Fang (2000:62) the most reliable analyses are ttMA in urine and benzene in blood at low exposure levels, and the ttMA/benzene-urine ratio may be an important index of susceptibility to benzene toxicity.

Urinary trans-trans-muconic acid was found to be elevated in a single individual, as was the exhaled breath concentration, following exposure to benzene-contaminated water (Buckley *et al.*, 1992). A study of six individuals exposed to benzene in environmental tobacco smoke (ETS) found they also had elevated urinary trans-trans-muconic acid compared to their urinary trans-trans-muconic acid on days without ETS exposure, but the use of urinary muconic acid as a biomarker in this study depended upon a knowledge of

both the background excretion rate of muconic acid and the background exposure to benzene from other sources, and thus cannot be generalized to use in the general population for short-term exposures (Yu *et al.*, 1996:453). A correlation between urinary benzene and the time-weighted 8-hr exposure among nonexposed, non-smokers, smokers, and occupationally exposed individuals has been reported, but the exposure levels were higher than would occur for environmental exposures (Ghittori *et al.*,1993:233). Thus urinary biomarkers of benzene exposures currently have limited utility in establishing low-level, distinct environmental exposures in individuals, particularly in single urine samples.

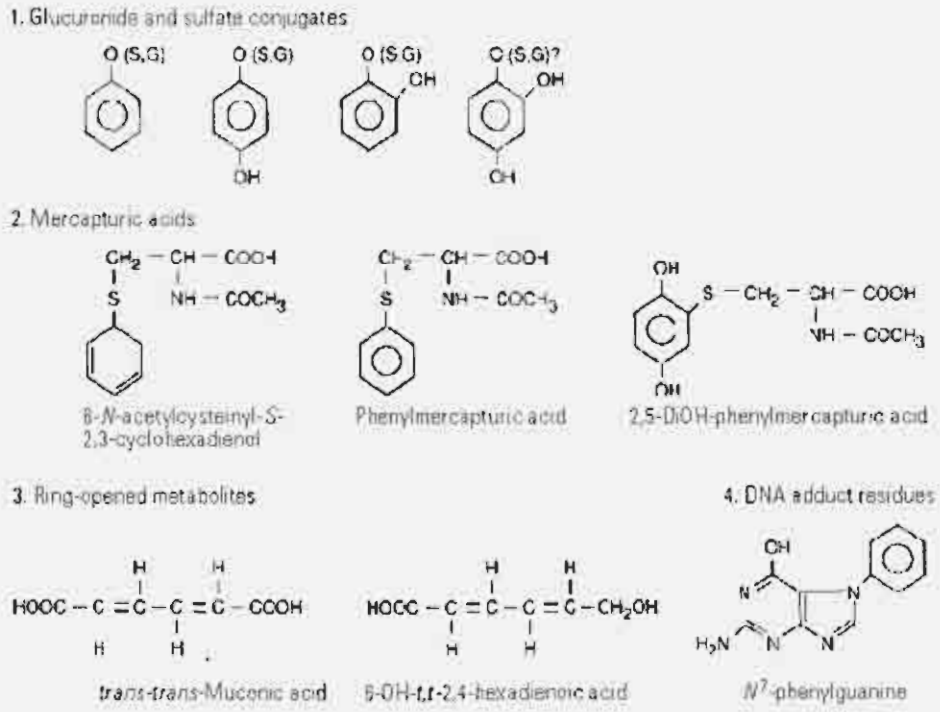

Figure 2.2. The urinary metabolites of benzene (Adapted from Snyder *et al.*, 1996:1170).

However, results based on recent studies by Wiwanitkit *et al.* (2001:399) conclude that a wider use of urine ttMA determination is recommended as a biomarker for occupational exposure to benzene.

2.2.3 Potential Mechanisms of Toxicity

The production of benzene metabolites, largely in the liver, is followed by their transport to the bone marrow and other organs. There are many possibilities for causing bone marrow toxicity. Irons *et al.* (1980:297) and Pfeifer *et al.* (1983:463) suggested that covalent binding of hydroquinone to spindle fibre protein could explain inhibition of cell replication by benzene. Damage to DNA could result in bone marrow depression leading to aplastic anemia, which in survivors leads to marrow dysplasia and ultimately to acute myeloid leukemia (Snyder *et al.*, 1994:177). Figure 2.3 illustrates two mechanisms by which benzene metabolites could cause damage to DNA. One pathway focuses on the metabolic activation of benzene to chemical species that covalently bind to DNA to produce mutagenic events that are expressed as leukemia. The second mechanism involves the production of metabolites that cause oxidative stress, subsequent oxidative damage to DNA, and a mutagenic effect that has the same consequences.

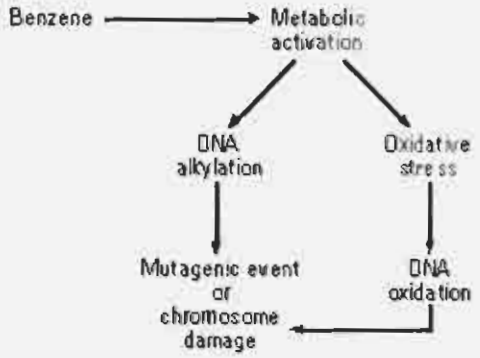

Figure 2.3. Potential pathways of DNA damage by benzene in bone marrow cells (Adapted from Snyder *et al.*, 1996:1171).

2.2.4. Biomarkers

Biomarkers are defined by Jendrychowski *et al.* (1992:7) as any parameter that can be used to measure an interaction between a biological system and an environmental agent, which may be chemical, physical or biological.

Biomarkers in the context of environmental health are therefore indicators of events in biological systems and samples. Figure 2.4 shows the progression from exposure to clinical disease.

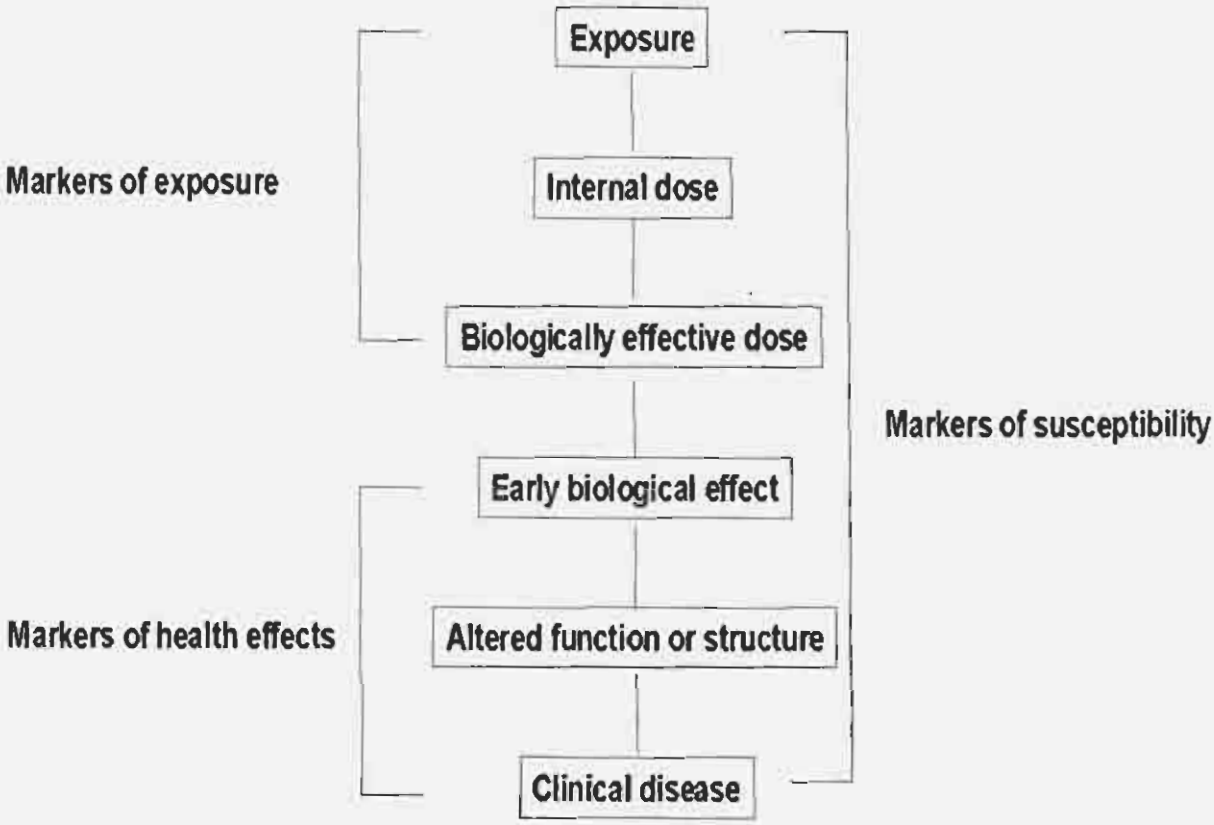

Figure 2.4. Kinds of Biological Markers (Adapted from Jendrychovski *et al.*, 1992:8).

These biomarkers can be divided into markers of exposure, markers of effect, and markers of susceptibility.

2.3 DNA damage and repair mechanisms

Numerous agents of endogenous and exogenous origin damage DNA in our genome. There are several DNA repair pathways that recognise lesions in DNA and remove them through a number of diverse reaction sequences. Defects in DNA repair proteins are associated with several human hereditary

syndromes, which show a marked predisposition to cancer (Schärer, 2003:2947).

2.3.1 Mutagenic and DNA damaging effects (genotoxicity) of carcinogens

The maintenance of the genomic integrity is of crucial importance for all organisms (Schärer, 2003:2947). Damage to the DNA that makes up our genes is constantly inflicted by a large number of agents and can have severe effects if it persists. Modification of DNA can lead to mutations, which alter the coding sequence of DNA and can lead to cancer in mammals. Mutations are of three general types: point, chromosomal or genomic (Glickman *et al.*, 1995:33). Other DNA lesions interfere with normal cellular transactions, such as DNA replication or transcription, and are deleterious to the cell. Cells have involved several ways to counteract these adverse effects of damaged DNA. There are various DNA repair pathways that can remove lesions from DNA (Schärer, 2003:2954).

Damage to DNA leads to a number of responses in the cell, which are tightly coordinated with DNA repair (Figure 2.5). The cell cycle is arrested in response to DNA damage to allow time for repair before replication and cell division (Rouse *et al.*, 2002:547). If the damage load is too large for a cell to be repaired, the cell may undergo programmed cell death (apoptosis) to avoid the propagation of highly defective cells (Rich *et al.*, 2000:777). Furthermore, some specialized DNA polymerases tolerate damage during replication and bypass a lesion in a process that either gives an accurate replication product or a mutation. The biological roles of these unusual DNA polymerases are not yet clear (Friedberg *et al.*, 2002:1627).

