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TITLE: Biomarkers of oxidative stress in models of schizophrenia.

YEAR: 2007

This work was submitted to- and approved by Robert Gordon University in partial fulfilment of the following degree:
Doctor of Philosophy, School of Pharmacy

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Biomarkers of Oxidative Stress in Models of Schizophrenia

Biomarkers of Oxidative Stress

in Models of Schizophrenia

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A thesis submitted in fulfilment of the requirements of

The Robert Gordon University

for the degree of

DOCTOR OF PHILOSOPHY

School of Pharmacy

The Robert Gordon University

Aberdeen

December 2007

DECLARATION

This thesis has been composed by myself and has not been submitted in any previous application for a higher degree. The work of which it is a record has been done chiefly by me, but where assistance has been provided; those individuals have been appropriately acknowledged. All verbatim extracts have been distinguished by quotation marks and the sources of information specifically acknowledged.

Julie Young

For Erin

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Acknowledgements

I would like to express my sincere thanks to the following people for their help, guidance and encouragement during this project. The human studies were collaborative activities which brought together the collective expertise of researchers at the School of Pharmacy, The Robert Gordon University (Aberdeen) and the Ness Foundation (Inverness), an associate partner of The University of the Highlands and Islands.

Dr Susanne Boyle and Professor Iain Glen were responsible research governance on the Aberdeen and Inverness site respectively. I would also like to thank Dr Susanne P Boyle, Director of Studies, for her advice, guidance and support throughout the project and the preparation of this thesis.

Ms Siofradh McKinney was the clinical research nurse responsible for trial recruitment, collection of blood and breath samples and completion of volunteer questionnaires.

Mr Ivor MacKenzie (Ness Foundation) was the research technician responsible for determination of breath volatiles by GC-MS.

Mr Raymond Reid (RGU) was the research technician responsible for determination of total plasma MDA by LC-MS and a measure of free plasma hydroxyalkenals by UV spectrophotometry.

I acknowledge the support of NHS staff at New Craig's Hospital, Inverness and the participation of the patients and volunteers in this study without which the research would not have been possible.

Professor Klaus Wahle, for his advice, guidance and help with the preparation of this thesis.

Dr Brian Ross, for his support and help during my first year.

RGU and Professor Terry Healy for financial support.

Dr Gordon Bell, for the cellular fatty acid determinations.

Dr Susan Duthie for her invaluable guidance and direction during the establishment of the comet assay.

Dr Gary Duthie's group for undertaking the analysis of the plasma vitamin C.

Dorothy Moir, Margaret Brown, Vivienne Hamilton, Heather Scott and Raymond Reid for their technical assistance.

Dr Marie Goua, for her help with cell culture.

Dr Anita Weidmann for all of those coffee breaks. I couldn't have got through this without them.

Abstract

Background

Increasing evidence indicates that oxidative injury exists in schizophrenia. Although it may not be the main cause, oxidative damage has been suggested to contribute to the pathophysiology and may account for deteriorating course and poor outcome in schizophrenia. There is increasing interest in the neuroprotective efficacy of antioxidants in modulating such processes with at least one polyphenolic being tested as a prophylactic in Alzheimer's disease. Beneficial effects of adjunctive ω-3 (n-3 series) polyunsaturated fatty acids with combined intakes of vitamin C and E on both the positive and negative symptoms of schizophrenia have been reported. Robust *in vitro* systems are desirable, enabling a mechanistic investigation of the molecular mechanisms underpinning such effects and identification of further potentially efficacious nutraceuticals.

Materials and Method

Comparative studies employing a lymphoblastoid cell line of schizophrenic origin, a neuroblastoma IMR-32 cell line and the lymphoma U937 cell line was undertaken. The cytoprotective effects of phenolic antioxidants and essential fatty acids in affording protection to cellular DNA, protein and lipids from an oxidative challenge were assessed in the three cell lines. In addition, two human studies were undertaken. The first study utilised the non-invasive technique of breath hydrocarbon analysis and the lipid peroxidation products in a population of schizophrenic patients were compared to a population of apparently healthy aged-matched control subjects, while the second study investigated possible differences in biomarkers of DNA, lipid

and protein oxidation in schizophrenic and control subjects. Plasma vitamin C levels were also compared in both groups.

Results and Conclusion

Cell Culture Studies: Pre-treatment of peripheral and neuronal cells with antioxidant or ω-3 fatty acids followed by an oxidative challenge significantly reduced the levels of DNA damage. Treatment with H_2O_2 alone and following pre-treatment with EPA or DHA had no effect on the levels of protein carbonyls in U937 cells, however, DHA supplementation did appear to reduce endogenous and H_2O_2-induced protein carbonylation. Marked differences in the uptake of fatty acids by the cell types were found and the IMR-32 cell line was most susceptible to the oxidant challenge. Hydroxytyrosol gave significant cytoprotection in all three cell lines and this possible neuroprotective efficacy warrants further investigation, both *in vitro* and *in vivo*. Treatment of the three cell lines with a high concentration of H_2O_2 for 30min or 4 hours did not induce a significant increase in MDA. U937 cells were supplemented for 24 hours with fatty acids followed by a 4 hour oxidative stress. Both EPA and DHA treatment appeared to reduce LOOH levels in the U937 cells but not significantly. Cytoplasmic PLA_2 activity in the three human cell lines was examined and the basal level of $cPLA_2$ activity was found to be comparable in the lymphoblastoid and IMR-32 cells but significantly lower than that measured in the U937 cells. Supplementation of the U937 cell line with EPA caused a significant decrease ($p<0.05$) in $cPLA_2$ activity relative to the vehicle treated control but neither EPA nor DHA supplementation appeared to have any significant effect on either total PLA_2 or $cPLA_2$ activity in IMR-32 or lymphoblastoid cell lines.

Human Studies: No significant difference was found between the levels of ethane and pentane in the breath from the schizophrenic patients and control samples. In addition, no significant difference in the levels of plasma MDA between the two groups was detected. Ethane levels and MDA levels were higher in the male schizophrenic samples than in the female schizophrenic samples but the results were not statistically significant. The pentane levels were higher in the female schizophrenic samples when compared to the male schizophrenia samples but again, these were not significantly greater. Finally, results of study 2 revealed that cellular DNA damage and plasma protein carbonyl levels were increased in the schizophrenic group compared to control subjects but not significantly. However, DNA damage in lymphocytes from the male schizophrenic group was significantly higher than the female group. Biomarkers of lipid peroxidation and plasma vitamin C levels also revealed no significant difference between the two groups under investigation, although a significant elevation in plasma vitamin C was observed in the female control group when compared to the male groups.

Treatment of cells with EPA, DHA and hydroxytyrosol to reduce levels of oxidative damage warrants further investigation. Ultimately, it is important to investigate a range of biomarkers to determine whether the measurement of oxidative damage to lipids, proteins and DNA has clinical significance. This will enable better understanding of the disease of interest and allow these biomarkers to become potentially useful clinical tools.

Abbreviations

4-HNE	4-hydroxynonenal
8-OHdG	8-oxo-2'deoxyguanosine
8-OH-Gua	8-hydroxyguanine
8-oxo-dGuo	8-oxo-2'-deoxyguanosine
8-oxoguanine, 8-oxoG	7,8-dihydro-8-oxoguanine
AA	Arachidonic acid
ABTS	2,2'azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
AD	Alzheimer's disease
AdrA	Adrenic acid
Akt	Akt1 or protein kinase B family of enzymes
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
AODS	Antioxidant defence system
ARE	Antioxidant response element
ATD	Automatic thermal desorption
BBB	Blood brain barrier
BER	Base excision repair
BHT	Butylated hydroxytoluene
BPRS	Brief psychiatric rating scale
BSA	Bovine serum albumin
CAT	Catalase
CM-DCF	5-(and-6)-chloromethyl-2',7'-dichlorofluorescine
CM-H_2DCF	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescin
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
CNS	Central nervous system
$cPLA_2$	Cytosolic phospholipase A_2
CSF	Cerebrospinal fluid
Cu,Zn-SOD	Copper, zinc-containing superoxide dismutase
CV	Coefficients of variation
CVD	Cardiovascular disease
Cys	Cysteine
D2	Dopamine
DAG	Diacylglycerol
DAPI	4'6-diamidine-2-phenylindole dihydrochloride
DCF	2',7'-dichlorofluorescein
DCFH	2',7'-dichlorofluorescein diacetate
DGLA	Dihomogamma-linolenic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribosenucleic acid
DNP	Dinitrophenylhydrazine
DNP	Dinitrophenylhydrazine
DNPH	2,4-dinitrophenylhydrazine

DPA	Docosapentaenoic acid
EC	Epigallocatechin
ECACC	European Collection of Cell Cultures
ECD	Electrochemical detection
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EI	Electron ionization
ELISA	Enzyme linked immunoabsorbent assay
EPA	Eicosapentaenoic acid
EPS	Extrapyramidal side effects
EPUFA(s)	Essential polyunsaturated fatty acid(s)
ERK1/2	Signalling cascade
ESCODD	European Standards Communication on Oxidative DNA Damage
ETA	Eicosatrienoic acid
ETOH	Ethanol
F_2-iP	F_2-isoprostanes
FAMES	Fatty acid methyl esters
FCS	Fetal calf serum
FPG	Formamidopyrimide glycosylase
FSC	Forward scatter
GBD	Global burden of disease
GC	Gas chromatography
GC-MS	Gas chromatograph-mass spectrometry
Glu	Glutamate
GSH	Glutathione
GSx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HAE	4-hydroxyalkenals
His	Histidine
HMP	High melting point
HPLC	High pressure liquid chromatography
HPLC-ECD	High pressure liquid chromatography-electrochemical detection
HPLC-MS	High pressure liquid chromatography-mass spectrometry
HRP	Horse radish peroxidase
IFNγ	Interferon gamma
IL-1β	Interleukin-1beta
IL-2	Interleukin-2
$iPLA_2$	Ca^{2+}-independent intracellular phospholipase A_2
LA	Linoleic acid
LC-MS	Liquid chromatography-mass spectrometry
LMP	Low melting point
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
LSM	Lymphocyte separation medium

Lys	Lysine
MAPK	Mitogen activating protein kinase
MDA	Malondialdehyde
Mn-SOD	Manganese-containing superoxide dismutase
MPA	Metaphosphoric acid
NA	Nervonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NER	Nucleotide excision repair
NF-kappa	Nuclear factor-kappa
NHS	National Health Service
NMP	Normal melting point
NRP	Non radical products
$OH^{\cdot}$	Hydroxy radical
PAF	Platelet-activating factor
PANSS	Positive and negative syndrome scale
PBS	Phosphate buffered saline
PCOs	Protein carbonyls
PD	Parkinson's disease
PKC	Protein kinase C
PLA2	Phospholipase A_2
PLD/C	Phospholipase D/C
PMSF	Phenylmethanesulfonyl fluoride
PMT	Photomultiplier tubes
PUFA(s)	Polyunsaturated fatty acid(s)
ROS	Reactive oxygen species
Rts	Retention times
SCGE	Single cell gel electrophoresis
SDS	Sodium dodecyl sulphate
SIR	Selected ion response
SOD	Superoxide dismutase
sPLA2	Ca^{2+}-dependent secretory phospholipase A_2
SSC	Side scatter
TBA	Thiobarbituric acid
TBARs	Thiobarbituric acid reactive substances
TCA	Trichloracetic acid
TNFα	Tumour necrosis factor alpha
UV	Ultraviolet
VOCs	Volatile organic compounds

CHAPTER 1

Introduction

1.1 What is Schizophrenia?

Schizophrenia is a major illness characterised by psychosis, apathy and social withdrawal and cognitive impairment which results in impaired functioning in work, school, parenting, self-care, independent living, interpersonal relationships, and leisure time (Mueser and McGurk 2004). The illness is characterised by three broad types of symptoms including psychotic symptoms, negative symptoms and cognitive impairment (Liddle 1987). Psychotic symptoms involve the loss of contact with reality, including false beliefs (delusions), perceptual experiences not shared by others (hallucinations) or bizarre behaviours. Negative symptoms are deficit states in which basic emotional and behavioural processes are diminished or absent. Cognitive impairment in schizophrenia includes problems in attention and concentration, psychomotor speed, learning and memory and executive functions (e.g. abstract thinking, problem solving) (Mueser and McGurk, 2004). The most common subtype of schizophrenia currently found in the UK is paranoid schizophrenia, where age of onset occurs later on in life and it has a more insidious course than the other subtypes (Holtam, 2000).

Diagnosis in psychiatry is not only crucial for understanding the disease process but also for making judgements about treatment and prognosis. However, for those psychiatric disorders whereby there are no clear-cut and differentiating biological markers, there has been less precision and agreement on diagnosis. Consequently psychiatrists have had to rely mainly on symptom expression and historical data, information that may vary depending on the skill of the interviewer and the scope and depth of inquiry (Kay *et al* 1991). The limitation of categorical diagnosis is most evident in psychiatric conditions that are heterogeneous and not uniformly responsive to drug therapy. Schizophrenia is a prime example as it is now accepted

as encompassing more than one disease process. The data from separate perspectives, such as premorbid history, genetic study, neurological findings and drug response, overlap to reveal distinct syndromes, such as those that have different origins and may require different treatments. The analysis of these syndromes reveals a profile of symptoms most notably the positive and negative features (i.e. following PANSS assessment, those patients who are categorized by the negative subscale and have predominantly negative symptoms as compared to the patients who fall within the positive subscale and exhibit predominantly positive symptoms, summarized according to the range of cognitive and emotional domains involved). As a consequence, the Positive and Negative Syndrome Scales (PANSS) has been developed as a broad-ranged, operationalized instrument to measure these and other features of schizophrenic symptomatology (Kay *et al* 1991).

1.2 Burden of Disease

The global burden of disease (GBD) 2000 methodology reports a point prevalence of 0.4% for schizophrenia. Schizophrenia causes a high degree of disability. In a recent 14-country study on disability associated with physical and mental conditions, active psychosis was placed as the third most disabling condition, higher than paraplegia and blindness, by the general population (Üstün *et al* 1999). In the global burden of disease study, schizophrenia accounted for 1.1% of the total disability-adjusted life years and 2.8% of years lost due to disability. The economic cost of schizophrenia to society is also high. It has been estimated that in 1991, the cost of schizophrenia to the United States was US$ 19 billion in direct expenditure and US$ 46 billion in lost productivity (The World Health Organisation Report 2001).

A study conducted by Guest and Cookson (1999) examining the cost of schizophrenia to UK society of an annual cohort of newly diagnosed patients with schizophrenia over the first 5 years following diagnosis, revealed that the total discounted cost to society was estimated at £862 million. The National Health Service (NHS) accounted for 38% of the total cost, of which hospital admissions accounted for 69%, hospital visits a further 26% and drugs accounting for 2%, Local Authorities for 12% and the Home Office for 1%. Indirect costs due to lost productivity accounted for 49%. The study concluded that NHS expenditure and lost productivity costs predominated, irrespective of disease course. The findings suggest that treatments that reduce hospitalisation and have the potential to enable patients to return to active employment could drastically reduce the societal burden of schizophrenia.

1.3 Oxidative Stress

Oxidative stress is implicated in the aetiopathogenesis of numerous diseases and is defined as “a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage” (Sies 1991). Oxidative stress often occurs following an increased production of free radicals, or when the antioxidant defence system is ineffective or a combination of both events and will lead to a free radical attack of protein, DNA and lipids. Oxidants comprise reactive oxygen species (ROS), reactive nitrogen species, sulphur-centred radicals and various others. Not all reactive species are radicals but the reactive non-radical species often become radicals damaging biomolecules by (one-electron) oxidation. This type of reaction results in the formation of oxidation products which are able to propagate the reaction leading to extensive damage.

1.3.1 Free Radicals

A free radical is any chemical species that contains one or more unpaired electrons. Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical, because they act as electron acceptors and essentially 'steal' electrons from other molecules. This loss of electrons is called oxidation and free radicals are referred to as oxidizing agents because they tend to cause other molecules to donate their molecules. When two free radicals come into contact, their unpaired electrons form a pair and both radicals are lost. But since most molecules found in living organisms do not have unpaired electrons, any free radicals that are produced will most likely react with non-radicals, generating new free radicals and creating a chain reaction (Figure 1.1). Unpaired electrons can be associated with many different atoms and molecules and so many different free radicals exist.

(1) ADDITION

$x^{\cdot} + y \rightarrow [x\text{-}y]^{\cdot}$

(2) ELECTRON DONATION

$x^{\cdot} + y \rightarrow y^{\cdot}\text{-} + x+$

(3) ELECTRON REMOVAL

$x^{\cdot} + y \rightarrow x\text{-} + y^{\cdot}+$

only when two radicals meet can chain reactions be terminated.

$x^{\cdot} + x^{\cdot} \rightarrow x2$

$x^{\cdot} + y^{\cdot} \rightarrow xy$

Figure 1.1. Free radical chain reaction.

In biological systems most attention has been focused on the oxygen radicals (Gutteridge and Halliwell 1994). Reduction of oxygen can produce two free radicals, superoxide and hydroxyl. If two hydroxyl radicals combine the joining of their unpaired electrons produce an oxygen-oxygen covalent bond creating hydrogen peroxide (H_2O_2), a product with no unpaired electrons:

$$OH^{\cdot} + OH^{\cdot} \rightarrow H\text{-}O\text{-}O\text{-}H$$

Because H_2O_2 has no unpaired electrons, it is not a free radical. However, if one extra electron is added, $OH^{\cdot}$ is generated. Although poorly reactive, H_2O_2 can make $OH^{\cdot}$ at any time if an electron is supplied to it, for example from iron via the Fenton reaction.

Free radicals and related molecules are often classified together as reactive oxygen species to signify their ability to promote oxidative changes within the cell (reviewed by Gilgun-Sherki *et al* 2001). Oxidants can be formed in various ways such as by ionizing radiation, chemical reactions, enzymatically, redox-catalysis involving free transition metal ions or metal ions bound to enzymes. Important cellular sources of oxidative stress are: (i) the formation of reactive oxygen species by incomplete reduction of oxygen in the respiratory chain of mitochondria; and (ii) the host defence systems which includes the 'oxidative burst' mediated by NADPH oxidase, producing superoxide radical and myeloperoxidase, leading to the formation of hypochlorous acid.

Monitoring oxidative stress in humans can be done indirectly at best, by assaying products of oxidative damage or by investigating the potential of an organism, tissue or body fluids to withstand further oxidation (Abuja and Albertini 2001).

1.3.2 Oxidative Stress and Neurological Disorders

The nervous system is particularly susceptible to ROS attack because of its high metabolic rate, its low levels of oxidant defence systems and its moderate cellular turnovers (Mahadik *et al* 2001). Oxidative stress, therefore, is currently regarded as a contributor to the initiation or progression of various neurological illnesses. Disorders such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis have been causally linked to the generation of ROS and oxidative stress and evidence suggests that oxidative neuronal injury accompanies other neurological brain disorders including schizophrenia (Mahadik *et al* 2001). It has also been reported that free radical-mediated abnormalities may contribute to the development of a number of clinically significant processes including prominent negative symptoms, tardive dyskinesia, neurological 'soft' signs and parkinsonian symptoms. Altered membrane dynamics and antioxidant enzyme activities in schizophrenia are thought to occur because of a free radical-mediated neurotoxicity in schizophrenia (Yao *et al* 2001). Earlier studies have also revealed that oxidized metabolites of catecholamines (aminochrome, derived from dopamine and related compounds) occur in the human brain and it has been suggested that some of the demonstrated cellular damage in the schizophrenic brain may be mediated by catecholamine o-quinones (Smythies 1997).

1.3.3 Oxidative Stress and Schizophrenia

Increasing evidence supports the view that a number of the neuropathological changes seen in schizophrenia may be the result of increased free radical-mediated or ROS-mediated neuronal injury (Mahadik and Mukherjee, 1996). Free radicals are recognised as causative agents of peroxidative damage to lipids, and numerous lipid peroxidation biomarkers exist in the majority of human diseases. Polyunsaturated fatty acids (PUFA), major components of membrane phospholipids, are at high risk of free radical attack and auto-oxidation to form peroxyradicals and lipid peroxide intermediates such as aldehydes (malondialdehyde (MDA), 4-hydroxynonenal (4-HNE)), pentane and ethane, isoprostanes and cholesteroloxides. Altered *in vivo* and *in vitro* phospholipid metabolism has been identified in some patients with schizophrenia. This altered lipid metabolism has been suggested to be a primary result of reduced levels of membrane phospholipid PUFAS. From this 'the membrane phospholipid hypothesis of schizophrenia' has arisen which suggests that altered phospholipid metabolism is the fundamental cause of schizophrenia (Horrobin, 1994). In particular, it is suggested that, in individuals who develop schizophrenia, there is an accelerated loss of unsaturated fatty acids, notably arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and dihomogamma-linolenic acid (DGLA) from the Sn2 position of the phospholipid.

1.4 The Phospholipid Membrane Hypothesis

Approximately 20% of the brain by weight consists of highly unsaturated fatty acids, in particular AA and DHA. The specific essential polyunsaturated fatty acid content of synaptic membranes can modify neuronal functioning and produce clinical effects through at least 2 mechanisms: changes in membrane EPUFA content alter the microenvironment and hence structure and function of membrane receptors; ion channels and enzymes and EPUFAs contribute to cell regulation by acting as a source of precursors for second messengers in intra- and inter-cellular signal transduction (Fenton *et al* 1999).

Feldberg (1976) and Horrobin (1977) introduced the phospholipid membrane hypothesis of schizophrenia from their findings that schizophrenia might be caused by a prostaglandin excess or deficiency. In neurones, the outstanding feature of the phospholipids is that in contrast to other tissues, there are only small amounts of the EPUFAs linoleic and α-linoleic acids. However, there are large amounts of AA and DHA in the sn2 position of the phospholipid with smaller amounts of dihomo-γ-linolenic acid, adrenic acid (AdrA), EPA and docosapentaenoic acid (DPA). The fatty acids may be released from the sn2 position usually by activation of one of the phospholipase A_2 group of enzymes and these then can be converted to a variety of signalling molecules known as eicosanoids including prostaglandins and leukotrienes.

This mixture of fatty acids provides the neuronal membranes their specific properties in relation to cell signalling and to modulation of structures and function of membrane-bound and membrane-associated proteins. In people who develop schizophrenia, it is thought that there is a rapid loss of unsaturated fatty acids,

notably AA, DHA, EPA and DGLA from the sn2 position (reviewed by Horrobin, 1999). When present to a mild degree, this increased rate of loss will be compensated by an increased role of incorporation, with no change in phospholipid composition. But when present to a greater degree, or when associated with problems of incorporation, there will be an actual change in membrane composition (Horrobin *et al* 1995).

An increased rate of loss of the key fatty acids will lead to changes in functioning of membrane-associated proteins and various cell signalling systems. A possible enzymatic basis for such increased loss is the over activity of one or more of the PLA_2 group of enzymes. This could result from the presence of an abnormal enzyme variant or from over expression of the normal enzyme. The increased rate of removal from phospholipids would make the free acids more susceptible to oxidation (Horrobin 1999).

For the development and function of the brain, three fatty acids are important: DHA from the ω-3 series, AA from the ω-6 series and nervonic acid (NA) from the ω-9 series (Assies *et al* 2001). The laying down of these fatty acids is greatest during brain growth in utero, the first months after birth and during puberty. Providing animals with oils that have a low α-linolenic content results in all brain cells and organelles and various organs having reduced amounts of 22:6ω-3, which is compensated for by an increase in 22:5ω-6. The speed of recovery from these abnormalities is extremely slow for brain cells, organelles and microvessels in contrast to other organs. Furthermore, prolonged PUFA deficiency leads to death in animals (Bourre *et al* 1992). The main idea of the phospholipid hypothesis is that normal neuronal phospholipid metabolism is essential for the normal development of

brain architecture in utero and in childhood, for the normal modulation of the architecture around the time of puberty, and for normal functioning of the adult nervous system. The phospholipid hypothesis therefore proposes that "in schizophrenia there are two major primary abnormalities in phospholipid metabolism and that the impact of these may be modified by other lipid-related genes and by environmental factors (such as oxidative stress). The two primary abnormalities are an increased rate of removal of EPUFAs especially AA and DHA from membrane phospholipids, coupled with a reduced rate of incorporation into phospholipids of these same fatty acids" (Horrobin 1999).

1.4.1 Lipids and Fatty Acids

Biological lipids are a chemically diverse group of compounds, although they are all insoluble in water. Furthermore, their biological functions are extremely diverse. Fats and oils are stored mainly as energy in many organisms and phospholipids and sterols make up about half the mass of biological membranes. Lipids have fundamental structural and functional roles in cell membranes in general and in the central nervous system in particular. Lipids form the matrix within which membrane proteins such as receptors and ion channels are embedded and to which membrane associated proteins such as those involved in second messenger systems may be attached. Lipids are also essential constituents of synaptic vesicles; most of the second messenger systems of the neurone depend on lipids such as free fatty acids, diacylglycerols, prostaglandins, leukotrienes and hydroxy-fatty acids.

The formation of membrane phospholipids from simple precursors requires: a) synthesis of the backbone molecule (glycerol or sphingosine); b) attachment of fatty acid(s) to the backbone via ester or amide linkage; c) addition of a hydrophilic head

group joined to the backbone through a phosphodiester linkage; and in some cases d) alteration or exchange of the head group to produce the final phospholipid product (Figure 1.2).

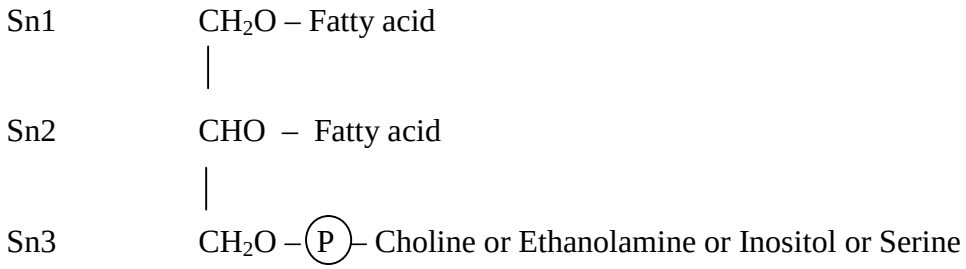


Figure 1.2: The general structure of a phospholipid. The three carbon atoms of the glycerol (or sphingosine) backbone are designated Sn1, Sn2 and Sn3.

In eukaryotic cells, phospholipid synthesis usually occurs at the surface of the smooth endoplasmic reticulum. Some stay within that membrane while others are inserted into different cell membranes in different proportions. Because membrane lipids are insoluble in water they cannot diffuse from their point of synthesis to their point of insertion. Instead they are transported in membrane vesicles that bud from the Golgi complex then move to and join to the target membrane. In addition, cytosolic proteins bind phospholipids and sterols and carry them from one cell membrane to another and from one face of a lipid bilayer to another. The combined action of transport vesicles and proteins produces the characteristic lipid composition of each organelle membrane.

Typical Western diets are made up of 10-20 fold more ω-6 (n-6) series fats than ω-3 (n-3) series. The high proportion of ω-6 fat is due to the large quantity of linoleic acid (LA) in the diet which is present in high concentrations in soy, corn, safflower

and sunflower oils and in the spreads and shortenings produced from them. In comparison, there is a low intake of the ω-3 homologue of LA, α-linolenic acid (ALA) found in leafy green vegetables, flaxseed, linseed and canola oils. When consumed, the 18-carbon fatty acids are desaturated and elongated *in situ* to 20-carbon ω-6 fatty acids. LA is converted to AA and ALA to eicosapentaenoic acid (EPA) and DHA, although DHA conversion is slower in humans. Compared with LA, there is little dietary intake of AA and EPA found in meat and fish respectively. Furthermore, unlike the 18-carbon ω-3 fatty acid ALA, oleic acid is consumed in considerable amounts in the typical Western diet but is not an essential fatty acid, and only small amounts of eicosatrienoic acid (ETA) are found in cells membranes.

Unsaturated fatty acids are not interconvertible but are metabolised by a common series of enzymes (elongases and desaturases) (Figure 1.3). The same series of enzymes that catalyze desaturation and elongation of ω–6 and ω–3 fatty acids also catalyze desaturation and elongation of ω–9 fatty acids. The substrate preference for these enzymes is ω–3 followed by ω–6 and finally, ω–9, thus competition between the ω–9 fatty acids and either the ω–6 or ω–3 fatty acids is rare. However, if the concentrations of 18:2ω–6 and 18:3ω–3 are low, 18:1ω–9 is readily desaturated and elongated to 20:3ω–9 (eicosatrienoic acid).

An increase in dietary ω-3 fatty acids results in reduction of AA levels in tissue lipids because synthesis from linoleate is inhibited. Alterations of dietary fatty acids, therefore, can lead to changes in fatty acid composition of tissue leading to changes in cellular responses (Hwang 2000). Because mammalian systems have lost the ability to insert a double bond between an existing double bond and the methyl or omega end of the fatty acyl chain, this will result in a) an omega-6 or omega-3 fatty

acid remaining such after metabolism *in situ*; b) fatty acids with the requisite double bond have to be ingested, i.e. are essential (Wahle 2000). Plant tissues and plant oils tend to be rich sources of LA and ALA and once consumed in the diet, LA can be converted via γ-linolenic and dihomo-γ-linolenic (DGLA) acids to AA. Using the same pathway, ALA can be converted to EPA and then DHA.

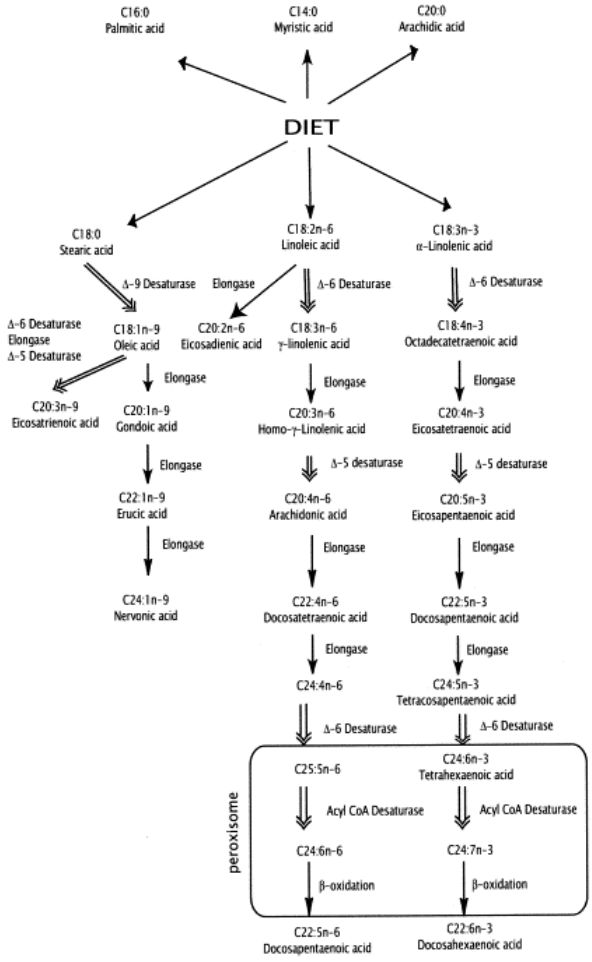


Figure 1.3. Pathways for the metabolism of ω-3, ω-6, and ω-9 polyunsaturated fatty acids. The steps are catalyzed by either an elongase (single arrow) or a desaturase (double arrow) enzyme (Assies *et al* 2001).

Marine plants such as phytoplankton carry out chain elongation and further desaturation of ALA to yield EPA and DHA. It is because of the production of these long ω-3 EPUFAs by marine algae and their transfer through the food chain that the tissues of some marine animals and fish are a particularly rich source of these fatty acids. Commercial products known as 'fish oils', a preparation of the body oils of cold water fish, contain high quantities of EPA and DHA. These are also found in high proportions in the oils extracted from the livers of other species of fish found in warmer waters.

PUFA supplementation has been widely used in human schizophrenia clinical trials since their benefits in reducing a variety of positive symptoms have been reported. However, although a review of the current research has not conclusivley recommended omega-3 PUFA as either a mono- or adjunctive-therapy in any mental illness, the available evidence is strong enough to justify continued study (Ross *et al* 2007). Therefore, by supplementing a variety of human cells with different concentrations of EPA and DHA, we sought to gain a better awareness of the effects that these fatty acids might have on DNA and protein molecules. For example, we were intestered in discovering whether levels of induced oxidative stress would be reduced or increased in the cells following supplementation of low, medium and high concentrations of fatty acids? Ultimately, could these changes in levels of oxidative damage be identified by changes to the DNA and protein, ie via biomarkers?

1.4.2 Phospholipase Enzymes

Most cells continually degrade and replace their membrane lipids. For each of the bonds in a glycerophospholipid, there is a specific hydrolytic enzyme. Phospholipases of the A type (A_1 and A_2) remove one of the two fatty acids, by hydrolyzing the ester bonds of the intact glycerophospholipid at C-1 and C-2 respectively of glycerol (Figure 1.4). Phospholipases C and D each split one of the phosphodiester bonds in the head group. Phospholipases D (PLDs) cleave phospholipids, preferably phosphatidylcholine releasing the free base (i.e. choline) and yielding phosphatidic acid. Although many aspects of PLD enzymology have been elucidated, the functions of mammalian PLDs remain less well understood. In the nervous system, PLD isoforms are present in neurons as well as in glial cells (such as astrocytes and oligodendrocytes) where they play a role in cellular proliferation, exo- and endocytosis and neurite formation (Klein 2005). The rapid hydrolysis of the membrane phospholipid phosphotidylinositol 4,5-bisphosphate is catalysed by phosphoinositide-specific phospholipase C isozymes resulting in the generation of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. These compounds mediate the release of Ca^{2+} from intracellular stores and action of protein kinase C (reviewed by Ochocka and Pawelczyk 2003).

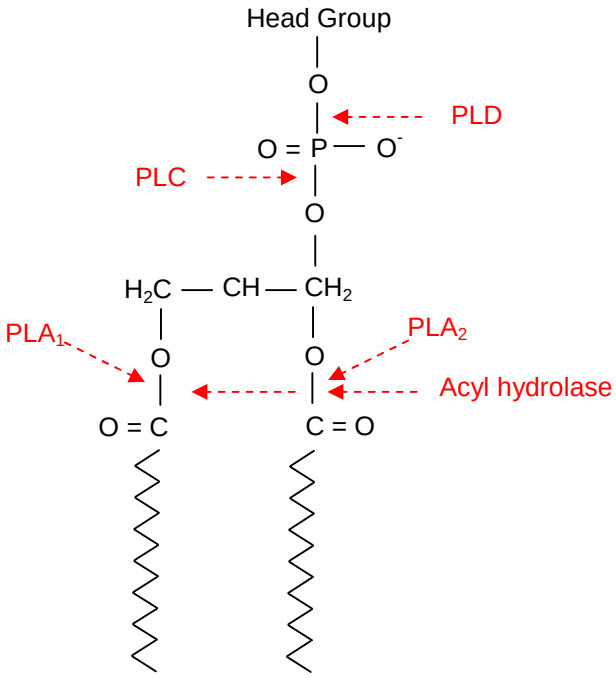


Figure 1.4. Sites of phospholipid hydrolysis by PLD, PLC, PLA_2 and PLA_1 and acyl hydrolase

The growing family of phospholipases A_2 are enzymes that specifically hydrolyze the acyl ester bond at the sn-2 position of glycerol in membrane phospholipids and produce free fatty acids and lysophospholipids. This provides the precursors for many of the lipid mediators involved in normal brain function and neuroinflammatory pathophysiological processes (Farooqui and Horrocks 2004). AA, one such precursor, is then converted to the inflammatory mediators, prostaglandins and leukotrienes. In theory, AA can be produced from numerous other pathways including the DAG lipase pathway as well as phospholipase D-generating phosphatidic acid. As well as being involved in eicosanoid synthesis, PLA_2 plays another important role in inflammation by releasing another compound the 2-lysophospholipid, whose action on membrane phospholipids can be utilized to form platelet-activating factor (PAF), a potential inflammatory mediator

(Chakraborti 2003). Any oxidative modification of membrane phospholipids is a damaging process which is best repaired by the selective cleavage of peroxidized fatty acid residues and their replacement by native fatty acids. Because polyunsaturated fatty acids are preferentially located in the sn-2 position of the membrane phospholipid, this position is especially prone to lipoxygenation or lipid peroxidation and PLA_2s are best designed for such a repair function (Nigam and Schewe 2000).

Several new PLA_2s have been identified based on their nucleotide gene sequences and have been classified mainly into three groups: a) cytosolic PLA_2 ($cPLA_2$); b) Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$); and c) Ca^{2+}-independent intracellular PLA_2 ($iPLA_2$) (Muralikrishna Adibhatla and Hatcher 2006). There is also a class of PLA_2 called PAF acetylhydrolases which appear to act on PAF and oxidized lipids (Six and Dennis 2000).

$cPLA_2$ is a high molecular weight enzyme (85kDa) ubiquitous enzyme found in the cytosol of most cell types. Because of its preference for phospholipid containing AA, it is thought to be associated with receptor-activated signalling cascades and is phosphorylated by kinases of the mitogen-activated protein kinases cascade. Following an increase in intracellular Ca^{2+}, $cPLA_2$ translocates to membranes via a Ca^{2+} lipid binding domain that is present within the protein (Gijon *et al* 2000).

Understanding the role that PLA_2 plays in schizophrenia is an area of research that is currently a 'hot topic'. The effect of EPA and DHA supplementation on the levels of PLA_2 and $cPLA_2$ in human schizophrenia patients has not been extensively explored. However, it has been shown that in human suffers of depression, there is strong epidemiological evidence that fish consumption reduces risk of becoming depressed

and evidence that cell membrane levels of n-3 PUFA are reduced and two placebo-controlled trials have shown a strong therapeutic effect of ethyl-EPA added to existing medication (Peet 2003). Furthermore, four out of five placebo-controlled double- blind trials of EPA in the treatment of schizophrenia have given positive findings (Peet 2003). The mode of action of EPA is currently unknown, but research suggests that AA, if it is of particular importance in schizophrenia, then the clinical improvement observed in patients with schizophrenic using EPA treatment, correlates with changes in AA (Peet 2003). Thus, if EPA and/or DHA supplementation is to be considered a possible future adjunctive therapy for the treatment of schizophrenia, then understanding the effect these fatty acids have on the phospholipase enzyme is important. We undertook a study to gain better understanding into whether different concentrations of fatty acids had any effect on the levels of total PLA_2 and $cPLA_2$ content in human cells.

1.5 Antioxidant Defence System

Aerobic organisms have evolved many types of antioxidant defence. An antioxidant is defined by Gutteridge and Halliwell (1994) as "any substance that delays or inhibits oxidative damage to a target molecule" (p 40). All molecules present in living organisms are potential targets of oxidative damage: lipids, proteins, nucleic acids and carbohydrates. However, antioxidants are thought to protect a potential target from attack by various mechanisms:

- scavenging oxygen-derived species, either by using enzymes or by direct chemical reaction;
- minimizing the formation of oxygen-derived species;

- binding metal ions needed to convert poorly reactive species (such as $O_2^{\cdot-}$ and H_2O_2) into more damaging species (such as $OH^{\cdot}$);
- repairing damage to the target;
- destroying badly damaged target molecules and replacing them with new ones.

The potential health promoting effects of a number of dietary antioxidants are widely reported with epidemiological studies suggesting that naturally occurring phenols and polyphenols have the potential to prevent oxidative damage in a variety of pathological conditions. The first line of antioxidant defence in cells is provided by enzymes. The antioxidant defence system (AODS) is made up of a series of enzymatic (endogenous) and non-enzymatic (exogenous) components.

1.5.1 Endogenous Antioxidants

The human body has a complex antioxidant defence system which includes the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SODs contain metals essential for their catalytic function. The metals bound to SOD catalyse the reaction of two $O_2^{\cdot-}$ molecules with H+ ions to form H_2O_2 and O_2. This reaction occurs slowly at pH 7.4 but SODs accelerate the reaction by 10,000 times. The manganese-containing SOD (Mn-SOD) found in human mitochondria is thought to remove $O_2^{\cdot-}$ produced as a result of electron leakage on to O_2 from the mitochondrial electron transport chain and by mitochondrial oxidase enzymes. The copper- and zinc-containing SOD (Cu,Zn-SOD) removes $O_2^{\cdot-}$ from cytosolic oxidases and from the cytochrome P_{450} enzymes which are located in the endoplasmic reticulum of the cells. Catalase is a large enzyme with haem-bound iron at its active site. It is located within peroxisomes and

may be found in mitochondria of heart tissue. It is responsible for removing H_2O_2 by converting it to O_2. Human tissues contain glutathione peroxidases as their major peroxide-removing enzymes. They remove H_2O_2 at a high rate through the process of the oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG). GSH also has antioxidant properties and has been shown to repair DNA damage following attack by $OH^{\cdot}$. Failure of this first line of antioxidant defence may lead to initiation of lipid peroxidation. Selenium-dependent GSH protects against lipid peroxidation through the conversion of H_2O_2 to H_2O or by converting toxic hydroperoxides to less toxic alcohols. Because of the importance of these enzymes, altered activity of one of them without compensatory changes in other enzymes may result in cell membranes being susceptible to attack.

1.5.2 Exogenous Antioxidants

1.5.2.1 Antioxidant Vitamins

A variety of intra- and extracellular antioxidants play important roles in controlling oxidative stress. Several studies suggest that antioxidant vitamins in the diet may partially protect the integrity of cellular DNA against ROS and so reduce the risk of some diseases. The non-enzymatic antioxidant components that are important in the antioxidant defence systems include those that react with activated oxygen species and prevent the propagation of free radical chain reactions. The most common are albumin, uric acid, bilirubin, GSH, d-α-tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene. The major scavenger inside human cell membranes is d-α-tocopherol. Tocopherols inhibit lipid peroxidation by scavenging lipid peroxyl radicals faster than these radicals can react with adjacent fatty acid side chains or with membrane proteins:

α-tocopherol + lipid- $O_2^{\cdot} \rightarrow$ -α-tocopherol$^{\cdot}$ + lipid-O_2H

The hydrogen atom from the hydroxyl group of the α-tocopherol is donated to the peroxyl radical converting it to a lipid peroxide, resulting in an unpaired electron on the $O^{\cdot}$ leaving behind α-tocopherol radical. α-tocopherol therefore is described as a chain-breaking antioxidant since it breaks the chain reaction of lipid peroxidation but is itself converted to a radical during the process and hence vitamin E is consumed.

Ascorbic acid is also a potent scavenger of several radicals. Found within the aqueous phase of plasma, it also has the ability to recycle α-tocopherol in lipoproteins and delays the onset of peroxidation in human low density lipoproteins. However, ascorbic acid can also exert pro-oxidant properties. In the presence of H_2O_2, ascorbate can speed up $OH^{\cdot}$ formation by the reduction of iron and copper resulting in Fe^{2+} and Cu^{+} which react with H_2O_2 to produce $OH^{\cdot}$.

1.5.2.2 Phenolic Antioxidants

Polyphenols are the most abundant antioxidants in our diets and are widely found in many types of foods and beverages. Consumption of fruit and vegetables, which are rich in antioxidants, prevents cancers and may also prevent stroke, whereas wine consumption might prevent coronary heart diseases and soy may protect against breast cancer and osteoporosis. Food and drinks that provide a good source of phenolic compounds, such as flavonoids (Figure 1.5) have also been associated with decreased risk of developing several diseases (Halliwell *et al* 2005). Interest has also grown in this group of compounds due to their antioxidant properties but phenolic compounds also have several other specific biological actions that are still poorly understood (Manach *et al* 2004).

Polyphenols are reducing agents and together with other dietary reducing agents such as vitamin C, vitamin E and carotenoids, they protect the body's tissues against oxidative stress. Many molecules containing a polyphenol structure (i.e. several hydroxyl groups on aromatic rings) have been identified in higher plants and a large number are also found in edible plants. These molecules are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or attack by pathogens. They are classified according to the number of phenol rings that they contain and defined according to the nature of their carbon skeleton and comprise phenolic acids, flavonoids, stilbenes and lignans.

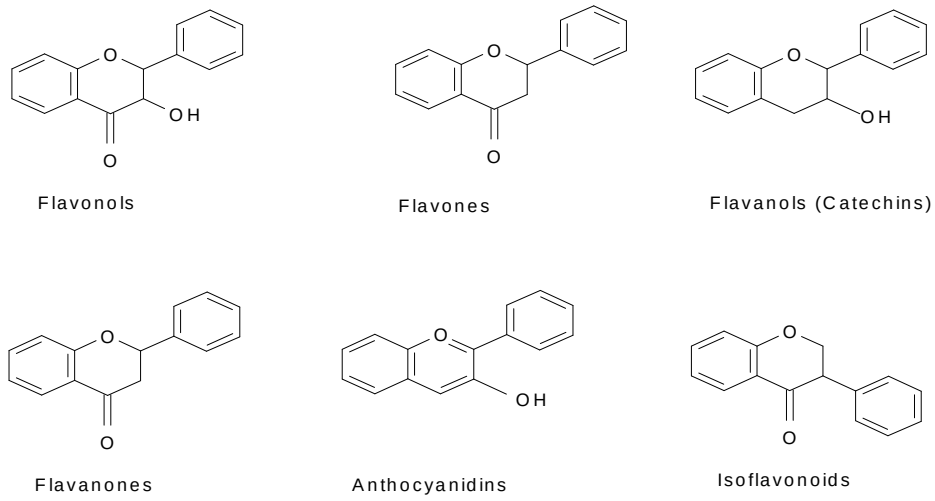


Figure 1.5. Structures of main classes of dietary flavonoids

The chemical structure of polyphenols will affect their biological properties i.e. bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes and other properties. The chemical structure of polyphenols also determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma, as well as determining the conjugation reactions with methyl, sulphate or glucuronide groups and the nature and the amounts of metabolites formed by the gut microflora absorbed at the colon level.

Experimental data has proposed that polyphenols may provide an indirect protection by activating endogenous defence systems. For example, resveratrol and 4-hexylresorcinol have been shown to induce an increase in glutathione, glutathione peroxidase and glutathione reductase (Yen *et al* 2003), while catechins (including epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) have been shown to have activity similar to or higher than that of vitamin C in vitamin E regeneration (Mukai *et al* 2005). Several reports have revealed that exogenous and endogenous antioxidants appear to act in a co-ordinated fashion (reviewed by Masella *et al* 2005). The authors suggest that this may be achieved partly via antioxidant response elements (ARE) located within the promoter regions of the genes induced by oxidative and chemical stress and that dietary polyphenols can stimulate antioxidant transcription and detoxification defence systems through ARE.

A study by McCreadie (2003) which examined the lifestyle of people with schizophrenia reported that fewer males compared with the general population, reached acceptable levels for consumption of fruit, vegetables, milk, potatoes and pulses. In an earlier study (McCreadie *et al* 1998) it was reported that as well as

having a lower fibre consumption and a higher saturated fat intake than the control population, more male schizophrenic patients than female patients tended to consumed less vitamin E. In addition, high dietary intake of sugar as well as saturated fat predicts poor outcome in schizophrenia (reviewed by Peet 2004). This is an important consideration since the consumption of foods rich in antioxidants, such as vitamin C, lycopene, and flavonoids has been shown to confer resistance against lymphocyte DNA oxidation (Boyle *et al* 2000). Traditional Mediterranean diets, as opposed to Northern European diets generally include a higher intake of plant foods which have been associated with lower risk for development of coronary heart disease and certain cancers (Visioli and Galli 2001). A major component of this type of diet is represented by olive oil which contains antioxidants such as tyrosol and hydroxytyrosol and recent data suggests that the components of dietary olive oil may have a greater role in disease prevention than previously thought (Owen *et al* 2000). For example, the content of oleic acid, a monounsaturated long-chain fatty acid is high in olive oil compared with the seed oils e.g. sunflower oil which is rich in polyunsaturated linoleic acid, a fatty acid that is prone to oxidation (Owen *et al* 2000). The involvement of excessive free radical production and the number of epidemiological studies linking antioxidant intake with such diseases as coronary heart disease, suggests that dietary antioxidants may play a protective role (Visioli and Galli 2001).

Recently, attention has focused more on the potential neuroprotective effects of the flavonoids against the neuronal deficits associated with ageing or age-related neurodegenerative diseases. However, to achieve high efficacy, antioxidants must penetrate through the blood brain barrier (BBB), an endothelial barrier that contains tight junctions, a low number of pinocytic vesicles and the expression of various

uptake and efflux transport systems and metabolic enzymes. The tight junctions of the BBB enable diffusion of minute quantities of water-soluble compounds while the large surface area of the lipid membranes of the endothelium provides an effective diffusive route for lipid-soluble agents. Essential nutrients such as glucose, purine bases, nucleotides, choline and certain large neutral amino acids are transported into the brain via specific membrane-transporting proteins. In addition, there also seem to be processes that are capable of shuttling macromolecules into the brain such as receptor-mediated and adsorptive endocytosis systems (Gilgun-Sherki *et al* 2001). Although flavonoids have been shown to possess neuroprotective properties, few studies have specifically examined entry into the central nervous system (CNS). Evidence for CNS entry is available for epigallocatechin gallate and epicatechin (i.e. the epicatechin conjugate and the O-methylated epicatechin conjugate) (El Mohsen *et al* 2002), found in tea and wine and the citrus flavonoids naringenin (and its glucuronide) and hesperetin (Youdim *et al* 2004). In their study, Youdim *et al* (2004) reports novel observations on the permeability of naringenin and quercetin at the BBB, the penetration of these flavonoids into different brain regions and the influence of efflux transports in limiting their entry. Resveratrol, found in relatively high quantities in grapes has been shown to protect against neuronal cell death following experimentally induced ischemia, demonstrating that it can cross the blood brain barrier and exert protective effects against cerebral ischemic injury over a prolonged period of time (Wang *et al* 2002). Nevertheless, despite increased awareness of the chemical forms in which antioxidants such as the flavonoids are able to enter the circulation, studies of the non-physiologically relevant forms are still undertaken. With this in mind Youdim *et al* (2004) have highlighted the flavonoids from particular families that are able to permeate the BBB whereas the

entry of others is limited by the actions of efflux transporters expressed at the endothelium surface.

Numerous studies have been undertaken examining levels of antioxidant enzymes in schizophrenic patients. The data is controversial and shows a variation for the activities of antioxidants. Red blood cell SOD activity has been found to be increased (Vaiva *et al* 1994), unchanged (Herken *et al* 2001) or decreased (Evans *et al* 2003; Ranjekar 2003; Akyol *et al* 2002; Mukerjee *et al* 1996) and CAT and GPx activities were found to be increased (Evans *et al* 2003; Herken *et al* 2001), unchanged (Evans *et al* 2003; Akyol *et al* 2002; Yao *et al* 1998) or decreased (Ranjekar 2003) in schizophrenic patients. Plasma bilirubin and albumin levels (Pae *et al* 2004; Reddy *et al* 2003; Yao *et al* 2000) and uric acid levels (Reddy *et al* 2003) have been shown to be significantly lower in schizophrenic patients compared to controls. Here, bilirubin was significantly lower in the negative subgroup of the patient group, suggesting antioxidant levels are associated with schizophrenia as well as with the clinical symptomatology and the treatment implications in schizophrenia.

Antioxidant supplementation is being considered as a possible adjunctive therapy in patients with schizophrenia, but there is limited knowledge concerning their effects at different concentrations on DNA, proteins and lipids in schizophrenia sufferers. In addition, when DNA, proteins and lipids are subjected to an oxidative challenge it is not clearly understood how antioxidant supplementation helps to protect the cell against ROS or free radical damage. However, what is known is that EGCG biovailability in the CNS has been demonstrated (El Mohsen *et al* 2002) and resveratrol has been shown to cross the BBB and protect against cerebral ischemia (Wang *et al* 2002). Similarly, tyrosol, has been examined in a rat model for cerebral

ischemia and was shown to have a neuroprotective effect which was dose-dependent in the range of 3-30 mg/kg body weight (Bu *et al* 2007). By examining human cells supplemented with a variety of antioxidants and subjected to an oxidative challenge, we aimed to gain a clearer understanding of the effect of antioxidants on a range of biomarkers of oxidative stress.

1.6 Biological Markers of Oxidative Stress

A 'biomarker' can be used to identify a molecular change in a biological molecule (Griffiths *et al* 2002). When studied in tissues, organelles or fluids, biomarkers can provide understanding concerning the nature of the denaturing radical, often along with the location of oxidative damage. As highly sensitive markers of oxidative damage in mammalian systems, biomarkers may provide information on three progressive levels to disease outcome: (a) as measurable endpoints of oxidative damage to proteins, amino acids, oxidised lipids, oxidised DNA bases; (b) as functional markers of blood flow, platelet aggregation, or cognitive function, for example and (c) as endpoints related to a specific disease (Griffiths *et al* 2002). Although clinical symptoms are considered to be endpoints of an illness, they are often inappropriate for early detection and disease prevention.

The peroxidation of PUFAs may be examined in a number of ways including determination of lipid peroxides (Chamblee *et al* 2000), determination of isoprostanes (Morrow *et al* 1990; Boyle *et al* 2000) determination of aldehydic end products [e.g. malondialdehyde and 4 hydroxy nonenal] (Boyle *et al* 2000; Claeson *et al* 2001) and by the determination of volatile hydrocarbons. Volatile hydrocarbon analysis is increasingly being adopted as a method of assessing lipid peroxidation both *in vivo* (Kivits *et al* 1981; Kovaleva *et al* 1989) and *in vitro* (North *et al* 1994).

Volatile hydrocarbons are produced as terminal catabolites of lipid and protein peroxides, ultimately being exhaled in breath. The volatile hydrocarbons that are typically monitored are ethane (C2) and pentane (C5) being derived from the oxidation of ω-3 and ω-6 fatty acids respectively. Ethane is often considered to be a better indicator of lipid peroxidation than pentane since it is less easily metabolized to carbon dioxide (Allerheiligen *et al* 1987). Furthermore, the between day biological variation of ethane in expired air has been noted to be less than that of pentane (24% CV vs. 38% CV) (Knutson *et al* 1999) with the greater variation in pentane rates possibly being due to changes in rates of pentane metabolism. Hydrocarbons are metabolized by hepatic monooxygenases (Frank *et al* 1980) and *in vitro* studies have indicated that pre-treatment of subjects with a blocker of hepatic monooxygenases decreased ethane exhalation by 70% and pentane by 1250% (Wade and van Rij 1985) demonstrating that pentane exhalation is more significantly influenced by hepatic metabolism and thus is a less reliable indicator of lipid peroxidation. Butane is believed to be a catabolite of protein oxidation and arises following the *in vitro* oxidation of haemoglobin (Clemens *et al* 1984).

Generally, oxidatively modified proteins resulting in new functional groups such as hydroxyls and carbonyls are not repaired and must be removed by proteolytic degradation. Amino acids capable of delocalising charge such as those containing aromatic and thiol side chains are more prone to oxidative attack and a large number of aliphatic residues are also subject to oxidation resulting in the generation of protein carbonyl moieties (Griffiths *et al* 2002). Ultimately, therefore, a decrease in the efficiency of proteolysis will lead to an increase in the cellular content of oxidatively modified proteins (Stadtman and Levine 2000). Elevated markers of protein oxidation have been associated with diseases such as Alzheimer's disease,

Parkinson's disease, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, rheumatoid arthritis and progeria (Mayne, 2003). The level of modified molecules can be quantitated by measurement of the protein carbonyl content by an enzyme-linked immunoabsorbent (ELISA) method (Buss *et al* 1997) which is based on the reaction of dinitrophenylhydrazine (DNP) with protein carbonyls.

Free radical attack generates a range of DNA lesions, including strand breaks and modified bases. Hydroxyl radical attack on DNA leads to a large number of pyrimidine- and purine-derived base changes. Some of these modified DNA bases have considerable potential to damage the integrity of the genome. 8-oxo-2'-deoxyguanosine (8-oxo-dGuo) is one of the most critical lesions. Unless repaired prior to DNA replication, 8-oxo-dGuo residues can result in base pair transversions (Griffiths *et al* 2002).

1.6.1 Membrane Phospholipid Peroxidation and Schizophrenia

Oxidative damage to lipids and proteins can result in a number of pathophysiological processes while changes in DNA may alter gene expression or cause cell death and lead to genetic modification and mutagenesis. Although the aetiology of schizophrenia remains unknown, investigation of the effects of oxidative stress in schizophrenia have largely focused on the determination of lipid oxidation products, (Scottish Schizophrenia Research Group, 2000; Ranjekar *et al* 2003). So far, studies of oxidative injury in schizophrenia have focused on membrane lipids, the rationale behind these studies stemming from the emergence of the membrane phospholipid theory of schizophrenia proposed by Horrobin *et al* (1994). In addition, reduced membrane PUFAs have been found to be associated with psychopathology (Glen *et al* 1994; Peet *et al* 1995; Doris *et al* 1998; Khan *et al* 2002), although in a recent

study by Peet *et al* (2004) no significant difference in red blood cell AA levels between unmedicated patients and controls was observed.

