Genotoxicity of haloacetic acids, aspirin and ibuprofen in human cells

Genotoxic effects of water disinfectant- by-products in human blood and sperm and bulk and nano forms of aspirin and ibuprofen in human blood of respiratory disease patients

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Abstract

This project focuses on two important topics which may pose hazards to human health. Firstly, drinking water disinfection by-products (DBPs), which are generated by the chemical disinfection of water have been investigated. What has not been shown is the effect of DBPs in human germ cells as well as somatic cells and whether oxidative stress is involved in the mechanism of genotoxic action. Three different DBPs (halo acetic acids: HAAs), together with the antioxidants – catalase and butylated hydroxyanisole (BHA), were investigated in peripheral blood cells and sperm from healthy individuals using the Comet assay and lymphocytes only using the micronucleus assay. Secondly, nanoparticles of the non-steroidal anti-inflammatory drugs (NSAIDs), aspirin and ibuprofen, have been investigated in patients with respiratory diseases, in the micronucleus assay and the Comet repair assay. NSAIDs inhibit cyclooxygenase enzyme activity, which plays part in tumour progression. In the Comet assay, BHA and catalase were able to reduce DNA damage in both cell types compared to HAAs alone. Similarly, in the micronucleus assay, micronuclei were reduced with the antioxidants, suggesting oxygen radical involvement in both assays. With the NSAIDs,

reductions were seen for DNA damage in the micronucleus assay with aspirin and ibuprofen nanoparticles compared to their bulk forms. Using the Comet repair assay, aspirin and ibuprofen nanoparticles aided repair of DNA to a greater extent than their bulk counterparts, which in turn showed better repair compared to samples repaired without NSAIDs. These observations show the importance of DBPs and NSAIDs in genotoxic public health issues.

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Contents

Abstract ..... **i**

Acknowledgments ..... **iii**

List of figures, tables and graphs ..... **x**

Figures: x

Tables: xi

Graphs: xii

Abbreviations ..... **xiii**

Chapter 1 - Introduction **1**

1.1 Overview of Project 2

1.2 History and increasing use of disinfectants 3

1.3 Emergence of DBPs 4

1.3.1 Halo acetic acids (HAAs) 5

1.3.2 Chemical reaction and Structure of Halo acetic acids (HAAs) 7

1.4 Health related issues of DBPs 8

1.5 Antioxidants: Catalase and butylated hydroxyanisole. 10

1.5.1 Uses and functions of catalase and butylated hydroxyanisole 10

1.5.2 Catalase and butylated hydroxyanisole impact on HAAs 12

1.6 Introduction to nanoparticles 13

1.6.1 Background to Nanomaterials 13

1.6.2 Uses of Nanoparticles in Industry / Types of Nano compounds 14

1.6.3 Properties of nanoparticles 15

1.7 Non-steroidal anti-inflammatory drugs (NSAIDs) 23

1.8 Genotoxicity Assays 32

1.8.1 Comet assay 32

1.8.2 Comet repair assay 34

1.8.3 Micronucleus assay 37

1.9 Aims of study (HAAs) 43

1.10 Aims of study (nano and bulk aspirin and ibuprofen) 45

Chapter 2 - Materials & Methods **48**

2.1 Ethical approval 49

2.2 Materials and Methods for testing HAAs using the Comet assay 49

2.2.1 Reagents. 49

2.2.2 Lymphocyte and Sperm sample collection 50

2.2.3 Cell Viabilities 51

2.2.4 Study Cohort for both lymphocyte and sperm Comet assays. 51

2.2.5 Single cell gel electrophoresis (SGCE) Comet assay 52
2.2.6 Embedding of cells in Agarose 54
2.2.7 Cell Lysis 55
2.2.8 Electrophoresis 56
2.2.9 Scoring of Slides and Statistical Analysis: 57
2.3 Materials and Methods for Cytokinesis block Micronucleus (CBMN) Assay for testing HAA compounds (chapter 4) 58
2.3.1 Reagents 58
2.3.2 Whole blood sample collection & Study Cohort. 59
2.3.3 Preparation of basic culture medium 60
2.3.4 Cell culture of Lymphocyte cultures. 60
2.3.5 Cell hypotonic shock treatment: 61
2.3.6 Fixation of cells 62
2.3.7 Slide Preparation, staining and fixation of slides. 62
2.3.8 Slide scoring 63
2.3.9 Data Analysis and statistics used 63
2.4 Methods for Preparation of NSAIDs - Aspirin nano & Ibuprofen nano compounds, (for chapters 5 & 6), sections 2.5 & 2.6 of Materials & methods. 64
2.4.1 Reagents. 64
2.4.2 Methodology for Aspirin & Ibuprofen nano suspensions. 64
2.4.3 Analysis of nano-suspension particle size 65
2.4.4 Zeta potential analysis of nano suspensions 66
2.5 Materials and Methods for Cytokinesis block Micronucleus (CBMN) Assay for testing aspirin & ibuprofen (chapter 5) 66
2.5.1 Reagents. 66
2.5.2 Whole blood sample collections & Study cohort 67
2.5.3 Cell culture of whole blood 68
2.5.4 Preparation of basic culture medium 68
2.5.5 Cell hypotonic shock treatment: 68
2.5.6 Fixation of cells 69
2.5.7 Slide Preparation, staining and slide fixing. 69
2.5.8 Slide scoring 69
2.5.9 Data Analysis and statistics used 69
2.6 Materials and Methods for testing ibuprofen and aspirin using the Comet repair assay. 69
2.6.1 Reagents. 69
2.6.2 Whole blood collection & study cohort 70

2.6.3 Cell Viabilities 70
2.6.4 Single cell gel electrophoresis (SGCE) Comet assay with repair mechanism 70
2.6.5 Embedding of cells in Agarose 73
2.6.6 Cell Lysis 73
2.6.7 Electrophoresis 74
2.6.8 Scoring of Slides and Statistical Analysis: 74
Chapter 3 – Genotoxic effects of HAAs and antioxidant treatments in the Comet assay 76
3.1 Introduction 77
3.2 Materials and Methods 79
3.2.1 Materials 79
3.2.2 Methods 79
3.2.4 Statistical Analysis 79
3.3 Results 81
3.3.1 Cell viability 81
3.3.2 Overview of results 81
3.3.3 Statistics: 82
3.3.4 Confounding factors: 83
3.4 Discussion 93
Chapter 4 – Exposure of haloacetic acids (HAAs) in human peripheral blood using Cytokinesis-block micronucleus assay 95
4.1 Introduction 96
4.2 Materials and Methods 99
4.2.1 Materials 99
4.2.2 Methods 99
4.2.4 Statistical Analysis. 99
4.3 Results 101
4.3.1 Determining concentrations of HAAs and antioxidants 101
4.3.2 Overview of results 101
4.3.3 Induction of micronuclei by iodoacetic acid (IAA) 102
4.3.4 Induction of micronuclei by bromoacetic acid (BAA) 103
4.3.5 Induction of micronuclei by chloroacetic acid (CAA) 105
4.3.6 General overview of all HAAs: 106
4.3.7 Statistics: 106
4.3.8 Confounding factors: 106

4.4 Discussion 111
Chapter 5 – Implications of nano-compounds in lymphocytes from on respiratory disease patients using the Cytokinesis-block Micronucleus assay 113
5.1 Introduction 114
5.2 Materials and Methods 117
5.2.1 Materials 117
5.2.2 Methods 117
5.2.3 Statistical Analysis. 117
5.3 Results 118
5.3.1 Particle size analysis 118
5.3.2 Preliminary work 118
5.3.3 General overview of data in Binucleated Cells (BiNC) 119
5.3.4 General overview of data in mononucleated Cells (MonoNC) 119
5.3.5 Exceptions in the general trends of data described above 120
5.3.6 Data for NDI, NPBs and NBUDs in cells 120
5.4 Discussion 128
Chapter 6 – Role of NSAIDs on the repair of DNA following exposure to bleomycin 131
6.1 Introduction 132
6.2 Materials and Methods 135
6.2.1 Materials 135
6.2.2 Methods 135
6.2.3 Study design 135
6.2.4 Statistical Analysis 136
6.3 Results 138
6.3.1 Particle size analysis 138
6.3.2 Cell viability 138
6.3.3 Preliminary investigations 139
6.3.4 Statistics 139
6.3.5 Overview of results 139
6.3.6 Olive tail moment data 140
6.3.7 Percentage (%) tail DNA data 142
6.4 Discussion 150
Chapter 7 – General Discussion 153
7.1 Discussion of Halo acetic acids (HAAs) 155

7.2 Methods of reducing DBP related health issues 157
7.3 Genotoxic effects of HAAs and antioxidant implications on their damage in the Comet assay 159
7.4 Micronuclei (MNi) induction following HAA exposure 161
7.5 Discussion of NSAIDs & nanoparticles 164
7.5.1 Respiratory diseases (Asthma, COPD & Lung cancer) 164
7.5.2 NSAIDs – aspirin and ibuprofen 165
7.6 Implications of ASP & IBU on lymphocytes from respiratory disease and cancer patients using the Cytokinesis-block Micronucleus assay (Chapter 5) .. 166
7.7 Role of NSAIDs on the repair of DNA following exposure to bleomycin (Chapter 6) 167
7.8 Conclusions 168
Chapter 8 – References 172

List of figures, tables and graphs

Figures:

Figures in Introduction (Chapter 1)

Figure 1: Structure of haloacetic acids……..……..……..……....7

Figure 2: Structure of butylated hydroxyanisole (BHA)…….……..…....12

Figure 3: Surface reactivity of nanoparticles……..……...16

Figure 4: Factors to consider for nanoparticles synthesis ……..17

Figure 5: Agglomeration of nanoparticles.……..……..……..…19

Figure 6: Role of cyclooxygenase enzymes in prostaglandin synthesis……..……...26

Figure 7: Image of a Comet cell……..……..……..…..33

Figure 8: Possible fates of cells after cell division……..……....40

Figure 9: Micronuclei (MNi) formation.……..…42

Figure 10: Types of MNi……..……..……..42

Figures in Results (Chapters 3, 5 and 6)

Figure 11: Image of lymphocyte Comet……..……..…..…….92

Figure 12: Image of sperm Comet …..…….....…….92

Figure 13: Aspirin nano particles (using TEM) ……..……..…..127

Figure 14: Ibuprofen nano particles (using TEM) ……..……..…...127

Figure 15: Aspirin nano particles (using TEM)..……..……149

Figure 16: Ibuprofen nano particles (using TEM)..……..…..……..…149

Tables:

Tables in Materials & Methods (Chapters 2)

Table 1a: List of lymphocyte samples used in the Comet assay……...…..…....52

Table 1b: List of sperm samples used in the Comet assay…….52

Table 2: List of whole blood samples used in the CBMN assay on HAAs……....59

Table 3: Treatment groups in the CBMN assay on HAAs……..……..……..…61

Table 4: List of blood samples used in the CBMN assay on nanoparticles...……..67

Table 5: Treatment groups in the CBMN assay on nanoparticles…...……..….....68

Tables in Results (Chapters 4 and 5)

Table 6: CBMN assay - iodoacetic acid (IAA) exposure to lymphocytes……...108

Table 7: CBMN assay - bromoacetic acid (BAA) exposure to lymphocytes……....109

Table 8: CBMN assay - chloroacetic acid (CAA) exposure to lymphocytes…….....110

Table 9a: Particle size of nano aspirin and ibuprofen……...124

Table 9b: Particle size of nano aspirin and ibuprofen…….……..……...124

Table 10: CBMN assay – treatments with NSAIDs……..…….125/126

Graphs:

Graphs in Results (Chapters 3, 5 and 6)

Graph 1a: Dose response curve of HAAs in lymphocytes (Olive tail moment)……84

Graph 1b: Dose response curve of HAAs in lymphocytes (% tail DNA)….....85

Graph 2a: Dose response curve of HAAs in sperm (Olive tail moment).....86

Graph 2b: Dose response curve of HAAs in sperm (% tail DNA)…….....87

Graph 3a: Antioxidant responses to HAAs in lymphocytes (Olive tail moment).....88

Graph 3b: Antioxidant responses to HAAs in lymphocytes (% tail DNA)…….....89

Graph 4a: Antioxidant responses to HAAs in sperm (Olive tail moment)…..…....90

Graph 4b: Antioxidant responses to HAAs in sperm (% tail DNA)……..…..... 91

Graph 5a: CBMN assay – MNi frequency in healthy individuals…….……122

Graph 5b: CBMN assay – MNi frequency in asthma patients…….……122

Graph 5c: CBMN assay – MNi frequency in COPD patients …….……123

Graph 5d: CBMN assay – MNi frequency in lung cancer patients……..…...…...123

Graph 6a: DNA repair in healthy individuals (Olive tail moment) ……..…145

Graph 6b: DNA repair in asthma patients (Olive tail moment) ……..145

Graph 6c: DNA repair in COPD Patients (Olive tail moment)…….....……146

Graph 6d: DNA repair in lung cancer patients (Olive tail moment) ……..……..146

Graph 7a: DNA repair in healthy individuals (% tail DNA) ……147

Graph 7b: DNA repair in asthma patients (% tail DNA) ……..……....……147

Graph 7c: DNA repair in COPD Patients (% tail DNA)……....148

Graph 7d: DNA repair in lung cancer patients (% tail DNA) ……..…...…..148

Abbreviations

Genomic and Cytogenetic assays:

CBMN: Cytokinesis-Block Micronucleus assay

SGCE: Single cell gel electrophoresis

Chemicals & enzymes:

ASP B: Aspirin bulk

ASP N: Aspirin nano

BAA: Bromoacetic acid

BHA: Butylated hydroxyanisole

BLM: Bleomycin

CAA: Chloroacetic acid

COX-1: Cyclooxygenase enzyme 1

COX-2: Cyclooxygenase enzyme 2

DBPs: Disinfection-by-products

HAAs: Haloacetic acids

IAA: Iodoacetic acid

IBU B: Ibuprofen bulk

IBU N: Ibuprofen nano

MMC: Mitomycin C

NSAIDS: Non-steroidal anti-inflammatory drugs

THMs: Trihalomethanes

Health condition:

COPD: chronic obstructive pulmonary disease

CBMN cells types:

BiNC: Binucleated cells

MonoNC: Mononucleated cells

MNi: Micronuclei

MultiNC: Multinucleated cells

NBUD: Nuclear buds

NPB: Nucleoplasmic bridges

CBMN statistics:

NDI: Nuclear Division Index

Chapter 1 - Introduction

Introduction

1.1 Overview of Project

This PhD project involves studies of the genotoxic damage to DNA caused in human cells that are diploid, more specifically, peripheral lymphocytes and whole blood cells or haploid, sperm. These cells are from healthy control individuals or patients and the general theme is to determine and analyse the impact certain chemicals have on DNA and cell damage. Blood cells are of interest because damage to the DNA could give rise to cancer, and damage to the DNA in germinal cells could give rise not only to cancer but also to heritable defects.

This project focuses on two important topics. The first one is the side effects of by-products of disinfectants used in the treatment of drinking water. These effects are investigated in human peripheral blood cells and sperm using the Comet assay as a means of testing genotoxicity by observing the % tail DNA and Olive tail moments of Comets. The micronucleus assay is also used to assess the cytogenetic damage of these by-products in lymphocytes by observing the number of micronuclei (MNi) in mononucleated and binucleated cells. Findings from these studies will help to establish health related implications for DBPs and will further contribute to the existing literature pointing to the needs for greater regulation of DBPs in drinking water. The second topic investigates the effects of nanoparticles in human peripheral blood cells. Nanoparticles of the non-steroidal anti-inflammatory drugs, aspirin and ibuprofen, have been investigated in patients with respiratory diseases, using the micronucleus assay and a Comet repair assay. Determining the

toxicity of nanoparticles is very important because nanotoxicology lags behind nanotechnology and recently there has been a boom in the latter.

1.2 History and increasing use of disinfectants

Water disinfection has become one of the biggest health improvements of the last century which has reduced the transmission of waterborne infectious diseases (Akin et al., 1982). Today millions of people around the world receive clean, safe and quality drinking water each day through their public water systems. Most developed nations have systems and procedures in place for treating water, such as the U.S EPA (Environmental protection agency) (Richardson et al., 2007). In untreated water, the transmission of pathogenic microorganisms like *Vibrio chloerae*, *Salmonella typhi* and *Enteromoeba histologica*, are responsible for the rise of waterborne infectious diseases such as cholera, typhoid fever, and amoebic dysentery, to name a few (Akin et al., 1982; Dziuban et al., 2006). Pathogenic transmissions can occur through drinking water, washing, bathing, swimming, ingestion, preparation and consumption of food with infected water. According to the World Health Organization (WHO), diarrhoeal infections account for 4.1% of the total DALY burden (Death and disability-adjusted life year) of disease in developing nations, causing 1.8 million deaths annually. The WHO also reports that 88% of the burden is due to, lack of hygiene, sanitation and unsafe/untreated water (World Health Organization., 2010b).

Fortunately, chemical disinfectants have helped to eliminate many of these diseases by effectively killing harmful microorganisms found in drinking water (Richardson et al., 2008; Richardson et al., 2007). These disinfectants are

capable of oxidising organic matter and naturally occurring bromide and iodide present in waters of reservoirs, lakes and rivers (Plewa et al., 2002; Richardson et al., 2008; Richardson et al., 2007). Chlorine is the most commonly used disinfectant to treat drinking water and is also used in swimming pools (Kogevinas et al., 2010). It inactivates bacterial, viral and protozoal pathogens and prevents algal/biological growth (Akin et al., 1982; Sadiq and Rodriguez, 2004). Other disinfectants include; chloramines, chlorine dioxide and ozone treatment (Plewa et al., 2002; Richardson et al., 2007). The benefit of treating drinking water has a major impact on improving health and wellbeing. However their use has led to the formation of unwanted drinking water disinfection by-products (DBPs). DBPs are formed from the reaction between the chemical disinfectant and organic constituents, bromide and iodide that are naturally present in water (Richardson, 2011).

1.3 Emergence of DBPs

In 1974, Rook and other researchers identified the first DBPs found in chlorinated drinking water. The first two classes identified were chloroform and trihalomethanes (THMs), and halo acetic acids (HAAs) were identified soon after. Over 30 years later, some 600 DBPs have been identified, as reviewed by Richardson et al. (2007).

There are several different classes of DBPs (Richardson *et al.,* 2007):

1. THMs – Trihalomethanes (chloroform, bromoform, bromodichloromethane and dibromochloromethane),
2. HAAs – halo acetic acids, including iodoacids (iodoacetic acid, bromoiodoacetic acid, chloroacetic acid, bromoacetic acid, dichloroacetic acid and trichloroacetic acid).

3. Other halo acids (bromochloroacetic acid, bromodichloroacetic acid), dibromochloroacetic acid and tribromoacetic acid).
4. Iodo-THMs (dichloroiodomethane, bromochloromethane)
5. Oxyhalides (bromate and chlorate).
6. MX Compounds (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone) and EMX compounds (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid).
7. Haloamides (iodoacetamide, bromoacetamide and chloroacetamide).
8. Haloacetnitriles (iodoacetnitrile, bromoacetnitrile and chloroacetnitrile).
9. Halopyroles, Nitrosamines and Aldehydes.

Although it has been possible to identify a large number of DBPs, only a few of these DBPs have been analysed for their cytotoxic and genotoxic potential and impact on health issues (Cemeli et al., 2006; Krasner et al., 2006; Muellner et al., 2007; Plewa et al., 2002; Plewa et al., 2008; Plewa et al., 2010; Plewa and Wagner, 2011; Plewa et al., 2004). As far as the regulations of DBPs are concerned, only a small number with genotoxic and cytotoxic potentials are regulated, however many DBPs with similar consequences remain unregulated (Plewa et al., 2010; Richardson et al., 2007). As with all common DBPs, HAAs can form from usage of chlorine, chloramine, chlorine dioxide and ozone treatment; however, amongst them chlorination use produces higher levels of HAAs (Krasner et al., 2006; Richardson et al., 2007).

1.3.1 Halo acetic acids (HAAs)

Several different classes of DBPs are found in finished drinking water as mentioned above but two of the well-known classes are the haloacetic acids (HAAs) (Bougeard et al., 2010; Cemeli et al., 2006; Plewa et al., 2002; Plewa et al., 2010) and the trihalomethanes (THMs) (Kogevinas et al., 2010; Liviac et al., 2010).

The US Environmental protection agency (EPA) regulates five out of nine HAAs to a maximum containment level of 60 μg/l. These are known as the HAA5 group consisting of; bromoacetic acid (BAA), dibromoacetic acid (DBAA), chloroacetic acid (CAA), dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA). The other four compounds include four further chloro-bromo-HAAs which form part of a larger HAA9 group (Plewa et al., 2010). In the UK, THMs are the only class of DBPs which are subject to regulation, regulated to levels <100 μg/L. Although HAAs are equally prevalent as THMs, they are not subject to regulation but the EU is considering starting regulating the HAA9 group to a maximum level of 80 μg/L, and there is further interest into HAAs calling for regulation (Bougeard et al., 2010).

Drinking water subject to chlorination contains HAAs at low to mid-range concentrations (μg/l), similar to regulated levels of THMs. Further to this, the total concentration of the entire HAA9 group is also comparable to that total concentration of the THM group (known as the THM4) (Krasner et al., 2006).

Drinking water typically contains chloro-HAAs, such as DCAA and TCAA, but if the source waters particularly contain high levels of bromine > 50 μg/L, then bromo-HAAs, such as the DBAA and BCAA are the sub types that dominate. However, other factors like residence time, pH, and temperature and disinfection procedure can also impact the types and compositions of HAAs (Cemeli et al., 2006; Plewa et al., 2010). Bougeard *et al.,* (2010) have also reported the shift to bromo-HAAs in source waters containing higher levels of bromine in the United Kingdom. Bromo-HAAs are more likely to be found in

source waters from coastal locations, due to saltwater intrusion (Cemeli et al., 2006; Nieuwenhuijsen et al., 2000).

HAAs, including iodoacetic acid (IAA), bromoacetic acid (BAA), and chloroacetic acid (CAA) have previously been reported to be genotoxic and cytotoxic in various studies, (Plewa et al., 2004; Richardson et al., 2007). These HAAs have been reported to be carcinogenic and toxic in experimental animals but there is no such evidence of similar effects in humans; however, many *in vitro* testings have demonstrated HAA's cytotoxic and genotoxic potential (DeAngelo et al., 1996; Richard and Hunter, 1996).

1.3.2 Chemical reaction and Structure of Halo acetic acids (HAAs)

HAAs are carboxylic acids where the hydrogen atoms from the alpha-carbon chain are replaced by one or more of halogen atoms. These electronegative halogens enhance and help stabilise the negative charge of the conjugate base, resulting in higher levels of acidity in these compounds (Plewa et al., 2010).

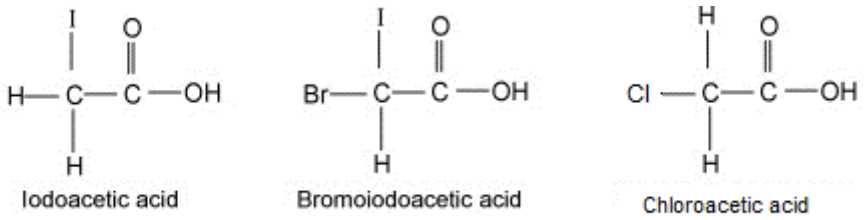


Figure 1: Structure of haloacetic acids: Figure shows the chemical structure of halo acetic acids, the halogen atoms replace the hydrogen atoms on the alpha carbon chain.

1.4 Health related issues of DBPs

DBPs are classed as environmentally hazardous chemicals which can have many health related implications in humans (Cemeli et al., 2006). Increased amounts of THMs and HAAs uptake have been detected in people through activities such as tap water ingestion, showering, bathing and swimming (Kogevinas et al., 2010; Nieuwenhuijsen et al., 2000). This is supported by several epidemiological studies that show a correlation between consumption of disinfected water with increased risk of bladder, kidney and brain cancers (Cantor et al., 1999; Koivusalo et al., 1994) and consumption of drinking tap water by pregnant women was found to be associated with early miscarriages and spontaneous abortion (Swan et al., 1998; Waller et al., 1998). However Arbuckle et al. (2002) have shown that epidemiological studies may pose shortcomings, for example the characterisation of DBPs exposures in all studies is not necessarily the same and other factors like, surface water, ground water, whether the water was chloraminated or chlorinated are different. All these factors can lead to inconsistencies; however, it was established that there was a greater need to keep these factors as similar as possible.

It has previously been demonstrated that the HAAs, - iodoacetic acid (IAA), bromoacetic acid (BAA) and chloroacetic acid (CAA) are cytotoxic and genotoxic DBPs when analysed in the Ames test, and in the mammalian CHO cell system (Plewa et al., 2002; Plewa et al., 2010; Richardson et al., 2008). Cemeli et al. (2006) have also shown the involvement of oxygen radicals with IAA in these cells. In addition, HAAs have also been found to be cytotoxic in

human lymphocytes but not genotoxic in the Micronucleus assay in TK6 cells (Liviac et al., 2010). IAAs have also been reported to induce chromosomal aberrations in CHO cells (Hilliard et al., 1998). Plewa et al. (2004) have shown responses amongst the HAAs for IAA, BAA and CAA when analysing HAA-induced genomic DNA damage in CHO cells. Their studies have shown that bromo-HAAs [brominated HAA] were more cytotoxic and genotoxic than chloro-HAAs [chlorinated HAA] analogues and that iodo-HAAs [iodinated HAA] analogues were more cytotoxic and genotoxic when compared to both bromo-HAAs and chloro-HAAs (Plewa et al., 2002; Plewa et al., 2004). These HAAs were toxic in the same order, following 24 hour exposure to mice (iodo-HAAs > bromo-HAAs > chloro-HAAs) for the induction of neural tube defects in mouse embryos. Exposure also produced malpositioned and/or hypoplastic pharyngeal arches and affected the heart and optic development (Hunter et al., 1996).

Toxicogenomic implications of HAAs were shown in studies in which mice were exposed to chloro and bromo-chloro-HAAs. Altered gene expression was found in pathways involving cell communication, cell cycle and proliferation, metabolism, signal transduction, spermatogenesis and male fertility in mice exposed to dichloroacetic acid (DCAA) (Thai et al., 2003). Mice that were subject to bromo-chloro acetic acid (BCAA) exposure, exhibited cellular damage and altered gene expression in different pathways involving fatty acid metabolism, angiogenesis, and tissue remodelling (Tully et al., 2005). Another study on HAA toxicogenomic showed that BAA was able to alter transcriptome profiles for genes involved in DNA repair, including the cell cycle regulation of double stranded DNA strand breaks (dd-DSBs) (Muellner et al., 2010). In non-

transformed human cells toxicogenomic analyses of the HAAs, the major pathways involved with altered gene expression were ATM, MAPK, p53, BRCA1, BRCA2, and ATR. These latter pathways highlight the involvement of DNA repair, especially the repair of double strand DNA breaks (Attene-Ramos et al., 2010).

In human lymphocytes, Kogevinas et al. (2010) reported damage with THMs in lymphocytes using the micronucleus assay, but not in the Comet assay. These studies involving HAAs in both *in vitro* and *in vivo* experiments, demonstrate the need for further studies to address and raise the importance of health implications posed by HAAs. The present study further determines toxic effects in *in vitro* of HAAs in human somatic cells and for the first time also in germ cells. Samples were taken from participating consented volunteers. The Comet assay and the micronucleus assay were used to detect any genotoxic damage in these cells.

1.5 Antioxidants: Catalase and butylated hydroxyanisole.

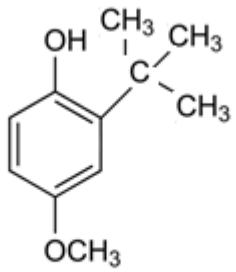
1.5.1 Uses and functions of catalase and butylated hydroxyanisole

Catalase, (also known as hydroperoxidases) is a common enzyme involved in the reactive oxygen species detoxification pathways. The enzyme has been a subject of study in a vast number of different organisms. The ubiquity of the enzyme, have made it an attractive protein to work with. It is used in foods and prevents oxidation of foods via its use in food packaging. Oxygen radical break down is split into two reactions: Metabolic reactions generate O• radicals which

are converted to hydrogen peroxide (H_2O_2) by super oxide dismutase. Catalase then works by catalysing the decomposition of toxic H_2O_2 to H_2O & O_2 (Chelikani et al., 2004).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Butylated hydroxyanisole (BHA) is a synthetic antioxidant. It consists of a mixture of two compounds, 3-tert-butyl-4-hydroxyanisole (3-BHA) and 2-tert-butyl-4-hydroxyanisole (2-BHA). BHA plays its role as a free radical scavenger. It contains a conjugated aromatic ring in its structure, which sequesters free radicals in order to stabilise them (See Figure 2). It has primarily been used as an antioxidant to preserve the freshness of food, food colouring and maintain flavour. BHA used in foods consists of 85% of 3-BHA and 15% 2-BHA (Williams *et al.,* 1999). BHA does not produce DNA reactivity in assays for DNA adduct detection and most genotoxic studies have shown negative responses with BHA induction, and BHA used at current food additive levels does not represent any cancer risk, as reviewed by Williams et al. (1999). Moreover, it has previously been suggested to reduce cancer and provide additional benefits (Williams et al., 1999).



butylated hydroxyanisole (3-BHA structure)

Figure 2: Structure of butylated hydroxyanisole (BHA): Figure shows the chemical structure of 3-tert-butyl-4-hydroxyanisole (3-BHA). The 2-tert-butyl-4-hydroxyanisole (2-BHA) simply has the $C(CH_3)_3$ group located on the third carbon of the aromatic ring (closer to the OCH_3 structure).

1.5.2 Catalase and butylated hydroxyanisole impact on HAAs

Cemeli et al. (2006) have shown that catalase and butylated hydroxyanisole were able to reduce IAA induced mutagenicity of *S. typhimurium* and IAA induced genotoxicity in CHO cells. It was reported that this reduction was significantly different (*** p = < 0.001), when compared to the IAA positive control. Catalase played a protective role in reducing mutagenicity in *S. typhimurium* by 26.8% and genotoxicity in CHO cells by 42.5%. This suggests that IAA-induced genomic DNA damage must have involved an oxidative stress mechanism, via increase in hydrogen peroxide as catalase was able to reduce this damage.

Butylated hydroxyanisole was found to be highly effective in comparison to catalase. It was able to act as an effective modulator of IAA induced DNA damage; there was 33.5% reduction of mutagenicity in *S. typhimurium* and 86.5% reduction of genotoxicity in CHO cells. Furthermore, Plewa et al. (2004), demonstrated the ranking order of monohalogenated acetic acids. It was reported that IAA was 2.6 × and 523.3 × more mutagenic in *S.*

typhimurium TA100 than BAA and CAA, respectively. IAA was found to be 2.0 × and 47.2 × more genotoxic than BAA and CAA, respectively in CHO cells. In Cemeli et al. (2006), their findings warrant the possibility of analysing similar conditions in lymphocytes and sperm to determine oxygen radical involvement by observing BHA levels via single cell-gel electrophoresis (known as the Comet assay) under alkaline conditions > pH 13. Following genotoxic analysis in the Comet assay, the cytotoxicity and genotoxicity of lymphocyte cells will be examined in the micronucleus assay.

1.6 Introduction to nanoparticles

1.6.1 Background to Nanomaterials

Nanomaterials, including nanoparticles have become an important part of genetic toxicology studies. Owing to their vast compound properties, they have become increasingly popular in recent years for commercial and medical products (Osman et al., 2010). Several consumer products are currently using NP, such as additives to cosmetics, surface-coatings of clothes, food colourants, substrates in electronics and many more. As such new products are regularly being launched on a daily basis. In genetic toxicology and most medical studies for that matter, they have an important role in oncology, molecular diagnostics, cardiovascular medicine and the discovery and delivery of drugs (Haase et al., 2012; Kubik et al., 2005; LaVan et al., 2003).

1.6.2 Uses of Nanoparticles in Industry / Types of Nano compounds

Nano compounds or particles are classified into two different groups; natural and anthropogenic nanoparticles (Dhawan and Sharma, 2010). Natural nanoparticles are those which have always existed in the environment, long before nanotechnology development work had started. Examples of some of these compounds include airborne nanocrystals of sea salts, fullerenes and soil (consisting of inorganic and organic nanoparticles like metal oxides, hydroxides and clay materials) (Nowack and Bucheli, 2007). Natural vegetation also contains organic nano compounds (Xia et al., 2010).

The anthropogenic class of nanoparticles are subdivided into further two classes: incidental and engineered. Incidental nanoparticles are those consisting of nanoparticles that have been produced unintentionally during manmade processes. Examples include: carbon nanotubes, carbon black, platinum and rhodium-containing nanoparticles from combustion by-products (Nowack and Bucheli, 2007). Engineered/manufactured nanoparticles are those produced intentionally due their desirable nano-specific characteristics and /or properties – examples of which are mentioned below.

Nanoparticles are found in variety of everyday consumer products, like tennis rackets and baseball bats to improve their strength and reduce weight. Others include cosmetics and sunscreens where the use of nanoparticles allows for greater transparency and efficacy. Nanomaterials have also found use within the textile industry where they have been deployed to produce clothes that are stain and wrinkle-free and water resistant (Thomas et al., 2006). The food

industry also makes use of nanoparticles such as silver and zinc oxide (ZnO) for food packaging (Bouwmeester et al., 2009). Engineered nanoparticles have been extensively investigated in the Comet assay and the micronucleus assay both *in-vivo* and *in-vitro* (Magdolenova et al., 2014).

1.6.3 Properties of nanoparticles

Nanoparticles have become popular because of their certain properties. The key feature being the difference in size of nano-compounds compared to their bulk equivalents. Nanocompounds are defined as those compounds or materials, whose size is below 100 nm, however the pharmaceutical industry considers sizes up to 1000 nm to be classified as nano compounds (Carlin, 2014; Teeguarden et al., 2007). As a consequence, their smaller size provides for a large surface area to volume ratio and together with their physiochemical properties, these particles are capable of easily passing through cell membranes (Figure 3). This has toxicological consequences, since the behaviour of compounds is unpredictable and a major cause of concern is the ability of these compounds to become lodged in the mitochondria (Foley et al., 2002), whilst some engineered nano compounds can be redox active (Carlin, 2014; Colvin, 2003). Thus, increasing usage and applications of nano compounds / materials in a wide range of fields can cause an increase of nano exposure to humans, which in turn may directly increase toxicological effects (Osman et al., 2010).

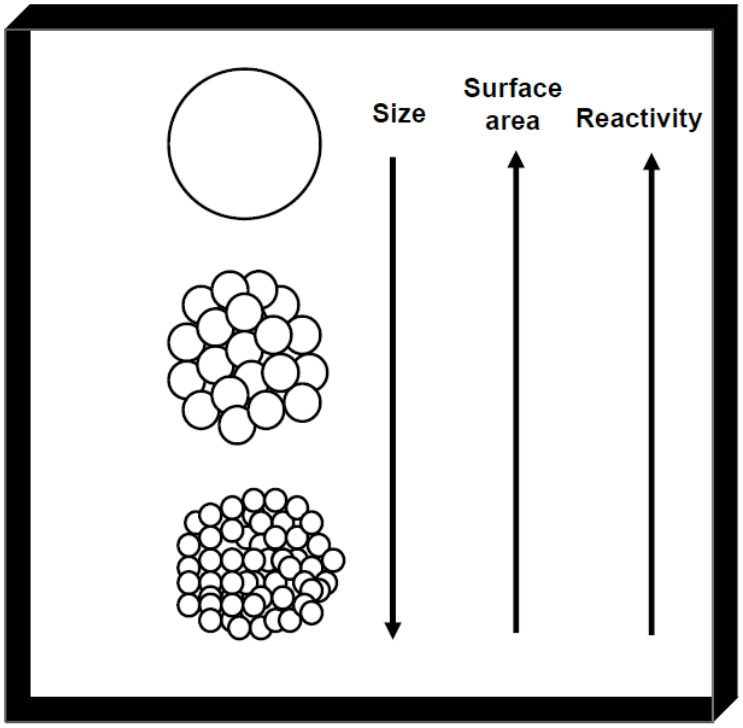


Figure 3: Surface reactivity of nanoparticles: Paradigm above, shows the size-surface area-reactivity for nanoparticles, with the downward and upward arrows representing decrease and increase of certain properties of nanoparticles, respectively as shown by Dhawan et al. (2009b) Generally, a reduction in size increases surface area and therefore leads to increased reactivity.

As has been discovered over the last decade, alongside the advantages of nanoparticles, these compounds also have disadvantages which have been documented in past studies (Goldston, 2007; Igarashi, 2008). Furthermore, increasing production and usage of nanoparticles have led to increased concerns and debates surrounding their implications in biological systems by public, scientific and regulatory authorities (Dhawan and Sharma, 2010). Investigating the challenges and issues presented by nanomaterials in biological systems has given rise to the term ‘nanotoxicology’ (Dhawan et al., 2009).

So far nanotoxicology has lagged behind nanotechnology due to a number of experimental challenges found in designing toxicological assessments of nanomaterials (Dhawan et al., 2009a; Dhawan and Sharma, 2010). Some of the most important challenges and issues such as the characterisation, agglomeration, surface coating, and dose-response and exposure assessment of nanoparticles are mentioned below.