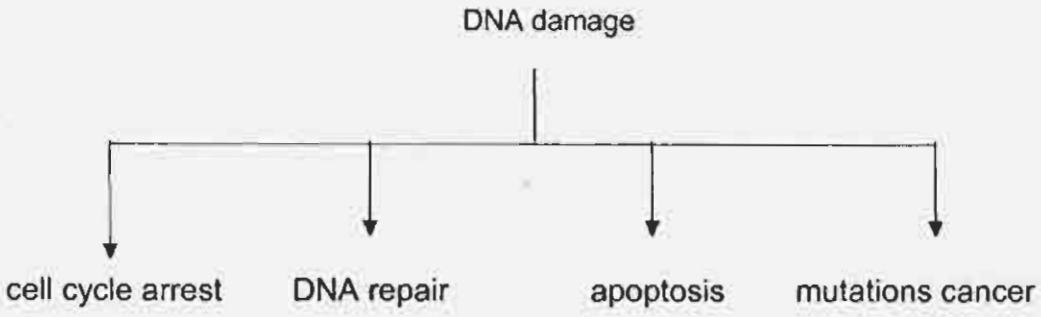

Figure 2.5. Responses to and consequences of DNA damage. (Adapted from Rouse *et al.*, 2002:549).

DNA is not indefinitely stable in aqueous solution and numerous sources of damaging agents of endogenous and exogenous origins additionally contribute to the decay and instability of DNA (Lindahl, 1993:709). Crude estimates of the number of DNA damage events in a single human cell range from $10^4 - 10^6$ per day, requiring therefore in an adult human (10^{12} cells) about $10^{16} - 10^{18}$ repair events per day (Friedberg *et al.*, 1995:698). Since alterations in only a small number of base pairs in the genome are in principle sufficient for the induction of cancer, it is clear that DNA repair systems effectively counteract this threat (Schärer, 2003:2948).

DNA contains many potentially reactive sites and its structure can be modified in a number of ways that will be summarised below.

2.3.1.1 Damage to the nucleobases of DNA

The simplest reaction that is potentially harmful to DNA is hydrolysis (Lindahl, 1993:711). The glycosidic bond of purine nucleotides is rather prone to acid-catalyzed hydrolysis. Abasic sites, which are the products of depurination, have lost the genetic coding information and can thus lead to mutations during replication (Loeb *et al.*, 1986:201).

2.3.1.2 Damage to the DNA backbone and double-strand breaks

Damage to the backbone of DNA occurs mostly through oxidation of the deoxyribose sugar, which has several positions that are highly reactive toward oxygen radical species (Pogozelski *et al.*, 1998:55). Oxidative degradation of the sugar moieties leads to a number of adducts. The pattern of adducts formed depends on the position of the hydrogen abstraction in the sugar. All these oxidative reactions lead to the formation of DNA breaks (Pogozelski *et al.*, 1998:58).

2.3.1.3 DNA interstrand crosslinks

Another class of lesions in which both DNA strands are damaged are interstrand crosslinks (ICLs) formed by a covalent connection of two DNA bases on opposite strands of DNA. ICLs are highly cytotoxic because they block all transactions that necessitate the separation of the two DNA strands, for example DNA replication and transcription (Schärer, 2003:2950).

2.3.2 Adduct formation

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 and is a property common to a wide variety of otherwise disparate carcinogens (Lawley, 1990:44; Singer and Grunberger, 1983:348; Cooper, 1990:604). Adducts range in size and complexity from simple alkyl groups (e.g. methyl, ethyl) to bulky multi-ring residues from chemicals such as polycyclic aromatic hydrocarbons and aromatic amines. Adducts can form links between adjacent bases on the same strand (intrastrand crosslinks) and can form interstrand crosslinks (Lawley, 1990:51).

Some chemicals known to be genotoxic, are intrinsically reactive and can form DNA adducts directly, either with DNA in solution in a test-tube, or with DNA in a living cell. These are called 'direct acting agents' and include alkylsulphonic esters, epoxides, aromatic *N*-oxides, aromatic nitro compounds, lactones, alkylnitrosoureas and alkylnitrosamides. These are all electrophilic – they acquire electrons during chemical reactions. DNA contains many nucleophilic centres – atoms that donate electrons. DNA-adduct formation occurs mainly by the reaction of electrophiles with nucleophilic centres in DNA – the attraction and bonding of positive to negative (Cooper, 1990:608).

2.4 DNA repair

Fifty years after discovery of the structure of DNA (Watson and Crick, 1953:737), DNA repair has become one of the most interesting topics in modern biology. The sequencing of the human genome yielded a first overview of the huge number of proteins involved in the protection of the genome (Lander *et al.*, 2001:861; Venter *et al.*, 2001:1305).

According to Christmann *et al.* (2003:1) DNA repair genes can be sub-grouped into genes associated with signaling and regulation of DNA repair on the one hand and on the other into genes associated with distinct repair mechanisms such as mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), direct damage reversal and DNA double-strand break repair (DSBR). Mutations in genes involved in DNA repair are responsible for the development of tumors and various hereditary diseases characterized by complex metabolic alterations. DNA repair genes and their corresponding proteins are also responsible for the development of cytostatic drug resistance in tumour cells.

The repair mechanisms mentioned above will now be discussed briefly.

2.4.1 Base excision repair (BER)

BER is responsible for removing DNA damaged bases, which can be recognized by specific enzymes, the DNA glycosylases. The main lesions subjected to BER are oxidized DNA bases, arising spontaneously within the cell, during inflammatory responses, or from exposure to exogenous agents, including ionizing radiation and long-wave UV light. Another main source of lesions repaired by BER is DNA alkylation induced by endogenous alkylating species and exogenous carcinogens such as nitrosamines (Christmann *et al.*, 2003:9).

2.4.2 Mismatch repair (MMR)

The MMR system is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, methylation and replication errors (Modrich *et al.*, 1996:101). The main targets of MMR are base mismatches such as G/T (arising from deamination of 5-methylcytosine), G/G, A/C and C/C (Fang *et al.*, 1993: 11838). MMR not only binds to spontaneously occurring base mismatches but also to various chemically induced DNA lesions. According to Christmann *et al.* (2003:5) the steps by which MMR proceeds are: recognition of DNA lesions, strand discrimination as well as excision and repair synthesis.

2.4.3 Nucleotide excision repair (NER)

Bulky DNA adducts, such as UV-light-induced photolesions, intrastrand cross-links, large chemical adducts generated from exposure to aflatoxine, benzo[a]pyrene and other genotoxic agents are repaired by nucleotide excision repair (NER) (Friedberg, 2001:22). In NER about 30 proteins are involved. According to Vermeulen *et al.* (1997:309) NER consists of two distinct pathways termed global genomic repair (GGR) and transcription-coupled repair (TCR).

2.4.4 DNA double-strand break repair (DSBR)

DNA double-strand breaks (DSBs) are highly potent inducers of genotoxic effects (chromosomal breaks and exchanges) and cell death (Lips *et al.*, 2001:579). In higher eukaryotes a single non-repaired DSB inactivating an essential gene can be sufficient for inducing cell death via apoptosis (Rich *et al.*, 2000:777). According to Christmann *et al.* (2003:11) there are two main pathways for DSB repair, homologous recombination (HR) and non-homologous end-joining (NHEJ), which are error-free and error-prone, respectively.

Many cytogenetic investigations have documented that occupational exposure to benzene caused a dose- and time-dependent increase of chromosome aberrations (CA) and impaired DNA repair mechanisms. Since benzene is a ubiquitous environmental contaminant, the general population is also exposed. Therefore, studies should be conducted to understand the carcinogenic property of benzene (Au *et al.*, 2002:155)

CHAPTER 3

COMET ASSAY

3.1 Introduction

In recent years, single cell gel electrophoresis (SCGE), referred to as the 'comet assay' after the shape of the images (comets) seen under the microscope, has been widely used to detect DNA strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites. It is a microgel electrophoresis technique which permits to measure DNA damage cell by cell. The alkaline version, introduced by Singh *et al.* (1988:184), detects DNA breakage, alkali labile sites, open repair sites and crosslinks. For this technique cells are mixed with molten agarose, which is spread onto a microscope slide. The cells are then lysed with high salt concentrations and detergents. The remaining nuclear DNA is then denaturated in an alkali buffer and electrophoresed in the same buffer. The DNA fragments migrate from what was the nucleus, towards the positive electrode. After electrophoresis, the slides are stained with a fluorochrome, like ethidium bromide, for UV imaging of the DNA. An image analysis system is used to measure several damage parameters. The most useful are tail length (TL), measured from the centre of the nucleus towards the end of the tail, the percentage of DNA in the tail (TD) and tail moment (TM = TL x TD). Figure 3.1 illustrates the migration process mentioned above.

Figure 3.1. DNA migration and characteristics of interest. (Adapted from Kassie *et al.*, 2000:16).

Measurements are taken at all five stages involved in the comet assay, which are stated in Table 3.2.

The technique suggested a positive role of the comet assay in the human monitoring of DNA damage from environmental and/or occupational exposure to carcinogenic and mutagenic agents, and has been shown to be a very sensitive method and a useful tool to detect genetic damage at the individual cell level and in human biomonitoring (Kassie *et al.*, 2000:13; Meller *et al.*, 2002:1007). Due to its sensitivity to detect genetic damage at the individual cell level and its potential application to virtually all eukaryotic cell types, the assay has quickly been adopted as a useful tool in short-term genotoxicity and human biomonitoring studies (Fairbairn *et al.*, 1995:37; Collins *et al.*, 1997:139). Table 3.1 summarizes practical characteristics of the comet assay method:

TABLE 3.1. Advantages and disadvantages of the comet assay method

Advantages	**Disadvantages**
• Cell/cell approach • Applicable on many cell types • No *in vitro* cultivation step required • Possible estimation of global repair capacity after *in vitro* challenging experiments • Relatively cheap running costs • Fast • Simplicity • Give some indication of apoptosis	• The detected DNA damage does not correspond to fixed mutations • Need of internal reference to avoid experimental variation during electrophoresis • Expensive fluorescence microscope

The comet assay method has been modified by incubating DNA of individual cells with specific repair enzymes that detect certain types of DNA lesions (Collins *et al.*, 1995:347). The method is then not only suitable for the detection of direct breaks but also of other types of DNA lesions, in particular oxidative DNA lesions and large DNA adducts. This significantly extends the number of applications of the method in research, in particular concerning genotoxicology, molecular epidemiology, diagnosis and therapy as in studying basic processes in the cell.