1.6.2 DNA Oxidation and Schizophrenia

Relatively little information has so far been directed towards determining the levels of oxidative damage to cellular proteins and DNA. Psimadas *et al* (2004) examined the effects of oxidative insult on the cellular DNA of a Greek sample of male schizophrenic patients and controls and no significant difference in DNA damage between groups was observed. Another recent study however has reported evidence for oxidative DNA damage and coordinated cell-cycle activation in elderly 'poor-outcome' schizophrenia (Nishioka and Arnold, 2004).

Investigation of DNA damage is important because it is a possible indicator of carcinogenic and mutagenic potential. It has been suggested in population based studies (Cohen *et al* 2002; Lichtermann *et al* 2001) that in the absence of confounding factors such as smoking, age, socioeconomic status and alcohol, the risk for some types of cancer (e.g. respiratory and lung cancer) in schizophrenic patients is reduced, although no investigation of possible gender differences has been reported, and no clear mechanism to explain this resistance has been identified.

1.6.3 Protein Oxidation and Schizophrenia

Oxidation of catecholamines to the corresponding *o*-quinones and their subsequent reduction to reactive species, which can induce redox cycling, has been postulated to be involved in neurodegeneration in the mesolimbic and nigro-striatal systems, and thus in the aetiology of schizophrenia and Parkinson's disease (Baez *et al* 1997; Smythies 1997). In addition, accumulation of modified protein disrupts cellular function either by loss of catalytic and structural integrity or by interruption of

regulatory pathways (Stadtman and Levine 2000). Tardive dyskinesia is a movement disorder which affects 20-40% of patients treated chronically with neuroleptic drugs. It has been suggested that neuroleptics increase striatal glutamatergic neurotransmission by blocking presynaptic dopamine receptors, leading to neuronal damage as a consequence of oxidative stress (Tsai *et al* 1998). Patients suffering from tardive dyskinesia have been shown to have significantly higher concentrations of N-acetylaspartate, N-acetyl-aspartylglutamate and aspartate in their cerebral spinal fluid and higher levels of protein-oxidized products associated with tardive dyskinesia (Tsai *et al* 1998).

1.7 Treatment of Schizophrena

1.7.1 Antipsychotics

At present there is no cure for schizophrenia. There are a range of drug treatments for schizophrenia (Table 1.1), although there is no one drug that is better than another. Each has different indications, for example, clozapine is the drug of choice for treatment resistant schizophrenia. Each of the typical antipsychotics and the atypicals risperidone, olanzapine, quetiapine and ziprasidone (but not clozapine) are used as treatment for early (‘first-episode’) schizophrenia and also for other stages of the illness. Older antipsychotics (also know as first generation, conventional, typical) are drugs that possess antipsychotic efficacy but are also associated with extrapyramidal side-effects (EPS). Although it is not clear how these drugs work, the proposed mechanism of action of both typical and atypical antipsychotics is that they block D2 receptors (reviewed by Jones and Buckley 2003). However, neuroleptics have been shown to have both pro-oxidant and antioxidant properties (Jeding *et al* 1995) and the antipsychotic response of a neuroleptic may depend on its pro- or

antioxidant property and the level of pre-existing oxidative stress in the patient (Mahadik *et al* 2001).

Clinical studies have indicated that antioxidant enzyme activities are connected with the use of neuroleptics provided during the treatment of schizophrenia (Abdalla *et al* 1986; Yao *et al* 1998), suggesting that the changes in the enzyme activity may be due to the neuroleptic treatment (Akyol *et al* 2002; Evans *et al* 2003). The neuroleptic Flupenthixol (Table 1.1) can accept electrons in competition with oxygen and therefore reduce the production of superoxide and inhibit cytochrome b5 reductase activities; alternatively, neuroleptics may react directly with superoxide (Whatley *et al* 1998).

Dakhale *et al* (2004) examined the effect of atypical antipsychotics on lipid peroxidation, SOD and ascorbic acid. Results revealed an increase in serum SOD, serum malondialdehyde and a decrease in plasma ascorbic acid in schizophrenic patients as compared to controls; however, the trend altered significantly following treatment with antipsychotics. In contrast, Taneli *et al* (2004) found that antipsychotic drugs did not significantly decrease levels of serum nitric oxide in schizophrenic patients after a 6-week treatment regimen. The authors suggest that longer therapy periods may prove beneficial. This is in agreement with the findings from another study (Evans *et al* 2003) who reported that following twelve weeks of antipsychotic treatment antioxidant levels were not significantly different from the low levels measured at baseline. Yet after 24 weeks, antipsychotic treatment almost normalized the levels of SOD but further increased the levels of CAT.

Class **Typical**	**Example**	**Class** **Atypical**	**Example**
Butyrophenone	Haloperidol	Dibenzodiazepine	Clozapine
Phenothiazine Aliphatic Piperidine Piperazine	 Chlorpromazine Thioridazine Trifluoperazine	Benzisoxazole	Risperidone
Thioxanthene	Fluphenthixol	Thienobenzodiazepine	Olanzapine
Diphenylbutylpiperidine	Pimozide	Dibenzothiazepine	Quetiapine
Substituted benzamide	Sulpiride	Benzisothiazolyl	Ziprasidone
		Phenylindol	Aripiprazole
		Phenylindol	Sertindole
		Dibenzothiepine	Zotepine

Table 1.1. Classes of antipsychotic medications.

1.7.2 Fatty Acids and Antioxidants as Adjunctive Therapy for Schizophrenia

Treatment of schizophrenia is most effectively accomplished with antipsychotics and recent treatment with newer antipsychotics appears to have therapeutic effects against core negative symptoms and cognitive deficits that are considered difficult to treat (Arvindakshan *et al* 2003). However, response to current pharmacological treatment of schizophrenia is variable with only 60% of patients responding favourably (Yao *et al* 2004) in addition EPS, limited efficacy and cost are serious limitations of the conventional antipsychotics. Treatment non-responders frequently have prominent cognitive deficits and persistent negative symptoms. Adjunctive use of EPA and DHA through dietary supplementation has shown hopeful results in decreasing some of the clinical symptoms of schizophrenia (Yao *et al* 2004; Emsley *et al* 2002; Peet *et al* 2002) and is based on altered membrane phospholipid

composition. EPA alone has been suggested to correct the membrane EPUFA deficits and improve the psychopathology of schizophrenia (Peet *et al* 2001). Yet, the combined use of EPA and DHA may still be a preferred supplement. A large proportion of EPA is converted to prostaglandins and may not be an adequate substrate for DHA, a major neuronal membrane EPUFA (Arvindakshan *et al* 2003).

Studies in schizophrenic patients have indicated that antioxidant defence is impaired and the content of membrane phospholipid EPUFAs is reduced and lipid peroxidation is increased. Dietary supplementation with antioxidants has been considered to prevent these changes and to improve some of the psychopathology (Mahadik and Mukherjee 1996; Reddy and Yao 1996). It is known that high doses of EPUFAs, if not balanced with dietary antioxidants can form high levels of peroxides. As the evidence is mounting for increased oxidative stress and oxidative cellular injury in schizophrenia, use of supplementation with a combination of ω-3 fatty acids and antioxidants may be a preferred supplement and has been shown to improve the outcome of schizophrenia (Arvindakshan *et al* 2003).

Mukherjee *et al* (1996) have reported that antioxidant enzyme defence is altered at the onset of psychosis and might be associated with poor clinical outcome and suggest that it might be important to use antioxidant co-treatment from the start of the therapeutic treatment to prevent progression of pathology predating the illness as well as its accentuation following treatment. Furthermore, persistent oxyradical-mediated injury might contribute to progressive deterioration resulting in deficit state in some schizophrenic patients as well as a relapsing chronic course of illness in others irrespective of treatment. It is likely that adjunctive antioxidant treatment

might ameliorate the development of negative symptoms and prevent progression of the defect state (Mahadik and Mukherjee 1996).

1.8 Project Aims

So far, little or no research has been carried out in the area of oxidation of DNA and protein in schizophrenia. The majority of the ongoing research of oxidative stress in schizophrenia, to date has focused on lipid peroxidation, arising from the idea that some neuropathological changes in schizophrenia may be the result of increased free radical-mediated or reactive oxygen species mediated neuronal injury (Mahadik and Mukherjee, 1996; Reddy and Yao, 1996). Numerous lipid peroxidation biomarkers exist in the majority of human diseases and as a major component of membrane phospholipids, PUFA are highly susceptible to free radical attack and undergo auto-oxidation to form peroxyradicals and lipid peroxide intermediates. Altered *in vivo* and *in vitro* phospholipid metabolism has been identified in some patients with schizophrenia, as a result of reduced levels of membrane phospholipid PUFA injury (Mahadik and Mukherjee, 1996; Reddy and Yao, 1996).

Limited research into the use of biomarkers of oxidative damage of DNA in schizophrenia has been undertaken. Furthermore, establishing whether protein carbonyls, biomarkers of protein oxidation are a characteristic of neurological diseases such as schizophrenia is a novel aspect of this study which will be explored. Another area to be investigated will include the investigation of the effects of antioxidants and fatty acids within human lymphocytes and IMR-32 neuroblastoma cells, neuronal-type cells. This novel work will be undertaken to establish whether the response of cultured lymphocytes and a neuronal cell line to an oxidative challenge are sufficiently similar to support the use of peripheral lymphocytes as biomarkers/predictors of *in vivo* neuronal cell response. Research into the use of neuroblastoma cells and in particular IMR-32 cells has shown that although they are an ideal model to assess cell changes in an Alzheimer's brain, the literature has

identified that they are also widely used to investigate the effect of free radical/reactive oxygen species damage in other neurological disorders, such as schizophrenia and Parkinson's Disease.

Breath analysis, a non-invasive technique used to assess largely lipid peroxidation but also protein oxidation, will be applied, to assess lipid peroxidation, as part of a collaborative project. Limited research has been undertaken on the use of this method in relation to schizophrenia. This technique will be carried out therefore, with the intention of investigating biomarkers of lipid and protein peroxidation in schizophrenic patients compared with healthy controls.

The question that was sought to be answered during the course of this research was: are levels of oxidative stress, as indicated by changes in a range of biomarkers, more enhanced in the models of schizophrenia compared to the matched controls?

CHAPTER 2

Examination of DNA oxidation in antioxidant and fatty acid treated human neuroblastoma and human peripheral cells

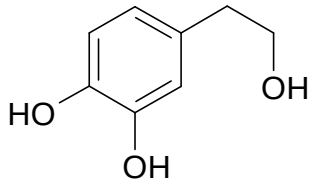
2.1 Introduction

2.1.1 Oxidative Stress and DNA Damage

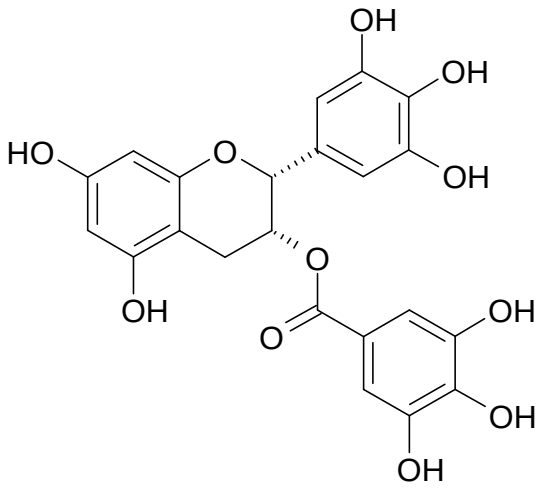
Oxidative stress and the damage that it can cause has been implicated in an enormous variety of natural and pathological processes including ageing, cancer, diabetes mellitus, atherosclerosis, neurological illnesses such as Alzheimer's disease, schizophrenia and autoimmune diseases such as arthritis. Oxidative stress was first recognised to cause damage to living organisms in 1952 by Conger and Fairchild (reviewed by Shackelford *et al* 2000) who demonstrated that an increase in oxygen pressure could cause chromosomal aberrations in pollen grains. Since then oxygen and ROS have been shown to induce many types of DNA damage, including single- and double-stranded DNA breaks, base and sugar modifications, DNA-protein crosslinks and depurination and depyrimidination. It has been suggested that approximately 2 x 10^4 DNA damaging events occur in every cell of the human body every day (Foray *et al* 2003) with a large proportion of the damage occurring as a result of ROS. Although at physiological levels ROS play a key role in regulating signalling pathways and gene expression (Durocher and Jackson 2001), excessive insult to the cell can be fatal.

In order to prevent attack from ROS and other free radicals, living cells use a number of defences such as low molecular weight compounds and antioxidants (for examples of antioxidants used in this study, see Figure 2.1) which scavenge free radicals. These antioxidants eventually become radicals themselves although they are much less reactive and prevent further damage to cellular biomolecules. More complex systems such as the enzymes superoxide dismutase, catalase and glutathione peroxidase have also evolved to limit the ROS-induced damage. In normally functioning cells there is a tendency for ROS to avoid these defences,

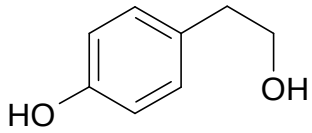
resulting in only a limited amount of damage to the cell. But if the balance between pro- and antioxidant processes becomes damaged in favour of the pro-oxidant systems, levels of cellular damage will initially increase. During turnover of lipids and proteins any modification of these molecules will be removed, but damage to DNA is required to be repaired since, unlike modified lipids and proteins, altered DNA cannot be replaced.



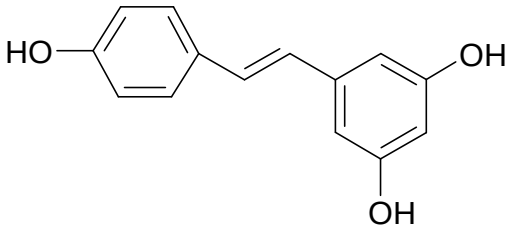
Hydroxytyrosol



EGCG



Tyrosol



Resveratrol

Figure 2.1. Antioxidant structures.

The ROS that cause oxidative damage can be divided into two categories: free oxygen radicals and non-radical ROS (Shackelford *et al* 2000). Common free oxygen radicals include $^{\bullet}OH$, $^{\bullet}NO$ and $O_2^{\bullet-}$. Nonradical ROS include molecules such as hydrogen peroxide (H_2O_2). Each of these ROS has the ability to damage most cellular components including DNA, either via direct action on DNA or through reaction with other cellular constituents to produce new ROS. H_2O_2 is produced by a variety of intracellular reactions particularly oxidative electron transport in the mitochondria and is normally present in cells at a concentration of roughly 1.0×10^{-8}M (reviewed by Shackelford *et al* 2000). H_2O_2 alone is relatively non-reactive toward DNA and at concentrations at or below 20-50μM has been reported as having limited cytotoxicity to many cell types (Halliwell *et al* 2000). Most of the H_2O_2-mediated DNA damage is due to the production of $^{\bullet}OH$ via events such as the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes (ferryl?)} \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

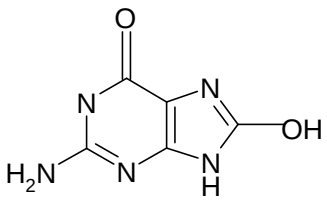
Catalase is then responsible for the deactivation of H_2O_2 via the reaction:

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

Hydrogen peroxide is generated *in vivo* by the dismutation of superoxide radical both non-enzymatically and catalyzed by superoxide dismutase enzymes. It is also directly produced by a collection of oxidase enzymes including glycollate and monoamine oxidases as well as by the peroxisomal pathway for β-oxidation of fatty acids (Halliwell *et al* 2000). Free radicals including $^{\bullet}OH$ react with organic compounds by addition and abstraction. Hydroxyl radical adds to double bonds of heterocyclic DNA bases and abstracts an H atom from the methyl group of thymine

and from each of the C-H bonds of 2'-deoxyribose (Evans *et al* 2004). Hydroxyl radical adds to the double bonds of pyrimidines and purines at diffusion-controlled rates and preferentially adds to the site with the highest electron density.

The most prevalent damage to purines results in 7,8-dihydro-8-oxoguanine (also known as 8-oxoguanine or 8-oxoG) and the most studied of oxidative DNA lesions, 8-hydroxyguanine (8-OH-Gua) (Figure 2.2). The hydroxyl radical first reacts with guanine to form a C8-OH adduct radical followed by the loss of an electron and proton to generate 8-oxo-G. The C8-OH adduct radical may also be reduced by uptake of an electron and a proton forming 7-hydro-8-hydroxyguanine which is then converted to 2,6-diamino-4-hydroxy-5-formamidopyrimidine, another major oxidation product of guanine (Slupphaug *et al* 2003). Generation of oxidised pyrimidines, such as thymine glycol also occurs from radical attacks, for example, hydroxyl radical reacts to produce 5-hydroxy-6-peroxyl-radical. In the absence of oxygen, the loss of an electron followed by uptake of water and then finally the loss of a proton results in the production of thymine glycol. If oxygen is present however, oxygen uptake at position 6 first yields a 5-hydroxy-6-peroxyl radical which is converted to thymine glycol through loss of a proton and $O_2^{\bullet -}$ and reaction with water (Slupphaug *et al* 2003).



8-Hydroxyguanine (8-OH-Gua)

Figure 2.2. Structure of 8-OH-Gua

Once formed it is essential that oxidative DNA lesions do not remain in the genome. There are multiple repair pathways that undertake this task and are categorised into base excision repair (BER) and nucleotide excision repair (NER) systems. The BER pathway is initiated by non-enzymatic base loss or by a DNA glycolase. The glycolase thought to be responsible for the removal of 8-OH-Gua in human cells is the human 8-OH-Gua glycosylase. Prior to base removal, repair is completed by short-patch repair (one nucleotide gap) or long-patch repair (a two to eight nucleotide gap), the modified base is removed leaving an apurinic-apyrimidinic site and the enzyme has specificity for 8-OH-Gua:C pairs present in double-stranded DNA (Cooke *et al* 2005). The activity of the glycolase is complemented by another enzyme which removes the 8-OH-Gua from the nascent strand in 8-OH-Gua:A or 8-OH-Gua:G pairs.

If they are not repaired prior to DNA replication, 8-OH-Gua residues can result in GC to TA transversions, which may ultimately lead to mutagenesis (Griffiths *et al* 2002). Levels of 8-OH-Gua in DNA have been assayed to determine the mechanisms of ageing or carcinogenesis and to evaluate the risk of carcinogenesis by chemical materials and these studies have shown 8-OH-Gua to be a useful biomarker for elucidating the role of reactive oxygen species (Tsurudome *et al* 2001).

2.1.2 Detecting Biomarkers of DNA Oxidative Damage

The single cell gel electrophoresis (SCGE) or comet assay is a technique that can be used for the measurement of oxidative DNA damage in individual cells. It is widely used and can be applied to any cell population provided they have been isolated from tissue without degradation (Lovell *et al* 1999), although at present human population studies tend to use lymphocytes as surrogate tissues. Lymphocytes are often used as

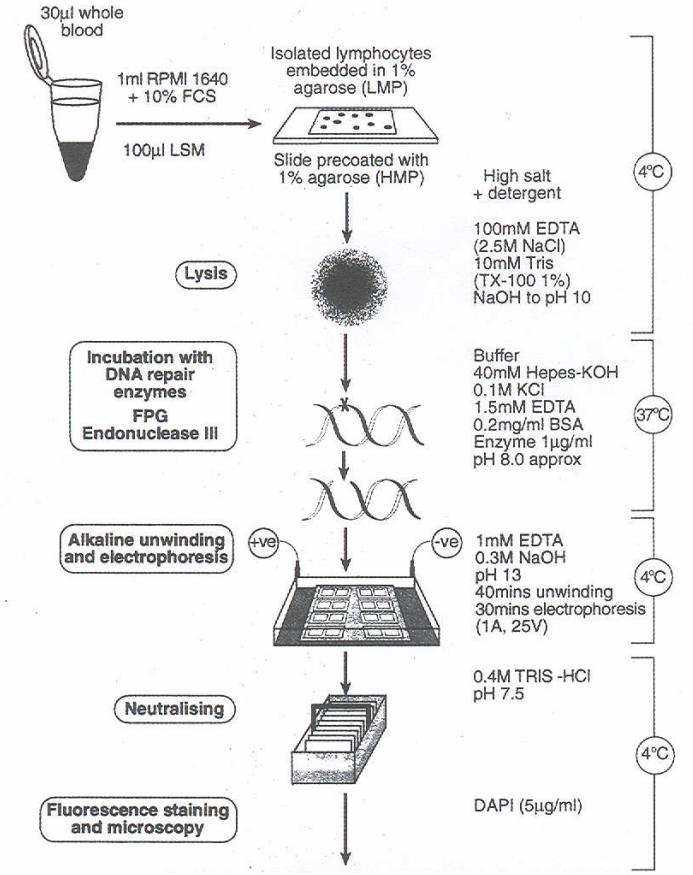
surrogate cells which are supposed to inform about oxidative stress, measured at a certain level of 8-OH-Gua in other tissues (Collins *et al* 1998). However, factors responsible for the formation of 8-OH-Gua and for oxidative stress may vary in different tissues (Griffiths *et al* 2002). These include, for example, the activity of key antioxidant enzymes which may affect oxidative stress in different organs (Gutteridge and Halliwell 1994). Few studies have examined the use of lymphocytes as surrogate markers but a direct correlation between DNA damage in human rectal cells and lymphocytes (Pool-Zobel *et al* 2004) and an age-associated increase of tissue and peripheral lymphocyte oxidative DNA damage in a rat model (Wolf *et al* 2004) has been identified.

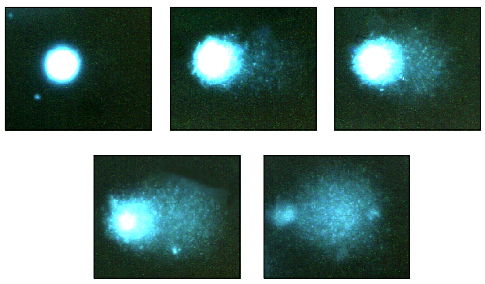
The comet assay, a sensitive method for detecting DNA strand breaks can also be used to measure oxidised bases, with the addition of a step in which DNA is incubated with bacterial repair endonucleases, enzymes which recognise and remove damaged bases and make nicks at the resulting abasic sites in the DNA. Chromatographic methods such as HPLC and GC-MS are also widely applied methods for the measurement of 8-OH-Gua in biological samples of DNA (Gedik *et al* 2002).

2.1.2.1 The Comet Assay

Rydberg and Johanson (1978) were the first to attempt to quantify DNA strand breaks using cells embedded in agarose on slides which were then lysed. Ostling and Johanson (1984) further developed a microelectrophoretic technique, the SCGE assay or comet assay. Following on from the earlier principle, cells were embedded in an agarose gel on a microscope slide, lysed by detergents and high salt solution, and following unwinding, and electrophoresis, the negatively charged DNA is pulled

towards the anode. However, only loops of DNA which contain breaks (either overt breaks or breaks resulting from repair enzyme digestion) are released from the supercoiling and migrate in the gel. These loops extend from the head of the nucleoid to form the comet tail. Further modifications to the assay were carried out by Singh *et al* (1988) whereby electrophoresis was performed under alkaline conditions (pH>13), which enabled detection of strand breaks as well as alkali-labile sites, DNA crosslinking, and incomplete excision repair sites. This is currently the most commonly used method (Figure 2.3) although additional modifications at various stages of the assay have also been applied. Incubation with specific bacterial repair endonucleases results in the recognition of the damaged bases; the enzymes create a nick at the resulting abasic sites in the DNA and the oxidised base is subsequently removed. Oxidised pyrimidines are detected via use of endonuclease III, while oxidised purines including 8-oxo-Gua, are recognised by formamidopyrimide DNA glycosylase (FPG) (Collins 2002). Furthermore, alkylation damage and misincorporated uracil can also be measured (Collins *et al* 1993; Collins *et al* 1998; Duthie and McMillan 1997).





Comet class:
0, 1, 2,
3, 4

Figure 2.3. Schematic of the comet assay protocol. Cell isolation, agarose embedding, lysis, enzyme digestion, unwinding, and electrophoresis, neutralizing, staining and scoring (Adapted from Duthie 2003). Key: Fetal calf serum (FCS), Lymphocyte Separation Medium (LSM), low melting point agarose (LMP), high melting point agarose (HMP), bovine serum albumin (BSA), formamidopyrimidine glycosylase (FPG), 4',6-diamidine-2-phenylindole dihydrochloride (DAPI).

Comets are viewed by fluorescence microscopy after staining with a suitable fluorescence DNA-binding dye. The relative intensity of fluorescence in the tail is a function of DNA breaks and can be assessed either visually or using computer-based image analysis. Cells with damaged DNA display increased migration of DNA fragments from the nucleus. The length of the migration indicates the amount of DNA breakage (Singh *et al* 1988), and DNA damage was often estimated based on the percent of damaged cells or by the migration of the tail length (Fairbairn *et al* 1995). Tail moment, the product of the amount of DNA in the tail and the mean distance of tail migration, is a more sensitive parameter (Olive *et al* 1990) and percentage of DNA in the tail occasionally represents the most accurate evaluation by image analyzers (McKelvey-Martin *et al* 1993; Olive *et al* 1993). It is difficult, however, to analyze many cells by image analysis because it is time-consuming to analyze the comets one by one. Manual scoring, on the other hand, a method that has not been widely used, is simple, less time consuming, and does not need special equipment (Kobayashi *et al* 1995).

2.1.2.2 Chromatography

High pressure liquid chromatography (HPLC) is widely used for the measurement of 8-oxo-Gua in biological samples of DNA such as animal tissue or white blood cells although the small extent of base oxidation requires the use of electrochemical detection (ECD) (Griffiths *et al* 2002). However, oxidation of guanine at any stage in sample preparation is the most significant problem. It can be improved by including antioxidants and by carrying out procedures in the absence of oxygen if possible, but, as problems of oxidation of DNA are resolved, the amount of 8-oxo-Gua detected in the DNA of normal human cells becomes even smaller and increasingly difficult to detect even with highly sensitive coulometeric electrochemical detection

(Griffiths *et al* 2002). Gas chromatography-mass spectrometry (GC-MS) is used to analyse DNA base products but this method is also prone to oxidation artefacts during sample preparation (Griffiths *et al* 2002).

For evaluation, internal standards of the analysed modifications labelled with stable isotopes are also necessary, along with the addition of appropriate antioxidants and the elimination of traces of oxygen (Griffiths *et al* 2002). A consortium of laboratories known as European Standards Committee on Oxidative DNA Damage (ESCODD, 2002) undertook a study highlighting the serious problem of oxidation artefact and concluded that GC-MS is not suitable for measuring basal 8-oxo-Gua levels in biological material. HPLC-MS has yet to yield convincing results while HPLC-ECD can measure induced damage accurately but still suffers from adventitious oxidation during sample preparation (Collins 2005).

Other approaches that are being employed in the detection of oxidised DNA include enzyme-linked immunoabsorbant assay (ELISA). HPLC and ELISA methods have been compared using placental tissue DNA. Values by both methods correlated well, but the ELISA values were higher than those determined by HPLC. This is thought to be due to the antibody cross reacting with compounds other than 8-oxo-Gua (Mayne 2003).

2.1.3 DNA Oxidative Damage and Schizophrenia

Although free radical pathology in schizophrenia is widely investigated, examination of ROS induced DNA damage is limited. However, evidence for DNA damage in the hippocampus of schizophrenic patients, detected by the presence of the 8-oxo-Gua biomarker, has recently been reported (Nishioka and Arnold 2004). Investigation of DNA damage is important because it is a possible indicator of carcinogenic and

mutagenic potential. In addition, there is accumulating evidence of altered antioxidant capacity in schizophrenia. Membrane dysfunction can be secondary to free radical-mediated pathology, and may contribute to specific aspects of schizophrenic symptomatology and complications of its treatment (Yao *et al* 2001). Specifically, free radical-mediated abnormalities may contribute to the development of a number of clinically significant consequences, including prominent negative symptoms, tardive dyskinesia, neurological 'soft' signs and parkinsonian symptoms (Yao *et al* 2001).

Investigation of the effect of polyunsaturated fatty acid supplementation on oxidative DNA damage in schizophrenia has not been undertaken to date. In cancer and ageing studies it appears that polyunsaturated fats may have a dual role, since they may inhibit oxidative stress and act as a protective agent, as well as provide a substrate for peroxidation reactions (Beeharry *et al* 2003, Quiles 2004). The role of antioxidants and fatty acids obtained from the diet in protection against disease is a topic of continuing interest and some controversy. It is widely recognised however, that people with schizophrenia make poor dietary choices and that consumption of antioxidants is low (McCreadie 2003) and PUFA intake is also low (Kemperman *et al* 2006) in this group of people.

2.1.4 Using Human Cells in Schizophrenia Studies

Whilst the importance of *in vivo* and *ex vivo* work cannot be over emphasised, *in vitro* models of clinical disease have much to offer in terms of elucidating molecular mechanisms and evaluating the efficacy of novel agents.

2.1.4.1 U937 Cells

The U937 cell line is a well characterised human lymphoma cell line of Caucasian origin. The use of this cell line has recently been adopted as an *in vitro* model of schizophrenia since it has been reported that these cells imitate in some ways, the lipid-related changes seen in suffers of schizophrenia (Gattaz *et al* 1990; Khan *et al* 2002) including very low levels of polyunsaturated fatty acids (Obermeier *et al* 1995), while a further *in vitro* observation obtained using the U937 monocyte derived cell line indicated that long term supplementation with EPA increased the stimulated release of AA from membrane phospholipids (Obajimi *et al* 2005). Studies investigating phospholipase A_2 have revealed that U937 cells express high levels of PLA_2 (Kramer *et al* 1991), and in particular, large amounts of $cPLA_2$ (Burke *et al* 1999) further evidence of some of the characteristics which mimic some of the biochemical changes reported in patients with schizophrenia. In addition, inhibitor studies (Balsinde *et al* 2002) utilising U937 cells, have suggested that the form of PLA_2 responsible for release of AA is calcium-dependent cytosolic PLA_2, while differentiation of U937 cells enables the cells to activate $cPLA_2$ to release arachidonate upon stimulation (Burke *et al* 1999). The cells have been used to better understand the biochemical features of AA incorporation into, and remodelling within phospholipid species of U937 cells (Balsinde *et al* 2002), an important process that is thought to function defectively in patients with schizophrenia.

2.1.4.2 IMR-32 Cells

The IMR-32 cell line is a human neuroblastoma cell line of Caucasian origin which is employed as a model for assessing neuronal cell damage, typically free radical mediated injury (Cova *et al* 1995). In addition, Cova *et al* (1995) also suggest that cultured neuronal cells of human origin are discriminating experimental systems,

sensitive to minor differences, of correlating *in vivo* and *in vitro* neurotoxicants. The IMR-32 cells have several features which are considered as useful properties in studies of oxidative stress, namely: they are of human origin; they can be grown in large quantities for protein studies, differentiated and maintained over several weeks and express large amounts of neurofilaments (reviewed by Cookson *et al* 1996). Neurofilaments are the intermediate filaments found only in neurones and are potential targets for free radical damage because of their abundance in motor neurones and their long biological half-life (Cookson *et al* 1996). IMR-32 cells have also been used specifically in studies of schizophrenia patients whereby antibodies to IMR-32 proteins were detected, suggesting an autoimmune factor in the pathogenesis of schizophrenia (Mazeh *et al* 1998).

2.1.4.3 Lymphoblastoid Cells

Lymphoblastoid cell lines were established by Epstein-Barr virus transformation of peripheral blood mononuclear cells derived from a schizophrenic patient. Since these cells were derived from a schizophrenic patient, it was anticipated that they would provide an accurate insight into the schizophrenic biochemical processes under investigation in our study. Furthermore, human population studies tend to use lymphocytes as surrogate tissues. Lymphocytes are often used as surrogate cells which are supposed to inform about oxidative stress, for example, levels of 8-OH-Gua in other tissues can be measured in lymphocytes and compared to levels in tissue elsewhere in the body (Collins *et al* 1998).

The primary focus of this research was a comparator study of these two cell lines with a further secondary, lymphoblastoid cell line of schizophrenic origin as a means of assessing the validity of monitoring peripheral lymphocytes as potential surrogate

markers of DNA damage in neuronal cell types. Additionally, the reducing ability of a range of antioxidants was assessed and in conjunction, the efficacy of a series of polyunsaturated fatty acids along with the same antioxidants was considered in affording protection to cellular DNA against an oxidative challenge examined.

2.2 Materials and Methods

2.2.1 Validation and Optimisation of the Comet Assay

2.2.1.1 Materials

Lymphoprep lymphocyte separation medium (LSM) (specific gravity 1.077 ± 0.001 g/ml) was supplied by Robins Scientific (Solihull, UK). Dutch Modified RPMI 1640 medium was obtained from Sigma (Poole, UK). Heat-inactivated fetal calf serum (FCS) was obtained from Invitrogen (Paisley, UK). Microscope slides and cover glasses were supplied from Fisher Scientific (Loughborough, UK). Low Melting Point (LMP) and Normal Melting Point (NMP) agarose were supplied by Gibco Life Technologies (Paisley, UK). 4',6-Diamidine-2-phenylindole dihydrochloride (DAPI) was obtained from Sigma (Poole, UK). Corning centrifuge tubes were supplied by Fisher Scientific (Loughborough, UK). Vacutainers were obtained from Aberdeen Royal Infirmary, (Aberdeen, UK). Resveratrol, epigallocatechin gallate (EGCG), 2-(4-hydroxyphenyl) ethanol (tyrosol), were supplied by Sigma (Poole, UK). 3,4-dihydroxyphenyl ethanol (hydroxytyrosol) was obtained from Alexis Corp (UK) Ltd (Nottingham, UK). 25cm^2 cell culture flasks and 96-well plates were supplied by Fisher Scientific (Loughborough, UK). Dimethyl sulfoxide (DMSO) was supplied by Sigma (Poole, UK).

2.2.1.2 Isolation and Cryopreservation of Human Lymphocytes

Venous blood (2 x 7ml) was collected by venepuncture. The whole blood was centrifuged at 2400x*g* for 15min at 4^{o}C, the buffy coats from each vacutainer (~1ml) were removed, combined into one centrifuge tube and diluted 1:1 with RPMI and layered onto an equal volume of LSM before centrifuging at 700x*g* for 30min at 20^{o}C. The lymphocytes were transferred to a fresh centrifuge tube, washed using RPMI medium and spun for a further 15min under the same conditions. The

supernatant was decanted, the cells resuspended in RPMI containing 10% heat-inactivated FCS and counted using a Neubauer Improved Haemocytometer. Isolated lymphocytes were either used immediately or were centrifuged and resuspended in 0.5ml aliquots at $3x10^6$cells/ml in freezing mix (90% v/v heat-inactivated FCS and 10% v/v DMSO), frozen at -1°C/min in polystyrene and stored at -70°C.

2.2.1.3 Hydrogen Peroxide-induced DNA Strand Breakage in Fresh Lymphocytes Measured Using the Comet Assay

Freshly isolated lymphocytes were incubated in microcentrifuge tubes with hydrogen peroxide (H_2O_2) (50, 100 or 200μM in phosphate-buffered saline (PBS) for 5min on ice), washed and suspended in 140μl of 1% (w/v) LMP agarose in PBS pH 7.4 at 37°C and immediately pipetted onto a glass microscope slide pre-coated with a layer of 1% (w/v) NMP agarose prepared in distilled water. The agarose was allowed to set for 5min at 4°C and the slides were incubated in lysis solution (2.5M NaCl, 10mM Tris, 100mM NA_2EDTA, 10M NaOH to pH 10.0 and 1% v/v Triton X-100) at 4°C for 1h to remove cellular proteins. After lysis, the slides were aligned in a 210mm wide horizontal electrophoresis tank containing buffer (1mM NA_2EDTA and 0.3M NaOH, pH 13.0) for 40min before electrophoresis at 21v for 30min (at an ambient temperature of 4°C with the temperature of the running buffer not exceeding 15°C). The slides were washed three times at 4°C for 5min each with neutralizing buffer (0.4M Tris-HCl, pH 7.5) before staining with 20μl of DAPI (1μg/ml).

2.2.1.4 Hydrogen Peroxide-induced DNA Strand Breakage in Cryopreserved Lymphocytes

Cryopreserved lymphocytes were thawed on ice until the last trace of ice had disappeared. The aliquots were centrifuged at 200x*g* for 3min to remove freezing mix and the lymphocyte pellet resuspended gently in 0.4ml of RPMI + 10% (v/v) heat-inactivated FCS. Lymphocytes were washed once in PBS and incubated in microcentrifuge tubes H_2O_2 (10, 25, 50, 200μM in PBS for 5min on ice). The cells were resuspended immediately in LMP agarose for detection of DNA strand breakage by Comet analysis.

2.2.1.5 Quantitation of the Comet Assay

Nucleoids were scored visually using a Leica fluorescence microscope. One hundred comets from each gel (scored at random) were classified into one of five classes according to the relative intensity of fluorescence in the tail and given a value of 0-4 (from undamaged, 0, to maximally damaged, 4). The total score for 100 comets can range from 0 (all undamaged) to 400 (all maximally damaged) and is expressed in arbitrary units. This method of visual classification has been extensively validated by comparison with comets selected using computerized image analysis.

2.2.1.6 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and one-way ANOVA and the Student's t-test was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

2.2.2 Determination of Antioxidant Activity in Poly and Monophenolic Antioxidants

2.2.2.1 Materials

Trolox, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS), diammonium salt, potassium persulphate, resveratrol, EGCG and tyrosol, were supplied by Sigma (Poole, UK). Hydroxytyrosol was obtained from Alexis Corp (UK) Ltd (Nottingham, UK).

2.2.2.2 ABTS Assay

The protocol used is an adaptation of Re *et al* (1999). 1mM Trolox was prepared in ethanol for use as a stock standard and fresh working standards were prepared on dilution with ethanol. Stock solutions of the antioxidants were prepared by dissolution in ethanol and subsequently diluted in ethanol for introduction into the assay system at a final concentration of 0.5mM. ABTS was dissolved in water to a 7mM concentration and ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with potassium persulfate and allowing the mixture to stand in the dark at room temperature overnight. The $ABTS^{\bullet+}$ solution was diluted with ethanol. Stock solutions of antioxidants were diluted such that, after introduction of a 10μl aliquot of each dilution into the assay, they produced between 20%-80% inhibition of the blank absorbance. After addition of 1ml of diluted $ABTS^{\bullet+}$ solution (A734nm = 0.701) to 10μl of antioxidant compound or Trolox standards the absorbance reading was taken at room temperature exactly 1min after initial mixing for up to 4min.

2.2.2.3 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and Student's t-test was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

2.2.3 Investigation of Phenolic Antioxidant Mediated Cytoprotection in Human Neuroblastoma IMR-32 and Human Peripheral (U937 and Lymphoblastoid) Cells

2.2.3.1 Materials

Dulbecco's Minimal Essential Medium Nutrient Fixed F-12 Ham media (DMEM), penicillin/streptomycin antibiotic, trypsin/EDTA and glucose were supplied by Sigma (Poole, UK). Heat-inactivated FCS was obtained from Invitrogen (Paisley, UK). RPMI 1640 media was supplied by Gibco (UK). EGCG was supplied by Sigma (Poole, UK). Hydroxytyrosol was obtained from Alexis Corp (UK) Ltd (Nottingham, UK). 25cm^2 culture flasks supplied by Fisher Scientific, (Loughborough, UK). DMSO was supplied by Sigma (Poole, UK). Neuroblastoma IMR-32 and lymphoblastoid cell lines were provided frozen by European Collection of Cell Cultures (ECACC) (Porton Down, Wiltshire). U937 cell line was provided growing in culture by ECACC (Porton Down, Wiltshire).

2.2.3.2 Culture of Neuroblastoma Cells

Complete neuroblastoma IMR-32 media was prepared under sterile conditions. 100ml DMEM medium containing 20% (v/v) heat-inactivated FCS, 1% 100U/ml penicillin, 100mg/ml streptomycin, 6g/L sterile filtered glucose were combined and stored at 4^{o}C. Neuroblastoma cells were resuscitated by incubating vials in a 37^{o}C water bath and agitated gently for 3-4min until completely thawed. The 1ml cell

suspension was pipetted into a 25cm^2 flask containing 8ml of complete DMEM media and incubated at 37^{o}C, 5% CO_2 until at least 80% confluent (approximately 3 days). Cells were passaged by washing twice with 1ml sterile PBS and adding 0.5ml trypsin/EDTA to ensure cells were dislodged from the flask. 5ml media was added to the flask and a needle and syringe was used to homogenise the cells. A further 4ml of media was added to the homogenised cells and 3ml of the cell suspension was decanted into 3 x 25cm^2 flasks containing 6ml of fresh media. Flasks were placed in the incubator and the media was replaced with 9ml of fresh media after 3 days of growth. Further passage of cells at a seeding density of 1 x 10^{6}cells/ml was undertaken and the cells were incubated in 9ml of fresh media in 25cm^2 flasks as described above. Cells were left to grow until 80-90% confluence was obtained. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

2.2.3.3 Culture of Peripheral Cells

Complete lymphocyte media was prepared under sterile conditions. RPMI medium containing L-glutamine, 10% (v/v) heat-inactivated FCS, 1% 100U/ml penicillin, 100mg/ml streptomycin, were combined and stored at 4^{o}C. U937 cells provided growing in culture, were incubated at 37^{o}C, 5% CO_2 until at least 80-90% confluent (approximately 2-3 days). Lymphoblastoid cells were resuscitated by thawing in a 37^{o}C water bath by gentle agitation for 3-4min and then pipetted into a 25cm^2 flask containing 9ml complete RPMI media. Cells were passaged by pouring off cell suspension into a centrifuge tube and centrifuging for 5min at 2000x*g*. The cell pellet was then washed twice with sterile PBS. The cell pellet was resuspended into 1ml of RPMI media and cells were counted using a haemocytometer and seeded at a density of 1 x 10^{6}cells/ml. Cells were incubated in fresh media in 12 well plates until 80-

90% confluent. Cell viability was determined as >90% as ascertained by the Trypan blue dye exclusion method of viability.

2.2.3.4 Antioxidant Treatment of Neuroblastoma Cells

10mM stock solutions of EGCG and hydroxytyrosol were prepared freshly in DMSO and sterile filtered. A final concentration of 10μM of each antioxidant or DMSO only was used. DMSO concentration was no more than 1% of the cell culture media. Antioxidant was added to the flask and cells were incubated for 30min and 6h at 37^{o}C, 5% CO_2. Following incubation, supernatant was poured off and the cells were washed 2 x with 1ml ice cold PBS. 0.5ml trypsin/EDTA was added to each flask to dislodge cells and the cell suspension was transferred into microcentrifuge tubes and placed on ice. Cells were washed with ice cold PBS, centrifuged at 200x*g* for 3min at 4^{o}C and stored on ice.

2.2.3.5 Antioxidant Treatment of Peripheral Cells

10mM stock solutions of EGCG and hydroxytyrosol were prepared freshly in DMSO. A final concentration of 10μM of each antioxidant or DMSO only was used. DMSO concentration was no more than 1% of the cell culture media. Antioxidant was added to the 12 well plates and cells were incubated for 30min and 6h at 37^{o}C, 5% CO_2. Following incubation, cell suspension was pipetted into a centrifuge tube and the cells were centrifuged for 5min at 200x*g* and washed twice with PBS. The cell pellet was resuspended in 1ml PBS and transferred into microcentrifuge tubes and placed on ice. Cells were washed with ice cold PBS, centrifuged at 200x*g* for 3min at 4^{o}C and stored on ice.

2.2.3.6 Hydrogen Peroxide-induced DNA Strand Breakage in Neuroblastoma Cells Measured Using the Comet Assay

Neuroblastomas were incubated in microcentrifuge tubes with 25μM H_2O_2 as described previously and the comet assay was carried out as described previously in section 2.2.1.3.

2.2.3.7 Hydrogen Peroxide-induced DNA Strand Breakage in Peripheral Cells Measured Using the Comet Assay

U937 and lymphoblastoid cells were incubated in microcentrifuge tubes with 100μM H_2O_2 as described previously and the comet assay was carried out as described previously in section 2.2.1.3.

2.2.3.8 Quantitation of the Comet Assay

Nucleoids were scored visually using a Leica fluorescence microscope as described previously in section 2.2.1.5. The slides were blinded so that treatment groups could not be identified.

2.2.3.9 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and two-way ANOVA was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

2.2.3.10 Acknowledgements

All experimentation work was carried out solely by me, and cells lines were cultured and maintained by me. However, during the establishment of the comet assay, I gratefully acknowledge the invaluable guidance and direction offered by Dr Susan J Duthie (Rowett Research Institute). In addition, I would also like to thank Dr Shauna

M Cunningham, School of Life Sciences, The Robert Gordon University for her insightful direction during the establishment of the IMR-32 cell line.

2.2.4 Investigation of Fatty Acid Mediated Cytoprotection in Human Neuroblastoma IMR-32 and Human Peripheral (U937 and Lymphoblastoid) Cells

2.2.4.1 Materials

DMEM, penicillin/streptomycin antibiotic, trypsin/EDTA, glucose were supplied by Sigma (Poole, UK). Heat-inactivated FCS was obtained from Invitrogen (Paisley, UK). RPMI 1640 media was supplied by Gibco (UK). Eicosapentaenoic acid (EPA, >95%) and docosahexaenoic acid (DHA, >90%) were supplied by Pronova, (Norway). 25cm^2 culture flasks and 12-well plates were supplied by Fisher Scientific, (Loughborough, UK). DMSO was supplied by Sigma (Poole, UK).

2.2.4.2 Culture of Neuroblastoma Cells

IMR-32 cells were cultured, maintained at a density of 1 x 10^{6}cells/ml and harvested as described previously in section 2.2.3.2.

2.2.4.3 Culture of Peripheral Cells

Human peripheral cells were cultured and maintained in 25cm^2 flasks. Upon reaching 80-90% confluence, cell suspension was poured into a centrifuge tube and centrifuged at 200x*g* for 3min. The cells were resuspended in 1ml media and counted. Cells were reseeded at a density of 1 x 10^{6}cells/ml in 12 well plates. Following treatment, cells were harvested as described previously in section 2.2.3.3.

2.2.4.4 Fatty Acid Treatment of Neuroblastoma Cells

100mM stock solutions of EPA and DHA were prepared in ethanol and stored under nitrogen at -20^{o}C. A final concentration of 10, 50 and 100μM of EPA and DHA was used. Fatty acid was added to the appropriate flasks and cells were incubated for 30min, 6h and 18h at 37^{o}C, 5% CO_2. Following incubation, cell media was poured off and the cells were washed twice with 1ml ice cold PBS. 0.5ml trypsin/EDTA was added to dislodge cells and the cell suspension was pipetted into microcentrifuge tubes and placed on ice. Cells were washed and centrifuged at 200x*g* for 3min at 4^{o}C with ice cold PBS.

2.2.4.5 Fatty Acid Treatment of Peripheral Cells

100mM stock solutions of EPA and DHA were prepared in ethanol and stored under nitrogen at -20^{o}C. Final concentrations of 10, 50 and 100μM of EPA and DHA were used. Fatty acid was added to the appropriate wells and cells were incubated for 30min, 6h and 18h at 37^{o}C, 5% CO_2. Following incubation, cell suspension was pipetted off into a centrifuge tube and the cells were washed twice with PBS and centrifuged at 200x*g* for 3min. The cell pellet was resuspended in 1ml PBS and transferred into microcentrifuge tubes and placed on ice. Cells were washed with ice cold PBS and centrifuged at 200x*g* for 3min at 4^{o}C.

2.2.4.6 Hydrogen Peroxide-induced DNA Strand Breakage in Neuroblastoma Cells Measured Using the Comet Assay

Neuroblastomas were incubated in microcentrifuge tubes with 25μM hydrogen peroxide as described previously and the comet assay was carried out as in section 2.2.1.3.

2.2.4.7 Hydrogen Peroxide-induced DNA Strand Breakage in Peripheral Cells Measured Using the Comet Assay

Peripheral cells were incubated in microcentrifuge tubes with 100μM hydrogen peroxide as described previously and the comet assay was carried out as in section 2.2.1.3

2.2.4.8 Quantitation of the Comet Assay

Nucleoids were scored visually using a Leica fluorescence microscope as described previously in section 2.2.1.5. The slides were blinded so that treatment groups could not be identified.

2.2.4.9 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and two-way ANOVA was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

2.3 Results

Validation of the comet assay was undertaken using freshly isolated and cryopreserved human lymphocytes, frozen up to 12 weeks at -80°C. Lymphocytes were treated with an increasing concentration of H_2O_2 for 5min on ice and the level of endogenous and exogenous cellular DNA damage was compared (Figure 2.4).

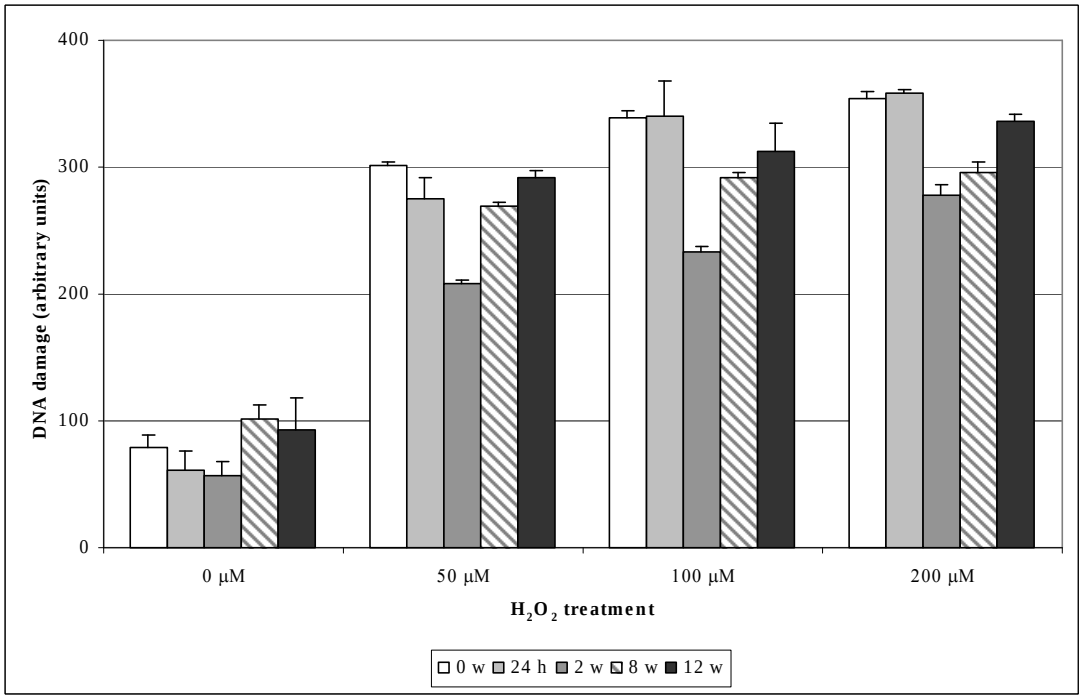


Figure 2.4. Comparison of endogenous and H_2O_2-induced DNA damage in fresh and cryopreserved human lymphocytes, frozen at -80°C for up to 12 weeks. Levels of DNA damage measured with the comet assay in freshly isolated and cryopreserved lymphocytes following 5min treatment of 0, 50, 100 and 200μM H_2O_2. Data show the mean DNA damage (n=2) ± sem.

Two-way ANOVA was used to investigate the effects of H_2O_2 treatment and time on the level of DNA damage. In addition, one-way ANOVA was used to make comparisons within the time and treatment means. Statistical analysis revealed no significant difference between the levels of DNA damage at each time point, ie. freezing lymphocytes at -80°C up to 12 weeks had no effect on the level of DNA

damage in either untreated or H_2O_2 treated cells. A treatment dose response was observed.

A comparison of the antioxidant activity of four phenolic antioxidants was determined by the ABTS assay and EGCG and hydroxytyrosol were shown to be the most potent radical quenchers examined (Table 2.1). One way ANOVA revealed that antioxidant potential in order of statistically significant ($p < 0.001$) decreasing effectiveness was EGCG > hydroxytyrosol > resveratrol > tyrosol. Based on these data, EGCG and hydroxytyrosol only were used during subsequent experimentation.

Test Compound 0.5mM	Mean % Quenching Radical Cation	SEM
EGCG	56.8	0.3
Hydroxytyrosol	35.3	0.3
Resveratrol	27.4	0.3
Tyrosol	7.2	0.1
Reference: Trolox (1mM)	24.4	0.9

Table 2.1. Comparison of phenolic antioxidant activity measured by ABTS assay. Data show the mean percentage quenching of radical cation (ie. antioxidant activity) (n=3) ± sem. Trolox is used as a standard reference.

The potency of the two test compounds (EGCG and hydroxytyrosol) mirrored the protective effects observed in the *in vitro* cell culture systems. A time course study (results not shown) was undertaken to determine the optimum incubation time required in which the antioxidants used in this study would display a protective

effect against H_2O_2-induced DNA damage and following 30min and 6h treatment with EGCG and hydroxytyrosol, human IMR-32 neuroblastoma cells were incubated with 25μM H_2O_2 at for 5min on ice. The mean extent of H_2O_2-induced DNA damage was measured and compared between H_2O_2 control and antioxidant+H_2O_2 treated cells. Two-way ANOVA was employed to investigate effect of time and treatment and analysis revealed a highly significant decrease in the level of DNA damage in both the EGCG+H_2O_2 and hydroxytyrosol+H_2O_2 treated IMR-32 cells after 30min and 6h of antioxidant incubation (Table 2.2). Furthermore, pre-treatment of U937 cells with EGCG or hydroxytyrosol for 30 minutes or 6 hours resulted in a highly significant reduction in the level of H_2O_2-induced DNA damage when compared to control cells. In contrast, pre-treatment of lymphoblastoid cells with hydroxytyrosol but not EGCG afforded significant protection of cellular DNA against an oxidative challenge, resulting in a 21% decrease in the level of DNA damage when compared to the H_2O_2 control. Although results are not shown, for completeness all cells were treated with antioxidants (or without ie. untreated and DMSO treated control) for 30min or 6h and without an H_2O_2 challenge. No difference in the level of DNA damage was observed between the untreated and antioxidant-treated peripheral and neuroblastoma cells and thus none of the test compounds elicited a pro-oxidant effect at the concentrations employed.

IMR-32 Neuroblastoma					U937					Lymphoblastoid				
Treatment	Mean DNA damage comet score (arb. units)		SEM		Treatment	Mean DNA damage comet score (arb. units)		SEM		Treatment	Mean DNA damage comet score (arb. units)		SEM	
	30 min	6 h	30 min	6 h		30 min	6 h	30 min	6 h		30 min	6 h	30 min	6 h
H_2O_2 control	217	228	7	7	H_2O_2 control	296	295	7	7	H_2O_2 control	302	304	11	8
DMSO+ H_2O_2	242	219	7	7	DMSO+ H_2O_2	294	298	10	11	DMSO+ H_2O_2	288	301	13	13
EGCG+ H_2O_2	142***	117***	8	8	EGCG+ H_2O_2	218***	228***	10	9	EGCG+ H_2O_2	274	293	11	5
OHTY+ H_2O_2	134***	139***	11	9	OHTY+ H_2O_2	173***	173***	8	6	OHTY+ H_2O_2	237***	240***	14	11

Table 2.2. Levels of DNA damage measured with the comet assay in human neuroblastoma and peripheral cells following 30min and 6h treatment with 10μM phenolic antioxidant and 25μM H_2O_2 (IMR-32) and 100μM H_2O_2 (U937 and lymphoblastoid). Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where $p < 0.001$. Key: EGCG = epigallocatechin gallate, OHTY = hydroxytyrosol.

As well as an examination of the efficacy of antioxidants in affording protection to cellular DNA against an oxidative challenge, the protective effects of two polyunsaturated acids were evaluated. Treatment of IMR-32 cells with 10, 50 and 100μM EPA or DHA after 30min, 6 and 24h was investigated and following incubation of cells with 25μM H_2O_2 for 5min, the mean extent of H_2O_2-induced DNA damage was measured and compared between H_2O_2 control and fatty acid+ H_2O_2 treated cells. Figure 2.5 shows significant reduction in the levels of DNA damage in EPA-treated IMR-32 cells between the ranges of 10-100 μM, after 30min and up to 24h. A significant level ($p < 0.001$) of cytoprotection was also observed when IMR-32 cells were treated with the same range of DHA concentrations between 30min and 24h (Figure 2.6). For completeness all cells were treated with fatty acids (or without ie. DMSO control and untreated) for 30min, 6h or 24h but

without an H_2O_2 challenge. No difference in the level of DNA damage was observed between the untreated and fatty acid treated neuroblastoma cells (results not shown).