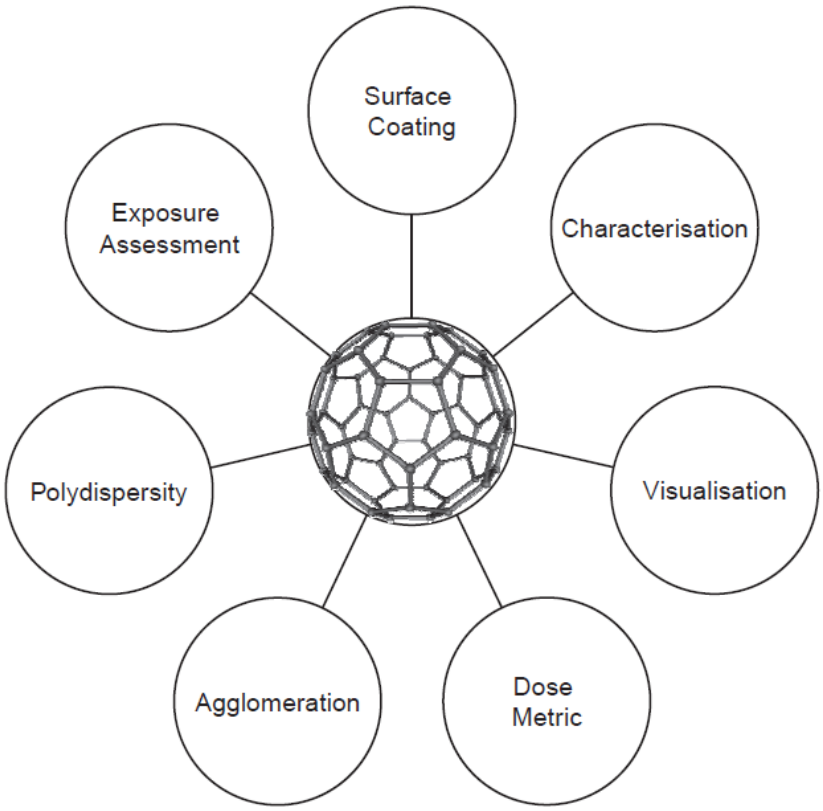


Figure 4: Factors to consider for nanoparticle synthesis: The diagram above highlights the key issues and challenges to address when designing new nanoparticles (Dhawan et al., 2009b).

1.6.3.1 Characterisation

Characterisation is the first step for nanomaterial preparation. A small amount of the test substance or sample of the nano material is characterised before

any toxicity screening is commenced. The process of characterising nanoparticles can be complex compared to their bulky chemical equivalents with known chemical compositions and purities because the exact properties of nanoparticles are poorly understood. More so, the reasons for nanoparticle toxicity are unknown and understood to a much lesser extent (Dhawan and Sharma, 2010). Thus, as part of an extensive characterisation, it is important to determine the shape, size, size distribution, surface area and chemistry, agglomeration state, and solubility etc. Determining these factors is imperative because it allows us to obtain the correct correlation between the physiochemical properties of the nanoparticles and the biological effects they elicit. A complete characterisation would also ensure reproducibility of experimental works as well as reliability i.e. working with ibuprofen nano particles, to check and characterise on a regular basis for consistency amongst data (Dhawan and Sharma, 2010; Warheit et al., 2009).

Commercially available nanoparticles can be purchased and used and researchers are also able to characterise nanoparticles for themselves. However, the process used by them can differ from the commercially produced ones, since researchers are more likely to characterise nanoparticles based on facilities available to them. The lack of comprehensive equipment, time and manual labour means that for researchers, the facilities they have are the deciding factor in the type and methods of characterisation performed (Warheit et al., 2009).

1.6.3.2 Agglomeration

Agglomeration is an important factor to consider when working with nanoparticles, Nanoparticles can agglomerate in both the dry form or in the wet/liquid form. Nanoparticles tend to agglomerate due to Van der Waal's forces, which have a greater influence due to the large surface area to volume ratio of nanoparticles (Dhawan et al., 2009b; Powers et al., 2007). Agglomeration also starts as soon as nanoparticles are produced, and when introduced in liquids, the agglomeration of particles is even greater (See Figure 5) (Murdock et al., 2008).

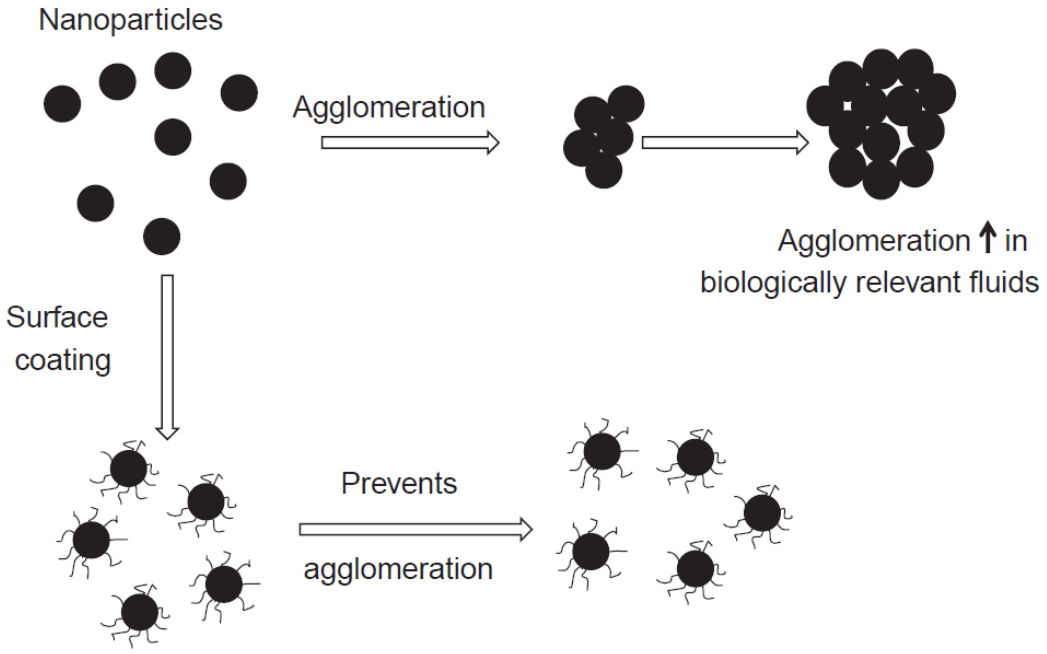


Figure 5: Agglomeration of nanoparticles: Diagram shows the process of agglomeration as shown by Dhawan et al. (2009b) and adapted from Murdock et al. (2008). Agglomeration can result in clumping of nano particles, however surface coating (as mentioned in the next section) of the nanoparticles can prevent particles from adhering to each other.

Nanoparticles agglomerate in Brownian motion. Particles move in random directions, colliding with each other and adhere together, increasing

successively, before settling downwards as larger clumps of particles due to gravitational forces. Consequently, the number and concentration of nanoparticles decreases, surface area decreases while the mass remains conserved, and these clumps diffuse much slower than freely moving single nanoparticles nearby. These parameters increase gravitational and buoyant forces, leading to rapid settlement in liquids (Dhawan et al., 2009b). Hence, it is important to consider size, surface area, and concentration of particles when addressing issues of agglomeration as they influence the toxicity of particles in solutions.

However, there are other additional factors that can also influence agglomeration. These are; the zeta potential, fluid characteristics, sample preparation methods, sonication timings and concentration of nanoparticles (high concentrations rapidly increase agglomeration due to greater particle interactions) (Teeguarden et al., 2007). Murdock et al. (2008) have studied nanoparticle agglomeration by examining different sizes of copper nanoparticles in deionised water, 1640- RPMI cell culture medium with and without serum and found that presences of serum in the RPMI-1640 culture medium decreases agglomeration. The proteins released from the serum reduce particle to particle interactions by coating over them.

Since agglomeration can greatly increase the overall size of particles (or the forming clump of particles), it is important to ensure that samples are regularly checked for their zeta potential and size to ensure they do not exceed beyond the nano meter (nm) range (1 nm – 1000 nm), since proceeding with samples above nm, work is performed on samples in the millimetre (mm) range, which

invalidates the purpose of studying for toxicity of nanoparticles (Balbus et al., 2007).

1.6.3.3 Surface coating

As mentioned previously, addressing nanoparticle agglomeration is a major challenge when designing and synthesising such particles. Surface coating is a key process by which this issue can be addressed. It involves coating by homogenously dispersing the particles in solutions prior to usage. Sometimes surface coating can also be used to reduce the surface reactivity's of nanoparticles and not just be used for agglomeration. For example zinc oxide nanoparticles constituting sunscreens, can be coated, to preventing oxygen radicals forming, when exposed to UV light absorption. Nanoparticles can be coated with polymers (such as 4-mercaptomethylstyrene coating of metal nanoparticles), alveolar surfactants in ionised or deionised solutions (Dhawan et al., 2009b; Farah et al., 2008).

Furthermore, dispersion capabilities of several medium were studied by Sager et al. (2007), who found that bronchoalveolar lavage fluid, was the best solution in which to suspend nanoparticles without affecting their toxic or inflammatory potential, when compared to other mediums such as phosphate buffer solution (PBS) or PBS containing mouse serum albumin. A study by Skebo et al. (2007) has shown that by adding 0.1 % sodium dodecyl sulphate (SDS) to silver nanoparticles, agglomeration can be reduced, with increased uptake of particles in cells. The addition of SDS also required washing of particles twice in ultrapure water in order to prevent SDS induced toxicity on the cell viability prior to introduction into cell cultures.

Surface coating, however does come with its own problems. Modifying nanoparticle surfaces may increase or decrease their effect in biological systems and the durability and stability of coating is another issue to address (Dhawan et al., 2009b).

1.6.3.4 Dose response and Exposure assessment

Dose-response relationships are an imperative aspect of all toxicological experiments. Dose-response testing, allows for the correct dosage to be established and used in toxicological studies. The dose metric is the critical factor that determines this dose-response relationship. This is usually based on exposure concentration of the chemical being investigated, whilst in nanoparticles finding the right dose-metric has been an issue of debate. This is because the relationships of nanoparticles and their physiochemical properties are not understood. Some studies, however, have considered the surface area to be the best parameter for assessing cellular response to exposure to nanoparticles, due to the reduction in size of particles resulting in an increased surface area, leading to increased reactivity. Thus the surface area is used as the dose metric choice for studies (Nel et al., 2006).

The safety and toxicity assessment of a nanoparticle is another challenging factor. The biological effects of a nanoparticle are more complex as this can differ based on the chemical composition, size, shape and structure of surface. It is also difficult to track nanoparticles within the body in *in vivo* experiments, although radiolabelling and fluorescent labelling can aid this to some degree (Dhawan and Sharma, 2010; Dhawan et al., 2009b).

All these factors amongst others are important to consider when working with nanoparticles. The nascent nature of nanoparticles, particularly rising over the last decade, has given rise to many uses of nano particles in everyday life. For toxicology, it has opened up a whole new means through which to test genotoxicity of compounds as part of finding new ways to help diagnose patients with disease and cancer. The potential of nanoparticle use has been demonstrated in this project. The studies where nanoparticle are concerned, have been conducted taking some of the above factors into account, such as sonication of nanoparticles before use and regular checking for zeta potential and size of nano particles.

1.7 Non-steroidal anti-inflammatory drugs (NSAIDs)

1.7.1 History of NSAIDs

Non-steroidal anti-inflammatory drugs, abbreviated as NSAIDs, are important drugs which were first introduced, starting with salicylic acid around 1860 as an anti-septic drug and as aspirin towards the end of the 19th century. NSAIDs have now become the most widely used and prescribed drugs, despite problems of patient tolerability and gastrointestinal toxicity (Donnelly and Hawkey, 1997). Aspirin and sodium salicylate were recognised in the early 1900s, as drugs that were anti-inflammatory, antipyretic and analgesic. Following their discovery, several drugs with similar functions such as acetaminophen (paracetamol) was discovered. These were later followed by recent discoveries like fenamates, ibuprofen and naproxen. These drugs have different structures, however share the same therapeutic properties. These drugs were also different from the glucocorticosterioids group of inflammation

treating drugs and they eventually became known as NSAIDs (Vane Dsc and Botting PhD, 1998). Today, most common drugs sold or prescribed are: diclofenac, ibuprofen, naproxen, celecoxib, mefenamic acid, etoricoxib, indomethacin and aspirin (Day and Graham, 2013; Peterson et al., 2010).

1.7.2 Impact of NSAIDs on health

NSAIDs help to reduce pain, reduce inflammation (swelling and redness), reduce body temperature and have antithrombotic effects. The drugs are commonly used to treat acute short term conditions of, headaches, back pain, toothaches, infections, common colds (in particular – symptoms of colds) and help treat soft tissue injuries such as sprains and strains. Chronic long term conditions like arthritis back and neck pain and minor ailments, can also be treated with these drugs (Peterson et al., 2010). However alongside the benefits, are the disadvantages of NSAIDs. NSAIDs can have adverse effects, including gastrointestinal bleeding (Traversa et al., 1995), hypertension, oedema (Johnson et al., 1994) and renal disease and more recently, including association to myocardial infarctions (Peterson et al., 2010).

1.7.3 Role of NSAIDs in Prostaglandin synthesis

These drugs act by inhibiting the enzymes cyclo-oxygenase-1 and cyclo-oxygenase-2 (COX-1 and COX-2) found in the joints and muscles of the body, which contribute to pain and inflammation of the body. COX-1 is constitutively expressed at high levels in cells and tissues such as platelets, monocytes, the endothelium, renal collecting tubules and seminal vesicles (Smith and Dewitt, 1996). In contrast, COX-2 is induced by inflammatory stimuli and cytokines

liposaccharides, interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) in migratory cells, like the endothelium, monocytes, macrophages and osteoclasts. Both, COX-1 & COX-2, are involved in prostaglandin synthesis (Day and Graham, 2013; Rao and Knaus, 2008).

There are two kinds of NSAIDs – non-selective NSAIDs, act by blocking both COX-1 & COX-2, causing a reduction in the production of prostaglandins. Another class of NSAIDs are known as the 'selective COX-2 inhibitors', which block the COX-2 enzymes only. The COX-2 inhibitors are known to be safer and effective than their non-selective equivalents (Donnelly and Hawkey, 1997). Blocking of COX-1 enzyme can cause bleeding, as COX-1 has a protective role at the lining of the stomach, which protects it from acids. Hence, NSAIDs, with selective COX-2 inhibition have the benefit of reducing pain and inflammation, but leave the stomach lining unaffected, by not blocking the COX-1 enzyme (Peterson et al., 2010).

Various NSAID drugs differ in their selectivity for COX-2, in terms of how much they affect the COX-2 enzyme, relative to the COX-1 enzyme (Peterson et al., 2010). NSAIDs such as aspirin, indomethacin and ibuprofen are non-selective inhibitors of COX enzymes, whereas celecoxib and rofecoxib are inhibitors which have a greater selection for COX-2 inhibition (Rao and Knaus, 2008).

COX-1 and COX-2 play a role in prostaglandin synthesis. Arachidonic acid (AA) is a precursor of prostaglandin synthesis. AA is an unsaturated 20-carbon fatty acid embedded in the cell membranes as a phospholipid ester, as shown in Figure 6. AA is released in response to stimuli, which is subsequently converted via the enzymes, lipoxygenase (LOX), COX and cytochrome P450

into lipid mediators called eicosanoids. The production of eicosanoids is dependent on the amount of freely available AA (Rao and Knaus, 2008; Smith et al., 2000).

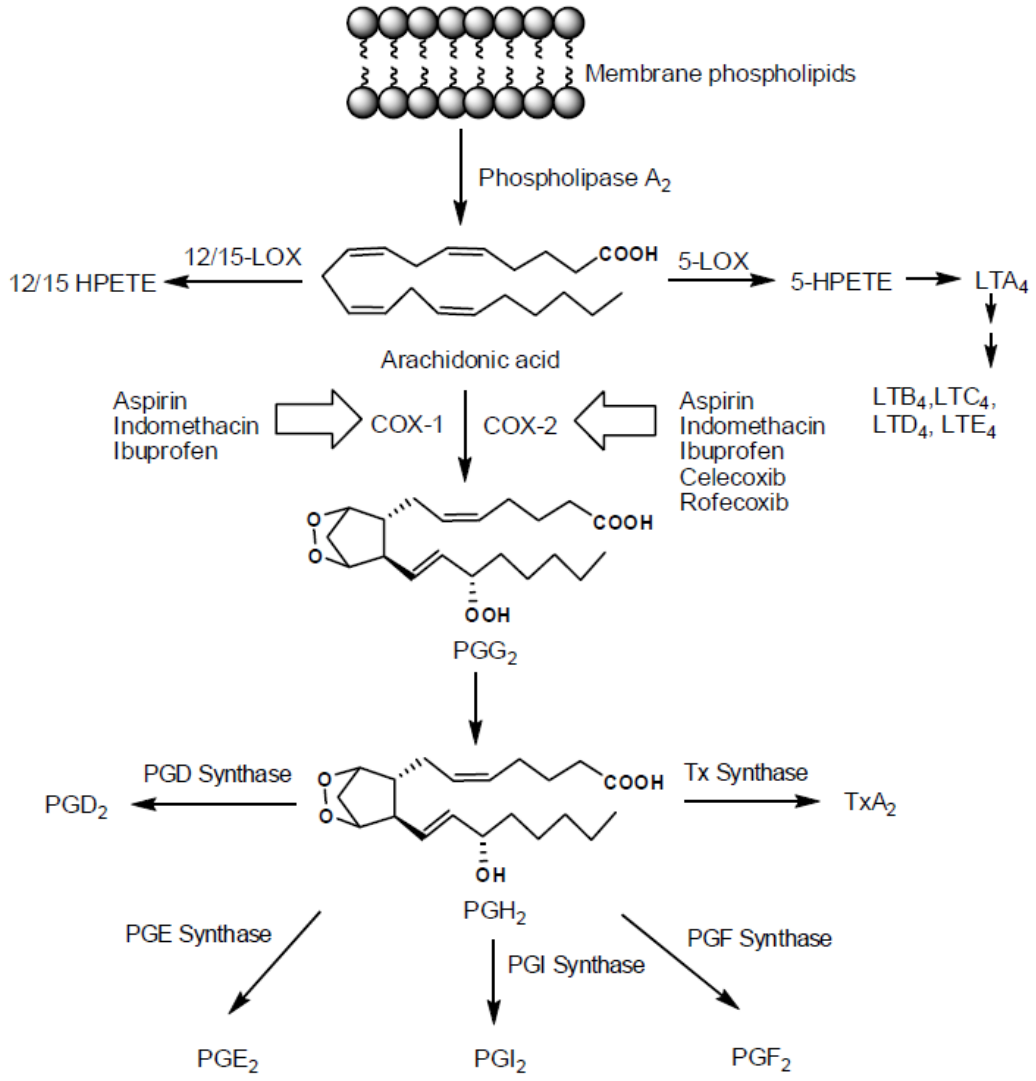


Figure 6: Role of cyclooxygenase enzymes in prostaglandin synthesis. Diagram above shows the pathway of prostaglandin biosynthesis from arachidonic acid and the role of COX-1 & COX-2 and prostaglandin synthase enzyme in the production of the final primary prostaglandins (Rao and Knaus, 2008).

The COX-1 and COX-2 enzymes catalyse two reactions as shown in Figure 6, above. In the first reaction, COX-1 & COX-2 convert AA to prostaglandin G2 (known as PGG2), a subsequent peroxidase reaction results in the reduction

of PGG2s to prostaglandin H2 (known as PGH2). Enzymes like synthases and isomerases then convert PGH2s into five primary and biologically active prostaglandins. These are prostaglandin D2 (known as PGD2), prostaglandin E2 (known as PGE2), prostaglandin F2α (known as PGF2α), prostacyclin (PGI2) and thromboxane A2 (known as TxA2) (Figure 6) (FitzGerald and Patrono, 2001; Rao and Knaus, 2008; Smith et al., 2000). The prostaglandins play a role in promoting tumour growth via increased cell proliferation and causing stimulation of angiogenesis (Skriver et al., 2005).

1.7.4 NSAIDs in Cancer and Respiratory disease.

The implications of NSAIDs in cancer have been well documented by various studies. In general, several studies have shown that NSAIDs exhibit anti-cancer activities on several hallmarks of cancer, such as, cell proliferation, evasion of apoptosis and cell cycle regulation (Burn et al., 2011; Park et al., 2014). The majority of studies on NSAIDs have been conducted on gastrointestinal cancer, such as colorectal, gastric and in the oesophagus, etc. NSAIDs have been investigated in much detail, such as a study, that has examined the potential role aspirin may play in improving survival and reducing recurrence in patients with a potentially curative colorectal cancer, with a particular focus on their effects at the local and systemic inflammatory responses (Mansouri et al., 2013).

Furthermore, some studies have shown, greater evidence of aspirin and other NSAIDs like sulindac and indomethacin all exhibiting a chemo preventative effect on colorectal cancer (Coghill et al., 2011; Cuzick et al., 2009; Waddell

and Loughry, 1983). Similar protective anti-cancer effects of NSAIDs have been found in prostate cancer (Kawahara et al., 2010), oesophageal cancer (Kang et al., 2013), and breast cancer (Brasky et al., 2011; Retsky et al., 2012). However, the association between renal cell carcinoma and NSAIDs was found to be inconsistent (Liu et al., 2013).

Since the discovery of COX-2 enzyme, many studies have shown that this isoform is highly expressed in cancers of the breast, pancreas and the prostate. Such expression has been associated with controlling many cellular processes in these cancers. As such, NSAIDs with inhibition for COX-2 have been well investigated in studies aimed at treatment and prevention of cancers (Kanaoka et al., 2007; Penning et al., 1997; Thill et al., 2014). For example, celecoxib – a selective COX-2 inhibitor induces apoptosis in human prostate cancer cell lines that are expressing COX-2, blocking the activation of anti-apoptotic kinase (Hsu et al., 2000; Liao et al., 2007).

This project focuses on respiratory diseases (asthma and COPD) and lung cancer and several studies have shown the implications of NSAIDs in patients from these groups. Harris et al. (2002), ran a case control study of NSAIDs among 489 lung cancer patients and 978 control subjects. Cases in the study were matched for age, gender, pack-years of cigarette smoking. In order to assess the effects of NSAIDs on tobacco carcinogenesis, only heavy smokers were included in the control group. Findings from the study indicated that daily intake of NSAIDs for at least 2 years prior to interview was associated with a 68% reduction in the relative risk of lung cancer, with a significance of $p = < 0.01$. Considering these results with molecular evidence suggested that regular NSAID intake may prevent carcinogenesis through COX-2 enzyme

blockade (Harris et al., 2002). The vast catalogue of studies appear to show protection of NSAIDs usage in colorectal and stomach cancers, however in people with respiratory diseases of asthma and COPD, the protective role of NSAIDs is unclear, with conflicting data, although many population studies indicate a relatively low risk of NSAIDs intake and incidence of these health conditions. The increasing developments of the nano industry, and therefore nano toxicology, has allowed for the testing of nanoparticles. Two of these nanoparticles are aspirin and Ibuprofen, which are discussed below as a summary and their capabilities as nanoparticles.

1.7.4 Aspirin

As mentioned above, aspirin, also known as acetylsalicylic acid, is non-steroidal anti-inflammatory drug (NSAID) that is used to relieve fevers, headaches, pain, and inhibits synthesis of prostaglandins, via the COX enzyme pathways. Various studies have reported reduction of cancer (including growth and metastasis) and mortality in association with aspirin intake, adding to the case for aspirin in prevention of cancer (Rothwell et al., 2012a; Rothwell et al., 2012b). Side effects of aspirin include, bleeding of the stomach lining, ringing in the ears of patients. In children and adolescents, aspirin is not recommended due to its association with Reye's syndrome – a condition that affects all organs of the body, however mostly the brain and the liver with raised intracranial pressure or liver failure (Macdonald, 2002). Aspirin is listed as one of the most essential medicines needed in a basic healthcare system, according to a report by WHO (World Health Organization., 2014).

1.7.5 Ibuprofen

Ibuprofen is the other NSAID that is the focus of this study. Like aspirin, ibuprofen is used to reduce fevers and pain and reduce inflammation, via its function as a cyclooxygenase inhibitor. Reduced cytokine production, modulation of leucocyte activity, inhibition of free radicals and signalling transduction as some of the anti-inflammatory properties of ibuprofen (Rainsford, 2003). Ibuprofen is a derivative of 2-(4-isobutylphenyl) propionic acid, which was first discovered over 50 years ago, as part of research into finding treatment for rheumatoid arthritis (Halford et al., 2012). Initially sold as a prescription medicine, further studies and better understanding of drug lead to it becoming the first licensed NSAID drug sold over the counter in the UK (Halford et al., 2012; Rainsford, 2003). Lung cancer is the leading cause of cancer in both males and females, accounting for 30% of all cancer-related deaths in the US (Jemal et al., 2010) and is much the same for Europe (Tyczynski et al., 2003). Epidemiological studies have shown that daily administration of ibuprofen reduces the risk of cancer (Harris et al., 2007) and in comparison to other NSAIDs, the side effects (like gastrointestinal, stomach ulcers, myocardial infarction) are significantly less (Cheng et al., 2014). Ibuprofen has also been found to have chemo protective effects of cancer and also helps in treating Alzheimer's disease. (Rainsford, 2003).

1.7.5 Aspirin and Ibuprofen nanoparticles and nano-carriers

The advantages of nanoparticles have been discussed above in a general context. Both aspirin and ibuprofen nanoparticles are of great interest to scientists, since the advantages of nanoparticles combined with the benefits

of NSAIDs have opened the door to whole new phenomena of research. Nanoparticles possess the unique capability to accumulate at the site of inflammation, and thus are suitable for targeted drug delivery (Elzoghby et al., 2012). Aspirin and ibuprofen, like many other nanoparticles, are delivered to their targeted site in the body via several nano-carriers. Polymeric nanoparticles, magnetic nanoparticles, ceramic nanoparticles, polymer drug conjugates, nanotubes and nanowires, to name some of these are being analysed for their capability to act as suitable drug carriers for nanoparticles (Elzoghby et al., 2012). Even protein such as albumin, collagen and whey protein have been studied for drugs and nutrient deliveries (Bryant and McClements, 1998; Elzoghby et al., 2011; Lu and Chen, 2004). Advantages of such systems are that they protect drugs from degradation and enhance drug absorption by facilitating diffusion through cell epithelium. They also allow modifications of pharmacokinetic properties and drug tissue profiles, and penetration of and better distribution of drugs. These systems can also withstand physiological stress or improved biological stability and alter the release pattern of the drug (Elzoghby et al., 2011). With these benefits in mind, this project examines how effective nanoparticles are compared to their bulk equivalents when introduced into cells. To develop nanoparticle solutions, suspension solutions of aspirin and ibuprofen are prepared with solid loads of 4% and 3% (w/w), respectively in deionised water as done in a method described by (Plakkot et al., 2011). The suspension solution of deionised water also contains hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone K-30 and sodium lauryl sulphate (Plakkot et al., 2011). This is followed by

process in which milling of these compounds are carried out using Lena Nanoceutics Technology DM-100 machine (Sulaiman 2007).

1.8 Genotoxicity Assays

1.8.1 Comet assay

1.8.1.1 Development and principles of Comet assay

The Comet assay, also known as the single-cell gel electrophoresis (SCGE), was first developed by Östling and Johanson in 1984 as a micro-electrophoretic technique for direct visualisation of DNA damage in mammalian cells (Ostling and Johanson, 1984). This was later modified by Singh et al. (1988) as the alkaline version. The Comet assay has been recognised for its time efficiency, simplicity, sensitivity, and cost effectiveness for analysing genotoxic DNA damage in sperm and lymphocytes as shown in previous studies (Anderson et al., 1997a; Anderson et al., 1998a; Anderson et al., 1997b; Anderson et al., 1997c; Anderson et al., 2003; Singh et al., 1988; Tice et al., 2000). It is considered to be an indicator for assessing and detecting genotoxic damage (Eastmond et al., 2009; Gopalan et al., 2011). In the Comet assay, DNA damage is measured following conversion of the DNA into double and single DNA strands. The process involves several stages. Firstly, cells are embedded in agar layers and “sandwiched” on slides coated between thin layers of agarose gel. Cells are then lysed with a detergent and salt which acts to remove all cellular protein content except the DNA. Nucleoids are formed containing supercoiled loops of DNA. Then, either under alkaline or neutral pH conditions, the DNA is allowed to unwind before being

electrophoresed. The electrophoresis creates an electric field in which the DNA fragments and loose DNA ends migrate away from the nucleus moving from cathode to anode. Under alkaline conditions only, the DNA double helix is denatured and the nucleoid becomes single stranded. Cells are then neutralised down to pH 7 with buffer. A DNA-intercalating fluorochrome, such as ethidium bromide (EtBr) is then applied to stain the DNA. The amount of fluorescence is then quantified within the head and the tail regions and assessed together with the length of the tail. EtBr based staining thus allows for the analysis of an image that resembles a Comet with a distinct head and tail. The head contains undamaged/intact DNA, whilst the tail consists of fragmented and/or damaged DNA segments (Collins, 2004). Thus, the quantity of DNA found in the tail region would indicate the amount of damage DNA in proportion to the undamaged DNA in the head region (Figure 7). When considering the length of the comet, then the parameter Olive tail moment (OTM) is used.

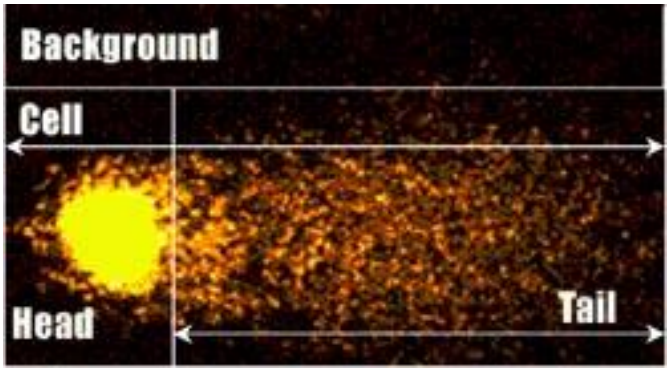


Figure 7: An image of a comet, showing parts of Head (undamaged DNA) and tail (damaged DNA). *Image is adapted from Comet assay India (Organisation).*

1.8.1.2 Genotoxic studies involving Comet assay.

The Comet assay has been used in many genotoxic studies, both *in vitro* and *in vivo* studies and in human monitoring to investigate genetic damage with the objective of measuring human exposure to genotoxic agents due to occupational exposure, drug treatments and environmental pollution (Faust et al., 2004). It has also been deployed to assess DNA repair ability, radiation biology, environmental bio-monitoring, genetic toxicology and human epidemiology (Faust et al., 2004; Wagner et al., 2003).

The Comet assay works well with most cell types, including lymphocytes, whole blood cells and sperm. The assay is ideal with these cells because of several advantages including; no requirement for large numbers of cells, much data can be generated in short amounts of time, these cells can easily be collected in relatively non-invasive procedures (Faust et al., 2004).

Lymphocytes, whole blood cells and sperm were used because DNA damage in somatic cells can potentially result in the development of cancer and damage to sperm cells can give rise to not only cancer, but can also result in heritable defects (Gopalan et al., 2011).

1.8.2 Comet repair assay

1.8.2.1 Repair of DNA.

The Comet or SGCE assay is a simple method for measuring DNA strand breaks in cells. The assay has been a useful method to access chemicals for their genotoxicity, usage in human biomonitoring, epidemiology, and

importantly, also in DNA damage and repair (Collins, 2004). Monitoring the repair of DNA is one of important determinants of cancer susceptibility (Gaivao et al., 2009). All cells possess mechanisms of repairing or correcting DNA damage before there are permanent changes made to DNA. The method of DNA cellular repair is also sometimes known as the challenge assay (Au et al., 2010; Azqueta et al., 2014).

There are various pathways which deal with DNA repair, the repair of single-strand breaks (SSBs) is rapidly achieved in most cells, consisting of a little more than ligation. One of these repair mechanisms is base excision repair (BER), which works to repair small altered bases, whilst the other process is the Nucleotide excision repair (NER), which recognises and repairs bulky adducts or helix distortions (Azqueta and Collins, 2013; Azqueta et al., 2014).

However the repair of double strand brakes (DSBs) is more complex, since the double helix of DNA is continuously disrupted. Homologous recombination restores the correct DNA sequence, using DNA from the homologous chromosome or sister chromatid as a template. On the other hand, non-homologous end-re-joining is less precise and can therefore result in loss of sequence.

1.8.2.2 Principle of the Comet repair assay

There are two ways of measuring the repair of cellular DNA damage. Firstly, the repair of DNA from cells is monitored by exposing cells to a DNA damage inducing agent and then the damage is measured after a short interval(s) of time (Collins, 2004). Usually cells undergoing treatment are exposed to chemicals such as hydrogen peroxide or ionising radiation, and the repair rate

is measured at certain time intervals, usually short period of time following treatment. This is the most common way of measuring lesions such SSBs in all cells. In addition to SSBs, other lesions like UV-induced pyrimidine dimers (using NER) and oxidised bases (using the BER) can be monitored by using specific enzymes that recognise the lesions and convert these into strand breaks (Azqueta and Collins, 2013; Azqueta et al., 2014). The enzyme formamidopyrimidine DNA glycosylase (FPG) is used to convert 8-oxoguanine and other oxidised purines into SSB, Alk A convert the alkylated bases and the T4 endonuclease V is used to convert cyclobutane pyrimidine dimers (induced by UV) (Collins, 2004). Thus, overall this assay allows us to monitor, SSB – re-joining (repair after exposure), BER (by monitoring removal of oxidised and alkylated bases) and NER (by monitoring removal of UV-induced cyclobutane dimers (Collins, 2014; Collins and Horvathova, 2001).

The Comet repair assay in this thesis monitors the repair rate of the re-joining of SSBs. DNA is organised as a series of (supercoiled) loops attached to the nuclear framework. Damage to the DNA, in the form of strand breaks, causes the relaxation of the supercoil. The alkaline nature of the Comet assay used, means that DNA can be damaged at higher pH, and in particular, the APsites are also converted to breaks. The greater the presence of breaks, the greater the relaxation of loops and therefore the fluorescence is even greater of the tail in the Comet, relative to the head region (nucleoid core). On the basis of observing this tail DNA damage, it would be possible to determine the amount of damage caused and subsequent repair of DNA following bleomycin exposure and additionally, also including exposure to NSAIDs, both in the bulk and the nano form (Azqueta and Collins, 2013).

1.8.3 Micronucleus assay

1.8.3.1 Principle of the Micronucleus assay

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably.

The understanding of DNA damage at the chromosome level is a key element of genetic toxicology. The mutations of chromosomes in carcinogenesis are an important event and the micronucleus assay is one particular technique that allows for the analysis of chromosomal damage (Fenech, 2000). The assay has been defined as a simple test designed to detect genotoxic damage by chemical exposure in cells at the interphase stage of the cell cycle. Certain chemicals can be mutagenic and may lead to the induction of micronuclei in cells at the interphase stage. This occurs as a consequence of interference with chromosome structure and/or segregation (Fenech, 2007).

1.8.3.2 How MN are formed in the assay

Micronuclei in erythrocytes have been identified for over 100 years as the Howell-Jolly bodies seen in haematology. The first induced micronuclei were first seen in *Vicia faba* root tips exposed to X-rays (Evans et al., 1959), whilst in 1975, the bone marrow micronucleus assay was used to examine micronuclei in immature young polychromatic erythrocytes and were found to be sensitive, simple and reliable (Schmid, 1975). Micronuclei found in

erythrocytes were achieved using an *in vivo* assay, whereas in the present studies, micronuclei have been observed using an *in vitro/ex vivo* approach.

The micronucleus test is essentially a mutagenic test system able to detect cytotoxicity of certain chemicals. The cytotoxicity is manifested through generation of DNA fragments seen within the cytoplasm in the interphase cells which are separate from the main nucleus (Fenech, 2002, 2007). These fragments originate from whole chromosomes/centric and acentric fragments, lacking centromere regions. Consequently, during the anaphase stage of the cell cycle division, they are unable to migrate in synchronisation with the rest of the chromosomes, thereby giving rise to micronuclei. Thus, micronuclei represent damage that has been transmitted to daughter cells. Chemical exposure to cells that results in micronuclei formation would hence demonstrate that they are genotoxic and affect the chromosome structure or alter segregation patterns. The number of micronuclei would represent the toxic potential of a chemical (Fenech, 2002; Kirsch-Volders et al., 1997; Kirsch-Volders et al., 2000).

1.8.3.3 Cell culture of Micronucleus assay

The general process of micronuclei involves 72 hour cell cycle division which includes stimulation by addition of the plant lectin and mitogen - phythaemagglutinin at 0 h., Cytochalasin B at 44 h., cell fixation at 72 h. and staining with dye, fixation of slides, followed by scoring under a microscope.

Cell culture is imperative when working with the Micronucleus assay, in order to obtain micronuclei at the interphase. The cells must undergo a nuclear division to show chromosome damage following exposure to a genotoxic

agent *in vitro*. Cell cultures are started off with the addition of lymphocytes (or other cells) to flasks containing medium and phythaemagglutinin (PHA). PHA stimulates cells, by selectively stimulating T lymphocytes to enter mitosis of cell division (Baumgartner et al., 2004; Fenech, 2000). Cytochalasin B, a microfilament-assemble inhibitor is added to cells *in vitro* at 44 h. to block cytokinesis, thus leading to formation of bi-nucleated cells. At 72h., cells undergo hypotonic shock followed by cell fixation. Cell fixation is performed as a methodology to preserve the cytoplasm, cytoplasmic boundaries and minimise clumping of cells (Kirsch-Volders et al., 2000). Cells are dropped onto slides according to methods used by Fenech (2002) Fenech (2007) and stained in Giemsa stain and scored under a microscope.

The addition of Cytochalasin B, as described above is imperative to halt cells division in the Micronucleus assay. This special method in known as the cytokinesis block micronucleus (CBMN) assay. The bi-nucleate presence of cells, confirms the cell has either divided in or following the presence of a test agent, and therefore it is possible to score the expressed micronuclei for these cells (Doherty, 2012; Fenech, 2002).

