



**Antioxidants or reduced oxygen tension as a
defense system in T cells *ex vivo* and *in vitro***

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Abbreviations

6-4 PP	6-4 pyrimidone photoproducts
ADP	Adenosine diphosphate
AP-1	Activator protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BER	Base excision repair
bp	Base pair
BR	Bilirubin
BSA	Bovine serum albumin
CAT	Catalase
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
CFSE	Carboxyfluorescein succinimidyl ester
CKI	Cyclin dependent kinase inhibitor
CPD	Cyclobutane pyrimidine dimer
CPL	Caeruloplasmin
Cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DCFDA	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTPP	Decyl triphenyl phosphonium cation
dGTP	8-hydroxy 2-deoxyguanosine
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
Endo III	endonuclease III
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
ETC	Electron transport chain
EU	European Union
FBS	Fetal bovine serum
Fe ²⁺	Ferrous ion
FPG	formamidopyrimidine glycosylase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
Gy	Gamma irradiated
H ³	Thymidine incorporation
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HDF	Human diploid fibroblast
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG ₂	Hepatocellular liver carcinoma cell line
HIF-1 α	Hypoxia inducible factor-1 α
HLA	Human leukocyte antigen

HLA-DR ⁺	Human leukocyte antigen- D related
HL-60	Human promyelocytic leukemia cell line
HPLC	High performance liquid chromatography
Hprt	Hypoxanthine guanine phosphoribosyltransferase
Hrs	Hours
HSCs	Haematopoietic stem cells
HSP	Heat shock protein
HUVECs	Human Umbilical Vein Endothelial Cells
IFN- γ	Interferon-gamma
IL	Interleukin
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LCA	Leucocyte common antigen
LDH	Lactate dehydrogenase leakage
M	Molar
μ M	Micromolar
mM	Millimolar
M2VP	1-methyl-2-vinylpyridinium trifluoromethane-sulfonate ¹
MACS	Magnetic cell separator
MAO	Monoamine oxidase
MAP	Mitogen activated protein
MBq	Mega becquerels
MDA	Malonyldialdehyde
Msr	Methionine sulfoxide reductase
MHC	Major Histocompatibility complex
Min	Minutes
MitoSOX	Mitochondrial superoxide indicator
MitoQ	Mitoquinone
MMR	Mismatch repair
MNNG	N-methyl- N-nitro- N-nitrosoguanidine
MSI	Microsatellite instability
mtDNA	Mitochondrial DNA
nM	Nanomolar
NAC	N-Acetyl L-Cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nDNA	nuclear deoxyribonucleic acid
NER	Nucleotide excision repair
NFkB cells	Nuclear factor kappa light chain enhancer of activated B cells
NK cells	Natural Killer cells
NKT cells	Natural Killer T cells
NO	Nitric oxide
NO ₂	Nitrogen dioxide
nt	Nucleotide
NTU	Nottingham Trent University
OECD	Organisation for economic co-operation and development
O ₂	Oxygen
O ₂ .	Superoxide anion
oxo8dG	8-hydroxy-2-deoxyguanosine

OXPHOS	Oxidative phosphorylation
PARP	Polyadenosine diphosphate ribose polymerase
PBN	<i>N-tert-butyl-α-phenylnitron</i> e
PBMCs	Peripheral blood mononuclear cells
PC-12	Pheochromocytoma cells
PD	Population doubling
PHA	Phytohemagglutinin
PP	Pyrimidone photoproducts
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SAPK	Stress activated protein kinase
S.D.	Standard deviation
SIPS	Stress induced premature senescence
SOD	Superoxide dismutase
Tc cell	Cytotoxic T cell
TCA	Tricarboxylic acid
TCIA	T cells in aging
TCR	T cell receptor
Th lymphocytes	T helper lymphocytes
TLR	Toll like receptor
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
UA	Uric acid
UV	Ultraviolet
UV-C	Ultraviolet irradiation
XO	Xanthine oxidase
Yr	Year

Abstract

Age-related deterioration and dysregulation of T cell function, termed 'immunosenescence', may lead to increased mortality and morbidity in humans through greater susceptibility to infections and disease. Previous research from the group of Barnett, suggested that oxidative stress may play a role in the immunosenescence process, through resultant genomic instability, cell cycle delay and arrest. The aim of this research programme was to investigate the effect of the antioxidants, ebselen, N-Acetyl L- Cysteine (NAC) or mitoQ or reduced oxygen (O₂) tension on markers of T cell function and integrity using CD4⁺ T cell clones and *ex vivo* polyclonal human peripheral blood derived mononuclear cells or CD4⁺ T cells derived from healthy young and older aged donors.

The results of this investigation revealed that 30µM ebselen or 7.5mM NAC supplementation significantly increased the lifespan and reduced levels of oxidative DNA damage in clones supplemented from a young *in vitro* age and in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* derived from either age group. The GSH:GSSG ratio was also significantly higher in supplemented clones and cells *ex vivo*. Ebselen or NAC were not able to bring about such biological effects in clones when supplemented from the midpoint of their *in vitro* lifespan. In this latter situation, age related changes in T cell physiology example: reduced DNA repair capacity, heat shock response and an accumulation of biomolecule damage may have contributed to these findings. Ebselen or NAC had similar antioxidant effects on polyclonal model system to those found for the *in vitro* clone model.

MitoQ, a mitochondria targeted antioxidant, at 0.5µM or 1µM concentrations, protected T cell genomic DNA from endogenously produced and exogenously applied oxidative stress evident by significantly higher GSH:GSSG ratio and significantly decreased levels of oxidative DNA damage on supplementation. 0.1µM or 0.25µM mitoQ protected genomic DNA from oxidative stress applied exogenously. The antioxidant effect on genomic DNA may be due to saturating concentrations in the mitochondria and the presence of mitoQ in the cytosol. The inability of mitoQ at lower concentrations to protect DNA against endogenously produced ROS may indicate that these lower concentrations of mitoQ may be taken up by the mitochondria, but they were not able to

reduce free radical release from the mitochondria explaining no significant differences in endogenous DNA damage levels and is reflected in the lack of changes to GSH:GSSG ratio. It may also be that mitochondrial ROS may not be the only source of oxidative genomic DNA damage.

Low O₂ culture conditions of 2% O₂ for T cells did not reveal an antioxidant potential in T cells *ex vivo* in a separate study evident by higher levels of oxidative DNA damage and lower GSH:GSSG ratio compared to T cells maintained at 20% O₂ culture conditions. The results of this PhD investigation suggest a potential *in vivo* anti-immunosenescent impact of these antioxidants at carefully selected concentrations.

CHAPTER-1

Introduction

1.1. Introduction

1.1.1. Ageing

Ageing can be described as a biological phenomenon resulting in changes in organs, tissues or cells in an organism leading to deterioration in their functional capacity in higher animals. Ageing has been described by biologists as a process with a significant genetic component, responsible for the varying lifespans among different species of animals, ranging from a few days to more than a hundred years. The ageing process causes changes in various cellular and tissue components resulting in altered physiological status leading to age related pathologies and ultimately death.

Ageing commonly results in changes in biomolecular structure in human body. These changes results in several physiological changes (Table 1.1). The rate of physiological alterations with age varies in different individuals in human beings. Each body system is detrimentally affected by the ageing process. The changes in hair and skin are the most common examples of a visual sign of this process. Ageing results in thinning, sagging, wrinkles, lentigo spots, dryness, broken blood vessels in the skin, greying and / or loss of hair. Lesser production of dermal connective tissue proteins, collagen and elastin with age can result in decreased elasticity and wrinkling of the skin. Ageing is also associated with loss in hair follicles, responsible for greying of hair. Ageing also results in slowing of wound healing.

In humans, ageing is further associated with a considerable deterioration of physical and psychological qualities of life and the appearance of a range of age related diseases such as cancer, heart diseases, dementia to name a few. Table 1.2 illustrates some of the age associated changes in systems in human body leading to pathological conditions (Barnett, 1994). Age related decline in the central nervous system may detrimentally affect intellectual functions, decrease orientation and may result in Alzheimer's or Parkinson's disease. The musculoskeletal system may also be affected resulting in sarcopenia (loss of muscle), thinning of cartilage in muscle joint resulting in osteoarthritis and overall bone loss.

Table 1.1 Physiological consequences associated with age related changes in biomolecular structure (Barnett, 1994)

Biomolecule	Changes / Alterations	Some of the physiological consequences as a result of alteration
Lipids	Lipid peroxidation	Oxidised membranes become rigid, lose permeability and finally cell death occurs
Proteins	Oxidation, Deamination	Alterations in long-lived proteins may result in ageing or associated pathologies, oxidation of proteins can affect cellular homeostasis
Carbohydrates	Fragmentation, Glucose auto-oxidation	Physical properties of connective tissues are altered and could result in osteoarthritis and age related joint disorders, Glycosylation of proteins can result in alteration of biological function
Nucleic acid	Strand breaks, base adducts	Damage could affect transcription, translation and DNA replication, capacity of the cells to synthesise important proteins and polypeptides.

Table 1.2 Age associated alterations in human body systems leading to pathological conditions (Barnett, 1994)

Body systems	Some of the age associated pathological conditions in human body system
Cardiovascular system	Atherosclerosis, Coronary heart disease
Central nervous system	Parkinson's disease, Alzheimer's disease
Endocrine system	Non-insulin dependent diabetes
Hemopoietic system	Anemia
Immune system	General decline in immune function, T cells in particular
Sense organs	Cataracts, diabetic retinopathy, glaucoma
Respiratory system	Interstitial fibrosis
Reproductive system	Decreased spermatogenesis
All systems	Cancer
Renal system	Glomerulosclerosis

A general decline in the function of the immune system, particularly T cells, has been found to result in increased occurrence of infectious and autoimmune diseases in elderly. It is a pre-requisite to investigate the complex changes in different sets of immune cell populations in a successfully aged population compared to an unsuccessfully aged population to implement better strategies to prevent age related illness culminating in death (Pawelec *et al.*, 2000).

Age related defects are often progressive and irreversible, resulting in an increase in the chance of death in all populations. The diseases of old age have led to an increase in physical and psychological decline in old aged people that result in them being a burden to their families and society. The world population has more than doubled in the last

half a century, evidenced by the rise in the number of people from two and a half billion in 1950s to approaching seven billion in 2010 according to a study carried out by the UN demographic society. Earlier demographic studies of ageing population has revealed an estimated 6% of the total world population were above the age of 65. The proportion is much higher in Europe (14%) and North America (13%) compared to 5% and 3% in Asia and Africa. Figure 1.1 shows the percentage of population of several developed countries above the age of 64 according a study carried out by an economic development organisation (OECD) in 2008. Further, a recent UN projection about the demographic changes have predicted a rise in share of populations over 60 years in developing countries as well (Algeria, Iran, South Africa, Egypt etc) from 5% to 15% in the near future. This means an increase in the number of old people with more pressure on the governments of the countries to address pensions, insurance demands and an increase in age related diseases. All these factors may contribute to the medical and economic burden of the country (Merson *et al.*, 2006).

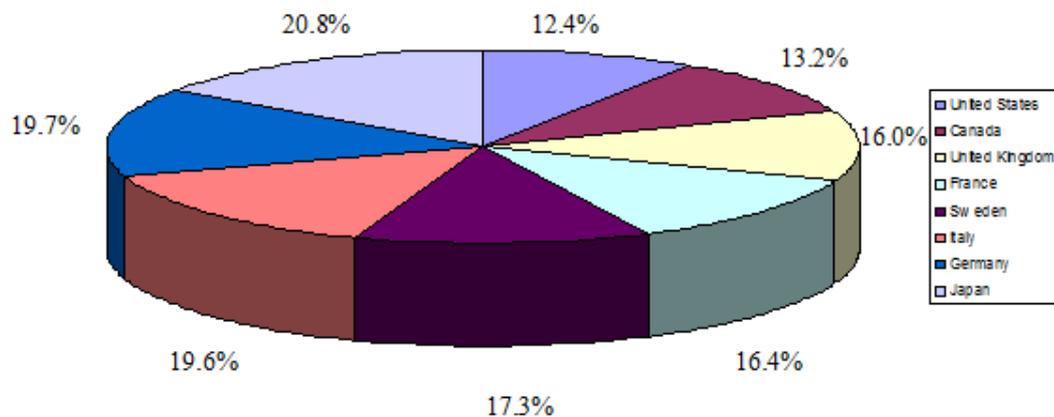


Figure 1.1. Percentage of population above the age of 64 in some of the developed countries in the world according to a demographic study carried out by an economic development organisation (OECD) in 2008.

Successful ageing research may enable the development of treatments or even identify interventions to help prevent age related diseases. If successful, ageing research would enable us to increase the healthy span of life in elderly. Although some humans have proven longevity escaping age related diseases and maintaining good physical health towards the later stages of life, their immune system is different from an unsuccessfully aged human (Engberg *et al.*, 2009). Some of the anti-ageing strategies include genetic manipulation or use of certain antioxidants (Ozawa, 1999). There have been different theories that have been proposed for explaining ageing.

1.1.1.1. The Free Radical Theory of Ageing

Reactive oxygen species (ROS) constitute free radicals and oxidants. Singlet oxygen is an example of an oxidant. ROS are highly reactive molecules that can originate from extracellular or intracellular sources and can damage cellular components in the body. ROS were first suggested as toxic agents by the group of Rebeca Gerschman (Gerschman *et al.*, 1954). The Free Radical Theory of Ageing, first proposed by Denham Harman, postulates ROS, produced mainly via aerobic metabolic pathway in the mitochondrion, is the major source of intrinsic oxidative damage for the cell that ultimately results in ageing (Harman, 1956; Sastre *et al.*, 2000; Muller *et al.*, 2007; Gruber *et al.*, 2008). ROS can cause cellular and tissue damage that impairs the function and proliferative capacity of the cells *in vivo* resulting ultimately in age related pathologies due to ageing (mainly as a result of the breakdown of protective mechanisms in the body) leading to death of the individual (Figure 1.2; Barnett, 1994).

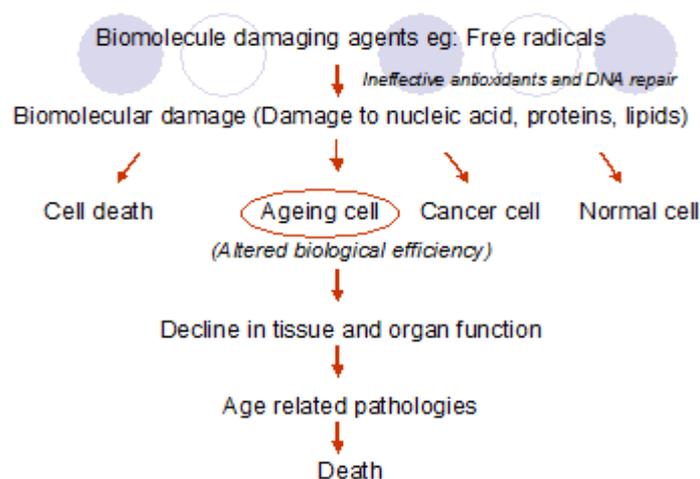


Figure 1.2. Free radicals leading to biomolecular damage resulting in ageing.

Although free radicals are seen as cellular damaging agents who are implicated in the aetiology and pathogenesis of degenerative diseases and ageing, they are involved in several intracellular signalling pathways generating huge amount of curiosity and interest in the field of ageing research. There are both endogenous and exogenous sources of free radicals (Table 1.3).

Table 1.3. Some examples of endogenous and exogenous sources of free radicals (Freeman, 1984; Halliwell, 1987)

Endogenous sources	Exogenous sources
Lipoxygenase, cyclooxygenase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in plasma membrane	Drug oxidation (Acetaminophen, cocaine)
Oxidases and flavoproteins as peroxisomes	Oxidising gases (O ₂ , Nitrogen dioxide (NO ₂))
Metal catalysed reactions	Xenobiotic elements
Haemoglobins, xanthine oxidase (XO), Monoamine oxidase (MAO)	Cigarette smoke, combustion products

The importance of free radicals in the field of ageing research has been strengthened further by their implication in several clinical conditions in humans. They are known to cause clinical conditions in skin, eye, and several other systems (Table 1.2) in human body. Solar radiation is an example of a source of free radical which could affect the skin in human beings.

1.1.1.2. Mitochondrial Theory of Ageing

The mitochondrial theory of ageing postulated by Harman in 1972, states that mitochondria are the major intracellular source of ROS. ROS contribute to cumulative damage to cellular constituents (Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), proteins and lipids) and this has been shown to play a role in ageing and eventual death (Harman, 1972; Brierley *et al.*, 1997; Miquel, 1998; Barja, 2000; Brand, 2000; Berdanier and Everts, 2000; Cadenas and Davies, 2000; Kowald and Kirkwood, 2000;

Melov, 2000; Rustin *et al.*, 2000; Sastre *et al.*, 2000; Salvioli *et al.*, 2001). The mitochondrial theory of ageing was further refined and developed by Jaime Miquel in 1980 (Miquel *et al.*, 1980).

One of the unique features that distinguish mitochondria (Figure 1.3) from other cell organelles is the presence of their own DNA called mitochondrial DNA (mtDNA). Mitochondria are present in virtually all of the cells in the human body with the exception of red blood cells. Their number varies from 20 to 2500 per cell (Pike *et al.*, 1984). The term mitochondria introduced by C. Benda around the turn of the century means 'thread like granules'. They are encapsulated by a double, outer and inner membrane. The inner membrane folds inwards to form extensions called cristae. Cristae protrude into the mitochondrial matrix and provide surface area necessary for mitochondria's vital functions. The ribosomes in the mitochondrial matrix are smaller than those of the cytoplasm and contain large calcium phosphate granules. The mtDNA genome is located in the matrix. Mitochondria are regarded as the powerhouse of the cell and play an important role in apoptosis and the production of adenosine triphosphate (ATP). ATP production within mitochondria occurs from the interaction of two metabolic cycles namely the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS) electron transport chain (ETC). Well functioning mitochondria is a pre-requisite for human life. Their number has been reported to increase with age and those surviving have been shown to increase in size after 60 years of age (Ozawa, 1997). The activity of mitochondrial enzymes involved in the ETC has been shown to decline with age. A decrease in the respiratory function of mitochondria in human tissues, increase in mtDNA damage and an exponential rise in the levels of the mutant form of 8-hydroxy-2-deoxyguanosine (dGTP), a product of free radical damage, are some of the age related molecular changes observed within mitochondria (Hayakawa *et al.*, 1993).

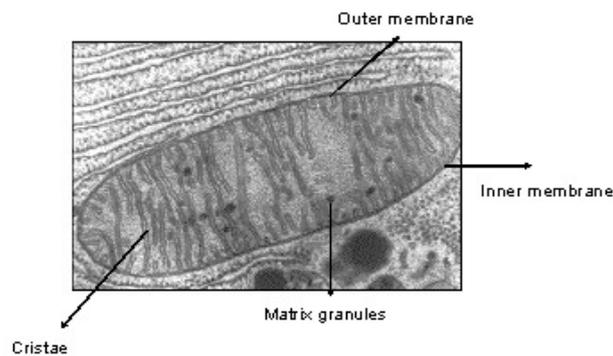


Figure 1.3. Electron microscopy of a human mitochondrion (Chapman and Hall, 1994).

1.1.1.2.1. Mitochondrial DNA genome

The human mitochondrial genome has been fully sequenced (Figure 1.4; Anderson *et al.*, 1981). It has a covalently closed circular double stranded genome of 16,569 bp. The mtDNA genome encodes for 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs) and 13 polypeptides. The polymorphic nature, maternal code of inheritance, relatively small genome and the lack of recombination of the mtDNA genome makes it an ideal template for pathogenic and anthropological studies venturing into human evolution, origin and patterns of migration (Torroni *et al.*, 1992; Torroni *et al.*, 1994; Torroni *et al.*, 1996; Cottrell *et al.*, 2000; Ingman *et al.*, 2000). Each mitochondrion contains between two to ten copies of mtDNA. Nuclear DNA (nDNA) is protected by histone proteins and various repair enzymes from free radicals or oxidants. mtDNA undergoes mutation five to ten times faster than nuclear DNA due to its proximity to the main site of ROS production, lack of protective histone proteins and an inefficient DNA repair mechanism (Richter, 1995). mtDNA damage often leads to the production of 8OHdG, an oxidised guanine base. A study carried out in ten healthy humans between the age of 42 and 97 years, analysing three brain regions, revealed a ten fold increase in 8OHdG formation in mtDNA compared to nDNA in all the subjects investigated, except those above 70 years of age who had a 15-fold increase. The increase in 8OHdG formation was correlated with increase in mtDNA deletions. Increase of mtDNA damage with age may ultimately lead to cellular energy crisis due to their detrimental effects on the functionality of ETC enzyme complexes that produce ATP (Richter, 1995).

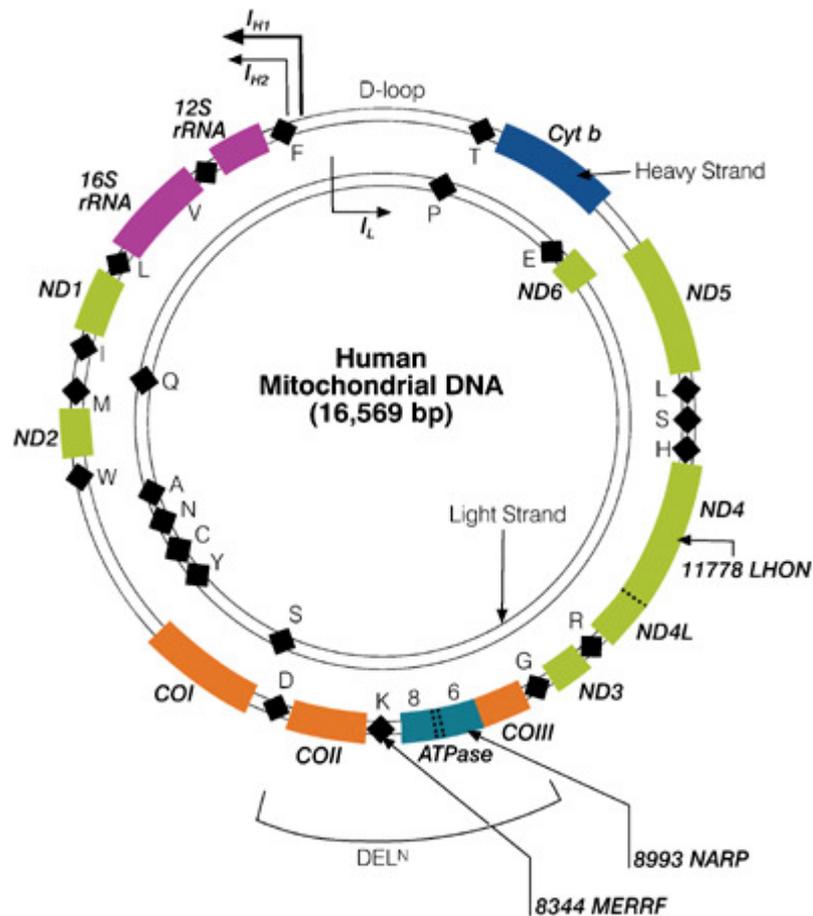


Figure 1.4. The 16,569 bp human mitochondrial DNA genome (Kyriakouli *et al.*, 2008).

1.1.2. Immune system

The immune system of the body protects individuals against foreign invaders such as pathogenic microorganisms and viruses. The immune system is made up of a network of cells, tissues and organs that protect the body. The conferred protection is called immunity. There are three types of immunity: innate, adaptive and passive immunity. Innate immunity (in detail in section 1.1.2.2) is the first line of defense system in the body, for example skin and mucous membranes that prevents pathogens or other invaders entering the body. Adaptive immunity (in detail in section 1.1.2.3) involves lymphocytes and develops throughout our lives and helps to protect against repetitive encounter of the same antigen. Passive immunity is gained from an external source and is temporary, for example: immunity conferred to an infant by antibodies in the mother's breast milk.

1.1.2.1. Cells of the immune system

The immune system comprises of several cells with assigned functions that protect the body against infectious organisms and invaders through a series of steps called the immune response. The majority of cells of the immune system, most importantly lymphocytes and phagocytes, are derived from stem cells in the bone marrow. Phagocytes circulate in the blood and develop into macrophages in tissues. Macrophages are tissue specific cells, for example alveolar macrophages in lungs and microglial macrophages in the brain. They scavenge non-functional cells in the body and engulf foreign pathogens, digesting their antigenic fragments. These antigens attract cytotoxic T cells that destroy the macrophages and the antigenic fragments facilitating a suicide attack. Lymphocytes enable the body to remember and recognise previous invaders and help the body to destroy them. The lymphocytes undergo maturation in primary lymphoid tissues, the thymus and the bone marrow. They circulate in the blood and through part of the lymphatic system such as lymph nodes, spleen and thymus. Lymph vessels are credited with the transportation and storage of lymphocytes within the body. The two major types of lymphocytes are T lymphocytes and B lymphocytes. Receptors on the surface of lymphatic cells help the cell to recognise a specific foreign antigen. They are very specific and can recognise only one specific antigen.

T cells are produced in the bone marrow and mature in the thymus. T cells are differentiated into two types namely helper T cells (Th cells) and killer T cells. Th cells are the main regulators of the immune defense. They enable the activation of B cells and killer T cells by the production of proteins. Proteins are produced by the activation of Th cells by their recognition of antigen presented by phagocytes (Figure 1.5). Killer T cells are the major surveillance cells in the body due to their ability to search every cell and recognise traces of antigen in some of the cells they encounter. This enables them to attack mutated cells and cells infected by foreign pathogens.

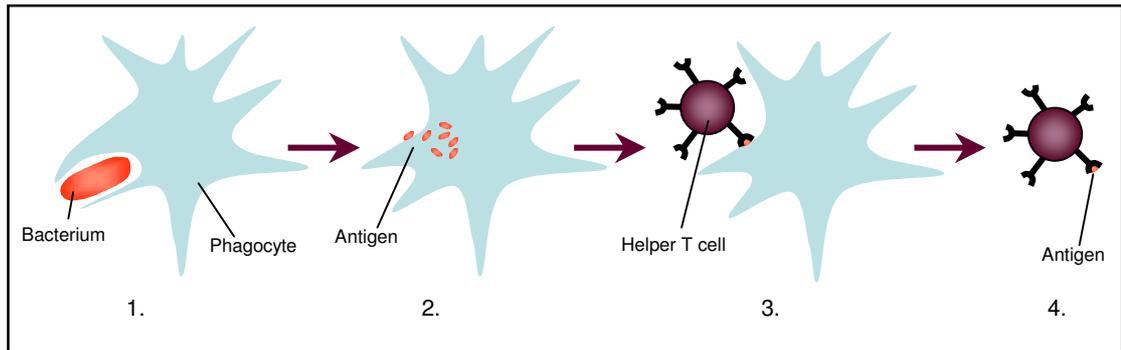


Figure 1.5. 1) A bacterium is engulfed by a phagocyte 2) Phagocyte digests the bacteria and presents the antigen on its surface 3) Th recognises the antigen presented by phagocyte on its surface 4) Antigen recognition results in activation of the Th cells.

The main function of B cells is to search for an antigen matching their receptors. Once proteins are produced by activated Th cells, B cells give rise to plasma cells and B memory cells. Plasma cells can produce antibodies capable of recognising the antigen bound to the B cell receptor enabling them to destroy any foreign pathogen. Antibodies produced by plasma cells are Y-shaped and have the ability to bind to multiple foreign antigens at the same time. B memory cells are associated with a prolonged lifespan and have the ability to recognise specific foreign pathogens that have attacked the body before. This strong memory associated with B cells enables them to confer immunity to the body against certain foreign antigens that were previously encountered (Figure 1.6).

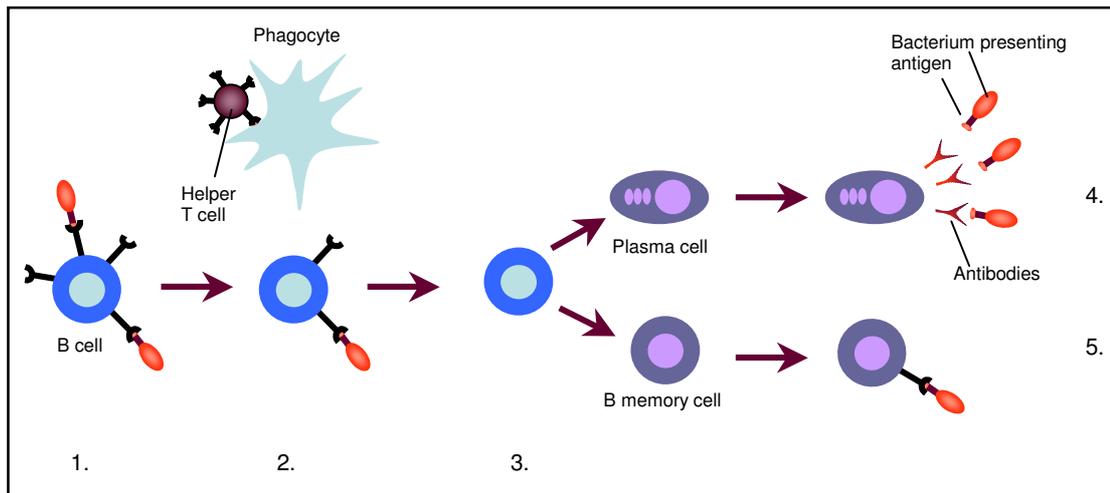


Figure 1.6. 1) A B cell binds to an antigen matching its receptors 2) Th cells recognise an antigen present on the surface of phagocytes resulting in production of proteins that enable the activation of the B cell 3) B cell activation results in its division giving rise to a plasma cell and a B memory cell 4) Plasma cells produce Y-shaped antibodies that attach to antigen 5) B memory cells confer immunity to the body by recognising foreign antigens that were already encountered previously.

Lymphatic cells express surface markers that broadly define their lineage and the stage of development. Haemopoetic cell markers are identified by cluster of differentiation (CD) numbers (Table 1.4). The two major sub-populations of T cells express CD4 and CD8. Lymphokines are secreted by CD4 and they act on other cells involved in immune responses. Cytotoxic CD8⁺ cells enable the lysis of infected cells.

Table 1.4. Some of the CD markers and their identity and function

CD (Cluster of differentiation)	Identity/function
CD3	T cell receptor (TCR) subunit (γ , δ , ϵ , ζ , η) – helps in antigen recognition and thereby activation of signal transduction pathways
CD4	Th cells – helps in activation of T cells on recognition of antigen presenting cells
CD8	Cytotoxic T cells (Tc cells) - T cells expressing CD8 markers can bind tightly to MHC Class I molecules expressing antigens due to presence of specific domains
CD28	Co-stimulatory TCR – required along with antigen specific signal for immune response
CD34	Bone marrow stem cell marker – Adhesion molecule helpful for T cells to enter the lymph nodes, expressed in endothelia in lymph nodes
CD38	Thymocyte activation marker – Regulates intracellular calcium by regulating the synthesis of cyclic ADP ribose
CD45	Leucocyte common antigen (LCA) – Protein tyrosine phosphatase receptor type C
CD69	Activation marker – cell surface glycoproteins involved in lymphocyte proliferation and differentiation

The two major types of immunity are detailed below.

1.1.2.2. Innate Immunity

The innate immune system deals with the day to day protection of the body from foreign pathogens. It is also referred to as the non-specific arm of the immune system and is credited with the release of cytokines and chemokines in the body. Natural Killer cells (NK), natural Killer T cells (NKT), macrophages and dendritic cells (DCs) are indispensable to the innate immune system. NK cells and NKT cells originate from the lymphoid progenitor cell; phagocytic cells (macrophages and DCs) originate from myeloid progenitor cells. The latter have the ability to bind to a variety of ligands due to the presence of surface receptors, which enable them to recognise pathogens. These receptors are called Toll like receptors (TLR) (Chaplin, 2003). TLR enable the stimulation of cells of the innate immune system, which acts in the following order: They recognise the pathogen, phagocytise it and eventually lyse it with complement molecules released by the innate immune system. DCs in the innate immune system have the ability to activate the adaptive immune system and to capture, process and present the antigen, through the major histocompatibility complex (MHC), to cells of the adaptive immune system. This forces the adaptive immune system to initiate a specific immune response against the antigen. Due to their efficiency, DCs are important in immune study for treating several infections.

1.1.2.3. Adaptive Immunity

The mechanism of immunity in the body starts with the innate arm of the immune system and terminates with its adaptive arm (Chaplin, 2003). The latter is more prone to the deleterious effects of ageing, compared to former. The adaptive arm primarily utilises the proteins and cells circulating in the body to provide protection from infections. T cell and B cell responses are a pre-requisite for the function of the adaptive immune system. B cells and antibodies are responsible for humoral responses that can present antigen to T lymphocytes and CTL or CD8⁺ T cells; CD4⁺ Th1 cells and CD4⁺ Th2 cells are the mediators of cellular responses. CD4⁺ Th1 cells enable activation of CTL, CTL recognise and eliminate infected cells and CD4⁺ Th2 cells help developing B cells. In adaptive immunity, DCs are attracted to the site of infection and phagocytose cell debris and apoptotic cell fragments, thereby intaking MHC molecules expressing peptides in the infected cell and migrate to the lymph nodes, resulting in interactions

with CD4⁺ Th1 cells and CD8⁺ T cells to form a trivalent complex. This complex enables DC maturation through cytokine and cell surface interactions between CD4⁺ Th1 and DCs that in turn results in interactions between DCs and CD8⁺ T cells resulting in the differentiation of CD8⁺ T cells into CTL. CTL migrate back to the site of infection along with the Th cells to instigate antigen specific killing of any infected cells presenting the antigen (Figure 1.7). CTL were pre-conditioned against the antigen and kill the infected cell through the secretion of cytotoxic granules or by stimulating certain receptors produced by the infected cells that enable its degradation (Chaplin, 2003). For an effective immune response, T cells are required to undergo rapid clonal expansion upon antigenic stimulation. Any factor which interferes with the ability of T cells to divide can therefore have the potential to contribute to a decline in T cell function resulting in a sub-optimal T cell mediated immune response.

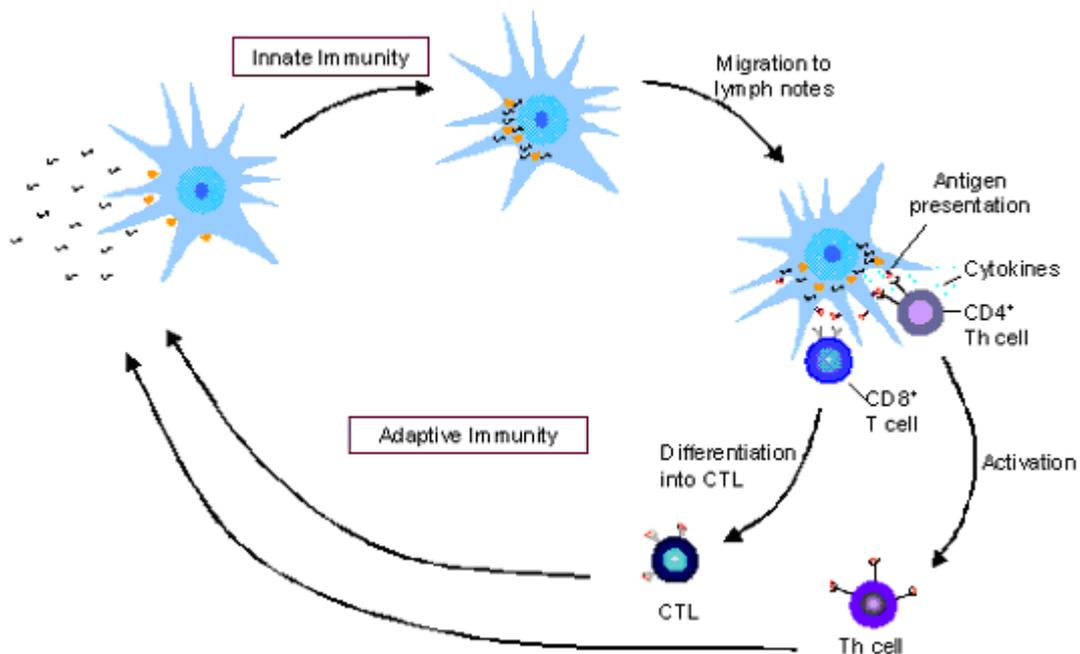


Figure 1.7. DCs are attracted to the site of infection and come in direct contact with infected cells. They phagocytose the cell debris and apoptotic cell fragments and migrate to the lymph node. DCs present the antigenic peptides expressed by MHC molecules on the cell surface for recognition by CD4⁺ Th1 cells and CD8⁺ T cells. This interaction results in the maturation of DCs. Interactions between DCs and CD8⁺ T cells result in differentiation of CD8⁺ T cells into CTL. CTL along with the activated Th cells migrate back to the site of infection resulting in antigen specific killing of any infected cells presenting antigen against which they are pre-conditioned.

1.1.3. Ageing and the immune system

Immunosenescence is a well characterised deterioration and dysregulation of T cell function with age. It can lead to increased mortality and morbidity in humans through greater susceptibility to infections and diseases (Pawelec, 2001). T cells are supposed to undergo rapid clonal expansion on antigenic stimulation to produce an effective immune response. DNA damage and mutation are some of the factors which may interfere with the ability of T cells to undergo replication. Defects in signalling pathways and decreased T cell response to antigens are some of the immunological abnormalities as a result of ageing (Gupta, 2003). A better understanding of the immunosenescence process may improve the healthy lifespan and quality of later stages of life.

1.1.3.1. Clinical relevance of immunosenescence

Clinical observations have indicated that severe and lethally infectious diseases, such as endocarditis, septicemia, tuberculosis are among those often contracted by elderly people due to lymphopenia (decline in immune function) (Ginaldi *et al.*, 2001). It is often associated with high mortality rate in old aged individuals (Gupta, 2003). Clinical evidence indicates a significant decline in the ability to raise a primary immune response against un-encountered antigens (Fagnoni *et al.*, 2000). Influenza is a major health problem among elderly people in industrialised countries to an extent that 90% of the deaths attributed to the influenza virus annually in the United States occur in people above 65 years of age (Castle, 2000). It was also noted that the extent of protection in response to influenza vaccine was considerably lower in old aged people, compared to young adults. The reasons for the increased rate of mortality may be due to one / or a number of immunological parameters (Table 1.5).

Table 1.5. Immunological parameters affected by age (Ginaldi *et al.*, 2001)

Parameters	Age associated alterations
CD8 ⁺ cytotoxic cell fraction	↑
T cell proliferative response	↓
CD4 ⁺ helper T cells	↓
CD19 B cells	↓

The increase in cytotoxic CD8⁺ cell fraction results in a low CD4:CD8 ratio (Saurwein-Teissl *et al.*, 2002). A study carried out on individuals aged 86-92 years showed three times higher mortality in individuals having low T cell proliferative response to mitogens, low B cell number and low CD4:CD8 ratio compared to individuals exhibiting high T cell proliferative response to mitogens, high B cell number and high CD4:CD8 ratio (Ferguson *et al.*, 1995; Wikby *et al.*, 1998).

1.1.3.2. Age associated alterations in the immune system

A strong immune system is a must to support healthy ageing. Ageing of the immune system involves large modifications of humoral, cell-mediated and innate immunity. Some of these summarised in table 1.6.

Table 1.6. Some of the age associated modifications of the immune system (Ginaldi *et al.*, 1999; 2001; Meyer and Soergel, 1999; Pawelec *et al.*, 1999)

Modifications of the immune system with age
↓ in number of naive T cells compared to memory T cells
↓ of interleukin-2 (IL-2) production
↓ in interferon-gamma- γ (IFN- γ) production
↓ of IL-4 production
↓ of production of pro-inflammatory cytokine IL-12
↑ of production and secretion of IL-10
↓ in function of neutrophils

An age associated decrease in naive T cells of the cell mediated immune response results in decreased production of IL-2, high affinity IL-2 receptors and a reduced T cell proliferative response to previously un-encountered antigens. The decrease in number of naïve T cells with age results in the retardation of the ability of the body to respond to previously un-encountered pathogens. IFN- γ are cell signalling proteins that are anti-viral agents and can fight tumours. It also increases the cell surface expression of MHC Class I and Class II molecules which enabled the activation of T cells to recognise the

antigens presented by them. IL-4 molecules enabling the differentiation of naïve T cells to Th2 cells and the production of IL-2 and TNF- α , which helps in combatting foreign pathogens, are also decreased with ageing of immune system.