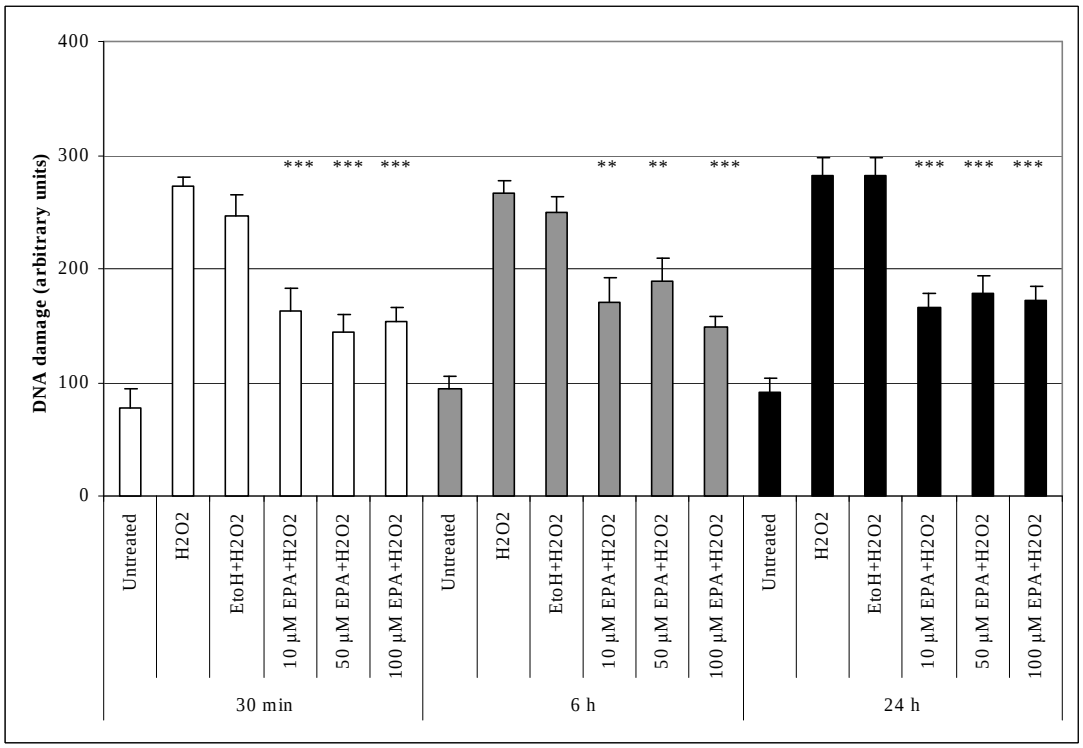


Figure 2.5. Evaluation of EPA cytoprotection in H_2O_2-induced DNA damage in human IMR-32 neuroblastoma cells. Levels of DNA damage measured with the comet assay in IMR-32 neuroblastoma cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of EPA and 25µM H_2O_2. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where $p < 0.001$ and ** where $p < 0.01$.

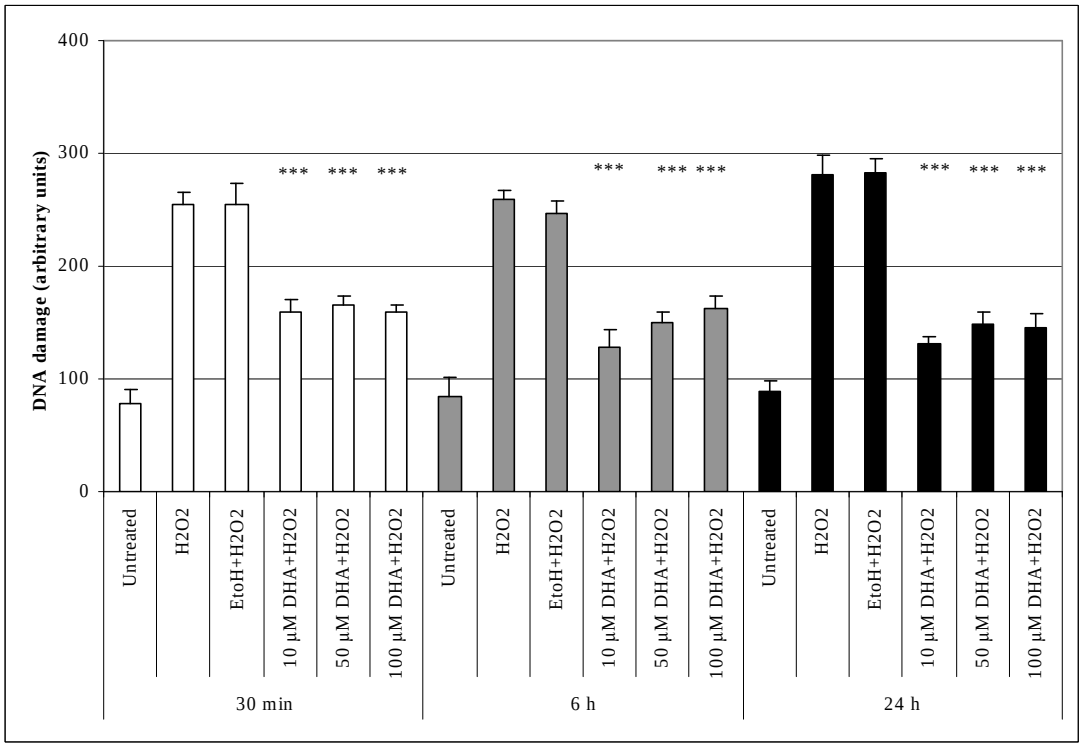


Figure 2.6. Evaluation of DHA cytoprotection in H_2O_2-induced DNA damage in human IMR-32 neuroblastoma cells. Levels of DNA damage measured with the comet assay in IMR-32 neuroblastoma cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of DHA and 25µM H_2O_2. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where p <0.001.

EPA or DHA treatment without H_2O_2 challenge was also evaluated in peripheral cells and the mean level of DNA damage was measured and compared between controls and fatty acid treated cells. No difference in the level of DNA damage was noted in the non-fatty acid treated and fatty acid treated U937 cells (results not shown). Conversely, when lymphoblastoid cells were treated with increasing concentrations of EPA or DHA, an increase in DNA damage was observed. This was only statistically significant ($p< 0.05$) when cells were treated with 100µM of DHA for 24h (Figure 2.7).

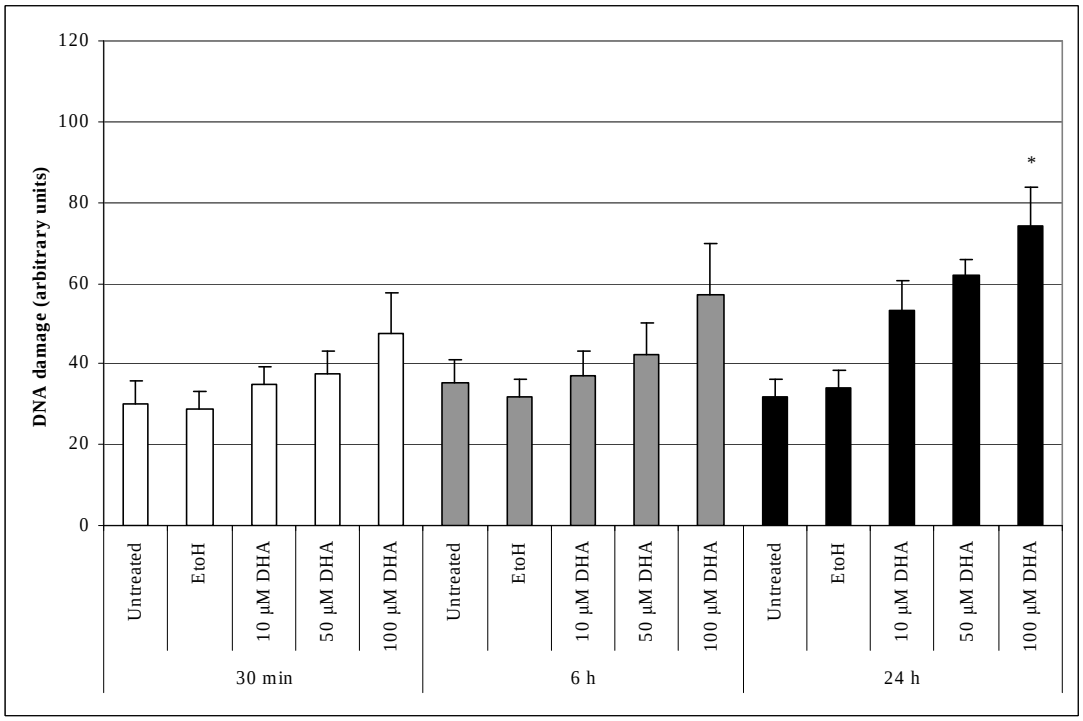


Figure 2.7. Evaluation of DHA cytoprotection in human lymphoblastoid cells. Levels of DNA damage measured with the comet assay in lymphoblastoid cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of DHA. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by * where $p < 0.05$.

A significant reduction in the levels of DNA damage in the U937 and lymphoblastoid cells treated with 10µM and 50µM EPA+ H_2O_2 (Figures 2.8 and 2.9) was observed after 30min and up to 24h when compared to H_2O_2 and ethanol+ H_2O_2 treatments. Furthermore, DHA pre-treated U937 and lymphoblastoid cells also offered a significant level of cellular protection against the H_2O_2 challenge following 30min and 24h incubations with the fatty acid (Figures 2.10 and 2.11).

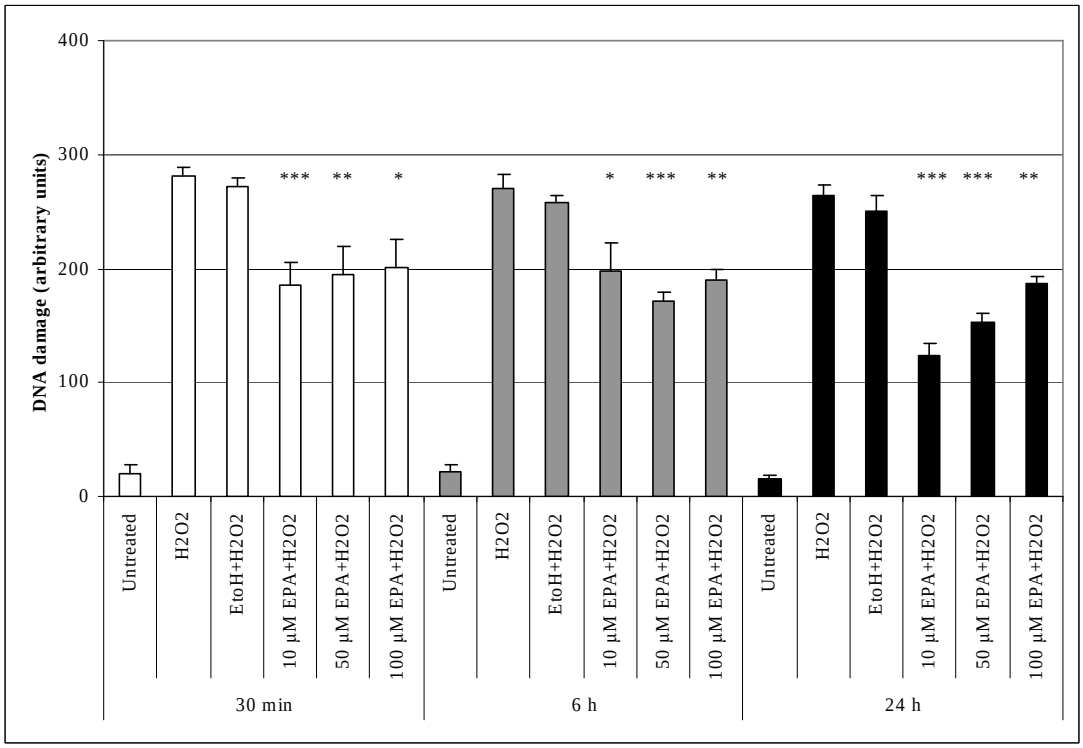


Figure 2.8. Evaluation of EPA cytoprotection in H_2O_2-induced DNA damage in human U937 cells. Levels of DNA damage measured with the comet assay in U937 cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of EPA and 100µM H_2O_2. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where $p < 0.001$, ** where $p < 0.01$ and * where $p < 0.05$.

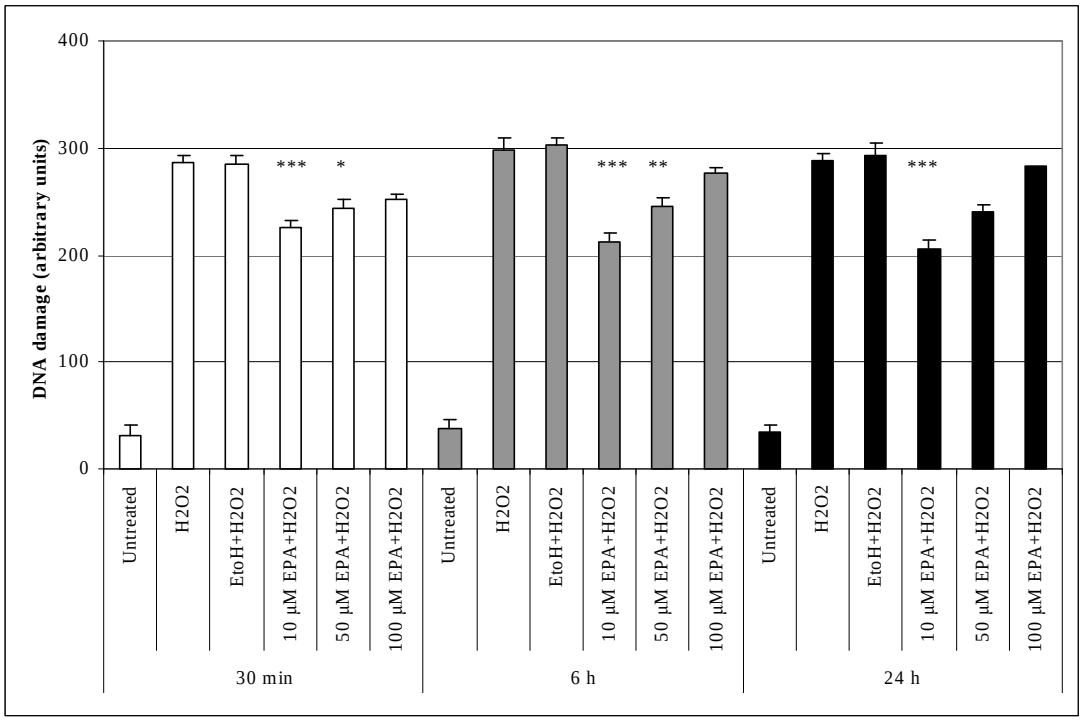


Figure 2.9. Evaluation of EPA cytoprotection in H_2O_2-induced DNA damage in human lymphoblastoid cells. Levels of DNA damage measured with the comet assay in lymphoblastoid cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of EPA and 100µM H_2O_2. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where $p < 0.001$, ** where $p < 0.01$ and * where $p < 0.05$.

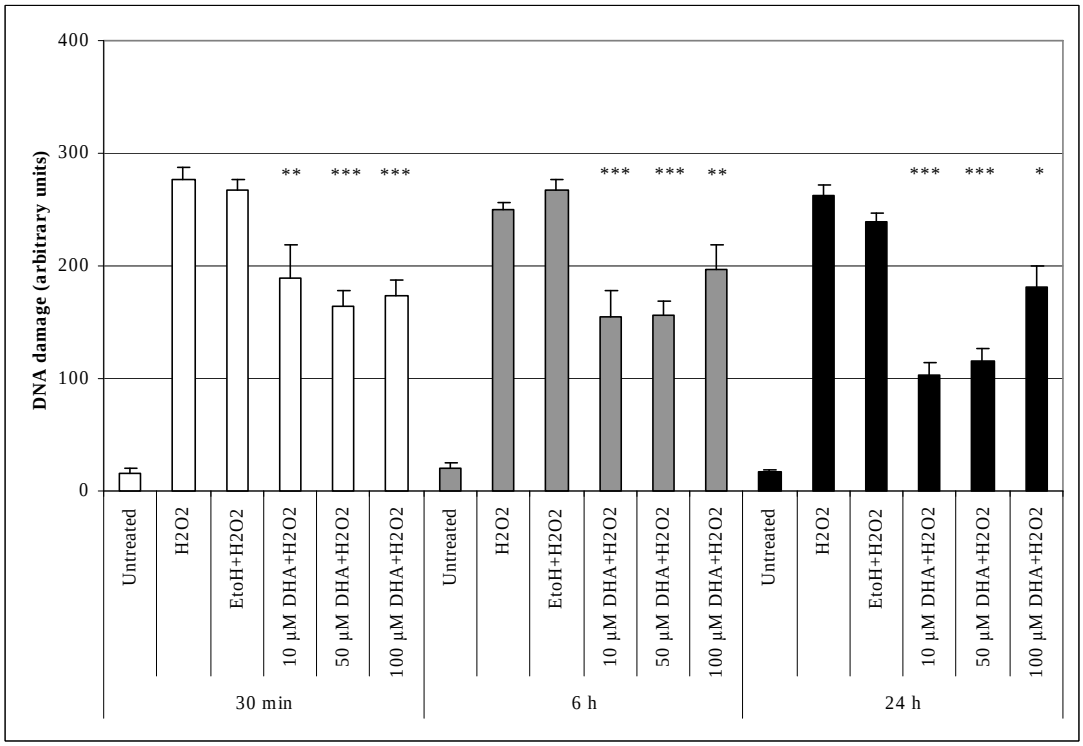


Figure 2.10. Evaluation of DHA cytoprotection in H_2O_2-induced DNA damage in human U937 cells. Levels of DNA damage measured with the comet assay in U937 cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of DHA and 100µM H_2O_2. Data show the mean DNA damage (n=3) ± sem. Significance is denoted by *** where $p < 0.001$, ** where $p < 0.01$ and * where $p < 0.05$.

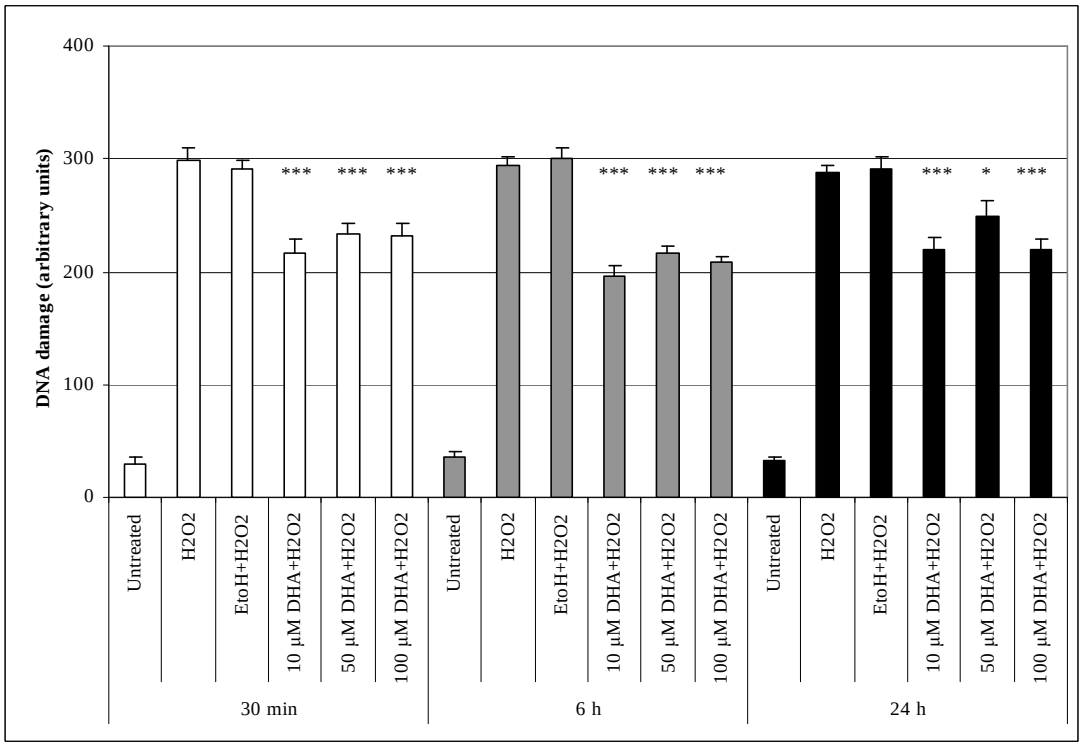


Figure 2.11. Evaluation of DHA cytoprotection in H_2O_2-induced DNA damage in human lymphoblastoid cells. Levels of DNA damage measured with the comet assay in lymphoblastoid cells following 30min, 6 and 24h treatment with 10, 50 and 100µM of DHA and 100µM H_2O_2. Data show the mean DNA damage (n=3) ±sem. Significance is denoted by *** where $p < 0.001$ and * where $p < 0.05$.

2.4 Discussion

The main aim of this chapter was a comparator study of two human peripheral cell lines and a neuroblastoma cell line as a means of assessing the validity of monitoring peripheral lymphocytes as potential surrogate markers of DNA damage in neuronal cell types. Furthermore, experimentation to test the susceptibility of each cell line to an oxidative challenge was also undertaken along with an examination of the potential protective effects of a series of antioxidants and fatty acids on DNA oxidative damage.

2.4.1 Comet Assay Validation

The comet assay was undertaken to investigate single-stranded DNA breaks as a biomarker of DNA oxidative damage. Results of the validation study reveal that freezing lymphocytes at different time points up to 12 weeks at -80^{o}C had no effect on the level of endogenous or exogenous cellular DNA damage. The effects of cryopreservation on endogenous and induced DNA damage in human peripheral blood lymphocytes has been measured in various studies (Duthie *et al* 2002; Visvardis *et al* 1997) where it was reported that DNA damage was the same as that seen in freshly isolated lymphocytes. In addition, long term storage was also shown to have no effect on endogenous and induced DNA damage.

2.4.2 ABTS Assay

In addition, the reducing ability of a range of antioxidants was assessed and in conjunction, the efficacy of these antioxidants and a series of polyunsaturated fatty acids were considered in affording protection to cellular DNA against an oxidative challenge. Numerous assays have been described in which antioxidant is added to a reaction mixture in which free radicals are generated. An example is the ABTS assay

in which ABTS is oxidised by 2,2'azobis(2-amidopropane). Any antioxidant present delays the appearance of the coloured product of this reaction. Two polyphenolic (EGCG and resveratrol) and two monophenolic (tyrosol and hydroxytyrosol) compounds were tested using the ABTS assay. EGCG and hydroxytyrosol (Table 2.1) were found to be the most potent radical quenchers examined.

Structure-activity comparisons suggest that antioxidant activity of phenolic compounds depends on the number and orientation of hydroxyl groups relative to the electron-withdrawing CO_2H, CH_2CO_2H or $(CH)CO_2CH$ functional groups (Rice-Evans *et al* 1996). When considering flavonoid (e.g. EGCG) antioxidant activity, the B-ring hydroxyl configuration is the most significant determinant of scavenging of reactive oxygen species. Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (Heim *et al* 2002). Functional differences between hydroxytyrosol and tyrosol have been attributed to the presence of only a single hydroxyl (reviewed by Quiles *et al* 2002) which may account for the greater antioxidant potential of hydroxytyrosol over tyrosol. These structural differences may explain why hydroxytyrosol and EGCG appear to have a greater antioxidant potential than tyrosol and resveratrol (Figure 2.1) since Masella *et al* (1999) suggest that the phenol chemical structure considerably influences the antioxidant activity as a consequence of both steric factors and those related to position and type of functional groups on the phenol ring. Furthermore, the antioxidant activity of biphenols depends mainly on the number of hydroxyl groups in the molecule (Masella *et al* 1999).

2.4.3 Antioxidant and Fatty Acid Protection against DNA Damage

2.4.3.1 DNA Damage and Antioxidant Protection

Several studies of schizophrenia have reported abnormalities in one or more antioxidant enzymes and/or in the levels of cerebrospinal fluid and plasma TBARs (Mahadik and Mukherjee, 1996; Reddy and Yao, 1996). Other studies have also reported a wide range of abnormalities in membrane phospholipids that may be a result of oxidative damage. These changes have been considered to be a result of altered metabolism (reduced synthesis and/or increased breakdown) of essential polyunsaturated fatty acids (EPUFAs). EPUFAs are the ω-3 and ω-6 series of fatty acids that must be consumed in the diet. Reduced membrane EPUFAs have been found to be associated with psychopathology (Glen *et al* 1994; Peet *et al* 1995).

Three human cell lines were used in this research as models of schizophrenia and cell supplementation studies were undertaken to assess potential antioxidant and fatty acid protection against ROS-induced DNA damage. Supplementation of neuroblastoma cells for 30min and 6 hours with EGCG and hydroxytyrosol of a concentration within human physiological range (ie. 10μM) resulted in a highly significant decrease in the level of DNA damage in cells treated additionally with H_2O_2. Furthermore, this same effect was observed in U937 cells. However, in only those lymphoblastoid cells treated with hydroxytyrosol was a significant protection of cellular DNA against an oxidative challenge observed.

Green tea is a widely consumed drink and has gained a reputation as a health-promoting dietary component due to the antioxidant activity of EGCG, its main phenolic constituent. Evidence is increasing however, that tea constituents can be cell damaging and pro-oxidant. These effects are suggested to be due to spontaneous

H_2O_2 generation by polyphenols in solution (Elbling *et al* 2005; Furukawa *et al* 2003). This pro-oxidant (or poor antioxidant) effect was perhaps more pronounced in the lymphoblastoid EGCG-treated cells used in this study, resulting in a higher overall ROS generation and greater level of DNA damage than seen in the U937 and IMR-32 EGCG-treated cells (Table 2.2). In contrast, the production of H_2O_2 in tea catechin-treated lymphoblastoid cells was compared by Sugisawa *et al* (2004) who suggested that the structure of EGCG is less likely to result in H_2O_2-induced chromosomal damage than EGC. EGC has a trihydroxyl structure with a B-ring similar to EGCG. Another catechin, ECG which has a gallate group linked to flavan-3-ol similar to EGCG, however, did not produce H_2O_2 (Sugisawa *et al* 2004). Nevertheless, tea catechins and polyphenols are effective scavengers of reactive oxygen species *in vitro* and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities (Higdon and Frei 2003).

Until recently, there was little understanding of the metabolism of flavonoids and their mode of entry into the systemic circulation after oral absorption. Although numerous studies have reported flavonoid-mediated neuroprotection, there is little information about the interaction of flavonoids or their circulating metabolites with the brain endothelial cells from the blood brain barrier (Youdim *et al* 2004). EGCG has been reported to enter the brain after oral administration (Suganuma *et al* 1998) and has more recently been investigated as a prophylactic for Alzheimer's disease (Rezai-Zadeh *et al* 2005).

2.4.3.2 DNA Damage and Fatty Acid Protection

Epidemiological studies have shown an apparent beneficial effect of fish oil containing high levels of ω-3 PUFA on mortality from heart disease and cancer

(Kikugawa *et al* 2003) and there is additional evidence to suggest that ω-3 fatty acids may also be important to mental health (Peet and Stokes, 2005). Conversely, a more recent systematic review (Hooper *et al* 2006) found no evidence of a clear benefit of ω-3 fats on health (in particular mortality, cardiovascular events, cancer or strokes). However, the benefits of using ω-3 fatty acids to protect DNA against oxidative damage, particularly in mental illness, have not been widely investigated. In this study human peripheral and neuroblastoma cells were incubated with increasing concentrations of EPA or DHA from 30min to 24h. Results revealed that there was no difference in the level of DNA damage in the U937 and IMR-32 cell lines treated with either EPA or DHA when compared to controls (i.e. non EPA or DHA treated cells) (results not shown). Conversely, examination of lymphoblastoid cells treated with the highest concentration of DHA after 24 hours (Figure 2.6) revealed a significant increase in DNA damage when compared to 30 minute and 24 hour controls.

Fatty acid treated peripheral and neuroblastoma cells were also subjected to a peroxide challenge. Results show that in fatty acid + H_2O_2-treated cells the level of DNA damage was significantly reduced in the U937 and IMR-32 cells at all time points compared to H_2O_2 control cells.

Although the use of fish oil has been suggested to have many beneficial effects, lipid peroxidation-mediated DNA damage is an undesirable consequence resulting from intake of high concentration of fish oil. *In vitro* studies have shown that peroxidized PUFA induce DNA chain breaking (Adam *et al* 1998) and 8-hydroxyguanosine formation (Kaneko and Tahara 2000). Contrasting studies have shown that ω-3 PUFA supplementation on oxidative stress-induced DNA damage in a variety of cell

types acts instead as a protective agent against oxidative stress rather than as a source of mutagenic metabolites (Beeharry *et al* 2003, Kikugawa *et al* 2003). The results of our study have also shown that EPA and DHA protect peripheral and neuroblastoma cells against oxidative stress induced DNA damage. It is worth noting that in their study, Kikugawa *et al* (2003) used fish oil supplemented with vitamin E. Since the purported health effects of fish oil are questioned by Hooper *et al* (2006), the reduction in oxidative stress-induced DNA damage reported by Kikugawa *et al* (2003) may well be attributable to the antioxidant rather than the fish oil. Nevertheless, these authors propose that although lipid peroxidation products formed from the use of high concentrations of ω-3 can damage DNA, the damage caused by these products may be significantly lower than those caused as a result of direct oxidative stress resulting from ROS.

Within our study, pure oils without added vitamin E were used as in the study reported by Beeharry *et al* (2003). In their study Beeharry *et al* (2003) suggest that the DNA-protective effects seen following incubation of human cells with the ω-6 PUFA linoleic acid may be as a result of the fatty acid acting as a substrate for lipid peroxidation. In addition, linoleic acid may also inhibit the induced oxidative stress that causes lipid peroxidation by blocking an early stage in the induction of damage. Interestingly, a preliminary study carried out by the same group has also shown DHA to be cytoprotective (Beeharry *et al* 2003).

In conclusion, the efficacy of a series of antioxidants and polyunsaturated fatty acids in affording protection against cellular DNA damage against an oxidative challenge has been investigated. The findings have shown that the antioxidants and PUFAs used within physiological concentrations and after a short incubation period are able

to significantly protect human peripheral and neuroblastoma cells against oxidative stress induced DNA damage. The validity of using peripheral cells as a potential surrogate marker of DNA damage in a neuronal cell type was also undertaken. Results have revealed that pre-treatment of peripheral cells with antioxidant or ω-3 fatty acids followed by an oxidative challenge significantly reduce the levels of DNA damage. Results have confirmed the same effects were observed with the neuronal cell type when provided with the same supplements under the same conditions. Further work should perhaps include a comparison investigation of a number of other neuronal cell types, using additional methods to detect oxidative-stress induced DNA damage. Nevertheless, the work carried out in this research appears to be the first to undertake such a comparator study and findings indicate that the use of peripheral cells can potentially be used as surrogate marker for neuronal cells when examining oxidative stress induced DNA damage.

CHAPTER 3

Examination of biomarkers of protein oxidation in fatty acid treated human U937 cells and detection of ROS in antioxidant treated U937 cells

3.1 Introduction

3.1.1 Oxidative Stress and Protein Damage

Although damage caused by oxidative stress to nucleic acids can be repaired by proficient excision/insertion mechanisms, repairing damaged proteins appears to be limited to the reduction of oxidised derivatives of the sulphur-containing amino acid residues (Stadtman and Levine 2000). Damaged proteins are degraded to amino acid elements by various endogenous proteases, while the collection of intracellular oxidised proteins involves pro-oxidant and antioxidant activities and the concentrations and activities of the proteases that degrade the oxidised forms of the proteins.

Proteins can be further damaged by pro-oxidant processes that influence environmental and dietary conditions. It is assumed that under normal conditions the metal-catalyzed oxidation systems are the basis of oxidative damage, arising through the interaction of ROS end-products, hydrogen peroxide and the alkylperoxides. When transition metals Fe(II) or Cu(I) are present, these relatively unreactive peroxides are transformed to the highly reactive hydroxyl radical (reaction 1) or the alkoxyl radical (reaction 2) which can react with most other organic substances.

$H_2O_2 + Fe(II)/Cu(I) \rightarrow HO\bullet + OH^- + Fe(III)/Cu(I)$ (1)

$ROOH + Fe(II)/Cu(I) \rightarrow RO\bullet + OH^- + Fe(III)/Cu(I)$ (2)

Any of the protein amino acids can be targeted for oxidation by OH• for example, by high concentrations of H_2O_2 and Fe(II) (Neuzil *et al* 1993). At physiological concentrations of H_2O_2, however, protein damage is probably limited to the

modification of amino acid residues at metal binding sites on the protein, which effectively concentrate the ions (Stadtman and Levine 2000).

3.1.2 Protein Oxidation as a Biomarker of Oxidative Stress

The earliest work concerning oxidation of a biologically important protein studied the effects of selected radicals generated by radiolysis of lysozyme (reviewed by Griffiths *et al* 2002). The revelation that the thiocyanate radical (a selective modifier of tryptophan) and the hydroxyl radical could inactivate the enzyme, suggested that tryptophan residues are essential for biological activity. Later, protein oxidation in respect of altered function was confirmed by oxidative modifications to histidine and lysine in low density lipoproteins leading to altered receptor recognition (Parthasarathy *et al* 1992). In addition, it was noted that the process of protein oxidation often introduced new functional groups such as hydroxyls or carbonyls which also play a role in altered function and turnover (Griffiths *et al* 2002).

ROS are capable of directly reacting with the protein or with molecules such as sugars and lipids, creating products that then react with the protein (Levine 2002). Either the protein peptide bond or side chain can be attacked. Following ROS damage, the protein may be cleaved producing lower molecular weight products or it may be cross-linked to give higher molecular weight products (Levine 2002). Oxidative modifications can be grouped into those which are quite specific, for example, those whose residue is oxidized and the product generated, and those which can alter multiple residues and may give rise to several products (Levine 2002). Of the covalent changes resulting from direct and indirect oxidative modification of proteins, one of the most common is the introduction of carbonyl groups into the side chains. Carbonyl groups are formed during the oxidation of the amino acid residues

arginine, lysine and proline (Berlett and Stadtman 1997). Protein carbonyl derivatives can also be formed via oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains. This results in formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative (Berlett and Stadtman 1997). Carbonyl groups can also arise from a further reaction with the primary oxidation products such as 4-hydroxy-2-nonenal (Levine 2002). As a consequence, oxidative changes can be measured by examining the carbonyl content of a specific protein or a tissue (Ciolino and Levine, 1997). Carbonyls can be generated in response to a wide variety of oxidising agents such as alkoxy and peroxy radicals illustrated in Figure 3.1.

While numerous studies of the formation of protein carbonyls have been undertaken, they have not identified those carbonyls produced through direct protein oxidation and those formed by the addition of previously oxidised molecules. For that reason protein carbonyls must be considered as a broad marker of oxidation (Dalle-Donne *et al* 2003). Compared with methionine sulphoxide and cysteinyl derivatives, the production of carbonyls is relatively difficult and as a result might be indicative of a more severe oxidative stress. In fact, elevated levels of protein carbonyls are generally a sign not only of oxidative stress but also of disease-derived protein dysfunction (Dalle-Donne *et al* 2003).

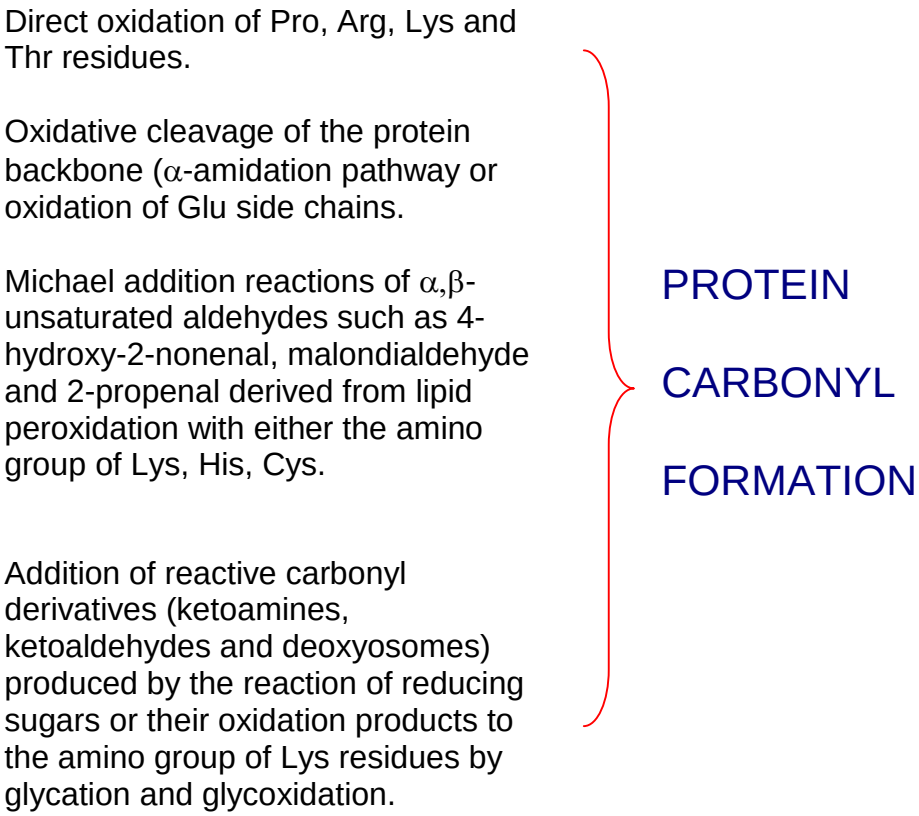


Figure 3.1. The production of protein carbonyls (aldehydes and ketones) (Adapted from Dalle-Donne *et al* 2003)

3.1.3 Detecting Biomarkers of Protein Oxidative Damage

Using protein carbonyls as a biomarker of oxidative stress in preference to lipid peroxidation products has the advantage that oxidised proteins are usually more stable (Dalle-Donne *et al* 2003). A study by Pantke *et al* (1999) demonstrated that protein carbonyls form early during coronary heart surgery (ie during the reperfusion stage) and serum protein carbonyl content was elevated for at least four hours indicating a much slower serum removal of oxidized protein when compared to MDA and glutathione disulphide.

Techniques for measurement of physiological protein oxidation vary from immunodetection by ELISA or Western blot to analytical HPLC. The most common and reliable method of detecting protein carbonyl groups is based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP) (Dalle-Donne *et al* 2003). The method was introduced by Levine *et al* (1990) and has since become one of the most widely used measures of protein oxidation in various human diseases (Dalle-Donne *et al* 2003).

3.1.3.1 Enzyme-linked Immunosorbent Assay

The ELISA method of measuring protein carbonyls as an index of oxidative injury was developed by Buss *et al* (1997) and the method was further amended by Winterbourn and Buss (1999). Protein samples are reacted with DNP, adsorbed to wells of an ELISA plate before probing with an antibody raised against protein-conjugated dinitrophenylhydrazine. The adsorbed protein is reacted with a biotinylated anti-DNP antibody followed by a streptavidin-biotinylated horseradish peroxidase. The ELISA is easier to use, less labour-intensive and handles more samples per day than the colorimetric assay (Dalle-Donne, 2003). In addition, the ELISA only requires microgram amounts (approximately 60μg) of protein, similar to that required for the HPLC method (Levine *et al* 1994).

3.1.3.2 Spectrophotometric DNPH Assay

The method devised by Levine *et al* (1994) involves the detection of protein carbonyl groups following their reaction with DNPH and spectrophotometric analysis of the acid hyrazones at 370nm. It can be used to quantify carbonyl content in a mixture of proteins in plasma, tissue homogenates, and cellular extracts or in isolated proteins. However, the DNPH assay has been shown to be unreliable in

samples containing high amounts of chromophore (e.g. haemoglobin, myoglobin, retinoids). Fagan *et al* (1999) improved upon the technique enabling the determination of carbonyl content to be made even in highly coloured tissue extracts and removed residual DNPH which was also found to interfere with the assay. The spectrophotometric detection of DNP-carbonyl derivatives after separation of the proteins by gel-filtration HPLC (reviewed by Dalle-Donne 2003) could provide information on the extent of oxidative damage to a particular protein in a complex mixture such as tissue homogenates or cellular extracts. Gel-filtration by HPLC has been shown to be a convenient and efficient technique in which DNP-carbonyl derivatised proteins are separated by molecular weight, allowing a more specific analysis of carbonyl content.

Other methods of detecting carbonyls include Western blot immunoassay (Shacter *et al* 1994) and an immunoblot technique developed by Robinson *et al* (1999) which involves a combination of DNPH derivatisation, the preparation of blanks by treatment with sodium borohydride and immunological detection. $NaBH_4$ reduces carbonyl to alcohols, eliminating immunostaining due to carbonyl groups and the difference between the staining intensities of a $NaBH_4$-treated and untreated sample constitutes a specific measure of the carbonyl content of the sample.

3.1.3.3 Detection of ROS

The specificity of the fluorescent probes used in flow cytometry is the most convenient method for measuring ROS generation and the importance of these probes was realised over 40 years ago when 2',7'-dichlorofluorescein diacetate (DCFH) was used to measure H_2O_2 in aqueous solution (reviewed by Walrand *et al* 2003). DCFH is a small non-polar, non-fluorescent molecule that diffuses into cells

where it is enzymatically deacetylated by intracellular esterases to the polar non-fluorescent compound, 2',7'-dichlorofluorescein (DCF) in the presence of the generated ROS (Szejda *et al* 1984). DCF then emits a fluorescent signal that is measured using a flow cytometer.

Flow cytometry is a method that enables the counting, examining and sorting of cells suspended in a stream of fluid. Simultaneous multiparametric analysis (ie the measurement of several characteristics of each cell or particle) of single cells flowing through an optical/electronic detection apparatus can also be carried out (BD Biosciences 2000). A laser light of a single frequency is directed onto a hydrodynamically focused stream of fluid and a variety of detectors are aimed at the point where the stream passes through the light beam. One in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter, SSC). FSC is proportional to cell-surface area or size while SSC is proportional to cell granularity or internal complexity (ie shape of the nucleus, membrane roughness). Correlated measurements of FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population (BD Biosciences 2000). Once a cell or particle passes through the laser light, emitted SSC and fluorescence signals are diverted to the photomultiplier tubes (PMTs) and a photodiode collects the FSC signals. PMTs detect fluorescence signals although they are often weak. Bandpass filters enable the optimisation of a particular fluorescent dye which allows only a narrow range of wavelengths to reach the detector. This spectral band of light is close to the emission peak of the fluorescent dye.

Light signals are created as the cells pass through the laser beam in a fluid stream. The light signals are then converted to electronic signals (voltages) by

photodetectors and are then allocated a channel number on a data plot. A voltage pulse is created when a particle enters the laser beam and starts to scatter light or fluoresce. When the light signals or photons come into contact with one side of the PMT they are converted into a proportional number of electrons that are multiplied creating a larger electrical current; this in turn travels to the amplifier and is converted to a voltage pulse. The voltage pulse is given a digital value representing 0-1,000mV channels. Cell populations can be displayed in several different formats. A single parameter such as FSC (FL1) can be displayed as a single parameter histogram where the horizontal axis represents the parameter's signal value in channel numbers and the vertical axis represents the number of events per channel number (Figure 3.2).

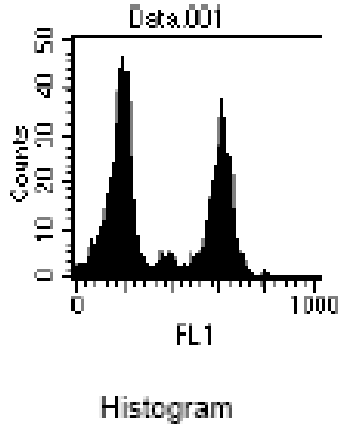


Figure 3.2. Histogram representation of flow cytometric data (BD Biosciences 2000).

Each event is placed in the channel that corresponds to its signal value. Signals with identical intensities collect in the same channel. Brighter signals are displayed in channels to the right of the less bright signals. The histogram enables a single parameter to be viewed against the number of events. A subclass control is used to

determine where the markers will be placed and these are used to specify a range of events for a single parameter (Figure 3.3). In the diagram, the first histogram M1 marker is placed around the negative peak of the subclass control while the M2 marker is placed to the right of M1 to show positive events. Statistical percentages of the negative and positive intensity peaks are used to quantify mean fluorescence intensities.

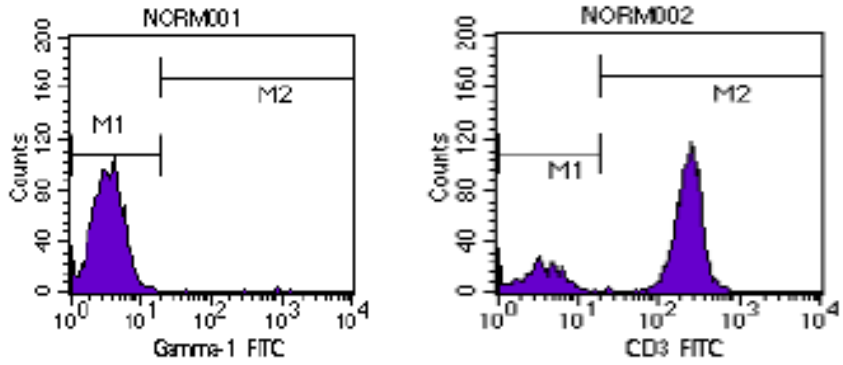


Figure 3.3. Histograms of subclass control (NORM001) and CD3 FITC (NORM002) with histogram markers M1 and M2 (BD Biosciences 2000).

3.1.4 Protein Oxidative Damage and Schizophrenia

There are numerous human diseases associated with elevated levels of carbonylated proteins. Neurological illnesses associated with this condition include Alzheimer's disease (AD), dementia with Lewy bodies and Parkinson's disease. Oxidative stress plays a major role in the development of AD typified by protein oxidation, lipid peroxidation and ROS formation (Dalle-Donne 2003). Moreover, chemical markers that are altered in AD have also been suggested to be abnormal in schizophrenia:

choline acetyltransferase, catecholamines and indolamines (reviewed by Powchik *et al* 1998).While schizophrenia shares many pathological similarities with AD and other psychiatric illnesses, increased levels of PCOs as a result of protein oxidative damage in schizophrenia has not been studied extensively before. Protein oxidation in a treatment-related disorder, called tardive dyskinesia, has been investigated. Tsai *et al* (1998) have hypothesised that neuroleptics enhance striatal glutamatergic neurotransmission by blocking presynaptic dopamine receptors, which causes neuronal damage as a consequence of oxidative stress. Patients suffering from tardive dyskinesia have been shown to have significantly higher concentrations of N-acetylaspartate, N-acetyl-aspartylglutamate and aspartate in their cerebral spinal fluid and higher levels of protein-oxidized products associated with tardive dyskinesia (Tsai *et al* 1998).

The aim of this study was to examine the effect of EPA or DHA supplementation with a pro-oxidant challenge on the U937 cell line and evaluate the use of protein carbonyl production as a marker of protein oxidation in a schizophrenic model. Cellular ROS status as assessed by flow cytometry was investigated and the efficacy of antioxidant treatment prior to a pro-oxidant challenge in U937 cells was also determined.

3.2 Materials and Methods

3.2.1 Examination of Biomarkers of Protein Oxidation in Fatty Acid Treated Human Peripheral (U937) Cells

3.2.1.1 Materials

Penicillin/streptomycin antibiotic, trypsin/EDTA, glucose and bovine serum albumin (BSA) were supplied by Sigma (Poole, UK). Heat-inactivated FCS was obtained from Invitrogen (Paisley, UK). RPMI 1640 media was supplied by Gibco (UK). EGCG was supplied by Sigma (Poole, UK). Hydroxytyrosol was obtained from Alexis Corp (UK) Ltd (Nottingham, UK). Eicosapentaenoic acid (EPA, >95%) and docosahexaenoic acid (DHA, >90%) were supplied by Pronova (Norway). Tris, EDTA and $25cm^2$ culture flasks were obtained from Fisher Scientific, (Loughborough, UK). DMSO, NaCl, EGTA, NaF, Na_3VO_4, $Na_4O_7P_2$, protease inhibitor cocktail, phenylmethanesulfonyl fluoride (PMSF), Triton-X-100, glycerol, sodium dodecyl sulphate (SDS) and trichloracetic acid (TCA) were supplied by Sigma (Poole, UK). Protein carbonyl ELISA kit components were supplied by Zenith Technology (Dunedin, New Zealand)

3.2.1.2 Culture of U937 Cells

All experimentation work was carried out solely by me, and cells lines were cultured and maintained by me. Human U937 cells were cultured, maintained in $25cm^2$ flasks and harvested as described previously in Chapter 2.

3.2.1.3 Fatty Acid Treatment of U937 Cells

U937 cells were incubated in 10ml media containing either 50μM of ethanol or 50μM fatty acids prepared in ethanol and the cells were incubated for 24h under

conditions described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

3.2.1.4 Hydrogen Peroxide Treatment of U937 Cells

Following 24h incubation, cells were washed three times with PBS and collected by centrifugation at 2000x*g* for 5min. Cells were resuspended in 1ml media containing 100μM H_2O_2 and incubated for 4h at 37^oC.

3.2.1.5 Protein Extraction

Cells were washed three times with ice cold PBS and collected by centrifugation, 2000x*g* for 5min at 4^oC. Supernatant was discarded and 0.2ml extraction buffer (comprising 1ml protein buffer (10mM Tris pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, 1% Triton-X-100, 10% Glycerol, 0.1% SDS) 10μl protein inhibitor and 3.3μl PMSF) was added to the pellet and vortexed for 10min. The samples were then centrifuged at 10,000x*g* for 15min at 4^oC. The supernatant was aliquotted into a new microcentrifuge tube and stored at -80^oC until ready for assay.

3.2.1.6 Protein Quantitation

The protein content of cell supernatants was determined by the Bradford method (Bradford 1976) using bovine serum albumin as a reference standard.

3.2.1.7 Low Protein Procedure

Cell protein extracts were concentrated to ensure a final concentration of 35-80g/L and the protocol was followed as per manufacturer's instructions (Zentech PC Test, Protein Carbonyl Enzyme Immunoassay Kit from Zenith Technology, Dunedin, New Zealand).

3.2.1.8 ELISA Experimental Procedure

Carbonyl groups were measured using the Zentech PC Test (Protein Carbonyl Enzyme Immunoassay Kit) from Zenith Technology (Dunedin, New Zealand). This kit follows the method outlined by Buss *et al* (1997) (as amended by Winterbourn and Buss 1999), which uses derivatization of protein carbonyls in samples and oxidized protein standards with dinitrophenylhydrazine, followed by ELISA with an anti-DNP antibody. Absorbance was read at 450nm on the BIO-TEK FL600 fluorescence plate reader (BIO-TEK® Instruments Inc, Vermont, USA).

3.2.1.9 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and Student's t-test and one-way ANOVA was undertaken using SPSS 11.5 for Windows. A level of $p< 0.05$ was used to determine significance.

3.2.2 Detection of ROS in Antioxidant Treated Human U937 Cells

3.2.2.1 Materials

α-tocopherol was supplied by Sigma (Poole, UK) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA) was supplied by Invitrogen, (Paisley, UK).

3.2.2.2 Culture of U937 Cells

Human U937 cells were cultured, maintained and harvested as described previously in Chapter 2.

3.2.2.3 Antioxidant Treatment of U937 Cells

U937 cells were incubated with 10μM antioxidant (EGCG, hydroxytyrosol and α-tocopherol) for 24h under the same conditions as described previously in Chapter 2.

Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

3.2.2.4 CM-H_2DCFDA Treatment of U937 Cells

Following 24h incubation, cells were collected by centrifugation at 2000x*g* for 5min and washed three times with PBS. Intracellular ROS, via the generation of H_2O_2 was estimated by resuspending cells in media containing 1mM of fluorescent probe (CM-H_2DCFDA) and incubated for 30min at 37^{o}C. CM-H_2DCFDA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to form non-fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescin (CM-H_2DCF) and is efficiently retained within cells (Liu *et al* 2001) which in turn is rapidly oxidised to form highly fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorofluorescine (CM-DCF) in the presence of ROS. The fluorescence of the end product is believed to increase with the amount of intracelluar ROS (Pérez-Pastén *et al* 2006).

3.2.2.5 ROS Analysis by Flow Cytometry

Cells were washed twice with PBS and collected by centrifugation (ie. 1000 – 2000x*g* for 10min at 4^{o}C). The cell pellet was resuspended in 0.750ml of PBS and then transferred to sampling tubes and vortexed thoroughly. Samples were analysed on a EPICS XL EXPO32 flow cytometer (Beckman Coulter UK Ltd, Bucks, UK) equipped with an argon laser (488nm emission), using EXPO32 analysis software. Cells were recognised on the basis of forward-angle light scatter and side-angle light scatter. The fluorescence of cells was recorded under 488nm excitation. The increase in intracellular oxidation was measured as an increase in FL1 or fluorescence through a 525nm band-pass filter recorded on a log scale for 10,000 events. ROS was quantified by mean CM-DCF intensity.

3.2.2.6 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and one-way ANOVA and Student's t-test was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

3.3 Results

3.3.1 Protein Carbonyl Analysis

The ELISA method was used to detect levels of protein carbonyls from cellular protein extracts, in particular from U937 cells incubated for 24h with or without polyunsaturated fatty acids followed by 4h incubation with H_2O_2. No difference in protein carbonyl content was found in U937 cells treated with H_2O_2 when compared to control cells (Figure 3.4a). Furthermore, there was no difference in the level of protein carbonyl content in the cells pre-treated with a PUFA but challenged with H_2O_2, when compared to H_2O_2 only treated cells.

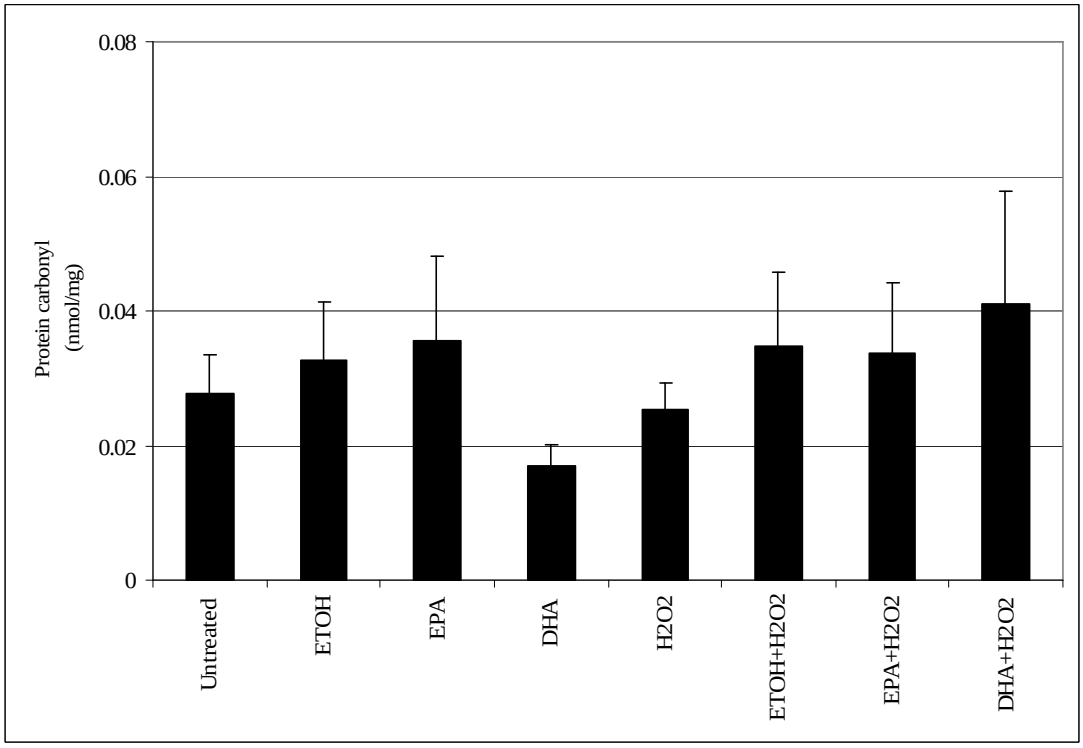


Figure 3.4a. Examination of protein carbonyl content in fatty acid and H_2O_2-treated human U937 cells. Cells were incubated with 50μM ETOH, EPA or DHA for 24h followed by 4h incubation at 37°C with 100μM H_2O_2. Data shows change in protein carbonyl production from triplicate samples as determined by two ELISA experiments ± sd.

The change in protein carbonyl levels was examined between experimental days. The data in Figure 3.4b reveals after completion of experiment 1, protein carbonyl levels have not increased significantly in any of the treatment groups compared to the untreated group. However, comparison of the data from experiment 2 shows a significant decrease in protein carbonyl levels following incubation with DHA, compared to the untreated sample. In addition, there is a significant increase in protein carbonyl levels in the cell lysate samples pre-treated with DHA+ H_2O_2 when compared to the DHA-only treated samples. Furthermore, there is significant increase in protein carbonyl levels in all samples between experiments, except in those samples pre-treated with DHA where protein carbonyl levels have significantly decreased in experiment 2 compared to experiment 1.