1.8.3.4 Advantages of CBMN Assay

The CBMN assay can be applied to various cell population types including primary or transformed cell lines such as mouse lymphoma cell line and primary human lymphocytes. Using general morphological criteria, the CBMN assay makes it possible to measure genotoxicity and cytotoxicity by observing: chromosome breakage, chromosome loss, chromosome rearrangement (including nucleoplasmic bridges), cell division, inhibition, apoptosis and

necrosis (Figure 8) (Fenech, 2000, 2002, 2006; Kirsch-Volders et al., 1997). The assay provides an alternative option to the chromosome aberration testing and the data obtained is not confounded by altered cell division kinetics caused by sub optimal cell culture conditions or cytotoxicity by agents tested. Interphase cells can be accessed relatively objectively, which means the cells are much faster to score, thus making it practical in scoring hundreds of cells per each treatment group, resulting in data with strong statistical power. Furthermore, automated processes can be used to reduce scoring time even further (Doherty, 2012; Fenech, 2002).

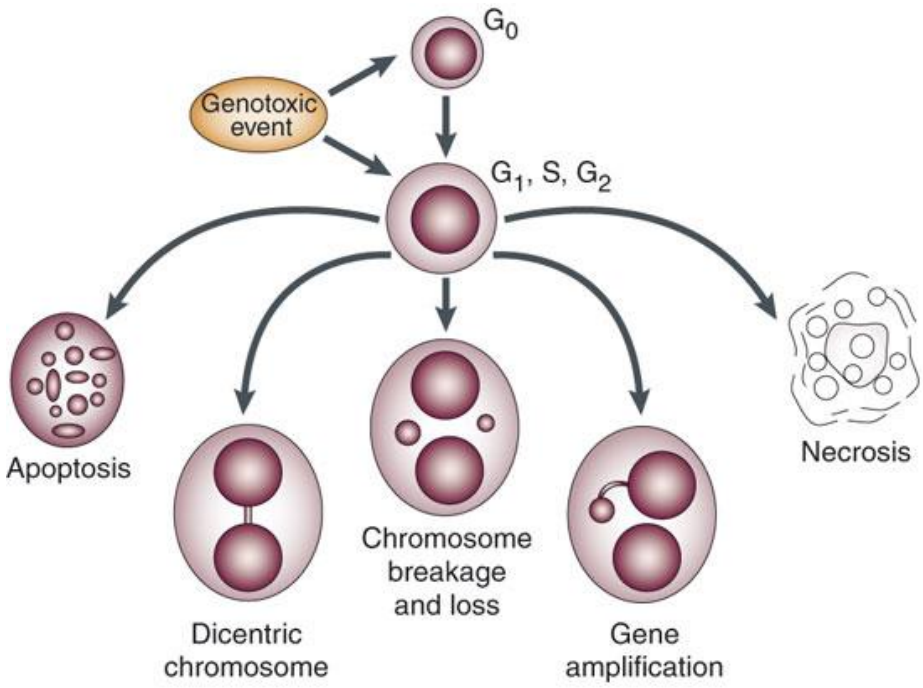


Figure 8: Possible fates of cells after division: Figure shows the various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents (Fenech, 2007).

The CBMN assay is therefore ideal for working with lymphocytes and micronuclei formation can be used as substitution to analyse and assess toxic damage posed by all three HAAs (IAA, BAA, and CAA).

1.8.3.5 Types of Micronuclei (MNi) in CBMN assay

There are various forms of MNi that can form. Firstly the cell type is determined. The cells can either be mono nucleated (contain one nucleus), bi nucleated (two nuclei) or multi nucleated (more than two nuclei). In addition, these cell types may contain one or several MNi (See Figures 9 and 10). In addition CBMN assay can be used to measure nuclear buds (NBUDs), nucleoplasmic bridges (NPBs) (Figure 10 for NBUD and Figure 9 and 10 for NPBs), and cell death (necrosis or apoptosis) as well as nuclear division rate (Fenech, 2002, 2006). NBUDs presence shows amplified DNA/ DNA repair complexes are absent whilst NBPs demonstrate DNA mis-repair and/or telomere end fusions (Thomas and Fenech, 2011).

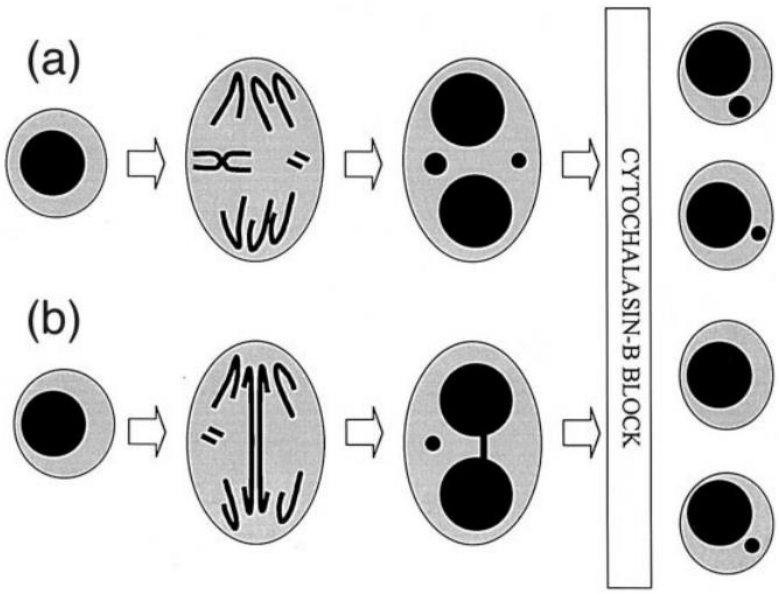


Figure 9: MNi formation: Figure 9a shows an example of the development of micronuclei from lagging whole chromosomes and acentric chromosome fragments at the anaphase stage. Figure 9b: A dicentric chromosome, centromeres are pulled to opposite ends eventually form into a nucleoplasmic bridge. The acentric parts in both figures 5a and 5b eventually become micronuclei (MNi). At 44 h. the addition of Cytochalasin B is used to prevent further cell divisions at the binucleate stage (Fenech, 2002).

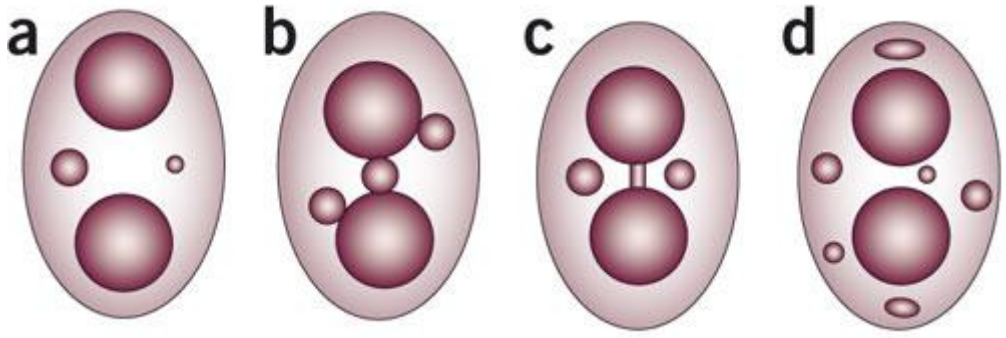


Figure 10: Types of MNI: Figure shows various types of MNi. The criteria for successfully scoring MNi is that it must be no more than a 1/3rd in diameter compared to the bi-nucleate but can be as small as 1/9th (a). MNi may also overlap but not touch the bigger nuclei (b), while MNi can also be found around bi-nucleated containing NPB (c). In other cases (d) there can be several MNi of different sizes, thought these are only typically found in cells that have been exposed to chemicals (Fenech, 2007).

1.9 Aims of study (HAAs)

Disinfection of drinking water gives rise to disinfection by products (DBPs). Various publications and literature have shown that DBPs have health implications and provide a cause for concern. DBPs have been associated with the development of certain cancers (kidney, brain bladder) (Villanueva et al., 2004; Villanueva et al., 2007). It has previously been demonstrated that the HAAs, - iodoacetic acid (IAA), bromoacetic acid (BAA) and chloroacetic acid (CAA) are cytotoxic and genotoxic DBPs when analysed in the Ames test, and in the mammalian CHO cell system (Plewa et al., 2002). Cemeli et al. (2006) have also shown the involvement of oxygen radicals with IAA in these cells. In addition, HAAs have also been found to be cytotoxic in human lymphocytes but not genotoxic in the Micronucleus assay in TK6 cells (Liviac et al., 2010). What has not been shown is the effect of the 3 HAAs in human somatic and germ cells and whether oxidative stress is involved in their mechanism of genotoxic action.

Contributing to a vast catalogue of information and genotoxicity posed by DBPs, the aim of this project was to investigate and examine both somatic and germ cells as peripheral blood lymphocytes and sperm. The effects of three HAA compounds, IAA, BAA and CAA have been investigated. After determining appropriate dose responses in lymphocytes and sperm, oxidative radical involvement has been investigated, with the anti-oxidant butylated hydroxanisole (BHA) and the enzyme catalase in the single cell gel-electrophoresis assay (Comet assay) under alkaline conditions, > pH 13.

The Comet assay was used in this study for its simplicity and being a sensitive assay for analysing genotoxic DNA damage in sperm and lymphocytes as shown in previous studies (Anderson et al., 1997a; Anderson et al., 1998a; Anderson et al., 1997b; Anderson et al., 2003; Singh et al., 1988; Tice et al., 2000). The Comet assay is considered to be an indicator for assessing and detecting genotoxic damage (Eastmond et al., 2009; Gopalan et al., 2011). These primary cells were used because the detection of DNA damage in somatic cells can potentially result in the development of cancer and damage to sperm cells can give rise to cancer, and could also cause heritable defects (Gopalan et al., 2011).

With lymphocytes, a similar approach using the CBMN assay was deployed as a means of analysing cytotoxicity and genotoxicity of these cells. Micronuclei formation will be observed and the aim will be to determine whether micronuclei are reduced following antioxidant induction to HAAs. This study may show findings that may contribute to existing literature arguing for the need to regulate HAAs, which are regulated in the United States but not the United Kingdom. With these notions in mind, important information can be obtained on these cells that is directly relevant to human beings, as damage detected *in-vitro* can predict damages *in-vivo* and help address issues facing health of human beings.

1.10 Aims of study (nano and bulk aspirin and ibuprofen)

A limited number of studies have shown direct effects and associations of non-steroidal anti-inflammatory drugs (NSAID) on tumour biology, with an increase of anti-tumour effects on several hallmarks of cancer, such cell proliferative capacity, evasion of apoptosis and cell cycle regulation and invasive capability of cancerous cells (Park et al., 2014). Clinical evidence has also shown that NSAIDs result in down-regulating systemic inflammatory responses, whilst favourably influencing the local inflammatory response within tumour microenvironment regions (Hussain et al., 2012). Generally, the majority of epidemiological studies have found that individuals who take NSAIDs have a reduced risk of colorectal adenomas and carcinomas. Such effects have been observed for aspirin (Burn et al., 2011; Cook et al., 2013).

Since most studies on NSAIDs have focussed on gastrointestinal cancers (such as colorectal, gastric and cancers of the oesophagus), this current investigation is concerned with the effect of two NSAIDs in blood samples from patients with respiratory diseases in two different studies.

In the first study, DNA damage in peripheral whole blood lymphocytes of healthy individuals and respiratory disease patients (asthma, chronic obstructive pulmonary disease (COPD) and lung cancer) have been compared following treatment with the bulk and nanoparticle versions of aspirin and ibuprofen in the micronucleus assay (Fenech, 2000, 2007). Further to this, the standard bulk versions of aspirin and ibuprofen were compared directly with their equivalent nano forms. The aim was to observe for MNi found in binucleated cells from controls and disease patients and therefore analyse this

information in order to determine whether the NSAIDs - aspirin and ibuprofen, show an increase or decrease in DNA damage. The objective of using the nano versions of these NSAIDs were to determine whether nano particles can cause a reduction or increase in DNA when compared to their respective bulk versions.

The second study similarly involves investigations of aspirin and ibuprofen and their effect on the repair of DNA using the Comet assay. Fellow member of the faculty (Dr. Mojgan Najafzadeh, University of Bradford - Paper currently in publication process) has investigated the DNA damage in peripheral lymphocytes of healthy individuals and respiratory disease patients (asthma, COPD and lung cancer), which have been compared before and after treatment with the bulk and nanoparticle versions of aspirin and ibuprofen using the SCGE or Comet assay (Tice et al., 2000).

The Comet repair assay also allows for DNA damage to be monitored by incubating cells after treatment with a damaging agent and measuring the damage remaining at different time intervals (Collins, 2004). For this part of the study, peripheral whole blood from healthy individuals and disease patients were obtained and treated with DNA damage inducing agent bleomycin and then repaired for a brief period of time with aspirin and ibuprofen, both bulk and nano forms, to determine whether these compounds can increase or reduce damage in cells and influence DNA repair of cells.

These three studies above are the first of their kind, to our knowledge in which the nano form of aspirin and ibuprofen NSAIDs, have been investigated at the cellular level *ex vivo/ in vitro*. The use of lymphocytes is supported by the

observations of the WHO/IPCS who reported that lymphocytes are suitable surrogate cells for the detection of cancer. Lymphocytes can be used as surrogates for somatic cells in *in vitro* genotoxicology tests. When challenged with UV as an external stressor they can also act as biomarkers to diagnose cancer of certain states (Najafzadeh et al., 2012).

Chapter 2 - Materials & Methods

2. Materials & Methods

2.1 Ethical approval

Ethical approval was obtained for performing both the Comet assay, Comet repair and the micronucleus assay for the studies on HAAs and NSAIDs, including sample collection of all cells. All work was undertaken after approval by the University of Bradford Subcommittee for Ethics in Research involving Human Subjects (Ethics Reference number: 0405/8). The study was also approved by Leeds (Central) Research Ethics Committee (REC Reference number: 09/H1313/37) and Research support & Governance office, Bradford Teaching Hospitals.

2.2 Materials and Methods for testing HAAs using the Comet assay

2.2.1 Reagents.

General Laboratory reagents and RPMI - 1640, FBS Medium was purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO, U.S.A). The three HAA compounds, (iodoacetic, bromoacetic and chloroacetic acids) were kindly gifted by Professor Michael Plewa, University of Illinois, Urbana, Illinois 61801, United States. HAAs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20^{O}C. The antioxidant, butylated hydroxyanisole (BHA) (CAS# 25013-16-5) was dissolved in DMSO and catalase (EC 1.11.1.6) in double distilled water (ddH_2O). Both antioxidants were purchased from Sigma Chemical Co. Ethidium Bromide was also purchased from Sigma chemical co. (Sigma, Poole, Dorset, UK: E-8751).

2.2.2 Lymphocyte and Sperm sample collection

Ethics: See section 2.1 (above) for ethical approval for the Comet assay.

2.2.2.1 Lymphocytes: Healthy volunteers donated blood. Isolation of lymphocytes was achieved by adding 6 ml of blood above 3 ml of LymphoprepTM (Axis-Shield, Oslo). Samples were centrifuged and lymphocytes that were concentrated at the interface were isolated. Isolated lymphocytes were then washed with 0.9% saline and centrifuged again. The supernatant was discarded and cells from each tube were suspended in foetal bovine serum (FBS) with 10 % DMSO (900 µl FBS, 100 µl DMSO) and transferred to 1.5 ml Eppendorf® tubes. The tubes were initially frozen at -20^{O}C before freezing at -80^{O}C for future use.

2.2.2.2 Sperm: Healthy volunteers provided semen samples through masturbation after abstinence for a minimum of 3 days, as recommended by WHO guidelines (World Health Organization., 2010a). The samples were brought to the laboratory within 30 minutes of ejaculation. Conventional semen analysis was carried out, according to WHO guidelines using a 100 µl sample. Using a phase-contrast microscope, the sample was analysed for motility, morphology, pH, concentration (in million cells per ml), and general appearance. The remainder of the sperm samples were transferred into Eppendorf® tubes in aliquots of 25 µl prior to flash freezing in liquid nitrogen and then subsequent storage at -80^{O}C for future use.

2.2.3 Cell Viabilities

For both lymphocytes and sperm, cell viability was measured in samples up to a cut-off point of 75% using trypan blue exclusion, so avoiding artefacts due to toxicity. Generally, the viability was in excess of 90%. Viability was measured in lymphocytes and sperm samples on the day they were freshly obtained and in lymphocytes (only) also checked before and after treatment. For sperm, after freezing, it was not possible to analyse and check viability due to reduced viabilities of frozen sperm samples (Gopalan et al., 2011; Henderson et al., 1998).

2.2.4 Study Cohort for both lymphocyte and sperm Comet assays.

In this study, samples were used from different donors in lymphocyte cells and sperm.

Comet assay lymphocytes: a total of 12 blood samples were used. These blood samples were obtained from healthy donors, and lymphocytes were isolated and stored at -80^{O}C in foetal calf-serum (FCS) and dimethylsolfoxide (DMSO). Frozen samples were thawed and then used for experiments. Four samples were used in succession to perform the Comet assay with one HAA (e.g. IAA). The results from the four experiments were then combined and averaged to create a data set (Table 1a).

Comet assay sperm: a spermiogram analysis was performed according to WHO guidelines (World Health Organization., 2010a) on samples from four donors (Four experiments were performed per HAA, and in each experiment,

one aliquoted sample from one of the donors was used, thus using all four different donors for each HAA experiment. Samples were aliquoted in amounts of 25 µl, immediately flash frozen in liquid nitrogen –at 196^{O}C, then transferred to a -80^{O}C freezer and later thawed before usage in the Comet assay. A small volume from each sample was retained to perform Spermiogram analysis immediately after samples were collected. The viability appeared not to be affected since we were able to score slides satisfactorily with both lymphocytes and sperm (Table 1b).

Table 1a: Samples used from Healthy, non-smoking donors.

Age	Sex
22	Female
24	Female
26	Male
27	Male
27	Female
28	Female
31	Female
33	Male
34	Male
37	Male
37	Male
39	Male

Table 1b: Sperm Samples used from healthy non-smoking donors.

Age
29
36
37
40

2.2.5 Single cell gel electrophoresis (SGCE) Comet assay

2.2.5.1 Cell Treatments:

2.2.5.1.1 Cell treatment of lymphocytes: Eppendorf® tubes were thawed in water before use. Tubes were centrifuged and the supernatant was discarded. 400 µl of fresh RPMI medium was used to dissolve the cell pellet and create a single cell suspension. 100 µl of lymphocytes were then added to each treatment tube (100 µl lymphocytes, 890 µl RPMI medium, plus 10 µl of

chemical or 900 µl for the negative control). Cells were treated with IAA, BAA, CAA, with and without antioxidants in RPMI-1640 medium for 30 min at 37^{O}C. (For details of antioxidant usage – see section 2.4.2).

2.2.5.1.2 Cell treatment of sperm: Eppendorf® tubes were thawed in water before use. 2µl of sperm were added to treatment tubes (2µl sperm, 988 µl medium, 10 µl chemical or 900 µl for negative control). Cells were treated with IAA, BAA, and CAA with and without antioxidants for 1 hr at 32^{O}C.

2.2.5.2 Concentrations of HAA treated samples

For each of the three HAAs, dose-responses were obtained and analysed to determine the dose that gave the maximum response without toxicity to the cells. Two controls with and without water and five different concentrations of the HAAs, IAA and BAA were used – 2 µM, 6 µM, 10 µM, 14 µM, 18 µM and 22 µM. For CAA, millimolar (mM) concentrations were used throughout at the same numerical concentrations but a 1000 fold higher, to align this study with previous studies, where CAA was investigated at mM level and IAA & BAA at µM level in the ranges of concentrations used in this study (Plewa et al., 2002).

The highest dose of each HAA was chosen to be the ideal choice as it was able to cause the highest damage without inducing cell cytotoxicity. However in this study a dose of 25 µM instead of 22 µM was used to compare with the study of Cemeli et al. (2006).

2.2.5.3 Antioxidant treated samples

Antioxidants with and without the highest dose of the HAAs were used to investigate the oxygen radical mechanism. Three different concentrations of the antioxidant butylated hydroxyanisole (BHA) (10, 50 and 100 µM) and a concentration of the enzyme, catalase (500 Units/ml) were used.

Treatment samples containing antioxidants in lymphocytes: 100 µl lymphocytes, 880 µl of RPMI medium, 10 µl of HAA, plus 10 µl of catalase or BHA were added. For antioxidant controls, only the antioxidants were added to lymphocytes and RPMI 1640 medium. For the negative controls, neither antioxidant nor HAAs were added.

Treatment samples containing antioxidants in sperm: 2 µl sperm, 978 µl of RPMI medium, 10 µl of HAA, plus 10 µl of catalase or BHA were added. For antioxidant controls, only the antioxidants were added to lymphocytes and RPMI 1640 medium. For the negative controls, neither antioxidant nor HAAs were added.

2.2.6 Embedding of cells in Agarose

After treatment, lymphocyte samples were centrifuged and the supernatant was discarded, with small amounts retained with the cell pellet. Next, 100 µl of 1% low melting agarose (LMP) (Invitrogen, Paisley, UK: 15517-022) was added to cell pellet to create a cell suspension. The cell suspension was transferred to slides pre-coated with 1% normal melting point (NMP) agarose. The slides were placed on an ice block for 5 min, after which 100 µl of 0.5%

LMP was added on top and slides were placed on ice for 5 min. In the case of sperm, the above process was repeated in a similar manner but with 1.5% NMP used for the creation of cell suspension and 1% LMP for the second layer.

2.2.7 Cell Lysis

Lymphocytes: Slides were placed in cold lysis solution for 1 hr. minimum. This was made up using 178 ml lysis stock solution plus 20 ml DMSO and 2 ml of Triton® X-100 (Sigma, Poole, Dorset, UK). The lysis solution had final concentrations of 2.5 M NaCl (VWR 27800.360), 100 mM EDTA (Sigma, Poole, Dorset, UK: E 3154), and 10 mM Trizma base (Sigma, Poole Dorset UK: T-6066). The pH of the solution was adjusted to pH10, with sodium hydroxide (NaOH).

Sperm: Slides were lysed in two different solutions, solutions A & B. In solution A, a cold lysis solution was prepared, consisting of 178 ml of lysis stock solution (as mentioned above), 20 ml H_2O and 2 ml of Triton® X-100 (Sigma, Poole, Dorset, UK). This solution also contained 10 mM dithiothretiol (DTT), (Sigma, Poole, Dorset, UK: D-9779). Slides were lysed in this solution for 1 h. After 1h., slides were lysed in solution B which was prepared as solution A, but instead of DTT, 0.125 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany: 1000144) was used. Samples were lysed in this solution for 1 hr; the total lysis period was 2 hrs.

2.2.8 Electrophoresis

Following lysis, electrophoresis was performed on lymphocytes and sperm samples based on the methods by Tice et al. (2000). Electrophoresis solution was prepared using 1930 ml of H_2O, 60 ml of NaOH (BDH: 301675N) and 10 ml of Na_2 EDTA (pH 13.5). The end concentrations for these were 300 mM and 1 mM, respectively.

Lymphocyte electrophoresis: Slides were placed in a tank for 30 min and electrophoresis was performed at 290-300 mA, at a constant voltage of 25 V for 30 min (0.71 V/cm).

Sperm electrophoresis: Slides were placed in a tank for 20 min and electrophoresis was performed at 290-300 mA, at a constant voltage of 25 V for 20 min (0.71 V/cm).

Slides were then washed three times for 5 min with cold neutralising buffer (pH 7.5) and then stained with 20 µg/ml ethidium bromide (Sigma, Poole, Dorset, UK: E-8751).

2.2.8.1 Calculating the Olive tail moment and % tail DNA

Following electrophoresis, two of the parameters used to score for DNA damage are Olive tail moment and % tail DNA.

Tail Length: Tail Length is used to describe the distance of DNA migration from the body of the nuclear core region (the head of Comet) and is used to evaluate the extent of DNA damage.

Olive tail moment: The tail moment is defined as the product of the tail length and the fraction of total DNA present in the tail. This also includes measurements of smallest detectable size of migrating fragmented DNA and relaxed and broken pieces of DNA.

The Olive tail moment is calculated as follows:

$$\text{Olive tail moment} = \frac{(\text{Mean Tail} - \text{Mean Head}) \times \% \text{ Tail DNA}}{100}$$

The % Tail DNA is calculated as follows:

Tail % DNA = 100 – Head percentage (%) DNA.

2.2.9 Scoring of Slides and Statistical Analysis:

All slides were coded before scoring and 50 cells were scored per observation using a microscope equipped with a CCD camera and Computer system (Andor Technology Ltd, Belfast, UK). Data were generated to measure Olive tail moment and % Tail DNA using Comet 6.0 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK). Mean data were generated with standard errors. Distribution of the response variable departed significantly from normality (Kolmogorov-Smirnov goodness of fit test) and thus, non-parametric statistics were considered adequate for the analysis of the data. The differences between groups were examined using the Kruskal-Wallis test and Mann-

Whitney U-test. Dose response relationships for IAA, BAA & CAA were determined by Pearson's correlation. A p-value of < 0.05 was considered significant. All analyses were performed using SPSS for windows statistical package (version 18.0).

2.3 Materials and Methods for Cytokinesis block Micronucleus (CBMN) Assay for testing HAA compounds (chapter 4)

2.3.1 Reagents

General Laboratory reagents were purchased from Fisher Scientific Co. (Itasca, IL). RPMI 1640 medium (with 2 mM L-glutamine and 25 mM HEPES). Cytochalasin B (Cat No. C6762) and mitomycin C (Cat No. M0503) were purchased from Sigma-Aldrich (Poole, UK). Phythaemagglutinin (PHA) (Cat No. 10576-015), penicillin-streptomycin solution (Cat No.15140-122) were purchased from Invitrogen Ltd, UK. Slides and coverslips were obtained from VWR international. The Fixogum rubber cement was purchased from (Marabu, Germany). As with the Comet assay, the three HAA compounds, (iodoacetic, bromoacetic and chloroacetic acids) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20^{O}C. The antioxidant, butylated hydroxyanisole (BHA) (CAS# 25013-16-5) was dissolved in DMSO and catalase (EC 1.11.1.6) in double distilled water (ddH_2O). Both antioxidants were purchased from Sigma Chemical Co (Sigma, Poole, Dorset, UK).

2.3.2 Whole blood sample collection & Study Cohort.

Ethics: See section 2.1 (above) for ethical approval for Micronucleus assay.

Micronucleus assay: 6 fresh blood samples were used from healthy donors (Table 2) Samples were immediately transferred to the laboratory and used for cell culture after collection in heparinised tubes. Fresh samples were used for the MN assay, since frozen samples do not provide satisfactory results for cell culture use. 500 cells were scored from each donor/sample, repeated twice (1000 cells in total). Two samples were tested each with IAA, BAA & CAA and the results were combined to create a data set for each compound.

Dose Responses: A range of concentrations were used starting with the Comet doses (diluting 1:1 each time). 6.25 µM was the optimal concentration for IAA and BAA and 62.5 µM for CAA. Catalase responded well; however BHA at 10 µM, 50 µM and 100 µM resulted in higher MNi frequencies compared to the negative control. Revised concentrations of 0.1 µM, 0.5 µM and 1.0 µM were used.

Table 2: Blood samples used in the micronucleus assay

Age	Sex
21	Female
22	Female
24	Female
32	Male
38	Male
38	Male

2.3.3 Preparation of basic culture medium

Under sterile conditions, basic culture medium was prepared. RPMI – 1640 medium (containing 25 mM HEPES and L-Glutamine) was supplemented with 15% foetal calf serum (FCS) and 1% penicillin-streptomycin solution. 100 ml of the prepared medium was used to aliquot 4.5 ml into T 25 cm^2 Corning culture flasks and stored at -20^{O}C. The T25 cm^2 flasks were equilibrated in a 37^{O}C incubator (5% CO_2) prior to cell culture use.

2.3.4 Cell culture of Lymphocyte cultures.

Under sterile conditions, using sterile plastics and solutions cell culture of lymphocytes was performed over a 72 hr period.

Time point 0 hr. /Culture start: Blood was obtained from healthy volunteers. 500 µl of blood was added to T25 cm^2 culture flask containing 4.5 ml medium, followed by 100 µl of phythaemagglutinin (PHA). In some flasks HAAs and /or antioxidants were added and mitomycin C was added for use as a positive control. For treatment types see table 3 below. Cultures were incubated at 37^{O}C in the presence of 5% CO_2 for 44 hours.

Table 3: shows the treatment groups in CBMN assay on HAAs (Chapter 4).The above 11 flasks represent the 11 different treatment types used when investigating each individual HAA at a time The concentrations for IAA & BAA were 6.25 µM and for CAA, 62.5 µM. The negative control contained no chemical and the positive control contained 0.4 µg/ml mitomycin C. Flasks 3-4 contained antioxidants only, while flask 7 was used for the HAA chemical only. Flasks 8-11 contained HAA and also antioxidants to determine the antioxidant response on the number of micronuclei (MNi).

Flask No.	Flask type	Blood (µl)	PHA (µl)	Chemical	Antioxidant
Flask 1	Negative Control	500 µl	100 µl	NONE	NONE
Flask 2	Positive Control	500 µl	100 µl	4 mM mitomycin C	NONE
Flask 3	Antioxidant Control	500 µl	100 µl	2,500 U/ml Catalase	NONE
Flask 4	Antioxidant Control	500 µl	100 µl	BHA 0.1 µM	NONE
Flask 5	Antioxidant Control	500 µl	100 µl	BHA 0.5 µM	NONE
Flask 6	Antioxidant Control	500 µl	100 µl	BHA 1.0 µM	NONE
Flask 7	Chemical only	500 µl	100 µl	HAA only	NONE
Flask 8	Chemical + Antioxidant	500 µl	100 µl	HAA	2,500 U/ml Catalase
Flask 9	Chemical + Antioxidant	500 µl	100 µl	HAA	BHA 0.1 µM
Flask 10	Chemical + Antioxidant	500 µl	100 µl	HAA	BHA 0.5 µM
Flask 11	Chemical + Antioxidant	500 µl	100 µl	HAA	BHA 1.0 µM

Time point 44 h. /Cytokinesis block: 30 µl of Cytochalasin B with an end concentration of 6 µg/ml was added to each flask to halt cytokinesis and arrest cells in the bi-nucleate form.

Time point 72 h. /End of cell culture: Flasks were removed from incubation and the contents from each flask were transferred to 15 ml falcon tubes.

2.3.5 Cell hypotonic shock treatment:

The 15 ml falcon tubes containing blood were centrifuged at 800 rpm/107 x *g* for 8 minutes. The supernatant was discarded using a vacuum pump, retaining above 500 µl. The pellet was re-suspended by gently patting the tube. 5 ml of cold ($4^{O}C$) 110 mM potassium chloride (KCl) solution was added to each tube on a vortex (gently). Tubes were stored at $4^{O}C$ for 15 minutes to allow for

hypotonic treatment of cells. Tubes were then centrifuged at 800 rpm/107 x *g* for 8 minutes. The supernatant was discarded using a vacuum pump, retaining around 500 µl. The pellet was re-suspended by gently patting the tube.

2.3.6 Fixation of cells

Cells were fixed three times using freshly prepared Carnoy's fixative (1 part of acetic acid was added to 3 parts of methanol) and 38% formaldehyde.

Fixation 1: 5 ml of fresh Carnoy's was added to each tube gently over a vortex, followed by 3 drops of 38% formaldehyde. Tubes were then centrifuged at 800 rpm/107 x *g* for 8 minutes. The supernatant was discarded using a vacuum pump, retaining above 500 µl. The pellet was re-suspended by gently patting the tube.

Fixation 2 & 3: The above process for fixation 1 was repeated twice without the addition of 38% Formaldehyde.

Tubes were stored overnight at $4^{O}C$.

2.3.7 Slide Preparation, staining and fixation of slides.

The following day, tubes were centrifuged at 800 rpm/107 x *g* for 8 minutes. The supernatant was discarded using a vacuum pump, retaining above 100 µl. Then according to cell density/pellet size around 200-600 µl of fresh Carnoy's solution was added. 20 µl of cell suspensions was dropped twice onto clean glass slides (centre left and centre right) and left to air dry. Three slides were used for each treatment group (6 drops total). After a short while,

the slides were stained for 5 minutes in Giemsa solution (5% Giemsa solution in phosphate buffer at pH 6.8. Slides were gently washed and left to air-dry.

Slides were fixed by using rubber Fixogum and placing a 24 mm × 50 mm coverslip on top.

2.3.8 Slide scoring

500 cells were scored per sample group using bright-field light microscope at 400 x magnification. The experiments were repeated twice for each HAA, resulting in a total of 1000 cells scored per each sample as recommended by guide lines for the assay (Fenech, 2000, 2002, 2007). MNi were scored per 500 cells each of binucleated (BiNC) and mononucleated (MonoNC) cells per slide. Statistical analysis was carried out using the chi-square test (χ^2) to determine significant differences with p value set at *** p = < 0.05 to compare samples.

2.3.9 Data Analysis and statistics used

The nuclear division index (NDI) can be used as an indicator of the cytotoxicity of HAAs and the calculation below was used to find the NDI

(Fenech, 2007).

NDI = (M1) + 2 (M2) + 3 (M3) /N

Where:
M1 = mononucleated cells
M2 = binucleate cells
M3 = multinucleated cells
N = the total number of viable cells scored.

Statistical analysis was carried out using the chi-square test (χ^2) to determine significant differences with *p* value set at *** *p* = < 0.001 for all treated samples

vs. the negative control and treatment groups containing both HAA and antioxidant treatments versus the HAA only group.

2.4 Methods for Preparation of NSAIDs - Aspirin nano & Ibuprofen nano compounds, (for chapters 5 & 6), sections 2.5 & 2.6 of Materials & methods.

2.4.1 Reagents.

Two non-steroidal anti-inflammatory drugs – ibuprofen and aspirin were used in this study. Aspirin and sodium lauryl sulphate (SLS) were purchased from Sigma-Aldrich, Poole, Dorset, United Kingdom. Ibuprofen was purchased from Albemarle Europe sprl, Belgium. Pharmcoat 606 (HPMC) was kindly donated by Shinetsu, Japan. Kollidon 30 (PVP K-30) was purchased from BASF, United Kingdom. Bulk and nano suspensions of Aspirin and Ibuprofen were kindly prepared by Lena Nanoceutics, Bradford, United Kingdom.

2.4.2 Methodology for Aspirin & Ibuprofen nano suspensions.

Suspensions of aspirin and ibuprofen with solid loads of 4% and 3% (w/w) respectively were prepared. The suspending medium consisted of: HPMC (0.5%, w/w), polyvinylpyrrolidone K-30 (0.5%, w/w) and sodium lauryl sulphate (SDS) (0.1%, w/w) in deionised water (Plakkot et al., 2011). HPMC was used as it is a lubricant, polyvinylpyrrolidone is a water soluble polymer and SDS is detergent that removes residues in the mixture. The millings of these compounds were carried out using Lena Nanoceutics technology DM-100 machine (Sulaiman 2007).

250 ml of each suspension were milled using 150 ml of 0.2 mm yttrium stabilised zirconium beads (Glen mills, USA). The materials were then recycled for 60 minutes in the milling machine before being discharged in an opaque glass bottle and stored in the refrigerator at (4°C) for the duration of the experiments. Nano-suspensions were then briefly sonicated for 15 minutes, prior to start of the micronucleus & Comet repair assays.

2.4.3 Analysis of nano-suspension particle size

Particle size of aspirin and ibuprofen were regularly checked for the duration of the micronucleus and Comet repair assay experiments. The particle size distribution of aspirin and ibuprofen nano-suspensions were determined using the dynamic light scattering technique of the Zetasizer Nano ZS (Malvern instruments, UK). Samples were measured at room temperature using disposable sizing cuvettes. All measurements were carried out in triplicates. The particle size of the stock suspensions were measured in triplicates immediately after milling and then rechecked at the end of the experiments to ensure no significant change in particle size occurred during the various phases of the experiments. The particle size of the bulk powder was measured using laser diffraction technique (Sympatec Helos, UK). Approximately 20 mg of each drug were transferred into the sample vial and the primary pressure was adjusted to 4 bars while the feeder speed was 40mm/s. Three samples of each drug were measured using R2 lens (0.25/0.45, 87.5µm).

Images were obtained using transmission electron-microscope (TEM). Samples were prepared by placing a drop of the suspension (Aspirin 4% w/w and Ibuprofen 3% w/w) on the TEM carbon grid. The drop was left on the grid

for 10 min then the grid was washed by dipping it in water. The grid was floated on a drop of Uranyl acetate for 10 min then washed again with water and left to dry. The grid was then placed in the TEM and the magnification power was 100, 000x. The beam intensity was 40 kV for Ibuprofen and 60 kV for Aspirin sample.

2.4.4 Zeta potential analysis of nano suspensions

The zeta potential for the suspensions was determined using Zetasizer Nano ZS (Malvern instruments, UK). The suspensions were diluted 1:100 using deionised water and measured at 25^{O}C. clear disposable zeta cells were used. Measurement duration was set as automatic with a minimum of 10 runs and a maximum of 100 runs. All measurements were made in triplicate.

2.5 Materials and Methods for Cytokinesis block Micronucleus (CBMN) Assay for testing aspirin & ibuprofen (chapter 5)

2.5.1 Reagents.

General Laboratory reagents for cell culture were purchased as described in section 2.3.1. In addition, the compounds of aspirin and ibuprofen were obtained as described in section 2.4.1. Bulk compounds were dissolved in Eppendorf's with ddH_2O and stored at 4^{O}C. Nano compounds were checked regularly using spectrophotometer (Zetasizer) to ensure the size of the particles did not exceed 400 nanometers (nm) (See section 2.4).

2.5.2 Whole blood sample collections & Study cohort

For ethics: See section 2.1 (above) for ethical approval for Micronucleus assay. Blood samples for asthma, lung cancer, COPD, COPD & Lung Nodule (combined) and lung cancer patients were obtained from Outpatient Respiratory Disease Clinic of Dr. Badie K. Jacobs (St Luke's Hospital, Bradford, UK) and healthy control samples were obtained from University of Bradford. Cell cultures were set up and run within 3 hours on the day of sample collection.