1.1.3.3. Age associated thymic involution

The thymus is a structure present in almost all vertebrates. It is located beneath the upper portion of the sternum and mostly consists of lymphatic tissue and to a smaller extent epithelial tissue known as Hassall's corpuscles. The human thymus increases in weight until puberty. Post-puberty, thymus gradually involutes and fat replaces lymphatic tissue of the thymus gland.

Age associated changes to the thymus are observed both in humans and mice. Increased levels of sex hormones have been suggested to cause thymic involution (Derhovanessian *et al.*, 2008). Haematopoietic stem cells (HSCs) are known to mature in thymus. Age associated thymic involution may affect their maturation. HSCs are characterised by their ability to self renew and to form multiple cell types. A compromised production or function of HSCs would affect the immune system as a whole. There is evidence for an association between HSCs decline and longevity (Geiger and Van, 2002). Hakim *et al.* (2005) demonstrated exceedingly low levels of thymic activity after the age of around 40 years in individuals after HSCs transplantation. The exact reason for thymic involution still remains unclear (Derhovanessian *et al.*, 2008). Thymic involution is also responsible for the compromise of T cell differentiation with age. Thymic function is also likely to be compromised by the presence of any underlying disease and its therapy.

1.1.3.4. Age associated alterations in T cell subsets

Mature T cells are subject to changes during ageing as a result of several factors including antigen exposure, clonal expansion and contraction. The varying proportions of T cell subsets in young and aged individuals is a particular example of this age related alteration in T cells. Old aged human donors have increased proportions of CD4⁺CD45RO⁺ memory phenotype cells and less CD45RA⁺ naive phenotype cells compared to young donors (Hannet *et al.*, 1992; Utsuyama *et al.*, 1992; Gabriel *et al.*, 1993). The increased proportions of CD4⁺ CD45RO⁺ memory phenotype cells enable the human body to respond to antigens encountered previously and lesser proportions of

CD45RA⁺ naive phenotype cells in peripheral blood (Fagnoni *et al.*, 2000) and immune organs such as lymph nodes (Lazuardi *et al.*, 2005) would result in the impairment of ability to respond to new antigens (Koch *et al.*, 2008). The functional integrity of both CD4⁺ and CD8⁺ T cell subsets decreases with age (Naylor *et al.*, 2005; Hadrup *et al.*, 2006). This contributes to a significant decrease in the ability of elderly to respond adequately to relapsing infections as well as their ability to respond to persistent infections due to immune exhaustion (Zinkernagel *et al.*, 1993).

1.1.3.5. Ageing of T cells

T cells are supposed to undergo rapid clonal expansion followed by clonal contraction to mediate an effective adaptive immune response. Prior to this step, T cells become activated by interactions with functional antigen presenting cells (APCs). The interactions of T cells with DCs depend on the number and structure of the cell-surface receptors and intracellular signalling pathways. Ageing is also associated with a decrease in the expression of the cell-surface molecule, CD28; commonly referred to as the biomarker for immunosenescence. The decrease in expression is attributed to the accumulation of CD28⁻ T cells within the CD8⁺ T cell subset in peripheral blood of elderly. The loss of CD28 expression *in vitro* is attributed to T cells undergoing differentiation and entering a state of replicative senescence (Effros *et al.*, 1994).

1.1.3.6. Age associated defects in efficacy of vaccinations

Depletion of T cell function with age is one of the main contributors detrimentally affecting the optimal efficiency of vaccines in the elderly. An age associated increase in pro-inflammatory factors contributes to poor response to vaccinations against pathogens causing influenza and pneumonia (Kovaiou *et al.*, 2007). Shorter-lasting effects of vaccinations and low levels of responses to secondary antigens are some of the examples of depleted efficacies of vaccinations in elderly. Boosting the efficacy of the vaccine may enhance the NK cell system which may in turn be associated with better vaccine responses (Mysliwska *et al.*, 2004). IL-2 pre-treated elderly subjects were better protected against influenza virus compared to controls (Provinciali *et al.*, 1994). Efficiency of IL-2 in boosting efficacy of vaccines was also revealed in an IL-2 supplemented liposomal vaccine study in 48 elderly people (Ben-Yehuda *et al.*, 2003).

1.1.3.7. Impact of ageing on cancer and autoimmune diseases

The reduced immune function in elderly, due to a compromised immune system, may result in increased cancer occurrence. The median age for the diagnosis of cancer in industrialised countries is approaching 70 years of age and is expected to increase (Ries *et al.*, 2003). There is no evidence to show an increase in occurrence of cancer with age. Normally, individuals are exposed to pathogens throughout their life time and exposure beyond an optimal load may result in immune exhaustion (compromise in immune system) leading to immunosenescence. Cancer antigen load is also believed to have a similar effect ultimately leading to immunosenescence (Derhovanessian *et al.*, 2008).

The impaired and inappropriate reaction of the immune system to self antigens, causing cell and tissue damage and acute or chronic inflammation processes, can result in autoimmune diseases, sometimes called as ‘autoaggression’. Rheumatoid arthritis (RA) is the most common example of autoimmune disease occurring in elderly. But it has been shown to also occur in young adults or teenagers (Witkowski, 2007).

1.1.3.8. Human T cell clones as a model for immune study

The use of monoclonal human T cell populations was a milestone in the field of ageing research, enabling the manipulation of immunosenescence *in vitro* and shedding insights into T cell immunosenescence *in vivo*. The T cell clone model *in vitro* is well documented (Pawelec *et al.*, 2000). The thorough understanding of the immunosenescence process may lead to the development of interventions aimed at lengthening the healthspan in old aged individuals.

T cell clones *in vitro* are useful for the study of factors which contribute to T cell senescence and for investigating age associated changes over the lifespan (Pawelec *et al.*, 2002). T cell clones can be maintained for extended periods in culture and have finite lifespans. At the end of the *in vitro* lifespan, T cell clones die by apoptosis. Since the T cell clone model is monoclonal, the behaviour of a single cell type can be followed. In polyclonal systems, changes observed may be due to alterations in the proportions of the different cells present (Pawelec *et al.*, 2002). Also in a polyclonal population, calculation of longevities can only be done by examining individual components cloned separately. Otherwise, only the longevity of the last remaining

surviving clone from the original polyclonal population can be measured. This has been a confounding factor in fibroblasts, one of the most extensively studied cell type, where the data available do not imply to the average longevity of the entire population, but only to the longest lived clone present in the study population. It is essential to know the *in vivo* age of the starting T cell population in a meaningful study of T cell ageing *in vitro*. This is impossible in a polyclonal population. So the isolation of precursors and enabling them to differentiate into T cell clones *in vitro* is the only way to make sure that all T cells studied are of the same age at the beginning of the experiment (Pohla *et al.*, 1993; Barnett and Barnett, 2000).

In spite of all the advantages, the T cell clone model has its limitations. Whilst on the one hand, one of the major assets of the *in vitro* model is its monoclonality; this clearly is not a true reflection of the *in vivo* situation, which is predominantly polyclonal. Furthermore, to maintain human T cell clones in culture throughout their lifespan is a relatively labour intensive and not cost effective process.

The growth characteristics and the longevity of human T cell clones depend on the age of the donor (Pawelec *et al.*, 2006). Clones derived from neonates, young adults (20-30 years (yr)) and elderly (70-90 yr) had 52, 40 and 32 population doublings (PDs) respectively (McCarron *et al.*, 1987). Extensive research carried by our group revealed similarities in overall patterns of growth in T cells derived from different origins with different ages (Pawelec *et al.*, 2002) (Table 1.7).

Table 1.7. Longevity is expressed as a percentage of established clones that survive to 20, 30 or 40 PDs. Origins: CD3 (young), mature peripheral T cells from healthy 30 yr old donors; (old), perfectly healthy SENIEUR- compliant donors over 80 yr; (cent), centenarian donors >100 yr (Pawelec *et al.*, 2006)

Clones/Origins	Percentage of clones reaching			Longest lived clone (PD)
	20PD	30PD	40PD	
CD3 (young)	47	24	15	90
CD3 (old)	48	26	16	77
CD3 (cent)	41	23	17	80

Human T cell clones can be easily generated and maintained in culture (Pawelec, 1993). The procedure involves a limiting dilution procedure of the cell suspension to be cloned and microwell culture of the diluted cells on an irradiated feeder cell layer in the presence of IL-2. In long term culture systems, T cells are stimulated weekly via the TCR using gamma irradiated (80 Gy) RJK 853 feeder cells. RJK 853 is an Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line with a complete deletion in the gene encoding hypoxanthine guanine phosphoribosyltransferase (HPRT). The T cell clones are maintained in X-Vivo 10 (Bio Whitaker), a serum free medium specifically designed for the cloning and propagation of lymphocytes. They are also supplemented once in every three days with 400U/ml recombinant human IL-2. The RJK cells are irradiated to prevent the interference / cross reaction of their intracellular functions with the T cell recognition. The RJKs express receptors on their surface that can be recognised by T cells. The age of the T cell clones can be tracked by calculating the number of PDs the cell has undergone. A different B lymphoblastoid cell line or human peripheral blood mononuclear cells can also be used as feeder cells instead of gamma irradiated (80 Gy) RJK 853 cells. Many important similarities have been found between the T cell clone model of immunosenescence and T cell ageing *in vivo* (Pawelec *et al.*, 2002) (Table 1.8).

Table 1.8. Similarities between T cell clones *in vitro* and T cell ageing *in vivo*

Similarities between T cell clone model of immunosenescence and T cells ageing <i>in vivo</i>
Age associated increase in DNA damage (Barnett <i>et al.</i> , 1999; Hyland <i>et al.</i> , 2000; 2001)
Decrease in telomere length
Decreased telomerase induction (Bodnar <i>et al.</i> , 1998)
Decrease in DNA repair capacity (Annett <i>et al.</i> , 2004; 2005)

1.1.4. The biological effects of oxidative stress

1.1.4.1. Effect of oxidative stress on intracellular redox status

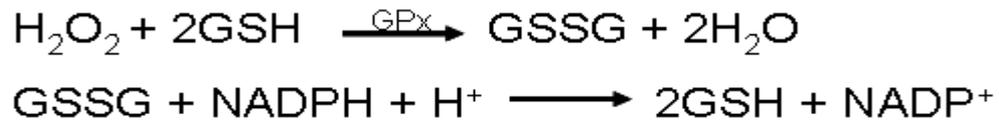
Intracellular redox status is an important mechanism credited for its role as a mediator in apoptosis in many cell systems (Cotgreave and Gerdes, 1998; Hall, 1999). Recent findings indicate depletion of intracellular reduced glutathione (GSH), a main determinant of intracellular redox status, before the onset of apoptosis (Beaver and Waring, 1995; Macho *et al.*, 1997; Ghibelli *et al.*, 1998). Some of the main factors leading to depletion of GSH are mentioned in table 1.9.

Table 1.9. Main factors leading to depletion of GSH

Main factors leading to depletion of GSH
The depletion of GSH could be due to increased oxidation of GSH to oxidised glutathione (GSSG) (Slater <i>et al.</i> , 1996)
Inhibition of GSH synthesis (Yang <i>et al.</i> , 2000)
Use of GSH by Glutathione transferases in detoxification of electrophilic compounds contributing to its depletion in the cell.

1.1.4.1.1. Glutathione function and redox balance

GSH is an abundant antioxidant in the cell (Cotgreave and Gerdes, 1998) which acts as an important biomarker of oxidative stress. It can exist in two forms: 1) Reduced monomer (GSH) 2) Oxidised dimer (GSSG). In normal conditions, GSH is present in cells at more than hundred fold concentration of GSSG. GSH is a major low molecular mass thiol in mammals (Halliwell and Gutteridge, 1999). The majority of GSH in plant and animal cells is present in the cytoplasm, where it is synthesised at concentrations between 0.5mM and 10mM. The reduced glutathione is a tripeptide with a free thiol group. It provides reducing equivalents for glutathione peroxidase (GPx) catalysed reduction of hydrogen peroxide (H₂O₂) and lipid hydroperoxides to water (H₂O) and the respective alcohol. This reaction results in the oxidation of GSH to GSSG. GSSG is then recycled into GSH by glutathione reductase (GR) and NADPH (Figure 1.8).



The activity of reductase increases in response to increase in concentrations of GSSG during oxidative stress (Jones, 2002). Excess or free GSSG can be eliminated from cells either by glutathionylation or through specific transporters exporting GSSG out of the cell.

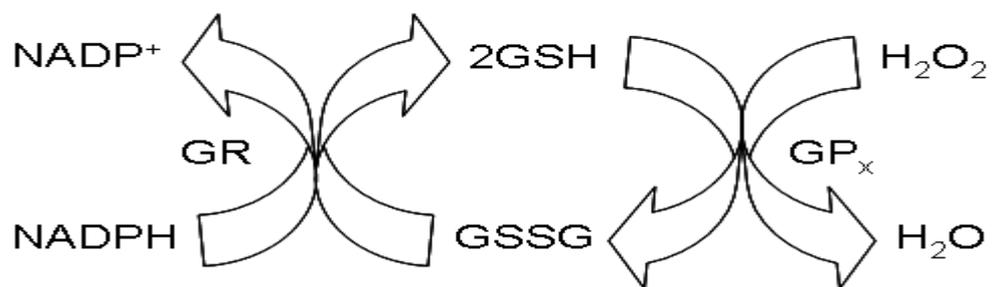


Figure 1.8. Oxidation of GSH to GSSG by GP_x and recycling of GSSG back to GSH by GR.

GSH recycling and biosynthetic pathways ensure its availability by up-regulation during oxidative stress and nitrosative stress (Griffith, 1999; Kondo *et al.*, 1999; Moellering *et al.*, 1999). Intracellular GSH is mainly regulated by the transport and availability of cysteine. The consecutive action of two enzymes, glutamate cysteine ligase and glutathione synthase are the pre-requisite for the synthesis of GSH in cells. The GSH:GSSG redox couple maintains the redox environment of the cell, it is abundant in the cell (Schafer and Buettner, 2001) and it serves as an indicator of the cellular redox environment. In biological systems, even a moderate level of oxidation can result in the formation of GSSG and mixed disulfides between protein sulfhydryl groups and GSH that would hinder the redox status of the cell. The decrease in levels of GSH can result in an increase in levels of GSSG and thereby lowering the GSH:GSSG ratio, which has been suggested to be responsible for several human diseases such as; lung inflammation, amyotrophic lateral sclerosis, chronic renal failure, malignant disorders, diabetes, alcoholism, Parkinson's disease, Alzheimer's disease and cataract formation (Lomaestro

and Malone, 1995; Herzenberg *et al.*, 1997; Navarro *et al.*, 1997; Samiec *et al.*, 1998; Spickett *et al.*, 1998; Cecchi *et al.*, 1999; Hadi Yasa *et al.*, 1999; Navarro *et al.*, 1999; Papp *et al.*, 1999; Bonnefont Rousselot *et al.*, 2000; Kharb, 2000; Lang *et al.*, 2000; Mills *et al.*, 2000; Pastore *et al.*, 2001). The underlying mechanism behind the cause of diseases may be the reduction in capacity of cells to detoxify free radicals due to reduced levels of glutathione peroxidises enzyme system or GSH depletion may greatly inhibit immune function of the body, thereby, increasing its vulnerability to infection (Droge *et al.*, 1994). Glutathione depletion has also been revealed to cause lipid peroxidation in patients suffering from Parkinson's disease (Lohr and Browning, 1995). But it has been found that during ageing GSH levels appear to decline in a number of tissues which increases the possibility of the cells succumbing to stress (Maher, 2005). Oxidative stress can be detected by evaluating the changes in redox tone (GSH:GSSG ratio).

1.1.4.2. Genetic damage in T cells *ex vivo*

DNA damage and mutation have been shown to increase as a function of age *ex vivo* in cultured lymphocytes from healthy male subjects in three different age groups (35-39, 50-54 and 65-69 years; Barnett and King, 1997). The basal levels of DNA damage were measured by the alkaline comet assay. Lymphocytes were also associated with an age-related decline in DNA repair capacity due to their inability to repair H₂O₂ induced DNA damage. This age related retardation of DNA repair capacity might result in an accumulation of oxidative DNA damage within T cells which might in turn result in age related decline in replicative capacity. Interestingly, subjects from the 75-80 year age group did not show an increase in DNA damage and decrease in DNA repair capacity levels were similar to that of 35-39 year old subjects.

Ageing was also associated with loss of X or Y chromosomes (Schneider, 1979), increase in development of micronuclei (French and Morley, 1985) and increase in frequency of mutations at various genetic loci (Morley *et al.*, 1982; Trainor *et al.*, 1984; Vijayalaxmi and Evans, 1984; Cole *et al.*, 1988; 1991; Barnett and King, 1995; King *et al.*, 1997). The mean mutant frequency at the *HGPRT* gene locus in lymphocytes isolated from healthy male subjects in the age group of 65-69 years was considerably higher than in the younger age groups of 35-39 years and 50-54 years (King *et al.*, 1994; 1997). The same can be said about chromosomal aberrations, when the frequency of

chromosomal aberrations in 75-80 year old subjects was fewer than in 50-69 year old subjects. It is also worth mentioning that the antioxidant status remained the same for age groups between 35-69 years. The factors above suggest DNA repair processes and antioxidant defense systems as major contributing factors for maintaining genomic stability *in vivo*, which in turn contribute to longevity (Barnett and Barnett, 1998).

1.1.4.3. Genetic damage in T cells *in vitro*

An increase in oxidative DNA damage (oxidised purine and pyrimidine DNA bases) in human CD4⁺ T cell clones *in vitro* as a function of age was revealed by Hyland *et al.*, (2000; 2001; Figure 1.9). DNA damage measured using the alkaline comet assay showed a significant increase in its levels of prior to the end of their lifespan. This increase in damage with age might interfere with the ability of T cells to undergo rapid clonal expansion on antigenic stimulation to produce an effective immune response resulting in immune responses to be sub-optimal. Human CD4⁺ T cell clones were also associated with an age related decline in the efficiency of DNA excision repair capacity (Table 1.10) and mismatch repair capacity (Table 1.12). These results were obtained in human CD4⁺ T cell clones derived from healthy 26-year old and 45-year old donors, but there was no decline in either DNA excision (Table 1.11) or mismatch (Table 1.12) repair capacity in CD4⁺ T cell clones from a healthy 80-year old donor (Annett *et al.*, 2004; 2005).

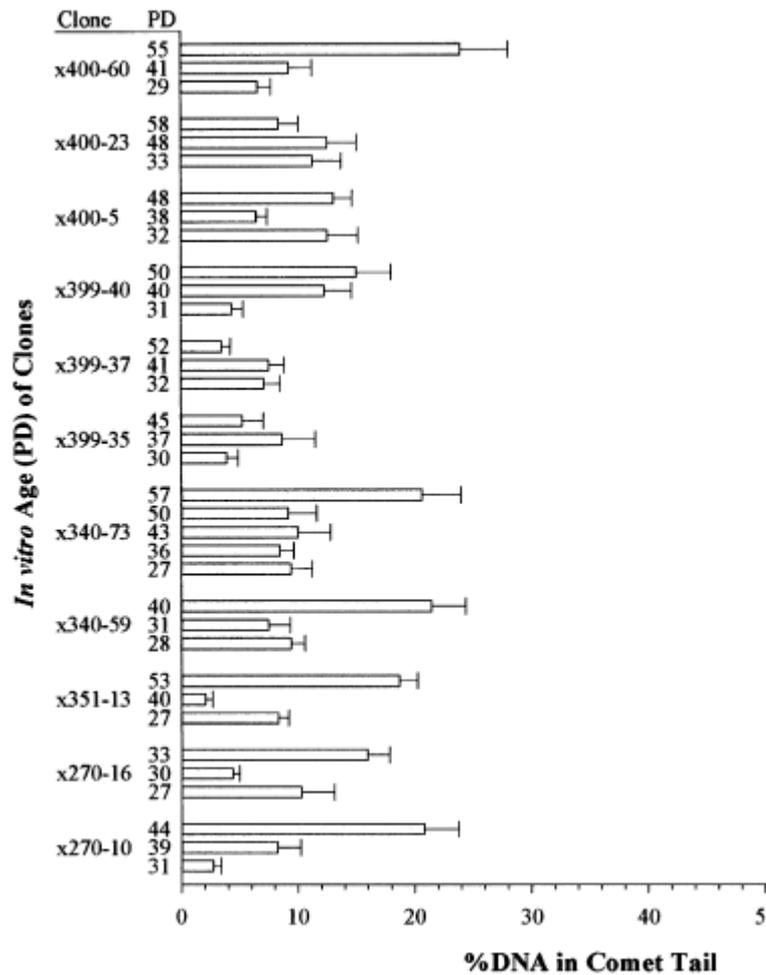


Figure 1.9. DNA damage levels in 11 peripheral blood derived CD4⁺ T cell clones as a function of their *in vitro* lifespan using alkaline comet assay revealed a typically greater percentage of DNA in the comet tail (>15%) at the last PD analysed in 7 out of 11 clones. Values represent mean standard error for each assay counting 50 cells per slide (Hyland *et al.*, 2001).

Table 1.10. Scale representation shows an age related decline in DNA repair capacity with age in T cell clones (400 series- 26 year old donor and 385 series- 45 year old donor) following induction of different types of DNA damage (Oxidation damage induced by H₂O₂, Alkylation damage induced by N-methyl- N-nitro- N-nitrosoguanidine (MNNG), ultraviolet irradiation (UV-C))

Clone	Type of DNA damage induced								
	Oxidation			Alkylation			UV-C		
	Young	Middle	Old	Young	Middle	Old	Young	Middle	Old
400-23	+++	++	+	++++	++++	+++	++	-	-
400-60	++	++	+	++	+++	++	++	++	+
385-2	+++	+++	++	+++	++	++	nd	nd	nd
385-7	++++	+++	+++	+++	+++	+++	nd	nd	nd

Table 1.11. Scale representation shows maintenance in DNA repair capacity of T cell clones (399 series- 80 year old donor) following induction of different types of DNA damage (Oxidation damage induced by H₂O₂, Alkylation damage induced by MNNG, UV-C)

Clone	Type of DNA damage induced								
	Oxidation			Alkylation			UV-C		
	Young	Middle	Old	Young	Middle	Old	Young	Middle	Old
399-35	+	++	-	+++	+++	++++	++	+	+
399-37	+++	+++	+++	++++	++++	++++	++++	++++	+++
399-40	++	+++	+++	+++	++	++++	nd	nd	nd

++++ Excellent repair, +++ Good repair, ++ Average repair, + Poor repair, - No repair, nd not determined (Adapted from Annett *et al.*, 2004).

Table 1.12. Age related decrease in mismatch repair capacity was observed in T cell clones derived from a 26 year old donor (400 series) and a 45 year old donor (385 series). There was no decline in mismatch repair capacity in T cell clones derived from an 80 year old donor (399 series) (Annett *et al.*, 2004)

Clone	Starting lifespan PD	End of lifespan PD	Repair rate at start of lifespan	Repair rate at end of lifespan
400-60	33.1	69.2	0.2%	0.05%
385-2	34.5	64.4	0.27%	0.10%
399-35	35.2	67.5	0.16%	0.21%

1.1.4.4. Oxidative DNA damage to T cells

ROS are considered as a major factor responsible for oxidative DNA damage in T cells. Free radicals, such as hydroxyl radicals and super-oxide radicals, are generated *in vivo* during normal cellular oxygen metabolism in the mitochondria (Ames *et al.*, 1993; Cardozo-Pelaez *et al.*, 2000). A variety of *in vivo* and *in vitro* experiments have revealed that ROS can induce various DNA lesions, such as DNA strand breaks [single-strand breaks and double-strand breaks] and base modifications (Dizdaroglu, 1998; Cadet *et al.*, 2003). DNA double stranded breaks etc, if un-repaired, can result in carcinogenesis, mutagenesis and other degenerative ageing diseases (Su *et al.*, 2006). Hydroxyl radicals have also been shown to be responsible for the attack on the sugar moiety of DNA molecules responsible for inducing backbone breaks (Dizdaroglu, 1998). 8-hydroxy-2-deoxyguanosine (oxo8dG) is a major mutagenic base lesion formed as a result of hydroxyl radical attack on DNA. Oxo8dG base pairs preferentially with adenosine, rather than cytosine, generating transversion mutations following replication.

1.1.4.5. Mitochondrial DNA damage and mutations in T cells

Human ageing has a peculiar effect on mitochondria. Ageing in various human tissues has been attributed to the occurrence and accumulation of different types of mutations (deletions, base substitutions and frame-shifts) in the mtDNA resulting in an established decline in mitochondrial bioenergetic function (Brierley *et al.*, 1998; Liu *et al.*, 1998;

Graff *et al.*, 1999; Michikawa *et al.*, 1999; Fernandez-Moreno *et al.*, 2000; Kopsidas *et al.*, 2000; Wang *et al.*, 2001). Deletions of mtDNA were found to be the most common mutation associated with human diseases and ageing process (Kovalenko *et al.*, 1997; Cormio *et al.*, 2000; Cottrell *et al.*, 2000; Raha and Robinson, 2000). 4977bp mtDNA deletion is the most commonly occurring deletion. It occurs between two 13bp direct repeats at nucleotide position nt8470-13447. mtDNA⁴⁹⁷⁷ deletions have been shown to accumulate with age in various human tissues including heart, brain and lung (Kao *et al.*, 1997; Liu *et al.*, 1998). Increased concentrations of mtDNA⁴⁹⁷⁷ have been consistently reported in the blood of patients suffering from Kearns-Sayre syndrome, Pearson's pancreas syndrome and mitochondrial myopathies (Biagini *et al.*, 1998; von Wurmb *et al.*, 1998). The general hypothesis is that, point mutations accumulate with age (Michikawa *et al.*, 1999; Wang *et al.*, 2001). However, previous studies from the group of Barnett did not find any age related accumulation of mutations in the mitochondria from T cell clones or *ex vivo* lymphocytes from elderly subjects compared to young controls (Ross *et al.*, 2002). This absence of an age related increase in mtDNA damage might be partly due to high turnover rate of blood cells which acts against the accumulation of mtDNA in any significant number (Meissner *et al.*, 2000).

1.1.5. Cellular defense systems in the body

Cellular defense mechanisms involve several systems, namely enzymatic antioxidants, non-enzymatic antioxidants, activation of nuclear proteins such as poly adenosine diphosphate (ADP) ribose polymerase (PARP) (protein involved in DNA repair processes and programmed cell death), stress proteins and DNA repair pathways.

1.1.5.1. Enzymatic antioxidant defense

Superoxide dismutase (SOD), GPx, catalase (CAT) and caeruloplasmin (CPL) are important enzymatic antioxidants capable of counteracting biomolecular damage by converting highly reactive ROS into less reactive moieties. SOD is a group of metalloenzymes that enables the conversion of superoxide anion (O_2^-) into O_2 and H_2O_2 . CAT enzymes convert H_2O_2 into H_2O and O_2 and GPx enzymes are credited with the removal of H_2O_2 from the body. Biomolecular damage can still occur irrespective of the presence of these enzymes and repair enzymes (for example: Proteasome, phospholipase-A2) may be needed to minimise the extent of damage. Those repair

systems are actually incapable of repairing the damage instead they remove or breakdown the damage proteins, lipids respectively.

1.1.5.2. Non-enzymatic antioxidant defense

Uric acid (UA), vitamin E (α -tocopherol), vitamin C (ascorbic acid) and stress proteins are some of the non-enzymatic antioxidants present *in vivo*. Hydrophilic vitamin C counteracts ROS induced DNA damage in cells. The lipophilic nature of vitamin E enables it to act in membranes and lipoproteins (Beckman and Ames, 1998).

Heat shock proteins (HSPs) are highly conserved nuclear proteins activated in response to oxidative stress or variable temperatures. Stress proteins have been credited with maintaining cellular homeostasis due to their ability to bind to and stabilise an unstable protein (molecular chaperones). Stress proteins also participate in the folding and correct assembly of unfolded or misfolded proteins, their transport to respective sub-cellular compartments and/or disposal by proteolytic degradation (Hartl, 1996). But the exact molecular targets of HSP protection still remain a mystery. HSP 70 is an important molecular chaperone and can act against cytoplasmic oxidative damage (Papp *et al.*, 2003). HSP 70 activity has been demonstrated to decline in response to increasing heat shock with age making the body more susceptible to stress induced damage (Favatier *et al.*, 1997).

1.1.5.3. Antioxidant status *in vivo*

The antioxidant system *in vivo* is a pre-requisite for maintaining genomic stability and thereby contributing to longevity. Centenarians (individuals who have successfully reached > 100 years of age) were shown to maintain their levels of non-enzymatic antioxidants, in particular vitamin E and vitamin C with age (Mecocci *et al.*, 2000). An age related decline in its efficiency has been well documented. King and Barnett (1997) showed an age related elevation in the levels of *in vivo* antioxidants (Gpx, CAT, CPL) in human lymphocytes from healthy 75 to 80 year old donors (Figure 1.10).

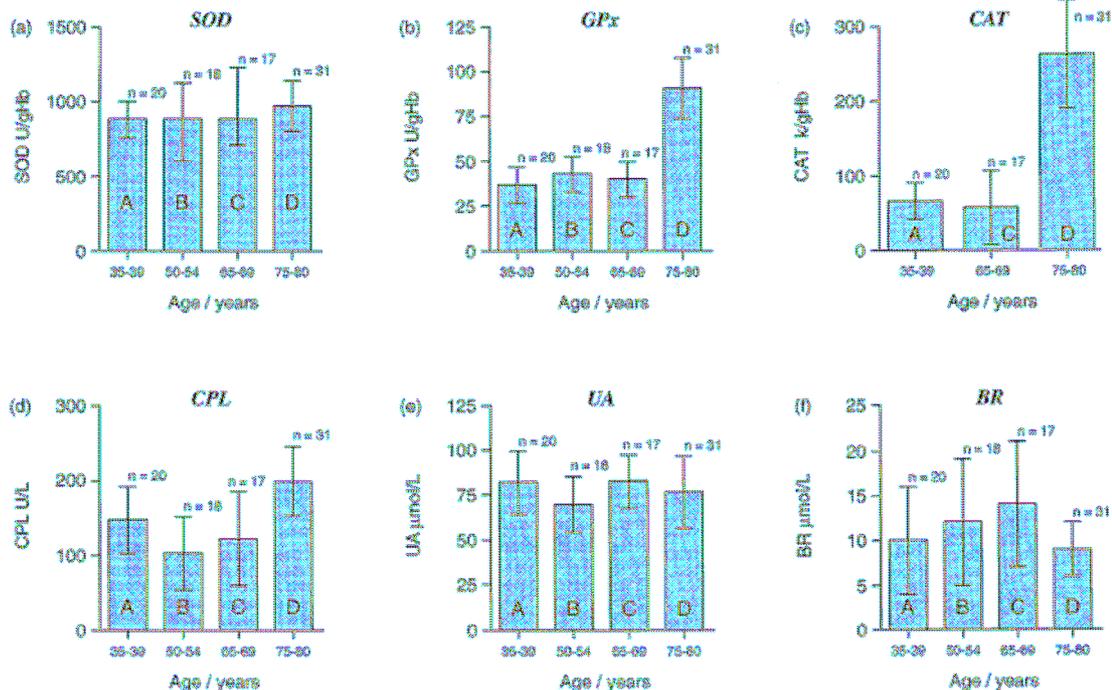


Figure 1.10. Shows an age related elevation in the levels of *in vivo* antioxidants (Gpx-(b), CAT-(c), CPL-(d)) in human lymphocytes from healthy 75 to 80 year old donors. There was no significant age related increase in the levels of *in vivo* antioxidants such as SOD, UA and Bilirubin (BR) (Barnett and King, 1997).

1.1.5.4. Cellular responses to DNA damage

A cell can respond to DNA damage in a number of ways; cell cycle arrest, repair of DNA damage, tolerate the DNA damage and continue to replicate or undergo apoptosis in response to critical levels of DNA damage (Hartwell and Kastan, 1994, Kaufmann and Paules, 1996). All these factors may lead to an effect on T cell mediated immune responses.

Cell cycle is a term for the period of time between one cell division and the next. G₁, S, G₂ and M are the four key phases of the cell cycle. For resting cells, the phase is G₀ in place of G₁. The progression from one phase to another of the cell cycle is under strict control by cell cycle check points. There can be several cell cycle check points in a cell cycle. The cell cycle check points are recognised as positions of control in a cell that ensures the order of events in the cell cycle and that integrate DNA repair with cell cycle progression (Hartwell and Kastan, 1994). The cell cycle check points respond in different ways to DNA damage. The DNA damage in cells within G₁ phase may cause

G₁ arrest; in S phase, DNA damage may result in replicon initiation inhibition and in G₂ phase, DNA damage may result in G₂ delay. These different types of responses by the cell to DNA damage are to facilitate time for the repair of DNA damage before it would result in DNA replication errors or mitotic errors (Kaufmann and Paules, 1996). DNA damage in T cells may hinder their ability to undergo rapid clonal expansion in response to the presence of an appropriate antigen, because of cell cycle arrest or delay. In this instance, the immune response may be sub-optimal due to inadequate number of T cells. Cell cycle arrest mechanisms are thought to be important in the process of replicative senescence.

Key regulators of the cell cycle are CDKs and CKIs, which regulate the progression of cells through the cell cycle. CDKs and CKIs detect DNA damage and initiate a series of molecular events resulting in cell cycle delay or arrest to allow sufficient time for the processing and repair before the next round of replication. CDKs and CKIs also enable the deletion of affected cells by apoptosis (Hyland *et al.*, 2001). T cells undergoing cell cycle arrest or delay will not be able to undergo rapid clonal expansion upon antigenic stimulation to produce an effective immune response *in vivo* and *in vitro*. In this case, the immune response may be sub-optimal due to inadequate production of T cells (Barnett and Barnett, 1998).

Cell cycle delay/arrest, DNA repair, replicative senescence and apoptosis are some of the complex responses to DNA damage in eukaryotic cells. The three important CKIs are p16, p21 and p27. p21 expression is associated with the cell senescence phenotype. p21 mediated cell cycle arrest is induced by DNA damage *via* the p53 pathway (Cox, 1997). In CD4⁺ T cell clones *in vitro*, the age related increase in DNA damage is not associated with proportionate increase in levels of p21 mRNA. This indicates that increased levels of oxidative DNA damage in T cell clones *in vitro* is probably not the major trigger of apoptosis *via* the p53/p21 pathway (Hyland *et al.*, 2001). p16 expression has been found to increase gradually during lifespan and persists at high levels during senescence (Li *et al.*, 1994; Rogan *et al.*, 1995; Alcorta *et al.*, 1996; Hara *et al.*, 1996; Reznikoff *et al.*, 1996; Zindy *et al.*, 1997). Short term induction of p16 expression has also been shown to induce reversible cell cycle arrest (Mitra *et al.*, 1999). But in most CD4⁺ T cell clones *in vitro*, mRNA levels of p16 were shown to decrease as a function of age suggesting, that they were not moving towards senescence (Hyland *et*

al., 2001). p27 expression functions as a mediator of extracellular antimitogenic signals and normally increases with lifespan, but there were no consistent pattern in the changes in levels of expression of p27 with age in CD4⁺ T cell clones *in vitro* (Hyland *et al.*, 2001).

Oxidative stress also results in detrimental effects on T cell replicative capacity *in vivo*. Peripheral blood mononuclear cell derived T cells may have less replicative capacity as they may have already undergone enough divisions or proliferation *in vivo* as part of an immune response. This means that, on transfer to an *in vitro* system, they are or may be nearer to their Hayflick Limit (irreversible state of growth arrest). The stability and the reproducibility of the loss of replicative potential have led to the belief that loss of replicative capacity and replicative senescence is controlled by an intrinsic cell division counting mechanism called the replicometer. Replicative senescence can be described as a permanent loss in replicative potential independent of the culture time. T cells more or less undergo a fixed number of cell divisions *in vitro* under constant culture conditions before they reach an irreversible state of growth arrest (Hayflick and Moorehead, 1965). T cell clones are associated with an age associated decrease in CD28 and IL-2 receptor expression and decreased IL-2 production (Watford *et al.*, 1981; Grubeck-Loebenstein *et al.*, 1994; Adibzadeh *et al.*, 1995). Individual T cell clones exhibit different *in vitro* lifespans and differences in the quality of other biomarker changes. These differences might be contributed to by some simple manipulations of tissue culture conditions that may be enough for certain T cells to successfully adapt to particular culture conditions, as well as the molecular and biochemical properties of individual T cell clones. Oxidative stress, leading to mortality due to complete loss of replicative potential, was detected in lymphocytes from elderly humans compared to young controls (Garcia Arumi *et al.*, 1998).

1.1.5.5. DNA repair process

The integrity of cellular DNA is under constant threat from a range of exogenous and endogenous DNA damaging agents (Toussaint *et al.*, 2002). The human body is equipped with a complex range of defense systems having the ability to counteract these agents and maintain genomic stability in the body, they include facilitation of cell cycle delay/arrest, DNA repair pathways and antioxidants *in vivo* that might reduce, remove or repair the DNA damage (Smith and Fornace, 1996). DNA damages, if not

counteracted, might lead to accumulation with age and may result in fixation of the damage leading to mutations. Previous studies from the group of Barnett focused mainly on two different DNA repair systems (Section 1.1.5.5.1 & Section 1.1.5.5.2).

1.1.5.5.1. Mismatch repair system

The mismatch repair system (MMR) is another DNA repair system that has been extensively investigated. It is a very effective system capable of identifying and subsequently rectifying errors in DNA replication (accurate process with <1 error per 10^{10} nucleotides synthesised) that escape DNA polymerase proofreading during the replication process (Kolodner, 1996). MMR enables the maintenance of genomic stability and helps in preventing spontaneous mutation that in turn prevents microsatellite instability (MSI). Microsatellites are short repetitive DNA sequences that are often copied incorrectly by DNA polymerases because their template daughter strands are particularly prone to misalignment during replication. An age related decrease in MMR capacity has been revealed in human $CD4^+$ T cell clones *in vitro* (Annett *et al.*, 2005) (Table 1.12). Earlier studies have shown a compromise in the activity of DNA polymerase contributing to increase in MSI with age (Srivastava and Busbee, 2003). In this case the MMR system was not able to counteract against DNA mismatch accumulation.

1.1.5.5.2. Excision repair pathways

The two different excision repair pathways identified are base excision repair (BER) and nucleotide excision repair (NER). BER is involved in repairing the damage that arises spontaneously. Instability, alkylation or oxidation of DNA is the major factors responsible for spontaneous DNA damage. NER is responsible for the removal of a variety of bulky DNA lesions such as Ultra violet (UV) rays induced cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PP) (Annett *et al.*, 2004). An age related decrease in DNA excision repair capacity has been demonstrated in human $CD4^+$ T cell clones *in vitro* (Annett *et al.*, 2004) (Table 1.10).

1.1.6. Exogenously applied antioxidants

Accumulation of genetic damage within T cells may contribute to an age related decline in T cell replicative capacity through cell cycle arrest, apoptosis or selection against T cells containing mutations (Barnett and Barnett, 1998). In case of accumulation of

genetic damage, T cells may not be able to undergo rapid clonal expansion upon antigenic stimulation resulting in a sub-optimal immune response. This phenomenon suggests a relationship between genomic stability and immunosenescence. This theory was further supported by the findings of high levels of genomic stability for the maintenance of healthy immune function in extremely old human subjects (Steinmann and Hartwig, 1995). Previous findings from our research group suggested maintenance of low levels of oxidative DNA damage and significantly higher plasma antioxidant capacity in Swedish nonagenarian (people between 90 and 100 years of age) subjects compared to controls (Hyland *et al.*, 2002), further emphasising the importance of a relationship between intact immune function and longevity. If oxidative damage to DNA in T cells results in decline in function with age, it is important to find a solution in which age related increase in oxidative damage to DNA *in vivo* can be counteracted. One way may be through the use of nutritional interventions and/or with antioxidant administration.