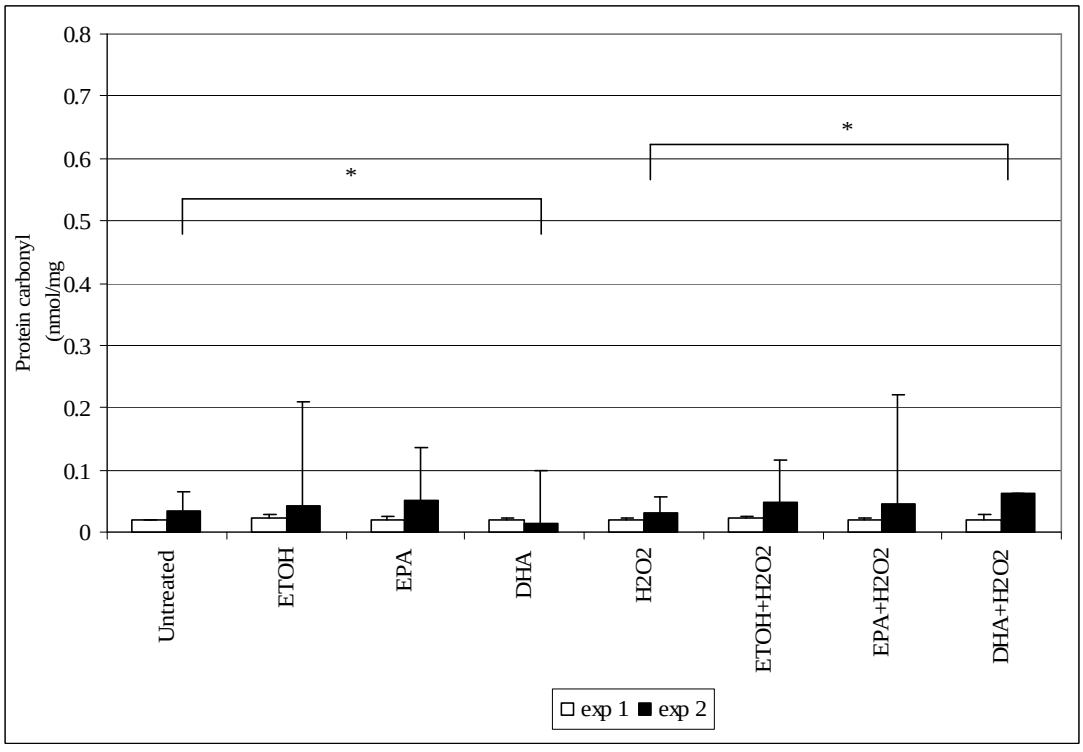


Figure 3.4b. Examination of protein carbonyl content in fatty acid and H_2O_2-treated human U937 cells in individual experiments. Cells on both days were incubated with 50μM ETOH, EPA or DHA for 24h followed by 4h incubation at 37°C with 100μM H_2O_2. Data shows change in protein carbonyl production from triplicate samples as determined by ELISA ± sd. Significance is denoted by * where $p < 0.05$.

The results of these experiments reveal that although the H_2O_2 challenge was high (100μM) the cells appear to be resistant to the pro-oxidant challenge since there is no statistically significant increase in protein carbonyl production. The lack of an effect seen following the H_2O_2 treatment, however, might be as a result of large intra/inter assay variation as shown by the error bars, rather than a high level of endogenous cytoprotection. Finally, treatment of U937 cells with either EPA or DHA alone induced no significant increase in protein carbonyl levels and so it would appear that there is no evidence of a pro-oxidant stress from either of the fatty acid treatments.

3.3.2 ROS Analysis

U937 cells pre-treated with hydroxytyrosol for 24h revealed a significant decrease ($p < 0.05$) in intracellular ROS when compared to untreated cells, although this reduction in ROS was not observed following EGCG treatment (Figure 3.5a). Furthermore, pre-treatment of cells for 24h with α-tocopherol revealed an increase in intracellular ROS levels compared to those treated with DMSO only, suggesting a pro-oxidant effect of this compound under these experimental conditions (Figure 3.5a). No difference in the level of intracellular ROS was noted following a 5min oxidant challenge with H_2O_2 when compared to untreated cells. The lack of challenge effect could be indicative of a high level of endogenous antioxidant or enzyme scavengers of radicals. The antioxidant effect of hydroxytyrosol was again observed when cells were pre-treated with this compound followed by an oxidant challenge. Results reveal a significant decrease in levels of ROS in these samples (Figure 3.5a)

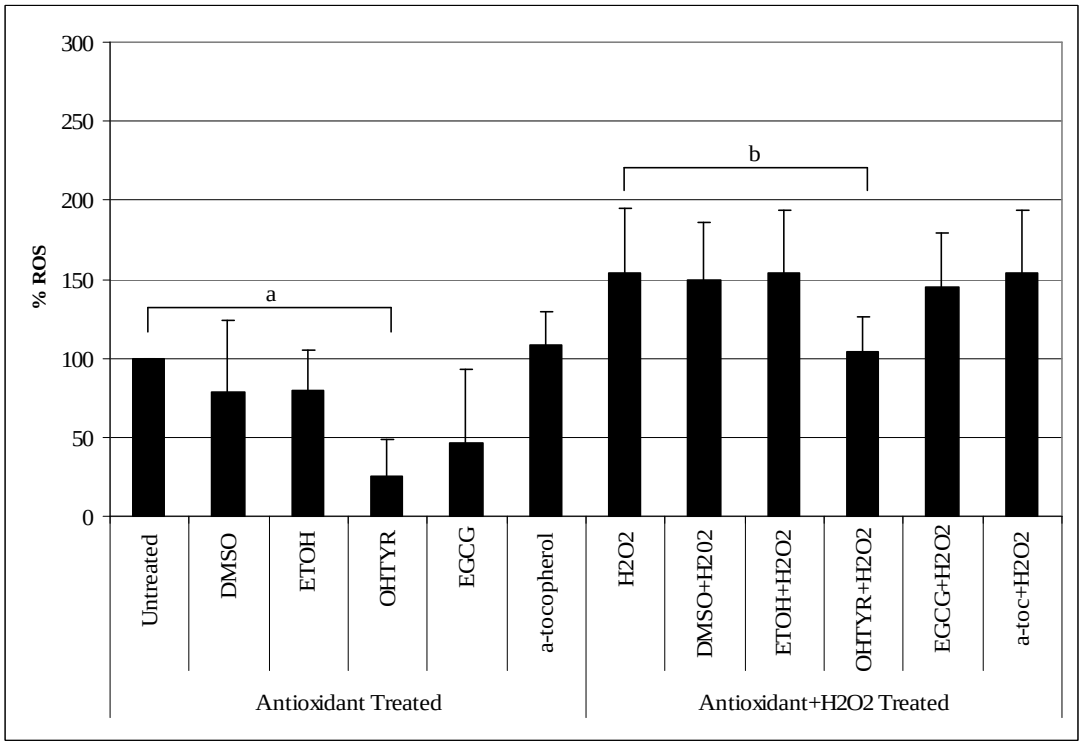


Figure 3.5a. Examination of ROS in antioxidant and H_2O_2-treated human U937 cells. Cells were incubated with 10μM OHTYR, EGCG or α-tocopherol for 24h followed by 30min incubation with 1mM CM-H_2DCFDA and 5min incubation with 100μM H_2O_2 at 37°C. Data shows mean % change in ROS production from triplicate samples as determined by 3 flow cytometry experiments ± %RSD. Significance is denoted by a and b where $p < 0.05$.

Between days variation of this assay was investigated. Although not indicated on the graph, there is significant difference in ROS levels between days. For example, there is significant difference between the levels of ROS on experimental day 1 and day 2 in untreated and DMSO pre-treated samples. However, there is no difference in the levels of cellular ROS in U937 cells pre-treated with or without antioxidant and H_2O_2 (Figure 3.5b).

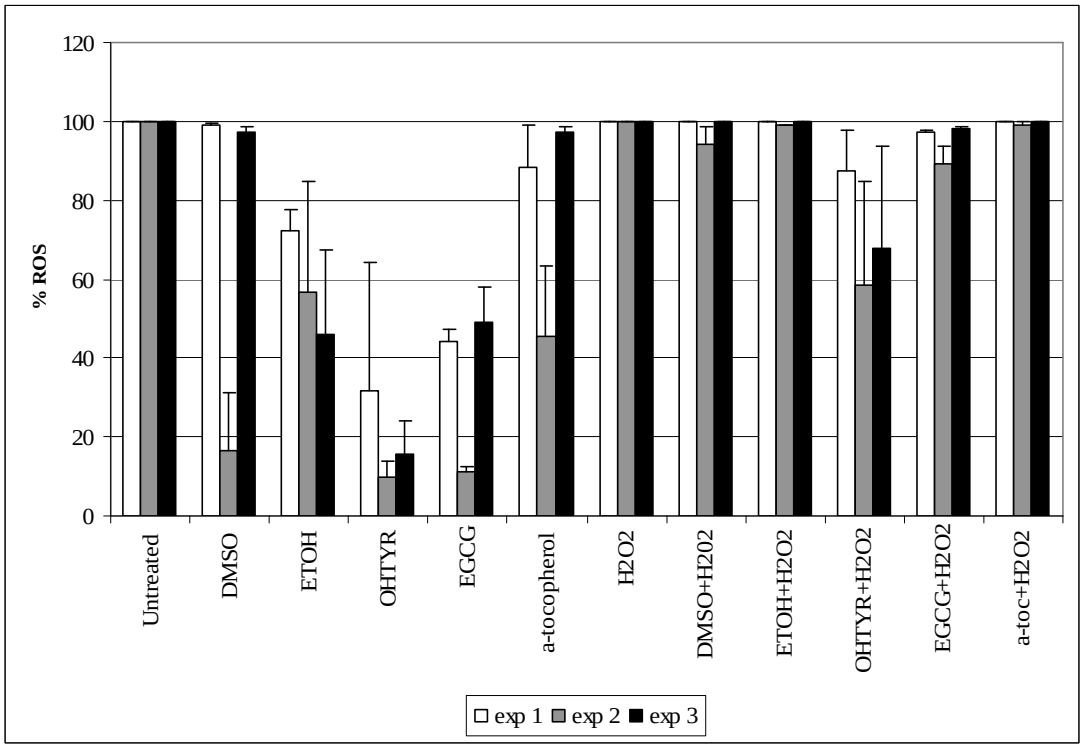


Figure 3.5b. Examination of ROS content in antioxidant and H_2O_2-treated human U937 cells in 3 individual experiments. Data shows mean ROS levels ± sd of triplicate samples.

The results of the above ROS experiments reveal significant between day variations in those cells that were not subjected to H_2O_2 treatment, particularly on experimental day 2 where ROS levels appear to be lower than on other experimental days. Despite this, hydroxytyrosol pre-treatment does appear to be effective in reducing ROS and H_2O_2-induced ROS, although the level of reduction did not reach statistical significance. Although a reduction in ROS is noted in cells pre-treated with EGCG, it is not significant and furthermore, although it does not appear to be an effective antioxidant treatment following cell treatment with H_2O_2, neither does this compound appear to act as a pro-oxidant.

3.4 Discussion

3.4.1 Protein Carbonyls

The evaluation of protein carbonyls as a potential marker of protein oxidation in the putative cell model for schizophrenia, U937 was undertaken. Treatment with H_2O_2 alone and following pre-treatment with EPA or DHA had no effect on the levels of protein carbonyls detected in the cell line by ELISA. Nevertheless, although the results were not statistically significant, DHA supplementation does appear to reduce endogenous and H_2O_2-induced protein carbonylation. Further *in vitro* and *in vivo* work would be merited since evaluation of the effect of fatty acid supplementation on the levels of protein oxidation in schizophrenia is limited. Indeed, the potential use of protein carbonyls as a biomarker of schizophrenia has not been widely examined until now.

The abnormal movements seen in tardive dyskinesia occur as a result of major late-onset chronic side effect of antipsychotic treatment. Oxidative stress and free radicals are thought to be associated with dopaminergic malfunctions (Sadan *et al* 2005) and oxidative stress-induced neurotoxicity in the striatal system is implicated in tardive dyskinesia (Shamir *et al* 2001). Several studies have reported that people with schizophrenia have abnormal levels of essential fatty acids in their blood cells and similar abnormalities have been recorded in association with the presence of tardive dyskinesia (reviewed by Vaddadi *et al* 1996). In their study, Vaddadi *et al* (1996) hypothesised that patients with schizophrenia would have lower levels of ω-3 and ω-6 EFAs than controls and that this abnormality would be more pronounced in those schizophrenic patients also suffering with tardive dyskinesia.

Studies of neurological diseases such as Parkinson's (Yang and Tiffany-Castiglioni 2005), mild cognitive impairment (Keller *et al* 2005) and multiple sclerosis (Bizzozero *et al* 2005) have reported an increase in levels of oxidative damage, including increased protein carbonyl levels. However, no evidence for increased oxidative stress to lipids, proteins or DNA in Huntington's disease was reported by Alam *et al* (2000), suggesting that oxidative stress is not a major component of this illness, or at least not to the extent that occurs in other neurodegenerative disorders. It is suggested that in pure chemical systems, the molecules most susceptible to free radical attack are PUFAs and in general the level of oxidation increases as the number of double bonds increases (Liu *et al* 1997). *In vivo*, the same relative oxidative susceptibility is thought to occur. That is, of the various biologically relevant PUFAs, long chain ω-3 fatty acids such as EPA and DHA oxidise more readily than do less saturated fatty acids such as linoleic acid (Wander and Du 2000). It is also well established that end-products of lipid peroxidation, such as MDA and 4-hydroxy-2-nonenal cause protein damage by means of reactions with lysine amino groups, cysteine sulphydryl groups and histidine imidazole groups (reviewed by Refsgaard *et al* 2000).

Contradictory results concerning the effect of the supplementation of EPA and DHA on oxidation have been reported both following *in vivo* and *in vitro* studies (reviewed by Wander and Du 2000) and this may be as a result of the different assays used to measure the extent of oxidation. Furthermore, although numerous assays are generally considered to measure lipid peroxides, various other products such as MDA are often measured and used as a surrogate for lipid peroxides (Wander and Du 2000). TBARs and MDA however, are reported to be inaccurate measures of lipid peroxidation (Esterbauer 1996; Janero 1990). The TBARs are measured via a

spectrophotometric assay that quantifies a chromogen produced by the reaction of thiobarbituric acid with MDA. Although easy to use, the assay is not specific in that many substances found in human biological fluids can react with TBA (Cherubini *et al* 2005). MDA can occur from degradation of endoperoxides and not from lipid peroxidation products and so this assay can give an overestimation of free radical damage. Even if the specificity of the measurement is improved by HPLC to separate the MDA-TBA adduct from interfering chromogens, there is still the possibility that part of the MDA present in the sample does not derive from oxidative damage of fatty acids (Cherubini *et al* 2005).

One assay that might have particular significance in assessing the extent of oxidative damage is the measure of protein oxidation and in particular, the protein carbonyl assay is useful as it evaluates 'general' protein damage (Wander and Du 2000). Yasuda *et al* (1998) reported that although mice fed on a diet rich in DHA-fish oil had significantly higher TBARs levels in the liver compared to those fed beef tallow, linoleic acid-rich safflower or α-linolenic acid-rich perilla oil, the protein carbonyl content in the liver was the same among the 4 dietary groups. A study by Wander and Du (2000) where oxidative stress produced due to the consumption of ω-3 fatty acids and vitamin E supplements in humans was measured, revealed although there was a modest increase in TBARs following consumption of fish oil, it was not modified by increased consumption of the vitamin E supplement. Furthermore, the study reported no increase in protein carbonyls after the consumption of fish oil. The authors concluded that if fish-oil consumption does not cause an increase in oxidation as measured by protein carbonyls, then an increased intake of vitamin E is not necessary. Consequently, EPA- and DHA-rich diets may not lead to increased oxidation *in vivo*.

It is also important to note the possible induction of glutathione peroxidase enzymes by fatty acids and the protective role that these antioxidant enzyme may play in cells and tissues against oxidative and inflammatory cytokine elicited damage (Crosby *et al* 1996). *In vitro* enrichment of peripheral blood monocytes with low concentrations of EPA and DHA have also been shown to induce an increase in glutathione peroxidase (Joulain *et al* 1994). Indeed schizophrenia may not just be linked to oxidative events and damage but rather to inflammatory events such as cytokine and eicosanoid production in the brain elicited by bacterial endotoxins and infections. For example, a recent study has reported that during critical stages of pregnancy maternal infection may lead to sensorimotor gating deficits in schizophrenia (Fortier *et al* 2007).

3.4.2 Reactive Oxygen Species

CM-H_2DCFDA-sensitive reactive oxygen species of the human U937 cell line along with the anti- or pro-oxidant effects of EGCG, hydroxytyrosol, α-tocopherol and H_2O_2 were evaluated by flow cytometry. Results from this study showed that the pre-treatment with the antioxidant hydroxytyrosol significantly reduced the level of endogenous ROS in U937 cells, while α-tocopherol appears to increase endogenous ROS levels. In addition, the beneficial effect of hydroxytyrosol was apparent following treatment of cells with H_2O_2 since it was observed that only hydroxytyrosol was effective in significantly reducing H_2O_2-induced cellular ROS (Figure 3.5).

Previous studies have suggested that ROS production may play a role in the pathogenesis of schizophrenia, and more recently, there is an emerging body of data indicating that schizophrenia may be associated with mitochondrial dysfunction

(Frey *et al* 2006). Animal studies have established that a global oxidative stress affects predominantly the brain (Mahadik and Mukherjee 1996). Brain vulnerability occurs because the brain is under higher oxidative stress than other organs since it produces very high levels of ROS due to its very high aerobic metabolism and blood perfusion (reviewed by Mahadik *et al* 2001). In addition, it is enriched in lipids that are preferentially susceptible to oxidative damage and the damaged neuronal DNA in the adult brain can not be effectively repaired since there is no DNA replication. Yet environmental ROS also attack the brain and inflammation is a major threat to brain function. Depending on the degree of oxidative stress and the developmental time, oxidative neuronal injury in the brain may cause abnormal neurodevelopment, neurodegeneration, or neuronal membrane impairment.

Gross *et al* (2003) report decreased production of reactive oxygen species in blood monocytes from schizophrenic patients following treatment with the neuroleptic clozapine. Neuroleptics have been shown to have both pro-oxidant and antioxidant properties (Jeding *et al* 1995) and the antipsychotic response of a neuroleptic may depend on its pro- or antioxidant property and the level of pre-existing oxidative stress in the patient (Mahadik *et al* 2001). Clinical studies have indicated that antioxidant enzyme activities are associated with the treatment of schizophrenic patients with neuroleptics (Abdalla *et al* 1986; Yao *et al* 1998), suggesting that the changes in the enzyme activity may be due in part to the neuroleptic treatment (Akyol *et al* 2002; Evans *et al* 2003), as well as genetics and environmental stimuli.

Because of its implied involvement in the pathogenesis of various neurological diseases, novel therapeutic approaches aimed at the inhibition of ROS-induced neurotoxicity, support the use of ROS scavengers, transition metal chelators and

antioxidant phenolics as part of an adjunctive therapy. The antioxidant activity of hydroxytyrosol has been demonstrated previously (Chapter 2) where it has been shown to lead to lower levels of hydroperoxides, DNA damage and mRNA levels of glutathione peroxidase (Quiles *et al* 2002). Hydroxytyrosol has also been shown to have greater antioxidant activity than α-tocopherol or butylated hydroxytoluene (BHT) (Trujillo *et al* 2006). It is also thought that hydroxytyrosol has greater antioxidant activity than tyrosol due to the presence of a single hydroxyl (reviewed by Quiles *et al* 2002) and that the phenol chemical structure considerably influences the antioxidant activity as a consequence of both steric factors and those related to position and type of functional groups on the phenol ring (Masella *et al* 1999). Other authors have suggested that hydroxytyrosol and olive oil waste water are potent scavengers of superoxide anions (Visioli *et al* 1998) while more recently Nousis *et al* (2005) found that the extracted hydroxytyrosol from the olive leaves and fruits themselves to be protective against hydrogen peroxide.

The potency of EGCG as an antioxidant is more controversial. Tea polyphenols have been reported to be potent antioxidants and beneficial in ameliorating oxidative stress related diseases (Higdon and Frei 2003; Frei and Higdon 2003; Lambert and Yang 2003; Waltner-Law 2002). On the other hand, pro-oxidant effects of tea polyphenols have been reported in cell culture systems (Johnson and Loo 2000). A recent study (Raza and John 2005) observed increased DNA breakdown and activation of apoptotic markers at high concentrations (>200μM) of EGCG in a cell culture system. At lower concentrations the study suggests that increased production of ROS may be scavenged by increased GSH and GST enzymes since no increase in peroxide formation was reported. At higher concentrations, an increase in ROS production might lead to the disruption of free radical chemistry, decrease in the

GSH pool and increased lipid peroxides. It has also been reported that EGCG has the ability to produce H_2O_2 and alter the free radical generation in the cell culture system which is known to induce oxidative stress (Chai *et al* 2003). However, flavonoid polyphenols such as EGCG have been shown to improve age-related cognitive decline and are neuroprotective in models of PD, AD and cerebral ischemia/reperfusion injuries (Mandel *et al* 2004). In addition, studies indicate that the radical scavenger property of green tea polyphenols is unlikely to be the sole explanation for their neuroprotective capacity and in fact, tea flavonoids have also been reported to have divalent metal chelating and anti-inflammatory activities, to penetrate the brain barrier and to protect neuronal death in a wide array of cellular and animal models of neurological diseases (reviewed by Mandel *et al* 2006). Furthermore, experimental studies have also revealed that compounds such as EGCG play a role in the regulation of molecules involved in the cell signal transduction pathways including NF-kappa, Akt, MAPK, p53, androgen receptor, and oestrogen receptor pathways. By modulating cell signalling pathways, these components, among other mechanisms, activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibition of cancer development and/or progression (Sarkar and Li 2004).

Limited work focusing on the metabolism of flavonoids and their mode of entry into the systemic circulation after oral absorption has been undertaken. Some studies have reported flavonoid-mediated neuroprotection, yet the interaction of flavonoids or their circulating metabolites with the brain endothelial cells from the blood brain barrier (Youdim *et al* 2004) is not widely understood. EGCG has been reported to enter the brain after oral administration (Suganuma *et al* 1998) and is more recently

being investigated as a prophylactic for Alzheimer's disease (Rezai-Zadeh *et al* 2005).

Although the possible potential beneficial effect of hydroxytyrosol in reducing the level of ROS, and the possible potential beneficial effect of DHA in reducing protein carbonyls in U937 cells was noted, a number of problems were encountered with these experiments. The U937 cells were kept under the same cell culture conditions throughout the experiment and were subjected to limited handling and exposure to the atmosphere. However it is possible that because of the length of time that the cells were in use, in particular, the number of passages might have lead to an increase in basal oxidative damage. Although cell viability was checked at each experiment only dead cells would have been highlighted and cells undergoing damage would not be obvious.

CHAPTER 4

Examination of biomarkers of lipid peroxidation, phospholipase A_2 activity and cellular lipid composition in fatty acid treated human neuroblastoma and peripheral cells

4.1 Introduction

4.1.1 Oxidative Stress and Lipid Damage

Lipid peroxidation (LPO) reactions are usually determined by free radical chain reactions whereby one radical can lead to the oxidation of a relatively large number of substrate molecules, which are represented by polyunsaturated fatty acids. Lipid oxidation progresses in a similar way to the oxidation of many other organic compounds and can be described in terms of initiation, propagation and termination processes. These processes often consist of a complex series of reactions. The chain reaction is initiated by the abstraction of a hydrogen atom from a reactive methylene group (LH, the carbon surrounded by double-bonds in a bisallylic double-bond) of a PUFA residue. Monounsaturated and saturated fatty acids are much less reactive and do not normally undergo LPO. This initiation is usually performed by a radical (R•) of sufficient reactivity:

$$LH + R\bullet \rightarrow L\bullet + RH \qquad (1)$$

Molecular oxygen is then added to the carbon-centred radical (L•) formed in this process resulting in a lipid peroxyl radical (LOO•):

$$L\bullet + O_2 \rightarrow LOO\bullet \qquad (2)$$

which in turn can abstract a hydrogen atom from another PUFA similarly as in equation 1:

$$LH + LOO\bullet \rightarrow L\bullet + LOOH \qquad (3)$$

This reaction is known as propagation suggesting that one initiation process can result in the conversion of numerous PUFA to lipid hydroperoxides (LOOH). LOOH

is the first relatively stable product of a lipid peroxidation reaction. In certain conditions, lipid peroxidation is continuously initiated but a termination reaction limits the extent of LPO resulting in non-radical products (NRP) and destroys two radicals simultaneously:

$$LOO\bullet + LOO\bullet \rightarrow NRP \qquad (4)$$

When transition metal ions are present, LOOH can lead to the generation of radicals which can re-initiate LPO by redox-cycling of these metal ions:

$$LOOH + Men^{+} \rightarrow LO\bullet + Me^{(n-1)+} \qquad (5)$$

$$LOOH + Me^{(n-1)+} \rightarrow LOO\bullet + Men^{+} \qquad (6)$$

Lipid hydroperoxides are also capable of giving rise to other products such as short and long chain aldehydes, phospholipid and cholesterol ester core aldehydes which can be used as biomarkers of lipid peroxidation within a biological system (Abuja and Albertini 2001).

4.1.2 Lipid Peroxidation as a Biomarker of Oxidative Stress

The products first formed as a result of lipid peroxidation are conjugated dienic hydroperoxides. These active substances are either decomposed into various aldehydes or into isoprostanes if the original fatty acid undergoing attack is arachidonic acid (Figure 4.1). All of these products of degradation and decomposition are used in determining oxidative stress (Dotan *et al* 2004).

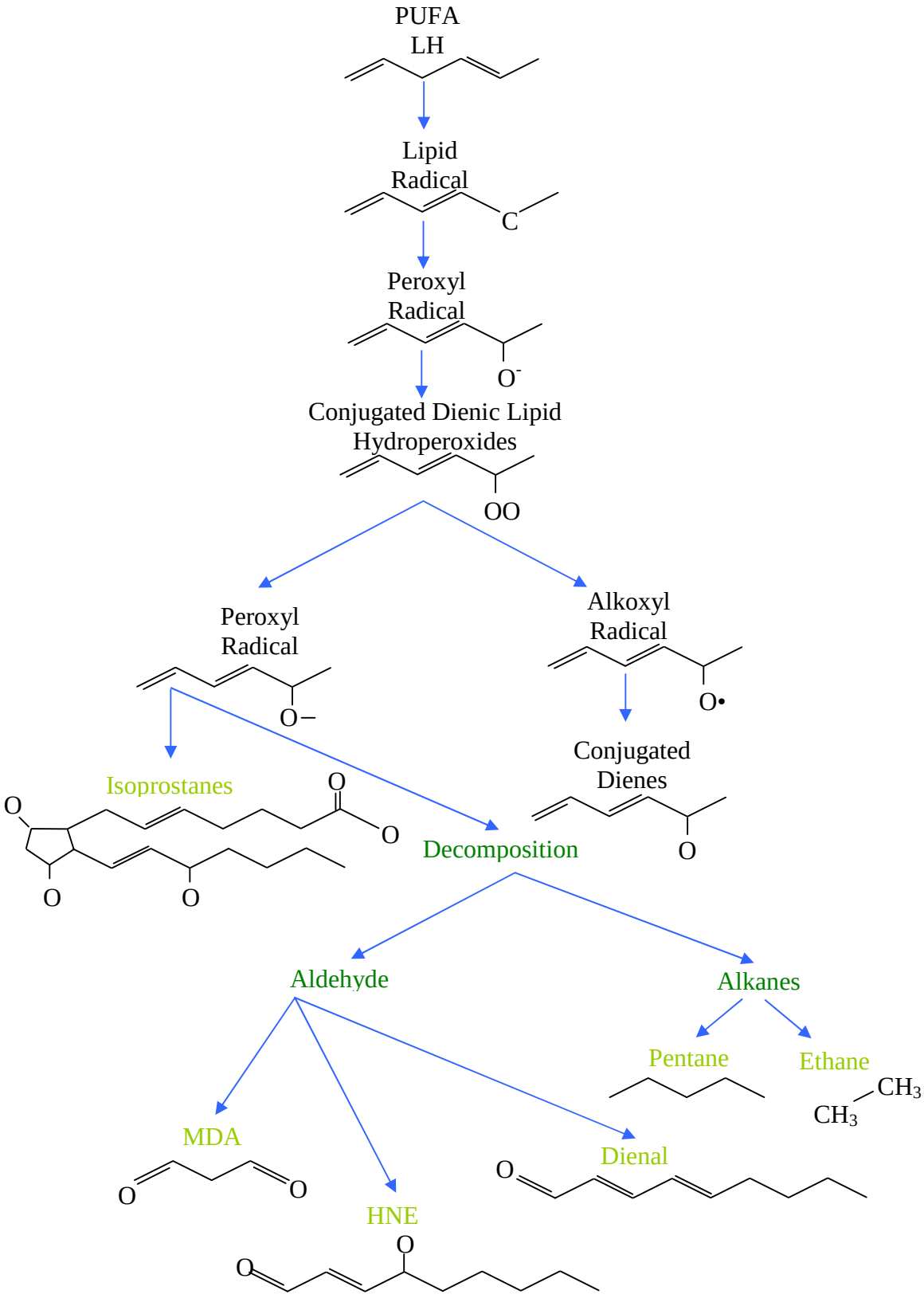


Figure 4.1. The products and pathways in the lipid peroxidation process (adapted from Dotan *et al* 2004). Key: MDA = malondialdehyde, HNE = hydroxynonenal.

Following peroxidation of ω-3 and ω-6 PUFAs relatively unstable fatty acid hydroperoxides are converted into more stable aldehydes. The major group of aldehydes that are formed following peroxidation of ω-6 fatty acids are 4-hydroxynonenal, MDA and hexanal. The peroxidation of ω-3 PUFAs leads to the formation of propanol and 4-HNE. These are the most commonly detected products found in biological tissues (reviewed by Meagher and FitzGerald 2000). Some of these products have been shown to react with biomolecules such as proteins and nucleic acids (Kautiainen *et al* 1993; Petersen and Doorn 2004). Aldehydes, however, are relatively stable in comparison to free radicals and are able to diffuse in or out of the cell to attack distant targets from the original site of free radical-initiated events (reviewed by De Zwart *et al* 1999).

4.1.3 Detecting Biomarkers of Lipid Peroxidation

Lipid peroxidation is probably the most examined process induced by free radicals. Because membrane phospholipids are in regular contact with radicals and are found at sites where ROS are formed, phospholipids are easy targets for attack. The PUFAs, in particular, are also vulnerable to attack by ROS (reviewed by De Zwart 1999). Peroxidation of fatty acids in lipids may result in a radical chain reaction resulting in the formation of many equivalents of lipid peroxides. Further propagation reactions in lipid membranes usually lead to the formation of a wide variety of alkanes and carbonyl compounds and some products such as hydroxyalkenals are toxic and may serve as second messengers for radical damage (reviewed by De Zwart 1999). Products of lipid peroxidation are therefore useful parameters for detecting radical damage.

4.1.3.1 Thiobarbituric Acid-Reacting Substance Assay

A widely used index of lipid peroxidation is the measurement of MDA by the thiobarbituric acid-reacting substance (TBARs) assay. The sample to be tested is heated with 2-thiobarbituric acid at low pH. Absorbance of pink chromogen is measured at 532nm or by fluorescence at 553nm. The TBARs assay is extremely easy to use but is limited by the fact that it is not specific since many substances that are present in human biological fluids can react with thiobarbituric acid (TBA) (Cherubini *et al* 2005). In combination with HPLC, the selectivity of the MDA-assay has been greatly improved. The use of a GC/MS assay for MDA has indicated that the commonly used TBARs assay overestimates the actual MDA levels by more than 10-fold, possibly resulting from cross reactivity with other aldehydes and the harsh conditions used in sample preparation such as high temperature conditions (Yeo *et al* 1994). More recent assays based on the measurement of MDA or HNE-lysine adducts are thought to be more applicable to biological samples since adducts of these reactive aldehydes are relatively stable (Moore and Roberts 1998).

4.1.3.2 Lipid Hydroperoxide Assay

Lipid hydroperoxide measurements are divided into those methods which detect more or less all –O-O-groups and methods which separate peroxides into chemical families of compounds (Abuja and Albertini 2001). One method to detect lipid hydroperoxides include the utilisation of xylenol orange (a dye specific for Fe^{3+}) and is based on the ability of transition metals in the reduced form to catalyze the reduction of peroxides to hydroxyl compounds while the metal is oxidised (Nourooz-Zadeh 1999). In this LOOH assay, the amount of Fe^{3+} formed is proportional to the content of total peroxides in the sample (Abuja and Albertini 2001).

Other methods that are used to detect biomarkers of lipid peroxidation include the measurement of volatile hydrocarbons. Hydrocarbon gases, such as ethane and pentane are produced from the peroxidation of ω-3 and ω-6 PUFAs (Aghdassi and Allard 2000). Measurement in the exhaled breath of these volatile alkanes not only enables the assessment of lipid peroxidation but also the effect of disease and antioxidant status with respect to oxidative stress (Aghdassi and Allard 2000). A series of prostaglandin F_2-like compounds (F_2-isoprostanes) capable of exerting potent biological activity is produced *in vivo* by free radical-induced lipid peroxidation (Awad *et al* 1993) which has been shown to increase profoundly in animal models of free radical injury and lipid peroxidation (Awad *et al* 1993). Although it may be considered a disadvantage, the quantification of F_2-iP requires techniques such as GC-MS and LC-MS methods (Wang *et al* 1995). However, quantification of F_2-isoprostanes provides an accurate assessment of oxidative stress both *in vivo* and *in vitro* and in a recent study F_2-iPs was shown to be the most reliable index of *in vivo* oxidant stress when compared to other well known biomarkers (Milne *et al* 2007). In addition, using F_2-iP as biomarkers has revealed that confounding factors are reduced since these products are not present as background in air or solvents (De Zwart *et al* 1999). GC/MS is considered the gold standard method for measurement of urinary F_2-isoprostanes but this method is laborious and costly which prohibits its use in large epidemiological studies (Il'yasova *et al* 2004). Immunoassays have been developed for specific F_2-iP (Patrignani *et al* 1996) but studies have reported mixed findings concerning the validity of its use compared to other methods of detecting F_2-iPs. One study reported a statistically weak agreement between the GC-MS and ELISA method in human urine (Il'yasova *et al* 2004); another observed that GC-MS and ELISA did not

measure the same compounds in human urine (Bessard *et al* 2001) while the application of the immunoassay method reported a linear correlation in the levels of F_2-iPs against an accepted marker of lipid peroxidation (Boyle *et al* 2000).

4.1.4 Phospholipase A_2 and Schizophrenia

Phospholipases A_2 make up a family of signal transduction enzymes which catalyse fatty acid hydrolysis from the Sn2 position of a phospholipid resulting in the production of lysophospholipid, a key event in the production of inflammatory lipid mediators. Historically, PLA_2s were named by activity location that is pancreatic, cytosolic or secretory (Law *et al* 2006). Later naming systems also included calcium requirement, although this was often misleading. Some calcium-dependent PLA_2s require calcium for catalytic activity, whereas others are constitutively active and calcium promotes binding to the phospholipid membranes (Law *et al* 2006).

The calcium-independent PLA_2 ($iPLA_2$) is one of the PLA_2 isoforms that does not seem to mobilise fatty acids, although both cytosolic ($cPLA_2$) and secretory ($sPLA_2$) are involved in total fatty acid turnover. The free fatty acid is then transformed by various eicosanoid-synthesising enzymes to lipoxygenase products and prostanoids (Chaitidis *et al* 1998). Cytosolic PLA_2 is activated via extracellular agonists acting mainly through G proteins, receptor tyrosine kinases or protein kinase C (PKC) (reviewed by Rashba-Step *et al* 1997). These signals are accompanied by increases in intracellular Ca^{2+} levels as part of a signalling mechanism. The signalling cascade enables $cPLA_2$ binding to membrane phospholipids. In addition, PKC- and ERK1/ERK2-mediated phosphorylation of $cPLA_2$ also modulates membrane binding and catalytic activity (Durstin *et al* 1994).

Secretory PLA_2 has been widely investigated with respect to its activity toward oxidised membranes; likewise, more and more information is being obtained concerning the catalytic activity of $cPLA_2$. $cPLA_{2alpha}$, the most extensively studied Group IV PLA_2 is widely expressed in mammalian cells and mediates the production of functionally diverse lipid products in response to extracellular stimuli (Ghosh *et al* 2006). For example, $cPLA_2$ requires submicromolar concentrations of calcium to enable its translocation from the cytosol to the membrane where its substrate is located and the translocation is mediated by its calcium-dependent phospholipid binding domain (Nafelski *et al* 1998) and phosphorylation has been shown to occur via various pathways (reviewed by van Rossum *et al* 2004). Moreover, $cPLA_2$ is maximally activated when it is first phosphorylated followed by Ca^{2+}-dependent translocation to membranes (Schalkwijk *et al* 1996). In their study van Rossum *et al* (2004) showed that in Her14 fibroblasts $cPLA_2$ is activated in a concentration-dependent manner by H_2O_2 which also induced a transient activation of $cPLA_2$ and $cPLA_2$ translocated to the membrane under calcium-free conditions with a concomitant increase in $cPLA_2$ activity.

$cPLA_{2alpha}$ has also been shown to have PLA_2 and lysophospholipase activities and is the only PLA_2 that has specificity for phospholipid substrates containing arachidonic acid. Furthermore, due to its role in initiating agonist-induced release of AA for the production of eicosanoids, $cPLA_{2alpha}$ activation is important in regulating normal and pathological processes in a variety of tissues (Ghosh *et al* 2006).

PLA_2 catalytic activity is thought to be enhanced following changes forced upon the membrane structure (Sweetman *et al* 1995). When there is an increased rate of loss of crucial fatty acids, changes in the functioning of membrane-associated proteins

and various cell signalling systems will occur. It is suggested that an enzymatic basis for such increased loss could be due to either the over activity of one or more of the PLA_2 enzymes as a result of the presence of an abnormal enzyme variant or from over expression of the normal enzyme, or because of sequential action of a phospholipase C and of a DAG lipase (Horrobin 1999). Either way, the increased rate of removal from phospholipids would make the free acids more susceptible to oxidation (Horrobin 1999). The hypothesis of the involvement of increased PLA_2 activity in schizophrenia initially received support from Gattaz *et al* (1990) and was later confirmed by a study conducted by Noponen *et al* (1993), although a study carried out in the same year found PLA_2 activity to be normal in schizophrenic patients (Albers *et al* 1993). Most studies measuring PLA_2 activity in psychotic disorders have used either a radiometric or a fluorometric method (reviewed by Law *et al* 2006), for example, a study by Macdonald *et al* (2004) using ELISA reported on a direct measure of the $cPLA_2$ protein and found increased levels of $cPLA_2$ in schizophrenia. An earlier report has suggested that the enzyme activity elevated in blood of schizophrenic patients points to the calcium-independent form (Ross *et al* 1997), while increased calcium-independent PLA_2 activity has also been reported more recently in first but not in multi-episode chronic schizophrenia (Smesny *et al* 2005), suggesting increased lipid turnover in the acute early phases of schizophrenia that is less obvious in chronic stages.

An absent response to the niacin skin test has been reported to occur in approximately 80% of schizophrenic patients as compared to 20% of healthy individuals (Tavares *et al* 2003). Niacin provokes redness in skin caused by a capillary vasodilation mediated by prostaglandins. The metabolism of prostaglandins is regulated by PLA_2 and several studies have reported increased PLA_2 activity in

schizophrenia. Tavares' study (2003) also supported the finding that absent response to niacin is more frequent in schizophrenic than in healthy individuals, although the magnitude of the difference was smaller than in the reported literature. Interest in the membrane phospholipid hypothesis has fuelled genetic association studies between PLA_2 genes and schizophrenia. The association between schizophrenia and the $cPLA_2$ variants in populations of different ethnicity and alternative statistical models have shown promising results, although many studies have not replicated these associations (Law *et al* 2006).

4.1.5 Lipid Peroxidation and Schizophrenia

Although observations of lipid peroxidation are limited in schizophrenic patients, reports have been consistent. Increased blood levels of MDA (Gama *et al* 2006; Zhang *et al* 2006; Dietrich-Muszalska *et al* 2005; Arvindakashan *et al* 2003; Khan *et al* 2002; reviewed by Yao *et al* 2001) and a similar increase in levels of lipid peroxides in cerebrospinal fluid (CSF) have been reported (Skinner *et al* 2005; reviewed by Yao *et al* 2001). Mahadik *et al* (1999) have also reported increased plasma lipid peroxide levels at the onset of psychosis in never-medicated, first-episode patients, suggesting the presence of oxidative stress early in the course of illness and independent of treatment. In addition, higher levels of pentane in the breath of schizophrenic patients compared to controls have also been reported (Phillips *et al* 1993; Kovaleva *et al* 1989).

Substantial evidence is available to indicate that membrane phospholipids and esterified EPUFAs are altered in schizophrenia (Muskiet and Kemperman 2006; Kemperman *et al* 2005; Reddy *et al* 2003; reviewed by Peet and Stokes 2005). The membrane phospholipid hypothesis suggests that disturbed phospholipid metabolism

is the fundamental cause of schizophrenia. It is thought that in people who develop schizophrenia, there is an increased rate of loss of unsaturated fatty acids, particularly AA, DHA, EPA and linolenic acid from the Sn2 position of phospholipids (Peet 1995; Horrobin *et al* 1994). When present to a mild degree the increased rate of loss will be balanced by an increased role of incorporation and no change in phospholipid composition. But if present to a greater degree or when associated with problems of incorporation, there will be a change in membrane composition (Horrobin *et al* 1995).

Unlike plasma membranes of non-neuronal cells, neuronal plasma membranes are approximately 70% lipids which constitute over 50% of the dry weight of human brain, and are enriched in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. These phospholipids, particularly in the cortex, hippocampus and basal ganglia are enriched in EPUFAs, primarily AA and DHA (Carlson 2001) that are selectively susceptible to lipid peroxidation due to the lowered bond dissociation energy of their allylic hydrogens. Hydrogen abstraction from their methylenic carbons has also been shown to occur in the presence of ROS (Gutteridge 1982).

The aim of this study was to examine any effect of H_2O_2-induced lipid peroxidation in pre-treated fatty acid human cells, used as models of schizophrenia. Because of its suggested involvement in schizophrenia, cytosolic PLA_2 content in neuroblastoma cells was investigated while total and $cPLA_2$ activity was also examined following fatty acid pre-treatment and a pro-oxidant challenge on the same human cell lines. Finally, the validity of using the fatty acid profiles of fatty acid-treated U937, lymphoblastoid and IMR-32 cells as models of schizophrenia, was investigated.

4.2 Materials and Methods

4.2.1 Examination of Biomarkers of Lipid Peroxidation, Phospholipase A_2 Activity and Fatty Acid Composition

4.2.1.1 Materials

DMEM, penicillin/streptomycin antibiotic, trypsin/EDTA, glucose were supplied by Sigma (Poole, UK). Heat-inactivated FCS was obtained from Invitrogen (Paisley, UK). RPMI 1640 media were purchased from Gibco (UK). EPA and DHA were supplied by Pronova (Norway). DMSO, thiobarbituric acid (TBA), trichloroacetic acid (TCA), tetramethoxypropane and acetic acid were supplied by Sigma (Poole, UK).

4.2.1.2 Evaluation of Lipid Peroxides (MDA) in Human Secondary Cells

4.2.1.2.1 Culture of Neuroblastoma Cells

IMR-32 cells were cultured, maintained and harvested as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

4.2.1.2.2 Culture of Peripheral Cells

Human peripheral cells were cultured, maintained and harvested as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

4.2.1.2.3 H_2O_2 Treatment of Neuroblastoma and Peripheral Cells

Fresh media mixed with 100μM H_2O_2 in PBS or PBS alone was added to the flasks and cells were incubated at 37^oC for 30min or 4h.

4.2.1.2.4 Sample Preparation – Cell Lysate

Cells were washed twice with PBS and collected by centrifugation (ie. 1000 – 2000x*g* for 10min at 4^oC). IMR-32 cells were harvested using a rubber policeman. The cell pellet was resuspended in 1ml of ice cold PBS and sonicated using a sonic probe for 5 cycles of 15sec at 50% power. Cells were stored on ice for assay.

4.2.1.2.5 TBARs Assay

1ml of cell sample was added to 1ml 0.05M H_2SO_4 in a screw-topped glass test tube and mixed. 0.5ml of 20% TCA was added and mixed and incubated at room temperature for 5min. Following centrifugation for 10min at 4000x*g* at 4^oC, 0.5ml of TBA was added to the tubes and incubated in a 100^oC water bath for 1h. The samples were left to cool.

4.2.1.2.6 TBARs Quantitation

For quantitation, calibration curves were constructed using 1,1,3,3 tetramethoxypropane as a standard, ranging from 0 to 3nmol. All measurements were performed in duplicate. Absorbance measurements of solutions were measured in 1ml plastic cuvettes in a Hewlett Packard spectrophotometer at 532nm.

4.2.1.2.7 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and one-way ANOVA was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

4.2.1.3 Evaluation of Lipid Peroxide (LOOH) Activity in Human Secondary Cells

4.2.1.3.1 Materials

Lipid Peroxidation Assay Kit II was supplied by Calbiochem, San Diego, CA, USA.

4.2.1.3.2 Culture of Peripheral Cells

Human peripheral cells were cultured, maintained and harvested as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

4.2.1.3.3 Fatty Acid Treatment of Peripheral Cells

Peripheral cells were incubated with 50μM of fatty acids for 24h under the same conditions as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

4.2.1.3.4 H_2O_2 Treatment of Peripheral Cells

Following 24h incubation, cells were washed three times with PBS and collected by centrifugation at 2000x*g* for 5min. Cells were resuspended in media containing 100μM H_2O_2 and incubated for 4h at 37^{o}C.

4.2.1.3.5 Sample Preparation – Cell Lysate

Cells were washed twice with PBS and collected by centrifugation (ie. 1000 – 2000x*g* for 10min at 4^{o}C). The cell pellet was resuspended in 1ml of ice cold PBS and sonicated using a sonic probe for 5 cycles of 15sec at 50% power. Cell lysate was frozen at -80^{o}C until ready to assay.

4.2.1.3.6 Determination of LOOH in Human Peripheral Cells

Cell lysate was thawed at room temperature. 10µl of catalase enzyme was added to each microcentrifuge tube and 90µl of sample was then added. Samples were mixed gently and incubated at room temperature for 2min. Blanks were prepared by adding 10µl of Reducing Agent and Test was prepared by adding 10µl of deionised water into a microcentrifuge tube. Samples were then covered and mixed by vortexing and incubated at room temperature for 30min. 900µl of Working Reagent (1 volume of Color Developer with 100 volumes of Chromogen) was added to each tube, covered and vortexed for 30sec and incubated at room temperature for 60min. Samples were then centrifuged at 10,000–12,000x*g* for 10min to remove all flocculated materials. Supernatants were transferred to spectrophotometric cuvettes and absorbance was measured at 560nm.

4.2.1.3.7 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and one-way ANOVA was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

4.2.1.4 Investigation of Total PLA_2 Activity and $cPLA_2$ Activity in Human Neuroblastoma and Peripheral Cells

4.2.1.4.1 Materials

$cPLA_2$ assay kit components were supplied by Cayman Chemical Company (Michigan, USA).

4.2.1.4.2 Culture of Neuroblastoma Cells

IMR-32 cells were cultured, maintained and harvested as described previously in Chapter 2.

4.2.1.4.3 Culture of Peripheral Cells

Human peripheral cells were cultured, maintained and harvested as described previously in Chapter 2.

4.2.1.4.4 Fatty Acid Treatment of Neuroblastoma and Peripheral Cells

U937 cells were incubated with 50μM of fatty acids for 24h under the same conditions as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability.

4.2.1.4.5 H_2O_2 Treatment of Neuroblastoma and Peripheral Cells

Following 24h incubation, cells were washed three times with PBS and collected by centrifugation at 2000x*g* for 5min. Cells were resuspended in media containing 100μM H_2O_2 and incubated for 4h at 37^oC.

4.2.1.4.6 Total PLA_2 Sample Preparation – Cell Lysate

Cells were washed twice with PBS and collected by centrifugation (ie. 1000 – 2000x*g* for 10min at 4^oC). IMR-32 cells were harvested using a rubber policeman. The cell pellet was resuspended in 1ml of ice cold PBS containing 1mM EDTA and sonicated using a sonic probe for 5 cycles of 15sec at 50% power. Cells were then stored on ice until ready to assay.

4.2.1.4.7 $cPLA_2$ Sample Preparation – Cell Lysate

Cells were washed twice with PBS and collected by centrifugation (ie. 1000 – 2000x*g* for 10min at 4^oC). IMR-32 cells were harvested using a rubber policeman.

The cell pellet was resuspended in 1ml of ice cold PBS containing 1mM EDTA and sonicated using a sonic probe for 5 cycles of 15sec at 50% power. Samples were then concentrated using a Millipore Centriprep®. Samples were centrifuged for 5min at 10,000x*g* and lysate containing molecules of >30,000 nominal molecular weight was decanted. The retained pellet was centrifuged again as above and lysate poured off once again. During this process, any residual $sPLA_2$ within the sample is also removed. To avoid any measurement of $iPLA_2$ activity in the remaining sample, 1ml samples were incubated with 5µl of 1 mM bromoenol lactone for 15min at 25^{o}C. Cells were then stored on ice until ready to assay.

4.2.1.4.8 PLA_2 Assay

Buffers and reagents were prepared as per protocol instructions. 15µl of assay buffer was added to the blank wells in triplicate as non-enzymatic controls. 10µl of PLA_2 and 5µl of assay buffer were added to the positive control wells in triplicate. 10µl of sample and 5µl of assay buffer were added to the sample wells in triplicate. Reactions were initiated by adding 200µl of substrate solution to all of the wells. The plate was shaken gently for 30sec to mix and covered and incubated for 60min at room temperature. Following incubation, the plate cover was removed and 10µl of reagent was added to each well to stop enzyme activity and to develop the reaction. The plate was gently shaken for 30sec to mix and incubated for 5min at room temperature. The plate was placed into a BIO-TEK FL600 fluorescence plate reader and absorbance of the wells was read at 405nm.

4.2.1.4.9 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and one-way ANOVA and Student's t-test were carried out as appropriate using SPSS 11.5

for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

4.2.1.5 Examination of Fatty Acid Composition in Human Neuroblastoma and Peripheral Cells

4.2.1.5.1 Cell Culture

Human neuroblastoma and peripheral cells were grown and maintained in 25cm^2 flasks at a seeding density of 1x10^{6}cells/ml, as previously described in Chapter 2.

4.2.1.5.2 Fatty Acid Treatment of Neuroblastoma and Peripheral Cells

Cells were incubated with 50μM of fatty acids for 24h under the same conditions as described previously in Chapter 2. Cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability. Following treatment cells were washed 3 times with PBS and the cell pellet was resuspended in 50μl PBS. The cell suspension was added to 2ml chloroform:methanol (2:1v/v) in glass vials and was purged for a few seconds with nitrogen gas. Samples were sealed and posted to Institute of Aquaculture, University of Stirling for fatty acid content analysis by GC. In brief, cell pellets were extracted by a variation of the Bligh and Dyer's (1959) method and the polar lipid fraction isolated by thin layer chromatography. Fatty acid methyl esters (FAMEs) were prepared by acid-catalysed trans-esterification of total lipids according to the method of Christie (1982). FAMEs were separated and quantified by GLC with flame ionisation detection using a 60m x 0.32mm i.d. capillary column (Phenomenex ZB Wax, Macclesfield, UK) and on-column injection. Hydrogen was used as carrier gas (flow rate 2ml/min) and temperature programming 50^{o}C to 150^{o}C at 40^{o}C/min and then to 225^{o}C at 2^{o}C/min

employed. Individual methyl esters were identified and quantified by comparison to known standards (Cyhlarova *et al* 2007).

4.2.1.5.3 Acknowledgement

I gratefully acknowledge the support offered by Dr J G Bell and his group at the Institute of Aquaculture, University of Stirling, in undertaking the cellular fatty acid determinations.

4.3 Results

4.3.1 Cellular Lipid Composition

Evaluation of the fatty acid content of the three human cells lines was undertaken using GC. Table 4.1 details in particular AA, EPA and DHA percentage values in untreated or EPA or DHA pre-treated cells. Analysis has revealed that in untreated cells, of the three PUFAs under investigation, all cell lines contain a higher percentage of AA than DHA or EPA. Following 24h incubation with EPA, levels of AA and DHA decrease when compared to the untreated cells. However, EPA content has increased by approximately 20% in EPA-treated U937 cells, 13% in lymphoblastoid cells and 9% in IMR-32 cells. Even more considerable is the percentage increase in the levels of DHA following cell treatment for 24h with DHA. In the U937 cell line, DHA content has increased by 46%, by 24% in lymphoblastoid cells and 13% in IMR-32 cells compared to DHA content in untreated cells. When compared to fatty acid content in untreated cells, AA levels in DHA-treated cells had decreased, while EPA content had increased marginally.

Total % saturated fatty acid has decreased in all cells lines pre-treated with DHA, but total percent saturated fatty acid level has increased marginally in IMR-32 cells pre-treated with EPA, while total percent monounsaturated fatty acid has decreased in the three cell lines pre-treated with either EPA or DHA. Total % ω-6 PUFA levels have also fallen following pre-treatment of cells with the chosen fatty acids while total ω-3 % PUFA levels have increased in the pre-treated fatty acid cells.

	U937			Lymphoblastoid			IMR-32		
	Untreated Cells	EPA Treated Cells	DHA Treated Cells	Untreated Cells	EPA Treated Cells	DHA Treated Cells	Untreated Cells	EPA Treated Cells	DHA Treated Cells
% AA	5.00	3.06	2.50	5.56	3.30	3.50	4.39	2.33	3.28
% EPA	0.21	20.58	0.90	0.39	13.71	1.00	0.29	9.13	1.16
% DHA	1.75	1.34	47.77	2.44	1.58	26.55	2.84	1.40	16.20
Total % Saturated FA	35.16	22.68	23.97	42.89	33.42	35.20	42.30	44.69	38.79
Total % Mono-unsaturated FA	45.15	19.05	16.14	36.57	20.62	23.85	39.17	25.77	29.94
Total % ω-6 PUFA	11.19	6.83	5.45	12.19	7.39	7.86	9.15	5.54	7.22
Total % ω-3 PUFA	3.71	49.32	52.59	4.40	36.01	30.14	5.15	21.66	20.43
ω-3/ω-6 Ratio	0.33	7.22	9.65	0.36	4.87	3.83	0.56	3.91	2.83

Table 4.1. Fatty acid profiles in untreated and 24h EPA and DHA 50μM treated human peripheral and neuroblastoma cell lines. Data show fatty acid composition (%) in $1x10^6$cells/ml following analysis by GC.

4.3.2 MDA Analysis

Cellular membrane components are highly susceptible to oxidation, thus the formation of lipid peroxides were investigated, whereby levels of MDA in particular were measured by using the TBARs assay. A validation study was undertaken to determine the specificity of the assay and involved the incubation of peripheral and neuroblastoma cells lines with an oxidant for 30 minutes or 4 hours. Linearity was demonstrated over a range of 0 -5 nm MDA of triplicate samples.

Figure 4.2 reports the effect of the treatment of 100μM H_2O_2 over both time points on MDA production in the three cell lines. Treatment with H_2O_2 at 30min or at 4h had no significant effect ($p < 0.05$) on the production of MDA in any of the three cell lines. In fact, a reduction in the level of MDA in the H_2O_2-treated cells at 30min and 4h was observed when compared to the untreated cells both in the U937 and IMR-32 cell lines. An increase in MDA production was only observed in the 30min H_2O_2-treated lymphoblastoid cells.

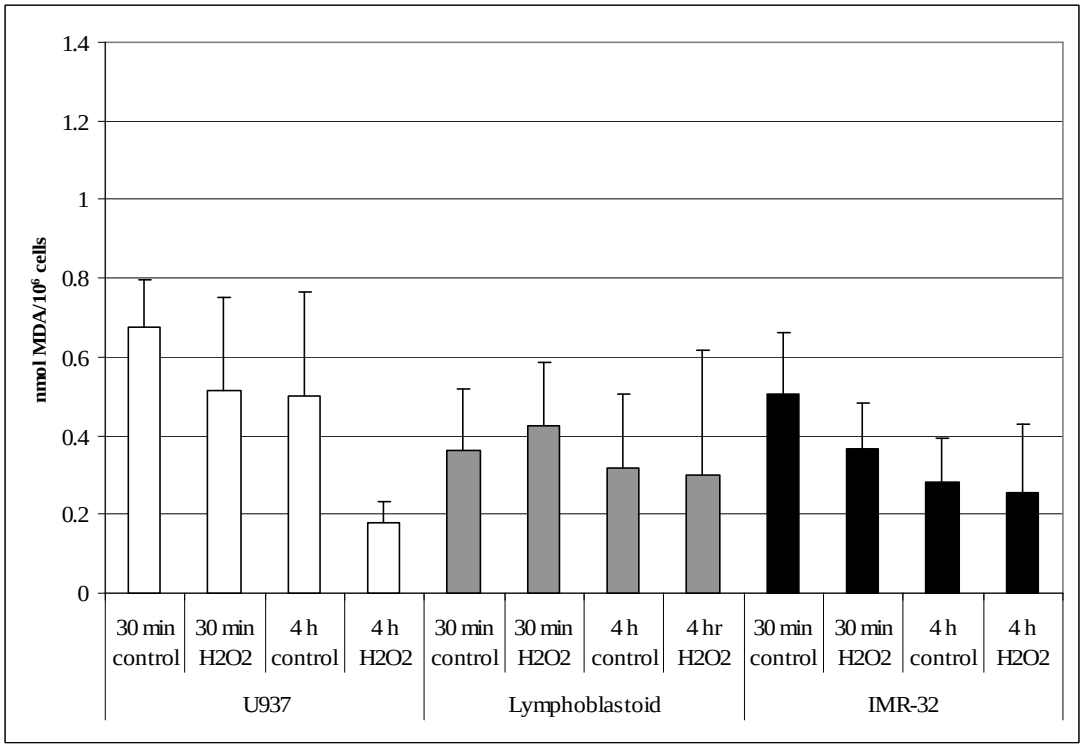


Figure 4.2. Investigation of MDA production in H_2O_2 treated human peripheral and neuroblastoma cells. Peripheral cells (U937 and lymphoblastoid) and IMR-32 neuroblastoma were treated for 30min or 4h with 100μM H_2O_2 and compared with MDA production in H_2O_2 untreated (control) samples measured by TBARs. Values are expressed as mean ± sem of three independent experiments.