Thirteen different blood samples were used in this study (Table 4). The samples consisted of: 3 healthy control individuals, 2 asthma patients, 2 COPD patients, 3 COPD & lung nodule patients (which were combined), and 3 lung cancer patients. The data for the latter two types were combined and considered as 6 samples in the group, for statistical purposes.

Table 4: Blood samples used from healthy non-smoking donors, asthma patients, COPD patients, COPD & Lung nodule patients and lung cancer patients.

Age	Sex	Ethnic Background	Non-Smoker/Smoker	Patient Group
47	Female	Asian	Non-smoker	Healthy Control
26	Female	Caucasian	Non-Smoker	Healthy Control
29	Male	Caucasian	Non-smoker	Healthy Control
24	Male	Asian	Non-smoker	Asthma
30	Male	Caucasian	Non-smoker	Asthma
63	Male	Caucasian	Non-smoker	COPD
55	Female	Caucasian	Smoker	COPD
63	Male	Caucasian	Smoker	COPD & Lung Nodule
65	Male	Caucasian	Non-smoker	COPD & Lung Nodule
63	Female	Caucasian	Smoker	COPD & Lung Nodule
65	Male	Caucasian	Smoker	Lung Cancer
68	Female	Caucasian	Non-smoker	Lung Cancer
55	Male	Caucasian	Smoker	Lung Cancer

2.5.3 Cell culture of whole blood

Under sterile conditions, basic culture medium was prepared as described in section 2.3.3

2.5.4 Preparation of basic culture medium

Under sterile conditions, using sterile plastics and solutions, the cell culture of lymphocytes was performed over a 72 hr period, generally in a similar manner as described in section 2.3.4. Nano compounds of aspirin & ibuprofen were sonicated using a sonicator for 15 min prior to their addition into flasks for cell culture. At the start of the cultures all flasks were prepared as follows for work with ibuprofen and aspirin (Table 5).

Table 5: shows the treatment groups in CBMN assay with nano compounds aspirin and ibuprofen (Chapter 5). The above 5 flasks represent the 5 different treatments types used to test for the effect of both the, bulk and nano form of aspirin and ibuprofen in asthma, COPD and lung cancer patients. The end concentrations for ASP B, ASP N, IBU B and IBU N in culture were 250 µg/ml.

Flask No.	Flask type	Blood (µl)	PHA (µl)	Chemical
Flask 1	Standard Negative Control	500 µl	100 µl	NONE
Flask 2	**Bulk** form of compound	500 µl	100 µl	250 µl Aspirin
Flask 3	**Nano** form of compound	500 µl	100 µl	250 µl Aspirin
Flask 4	**Bulk** form of compound	500 µl	100 µl	250 µl Ibuprofen
Flask 5	**Nano** form of compound	500 µl	100 µl	250 µl Ibuprofen

2.5.5 Cell hypotonic shock treatment:

Cells were treated as described in Section 2.3.5

2.5.6 Fixation of cells

Cells were treated as described in Section 2.3.6

2.5.7 Slide Preparation, staining and slide fixing.

Slides were prepared, stained and fixed as described in Section 2.3.7

2.5.8 Slide scoring

Slides were scored as described in Section 2.3.8

2.5.9 Data Analysis and statistics used

Data analysis and statistics were performed as described in section 2.3.9

2.6 Materials and Methods for testing ibuprofen and aspirin using the Comet repair assay.

2.6.1 Reagents.

General Laboratory reagents were purchased as described in section 2.2.1. Aspirin and Ibuprofen were purchased as described in section 2.4.1. Bleomycin sulphate was purchased from Enzo Life Sciences Ltd, Exeter, United Kingdom (CAS# 9041-93-4). Bleomycin stock solutions was prepared using ddH_2O and stored at -20^{O}C. Different working solutions (concentrations) were also prepared using ddH_2O and stored at 4^{O}C.

2.6.2 Whole blood collection & study cohort

Samples were obtained as described in the previous section, above (2.5.2). All patient samples (for asthma, COPD & lung cancer) came from St. Luke's Hospital. Bradford, UK. Control samples came from Healthy individuals from University of Bradford, UK. In this study 25 different whole blood samples were used. The samples consisted of: 10 healthy control individuals, 5 asthma patients, 5 COPD patients and 5 lung cancer patients.

2.6.3 Cell Viabilities

For whole blood samples, cell viability was measured in samples up to a cut-off point of 70% using trypan blue exclusion, so avoiding artefacts due to toxicity. Generally, the viability was in excess of 90%. Viability was measured in whole blood samples before and after treatment (Gopalan et al., 2011; Henderson et al., 1998).

2.6.4 Single cell gel electrophoresis (SGCE) Comet assay with repair mechanism

2.6.4.1 Cell treatment of Whole blood cells (General overview): Eppendorf® tubes containing whole blood of approximate 1000 µl total volume were obtained for each experiment. Seven tubes were used for the Comet repair assay; one negative control, two positive controls (Positive controls 1 & 2) containing bleomycin only and four tubes containing bleomycin and either aspirin bulk, aspirin nano, ibuprofen bulk or ibuprofen nano, to study the effects of the compounds on the repair of DNA. Bleomycin was used as challenger to

induce DNA damage. With the exception of the sample that contained bleomycin only (positive control 1), all cells in each tube were first treated for 30 minutes incubation at 37^{O}C in 5% CO_2 and then the cells were left for a further 15 minutes incubation at 37^{O}C in 5% CO_2 (45 minutes total incubation) to allow DNA repair in the presence of NSAIDs before being transferred to Agarose coated slides. The cells in the tube containing bleomycin only (positive control 1) were treated for 30 minutes incubation at 37^{O}C in 5% CO_2 before being transferred to Agarose coated slides with no repair time incubation included. The second positive control (positive control 2) – was treated in the same way as the positive control 1, but the cells were left for a further 15 minutes of incubation to allow DNA repair with no NSAIDs added.

2.6.4.2 Treatment over duration of 45 minutes

Start of first Incubation/Time point 0 hrs: For each treatment tube (6 tubes) - 100 µl of whole blood cells, 890 µl RPMI medium, plus 10 µl of bleomycin (end concentration 7.5 µg/ml) were added. For the negative control tube – 100 µl of whole blood cells, 900 µl RPMI medium were added. Tubes were incubated for 30 min at 37 ^{O}C in 5% CO_2.

End of first Incubation/Time point 0hrs, 30 min: All tubes were briefly centrifuged at 2000 rpm/107 x *g* for 3 minutes.

For Bleomycin tube with no further repair: 950 µl of supernatant was discarded, and the cell pellet was mixed in 0.5 % LMP Agarose and immediately transferred to agarose coated slide, covered on top with a cover-

slip and placed on ice for 5 min. Cover slip was removed and the slide was placed in lysis solution.

For the negative control, and bleomycin tube with further repair (for 15 minutes (45 minutes total)): For the negative control and the other five treatment tubes, 950 µl of supernatant was discarded and 950 µl of fresh 1640-RPMI medium was added again to each tube (for cell washing). Cell pellets were very gently mixed and centrifuged at 2000 rpm/107 x *g* for 3 minutes. This process was repeated twice and after the second centrifugation, cells were then treated with nano compounds.

Treatment with NSAID compounds (for 15 minutes (45 minutes total)):

For the negative control tube, 950 µl of fresh RPMI was added to the washed cell pellet and gently mixed. The same was done for bleomycin with further repair but no NSAID addition. To the remaining tubes, (also previously treated with Bleomycin), fresh RPMI medium was added, as well as NSAID Compounds, see below).

For treatment of aspirin bulk: 940 µl of Fresh RPMI and 10 µl aspirin bulk was added to the cell pellet and gently mixed (end concentration = 250 µg/ml).

For treatment of aspirin nano: 933.3 µl of Fresh RPMI and 16.66 µl aspirin nano was added to the cell pellet and gently mixed (end concentration = 250 µg/ml).

For treatment of ibuprofen bulk: 940 µl of Fresh RPMI and 10 µl ibuprofen bulk was added to the cell pellet and gently mixed (end concentration = 250 µg/ml).

For treatment of ibuprofen nano: 937.5 µl of Fresh RPMI and 12.5 µl ibuprofen nano was added to the cell pellet and gently mixed (end concentration = 250 µg/ml)

Start of Second Incubation/Time point 0hrs, 30 min:

Cells were repaired from minutes (30-45) via incubation for 15 min at 37 ^{O}C in 5% CO_2 (see above).

2.6.5 Embedding of cells in Agarose

After the repair period, all tubes were centrifuged and the supernatant was discarded, with small amounts retained with the cell pellet. Then, 100 µl of 0.5% low melting agarose (LMP) (Invitrogen, Paisley, UK: 15517-022) was added to cell pellet to create a cell suspension. The cell suspension was transferred to slides pre-coated with 1% normal melting point (NMP) agarose. The slides were placed on an ice block for 5 min.

2.6.6 Cell Lysis

After 5 min, coverslips were removed from the slides and the slides were placed in cold lysis solution and stored overnight. This was made up using 178 ml lysis stock solution plus 20 ml DMSO and 2 ml of Triton® X-100 (Sigma, Poole, Dorset, UK). The lysis solution had final concentrations of 2.5 M NaCl (VWR 27800.360), 100 mM EDTA (Sigma, Poole, Dorset, UK: E 3154), and 10 mM Trizma base (Sigma, Poole Dorset UK: T-6066). The pH of the solution was adjusted to pH10, with sodium hydroxide (NaOH).

2.6.7 Electrophoresis

Following lysis, electrophoresis was performed on whole blood cells based on methods by Tice *et al.,* (2000). Electrophoresis solution was prepared using 1930 ml of H_2O, 60 ml of NaOH (BDH: 301675N) and 10 ml of Na_2 EDTA (pH 13.5). The end concentrations for these were 300 mM and 1 mM, respectively. Slides were placed in a tank for 30 min and electrophoresis was performed at 290-300 mA, at a constant voltage of 25 V for 30 min (0.71 V/cm).

Slides were then washed three times for 5 min with cold neutralising buffer at pH 7.5 and then stained with 20 µg/ml ethidium bromide (Sigma, Poole, Dorset, UK: E-8751).

2.6.8 Scoring of Slides and Statistical Analysis:

All slides were coded before scoring and 100 cells were scored per observation using a microscope equipped with a CCD camera and Computer system (Andor Technology Ltd, Belfast, UK). Data were generated to measure Olive tail moment and % Tail DNA using 6 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK). Each experiment was repeated ten times for control group, and five times each for asthma, COPD and lung cancer groups. Mean data were generated with standard errors. Distribution of the response variable departed significantly from normality (Kolmogorov-Smirnov goodness of fit test) and thus, non-parametric statistics were considered adequate for the analysis of the data. The differences between groups were examined using the Kruskal-Wallis test and Mann-Whitney U-test. Dose response relationships

for both NSAIDs were determined by Pearson's correlation. A p-value of < 0.05 was considered significant. All analyses were performed using SPSS for windows statistical package (version 18.0).

Chapter 3 – Genotoxic effects of HAAs and antioxidant treatments in the Comet assay

3.1 Introduction

It is important to treat source waters in order to provide clean, odourless and tasteless, high quality water for household use. Water treatments commonly involve the use of chemical disinfectants. Over the last century, increasing use of these disinfectants has given rise to disinfection by products (DBPs) (Akin et al., 1982; Plewa et al., 2002) Chlorination is the most commonly used disinfectant due its low cost and germicidal potency (Qi et al., 2004). The chemical reaction between chlorine and natural organic constituents in source waters gives rise to two well-known classes of DBPs, THMs and HAAs (Plewa et al., 2002; Plewa et al., 2010). While disinfection of the drinking water has helped to eliminate and control water borne infectious diseases (Akin et al., 1982) the undesired presence of DBPs has led to health issues and these DBPs have been identified as carcinogenic (Cantor et al., 1999; Koivusalo et al., 1994; Qi et al., 2004; Villanueva et al., 2004).

In this chapter the three chemicals of the HAA DBP group: iodoacetic acid (IAA), bromoacetic acid (BAA) and chloroacetic acid (CAA) have been discussed. Previous studies have demonstrated that these HAAs are cytotoxic and genotoxic DBPs when analysed in the Ames test, and in the mammalian CHO cell system (Plewa et al., 2002). Cemeli et al. (2006) have also shown the involvement of oxygen radicals with IAA in these cells.

This chapter focuses on previously unreported effects of these HAAs in human somatic and germ cells and whether oxidative stress is involved in the HAA

mechanism of genotoxic action using the Comet assay. In this study somatic cells have been examined in the form of peripheral blood lymphocytes and germ cells using human sperm. After determining appropriate dose responses in lymphocytes and sperm (See results section 3.3, Figures 1a, 1b, 2a, and 2b), oxidative radical involvement has been investigated, with the anti-oxidant butylated hydroxanisole (BHA) and the enzyme catalase in the single cell gel-electrophoresis assay (Comet assay) under alkaline conditions, > pH 13.

The single cell gel electrophoresis (SCGE) or Comet assay, (so-called due to appearance of Comet like images), is an assay designed to measure DNA single-strand breaks in lymphocytes and sperm (Ostling and Johanson, 1984; Singh et al., 1988; Wu et al., 2001). Lymphocytes and sperm embedded in agarose gel are placed in an electric field at alkaline conditions. The fragments of negatively charged single-strand DNA move through the gel from cathode (negative) towards the (positive) anode. DNA, which is undamaged, will move relatively slowly, forming the head of the Comet, while DNA that is fragmented or damaged will move through much faster giving the appearance of tail-like structure. The Comet assay has been used to analyse two kinds of damage in lymphocytes and sperm: Olive tail moment and % tail DNA. These parameters have been described in further detail in section 2.2.8.1 in the Materials and Methods chapter.

3.2 Materials and Methods

3.2.1 Materials

The materials required for this study have been described in section 2.2.1

3.2.2 Methods

Sample collection and isolation of lymphocytes has been described in section 2.2.2.1. Collection of sperm sample(s) and spermiogram analysis has been described in section 2.2.2.2. Comet assay was deployed under alkaline conditions as described in sections 2.2.4-2.2.8. Treatment groups for lymphocytes and sperm have been described in sections 2.2.5.1 and 2.2.5.2, respectively. Antioxidant treated samples have been described in section 2.2.5.3

3.2.4 Statistical Analysis

All slides were coded and scored using a microscope equipped with a CCD camera and Computer system (Andor Technology Ltd, Belfast, UK) as described in section 2.2.9.

Data from Olive tail moment and % Tail DNA values were used to perform SPSS statistics (Version 19). Most data were normally distributed, as determined using the Mann-Whitney and Kolmogorov-Smirnov Z tests. For data that were not normally distributed using these tests, their normal distribution was confirmed using non-parametric - 2 independent samples test.

One Way ANOVA (Bonferroni) was then performed for significance of data to determine significant differences of all treatment groups, including antioxidant controls to the negative control group and significant difference of HAA and antioxidant treatment groups to the HAA only treatment group.

3.3 Results

3.3.1 Cell viability

Cell survival was above 90% for lymphocytes and above 75% for sperm. Spermiogram analysis on average from four sperm donors, gave the following results:

Sperm Morphology: 79% normal, 21% abnormal.

Sperm Motility:

44% fast-progressive
26% slow-progressive
13% non-progressive,
17% immobile (no tail movement).

Sperm pH = 8.5.

Sperm Concentration = 23.45 million per ml.

3.3.2 Overview of results

Graphs 1a, 1b and 2a, 2b, show the responses of the 3 HAAs over the repeated dose ranges in lymphocytes and sperm, respectively. All these HAAs showed similar response patterns. In the presence of antioxidants, BHA and catalase, results showed decreased responses in lymphocytes (Graphs 3a & 3b) and sperm (Graphs 4a & 4b), respectively. BHA and catalase showed no significant increases above the control values on their own. Both lymphocytes and sperm showed highest damage with HAA ($p = *** < 0.001$), but reduced levels of damage were seen in presence of catalase. In the case of BHA, less damage was found in cells that were exposed to the 100 µM antioxidant dose,

but damage increased as antioxidant dosage was reduced across both cell types, where there was more cell DNA damage with 50 µM BHA and even greater damage with 10 µM BHA. Catalase also appeared to cause a greater reduction in both sperm and lymphocyte DNA damage than in cells that were treated with 10 µM BHA except in CAA treated lymphocytes (Graphs 4A & 4B) show otherwise. However in all HAAs, cells treated with 50 µM BHA showed further decreases in cell DNA damage and at 100 µM BHA, showed the greatest reduction in DNA damage, suggesting BHA at this dosage is very effective in the reduction of damage and overall indicates the presence of oxygen radical involvement. In general, both cell types showed similar response patterns, with slightly higher levels of damage in sperm than lymphocytes. Figures 11 & 12 show images of lymphocyte and sperm Comets, respectively.

3.3.3 Statistics:

Both Lymphocytes and sperm: In general, there is no significance difference by comparison with the negative control for the antioxidants on their own (ns) but with the HAAs positive responses are generally shown (* p = < 0.05, ** p = < 0.01, *** p = < 0.001). When the HAAs are treated with the antioxidants, there is a reduction in response. These reductions are generally significant compared to the HAAs alone (Δ p = <0.05, ΔΔ p = < 0.01, ΔΔΔ p = < 0.001) or at the highest dose of BHA not significant (ns Δ). Both graphs show similar response patterns.

Exceptions include:

Lymphocytes - Olive tail moment: There is no significance (ns) of all three HAAs with the highest dose of BHA (100 µM) in comparison to the negative control (Graph 3a).

Lymphocytes - % tail DNA: There is no significance (ns) of BAA & CAA with the highest dose of BHA (100 µM) in comparison to the negative control (Graph 3b).

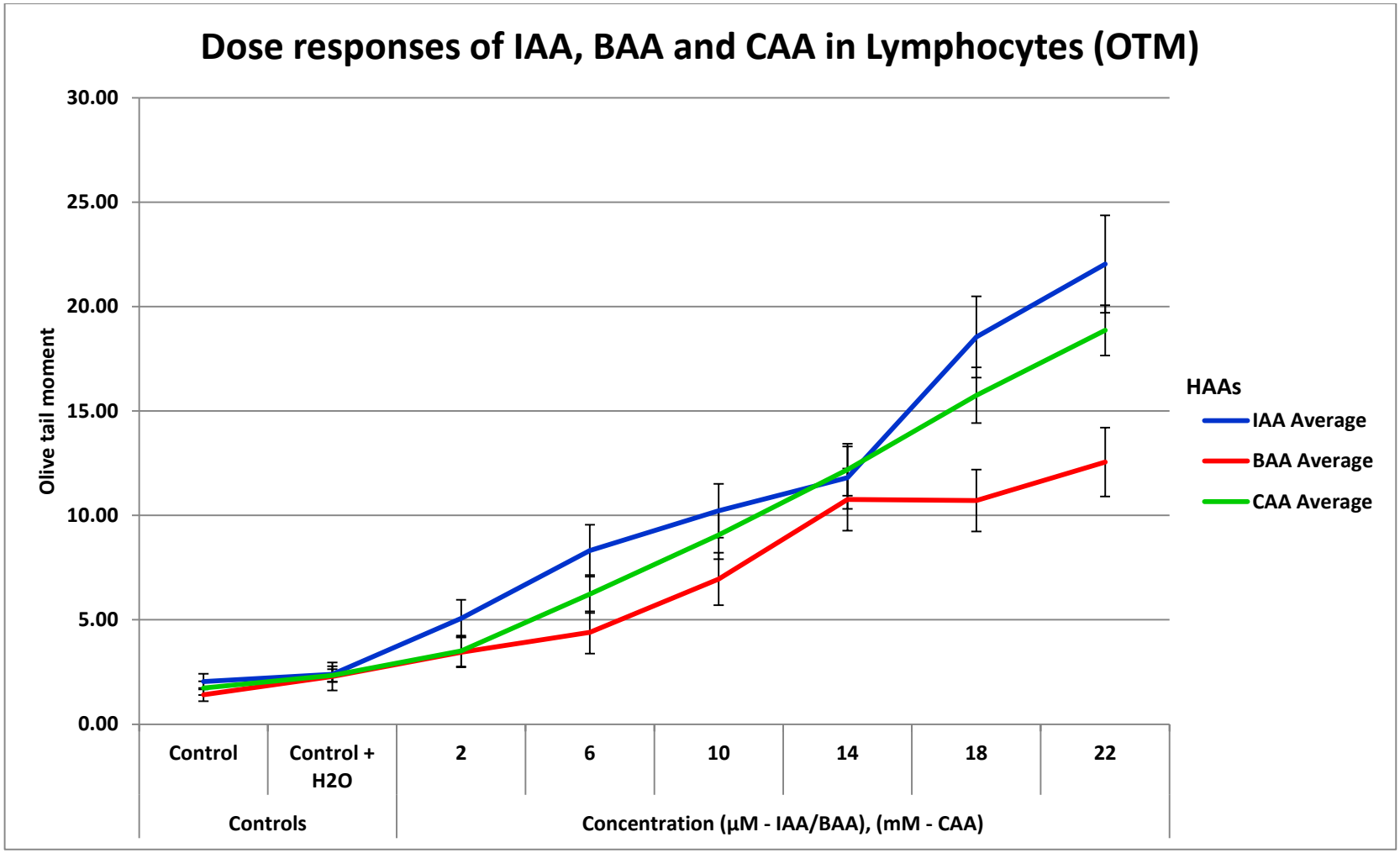
Sperm - Olive tail moment: There is no significance (ns) of IAA & CAA with the highest dose of BHA (100 µM) in comparison to the negative control (Graph 4a).

When the HAAs are treated with the antioxidants, there is a reduction in response. However some treatments groups are not significant (ns Δ) compared the HAAs alone. These treatment groups are; CAA with 500 Units/ml Catalase, IAA & CAA with 10 µM BHA.

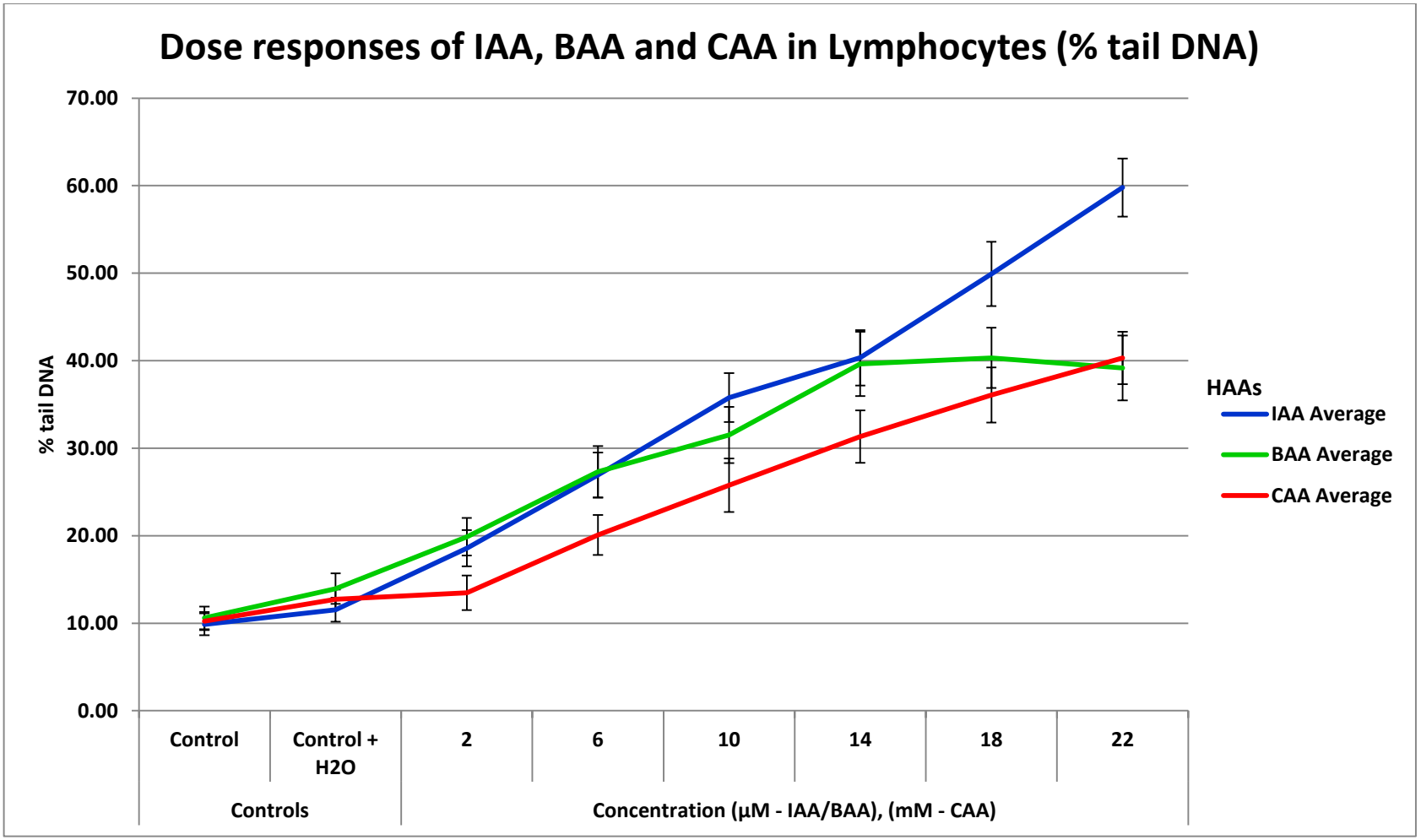
Sperm - % tail DNA: There is no significance (ns) of IAA & CAA with the highest dose of BHA (100 µM) in comparison to the negative control.

3.3.4 Confounding factors:

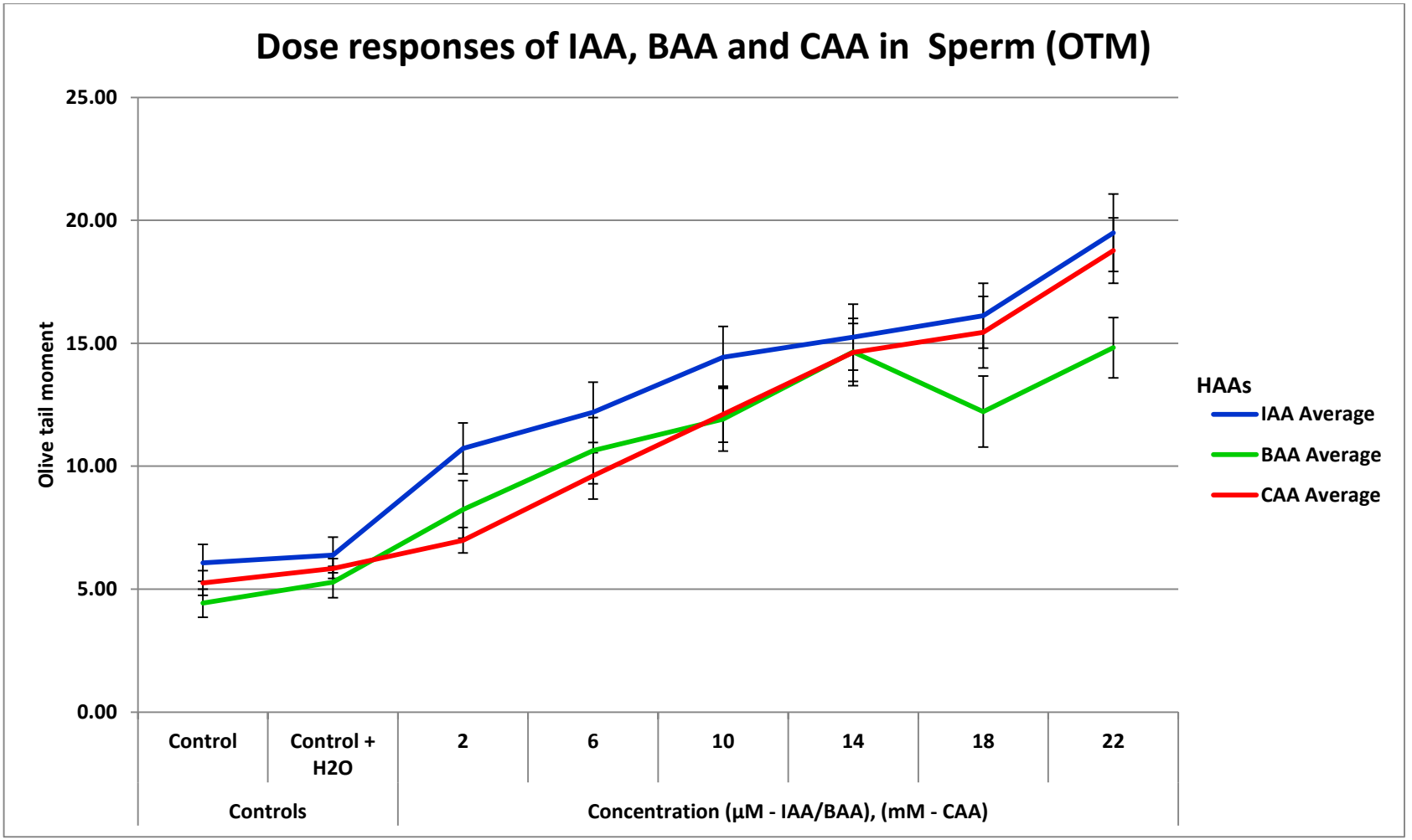
There were no confounding effects. All individuals were non-smokers. Approximately, 50 % were female and 50 % were male for the Comet assay and there appeared to be no differences due to gender. The age of the volunteers ranged from 21-40 years and the results for all individuals gave similar responses, regardless of age.



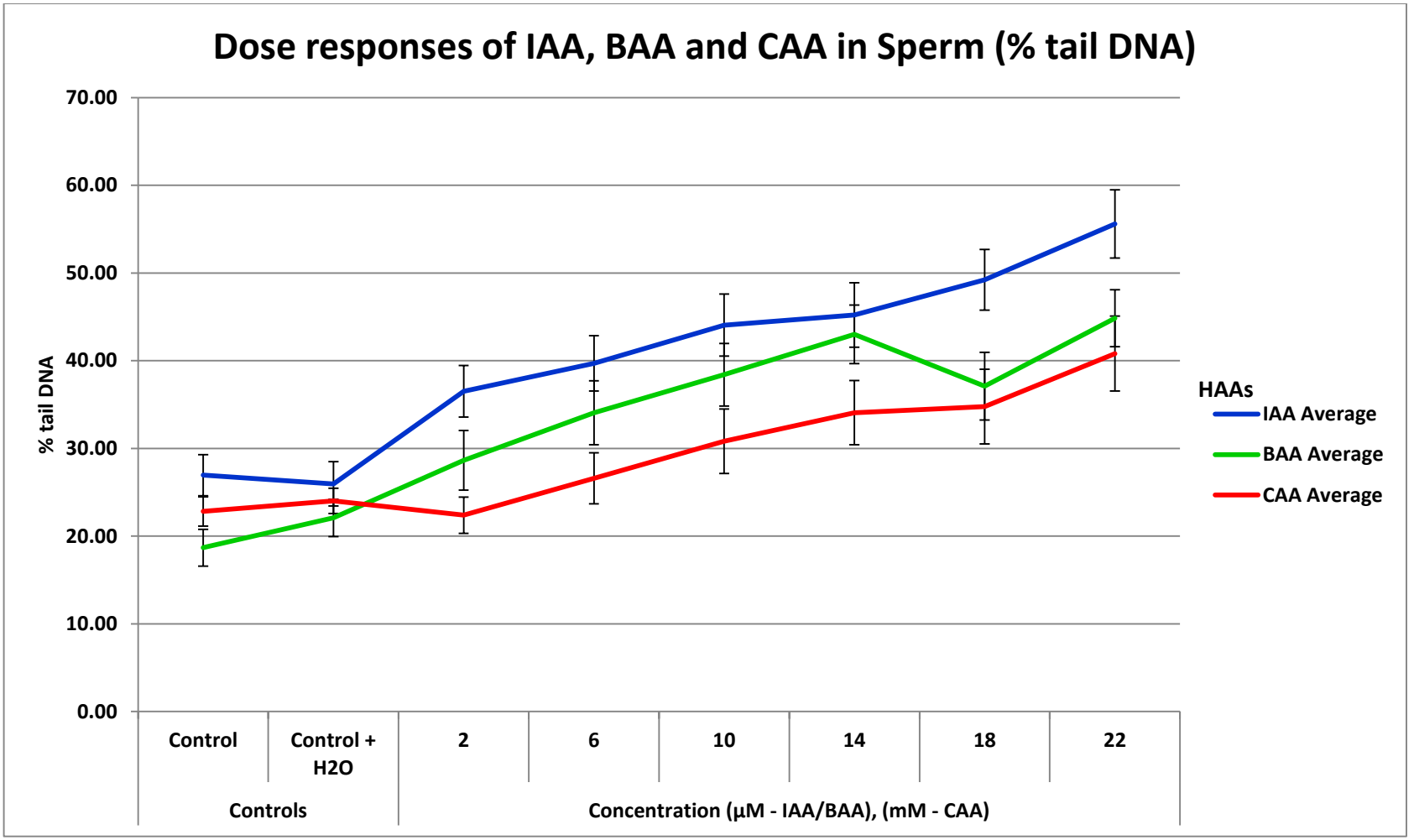
Graph 1a: Olive Tail moment: Graph shows the response of **lymphocytes** to IAA, BAA and CAA in the form of Olive tail moments. All curves increase over the range from 2µM to 22µM (mM For CAA) respectively after treatment with the three HAAs.



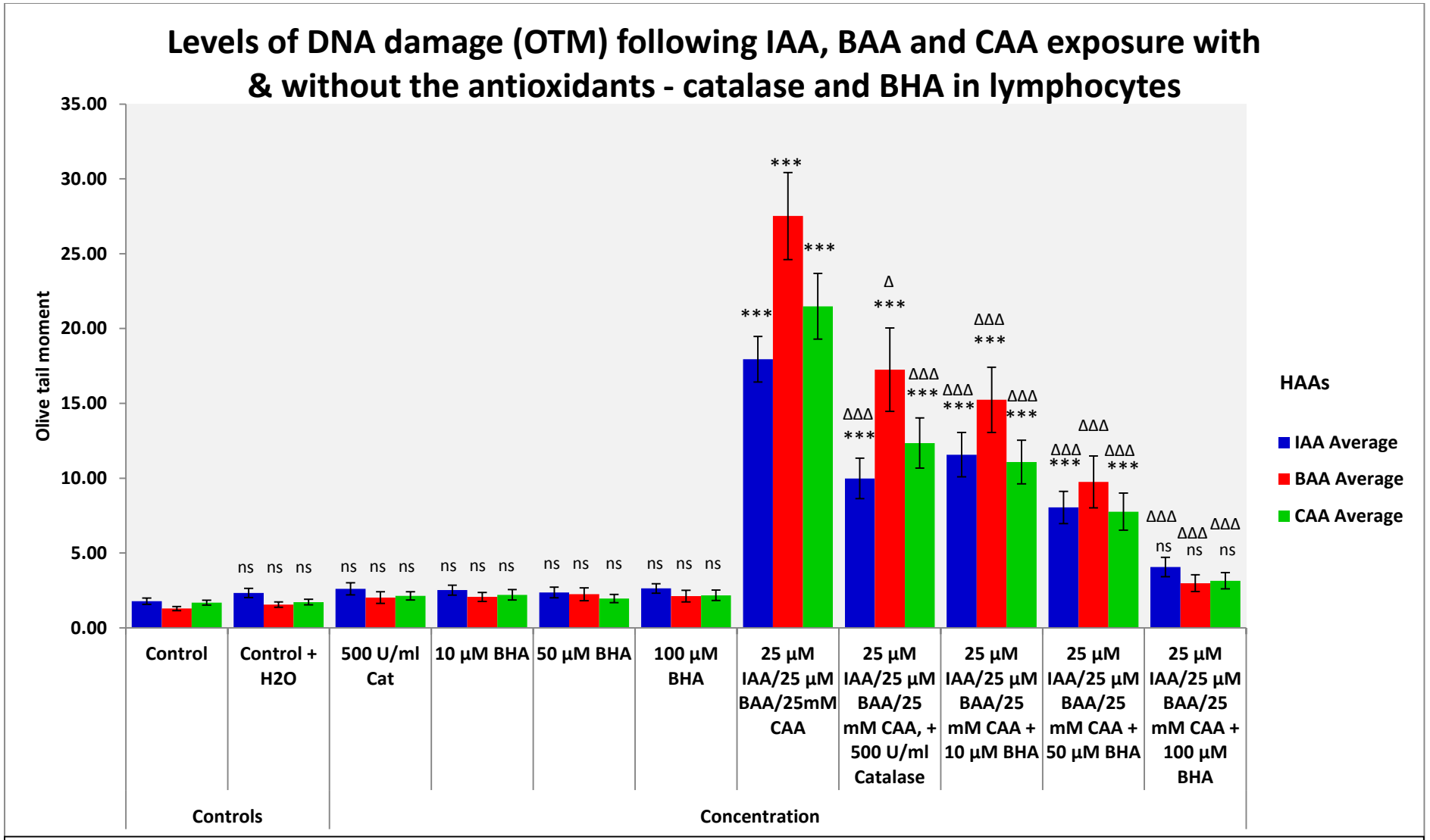
Graph 1b: % tail DNA: Graph shows the response of **lymphocytes** to IAA, BAA and CAA in the form of % tail DNA. All curves increase over the range from 2µM to 22µM (mM For CAA) respectively after treatment with the three HAAs.