3.2 Methods, materials and procedures used in the present study

Measurement of DNA single and double strand breaks was performed by the comet assay (single cell gel electrophoresis) essentially as described by Singh *et al.* (1988:186). Human lymphocytes were collected by centrifugation through a Hypaque gradient, washed twice with phosphate-buffered saline (PBS) and resuspended in PBS.

The cells were dissolved in low melting point agarose and spread on an agarose precoated microscopic slide.

Table 3.2 displays the five stages of chemical treatment involved in the comet assay.

TABLE 3.2. Five stages of chemical treatment of the comet assay

Stage 1	Control blood cells
Stage 2	H_2O_2 treatment
Stage 3	10 minute repair
Stage 4	20 minute repair
Stage 5	30 minute repair

After solidification, slides were immersed in the lysing solution (5M NaCl, 0.4 M EDTA, 10 ml Triton X-100 and 10% DMSO) overnight. Slides were then washed and transferred to an electrophoresis chamber and soaked in alkaline electrophoresis buffer (0.6M NaOH and 0.05 M EDTA, pH 13) for 25 min. Electrophoresis were performed for 25 minutes.

After neutralization with Tris buffer (0.5 M Tris.Cl, pH 7.5) nuclei were stained with ethidium bromide (20 μg/ml) and the Tail DNA% was measured using an Olympus X70 fluorescence microscope and the CASP software program. Use of software makes the analysis simple, faster and uniform. (CASP is a computer program used to score the comets). After scoring, the data is automatically placed in an Excel document, to be analysed. The picture below is an example of how the program operates:

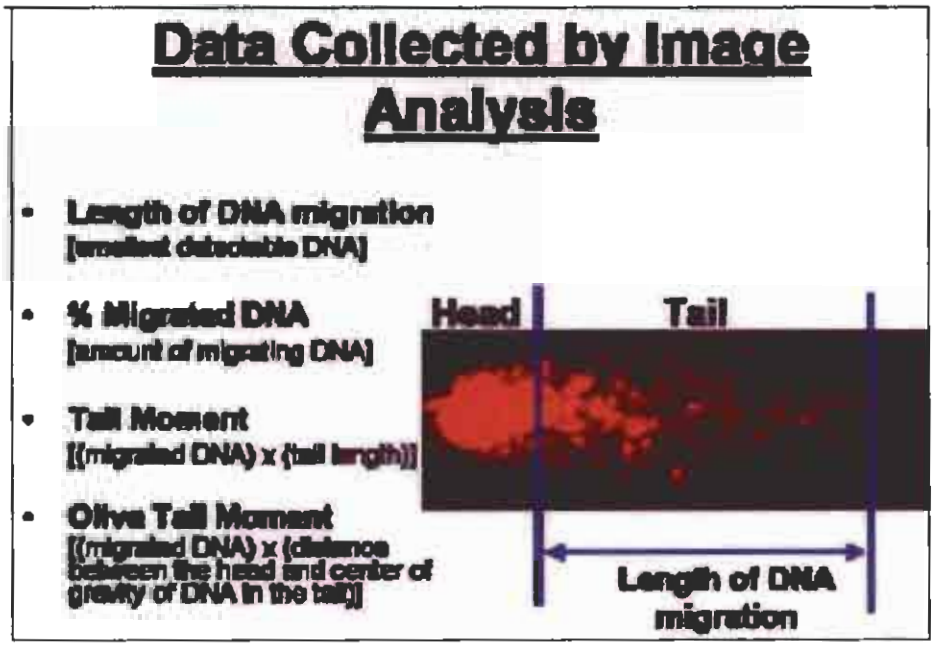

Figure 3.2. Data collected by image analysis (Adapted from Collins *et al.*, 1997:141).

The severity of genotoxicity was performed by a scoring system whereby cells were placed in classes based on the Tail DNA%, as is shown in Table 3.3. The cells are classified as being damaged or undamaged extending the visual

classification of DNA damage into five categories, i.e. from Class 0 (no damage) to Class 4 (highly damaged).

TABLE 3.3. Classification of Tail DNA%

Category	Tail DNA%
0	0-6
1	6.1-17
2	17.1-34
3	34.1-60
4	60.1-100

Most software gives a multitude of measurements of each comet DNA that has been analysed. The most commonly used are:

i) Tail DNA% (% migrated from the head)
ii) Olive Tail Moment (% DNA x distance of centre of gravity of DNA)
iii) Tail Length (Distance between the Head and the last DNA fragment)
iv) Tail Moment (%DNA x Tail Length)

We accept that %DNA x Tail Length (i.e. Tail Moment) is a reliable indicator of DNA damage, and will use both %DNA and Tail Moment in analysis. Table 3.4 displays a fractional excerpt from one experimental subject's dataset, as a typical example of the way the scored data is presented by the CASP software program. Thirteen variables were scored in total, of which 8 are displayed.

TABLE 3.4. Representation of the way the computer program interprets the scoring

NAME-CODES	Head Area	Head DNA	Tail DNA%	Head Radius	Tail Length	Comet Length	Tail MeanX	Tail Moment
MBU-NAT 0-1.tif	1710	360.304	8.09323	23	28	75	97.4388	2.2661
MBU-NAT 0-1.tif	2059	498.682	6.52219	25	38	89	164.647	2.47843
MBU-NAT 0-1.tif	1888	397.67	12.9854	24	58	107	99.7569	7.53152

MBU-NAT 0-1.tif	1881	396.326	12.7475	24	57	106	99.5423	7.2661
MBU-NAT 0-1.tif	1838	370.111	7.68301	24	25	74	198.044	1.92075
MBU-NAT 0-1.tif	1592	339.449	14.6967	22	63	108	190.029	9.25892
MBU-NAT 0-1.tif	1827	256.198	13.4141	24	58	107	138.953	7.7802
MBU-NAT 0-1.tif	2184	509.743	6.31917	26	40	93	163.016	2.52767
MBU-NAT 0-1.tif	1706	368.023	10.5096	23	51	98	74.3107	5.35988
MBU-NAT 0-1.tif	1710	362.005	8.18934	23	28	75	69.4744	2.29301
MBU-NAT 0-1.tif	2047	490.249	6.23563	25	46	97	129.126	2.86839
MBU-NAT 0-1.tif	1714	416.51	11.2281	23	56	103	93.9412	6.28773
MBU-NAT 0-1.tif	1753	418.887	11.2792	23	56	103	84.887	6.31637
MBU-NAT 0-1.tif	1834	425.999	10.7515	24	62	111	88.0664	6.66591
MBU-NAT 0-1.tif	1994	467.728	4.10878	25	31	82	175.241	1.27372

The columns representing Tail DNA% as well as Tail Moment were used for statistical analysis, and the other columns are an example of additional measurements that the CASP software program provides.

Further discussion of the method of research and relevant issues as the application of the comet assay to the present research problem will follow in Chapter 4.

CHAPTER 4

PRESENT RESEARCH AND METHODS

4.1 Introduction

From the investigations mentioned in the previous chapters it became clear that negative effects are associated with occupational exposure to benzene in the health of individuals and the general population. Therefore, the present study is justified and it will involve the following research processes:

4.1.1 Problem statement

The following question is investigated: Does DNA damage occur in workers at the fuel section of Sasol Synfuels at Secunda? If so, to which extent does DNA damage occur and in which way is the DNA repair influenced?

4.1.2 Aims of study

The aim of this study is to investigate empirically whether DNA damage occurs in workers of the fuel section of Sasol Synfuels at Secunda. Furthermore, if this is found to be true, the level of DNA damage, as well as the level of DNA repair, will have to be determined. The genotoxic potential of an environment where volatile organic compounds (VOCs) are present, is therefore investigated.

4.1.3 Hypothesis

A working environment where workers are exposed to petroleum and other VOCs causes DNA damage and delayed DNA repair.

4.2 Method of research

We will now proceed with a detailed discussion of the method of research and relevant issues.

4.2.1. Experimental and control subjects

An experimental and a control group of persons (subjects) are used for the study. Following the advice of a statistician, 30 subjects are planned to be used for the experimental group and 10 subjects for the control group. This realized in 27 experimental subjects and 9 control subjects, due to practical circumstances. In order to keep the two groups homogeneous as far as possible, and to maintain the exclusion criteria, the experimental and control groups are chosen according to a suitable questionnaire that is completed by factory and mine workers.

Table 4.1 below summarised information of the two groups:

TABLE 4.1. Information regarding the experimental and control groups

Experimental group	**Control group**
27 subjects	9 subjects
Work at fuel sections of Sasol Synfuels, Secunda	Work at Sasol mines, Secunda
VOC-exposure	No VOC-exposure

4.2.2. Methods of measuring

a) For this study only biological monitoring is essential.
b) Two samples, viz. a blood and a urine sample, are obtained from each of the experimental and control groups.
c) Blood sample:
   (i) 20 ml blood from each subject of the experimental and the control groups are obtained as follows: one blood sample of 10 ml is taken from each subject from both groups prior to the start of the work

shift, and one blood sample of 10 ml is obtained from each experimental and control subject at the end of a work shift.

(ii) Blood is extracted from the subjects by a nurse working at Sasol and one working at a private laboratory in Secunda, by using heparin tubes (heparin tubes contain an anti-coagulator).

(iii) Thereafter the blood is analised with the comet assay method, by myself and a scientist employed by the Division of Biochemistry of the Potchefstroom University.

d) Urine sample:

i) 50 ml urine from each subject of the experimental and the control groups are obtained at the end of a work shift, by a qualified nurse.

ii) The urine samples are then analised by Ampath laboratories, Pretoria for ttMA (a metabolite of benzene).

e) Finally, the results of the biological monitoring of the experimental and control groups are brought into relation with possible DNA damage and DNA repair ability as is explained in Chapter 3.

4.2.3. Methods of analysis

a) For the analysis of the blood samples, the so-called comet assay method is used. This method is suitable for the measurement of DNA damage (and DNA repair) after exposure to VOCs. (Pitarque *et al.*, 1999:197; Meller *et al.*, 2002:1009). As was mentioned in Chapter 3, the following should be kept in mind regarding the comet assay method:

(i) The comet assay has been identified to be a very sensitive method to determine DNA damage and DNA repair (Singh *et al.*, 1988:185).

(ii) Due to this method's sensitivity to indicate the damage of genetic material, the comet assay is very popular for bio-monitoring studies (Pitarque et al., 1999:197).

(iii) Any information that is suppressed regarding medication (e.g. anti-oxidants) will influence the comet assay.

(iv) Any experimental variation (e.g. day-to-day inherent differences regarding an experimental or control subject) will influence the comet assay method as well.