Vitamin E, ascorbic acid, carnosine and *N-tert-butyl- α -phenylnitron* (PBN) are some of the exogenous antioxidants successfully tested in humans. Several studies have pointed out the possibility that vitamin E may be protective against cancer (Duthie *et al.*, 1996; Yamashita *et al.*, 1998). Previously, our research group has successfully used Carnosine (β -alanyl-L-histidine) and PBN, a spin-trap agent, as antioxidants in human T cell clones. Carnosine has raised considerable attention due to its ability at physiological concentrations of 20mM to significantly increase the lifespan and lower levels of oxidative DNA damage in T cell clones derived from a healthy young subject (Hyland *et al.*, 2000). 250 μ M PBN increased the longevity and significantly lowered the levels of DNA damage in T cell clones compared to controls. The replicative capacity of T cell clone remained unchanged on supplementation with PBN.

In this investigation, the impact of long term exposure (beyond 24 hours of supplementation) of a range of concentrations of ebselen or NAC in human peripheral blood derived CD4⁺ T cell clones and human peripheral blood mononuclear cells was investigated.

1.1.6.1. Ebselen

Ebselen (2-phenyl-1,2-benzisoxazol-3(2H)-one; Figure 1.11) is an organoselenium compound mimicking glutathione peroxidase, an antioxidant selenoenzyme, in reducing H_2O_2 (Griffiths *et al.*, 1992; Yang *et al.*, 1999). This ability enables ebselen to minimise the deleterious effect of ROS (Muller *et al.*, 1984; Wendel *et al.*, 1984). The catalytically active selenium has the ability to reduce H_2O_2 . Selenium in ebselen can reduce H_2O_2 to H_2O and O_2 (Coffey *et al.*, 1995). The presence of the selenol group has been mainly attributed for the antioxidant properties in ebselen. Its protective effects against lipid peroxidation have been demonstrated *in vitro* and *in vivo* (Ozaki *et al.*, 1997). It has also been shown to be an efficient scavenger of free radicals such as peroxy radical and peroxy nitrite (Sies and Masumoto, 1997; Arteel *et al.*, 1999) and to inhibit the release of apoptotic factor cytochrome c (Boireau *et al.*, 2000). A recent study has shown the ability of ebselen to reduce the levels of peroxides enabling it to inhibit radiation induced apoptosis (Ramakrishnan *et al.*, 1996).

Ebselen has been successfully used in several cell types namely human hepatocellular liver carcinoma cell line (HepG₂) (Yang *et al.*, 1999), human promyelocytic leukemia cell line (HL-60) (Ji *et al.*, 2000) and pheochromocytoma cells (PC12) (Yoshizumi *et al.*, 2002). In HepG₂ cells, ebselen has the ability to decrease intracellular ROS, thereby reducing hydroxyl radical formation which may explain the decrease in DNA damage on supplementation. The ability of ebselen to decrease hydroxyl radical formation may explain the increase in cell growth and reduction of lactate dehydrogenase (LDH) leakage, facilitating a block in lipid peroxidation in HepG₂ cells (Yang *et al.*, 1999). Apart from all the advantages, ebselen has some disadvantages such as its H_2O insolubility. Prior to this investigation, the effects of ebselen in human T cells *in vitro* and *ex vivo* had yet to be explored.

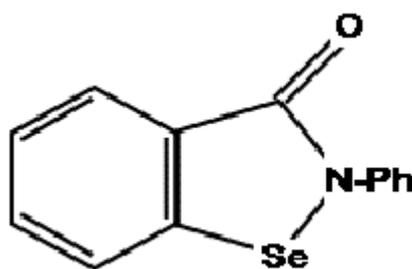


Figure 1.11. Schematic representation of ebselen

1.1.6.2. N-Acetyl L-Cysteine

N-Acetyl L-Cysteine (NAC; $C_5H_9NO_3S$; Figure 1.12), a precursor of GSH is an antioxidant involved in cellular detoxification (Meister and Anderson, 1983). It is an acetylated form of the amino acid L-cysteine and has a thiol group that functions as a pre-cursor of glutathione synthesis. The presence of cysteine in NAC serves as a source to synthesise glutathione (Su *et al.*, 2006) and sulfhydryl groups. NAC also acts as a stimulator of cytosolic enzymes involved in glutathione regeneration (Banaclocha, 2001). It is H_2O soluble and its intake is the most cost-effective way of boosting glutathione levels in the body. NAC is a low molecular weight compound that can readily pass through the blood brain barrier. NAC has also been shown to protect against oxidative damage with ROS (Aruoma *et al.*, 1989). Initially it was used as a mucolytic agent (Cotgrave, 1997). It has been found to have a protective effect against cigarette smoke (Ozaras *et al.*, 2003) and has a beneficial role in prostrate and respiratory conditions. Under normal conditions GSH peroxidases decomposes organic and inorganic peroxides in the cell using GSH as a substrate (Flohe, 1989). But peroxide accumulation may result in depletion of GSH thereby impairing the function of GSH peroxidase. The mechanism behind the antioxidant effect of NAC may be the presence of a free sulfhydryl group that enables scavenging of ROS and NO thereby blocking lipid peroxidation by peroxynitrate (Han *et al.*, 1997). Glutamate and cysteine share the same transporter in the body and elevation in levels of extracellular glutamate competitively inhibit cysteine transport resulting in depletion of intracellular GSH synthesis. The ability of NAC to raise GSH levels due to its capacity to donate cysteine amino acid may also supplement its antioxidant potential (Han *et al.*, 1997; Wagner *et al.*, 1998).

It would be interesting to investigate the effect of NAC in human T cells *ex vivo* using human peripheral blood mononuclear cells and human peripheral blood derived $CD4^+$ T cell clones *in vitro* at 20% O_2 tension. If ebselen or NAC could reveal an antioxidant capacity under physiological O_2 tension, they may have potential anti-immunosenescent effects *in vivo*.

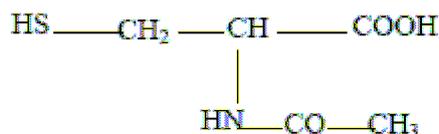


Figure 1.12. Schematic representation of NAC (Toussaint *et al.*, 2000).

1.1.6.3. MitoQ- a mitochondria targeted antioxidant

Mitochondria have a pre-dominant role in the life and death of a cell. Damage to mitochondria has been widely implicated as a major reason behind a wide range of diseases such as Diabetes, Huntington, Parkinson's, disorders associated with mitochondrial DNA mutations, apoptosis in cancer, pathophysiology of ageing to name a few (Nishikawa *et al.*, 2000; Tabrizi *et al.*, 2000; Smith *et al.*, 2003). One of the main problems encountered in treating diseases caused by mitochondrial dysfunction is the inability to target bioactive molecules to mitochondria *in vivo*. This problem was rectified by the development of a strategy to target bioactive molecules specifically to mitochondria by their attachment to lipophilic triphenyl phosphonium cation through an alkyl linker (Murphy, 1997; Murphy and Smith, 2000; Kelso *et al.*, 2001). The lipophilic triphenylphosphonium cation can rapidly permeate lipid bilayers due to a delocalised positive charge and the large mitochondrial membrane potential enables the cation to accumulate several hundred-fold inside isolated mitochondria and within mitochondria in cultured cells (Figure 1.15) (Sanjuan-Pla *et al.*, 2005). This led to the development of mitoubiquinone (MitoQ), a mitochondria targeted antioxidant.

MitoQ is a mixture of two redox forms of mitochondrially targeted ubiquinone derivatives 1) Mitoquinol (reduced form) (Figure 1.13) and 2) Mitoquinone (oxidised form) (Figure 1.14). The former, is the active antioxidant form of mitoQ that can detoxify ROS thereby enabling blockage of mitochondrial oxidative damage (Echtay *et al.*, 2002; Jauslin *et al.*, 2003; Schafer *et al.*, 2003; Saretzki *et al.*, 2003). This theory was further enhanced by the ability of mitoquinol to block lipid peroxidation compared to mitoquinones inability in human osteosarcoma 143B cells (Kelso *et al.*, 2001). The ability of mitoQ to selectively accumulate within mitochondria enables the study of its effect on mitochondrial derived ROS and their signalling properties (Sanjuan-Pla *et al.*, 2005). The selective uptake of mitoQ by mitochondria increases the efficacy and

specificity of this bioactive molecule, designed to interact with mitochondria. The large mitochondrial membrane potential enables mitoQ to accumulate within mitochondria inside cells due to the attached cation. The ubiquinone moiety present in the lipid bilayer is reduced to an ubiquinol derivative by the respiratory chain. Ubiquinone buried within the lipid core of the inner membrane accepts two electrons from II resulting in reduction to ubiquinol. The ubiquinol derivative acts as an effective antioxidant by inhibiting lipid peroxidation and thereby preventing the leakage of superoxide from mitochondrial respiratory chain which has been mentioned as an important source of oxidative stress (Raha and Robinson, 2000). Ubiquinol donates a hydrogen atom from its hydroxyl groups to a lipid peroxy radical enabling a decrease in lipid peroxidation within mitochondrial inner membrane (Takada *et al.*, 1984; Ernster *et al.*, 1992; Ingold *et al.*, 1993). This results in either ubisemiquinone radicals disproportionating to ubiquinone and ubiquinol (Land and Swallow, 1970) or reacting with O₂ to form superoxide and ubiquinone, which will be transferred to aqueous phase and will be detoxified by superoxide dismutase and peroxides (Maguire *et al.*, 1989; Ingold *et al.*, 1993). The respiratory chain can regenerate the ubiquinol derivative enabling the recycling of its antioxidant activity (Kelso *et al.*, 2001). The above mechanism enables mitoQ to protect mitochondria from oxidative damage (Kagan *et al.*, 1994; Lass and Sohal, 1998). There is also evidence of mitoQ decreasing the release of ROS from mitochondria (Hwang *et al.*, 2001).

The effects of mitoQ has previously been investigated in human 143B osteosarcoma cells and concentrations upto 10µM mitoQ had little effect on the membrane potential of isolated mitochondria. Higher concentrations (25µM) decreased mitochondrial membrane potential and concentrations of 25-50µM were found to inhibit the respiration of the cells resulting in cell death. Cell viability remained unaffected at concentrations of 10µM (Kelso *et al.*, 2001). Lower concentrations of mitoQ prevented oxidation of cisparinic acid by H₂O₂ and Ferrous ions (Fe²⁺) on co-incubation. Lower concentrations of mitoQ also blocked lipid peroxidation, evidenced by low levels of malonyldialdehyde (MDA) accumulation, and prevented disruption to mitochondrial function. Accumulation of caspases (cysteine dependent aspartate proteases known to be involved as a key executor of cell death) by H₂O₂ induced cytochrome c release from mitochondria into cytoplasm has been found to be a stimulus for apoptosis (Hampton

and Orenius, 1997). Pre-incubation with low concentrations of mitoQ completely blocked H_2O_2 induced caspase activation and apoptotic cell death in jurkat cells (Kelso *et al.*, 2001). MitoQ, at concentrations of 50-100nm inhibited the growth of human foetal lung (MRC-5) fibroblasts in long term culture. But lower concentrations of -20nm needed an adaptation period of 1 week under normoxic (normal O_2) conditions. At 40% hyperoxic (increase in O_2) conditions, 10-20nm mitoQ completely prevented the rise in telomere shortening rate (Saretzki *et al.*, 2003).

Although the previous findings suggested low levels of point mutation (mtDNA⁴⁹⁷⁷) in mitochondrial DNA with age (Ross *et al.*, 2002), the effect of mitoQ, on endogenous or exogenously applied ROS in human T cell samples had still to be investigated.

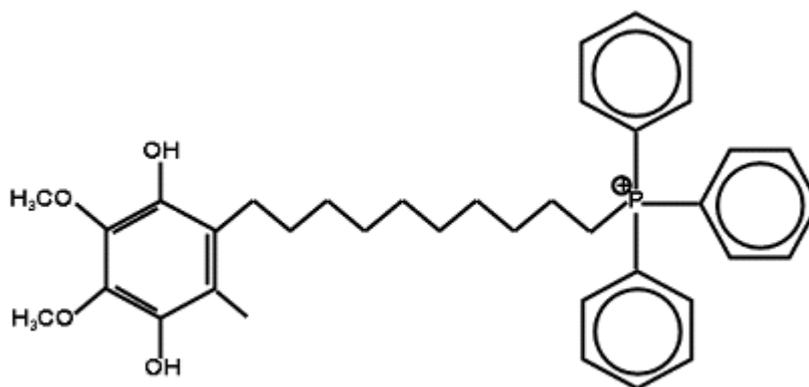


Figure 1.13. MitoQ (reduced) (Smith *et al.*, 2003).

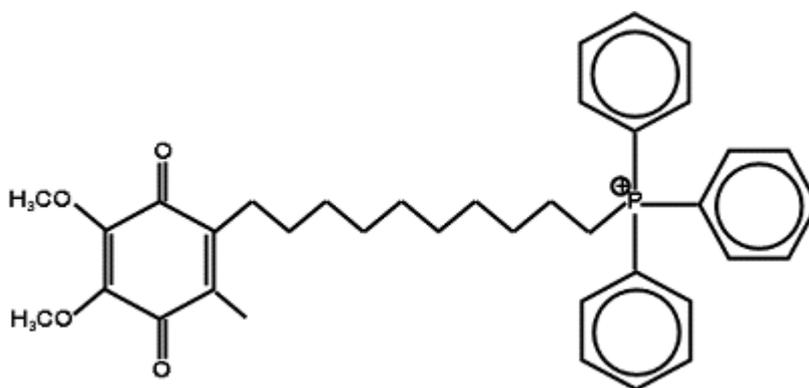


Figure 1.14. MitoQ (oxidised) (Smith *et al.*, 2003).

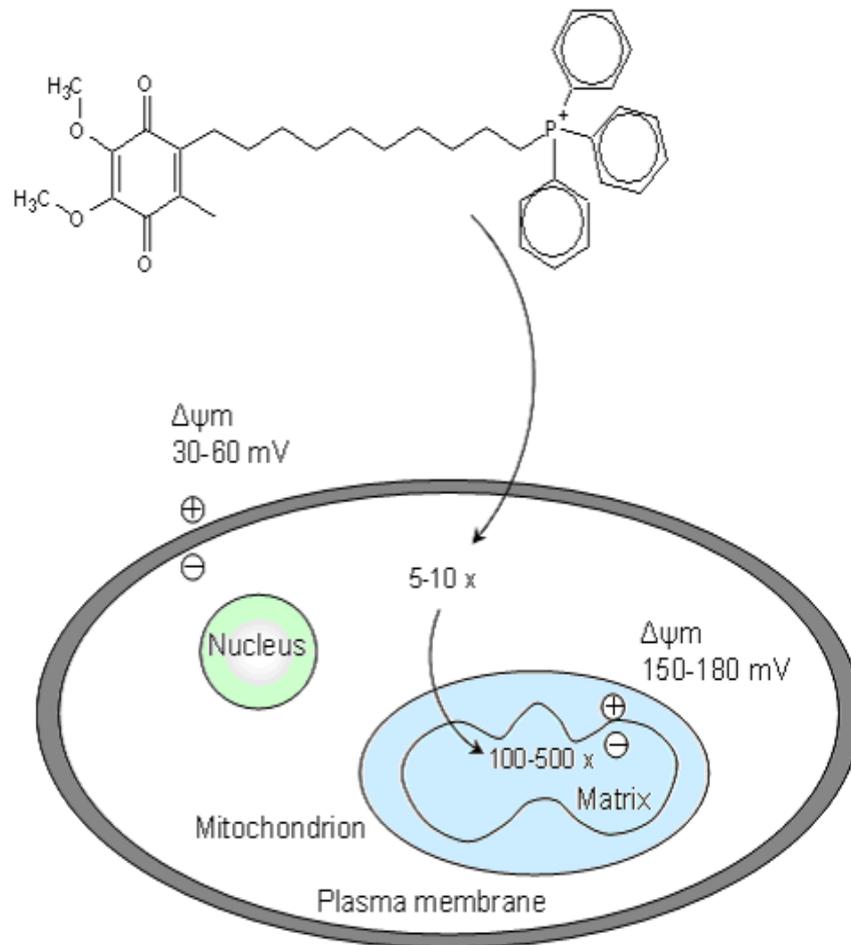


Figure 1.15. The uptake of alkyltriphenylphosphoniumcation containing biologically active molecule by the lipid bilayer in mitochondria. The cation containing the biologically active molecule is accumulated 100 to 500 fold into the mitochondrial matrix due to high mitochondrial membrane potential (Adapted from Smith *et al.*, 2003).

1.1.6.4. Effect of reduced O₂ tension as defense system

A number of investigations have reported on the impact of O₂ tension, cellular senescence and lifespan. Previous investigations have revealed a 25% increase in lifespan under 10% O₂ tension in human diploid fibroblasts (HDF) (Packer and Feuheur, 1977). A number of investigations have been carried out in HDF in reduced O₂ tension culture conditions. Lately, long term culture of HDF at a physiological concentration of 3% O₂ *in vitro* significantly extended the lifespan of fibroblasts as well as increased the rates of proliferation in the cells, when compared to those cultured at 20% O₂ tension (Chen *et al.*, 1995). However, T cell clones cultured at low O₂ tension (6%) had shorter lifespan (Table 1.13) and reduced replicative (proliferative) capacities (Table 1.14)

compared to the clones cultured at 20% O₂ tension. The oxidative damage to DNA remained low throughout their lifespan (Figure 1.16). This phenomenon was observed for clones derived from young and old donors (Duggan *et al.*, 2004). The shorter lifespan and low oxidative damage may be due to low levels of ROS in a low O₂ tension environment in the culture system. Even though ROS is pre-dominantly known for its detrimental effect on cellular biomolecules, they also act as intracellular signaling molecules within T cells (Schreck *et al.*, 1992; Kamata and Hirata, 1999). They are believed to act as signals/mediators to changes in cellular processes including cell function, proliferation, differentiation, cell damage and death. ROS are known to use several signaling molecules such as calcium, protein tyrosine kinases (PTKs), protein tyrosine phosphatases (PTPs), serine/threonine kinases, phospholipases in signalling pathways. Mitogen activated protein (MAP) kinases; a prominent family of protein kinases has several pathways identified such as extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK), P38 kinase. These pathways are involved in proliferation, differentiation and apoptosis. Preliminary work from our group suggested an up-regulation of stress activated protein kinases (SAPK) also known as JNK and P38 kinases at low O₂ tension culture conditions in *in vitro* T cell clones. It was not clear whether the up-regulation was age dependent (Figure 1.17). T cell signalling events such as protein tyrosine phosphorylation and activation of JNK, cellular proliferation induced by lectin are some of the few instances that require the presence of ROS (Pani *et al.*, 2000). This reduced ROS levels might interfere with the signalling pathways involved in T cell activation and proliferation, example: redox sensitive activation of transcription factors such as nuclear factor kappa light chain enhancer of activated B cells (NFκB) or activator protein-1 (AP-1) involved in T cell activation and proliferation (Tatla *et al.*, 1998). The results of the above studies warrant further investigation and it will be interesting to see the effect of reduced O₂ tension in T cell clones and *ex vivo* human peripheral blood mononuclear cells under varying culture conditions.

Table 1.13. Cumulative PD and the average lifespan of different T cell clones achieved overall under each culture conditions. Cumulative PD of T cell clones cultured under standard O₂ tension are clearly higher in comparison to T cell clones maintained at reduced O₂ tension (Adapted from Duggan *et al.*, 2004)

Clone	Standard O₂ tension	Reduced O₂ tension
385-2	69.9	61.6
385-7	73.5	71.5
399-35	72.1	45.0
399-37	78.1	51.9
400-23	80.7	51.7
Mean lifespan (PD)	74.9 ± 4.4	56.3 ± 10.3*

* Significantly lower, p<0.05

Table 1.14. Mean proliferative capacity per week of different T cell clones achieved overall under each culture conditions. Mean proliferative capacity of T cell clones cultured under standard O₂ tension are clearly higher in comparison to T cell clones maintained at reduced O₂ tension (Adapted from Duggan *et al.*, 2004)

Clone	Standard O₂ tension	Reduced O₂ tension
385-2	1.9 ± 0.6	1.1 ± 0.6
385-7	2.1 ± 0.7	1.2 ± 0.6
399-35	2.1 ± 0.9	0.8 ± 0.5
399-37	2.2 ± 0.8	0.9 ± 0.6
400-23	2.1 ± 1.0	0.9 ± 0.6
Mean PD/week	2.1 ± 0.1	1.0 ± 0.2*

* Significantly lower, p<0.05

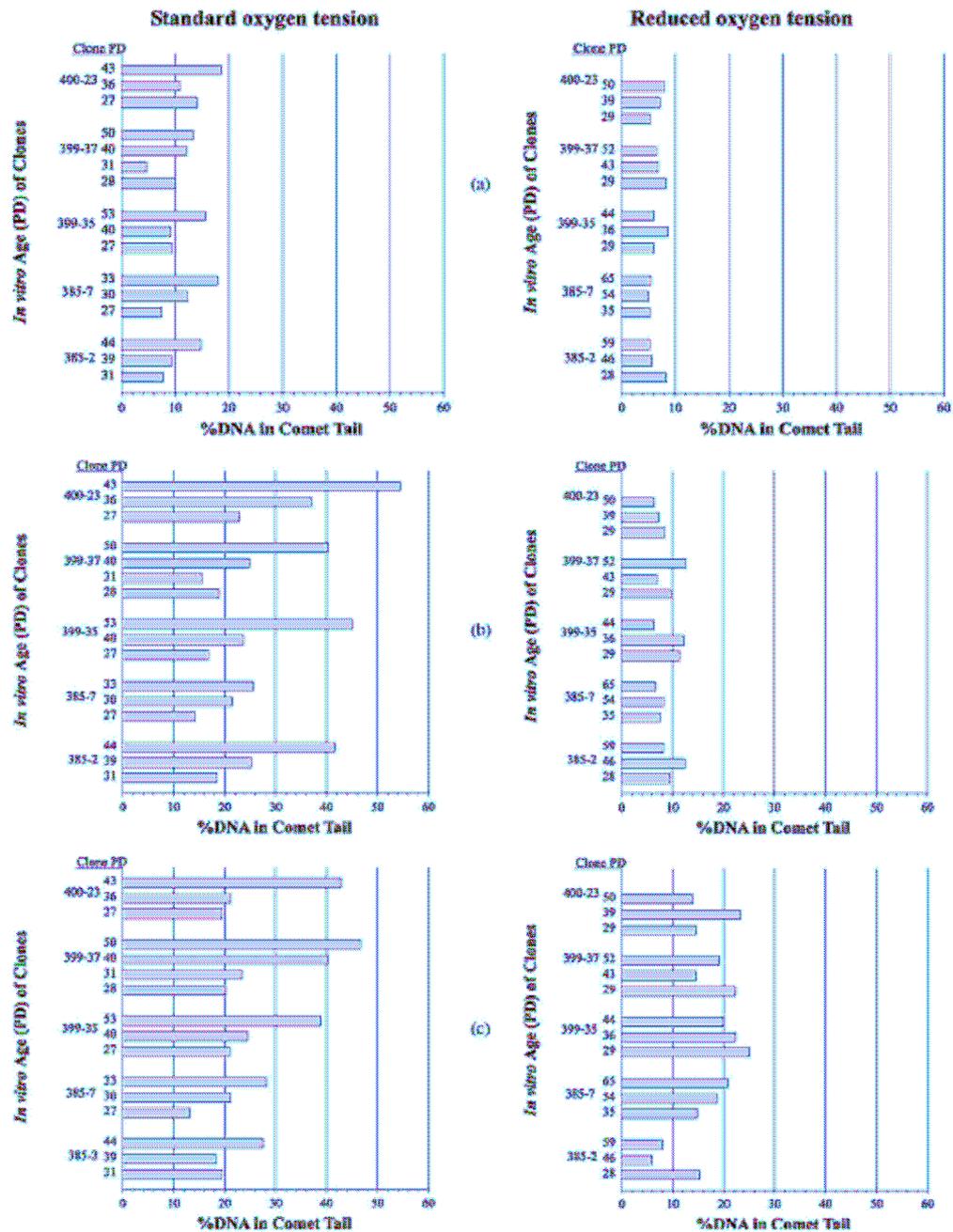


Figure 1.16. Oxidative DNA damage levels of T cell clones maintained under standard and reduced O₂ tension conditions. a) DNA strand breaks and alkali labile sites b) oxidatively damaged pyrimidine bases plus DNA strand breaks and alkali labile sites c) oxidatively damaged purine bases plus DNA strand breaks and alkali labile sites (Adapted from Duggan *et al.*, 2004).

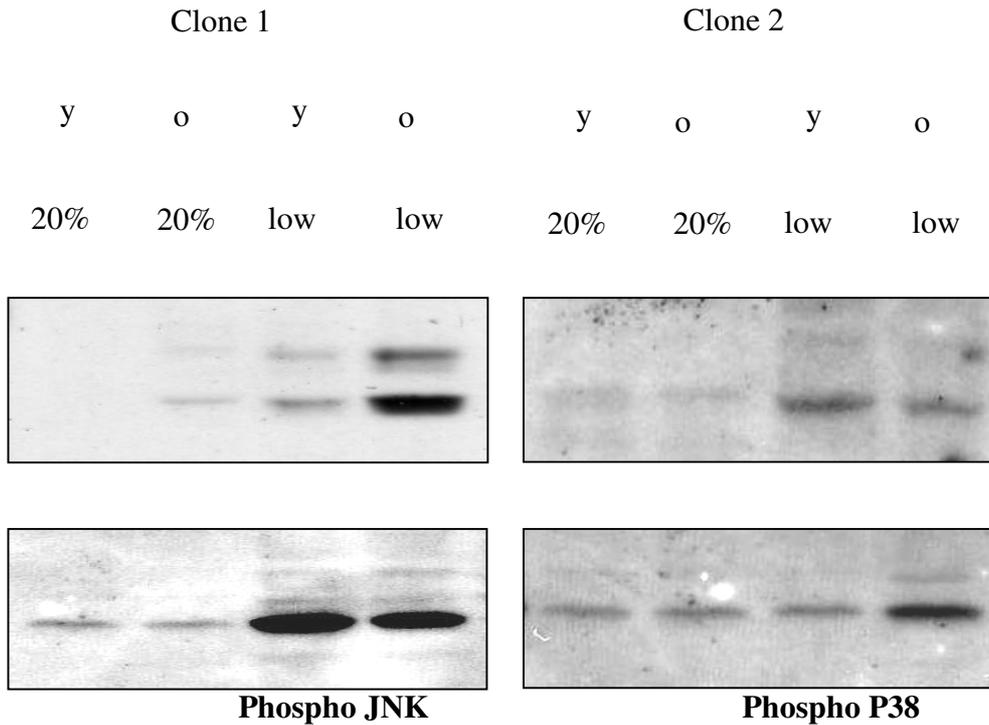


Figure 1.17. Up-regulation of MAP kinases (phospho JNK and phospho P38) at low O₂ tension conditions in T cell clones independent of age.

y- human peripheral blood derived CD4⁺ T cell clone derived from a healthy 26 year old donor, o- human peripheral blood derived CD4⁺ T cell clone derived from a healthy 80 year old donor.

Adapted from work carried out by Duggan *et al.*, (2004).

1.1.7. Aim of this investigation

The aim of this PhD programme was to investigate the effects of antioxidants or low O₂ tension on T cell integrity and function using T cell clones and human peripheral blood mononuclear cells from donors of different age groups.

The antioxidants used in this study were those which have potential applicability *in vivo*: 1) Ebselen 2) NAC and 3) MitoQ. The effect of low O₂ tension (2%) as a defense system on various aspects of T cells *ex vivo* was also investigated in this study.

A number of bioassays were set up to analyse the impact of altered culture conditions on T cell integrity and function:

- 1) Levels and types of oxidative damage to DNA were determined using a range of comet assay techniques.
- 2) Intracellular redox tone (GSH:GSSG ratio) was measured using GSH:GSSG ratio assay kit.
- 3) Total glutathione level were determined using GSH:GSSG ratio assay kit.
- 4) Mitochondrial membrane permeability was determined using JC-1 dye.

The specific biological markers of T cell function examined were:

- 1) Proliferative capacity. In T cell clones *in vitro*, the proliferative capacity was determined by calculating the PDs per week of the clones during their span in the culture. PDs were calculated by counting the number of live cells using the Trypan Blue Exclusion Test. For human peripheral blood mononuclear cells *ex vivo*, the capacity to proliferate was evaluated using thymidine incorporation.
- 2) Lifespan of T cell clones *in vitro* determined by calculating cumulative PDs during their span in the culture.

CHAPTER-2

Materials and Methods

2.1. Materials and Methods

2.1.1. Human peripheral blood derived CD4⁺ T cell clones

Human peripheral blood derived CD4⁺ T cell clones are accepted as a relevant system to allow the study of factors contributing to T cell senescence (Pawelec *et al.*, 2002). The T cell clones used in this study were established using a limited dilution protocol (Pawelec, 1993) and were kindly supplied by Professor Graham Pawelec, University of Tübingen, Germany. Twelve independently derived T cell clones were examined in this investigation. The 400-series clones were obtained from a 26 year old overtly healthy laboratory worker and the 385-series clones were obtained from a 45 year old overtly healthy laboratory worker (Chapter-1, Section 1.1.3.8).

2.1.1.1. Culture of T cell clones

Human peripheral blood derived CD4⁺ T cell clones (expressing T cell receptors for antigen; TCR, CD3⁺, CD4⁺) were maintained in culture in a serum-free medium, X-Vivo 10 (BioWhittaker), in 24 well plates (5 wells, 2 ml medium per well) at concentrations of 2-4 x 10⁵ cells per well, along with 2 x 10⁵ gamma-irradiated (80Gy) RJK853 cells per well (EBV-transformed B-lymphoblastoid cell line with complete hprt deletion, a gift from Dr. Jane Cole, MRC Cell Mutation Unit, University of Sussex, UK) as feeder cells. Clones were maintained in a 7-day cycle at 37°C under conditions of 5% CO₂ and 95% air atmosphere. They were supplemented with 400U/ml recombinant IL-2 (Chiron, UK) on days 1 and 4 of the cycle. On day 7, a viable cell count was performed on harvested cells using a Neubauer Counting Chamber, and a new culture cycle was set up with fresh medium and RJK853 feeder cells (Hyland *et al.*, 2000, 2001).

2.1.1.2. Determination of T cell clone proliferative capacity and lifespan

The number of population doublings (PD) that a clone underwent in a week in culture is referred to as the proliferative capacity of the clone. The cumulative and total number of population doublings the clones underwent during culture is defined as lifespan.

A peripheral blood derived T cell clone has to expand up to 26 PD to have sufficient number of clones for analysis or sufficient T cells for stock reserves, due to constraints

imposed by the cloning process. The earliest stage in the lifespan of T cell clones used in this analysis was 26-28 PD. Figure 2.1 demonstrates an example of growth of a T cell clone.

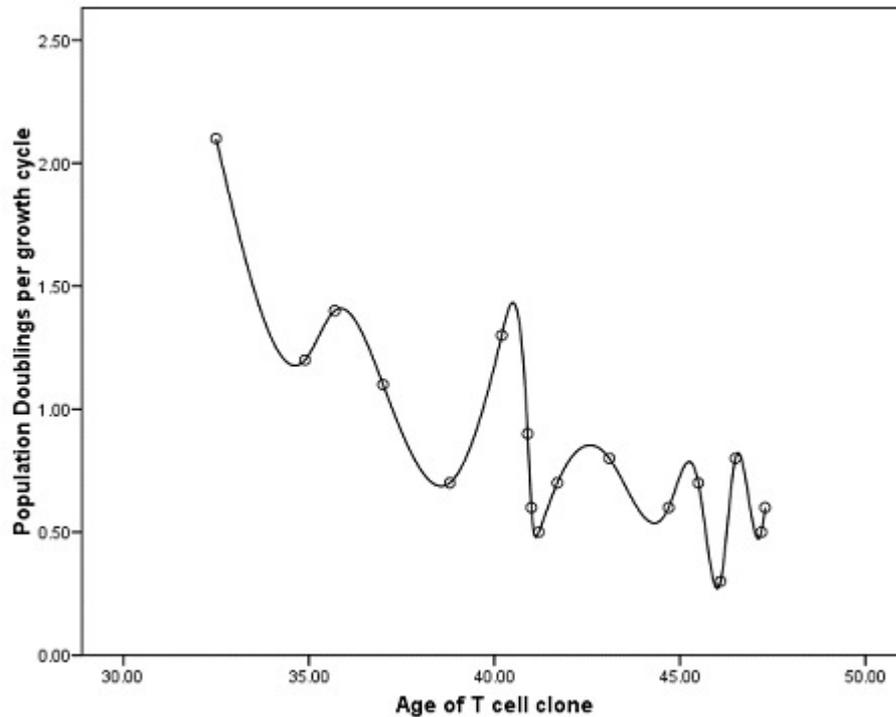


Figure 2.1 Proliferative capacity of 385-7b clone throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms.

The viable count of T cell clones was determined on day four and at the end of every 7 day cycle, with the trypan blue exclusion dye test used to assess viability. This count was used to calculate the number of PD the clones had undergone using the following formula:

$$PD = \text{Log}_2(\text{cell count at the end of the cycle}) - \text{Log}_2(\text{number of cells at the start of the cycle}).$$

The samples were then cryopreserved in a medium made up of 10% DMSO (Sigma), 20% FBS (Invitrogen) and 70% X-Vivo 10 and stored in liquid nitrogen for further analysis at the end of a 7 day growth cycle (Hyland *et al.*, 2000).

The clones used in this investigation, mean PD achieved by them in a seven day cycle and the cumulative PD of each of the clones is illustrated in chapter 3 (Table 3.1).

Previous work carried out using T cell clones determined the relationship between PD/week and the cumulative population doublings or the *in vitro* age of the clones using linear regression analysis. The results of the growth of the T cell clones revealed considerably higher levels of growth at the start of the culture compared to the later stages of their life span in culture. The data of the previous studies suggested a decreased PD capacity with age. After an initial exponential phase of growth, the remainder of the lifespan of the clones transitioned into a steady state indicating the maintenance of PD capacity throughout the lifespan of the clones (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

2.1.2. Ebselen or NAC supplementation

Initial studies were carried out by supplementing T cell clones from the midpoint of their *in vitro* lifespan with a range of doses of ebselen (0-100 μ M) (Table 2.1) (Sigma) and NAC (0-10mM) (Table 2.1) (Sigma) in X-Vivo 10. The results of the impact of ebselen or NAC on T cell clones from the midpoint of their *in vitro* lifespan were taken into consideration before choosing the dose range of 0-30 μ M for ebselen and 0-7.5mM for NAC for the studies investigating the impact of these antioxidants when supplemented from a young *in vitro* age. Clones were supplemented with ebselen or NAC on days one and four of the growth cycle. The protocol for the supplementation of the antioxidants were done similar to that previously used by the group of Barnett with studies on the antioxidant carnosine (Hyland *et al.*, 2000). Previously, no studies have been carried out to investigate the impact of long term exposure (beyond 24 hours) to ebselen or NAC in human T cell clones or in any other cell lines. The ability of ebselen to protect HepG₂ cells (Yang *et al.*, 1999), human HL-60 (Li *et al.*, 2000) and PC-12 cells (Yoshizumi *et al.*, 2002) against H₂O₂ induced cell death and DNA damage and the proven ability of NAC to act as an efficient antioxidant in HeLa cells (Hansen *et al.*, 2004) and HepG₂ cells (Yang *et al.*, 1999) were taken into consideration before choosing the dose range of the antioxidants in this investigation (Chapter-4, Section 4.1.2.4).

Table 2.1 denotes the different concentrations of ebselen or NAC used in this investigation

Ebselen (μM)	0	5	10	15	20	30	40	60	80	100
NAC (mM)	0	0.312	0.625	1.25	2.5	5	7.5	10		

2.1.3. Human peripheral blood mononuclear cells

The antioxidant potential of ebselen or NAC in a polyclonal population of human peripheral blood mononuclear cells was assessed. These results were compared with the data obtained for the T cell clone model, a monoclonal population.

2.1.3.1. Subject selection

Ethical approval was granted by NTU based on the consent obtained from all the study subjects prior to this study. The human blood samples for the present study were drawn from ten healthy, non-smoking males fasted overnight, five each from the age group of 25-30 years (n=5) and 55-60 years (n=5). Total number of donors from the age group of 25-30 years and 55-60 years = 10. The subjects were not on any form of medication or had not suffered any recent illness. Other lifestyle factors such as alcohol consumption and exercise regime were also taken into consideration. Previous studies in human peripheral blood mononuclear cells isolated from the donors of different age groups carried out by the group of Barnett revealed that human peripheral blood mononuclear cells derived from older aged donors (age group of 65-69 years) had higher levels of mutations and DNA damage and had lower DNA repair capacity and altered *in vivo* antioxidant status compared to donors derived from the younger age groups (35-39 years). The decrease in DNA repair capacity or alterations in *in vivo* antioxidant status may have contributed to the increase in mutation load with age in mononuclear cells derived from donors in the age group of 65-69 years (Barnett and King, 1995). The result of this study was taken into consideration before choosing the two age groups of donors (25-30 years & 55-60 years) in this investigation.

2.1.3.2. Sample collection

Human blood samples were drawn in the morning between 8.00 and 9.00 am by a licensed individual and collected in a tube containing heparin to prevent coagulation. The samples were kept on ice and taken to the laboratory within 5 minutes for separation of peripheral blood mononuclear cells.

2.1.3.3. Preparation, maintenance of culture and storage of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from fresh heparinised venous blood by density gradient centrifugation on Ficoll-Isopaque (PAA Laboratories) in LeucoSep (Lymphocyte separation tubes). The *ex vivo* T cell samples were maintained in culture separately using a well established method optimised previously (Warnock *et al.*, 1999; Barnett and Barnett, 2000). The peripheral blood mononuclear cells were washed twice in phosphate buffered saline or X-Vivo 10 media and then maintained in culture in a serum-free medium, X-Vivo 10, in 24 well plates (5 wells, 2 ml medium per well) at concentrations of $2-4 \times 10^5$ cells per well, along with 2×10^5 gamma-irradiated (80Gy) RJK853 cells per well (EBV-transformed B-lymphoblastoid cell line with complete hprt deletion) as feeder cells. Peripheral blood mononuclear cells were maintained in a 7-day cycle at 37°C under conditions of 5% CO₂ and 95% air atmosphere. They were supplemented with 400U/ml recombinant IL-2 on days 1 and 4 of the cycle. On day 7 a new culture cycle was set up with fresh medium and RJK853 feeder cells. The samples required for further analysis were then cryopreserved in a medium made up of 10% DMSO (Sigma), 20% FBS (Invitrogen) and 70% X-Vivo 10 and stored in liquid nitrogen for further analysis at the end of a 7 day growth cycle (Hyland *et al.*, 2000).

2.1.4. Ebselen or NAC supplementation

The impact of a range of doses of ebselen or NAC in human peripheral blood derived CD4⁺ T cell clones supplemented from a young *in vitro* age or the midpoint of their *in vitro* lifespan (monoclonal population) (Chapter-4) were taken into consideration before investigating the biological effect of the antioxidants on human peripheral blood mononuclear cells (a polyclonal population) (Chapter-5, Section 5.1.2.5). The human peripheral blood mononuclear cells from ten healthy, non-smoking males fasted overnight, five each from the age groups of 25-30 years and 55-60 years were

supplemented separately with 30 μ M ebselen or 5mM / 7.5mM NAC in X-Vivo 10. The *ex vivo* T cells (peripheral blood mononuclear cells) were supplemented with ebselen or NAC on days one and four of the growth cycle (Hyland *et al.*, 2000).

2.1.5. Negative selection of CD4⁺ T cells from peripheral blood mononuclear cells

The biological effects of a broad range of doses of ebselen or NAC on human peripheral blood derived CD4⁺ T cell clones, a monoclonal population were investigated (Chapter-4). The results from that investigation led to the analysis of the impact of the two antioxidants on human peripheral blood mononuclear cells, a polyclonal population (Chapter-5).