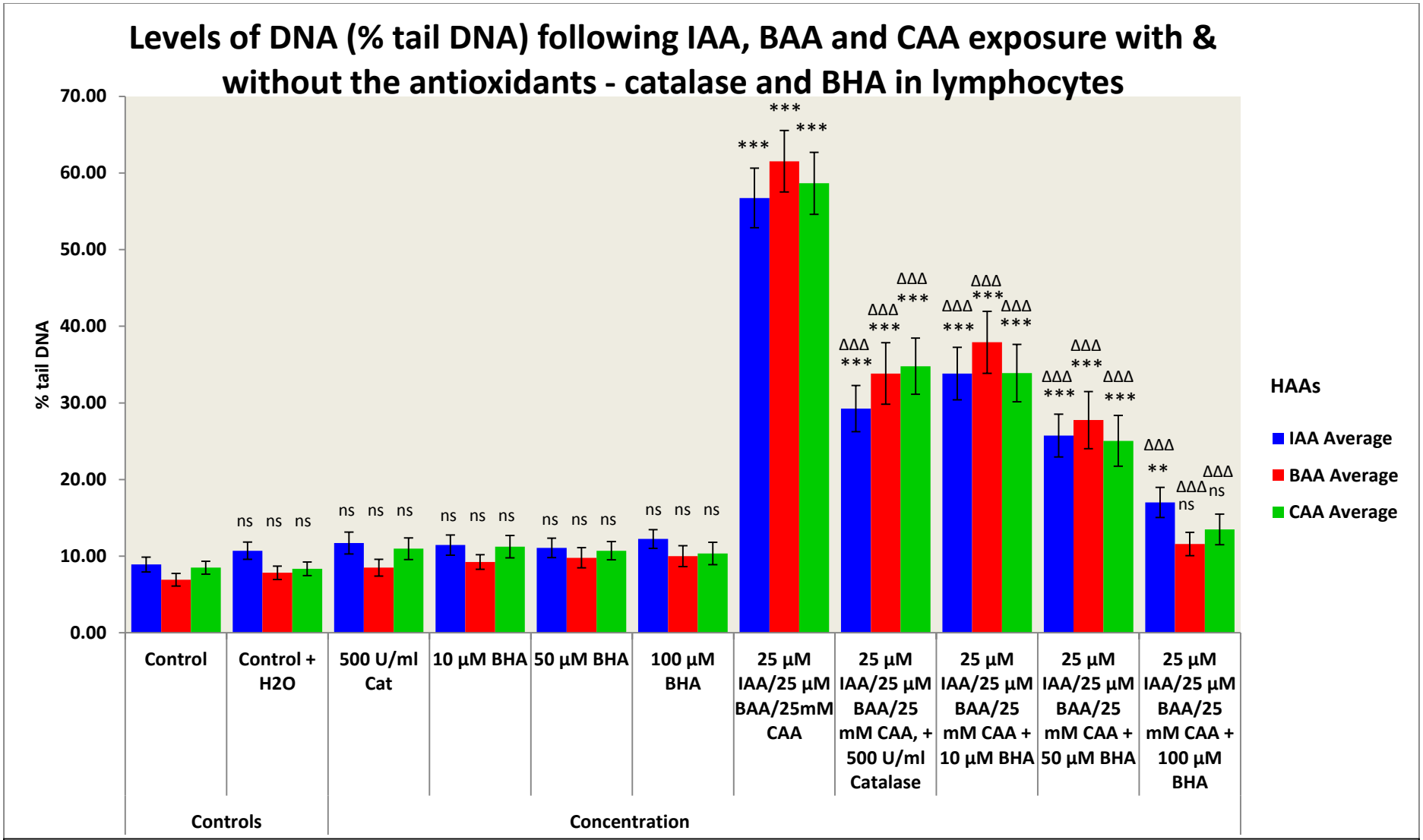
Graph 2a: Olive tail moment: Graph shows the response of **sperm** to IAA, BAA and CAA in the form of Olive tail moments % tail DNA. All curves increase over the range from 2µM to 22µM (mM For CAA) respectively after treatment with the three HAAs.



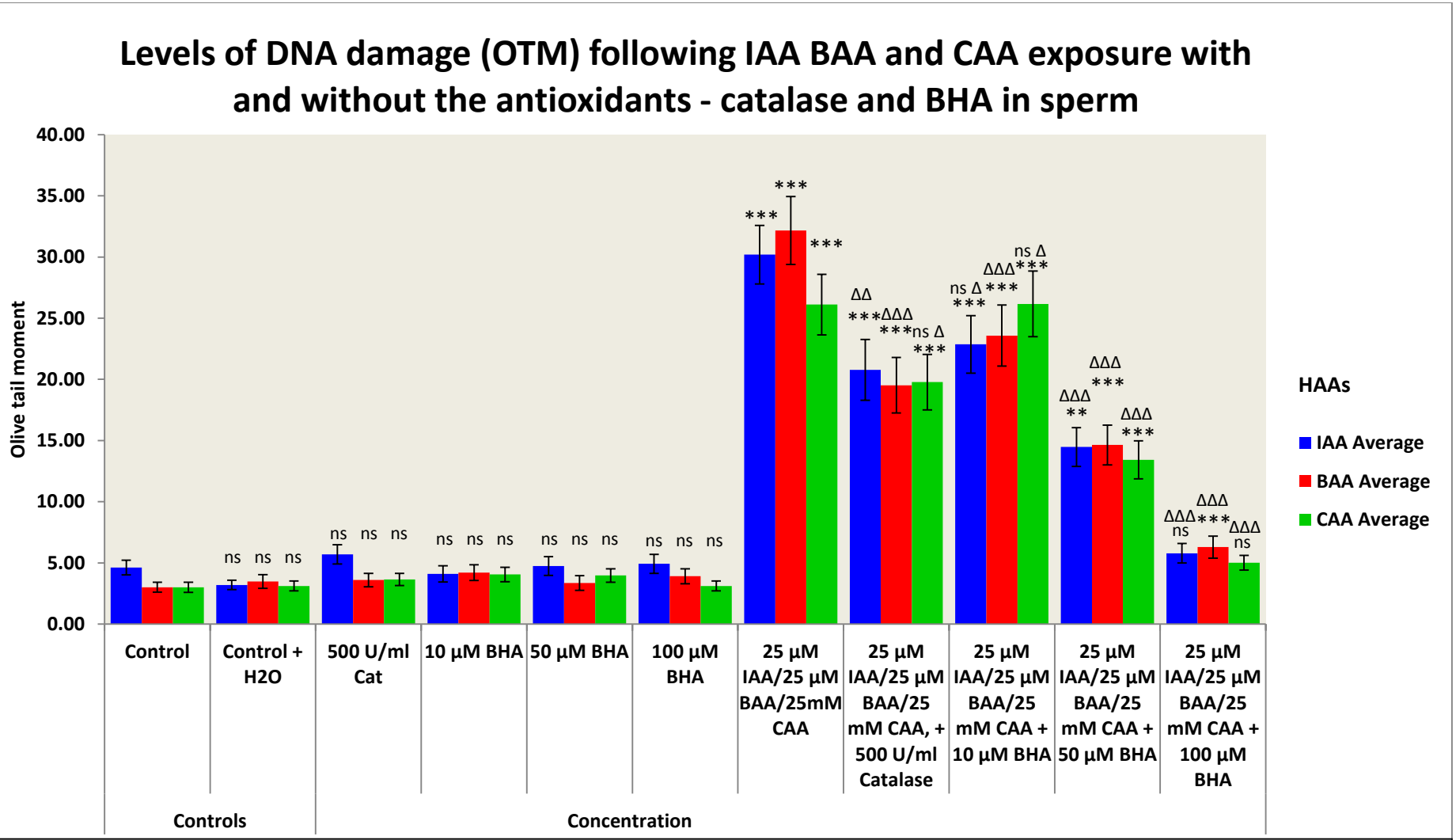
Graph 2b: % tail DNA: Graph shows the response of **sperm** to IAA, BAA and CAA in the form of % tail DNA. All curves increase over the range from 2µM to 22µM (mM For CAA) respectively after treatment with the three HAAs.



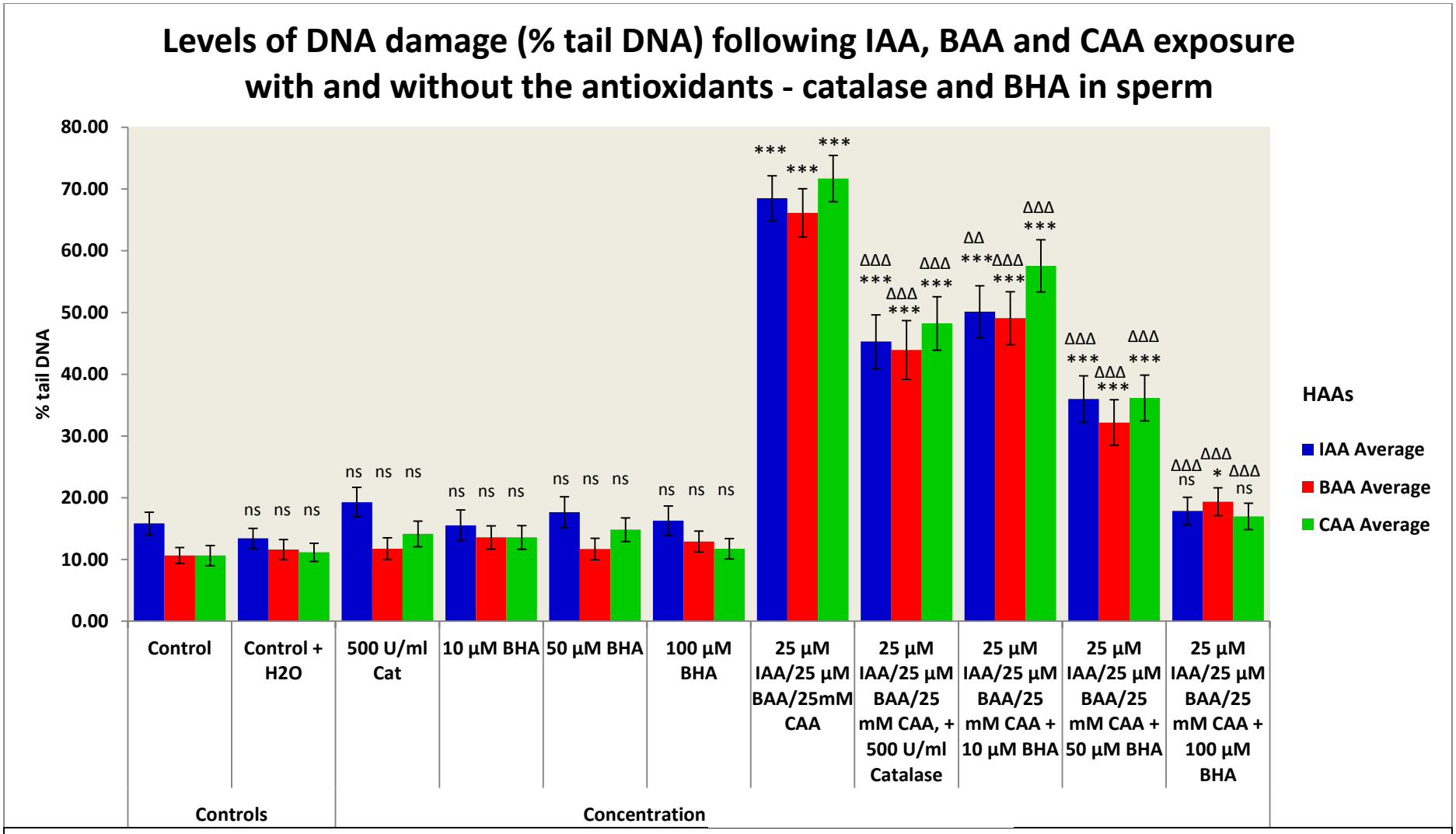
Graph 3a: Olive tail moment: Bar chart shows the response of **lymphocytes** to IAA, BAA and CAA. The HAA treatment group contains the highest DNA damage compared to controls. HAA treatment groups containing antioxidant result in decreased damage.



Graph 3b: % tail DNA: Bar chart shows the response of **lymphocytes** to IAA, BAA and CAA. The HAA treatment group contains the highest DNA damage compared to controls. HAA treatment groups containing antioxidant result in decreased damage.



Graph 4a: Olive tail moment: Bar chart shows the response of **sperm** to IAA, BAA and CAA. The HAA treatment group contains the highest DNA damage compared to controls. HAA treatment groups containing antioxidant result in decreased damage.



Graph 4b: % tail DNA: Bar chart shows the response of **sperm** to IAA, BAA and CAA. The HAA treatment group contains the highest DNA damage compared to controls. HAA treatment groups containing antioxidant result in decreased damage.

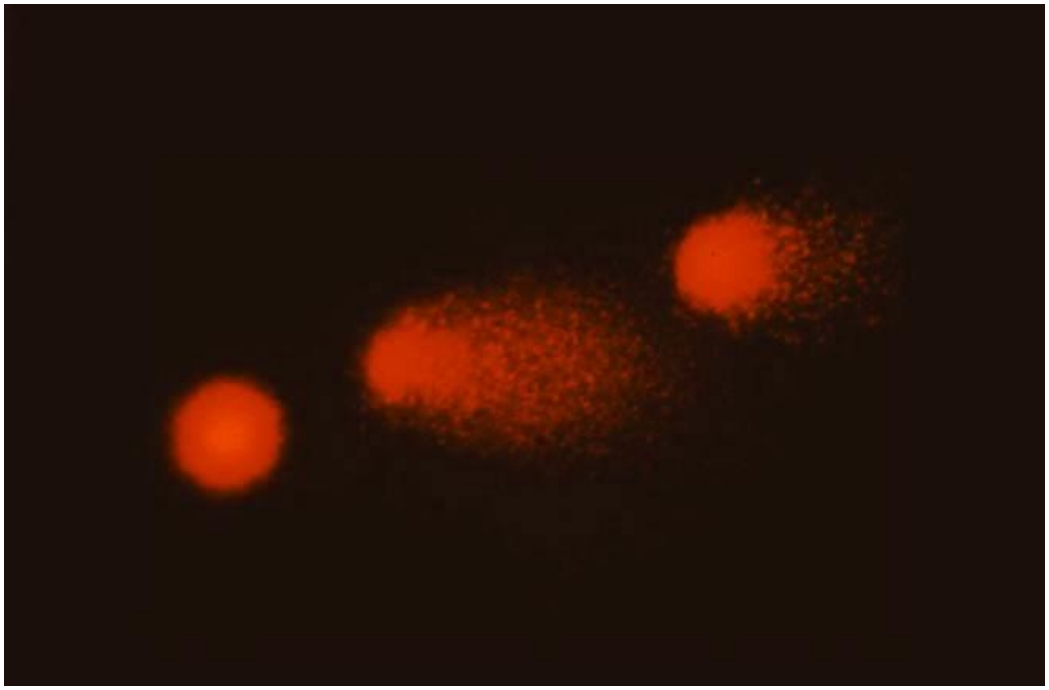


Figure 11: Typical image of lymphocyte Comets. Comets can be seen with minimal tail DNA damage (Left), high levels of damage (centre) and intermediate damage (right) (Image sourced from Professor Diana Anderson, University of Bradford, UK).

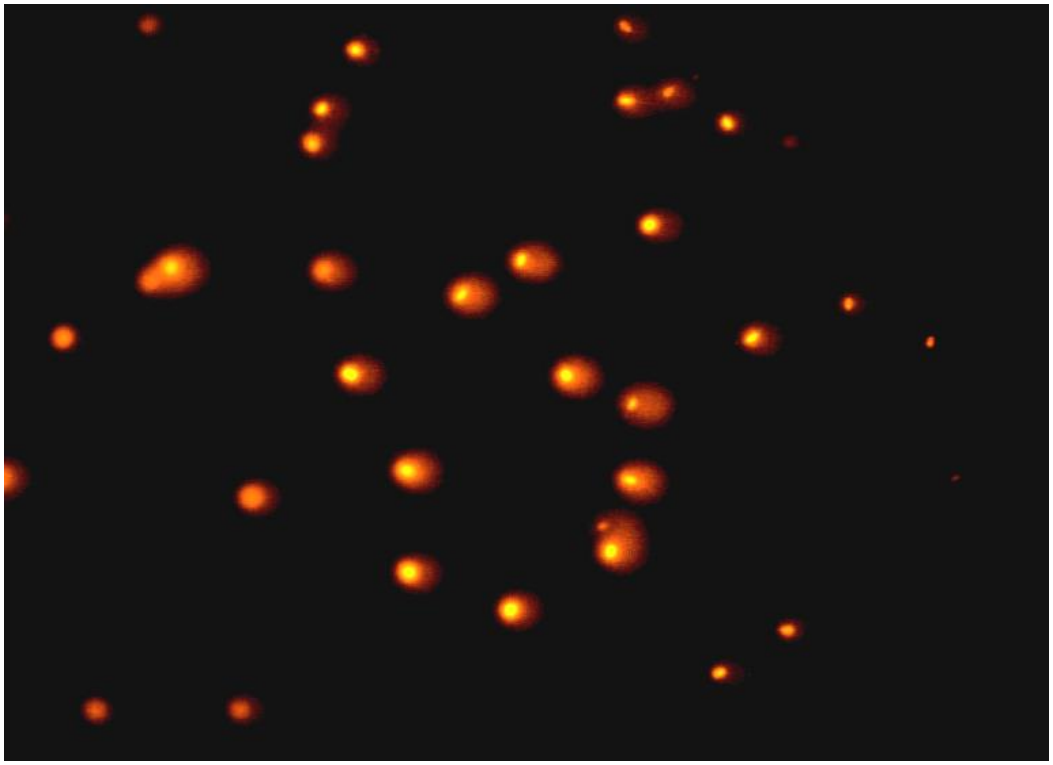


Figure 12: Typical image of sperm Comets (Image sourced from Professor Diana Anderson, University of Bradford, UK).

3.4 Discussion

In recent years, considerable interest has been shown in DBP genotoxicity, in particular the THM and HAA sub groups, some of which have been extensively identified (Richardson et al., 2008; Richardson et al., 2007). Genotoxicity assays have been used to determine the exact mechanisms on genotoxic actions posed by DBPs. HAAs are a major class of DBPs. This nine-membered HAA group is regulated to some extent in the United States but not in the United Kingdom (Bougeard et al., 2010). Furthermore, the list of DBPs is increasing by the day and the mechanisms and genotoxicity of most of them are yet to be identified (Richardson et al., 2007).

This investigation followed on the basis of the study by Cemeli et al. (2006) who has shown the involvement of oxygen radicals with IAA in these cells. In further developing our understanding of halo acetic acids, IAA was investigated in lymphocytes and sperm. The antioxidants butylated hydroxyanisole and catalase were also investigated to determine any oxygen radical involvement. To determine whether this applies to other HAAs in general and not just iodo haloacetic acid, two additional compounds - BAA and CAA were also included in this study.

Findings from this study suggest there are potential genotoxic effects involved and that there is oxygen radical involvement in both lymphocytes and sperm.

Results showed, all HAAs responded in a similar manner, producing similar dose responses over the same dose range in lymphocytes and sperm. In each

HAA, the highest dose in the range (22 µM for IAA and BAA, 22 mM for CAA) showed the greatest Olive tail moment and % tail DNA values compared to controls. The data suggested it would be feasible to work with the highest dose of each compound. For comparative purposes a concentration of 25 µM for IAA and BAA, 25 mM for CAA were selected. Antioxidants – BHA and catalase were used at same concentrations as done in the study of Cemeli et al. (2006).

After treatment with the anti-oxidants, all three HAAs were observed to respond in a similar way, with slightly more damage in Olive tail moment and % tail DNA in the sperm than in lymphocytes. A similar response pattern with sperm being more sensitive than lymphocytes has recently been shown by Baumgartner et al. (2012) with 12 genotoxic compounds. Also, the lymphocytes have shown a similar response in CHO cells when treated with IAA and BHA and catalase and our results thus support these earlier findings in mammalian cells (Cemeli et al., 2006).

These observations are of concern to public health since both somatic and germ cells show positive responses. These results open up the possibility of continuing research of these genotoxic compounds in *in*-vitro and *in-vivo* studies to try and develop a greater understanding of these compounds. Further testing of these cells with other similar HAAs such as diodoacetic acid, dibromoacetic, tribromoacetic acid, dichloroacetic acid, and trichloroacetic acid could enhance our understanding of HAAs.

Chapter 4 – Exposure of haloacetic acids (HAAs) in human peripheral blood using Cytokinesis-block micronucleus assay

4.1 Introduction

In the previous chapter (Chapter 3), results from the DBP group - HAAs: IAA, BAA and CAA showed the genotoxic DNA damage potential of these compounds, as seen via increases in Olive tail moment and % tail DNA using the Comet assay. The endogenous antioxidant – catalase, and the exogenous antioxidant – BHA, were both able to reduce the damage suggesting an oxygen radical involvement with these chemicals. With the Comet assay, it has been possible to detect genomic damage and these findings therefore indicate a need to investigate the same effect in cytogenetic assays. Therefore the aim of this chapter is to assess somatic cells – lymphocytes for cytogenetic damage using the cytokinesis-block micronucleus (CBMN) assay as an end-point.

The cell growth cycle is the process by which cells replicate their DNA, allowing them to divide into two daughter cells and eventually enter programmed cell death or apoptosis at the end of the life cycle. In eukaryotes it occurs over a 24 hr period. The cell cycle consists of four stages: - G1 phase (gap 1 phase), S phase (synthesis) of DNA, G2 phase (gap 2 phase) and M phase (mitosis) (Collins et al., 1997). The G1, S and G2 phases are classed as the interphase. In the G1 phase the cells grow and prepare for chromosome replication by synthesising enzymes necessary for S phase. During the S phase chromosomes are replicated to produce two identical copies and the G2 phase works to prepare the cells for cell division and synthesises cellular components needed for the M phase. The next stage, the M phase, is the most

important as cells undergo nuclear division through sub processes consisting of; prophase, prometaphase, metaphase, anaphase and telophase (Norbury and Nurse, 1992). Finally, the cycle comes to an end when the cell splits into two daughter cells (cytokinesis) (Vermeulen et al., 2003).

The CBMN assay uses cells undergoing the cell cycle division, such as binucleated cells (BiNC). These are cells that have undergone mitosis after being blocked at the cytokinesis stage with Cytochalasin B – which inhibits the microfilaments ring assembly important to end the process of cytokinesis (Fenech and Morley, 1985). Chemicals and exogenous agents can have mutagenic consequences on cells and interfere with chromosome structure and separation during the cell cycle (Fenech, 2007). As a consequence, micronuclei (MNi) arise and originate from acentric or whole chromosomes that lag behind the anaphase stage of mitosis. MNi induction in BiNC cells indicates cytogenetic damage. MNi can also be found in mononucleated (MonoNC) cells and multinucleated (MultiNC) cells; However scoring for MNi in BiNC cells is particularly important since restricting the scoring of MNi in BiNC cells prevents confounding effects caused by altered or suboptimal cell division kinetics, which is a major variable in the CBMN assay (Fenech, 2000, 2006).

In more recent developments, research suggests that the CBMN assay can be used to measure nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NBPs form a small narrow ‘bridge-like’ structure between two nuclei of a binucleated cell. NPBs act as a biomarker for dicentric chromosomes

which arise due to DNA mis-repair or telomere end-fusions. It is recommended to score these as they provide measures of chromosome rearrangement, which cannot be measured based on scoring MNi on their own (Umegaki and Fenech, 2000). NBUDs act as a biomarker for detecting gene amplification. Amplified DNA is localised at specific locations within the nuclei. During the S phase this amplified DNA is eliminated from the periphery of the nuclei via a nuclear 'budding' mechanism to form MNi-like buds. These buds remain linked to the main nuclei through nucleoplasmic material (Fenech, 2007).

MNi inductions in lymphocytes have been well documented in the CBMN assay (Fenech and Morley, 1985). The CBMN assay was performed to detect MNi inductions following HAA exposure, albeit at lower concentrations than in the Comet assay. In addition, lower catalase and BHA concentrations were also used (see chapter 3, results section 3.3) and mitomycin C was used as positive control. MNi induction was scored in BiNC, including BiNC with NPBs and BiNC with NBUDs as well as in MonoNC. All types of cells up to 500 were scored from which percentages of BiNC and MultiNC were calculated and together with the number of MonoNC, the nuclear division index (NDI) was calculated.

4.2 Materials and Methods

4.2.1 Materials

The materials required for this study have been described in section 2.3.1

4.2.2 Methods

The sample collection of whole blood was obtained as described in section 2.3.2 (see Table 2). The CBMN assay was deployed in three different experiments (each with different HAA) as described in sections 2.3.3 - 2.3.8. Treatment groups, including antioxidant treated groups per each experiment are described in section in section 2.3.4 (See Table 3).

4.2.4 Statistical Analysis.

All slides were coded and scored as described in protocols (Fenech, 2007). 500 of all cell types were scored for each sample to determine the percentages and NDI values (i.e. every mono, bi and multinucleate cells found in a total of 500 cells using a multiple counter). BiNC and MonoNC were then scored further until 500 cells each, observing for MNi found per 500 cells. A standard Chi-Squared test was performed for statistical analysis for MNi induction using the equation:

$$\chi^2 = \sum \frac{(o - e)^2}{e}$$

Where, o represents frequency of observed MNi, e represents frequency of expected MNi and ∑ represents the sum of (observed – expected MNi) ^{2} / 500 (The total possible MNi value achievable/ 500 BiNC and MonoNC scored.

The Chi-squared test was performed for significance of data to determine significant differences of all treatment groups to the negative control group and significant difference of HAA with antioxidant treatment groups to the HAA only treatment group. A *p* value was set at *** $p = < 0.001$.

4.3 Results

4.3.1 Determining concentrations of HAAs and antioxidants

Based on the Comet assay data, CBMN assay was initially performed using same doses, adjusting in preparation to take into account the end volume in T25 cm^2 flask at 5 ml. The concentrations of 25 μM for IAA and BAA were toxic and it was not possible to score the cells. CAA at 25 mM level was also toxic and cells could not be scored. A range of concentrations of these HAAs were used (diluting 1:1 each time) and it emerged 6.25 μM was the optimal concentration for IAA and BAA and 62.5 μM for CAA. Catalase responded well; however BHA at 10 μM, 50 μM and 100 μM were resulting in higher MNi compared to the negative control. These were revised until concentrations of 0.1 μM, 0.5 μM and 1.0 μM were deemed optimal.

4.3.2 Overview of results

Tables 6, 7 and 8 (after section 4.3.8, below) summarise the responses of IAA, BAA and CAA, respectively. In general, results are similar for all the three HAAs, with the greatest MNi inductions seen in BiNC in HAA treatment groups when compared to the negative control group and a reduction in the presence of catalase and/or BHA when compared to HAA alone. MNi induction caused by all three HAAs was less, but near to levels of MNi induction following exposure to mitomycin C (positive control). MNi in MonoNC were also evaluated and the results showed similar patterns to MNi in BiNC. However statistics from the Chi-squared showed some unusual results but generally indicate MNi reduction in all HAAs following antioxidant exposure. For NDI

values, IAA and CAA are more comparable, unlike BAA which shows higher NDIs and greater % of binucleated cells. The NDI values decrease in the order BAA > IAA > CAA (see individual sections 4.3.3-4.3.5).

4.3.3 Induction of micronuclei by iodoacetic acid (IAA)

Micronuclei were generated following genotoxic exposure to iodoacetic acid (see table 6, after section 4.3.8). A summary of the 11 treatment groups used in the experiment is summarised in the Materials and Methods section, see section (2.3.4, Table 3). In this experiment cells were treated with antioxidants only, 0.4 μM for MMC, 6.25 μM of IAA and 6.25 μM of IAA containing either catalase at 2,500 units/ml or BHA at 0.1 μM, 0.5 μM and 1.0 μM. For each treatment group, the number of MNi was also determined in 500 cells each of BiNC and MonoNC. In addition, the NDI index values were calculated and % of BiNC and MultiNC were determined in a total of 500 cells. Each experiment was repeated, twice (1000 cells total).

In the control group, there were approximately 59.2% of BiNC and 10.0% of MultiNC. Treatment with iodoacetic acid resulted in a decrease in both % of BiNC (2-fold less at 28%) and MultiNC (10 fold less at 1.6%), including IAA groups containing antioxidants. MMC exposure also resulted in a decreased % BiNC but the percentage was greater than most IAA/IAA with antioxidant groups. The % of MultiNC with MMC however, was low and comparable to all these groups. These results are reflected in the NDI values which show the negative control group having the highest NDI at 1.79 compared to all other treatment groups that have NDIs in the range of 1.40-1.64. All NDI values fall

in the normal expected range of 1.3-1.8 with lymphocyte cultures as described by Fenech (2006).

The effect of iodoacetic acid on MNi formation was measured in 500 cells of BiNC and MonoNC (See Table 6). The Chi-Squared test was performed to validate the significance of the data amongst individual treatment groups.

In BiNC cells, no significance difference of MNi was found by comparison with the negative control for the antioxidants on their own (ns), or IAA containing catalase and IAA containing 0.1, 0.5 and 1.0 μM BHA. With, MMC and IAA alone there is a significant difference of *** $p = < 0.001$ and ** $p = < 0.01$, respectively when compared to the negative control. This shows that catalase and all three doses of BHA (0.1, 0.5 and 1.0 μM) are able to reduce MNi formation. When IAA is treated with catalase or the highest dose of BHA (1.0 μM), there is a reduction in MNi compared to IAA alone († $p = < 0.05$), but in IAA with BHA 0.1 μM and IAA with 0.5 μM BHA there are no significances (ns Δ) compared to IAA alone.

In MonoNC, the results are generally similar to BiNC. Other findings show a smaller number of BiNPB in some groups.

4.3.4 Induction of micronuclei by bromoacetic acid (BAA)

BAA shows results similar to those obtained with exposure to IAA (See Table 7). In the control group, there were approximately 59.3% of BiNC and 13.2% of MultiNC. Unlike IAA, treatment with bromoacetic acid did not cause a

decrease of both % of BiNC and MultiNC. The % is generally 45-60% BiNC and 5-15% MultiNC for each group. These results are reflected in the higher NDI values compared to IAA and CAA (see section below) where all groups including negative control show NDIs in the range of 1.63-1.83 with exception of groups with catalase (1.50). All NDI values fall in the normal expected range of 1.7-1.85 with lymphocyte cultures as described by Fenech (2006).

The effect of bromoacetic acid on MNi formation was measured in 500 cells of BiNC and MonoNC. In BiNC cells, no significant difference of MNi was found by comparison with the negative control for the antioxidants on their own (ns), or BAA containing catalase and all three doses of BHA (0.1, 0.5 and 1.0 μM). With, MMC and BAA alone there is a significant difference of *** p = < 0.001 and ** p = < 0.01, respectively when compared to the negative control. This shows that catalase and all three doses of BHA (0.1, 0.5 and 1.0 μM) are able to reduce MNi formation as is case with the IAA. When BAA is treated with catalase there is a reduction in MNi compared to BAA alone († p = <0.05) while BAA with the 0.1 and 0.5 μM doses of BHA are not significant (ns Δ) compared to BAA alone. However, the higher dose of BHA (1.0 μM) is significant († p = <0.05).

In MonoNC, the results are generally similar to BiNC, where treatment of samples with BAA alone is significantly different compared to the negative control at († p = < 0.05). BAA treated with catalase and 0.1 μM BHA are not significantly different (Δ p = < 0.05) compared to BAA alone, but higher doses of BAA and BHA (0.5 μM and 1.0 μM) are significant († p = < 0.05). Like IAA, in addition to NPBs, NBUDs were also observed in some treatment groups.

4.3.5 Induction of micronuclei by chloroacetic acid (CAA)

CAA shows similar patterns of results to IAA (See Table 8). In terms of % BiNC and % MultiNC, like IAA, the negative control group contain more binucleated cells - 52% of BiNC, slightly more compared to most other treatment groups including the positive control. The % of MultiNC, is 11.3%, slightly more than all other treatment groups. The NDI value of negative control is 1.74, while the NDI values for rest of all treatment groups is in the range of 1.45-1.70, falling within normal NDI range for lymphocytes.

The effect of chloroacetic acid at the micromole (μM) level on MN formation was measured in 500 cells of BiNC and MonoNC. In BiNC cells, no significant difference of MNi was found by comparison with the negative control for the antioxidants on their own (ns), or CAA containing catalase and CAA containing and all three doses of BHA (0.1, 0.5 and 1.0 μM). With, MMC and CAA alone there is a significant difference of *** $p = < 0.001$ and ** $p = < 0.01$, respectively when compared to the negative control. When CAA is treated with catalase or the highest dose of BHA (1.0 μM), there is a reduction in MNi compared to CAA alone († $p = <0.05$) but CAA with BHA 0.1 μM and CAA with 0.5 μM BHA are not significant (ns †) compared to CAA alone, similar to IAA and BAA.

In MonoNC, the results are generally similar to BiNC. MMC and CAA are significantly different compared to the negative control at *** $p = < 0.001$ and * $p = < 0.05$, respectively. Unlike BAA in MonoNC, neither antioxidant is able to reduce MN formation (ns †).

4.3.6 General overview of all HAAs:

In general, results are similar for all three HAAs, with the greatest micronuclei (MNi) induction seen in bi-nucleated (BiNC) cells in the HAA treated samples when compared to the negative control group ($p = ** < 0.01$) and a reduction in the presence of catalase and/or BHA when compared to HAA alone. MNi induction caused by all three HAAs was less, but near to levels of MNi induction following exposure to MMC (positive control), which showed the greatest significant difference for all three HAAs experiments of $p = *** <0.001$.

4.3.7 Statistics:

Statistics from the Chi-squared test generally indicated MNi reduction in all HAAs following antioxidant exposure to catalase or BHA 1.0 µM with a significant difference of $p = † < 0.05$ in BiNC cells. The nuclear division index (NDI) values are the same for all three HAAs, within the normal expected range of 1.3-2.0.

4.3.8 Confounding factors:

There were no confounding effects. All individuals were non-smoking. Approximately, 50 % were female and 50 % were male for the micronucleus assay and there appeared to be no differences due to gender. The ages

ranged from 21-40 years and results for all individuals gave similar responses, regardless of age.

Table 6: shows various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC and MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes following exposure to iodoacetic acid. In general, MNi induction increase in presence of IAA but reduce with addition of antioxidants as shown by the above cytological scoring parameters.

	Treatment Group	NDI	% BiNC	% Multi	MNi in BiNC cells						Mono	
					BiMNi		BiNPB		BiBuds		MNi	
IAA	Control	1.79 ± 0.02	59.20 ± 2.60	10.10 ± 2.10	**2.5**	± 2.50	0	± 0.00	0	± 0.00	**1**	± 1.00
	MMC (0.4 µM)	1.61 ± 0.03	49.10 ± 4.30	5.30 ± 1.30	**62.00*** **	± 4.00	0	± 0.00	0.5	± 0.50	**21.00 ns**	± 14.50
	Catalase (2,500 U/ml)	1.57 ± 0.13	50.40 ± 15.00	3.20 ± 1.40	**0.50 ns**	± 0.50	0	± 0.00	0	± 0.00	**1.00 ns**	± 1.00
	BHA (0.1 µM)	1.63 ± 0.02	46.70 ± 3.70	8.00 ± 0.60	**7.50 ns**	± 2.50	0.5	± 0.50	0	± 0.00	**4.00 ns**	± 2.50
	BHA (0.5 µM)	1.64 ± 0.28	47.10 ± 13.50	8.30 ± 7.10	**2.00 ns**	± 1.00	0	± 0.00	0	± 0.00	**1.00 ns**	± 4.50
	BHA (1 µM)	1.60 ± 0.15	44.70 ± 11.50	7.70 ± 1.70	**5.50 ns**	± 1.50	1	± 1.00	1	± 1.00	**0.00 ns**	± 3.50
	IAA (6.25 µM)	1.40 ± 0.09	40.80 ± 12.80	1.50 ± 0.10	**48.00****	± 3.00	0	± 0.00	0	± 0.00	**28.00 ns**	± 11.50
	IAA (6.25 µM) + Catalase (2,500 U/ml)	1.44 ± 0.11	42.90 ± 11.10	0.23 ± 0.18	**15.50 ns (†)**	± 1.50	0	± 0.00	0	± 0.00	**8.00 ns (ns†)**	± 14.00
	IAA (6.25 µM) + BHA (0.1 µM)	1.55 ± 0.16	40.10 ± 7.70	7.80 ± 4.20	**28.50 ns (ns†)**	± 14.50	3	± 3.00	0.5	± 0.50	**2.00 ns (ns†)**	± 22.00
	IAA (6.25 µM) + BHA (0.5 µM)	1.43 ± 0.06	39.90 ± 8.10	1.25 ± 1.15	**28.50 ns (ns†)**	± 11.50	1.5	± 1.50	0.5	± 0.50	**2.00 ns (ns†)**	± 19.50
	IAA (6.25 µM) + BHA (1.0 µM)	1.59 ± 0.07	52.60 ± 4.80	3.10 ± 1.50	**15.00 ns (†)**	± 3.00	1	± 1.00	0	± 0.00	**4.00 ns (ns†)**	± 5.50

NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MonoNC = Mononucleated cells
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC and MonoNC

Chi-Squared Statistics X^2 was used determine significant differences:
Where ***** is used to compare all groups to the negative control. ***** p = < 0.05, ****** p = < 0.01, ******* p = < 0.001 and * ns '(ns*****)' = not significant.
Where † is used to compare IAA with antioxidant to IAA alone. † p = < 0.05, † p = < 0.01, † p = < 0.001 and † ns '(ns†)' = not significant.

Table 7: shows various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, and MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes following exposure to iodoacetic acid. In general, MNi induction increase in presence of BAA but reduce with addition of antioxidants as shown by the above cytological scoring parameters.