(v) Experimental subjects are not exposed to the same concentration VOCs during each work shift, due to routine in the work environment. This ought to influence the comet assay and the results should be interpreted in the light of this phenomenon.

b) Urine samples were analysed for a metabolite of benzene, urinary trans-trans-muconic acid (ttMA), with the aid of a HPLC/UV detector.

c) ttMA is used as an indicator of exposure of the experimental group to the aromatic components of petrol.

4.2.4. Ethical aspects

a) Written approval has been obtained from the ethical committee of the Potchefstroom University for CHE to conduct this study.

b) Persons participating in the study have given permission to be subjects of the study and signed appropriate consent forms, after they have been informed about the purpose and nature of the procedures.

4.2.5. Exclusion criteria

People excluded from this study are employees who are:

a) on chronic medication (e.g. employees suffering from diabetes-mellitus)

b) experiencing a family history of blood illness

c) excessive users of alcohol

d) smoking (smokers are already exposed to VOCs)

e) for some reason already exposed to VOCs

f) active sports people, exercising regularly.

4.2.6. Questionnaire

Information is obtained regarding the following aspects:

a) smoking habits

b) alcohol abuse

c) exercise habits

d) medication that is used

e) chronic disease that employees may suffer from

f) hobbies and after-hours activities

g) work history regarding exposition to VOCs employment (number of years employed) and previous occupations and work places.

Remark: The complete questionnaire is listed below to provide the reader with a better opportunity of understanding of the exclusion criteria.

Demographic and lifestyle questionnaire

All information given in this questionnaire is confidential

1. Subject number		
2. Name & Surname		
3. Date		
4. Age:		
5. Age	20 – 24	(1)
	25 – 29	(2)
	30 – 34	(3)
	35 – 39	(4)
	40 – 44	(5)
	45 +	(6)

6. What is your occupation?		
7. Do you work shifts?	No (1)	Yes (2)
If so, how long is each shift?		
How long is the rest period between shifts?		

Do you suffer from any of the following ?		
8. Blood diseases	No (1)	Yes (2)
9. Diabetes	No (1)	Yes (2)
10. Gout	No (1)	Yes (2)
11. Arthritis	No (1)	Yes (2)
12. Malaria	No (1)	Yes (2)
13. Chronic diseases (e.g. bronchitis, eczema, respiratory disease)	No (1)	Yes (2)

14. Any familial and/or hereditary conditions? If so, specify.	No (1)	Yes (2)
15. Do you take any medication and/or diet supplements (e.g. vitamins)?	No (1)	Yes (2)
16. If yes, please list details: _____ _____ _____ _____		
17. Do you smoke ?	No (1)	Yes (2)
18. For how long are you smoking (years)? _____		
19. If you don't smoke at the moment, were you a regular smoker previously?	No (1)	Yes (2)
20. If yes, for how long did you smoke previously? _____		
21. Do you use alcohol?	No (1)	Yes (2)
22. How much alcohol do you consume per week?		

23. Do you do physical exercises?	a) Never	b) Seldom	c) Regularly	d) Often
If so, mark the type of exercise and specify the hours per week:				
24. Gymnasium exercises (e.g. weights)				(2)
25. Training for long distance running (e.g. gym or road)				(2)
26. Cycling				(2)
27. Other (Specify)				(2)

28. Do you do any of the following at home on a regular basis:	No (1)	Yes (2)
a) Painting?		
b) Car repairs?		
c) Come into contact with any solvents?		

CHAPTER 5

STATISTICAL ANALYSIS

5.1 Data description

Data for the experimental group (27 persons) and control group (9 persons) were obtained as was described in Chapter 3 and in paragraph 4.2.2, and were presented as Excel data sheets. Pre-shift and after-shift data were available for both groups for the five stages of chemical treatment required in the comet assay. Due to a variety of reasons such as hemolysis and relatively long waiting and travelling times the data were not balanced (i.e. not the same number of scores were available for each stage for each person) and provision was made for missing data in some cases. The data were imported into the S-PLUS software program which was used for data-analysis, together with a newly developed Fortran code.

5.2 Comparisons

To visualize trends in the data, graphical methods are employed, followed by formal statistical inference.

In both Figures 5.1 and 5.2 below four sets of side-by-side barplots are used illustratively to display the means of the Tail DNA%-data and Tail Moment-data respectively in a comparative way, for all five stages involved in the comet assay, i.e., control, H_2O_2 treatment, repair times of 10, 20 and 30 minutes respectively. Furthermore, formal two-sample t-tests are performed to test the null hypothesis

H_0:The means are equal

against the alternative hypothesis:

H_A:The mean of the after-shift data *exceeds*

the mean of the pre-shift data.

Indications of statistical significant difference from H_0 are denoted above the columns with the associated p-values. (The p-value denotes the smallest probability for rejecting H_0 wrongly). If it is clear from the barplots that H_A is obviously not true (as is the case in Figure 5.2), H_0 is tested against the alternative hypothesis:

H_A:The mean of the after-shift data *is less than*
(instead of *exceeds*) the mean of the pre-shift data.

In this case, statistical significance is denoted with the appropriate p-value together with the indication 'less'.

Although the data were not always approximately symmetrically distributed for all the subgroups, analysis based on medians (instead of means) produced similar results as results based on the means of the sub-groups. Also, non-parametric two-sample Wilcoxon tests were performed, which provided the same conclusions as the two-sample t-tests. These additional results are not reported in this study.

Standard errors for the means of the sub-groups of the data were determined and are presented on all barplots as green bars at the top of each column, indicating accuracy of estimation procedures. (Standard errors are accuracy measures which are useful in determining, for example, lengths of confidence intervals for parameters, such as the population mean, should it be requested).

Figure 5.1 displays differences and/or patterns of behaviour between pre-shift and after-shift data. The means of both the Tail DNA%-data and Tail Moment-data are studied, for the following data set groupings:

(a) Pre-shift and After-shift data for the Experimental group
(b) Pre-shift and After-shift data for the Control group.

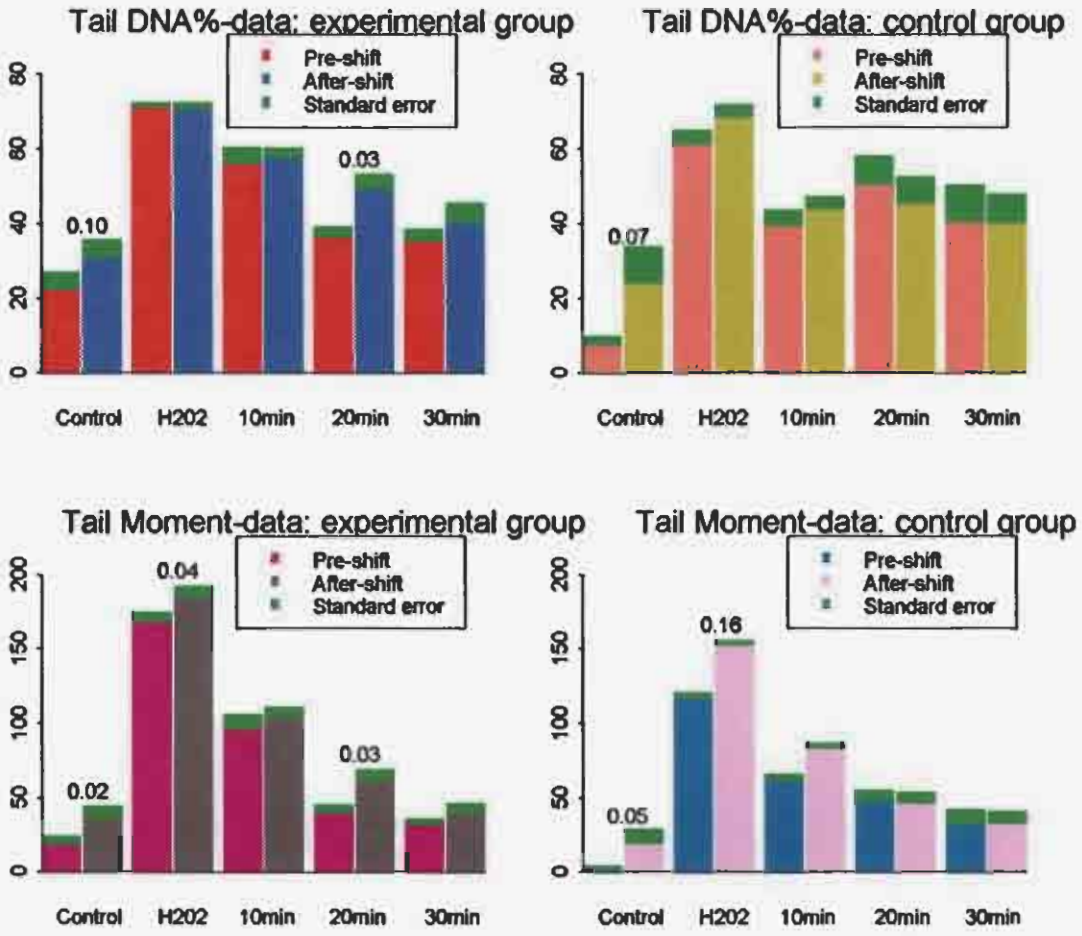

Figure 5.1. Pre-shift and After-shift comparisons for Tail DNA%-data as well as Tail Moment-data for both the experimental group and control groups. Statistical significant differences are supplied where it is appropriate. Standard errors of the data are displayed with green top-bars.

Figure 5.2 displays differences and/or patterns of behaviour between the experimental and control groups, regarding the means of both the Tail DNA%-data and Tail Moment-data, for the following data set groupings:

(c) Pre-shift data of the Experimental and Control groups

(d) After-shift data of the Experimental and Control groups.