4.3.3 LOOH Analysis

U937 cells pre-treated with 50μM fatty acid for 24h were incubated with or without 100μM H_2O_2 for 4h (Figure 4.3) and cell viability was determined as 90-95% as ascertained by the Trypan blue dye exclusion method of viability. The frozen cell lysate was used in the Calbiochem LOOH assay and absorbance of the samples was read at 560nm. ANOVA analysis revealed no significant difference in the levels of LOOH in either the fatty acid pre-treated H_2O_2 incubated cells or the fatty acid treated cells without H_2O_2 challenge.

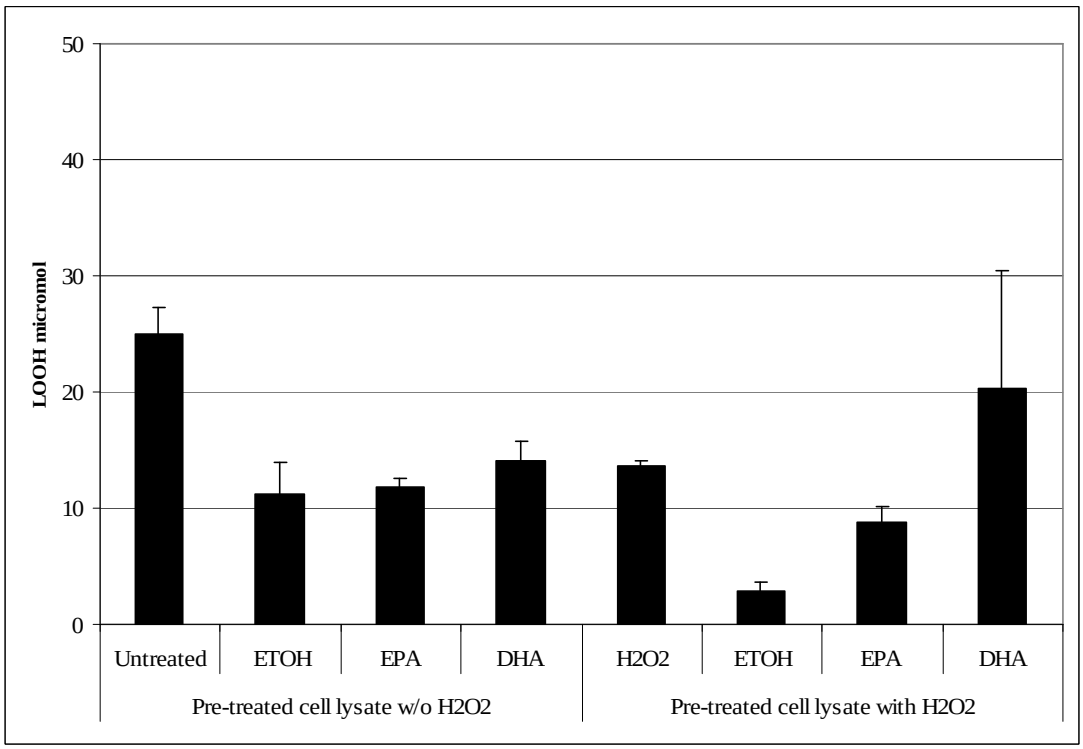


Figure 4.3. Evaluation LOOH in fatty acid treated and untreated U937 cell lysate, with and without H_2O_2 treatment. U937 cells were treated for 24h with fatty acid followed by 100μM H_2O_2 treatment for 4h. Values are expressed as mean ± sem of two independent experiments.

Analysis of this experiment suggests that although a high H_2O_2 challenge was used, the cells appear to be resistant to the pro-oxidant challenge since there is no statistically significant increase in lipid peroxides. Furthermore, treatment of U937 cells with either EPA or DHA alone induced no significant increase in LOOH levels and so it would appear that there is no evidence of a pro-oxidant stress from either of the fatty acid treatments. Untreated cells appear to have a high level of oxidative stress although all cells were subjected to the same culture conditions.

4.3.4 Total PLA_2 Activity Analysis

The cytosolic PLA_2 enzyme catalyses the hydrolysis of fatty acids at the Sn2 position of glycerophospholipids and its activity can be determined in cell cultures. The Cayman $cPLA_2$ assay kit was used to determine total and cytosolic PLA_2 enzyme activity in human peripheral and neuroblastoma cell lines.

In the first experiment the effect of H_2O_2 treatment over time was investigated (Figure 4.4). The three cell lines were incubated with or without 100μM H_2O_2 for a period of 30min or 4h and thawed cell lysates were used in the assay. One-way ANOVA was used to determine any effect of the H_2O_2 treatment over time. A highly significant increase in PLA_2 activity was observed at 4h within the U937 H_2O_2 treated samples (Figure 4.4). In addition, significant increase in PLA_2 activity was also observed after 4h in the H_2O_2 treated IMR-32 cells compared to the 4h untreated IMR-32 cells. No difference in enzyme activity was noted between the 30min or 4h untreated and H_2O_2-treated lymphoblastoid samples.

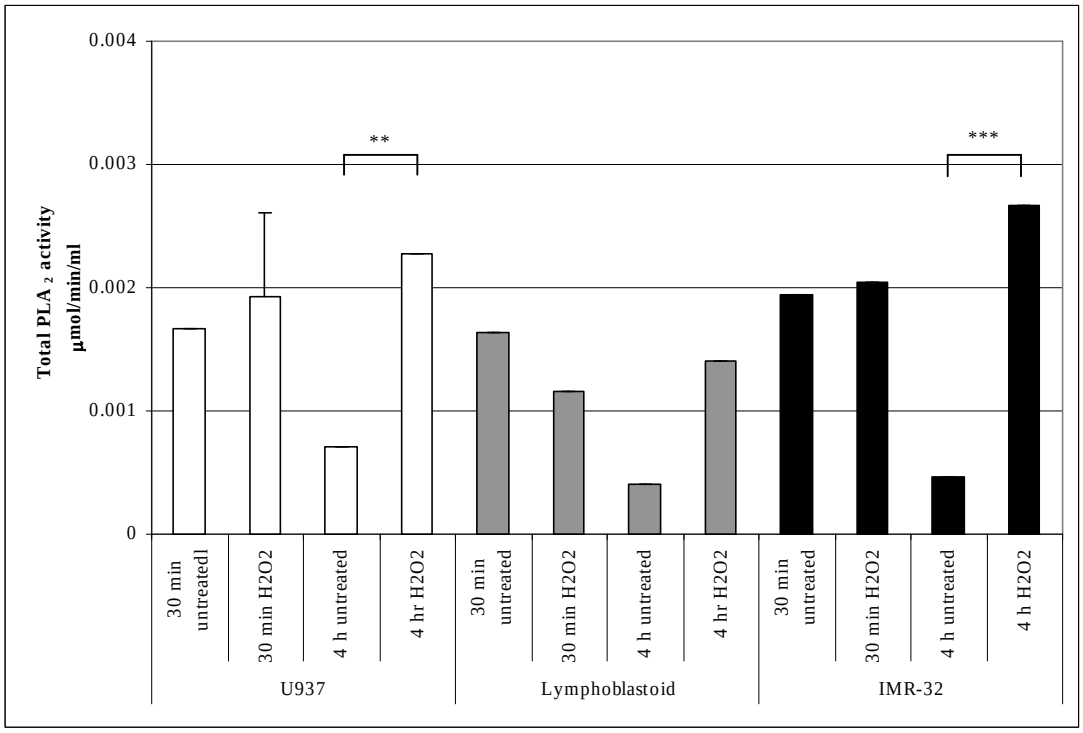


Figure 4.4. Determination of PLA_2 activity in human peripheral and neuroblastoma cells. Peripheral cells (U937 and lymphoblastoid) and IMR-32 neuroblastoma were treated for 30min or 4h with 100µM H_2O_2 and activity was compared to H_2O_2 untreated (control) samples. Values are expressed as mean ± sem. of two independent experiments (n=2). Significance is denoted by *** where $p < 0.001$ and ** where $p < 0.01$. If not shown, the error bars are too small to be visible.

Total PLA_2 activity was further examined in all three cell lines. Cells were pre-treated with fatty acids for 24h followed by H_2O_2 treatment for 4h and fresh cell lysate was used in the assay. In this experiment, a high variance was observed within the untreated cells which lead to the adoption of the ethanol-treated cells as the control set. In addition, the large error bars recorded in this experiment in particular may be due to the possible lack of intra-assay precision.

As observed in Figure 4.5, pre-treatment of U937 and IMR-32 cells with DHA for 24h appeared to increase mean total PLA_2 activity when compared to the ETOH-treated cells, but there is no comparable difference in the level of total PLA_2 activity

in the lymphoblastoid DHA-treated cells. U937 and lymphoblastoid cells pre-incubated with EPA report a decrease in total PLA_2 activity when compared to ETOH-treated cells, except in IMR-32 cells pre-incubated with EPA where an increase in total PLA_2 activity is observed. A decrease in mean total PLA_2 activity can be seen in all cell lines challenged with H_2O_2 compared to ETOH-treated cells, although U937 and IMR-32 cells pre-treated with ETOH followed by the H_2O_2 treatment reveal no difference in mean total PLA_2 levels and only an increase in PLA_2 activity is noted in the lymphoblastoid cells. H_2O_2 treatment of fatty acid pre-treated U937 cells reveal no marked change in mean total PLA_2 activity levels when compared to H_2O_2 and ETOH/H_2O_2 treated cells, although the hydrogen peroxide treatment does appear to increase PLA_2 activity in EPA pre-treated cells when compared to those EPA pre-treated cells not subjected to the H_2O_2 treatment. Conversely, the H_2O_2 challenge of pre-treated DHA cells appears to decrease mean total PLA_2 activity when compared to pre-treated DHA only U937 cells.

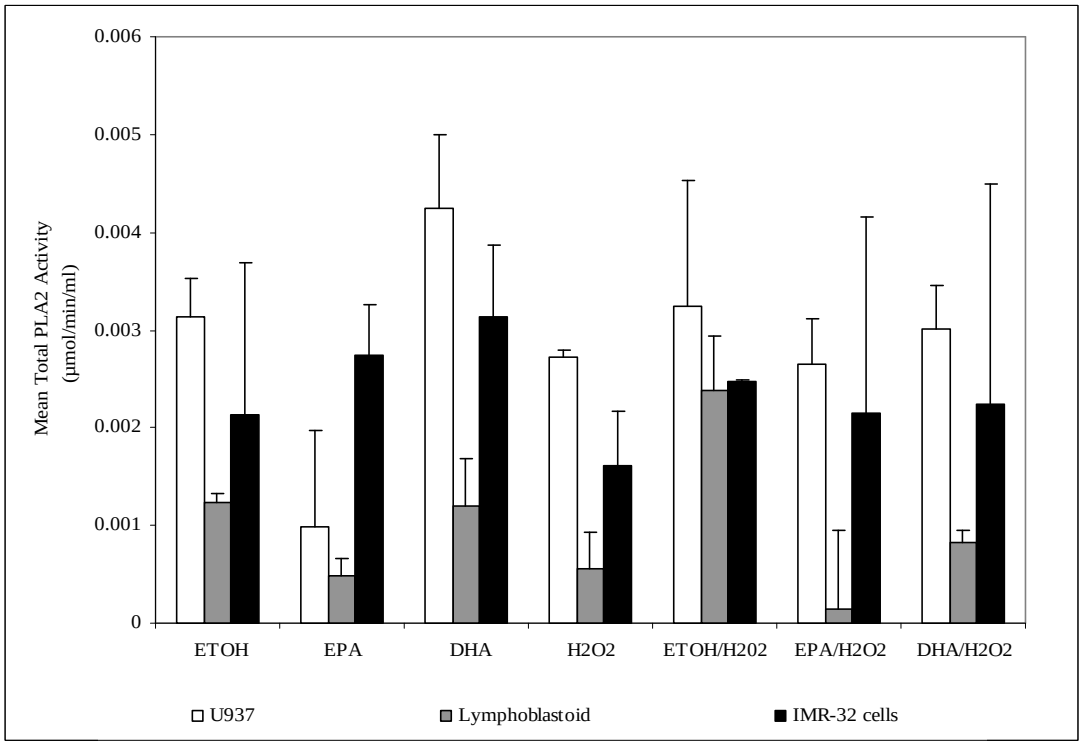


Figure 4.5. Examination of mean total PLA_2 activity in fatty acid and H_2O_2-treated peripheral and neuroblastoma cells. Cells were pre-treated for 24h with 50µM fatty acid and for 4h with or without 100µM H_2O_2. Values are expressed as mean ± sem of triplicate samples of one experiment ± sem.

Mean total PLA_2 activity is increased in lymphoblastoid cells pre-treated with ETOH followed by H_2O_2 in comparison to ETOH-only treated cells but following the H_2O_2 treatment of the pre-treated fatty acid lymphoblastoid cells, a decrease in PLA_2 activity is observed. Moreover, mean total PLA_2 activity appears to be further decreased in lymphoblastoid cells pre-treated with fatty acid followed by H_2O_2 stimulation when compared to the lymphoblastoid cells pre-treated with fatty acids only. PLA_2 activity in H_2O_2 treated IMR-32 cells is slightly reduced in contrast to ETOH pre-treated cells, however, there is no apparent difference in levels when comparing ETOH/H_2O_2 treated cells to ETOH pre-treated cells. In addition there is a marginal decrease in mean total PLA_2 activity in IMR-32 cells pre-treated with fatty

acid followed by the H_2O_2 challenge and without the H_2O_2 treatment in comparison to the pre-treated ETOH/H_2O_2 cells.

Results from Figure 4.5 suggest that pre-treatment of U937 and lymphoblastoid cells with EPA appeared to decrease PLA_2 activity while pre-treatment of U937 cells with DHA and IMR-32 with EPA and DHA appeared to increase total PLA_2 activity. However, not only is mean total PLA_2 activity decreased when cells are subjected to a high oxidative stress, pre-treatment of cells with EPA or DHA followed by an oxidative challenge decreased PLA_2 activity levels or had no effect on the mean total PLA_2 activity in these cell lines.

Figure 4.6 shows mean $cPLA_2$ activity in peripheral and neuroblastoma cells. Again, due to experimental error, ethanol-treated cells were used as controls. All experiments would have been repeated had time permitted. U937 cells pre-treated with ethanol appear to have the highest level of $cPLA_2$ activity. Yet, pre-treatment of these cells with fatty acid reduced $cPLA_2$ activity substantially and as can be seen, this reduction is more evident following pre-treatment of cells with EPA followed by a H_2O_2 treatment. However, U937 DHA pre-treated cells challenged with H_2O_2 reveal a slight increase in $cPLA_2$ activity in comparison to DHA pre-treated cells not challenged with H_2O_2. Evaluation of $cPLA_2$ activity in the lymphoblastoid cell line reveals a decrease in activity levels in cells pre-treated with fatty acids when compared to ETOH pre-treated cells. However, treatment of lymphoblastoid cells with H_2O_2 and pre-treatment with ETOH followed by H_2O_2 challenge reveals no change in $cPLA_2$ activity levels in comparison to ETOH pre-treated cells while the fatty acid pre-treated cells subjected to the oxidative stress show a decrease in mean $cPLA_2$ activity in contrast to the H_2O_2 and ETOH/H_2O_2 treated lymphoblastoid cells.

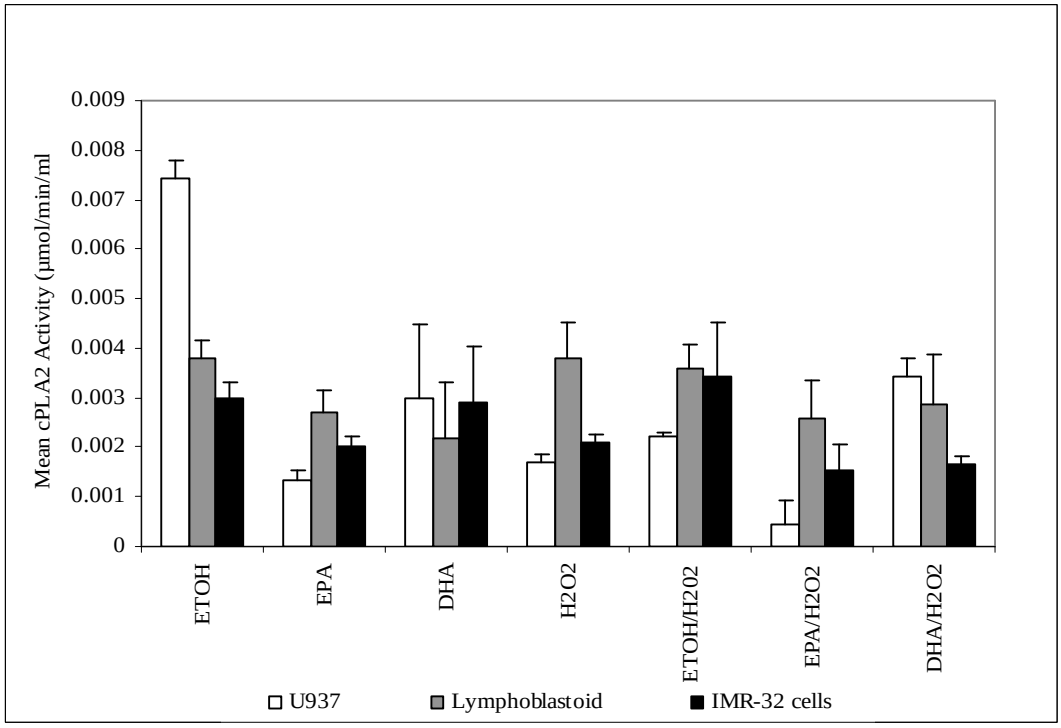


Figure 4.6. Examination of $cPLA_2$ activity in fatty acid and H_2O_2-treated peripheral and neuroblastoma cells. Cells were pre-treated for 24h with 50µM fatty acid and for 4h with or without 100µM H_2O_2. Values are expressed as mean of triplicate samples of one experiment ± sem.

DHA pre-treatment in IMR-32 cells appears to have no effect on $cPLA_2$ activity while EPA pre-treatment appears to have reduced $cPLA_2$ activity. The oxidative stress challenge on the pre-treated ETOH cells has increased $cPLA_2$ activity marginally in comparison to the ETOH unchallenged IMR-32 cells, however the fatty acid pre-treatment in combination with the H_2O_2 stress appears to have further decreased $cPLA_2$ activity levels.

Analysis of the data suggests that pre-treatment of cells with fatty acid seems to reduce $cPLA_2$ activity and this activity may be reduced further with the additional treatment of H_2O_2. Cytosolic PLA_2 activity in these cell lines therefore, does not appear to be enhanced by fatty acid supplementation nor by the treatment of the ROS generating H_2O_2.

4.4 Discussion

The aim of this chapter was to evaluate the effect of modifying cellular membrane fatty acid profiles by fatty acid supplementation trials and ROS-induced oxidative stress in putative cell models of schizophrenia, and in particular on biomarkers of lipid oxidation. Finally, total PLA_2 and $cPLA_2$ activity levels were assessed following EPA or DHA and H_2O_2 treatment in human neuroblastoma and peripheral cells in order to determine the extent to which EPA and DHA altered cell fatty acid composition.

4.4.1 Cellular Lipid Composition

Examination of the cellular lipid composition of the U937, lymphoblastoid and IMR-32 cells revealed the basal level of ω-3 fatty acids in the U937 cell line to be lower in comparison to the lymphoblastoid or IMR-32 cell lines. This observation strengthens the validity of the U937 cell line as a suitable system *in vitro* for examining cellular mechanism in schizophrenia. The similarity of the values and the lower ω-3/ω-6 ratio between the U937 and lymphoblastoid cell lines can be clearly seen compared to that observed in the IMR-32 cell line. The ω–3 fatty acid content expressed as a percentage of total fatty acids increased markedly in each cell line following treatment with either EPA or DHA but in both instances the U937 cells showed the greatest increase. Similarly, following treatment with DHA the % ω-3 fatty acid content was 52.59%, 30.14% and 20.43% respectively for the U937, lymphoblastoid and IMR-32 cells. As a result, all 3 cell lines were responsive to supplementation with DHA and EPA but the U937 cell line significantly more so.

Enrichment of ω-3 PUFA content was even more marked following DHA supplementation. Following 24 hour treatment, the DHA content in the U937 cell line had increased by 46%, by 24% in lymphoblastoid cells and by 13% in IMR-32 cells compared to DHA content in untreated cells. Astrocytes grown in medium supplemented with DHA and/or AA plus α-tocopherol have been shown to incorporate large amounts of long chain PUFA so that the ω-6/ω-3 PUFA compositions of the phosphatidylethanolamine and phosphatidylcholine, the two main classes of membrane phospholipids, were greatly altered (Champeil-Potokar *et al* 2004). Furthermore, the astrocytes cultured in medium plus DHA had a more physiological ω-3 status, grew better and retained their astrocyte phenotype (Champeil-Potokar *et al* 2004).

The profile of essential fatty acids in neuronal membranes has been suggested to affect normal physiological events in the brain. For example ω-6 and ω-3 PUFA imbalance early in life leads to irreversible changes in hypothalamic composition (Li *et al* 2006). DHA, the main ω-3 polyunsaturated fatty acid in membranes is particularly abundant in brain cells and decreased cerebral concentrations of DHA are associated with impaired cognitive function (Champeil-Potokar *et al* 2004). Moreover the fatty acid composition of neuronal cell membrane phospholipids reflects their intake in the diet (Li 2006; Haag 2003). Dietary fatty acids, therefore, may induce changes in neurophysiological and psychopathological parameters (Haag 2003; Evans *et al* 2003) while animal studies have shown that diet-induced lack of DHA in the brain leads to alterations in cognitive processes (Vancassel *et al* 2007). A diverse range of biological actions of DHA, a major component of brain membrane phospholipids, is involved in neuronal function, gene regulation and

maintenance of the structural integrity of neurones. DHA is also thought to have a protective role in a cell culture model of apoptosis (Salem *et al* 2001) and may play an important role in the regulation of cell signalling and in cell proliferation (Salem *et al* 2001). Similarly, EPA plays a role in many biological activities and a variety of actions have been reported. These include inhibition of phospholipase A_2 (El Azher *et al* 2000), inhibition of the apoptotic pathway (Englebrecht *et al* 2005; Martin *et al* 2002), the induction of apoptotic cell death (Schley 2005; Heimli 2002), the indirect neuroprotective effects of metabolites such as D series resolvins that have potent anti-inflammatory actions (Das 2006), and the ability to exert a number of positive actions against atherosclerosis and its complications (von Schacky 2006).

4.4.2 Lipid Peroxidation

Lipid peroxides are compounds that are unstable and have the ability to rapidly degrade into numerous products such as short chain alkanes and aldehydes. Examples of lipid peroxide end products that are used to investigate clinical disease include TBARs, exhalation of the alkanes pentane and ethane and cytotoxic aldehydes (Meagher and FitzGerald 2000). In this study MDA analysis was undertaken using the TBARs assay while lipid hydroperoxide analysis was completed using a commercial kit. Treatment of the three cell lines with a high concentration of H_2O_2 for 30min or 4 hours did not induce a significant increase in MDA. The human cell line U937 was then supplemented for 24 hours with fatty acids followed by a 4 hour oxidative stress. Both EPA and DHA treatment appeared to reduce LOOH levels in the U937 cells but not significantly. Furthermore, pre-treatment of the cells with the fatty acids followed by the H_2O_2 treatment did not further induce an increase in LOOH levels. The results suggest that not only is there no pro-oxidant stress arising from the supplementation of the chosen fatty acids but

the cells also appear to be resistant to the high ROS challenge in the presence of the PUFA. It is also possible that enhanced antioxidant enzyme activity induced by ω-3 fatty acid supplementation of the U937 cells caused a decrease in LOOH production. Crosby *et al* (1996) reported an induction of glutathione peroxidase activity in HUVEC treated with EPA and DHA for 24h, and consequently a reduction in the production of conjugated dienes, a marker of lipid peroxidation.

Further review of the literature revealed that there is limited data examining the supplementation of U937 cells with EPA and DHA and their effects on LOOH levels. However, a study examining retinal fatty acid binding protein also noted that lipid hydroperoxides stimulated by long-chain fatty acids were reduced on rod outer segments (Guarjado *et al* 2002). In contrast, Udilova *et al* (2003) reported that peroxidation of biomembranes can be initiated by LOOH from heated dietary oils and more recently the same group reported the production of LOOH from unsaturated oils as a key factor in colon carcinogenesis (Jurek *et al* 2005).

One of the most commonly used methods in free radical research is the determination of the TBA adduct and MDA in plasma. MDA has been found to be elevated in patients with schizophrenia when compared with normal controls (Zhang *et al* 2006; Dakhale *et al* 2005; Kuloglu *et al* 2002; Ravikumar *et al* 2000), as well as in animal models (Ozyurt *et al* 2007), although a recent human study (Zhang *et al* 2007) found significantly lower MDA plasma levels from male schizophrenic subjects who smoked compared to the non-smokers. While reports of increased levels of lipid peroxides in patients with schizophrenia have been observed (Li *et al* 2006, Evans *et al* 2003), and increased LOOH levels in patients with psychiatric disease have been reported (Balkan *et al* 2005; Karelson *et al* 2001), studies of lipid

hydroperoxides in particular, in patients with schizophrenia is limited. In our recent study, however, (Young *et al* 2007) we reported no significant differences in the levels of LOOH in either the schizophrenic or control group.

4.4.3 Phospholipase A_2

Phospholipases A_2 are important enzymes involved in the process of AA metabolism and are activated during monoaminergic neurotransmission (Berger *et al* 2006). Reduced membrane AA levels and an altered activity of PLA_2 have been found in peripheral membranes of drug-naïve patients with schizophrenia with some conflicting results in more chronic patient populations (reviewed by Berger *et al* 2006). Analysis of post-mortem brain tissue has further reported a decrease in calcium-dependent PLA_2 activity rather than an increase (Ross *et al* 1999). Furthermore, it has been suggested that genes expressing cytosolic PLA_2 may be involved in contributing to the aetiology of schizophrenia (Tao *et al* 2005).

Initial studies revealed significantly increased total PLA_2 levels in the U937 and IMR-32 cells following 4 hour treatment with H_2O_2 alone. An increase in total PLA_2 activity was also seen in lymphoblastoid cells although this did not reach significance. However, further experimentation with or without pre-treatment of cells with ω-3 fatty acids revealed total PLA_2 levels to be similar in all the cell lines and no change in activity levels was observed following challenge with H_2O_2. These findings are in contrast to the reports that H_2O_2 activates cytosolic phospholipase A_2 (van Rossum *et al* 2004) and it is difficult to provide an explanation for this discrepancy at present.

In an additional exploratory study, the $cPLA_2$ activity in the three human cell lines was examined and the basal level of $cPLA_2$ activity was found to be comparable in the lymphoblastoid and IMR-32 cells but lower than that measured in the U937 cells. However, because only one study was carried out, the results cannot be deemed significant since no statistical analysis was undertaken. In this experiment, a high variance was observed within the untreated cells which lead to the adoption of the ethanol-treated cells as the control set. In addition, the large error bars recorded in this experiment in particular may be due to the possible lack of intra-assay precision.

An enhanced level of $cPLA_2$ activity in the U937 cell line has been previously reported by Obajimi *et al* (2005) and is a feature which has led to the suggestion that this cell line is a suitable *in vitro* model for studying cellular mechanisms of schizophrenia. Supplementation of the U937 cell line with EPA appeared to reduce $cPLA_2$ activity relative to the vehicle treated control, an outcome which again appears to mimic some of the biochemical changes reported following EPA supplementation of Asperger's syndrome sufferers (Bell *et al* 2004). Neither EPA nor DHA supplementation appeared to have any significant effect on either total PLA_2 or $cPLA_2$ activity in IMR-32 or lymphoblastoid cell lines. However, it is also reported that ω-3 PUFAs modulate T-cell functions such as T-cell proliferation and cytokine secretion (Calder 2001). This is important since inflammatory cytokines such as IL-1β, TNFα and IFNγ, play a role in the activation of $cPLA_2$ (Xu *et al* 2003). A more recent study also reported that IL-2 increased $cPLA_2$ activity in the shell of the nucleus accumbens but IL-1-induced changes were significantly attenuated by EPA treatment (Song *et al* 2007).

In conclusion, although this study has attempted to evaluate the use of LOOH and PLA_2 as markers of oxidative stress in models of schizophrenia, the results are quite inconclusive. It is possible that the number of passages and general maintenance of the cells lead to the 'poor' results although this was not as a result of cell loss. The viability of the cells was checked before and after each treatment of every experiment and the cell viability was not changed so it seems unlikely that the inconclusiveness of the results was because of the treatments. Had resources and time permitted it would have been ideal to have carried out the experiments again, perhaps with minimal passages and minimal interference to the cells.

CHAPTER 5

Evaluation of biomarkers of lipid peroxidation in schizophrenic and control subjects. Human trial part 1.

5.1 Introduction

Lipid peroxidation in biological tissues can be measured by numerous peroxidation products including lipid hydroperoxides, conjugated dienes and malondialdehyde. Lipid peroxidation also produces hydrocarbon gases, such as ethane and pentane which are produced from the peroxidation of ω-3 and ω-6 PUFAs. Measurement in the exhaled breath of these volatile alkanes is another way of assessing lipid peroxidation and the effect of disease and antioxidant status with respect to oxidative stress (Aghdassi and Allard 2000).

5.1.1 Volatile Breath Hydrocarbons as a Biomarker of Lipid Peroxidation

Analysis of exhaled air products enables the observation of biochemical processes in the body via a non-invasive procedure. Gas chromatographic analysis of exhaled air was first investigated over 4 decades ago by Aulik (1966) and further by Pauling *et al* (1971) who determined more than 200 components in human breath. Evidence that volatile hydrocarbons are generated after peroxidation of unsaturated fats was provided by *in vitro* studies where it was shown that hydrocarbon gases evolved during the decomposition of fatty acid hydroperoxides by β-scission (Dumelin and Tappel 1977) leading to the formation of an alkyl radical and an aldehyde. In many studies the formation of alkanes in the C2-C5 range is analysed (Griffiths *et al* 2002), but as a result of the physiological ratio of ω-3 to ω-6 fatty acids, four times more pentane than ethane is generated through lipid peroxidation (Miekisch *et al* 2004) and so these gases are more often measured.

Lipid peroxidation is a chain reaction which is initiated by the removal of an allylic hydrogen atom through ROS. The radical generated in this way is conjugated,

peroxidized by oxygen and undergoes further reactions. Eventually saturated hydrocarbons such as ethane and pentane are produced in this way from ω-3 and ω-6 fatty acids respectively, as well as MDA from the same pathway. Animal and clinical studies have demonstrated a close correlation between clinical conditions whereby dietary antioxidants and lipid sources significantly influenced ethane and pentane output, supporting the idea that breath alkane output is a useful index of lipid peroxidation (Dillard *et al* 1977). In addition, levels of exhaled pentane and ethane have also been shown to correlate well with other lipid peroxidation markers such as lipid peroxides and F_2-isoprostanes (Aghdassi *et al* 2003).

Although originally difficult to quantify, the non-invasive measure of breath alkane output was finally standardised and validated as a measure of lipid peroxidation in humans (Dumelin and Tappel 1977; Filser *et al* 1983; Lemoyne *et al* 1987). Hydrocarbons as stable end products of lipid peroxidation show only low solubility in blood and are excreted into breath within minutes of their formation in tissues (Miekisch *et al* 2004). Therefore, exhaled concentrations of ethane and pentane can be used to monitor the degree of oxidative damage in the body (Risby and Sehnert 1999).

5.1.2 Breath Collection and Analysis

Because real-time monitors for breath biomarkers are unavailable at the present time, exhaled breath must be collected and transported to the laboratory for analysis. Most published studies have collected total exhaled breath in inert gas sampling bags and analysis is performed as soon as possible after collection. The requirements for gas sampling bags are that the bags must be inert and not adsorb analyte molecules (Risby and Sehnert 1999). Furthermore, the bags should have a volume that

corresponds to at least twice the amount of breath collected, minimizing any back pressure from the collected gas that could restrict the exhalation of the study subject. In addition, the inlet to the gas sampling bag should have sufficient dimensions to restrict any pressure drop across the inlet to the gas sampling bag (Risby and Sehnert 1999). More recently, thermal desorption tubes packed with adsorbents have been used to collect expiratory gases. These devices collect known volumes of the exhaled breath at controlled flow rates which results in no resistance to breathing and enabling the collected breath sample to be transported from sample site to laboratory (Risby and Sehnert 1999).

Separation of volatile compounds has been carried out using gas-liquid chromatography and gas-solid chromatography. Hydrocarbons in the nmol/l-pmol/l range such as ethane, pentane or isoprene usually are determined using gas chromatography coupled to flame ionisation or mass selective detection (reviewed by Miekisch *et al* 2004). Currently, the analytical method that is most frequently used is a capillary GC method with an adsorption/desorption sample-handling method which enables the preconcentration of small volumes of exhaled breath. For further analysis, volatile substances have to be released from the adsorption material. This can be achieved by heating the trap (thermal desorption or by means of microwave energy (Miekisch *et al* 2004) and can be carried out automatically or by commercially available devices. Breath volatiles can also be preconcentrated by means of solid phase microextraction (Grote and Pawliszyn 1997). The method involves extraction and preconcentration with a fused silica fibre, coated with a polymeric stationary phase. Desorption of the volatile compounds from these coated fibres is carried out by direct heating at very high temperatures and assayed by GC-MS.

The choice of the separation column is fundamental. Chromatographic separation of pentane and isoprene in human breath has proved difficult with the sum of isoprene and pentane being determined as pentane (Springfield and Levitt 1994). In addition, care is required in the measurement of breath pentane so that pentane and its isomer, isopentane are not co-eluted (Mitsui *et al* 2000). Although there are other potential sources of hydrocarbons in the body such as protein oxidation and colonic bacterial metabolism, these appear to be of limited importance and do not interfere with the interpretation of hydrocarbon breath test for ethane and pentane (Kneepkens *et al* 1994). Propane and butane are mainly derived from protein oxidation and faecal flora and their role as markers of lipid peroxidation is uncertain (Miekisch *et al* 2004)

5.1.3 Volatile Hydrocarbons and Schizophrenia

Clinical studies have shown that the presence of abnormal volatiles can aid diagnosis of certain diseases. For example, breath isoprene levels have been reported to be altered in a number of clinical conditions; although the physiological meaning of these changes has not yet been established (Salerno-Kennedy and Cashman 2005). Various lines of evidence suggest that isoprene is related to cholesterol biosynthesis. Therefore, breath isoprene measurements could potentially be used for screening of lipid disorders (Salerno-Kennedy and Cashman 2005).

It is thought that hydrocarbons found in breath may result from increased lipid peroxidation caused by oxidative stress (Ward 2000). Since it is suggested that oxidative stress may be a contributory factor in neurodevelopmental disorders (Ross 2000) the non-invasive breath test may have a role as a diagnostic tool in oxidative stress-related diseases.

The aim of this study was to utilise the non-invasive technique of breath hydrocarbon analysis. The results of the hydrocarbon analysis will be compared with another lipid biomarker in a population of schizophrenic patients compared to a population of apparently healthy aged-matched control subjects. In addition, a correlation of the lipid peroxidation products will be undertaken against the findings of the psychiatric rating scales.

5.2 Materials and Methods

5.2.1 Investigation of Biomarkers of Lipid Peroxidation in Breath Exhalant from Schizophrenia Patients and Control Subjects

5.2.1.1 Ethical Approval and Recruitment of Participants

Ethical approval was obtained from the NHS Highland Ethics Committee and all procedures were carried out in accordance with the Helsinki Declaration (1975) and the Data Protection Act (1998). Schizophrenic patients and apparently healthy controls were recruited from in- and out- patients and staff at the New Craigs Hospital, Inverness and written informed consent obtained after a full explanation of the study was given. All participants with schizophrenia were diagnosed according to DSM-IV criteria (American Psychiatric Association, 1994). Patients with schizophrenia were assessed using the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham 1962) and the Positive and Negative Symptom Scale (PANSS) (Kay *et al* 1987) (Table 5.3). The age of participants with schizophrenia did not differ significantly from apparently healthy control subjects. Mean age ± sem were 36.6 ± 1.9 and 37.4 ± 3.2 in the healthy control and schizophrenic groups respectively.

In each study (human trial part 1 and part 2) the volunteer sample size was small (ie. the number of volunteers in the control and patient group was 17 or less). However, the population recruited to our breath alkane study was comparable to those employed in a previous study by our collaborators who reported a significant elevation of breath ethane levels in their schizophrenic patient population (Glen et al 2003). Furthermore, our studies were exploratory since to date, there has been no published data examining the investigation of various biomarkers of oxidative stress in schizophrenia. Thus, at this stage, the results were not confirming any earlier or

previously published work. Additional factors that had to be considered were cost and resources. Funding was limited and had to be stretched over a 3-year research period which meant as a consequence resources and time was also limited. Subsequently, the smaller the sample size, the more manageable the tasks were. Furthermore, although additional help was provided throughout the human studies, large parts of the experimental work were completed solely by me. This meant that in order to carry out the experiments within in a reasonable time and to ensure that frozen samples were not held over a long time-frame to invalidate any result, it was important that the sample size was manageable. Finally, it has been noted that exploratory human trials have been undertaken and successfully published using small sample sizes (Puri et al 2007). As a result, we felt that the sample size chosen here was practical and acceptable.

5.2.1.2 Collection of Exhalants

All smokers were required to abstain from cigarette inhalation for at least 45 minutes prior to collection of the exhalant sample. Exhalant samples were obtained from seated subjects using a syringe system of volume 130ml equipped with a disposable mouthpiece. The subject was instructed to exhale one long breath into the OSI PanBag (Figure 5.1) until they could no longer breathe to collect the alveolar (end expired) air from the lungs. Once sample collection was complete, the OSI PanBag was sealed by means of a goggle clamp. Samples of ambient hydrocarbons were also collected and analysed thus enabling a correction for high atmospheric backgrounds to be introduced where appropriate.

5.2.1.3 Storage of Exhalant Samples

Samples were stored for a maximum of 48 hours at ambient temperature prior to GC-MS analysis.

5.2.1.4 Transfer of Breath Sample from OSI PanBag to ATD Tube

The OSI PanBag and contents were cooled rapidly to ensure that any moisture present in the breath sample was effectively trapped in the OSI PanBag, reducing the potential for interferents in the ATD tube. The OSI PanBag was immersed in dry ice with the exit tube remaining above the ice level (Figure 5.2). A glass syringe fitted with a stopcock was attached to the exit pipe of the PanBag via a PTFE connector. Both the goggle clamp on the PanBag and the exit valve on the syringe were opened and 100 ml sample was drawn from the PanBag into the syringe. The syringe was then closed and inserted into the ribbed end of the ATD tube. The stopcock of the syringe was opened and 100ml sample injected into the tube.

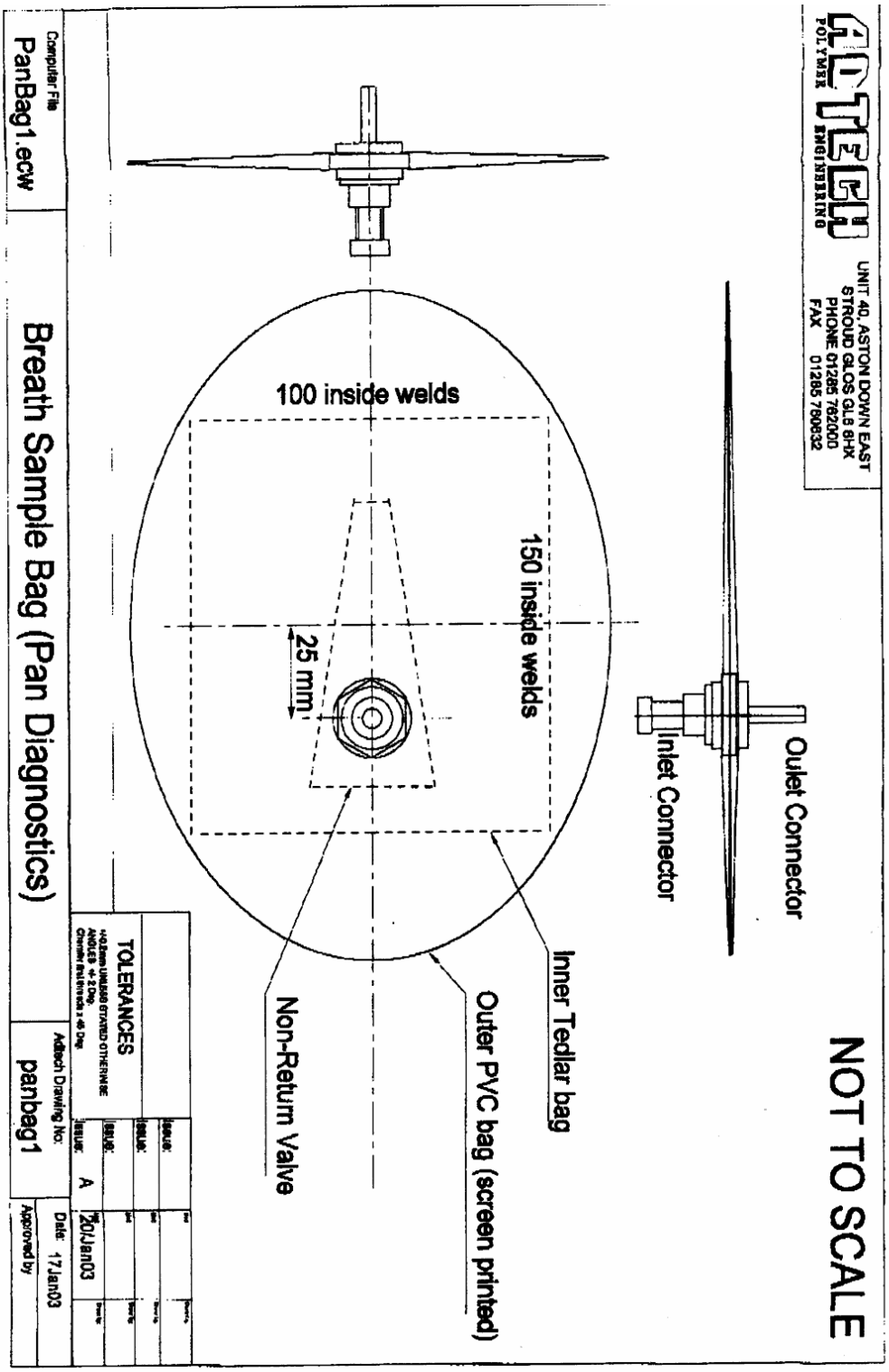
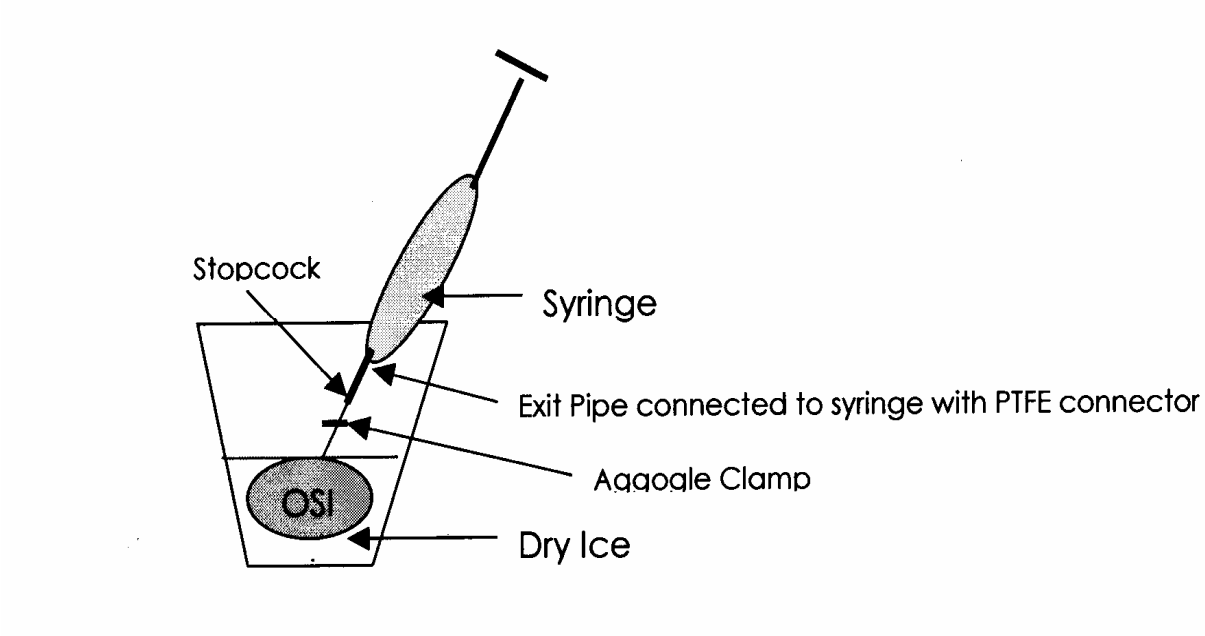


Figure 5.1. Illustration of OSI PanBag



Procedure

- PanBag is covered in dry ice, for approximately 20 minutes, with exit tube remaining above ice level.
- Syringe is attached to exit pipe of PanBag with PTFE connector.
- Ensure that stopcock at end of syringe is closed.
- A goggle clamp on PanBag is opened along with turn valve on syringe.
- 100ml volume of sample is removed and the turn valve on the syringe is closed.
- A goggle clamp is now closed and the syringe can now be detached from the PanBag.
- Syringe is inserted to ribbed end of ATD tube.
- Stopcock of syringe is opened and 100ml sample injected into tube.

Figure 5.2: Process of breath transfer from OSI PanBag to glass syringe

5.2.1.5 Determination of Exhalants by GC-MS/ATD Analysis

The measured volume of expelled air was injected into a Perkin-Elmer (UK) automatic thermal desorption (ATD) tube packed with carbotrap 300 (Perkin-Elmer, UK) via a sodium sulphate drying cartridge (International Sorbent Technology Ltd., UK). Gas samples were analysed using a Perkin-Elmer autosystem XL equipped

with a Turbo Mass spectrometer and ATD 400 automatic thermal desorption. ATD tubes were desorbed onto the cold trap at 320^{o}C, the cold trap being held at 5^{o}C. The trap was then rapidly heated to 350^{o}C and the liberated volatiles injected onto a 30m x 0.32mm PLOT GQ column (Perkin-Elmer, UK) with helium at 2ml/min. The oven was set at 45^{o}C for 3min and ramped at 14^{o}C per minute to 200^{o}C where it was held for 8min, yielding an analysis time of 22.07min.

5.2.1.6 Mass Spectrometer Conditions

The mass spectrometer was operated in EI mode with data collection enabled from 0.02 seconds to 18 minutes. Selected ion response (SIR) was included in the method to enable detection and identification of the analytes of interest. The timed windows for ion monitoring were as follows:

Time period	SIR/mass (Da)
0.02sec – 5min	30.00 for ethane
6.30min – 13.00min	67.00 for isoprene
6.30min – 13.00min	57.00 for pentane

5.2.1.7 Quantification

Quantification was by reference to calibration curves (0-60pmol) constructed using an authentic C1-C6 alkane standard mix (Supelco, UK).

5.2.1.8 Statistical Analysis

Statistical Analysis was carried out using Minitab. As anticipated there was significant inter-individual variation in the profile and level of ethane and pentane noted in the exhalant samples of trial participants. Both datasets are not normally

distributed, failing the Anderson Darling tests. Lack of normality implied that non-parametric tests, comparing median levels for patient and control groups, were appropriate and statistical significance was therefore tested by Mann-Whitney tests.

5.2.1.9 Determination of Total Plasma Malondialdehyde

The method is a modification of Grotto *et al* (2007) which has been validated internally and enables the determination of total plasma MDA with quantification by both mass spectrometric and diode array detection. Free malondialdehyde was prepared fresh by the acid hydrolysis of 1,1,3,3 tetramethoxypropane and a series of 10 calibrants over the range of 3 to 30µM employed in the calibration line. Plasma MDA determinations were performed in replicate (n=5) using 0.1ml aliquots of sample. Alkaline hydrolysis and protein precipitation enabled the determination of total MDA measured as a conjugate of thiobarbituric acid (Grotto *et al* 2007).

5.2.1.10 Determination of MDA by HPLC with Diode Array and MS Detection

An Agilent Technologies 6130 Quadrupole LC-MS was employed which had an auto-sampler and on-line diode array detection. Separation was achieved on a Phenomenex Hyperclone BDS C18 column (150 x 2mm, 3µm) with a 15% acetonitrile: 85% formic acid (0.1 % v/v) mobile phase at a flow rate of 0.25ml/min. The column oven was held at 35^{o}C and an injection volume of 50µl used.

The diode array detector was set at 532nm (λmax for TBA-MDA conjugate) and spectra were measured over a range of 500 to 580nm. The MS detector was set to measure in the negative mode and used a scan over the mass range of 300 – 350amu. Single ion monitoring was set at 323amu (characteristic of TBA-MDA conjugate). Analysis of an MDA calibration standard produced a single peak by LC-UV with a

typical retention time of 2.31 minutes. Linearity was established over the range of 3 – 30mM MDA and fresh calibration lines were prepared and analyzed daily.

5.2.1.11 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and the Mann-Whitney test was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

5.2.1.12 Acknowledgements

This research was a collaborative activity which brought together the collective expertise of researchers at the School of Pharmacy, The Robert Gordon University (Aberdeen) and the Ness Foundation (Inverness), an associate partner of The University of the Highlands and Islands. Ms Siofradh McKinney was the clinical research nurse responsible for trial recruitment, collection of blood and breath samples and completion of volunteer questionnaires. Mr Ivor MacKenzie (Ness Foundation) was the research technician responsible for determination of breath volatiles by GC-MS. I am also grateful to Raymond Reid, School of Pharmacy, The Robert Gordon University for his help with the validation and development of the MDA by HPLC. We acknowledge the support of NHS staff at New Craig's Hospital, Inverness and the participation of the patients and volunteers in this study without which the research would not have been possible.

5.3 Results

5.3.1 Validation of GC-MS Assay

5.3.1.1 Analyte Resolution and Identification

Method development and validation included an optimization of chromatographic conditions to ensure the resolution of key analytes and an assessment of assay linearity, sensitivity and specificity.

Figure 5.3 depicts typical chromatograms illustrating the resolution of the volatile hydrocarbons when analysed in a standard mix. Peak assignation resulted from a consideration of the fragmentation patterns under each chromatographic peak. Ethane eluted at 1.49min and was identified at m/z 30 (Figure 5.3), butane eluted at 7.3min and was identified at m/z 43 (Figure 5.3) whilst pentane eluted at 9.7min and was identified at m/z 43 (Figure 5.3). The resolution of isoprene (Rt = 9.77min), m/z 67 (Figure 5.3) from pentane (Rt = 9.70min) is not achieved in this method but by using selection ion monitoring it is possible to accurately quantify pentane in the presence of isoprene. The associated mass fragmentation patterns for ethane, butane, pentane and isoprene are depicted in Figures 5.4a-5.4d respectively. By employing on-line mass spectrometric analysis the specificity of the assay for each of the analytes of interest is ensured.

5.3.1.2 Linearity of Alkane Determination

Quantification was by reference to calibration curves (0-60pmol) constructed using an authentic C1-C6 alkane standard mix (Supelco, UK). Typical calibration lines for ethane, butane, pentane and isoprene are depicted in Figures 5.5-5.8 respectively and a summary of the regression equations and correlation coefficients is presented in Table 5.1.

A high measure of linearity was noted in all calibration analyses with a mean correlation coefficient of the order of 0.9974 (n=18) and a mean precision of 0.33% (%CV, n=18) being obtained (Table 5.1). There was significant inter-day variation in the assay response for each of the analytes with % coefficients of variation ranging (% CV) from 25.9 % for ethane (n=6) to 126.9% for butane (n=6). Such between day variations in detector response is corrected for by the inclusion of a range of calibration standards in each chromatographic run.

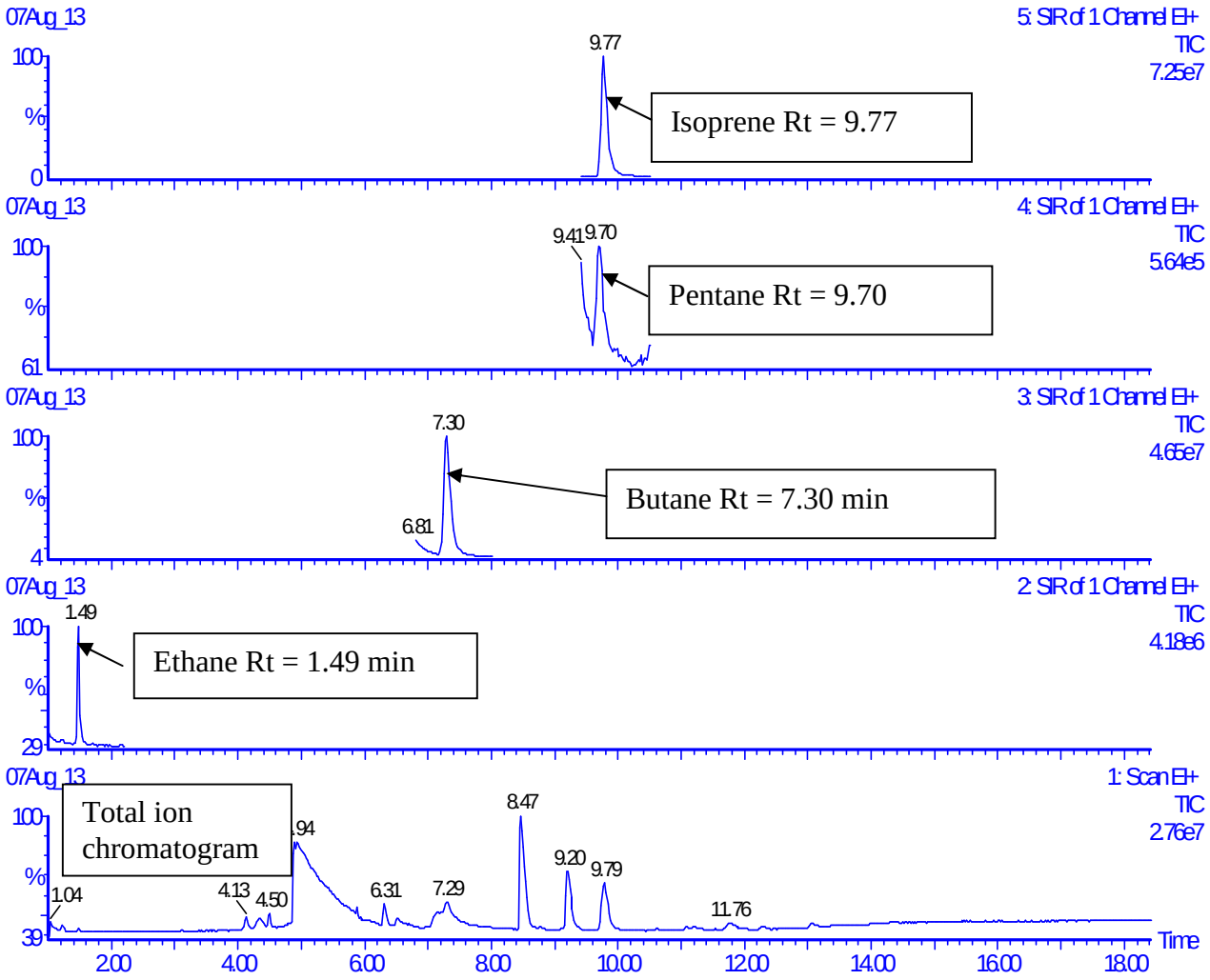


Figure 5.3: Chromatograms illustrating the gas chromatographic separation of ethane, propane butane, pentane and isoprene following analysis of a standard mix.