	Treatment Group	NDI	% BiNC	% Multi	MNi in BiNC cells			Mono
					BiMNi	BiNPB	BiBuds	MNi
BAA	Control	1.86 ± 0.03	59.30 ± 2.70	13.20 ± 3.20	**3** ± 1.00	0 ± 0.00	0 ± 0.00	**0.5** ± 0.50
	MMC (0.4 µM)	1.74 ± 0.11	56.60 ± 4.80	12.50 ± 1.10	**56.00 ***** ± 3.00	1.5 ± 1.50	0.5 ± 0.50	**21.50 ns** ± 14.50
	Catalase (2,500 U/ml)	1.68 ± 0.25	53.40 ± 15.00	7.50 ± 4.90	**3.00 ns** ± 2.00	0 ± 0.00	0 ± 0.00	**1.00 ns** ± 1.00
	BHA (0.1 µM)	1.73 ± 0.10	55.80 ± 0.80	16.50 ± 0.50	**1.50 ns** ± 0.00	0 ± 0.00	0 ± 1.50	**1.50 ns** ± 11.10
	BHA (0.5 µM)	1.82 ± 0.02	67.90 ± 4.10	12.20 ± 6.20	**2.00 ns** ± 1.00	0 ± 0.00	0 ± 0.00	**0.50 ns** ± 0.50
	BHA (1 µM)	1.74 ± 0.10	66.70 ± 5.70	7.50 ± 6.30	**3.00 ns** ± 1.00	0.5 ± 0.50	0 ± 0.00	**0.00 ns** ± 0.00
	BAA (6.25 µM)	1.69 ± 0.08	57.40 ± 4.20	6.80 ± 3.20	**50.00 **** ± 2.00	1.5 ± 0.50	0.5 ± 0.50	**36.00 *** ± 8.00
	BAA (6.25 µM) + Catalase (2,500 U/ml)	1.50 ± 0.08	44.00 ± 4.40	3.70 ± 0.90	**16.50 ns (†)** ± 1.50	0 ± 0.00	1 ± 1.00	**8.50 ns (ns†)** ± 2.50
	BAA (6.25 µM) + BHA (0.1 µM)	1.74 ± 0.09	55.10 ± 4.90	15.50 ± 9.70	**32.00 ns (ns†)** ± 10.00	0.5 ± 0.50	0 ± 0.00	**21.50 ns (ns†)** ± 6.50
	BAA (6.25 µM) + BHA (0.5 µM)	1.76 ± 0.00	62.70 ± 3.70	11.40 ± 3.00	**24.50 ns (ns†)** ± 12.50	0.5 ± 0.50	0 ± 0.00	**3.50 ns (†)** ± 0.50
	BAA (6.25 µM) + BHA (1.0 µM)	1.78 ± 0.02	51.30 ± 2.70	13.10 ± 2.10	**18.00 ns (†)** ± 8.00	0.5 ± 0.50	0 ± 0.00	**7.50 (ns †)** ± 7.50

NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MonoNC = Mononucleated cells
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC and MonoNC

Chi-Squared Statistics X^2 was used determine significant differences:
Where ***** is used to compare all groups to the negative control. ***** p = < 0.05, ****** p = < 0.01, ******* p = < 0.001 and * ns '(ns*****)' = not significant.
Where † is used to compare BAA with antioxidant to BAA alone. † p = < 0.05, † p = < 0.01, † p = < 0.001 and † ns '(ns†)' = not significant.

Table 8: shows various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, and MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes following exposure to iodoacetic acid. In general, MNi induction increase in presence of CAA but reduce with addition of antioxidants as shown by the above cytological scoring parameters.

	Treatment Group	NDI		% BiNC		% Multi		MNi in BiNC cells						Mono	
								BiMNi		BiNPB		BiBuds		MNi	
CAA	Control	1.74	± 0.28	52.00	± 6.20	11.30	± 10.90	**3**	± 1.00	0	± 0.00	0	± 0.00	**1.5**	± 1.50
	MMC (0.4 µM)	1.62	± 0.27	47.90	± 12.90	7.10	± 6.50	**58.50*** **	± 4.50	3	± 1.00	1.5	± 0.50	**57.50*** **	± 33.50
	Catalase (2,500 U/ml)	1.59	± 0.21	45.20	± 12.80	7.10	± 4.10	**1.50 ns**	± 0.50	0	± 0.00	0	± 0.00	**1.50 ns**	± 1.50
	BHA (0.1 µM)	1.56	± 0.22	43.20	± 12.80	5.90	± 5.10	**3.50 ns**	± 0.50	0	± 0.00	0	± 0.00	**3.00 ns**	± 0.00
	BHA (0.5 µM)	1.61	± 0.23	44.80	± 7.20	8.90	± 7.10	**3.50 ns**	± 1.50	0	± 0.00	0	± 0.00	**3.00 ns**	± 3.00
	BHA (1 µM)	1.47	± 0.05	44.80	± 11.20	2.90	± 1.30	**3.00 ns**	± 0.00	0	± 0.00	0	± 0.00	**3.00 ns**	± 0.00
	CAA (62.5 µM)	1.59	± 0.27	44.40	± 13.20	7.50	± 6.70	**45.00** **	± 7.00	1	± 1.00	1	± 1.00	**40.50***	± 20.50
	CAA (62.5 µM) + Catalase (2,500 U/ml)	1.55	± 0.17	44.00	± 12.20	5.30	± 2.50	**14.00 ns (†)**	± 2.50	1.5	± 1.50	0.5	± 0.50	**24.00 ns (ns†)**	± 11.00
	CAA (62.5 µM) + BHA (0.1 µM)	1.59	± 0.21	46.60	± 9.00	6.20	± 6.20	**24.50 ns (ns†)**	± 11.50	0	± 0.00	0.5	± 0.50	**22.50 ns (ns†)**	± 17.50
	CAA (62.5 µM) + BHA (0.5 µM)	1.51	± 0.10	38.90	± 5.10	5.80	± 2.60	**21.00 ns (ns†)**	± 18.00	0.5	± 0.50	1.5	± 1.50	**23.50 ns (ns†)**	± 19.50
	CAA (62.5 µM) + BHA (1.0 µM)	1.56	± 0.18	43.60	± 10.00	6.20	± 4.20	**12.50 ns (†)**	± 8.50	1.5	± 0.50	0.5	± 0.50	**21.00 ns (ns†)**	± 19.50

NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MonoNC = Mononucleated cells
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC and MonoNC

Chi-Squared Statistics X^2 was used determine significant differences:
Where ***** is used to compare all groups to the negative control. ***** p = < 0.05, ****** p = < 0.01, ******* p = < 0.001 and * ns '(ns*****)' = not significant.
Where † is used to compare CAA with antioxidant to CAA alone. † p = < 0.05, † p = < 0.01, † p = < 0.001 and † ns '(ns†)' = not significant.

4.4 Discussion

The CBMN assay is a test deployed to measure the ability of genotoxic agents to induce both clastogenic and/or aneugenic effects in cells (Fenech, 2000; Liviac et al., 2010). Haloacetic acids have not been examined or reported as such using the Cytokinesis-block Micronucleus assay, in particular with lymphocytes. This opened up the feasibility to explore and establish MNi induction in lymphocytes exposed to HAAs using this technique. As discussed in the introduction, ingestion, showering, bathing and swimming are alternative routes of exposure to DBPs in addition to tap water consumption (Kogevinas et al., 2010; Nieuwenhuijsen et al., 2000). The closely related DBPs group – the trihalomethanes (THMs) have been studied by Kogevinas et al. (2010). Blood obtained from swimmers exposed to chlorinated water was obtained before and an hour after swimming and it was found that MNi frequency was increased in lymphocytes after swimming, with higher concentrations of brominated THMs. However, another study has demonstrated that five doses each of IAA, BAA and CAA were not able to increase significantly the frequency of MNi in BiNC TK6 cells (Liviac et al., 2010).

In this study, results from the CBMN assay performed on lymphocytes exposed to IAA, BAA and CAA has demonstrated MNi induction with all HAAs, unlike in TK6 cells. Together with the findings of (Kogevinas et al., 2010) these results suggest that MNi can form in lymphocytes when exposed to DBPs and whilst in TK6 lymphoblastoid cells this is not the case. However, further work will be needed to confirm this point (repeating of experiments), since the data for the micronucleus test only act as an indicator at this point. Although the

general trend shows greater MNi frequency in HAA alone and a reduction in the presence of either catalase or BHA or both, there are differences of MNi in binucleated cells and mononucleated cells, where for example with CAA, MNi in BiNC are significantly reduced with exposure to CAA in the presence of catalase or 1.0 μM BHA, but not in the MonoNC and there are cases of the opposite effects as in IAA and BAA, suggesting cell division kinetics may be a factor. However as mentioned by Fenech (2006), restricting MNi data in BiNC is important as it addresses any confounding effects due to suboptimal cell division kinetics. The Chi-squared data showed catalase as being effective antioxidant in reducing MNi in all three HAAs; however, data for BHA showed mixed results but nevertheless the results provides a good indication and direction for further investigation.

Chapter 5 – Implications of nano-compounds in lymphocytes from on respiratory disease patients using the Cytokinesis-block Micronucleus assay

5.1 Introduction

Good health and wellbeing is an important aspect of life. Healthcare scientists and professionals around the world work to address health issues and find solutions, diagnosis and treatments to diseases. Respiratory diseases, like asthma and lung cancer are amongst the most common health conditions affecting individuals. In this project respiratory diseases (asthma, COPD and lung cancer) have been the focus of the study.

Asthma is a common and potentially serious chronic disease, affecting people of all ethnic backgrounds, children and adults alike. The disease is characterised by reversibility of airways constriction, with recurring symptoms, like coughing, wheezing, tightness of the chest and shortness of breath. Serious consequences of the condition can lead to individuals with the conditions to have, limitations to activity, and asthma attacks and can prove fatal. The disease itself is affected by genetics and/or the environmental factors and often the inflammatory nature of this disease affects people with asthma at night or in the morning (Buist, 2002; Masoli et al., 2004).

Chronic obstructive pulmonary disease (COPD), is also a chronic inflammatory disease, and has similar features to asthma. These include, small airways, limited airflow, and also arises due to genetic and environmental factors and is characterised by mucus and bronchoconstriction (Buist, 2002). Cigarette smoking is the major cause of COPD (Bhattacharya et al., 2011). However, the difference between asthma and COPD patients is the type of inflammation which can subsequently affect the response to pharmacological agents. In asthma the airway can be remodelled, with partly

reversible airflow obstruction, but COPD is a disease, in which the air flow limitation is not fully reversible. This limitation is slowly progressive and usually results from an abnormal response of the lungs to noxious particles or gas (Bhattacharya et al., 2011; Buist, 2002)

Lung cancer is a leading cause of cancer, with 1.4 million deaths worldwide (Thun et al., 2008). In lung cancer, uncontrolled hallmarks of cancer, like cell proliferation are triggered by exposure to chemicals in cigarette smoke, either through primary exposure (smoking) or indirect exposure to second hand smoke. Common symptoms include, weight loss, coughing, wheezing and shortness of breath (Thun et al., 2008). If there is another factor involved in combination with smoking, such as exposure to asbestos, the chance of lung cancer incidence greatly increases (O'Reilly et al., 2007). Hence, on-going research is important to help and manage individuals with these conditions. In this chapter, blood from healthy control individuals, asthma, COPD and lung cancer patients have been analysed using the CBMN assay after exposure to NSAIDs.

The significance of the Cytokinesis-block micronucleus assay (CBMN) assay, has been described in the previous chapter (4), discussing the importance of using the cell cycle regulation as a means of detecting damaged DNA in cells that have been exposed to genotoxic agents (Collins et al., 1997; Fenech, 2000). The assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably. Certain chemicals can be mutagenic and may lead to the induction of micronuclei in cells at the interphase stage. This occurs as a consequence of interference with

chromosome structure and/or segregation (Fenech, 2002, 2007; Kirsch-Volders et al., 1997; Kirsch-Volders et al., 2000).

Various studies have shown the direct effects of NSAIDs on tumour biology, with an anti-tumour effect on several of the hallmarks of cancer, including proliferative capacity, evasion of apoptosis and cell cycle regulation, and invasive capability of tumour cells (Park et al., 2014). The focus of this study is to observe the effects of NSAIDs in patients with respiratory diseases. DNA damage in peripheral whole blood of healthy individuals and respiratory disease patients (asthma, chronic COPD and lung cancer) have been compared following the exposure to the bulk and nanoparticle versions of aspirin and ibuprofen in the and in the micronucleus assay and the nano compounds were compared to their bulk equivalents for each blood sample. This is the first study to our group's knowledge that the nano forms of these compounds have been investigated at the cellular level *ex-vivo* / *in-vitro*.

The CBMN assay was performed to detect MNi inductions following exposure to the NSAIDs - ASP B, ASP N, IBU N and IBU B at concentrations of 250 µg/ml. 13 samples were used in this study, 3 healthy control individuals, 2 asthma patients, 2 COPD patients, 3 COPD & lung nodule patients (which were combined), and 3 lung cancer patients. MNi induction was scored in BiNC, including BiNC with NPBs and BiNC with NBUDs as well as in MonoNC. All types of cells up to 1000 were scored, from which percentages of BiNC and MultiNC were calculated and together with the number of MonoNC, the nuclear division index (NDI) was calculated.

5.2 Materials and Methods

5.2.1 Materials

The materials required for this study have been described in section 2.4 of Materials and Methods chapter.

5.2.2 Methods

The sample collection of whole blood was obtained as described in section 2.5.2. The CBMN assay was deployed in thirteen different experiments as described in sections 2.5.3 -2.5.8. Treatment of standard controls, ASP B, ASP N, IBU B and IBU N for each experiment are described in section in section 2.5.4 (See Table 5).

5.2.3 Statistical Analysis.

All slides were coded and scored as described in protocols (Fenech, 2007). A total of 1000 of all cell types (mononucleated, binucleated and multinucleated cells) were scored to determine percentages and NDI values. BiNC and MonoNC were then scored further until 1000 cells each, observing for MNi found per 1000 cells. A standard Chi-Squared test was performed for statistical analysis for MNi induction using the equation as described in chapter 4.

5.3 Results

5.3.1 Particle size analysis

The particle size distribution of aspirin and ibuprofen nano-suspensions (Table 9a, below) and bulk powders (Table 9b, below) were determined using the dynamic light scattering technique of the Zetasizer Nano ZS machine, (See Table 9a and 9b below).

Images of the nanoparticles were obtained using transmission electron microscopy (TEM), as described in Materials and Methods (Section 2.4.2 and 2.4.3). The images showed that the aspirin crystals are slightly larger than those of ibuprofen as was shown by the laser diffraction measurements (see Figures 13 & 14 below). The images show some smaller particles on the side which might be small crystals of the drugs or it could be aggregates of the polymer used to stabilise the crystals.

5.3.2 Preliminary work

Three different dosages of both bulk and nano, of aspirin and ibuprofen each, were used to test for MNi frequency in blood samples. The number of MNi is an indicator of DNA damage. The doses used were 250, 500, 1000 µg/ml. In the tests it was determined that the 250 µg/ml dosage was optimum to use for all four compounds without inducing total cell cytotoxicity. Graph(s) 5a-b (below), are a visual representation of the MNi frequencies observed in BiNC and MonoNC cells, and Table 10 (also below) is the tabulated form of these data. This shows additional information like the NDI, percentages of binucleated cells (BiNC) and multi-nucleate cells (MultiNC), the number of MNI

in BiNC cells, and also the cells which have nuclear budding or nuclear plasmid bridges (Fenech, 2007).

5.3.3 General overview of data in Binucleated Cells (BiNC)

For healthy control individuals and all patients groups (See graphs 5a-5d) there was an increase in the number of MNi in BiNC cells treated with both aspirin and ibuprofen, in the bulk form when compared to the negative control. When blood samples were treated with the nano form, the number of MNi was generally similar to control values in healthy individuals and all three patient groups. Generally, there is a pattern, in the form of reduction in the number of MNi seen for the nano form of the aspirin compared to the bulk form and this is much the same for ibuprofen with a reduction of MNi frequencies for the nano form compared to its bulk equivalent.

5.3.4 General overview of data in mononucleated Cells (MonoNC)

For healthy control individuals and all patient groups, the number of MNi is generally higher in MonoNC cells compared to BiNC cells, for all samples. There is an increase in the number of MNi found in cells treated with both aspirin and ibuprofen, in the bulk form when compared to the negative control. For comparisons between the bulk and the nano form, there is a reduction for the nano form of aspirin and ibuprofen, when compared to their bulk equivalents, except in the Lung Cancer group with contrasting results that show reduction for the bulk form, compared to the nano form.

5.3.5 Exceptions in the general trends of data described above

Performing Chi-squared tests was not possible due to the nature of large sample size (1000 cells), but fewer patient numbers. Graphs 5a-d were constructed and standard errors were calculated. These standard errors can be seen as error bars on the graphs. As can be seen in many sample groups, the upper and lower error bars for some samples do not overlap, demonstrating a strong indication for differences between these samples.

Alongside the similar trends of results in healthy individuals and all asthma, COPD and lung cancer patients, there were some contrasting results. For the patient groups of asthma and COPD, it can be seen that in BiNC cells, there are lower MNi frequencies found in samples that were treated with Aspirin nano compared to the negative controls, suggesting that nanoparticle of aspirin may show some benefits in reducing DNA damage. These data are based on MNI recorded per 1000 cells observed – a strong statistical power with high number of cells scored (Fenech, 2007).

5.3.6 Data for NDI, NPBs and NBUDs in cells

Table 10 further below, shows the data for various cytological scoring parameters on each sample, per treatment group.

Nuclear division index (NDI): NDI values for almost all cell cultures are between 1.3-1.6, which is within the expected NDI range of 1.3-2.0, indicating that the cell cultures were satisfactory to use with a good rate of cell divisions. The only minor exception is the aspirin nano samples of healthy individuals,

which was 1.27, based on an average of three blood samples. In these samples, scoring was still possible as scoring had been achieved up to 1000 cells on all three control samples.

Percentage of dividing cells: The percentage of BiNC cells out of 1000 cells are between ~20% to 45%, and MultiNC cells, percentage ranges from between ~1.1% to 10 % out of 1000 cells, sufficient to enable scoring of all samples.

BiNC cells with nucleoplasmic bridges (NBPs): NPBs – a biomarker for dicentric chromosomes, were observed in all samples. In all samples the number ranges from 1-8 NPBs, showing some evidence of chromosome rearrangements occurring during cell division.

BiNC cells with nuclear buds (NBUDs): NBUDs – a biomarker for gene amplification, were also observed and their presence was even less than NPBs, between 1-2 NBUDs were found in some samples in patient groups, but not in the healthy individuals group.

Graph(s) 5a-d: show the MNI frequency data in BiNC and MonoNC for the control group (Figure 5a), asthma (Figure 5b), COPD (Figure 5c) and Lung cancer patients (Figure 5d) with mean standard error values ±.

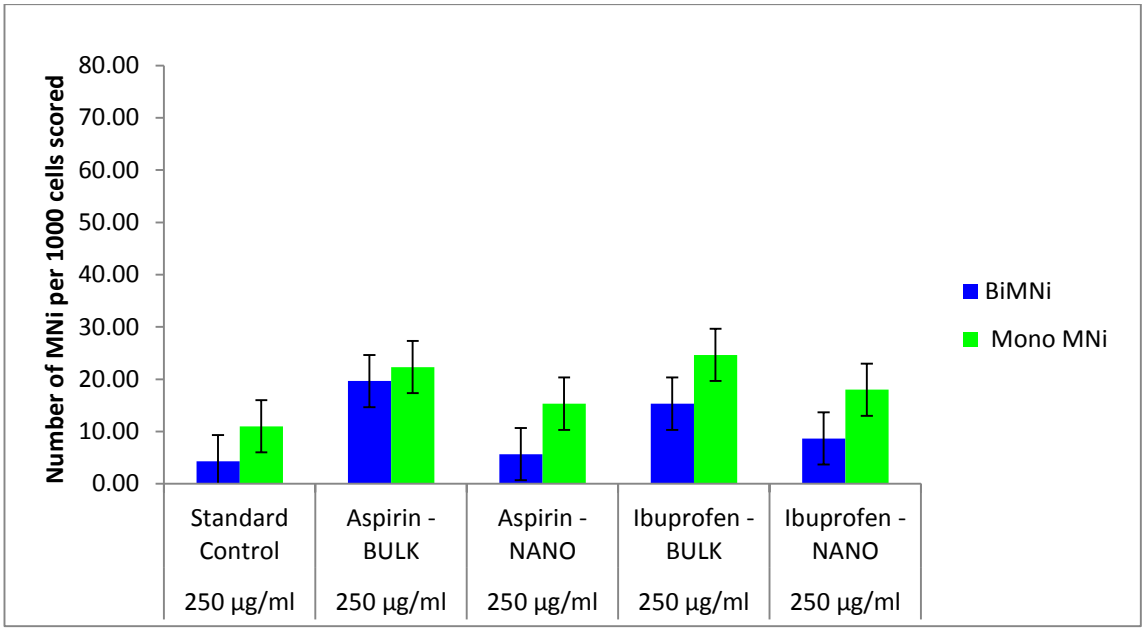


Figure 5a: Healthy control samples treated with ASP B, ASP N, and IBU B & IBU N: Negative standard control show the lowest MNi frequency per 1000 cells in healthy individuals, with ASP N, showing also similar levels to the control sample. Both ASP N and IBU N have a lower MNi frequency than their respective bulk equivalents (ASP B& IBU B).

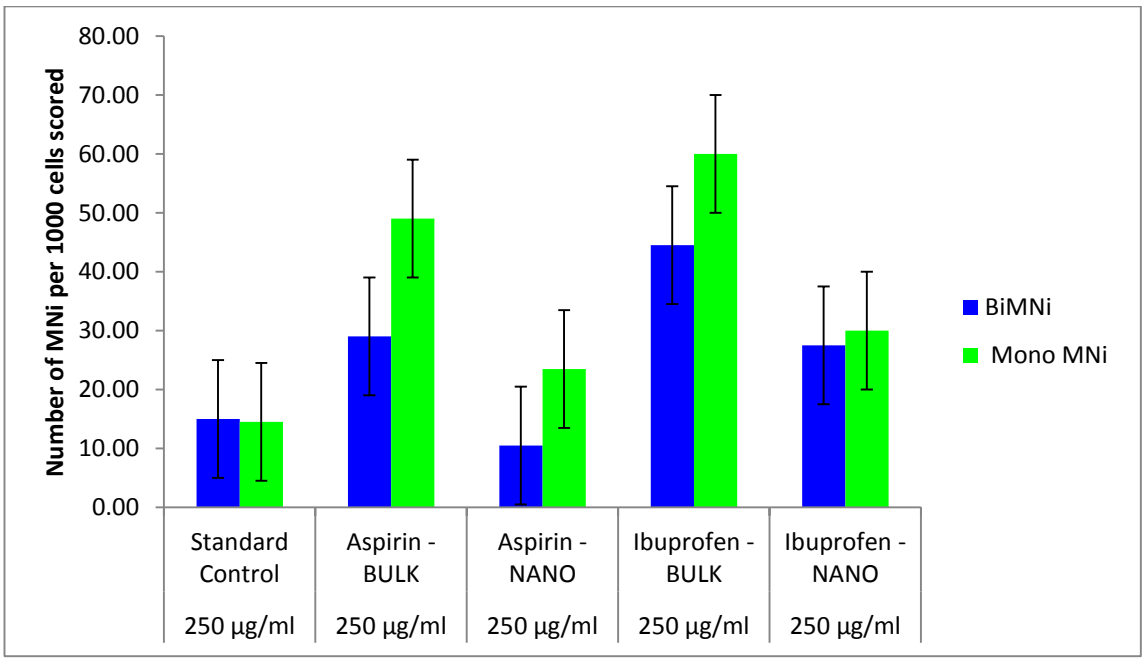


Figure 5b: Asthma patient samples treated with ASP B, ASP N, and IBU B & IBU B: In asthma patients, the lowest MNi frequency per 1000 BiNC cells is seen in sample treated with ASP N, lower than the negative control. All other NSAID samples have higher MNi frequencies than the negative control. Both ASP N and IBU N also have a lower MNi frequency than their respective bulk equivalents (ASP B & IBU B).

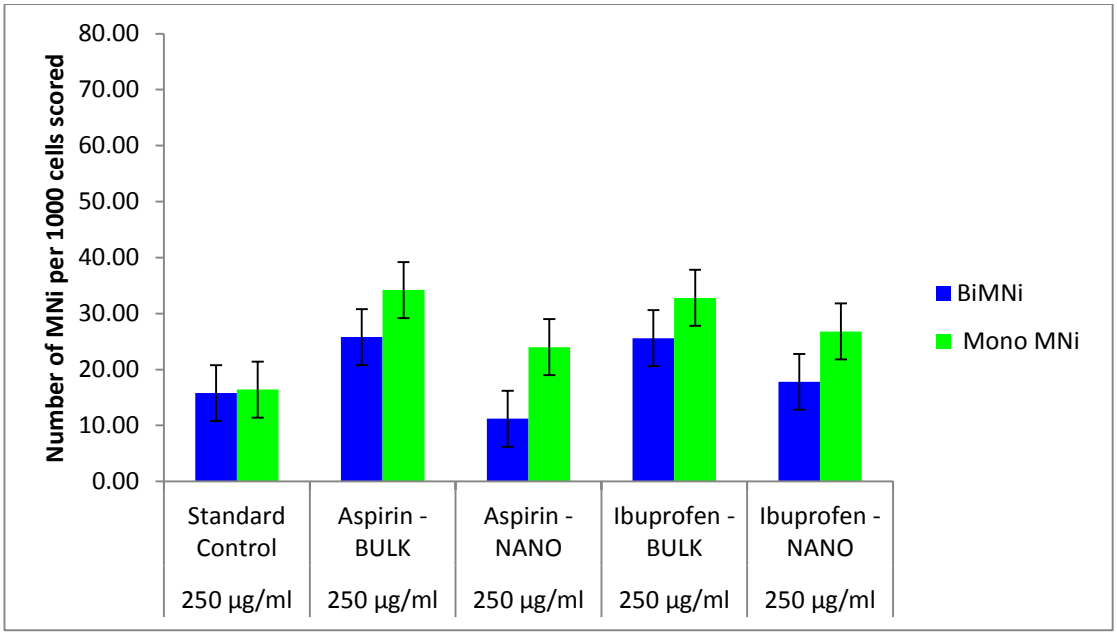


Figure 5c: COPD Patient samples (including Lung Nodule) treated with ASP B, ASP N, and IBU B & IBU B: In COPD patients, the lowest MNi frequency per 1000 BiNC cells is seen in sample treated with ASP N, lower than the negative control. All other NSAID samples have higher MNi frequencies than the negative control. However, IBU N in BiNC cells not so by much. Both ASP N and IBU N also have a lower MNi frequency than their respective bulk equivalents (ASP B & IBU B).

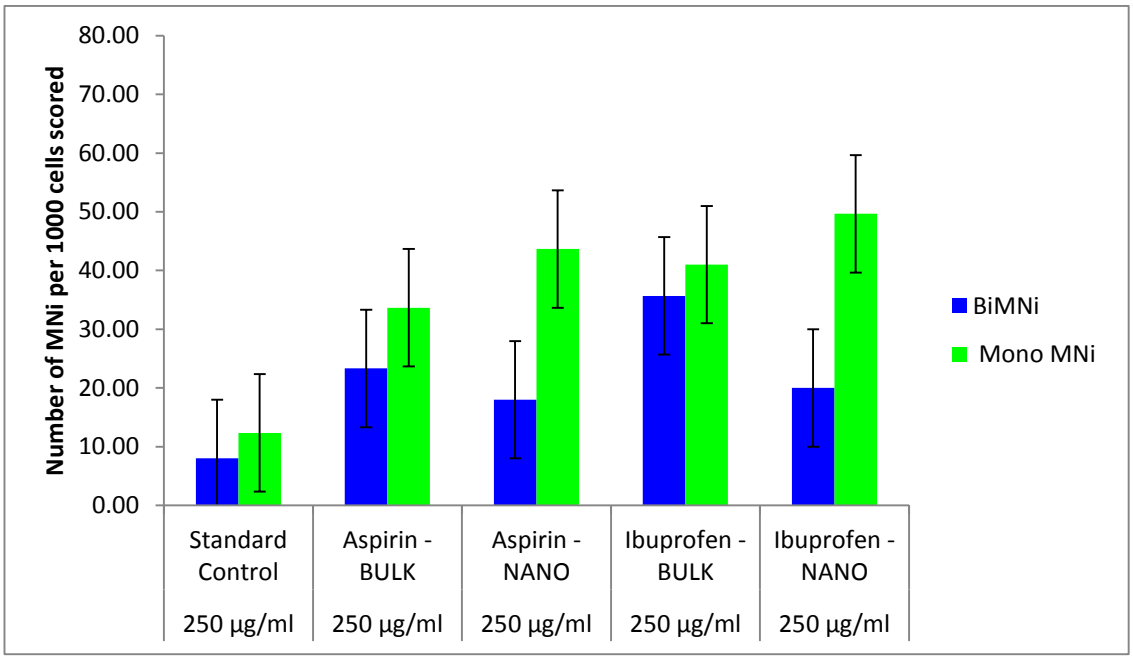


Figure 5d: Lung cancer patients samples treated with ASP B, ASP N, and IBU B & IBU B: Negative control levels show the lowest MNi frequency per 1000 cells in lung cancer samples, with ASP N and IBU N showing higher MNI frequencies. Both ASP N and IBU N also have a lower MNi frequency than their respective bulk equivalents (ASP B & IBU B).

Table 9a: Average particle size, polydispersity index and zeta potential values of the nano-suspensions. (n=3). This table was provided by Mohammed Isreb, School of Pharmacy, University of Bradford.

Suspension name	Time of measurement	Average particle size (nm)	Polydispersity index	Zeta potential value (mV)
Ibuprofen nano-suspension 3%	Before cells treatment	323±6.4	0.2± 0.01	-2.1
	After cells treatment	340±1.2	0.3±0.001	
Aspirin nano-suspension 4%	Before cells treatment	289±3	0.3±0.03	-6.1
	After cells treatment	299±6.3	0.3 ± 0.05	

Table 9b: Average particle size (x_{90}) and the volume mean diameter of the bulk powder (as received) of aspirin and ibuprofen (n=3). This table was provided by Mohammed Isreb, School of Pharmacy, University of Bradford.

Suspension name	Average particle size (µm)	Volume Mean Diameter(µm)
Ibuprofen	52.80 ± 4.37	20.50
Aspirin	78.30 ± 0.23	44.57

Table 10 (on the next page) shows various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, and MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes following exposure to both bulk and nano forms of aspirin and ibuprofen. Control samples for each patient group exhibit fewer MNi than found in samples treated with aspirin and ibuprofen. Table 9 also shows that in general, when observing numbers of MNi in both cell types, there are less MNi in the nano form of aspirin and ibuprofen compared to the bulk form, thus indicating that nano compounds show a reduction in DNA damage, levels of which are comparable to control levels in some cases.

Subject	Treatment Group (Conc. 250 mg/ml)	NDI	% BiNC	% Multi	MNi in BiNC cells			Mono
					BiMNi	BiNPB	BiBuds	MNi
Healthy individuals	Untreated Lymphocytes	1.56 ± 0.06	45.6 ± 6.21	5.2 ± 1.67	**4.33** ± 1.20	0 ± 0.00	0 ± 0.00	**11** ± 4.04
	Aspirin - B	1.34 ± 0.03	21.47 ± 3.67	6.43 ± 0.81	**19.67** ± 0.67	0.67 ± 0.67	0 ± 0.00	**22.33** ± 2.19
	Aspirin - N	1.27 ± 0.02	21.27 ± 1.13	2.47 ± 0.73	**5.67** ± 2.33	0 ± 0.00	0 ± 0.00	**15.33** ± 1.20
	Ibuprofen - B	1.41 ± 0.03	26.67 ± 4.29	7.33 ± 2.47	**15.33** ± 4.48	0.67 ± 0.67	0 ± 0.00	**24.67** ± 5.78
	Ibuprofen - N	1.32 ± 0.06	23.27 ± 2.17	4.53 ± 2.54	**8.67** ± 2.03	0 ± 0.00	0 ± 0.00	**18** ± 2.52
Asthma	Untreated Lymphocytes	1.59 ± 0.07	34.9 ± 6.50	11.9 ± 6.90	**15** ± 9.00	1.5 ± 0.50	0 ± 0.00	**14.5** ± 9.50
	Aspirin - B	1.4 ± 0.10	34 ± 1.70	12.7 ± 13.00	**29** ± 13.00	0.5 ± 0.50	1.5 ± 1.50	**49** ± 26.00
	Aspirin - N	1.41 ± 0.02	21 ± 1.60	10 ± 1.60	**10.5** ± 3.50	0.5 ± 0.50	0 ± 0.00	**23.5** ± 6.50
	Ibuprofen - B	1.37 ± 0.14	24.25 ± 6.15	6.55 ± 6.15	**44.5** ± 1.50	8 ± 8.00	4 ± 4.00	**60** ± 6.00
	Ibuprofen - N	1.42 ± 0.06	25.85 ± 4.40	8 ± 4.40	**27.5** ± 15.50	6 ± 6.00	0.5 ± 0.50	**30** ± 8.00
Lung Cancer	Untreated Lymphocytes	1.4 ± 0.06	32.67 ± 6.91	3.73 ± 0.48	**8** ± 2.08	0.33 ± 0.33	0 ± 0.00	**12.33** ± 4.41
	Aspirin - B	1.32 ± 0.06	20.53 ± 0.67	5.87 ± 2.77	**23.33** ± 1.33	0.67 ± 0.33	1 ± 0.58	**33.67** ± 7.75
	Aspirin - N	1.35 ± 0.12	19.33 ± 0.27	8 ± 5.94	**18** ± 6.51	1.67 ± 1.20	3 ± 2.00	**43.67** ± 4.63
	Ibuprofen - B	1.31 ± 0.02	24.53 ± 5.09	3.57 ± 1.70	**35.67** ± 6.67	2 ± 2.00	0 ± 0.00	**41** ± 16.46
	Ibuprofen - N	1.28 ± 0.34	22.87 ± 0.34	2.6 ± 2.48	**20** ± 7.25	1.67 ± 0.87	0.33 ± 0.96	**49.67** ± 10.62
COPD & COPD with Lung Nodule	Untreated Lymphocytes	1.43 ± 0.05	34.92 ± 7.10	3.88 ± 1.71	**15.8** ± 2.52	2 ± 1.30	2.2 ± 1.50	**16.4** ± 3.41
	Aspirin - B	1.34 ± 0.04	27.28 ± 1.62	3.12 ± 1.11	**25.8** ± 5.89	2.4 ± 1.29	1 ± 0.55	**34.2** ± 4.31
	Aspirin - N	1.37 ± 0.03	20.84 ± 2.06	8.2 ± 1.67	**11.2** ± 4.09	0.8 ± 0.37	0.2 ± 0.20	**24** ± 8.30
	Ibuprofen - B	1.35 ± 0.06	30.56 ± 6.61	2.32 ± 0.50	**25.6** ± 3.28	1.2 ± 0.37	2.4 ± 1.94	**32.8** ± 9.44
	Ibuprofen - N	1.47 ± 0.05	33.62 ± 9.72	7.92 ± 2.81	**17.8** ± 4.21	0.6 ± 0.40	0.4 ± 0.40	**26.8** ± 5.30

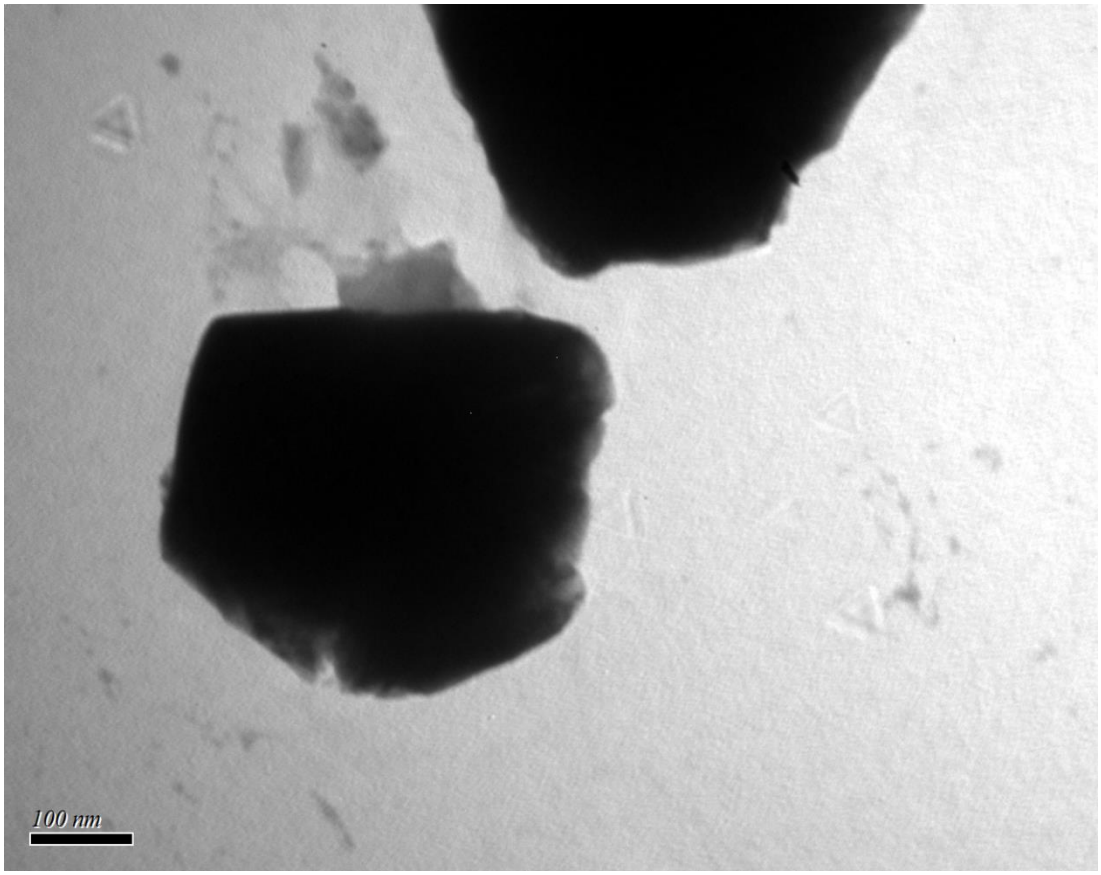


Figure: 13: TEM image of aspirin nanoparticles.