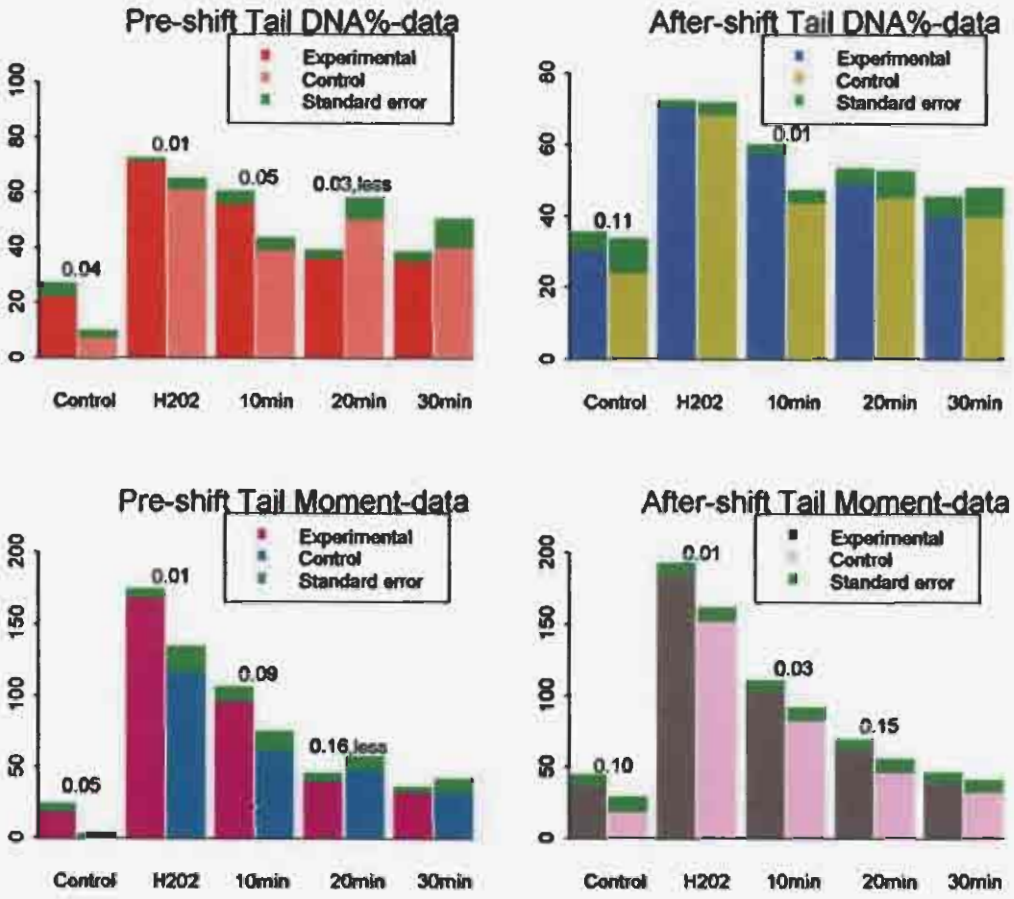

Figure 5.2. Comparisons between Experimental group and Control group for both the Tail DNA%-data as well as Tail Moment-data, pre-shift and after-shift. Statistical significant differences are supplied where it is appropriate, also when the mean of the experimental group was significantly *less than* the mean of the control group. Standard errors of the data are displayed with green top-bars.

5.3 Distribution theory: the Weibull density

The comet assay has, since its invention in 1984 by Östling *et al.* (1984:291) as a technique for the direct visualization of DNA damage and repair in individual cells, proved to be a useful method for studying genotoxicity in cells exposed to several agents. According to Ejchart *et al.* (2003:85) there is no standard statistical method for detecting and comparing the response of cells exposed to a variety of physical and chemical agents, in spite of the wide applications and increasing popularity of the comet assay. Ejchart *et al.*

(2003:85) proposed a method of critical evaluation of comet assay test results, which will be applied to the data obtained from the methods described in the paragraphs above, together with standard statistical methods.

The results of the comet assay are in general presented by estimating the distribution of the data by histograms, i.e. by displaying frequencies of interest as a function of some cell property, e.g. tail moment or Tail DNA%. In the following paragraphs histograms will be used illustratively, followed by formal test procedures of hypotheses.

The shape of a histogram, however, depends on several parameters. For example, different number of classes (bins) and the sizes of the classes allowed for the data of interest, will result in different forms of histograms. Statistical research has shown that more accurate data analysis can be achieved by estimating the distribution of the data by some probability density function $f(x)$ of known form, with known or unknown parameters, which can be estimated from the data or from other standard statistical methods (Silverman, 1986:1-25).

In this regard, Ejchart *et al.* (2003:85) pointed out that the versatile two-parameter Weibull density can be applied successfully in analysing comet assay-data, and discuss the applicability of this distribution to comet assay-data together with interesting characteristics resulting from applying density functions to comet assay data in general.

Johnson *et al.* (1970:250) define and discuss all the major properties and characteristics of the general Weibull density.

In the present study the Weibull density function will be presented by

$$f(x)=\frac{\beta}{\alpha}\left(\frac{x}{\alpha}\right)^{\beta-1}e^{-\left(\frac{x}{\alpha}\right)^{\beta}},$$

with α and β the unknown parameters of the distribution. β is known as the 'shape parameter' of the distribution, and α is the so-called 'scale parameter'. Both parameters was estimated from the data, according to the methods described in Johnson *et al.* (1970:261). Fortran code was developed to estimate these parameters and Table 5.1 displays the values of α and β for the various subgroups of data involved in the analysis.

TABLE 5.1. Weibull estimators for the Tail DNA%-data and Tail Moment-data for the Experimental and Control groups.

			Tail DNA%		**Tail Moment**	
		Stage	α	β	α	β
Experimental	Pre-shift	0	22.64	1.03	14.67	0.69
		1	73.5	13.83	181.74	5.53
		2	62.53	3.39	112.89	2.05
		3	40.22	3.53	46.02	2.07
		4	39.28	3.46	37.42	2.09
	After-shift	0	33.52	0.81	36.56	0.45
		1	73.75	12.30	198.91	6.40
		2	61.91	6.19	114.70	4.31
		3	54.90	3.53	71.22	2.69
		4	45.92	2.37	46.15	1.70
Control	Pre-shift	0	8.56	0.93	2.05	0.53
		1	65.45	6.32	139.20	1.89
		2	43.49	3.95	73.40	1.92
		3	59.87	1.70	57.04	1.30
		4	46.87	0.97	35.27	0.68
	After-shift	0	23.91	0.82	15.24	0.50
		1	72.27	8.12	161.94	6.98
		2	47.13	5.71	90.98	4.40
		3	52.70	1.83	55.03	1.42
		4	46.90	1.37	38.16	1.08

According to Johnson *et al.* (1970:252), the estimated value of α can be interpreted and used in the following interesting practical way (due to a property of the Weibull(α, β) distribution):

For all possible values of β, it is true that the probability is more or less 63% that a data point is less than the value of α. This implies that about 63 out of 100 data points are less in value than the value of α, and 37 are exceeding the value of α. If, for example, for Tail DNA%-data, the value of α is estimated to be equal to 60, then (recalling the information displayed in Table 3.3), about 37 of the data points fall into the category associated with serious cell damage. However, the value of α should not be confused in general with the critical 60%-point of seriously damaged status. However, for Tail DNA%-data, the distance between α and any of the boundaries contained in Table 3.3 should always be kept in mind. For example, if α is estimated to be 35, then it is clear that at least 37% of the data points fall into categories 3 and 4 of Table 3.3, implying considerable cell damage. This characteristic of the Weibull distribution will be used conclusively in paragraph 5.4. For the (relatively small samples) involved in this study, this phenomenon proves to be remarkable true.

For the various stages of the comet assay (Table 3.2), Weibull distributions presenting the Tail DNA%-data and Tail Moment-data of the experimental group, are displayed in Figure 5.3 for both pre-shift and after-shift data. Visual inspection of the gaphic material shows that differences and/or patterns of behaviour between pre-shift and after-shift data are easily detected from visual inspection. These observations will be discussed in paragraph 5.4.

Remark 5.3.1

A very useful characteristic of density functions is that the area below the function, between two boundaries, denote the probability for the variable of interest to be between the boundaries. Also, given any known value x (e.g., x=0.4) on the horizontal axis, then the area under the function, to the right-side of x, denotes the probability that the variable *exceeds* the value x. This characteristic will be used extensively in the discussions.

It should be kept in mind that the experimental and control groups are respresentatives of worker populations (exposed or not exposed to benzene, respectively, during working hours). The following Weibull densities (obtained from the data) estimate the distributions of these populations and subpopulations.

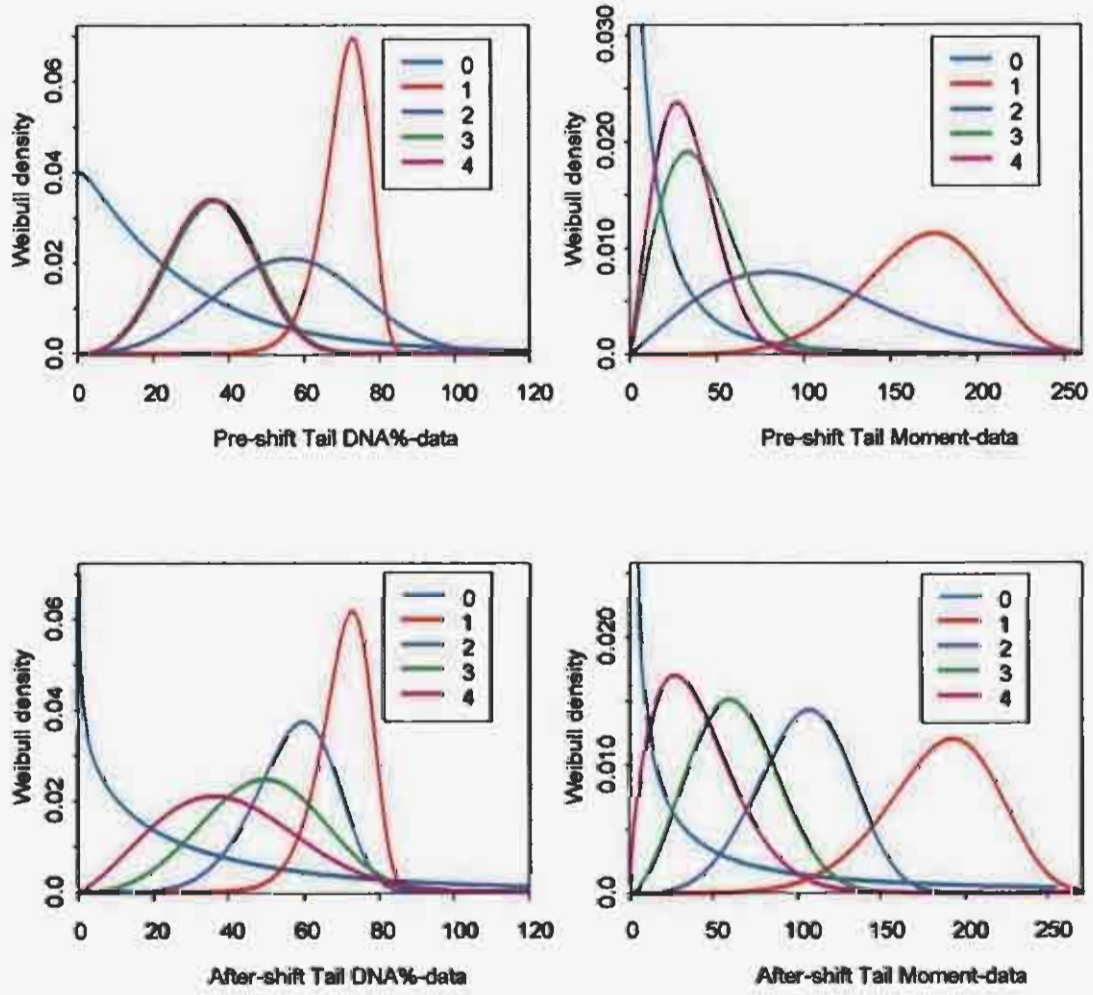

Figure 5.3. Weibull distribution plots for the Tail DNA%-data and Tail Moment-data of the experimental group for the five stages (0-4) of the comet assay (Table 3.2).