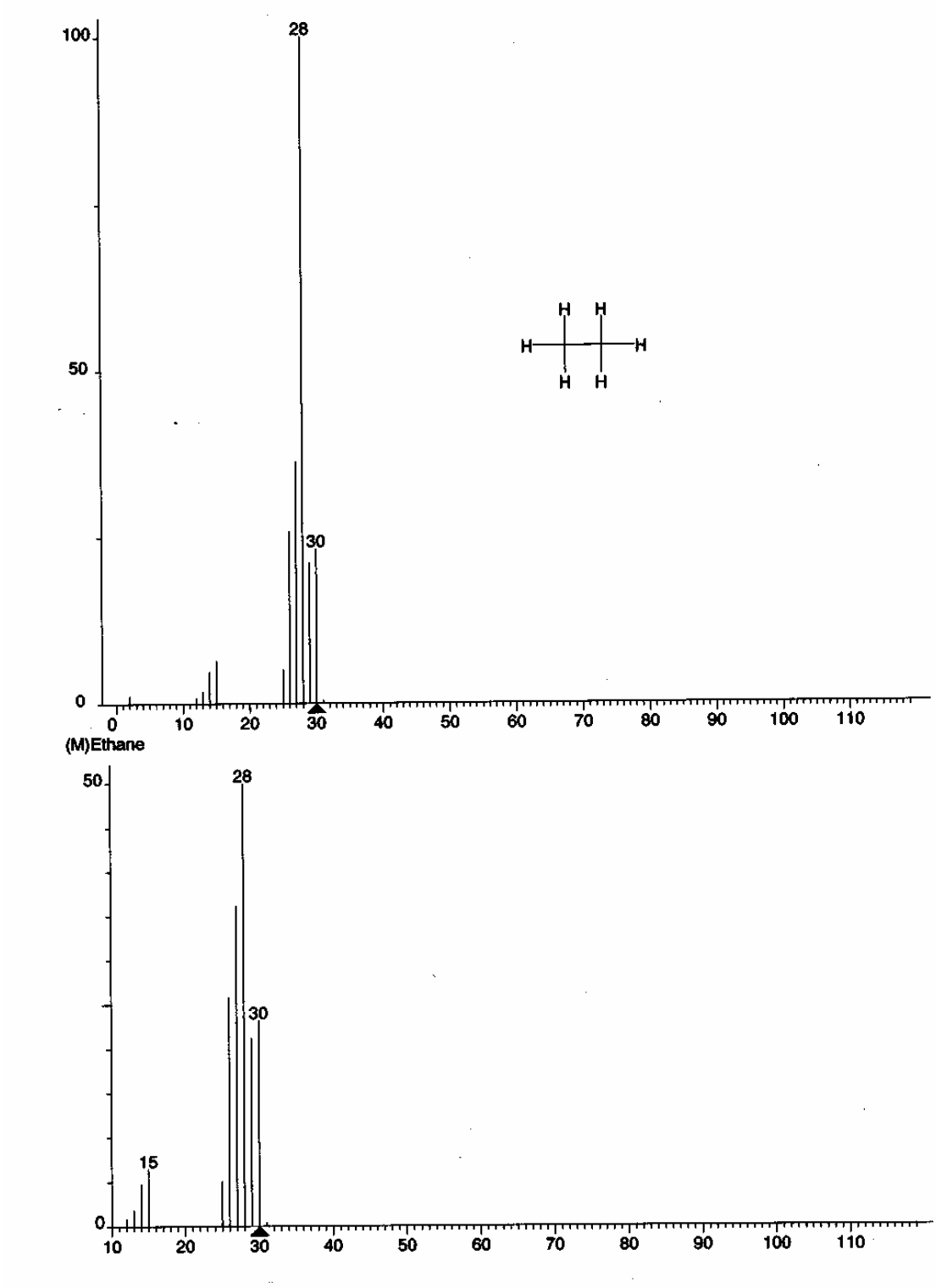


Figure 5.4a: Typical mass fragmentation pattern for ethane. Ethane (C2H6, Mr=30) was the predominant chromatographic peak in the range of 1.00-2.00 minutes and was identified by the presence of m/z = 30.

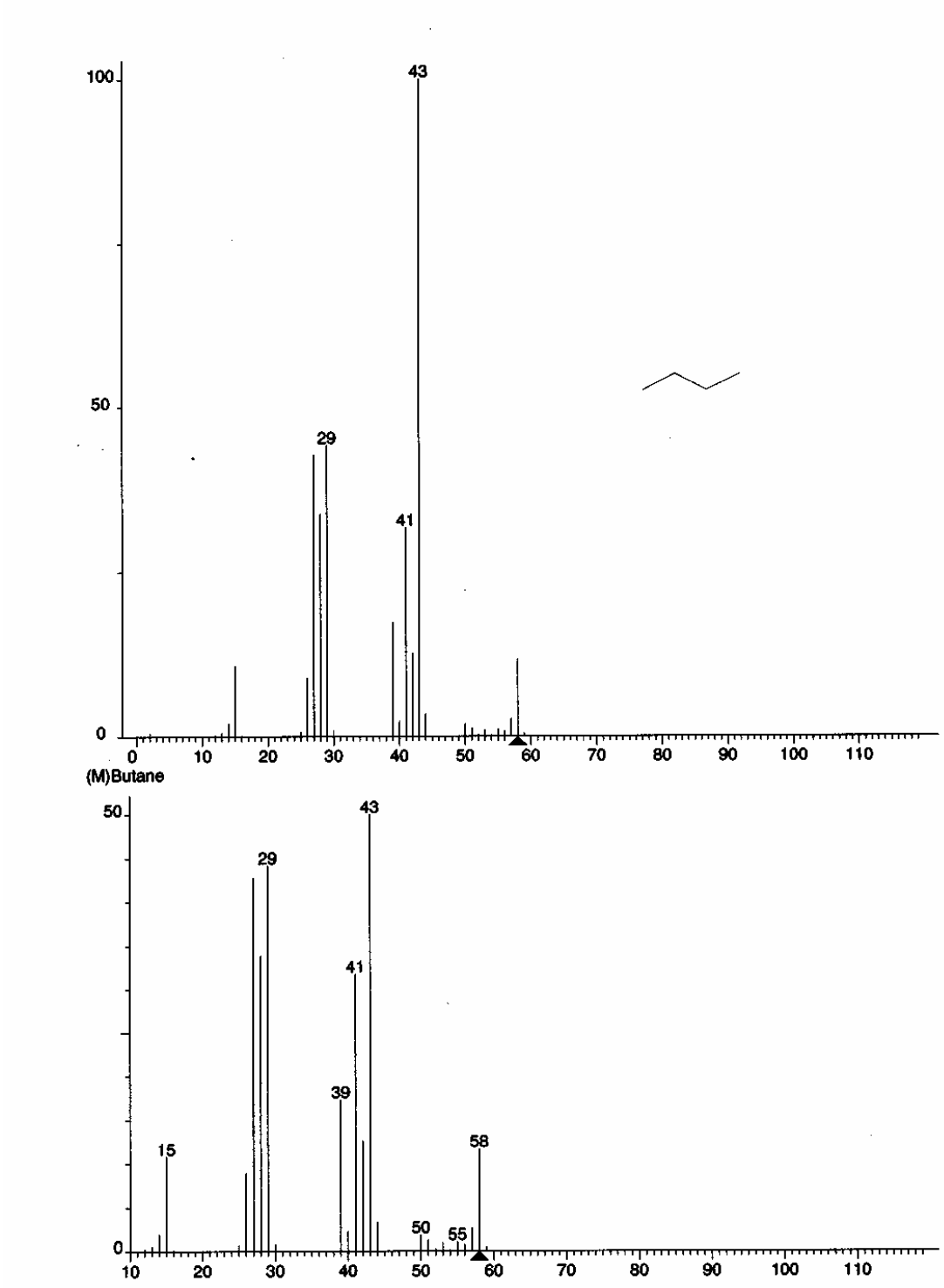


Figure 5.4b. Typical mass fragmentation pattern for butane. Butane (C4H10, Mr=58) was the predominant chromatographic peak in the range of 7.0-8.2 minutes and was identified by the presence of m/z=58.

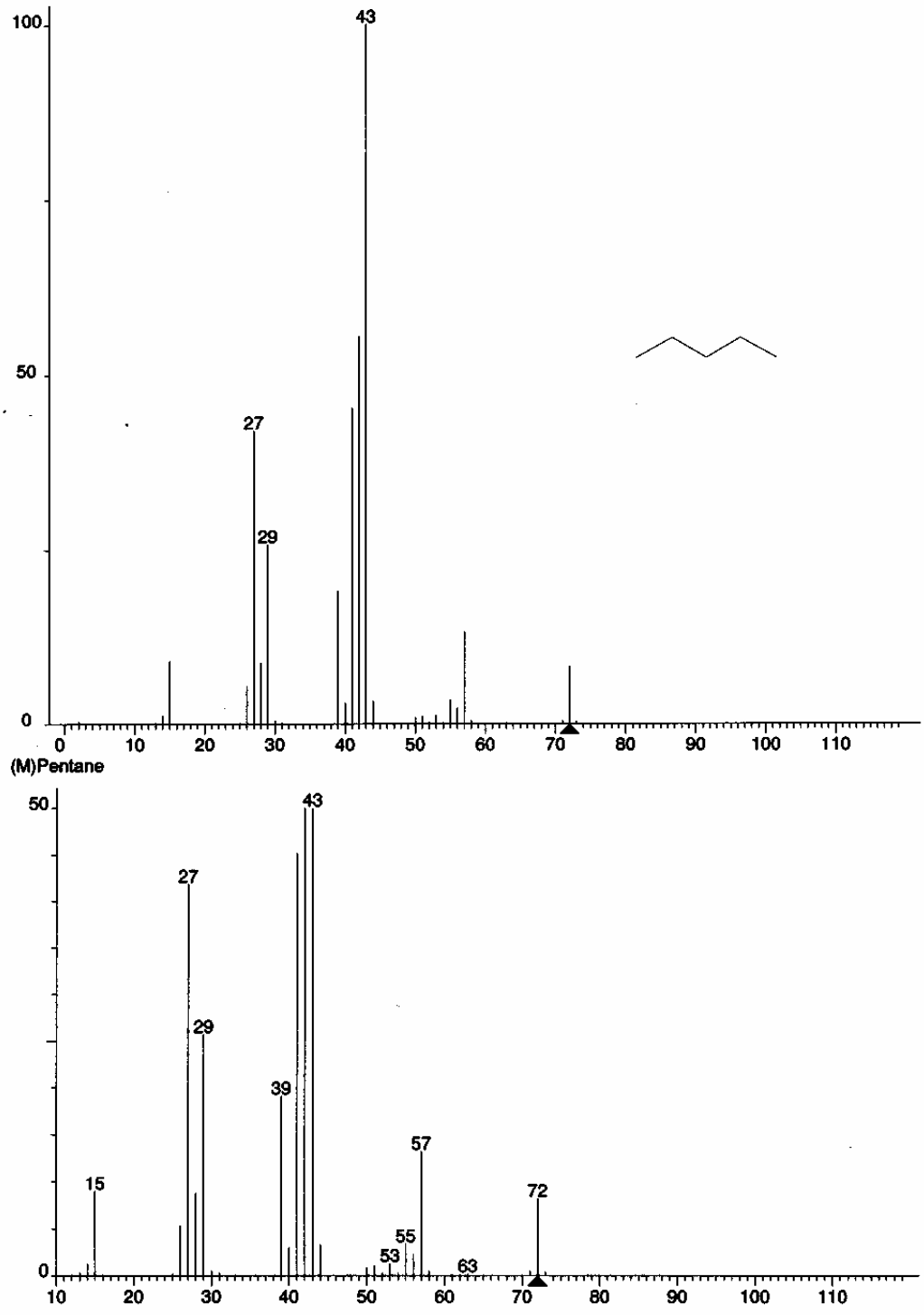


Figure 5.4c. Typical mass fragmentation pattern for pentane. Pentane (C5H12, Mr = 72) was the predominant chromatographic peak in the range of 9.00 – 11.00 minutes and was identified by the presence of m/z = 72.

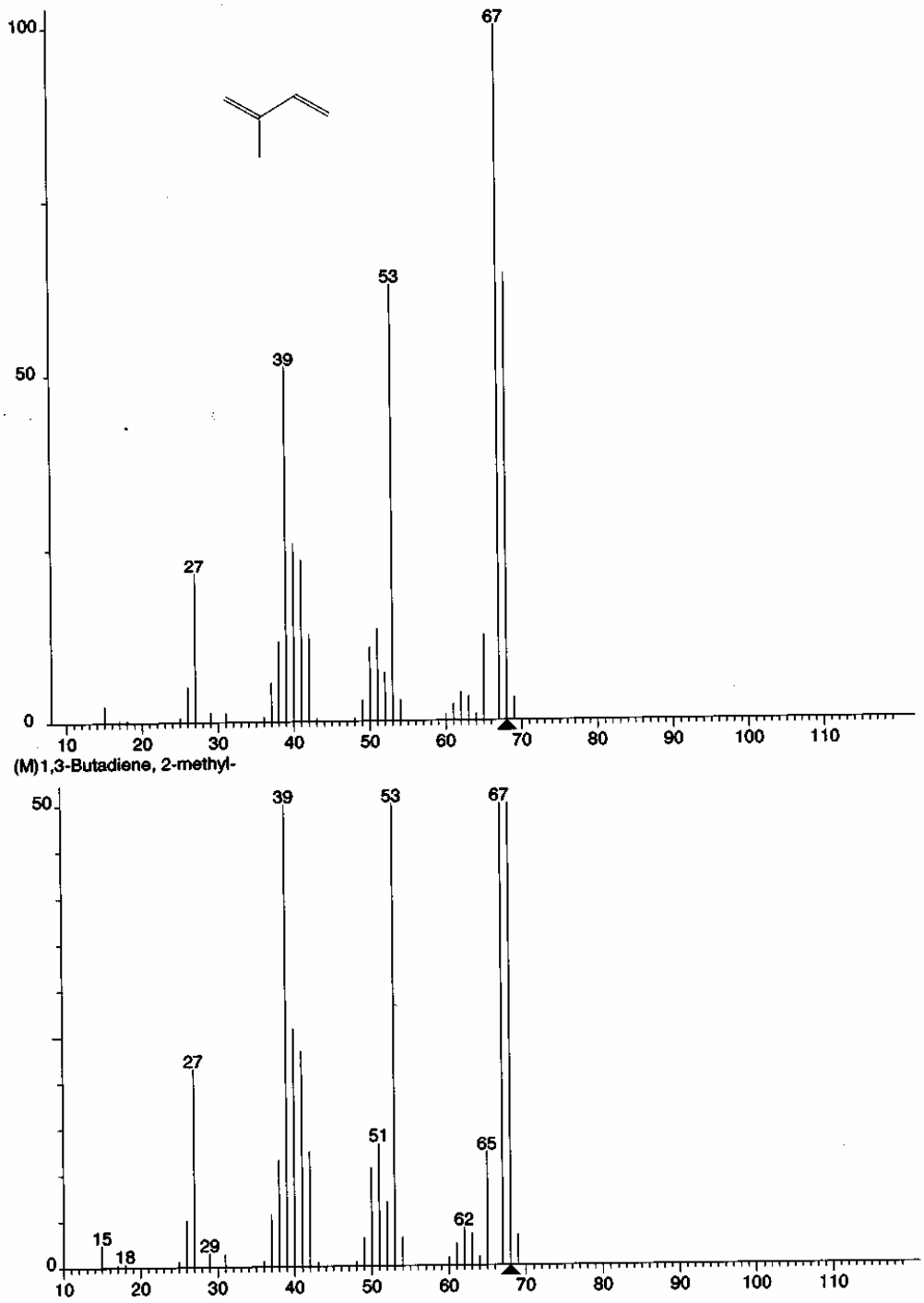


Figure 5.4d. Typical mass fragmentation pattern isoprene. Isoprene (C5H8, Mr = 68) was the predominant chromatographic peak in the range of 9.0 – 11.0 minutes and was identified by the presence of m/z = 67.

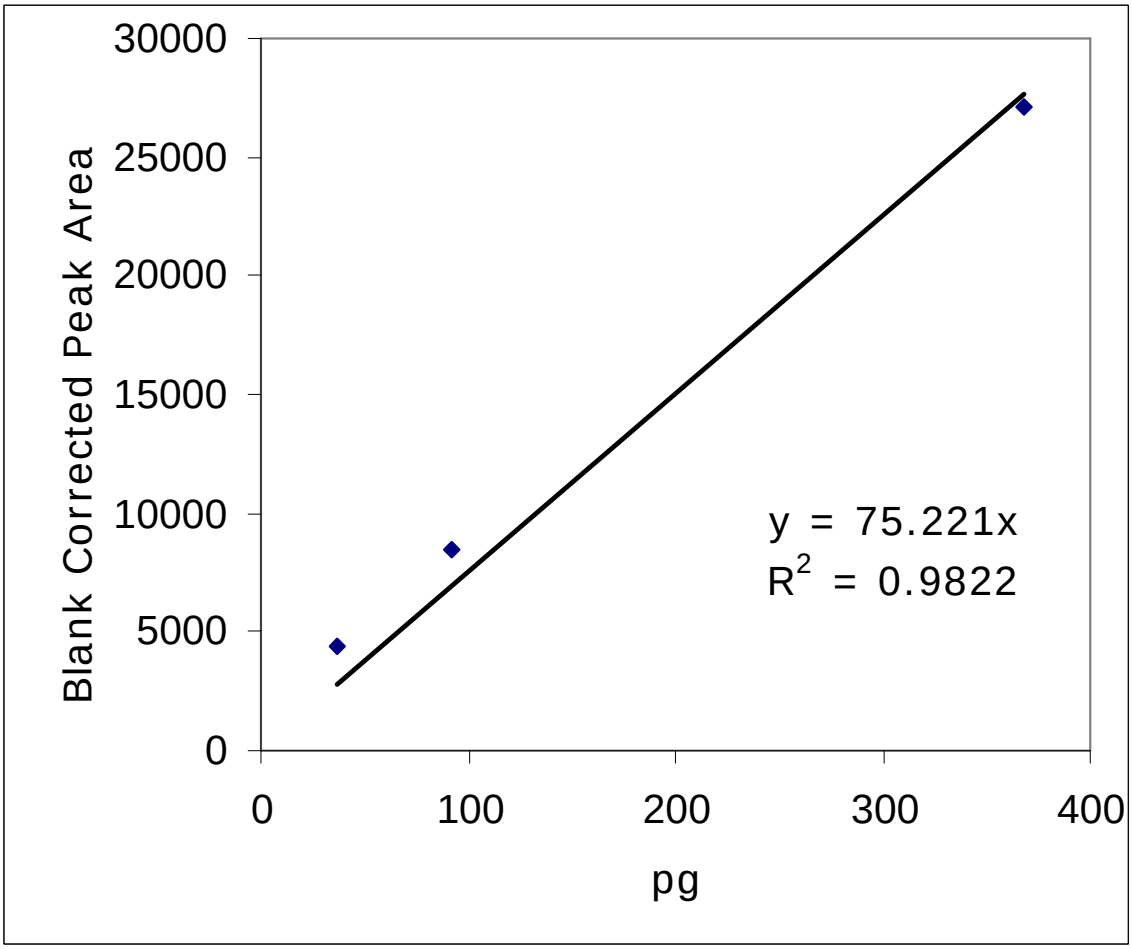


Figure 5.5. Typical calibration line for the determination of ethane by GC-MS. The linearity of assay response to ethane was determined by GC-MS analysis of a series of ethane calibrants (n=3). Calibration lines were included in the analysis of every batch of trial samples.

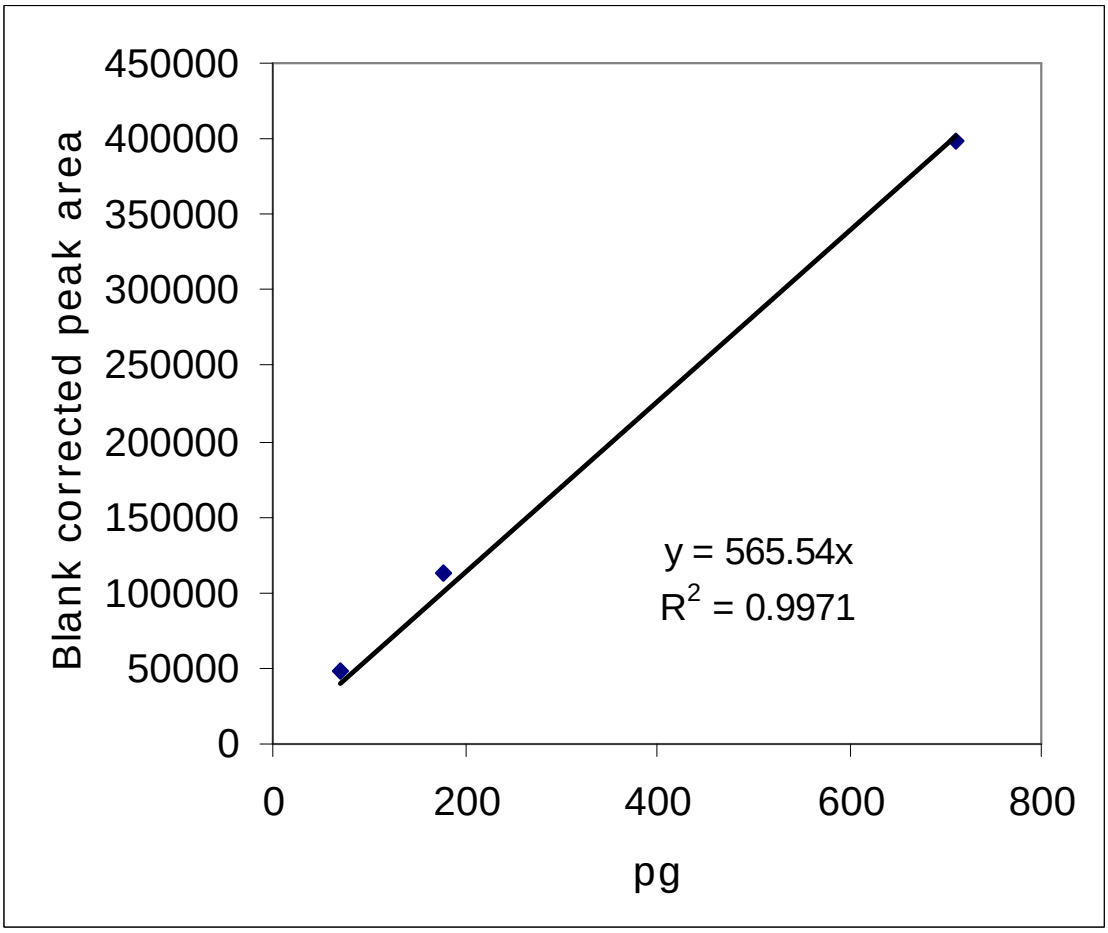


Figure 5.6. Typical calibration line for the determination of butane by GC-MS. The linearity of assay response to butane was determined by GC-MS analysis of a series of butane calibrants (n=3). Calibration lines were included in the analysis of every batch of trial samples.

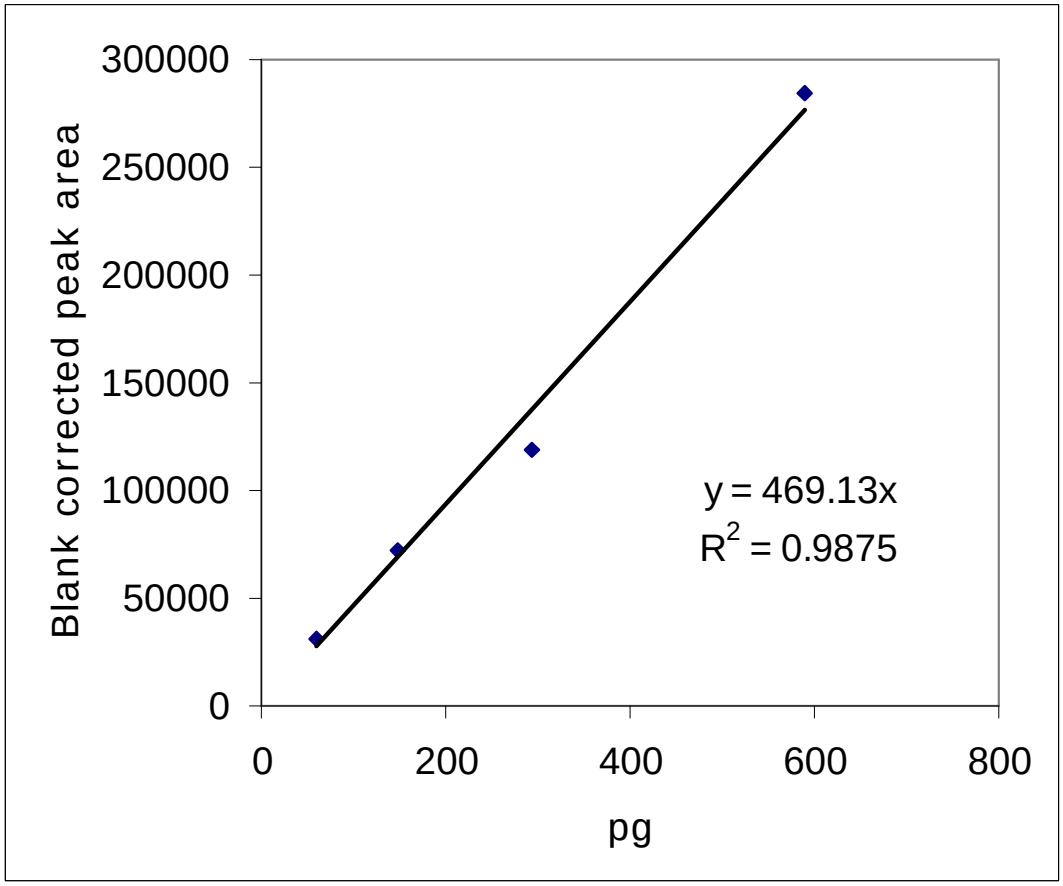


Figure 5.7. Typical calibration line for the determination of pentane by GC-MS. The linearity of assay response to pentane was determined by GC-MS analysis of a series of pentane calibrants (n= 4). Calibration lines were included in the analysis of every batch of trial samples.

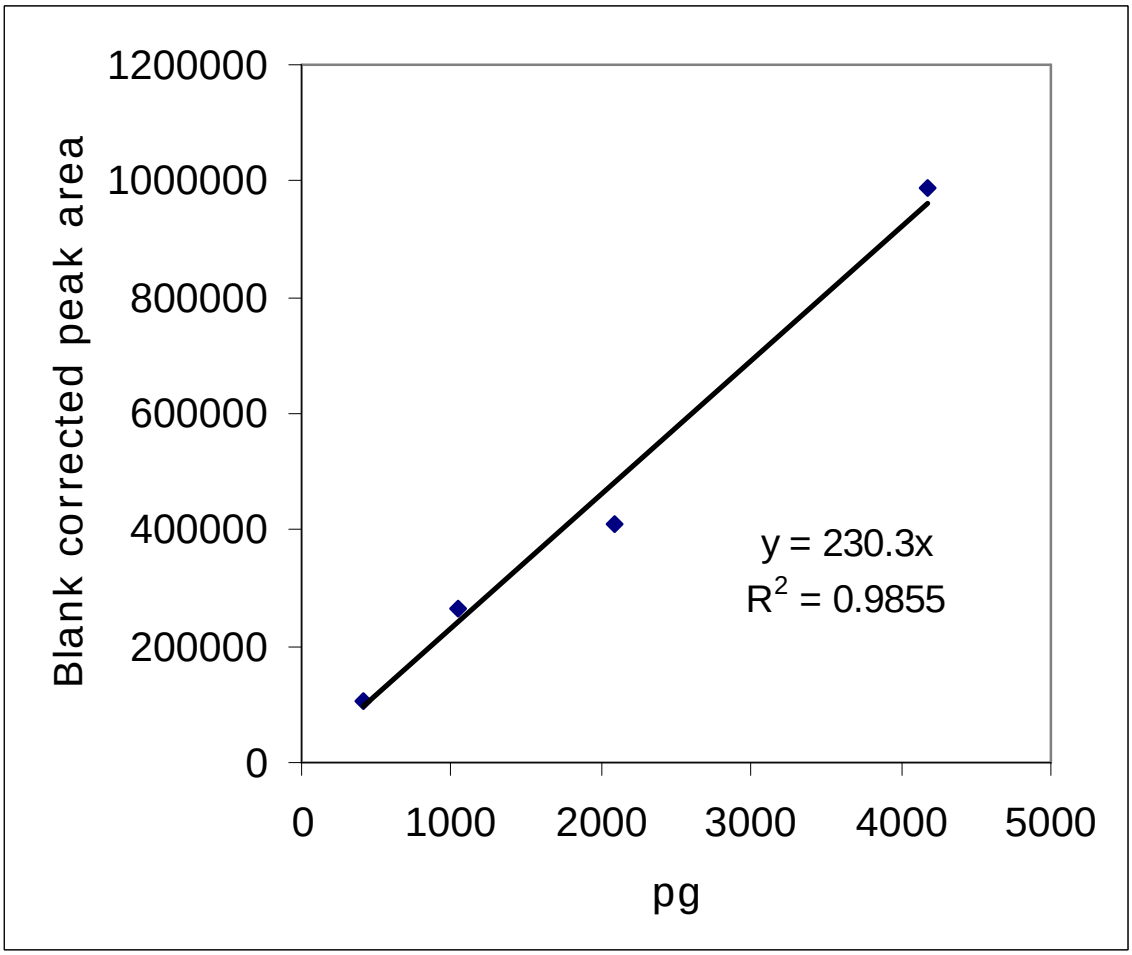


Figure 5.8. Typical calibration line for the determination of isoprene by GC-MS. The linearity of assay response to isoprene was determined by GC-MS analysis of a series of isoprene calibrants (n=4). Calibration lines were included in the analysis of every batch of trial samples.

Analyte	Regression Equation	Correlation Coefficient
ETHANE	y = 75.21x	0.9998
	y = 41.946x	0.9996
	y = 70.374x	0.9972
	y = 56.181x	0.9941
	y = 56.513x	0.9942
	y = 38.653x	0.9933
Mean gradient	56.48	0.9964
%RSD (n=6)	25.9%	0.29%
BUTANE	y = 565.54x	0.9996
	y = 297.96x	0.9991
	y = 539.95x	0.9992
	y = 3229.6x	0.9984
	y = 486.08x	0.9995
	y = 298.3x	0.9966
Mean gradient	902.88	0.9987
%RSD (n=6)	126.9%	0.11%
PENTANE	y = 469.13x	0.9879
	y = 209.28x	0.9994
	y = 405.98x	0.9985
	y = 322.33x	0.9977
	y = 373.74x	0.9990
	y = 212.19x	0.9998
Mean gradient	332.11	0.9970
%RSD (n=6)	31.7%	0.48%

Table 5.1. Statistical summary of precision of assay response and assay linearity following GC-MS analysis of a series of ethane, butane and pentane calibrations standards. Summary of typical between day variation in the regression equation and correlation coefficients obtained following the GC-MS analysis of a series of ethane, butane and pentane calibration standards.

5.3.1.3 Assay Sensitivity

The limits of detection for ethane, butane and pentane were determined to be 0.02ppb. In the adult population, the 95% reference range for breath ethane levels using this technique is from 0.05 to 2.1 parts per billion (n=70 subjects) (Glen *et al* 2003).

5.3.2 Clinical Rating Scales (PANSS, BPRS)

Clinical rating scales, as assessed by a psychiatric nurse, using PANSS and BPRS questionnaires were carried out with the consent of all participating volunteer schizophrenia patients resident at New Craig's Hospital. The findings are detailed in Table 5.2. (Note: there are more BPRS results than patient breath samples as some breath samples were not able to be analysed post collection).

Volunteer	Positive Symptoms	Negative Symptoms	General Psychopathology	BPRS
1	9	13	24	8
2	NA	NA	NA	NA
3	25	14	43	32
4	15	19	32	19
5	18	24	38	25
6	26	28	47	32
7	27	15	23	32
8	27	17	40	32
10	26	23	45	25
12	21	24	46	32
13	28	29	50	37
14	19	21	56	34
15	25	19	43	26
16	26	20	56	36
17-21	NA	NA	NA	NA
22	22	17	41	23
23-29	NA	NA	NA	NA
30	25	33	48	35
31-33	NA	NA	NA	NA
34	30	27	24	39
37-38	NA	NA	NA	NA
38	27	27	56	38
39	34	38	24	48
41	30	27	53	40
43-44	NA	NA	NA	NA
46	29	15	49	45
47-48	NA	NA	NA	NA
49	15	16	41	33
50	30	35	67	52
51-52	NA	NA	NA	NA
53	24	28	64	44
55	NA	NA	NA	NA
56	24	22	47	35
57	22	12	35	22
Total	604	563	1092	824
Mean	24.16	22.52	43.68	32.96
Standard Dev	6.51	7.05	12.03	9.61

Table 5.2: Clinical rating scales (PANSS, general psychopathology and BPRS) of all volunteer subjects. NA = not applicable.

Statistical analysis of PANSS and BPRS clinical ratings with breath and blood biochemical variables, ie ethane, pentane and total plasma MDA was undertaken (Table 5.3) and Pearson's correlation coefficient was determined. Using this test, r gives a measure of the strength of the relationship of the variables under examination (r =1 for a perfect straight line with upward slope, r = -1 for a perfect straight line with a downward slope). The results show that there is no correlation between the psychiatric clinic rating scales with the biochemical variables (Figure 5.9).

Correlations

		Positive Symptoms	Negative Symptoms	General Psychopathology	BPRS	Ethane	Pentane	Mean Total Plasma MDA (mM)
Positive Symptoms	Pearson Correlation	1	.537**	.214	.771**	.197	-.221	.299
	Sig. (2-tailed)		.006	.305	.000	.482	.490	.278
	N	25	25	25	25	15	12	15
Negative Symptoms	Pearson Correlation	.537**	1	.319	.637**	-.026	.005	.402
	Sig. (2-tailed)	.006		.120	.001	.928	.987	.137
	N	25	25	25	25	15	12	15
General Psychopathology	Pearson Correlation	.214	.319	1	.493*	.022	.080	.139
	Sig. (2-tailed)	.305	.120		.012	.938	.806	.622
	N	25	25	25	25	15	12	15
BPRS	Pearson Correlation	.771**	.637**	.493*	1	.092	-.241	.491
	Sig. (2-tailed)	.000	.001	.012		.744	.450	.063
	N	25	25	25	25	15	12	15
Ethane	Pearson Correlation	.197	-.026	.022	.092	1	-.012	.096
	Sig. (2-tailed)	.482	.928	.938	.744		.956	.634
	N	15	15	15	15	27	23	27
Pentane	Pearson Correlation	-.221	.005	.080	-.241	-.012	1	-.371
	Sig. (2-tailed)	.490	.987	.806	.450	.956		.082
	N	12	12	12	12	23	23	23
Mean Total Plasma MDA (mM)	Pearson Correlation	.299	.402	.139	.491	.096	-.371	1
	Sig. (2-tailed)	.278	.137	.622	.063	.634	.082	
	N	15	15	15	15	27	23	27

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 5.3. Statistical analysis using Pearson's correlation coefficient r, to determine correlation with the findings of the clinical rating scales and the biochemical variables.

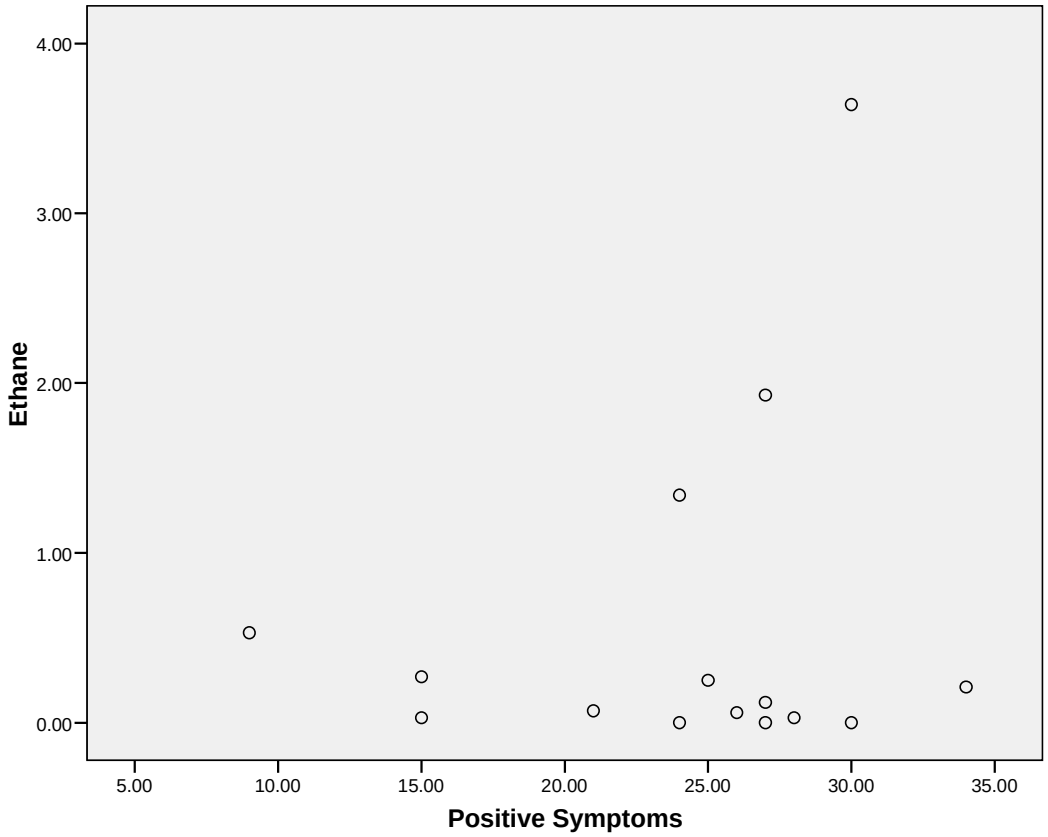


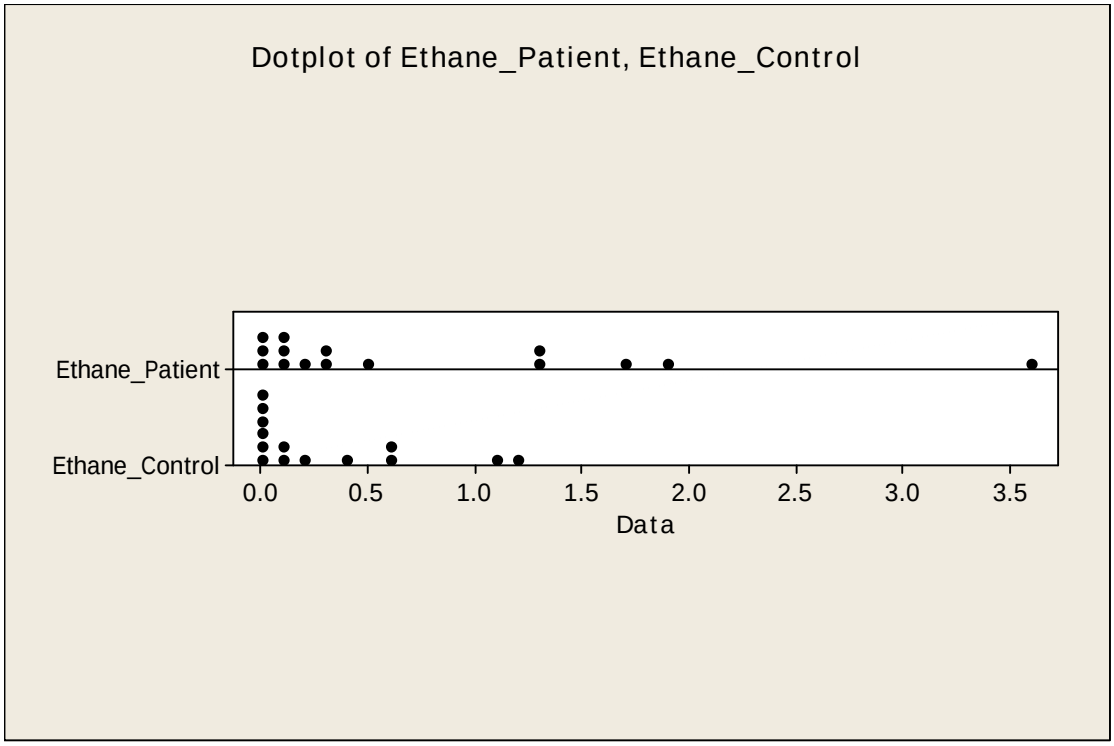
Figure 5.9. Scatter diagram detailing the random, non-linear relationship (correlation = 0.197, significance = 0.482) between the biochemical variable (ethane) with the findings from the rating scales (positive symptoms) of the patients with schizophrenia as determined by assessment using the PANSS questionnaire.

5.3.3 Determination of Breath Volatiles in Patient and Control Samples

This validated method was used to determine the level of exhalants in samples from trial participants. Tentative identification was made on comparison of Retention times (Rts) between calibration standards and participant samples, with definitive peak identity being assigned upon consideration of the fragmentation pattern and presence of the characteristic m/z of the characteristic ion.

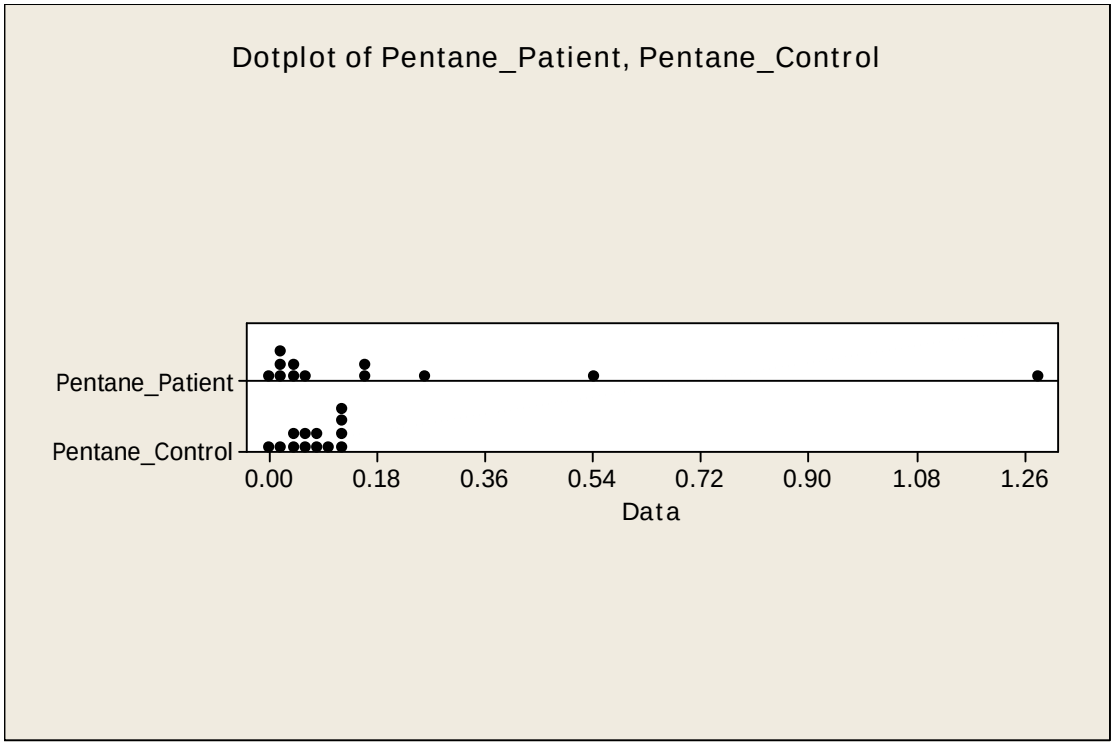
5.3.3.1 Breath Exhalants

Figures 5.10/11 shows dotplots for the ethane groups and pentane groups, revealing the existence of some extreme levels in the patient groups.



Median data:
Ethane_Patient (n=15) 0.250
Ethane_Control (n=14) 0.120

Figure 5.10. The dotplot shows mostly similar levels of ethane in both patient and controls groups with the exception of 3 patients who have ethane levels in excess of 1.5 and one patient reaching a level of 3.64.



Median
Pentane_Patient (n=12) 0.0480
Pentane_Control (n=13) 0.080

Figure 5.11. The dotplot reveals similar levels of pentane between the patient and control groups with the exception of 5 patients who show pentane levels in excess of 0.16. One patient has a pentane level of 0.54 and another at 1.28.

In conclusion, there was marginal evidence (P = 0.0840) that patients have a higher median level of ethane than the control group. There is no evidence of any difference in medians for pentane levels (P = 0.4458). However, for both ethane and pentane there are incidences of much higher levels for certain individuals in the patient groups.

5.3.4 Determination of Total Plasma Malondialdehyde in Patient and Control Samples

Patient and control total plasma MDA levels were measured utilising the TBARs assay and HPLC and the results are illustrated in Figure 5.12. Results of the Mann-Whitney test reveal that the level of MDA is similar between the patients and controls and therefore no statistical significant difference in the MDA levels can be seen between the two groups.

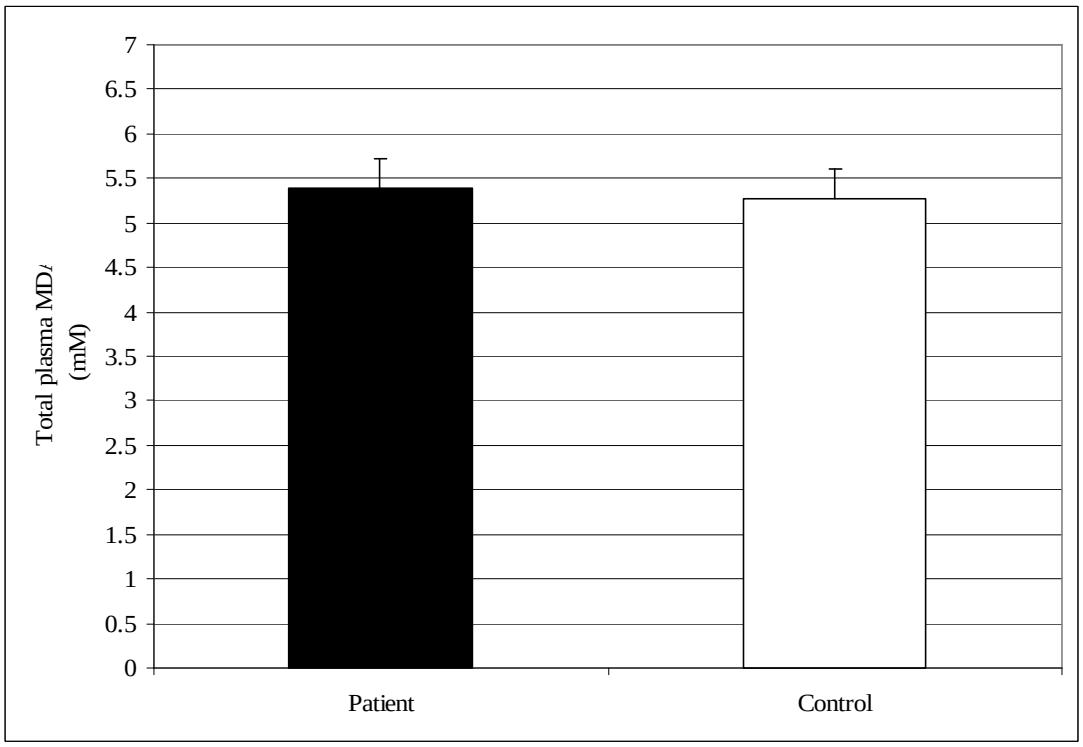


Figure 5.12. Investigation of total plasma MDA production in human schizophrenic patients (n=15) and control (n=14) samples as measured by TBARs and HPLC. Values are expressed as mean ± standard deviation of 5 assay replicates.

5.3.5 Comparison of Ethane, Pentane and Total Plasma Malondialdehyde in Male and Female Patient Samples

Levels of ethane, pentane and total plasma MDA were compared in the male and female patient samples (Figure 5.13). Although not statistically significant, on average, a higher level of ethane and MDA was detected in the male group than the female patient group. However, a comparison of the pentane levels in the two groups revealed that the female patient group had a higher average pentane level than the male patient group but again the results were not statistically significant.

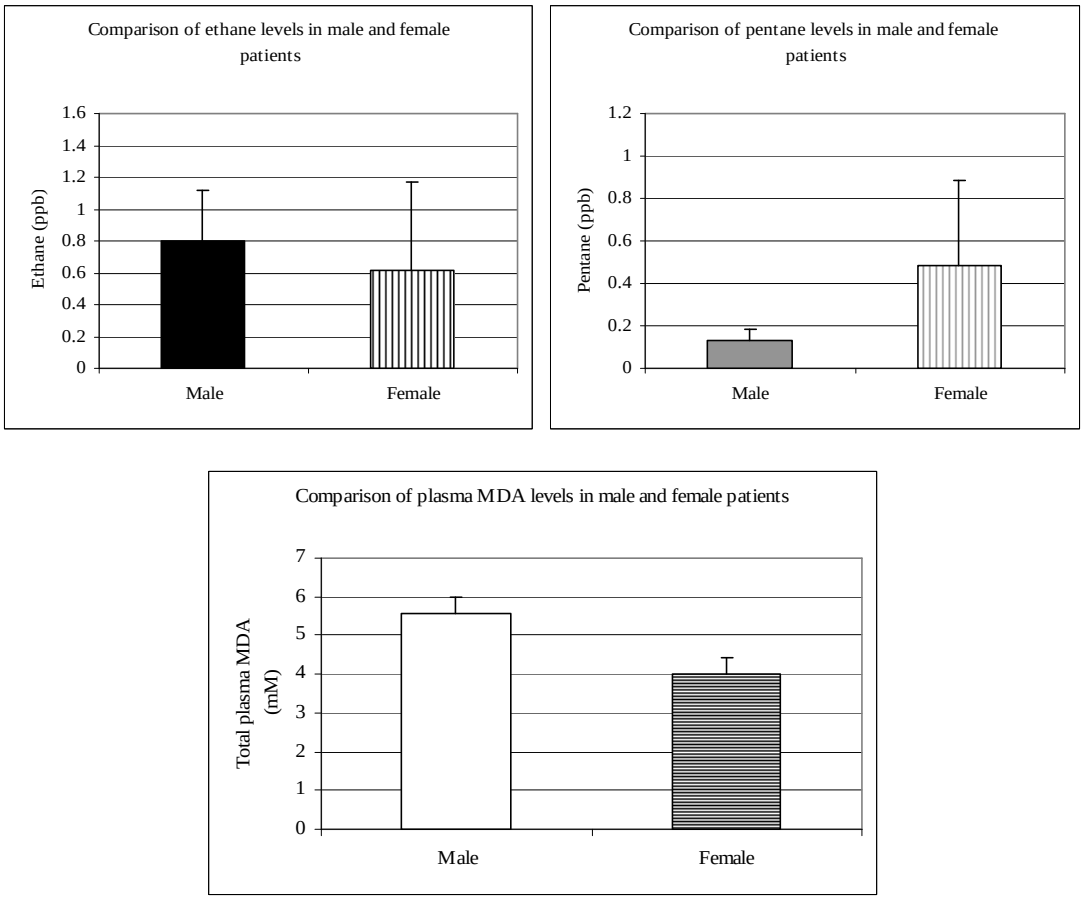


Figure 5.13. Evaluation of ethane and pentane levels measured by GC-MS and MDA levels measured by TBARs and HPLC in the patient male (n=12) and female (n=3) groups. Values are expressed as mean ± sem of 5 replicates.

5.4 Discussion

5.4.1 Breath Analysis

Breath analysis is a non-invasive and easy to use method to assess lipid peroxidation. In the first part of the human trial undertaken during this project, the level of breath hydrocarbons measured by gas chromatography-mass spectrometry (GC-MS) was compared in schizophrenic and control subjects. Although there were incidences of higher levels of both ethane and pentane in the patient group, suggesting that these individuals may be subjected to greater oxidative stress and hence greater levels of lipid peroxidation than individuals in the control group, no significant difference was found between the levels of ethane and pentane in the breath from the schizophrenic patients and control samples on the whole. In addition, plasma MDA levels were evaluated in the schizophrenic patients and controls but again there was no significant difference in the levels of plasma MDA between the two groups. Further analysis of the breath and MDA data from the schizophrenic group alone was undertaken to determine any gender differences and although ethane levels and MDA levels were higher in the male schizophrenic samples the data was not statistically significant. Only the pentane levels were higher in the female schizophrenic samples when compared to the male samples but these were not significantly greater. The lack of significance may be as a result of the small sample sizes, although it is possible that there is just no definite difference in the levels of oxidative stress between the two groups. However, any future work that may be carried out would probably benefit from larger sample sizes.

During the lipid peroxidation chain reaction process, saturated hydrocarbons such as ethane and pentane are eventually formed from the ω-3 and ω-6 fatty acids along with aldehydes such as MDA and *in vitro* studies have shown that ethane and

pentane are generated when cell cultures are exposed to ROS (reviewed by Miekisch *et al* 2004). As stable end products of lipid peroxidation, hydrocarbons show low solubility in blood and are excreted into the breath minutes following their formation in tissues and exhaled concentrations of ethane and pentane therefore can be used to monitor the degree of oxidative stress within the body (Risby and Sehnert 1999). Animal and clinical studies have also reported close correlation between clinical conditions where high levels of lipid peroxidation or inflammation occur and the exhalation of ethane and pentane generated through ROS attack on lipid membrane structures (Aghdassi *et al* 2000) while the same authors have also shown that levels of exhaled pentane and ethane correlate well with other markers of lipid peroxidation including MDA and TBARs (Aghdassi *et al* 2003). Although the comparison was made, we were unable to demonstrate a correlation between the two different methods of determining lipid peroxidation or plasma MDA levels, or any correlation between these variables and the clinical rating scales. Furthermore, we did not observe any differences in the mean population levels of breath hydrocarbons or MDA between the patient and control group.

As mentioned earlier incidences of higher levels of ethane and pentane were noted in the patient group, however, only one patient had an ethane level falling outside the 95% reference range for breath ethane levels as determined by Glen *et al* 2003. Why this patient appears to produce higher levels of breath ethane is unknown. Volatile organic compounds (VOCs) such as ethane and pentane may be produced endogenously ie in the body or they may be exogenously generated ie absorbed as contaminants from the environment. Furthermore, the composition of VOCs in breath varies hugely between individuals both qualitatively and quantitatively (Cao and Duan 2006). Increased levels of oxidative stress might be a cause and perhaps

the type of medication that the patient is receiving may also be a factor. Gama *et al* (2006) recently reported serum TBARs to be significantly higher in patients receiving clozapine than those under haloperidol treatment although it is possible that the increase in TBARs levels may be as a result of the course of the disease rather than the medication, as may be the case with our patient.

Markers of oxidative stress have been reported in the breath of patients with schizophrenia by Phillips *et al* (1993), and Glen *et al* (2003). Breath analysis is also being used more commonly to determine oxidative stress and inflammation in a variety of diseases such as asthma (Paredi *et al* 2000), lung diseases (Kanoh *et al* 2005) and Crohn's disease (Aghdassi *et al* 2003) where increased levels of both ethane and pentane have been reported and the method is receiving support as a non-invasive method of determining lipid peroxidation. But if the technique is such an easy one to use, why is it not even more commonplace in clinical practice? It would seem that there are some obstacles, mainly technical problems. Yet, there appears to be some improvement of these problems since development and technical progress is being made which will enable improved and simplified analytical techniques being utilised for routine use in the future (Miekisch *et al* 2004). There are however, a number of advantages of using breath analysis over the existing serum and urine analytical techniques such as i) breath samples closely reflect the arterial concentrations of biological substances and may help to prevent the more difficult collection of arterial blood samples; ii) breath is a less complicated mixture than serum or urine and a complete analysis of all its compounds is possible; iii) breath analysis provides direct information on respiratory function that is not obtainable by other means and iv) it is easy to use and non-invasive (reviewed by Cao and Duan 2006).

5.4.2 Thiobarbituric Acid Reactive Substances

The measurement of MDA by the TBARs method is widely debated but although it has its pitfalls, it is still one of the most commonly reported indices of oxidative damage. Plasma MDA levels have been found to be elevated in patients with schizophrenia when compared to normal controls (Zhang *et al* 2007; Medina-Hernandez *et al* 2007; Gama *et al* 2006; Zhang *et al* 2006; Khan *et al* 2002; Kuloglu *et al* 2002), while only one recent study (Skinner *et al* 2005) reported lower levels of MDA measured by TBARs in CSF from schizophrenic patients compared to controls. These are in contrast to our study which has shown no difference in the level of plasma MDA from schizophrenic patients compared to controls, although the MDA levels in our volunteers appear to be much higher than the reported normal ranges of <1.0 micromol/litre as reported by Kropp *et al* 2005. Nevertheless, these results are similar to our earlier findings (Young *et al* 2007) where we reported no significant difference in the levels of MDA and hydroxyalkenals in cryopreserved plasma samples from control and schizophrenic samples. Furthermore, between sex comparisons of the level of plasma MDA and hydroxynonenal from control and schizophrenic patients also revealed no statistically significant difference (Young *et al* 2007).