Investigating the impact of these two antioxidants on CD4⁺ T cells *ex vivo* isolated from human peripheral blood mononuclear cells, allowed for a comparison of their effects on a polyclonal population CD4⁺ T cell population versus a monoclonal CD4⁺ T cell population. In this investigation a CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) was used for the separation of CD4⁺ T cells from human peripheral blood mononuclear cells.

2.1.5.1. Principle of the kit

The CD4⁺ T Cell Isolation Kit (Miltenyi Biotech) is a magnetic labelling system for the isolation of CD4⁺ T helper cells from human peripheral blood mononuclear cells. Non-CD4⁺ T cells are magnetically labelled by using a cocktail of biotin-conjugated antibodies against non-CD4⁺ T cells and these cells can subsequently be depleted by labelling with anti-biotin microbeads. The cell suspension is passed through the column in a MACS separator and CD4⁺ T cells are collected in the bottom tube.

2.1.5.2. Isolation procedure and culture of CD4⁺ T cells

10 x 10⁶ human peripheral blood mononuclear cells from each of the donor samples were separately treated with a cocktail of biotin conjugated antibodies against non-CD4⁺ T cells and were subsequently depleted by labelling with anti-biotin microbeads. The different cell suspensions were then allowed to pass separately through a PBS (Oxoid Ltd) pre-rinsed column placed in the magnetic field of a suitable MACS separator. CD4⁺ T cell elutes were collected in the bottom tube. The CD4⁺ T cell elutes

was then washed in phosphate buffered saline or X-Vivo 10 media and then maintained in culture separately, as described in section 2.1.5.2.

2.1.6. Ebselen or NAC supplementation

CD4⁺ T cells *ex vivo* from ten healthy, non-smoking males fasted overnight, five each from the age groups of 25-30 years and 55-60 years were supplemented separately with 30µM ebselen or 5mM / 7.5mM NAC in X-Vivo 10. The *ex vivo* T cells (CD4⁺) were supplemented with ebselen or NAC on days one and four of the growth cycle, in line with the previous work carried out by the group of Barnett using other antioxidants in CD4⁺ T cell clones (Hyland *et al.*, 2000). There is no information on the length of stability of ebselen or NAC in any cell lines in the existing scientific literature. Previous work has demonstrated the impact of ebselen or NAC upto 24 hours of supplementation (Yoshizumi *et al.*, 2002; Hansen *et al.*, 2004).

2.1.7. Impact of mitoQ on human peripheral blood mononuclear cells *ex vivo*

It has been shown that mitochondria accumulate oxidative damage more rapidly than the rest of the cell. Such damage is thought to contribute to mitochondrial dysfunction and cell death and lead to the development of degenerative diseases and ageing. The main reason behind this accumulation of oxidative damage in mitochondria is due to the presence of the mitochondrial respiratory chain which is a major source of reactive oxygen species (Kelso *et al.*, 2002). A challenge to treating diseases resulting from mitochondrial dysfunction has been the inability to target bioactive molecules to mitochondria *in vivo*. This has been overcome by the use of mitoQ (Chapter-1, Section 1.1.6.3).

Previous work, carried out in Jurkat cells, revealed the ability of 1µM mitoQ to completely block 150µM H₂O₂ induced caspase activation and substantially decreased apoptotic induced cell death after 4 to 6 hours (Kelso *et al.*, 2001). The accumulation of caspases following H₂O₂ exposure has been found to be a stimulus for apoptosis (Hampton and Orenius, 1997). Other studies have indicated that mitochondrial oxidative stress may play a significant role in H₂O₂ induced apoptosis via its effects on mitochondrial membrane transition pores resulting in a decrease in mitochondrial

transmembrane potential in Jurkat cells at 100 μ M H₂O₂ (Dumont *et al.*, 1999). MitoQ concentrations of up to 10 μ M did not have an effect on mitochondrial membrane potential or cell viability in isolated mitochondria from human 143B cells. But higher concentrations, 25-50 μ M, decreased the mitochondrial membrane potential and resulted in cell death as revealed by the release of LDH into the culture medium. In this investigation, decyl triphenyl phosphonium cation (DTPP, a non-antioxidant mitochondria-targeted compound) and vitamin E (a lipophilic, non-mitochondria-targeted antioxidant) were used as controls. The dose range of DTPP used in this investigation was the same as for mitoQ. Vitamin E at 37 μ M concentrations has been shown to reduce H₂O₂ induced toxicity in murine splenocytes (Horton *et al.*, 2007). This study was taken into consideration before choosing the dose range for Vitamin E in this investigation.

In terms of long term exposure to mitoQ, concentrations of 50-100nM inhibited the growth of human foetal lung (MRC-5) fibroblasts in long term culture. Concentrations of 10-20nM needed an adaptation period of 1 week under normoxic (normal O₂) conditions before revealing its antioxidant potential. In MRC-5 cells, 10-20nM mitoQ completely prevented the rise in telomere shortening rate at 40% hyperoxic (increase in O₂) conditions (Saretzki *et al.*, 2003). The results of these studies were taken into consideration before finalising the dose range of mitoQ, DTPP, vitamin E and H₂O₂ in this investigation.

2.1.7.1. Acute exposure of mitoQ in human peripheral blood mononuclear cells *ex vivo*

Freshly isolated human peripheral blood mononuclear cells from ten healthy, non-smoking males fasted overnight, five each from the age groups of 25-30 years and 55-60 years were washed separately in phosphate buffered saline or X-Vivo 10 media and each of the samples were maintained separately in culture in a serum-free medium, X-Vivo 10, in 24 well plates (5 wells, 2 ml medium per well) at concentrations of 2-4 x 10⁵ cells per well, along with 2 x 10⁵ gamma-irradiated (80Gy) RJK853 cells per well (EBV-transformed B-lymphoblastoid cell line with complete hprt deletion) as feeder cells. The samples were then incubated *in vitro* with various doses of mitoQ (a gift from Dr. Mike Murphy, MRC- Dunn Human Nutrition Unit, Cambridge, UK) for 3 or 6

hours. Following mitoQ treatment, the cells were challenged with a range of doses of H₂O₂ (0-250µM) (Sigma) at two time intervals, after 3 or 6 hours. The samples were frozen down within 15 minutes of H₂O₂ treatment. The entire H₂O₂ treatment was performed at 4°C to minimise DNA repair. DTPP and vitamin E (Sigma) controls were similarly processed in parallel to the mitoQ samples. The samples required for further analysis were frozen down.

2.1.7.2. Doses of mitoQ / DTPP / vitamin E supplementation

Peripheral blood mononuclear cells from ten healthy, non-smoking males fasted overnight, five each from the age groups of 25-30 years and 55-60 years were incubated separately with 0-1µM mitoQ or DTPP or 37µM- 74µM Vitamin E for 3 or 6 hours. 0-250µM H₂O₂ was exogenously applied after 3 or 6 hours. H₂O₂ (kept on ice) was maintained at 4°C prior to treatment and the samples treated with H₂O₂ were frozen down within 15 minutes of treatment, in order to maximise damage through reducing DNA repair (Annett *et al.*, 2004).

The impact of chronic exposure (upto three weeks after supplementation) of mitoQ was also investigated in this study. Freshly isolated human peripheral blood mononuclear cells from six healthy, non-smoking males fasted overnight, three each from the age groups of 25-30 years and 55-60 years were maintained in culture separately as described in section 2.1.3.3. The human peripheral blood mononuclear cells were supplemented with 0-30nM mitoQ or DTPP in X-Vivo 10. The *ex vivo* T cells (peripheral blood mononuclear cells) were supplemented with mitoQ or DTPP on days one and four of the growth cycle.

2.1.8. JC-1 staining for analysing mitochondrial membrane permeability in *ex vivo* human peripheral blood mononuclear cells

The JC-1 assay kit (Invitrogen) was used to measure the mitochondrial membrane potential, due to the potential dependent accumulation of the dye in the mitochondria. It is a well established method successfully used in human peripheral blood mononuclear cells (Ostan *et al.*, 2006). In this investigation, JC-1 dye was used to investigate the impact of mitoQ on mitochondrial membrane potential + / - H₂O₂.

2.1.8.1. Principle of the assay and quantification of mitochondrial membrane potential in *ex vivo* human peripheral blood mononuclear cells using JC-1 staining assay kit

The loss of mitochondrial membrane potential is a characteristic that normally precedes apoptosis. The loss of potential occurs through the formation of pores in the mitochondria by pro-apoptotic proteins such as Bax. These pores result in the collapse of the electrochemical gradient across the mitochondrial membrane and so resulting in loss of membrane potential. JC-1 assay kit measures the mitochondrial membrane potential in cells using the dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethylbenzimidazolylcarbocyanine iodide). The JC-1 exists as a monomer in the cytosol (green) and forms aggregates in the mitochondria, staining them red in healthy cells. The dye enters the mitochondria, due to their delocalised positive charge, and the negative charge established by the intact mitochondrial membrane potential. J-aggregates form when the critical concentration of the dye increases in the mitochondria and stains them fluorescent red. In apoptotic cells, the loss of mitochondrial membrane potential results in the inability of the dye to accumulate in the mitochondria, and they stain the cytosol fluorescent green. The JC-1 stained cells are analysed using flow cytometer at absorption maxima of 488nm.

Freshly isolated human peripheral blood mononuclear cells from six healthy, non-smoking males fasted overnight, three each from the age groups of 25-30 years and 55-60 years were washed twice in phosphate buffered saline or X-Vivo 10 media and maintained separately in culture in a serum-free medium, X-Vivo 10 as explained in section 2.1.7.1. The samples were then incubated *in vitro* with mitoQ (0.1 μ M or 0.5 μ M) for 6 hours. Following mitoQ treatment, the cells were challenged with H₂O₂ (0-250 μ M) maintained at 4°C (0-250 μ M). The H₂O₂ treated samples were then frozen down for further analysis within 15 minutes of treatment.

The JC-1 staining kit assay was then optimised using a range of cells (0.5 x 10⁶ to 2 x 10⁶ cells/ml). After optimisation, 1.5 x 10⁶ human peripheral blood mononuclear cells *ex vivo* was used for analysis using JC-1 staining kit. Human peripheral blood mononuclear cells *ex vivo* were suspended in warm PBS and stained with JC-1 dye at final concentration of 2 μ M. The cells were maintained at 37°C at 5% CO₂ for 30

minutes. The cells were then re-suspended in fresh warm PBS or X-Vivo media and the mean fluorescent intensity of green signal (percentage of apoptotic cells) was compared to the mean fluorescent intensity of red signal (percentage of live cells) following analysis with flow cytometer at an absorption maxima of 488nm. The results were expressed as mean ratio values. Mean value represents the mean fluorescent intensity giving the highest number of counts in a flow cytometer.

The mean ratio values are calculated using the following formula:

$$\text{Mean ratio} = \frac{\text{Mean value of green signal}}{\text{Mean value of red signal}}$$

Higher mean ratio represents lower mitochondrial membrane potential and a lower mean ratio or ratio closer to basal levels represents higher mitochondrial membrane potential.

2.1.9. T cell proliferation assays

The radioactive thymidine incorporation proliferation assay is a well established assay used for determining the proliferation capacity of human peripheral blood mononuclear cells (Pawelec *et al.*, 2000). In this investigation the effects of the antioxidants ebselen or NAC on the proliferation capacity of human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo* were examined.

The proliferation assay involved the incubation of cells with radioactive thymidine. The cells incorporate radioactive thymidine into their DNA with each cell division or proliferation during the incubation period. Higher proliferation rate is associated with higher amounts of thymidine incorporation (higher radioactivity). After incubation, the cells are washed with distilled water using force that sets the DNA free. The cell organelles and DNA is then filtered through a membrane of 1.5mm diameter. Intact DNA is collected on the filter membrane. The radioactivity is then measured using a scintillation counter.

Human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo* from ten healthy, non-smoking males fasted overnight, five each from the age groups of 25-30 years and 55-60 years, with or without the antioxidants, were counted separately at 1×10^6 cells per well in a 24 well plate. Tritiated thymidine was added 18 hours prior to harvesting at a final concentration of 0.037MBq/ml. Cells were transferred onto a 96 well plate the next day and were harvested using a 96-well harvester onto a 96-well filter plate and 40µl of scintillation fluid was added to each of the filter wells. Filters were counted for 1 minute per well with a Top-Count scintillation counter and the amount of radioactivity incorporated (proliferation capacity of the cells) was expressed in thymidine incorporation (H^3) counts per minute (cpm). All the samples were analysed in duplicate.

2.1.10. Assessment of levels of oxidative damage to DNA

In this study the alkaline comet assay was used to determine levels of oxidative damage to DNA. This method has been successfully used in the group of Barnett to measure DNA damage in T cells (Hyland *et al.*, 2000; 2001; Duggan *et al.*, 2004).

The use of the Comet Assay was first introduced in NTU with this study. In this investigation, the alkaline comet assays were used to determine the effect of the antioxidants (ebselen, NAC, mitoQ & vitamin E) on the levels of oxidative damage to DNA in T cells *in vitro* and *ex vivo*.

The comet assay, also known as single cell gel electrophoresis assay, is a method used to measure DNA strand breaks in eukaryotic cells. The alkaline comet assay involves analysing stained cells embedded in agarose in a microscopic slide using a fluorescent microscope. Prior to staining, the cells are lysed with detergent and high salt resulting in nucleoids containing super-coiled loops of DNA linked to the nuclear matrix. On electrophoresis, the loops containing a break lose their super-coiling and extend towards the anode. These loops give structures similar to comets. This technique involves electrophoresis at high pH that results in structures similar to comets viewed using a fluorescent microscope. The number of DNA breaks can be assessed by quantifying the intensity of comet tail compared to the head (Collins, 2004; Figure 2.2). The alkaline comet assay is performed to detect DNA single strand breaks and alkali-labile lesions. Modified comet assay involves the treatment of cells with enzymes endonuclease III (Endo III) (Asahara *et al.*, 1989) or formamidopyrimidine glycosylase (FPG) (Boiteux

alkaline unwinding of the DNA. The slides were then electrophoresed in a pre-chilled tank at 25V, 300mA for 20 minutes. Then the slides were neutralised with three 5 minutes washes in neutralising buffer (0.4M Tris pH 7.5). Finally, the gels were stained with 50µg/ml ethidium bromide (Sigma) and digitally analysed using a fluorescent microscope and the comet analysis was performed on the cells detected using computer image analysis software (Komet 5.5, Andor Technology, UK), counting 50 cells per slide. The percentage of DNA damage in the comet tail was used as the measure of DNA damage (Hyland *et al.*, 2000, 2001). The reagents for the assay were purchased from Sigma. All the samples were performed in duplicate.

2.1.11. Assessment of GSH:GSSG ratio and total glutathione level in human peripheral blood mononuclear cells / T cell clones

The intracellular redox status (GSH:GSSG ratio) plays an important role as a mediator in apoptosis in many cell systems (Hall, 1999). Previous findings have demonstrated the depletion of intracellular reduced GSH, a major determinant of an altered intracellular redox status, prior to the onset of apoptosis (Ghibelli *et al.*, 1998). The depletion of intracellular reduced GSH may be due to increased oxidation of GSH to GSSG. The oxidation of even a small amount of GSH may hinder the redox status of the cell resulting in a sub-optimal GSH:GSSG ratio. A low GSH:GSSG ratio *in vivo* has been associated with several human diseases (more in detail in chapter-1, Section 1.1.4.1). In this study, a GSH:GSSG ratio assay kit (Calbiochem) was used, after a period of time was spent unsuccessfully setting up an HPLC system.

The GSH:GSSG ratio assay kit has been used previously in assessing the GSH:GSSG ratio in human peripheral blood mononuclear cells (Yamada *et al.*, 2006). The assay was optimised for use in T cell clones in this investigation by trying a range of cells from 0.5×10^6 to 3×10^6 cells/ml for assessing the levels of total glutathione and 2×10^6 to 7×10^6 cells/ml for assessing the levels of GSSG. Higher number of cells was used for GSSG measurement, since it is present in such low concentration in human tissue. The same procedure was carried out for human peripheral blood mononuclear cells *ex vivo*. In this investigation, GSH:GSSG ratio assay kit was used to determine the effect of antioxidants (ebselen, NAC, mitoQ & vitamin E) on the redox status of T cells *ex*

vivo and *in vitro*. GSH is a tripeptide having a free thiol group and acts a major antioxidant in human tissues. GSH provides reducing equivalents for GPx catalysed reduction of H_2O_2 and lipid hydroperoxides to water and respective alcohol. This reduction results in the oxidation of GSH to form GSSG. GR and NADPH helps in the recycling of GSSG to GSH. An increase in oxidative stress is normally associated with the oxidation of GSH to GSSG, resulting in GSSG accumulation and an associated decrease in the GSH:GSSG ratio. The GSH:GSSG ratio is also used to analyse the effectiveness of antioxidant treatments. The accurate measurement of GSSG level in cells has proved to be very difficult due to a low concentration in human tissues (Griffith, 1980). The main reason for this is the absence of a method which can effectively prevent the oxidation of GSH to GSSG. The assay in this investigation relies on 1-methyl-2-vinylpyridine trifluoromethane sulfonate (M2VP), a thiol scavenging reagent that scavenges GSH, to prevent its oxidation to GSSG. M2VP does not interfere with the GR assay (Figure 2.3).

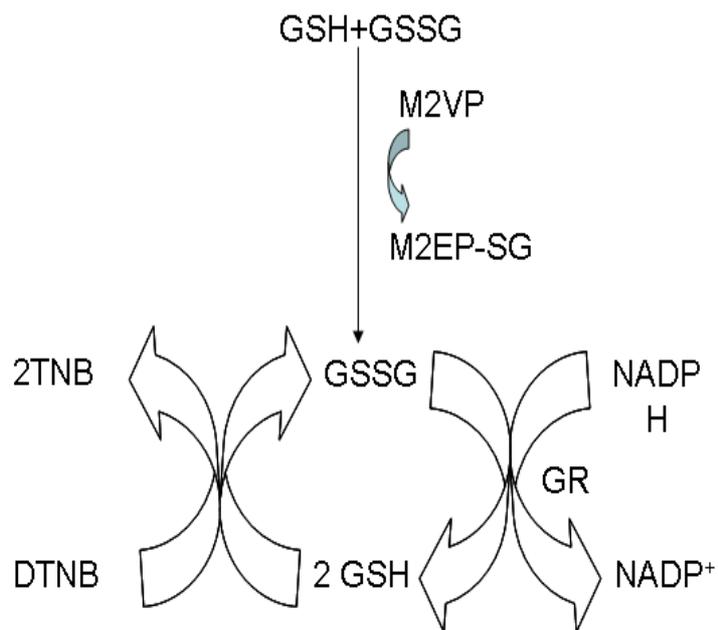


Figure 2.3. M2VP scavenges GSH in the T cell sample resulting in GSSG. GR and NADPH recycle GSSG to 2 GSH. GSH then reacts with 5,5¹-Dithiobis-2-nitrobenzoic acid (DTNB), a chromogen that oxidises 2GSH resulting in GSSG enabling its measurement. M2VP is not involved in the experiment involving the measurement of total glutathione (GSH + GSSG).

After assessing the levels of GSH + GSSG and GSSG in the T cell sample, the GSH in the sample can be calculated by subtracting GSSG from GSH + GSSG.

The GSH:GSSG ratio is calculated using the formula:

$$\frac{(GSH + GSSG) - 2 (GSSG)}{GSSG}$$

The cell extracts for analysis were prepared from harvested cells (2×10^6 cells/ml for GSH and 5×10^6 cells/ml for GSSG) according to the manufacturers instructions. The GSH:GSSG ratio and the total glutathione levels were calculated from the GSH and GSSG concentrations of the T cells *ex vivo* or *in vitro* were photometrically measured at 412 nm in μ M concentrations. The GSH:GSSG ratio was calculated using the formulae described in above. All the samples were analysed in duplicate.

2.1.12. Statistical analysis of the samples

The results were tested for significance using paired two-sample Student's t-tests assuming equal variances; p values are presented as appropriate. The type 2 student's t-test was chosen because in one experiment there are two samples of the same cell population having similar variance or the variations between the values will be the same since they are of the same cell population.

CHAPTER-3

**The growth characteristics of
human peripheral blood derived
CD4⁺ T cell clones *in vitro***

3.1. The growth characteristics of human peripheral blood derived CD4⁺ T cell clones *in vitro*

3.1.1. Introduction

T cells are supposed to undergo rapid clonal expansion upon antigenic stimulation to produce an effective immune response. A successful stimulation, co-stimulation and an appropriate cytokine environment are a pre-requisite for T cell clonal expansion that in turn results in a successful T cell immune response. The clonal expansion is followed by contraction in the absence of antigen and almost all of the T cells die by apoptosis. Subsequent antigenic challenge can result in the repetition of the process. Any factor that interferes with the ability of the T cell clones to proliferate may deleteriously affect the immune response, thus limiting the clonal expansion. Replicative senescence and / or genetic damage can be an interfering factor (Effros and Pawelec, 1997; Barnett and Barnett, 1998). It is also worth mentioning the decreased proliferative potential of T cells that may have previously proliferated *in vivo* (Pawelec *et al.*, 1999).

The *in vitro* T cell clone model of immunosenescence is an invaluable model for determining age associated changes to T cell function and replicative senescence. The behaviour of a single cell type can be longitudinally followed using this model. The observed changes in a polyclonal model system may be due to the alterations in the proportions of different clones present. In terms of calculating longevities, polyclonal model systems have mixed populations and only the longevity of the remaining surviving clone can be calculated. For a proper clonal study in a mixed population, longevities of each of the clones have to be examined separately. This is technically very challenging. Cellular and molecular changes associated with T cell clone ageing *in vitro* have been found to be similar to the ageing T cells *in vivo*. The *in vitro* T cell clone model has important similarities to ageing of T cells *in vivo* (Chapter-1, Table 1.8; Pawelec *et al.*, 2002). T cell clones are known to have a finite lifespan and can be maintained for extended periods in culture. The cloning efficiency of T cell clones from donors of varying ages, longevities of individually cultured T cell clones, their cytokine secretion and surface marker profiles have previously been examined (Pawelec *et al.*, 1999). This chapter reports on the growth characteristics of the T cell clones used in this investigation during *in vitro* culture and the impact of age on their ability to replicate.

3.1.2. Materials and Methods

Twelve human peripheral blood derived CD4⁺ T cell clones were cultured as described previously by (Hyland *et al.*, 2000, 2001; Chapter-2, Section 2.1.1.1). The 400-series clones were obtained from a 26 year old overtly healthy laboratory worker and the 385-series clones were obtained from a 45 year old overtly healthy laboratory worker. Viable cell counts were determined at the end of each 7 day culture cycle. The viable cell counts were used to track the PDs each clone had undergone during their span in culture. Viability of the T cell clones was assessed by trypan blue exclusion test. The population doublings each clone had undergone per week or the proliferative capacity was calculated using the following formula;

$$\text{PD} = \text{Log}_2(\text{Cell count at the end of cycle}) - \text{Log}_2(\text{Cell count at the start of cycle}).$$

Cumulative PD or total PD of the clones at the end of their span in culture was used to track the *in vitro* age of the clones.

3.1.3. Results and Discussion

The range of PD per week of the T cell clones used in this investigation, during their lifespan was 0.7-1.1 (Table 3.1). The relationship between PD/week and the cumulative population doublings or the *in vitro* age of the clones was determined by linear regression analysis. The T cell clones used in this investigation revealed considerably higher levels of growth at the start of the culture compared to the later stages of their life span in culture (Appendix- I, Figures 1-12). The regression analysis data (as revealed in table 3.2) shows a significant downward slope reaching statistical significance for the complete lifespan data, suggesting decreased PD capacity with age. However, post the initial peak values, the remainder of the lifespan of the clones transitioned into a steady state. This result indicates the maintenance of PD capacity throughout the lifespan of the clones. The results of this investigation were in line with the previous findings of the group of Barnett using human peripheral blood derived CD4⁺ T cell clones (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

Table 3.1. Average PD/week and cumulative PDs of twelve human peripheral blood derived CD4⁺ T cell clones during their *in vitro* lifespan

Clone	Mean PD per seven day growth cycle	Maximum PD achieved
400-23a	0.7	44.9
400-23b	0.9	49.1
400-23c	1.1	83.6
400-23d	0.8	77.6
400-5a	0.9	57.0
400-5b	0.8	53.2
385-7a	0.9	44.4
385-7b	1.0	47.2
385-7c	1.1	78.2
385-7d	0.9	74.4
385-2a	0.8	50.5
385-2b	0.7	48.1
Mean	0.9	59.0

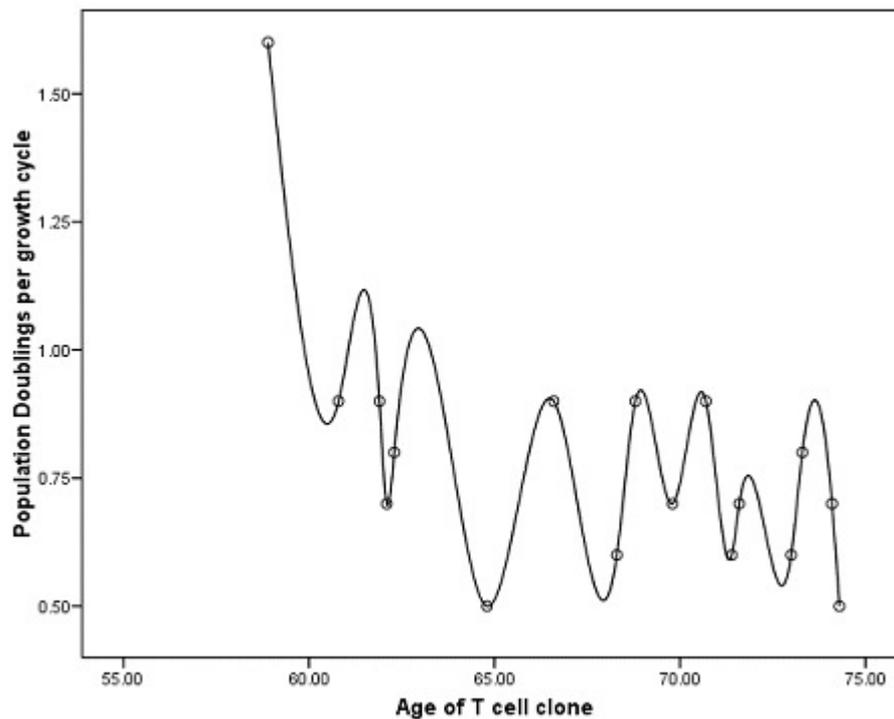


Figure 3.1. Proliferative capacity of 385-7d clone throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms.

The SPSS software was used for creating spline curves and the software is designed to interpolate (create new points) for a smooth transition between two points. The software uses a polynomial (finite length of expression constructed from variables) function to create spline curves. Figure 3.1 illustrates an example for how the T cell clones grew during their span in culture. All the twelve clones examined in this investigation, grew in waves of maximal and minimal rates of proliferation variable from one to several growth cycles in length during the early part of their lifespan (as indicated in figure 3.1 and figures 1-12 in appendix-I). However, the size or length of the waves decreased and the wave frequency increased during the later stages of their lifespan. This finding was similar to the previous findings from the group of Barnett (Hyland *et al.*, 2000). This phenomenon observed in T cell clones may be an indication of a frequency in cell cycle arrest due to age-related accumulation of oxidative DNA damage in T cell clones (Barnett and Barnett, 1998; Hyland *et al.*, 2000; 2001; Chapter-4) *in vitro* during the later stages of the lifespan of T cell clones. This increase in DNA damage when critical would result in the failure of T cells to proliferate due to DNA damage mediated cell cycle arrest; decreased rates of proliferation as a consequence of selection *in vivo* against cells carrying mutations and finally may result in apoptosis (Barnett and Barnett, 1998). The increase in frequency of the waves towards the later stages of a T cell clones lifespan data can be implied as their ability to retain their responsiveness to proliferative stimulation (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

Table 3.2. Linear regression model statistics analysing the relationship between PD/growth cycle and the *in vitro* age in twelve human peripheral blood derived CD4⁺ T cell clones. Slope indicates the decrease in PD achieved per growth cycle. The data in this table is the average of twelve clones used in this investigation

n=12	Slope	r ²	p value
Full Lifespan data	-.04701	0.1925	<0.01
Lifespan data subsequent to initial crest	-.0084	0.009	0.4486

The results of the analysis described in this chapter reveal that even though the T cell clones maintain their ability to proliferate with age, their ability to sustain proliferation during the late stages of their lifespan is reduced due to age related stresses. The relative proportion of the cells undergoing cell cycle arrest / senescence during the lifespan of the clone in culture was not investigated.

CHAPTER-4

**An investigation of effects of the
antioxidants, ebselen or NAC, on
cultured human peripheral blood
derived CD4⁺ T cell clones**

4.1. An investigation of effects of the antioxidants, ebselen or NAC, on cultured human peripheral blood derived CD4⁺ T cell clones

4.1.1. Introduction

T cell function has been shown to decline with age, resulting in an increase in susceptibility to infection, cancer and a variety of diseases. Naïve/memory T cells are supposed to undergo rapid clonal expansion on contact with the antigen, for which they carry unique antigen receptors, to produce an effective immune response. Any factor that interferes with the ability of T cells to clonally expand may impact on the effectiveness of an immune response with the potential to render it sub-optimal.

Previous work from the group of Barnett has demonstrated an age associated increase of DNA damage in T cell clones *in vitro* under standard culture conditions of 20% O₂ tension (Barnett *et al.*, 1999; Hyland *et al.*, 2000, 2001). The major source of DNA damage *in vivo* has been attributed to ROS from a wide variety of intrinsic and extrinsic sources (Ames *et al.*, 1993). ROS have been implicated in several human degenerative diseases of ageing (Naik *et al.*, 2006). T cells are also exposed to high concentrations of ROS and reactive nitrogen intermediates, produced as a normal part of an immune response, at sites of inflammation. Such exposure may ultimately result in damage to the T cells. *In vivo* antioxidants and DNA repair systems are two types of T cell defense that can counteract the occurrence of damage (Hyland *et al.*, 2000). An age related decline of DNA repair capacities in T cells has been previously reported in human peripheral blood derived CD4⁺ T cell clones cultured *in vitro* under standard O₂ tension (Annett *et al.*, 2004, 2005). The decline in DNA repair capacities may result in an accumulation of DNA damage to critical levels within T cells, resulting in cell cycle arrest or even apoptosis (Barnett and Barnett, 1998). The potential anti-ageing effect of antioxidants in T cells is yet to be thoroughly investigated. This chapter describes the results of such an investigation with the antioxidants ebselen and NAC.

Ebselen is a synthetic, lipid soluble seleno-organic compound having potent antioxidant capacity. It is also a novel anti-inflammatory agent having glutathione peroxidase like

activity, first described in 1984 (Parnham *et al.*, 1991; Sies, 1993; Yang *et al.*, 1999). A neuroprotective effect of ebselen in a variety of *in vitro* and *in vivo* animal models and several pre-clinical studies has been well documented (Imai *et al.*, 2003; Porciuncula *et al.*, 2003; Moretto *et al.*, 2005). Ebselen has also been reported to protect HepG₂ cells (Yang *et al.*, 1999), human HL-60 (Li *et al.*, 2000) and PC-12 cells (Yoshizumi *et al.*, 2002) against H₂O₂ induced cell death and DNA damage.

NAC is an important antioxidant containing an acetylated form of the amino acid L-cysteine that functions as a precursor of glutathione synthesis (Pivetta *et al.*, 2005). Glutathione is an important thiol involved in cellular detoxification (Meister and Anderson, 1983). The presence of sulfhydryl groups in NAC also enables the neutralisation of free radicals. It is water soluble and its intake is the most cost-effective way of boosting glutathione levels in the body. NAC is a low molecular weight compound that can readily pass through the blood brain barrier (Martinez 2000). The aim of this chapter is to describe the results of an investigation on the impact of exposure to different concentrations of ebselen or NAC on cultured human peripheral blood derived *in vitro* CD4⁺ T cell clones.

4.1.2. Materials and Methods

4.1.2.1. Human peripheral blood derived CD4⁺ T cell clones

Human peripheral blood derived CD4⁺ T cell clones (Chapter-1, Section 1.1.3.8) is a well established monoclonal *in vitro* model which has been used in many studies on immunosenescence (Pawelec *et al.*, 2006). Twelve independently derived T cell clones were examined in this investigation. The 400-series clones were obtained from a 26 year old overtly healthy laboratory worker and the 385-series clones were obtained from a 45 year old overtly healthy laboratory worker (Chapter-2, Section 2.1.1).

4.1.2.2. Culture of T cell clones

Human peripheral blood derived CD4⁺ T cell clones (expressing T cell receptors for antigen; TCR, CD3⁺, CD4⁺) were maintained in culture in a serum-free medium, X-Vivo 10 (BioWhittaker), in 24 well plates as detailed in Hyland *et al.*, (2000; 2001) and (Chapter-2, Section 2.1.1.1).

4.1.2.3. Determination of T cell clone proliferative capacity and lifespan

Proliferative capacity was calculated as the number of PDs a clone underwent in a week in culture. PDs can be calculated using the following formula.

$PD = \text{Log}_2(\text{cell count at the end of the cycle}) - \text{Log}_2(\text{number of cells at the start of the cycle})$.

Lifespan was defined as the cumulative number of population doublings the clones underwent during their lifespan in culture. The required samples for analysis were cryopreserved using the protocol detailed previously in Hyland *et al.*, (2000, 2001) and (Chapter-2, Section 2.1.1.2).

4.1.2.4. Ebselen or NAC supplementation

Initial studies were carried out by supplementing T cell clones from the midpoint of their *in vitro* lifespan with a range of doses of ebselen (0-100 μ M) (Table 2.1) (Sigma) and NAC (0-10mM) (Table 2.1) (Sigma) in X-Vivo 10. The results of the impact of ebselen or NAC on T cell clones from the midpoint of their *in vitro* lifespan were taken into consideration before choosing the dose range of 0-30 μ M for ebselen and 0-7.5mM for NAC for the studies investigating the impact of these antioxidants when supplemented from a young *in vitro* age. Clones were supplemented with ebselen or NAC on days one and four of the growth cycle (Chapter-2, Section 2.1.2).

Previous studies with ebselen or NAC investigated their impact on acute exposure in different cell lines. The concentration range of ebselen used in this novel investigation was chosen on the basis of earlier studies of ebselen carried out in HepG₂ and HL-60 cell lines. HepG₂ cells have been successfully used in several cancer and apoptotic studies mainly dealing with signalling pathways (Castaneda and Rosin-Steiner, 2006). Yang *et al.* (2000) demonstrated an increase in percentage of viability in HepG₂ cells on treatment with 50 μ M ebselen for 24hrs. 25 μ M ebselen had no effect on cell viability and the percentage of viability significantly decreased at concentrations of 75 μ M or 100 μ M of ebselen. A concentration dependent increase in apoptosis and decrease in intracellular GSH concentrations after 24hrs were demonstrated with ebselen (50-100 μ M) supplementation in HepG₂ cells (Yang *et al.*, 2000) after 24 hrs. Previous work

in HepG₂ cells demonstrated the ability of ebselen (1-25µM) to stimulate cell growth, inhibit H₂O₂ induced suppression, significantly reduce LDH leakage and lipid peroxidation, almost completely inhibit DNA damage and significantly reduced intracellular ROS levels in a concentration dependent manner. Concentrations above 50µM proved cytotoxic to cells (Yang *et al.*, 1999). Ebselen (1-20µM) has also been demonstrated to inhibit H₂O₂ induced suppression of cell growth, lipid peroxidation, reduction of DNA damage and intracellular ROS levels in a concentration dependent manner in HL-60 cells. Concentrations above 20µM were shown to be cytotoxic to cells (Ji *et al.*, 2000).

In terms of NAC, the following studies were taken into consideration before choosing the dose range in this investigation. Previous studies investigating the effect of a range of NAC concentrations (0.5-2mM) in HeLa cells have shown the ability of 2mM NAC to increase intracellular GSH by 15% compared to controls after 24hrs of incubation (Hansen *et al.*, 2004). In HepG₂ cells, the increase in intracellular GSH level was highest at 5mM NAC supplementation for 24hrs (Yang *et al.*, 2000).

4.1.2.5. Determination of levels of oxidative damage to DNA in human CD4⁺ peripheral blood derived T cell clones

The levels of oxidative damage to DNA, plus DNA single strand breaks and alkali labile sites, in T cell clones were determined at four time points of their lifespan in culture, using the modified alkaline comet assay as previously described (Collins *et al.*, 1993; Hyland *et al.*, 2000, 2001; Duggan *et al.*, 2004; Chapter-2, Section 2.1.10). This assay uses two different enzymes namely, FPG (New England Biolabs) that recognises oxidatively modified purines (Boiteux *et al.*, 2002), and Endo III (New England Biolabs) which recognises oxidatively modified pyrimidines (Asahara *et al.*, 1989). The enzymes nick DNA at the site of oxidatively damaged nucleotides, creating single strand breaks that can be detected by the assay. Computer image analysis software, Komet 5.5 (Andor Technology, UK) used to perform analysis of cells in the slides. Comet assays for the samples were performed in duplicate.

4.1.2.6. Quantitative determination of intracellular GSH:GSSG and total glutathione levels

The ratio of the reduced and oxidised form of glutathione and total glutathione levels were assessed using a GSH:GSSG ratio assay kit (Calbiochem, UK) (Chapter-2, Section 2.1.11). This technique incorporates a thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethane-sulfonate1 (M2VP) at a level that rapidly scavenges GSH, but does not interfere with the glutathione reductase in recycling GSSG into GSH. Whole cell extracts were prepared from harvested cells (2×10^6 cells/ml for GSH and 5×10^6 cells/ml for GSSG), according to the manufacturers instructions. The total glutathione levels and the GSH:GSSG ratio were calculated from the GSH and GSSG concentrations of the clones, photometrically measured at 412 nm in μM concentrations. The samples were analysed in duplicate.

4.1.2.7. Statistical analysis

The results were tested for significance using paired two-sample Student's t-tests assuming equal variances; p values are presented as appropriate.

4.1.3. Results

4.1.3.1. The impact of ebselen or NAC on *in vitro* proliferative capacity and lifespan of human CD4⁺ T cell clones

The human CD4⁺ T cell clones used in this study underwent apoptosis through deletion at the end of their lifespan after undergoing a number of PDs. This was in line with previous reports (Grubeck-Leobenstein *et al.*, 1994; Hyland *et al.*, 2000).

The effect of different concentrations of ebselen (Tables 4.1A & 4.1B) or NAC (Tables 4.2A & 4.2B) on the proliferative capacity and *in vitro* lifespan of CD4⁺ T cell clones was investigated by supplementing them from a young *in vitro* age (34.5 and 41.0 PD) until the end of their lifespan. The effect of different concentrations of ebselen (Tables 4.3A & 4.3B) or NAC (Tables 4.4A & 4.4B) on the proliferative capacity and *in vitro* lifespan of CD4⁺ T cell clones supplemented from the midpoint of their *in vitro* lifespan (58.7 and 63.4 PD) until the end of their lifespan was also investigated. Control clones were grown in parallel to the ebselen or NAC supplemented clones and proliferative

capacity and lifespan were determined in both supplemented and non-supplemented clones.

Table 4.1. The effect of ebselen on the proliferative capacity and lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.1A) and a healthy 45 year old donor (4.1B) supplemented from a young *in vitro* age

*** Significantly higher, p < 0.05**

4.1A

**Clone x 400-23 26-year old donor
Initial PD- 34.5**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	0.7	44.9
10µM ebselen	0.8	46.9
30µM ebselen	1.2	56.2*

4.1B

**Clone x 385-7 45-year old donor
Initial PD- 31.0**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	0.9	44.4
10µM ebselen	0.9	44.9
30µM ebselen	1.4	50.8*

Tables 4.1A and 4.1B indicates a significant increase in lifespan of CD4⁺ T cell clones derived from a young donor and a middle aged donor on supplementation with 30µM ebselen from a young *in vitro* age, compared to non-supplemented clones. 10µM ebselen did not significantly alter the lifespan of CD4⁺ T cell clones derived from donors of either age group, compared to non supplemented clones.