Similar Weibull distributions, obtained from estimating the parameters in the same way as before, presenting the Tail DNA%-data and Tail Moment-data of the control group, are displayed in Figure 5.4 for both pre-shift and after-shift data. Discussion of the results will follow in Chapter 5.4.

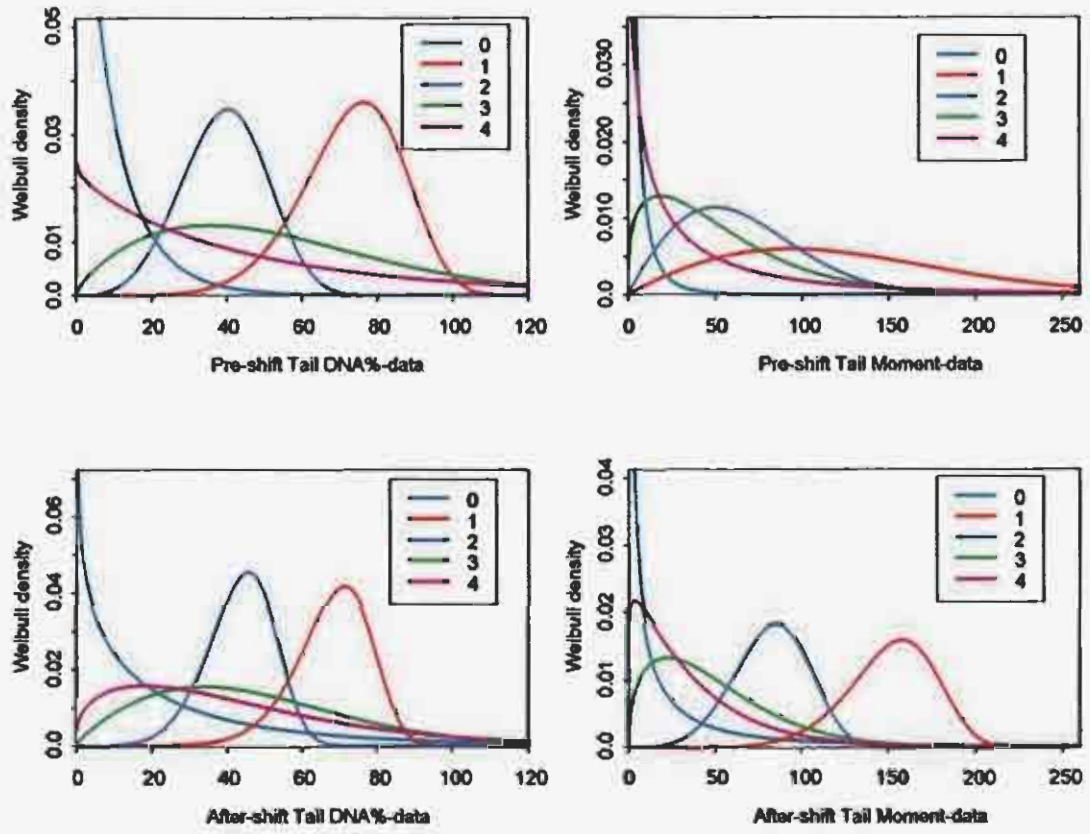

Figure 5.4. Weibull distribution plots for the Tail DNA%-data and Tail Moment-data of the control group for the five stages of the comet assay.

Figures 5.5-5.9 displays pairwise images of the experimental and control groups, for both the Tail DNA%-data and the Tail Moment-data (pre-shift and after-shift), for the five stages (separately) involved in the comet assay. Tendencies observed in these graphs, correspond to behaviours observed from Figures 5.1 and 5.2. Discussions about the graphs follow in paragraph 5.4.

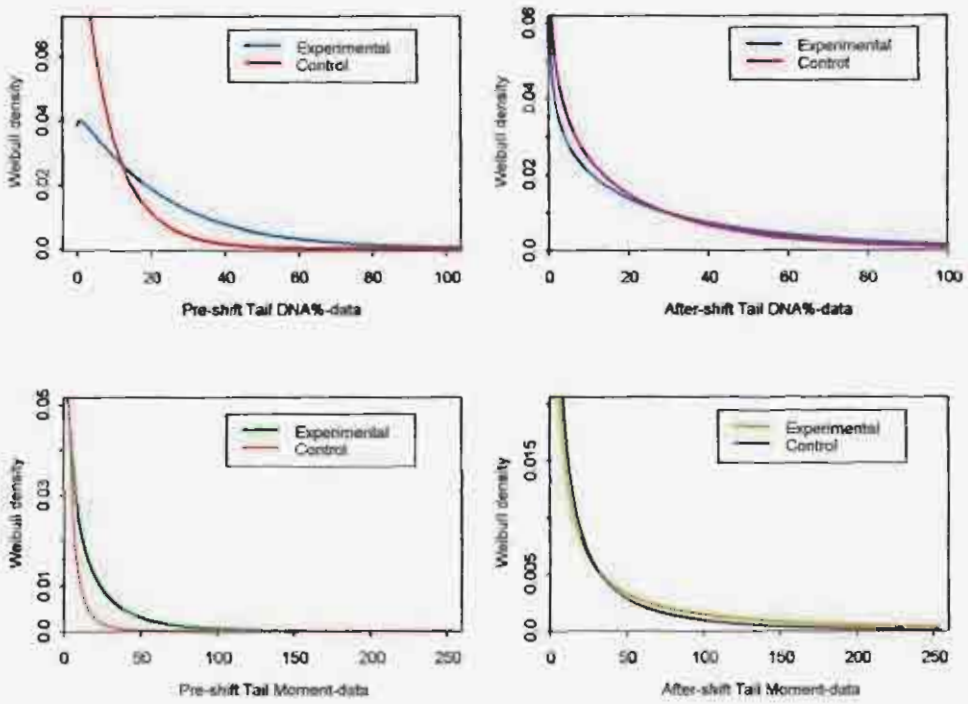

Figure 5.5. Weibull distribution plots for Tail DNA% and Tail Moment data for stage 0 (control) of the comet assay.

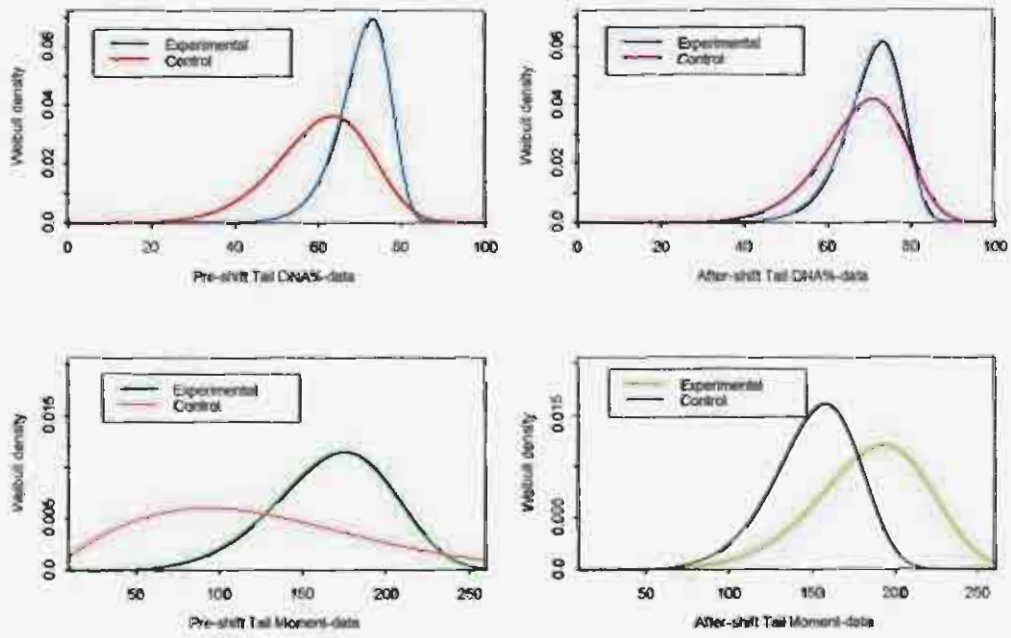

Figure 5.6. Weibull distribution plots for Tail DNA% and Tail Moment data for H_2O_2 treatment of the comet assay.

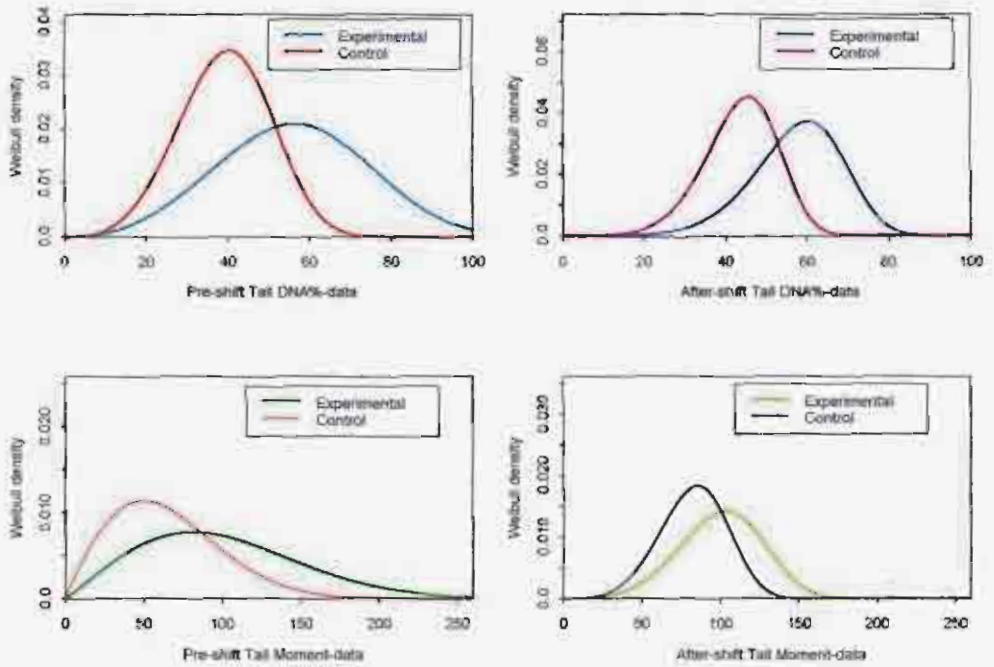

Figure 5.7. Weibull distribution plots for Tail DNA% and Tail Moment data , after 10 minutes of repair time.