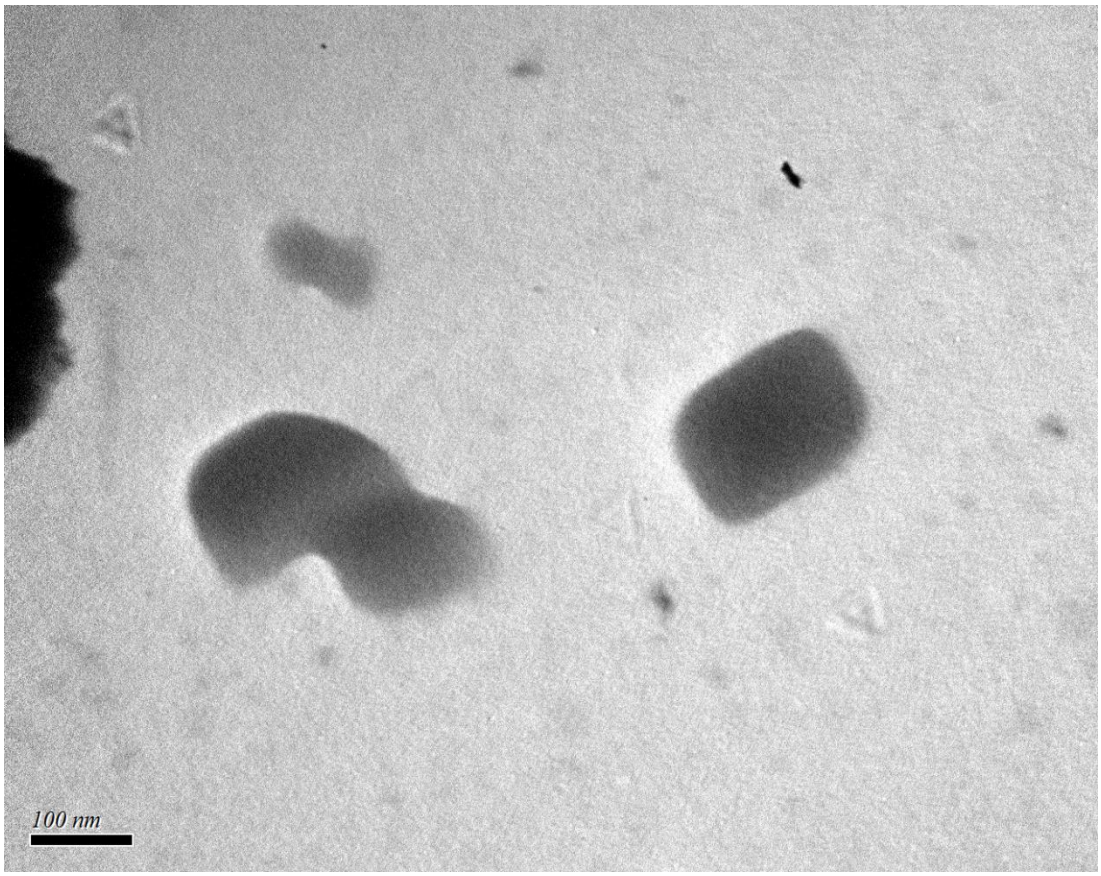


Figure 14: TEM image of ibuprofen nanoparticles.

5.4 Discussion

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzyme activity, a significant mechanism of action of NSAIDs. The COX enzyme consists of two isoforms, the COX-1 enzyme and the COX-2 enzyme. They play a significant role in the synthesis of prostaglandins, which plays a role in tumour progression (Day and Graham, 2013; Rao and Knaus, 2008). Several studies have shown that NSAIDs, like aspirin and ibuprofen exhibit anti-cancer activities on several hallmarks of cancer, such as cell proliferation, evasion of apoptosis and cell cycle regulation (Burn et al., 2011; Mansouri et al., 2013; Park et al., 2014).

This focus of this study was to investigate aspirin and ibuprofen compounds in blood samples from healthy individuals and respiratory disease patients. Nanotoxicology has become a vast significant area of interest to scientists, who are using this technology to find better ways to improve and/or develop current or new forms of diagnosis or treatment. Nanoparticles have the biggest advantage of having a smaller size, resulting in a large surface area to volume ratio, thereby increasing the reactivity of cells (Dhawan and Sharma, 2010). Such particles, are those that are defined as being under 100 nm, however the pharmaceutical industry considers sizes up to 1000 nm to be classified as nano compounds (Carlin, 2014; Teeguarden et al., 2007). Nanoparticles possess the unique capability to accumulate at the site of inflammation, and thus are suitable for targeted drug delivery (Elzoghby et al., 2012). Nanoparticles are delivered to the targeted site via several nano-carriers which protect the drugs from degradation and enhance drug absorption by facilitating

diffusion through cell epithelium. These systems can also withstand physiological stress or improved biological stability and alter the release pattern of the drug (Elzoghby et al., 2011).

In this study, suspension solutions of aspirin and ibuprofen were prepared with solid loads of 4% and 3% (w/w), respectively in deionised water using a method described by (Plakkot et al., 2011). The suspension solution of deionised water also contained HPMC, polyvinylpyrrolidone K-30 and sodium lauryl sulphate (Plakkot et al., 2011). This is followed by a process in which milling of these compounds were carried out using Lena nanoceutics technology DM-100 machine (Sulaiman 2007).

Data from the CBMN assay showed an increase in the number of MNi in BiNC cells treated with both aspirin and ibuprofen, in the bulk (ASP B & IBU B) form when compared to the negative control. In the blood samples that were treated with the nano forms of aspirin (ASP N) and ibuprofen (IBU N), the number of MNi was generally similar to control values for each of the healthy individuals and all three patient groups. Generally, there is a pattern, with lower MNi frequency in ASP N compared to ASP B and this is much the same for ibuprofen with a reduction of MNi frequency in IBU N compared to IBU B. Cells were observed for MNi seen in BiNC and MonoNC, where up to 1000 cells were counted.

The data for MNi frequencies in BiNC was very similar to those in MonoNC. The MonoNC cells had higher numbers of MNi in comparison BiNC cells. However, the main result of interest is MNi numbers found in BiNC, since this constitutes damage occurring after exposure to NSAIDs and during the cell

cycle at the cell culture stage. The NDI numbers were found to be within the normal expected range, indicating successful division of cells, which were later used to score for the MNi data.

An interesting result seen was that in asthma and COPD patients, where the number of MNi was less than the number of MNi at the control levels in BiNC, thus providing some early indication that those nanoparticles may have an advantage in reducing DNA damage. A parallel study was conducted in the Comet assay by Dr. Mojgan Najafzadeh (Researcher at University of Bradford). That investigation also showed a reduction in ASP N and IBU N, compared to ASP B and IBU B, respectively. Similarly, ASP N had less DNA damage compared to the negative controls in healthy individuals, COPD and lung cancer patients. Similar reductions were also found with IBU N compared to the negative controls in healthy individuals and lung cancer group. This study in the CBMN assay and also the one on the Comet assay provide some interesting results on NSAID nanoparticles in peripheral lymphocytes (Comet) and whole blood (CBMN) and show some potential benefits of nanoparticles. Further work and study will be needed to verify and strengthen these data.

Chapter 6 – Role of NSAIDs on the repair of DNA following exposure to bleomycin

6.1 Introduction

The Comet assay is a micro-electrophoretic technique used for direct visualisation of DNA damage in mammalian cells (Ostling and Johanson, 1984). The Comet assay has been recognised for its time efficiency, simplicity, sensitivity, and cost effectiveness for analysing genotoxic DNA damage in sperm and lymphocytes as shown in previous studies (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et al., 1998b; Anderson et al., 2003; Singh et al., 1988; Tice et al., 2000). The assay has been a useful method to access chemicals for their genotoxicity, usage in human biomonitoring, epidemiology, and importantly, also in DNA damage and repair (Collins, 2004). Monitoring the repair of DNA is one of the important determinants of cancer susceptibility (Gaivao et al., 2009). All cells possess mechanisms of repairing or correcting DNA damage before there are permanent changes made to DNA. The method of DNA cellular repair is also sometimes known as the challenge assay (Au et al., 2010; Azqueta et al., 2014).

One approach to accessing DNA repair of cells is a process called the *in-vitro* cellular repair assay (also known as the mutagen challenge assay). In this method, a genotoxic agent such as mitomycin C, doxorubicin, or bleomycin are used to treat cells in order to induce DNA damage to cells, which are then incubated. This allows for the monitoring of single stand breaks that re-join (and repair themselves) (Azqueta and Collins, 2013; Azqueta et al., 2014). After incubation, cells were transferred into fresh buffer and further incubations with short time intervals (like 10, 15 minutes etc.) were allowed for DNA repair to occur in the absence of genotoxic agents. Levels of DNA damage are then

measured from samples at the end of repair and compared to those samples not repaired, by analysing and comparing DNA damage repair capabilities in the form of a reduction of the DNA damage parameters a reduction of the DNA damage parameters - Olive tail moment and % tail DNA. Other than observing the repair of single strand breaks, the monitoring of other lesions, like oxidised bases (using base excision repair) or altered bases (using nucleotide excision repair) can be made possible, by using enzymes which recognise these lesions and subsequently, convert these lesions into strand breaks (Azqueta and Collins, 2013; Azqueta et al., 2014).

This study design looks at the former methodology as mentioned above. Cells from healthy individuals, asthma, and COPD and lung cancer patients are challenged using bleomycin and then allowed to repair DNA damage with no bleomycin (BLM) present. Some samples have been allowed to repair DNA damage in the presence of NSAIDs. Previous work on NSAIDs using the micronucleus assay (Chapter 5) and the Comet assay (personal communication with Dr. Mojgan Najafzadeh, University of Bradford) have shown the reductions in DNA damage when treated with NSAIDs and to a greater extent with the nanoparticle versions. Investigating the influence of DNA repair of cells, this study is designed to determine whether these compounds also facilitate the repair of DNA or have the opposite effect.

Bleomycin was used in this study as it has been extensively used in other repair assay studies. Several studies have investigated DNA damage and repair in cells from cancer patients following bleomycin exposure, such as lung cancer and psoriasis patients with basal carcinomas, to name a few (Moller et al., 2000; Wei et al., 2005; Zheng et al., 2003). Bleomycin, first discovered in

1966, is glycopeptide antibiotic agent that was first isolated from *Streptomyces verticillus*. Bleomycin has been extensively studied in cancer studies because of its anti-tumour activities (Chen and Stubbe, 2005; Kawai and Akaza, 2003; Umezawa et al., 1966). Bleomycin is commonly used as part of chemotherapy regimens for various cancer treatments, such as testicular cancer (Chen and Stubbe, 2005; Kawai and Akaza, 2003). Bleomycin that is administered in chemotherapy treatment, uses two separate forms of the drug – bleomycin A (60%) and bleomycin B (30%), with the addition of other components (Chen and Stubbe, 2005). Bleomycin causes damage to the DNA by binding to the transition metals (Fe(II) or Cu(I)) and oxygen. In the presence of one-electron reductant, the drug is then able to catalyse the formation of single stranded breaks (SSBs) and double stranded DNA lesions (Chen and Stubbe, 2005).

6.2 Materials and Methods

6.2.1 Materials

The materials required for this study have been described in section 2.6.1

6.2.2 Methods

Sample collection and isolation of whole blood cells has been described in section 2.6.2. The Comet assay was deployed under alkaline conditions as described in sections 2.6.4-2.6.7. Treatment groups for all samples have been described within this section. Scoring of cells and statistical analysis has been described in section 2.6.8

6.2.3 Study design

This study was conducted to determine the DNA repair capabilities of whole blood cells, following bleomycin exposure from healthy control individuals and asthma, COPD and lung cancer patients. Following bleomycin exposure for 30 minutes, cells were allowed to carry out DNA repair in the presence of or in the absence of aspirin and ibuprofen, each in nano form and the standard bulk form. The aim was to determine the effect of these compounds on the repair of DNA Comet cells, by comparing these to two samples; one treated with bleomycin and not repaired, and the other treated with bleomycin and repaired without any NSAIDs. Three different kinds of statistical analysis were deployed (see section 6.2.4 below).

6.2.4 Statistical Analysis

All microgels were coded before scoring and 100 cells were scored per observation using a microscope equipped with a CCD camera and Computer system using Comet Kinetic Imaging Software© 6.0, Liverpool/Andor Technology, Belfast, UK. Each experiment was repeated ten times for samples from healthy individuals and five times each for asthma, COPD and lung cancer samples. Mean data were generated with standard errors.

Data from Olive tail moment and % Tail DNA values were used to perform SPSS statistics (Version 20). Most data were normally distributed, as Gaussian normality did not depart for normality for most treatment samples in all groups (controls and asthma, COPD and lung cancer patients), as determined using Smirnov goodness of fit test. For data that were not normally distributed using these tests, their normal distribution was confirmed using non-parametric - 2 independent samples test.

Three different kinds of significance were determined:

Firstly, the significance was compared from all samples to the negative control sample, including the bleomycin sample where DNA repair was disallowed (*** p = < 0.001, ** p = < 0.01, * p = < 0.05, ns* = > 0.05 not significant).

Secondly, the five samples, (4 samples treated with bleomycin and allowed for repair in the presence of NSAIDs (ASP B, ASP N, IBU B & IBU N) and one sample that was treated with bleomycin allowing DNA repair in the absence of

all four NSAIDs), were all compared to the sample treated with bleomycin without allowing DNA repair (††† p = < 0.001, †† p = < 0.01, † p = < 0.05, ns† = > 0.05 not significant).

Finally, the samples that were treated with bleomycin and repaired in the presence of NSAIDs were compared with each other. ASP N was compared to ASP B and IBU N was compared to IBU B (ΔΔΔ p = < 0.001, ΔΔ p = < 0.01, Δ p = < 0.05, nsΔ = > 0.05 not significant).

6.3 Results

6.3.1 Particle size analysis

The particle size distribution of aspirin and ibuprofen nano-suspensions (Table 9a) and bulk powders (Table 9b) were determined using the dynamic light scattering technique of the Zetasizer Nano ZS machine, (See Table 9a and 9b in the previous chapter).

Images of the nanoparticles were obtained using transmission electron microscopy (TEM), as described in Materials and methods (Section 2.4.2 and 2.4.3). The images showed that the aspirin crystals are slightly larger than those of ibuprofen as was shown by the laser diffraction measurements (see Figures 15 & 16 below). The images show some smaller particles on the side which might be small crystals of the drugs or it could be aggregates of the polymer used to stabilise the crystals.

6.3.2 Cell viability

Cell survival was measured before samples were used. Cell viability was measured in samples up to a cut-off point of 75% using trypan blue exclusion, so avoiding artefacts due to toxicity. Generally, the viability was in excess of 90%. Viability was measured in samples immediately before and after treatment. (Gopalan et al., 2011; Henderson et al., 1998).

6.3.3 Preliminary investigations

Dose response experiments with bleomycin (BML) were implemented, to find the dose that gave the maximum repair difference, from the unrepaired samples (incubation only) and to those incubated and repaired. End-concentration dose ranges of 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml and 10 µg/ml were used in dose response experiments, which were repeated twice and 7.5 µg/ml of bleomycin was found to be the most appropriate concentration to deploy with NSAIDs.

6.3.4 Statistics

Data did not depart from normality using Gaussian normality for most treatment samples in all groups, as determined using Smirnov goodness of fit test. For samples where Gaussian normality was violated (non-normally distributed samples), non-parametric statistics were considered adequate for the analysis of the data. A p-value of < 0.05 was considered significant. All analyses were performed using SPSS for windows statistical package (version 20).

6.3.5 Overview of results

In general, the data for Olive tail moments and % tail DNA are similar for all groups of patients and the individual treatment types within them. All treatment samples have greater levels of DNA damage which are, significantly different compared to the negative control sample ($p = ** < 0.01$ or $p = *** < 0.001$), with the exception to both nanos (nano aspirin & nano ibuprofen). Statistics are described below (Section 6.3.5). The reduction in DNA damage after repair is

greater to the extent that DNA damage found in these groups is not significantly different from DNA damage in the control groups. However, there are some individual differences between results, which are described below.

6.3.6 Olive tail moment data

Healthy control individuals:

Results were analysed using Olive tail moment data. In healthy individuals (Graph 6a), all samples showed significant increase of DNA damage compared to the negative control at a level of significance of *** p = < 0.001, except for ASP B was significantly different at ** p = < 0.01 and both ASP N and IBU which are not significant (ns*) when compared to the negative control, suggesting the nanoparticles are able to increase DNA repair. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significant with a significance of ††† p = < 0.001. When the nanoparticles were compared to the bulk samples, DNA damage in ASP N was significantly lower compared to ASP B (ΔΔ p = < 0.01), however IBU N compared to IBU B was not significant (nsΔ), although a reduction can be seen.

Asthma patients:

In asthma patients (Graph 6b), all samples were significantly different compared to the negative control with a significance of *** p = < 0.001, except for ASP B, ASP N and IBU N, which were not significant (ns*) when compared to the negative control. This suggests, that the nanoparticles are able to increase DNA repair, as is the bulk form of aspirin. All samples were then

compared to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001, expect for sample with bleomycin treatment and repair without NSAIDs, which was significantly different by †† p = < 0.01. When the nanoparticles were compared to the bulk samples, DNA damage in ASP N was lower compared to ASP B but not significant (nsΔ), however, IBU N when compared to IBU B was significant (ΔΔ p = < 0.01).

COPD patients:

In COPD patients (Graph 6c), all samples were significantly different compared to the negative control with a significance of *** p = < 0.001, except for ASP B, ASP N and IBU N, which are not significant when compared to the negative control, suggesting the nanoparticles are able to increase DNA repair, as is the bulk form of aspirin. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001. When the nanoparticles were compared to the bulk samples, DNA damage in ASP N, IBU N was lower compared to ASP B and IBU B, respectively, although not significant for Olive tail moment data (nsΔ).

Lung cancer patients:

In lung cancer patients (Graph 6d), all samples are significantly different compared to the negative control with a significance of *** p = < 0.001, except for all NSAIDs (ASP B, ASP N, IBU N and IBU B) which are not significant (ns*) when compared to the negative control. All samples were then compared

to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001, expect for sample with bleomycin treatment and repair without NSAIDs, which was not significant (ns†). As with COPD data above, when the nanoparticles were compared to the bulk samples, DNA damage in ASP N, IBU N was lower compared to ASP B and IBU B, respectively, although not significant for Olive tail moment data (nsΔ).

6.3.7 Percentage (%) tail DNA data

Healthy control individuals:

Results were analysed using % tail DNA data. In healthy individuals (Graph 7a), all samples were significantly different compared to the negative control with a significance of *** p = < 0.001, except for ASP N which is not significant (ns*) when compared to the negative control, suggesting ASP N is able to increase DNA repair. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significant with a significance of ††† p = < 0.001. When the nanoparticles were compared to the bulk samples, DNA damage in ASP N and IBU N were significantly lower compared to their bulk equivalents - ASP B and IBU B, respectively (ΔΔΔ p = < 0.001).

Asthma patients:

In asthma patients (Graph 7b), all samples are significantly different compared to the negative control with a significance of *** p = < 0.001, except for ASP B which is significantly different by * p = < 0.05 and both ASP N and IBU N which

are not significant (ns*) when compared to the negative control. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001). When the nanoparticles were compared to the bulk samples, DNA damage in ASP N was lower compared to ASP B but not significant (nsΔ), however IBU N when compared to IBU B was significant (ΔΔΔ p = < 0.001).

COPD patients:

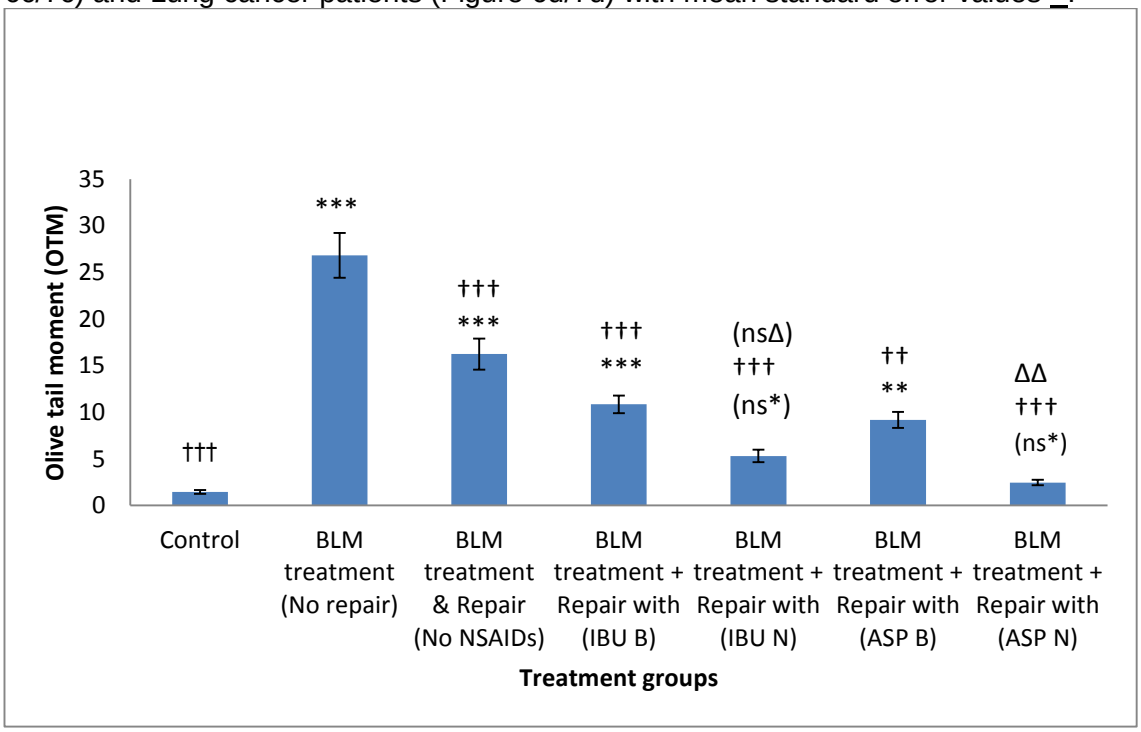
In COPD patients (Graph 7c), all samples were significantly different compared to the negative control with a significance of *** p = < 0.001, except for IBU N which is significantly different at * p = < 0.05, IBU B which is significantly different by ** p = < 0.01, and ASP B and ASP N, which are not significant when compared to the negative control. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001 except IBU N (†† p = < 0.01). When the nanoparticles were compared to the bulk samples, DNA damage in ASP N and IBU N was lower compared to ASP B and IBU B, respectively, although not significant for % tail DNA data (nsΔ).

Lung cancer patients:

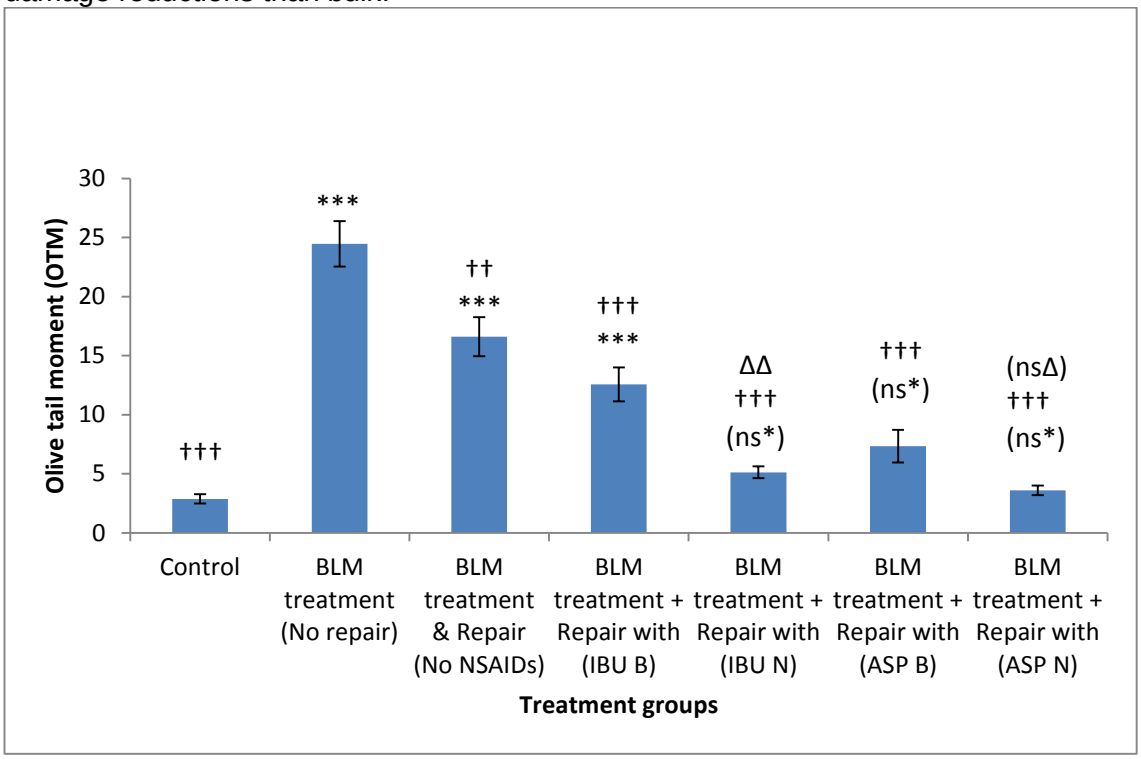
In lung cancer patients (Graph 7d), all samples were significantly different compared to the negative control with a significance of *** p = < 0.001, except for IBU B which was significantly different at ** p = < 0.01, and ASP B, ASP N and IBU B which were not significant (ns*) when compared to the negative control, suggesting both the bulk and nanoparticles are able to increase DNA

repair. All samples were then compared to the sample treated with bleomycin only (no repair), and all were significantly different with a significance of ††† p = < 0.001, expect for the sample with bleomycin treatment and repair without NSAIDs, which was not significantly (ns†). As with COPD data above, when the nanoparticles were compared to the bulk samples, DNA damage in ASP N, IBU N was lower compared to ASP B and IBU B, respectively, although not significant for Olive tail moment data (nsΔ).

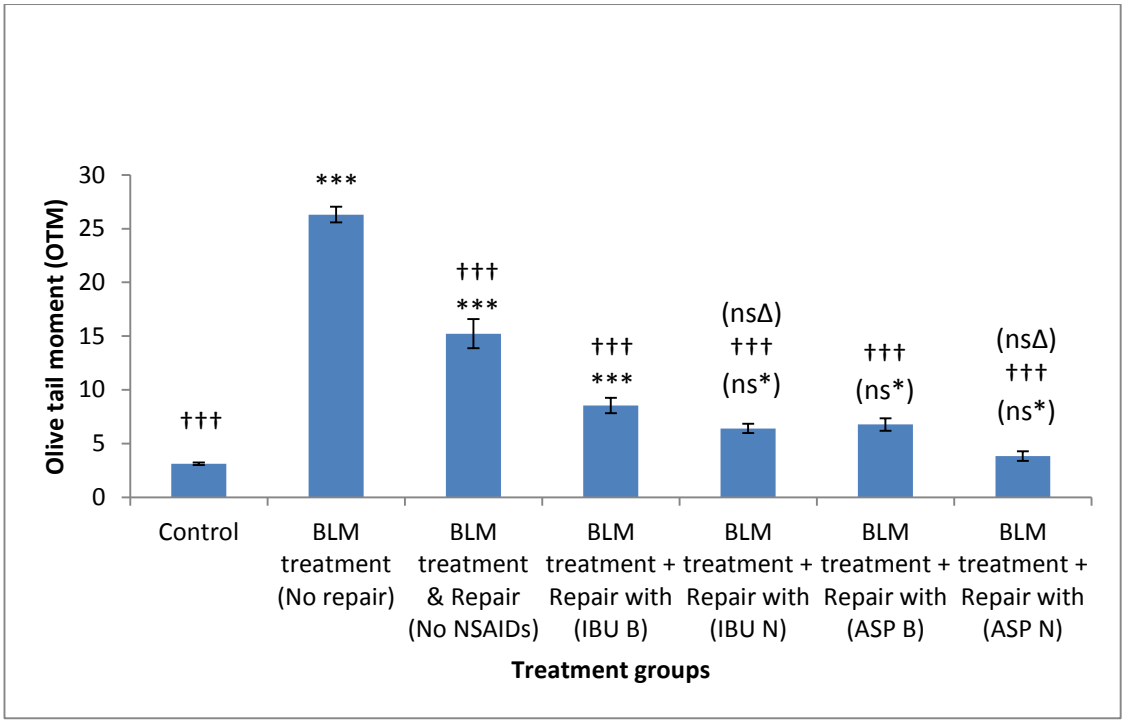
Graph(s) 6 (OTM)/ 7 (%tail DNA) a-d: show the levels of DNA damage for the healthy individuals group (Figure 6a/7a), asthma (Figure 6b/7b), COPD (Figure 6c/7c) and Lung cancer patients (Figure 6d/7d) with mean standard error values ±.



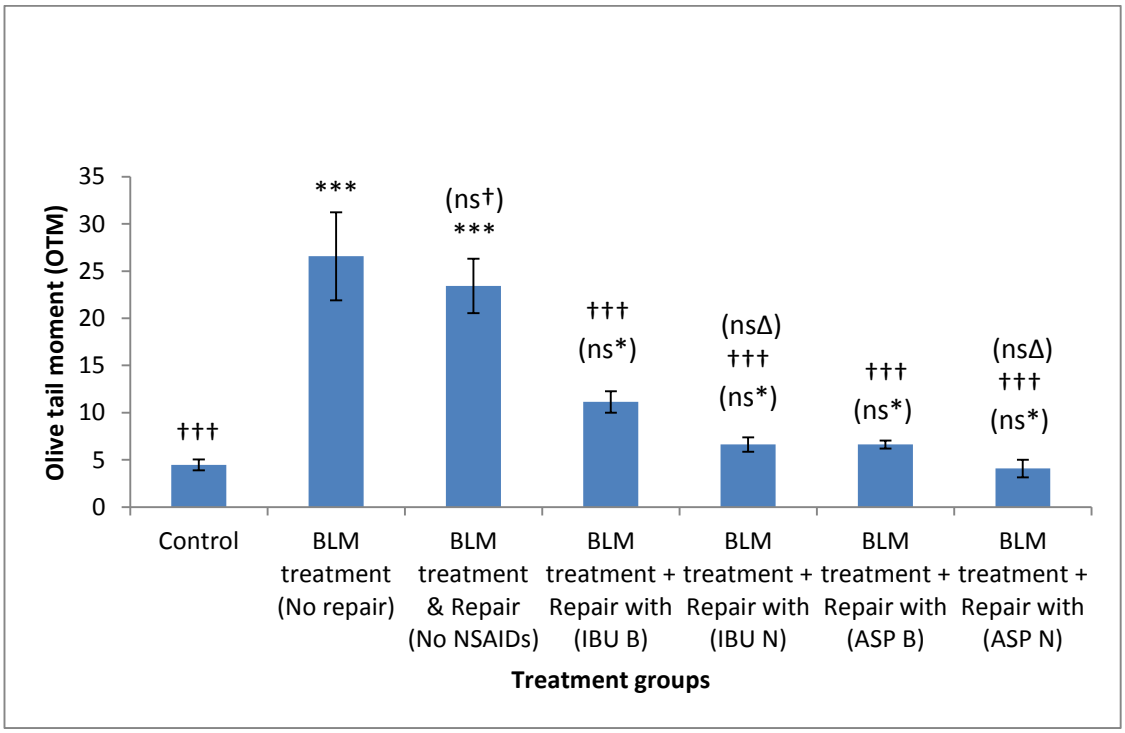
Graph 6a (above): Healthy individuals: Repair of DNA, in the presence and absence of NSAIDs following treatment with bleomycin. NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk.



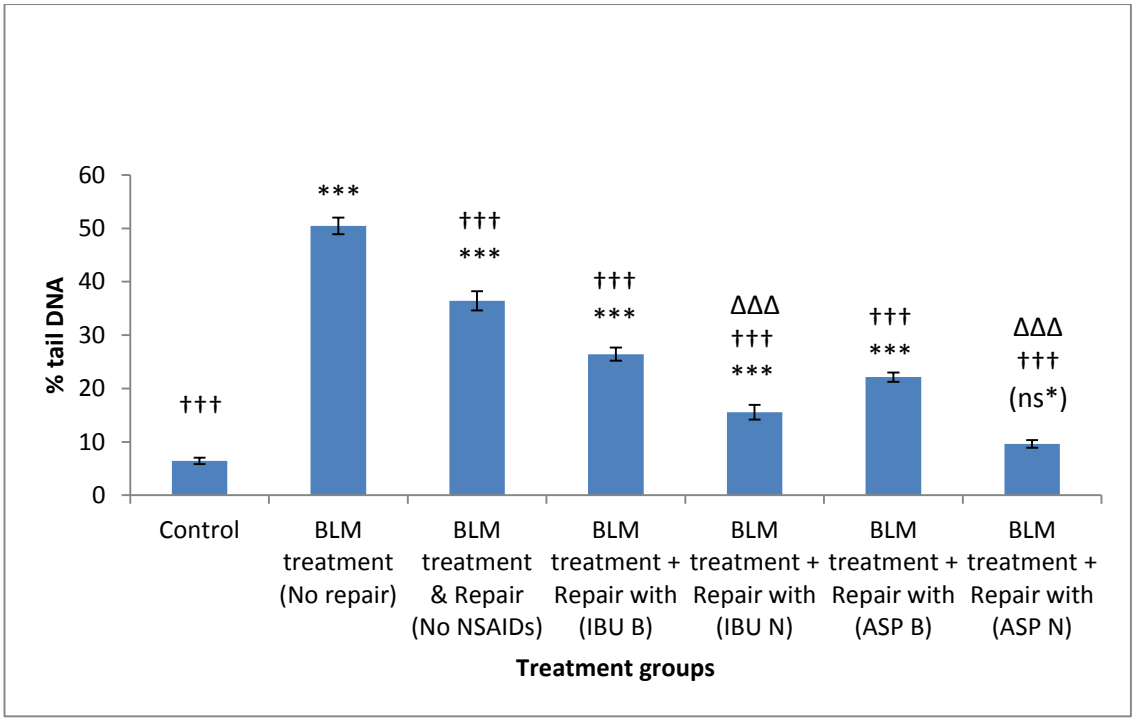
Graph 6b (above): Asthma patients: Repair of DNA, in the presence and absence of NSAIDs following treatment with bleomycin. NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk.



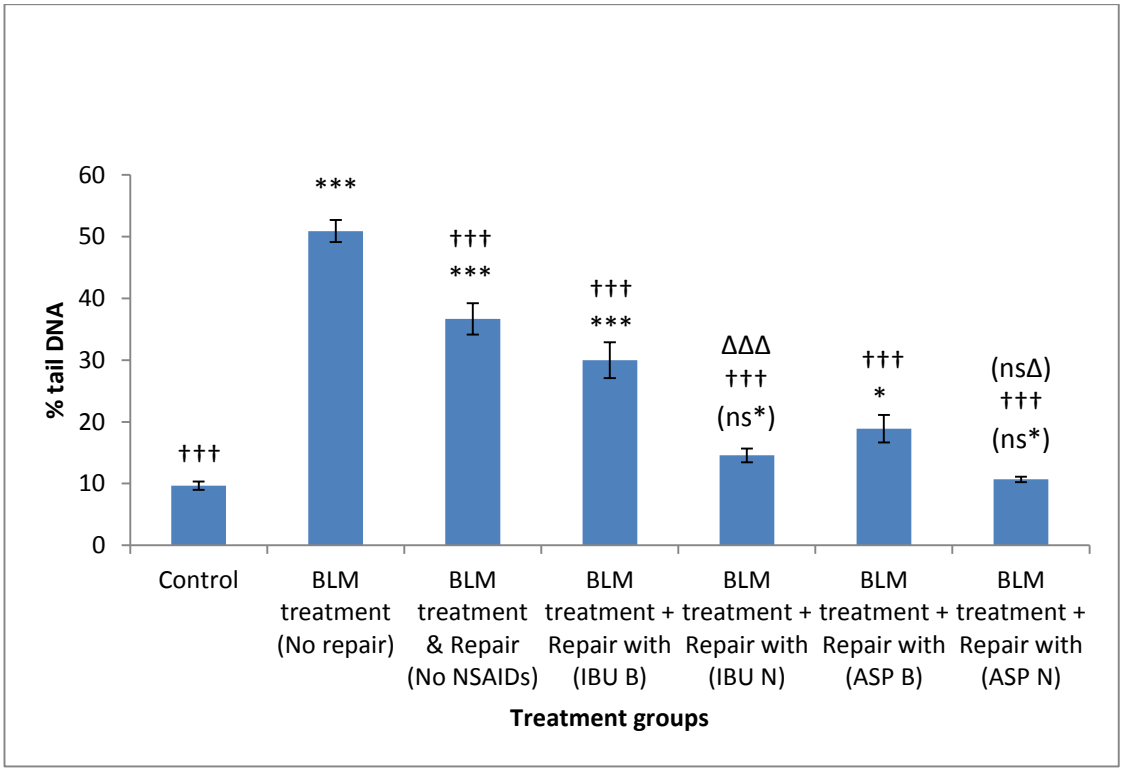
Graph 6c: COPD patients: Repair of DNA, in the presence and absence of NSAIDs following treatment with bleomycin. NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk.