Table 4.2. The effect of NAC on the proliferative capacity and lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.2A) and a healthy 45 year old donor (4.2B) supplemented from a young *in vitro* age

*** Significantly higher, p < 0.05**

4.2A

**Clone x 400-23 26-year old donor
Initial PD- 34.5**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	0.9	49.1
1.25mM NAC	0.8	48.4
5mM NAC	1.1	53.5
7.5mM NAC	1.2	56.1*

4.2B

**Clone x 385-7 45-year old donor
Initial PD- 31.0**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	1.0	47.2
1.25mM NAC	1.0	48.7
5mM NAC	1.3	52.8
7.5mM NAC	1.4	55.8*

Tables 4.2A and 4.2B summarises a significant increase in lifespan of CD4⁺ T cell clones derived from a young donor and a middle aged donor on supplementation with 7.5mM NAC from a young *in vitro* age, compared to non-supplemented clones. 5mM NAC supplementation resulted in a marginal increase in proliferation capacity and lifespan, compared to controls in both clones. There were no significant differences in proliferative capacity and lifespan of CD4⁺ T cell clones derived from a healthy 26 year old donor compared to the T cell clones derived from a healthy 45 year old donor on supplementation with 1.25mM NAC.

Table 4.3. The effect of ebselen on the proliferative capacity and lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.3A) and a healthy 45 year old donor (4.3B) supplemented from the midpoint of their *in vitro* lifespan

4.3A

**Clone x 400-23 26-year old donor
Initial PD- 63.4**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	1.2	83.6
5µM ebselen	0.8	80.8
15µM ebselen	0.7	79.5
30µM ebselen	0.9	82.8

4.3B

**Clone x 385-7 45-year old donor
Initial PD- 58.7**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	1.1	78.2
5µM ebselen	0.8	77.1
15µM ebselen	0.8	77.8
30µM ebselen	0.7	74.0

Tables 4.3A and 4.3B shows that there were marginal reductions in proliferative capacity and lifespan CD4⁺ T cell clones derived from a young donor and a middle aged donor, supplemented with different concentrations of ebselen from the midpoint of their *in vitro* lifespan, compared to control clones. The reductions in proliferative capacity and lifespan in supplemented clones were not statistically different compared to controls.

Table 4.4. The effect of NAC on the proliferative capacity and lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.4A) and a healthy 45 year old donor (4.4B) supplemented from the midpoint of their *in vitro* lifespan

4.4A

**Clone x 400-23 26-year old donor
Initial PD- 63.4**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	1.0	77.6
0.312mM NAC	0.8	76.4
1.25mM NAC	0.7	75.8
5mM NAC	0.9	77.2

4.4B

**Clone x 385-7 45-year old donor
Initial PD- 58.7**

Concentration	Average PD per week	Cumulative PD achieved at the end of lifespan in culture
Control	1.0	74.4
0.312mM NAC	0.7	72.8
1.25mM NAC	0.7	71.6
5mM NAC	0.8	73.5

Tables 4.4A and 4.4B shows that there were marginal reductions in proliferative capacity and lifespan CD4⁺ T cell clones derived from a young donor and a middle aged donor, supplemented with different concentrations of NAC from the midpoint of their *in vitro* lifespan, compared to control clones. The reductions in proliferative capacity and lifespan in supplemented clones were not statistically different compared to controls.

4.1.3.2. The impact of ebselen or NAC on levels of oxidative DNA damage in human CD4⁺ T cell clones as a function of *in vitro* age

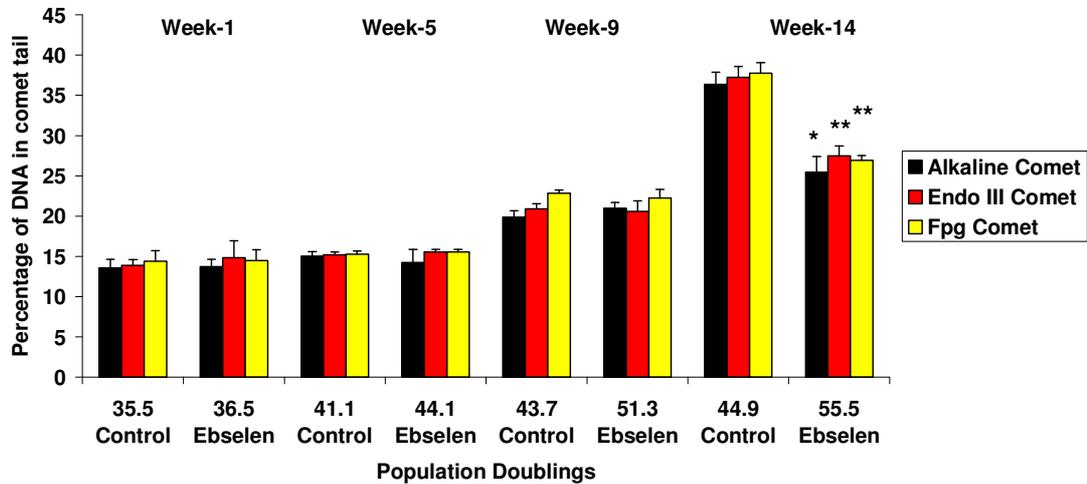
Aliquots of T cell clone samples were taken from culture at various time points and the effect of different concentrations of ebselen or NAC on the levels of oxidative DNA damage within the T cells was determined. In control samples, levels of oxidative damage to DNA increased as a function of age, as measured by the modified Endo III and FPG comet assays, in line with previously published findings (Barnett *et al.*, 1999; Hyland *et al.*, 2000).

Figure 4.1 shows the results of the effect of 30µM ebselen on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.1A) and a healthy 45 year old donor (4.1B) supplemented from a young *in vitro* age, compared to the non-supplemented clones. Figure 4.2 shows the results of the effect of 7.5mM NAC on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.2A) and a healthy 45 year old donor (4.2B) supplemented from a young *in vitro* age, compared to the non-supplemented clones. Figure 4.3 shows the results of the effect of 30µM ebselen on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.3A) and a healthy 45 year old donor (4.3B) supplemented from the midpoint of their *in vitro* lifespan, compared to the non-supplemented clones. Figure 4.4 shows the results of the effect of 5mM NAC on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.4A) and a healthy 45 year old donor (4.4B) supplemented from the midpoint of their *in vitro* lifespan, compared to the non-supplemented clones.

4.1A

Clone x 400-23 - 26 year old donor

30 μ M Ebselen



4.1B

Clone x 385-7 - 45 year old donor

30 μ M Ebselen

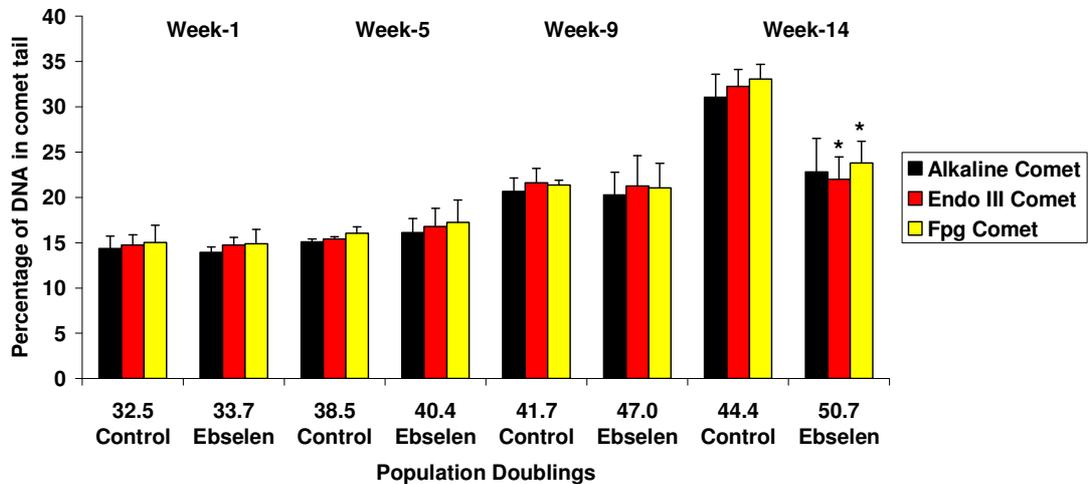


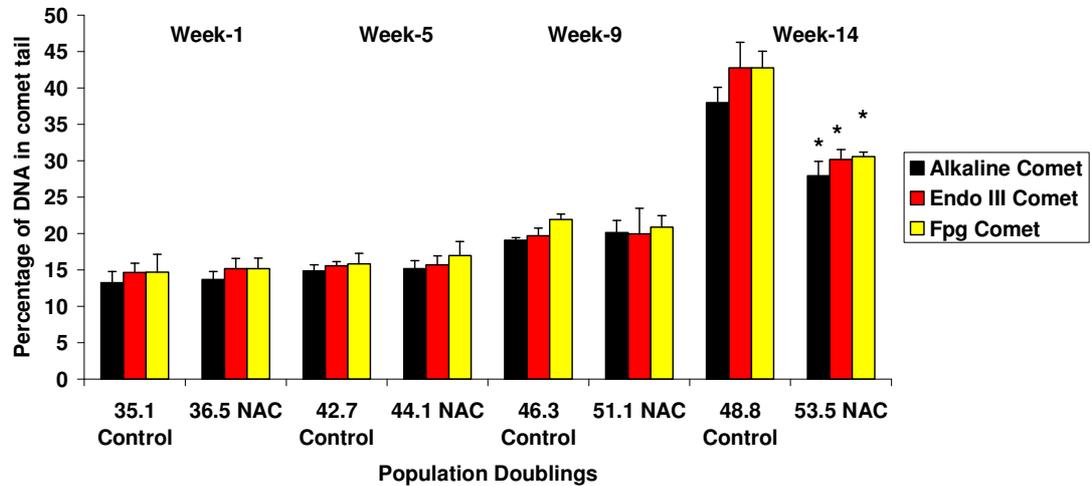
Figure 4.1 The impact of 30 μ M ebselen on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.1A) and a healthy 45 year old donor (4.1B) supplemented from a young *in vitro* age. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

* $p < 0.05$, ** $p < 0.01$ - Significantly lower than control levels

4.2A

Clone x 400-23 - 26 year old donor

7.5mM NAC



4.2B

Clone x 385-7 - 45 year old donor

7.5mM NAC

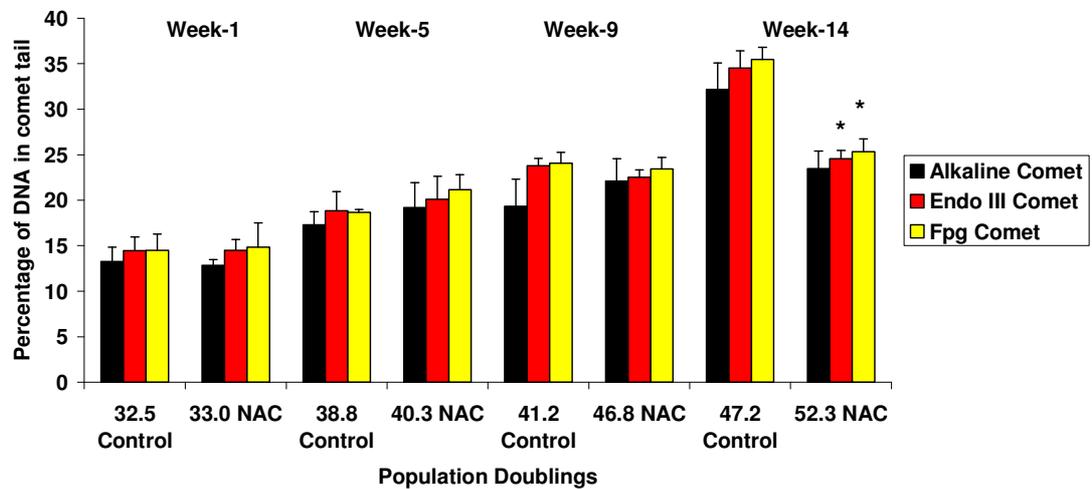


Figure 4.2 The impact of 7.5mM NAC on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.2A) and a healthy 45 year old donor (4.2B) supplemented from a young *in vitro* age. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

* $p < 0.05$ - Significantly lower than control levels

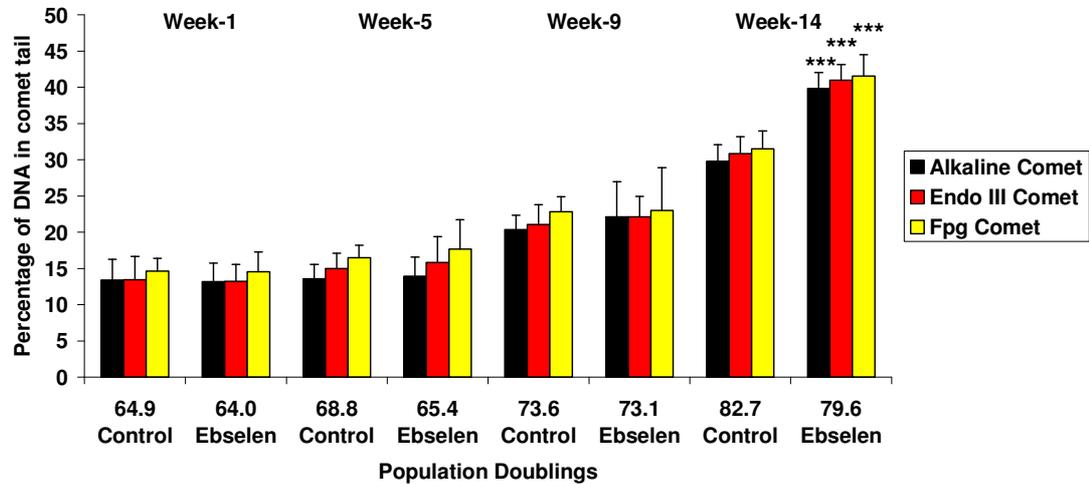
Figures 4.1A, 4.1B, 4.2A & 4.2B reveal an increase in levels of oxidative DNA damage with increase of time in culture in both supplemented and non-supplemented clones. Supplemented clones underwent more PDs after 14 weeks in culture, compared to non-supplemented clones. When compared to non-supplemented clones, supplementation from a young *in vitro* age with 30 μ M ebselen (Figure 4.1) or 7.5mM NAC (Figure 4.2) resulted in no significant differences in the levels of oxidative DNA damage after one, five and nine weeks culture, in the T cell clones derived from both donors. At the nine week point the reduction in levels of oxidative damage to DNA in supplemented clones reached statistical significance at the fourth time point (after fourteen weeks) (Student's t-test, 95% confidence levels).

Furthermore, the impact of other doses of ebselen or NAC on the levels of oxidative DNA damage was investigated. 10 μ M ebselen or 5mM NAC did not significantly alter the levels of oxidative DNA damage in CD4⁺ T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor, compared to non supplemented clones in any of the four time points investigated. There were no significant differences in levels of oxidative damage to DNA in T cell clones derived from a young donor when compared with levels in cells from a middle aged donor.

4.3A

Clone x 400-23 - 26 year old donor

30 μ M Ebselen



4.3B

Clone x 385-7 - 45 year old donor

30 μ M Ebselen

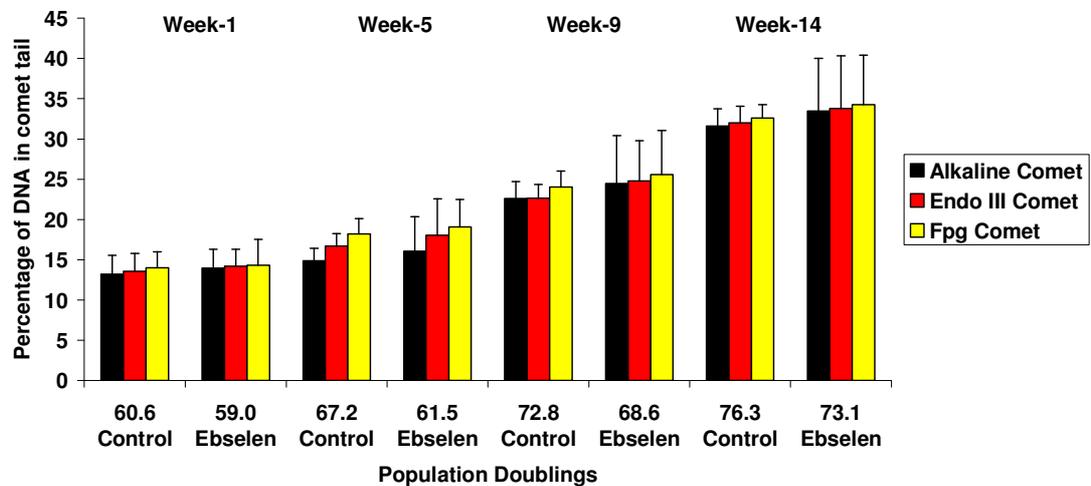


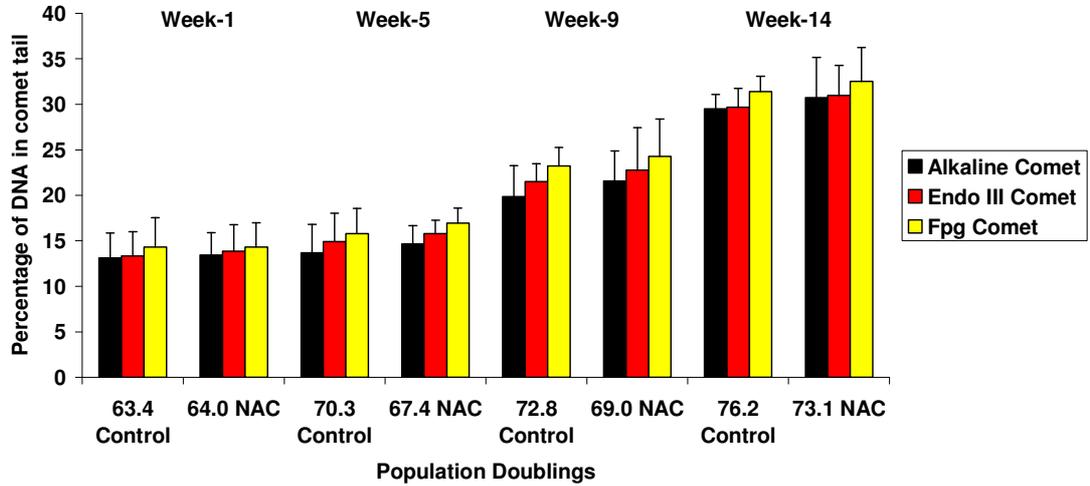
Figure 4.3 The impact of 30 μ M ebselen on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.3A) and a healthy 45 year old donor (4.3B) supplemented from the midpoint of their *in vitro* lifespan. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

*** Significantly higher than control levels, $p < 0.001$

4.4A

Clone x 400-23 - 26 year old donor

5mM NAC



4.4B

Clone x 385-7 - 45 year old donor

5mM NAC

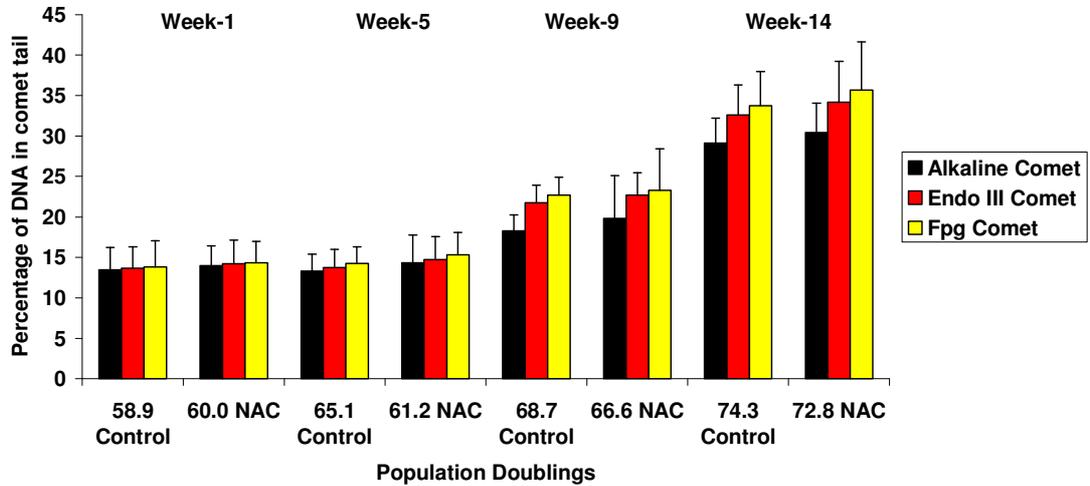


Figure 4.4 The impact of 5mM NAC on oxidative DNA damage levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.4A) and a healthy 45 year old donor (4.4B) supplemented from the midpoint of their *in vitro* lifespan. The bars indicate the mean \pm S.D.

Supplementation from the midpoint of their *in vitro* lifespan with 30 μ M ebselen (Figures 4.3A & 4.3B) or 5mM NAC (Figures 4.4A & 4.4B) resulted in no reductions in the levels of oxidative damage to DNA at the any of the time points examined in the T cell clones derived from a young donor (Figures 4.3A & 4.4A) as well as clones derived from a middle aged donor (Figures 4.3B & 4.4B). The levels of oxidative damage to DNA were marginally higher on supplementation with 30 μ M ebselen (Figures 4.3A & 4.3B) or 5mM NAC (Figures 4.4A & 4.4B), compared to non-supplemented clones. The levels of oxidative damage to DNA on supplementation with 30 μ M ebselen (Figure 4.3A) were significantly higher than the non-supplemented clones at the fourth time point (Student's t-test, 95% confidence levels). In all the above cases, levels of oxidative DNA damage increased with increase of time in culture in both supplemented and non-supplemented clones. The non-supplemented clones underwent more PDs compared to supplemented clones after 14 weeks in culture as revealed in figures 4.3A, 4.3B, 4.4A & 4.4B. There were no significant differences in levels of oxidative damage to DNA in T cell clones derived from a young donor when compared to those measured in T cells derived from a middle aged donor.

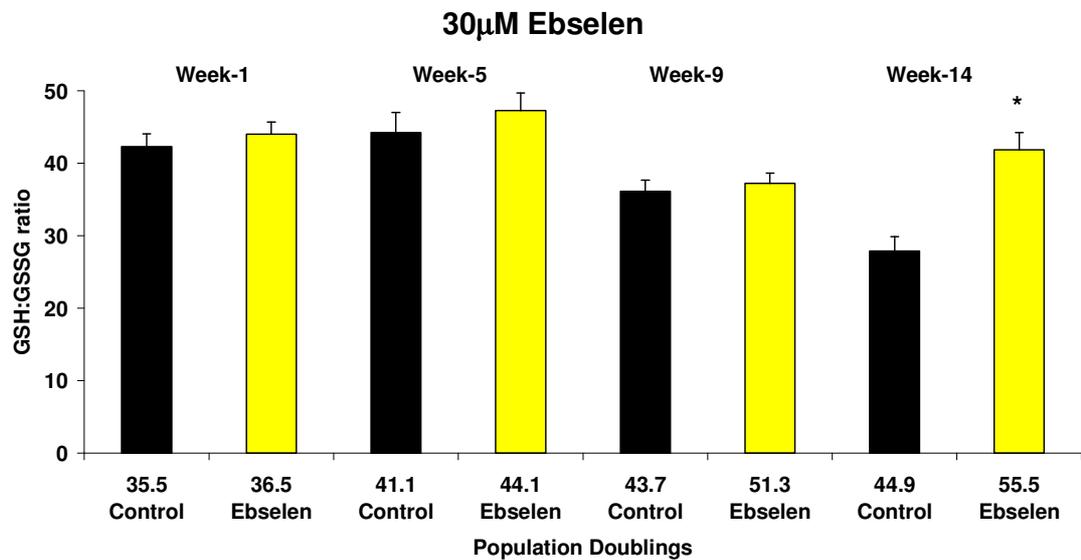
4.1.3.3. The impact of ebselen or NAC on intracellular redox status (GSH:GSSG ratio) and total glutathione levels in human peripheral blood derived CD4⁺ T cell clones *in vitro*

T cell clone samples were taken from the culture at various time points of their lifespans and the effect of different concentrations of ebselen or NAC on intracellular redox status (GSH:GSSG ratio) and total glutathione levels within the T cells were determined. Figure 4.5 shows the results of the effect of 30 μ M ebselen on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (Figure 4.5A) and a healthy 45 year old donor (Figure 4.5B) supplemented from a young *in vitro* age, compared to non-supplemented clones. Figure 4.6 shows the results of the effect of 7.5mM NAC on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (Figure 4.6A) and a healthy 45 year old donor (Figure 4.6B) supplemented from a young *in vitro* age, compared to non-supplemented clones. Figure 4.7 shows the results of the effect of 30 μ M ebselen on GSH:GSSG ratio at various time points during the lifespan of human

peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (Figure 4.7A) and a healthy 45 year old donor (Figure 4.7B) supplemented from the midpoint of their *in vitro* lifespan, compared to non-supplemented clones. Figure 4.8 shows the results of the effect of 5mM NAC on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (Figure 4.8A) and a healthy 45 year old donor (Figure 4.8B) supplemented from the midpoint of their *in vitro* lifespan, compared to non-supplemented clones. Figure 4.9 shows the results of the effect of 30μM ebselen (Figure 4.9A) or 7.5mM NAC (Figure 4.9B) on total glutathione levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor supplemented from a young *in vitro* age, compared to non-supplemented clones.

4.5A

Clone x 400-23 - 26 year old donor



4.5B

Clone x 385-7 - 45 year old donor

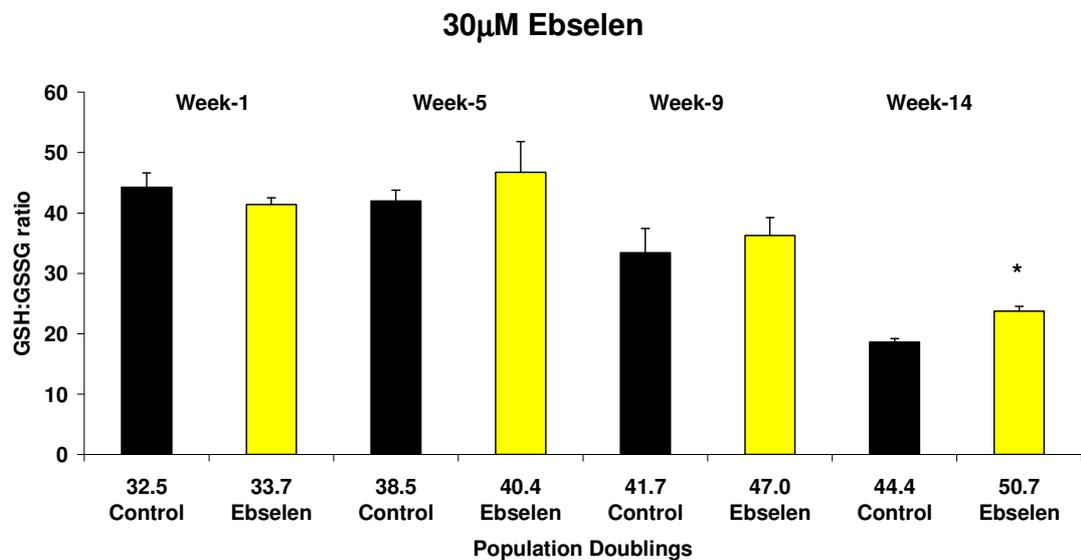


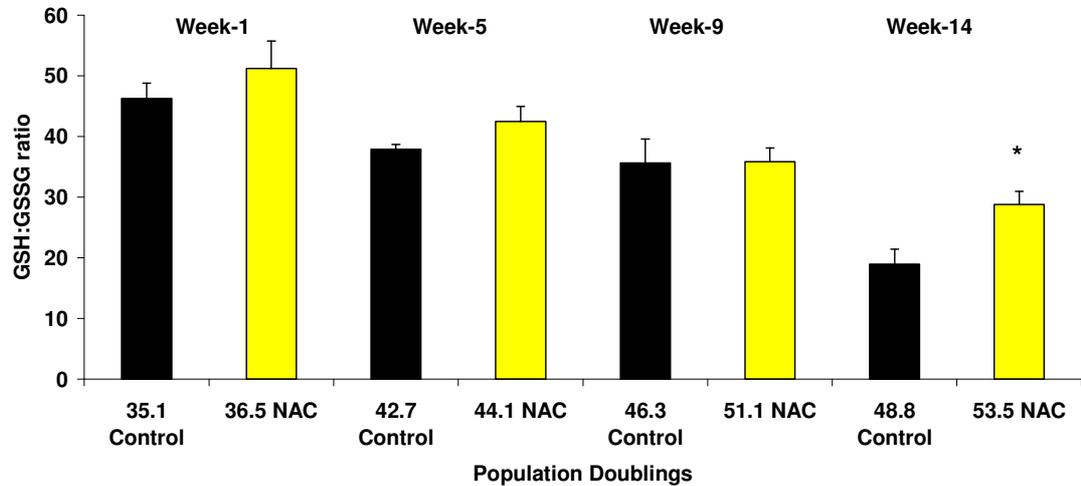
Figure 4.5 The impact of 30 μ M ebselen on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.5A) and a healthy 45 year old donor (4.5B) supplemented from a young *in vitro* age. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

* Significantly higher than the ratio of non-supplemented clones, $p < 0.05$

4.6A

Clone x 400-23 - 26 year old donor

7.5mM NAC



4.6B

Clone x 385-7 - 45 year old donor

7.5mM NAC

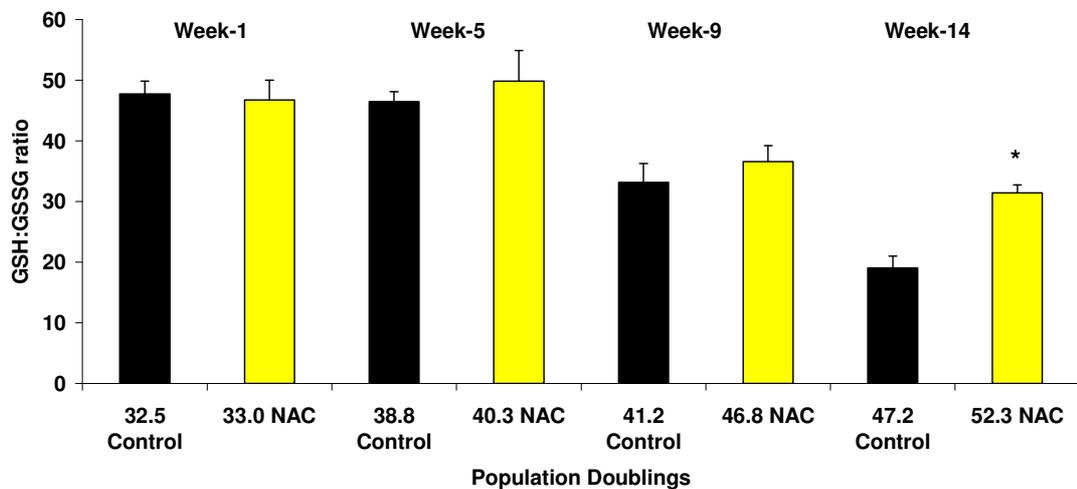


Figure 4.6 The impact of 7.5mM NAC on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.6A) and a healthy 45 year old donor (4.6B) supplemented from a young *in vitro* age. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

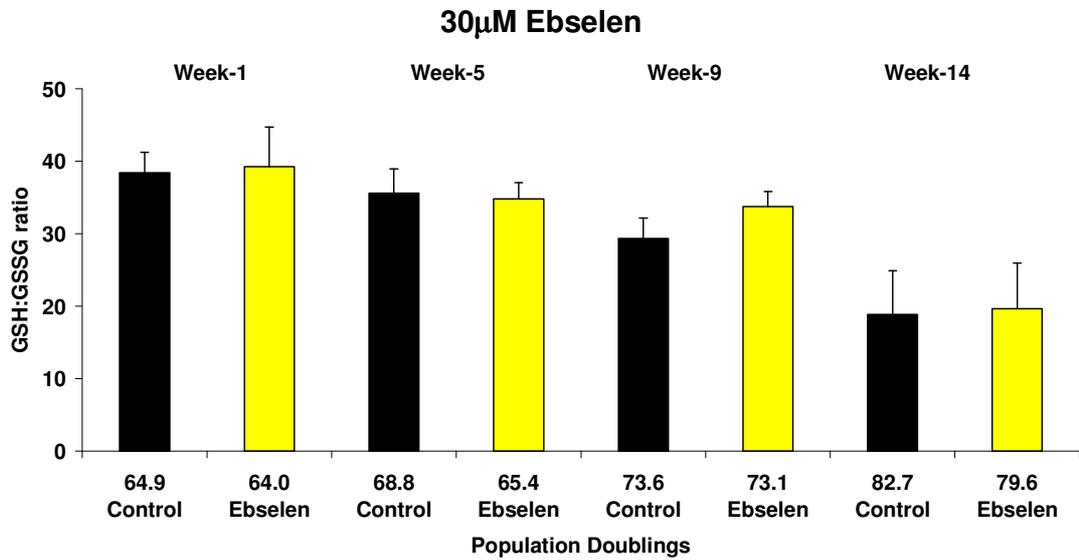
* Significantly higher than the ratio of non-supplemented clones, $p < 0.05$

The supplementation from a young *in vitro* age with 30 μ M ebselen (Figures 4.5A & 4.5B) or 7.5mM NAC (Figures 4.6A & 4.6B) resulted in reduction of radical levels evidenced by a significantly higher GSH:GSSG ratio that reached statistical significance at the fourth time point (after 14 weeks of supplementation) (Student's t-test, 95% confidence levels) in T cell clones derived from a young donor (Figures 4.5A & 4.6A) as well as in the clones derived from a middle aged donor (Figures 4.5B & 4.6B), compared to non-supplemented clones. The changes in GSH:GSSG ratio of 30 μ M ebselen or 7.5mM NAC supplemented clones at one, five and nine weeks in culture were marginal, compared to non-supplemented clones (Figures 4.5A, 4.5B, 4.6A & 4.6B).

Investigation of the impact of other doses of ebselen or NAC on GSH:GSSG ratio in T cell clones revealed the following. 10 μ M ebselen did not significantly alter the GSH:GSSG ratio of CD4⁺ T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor compared to non supplemented clones. Following NAC supplementation, there were no significant differences in the GSH:GSSG ratio in CD4⁺ T cell clones derived from a healthy 26 year old donor compared to a T cell clone derived from a healthy 45 year old donor on supplementation with 1.25mM NAC or 5mM NAC. There were no significant differences in the GSH:GSSG ratio in T cell clones derived from a young donor compared to the ratio in T cells derived from a middle aged donor.

4.7A

Clone x 400-23 - 26 year old donor



4.7B

Clone x 385-7 - 45 year old donor

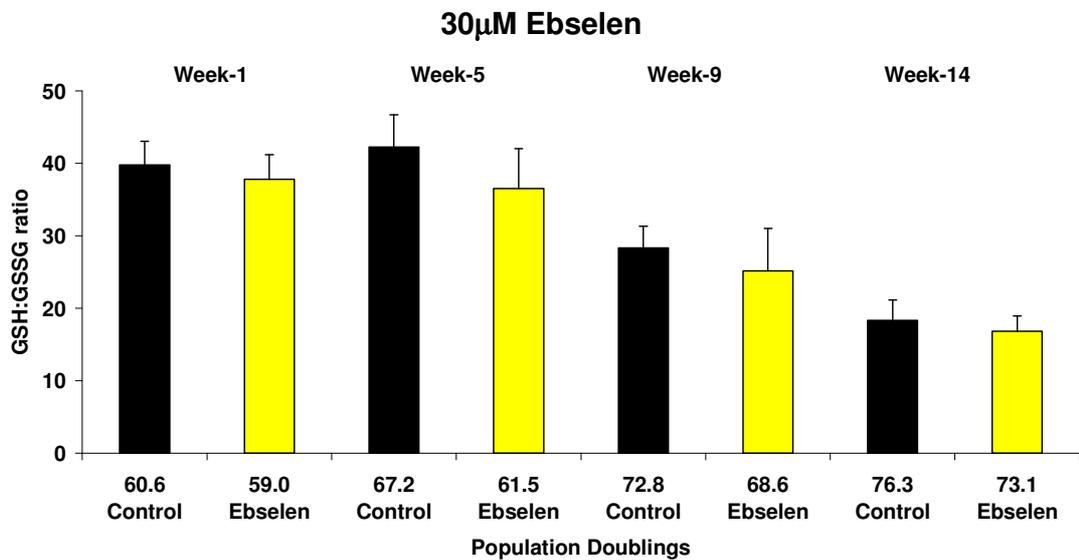
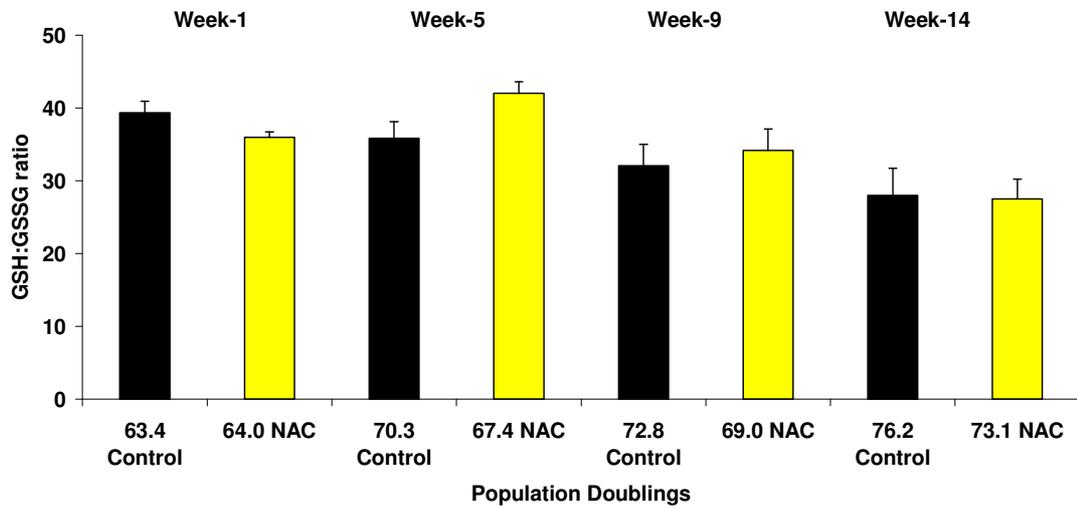


Figure 4.7 The impact of 30 μ M ebselen on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.7A) and a healthy 45 year old donor (4.7B) supplemented from the midpoint of their *in vitro* lifespan. The bars indicate the mean \pm S.D.