Whilst a few studies have reported evidence of elevated TBARs in drug naïve patients (Skinner *et al* 2005; Arvindakshan *et al* 2003) most studies reporting an increase in TBARs have focused on schizophrenic patients treated with typical neuroleptic (eg haloperidol) regimes (Gama *et al* 2006; Kropp *et al* 2005). In our study, patients were treated with a range of psychotropic agents, both typical and atypical neuroleptics. Parikh *et al* (2003) reported an increase in lipid peroxidation in the brains of rats chronically treated with haloperidol but not in animals chronically

treated with risperidone, olanzapine or clozapine, while Kropp *et al* (2005) observed MDA levels in schizophrenic patients receiving clozapine, quetiapine, amisulpride and risperidone were significantly lower than those group of patients treated with first generation (typical) antipsychotics. In contrast, Gama *et al* (2006) found that serum TBARs was significantly higher ($p = 0.008$) in schizophrenic patients treated with clozapine than those treated with haloperidol. Thus the effects of neuroleptic treatment are unclear, effects of species and tissue further confound the field and essentially further investigations are required, the design of which should be sufficiently large as to enable a detailed examination of treatment effects on a profile of oxidative indices.

CHAPTER 6

Examination of biomarkers of oxidative stress in schizophrenia patients and control subjects.

Human trial part 2.

6.1 Introduction

Oxidative stress occurs when there is disequilibrium between pro-oxidant processes and the antioxidant defence system in favour of the former. Oxidative stress often occurs as a result of increased production of free radicals, or even when the antioxidant defence system is ineffective or a combination of both events and will lead to a free radical attack of protein, DNA and lipids. Direct measurement of reactive species and free radicals is impractical because they are short-lived, due to their highly reactive nature. Thus, biomarkers have been used to reflect the degree of oxidative damage in light of a particular clinical or research interest ie. disease or disorder state (Peoples and Karnes 2005).

6.1.1 Biomarkers

A biomarker enables the identification of a modified biological molecule and provides understanding of the nature of the denaturing radical along with the location of oxidative damage. As highly sensitive markers of oxidative damage in mammalian systems, biomarkers may provide information on three progressive levels to disease outcome: a) as measurable endpoints of oxidative damage to proteins, amino acids, oxidised lipids, oxidised DNA bases; b) as functional markers of blood flow, platelet aggregation, or cognitive function, for example and c) as endpoints related to a specific disease (Griffiths *et al* 2002). Although clinical symptoms are considered to be endpoints of an illness, they are often inappropriate for early detection and disease prevention. Increasing evidence supports the view that a number of neuropathological changes seen in schizophrenia may be the result of increased free radical-mediate or ROS mediated neuronal injury (Mahadik and Mukherjee 1996).

6.1.2 Lipid Biomarkers

The process of lipid peroxidation includes oxidative chain reactions of fatty acids where several measurable products may be produced and numerous lipid peroxidation biomarkers exist in the majority of human diseases. The peroxidation of PUFAs may be monitored in a number of ways including determination of lipid peroxides (Chamblee *et al* 2000), determination of aldehydic end products such as MDA and 4-HNE (Boyle *et al* 2000; Claeson *et al* 2001), determination of volatile hydrocarbons and by determination of isoprostanes (Morrow *et al* 1990; Boyle *et al* 2000). The prostaglandin-like isoprostanes are thought to be specific markers of lipid peroxidation since their production is non-enzymatic. F_2-isoprostanes formed from the peroxidation of arachidonic acid, have represented the bulk of isoprostane research. Recently there has been a greater focus on F_4-neuroprostanes which originate from peroxidation of docosahexaenoic acid, the major fatty acid in the brain (Reich *et al* 2000). F_4-neuroprostanes may therefore reflect oxidative injury to nervous system tissue (reviewed by Peoples and Karnes 2005).

6.1.3 Protein Biomarkers

The measurement of protein markers as indicators of oxidative stress may involve several products due to the fact that there are 20 amino acids available for oxidative attack. Oxidatively modified proteins that result in new functional groups such as hydroxyls and carbonyls are not repaired and must be removed by proteolytic degradation. Amino acids capable of delocalising charge such as those containing aromatic and thiol side chains are more open to oxidative attack and numerous aliphatic residues are also the focus of oxidation resulting in the generation of protein carbonyl moieties (Griffiths *et al* 2002). Ultimately therefore, a decrease in the efficiency of proteolysis will lead to an increase in the cellular content of

oxidatively modified proteins (Stadtman and Levine 2000). The level of modified molecules can be quantitated in a variety of ways, for example by the measurement of the protein carbonyl content of an ELISA (Buss *et al* 1997). Elevated markers of protein oxidation have been associated with diseases such as Alzheimer's disease, Parkinson's disease, Duchenne muscular dystrophy, amoytrophic lateral sclerosis, rheumatoid arthritis and progeria (Mayne 2003).

6.1.4 DNA Biomarkers

Oxygen and ROS have been shown to induce many types of DNA damage. ROS (ie. hydroxyl radical) can alter the deoxyribose-phosphate backbone, cause DNA-protein cross-links and modify both purine and pyrimidine bases. It has been suggested that approximately 2 x 10^4 DNA damaging events occur in every cell of the human body every day (Foray *et al* 2003) with a significant proportion of the damage occurring as a result of ROS. Although at physiological levels ROS play a key role in regulating signalling pathways and gene expression (Durocher and Jackson 2001), excessive insult to the cell can be fatal. Repair of oxidized DNA *in vivo* is accomplished by glycosylases (bases) and endonucleases (deoxynucleotides) (Figure 6.1). Nevertheless, some of these modified DNA bases have considerable potential to damage the integrity of the genome. Guanine most readily undergoes oxidative attack, possessing the lowest oxidation potential of the four bases (Peoples and Karnes 2005). Consequently, the nucleoside 8-oxo-2'deoxyguanosine (8-OHdG) is one of the most critical lesions and most studied biomarker of oxidative damage (Wu *et al* 2004).

Evidence has indicated that urinary 8-OHdG not only is a biomarker of generalized, cellular oxidative stress but might also be a risk factor for illnesses such as cancer,

atherosclerosis and diabetes. For example, elevated level of urinary 8-OHdG has been detected in patients with various cancers (Kuo *et al* 2007; Wada *et al* 2006). Elevated urinary 8-OHdG and leukocyte DNA have also been detected in diabetic patients with hyperglycaemia, with the level of urinary 8-OHdG in diabetes correlating with the severity of diabetic nephropathy and retinopathy (Wu *et al* 2004). Upon DNA repair, 8-OHdG is excreted in the urine; however, unless repaired prior to DNA replication, 8-oxo-2'deoxyguanosine residues can result in base pair transversions (Griffiths *et al* 2002) (Figure 6.1). Rydberg and Johanson (1978) were the first to attempt to quantify DNA strand breaks while Ostling and Johanson (1984) further developed a microelectrophorectic technique, the SCGE assay or comet assay.

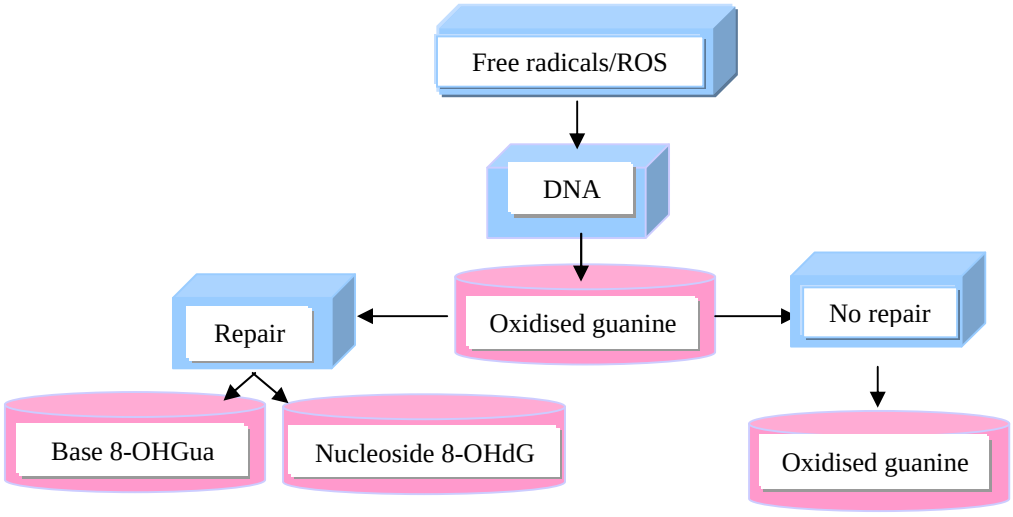


Figure 6.1. Pathway of commonly measured biomarkers of oxidative stress (adapted from Peoples and Karnes 2005).

6.1.5 Oxidative Stress in Schizophrenia

Although the aetiology of schizophrenia remains unknown, investigation of the effects of oxidative stress in schizophrenia has largely focused on the determination of lipid oxidation products (Scottish Schizophrenia Research Group 2000; Khan *et al* 2002). So far, studies of oxidative injury in schizophrenia have focused on membrane lipids with relatively little information available on oxidative damage to cellular proteins and DNA. To date, only one study has examined the effects of oxidative insult on the cellular DNA of a Greek sample of male schizophrenic patients and controls (Psimadas *et al* 2004). The authors did not observe any difference in basal levels of DNA damage between schizophrenic and normal populations in cryopreserved cells. However, the research reported here is the first to investigate the effects of oxidative stress, on the level of DNA damage in peripheral blood lymphocytes, in a British population. Previous work has noted a disparity in erythrocyte PUFA stability when stored at -20^{o}C (Fox *et al* 2003). Consequently, the research reported here determined the level of DNA damage in both fresh and cryopreserved lymphocyte samples.

The main aim of this study therefore, was to try to ascertain whether schizophrenic subjects exhibit enhanced levels of oxidative stress as indicated by changes in a profile of biomarkers compared to healthy matched controls. Equally importantly, the effects of diet on schizophrenia outcome has recently been addressed (Peet 2004) and since there are clear differences between the habitual diet of a Greek and British population, the research sought to examine the effects of disease state on the levels of endogenous and H_2O_2-induced DNA damage and plasma vitamin C levels in an indigenous British population.

6.2 Materials and Methods

6.2.1 Examination of Biomarkers of DNA Oxidation in Peripheral Blood Lymphocytes from Schizophrenia Patients and Control Subjects

6.2.1.1 Materials

Lymphoprep lymphocyte separation medium (LSM) (specific gravity 1.077 ± 0.001g/ml) was supplied by Robins Scientific (Solihull, UK). Dutch Modified RPMI 1640 medium was obtained from Sigma (Poole, UK). Heat-inactivated FCS was obtained from Invitrogen (Paisley, UK). Microscope slides and cover glasses were supplied from Fisher Scientific (Loughborough, UK). LMP and NMP agarose were supplied by Gibco Life Technologies (Paisley, UK). DAPI was obtained from Sigma (Poole, UK). Corning centrifuge tubes were supplied by Fisher Scientific (Loughborough, UK). Vacutainers were obtained from Aberdeen Royal Infirmary, (Aberdeen, UK).

6.2.1.2 Study Design – Lymphocyte Collection, Storage and Analysis

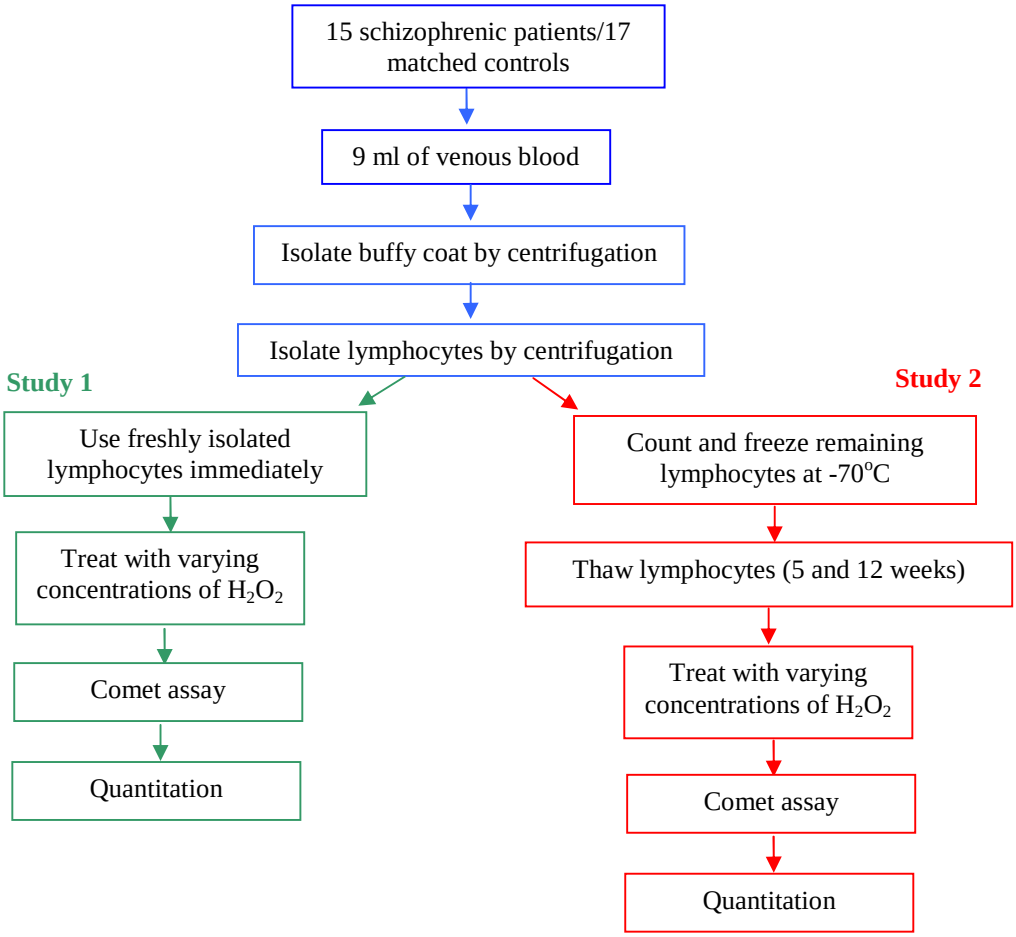


Figure 6.2. Human study, study 1 and 2. Experimental design for lymphocyte collection, storage and analysis of DNA damage.

6.2.1.3 Ethical Approval and Consent

Ethical approval was obtained from the LREC and all procedures were carried out in accordance with the Helsinki Declaration (1975) and the Data Protection Act (1998). Schizophrenic patients and apparently healthy controls were recruited and informed consent obtained from ward patients and staff at the New Craigs Hospital, Inverness. The trial protocol was reviewed by ward consultants at the hospital and participants matched for age, gender and smoking status.

6.2.1.4 Subjects

Of the 32 volunteers (27 smokers and 5 non-smokers), the schizophrenic group comprised 11 male and 4 female patients (average age 37.9 ± 11.0 years) and 12 male and 5 female healthy controls (average age 38.9 ± 9.2 years). Smoking habit was assessed by a self-reported lifestyle questionnaire. There was a range of smokers and non-smokers in the male and female populations with the maximum smoking habit in each population being 30 cigarettes per day. This equated to an average (self-reported) daily intake of cigarettes of 12 and 14 in the female and male populations, respectively. No significant difference in the smoking habit of the patient/control groups was noted and similarly there was no significant difference in the smoking habit of male and female schizophrenic patients.

6.2.1.5 Drug Therapy

The schizophrenic group comprising 12 male and 4 female patients were on a range of drug therapies which for the male patients included antipsychotics (n=3, Zuclopenthioxol Decanoate; n=1 Haloperidol; n=1 Chlorpromazine), atypical antipsychotics (n = 3 Clozapine; n = 4 Risperidone; n = 1 Olanzapine), SSRIs (n = 2 Fluoxetine), antianxiety/antipsychotic (n=1 Trifluoperazine), H2-receptor antagonists (n=1 Cimetidine), b blockers (n=1 Lorazepam and Diazepam; n=1 Diazepam; n=1 Lorazepam) and antidepressant therapy (Venlafaxine XL). Whilst the female patients were being treated with a range of drug therapies including antipsychotics (n=2 Zuclopenthixol Decanoate), atypical antipsychotics (n=2 Quietapine), SSRIs (n=2 Fluoxetine), antianxiety/antipsychotic (n=2 Trifluoperazine), anxiolytics (n=1 Diazepam) and antimuscarinic therapy (n=1 Procyclidine).

6.2.1.6 Isolation and Cryopreservation of Human Lymphocytes

Venous blood (1 x 9ml) was collected by venepuncture at the New Craigs Hospital. The whole blood was centrifuged at 2400x*g* for 15min at 4^{o}C, the buffy coat (~2ml) removed and diluted 1:1 with RPMI. Samples were retained on ice until transportation to the laboratory enabled the remainder of the separation procedure to be completed. The buffy coat was layered onto an equal volume of LSM before centrifuging at 700x*g* for 30min at 20^{o}C. The lymphocytes were transferred to a fresh centrifuge tube, washed using RPMI medium and spun for a further 15min under the same conditions. The supernatant was decanted, the cells resuspended in RPMI containing 10% heat-inactivated FCS and counted using a Neubauer Improved Haemocytometer. Isolated lymphocytes were either used (or treated) immediately (freshly isolated lymphocytes), (study 1, Figure 6.2) as described previously or were centrifuged and resuspended in 0.5ml aliquots at 3x10^{6} cells/ml in freezing mix (90% v/v heat-inactivated FCS and 10% v/v DMSO), frozen at -1^{o}C/min in polystyrene and stored at -70^{o}C (study 2, Figure 6.2).

6.2.1.7 Hydrogen Peroxide-induced DNA Strand Breakage in Fresh Lymphocytes Measured Using the Comet Assay (Study 1)

Freshly isolated lymphocytes were incubated in microcentrifuge tubes with hydrogen peroxide (H_2O_2) (50, 100 or 200μM in phosphate-buffered saline (PBS) for 5min on ice), washed and suspended in 140μl of 1% (w/v) LMP agarose in PBS pH 7.4 at 37^{o}C and immediately pipetted onto a glass microscope slide pre-coated with a layer of 1% (w/v) NMP agarose prepared in distilled water. The agarose was allowed to set for 5min at 4^{o}C and the slides were incubated in lysis solution (2.5M NaCl, 10mM Tris, 100mM NA_2EDTA, 10M NaOH to pH 10.0 and 1% v/v Triton X-100) at 4^{o}C for 1h to remove cellular proteins. After lysis, the slides were aligned in a

210mm wide horizontal electrophoresis tank containing buffer (1mM NA_2EDTA and 0.3M NaOH, pH 13.0) for 40min before electrophoresis at 21v for 30min (at an ambient temperature of 4^{o}C with the temperature of the running buffer not exceeding 15oC). The slides were washed three times at 4^{o}C for 5min each with neutralizing buffer (0.4M Tris-HCl, pH 7.5) before staining with 20µl of DAPI (1µg/ml).

6.2.1.8 Hydrogen Peroxide-induced DNA Strand Breakage in Cryopreserved Lymphocytes (Study 2)

Lymphocytes cryopreserved for 5 and 12 weeks were thawed on ice until the last trace of ice had disappeared. The aliquots were centrifuged at 200x*g* for 3min to remove freezing mix and the lymphocyte pellet resuspended gently in 0.4ml of RPMI + 10% (v/v) heat-inactivated FCS. 5 week cryopreserved lymphocytes were washed once in PBS and incubated in microcentrifuge tubes H_2O_2 (10, 25, 50, 200µM in PBS for 5min on ice). A change in the study design meant that 12 week cryopreserved lymphocytes were washed once in PBS and incubated in microcentrifuge tubes H_2O_2 (10, 25, 50µM concentrations only, in PBS for 5min on ice). The cells were resuspended immediately in LMP agarose for detection of DNA strand breakage by Comet analysis.

6.2.1.9 Quantitation of the Comet Assay

Nucleoids were scored visually using a Leica fluorescence microscope. One hundred comets from each gel (scored at random) were classified into one of five classes according to the relative intensity of fluorescence in the tail and given a value of 0-4 (from undamaged, 0, to maximally damaged, 4). The total score for 100 comets can range from 0 (all undamaged) to 400 (all maximally damaged) and is expressed in arbitrary units. This method of visual classification has been extensively validated by

comparison with comets selected using computerized image analysis. The slides from Study 1 and Study 2 were blinded so that treatment groups could not be identified.

6.2.1.10 Statistical Analysis

The Kolmogorov-Smirnof test, Student's t-test and one-way ANOVA were carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

6.2.2 Investigation of Biomarkers of Protein Oxidation in Plasma from Schizophrenia Patients and Control Subjects

6.2.2.1 Materials

Protein carbonyl ELISA kit components were supplied by Zenith Technology (Dunedin, New Zealand) and contained: ELISA 96-well plate and sealing tape, EIA buffer powder, blocking reagent, dinitrophenylhydrazine (DNP), guanidine hydrochloride diluent, anti-DNP-biotin-antibody, streptavidin-horseradish-peroxidase, chromatin reagent, stopping reagent, standards (5) and carbonyl control samples (low, medium and high).

6.2.2.2 Study Design – Plasma Collection, Storage and Analysis

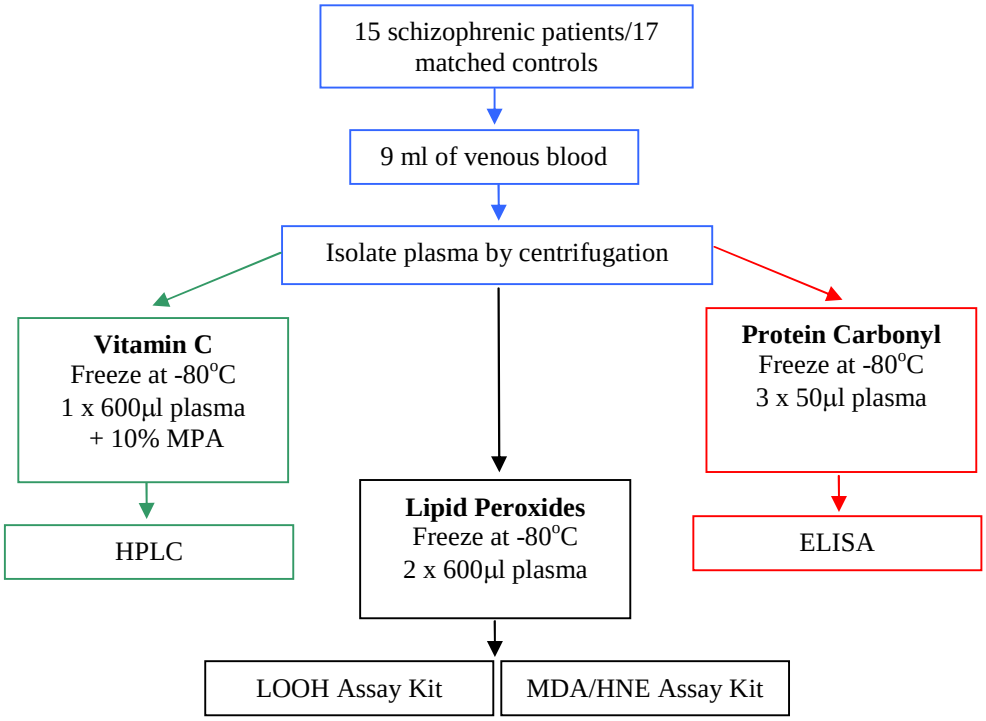


Figure 6.3. Human study. Experimental design for plasma collection, storage and analysis of vitamin C, protein carbonyls and lipid peroxides.

6.2.2.3 Collection and Storage of Human Plasma

Venous blood (1 x 9ml) was collected by venepuncture at the New Craigs Hospital. The whole blood was centrifuged at 2400x*g* for 15min at 4°C and the plasma (~1.5ml) was aliquotted into a microcentrifuge tube. From this aliquot, 50µl of plasma per tube was transferred into 3 microcentrifuge tubes and kept on dry ice until samples were transported back to the lab and placed in storage at -80°C.

6.2.2.4 Pre-assay Preparation

Buffers and reagents were prepared as per protocol instructions. Plasma samples were allowed to thaw at room temperature and centrifuged before use.

6.2.2.5 Sample Derivatisation with DNP

1.5ml microcentrifuge tubes were labelled as appropriate and 200µl of diluted DNP-solution was added to each tube. 5µl of each sample, standard or control was added to the appropriate tube and contents were mixed and incubated for 45min. A complimentary set of 1.5ml microcentrifuge tubes for samples, standards and controls were prepared and 1ml of EIA buffer was added to each tube.5µl of each DNP-treated sample was added to the appropriate tube and mixed thoroughly.

6.2.2.6 ELISA Procedure

200µl of each sample in EIA-buffer (plasma from schizophrenic or control subjects, blinded to the examiner) was added into each of the assigned ELISA-plate wells, covered with sealing tape and incubated for 2h at 37^{o}C. Following incubation, the plate was washed with EIA buffer (5 x approximately 300µl per well). 250µl of diluted blocking solution was added per well and incubated for 30min at room temperature. The plate was washed with EIA buffer as before. 200µl of diluted anti-DNP-biotin-antibody was added to each well and incubated for 1h at 37^{o}C. Following plate washing as before, 200µl of diluted streptavidin-HRP was added to each well and incubated for 1h at room temperature. The plate was washed for a final time as before.

6.2.2.7 Colour Development and Measurement

Chromatin reagent (200µl) was added to each well and the colour was left to develop for 4-7min. The reaction was stopped by adding 100µl of stopping reagent to each well to ensure that all wells were exposed to the chromatin reagent for the same amount of time. The plate was shaken gently to mix reagents and absorbance at

450nm was read directly after the addition of the stopping reagent. All measurements were performed in triplicate.

6.2.2.8 Statistical Analysis

The Kolmogorov-Smirnof test was used to examine the normality of the data and non-parametric tests, ie. the Mann-Whitney and Kruskal-Wallis tests were carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

6.2.3 Investigation of Biomarkers of Lipid Peroxidation in Plasma from Human Schizophrenic and Control Subjects

6.2.3.1 Materials

Lipid Peroxidation Assay Kit was supplied by EMD Biosciences Inc, La Jolla, CA, USA and Lipid Peroxidation Assay Kit II was supplied by Calbiochem, San Diego, CA, USA.

6.2.3.2 Collection and Preparation of Human Plasma

From the 1.5ml of collected plasma (section 6.2.2.1.3), 600µl of plasma per tube was aliquotted into 2 microcentrifuge tubes and kept on dry ice until samples were transported back to the lab and placed in storage at -80^{o}C.

6.2.3.3 Determination of Lipid Peroxidation in Cryopreserved Human Plasma

MDA and HAE are end products derived from peroxidation of PUFA and related esters. Measurement of such aldehydes provides a convenient index of lipid peroxidation. The Calbiochem Lipid Peroxidation Assay Kit employs a chromogenic reagent which reacts with MDA and HAE at 45^{o}C. Condensation of one molecule of

either MDA or HAE with 2 molecules of chromogenic reagent yields a stable chromophore with maximal absorbance at 586nm. Lipid hydroperoxides were measured using the Lipid Peroxidation Assay Kit II which utilises a simple direct colorimetric measurement of lipid hydroperoxides measured by UV detection at 560nm.

6.2.3.4 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data. Student's t-test and one-way ANOVA was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

6.2.4 Investigation of Vitamin C Content in Plasma from Schizophrenic and Control Subjects

6.2.4.1 Collection and Preparation of Human Plasma

From the 1.5ml of collected plasma, 600µl of plasma per tube was aliquotted into a microcentrifuge tube and mixed with 10% w/v metaphosphoric acid (MPA). Samples tubes were kept on dry ice until samples were transported back to the lab and placed in storage at -80^{o}C.

6.2.4.2 HPLC Analysis

Cryopreserved human schizophrenic and control subject plasma samples treated in MPA were transported to Dr Garry Duthie, Molecular Nutrition Group, Rowett Research Institute, Aberdeen, on ice. On arrival samples were transferred to -80^{o}C freezer for storage. Plasma samples were analysed by reverse-phase HPLC with UV detection at 263nm (Boyle *et al* 2000).

6.2.4.3 Statistical Analysis

The Kolmogorov-Smirnof test was used to test the normality of the data and Student's t-test was carried out as appropriate using SPSS 11.5 for Windows. For statistical analysis, a level of 0.05 was used to determine significance.

6.2.4.4 Acknowledgements

I gratefully acknowledge the assistance of Siofradh McKinney, The Ness Foundation, Inverness for recruiting volunteers, undertaking the PANSS interviews and for her help with the collection of blood samples. In addition I acknowledge the assistance provided by Dr G G Duthie's group, Rowett Research Institute in undertaking the contract analysis of plasma vitamin C.

6.3 Results

6.3.1 Examination of Biomarkers of DNA Oxidation in Peripheral Blood Lymphocytes from Schizophrenia Patients and Control Subjects

Following 5 minutes treatment with H_2O_2 at concentrations of 0, 50 and 200μM, the mean extent of endogenous and H_2O_2-induced DNA damage in freshly isolated lymphocytes was measured and compared in control and schizophrenic subjects. There is a small difference in the extent of DNA damage between schizophrenics and the control group (Figure 6.4), although not statistically significant.

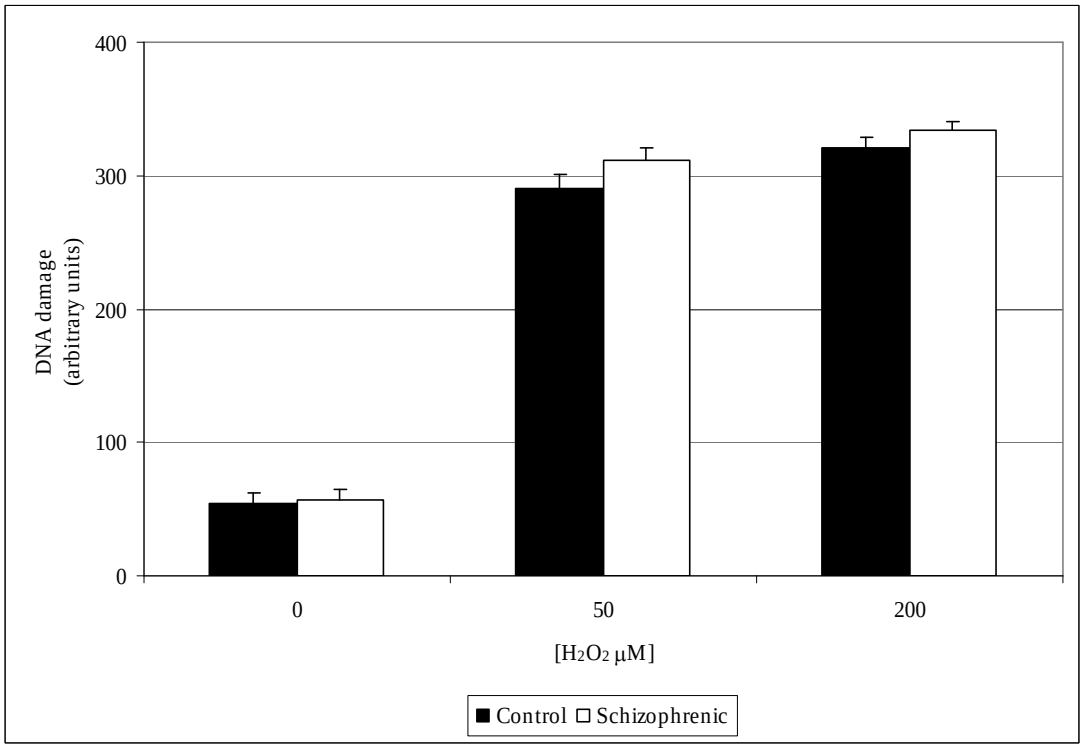


Figure 6.4 Study 1. Comparison of endogenous and H_2O_2-induced DNA damage in freshly isolated lymphocytes from control subjects and schizophrenic patients. Levels of DNA damage measured with the comet assay in freshly isolated control and schizophrenic lymphocytes following treatment with 0, 50 and 200μM of H_2O_2. Data show the mean ± sem DNA damage of control (n=17) and schizophrenic samples (n=15).

Examination of endogenous and H_2O_2-induced DNA damage in the freshly isolated lymphocytes of male and female schizophrenic patients, however, revealed significantly greater ($p < 0.05$) DNA damage in male than female patients in the levels of both basal and H_2O_2-induced DNA damage (Figure 6.5).

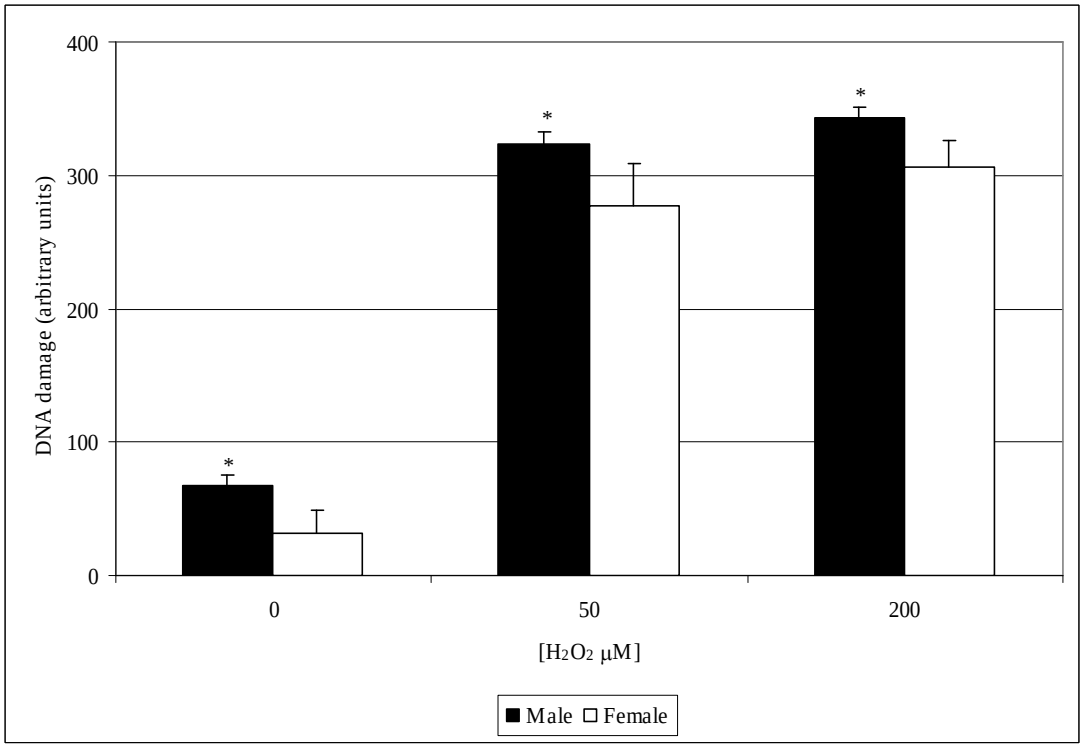


Figure 6.5 Study 1. Comparison of endogenous and H_2O_2-induced DNA damage in freshly isolated lymphocytes of male and female schizophrenia patients. Levels of DNA damage measured with the comet assay in freshly isolated male and female schizophrenic lymphocytes following treatment with 0, 50 and 200µM of H_2O_2. Data show the mean ± sem DNA damage of male (n=11) and female (n=4) schizophrenic samples. Significance is denoted by * where $p < 0.05$.

6.3.2 H_2O_2-induced DNA Strand Breakage in Cryopreserved Lymphocytes from Control Subjects and Schizophrenia Patients (Study 2)

Thawed cells were treated for 5 minutes with H_2O_2 at concentrations of 0, 10, 25, 50 and 200μM. Endogenous and H_2O_2-induced DNA damage in cryopreserved lymphocytes was measured and compared in the control and schizophrenic samples. No significant difference was found between the levels of endogenous DNA damage in the control and schizophrenic groups as illustrated in Figure 6.6. Similarly there was no difference in the susceptibility of DNA damage from control or schizophrenic subjects at each of the H_2O_2 concentrations examined (Figure 6.6).

Following statistical analysis using ANOVA, no significant differences were found in levels of endogenous and H_2O_2-induced DNA damage in the male and female schizophrenic patients' cryopreserved lymphocytes (Figure 6.7). However, a student's t-test did reveal that after 5 weeks cryopreservation, the level of DNA damage in 50μm H_2O_2-induced cells was statistically significantly greater in males than females (Figure 6.7).

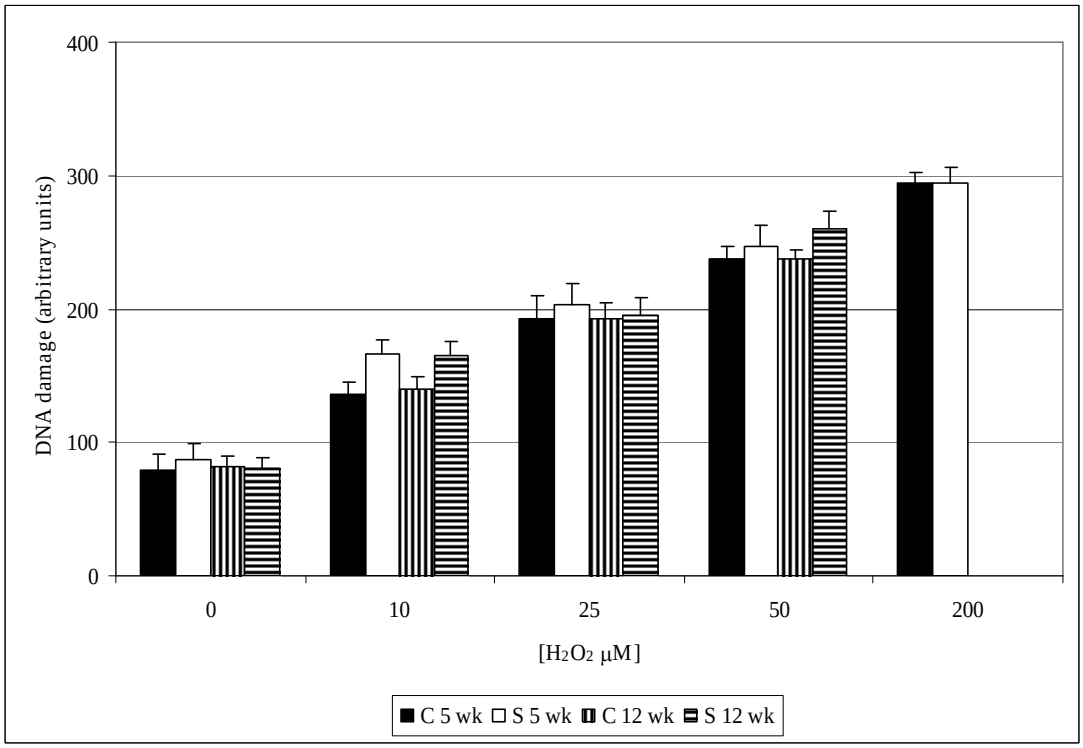


Figure 6.6 Study 2. Comparison of endogenous and H_2O_2-induced DNA damage in cryopreserved lymphocytes from control subjects and schizophrenia patients. Levels of DNA damage measured with the comet assay in cryopreserved lymphocytes from control subjects and schizophrenia patients following treatment with 0, 10, 25, 50 and 200μM of H_2O_2. Data show the mean ± sem DNA damage of 16 control and 15 schizophrenic samples. Key: C 5wk = control lymphocytes cryopreserved for 5 weeks, S 5wk = schizophrenic lymphocytes cryopreserved for 5 weeks, C 12wk = control lymphocytes cryopreserved for 12 weeks, S 12wk = schizophrenic lymphocytes cryopreserved for 12 weeks.

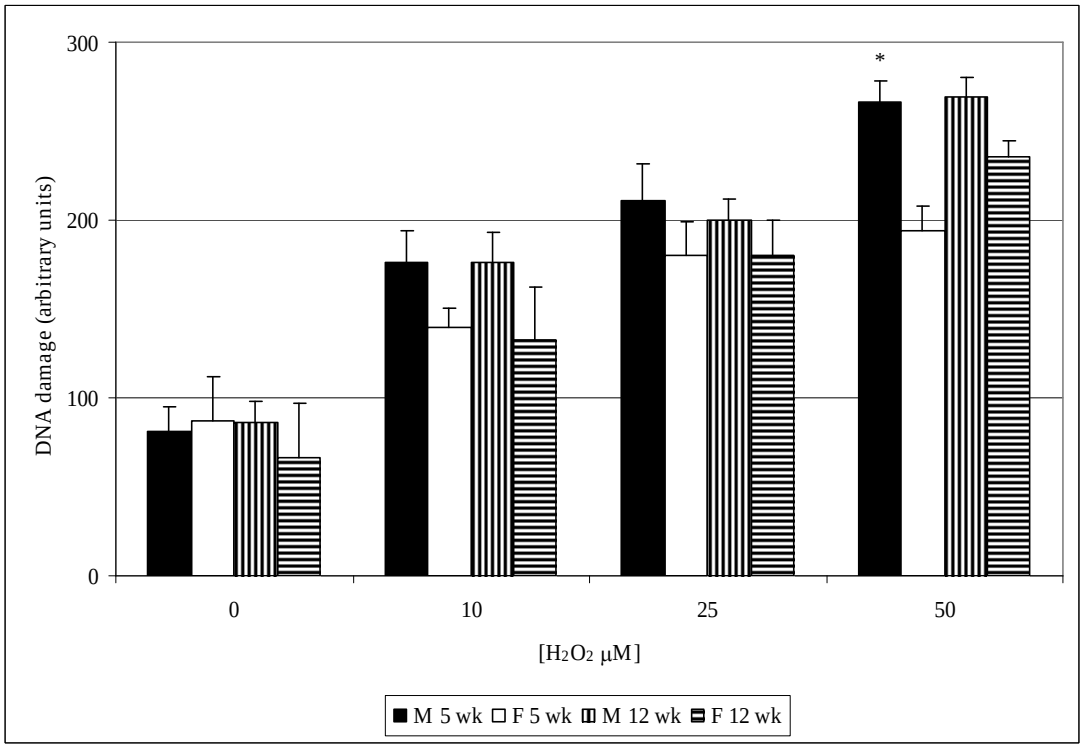


Figure 6.7 Study 2. Comparison of endogenous and H_2O_2-induced DNA damage in cryopreserved lymphocytes from male and female schizophrenia patients. Levels of DNA damage measured with the comet assay in cryopreserved lymphocytes from male and female schizophrenia patients following treatment with 0, 10, 25, 50 and 200μM of H_2O_2. Data show the mean DNA damage of 11 male and 4 female samples ± sem. Key: M 5wk = male lymphocytes cryopreserved for 5 weeks, F 5wk = female lymphocytes cryopreserved for 5 weeks, M 12wk = male lymphocytes cryopreserved for 12 weeks, F 12wk = female lymphocytes cryopreserved for 12 weeks. Significance is denoted by * where $p < 0.05$.

6.3.3 Determination of Protein Carbonyl Levels in Plasma from Control Subjects and Schizophrenia Patients

The ELISA method was used to detect levels of protein carbonyls in plasma from control and patient subjects. A comparison of the level of protein oxidation in plasma from the control and schizophrenic group revealed a slight increase in protein carbonyl levels in the schizophrenic group. However, Mann-Whitney analysis revealed that this increase in carbonyl levels in the schizophrenic group was not statistically significant (Figure 6.8).

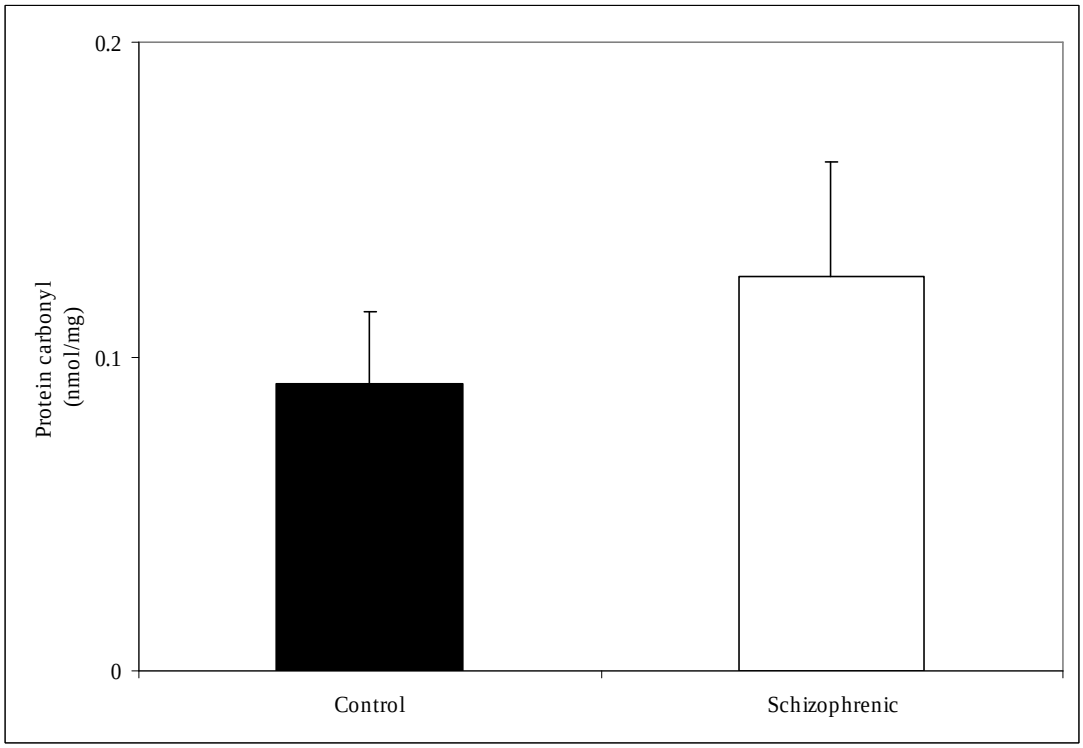


Figure 6.8. Determination of protein oxidation in plasma from control subjects and schizophrenia patients. Levels of protein carbonyls detected in human cryopreserved plasma from control (n=17) and schizophrenic (n=12) subjects. Data shows mean ± sem. of protein carbonyl production from triplicate samples as determined by ELISA.

Further analysis of the two test groups, specifically investigating any gender effect reported an increase in carbonyl levels in both male groups when compared to the female groups with a noticeable increase in plasma protein carbonyl levels from the male schizophrenic group. Using the Kruskal-Wallis test, analysis of the samples revealed that the observed increased levels of protein carbonyls in the male schizophrenic group in particular, were not statistically significantly higher than any other group (Figure 6.9).

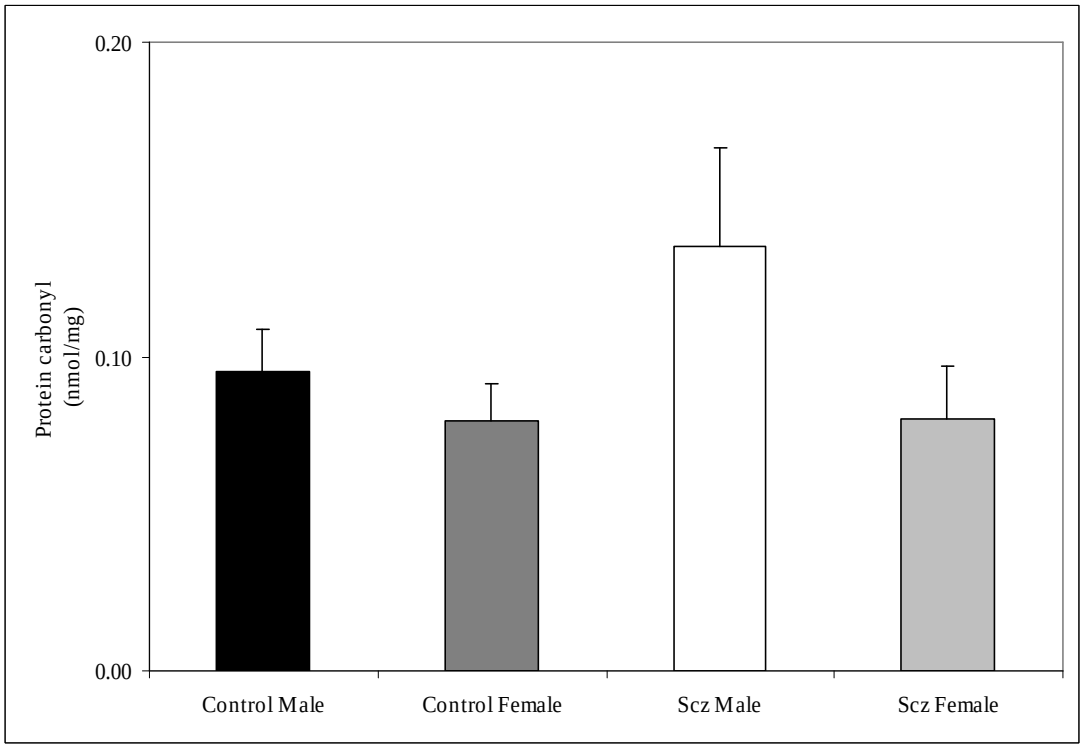


Figure 6.9. Determination of protein oxidation in plasma from control and schizophrenic male and female subjects. Levels of protein carbonyls detected in human cryopreserved plasma from control male (n=12), control female (n=5) and male schizophrenic (n=10) and female schizophrenic (n=2) subjects. Data shows mean ± sem of protein carbonyl production from triplicate samples as determined by ELISA.

6.3.4 Determination of Lipid Peroxidation in Plasma from Control Subjects and Schizophrenia Patients

Cryopreserved human plasma samples were used to measure levels of lipid hydroperoxides and a comparison in the levels of LOOH in duplicate samples were investigated in the control and schizophrenic samples (Figure 6.10). Student's t-test revealed no statistically significant difference in the levels of LOOH between the two groups under investigation. Comparison of the levels of plasma LOOH from control and schizophrenic male and female subjects also revealed no statistically significant difference (Figure 6.11).

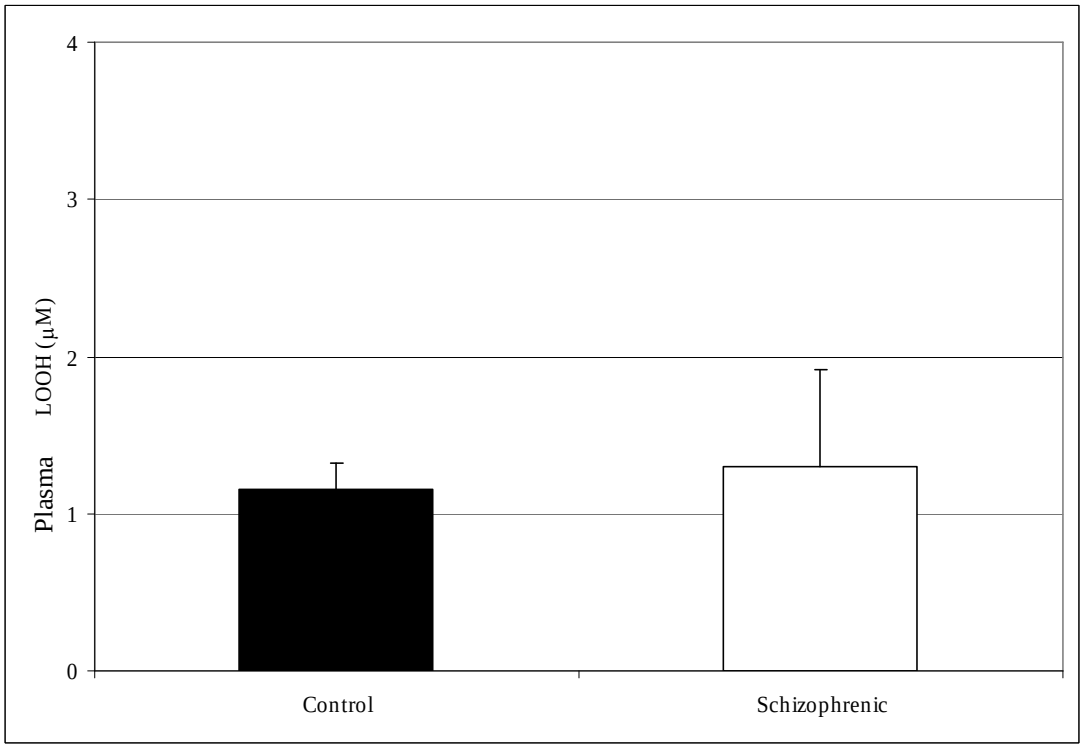


Figure 6.10. Comparison of lipid hydroperoxide levels in cryopreserved plasma from control subjects and schizophrenia patients. Levels of plasma LOOH detected in human cryopreserved plasma from control (n=17) and schizophrenic (n=16) subjects. Data shows mean ± sem of LOOH production from duplicate samples as determined by LOOH assay.

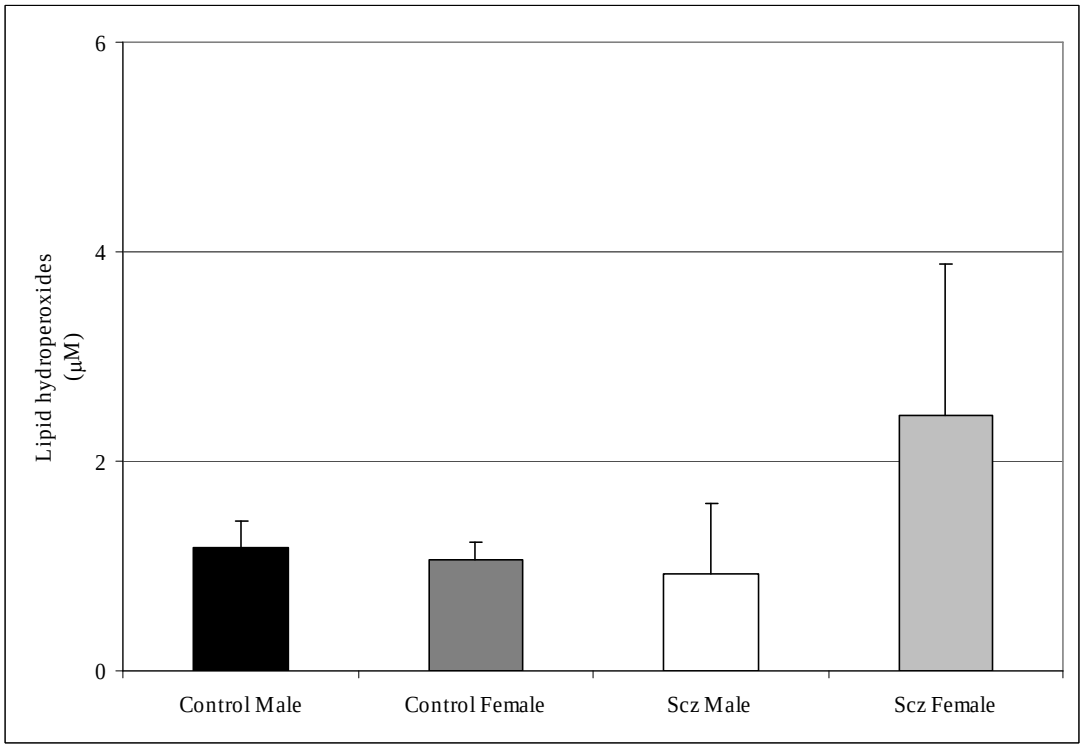


Figure 6.11. Comparison of lipid hydroperoxide levels in cryopreserved plasma from control and schizophrenic male and female subjects. Levels of LOOH detected in human cryopreserved plasma from control male (n=12), control female (n=5) and schizophrenic male (n=12) and schizophrenic female (n=4) subjects. Data shows mean ± sem of LOOH production from duplicate samples as determined by LOOH assay.

Levels of MDA and HNE were examined in cryopreserved plasma samples from the control and schizophrenic samples (Figure 6.12). Student's t-test revealed no statistically significant difference in lipid peroxide levels between the control and schizophrenic groups. Comparison of the levels of plasma MDA+HNE from control and schizophrenic male and female subjects also revealed no statistically significant difference (Figure 6.13).

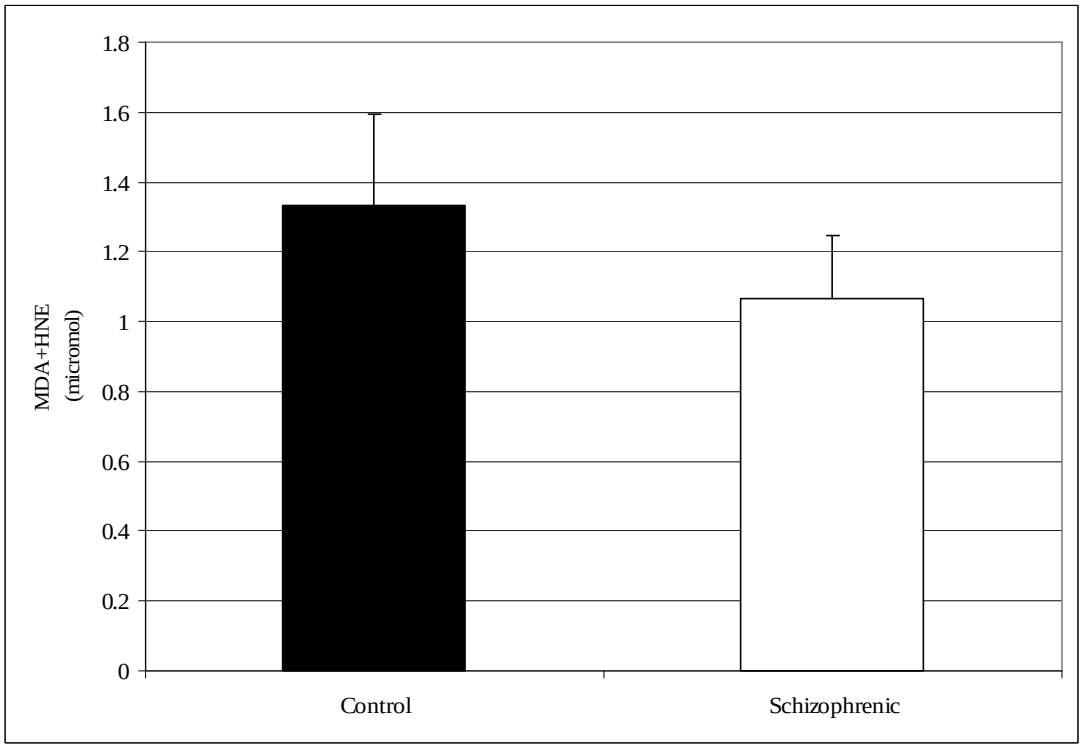


Figure 6.12. Comparison of MDA+HNE levels in cryopreserved plasma from control and schizophrenic subjects. Levels of lipid peroxides detected in human cryopreserved plasma from control (n=17) and schizophrenic subjects (n=16) Data shows mean ± sem of duplicate samples.