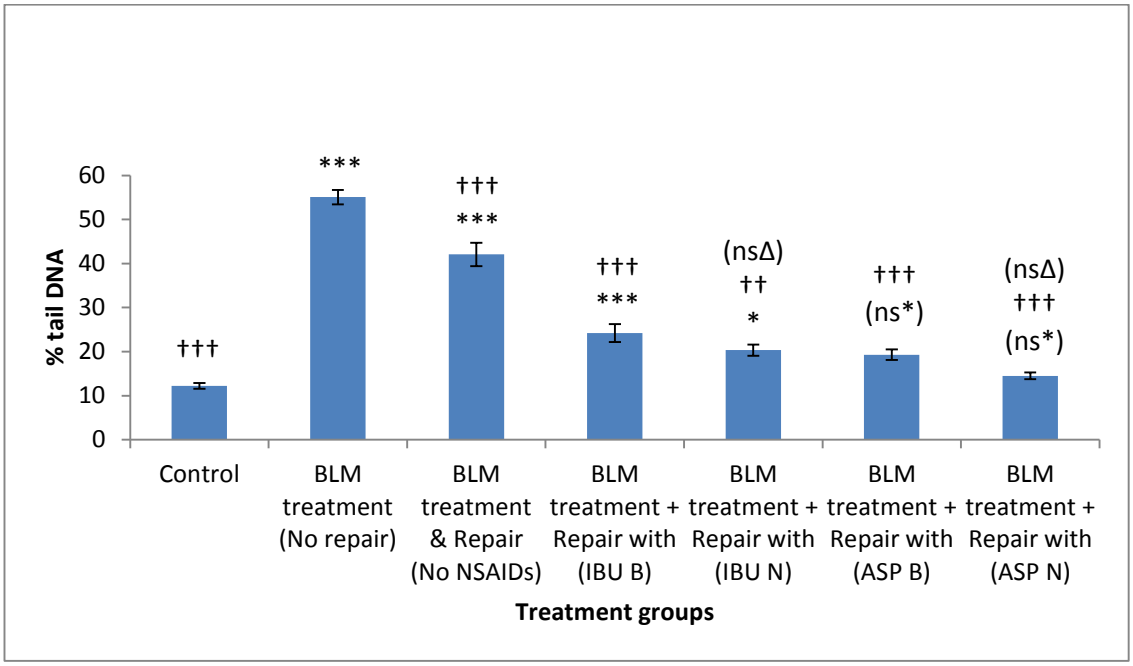
Graph 6d: Lung cancer patients: Repair of DNA, in the presence and absence of NSAIDs following treatment with bleomycin. NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk.



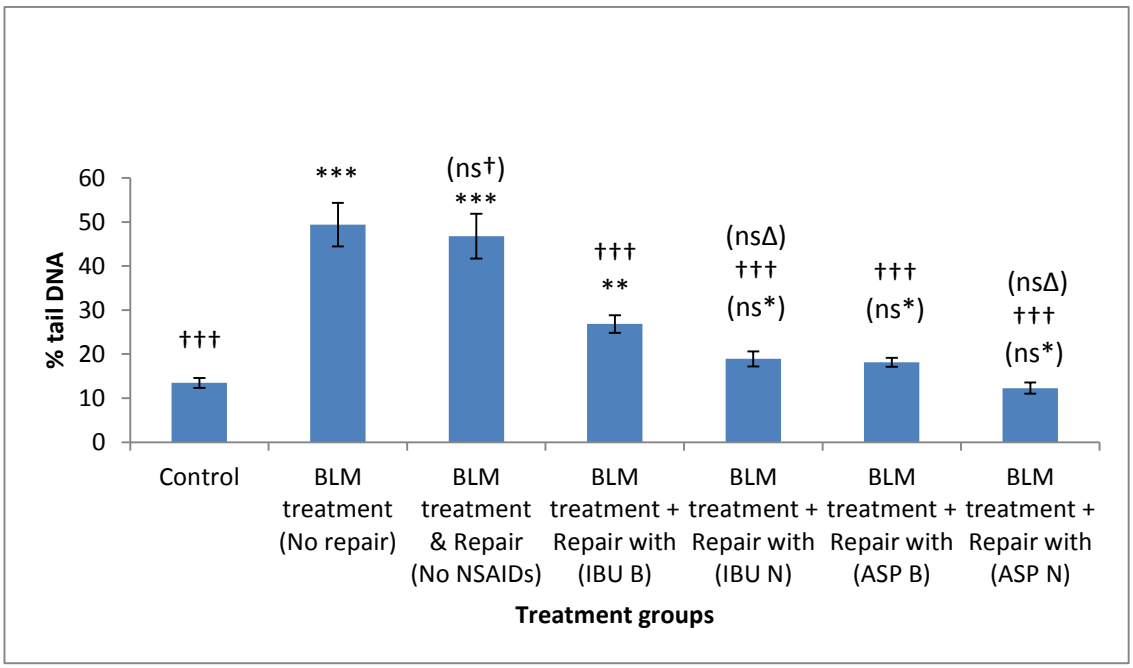
Graph 7a: Healthy individuals: NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk equivalents.



Graph 7b: Asthma patients: NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk equivalents.



Graph 7c: COPD patients: NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk equivalents.



Graph 7d: Lung cancer patients: NSAIDs compounds reduce DNA damage during the repair process. The nano versions showed greater DNA damage reductions than bulk equivalents.

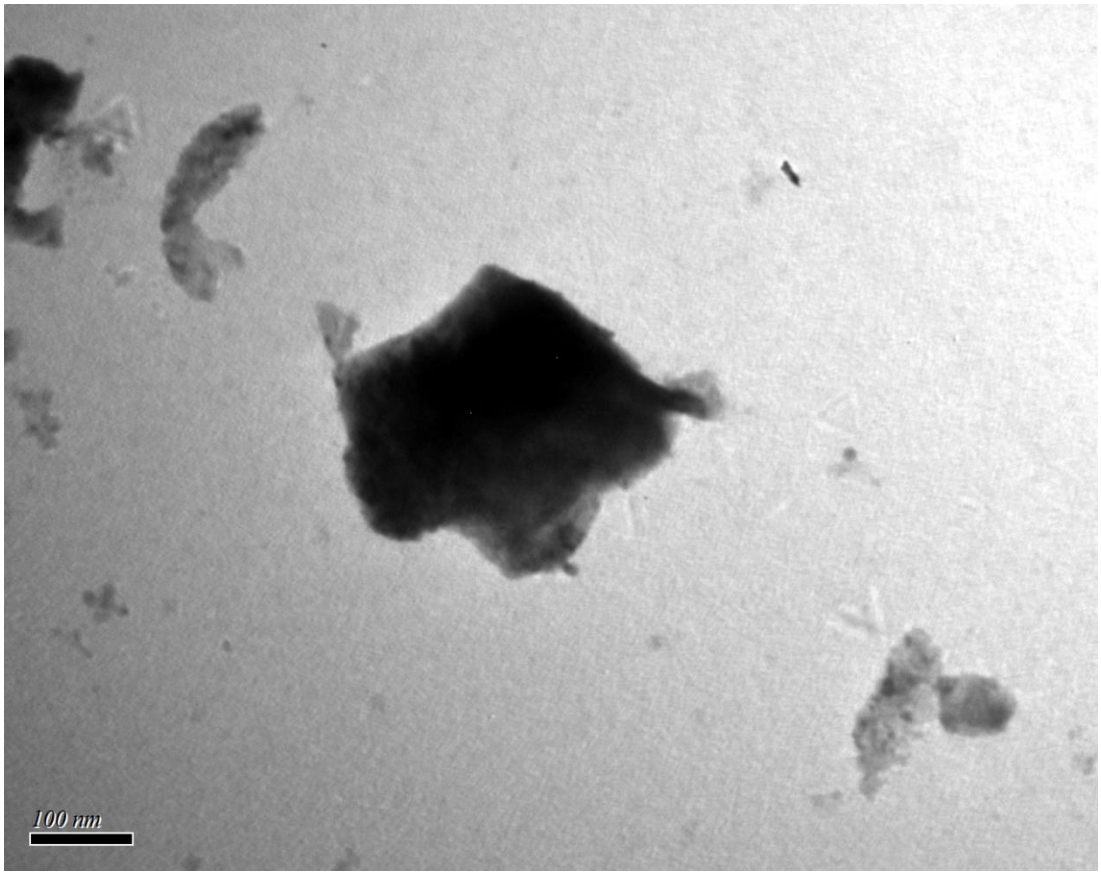


Figure: 15: TEM image of aspirin nanoparticles.

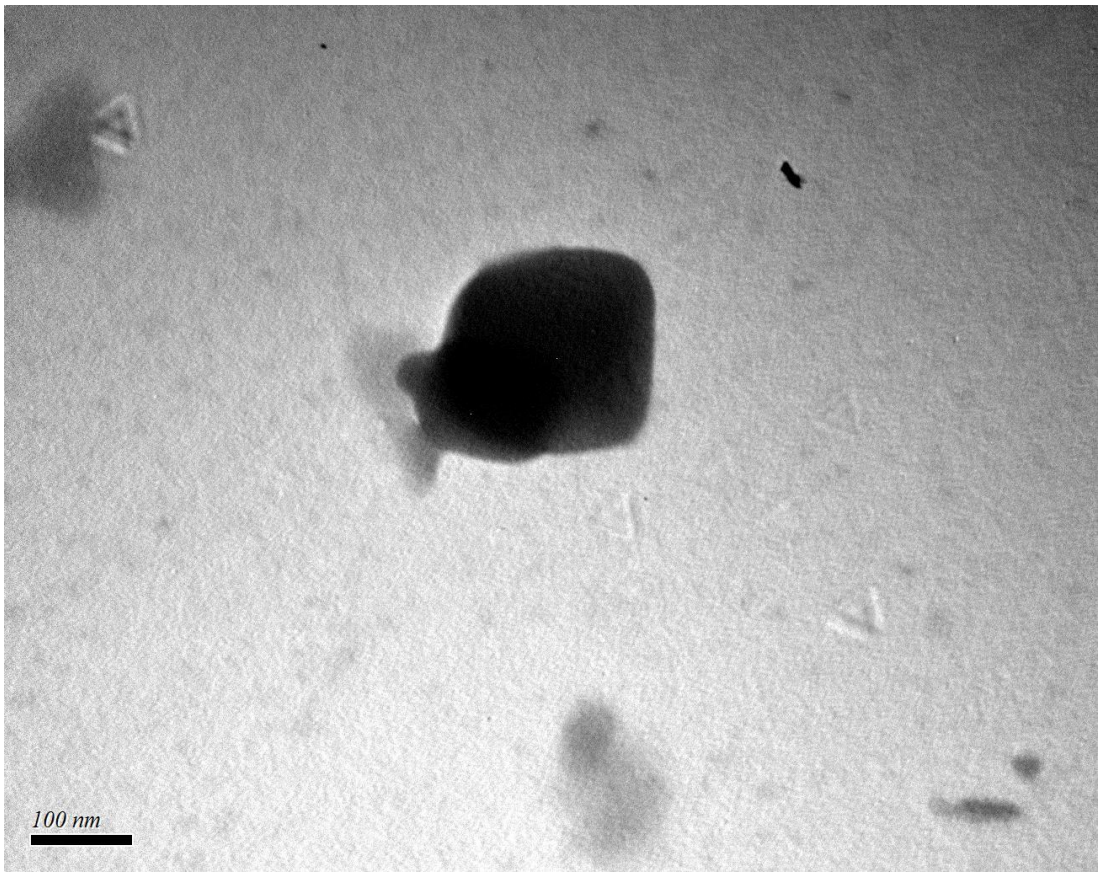


Figure 16: TEM image of ibuprofen nanoparticles.

6.4 Discussion

The present study has examined DNA damage in whole blood cells from healthy control individuals and patients of respiratory disease (asthma, COPD and lung cancer). Cells were subjected to DNA damage with bleomycin using the Comet assay. Cells were treated and allowed to carry out DNA repair without the genotoxins present in absence and presence of NSAIDs as previously discussed. In the Comet assay, the process of lysis ensures the nuclear region of all whole blood cells were kept intact. These are regions that contain the DNA, both damaged and undamaged DNA. Through electrophoresis, the damaged DNA (lose pieces which move under electric gradient), could be identified and compared as percentage or fraction in relation to undamaged DNA (Singh et al., 1988; Tice et al., 2000).

All cells are capable of DNA repair, and mutagens like bleomycin and ultra-violet C (UVC) have been used as clastogens to induce DNA damage in cells to test repair abilities (Wei et al., 2005; Zheng et al., 2004). Bleomycin plays an important role in treating cancers with its anti-tumour effects and NSAIDs (aspirin and ibuprofen) have previously been shown to inhibit prostaglandin synthesis – a promoter of cancer, via inhibition of cyclooxygenase enzymes (Peterson et al., 2010). The present study is able to combine the effects of bleomycin with NSAIDs.

In this study some interesting results have been found after treating cells with 7.5 µg/ml of bleomycin (end concentration). The data for both Olive tail moment and % tail DNA was found to be similar for all groups. Olive tail moment and % tail DNA were used as DNA damage is reflected more

accurately in results obtain using these two parameters for the measurements of Comets and there are no statistical differences between them (Dusinska and Collins, 2008; Kumaravel and Jha, 2006).

Generally, data from all groups showed that all treated bleomycin samples had greater levels of DNA damage (OTM & % tail DNA) which were, significantly different compared to the negative control sample ($p = ** < 0.01$ or $p = *** < 0.001$), with the exception to both ASP N and IBU N, which showed that in the presence of these two compounds for 15 minutes, most DNA was able to repair itself as the majority of samples show no significant difference compared to the control.

For healthy individuals, asthma and COPD patients, reductions in DNA damage can be seen in the sample treated with bleomycin and repaired in the absence of NSAIDs, compared to the bleomycin treated sample with no repair. ASP and IBU samples show even greater reductions in DNA damage compared to the bleomycin with no repair, but the biggest reductions in DNA damage are those of the nano versions of aspirin and ibuprofen as mentioned above. Data for asthma and COPD patients show similar results, which could possibly be linked to the similar nature of both of these conditions (bronchorestriction and blockage of respiratory airways) (Buist, 2002; Masoli et al., 2004).

For lung cancer patients the general pattern of the results are the same as with healthy individuals, asthma and COPD groups. However samples of bleomycin treated cells which were repaired in the absence of NSAIDs were unable to show any reductions in DNA damage compared to the treatment

with bleomycin (and no repair), with no significant difference between them. When samples are treated in the presence of the ASP B & IBU B, there are reductions in DNA damage and even greater reductions with the nano versions (ASP N & IBU N), demonstrating the importance of NSAID compounds in reducing DNA damage and especially with the increased surface reactivity of nanoparticles as result of their large surface area to volume ratio. Although the present system is an *in-vitro* study, nevertheless, these data are thus early indicators of showing the potential of nano compounds. Perhaps, the usage of bleomycin in combination with nanoparticle NSAIDs could be a future avenue for research.

Chapter 7 – General Discussion

7. Discussion

Genotoxicity studies play an important role in the diagnosis or the prevention of disease. The focus of this PhD thesis has been to investigate the genotoxic damage to the DNA in human cells that are diploid, more specifically, peripheral lymphocytes and whole blood cells or haploid in the form of sperm cells. These cells are from healthy control individuals or patients and the general theme was to determine and analyse the impact HAAs and NSAIDs have on DNA and cell damage. Blood cells are of interest because damage to the DNA could give rise to cancer, and damage to the DNA in sperm could give rise not only to cancer but also to heritable defects.

This discussion focuses on two important topics. The first is based on side effects of by-products following treatment of drinking water with disinfectants. The Comet assay has been used as a means of testing genotoxicity by observing the % tail DNA and Olive tail moments of Comets. The micronucleus assay is also used to assess the cytotoxic and cytogenetic potential of these by-products in lymphocytes by observing the number of micronuclei (MNi) in mononucleated and binucleated cells (See sections 7.1-7.4 below).

The second part of the discussion focuses on nanoparticles in human peripheral blood cells. Nanoparticles of the non-steroidal anti-inflammatory drugs, aspirin and ibuprofen, have been investigated in patients with respiratory diseases, using the micronucleus assay and the Comet repair assay (See sections 7.5-7.7). This is followed by conclusions (Sections 7.8).

7.1 Discussion of Halo acetic acids (HAAs)

In today's society, many people are at different levels of health. The Scientific and Research industries work together to address current health issues, to try to find solutions and cures for diseases and various other ailments. This is in order to develop a better picture of good health essential for human welfare. The World Health Organization (WHO), is an established globalised agency for the United Nations, responsible for international public health matters across many countries. It works to provide leadership guidelines on health matters, shaping health and research agendas, setting rules and regulations in research, provides support and assesses and monitors health trends worldwide. The purpose of which is to globally provide a sustainable social and economic benefit for all (WHO, 2010a). A branch of the WHO focuses on environmental health, which considers all aspects of human health and disease that are determined by factors in the environment. The environment plays a critical role in health and disease and in general several factors such as; air quality, climate change, housing and health, noise, occupational health, water and sanitation all contribute to health and disease.

According to WHO, almost 20% of deaths in Europe and worldwide involve an environmental factor. Water sanitation is a major issue to address; in particularly in developing nations where in some parts there is a lack of clean, safe and quality drinking water, exposing people to pathogens and disease causing organisms present in the water. In order to disinfect water, water companies and suppliers make use of disinfectants, mainly chlorine but also including alternatives: chloramines, chlorine dioxide and ozone (Plewa *et al.,*

2002; Richardson *et al.,* 2007; Zhang *et al.,* 2000). While disinfectants have provided a novel method as a means to clean water, their usage leads to the formation of unwanted drinking water disinfection by-products (DBPs). DBPs form as a consequence of reactions between the chemical disinfectant and organic constituents, bromide and iodide that are naturally present in water (Cemeli *et al.,* 2006, Plewa *et al.,* 2010).

DBPs are a major public health concern and they have become one of the important areas in investigations of toxicology. In epidemiological studies, consumption or exposure to water above the maximum containment levels of DBPs in water have been associated with problems of liver, kidney, the central nervous system and increased risks of bladder, and colorectal cancers (Bull et al., 2011; Rahman et al., 2010; Villanueva et al., 2004; Villanueva et al., 2007). Other studies have reported adverse pregnancy outcomes; DBP exposure has been associated with spontaneous abortions, low birth weight, still births and late pregnancy preterm delivery (Grellier et al., 2010; Hinckley et al., 2005; Wright et al., 2003; Yang et al., 2007).

These findings demonstrate an important cause for concern, suggesting the need to further study the effect of DBPs in cells that have not been reported in current literature. Both the Comet assay and the micronucleus assay have shown positive results of HAA damage in lymphocytes and sperm. Damage to somatic and germ cells can give rise to cancer, but in germ cells damage could also produce heritable defects. There is indirect evidence that shows this is so

(Baumgartner et al., 2012; Dubrova et al., 1996; Gardner et al., 1990a; Gardner et al., 1990b).

7.2 Methods of reducing DBP related health issues

The prime method of reducing DBP-related health issues is by using new approaches in treating water. Therefore adopting new methods to disinfect water might be one avenue for researchers in this field. Some of these methods have demonstrated the benefits. One suggestion is targeting the removal of DBP precursors. The major advantage in targeting DBP precursors over managing the DBPs is that removing precursors will minimise DBP formation that includes both known and unknown DBPs, either regulated or unregulated. Over 600 DBPs have been identified and the toxicological effects of many of these compounds are not exactly known, especially as it deemed not feasible to monitor and assess every DBP. Hence targeting DBP precursor is more appropriate and a simple approach to use (Watson et al., 2012).

Other strategies to remove DBP precursors (present in natural organic matter) from source waters include membrane techniques such as membrane filtration including; reverse osmosis, nano-filtration, ion exchange membrane and electro dialysis that been determined as effective in removing natural organic matter – important for controlling DPB precursors, However, these techniques remain expensive and energy inefficient but other approaches like absorption techniques; carbon aerogels; ion-exchange resins and aluminium coagulation

appear to show better progress in a bid to eradicate DBP-precursors (Watson et al., 2012).

Finally, further research has shown some interesting findings in DBPs found from tap water in households. DBPs can vary indoors, as people may additionally, generally use indoor water handling strategies in a bid to improve taste and smell of water and reduce chances of microbiological contamination that includes: boiling of water before use, use of water filtration units, refrigeration and storage and these can all contribute in reduction of DBPs (Levesque et al., 2006). Trihalomethanes (THMs) levels were greatly reduced in two studies after boiling water for between 1-5 minutes (Batterman et al., 2000; Krasner and Wright, 2005). Similar findings have shown reductions in other DBPs; chloroform and HAAs reductions after boiling water and using carbon filters (Weinberg et al., 2006; Wu et al., 2001).

Wu et al. (2001) observed an 83% reduction of chloroform after 5 min of boiling, a two-fold increase of dichloroacetic acid (DCAA) levels and a 30% decrease of trichloroacetic acid (TCAA) after 1 min of boiling. Weinberg et al. (2006) reported that different types of activated carbon filters removed from 93% to 99% of THM levels and from 68% to more than 95% of HAA levels.

7.3 Genotoxic effects of HAAs and antioxidant implications on their damage in the Comet assay

The Comet assay is a micro-electrophoretic technique for the direct visualisation of DNA damage in mammalian cells (Ostling and Johanson, 1984). The damage was measured by investigating the proportion of head DNA (undamaged DNA) with respect to Tail DNA (damaged DNA) (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et al., 1998b; Anderson et al., 1997c; Anderson et al., 2003; Singh et al., 1988; Tice et al., 2000).

In this study, dose response experiments demonstrated that the increasing dosage of all three chemicals was proportional to DNA damage, and hence, reaffirms the necessity to keep HAAs regulated in line with THMs. As the antioxidants, catalase and BHA were able to reduce damage in CHO cells, the findings of this investigation contribute to these findings, suggesting catalase and BHA may be able reduce HAA-induced DNA damage in other mammalian cells.

HAAs can have various modes of action in cells. These include; induction of uncontrolled cell proliferation, the accumulation of endogenous mutations by sustained DNA synthesis during hyperplasia, and the generation of reactive oxygen species (ROS) as a consequence of imbalanced production of peroxisomal enzymes (Cemeli et al., 2006). ROS can cause DNA damage and can induce DNA strand breaks and damage bases such as 8-hydroxydeoxyguanosine (8-OH-G) that can lead to tumour progression. Male mice B6C3F1 exposed to HAAs had expressed a dose-dependent increase of 8-OH-G isolated from nuclei of liver cells (Parrish et al., 1996)

Catalase is present in almost every cell of the human body where it works to prevent oxygen radicals (ROS) forming by breaking down H_2O_2 into harmless H_2O and O_2 (Zamocky et al., 2008). BHA is an exogenous antioxidant that functions as a free radical scavenger. Its aromatic ring is able to stabilise ROS and sequester them. Previous studies have shown dietary administration of BHA increases hepatic glutathione (GSH) (Eaton and Hamel, 1994). GSH is an important reagent with antioxidant properties, protecting cells from toxic substances such as carcinogens, pollutants and drugs. Importantly, GSH also regulates the nitric oxide cycle (Hogg *et al.,* 1996). The dipeptide - gamma-glutamylcysteine plays a vital role in bio synthesis of GSH (Sun et al., 1996). Eaton and Hamel (1994), found the rate-limiting enzyme gamma-glutamylcysteine ligase and GSH to be elevated in mice following BHA diet. In addition, another study showed BHA levels increased GSH-S-transferase activity in mice hepatocytes induced with a carcinogen - 3-methylcholantherene (3MC) following BHA induction, suggesting BHA has a detoxifying effect on chemical carcinogens and the GSH—transferase levels can be used as diagnostic biomarker in cancer (Chen, 1990).

Although, catalase and BHA were able to reduce DNA damage they were unable to entirely eradicate the damage in lymphocytes and sperm, as is the case of IAA in CHO cells. This indicates there are other routes involved in the mechanism of genotoxic action. If oxygen radical involvement was the only mechanism, this would have been demonstrated by no damage in the presence of antioxidants. However a considerable reduction in IAA, BAA and CAA-induced genomic damage in the presence of these antioxidants does in itself highlight the importance of ROS damage to genomic DNA. The

implications of these findings suggest that, perhaps, it could also be appropriate to increase the units of catalase and increase dosage of BHA and determine if there is a complete eradication of DNA damage. Moreover, GSH levels could be investigated and used as a biomarker for antioxidant effect as it is known be elevated by BHA. Arbuckle et al. (2002) have also mentioned the possibility of using GSH as a biomarker, as DBP-metabolising enzymes like gamma-glutamylcysteine is polymorphically expressed in humans.

7.4 Micronuclei (MNi) induction following HAA exposure

Genomic DNA damage was prevalent in peripheral lymphocytes using the Comet assay. The micronucleus test was deployed as a cytogenetic end point using these cells. The mitosis stage of the cell cycle growth can lead to the creation of small distinct extra-nuclear bodies called micronuclei (MNi) (Fenech, 2006, 2007). MNi develop from acentric parts or whole chromosomes that fail to interact with metaphase spindles during mitosis resulting from chromosomal damage and cell damage and as a consequence, they do not form part of the two newly divided daughter cells. In this study, amongst various types of cell scoring, particular attention was emphasised on MNi presence in treatment groups since MNi presence equates signs of chromosomal loss, breakage, rearrangement, non-disjunction and apoptosis (Fenech, 2007).

Related DBPs - THMs have shown an increase of MNi induction in lymphocytes; the findings from this investigation agree with these results, but

they contrast with those of another study using HAAs, which has found no increase in MNi frequency in the TK6 cell line (Liviac et al., 2010). In this study, HAAs were initially tested at same doses as deployed in the Comet assay and all three HAAs were found be too toxic and thus cells could not be scored. Subsequently, dose-response experiments were performed, by halving dosage each time) to determine the highest dose that could be used to score for genotoxicity as that immediately lower than the dose-inducing total cytotoxicity. With IAA and BAA a dose of 6.25 μM was used, which compared to the range investigated by Liviac et al. (2010) in TK6 cells. Our findings differ for CAA where a concentration of 62.5 μM was used, lower than the minimum concentration of 100 μM used in TK6 cells, as investigated by Liviac et al. (2010), possibly indicating cell-specific differences.

In this study, all three HAAs caused MNi inductions but for all three this was lower than the positive control MMC. MMC is a compound of the mitomycin family of drugs, which was first discovered in 1956 (Hata et al., 1956). This group contains several functional groups, including aminobenozquinone and aziridine-ring systems (Mao et al., 1999). MMC works as a potent DNA cross linker that has absolute specificity for the DNA sequence CpG and has been shown to have the highest tumour-specificity and has an important clinical use as a chemotherapeutic agent, due to its anti-cancer antibiotic activity (Tomasz and Palom, 1997). In all three HAA experiments, MMC showed the highest frequency of MNi.

In the micronucleus assay, it must be noted that other factors can play a contributory role to the results obtained. For example there are issues with the

experimental design of the CBMN assay and results can vary due to individual factors. Firstly the age and sex of the blood donor can impact on MNi scored in the CBMN assay. MNi frequency increases with age and in females there is a higher frequency of MNi by approx. 1.4 fold as a result of random loss in the inactive X chromosome, which like acentric and other whole chromosome fragments may lag at the anaphase (Fenech and Morley, 1985; Hando et al., 1994). Secondly, hypotonic treatment is used as a method to remove red blood cells, but it can also alter cell morphology and structural detail of viable cells, which can have negative implications for results (Fenech, 2002). Also results from the Human MicroNucleus (HUMN) project, a worldwide collaboration of 30 laboratories aiming at improving application and understanding of the lymphocyte CBMN assay, has shown that even if all laboratories follow and use the same scoring procedure, from same cell cultures prepared in the same manner, there would still be large differences found amongst the results between each scorer and the laboratories reflecting differences in visual discrimination of the cell structures scored and their interpretation of the scoring criteria (Fenech, 2002). This might perhaps, explain why Chi-Squared data showed different results of BHA in reducing HAA-induced damage on lymphocytes, compared to catalase, further repeat experimentations should resolve this issue.

7.5 Discussion of NSAIDs & nanoparticles

7.5.1 Respiratory diseases (Asthma, COPD & Lung cancer)

Cancer and disease are the biggest threats to the health and wellbeing of animals and humans. The factors that trigger the onset of these diseases are often through the varying factors of lifestyle (mutagens and carcinogens like smoking), diet, occupational health, environment (exposure to chemicals) and genetics. As humans there are ways on addressing some of these issues by taking everyday approaches, such as maintaining a good diet with regular exercise and avoid smoking for example, however sometimes the dogma of disease and cancer is such that these external factors are insufficient to prevent their onset. As such understanding of health and disease is critical to survival.

The discovery of deoxyribonucleic acid (DNA) several decades ago has opened a wealth of knowledge and much has been done to gain a better understanding of DNA which encodes genetic information and has an important role in the development and functions of all cells. Certain chemicals can be mutagenic to the DNA in cells and this can cause changes to the cell and it's functioning, sometimes leading to cancer. This gives rise to the term, genotoxicity – which involves studies of these chemicals possessing the capability of causing damage to the DNA or chromosomes (Phillips and Arlt, 2009). Damage to the DNA can be in the form of strand breaks (single and double), loss of repair (BER, NER), point mutations and chromosomal aberrations (Phillips and Arlt, 2009).

Several assays like the Ames test and the Comet and micronucleus assays have been developed to aid better understanding of the potential of chemical mutagens which can ultimately cause cancer, by causing changes to the DNA (Ames et al., 1973; Phillips and Arlt, 2009; Singh et al., 1988; Tice et al., 2000). Two of these assays – the micronucleus and the Comet assays, have been deployed in *in-vitro* experiments, testing the role of non-steroidal anti-inflammatory drugs (NSAIDs) on the blood from respiratory disease and cancer patients.

In patients with respiratory diseases, conditions like asthma, COPD, the organs and tissue involved in the gas exchange of inhaled and exhaled air is greatly affected. These diseases have serious consequences, since they can cause common-colds, to life threatening conditions of pulmonary embolism, pneumonia and even lung cancer.

7.5.2 NSAIDs – aspirin and ibuprofen

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzyme activity, a significant mechanism of action of NSAIDs. These enzymes play a significant role in the modulation of prostaglandin synthesis and subsequently, tumour progression (Day and Graham, 2013; Rao and Knaus, 2008). Several studies have shown that NSAIDs, like aspirin and ibuprofen exhibit anti-cancer activities on several hallmarks of cancer, such as cell proliferation, evasion of apoptosis and cell cycle regulation (Burn et al., 2011; Mansouri et al., 2013; Park et al., 2014). Some of these consist of a large number of clinical and

preclinical studies that have presented strong data to show NSAIDs could inhibit various types of cancers as described earlier (Brady et al., 2011; Coghill et al., 2011; Coghill et al., 2012; Kang et al., 2013; Kawahara et al., 2010; Retsky et al., 2012; Seufert et al., 2013; Thapa et al., 2010).

Nanotoxicology has become a vast significant area of interest to scientists, who are using this technology to find better ways to improve / develop current or new forms diagnosis or treatment. Nanoparticles (100 nm – 1000 nm) have the biggest advantage of having a smaller size, resulting in a large surface area to volume ratio, thereby increasing the reactivity of cells (Dhawan and Sharma, 2010). Nanoparticles are better candidates in accumulating at the site of inflammation, and thus are suitable for targeted drug delivery (Elzoghby et al., 2012).

7.6 Implications of ASP & IBU on lymphocytes from respiratory disease and cancer patients using the Cytokinesis-block Micronucleus assay (Chapter 5)

The ability of NSAID's anticancer properties combined with effectiveness of nanoparticles was tested using the micronucleus assay in peripheral blood lymphocytes of patients with respiratory diseases and healthy individuals using nanoparticle and bulk versions of the NSAIDs, aspirin and ibuprofen.

This investigation has showed a reduction of MNi in binucleated cells in the nano forms of aspirin and ibuprofen, compared to aspirin and ibuprofen bulk forms, respectively. For aspirin and ibuprofen nano, the MNi frequency was found to be generally similar to the negative controls and in asthma and COPD

patients, the MNi frequency was even lower, demonstrating possible benefits of using nanoparticles.

Similar findings were found using Comet assay as part of a parallel study in our laboratory (conducted by Dr. Mojgan Najafzadeh, University of Bradford).

7.7 Role of NSAIDs on the repair of DNA following exposure to bleomycin (Chapter 6)

Previous works on NSAIDs using the micronucleus assay (Chapter 5) and the Comet assay (by Dr. Mojgan Najafzadeh, University of Bradford) have shown the reductions in DNA damage in lymphocytes when treated with NSAIDs and to a greater extent with the nanoparticle versions. With these findings, this study was performed to investigate the influence of DNA repair of cells in the presence and absence of NSAIDs in order to determine whether these compounds facilitate the repair of DNA or have the opposite effect or no effect.

Monitoring the repair of DNA is one of important determinants of cancer susceptibility (Gaivao et al., 2009). Cells from healthy individuals, asthma, and COPD and lung cancer patients are challenged using bleomycin and then allowed for DNA repair without bleomycin (BLM). Some have been allowed to carry out DNA repair in the in the presence of NSAIDs, as previously discussed.

Following exposure to bleomycin, the greatest efficiency in the repair of DNA was found in samples which were allowed for repair in the presence of NSAIDs. The nano forms in particular, showed the best repair of DNA

compared to their bulk equivalent forms, where DNA damage had reduced to negative control levels.

In lung cancer patients, most DNA was still not repaired in the samples where DNA repair was allowed in the absence of NSAIDs, however reductions in DNA damage is seen in the presences of the bulk versions of the NSAIDs, and this reduction is even greater in presence of the nano versions. These results demonstrate the importance of NSAIDs compounds in reducing DNA damage and especially with the increased surface reactivity of nanoparticles as result of their large surface area to volume ratio. Although the present system is an *in-vitro* study, these data obtained are thus early indicators of the potential of nano compounds. Perhaps, the usage of bleomycin in combination with nanoparticle NSAIDs for cancer treatments could be a future avenue for research.

7.8 Conclusions

Data from the Comet assay has shown that HAAs responded in a similar manner, producing similar dose responses over the same dose range in lymphocytes and sperm. After treatment with the antioxidants, all three HAAs responded in a similar way, with slightly more damage in both the Olive tail moment and % tail DNA in the sperm than in lymphocytes, suggesting that haploid cells maybe more sensitive to genotoxic events. Data with the micronucleus assay showed a similar pattern in the form of micronuclei. Frequency of micronuclei increased in presence of HAAs but reduced with catalase, with reductions also seen in some dosages of BHA, following a

similar trend to results in the Comet assay where the DNA damage may initially be greater but generally less as primary DNA is able to repair itself over time. However, here we have shown fixed DNA damage as a consequence of exposure to HAAs. Our study, thus demonstrates the genotoxic effects of these compounds to both somatic and germ cells.

These observations are of concern to public health since both somatic and germ cells show positive responses and further contribute to existing literature demonstrating the importance DBPs have in relation to health and the environment, in particular HAAs which are not regulated in the United Kingdom. A joint project – HIWATE (Health Impacts of long term exposure to WATEr) has recently been established in the European Union to investigate potential human health risks associated with long term exposure to DBPs (Nieuwenhuijsen et al., 2009). This should provide greater insight and better understanding of DBPs and their impact on human health and environment. These were the first studies to show that all three HAAs act through an oxygen radical involvement. (Cemeli et al. (2006)), had previously shown that IAA acted in this way. No enzyme treatments were necessary in the Comet assay, since the antioxidant effect was detectable without the necessity of these enzymes, so strengthening the case of the antioxidant response.

Similarly, investigating NSAID nanoparticles have also yielded some interesting findings using both the micronucleus and the Comet repair assays in healthy individuals, asthma, and COPD and lung cancer patients. Using the micronucleus assay we are able to show that nanoparticle NSAIDs (aspirin

and ibuprofen) have beneficial effects. Micronuclei (MNi) – an indicator of DNA damage in fixed cells, was found to be in lower numbers compared to their equivalent bulk standard sized equivalents (aspirin and ibuprofen). With the Comet repair assay we were able to show that NSAIDs bulks greatly enhance the repair of DNA, following exposure to bleomycin. This enhancement is increased further, when the repair of cells occurs with the addition of NSAID nanoparticles. NSAIDs are known for their anti-cancer effects and nanoparticles are known for their benefits, via their physiochemical properties and effective drug delivery (Osman et al., 2010). With this in mind, findings from these two *in-vitro* studies have provided some useful information that might be of relevance for further research, including research *in-vivo.*

The doses that are used in all four of these studies (Chapters 3, 4, 5 & 6) might be higher than those found in real life situations. They have been optimised to detect a response as is the case in all genotoxicology assays. We do not know the situations for water samples in all countries around the world, but our studies indicate the potential for a positive response with these chemical in both somatic and germ cells, and therefore should be considered with caution.

These data have been generated *in vitro*. As for all *in vitro* experiment, it is difficult to extrapolate from in vitro to the *in vivo* situation. These *ex vivo* / *in vitro* are the next best approach to extrapolating to the *in vivo* situation. We know from many experiments of this nature conducted in our laboratory that lymphocytes and sperm possess some metabolic activity relating to the human situation since cells are freshly removed from the human body. Whilst we

cannot directly do experiments in man, this approach gives some indication of what might be expected if man were exposed directly to these compounds.

Future work

It would be interesting to determine the nature of chromosome damage lesions using micronucleus FISH (Fluorescence *in situ* hybridisation) in both studies on HAAs and NSAIDs. FISH in combination with micronucleus assay, consists of a probe that is used to label the pan-centromeric regions of the chromosome. This technique allows for discrimination between micronuclei containing a whole chromosome (centromere positive micronucleus) and a centric chromosome fragment (centromere negative micronucleus).

Chapter 8 – References

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