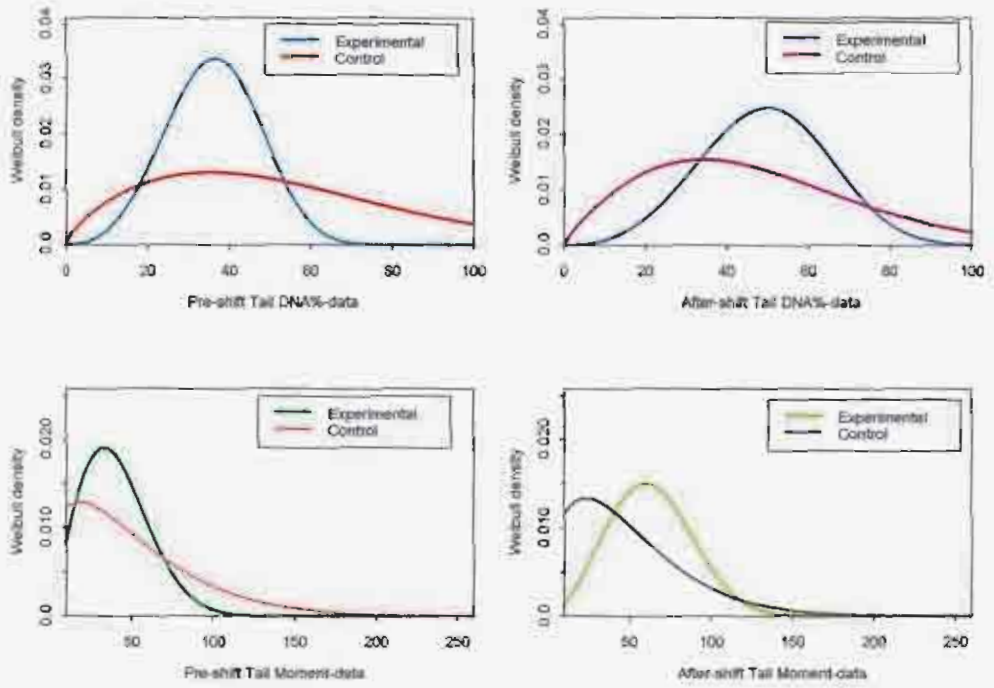

Figure 5.8. Weibull distribution plots for Tail DNA% and Tail Moment data, after 20 minutes of repair time.

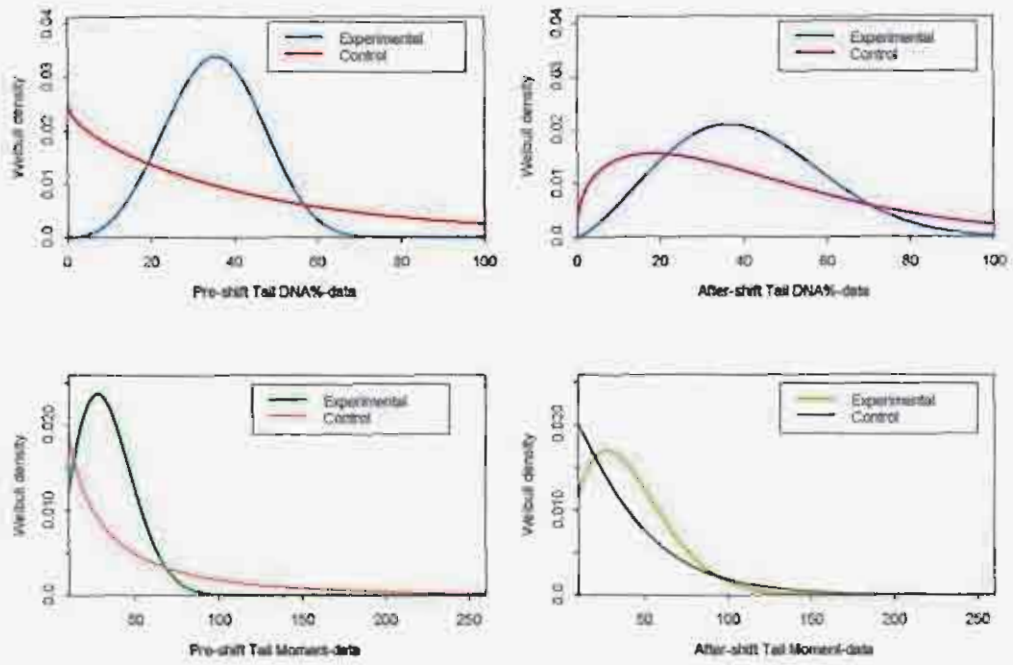

Figure 5.9. Weibull distribution plots for Tail DNA% and Tail Moment data, after 30 minutes of repair time.

5.4 Discussions of the comet assay results

5.4.1 Graphical and formal interpretations

a) Visual inspection of Figure 5.1 shows for both Tail DNA%-data and Tail Moment-data that, for the experimental group, after-shift data exceeds pre-shift data in general. This is not true for the control group, as is pointed out at stage 3 of the control group Tail DNA%-graph. However, the Tail Moment – graph does not support this phenomenon, which may imply that an outlier-value (due to the presence of other damaging agents present in a control person) may be responsible for this reaction. Alternatively, other causes are factors such as diet, age and genetic susceptibility (Au *et al.*, 2002:157). It is clear that experimental and control data shows different behaviour in the repair process. This becomes more clear in later discussions. Formal statistical tests points out that after-shift data exceeds pre-shift data for the

experimental as well as the control groups (p-values are acceptably small). This may be the result of a normal working day. The point to notice in this case, is that the pre-shift value of the experimental group, is much higher (about 9%) than that of the control group, which may be an indication of a carried-over effect which may exist from the previous shift, due to the exposure to benzene. Standard errors for both groups are sufficiently small.

b) Visual inspection of Figure 5.2 shows clearly that the experimental data are initially (pre-shift) higher than control data, as was mentioned in point a) above. The irregular behaviour at stage 3 of the Tail DNA%-data, was also discussed in a) above. The Tail Moment-data, however, shows regular behaviour during the repair process, which may be contributed to the variable 'Tail Length', which is a component of Tail Moment.

From a) and b) above it is evident that the experimental group starts off initially with high pre-shift data-values (which are much lower for the control groups), which imply that the time allowed between shifts may not be long enough to enable repair mechanisms to function successfully. Also, initial after-shift values for the control group exceeds expectations, which may be the result of a tiresome working day or other social factors such as exposure to inactive smoking due to social contact during working hours (Vineis, 2001:490). However, the repair mechanisms of the control group seem to function successfully between shifts, since the pre-shift data of the control group are very low in the zero class of Table 3.3.

c) The practical interpretation of the α values (displayed in Table 5.1) was discussed in paragraph 5.3. For example, for the experimental group (representing the population of workers exposed to benzene), at stage 0, α = 22.64 indicating that 37% of the Tail DNA%-data exceeds 22.64 , which is half-way situated in Class 2 (Table 3.3). After-shift, 37% of the workers exceeds 33.5, which represents classes 3 and 4 of DNA damage. For the control group, the pre-shift value of α is only 8.56, which is just out of class 0.

The after-shift value is much lower than 33.5. These deductions points out numerically that higher health risks exist for the experimental population. Interpretations for stage 4 from Tables 3.3 and 5.1, similarly points out the differences in risk regarding DNA repair for the experimental and control groups. For the experimental group (pre-shift), $\alpha = 39.28$ and for the control group $\alpha = 46.87$. After-shift α-values are about equal, but between shifts, the control group repair goes to class 1, but the pre-shift α-value of the experimental group, is much higher, indicating a carried-over effect.

d) The DNA repair processes discussed in Chapter 2, are clearly indicated by Figures 5.3 and 5.4. Different behaviour patterns are evident between pre-shifts and after-shifts. For example, for Figure 5.3, the area between 0 and 20% below the blue Weibull-curve in the pre-shift data is larger than the corresponding area under the blue Weibull-curve representing the after-shift data. This means that a larger proportion of small %DNA-values exists for the pre-shift data than for the after-shift data, implying that after-shift data are initially higher than pre-shift data. Furthermore, close inspection of stages 2-4 of the pre-shift and after-shift Weibull-distributions, reveals different shapes for stages 2. Also, stages 3 and 4 of the pre-shift data are almost identical, while there is a definite difference between stages 3 and 4 of the after-shift data. The after-shift data also shows a greater variation for stages 2-4 than the pre-shift data, which indicates a tendency to remain in the larger values for the after-shift data. Different behaviour is also observed for experimental and control data. Although stage 0 and 1 appear to be similar for both data-groups, different tempo's of repair are evident for stages 2-4. It is important to notice that, for the control graphs (Figure 5.4), stages 4 and 0 are closely related, indicating almost total repair for the control group. For the experimental group (Figure 5.3), these stages are represented by totally different Weibull-distributions, indicating that DNA repair was not completed for the experimental group.

e) Using Figures 5.5-5.9, and Remark 5.3.1, it is true that for most of the graphs, a higher probability is estimated for the population of exposed workers to be in a high class of DNA damage, than is estimated for non-exposed workers. This is evident by using Figure 5.5-5.9, and by calculating (or by doing visual inspection) areas below the estimated Weibull density functions. From Figure 5.7, it seems reasonable to deduce that the experimental group suffers from impaired repair mechanisms, in the sense that these mechanisms appear to be delayed.

5.5 Analysis of the urine samples

5.5.1 Biomarker of exposure (ttMA)

According to Wiwanitkit *et al.* (2001:399), environmental exposure to benzene causes an increased body burden, which is reflected in several biomarkers, e.g., urine trans-trans muconic acid (ttMA).

In this study benzene exposure was monitored by high-performance liquid chromatography (HPLC) of urine ttMA in 36 persons, including 9 controls and 27 exposed workers. Figure 5.10 displays the distributions of the data for the two groups on the same scale. The red line on each graph indicates the maximal permissible concentration. According to Lauwereys *et al.* (2001:724) this maximum permissible concentration is 1.5 mg/g of creatinine. The exposed workers showed a ttMA mean value of 3.78 ± 0.16 mg/g of creatinine, and the control persons showed a ttMA mean value of 2.84 ± 0.25 mg/g of creatinine. Without the obvious outlier in the control group (who has a ttMA value of 8.55mg/g), the ttMA mean value of the control group is 2.13 ± 0.25. The two-sample t-test confirms a significant difference in the ttMA mean value between the control persons and the exposed workers (the p-value is 14%, which is adequately significant for small samples). Figure 5.10 also shows that ttMA successfully served as biomarker of benzene exposure. Discussions of the results obtained will follow in Chapter 6.