4.8A

Clone x 400-23 - 26 year old donor

5mM NAC



4.8B

Clone x 385-7 - 45 year old donor

5mM NAC

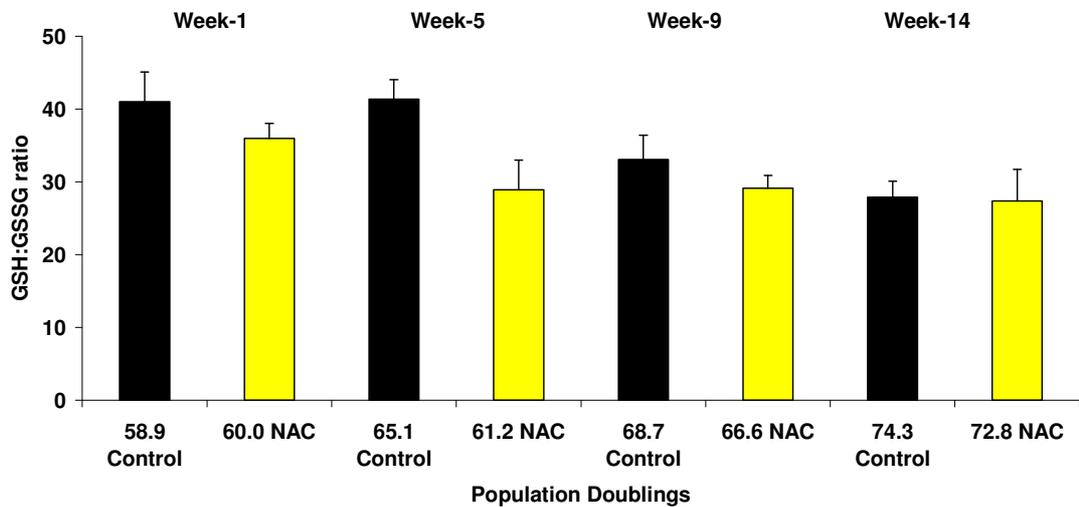
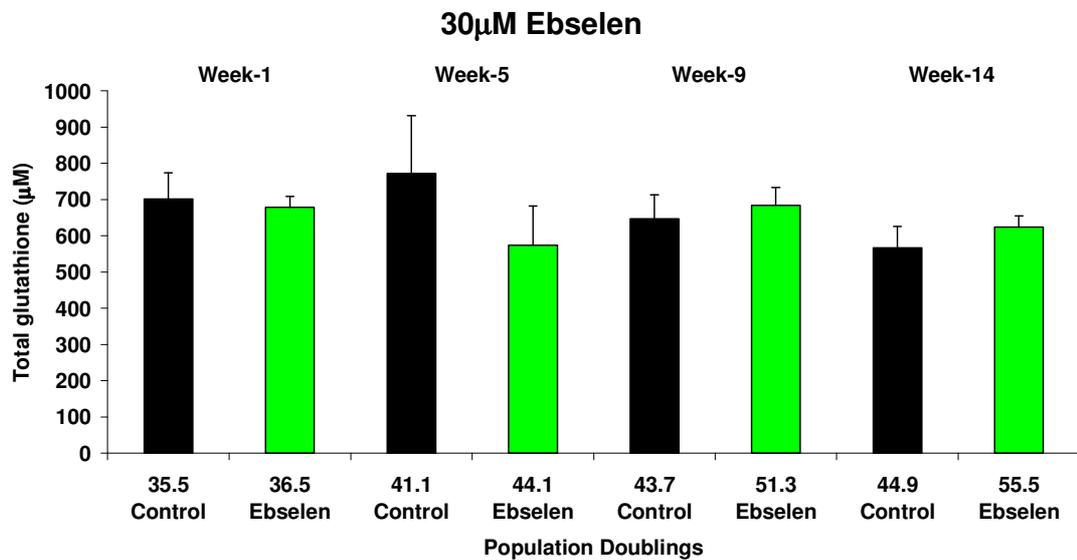


Figure 4.8 The impact of 5mM NAC on GSH:GSSG ratio at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor (4.8A) and a healthy 45 year old donor (4.8B) supplemented from the midpoint of their *in vitro* lifespan. The bars indicate the mean ± S.D.

The supplementation of CD4⁺ T cell clones from the midpoint of their *in vitro* lifespan with 30 μ M ebselen (Figures 4.7A & 4.7B) or 5mM NAC (Figures 4.8A & 4.8B) did not result in any significant changes in GSH:GSSG ratio, compared to non-supplemented clones at any of the four time points investigated. There were no significant differences in GSH:GSSG ratio in T cell clones derived from a young donor on comparison to the T cell clones derived from a middle aged donor.

4.9A

Clone x 400-23 - 26 year old donor



4.9B

Clone x 400-23 - 26 year old donor

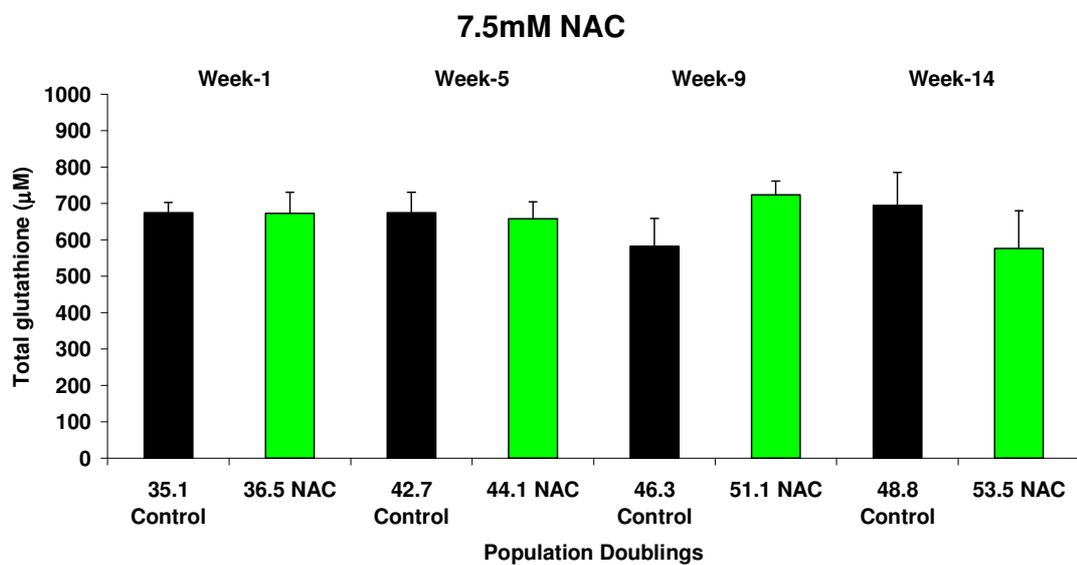


Figure 4.9 The impact of 30 μ M ebselen (4.9A) or 7.5mM NAC (4.9B) on total glutathione levels at various time points during the lifespan of human peripheral blood derived CD4⁺ T cell clones derived from a healthy 26 year old donor supplemented from a young *in vitro* age. The bars indicate the mean \pm S.D.

The supplementation from a young *in vitro* age with 30 μ M ebselen (Figure 4.9A) or 7.5mM NAC (Figure 4.9B) resulted in no significant changes in total glutathione levels at any of the four time points in CD4⁺ T cell clones derived from a healthy 26 year old donor (Figures 4.9A & 4.9B) and a healthy 45 year old donor compared to non-supplemented clones. Furthermore, supplementation with other concentrations of ebselen (10 μ M) or NAC (1.25mM, 5mM) also resulted in no significant impact on total glutathione levels in human CD4⁺ T cell clones derived from both donors compared to the non-supplemented clones.

Studies investigating the supplementation of CD4⁺ T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor with 30 μ M ebselen or 5mM NAC from the midpoint of their *in vitro* lifespan did not result in any significant changes in total glutathione levels, compared to non-supplemented clones. Similar set of results were derived for human CD4⁺ T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor supplemented with other concentrations of ebselen (5 μ M, 15 μ M) or NAC (0.312mM, 1.25mM). The results did not show any significant changes in total glutathione levels, when supplemented from the mid point of their *in vitro* lifespan. There were no significant differences in total glutathione levels in T cell clones derived from a young donor compared to the ratio in T cell clones derived from a middle aged donor.

4.1.4. Discussion

Recent studies have suggested that ebselen, an organoselenium compound, has the ability to minimise the deleterious effects of ROS (Muller *et al.*, 1984; Wendel *et al.*, 1984) by reducing H₂O₂ in human plasma (Griffiths *et al.*, 1992) and HepG₂ cells (Yang *et al.*, 1999). The catalytically active selenium in ebselen has the ability to reduce H₂O₂ to H₂O and O₂ (Coffey *et al.*, 1995; Sarma and Mugesh, 2005). The presence of the selenol group has been mainly attributed for the antioxidant properties in ebselen. Its protective effects against lipid peroxidation have also been demonstrated *in vitro* and *in vivo* (Ozaki *et al.*, 1997). Earlier studies have revealed the ability of ebselen to scavenge hydroxyl radicals and ROS which are primarily responsible for lipid peroxidation ultimately resulting in DNA damage. This scavenging ability of ebselen results in

blockage of lipid peroxidation and decrease in levels of oxidative damage to DNA (Yang *et al.*, 1999).

NAC has also been shown to protect against oxidative damage with ROS in mice (Aruoma *et al.*, 1989). NAC has also been shown to decrease H₂O₂ induced damage in calf thymus DNA in a concentration dependent manner (Izzotti *et al.*, 1997). The presence of sulfhydryl group and cysteine amino acid residue contributes to the antioxidant potential of NAC. The free sulfhydryl group enables scavenging of ROS and NO thereby blocking lipid peroxidation by peroxynitrate (Han *et al.*, 1997). NAC has the ability to donate cysteine amino acid thereby raising GSH levels ultimately resulting in glutathione synthesis (Han *et al.*, 1997; Wagner *et al.*, 1998; Su *et al.*, 2006). NAC also acts as a stimulator of the cytosolic enzymes involved in glutathione regeneration (Banaclocha, 2001). It was of interest in this current investigation to analyse whether ebselen or NAC supplementation would exhibit antioxidant potential in a monoclonal T cell population.

The use of human peripheral blood derived *in vitro* T cell clones enables the study of age associated changes over their lifespan (Pawelec *et al.*, 2002) and the identification of factors contributing to T cell senescence. In this investigation, supplementation of CD4⁺ T cell clones derived from a healthy 26 year old donor (400-23) and a healthy 45 year old donor (385-7) with 30µM ebselen (Tables 4.1A & 4.1B) or 7.5mM NAC (Tables 4.2A & 4.2B) from a young *in vitro* (34.5 – 31.0 Initial PD) age significantly extended their lifespan (cumulative PD at the end of their lifespan in culture). T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor supplemented with 30µM ebselen survived for an additional three and two weeks respectively before apoptosis, compared to non-supplemented clones. 7.5mM NAC supplementation enabled the T cell clones from a healthy 26 year old donor and a healthy 45 year old donor to extend their lifespan evidenced by their additional 2 weeks in culture before apoptosis, compared to the non-supplemented clones.

Further, to investigate the extent of the antioxidant / free radical scavenging properties of ebselen or NAC, modified alkaline comet assays were used to determine the levels of oxidative DNA damage in CD4⁺ T cell clones derived from a healthy 26 year old donor

(400-23) and a healthy 45 year old donor (385-7). 30 μ M ebselen (Figures 4.1A & 4.1B) or 7.5mM NAC (Figures 4.2A & 4.2B) significantly decreased the levels of oxidative damage to DNA after 14 weeks of supplementation in both CD4⁺ T cell clones when supplemented from their young *in vitro* age, compared to non-supplemented clones. The impact of supplementation on intracellular redox status (GSH:GSSG ratio) and total glutathione levels was also determined. Intracellular redox status is an important mechanism having an invaluable role as a mediator in apoptosis in many cell systems (Slater *et al.*, 1996; Cotgreave and Gerdes, 1998; Hall, 1999). Recent findings reveal that intracellular reduced glutathione (GSH), a main determinant of intracellular redox status, is depleted before the onset of apoptosis (Beaver and Waring, 1995; Macho *et al.*, 1997; Ghibelli *et al.*, 1998). The GSH:GSSG redox couple maintains the redox environment of the cell and GSH is abundant in the cell (Schafer and Buettner, 2001) serving as an indicator of the cellular redox environment. Redox status of the cell is hindered on oxidation of even a small amount of GSH. This oxidation results in the formation of GSSG and mixed disulfides between protein sulfhydryl groups in biological systems. It will also decrease the levels of GSH resulting in an increase in levels of GSSG and so lowering the GSH:GSSG ratio, which has been suggested to be responsible for several human diseases (Bonnefont Rousselot *et al.*, 2000; Kharb, 2000; Lang *et al.*, 2000; Mills *et al.*, 2000; Pastore *et al.*, 2001). 30 μ M ebselen (Figures 4.5A & 4.5B) or 7.5mM NAC (Figures 4.6A & 4.6B) reduced radical levels evidenced by significantly higher GSH:GSSG ratio after 14 weeks of supplementation in CD4⁺ T cell clones derived from a healthy 26 year old donor as well as a healthy 45 year old donor when supplemented from their young *in vitro* age, compared to non-supplemented clones. The reduction of radical levels evidenced by significantly higher GSH:GSSG ratio on supplementation with 30 μ M ebselen or 7.5mM NAC were in parallel to the significant decreases in oxidative DNA damage after 14 weeks of supplementation in CD4⁺ T cell clones from donors of both age groups, compared to the non-supplemented clones proving that the increased antioxidant status of the supplemented clones resulted in a significant reduction in the accumulation of oxidative DNA damage. The antioxidant potential of ebselen evident by their effect on markers of T cell integrity and function investigated above is a sign of fewer radicals present in culture or the radical scavenging capacity of ebselen. In terms of NAC, cysteine amino acid present is a derivative of GSH formation explaining the higher GSH:GSSG ratio on

supplementation. The significantly higher GSH:GSSG ratio on supplementation with ebselen or NAC, represents an increased antioxidant state. The increase in antioxidant status further explains the decrease in levels of oxidative DNA damage that in turn explains a lower apoptotic state explained by the extended lifespan of the clones on antioxidant supplementation. In terms of total glutathione levels, neither of the antioxidants (Figures 4.9A & 4.9B) significantly altered the total glutathione levels in either of the CD4⁺ T cell clones. This result reveals that none of the antioxidant supplements in any way had an impact on glutathione pathways, and therefore failed to initiate a significant change in glutathione levels, in the T cell clones examined.

There were no significant changes in proliferative capacity and lifespan of T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor on supplementation from the midpoint of their *in vitro* age (58.7 & 63.4 Initial PD) with any of the concentrations of ebselen (Tables 4.3A & 4.3B) or NAC (Tables 4.4A & 4.4B). In terms of the levels of oxidative damage to DNA, antioxidant supplementation did not significantly alter the levels of DNA damage in clones (Figures 4.3B, 4.4A & 4.4C), compared to non-supplemented clones at any of the four time points. An exception being for 30µM ebselen (Figure 4.3A) supplemented clones derived from a healthy 26 year old donor where there were significantly higher levels of oxidative damage to DNA, compared to controls after 14 weeks of supplementation from the midpoint of their *in vitro* lifespan. The results of the analysis of GSH:GSSG ratios revealed that 30µM ebselen (Figures 4.7A & 4.7B) supplementation did not significantly alter the GSH:GSSG ratio at any of the time points investigated in CD4⁺ T cell clones derived from a healthy young donor and a healthy middle aged donor when supplemented from the midpoint of their *in vitro* lifespan. In terms of NAC, at concentrations of 5mM (Figures 4.8A & 4.8B), the GSH:GSSG ratio in CD4⁺ T cell clones derived from a healthy young donor and a healthy middle aged donor when supplemented from the midpoint of their *in vitro* lifespan were similar to the non-supplemented clones.

The results of proliferation capacity, lifespan and oxidative damage to DNA on supplementation with either of the antioxidants from the midpoint of their *in vitro* lifespan were in line with the previous findings from the group of Barnett that revealed no significant changes in longevity in CD4⁺ T cell clones on long term culture with

20mM Carnosine from the midpoint of their *in vitro* lifespan (Hyland *et al.*, 2000). The suggested reason for the failure of carnosine to reveal its antioxidant potential may have been the high background of biomolecule damage that already existed in these T cells accumulated during earlier stages of their lifespan under conditions of 20% O₂ that may have decreased the antioxidant or free radical scavenging potential of carnosine. There may be more radicals in older T cell clones that may affect the function of defense systems in the body. So the antioxidants at specific concentrations, able to reveal an antioxidant potential in T cell clones supplemented from a young *in vitro* age may not be enough to reveal an antioxidant potential when supplemented from the midpoint of their lifespan. This could be the reason for the failure of ebselen or NAC at specific concentrations to reveal an antioxidant potential in aged T cell clones. It would also be worth noting the decrease in other markers of T cell integrity with age revealed in the existing scientific literature for example: DNA repair capacity and HSPs. The DNA repair capacity in T cells *in vitro* has been shown to decline with age, revealed in previous studies by the group (Annett *et al.*, 2004; 2005; Chapter-1, Section 1.1.5.5.1 & 1.1.5.5.2). Furthermore, HSPs activity has been demonstrated to decline in response to age making the body more susceptible to stress induced damage (Favatier *et al.*, 1997; Chapter-1, Section 1.1.5.2).

Analysis of the percentage or levels of oxidative DNA damage in clones supplemented from the midpoint of their *in vitro* lifespan independent of the donor age compared to the clones supplemented from a young *in vitro* age were not significantly different. In terms of GSH:GSSG ratio and total glutathione levels, there were only marginal differences in their levels between the clones supplemented from a young *in vitro* age, compared to clones supplemented from the midpoint of their *in vitro* lifespan.

Previous findings from the group of Barnett have demonstrated an age related increase in levels of oxidised purine and pyrimidine bases within T cells (Barnett *et al.*, 1999) which is in line with our findings. The introduction of new culture conditions by the supplementation of antioxidants from the midpoint of the *in vitro* lifespan of the clones may have altered their redox status resulting in the failure of the antioxidants to reveal their antioxidant potential. Long term supplementation from a young *in vitro* age of CD4⁺ T cell clones derived from a healthy young donor or a healthy middle aged donor with 30µM ebselen or 7.5mM NAC at 20% O₂ tension used in this investigation

revealed mild antioxidant / free radical scavenging potential that may be more significant under physiological O₂ tension.

Interestingly, the impact of long term supplementation of ebselen or NAC has not yet been revealed in other cell lines. The previous studies investigating the impact of ebselen in HepG₂ (Yang *et al.*, 2000), HL-60 (Ji *et al.*, 2000) and glioblastoma cell lines (Sharma *et al.*, 2008) or the impact of NAC in HepG₂ (Yang *et al.*, 2000) and HeLa (Hansen *et al.*, 2004) cell lines to name a few, revealed their potential impact upto 24 hours after the antioxidant supplementation. In this investigation, T cell clones were supplemented with ebselen or NAC on day one and day four of every seven day cycle till the end of their lifespan. The viability of the clones when investigated on day four using trypan blue exclusion test revealed no significant difference in the number of live cells compared to the live cell number at the start of the cycle.

Furthermore, on investigation of the effect of higher concentrations of ebselen (60-100µM) or NAC (10mM) on human peripheral blood derived CD4⁺ T cell clones from a healthy young or middle aged donor revealed complete inhibition of the growth of T cell clones. It may be that higher concentrations of ebselen or NAC may have completely scavenged intracellular oxygen free radicals contributing to the inhibition of the growth of the CD4⁺ T cell clones. Although ROS are generally thought as harmful molecules, they play an important role in T cell signalling events such as protein tyrosine phosphorylation and activation of JNK (Pani *et al.*, 2000). One of the prominent families of protein kinases is the MAP kinases. ERK, JNK (SAPK) and P38 kinase are some of the important pathways in MAP kinases that have been identified. These pathways are involved in proliferation, differentiation and apoptosis in cells. Previous work from the group of Barnett suggested an increase in JNK phosphorylation and P38 phosphorylation at 6% O₂ tension (Duggan *et al.*, 2004). It would be interesting to examine the impact of ebselen or NAC on these pathways. Previous studies have demonstrated a significant increase in intracellular GSH depletion with an increase in ebselen concentration in HepG₂ cells (Yang *et al.*, 2000). Ebselen has the ability to bind with GSH to form ebselen selenyl sulphide (Haenen *et al.*, 1990). Ebselen selenyl sulphide reacts with excess of GSH to form ebselen selenol intermediate that in turn reacts with peroxides (Morgenstern *et al.*, 1992). Ebselen selenol intermediate can further react with ebselen selenyl sulphide to form ebselen diselenide. Ebselen

diselenide can further react with peroxides resulting in intracellular GSH depletion that leads to induction of apoptosis (Yang *et al.*, 2000) or cells succumbing to stress (Maher, 2005). This may also be the reason for the complete inhibition of growth of T cell clones on supplementation with higher concentrations of ebselen. In terms of NAC, supplementation with high concentrations of 10mM, may have completely scavenged the ROS which are important in less critical levels, for cell proliferation, differentiation and apoptosis.

In summary, the overall data of the current study revealed that for an optimal set of conditions, 30 μ M ebselen or 7.5mM NAC supplementation among a range of concentrations of ebselen or NAC investigated showed antioxidant potential, evidenced by the significant increase in lifespan; significantly higher GSH:GSSG ratio and a significant decrease in levels of oxidative damage to DNA in CD4⁺ T cell clones, when supplemented from a young *in vitro* age.

CHAPTER-5

Impact of ebselen or NAC in human peripheral blood mononuclear cells

5.1. Impact of ebselen or NAC in human peripheral blood mononuclear cells

5.1.1. Introduction

The general decline in T cell function with age has been associated with the susceptibility of the human body to a wide range of infection and diseases (Remarque and Pawelec, 1998). The ability of very small clonal populations of naïve / memory T cells to multiply rapidly on contact with the antigen for which they carry unique receptors determines the immune response. The ability of T cells to clonally expand may be threatened by several factors, including DNA damage (Barnett and Barnett, 1998).

The levels of DNA damage and mutation have been shown to increase with age in humans (Vijg, 1995). Previous work from the group of Barnett has established an age-related increase of DNA damage and mutation in human lymphocytes (King *et al.*, 1994; Barnett and King, 1995; King *et al.*, 1997; Hyland *et al.*, 2000). ROS, intrinsically and extrinsically derived, have been implicated as a major source of DNA damage *in vivo* (Ames *et al.*, 1993). As part of the normal immune system, T cells are additionally exposed to high concentrations of ROS and reactive nitrogen intermediates produced from activated neutrophils and macrophages. An ineffective *in vivo* defense system (*in vivo* antioxidants and DNA repair pathways) could result in DNA damage. Previous work from Barnett *et al.* has demonstrated an age-related decline in DNA repair capacity against DNA damage induced by ROS in lymphocytes derived from healthy male subjects between the age group of 35-69 years (Barnett and King, 1995; King *et al.*, 1997). The increase in levels of genetic damage over a period of time may result in cell cycle arrest and/or T cell apoptosis (Barnett and Barnett, 1998), thus rendering the immune response sub-optimal.

In this chapter the impact of long term exposure of ebselen (30µM) or NAC (5mM / 7.5mM) on certain markers of T cell integrity and function in a polyclonal population of human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* was examined.

Ebselen is a synthetic, seleno-organic compound scavenging ROS and having a glutathione peroxidase like activity. 30µM ebselen has been demonstrated to significantly decrease levels of oxidative damage to DNA and increase the proliferative capacity, lifespan and GSH:GSSG ratio in CD4⁺ T cell clones *in vitro*, when supplemented from a young *in vitro* age, as previously described in chapter-4. NAC is an important antioxidant that can act as a precursor for the synthesis of glutathione. It has also been demonstrated to stimulate cytosolic enzymes that enable glutathione regeneration in rats (Sleven *et al.*, 2006). NAC contains an acetylated form of the aminoacid L-cysteine, a pre-cursor of glutathione synthesis (Pivetta *et al.*, 2005; Su *et al.*, 2006). The presence of sulfhydryl groups also serves to support its antioxidant potential. 7.5mM NAC demonstrated antioxidant potential in CD4⁺ T cell clones *in vitro*, when supplemented from a young *in vitro* age evidenced by the significantly decreased levels of oxidative damage to DNA, significantly increased lifespan and significantly higher GSH:GSSG ratio, compared to controls. In the previous study carried out in CD4⁺ T cell clones *in vitro*, 5mM NAC supplementation marginally increased the lifespan of the clones when supplemented from a young *in vitro* age. Furthermore, among the range of doses of NAC investigated in clones supplemented from the midpoint of their *in vitro* lifespan (Chapter-4), dosage of 5mM NAC had similar effects to controls, compared to other doses, on markers of T cell integrity and function investigated (Chapter-4).

5.1.2. Materials and Methods

5.1.2.1. Subject selection

Ethical approval was granted by NTU based on the consent obtained from all study subjects prior to this study. The human blood samples for the present study were drawn from ten healthy, non-smoking males fasted overnight, five each in the age group of 25-30 years and 55-60 years. The subjects were not on any form of medication or had not suffered any recent illness. Other lifestyle factors such as alcohol consumption and exercise regime were also taken into consideration. The results of the previous studies in human peripheral blood mononuclear cells carried out by the group of Barnett were taken into consideration before choosing the two age groups of donors in this study (Barnett and King, 1995; Chapter-2, Section 2.1.3.1).

5.1.2.2. Sample collection

Human blood samples were drawn in the morning between 8.00 and 9.00 am by a licensed individual and collected in a tube containing heparin to prevent coagulation. The samples were kept on ice and taken to the laboratory within 5 minutes for separation of peripheral blood mononuclear cells.

5.1.2.3. Preparation, maintenance of culture and storage of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from fresh heparinised venous blood by density gradient centrifugation on Ficoll-Isopaque (PAA Laboratories) in LeucoSep (Lymphocyte separation tubes). The peripheral blood mononuclear cells from the different donors were washed twice in phosphate buffered saline or X-Vivo 10 (BioWhittaker) media and then maintained in culture separately in a serum-free medium, X-Vivo 10, in 24 well plates (5 wells, 2 ml medium per well) at concentrations of $2-4 \times 10^5$ cells per well, along with 2×10^5 gamma-irradiated (80Gy) RJK853 cells per well (EBV-transformed B-lymphoblastoid cell line with complete hprt deletion, a gift from Dr. Jane Cole, MRC Cell Mutation Unit, University of Sussex, UK) as feeder cells. Peripheral blood mononuclear cells were maintained in a 7-day cycle at 37°C under conditions of 5% CO₂ and 95% air atmosphere. They were supplemented with 400U/ml recombinant IL-2 (Chiron, UK) on days 1 and 4 of the cycle. On day 7, a viable cell count was performed on harvested cells using a Neubauer Counting Chamber, and a new culture cycle was set up with fresh medium and RJK853 feeder cells. The samples required for further analysis were then cryopreserved in a medium made up of 10% DMSO (Sigma), 20% FBS (Invitrogen) and 70% X-Vivo 10 and stored in liquid nitrogen for further analysis at the end of a 7 day growth cycle (Warnock *et al.*, 1999; Barnett & Barnett, 2000; Hyland *et al.*, 2000, 2001).

5.1.2.4. Isolation of CD4⁺ T cells from peripheral blood mononuclear cells and maintenance in culture

CD4⁺ T cells *ex vivo* were isolated separately from ten healthy, non-smoking males fasted overnight, five each in the age group of 25-30 years and 55-60 years, using the CD4⁺ T cell isolation kit II (Miltenyi Biotec) (Chapter-2, Section 2.1.5.2). The CD4⁺ T

cells *ex vivo* were then washed separately in phosphate buffered saline or X-Vivo 10 media and maintained in culture separately as detailed above (Section 5.1.2.3).

5.1.2.5. Ebselen or NAC supplementation

The effect of a range of concentrations of ebselen or NAC on CD4⁺ T cell clones was taken into consideration before choosing the doses of ebselen or NAC. 30µM ebselen or 7.5mM NAC revealed significant antioxidant potential in human peripheral blood derived CD4⁺ T cell clones supplemented from a young *in vitro* age (Chapter-4). Preliminary studies in human peripheral blood derived CD4⁺ T cell clones supplemented from the midpoint of their *in vitro* lifespan prior to the studies on supplementation of clones from a young *in vitro* age, revealed that T cell clones supplemented with 5mM NAC had a longer lifespan compared to clones supplemented with other concentrations of NAC (Chapter-4). These results from the previous studies lead to the investigation of the effect of 5mM / 7.5mM NAC in human peripheral blood mononuclear cells, which would enable the differentiation of antioxidant potential or pro-oxidant potential of NAC at different concentrations to be determined in this study. NAC at higher concentrations (10mM) completely inhibited the growth of T cell clones in the previous study (Chapter-4, Section 4.1.4). The peripheral blood mononuclear cells or CD4⁺ T cells from ten healthy, non-smoking males fasted overnight, five each in the age group of 25-30 years and 55-60 years were supplemented separately with 30µM ebselen (Sigma) or 5mM / 7.5mM NAC (Sigma) in X-Vivo 10 media. The different *ex vivo* T cell samples (peripheral blood mononuclear cells / CD4⁺ T cells) were supplemented with ebselen or NAC on days one and four of the growth cycle. The supplementation of the antioxidants in this investigation was performed in the same way to the previous studies carried out by the group of Barnett (Hyland *et al.*, 2000) and chapter-4 (Section 4.1.2.4). Existing scientific literature was consulted before the investigation. Previous studies have demonstrated the ability of ebselen (dose ranges detailed in chapter-4, Section 4.1.2.4) on acute exposure (up to 24 hours after supplementation) to protect HepG₂ cells (Yang *et al.*, 1999), human HL-60 (Li *et al.*, 2000) and PC-12 cells (Yoshizumi *et al.*, 2002) from H₂O₂ induced cell death and DNA damage. NAC has also been proven to act as an efficient antioxidant on acute exposure in HeLa cells (Hansen *et al.*, 2004) and HepG₂ cells (Yang *et al.*, 1999) (Chapter-4, Section 4.1.2.4). In this PhD, the effect of chronic exposure (beyond 24 hours of supplementation) to ebselen or NAC in human T cells *ex vivo* has been investigated.

5.1.2.6. Proliferation assays for peripheral blood mononuclear cells *ex vivo* / CD4⁺ T cells

Human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo* were seeded separately at 1×10^6 cells per well in a 24 well plate. Tritiated thymidine at a final concentration of 0.037MBq/ml was added to each of the *ex vivo* T cell samples 18 hours prior to harvesting. Cells were transferred onto a 96 well plate the next day and were harvested using a 96-well harvester onto a 96-well filter plate and 40 μ l of scintillation fluid was added to each of the filter wells. Filters were counted for 1 minute per well with a Top-Count scintillation counter. Proliferation assays (Chapter-2, Section 2.1.9) were performed in duplicate for both human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*. Control (untreated) human peripheral blood mononuclear cells were grown in parallel to the ebselen or NAC supplemented peripheral blood mononuclear cells and proliferation capacity was determined in both supplemented and non-supplemented cells.

5.1.2.7. Assessment of levels of oxidative damage to DNA

The levels of oxidative DNA damage within human peripheral blood mononuclear cells or CD4⁺ T cells *ex vivo* were determined separately using the modified alkaline comet assay (Collins *et al.*, 1993; Chapter-2, Section 2.1.10). The modified alkaline comet assay involves the pre-treatment of cells with two enzymes namely FPG (New England Biolabs) and Endo III (New England Biolabs) which recognise oxidatively modified purines or oxidatively modified pyrimidines, respectively. The enzymes nick DNA at the site of oxidatively modified nucleotides, creating single stranded breaks which can be detected by the comet assay. Comet analysis was performed on the cells counting 50 cells per slide using Komet 5.5 computer image analysis software (Andor Technology, UK). The comet assays were performed in duplicates for both human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* derived.

5.1.2.8. Quantitative determination of intracellular GSH:GSSG and total glutathione levels

A GSH:GSSG ratio assay kit (Calbiochem, UK) (Chapter-2, Section 2.1.11) was used to assess the ratio of reduced to oxidised form of glutathione and total glutathione levels in peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo*. This technique incorporates

a thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethane-sulfonate (M2VP) at a level that rapidly scavenges GSH, but does not interfere with the glutathione reductase in recycling GSSG into GSH. The whole cell extract was prepared from harvested cells (2×10^6 cells/ml for GSH and 5×10^6 cells/ml for GSSG) according to the manufacturers instructions. The GSH:GSSG ratio and the total glutathione levels were calculated from the GSH and GSSG concentrations of the peripheral blood mononuclear cells photometrically measured at 412 nm in μM concentrations. The assays were performed in duplicate.

5.1.2.9. Statistical analysis

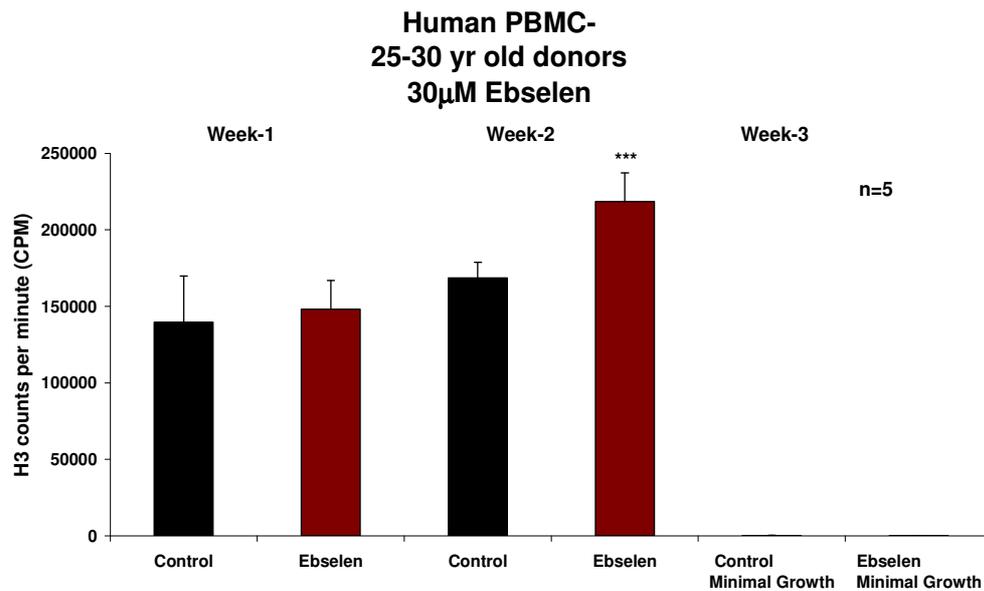
The results were tested for statistical significance using paired two-sample Student's t-tests assuming equal variances; p values are presented as appropriate.

5.1.3. Results

5.1.3.1. The impact of ebselen or NAC on proliferation capacity in human peripheral blood mononuclear cells and CD4^+ T cells *ex vivo*

The effect of $30\mu\text{M}$ ebselen (Figures 5.1A & 5.1B) or 7.5mM NAC (Figures 5.2A & 5.2B) on the proliferation capacity of ten different human peripheral blood mononuclear cells, isolated from fresh human blood, was investigated. Figures 5.3A & 5.3B and figures 5.4A & 5.4B denote the effect of $30\mu\text{M}$ ebselen or 7.5mM NAC on the proliferation capacity in human CD4^+ T cells isolated from fresh human blood.

5.1A



5.1B

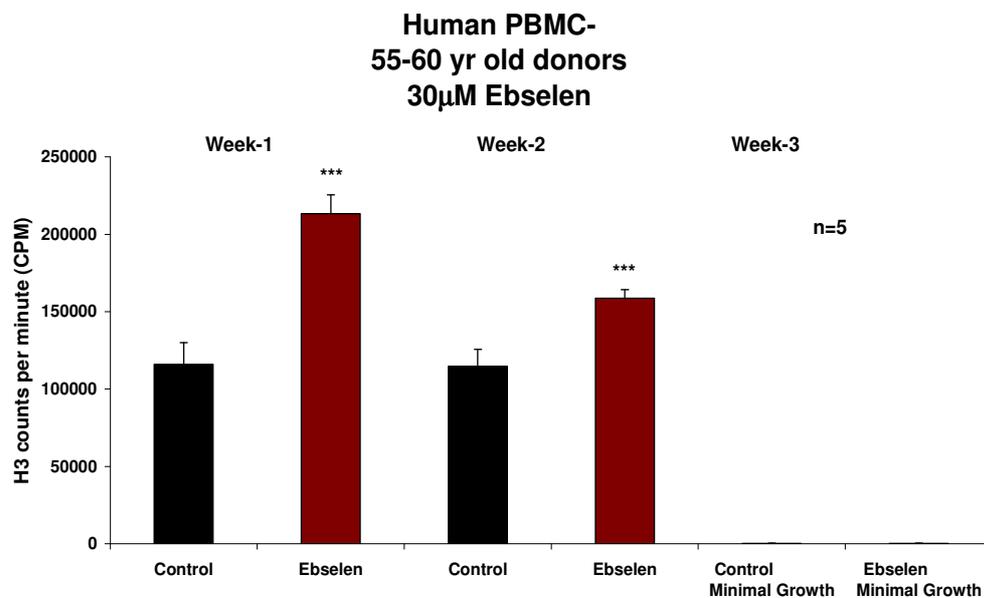
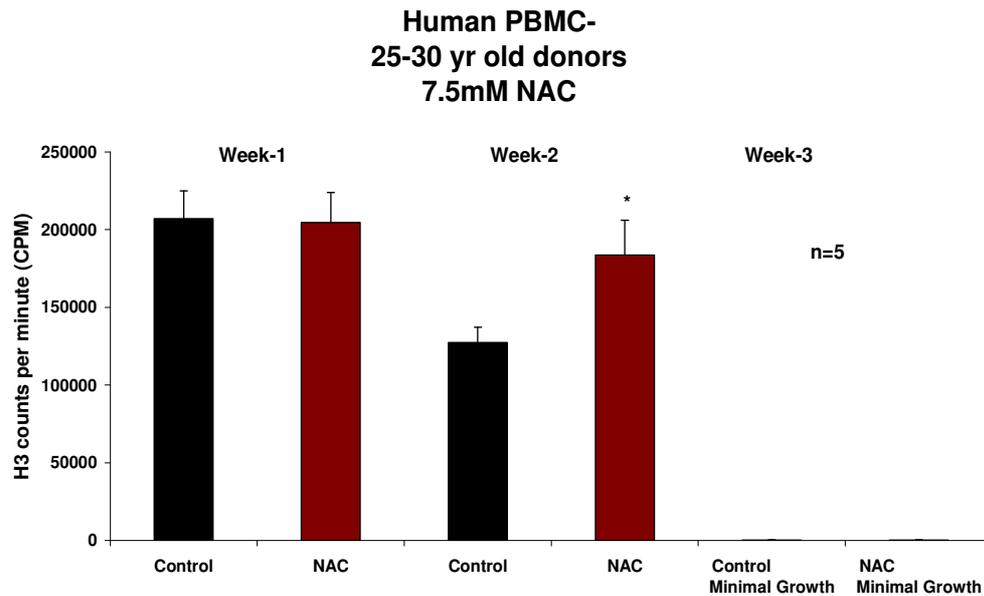


Figure 5.1 The effect of 30 μ M ebselen on the proliferation capacity of human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.1A) and 55-60 years (5.1B) supplemented *ex vivo* for 3 weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

******* Significantly higher proliferation capacity compared to controls, $p < 0.001$

5.2A



5.2B

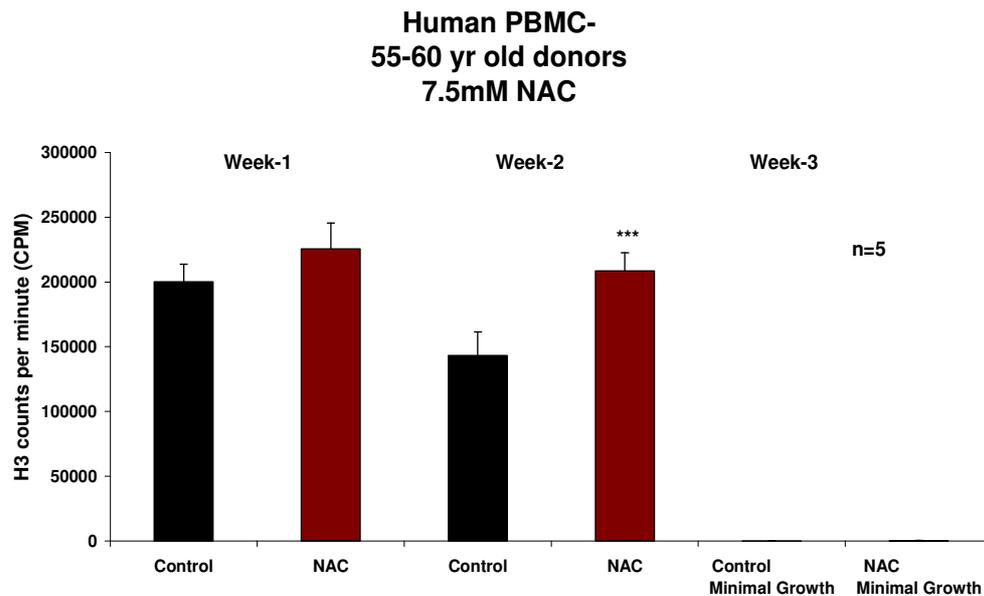
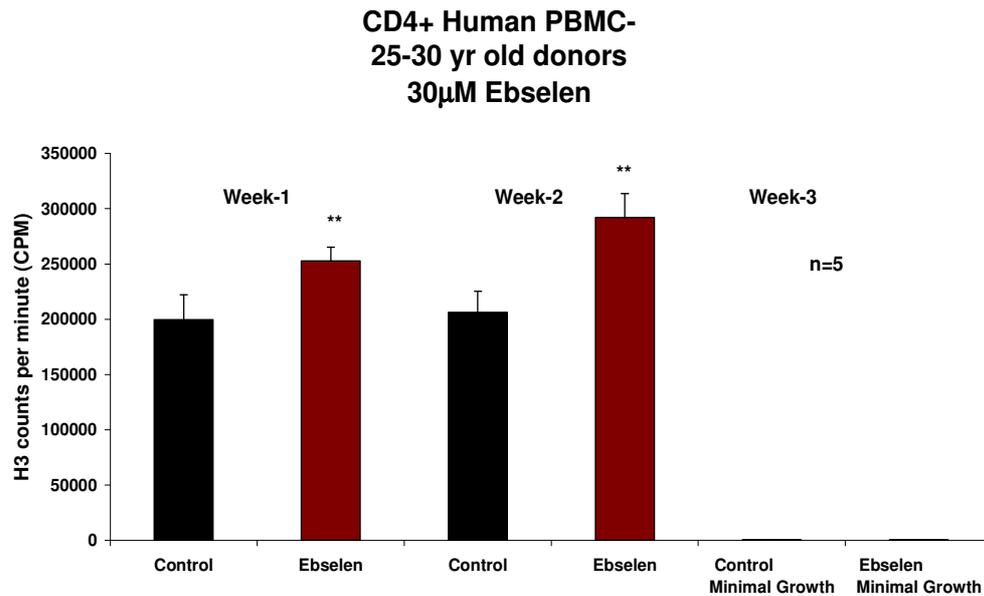


Figure 5.2 The effect of 7.5mM NAC on the proliferation capacity of human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.2A) and 55-60 years (5.2B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

* $p < 0.05$, *** $p < 0.001$ - Significantly higher proliferation capacity compared to controls

Figure 5.1A shows a significant increase (Student's t-test, 95% confidence levels) in the proliferation capacity of human peripheral blood mononuclear cells derived from donors from the 25-30 years age group after the second week of supplementation *ex vivo* with 30 μ M ebselen, compared to non-supplemented peripheral blood mononuclear cells. Figure 5.1B denotes a significant increase of proliferation capacity in human peripheral blood mononuclear cells derived from donors from the 55-60 years age group from one week of supplementation *ex vivo* with 30 μ M ebselen, compared to non-supplemented peripheral blood mononuclear cells. In the case of 7.5mM supplementation, the increase in proliferation capacity reached statistical significance after 2 weeks of supplementation in samples from both age groups (Figures 5.2A & 5.2B). The results on supplementation of *ex vivo* T cells with 5mM NAC were not significantly different to the results on supplementation with 7.5mM NAC in human peripheral blood mononuclear cells derived from donors of both age groups. The number of live cells was too low after three weeks of supplementation to carry out any analysis (Warnock *et al.*, 1999). Furthermore, the rate of proliferation capacity observed among human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.1A, 5.1B, 5.2A & 5.2B).