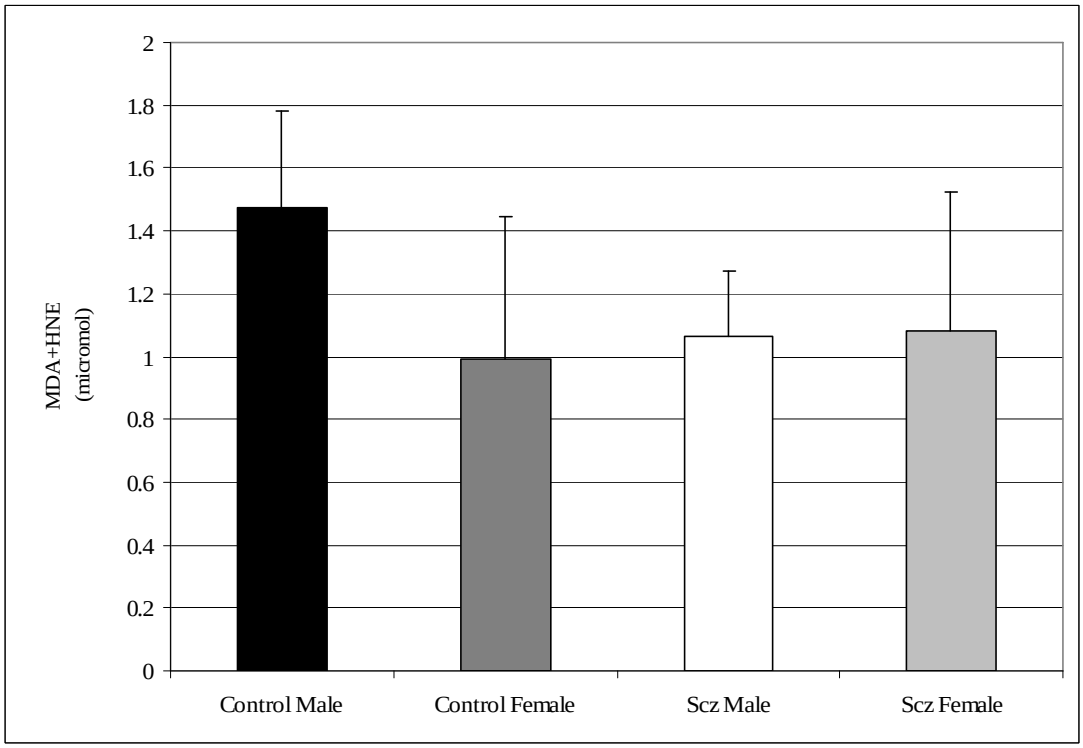


Figure 6.13. Comparison of MDA+HNE levels in cryopreserved plasma from control and schizophrenic male and female subjects. Levels of lipid peroxides detected in human cryopreserved plasma from control male (n=12), control female (n=5) and schizophrenic male (n=12) and schizophrenic female (n=4) subjects. Data shows mean ± sem of duplicate samples.

6.3.5 Determination of Vitamin C in Plasma from Control Subjects and Schizophrenia Patients

A comparison of the level of vitamin C in plasma from the control and schizophrenic groups was analysed by HPLC. Results show an increase in vitamin C levels in the control group when compared to the schizophrenic group (Figure 6.14) although this increase did not reach statistical significance.

Examination of the vitamin C levels from male and female plasma samples within the two test groups reported a highly significant ($p < 0.01$) increase in the level of vitamin C in the control female group when compared to the male groups (Figure 6.15).

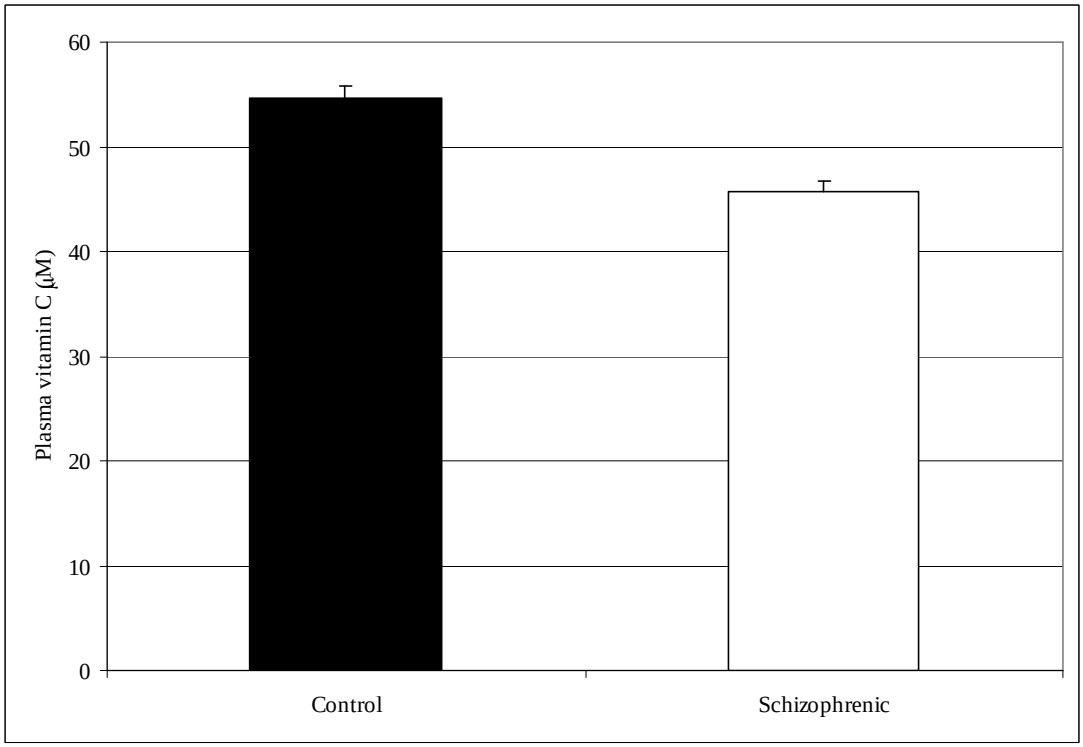


Figure 6.14. Evaluation of vitamin C levels from plasma of control subjects and schizophrenia patients. Levels of vitamin C detected in human cryopreserved plasma from control (n=17) and schizophrenic (n=16) subjects. Data shows mean ± sem of vitamin C production from duplicate samples as determined by HPLC.

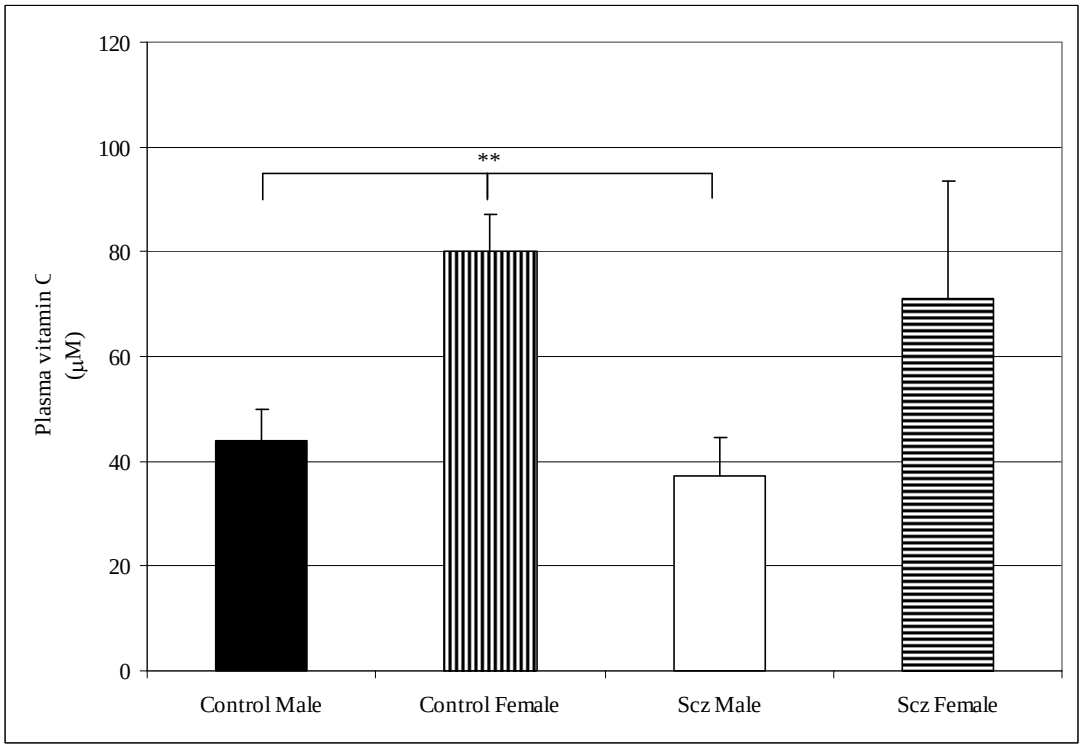


Figure 6.15. Evaluation of vitamin C levels from plasma of male and female control subjects and schizophrenia patients. Levels of vitamin C detected in human cryopreserved plasma from control male (n=12), control female (n=5) and schizophrenic male (n=12) and schizophrenic female (n=4) subjects. Data shows mean ± s.d. of vitamin C production from duplicate samples as determined by HPLC. Significance is denoted by ** where $p < 0.01$.

6.4 Discussion

Although free radical pathology in schizophrenia is widely investigated, examination of ROS induced DNA and protein damage is limited. The aim of this research was to investigate all biomarkers of oxidative stress in schizophrenic and control subjects including not only biomarkers of lipid peroxidation but also biomarkers of DNA and protein oxidation.

6.4.1 Biomarkers of DNA Oxidation in Schizophrenia

Differences in endogenous and H_2O_2-induced DNA damage in freshly isolated and cryopreserved peripheral blood lymphocytes from male and female control and schizophrenic subjects was first examined using the comet assay. The results of this study reveal no significant difference in the level of DNA damage, neither endogenous nor H_2O_2-induced in normal and schizophrenic subjects when assessed in freshly isolated lymphocytes. Furthermore, no significant difference in either endogenous or H_2O_2-induced DNA damage was noted in lymphocytes from control and schizophrenic subjects that were cryopreserved for up to 12 weeks. These results are identical to findings observed by Psimadas *et al* (2004) in their study using cryopreserved lymphocytes.

Our study sample differed, however, in that the control and patient group consisted of both male and female schizophrenics. We observed that in the freshly isolated lymphocytes, the level of DNA damage in male schizophrenic subjects was significantly greater than in female schizophrenic subjects ($p < 0.05$). Furthermore, a trend emerged whereby endogenous and H_2O_2-induced DNA damage in cryopreserved lymphocytes was greater in male than in female schizophrenic subjects. In addition, after 5 weeks of cryopreservation, the level of induced DNA

damage at a concentration of 50 μM H_2O_2 in the male schizophrenic group was also statistically significantly ($p < 0.05$) greater than in the female schizophrenic group.

6.4.4.1 Effect of Cryopreservation

The effects of cryopreservation on endogenous and induced DNA damage in human peripheral blood lymphocytes has been measured in various studies (Duthie *et al* 2002; Visvardis *et al* 1997) where it was reported that DNA damage was the same as that in freshly isolated lymphocytes. In addition, long term storage was also shown to have no effect on endogenous and induced DNA damage. In our investigation, cryopreservation did not appear to have an effect on the level of DNA damage in lymphocytes from either group up to 5 weeks storage at -80^{o}C. However, statistical differences at the level of DNA damage were observed following 12 weeks of cryopreservation, suggesting that possible changes in lymphocyte environment may occur following storage for this length of time.

6.4.4.2 Gender Differences

The risk of schizophrenia was originally thought to be the same in men and women. However, more recent studies have reported the risk to be higher in men (reviewed by Leung and Chue 2000). Furthermore, the existence of gender differences in schizophrenics has received wide empirical support with respect to age at onset, premorbid functioning, course of illness and outcome, familial transmission and neurobiological factors. Uncertainties concerning the gender differences remain although a number of theories to explain the sex differences in schizophrenia have been proposed. These include the effects of oestrogen, differential vulnerability to sub-types of schizophrenia, neurodevelopment and lateralization differences (Leung and Chue 2000).

6.4.5 Biomarkers of Lipid Peroxidation in Schizophrenia

One of the most commonly used methods in free radical research is the determination of the TBA and MDA adduct in plasma since MDA has been found to be elevated in patients with schizophrenia when compared with normal controls (Zhang *et al* 2006; Dakhale *et al* 2005; Kuloglu *et al* 2002; Ravikumar *et al* 2000). This is in contrast to the findings here whereby plasma MDA levels in our schizophrenic group, measured using a lipid peroxidation assay was determined as not being significantly different from those recorded in the control group. Ravikumar *et al* 2000 also reported in their study elevated levels of lipid hydroperoxides in plasma from schizophrenic subjects. Our findings reveal no significant differences in the levels of plasma LOOH in either the schizophrenic and control group.

6.4.6 Biomarkers of Protein Oxidation in Schizophrenia

This study is the first to investigate the use of protein carbonyls as a marker of oxidative stress in human schizophrenic patients. Observations have revealed no significant difference in the levels of production of plasma protein carbonyls between the schizophrenic and control group. Moreover, although a marked increase of protein carbonyl levels was noted in the male schizophrenic group when compared to other male and female groups, the increase was not statistically significant.

Accumulation of modified protein disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways (Stadtman and Levine 2000). Oxidation of catecholamines to the corresponding o-quinones and their subsequent reduction to reactive species, which can induce redox cycling, has been postulated as being involved in neurodegeneration in the mesolimbic and nigro-

striatal systems and thus in the aetiology of schizophrenia and Parkinson's disease (Baez *et al* 1997; Smythies *et al* 1997). Tardive dyskinesia is a movement disorder which affects 20-40% of patients treated chronically with neuroleptic drugs and it has been hypothesised that neuroleptics enhance striatal glutamatergic neurotransmission by blocking presynaptic dopamine receptors which causes neuronal damage as a consequence of oxidative stress (Tsai *et al* 1998).

6.4.6.1 Neuroleptics

Neuroleptics have also been shown to have both pro-oxidant and antioxidant properties (Jeding *et al* 1995) and the antipsychotic response of a neuroleptic may depend on its pro- or antioxidant property and the level of pre-existing oxidative stress in the patient (Mahadik *et al* 2001). Clinical studies have indicated that antioxidant enzyme activities are associated with the treatment of schizophrenic patient with neuroleptics (Abdalla *et al* 1986; Yao *et al* 1998), suggesting that the changes in the enzyme activity may be due to the neuroleptic treatment (Akyol *et al* 2002; Evans *et al* 2003). The neuroleptic Flupenthixol can accept electrons in competition with oxygen and therefore reduce the production of superoxide and inhibit cytochrome b5 reductase activities; alternatively, neuroleptics may react directly with superoxide (Whatley *et al* 1998). A recent study (Dakhale *et al* 2004) examined the effect of atypical antipsychotics on lipid peroxidation, SOD and ascorbic acid. Results revealed an increase in serum SOD, serum MDA and a decrease in plasma ascorbic acid in schizophrenic patients as compared to controls, however, the trend altered significantly following treatment with antipsychotics and results of the Brief Psychiatry Rating Scale for schizophrenia also improved.

6.4.7 Levels of Plasma Vitamin C in Schizophrenia

The study of plasma vitamin C levels was reported in mentally ill and schizophrenic patients over two decades ago (Morgan and Hullin 1982; Schorah *et al* 1983; Suboticanec *et al* 1986; Suboticanec *et al* 1990) where lower levels of vitamin C were noted in patients compared to control subjects. More recent studies have also recorded similar findings (Dakhale *et al* 2004; Dakhale *et al* 2005) and these and other supplementation studies have reported reduction in oxidative stress (Dakhale *et al* 2005) and improved outcome in schizophrenia (Arvindakshan 2003; Dakhale *et al* 2005). Our results recorded a decrease in the level of plasma vitamin C in the schizophrenic group in comparison to the control group but this was not statistically significant. Further examination of plasma vitamin C between the sexes, revealed a significantly higher concentration of vitamin C in the female control group than in the male groups. This pattern was also observed in the schizophrenic group but statistical analysis revealed no significant difference between the schizophrenic male and female vitamin C levels.

Vitamin C is the first line of defence against oxygen radicals in the water-soluble compartment (Nordberg and Arner 2001). This vitamin reacts directly with superoxide, hydroxyl radical and singlet oxygen and serves to prevent lipid hydroperoxide formation in plasma lipoproteins by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals. Under conditions of protective plasma vitamin C concentrations (>50μmol/l) significantly lower values of DNA, protein and lipid oxidative damage have been found in comparison with a vitamin C-deficient group (<50μmol/l) (Krajčovičová-Kudláčková *et al* 2006). Our results revealed a parallel between the levels of vitamin C and levels of cellular DNA damage. DNA damage in lymphocytes from schizophrenic male subjects was higher

than in lymphocytes from schizophrenic females. However, mean plasma vitamin C levels were found to be higher in the female schizophrenic patients (~70μmol/l) than in male schizophrenic patients (~38μmol/l).

6.4.7.1 Dietary Factors

Nutrition may also play an important role in the outcome of schizophrenia. A dietary intake profile of patients with schizophrenia (Henderson *et al* 2006) reports differences in diet compared to controls. The schizophrenia group were found to consume significantly fewer calories, carbohydrate, protein total fat, saturated fat, monounsaturated fatty acids, PUFA, fibre, folate, sodium and alcohol but significantly more caffeine than the control group. A study by McCreadie (2003) which examined the lifestyle of people with schizophrenia reported that fewer males compared with the general population, reached acceptable levels for consumption of fruit, vegetables, milk, potatoes and pulses. In an earlier study (McCreadie *et al* 1998), the authors reported that as well as consuming less fibre and consuming more saturated fat than the control population, more male schizophrenic patients than female patients consumed less vitamin E.

In conclusion, this human study has examined biomarkers of lipid peroxidation, DNA and protein oxidation to assess oxidative stress status in schizophrenia compared to controls. The findings have revealed no significant differences in the levels of biomarkers of lipid, DNA and protein oxidation between the patient and control groups. However, a trend was seen whereby the level of oxidatively damaged DNA and proteins was greater in male schizophrenic patients compared to the female patients. In addition, it was also noted that the sub-population with the lowest level of DNA damage ie. female controls also had the highest level of plasma

vitamin C suggesting that an intrinsic protection may have arisen from an increased dietary intake of this essential dietary vitamin. Ultimately, it is important to investigate a range of biomarkers to gain vital information concerning free radical damage and antioxidant status and to be able to better understand the disease of interest.

CHAPTER 7

General Discussion

This research looked at investigating whether levels of oxidative stress, indicated by a variety of biomarkers was increased in the models of schizophrenia compared to controls. The study was mostly inconclusive in its findings regarding lipid peroxidation and protein oxidation biomarkers; however, our observations were more easily interpreted following evaluation of DNA damage.

7.1 Evaluation of DNA Damage in Models of Schizophrenia

7.1.1 DNA Damage in Human Cell Cultures

A comparator study of two human peripheral cells lines and a neuroblastoma cell line as a means of assessing the validity of monitoring peripheral lymphocytes as potential surrogate markers of DNA damage in neuronal cell types was undertaken. Furthermore, experimentation to test the susceptibility of each cell line to an oxidative challenge was also undertaken along with an examination of the potential protective effects of a series of antioxidants and fatty acids on DNA oxidative damage. During the investigation of both models of schizophrenia ie the cell culture models and the human peripheral lymphocytes extracted from volunteer blood samples, it appeared that freezing of the lymphocytes at -80^{o}C had no effect on the basal level of DNA damage as assessed by the comet assay, a method used to detect DNA strand breaks. Furthermore, although no significant difference in the level of cellular DNA damage was noted in the lymphocytes from either the schizophrenic or control groups, and a possible confounding factor may present due to differences in antioxidant status, either endogenous or dietary derived, an apparent difference between sexes in terms of DNA damage within the schizophrenic group was noted which reached a level of significance.

7.1.2 DNA Damage and Antioxidant Protection in Human Cell Cultures

Several studies of schizophrenia have reported abnormalities in one or more antioxidant enzymes and/or in the levels of cerebrospinal fluid and plasma TBARs (Mahadik and Mukherjee, 1996; Reddy and Yao, 1996). Other studies have also reported a wide range of abnormalities in membrane phospholipids that may be a result of oxidative damage. These changes have been considered to be a result of altered metabolism (reduced synthesis and/or increased breakdown) of essential polyunsaturated fatty acids (EPUFAs). EPUFAs are the ω-3 and ω-6 series of fatty acids that must be consumed in the diet. Reduced membrane EPUFAs have been found to be associated with psychopathology (Glen *et al* 1994; Peet *et al* 1995).

Three human cell lines were used in this research as models of schizophrenia and cell supplementation studies were undertaken to assess potential antioxidant and fatty acid protection against ROS-induced DNA damage. Supplementation of neuroblastoma cells for 30min and 6 hours with EGCG and hydroxytyrosol of a concentration within human physiological range (ie. 10μM) resulted in a highly significant decrease in the level of DNA damage in cells treated additionally with H_2O_2. Furthermore, this same effect was observed in U937 cells. However, in only those lymphoblastoid cells treated with hydroxytyrosol was a significant protection of cellular DNA against an oxidative challenge observed.

The effect of hydroxytyrosol observed in this study is interesting. It is known that 3,4-dihydroxyphenylethanol, also known as hydroxytyrosol, is an endogenous metabolite of dopamine (Hashimoto *et al* 2004) and has been shown to protect cells against H_2O_2 induced damage (Grasso 2007; Hashimoto *et al* 2004) as well as being

protective against the peroxynitrite-dependent nitration of tyrosine and DNA damage by peroxynitrite *in vitro* (Deiana *et al* 1999). More recently, hydroxytyrosol has been shown to protect brain cells *in vitro* and *ex vivo* against Fe^{2+} and nitric oxide-induced cytotoxicity (Schaffer *et al* 2007) and the study by Schaffer *et al* provides the first evidence of the neuroprotective effects of oral hydroxytyrosol intake. With this in mind when thinking of future work, it might be beneficial to use the antioxidant hydroxytyrosol as a supplement alone or in conjunction with EPA and/or DHA in human schizophrenic patient studies and in further cell culture studies. However, because increased dopamine activity is thought to lead to the psychotic symptoms associated with schizophrenia, it is worth considering the role, if any, that the dopamine metabolite 3,4-dihydroxyphenylethanol might play in the psychotic symptoms, regardless of its antioxidant properties.

Green tea is a widely consumed drink and has gained a reputation as a health-promoting dietary component due to the antioxidant activity of EGCG, its main phenolic constituent. Evidence is increasing however, that tea constituents can be cell damaging and pro-oxidant. These effects are suggested to be due to spontaneous H_2O_2 generation by polyphenols in solution (Elbling *et al* 2005; Furukawa *et al* 2003). This pro-oxidant effect was perhaps more pronounced in the lymphoblastoid EGCG-treated cells used in this study, resulting in a higher overall ROS generation and greater level of DNA damage than seen in the U937 and IMR-32 EGCG-treated cells (Table 2.2). In contrast, the production of H_2O_2 in tea catechin-treated lymphoblastoid cells was compared by Sugisawa *et al* (2004) who suggested that the structure of EGCG is less likely to result in H_2O_2-induced chromosomal damage than EGC. EGC has a trihydroxyl structure with a B-ring similar to EGCG. Another catechin, ECG which has a gallate group linked to flavan-3-ol similar to EGCG,

however, did not produce H_2O_2 (Sugisawa *et al* 2004). Nevertheless, tea catechins and polyphenols are effective scavengers of reactive oxygen species *in vitro* and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities (Higdon and Frei 2003).

Until recently, there was little understanding of the metabolism of flavonoids and their mode of entry into the systemic circulation after oral absorption. Although numerous studies have reported flavonoid-mediated neuroprotection, there is little information about the interaction of flavonoids or their circulating metabolites with the brain endothelial cells from the blood brain barrier (Youdim *et al* 2004). EGCG has been reported to enter the brain after oral administration (Suganuma *et al* 1998) and is more recently being investigated as a prophylactic for Alzheimer's disease (Rezai-Zadeh *et al* 2005).

7.1.3 DNA Damage and Fatty Acid Protection in Human Cell Cultures

Epidemiological studies have shown an apparent beneficial effect of fish oil containing high levels of ω-3 PUFA on mortality from heart disease and cancer (Kikugawa *et al* 2003) and there is additional evidence to suggest that ω-3 fatty acids may also be important to mental health (Peet and Stokes, 2005). Conversely, a more recent systematic review (Hooper *et al* 2006) found no evidence of a clear benefit of ω-3 fats on health (in particular mortality, cardiovascular events, cancer or strokes). However, the benefits of using ω-3 fatty acids to protect DNA against oxidative damage, particularly in mental illness, have not been widely investigated. In this study human peripheral and neuroblastoma cells were incubated with increasing concentrations of EPA or DHA from 30min to 24h. Results revealed that there was no difference in the level of DNA damage in the U937 and IMR-32 cell lines treated

with either EPA or DHA when compared to controls (i.e. non EPA or DHA treated cells) (results not shown). Conversely, examination of lymphoblastoid cells treated with the highest concentration of DHA after 24 hours (Figure 2.6) revealed a significant increase in DNA damage when compared to 30 minute and 24 hour controls.

Fatty acid treated peripheral and neuroblastoma cells were also subjected to a peroxide challenge. Results show that in fatty acid + H_2O_2-treated cells the level of DNA damage was significantly reduced in the U937 and IMR-32 cells at all time points compared to H_2O_2 control cells.

Although the use of fish oil has been suggested to have many beneficial effects, lipid peroxidation-mediated DNA damage is an undesirable consequence resulting from intake of high concentration of fish oil. *In vitro* studies have shown that peroxidized PUFA induce DNA chain breaking (Adam *et al* 1998) and 8-hydroxyguanosine formation (Kaneko and Tahara 2000). Contrasting studies have shown that ω-3 PUFA supplementation on oxidative stress-induced DNA damage in a variety of cell types acts instead as a protective agent against oxidative stress rather than as a source of mutagenic metabolites (Beeharry *et al* 2003, Kikugawa *et al* 2003). The results of our study have also shown that EPA and DHA protect peripheral and neuroblastoma cells against oxidative stress induced DNA damage. It is worth noting that in their study, Kikugawa *et al* (2003) used fish oil supplemented with vitamin E. Since the purported health effects of fish oil are questioned by Hooper *et al* (2006), the reduction in oxidative stress-induced DNA damage reported by Kikugawa *et al* (2003) may well be attributable to the antioxidant rather than the fish oil. Nevertheless, these authors propose that although lipid peroxidation products formed

from the use of high concentrations of ω-3 can damage DNA, the damage caused by these products may be significantly lower than those caused as a result of direct oxidative stress resulting from ROS.

Within our study, pure oils without added vitamin E were used as in the study reported by Beeharry *et al* (2003). In their study Beeharry *et al* (2003) suggest that the DNA-protective effects seen following incubation of human cells with the ω-6 PUFA linoleic acid may be as a result of the fatty acid acting as a substrate for lipid peroxidation. In addition, linoleic acid may also inhibit the induced oxidative stress that causes lipid peroxidation by blocking an early stage in the induction of damage. Interestingly, a preliminary study carried out by the same group has also shown DHA to be cytoprotective (Beeharry *et al* 2003).

7.1.4 Reducing Abilities of Antioxidants in Human Cell Cultures

The reducing ability of a range of antioxidants was assessed and in conjunction, the efficacy of these antioxidants and a series of polyunsaturated fatty were considered in affording protection to cellular DNA against an oxidative challenge. Numerous assays have been described in which antioxidant is added to a reaction mixture in which free radicals are generated. An example is the ABTS assay in which ABTS is oxidised by 2,2'azobis(2-amidopropane). Any antioxidant present delays the appearance of the coloured product of this reaction. Two polyphenolic (EGCG and resveratrol) and two monophenolic (tyrosol and hydroxytyrosol) compounds were tested using the ABTS assay. EGCG and hydroxytyrosol were found to be the most potent radical quenchers examined.

Structure-activity comparisons suggest that antioxidant activity of phenolic compounds depends on the number and orientation of hydroxyl groups relative to the electron-withdrawing CO_2H, CH_2CO_2H or $(CH)CO_2CH$ functional groups (Rice-Evans *et al* 1996). When considering flavonoid (e.g. EGCG) antioxidant activity, the B-ring hydroxyl configuration is the most significant determinant of scavenging of reactive oxygen species. Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (Heim *et al* 2002). Functional differences between hydroxytyrosol and tyrosol have been attributed to the presence of only a single hydroxyl (reviewed by Quiles *et al* 2002) which may account for the greater antioxidant potential of hydroxytyrosol over tyrosol. These structural differences may explain why hydroxytyrosol and EGCG appear to have a greater antioxidant potential than tyrosol and resveratrol since Masella *et al* (1999) suggest that the phenol chemical structure considerably influences the antioxidant activity as a consequence of both steric factors and those related to position and type of functional groups on the phenol ring. Furthermore, the antioxidant activity of biphenols depends mainly on the number of hydroxyl groups in the molecule (Masella *et al* 1999).

7.1.5 DNA Damage in Human Schizophrenia and Control Subjects

Differences in endogenous and H_2O_2-induced DNA damage in freshly isolated and cryopreserved peripheral blood lymphocytes from male and female control and schizophrenic subjects was examined using the comet assay. The results of this study revealed no significant difference in the level of DNA damage, neither endogenous nor H_2O_2-induced in normal and schizophrenic subjects when assessed in freshly isolated lymphocytes. Furthermore, no significant difference in either endogenous or

H_2O_2-induced DNA damage was noted in lymphocytes from control and schizophrenic subjects that were cryopreserved for up to 12 weeks. These results are identical to findings observed by Psimadas *et al* (2004) in their study using cryopreserved lymphocytes.

Our study sample differed, however, in that the control and patient group consisted of both male and female schizophrenics. We observed that in the freshly isolated lymphocytes, the level of DNA damage in male schizophrenic subjects was significantly greater than in female schizophrenic subjects ($p < 0.05$). Furthermore, a trend emerged whereby endogenous and H_2O_2-induced DNA damage in cryopreserved lymphocytes was greater in male than in female schizophrenic subjects. In addition, after 5 weeks of cryopreservation, the level of induced DNA damage at a concentration of 50μM H_2O_2 in the male schizophrenic group was also statistically significantly ($p < 0.05$) greater than in the female schizophrenic group.

7.1.6 Effect of Cellular Cryopreservation

The effects of cryopreservation on endogenous and induced DNA damage in human peripheral blood lymphocytes has been measured in various studies (Duthie *et al* 2002; Visvardis *et al* 1997) where it was reported that DNA damage was the same as that in freshly isolated lymphocytes. In addition, long term storage was also shown to have no effect on endogenous and induced DNA damage. In our investigation, cryopreservation did not appear to have an effect on the level of DNA damage in lymphocytes from either group up to 5 weeks storage at -80^{o}C. However, statistical differences at the level of DNA damage were observed following 12 weeks of cryopreservation, suggesting that possible changes in lymphocyte environment may occur following storage for this length of time.

7.1.7 Gender Differences

The risk of schizophrenia was originally thought to be the same in men and women. However, more recent studies have reported the risk to be higher in men (reviewed by Leung and Chue 2000). Furthermore, the existence of gender differences in schizophrenics has received wide empirical support with respect to age at onset, premorbid functioning, course of illness and outcome, familial transmission and neurobiological factors. Uncertainties concerning the gender differences remain although a number of theories to explain the sex differences in schizophrenia have been proposed. These include the effects of oestrogen, differential vulnerability to sub-types of schizophrenia, neurodevelopment and lateralization differences (Leung and Chue 2000).

We sought to investigate DNA damage in schizophrenia because it had not been investigated until recently. Psimadas *et al* (2004) also reported no difference in levels of H_2O_2-induced DNA damage in lymphocytes from their schizophrenic patients and controls, yet our study (Young *et al* 2007) was the first to report differences in the level of DNA damage in lymphocytes from male and female schizophrenic patients. Gender issues in relation to DNA damage were mentioned earlier but it is important to note that in general, women require lower doses of medication than men during both acute and maintenance phases of the illness at least until menopause (reviewed by Afifi 2007). It is worth considering therefore, whether the difference in the medication dosage might play a role in the differences in levels of DNA damage reported here. In addition, the type of neuroleptic medication received by the patient has also been reported as having an effect on levels of lipid peroxidation. Again, perhaps it is possible that the type of antipsychotic treatment might also have an effect on the level of DNA damage. In future research

nevertheless, where gender is being considered in schizophrenia studies, it would be advisable to ensure that the numbers of male and female volunteers are equally matched since the significant results reported in our study might be as a result of the small sample of women volunteers compared to the larger sample of male volunteers.

7.2 Evaluation of Protein Oxidation in Models of Schizophrenia

7.2.1 Protein Carbonyls in Human Cell Cultures

The evaluation of protein carbonyls as a potential marker of protein oxidation in the putative cell model for schizophrenia, U937 was undertaken. Treatment with H_2O_2 alone and following pre-treatment with EPA or DHA had no effect on the levels of protein carbonyls detected in the cell line by ELISA. Nevertheless, although the results were not statistically significant, DHA supplementation does appear to reduce endogenous and H_2O_2-induced protein carbonylation. Further *in vitro* and *in vivo* work would be merited since evaluation of the effect of fatty acid supplementation on the levels of protein oxidation in schizophrenia is limited. Indeed, the potential use of protein carbonyls as a biomarker of schizophrenia has not been widely examined until now.

The results of our evaluation of the levels of protein carbonyls in cultured human cells showed that although not statistically significant, pre-treatment of the cultured cells with DHA might reduce protein oxidation. If this is the case it would appear that male human schizophrenic patients may benefit from DHA supplementation since our human study revealed this group to have the highest levels of plasma protein carbonyls. Since this work was carried out, further studies have shown the advantages of omega-3 supplementation. In an animal study, male Wistar rats were

shown to have increased levels of protein carbonyls and MDA following chemically induced psychosis (Ozyurt *et al* 2007), while another study by the same group describes a decrease in protein carbonyls in the rat prefrontal cortex following omega-3 (EPA and DHA combined) treatment (Ozyurt *et al* 2007).

7.2.2 Protein Carbonyls in Schizophrenia Patients and Control Subjects

This study was the first to investigate the use of protein carbonyls as a marker of oxidative stress in human schizophrenic patients. Observations have revealed no significant difference in the levels of production of plasma protein carbonyls between the schizophrenic and control group. Moreover, although a marked increase of protein carbonyl levels was noted in the male schizophrenic group when compared to other male and female groups, the increase was not statistically significant.

The abnormal movements seen in tardive dyskinesia occur as a result of major late-onset chronic side effect of antipsychotic treatment. Oxidative stress and free radicals are thought to be associated with dopaminergic malfunctions (Sadan *et al* 2005) and oxidative stress-induced neurotoxicity in the striatal system is implicated in tardive dyskinesia (Shamir *et al* 2001). Several studies have reported that people with schizophrenia have abnormal levels of essential fatty acids in their blood cells and similar abnormalities have been recorded in association with the presence of tardive dyskinesia (reviewed by Vaddadi *et al* 1996). In their study, Vaddadi *et al* (1996) hypothesised that patients with schizophrenia would have lower levels of ω-3 and ω-6 EFAs than controls and that this abnormality would be more pronounced in those schizophrenic patients also suffering with tardive dyskinesia.

During conditions of oxidative stress protein modification can lead to protein dysfunction or tissue damage and disease progression. Carbonylation of proteins is an irreversible oxidative damage, often leading to a loss of protein function, which is considered a widespread indicator of severe oxidative damage and disease-derived protein dysfunction (Dalle-Donne 2006). Although moderately carbonylated proteins are degraded by the proteasomal system, those that have been heavily carbonylated form high-molecular-weight aggregates that are resistant to degradation and accumulate as damaged or unfolded proteins while masses of carbonylated proteins can inhibit proteasome activity. It is widely accepted that a variety of neurodegenerative diseases are linked to the accumulation of proteolysis-resistant aggregates of carbonylated proteins in tissues. If this is the case, identification of specific functionally impaired protein carbonyls and the development of particular carbonyl inhibitors might result in a better understanding of the role of protein carbonylation in disease onset and/or progression, possibly providing new therapeutic approaches (Dalle-Donne 2006). For example, model studies are now able to reveal the structure of adducts that can be more readily identified in mass spectrometric studies on proteins exposed to the various pure aldehydes or to peroxidized PUFAs (Sayre *et al* 2006).

7.2.3 ROS in Human Cell Cultures

CM-H_2DCFDA-sensitive reactive oxygen species of the human U937 cell line along with the anti- or pro-oxidant effects of EGCG, hydroxytyrosol, α-tocopherol and H_2O_2 were evaluated by flow cytometry. Results from this study showed that the pre-treatment with the antioxidant hydroxytyrosol significantly reduced the level of endogenous ROS in U937 cells, while α-tocopherol appears to increase endogenous

ROS levels. In addition, the beneficial effect of hydroxytyrosol was apparent following treatment of cells with H_2O_2 since it was observed that only hydroxytyrosol was effective in significantly reducing H_2O_2-induced cellular ROS

Previous studies have suggested that ROS production may play a role in the pathogenesis of schizophrenia, and more recently, there is an emerging body of data indicating that schizophrenia may be associated with mitochondrial dysfunction (Frey *et al* 2006). Animal studies have established that a global oxidative stress affects predominantly the brain (Mahadik and Mukherjee 1996). Brain vulnerability occurs because the brain is under higher oxidative stress than other organs since it produces very high levels of ROS due to its very high aerobic metabolism and blood perfusion (reviewed by Mahadik *et al* 2001). In addition, it is enriched in lipids that are preferentially susceptible to oxidative damage and the damaged neuronal DNA in the adult brain can not be effectively repaired since there is no DNA replication. Yet environmental ROS also attack the brain and inflammation is a major threat to brain function. Depending on the degree of oxidative stress and the developmental time, oxidative neuronal injury in the brain may cause abnormal neurodevelopment, neurodegeneration, or neuronal membrane impairment.

Although the possible potential beneficial effect of hydroxytyrosol in reducing the level of ROS, and the possible potential beneficial effect of DHA in reducing protein carbonyls in U937 cells was noted, a number of problems were encountered with these experiments. The U937 cells were kept under the same cell culture conditions throughout the experiment and were subjected to limited handling and exposure to the atmosphere. However it is possible that because of the length of time that the cells were in use, in particular, the number of passages might have lead to an

increase in basal oxidative damage. Although cell viability was checked at each experiment only dead cells would have been highlighted and cells undergoing damage would not be obvious.

7.3 Evaluation of Lipid Peroxidation in Models of Schizophrenia

7.3.1 Lipid Peroxidation in Human Cell Cultures

In contrast to many other studies whereby increased levels of lipid peroxidation have been observed in plasma from schizophrenic patients, our study has reported no significant difference in lipid peroxidation in our models of schizophrenia compared to the controls, as determined by a range of biomarkers. Furthermore, even after supplementation with fatty acids, the experiments utilising cultured cells did not report any clear cut results, particularly in those seeking to evaluate phospholipase A_2 levels.

There are a number of reasons why the results did not draw any concise conclusions and it is likely that the main limitation of the cell culture experiments was the maintenance of the cells. During the course of these experiments the cells were cultured in different laboratories with the risk of bacterial and fungal contamination being an unfortunate added factor. It is also likely that the number of passages of the cells might have resulted in them being less viable and hence led to the production of unexpected results. Nevertheless, the viability of the cells was checked routinely with the Trypan blue assay and solutions and reagents were freshly made before each experiment. In addition, the use of the assay kits may have played a role in producing ambiguous data. Although the kits are quick and relatively straightforward to use, they can be expensive, have a limited shelf life and the number of experiments that can be carried out is restricted. Had time and resources permitted, it

would have been ideal to have repeated the experiments a number of times, not only using fatty acid supplementation but also with antioxidant treatment. On the other hand, it was possible to optimise and validate the GC-MS and TBARs methods and using these methods seemed to be more straightforward and produced more conclusive data. Whether this was down to the method itself or the cultured cells that were used with the kits is difficult to pinpoint.

It is now clear that increased lipid peroxidation and failure of antioxidant systems can lead to neuronal damage in schizophrenic patients and moreover, oxidative stress may be exacerbated by the treatment with antipsychotics with pro-oxidant properties. Quantification of MDA still remains the most measured and reliable biomarker of lipid peroxidation in schizophrenia studies, although there is scope for more breath analysis to be undertaken, and recent novel work has revealed that increased serum MDA and 4-hydroxynonenal levels are found in treatment refractory schizophrenic patients when compared to non-refractory treatment schizophrenics. In addition, these increased lipoperoxidation products correlated with worsening of some the psychiatric symptoms (Medina-Hernandez *et al* 2007). Conversely, short term (6 week) antipsychotic treatment has been shown to have no effect on oxidative-antioxidative system parameters (Sarandol *et al* 2007).

7.3.2 Phospholipase A_2 in Human Cell Cultures

Recognition of the phospholipid hypothesis of schizophrenia is growing since the observations made by numerous groups who report increased blood and brain PLA_2 activity in schizophrenia (Barbosa *et al* 2007), while findings from the niacin skin flush test, PUFA supplementation studies and biochemical studies for the phospholipid metabolism pathway also suggest a disordered phospholipid

metabolism in the disease (Liu *et al* 2007). In addition, a genetic variant of a cytosolic PLA_2 gene has been reported to be associated with schizophrenia. These data indicate that variants of PLA_2 encoding genes are plausible candidates for increasing the susceptibility for schizophrenia (Barbosa *et al* 2007).

Initial studies revealed significantly increased total PLA_2 levels in the U937 and IMR-32 cells following 4 hour treatment with H_2O_2 alone. An increase in total PLA_2 activity was also seen in lymphoblastoid cells although this did not reach significance. However, further experimentation with or without pre-treatment of cells with ω-3 fatty acids revealed total PLA_2 levels to be similar in all the cell lines and no change in activity levels was observed following challenge with H_2O_2. These findings are in contrast to the reports that H_2O_2 activates cytosolic phospholipase A_2 (van Rossum *et al* 2004) and it is difficult to provide an explanation for this discrepancy at present.

In an additional exploratory study, the $cPLA_2$ activity in the three human cell lines was examined and the basal level of $cPLA_2$ activity was found to be comparable in the lymphoblastoid and IMR-32 cells but lower than that measured in the U937 cells. However, because only one study was carried out, the results cannot be deemed significant since no statistical analysis was undertaken. In this experiment, a high variance was observed within the untreated cells which lead to the adoption of the ethanol-treated cells as the control set. In addition, the large error bars recorded in this experiment in particular may be due to the possible lack of intra-assay precision. Although our study did not conclude any definitive findings, because of the importance the PLA_2 enzyme plays in the metabolism of fatty acids and its suggested causal role in the aetiology of schizophrenia, it would be interesting to repeat our

earlier experiments with human blood samples if possible, and also peripheral blood cells and neuronal cells, treated with EPA or DHA or a combination of both PUFAs.

An enhanced level of $cPLA_2$ activity in the U937 cell line has been previously reported by Obajimi *et al* (2005) and is a feature which has led to the suggestion that this cell line is a suitable *in vitro* model for studying cellular mechanisms of schizophrenia. Supplementation of the U937 cell line with EPA appeared to reduce $cPLA_2$ activity relative to the vehicle treated control, an outcome which again appears to mimic some of the biochemical changes reported following EPA supplementation of Asperger's syndrome sufferers (Bell *et al* 2004). Neither EPA nor DHA supplementation appeared to have any significant effect on either total PLA_2 or $cPLA_2$ activity in IMR-32 or lymphoblastoid cell lines. However, it is also reported that ω-3 PUFAs modulate T-cell functions such as T-cell proliferation and cytokine secretion (Calder 2001). This is important since inflammatory cytokines such as IL-1β, TNFα and IFNγ, play a role in the activation of $cPLA_2$ (Xu *et al* 2003). A more recent study also reported that IL-2 increased $cPLA_2$ activity in the shell of the nucleus accumbens but IL-1-induced changes which were significantly attenuated by EPA treatment (Song *et al* 2007).

7.3.3 Breath Analysis in Schizophrenia Patients and Control Subjects

Breath analysis is a non-invasive and easy to use method to assess lipid peroxidation. In the first part of the human trial undertaken during this project, the level of breath hydrocarbons measured by GC-MS was compared in schizophrenic and control subjects. Although there were incidences of higher levels of both ethane and pentane in the patient group, suggesting that these individuals may be subjected to greater oxidative stress and hence greater levels of lipid peroxidation than individuals in the

control group, no significant difference was found between the levels of ethane and pentane in the breath from the schizophrenic patients and control samples on the whole. In addition, plasma MDA levels were evaluated by TBARs and HPLC in the schizophrenic patients and controls, but again, there was no significant difference in the levels of plasma MDA between the two groups. Further analysis of the breath and MDA data from the schizophrenic group alone was undertaken to determine any gender differences and although ethane levels and MDA levels were higher in the male schizophrenic samples the data was not statistically significant. Only the pentane levels were higher in the female schizophrenic samples when compared to the male samples but these were not significantly greater. The lack of significance may be as a result of the small sample sizes, although it is possible that there is just no definite difference in the levels of oxidative stress between the two groups. However, any future work that may be carried out would probably benefit from larger sample sizes.

During the lipid peroxidation chain reaction process, saturated hydrocarbons such as ethane and pentane are eventually formed from the ω-3 and ω-6 fatty acids along with aldehydes such as MDA and *in vitro* studies have shown that ethane and pentane are generated when cell cultures are exposed to ROS (reviewed by Miekisch *et al* 2004). As stable end products of lipid peroxidation, hydrocarbons show low solubility in blood and are excreted into the breath minutes following their formation in tissues and exhaled concentrations of ethane and pentane therefore can be used to monitor the degree of oxidative stress within the body (Risby and Sehnert 1999). Animal and clinical studies have also reported close correlation between clinical conditions where high levels of lipid peroxidation or inflammation occur and the exhalation of ethane and pentane generated through ROS attack on lipid membrane

structures (Aghdassi *et al* 2000) while the same authors have also shown that levels of exhaled pentane and ethane correlate well with other markers of lipid peroxidation including MDA and TBARs (Aghdassi *et al* 2003). Although the comparison was made, we were unable to demonstrate a correlation between the two different methods of determining lipid peroxidation or plasma MDA levels, or any correlation between these variables and the clinical rating scales. Furthermore, we did not observe any differences in the mean population levels of breath hydrocarbons or MDA between the patient and control group.

As mentioned earlier incidences of higher levels of ethane and pentane were noted in the patient group, however, only one patient had an ethane level falling outside the 95% reference range for breath ethane levels as determined by Glen *et al* 2003. Why this patient appears to produce higher levels of breath ethane is unknown. Volatile organic compounds (VOCs) such as ethane and pentane may be produced endogenously ie in the body or they may be exogenously generated ie absorbed as contaminants from the environment. Furthermore, the composition of VOCs in breath varies hugely between individuals both qualitatively and quantitatively (Cao and Duan 2006). Increased levels of oxidative stress might be a cause and perhaps the type of medication that the patient is receiving may also be a factor. Gama *et al* (2006) recently reported serum TBARs to be significantly higher in patients receiving clozapine than those under haloperidol treatment although it is possible that the increase in TBARs levels may be as a result of the course of the disease rather than the medication, as may be the case with our patient.

7.3.4 LOOH and MDA in Schizophrenia Patients and Control Subjects

The measurement of MDA by the TBARs method is widely debated but although it has its pitfalls, it is still one of the most commonly reported indices of oxidative damage. Plasma MDA levels have been found to be elevated in patients with schizophrenia when compared to normal controls (Zhang *et al* 2007; Medina-Hernandez *et al* 2007; Gama *et al* 2006; Zhang *et al* 2006; Khan *et al* 2002; Kuloglu *et al* 2002), while only one recent study (Skinner *et al* 2005) reported lower levels of MDA measured by TBARs in CSF from schizophrenic patients compared to controls. These are in contrast to our study which has shown no difference in the level of plasma MDA from schizophrenic patients compared to controls, although the MDA levels in our volunteers appear to be much higher than the reported normal ranges of <1.0micromol/litre as reported by Kropp *et al* 2005. Nevertheless, these results are similar to our earlier findings (Young *et al* 2007) where we reported no significant difference in the levels of MDA and hydroxyalkenals in cryopreserved plasma samples from control and schizophrenic samples. Furthermore, between sex comparisons of the level of plasma MDA and hydroxynonenal from control and schizophrenic patients also revealed no statistically significant difference (Young *et al* 2007).

7.4 Evaluation of EPUFA Treatment in Cell Models of Schizophrenia

The basal level of ω-3 fatty acids in the U937 cell line was depleted when compared to the lymphoblastoid and IMR-32 cell lines and therefore confirmed the validity of the U937 cell line as an appropriate model for investigating the effects of ω-3 supplementation on cellular mechanisms in schizophrenia. The ω-3 and ω-6 ratio in the U937 and lymphoblastoid cells lines are highly comparable and significantly lower than that seen in the IMR-32 cell line. Following treatment with EPA or

DHA, the percentage of ω-3 fatty acid increased in each cell line but the U937 cells showed the largest increase. In all the cell lines 24h supplementation with EPA resulted in a decrease in AA and DHA levels when compared to the untreated cells, but lead to an increase in EPA content in all cell lines. 24h supplementation of the three cell lines with DHA also revealed an increase in DHA levels with an increase in all the cell types, compared to DHA content in untreated cells. It has been suggested that the profile of essential fatty acids in neuronal membranes affects normal physiological events in the brain and that the fatty acid composition of neuronal cell membrane phospholipids reflects their intake in the diet (Haag 2003). Furthermore, the effects of dietary fatty acids may result in changes in neurophysiological, cognitive and psychophathological parameters (Haag 2003; Evans *et al* 2003) although it is thought that oxidative stress may play its part as an associated event (Mahadik *et al* 2001). As a major component of brain phospholipids, DHA has a wide range of biological actions and is also thought to play an important role in the regulation of cell signalling and in cell proliferation (Salem *et al* 2001). Similarly, EPA plays a role in many biological activities and has a variety of actions. Epidemiological studies have not only reported the beneficial effects of ω-3 PUFAs with respect to mortality from heart disease to cancer (Jude *et al* 2006) but there is also growing evidence to suggest a beneficial role in mental health (Peet and Stokes 2005).

Although PUFA supplementation might be expected to increase the possibility of oxidation in the cells, no differences in the level of DNA damage in the U937 or IMR-32 cells treated with PUFA was noted when compared to the untreated cells. Only supplementation with a higher concentration (100μM) of DHA for 24h resulted in an increase in DNA damage compared to the control cells suggesting the expected

pro-oxidant effect of this fatty acid in this cell line. However, both EPA and DHA significantly attenuated the DNA damage elicited by an induced oxidative stress in all cell lines and this protective effect was observed at low concentrations of the fatty acids than could possibly be achieved through dietary ω-3 PUFA supplementation to humans *in vivo*. Omega-3 intake has also been shown to attenuate inflammatory cytokine production in various cells which may also be regarded as beneficial to brain function since the cytokines are reported to induce adverse effects in brain chemistry (Martin *et al* 2002).

It is important to note the possible induction of glutathione peroxidase enzymes by fatty acids and the protective role that these antioxidant enzyme may play in cells and tissues against oxidative and inflammatory cytokine elicited damage (Crosby *et al* 1996). *In vitro* enrichment of peripheral blood monocytes with low concentrations of EPA and DHA have also been shown to induce an increase in glutathione peroxidase (Joulain *et al* 1994). Indeed schizophrenia may not just be linked to oxidative events and damage but rather to inflammatory events such as cytokine and eicosanoid production in the brain elicited by bacterial endotoxins and infections. For example, a recent study has reported that during critical stages of pregnancy maternal infection may lead to sensorimotor gating deficits in schizophrenia (Fortier *et al* 2007).

7.5 Oxidative Stress, Diet and Schizophrenia

Oxidative stress increases when the level of reactive oxygen species exceeds the cellular antioxidant defence capacity. The primary antioxidant defence is enzymatic. However, non-enzymatic antioxidant defence which also plays a significant role in control of oxidative injury constitutes dietary supplements such as antioxidant

vitamins (A, C, E and Q), flavones, quinines, lycopenes, β-carotene and uric acid (Mahadik *et al* 1999). Numerous studies assessing diet in schizophrenic subjects report the lack of consumption of fruit and vegetables and fibre but increased consumption of saturated fats and sugar in comparison to controls (Roick *et al* 2007; Amani 2007). Conversely, obesity in schizophrenia patients is not solely related to food consumption, but may be due to other factors including side effects of medication and reduced physical activity (Henderson *et al* 2006). However, people with schizophrenia are at greater risk of obesity, Type 2 diabetes, dyslipidaemia and hypertension than the general population. This results in an increased incidence of cardiovascular disease (CVD) and reduced life expectancy, over and above that imposed by their mental illness through suicide (Barnett *et al* 2007). Evidence from data linkage analyses to clinical trials show that treatment-related metabolic disturbances are widespread in this patient group, and that the use of certain second-generation antipsychotics may increase the risk of developing the metabolic syndrome and CVD. In addition, smoking, poor diet, reduced physical activity and alcohol or drug abuse is prevalent in people with schizophrenia and contribute to the overall CVD risk (Barnett *et al* 2007). Had resources permitted, our study may have benefited from a dietary assessment to gain a better understanding of the role that dietary choices and the impact on oxidative stress that these choices may play in the disease.

7.5.1 Vitamin C in Schizophrenia Patients and Control Subjects

The study of plasma vitamin C levels was reported in mentally ill and schizophrenic patients over two decades ago (Morgan and Hullin 1982; Schorah *et al* 1983; Suboticanec *et al* 1986; Suboticanec *et al* 1990) where lower levels of vitamin C were noted in patients compared to control subjects. More recent studies have also

recorded similar findings (Dakhale *et al* 2004; Dakhale *et al* 2005) and these and other supplementation studies have reported reduction in oxidative stress (Dakhale *et al* 2005) and improved outcome in schizophrenia (Arvindakshan 2003; Dakhale *et al* 2005). Our results recorded a decrease in the level of mean plasma vitamin C in the schizophrenic group in comparison to the control group but this was not statistically significant. Further examination of plasma vitamin C between the sexes, revealed a significantly higher concentration of vitamin C in the female control group than in the male groups. This pattern was also observed in the schizophrenic group but statistical analysis revealed no significant difference between the schizophrenic male and female vitamin C levels.

Vitamin C is the first line of defence against oxygen radicals in the water-soluble compartment (Nordberg and Arner 2001). This vitamin reacts directly with superoxide, hydroxyl radical and singlet oxygen and serves to prevent lipid hydroperoxide formation in plasma lipoproteins by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals. Under conditions of protective plasma vitamin C concentrations (>50μmol/l) significantly lower values of DNA, protein and lipid oxidative damage have been found in comparison with a vitamin C-deficient group (<50μmol/l) (Krajčovičová-Kudláčková *et al* 2006). Our results revealed a parallel between the mean levels of vitamin C and levels of cellular DNA damage. DNA damage in lymphocytes from schizophrenic male subjects was higher than in lymphocytes from schizophrenic females. However, mean plasma vitamin C levels were found to be higher in the female schizophrenic patients (~70μmol/l) than in male schizophrenic patients (~38μmol/l).

In summary, this research aimed to evaluate whether levels of oxidative stress, via a range of biomarkers are increased in models of schizophrenia and in patients with schizophrenia compared to the matched controls. With respect to lipid peroxidation and protein oxidation biomarkers, no significant difference in the levels of oxidative stress between the human schizophrenic and control groups was detected, while the results from the cell culture studies were inconclusive. However, treatment of cells with EPA, DHA and hydroxytyrosol to reduce levels of oxidative damage may warrant further research. Investigation of DNA damage suggests that hydrogen peroxide-induced DNA damage can be significantly reduced *in vitro* by certain ω-3 fatty acids and phenolic antioxidants while DNA damage in human subjects was not significantly different between the two study groups. Ultimately, it is important to investigate a range of biomarkers to determine whether the measurement of oxidative damage to lipids, proteins and DNA has clinical significance. This will enable better understanding of the disease of interest and allow these biomarkers to become potentially useful clinical tools.

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