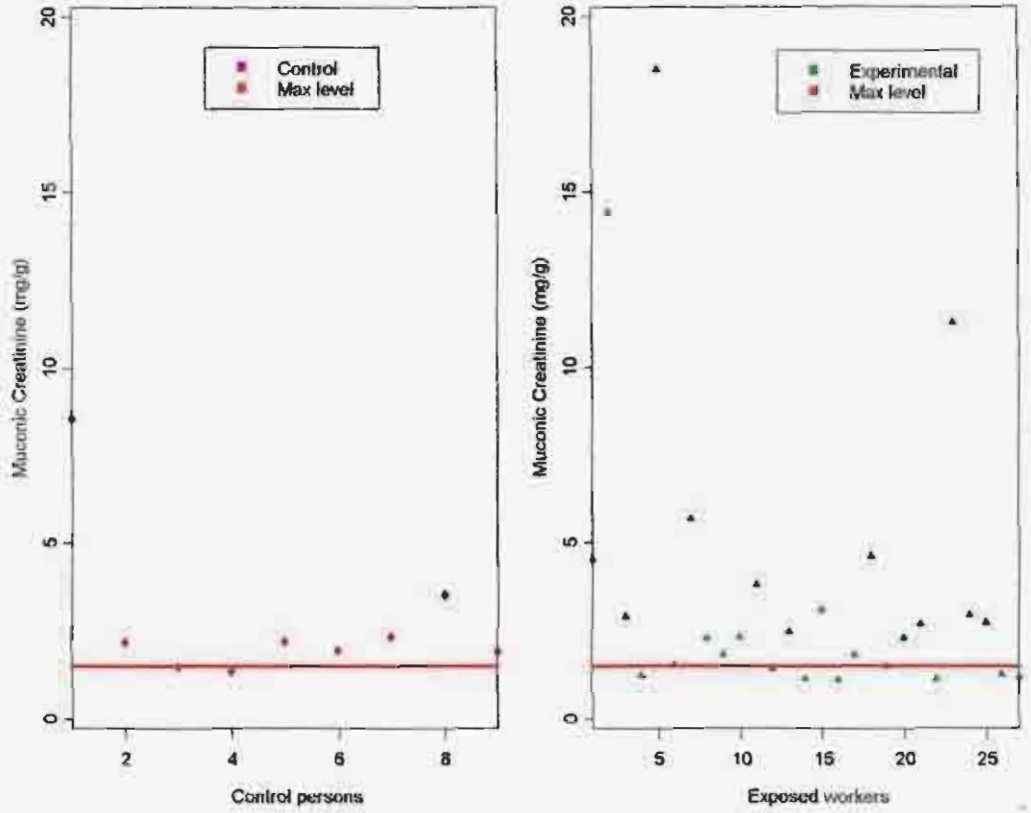

Figure 5.10. Scatterplots of ttMA data for the control persons and exposed workers respectively.

CHAPTER 6
CONCLUSIONS

6.1 Introduction

The induction of health problems by environmental mutagenic chemicals such as benzene is frequently mediated by the induction of persistent effects in cellular macromolecules (Loeb, 2001:3230). Whether a mutagenic chemical can induce persistent effects and whether persistent effects can lead to the development of health problems are influenced by a variety of extrinsic and intrinsic factors. Human intrinsic factors such as diet, aging, previous exposure history, genetic susceptibility and health status, can influence the toxicity of environmental agents. According to Au *et al.* (2002:160), data for health risk assessment can significantly be affected by factors such as target organ specific responses, interactions with other toxic agents and differences in exposure conditions in humans (e.g. chronic versus acute exposures, inhalation versus ingestion).

The presence of toxic benzene metabolites is normally used to indicate exposure to benzene among workers. However, these metabolites are also frequently found in blood and urine among the general population, even in subjects with no known exposure to benzene. The high but varying background levels of phenol and ttMA in control individuals can be contributed by various factors such as the consumption of diets rich in meat and protein, the use of antibiotics which changes gastrointestinal flora make-up, and variant metabolic enzyme activities (Vineis, 2001:485).

6.2 Summary of results

In this study the vital status was determined for 27 experimental (workers exposed to organic solvents) and 9 control human subjects in order to investigate empirically whether DNA damage occurs in the exposed workers of the refinery section of Sasol Synfuels at Secunda. If DNA damage is found

to be present, the level of DNA damage, as well as the level of DNA repair, were also to be determined.

The following deductions can be made from the analysis:

- From Figures 5.1 and 5.2 it became evident by inspection of both Tail DNA%-data and Tail Moment-data and by formal comparative two-sample tests (t-test and Wilcoxon-test) that DNA damage was indeed present at the exposed (experimental) group of workers. For the experimental group, after-shift data exceeded pre-shift data in general. The control group-data showed different behaviour at stage 3 of the comet assay process, which may be explained by factors mentioned in the introduction above. From these two graphs, different behaviour of the experimental and control-data during the repair process is also clear. The important point to notice from these graphs is that the initial pre-shift data-values of the experimental group, is much higher (about 9%) than that of the control group, which may be an indication of a carried-over effect which may exist from the previous shift, due to the exposure to benzene.

- Inspection of Figure 5.2 shows clearly that the pre-shift experimental data are initially higher than the initial control data, which implies that, for the experimental group, the time allowed between shifts may not be long enough to enable repair mechanisms to function successfully. Reasons for the high initial after-shift values for the control group are surprising and deserve attention in future research projects. The low initial pre-shift data-values of the control group show that the repair mechanisms of this group seem to function successfully between shifts, in contrast to the results obtained from the experimental group.

- The practical interpretation of the high estimated first parameter (α-values) of the Weibull distribution (displayed in Table 5.1) together with the information contained in Table 3.3 imply that higher health risks exist for the experimental population than for the control population, as is explained in paragraph 5.4.1. Between shifts, parameter-estimates indicate that the DNA

repair for the control group goes back to class 1 of Table 3.3, while the pre-shift α-value of the experimental group is much higher, indicating a carried-over effect.

- Regarding the DNA repair processes, Figures 5.3 and 5.4 indicate different behaviour patterns between pre-shifts and after-shifts, which are dicussed in paragraph 5.4.1. A larger proportion of small %DNA-values exists for the pre-shift data than for the after-shift data, implying that after-shift data are initially higher than pre-shift data, emphasising the results mentioned before. Furthermore, after-shift data also shows a greater variation for stages 2-4 of the comet assay than the pre-shift data, indicating a delay in the repair process. Different behaviour is also observed for experimental and control data. The fact that stages 4 and 0 are closely related for the control group, points to almost total repair for the control group. For the experimental group (Figure 5.3), these stages are represented by totally different Weibull-distributions, indicating that DNA repair was not completed for the experimental group, which was also deduced from Figures 5.1. and 5.2.

- Using Figures 5.5-5.9, and Remark 5.3.1 it is found throughout that a higher probability is estimated for the population of less-exposed workers to be in a higher class of DNA damage, than is estimated for exposed workers. Figure 5.7 also shows that it seems reasonable to deduce that the experimental group suffers from impaired repair mechanisms, in the sense that these mechanisms appear to be delayed.

- From Figure 5.10 and the p-value associated with the two-sample t-test it is clear that the exposed workers show a markedly higher (1,8 times) ttMA mean value than the control persons. A distressing observation is the fact that the mean ttMA values for both the control persons and the exposed workers exceed the maximal permissible concentration value (1.5 mg/g of creatinine). The high ttMA values may imply that also the control group are excessively exposed to benzene, but various other factors could have contributed to these high ttMA values. For example, a known alternative source of urinary ttMA is sorbic acid ($CH_3CH=CHCH=CHCOOH$). This

preservative is used in certain foods, cosmetics and pharmaceutical products and its metabolism leads to ttMA (Melikian *et al.*,1999:719). It may also imply that the genotoxic potential was increased due to a general environment which was more contaminated than was expected.

6.3 Achievement of research aims

The following questions defined the aims and purpose of this study:

- Does DNA damage occur in workers at the fuel section of Sasol Synfuels at Secunda?
- If so, to which extent does DNA damage occur and in which way is the DNA repair influenced?

The genotoxic potential of an environment where volatile organic compounds (VOCs) are present was therefore investigated.

From the observations and analyses derived in Chapter 5 and the conclusions contained in paragraph 6.2. it is clear that the research questions have been addressed successfully and the research aims achieved.

6.4 Recommendations

As far as the workers of the refinery section of Sasol Synfuels at Secunda is concerned, various forms of human intervention (of which some are unpractical and not convenient or comfortable) are possible to assist the health conditions of the exposed workers. The following procedures can be applied and explored:

- Primary prevention:

Theoretically, the source of emission (benzene) should be removed, i.e. workers must be separated from exposure to benzene. Since this step is not practically possible, secondary preventions steps should be applied. Also, the localization of sources of benzene must be identified for regular monitoring purposes.

- Secondary prevention:

Secondary prevention involves the distribution of personal protective equipment (PPE) to the workers during shifts. For example, the use of gas masks during shifts will possibly be protective but uncomfortable, and the masks will gradually become unpopular during long shifts (8 hours).

- Tertiary prevention:

Regular monitoring (by an alert medical team) of exposed workers, consisting of frequent medical check-ups, immediate treatment of exposure related illnesses when it is suspected and follow-up monitoring during healthy periods, form part of this cycle. Also, regular monitoring by an experienced occupational hygienist of the immediate problematic environment can be invaluable in preventing unacceptable levels of contamination.

- Additional remarks:

The following practical steps are also recommended:

a) Reorganising shift schedules to enable DNA repair mechanisms to recover, is an attainable option to consider. Also, the effectiveness of longer rest periods between shifts can be investigated.

b) Rotation of workers within shifts and within the refinery to minimise the exposure to organic solvents, is another obtainable option to apply.

c) Issuing exposed workers with controlled quantities of supplements such as anti-oxidants to assist DNA repair mechanisms may be costly. However, the impact of this intervention can not be overemphasized. The effectiveness of such an intervention, as well as the time of administering (pre-shift or after-shift) and quantities of administered supplements, is a matter for further research.

d) Long term follow up studies of benzene exposed workers are essential, to determine patterns of negative health conditions such as predictive risk of cancer.

e) Epidemiological studies regarding possible consequences such as typical illnesses associated with exposure (i.e., occurrence of cancer in retired workers) should be conducted continually as part of a monitoring program.

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