5.3A



5.3B

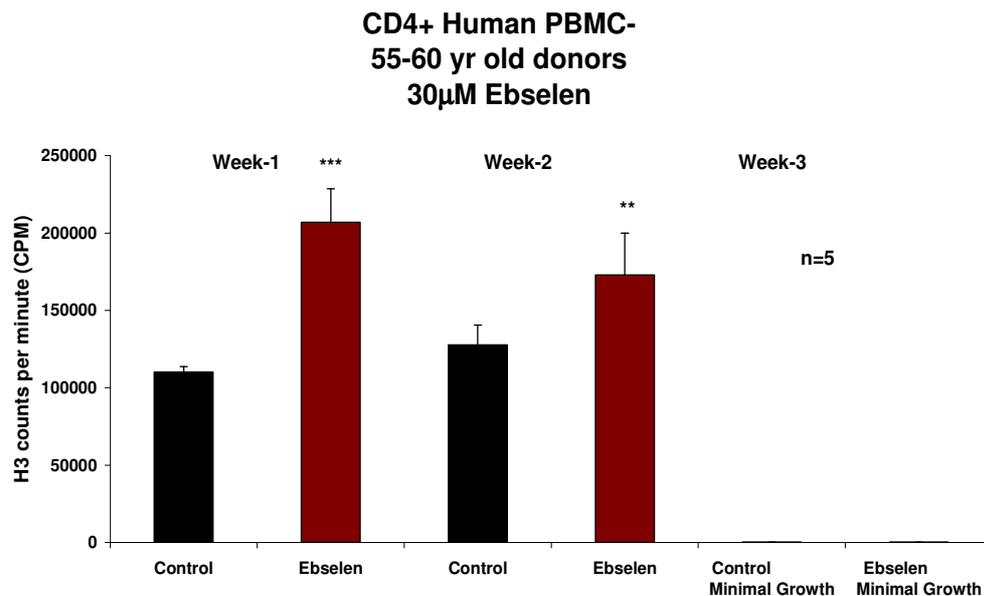
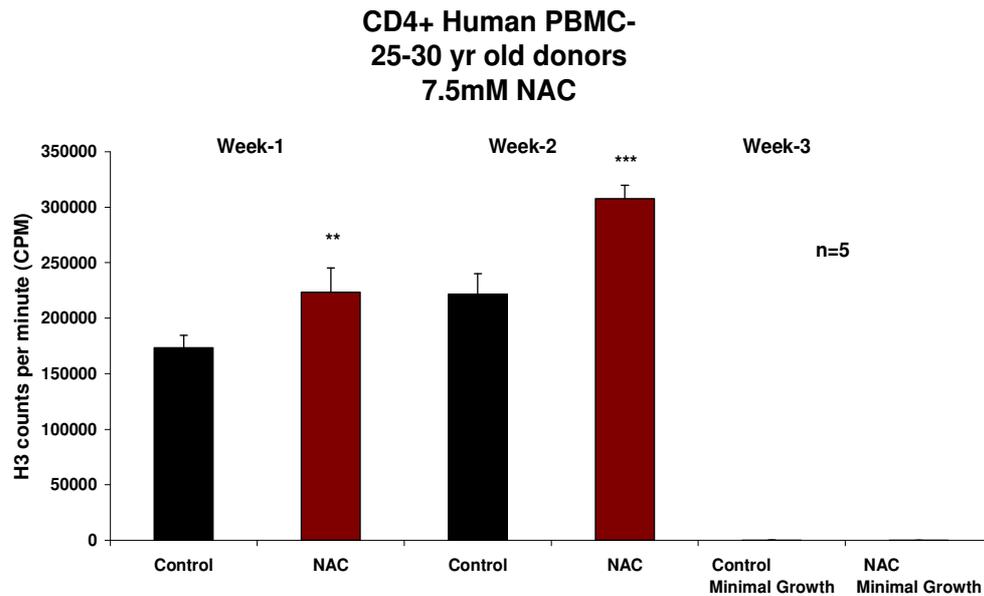


Figure 5.3 The effect of 30 μ M ebselen on the proliferation capacity of CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.3A) and 55-60 years (5.3B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

**** p < 0.01, *** p < 0.001 - Significantly higher proliferation capacity compared to controls**

5.4A



5.4B

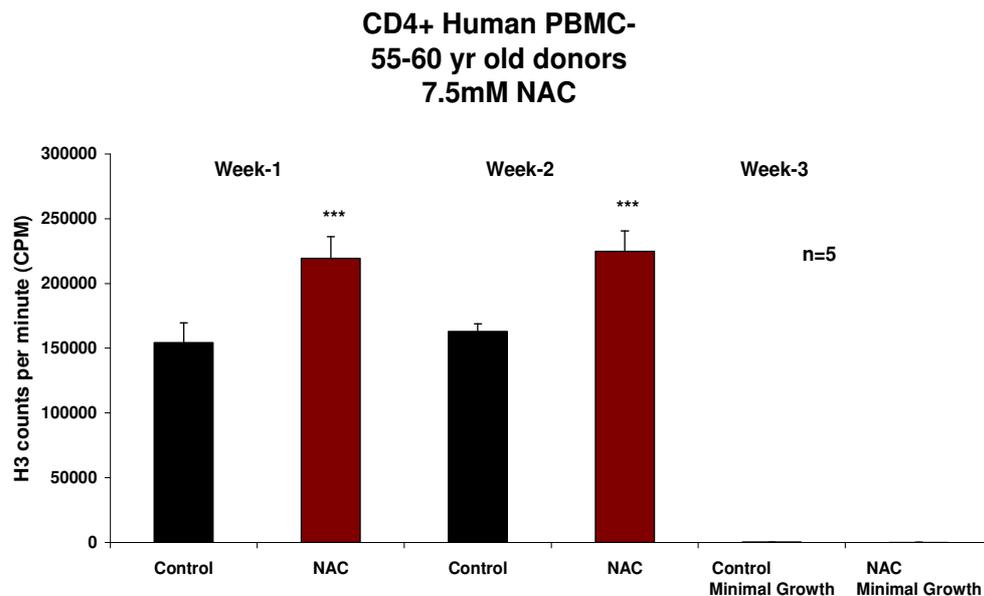


Figure 5.4 The effect of 7.5mM NAC on the proliferation capacity of CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.4A) and 55-60 years (5.4B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

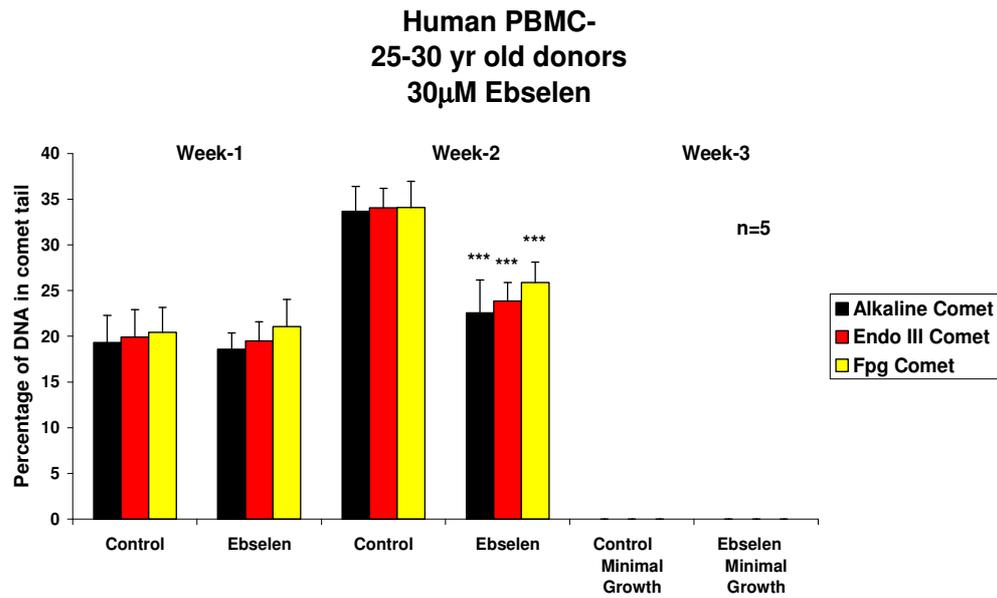
**** p < 0.01, *** p < 0.001 - Significantly higher proliferation capacity compared to controls**

Figures 5.3A and 5.3B show a significant increase (Student's t-test, 95% confidence levels) in proliferative capacity of CD4⁺ T cells derived from donors from the two age groups studied after one and two weeks of supplementation *ex vivo* with 30μM ebselen, compared to non-supplemented CD4⁺ T cells. Figures 5.4A and 5.4B show a significant increase in proliferative capacity of CD4⁺ T cells derived from donors from the two age groups studied from one week of supplementation *ex vivo* with 7.5mM NAC, compared to non-supplemented CD4⁺ T cells. In terms of supplementation with 5mM NAC, an increase in proliferation capacity was observed which reached statistical significance from one week of supplementation in CD4⁺ T cells compared to non-supplemented CD4⁺ T cells *ex vivo* in donors from both age groups. The antioxidant potential of 5mM NAC from one week of supplementation was not significantly different from the antioxidant potential of 7.5mM NAC after one or two weeks of supplementation of human CD4⁺ T cells from donors of both age groups. The number of live cells was too low after three weeks of supplementation to carry out any analysis. Furthermore, the rate of proliferation capacity observed among CD4⁺ T cells *ex vivo* derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.3A, 5.3B, 5.4A & 5.4B).

5.1.3.2. The impact of ebselen or NAC on levels of oxidative DNA damage in human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo*

Ex vivo T cell samples were taken from culture at various time points and the effect of 30μM ebselen or 7.5mM NAC on the levels of oxidative DNA damage was determined. In all cases, levels of oxidative damage to DNA increased as a function of age, as measured by the modified Endo III and FPG comet assays. Figures 5.5 and 5.6 show the results of the effects of 30μM ebselen or 7.5mM NAC on levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from donors from the 25-30 years age group (Figures 5.5A & 5.6A) and 55-60 years age group (Figures 5.5B & 5.6B). Figures 5.7 and 5.8 illustrate the results of the effect of 30μM ebselen or 5mM NAC on oxidative DNA damage in CD4⁺ T cells isolated from fresh human blood derived from donors from 25-30 years (Figures 5.7A & 5.8A) age group and 55-60 years age group (Figures 5.7B & 5.8B).

5.5A



5.5B

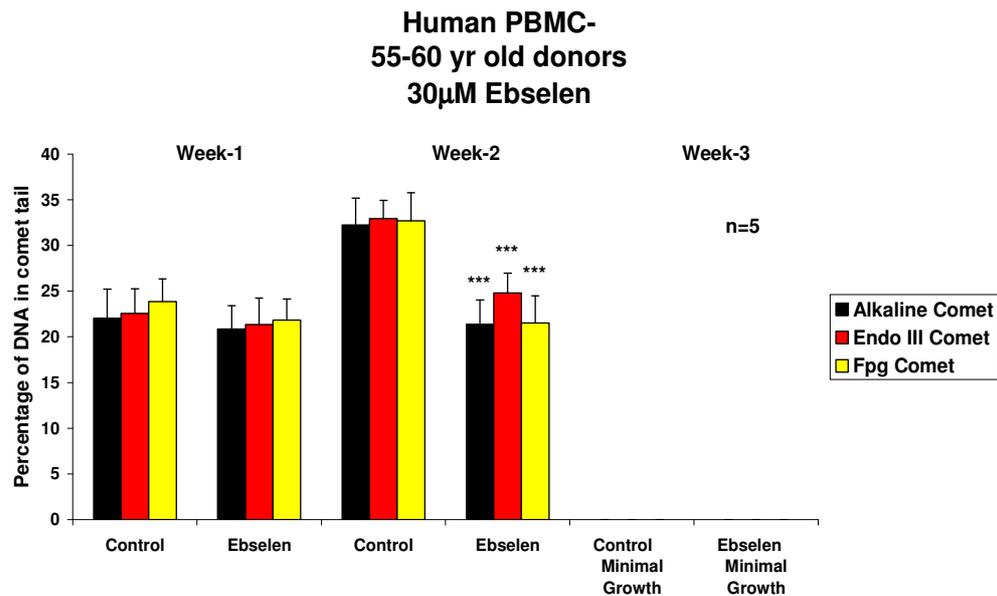
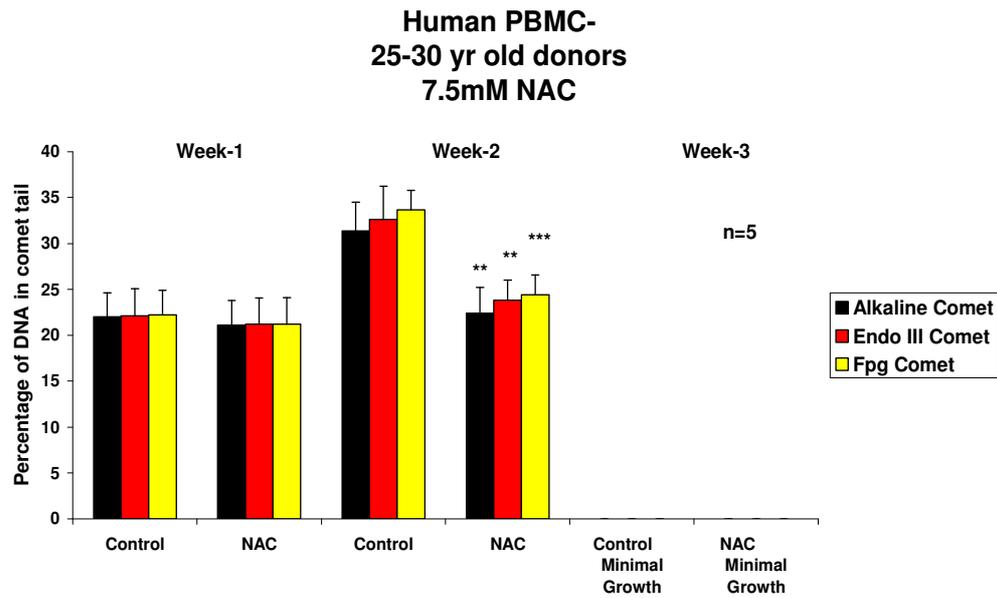


Figure 5.5 The effect of 30 μ M ebselen on levels of oxidative damage to DNA in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.5A) and 55-60 years (5.5B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

*** Significantly lower than control levels, $p < 0.001$

5.6A



5.6B

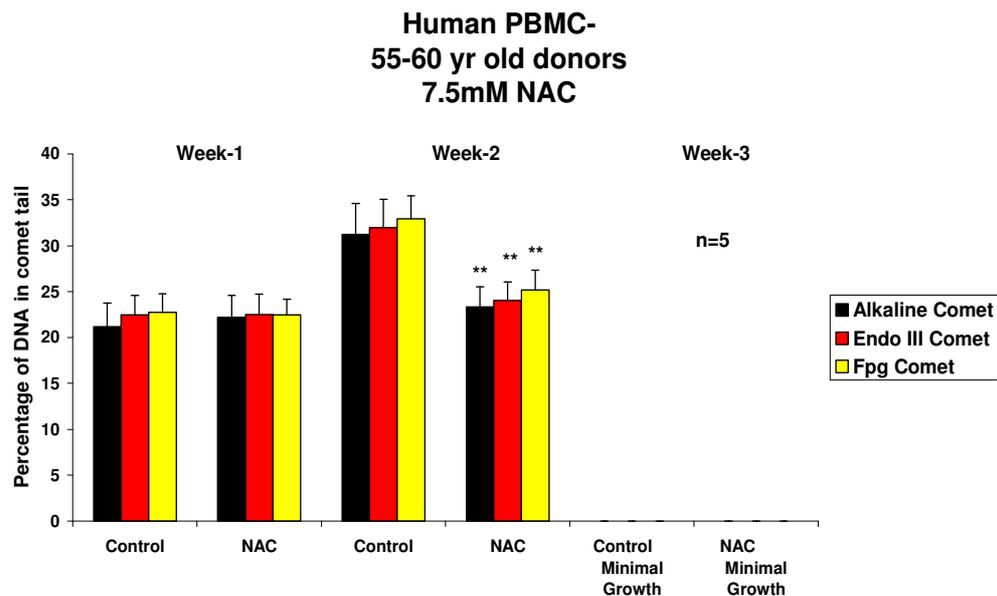
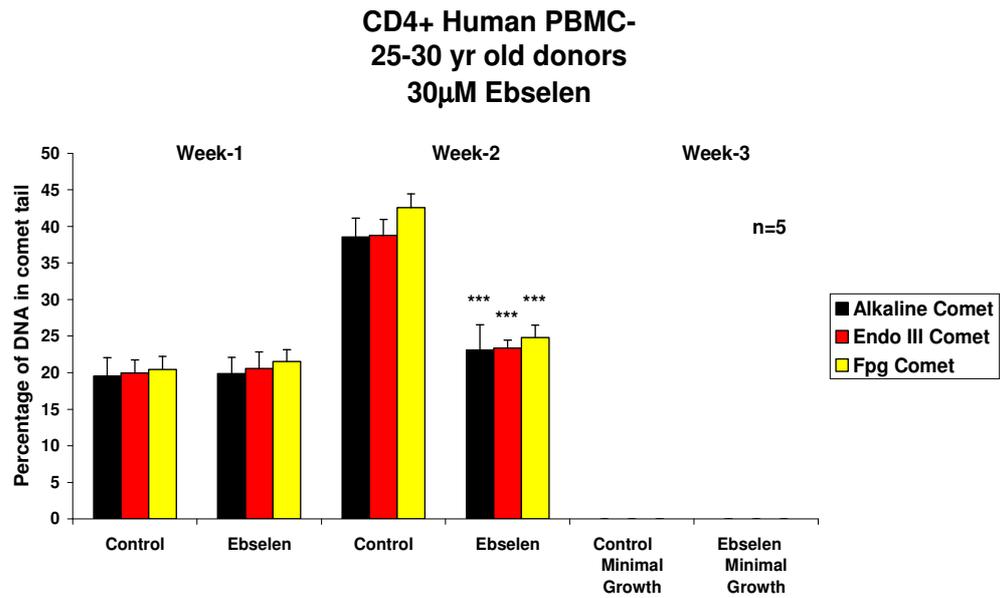


Figure 5.6 The effect of 7.5mM NAC on levels of oxidative damage to DNA in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.6A) and 55-60 years (5.6B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

** $p < 0.01$, *** $p < 0.001$ - Significantly lower than control levels

Figures 5.5A and 5.5B show a significant decrease (Student's t-test, 95% confidence levels) in levels of oxidative damage to DNA in human peripheral blood mononuclear cells derived from donors from both age groups (25-30 years; 5.5A and 55-60 years; 5.5B) after two weeks of supplementation *ex vivo* with 30 μ M ebselen, compared to non-supplemented cells. 7.5mM NAC supplementation led to a significant decrease in levels of oxidative damage to DNA in human peripheral blood mononuclear cells derived from donors from both age groups (25-30 years; 5.6A and 55-60 years; 5.6B) after two weeks of supplementation *ex vivo*, compared to non-supplemented cells. The supplementation of human peripheral blood mononuclear cells with 5mM NAC resulted in a significant decrease in levels of oxidative DNA damage after two weeks of supplementation, compared to non-supplemented peripheral blood mononuclear cells from donors of both age groups. The levels of oxidative DNA damage in 5mM supplemented human peripheral blood mononuclear cells were not significantly different from the levels of oxidative DNA damage in *ex vivo* T cells from either age group supplemented with 7.5mM NAC. All four figures (5.5A, 5.5B, 5.6A & 5.6B) show an increase in levels of oxidative DNA damage with age (in line with the findings in chapter-4 [Section 4.1.3.2] with T cell clones). There were no significant differences in the levels of oxidative damage to DNA in donors from the 25-30 years age group compared to donors from the 55-60 years age group. The number of live cells was too low after three weeks of supplementation to carry out any analysis. Furthermore, the levels of oxidative DNA damage observed among human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.5A, 5.5B, 5.6A & 5.6B).

5.7A



5.7B

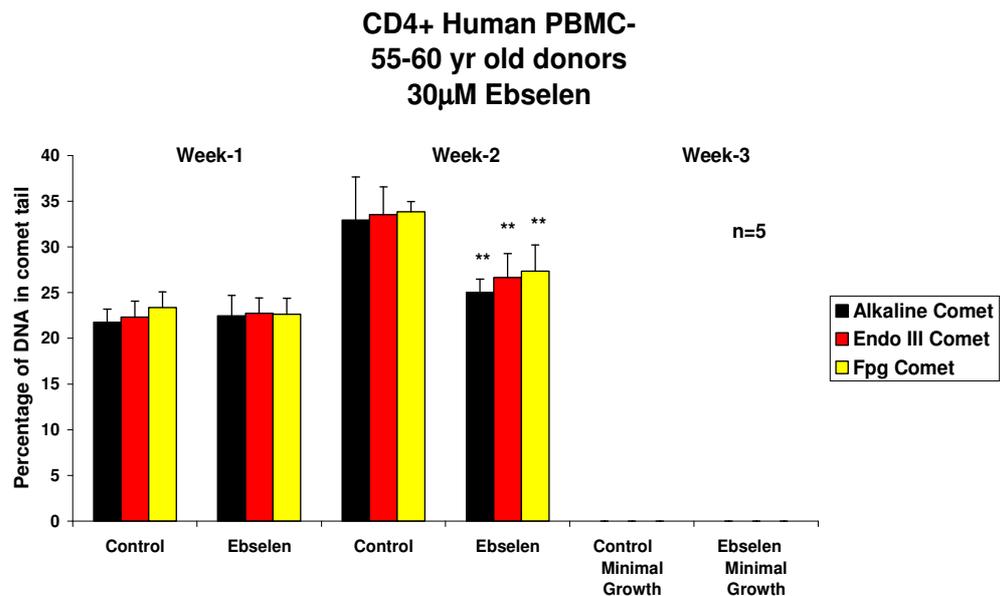
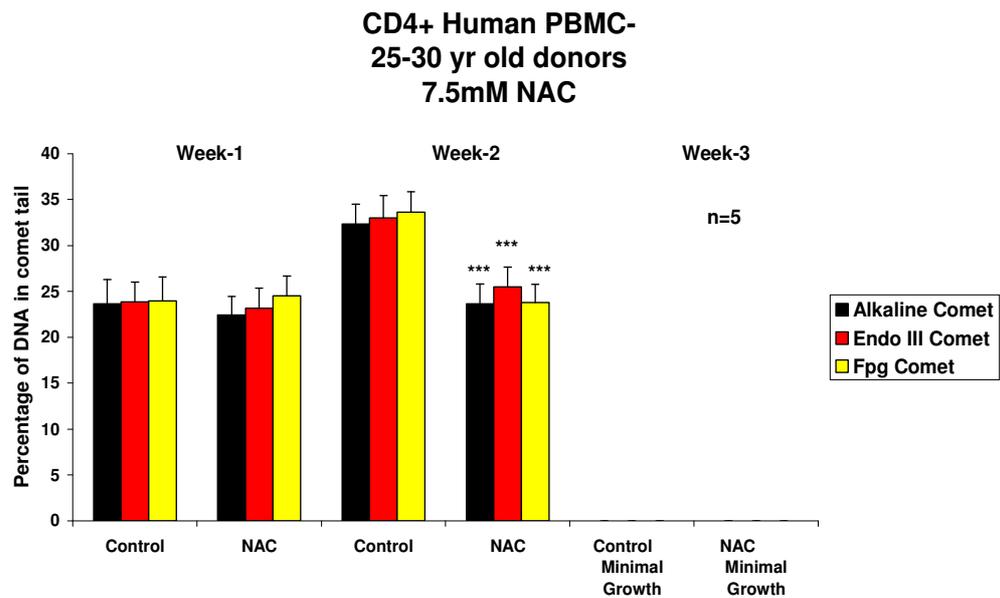


Figure 5.7 The effect of 30µM ebselen on levels of oxidative damage to DNA in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.7A) and 55-60 years (5.7B) supplemented *ex vivo* for three weeks. The bars indicate the mean ± S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

** p<0.01, *** p < 0.001- Significantly lower than control levels

5.8A



5.8B

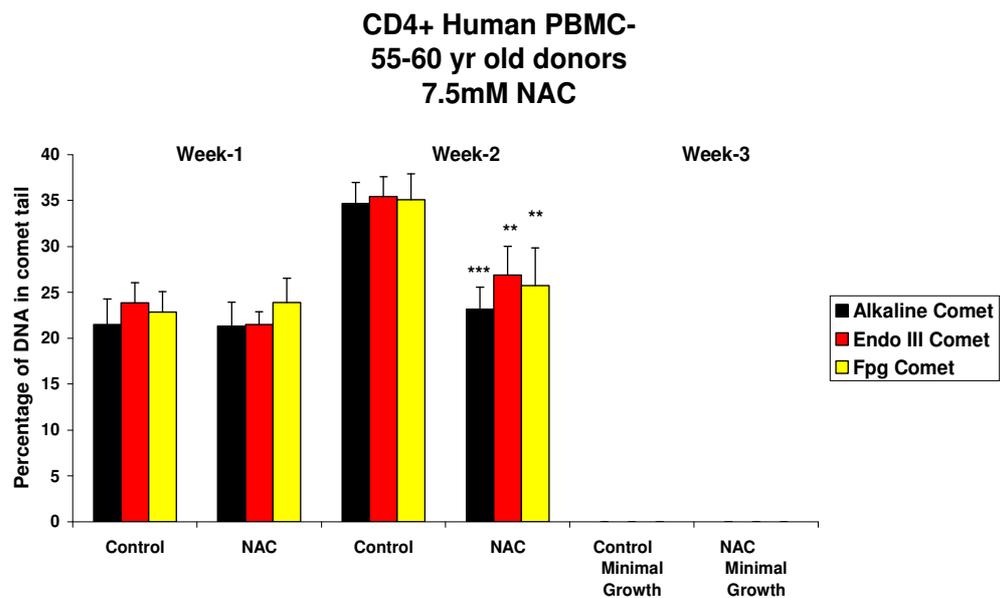


Figure 5.8 The effect of 7.5mM NAC on levels of oxidative damage to DNA in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.8A) and 55-60 years (5.8B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

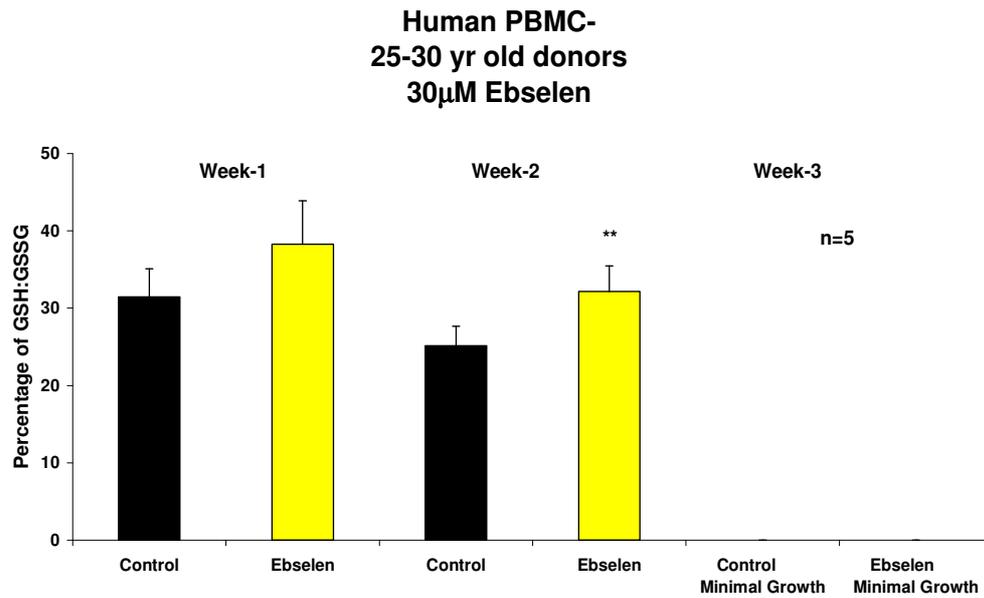
** p<0.01, *** p < 0.001- Significantly lower than control levels

Figures 5.7A and 5.7B show a significant decrease (Student's t-test, 95% confidence levels) in levels of oxidative damage to DNA in CD4⁺ T cells derived from donors from the 25-30 years age group (Figure 5.7A) and 55-60 years age group (Figure 5.7B) after two weeks of supplementation *ex vivo* with 30µM ebselen compared to non-supplemented CD4⁺ T cells. 7.5mM NAC supplementation also revealed a significant decrease in levels of oxidative damage to DNA in CD4⁺ T cells derived from donors of both age groups after two weeks of supplementation *ex vivo*, compared to non-supplemented CD4⁺ T cells. The supplementation of CD4⁺ T cells with 5mM NAC resulted in a significant decrease in levels of oxidative DNA damage after two weeks of supplementation compared to non-supplemented CD4⁺ T cells in donors from both age groups. The levels of oxidative DNA damage in 5mM supplemented CD4⁺ T cells were not significantly different from the levels of oxidative DNA damage in CD4⁺ T cells *ex vivo* from either age group supplemented with 7.5mM NAC. All the four figures (Figures 5.7A, 5.7B, 5.8A & 5.8B) show an increase in levels of oxidative DNA damage with age (in line with the findings in chapter-4 (Section 4.1.3.2) with T cell clones). There were no significant differences in the levels of oxidative damage to DNA in donors from the 25-30 years age group compared to donors from the 55-60 years age group. The number of live cells was too low after three weeks of supplementation to carry out any analysis. It was also worth noting that the levels of oxidative DNA damage observed among CD4⁺ T cells *ex vivo* derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.7A, 5.7B, 5.8A & 5.8B).

5.1.3.3. The impact of ebselen or NAC on intracellular redox status (GSH:GSSG ratio) in human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo*

T cell samples were taken from culture at various time points and the effect of 30µM ebselen or 7.5mM NAC on intracellular redox status (GSH:GSSG ratio) was determined. Figures 5.9 and 5.10 illustrate these results. Figures 5.11 and 5.12 show the results of the effect of 30µM ebselen or 7.5mM NAC on GSH:GSSG ratio in CD4⁺ T cells derived from donors from 25-30 years age group (Figures 5.11A & 5.12A) and 55-60 years age group (Figures 5.11B & 5.12B), when supplemented *ex vivo* compared to non-supplemented CD4⁺ T cells.

5.9A



5.9B

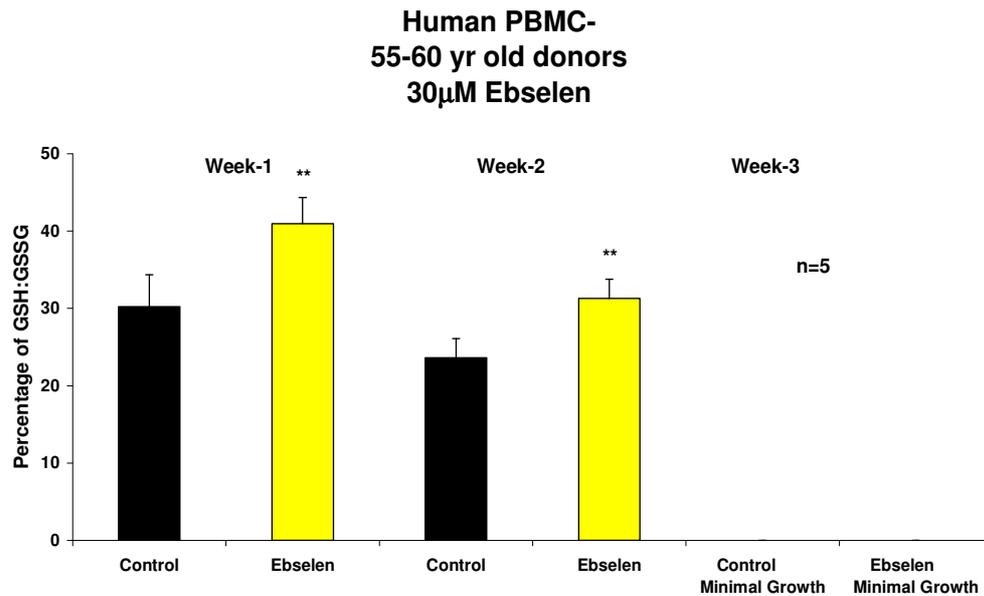
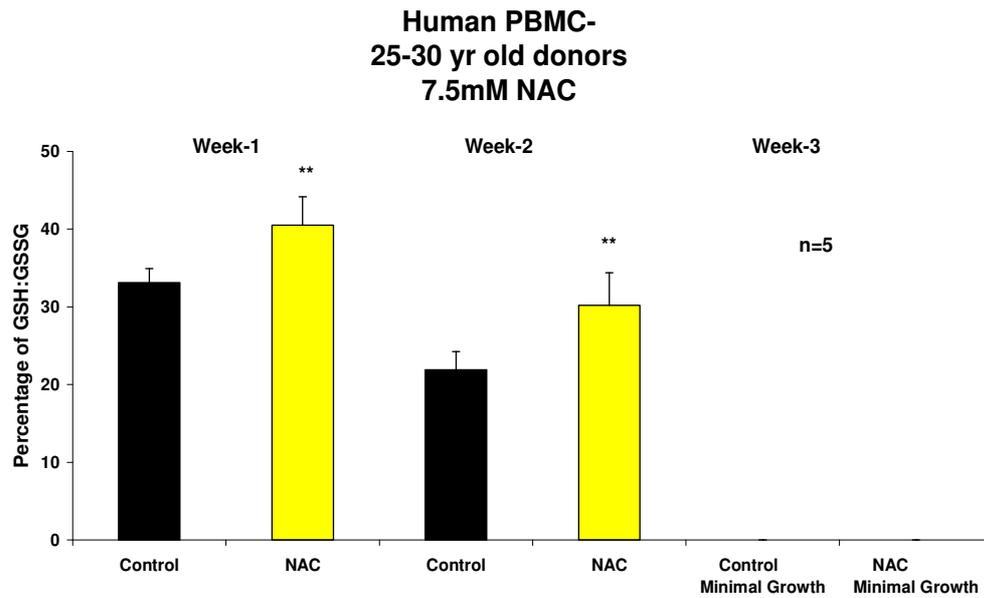


Figure 5.9 The effect of 30 μ M ebselen on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.9A) and 55-60 years (5.9B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

**** Significantly higher than the ratio of non-supplemented mononuclear cells, $p < 0.01$**

5.10A



5.10B

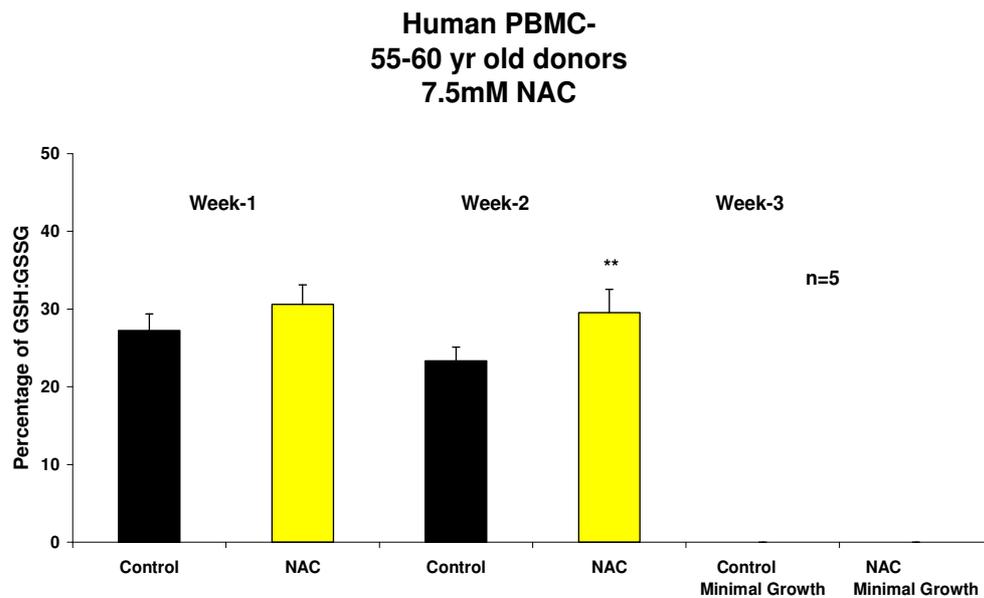
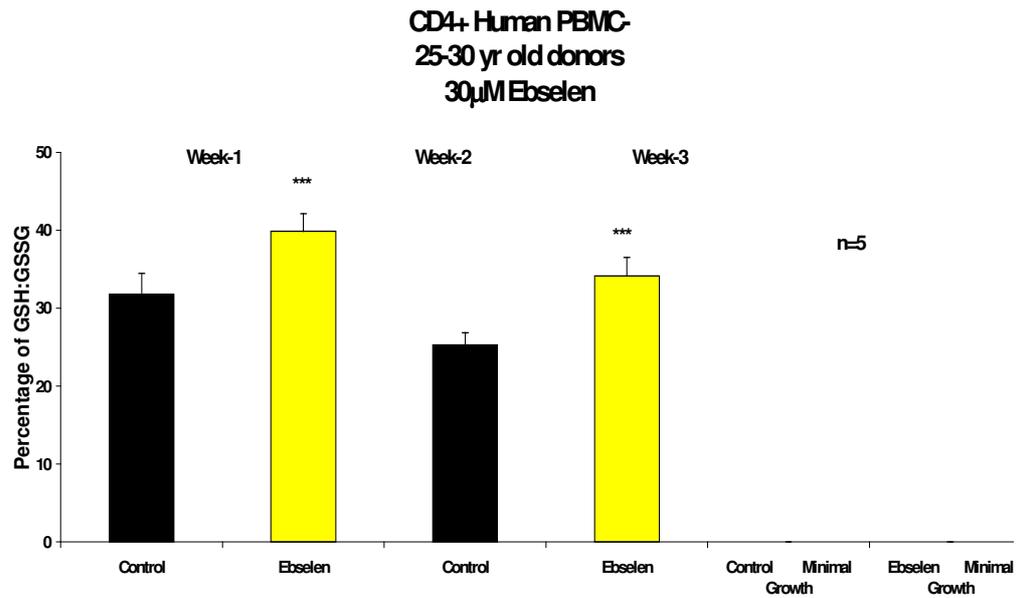


Figure 5.10 The effect of 7.5mM NAC on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.10A) and 55-60 years (5.10B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

**** Significantly higher than the ratio of non-supplemented mononuclear cells, $p < 0.01$**

Figure 5.9A shows a significantly higher (Student's t-test, 95% confidence levels) GSH:GSSG ratio in human peripheral blood mononuclear cells derived from donors from the 25-30 years age group after two weeks of supplementation *ex vivo* with 30 μ M ebselen, compared to non-supplemented peripheral blood mononuclear cells. Figure 5.9B denotes a significantly higher GSH:GSSG ratio of human peripheral blood mononuclear cells derived from donors from the 55-60 years age group after one week as well as two weeks of supplementation *ex vivo* with 30 μ M ebselen, compared to non-supplemented peripheral blood mononuclear cells. Figure 5.10A denotes a significantly higher GSH:GSSG ratio in human peripheral blood mononuclear cells derived from donors from the 25-30 years age group after one week as well as two weeks of supplementation *ex vivo* with 7.5mM NAC, compared to non-supplemented peripheral blood mononuclear cells. Figure 5.10B indicates a significantly higher GSH:GSSG ratio of human peripheral blood mononuclear cells derived from donors from the 55-60 years age group after two weeks of supplementation *ex vivo* with 7.5mM NAC, compared to non-supplemented peripheral blood mononuclear cells. Following supplementation with 5mM NAC, there was a significantly higher GSH:GSSG ratio after both one week and two weeks of supplementation in cells derived from both age groups compared to non-supplemented peripheral blood mononuclear cells. There were no significant differences in GSH:GSSG ratio in human peripheral blood mononuclear cells supplemented with 5mM NAC or 7.5mM NAC and derived from either age group. There were no significant differences in GSH:GSSG ratio in donors from the 25-30 years age group compared to donors from the 55-60 years age group. The number of live cells was too low after three weeks of supplementation to carry out any analysis. Furthermore, the GSH:GSSG ratios observed among human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.9A, 5.9B, 5.10A & 5.10B).

5.11A



5.11B

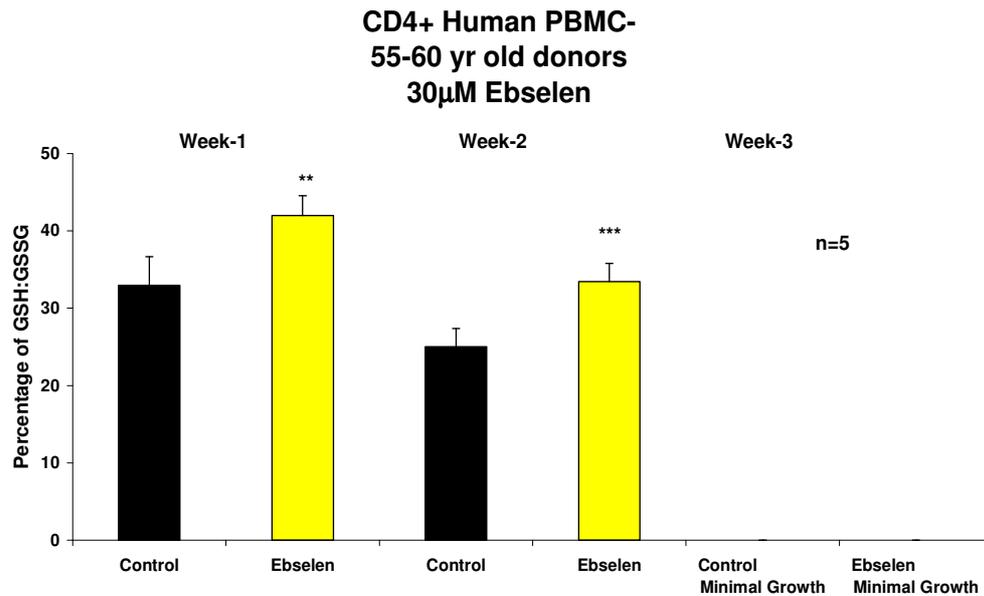
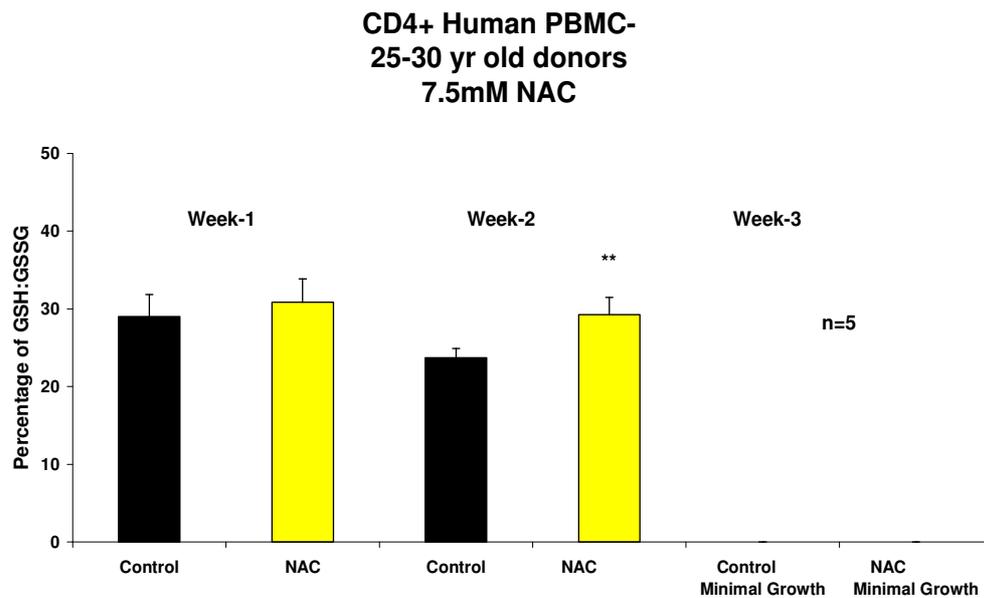


Figure 5.11 The effect of 30 μ M ebselen on intracellular GSH:GSSG ratio in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.11A) and 55-60 years (5.11B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

** p<0.01, *** p < 0.001- Significantly higher than the ratio of non-supplemented CD4⁺ T cells

5.12A



5.12B

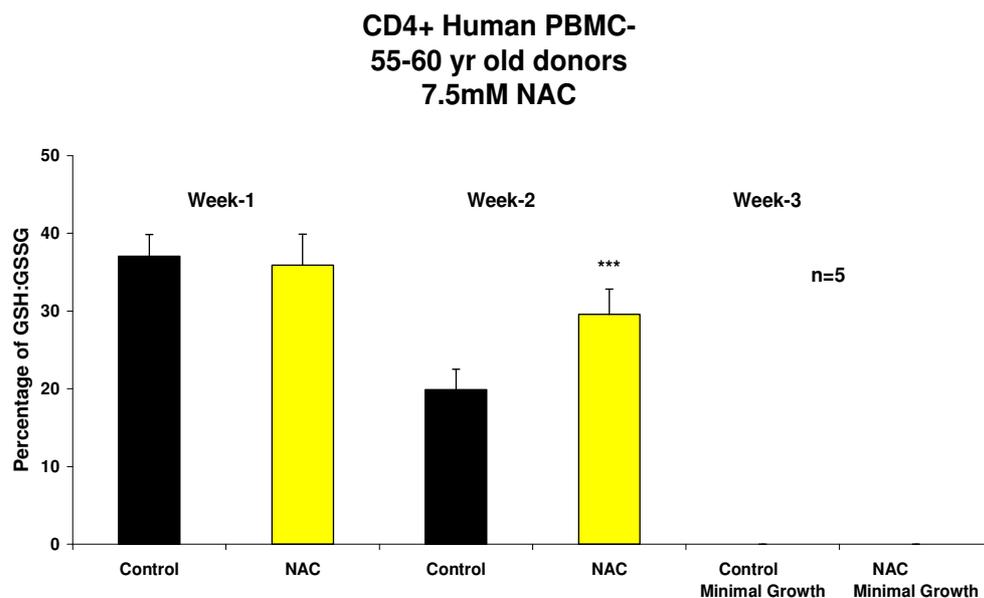


Figure 5.12 The effect of 7.5mM NAC on intracellular GSH:GSSG ratio in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.12A) and 55-60 years (5.12B) supplemented *ex vivo* for three weeks. The bars indicate the mean ± S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

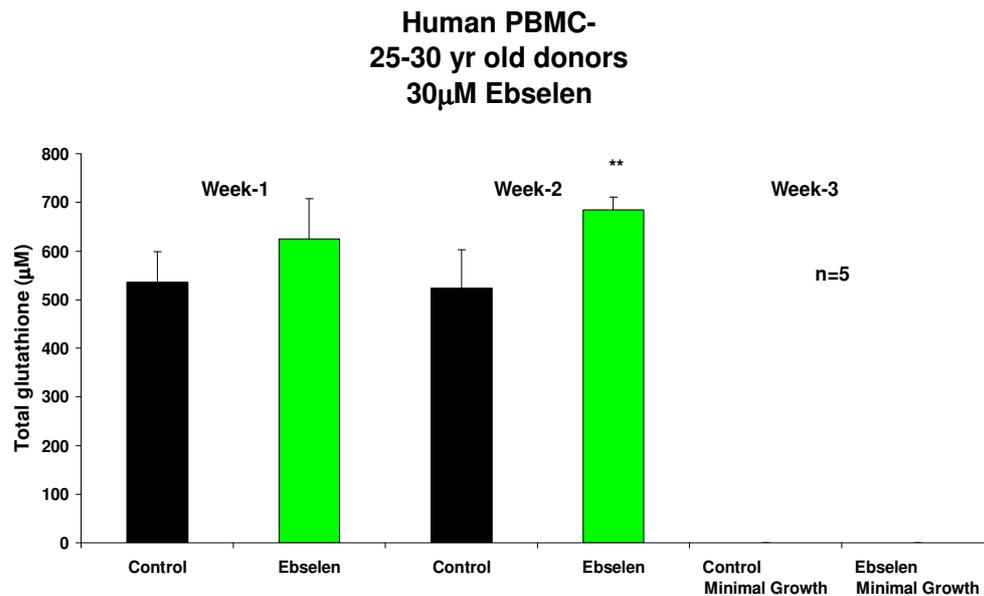
** p<0.01, *** p < 0.001- Significantly higher than the ratio of non-supplemented CD4⁺ T cells

Figures 5.11A and 5.11B show a significantly higher (Student's t-test, 95% confidence levels) GSH:GSSG ratio of CD4⁺ T cells derived from donors from the 25-30 years age group and 55-60 years age group, after one week and two weeks of supplementation *ex vivo* with 30μM ebselen compared to non-supplemented CD4⁺ T cells. Figures 12A and 12B denote a significantly higher GSH:GSSG ratio of CD4⁺ T cells derived from donors of 25-30 years age group and 55-60 years age group, after two weeks of supplementation *ex vivo* with 7.5mM NAC compared to non-supplemented CD4⁺ T cells. The results of CD4⁺ T cells supplemented with 5mM NAC were not significantly different from the results of CD4⁺ T cells supplemented with 7.5mM NAC in donors from both age groups. The supplementation of 5mM NAC resulted in a significantly higher GSH:GSSG ratio after two weeks of supplementation in donors from both age groups. There was no significant increase in GSH:GSSG ratio in CD4⁺ T cells after one week of supplementation with 5mM / 7.5mM NAC in donors from either age groups compared to non-supplemented CD4⁺ T cells *ex vivo*. The number of live cells was too low after three weeks of supplementation to carry out any analysis. The results of the analysis also revealed that the GSH:GSSG ratios observed among CD4⁺ T cells *ex vivo* derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.11A, 5.11B, 5.12A & 5.12B).

5.1.3.4. The impact of ebselen or NAC on total glutathione levels in human peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo*

T cell samples were taken from the culture at various time points of their lifespan and the effects of 30μM ebselen or 7.5mM NAC on total glutathione levels within the T cells *ex vivo* were examined. Figures 5.13 & 5.14 show the results of the effect of 30μM ebselen or 7.5mM NAC on total glutathione levels in human peripheral blood mononuclear cells derived from donors from the 25-30 years age group and 55-60 years age group supplemented *ex vivo*, compared to non-supplemented peripheral blood mononuclear cells. Figures 5.15 & 5.16 show the results of the effect of 30μM ebselen or 7.5mM NAC on total glutathione levels in CD4⁺ T cells derived from donors from the 25-30 years age group and 55-60 years age group supplemented *ex vivo*, compared to non-supplemented CD4⁺ T cells.

5.13A



5.13B

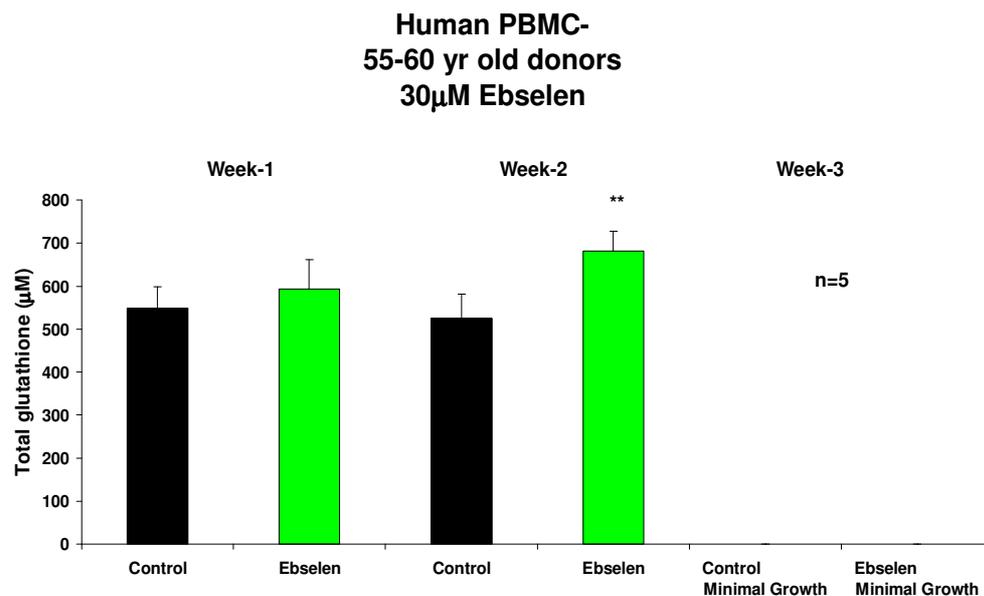
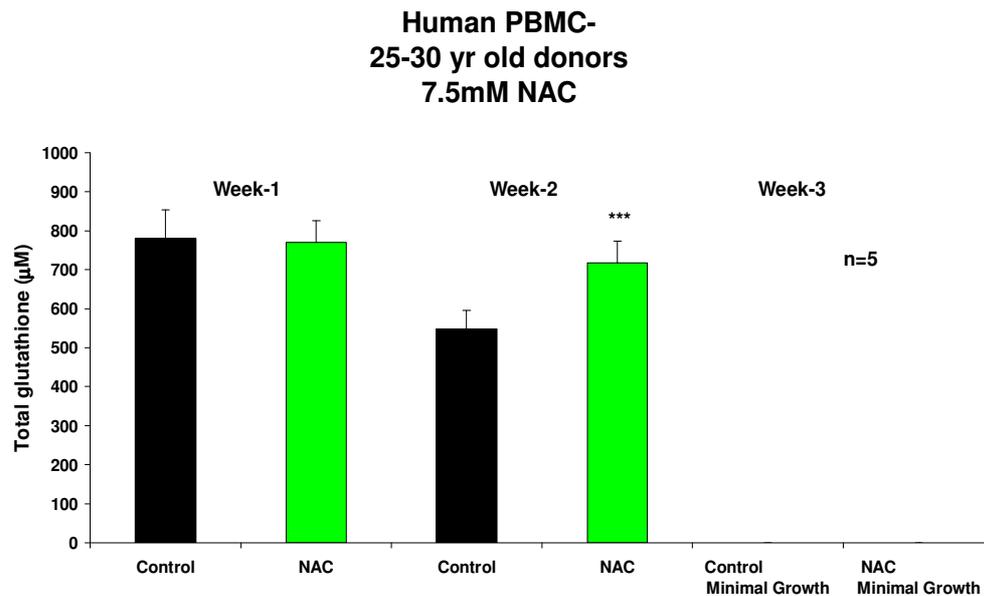


Figure 5.13 The effect of 30 μ M ebselen on total glutathione levels in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.13A) and 55-60 years (5.13B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

**** p<0.01 - Significantly higher than the non-supplemented controls**

5.14A



5.14B

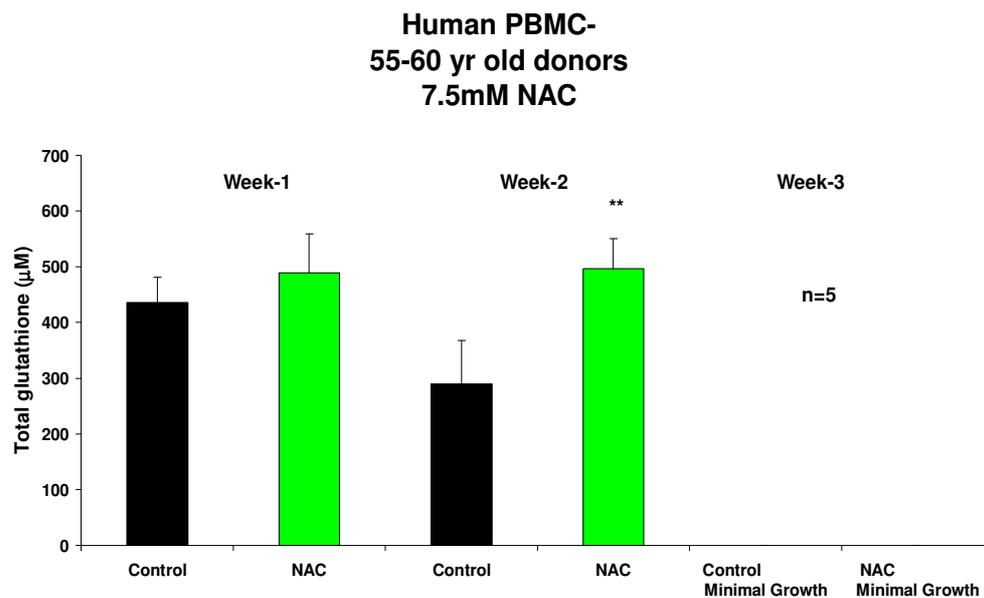
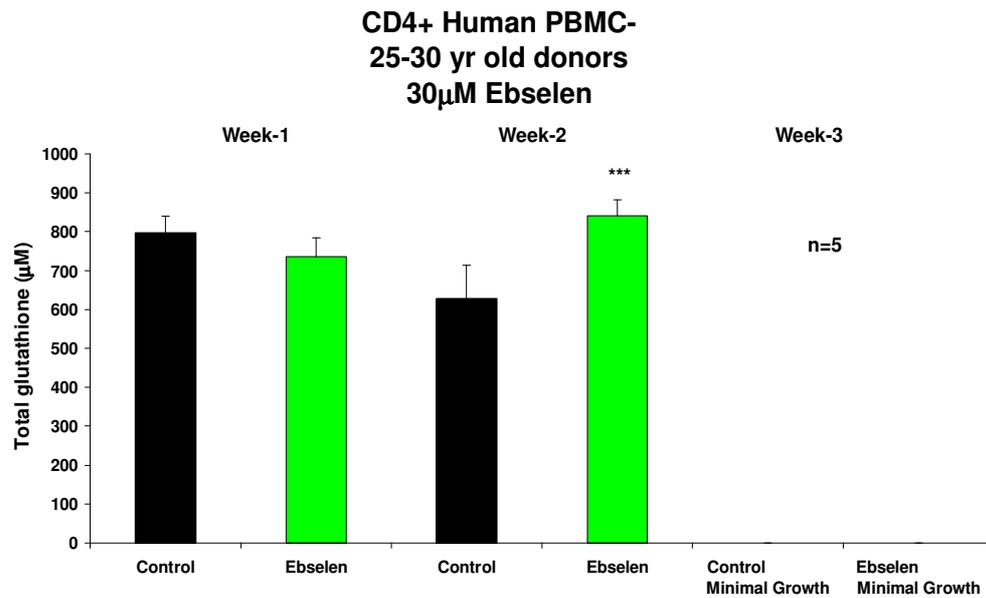


Figure 5.14 The effect of 7.5 mM NAC on total glutathione levels in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.14A) and 55-60 years (5.14B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

**** p<0.01, ***p<0.001 - Significantly higher than the non-supplemented controls**

Figures 5.13A, 5.13B, 5.14A & 5.14B indicate a significant increase (Student's t-test, 95% confidence levels) in total glutathione levels in human peripheral blood mononuclear cells derived from donors from the 25-30 years age group and 55-60 years age group, after two weeks of supplementation *ex vivo* with 30 μ M ebselen (Figures 5.13A & 5.13B) or 7.5mM NAC (Figures 5.14A & 5.14B), compared to non-supplemented peripheral blood mononuclear cells. The impact on total glutathione levels in human peripheral blood mononuclear cells supplemented with 5mM NAC was not significantly different from the impact of 7.5mM NAC supplementation in peripheral blood mononuclear cells. 5mM NAC supplementation resulted in a significant increase in total glutathione levels after two weeks of supplementation compared to non-supplemented peripheral blood mononuclear cells in donors from both age groups. There were no significant differences in total glutathione levels in donors from the 25-30 years age group compared to donors from the 55-60 years age group. The number of live cells was too low after three weeks of supplementation to carry out any analysis. Furthermore, the results of the analysis also revealed that the total glutathione levels observed among human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.13A, 5.13B, 5.14A & 5.14B).

5.15A



5.15B

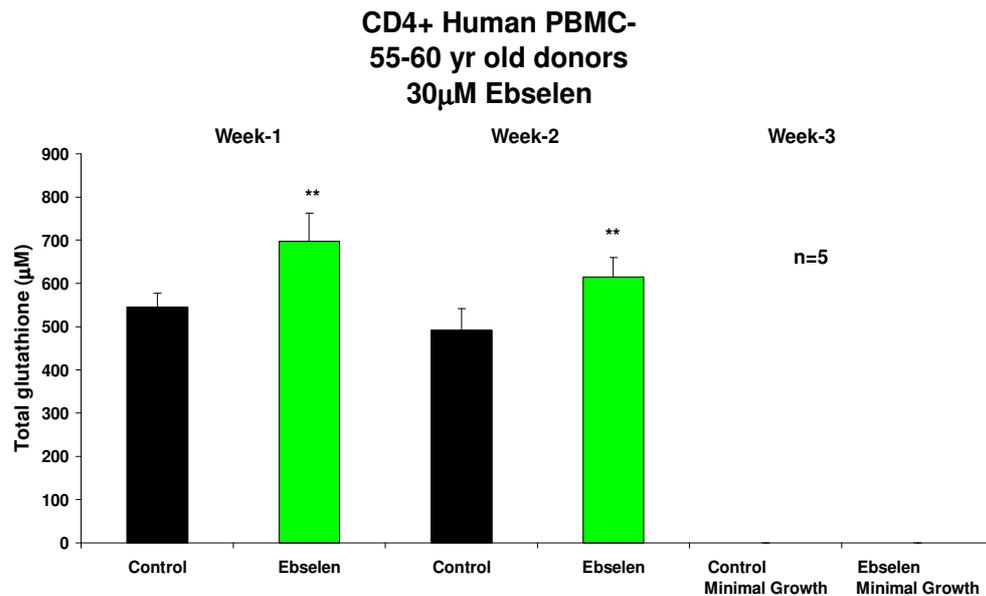
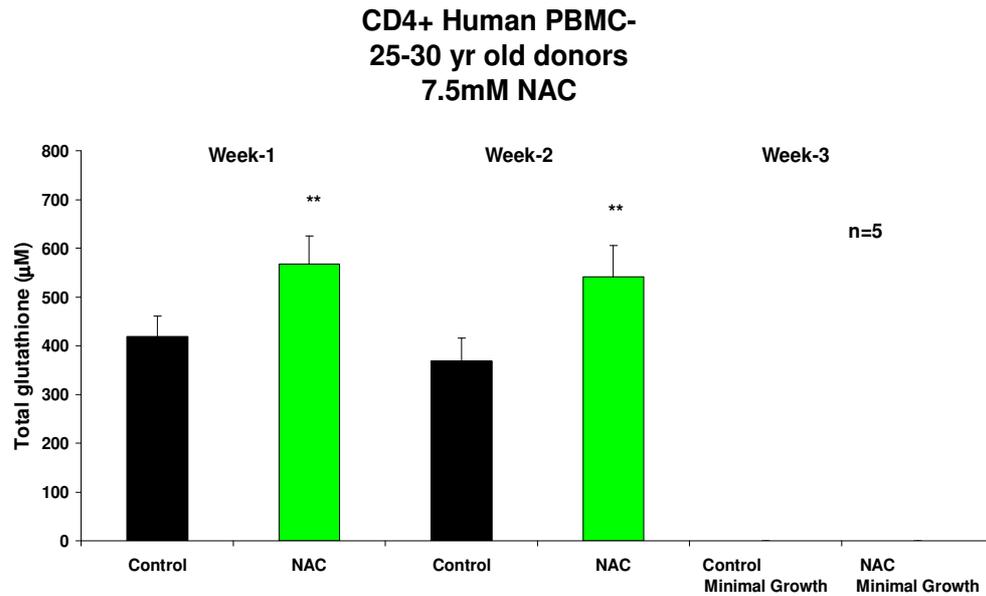


Figure 5.15 The effect of 30 μ M ebselen on total glutathione levels in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.15A) and 55-60 years (5.15B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

**** p<0.01, ***p<0.001 - Significantly higher than the non-supplemented controls**

5.16A



5.16B

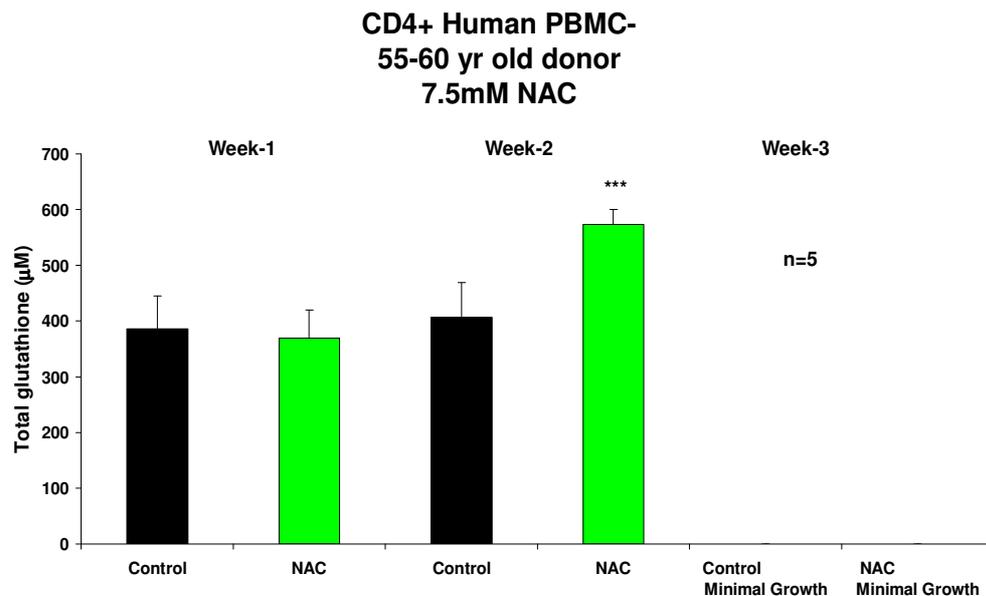


Figure 5.16 The effect of 7.5mM NAC on total glutathione levels in CD4⁺ T cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (5.16A) and 55-60 years (5.16B) supplemented *ex vivo* for three weeks. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

** p<0.01, ***p<0.001 - Significantly higher than the non-supplemented CD4⁺ controls

Figure 5.15A indicates a significant increase (Student's t-test, 95% confidence levels) in total glutathione levels after two weeks of supplementation *ex vivo* with 30 μ M ebselen in CD4⁺ T cells derived from donors from the 25-30 years age group and the increase in total glutathione levels reached statistical significance in donors from the 55-60 years age group (Figure 5.15B) after both weeks of supplementation compared to non-supplemented CD4⁺ T cells. In the case of supplementation with 7.5mM NAC, the increase in total glutathione levels reached statistical significance after the first and second week of supplementation in donors from the 25-30 years age group (Figure 5.16A) and the increase in total glutathione levels significantly increased after the second week of supplementation in donors from the 55-60 years age group. 5mM NAC supplementation resulted in a significant increase in total glutathione levels after both one week and two weeks of supplementation in CD4⁺ T cells compared to non-supplemented CD4⁺ T cells in donors from both age groups. The number of live cells was too low after three weeks of supplementation to carry out any analysis. The results also revealed that the total glutathione levels observed among CD4⁺ T cells *ex vivo* derived from each of the five different donors in each of the above cases were not significantly different (Figures 5.15A, 5.15B, 5.16A & 5.16B).

5.1.4. Discussion

In this study the impact of ebselen or NAC as antioxidants on *ex vivo* human peripheral blood mononuclear cells and *ex vivo* CD4⁺ T cells (both polyclonal populations) has been investigated. The study has also allowed a comparative analysis of the impact of these antioxidants in human CD4⁺ T cells *ex vivo* compared to their impact in human peripheral blood derived CD4⁺ T cell clones *in vitro*, a monoclonal population (Chapter-4). The impact of age on markers of T cell integrity and function was also determined by comparing the proliferative capacity, the levels of oxidative DNA damage, GSH:GSSG ratio and total glutathione levels in peripheral blood mononuclear cells derived from donors of the 25-30 years age group compared to the cells derived from donors of the 55-60 years age group.

The ability of ebselen or NAC to minimise or scavenge ROS is well documented (Chapter-1, Section 1.1.6.1 & 1.1.6.2). The ability of ebselen to scavenge hydroxyl radicals and peroxy radicals, responsible for lipid peroxidation and leading to DNA damage, contributes to its antioxidant potential (Yang *et al.*, 1999). The free sulfhydryl

group scavenging ROS and NO and the ability to raise GSH levels by cysteine donation supplements the antioxidant potential of NAC (Han *et al.*, 1997; Wagner *et al.*, 1998). Supplementation of human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* derived from donors from either age groups, with 30µM ebselen or 7.5mM NAC resulted in a significantly higher GSH:GSSG ratio (Figures 5.9A, 5.9B, 5.10A, 5.10B, 5.11A, 5.11B, 5.12A & 5.12B), total glutathione levels (Figures 5.13A, 5.13B, 5.14A, 5.14B, 5.15A, 5.15B, 5.16A & 5.16B) and proliferative capacity (Figures 5.1A, 5.1B, 5.2A, 5.2B, 5.3A, 5.3B, 5.4A & 5.4B) at various time points during their span in culture compared to non-supplemented human peripheral blood mononuclear cells. Previous studies have demonstrated the ability of ebselen at concentrations of 1-25µM to stimulate cell growth, inhibit H₂O₂ induced suppression, and significantly reduce LDH leakage and lipid peroxidation and significantly reduced intracellular ROS levels in HepG₂ cells in a concentration dependent manner due to its ability to scavenge hydroxyl radicals and other ROS (Yang *et al.*, 1999). The free radical scavenging ability of ebselen has also been successfully demonstrated in HL-60 cells (Ji *et al.*, 2000). In terms of NAC, other studies investigating the effect of a range of NAC concentrations (0.5-2mM) in HeLa cells have shown that 2mM NAC increased intracellular GSH by 15% compared to controls after 24hrs of incubation (Hansen *et al.*, 2004). In HepG₂ cells, the increase in intracellular GSH level was highest after 5mM NAC supplementation for 24hrs (Yang *et al.*, 2000).

The ability of ebselen or NAC to scavenge radicals and thereby elevate the GSH:GSSG ratio is of significance considering the important role of intracellular redox status (GSH:GSSG ratio) as a mediator of apoptosis in many cell systems (Slater *et al.*, 1996; Cotgreave and Gerdes, 1998; Hall, 1999). Intracellular reduced glutathione (GSH), a main determinant of intracellular redox status, is known to become depleted before the onset of apoptosis (Beaver and Waring, 1995; Macho *et al.*, 1997; Ghibelli *et al.*, 1998). The GSH:GSSG redox couple maintains the redox environment of the cell and GSH is abundant in the cell (Schafer and Buettner, 2001), serving as an indicator of the cellular redox environment. Oxidation of even a small amount of GSH may hinder the redox status of the cell. This oxidation results in the formation of GSSG and mixed disulfides between protein sulfhydryl groups in biological systems. It will also result in a decrease in the levels of GSH and so lead to a lowering of the GSH:GSSG ratio, which has been suggested to be responsible for several human diseases (Chapter-1, Section 1.1.4.1.1).

Thus the higher GSH:GSSG ratio represents an improved antioxidant state for the ebselen or NAC treated cells.

The results of this investigation also revealed a significant decrease in the levels of oxidative DNA damage (Figures 5.5A, 5.5B, 5.6A, 5.6B, 5.7A, 5.7B, 5.8A & 5.8B) at various time points in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* during their span in culture, when compared to non-supplemented peripheral blood mononuclear cells. The ability of ebselen to almost completely inhibit oxidative DNA damage has been previously demonstrated in HepG₂ cells (Yang *et al.*, 1999) and HL-60 cells (Ji *et al.*, 2000), attributed to their ability to scavenge hydroxyl and peroxide radicals. In terms of NAC, concentrations of 100-300µM NAC, significantly lower than the concentrations used in this investigation, reduced the DNA damage compared to controls in PC-12 cells (Yan and Greene, 1998).

Further studies on the effect of ebselen or NAC supplementation on total glutathione levels revealed a significant increase, on supplementation with ebselen (30µM) or NAC (7.5mM) in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*.

The significant increase in total glutathione levels on supplementation with 7.5mM NAC demonstrates the ability of NAC to contribute glutathione to the cell thereby supplementing the *in vivo* antioxidant status. A significant increase in total glutathione levels was also observed on supplementation with 30µM ebselen. Ebselen is a GPx mimetic. GPx has the ability to scavenge organic and inorganic peroxides using GSH as substrate. GSH is depleted during peroxide accumulation. Ebselen has a proven ability to scavenge hydroxyl radicals and ROS that initiates lipid peroxidation as a result of peroxide accumulation. In this study, ebselen may have scavenged the radicals present in the cell system. The fewer the radicals, the lesser will be the need for GSH to scavenge them. Since the GSH is not consumed to scavenge the radicals, levels of GSH may appear greater in the cell system on supplementation with ebselen.

The results of this chapter clearly reveal the antioxidant potential of 30µM ebselen or 7.5mM NAC, against endogenously generated oxidants and those associated with the 95% air, 5% CO₂ culture conditions in human peripheral blood mononuclear cells and

CD4⁺ T cells *ex vivo* at various time points during their span in culture. The higher GSH:GSSG ratio represents an increased antioxidant state on supplementation with ebselen or NAC. The increase in antioxidant status explains the decrease in levels of oxidative DNA damage that further explains a lower apoptotic state explained by the increase in proliferative capacity of the cells on antioxidant supplementation. 5mM NAC supplementation investigated in this study also revealed a significant antioxidant potential, similar to supplementation with 7.5mM NAC, in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*, considering their effect on the markers of T cell function and integrity investigated in this study.

Apart from the potentially detrimental effects, ROS play an important role in T cell signalling events such as protein tyrosine phosphorylation and activation of JNK (Pani *et al.*, 2000). ERK, JNK (SAPK) and P38 are some of the important pathways of mitogen activated protein (MAP) kinases, a prominent family of protein kinases, involved in proliferation, differentiation and apoptosis in cells. Previous work from the group of Barnett suggested an increase in JNK phosphorylation and P38 phosphorylation at 6% O₂ tension (Duggan *et al.*, 2004). Other studies revealed inhibition of H₂O₂ induced P38 MAP kinase activation and cytochrome c release (cytochrome c plays an important role in apoptotic cell death and is released from the mitochondria in response to apoptotic signals) by 0.3µM ebselen supplementation in HUVECs and inhibition of the H₂O₂ induced activation of JNK signalling pathway by ebselen in PC-12 cells (Yoshizumi *et al.*, 2002). This inhibition of P38 MAP kinase was later revealed as the major factor for endothelial cell death. This finding was implied as useful in prevention or treatment of endothelial dysfunction, suggested to be the first step in the development of atherosclerosis (Ali *et al.*, 2004). The inhibition of H₂O₂ induced activation JNK pathway by ebselen may be useful for the treatment of ischaemic cerebral diseases (Yoshizumi *et al.*, 2002). JNK and P38 MAP kinase has been proven to be activated on cytotoxic insult and are often associated with apoptosis on activation (Ali *et al.*, 2004). The inhibition of H₂O₂ induced activation of the MAP kinase pathways by ebselen has been attributed to its hydroxyl radical scavenging capacity. It will be interesting to examine the impact of ebselen or NAC on these pathways in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*.

Furthermore, when the effects of 30 μ M ebselen or 7.5mM NAC in human CD4⁺ T cells *ex vivo* was compared to the effects on human peripheral blood derived CD4⁺ T cell clones *in vitro*, there were no significant differences found across all markers of T cell integrity and function. Interestingly, 5mM NAC supplementation did not reveal any significant antioxidant potential in CD4⁺ T cell clones *in vitro* (Chapter-4), unlike the potential demonstrated in the *ex vivo* polyclonal peripheral blood mononuclear cell population and CD4⁺ T cells *ex vivo*. Human T cell clones are maintained in culture throughout their lifespan over several months. This is not the case in cells *ex vivo*. It may be that the longer span of culture *in vitro* may have altered certain aspects of the biochemical / molecular status of the cells. The *in vitro* T cell clones may have steadily accumulated biomolecule damage during their lifespan under conditions of 20% O₂ that may have decreased the antioxidant or free radical scavenging potential of NAC at this specific concentration on long term supplementation. Under these extensive culture conditions, antioxidant or free radical scavenging effects of NAC at 5mM concentrations may be insufficient to reveal any significant antioxidant potential in T cell clones *in vitro*.

Previous findings from the group of Barnett revealed that human peripheral blood mononuclear cells derived from donors of 65-69 years had higher levels of mutations and DNA damage and had lower DNA repair capacity and altered *in vivo* antioxidant status compared to cells derived from donors aged 35-39 years. The decrease in DNA repair capacity or alterations in *in vivo* antioxidant status may have contributed to the increase in mutation load in mononuclear cells derived from donors of 65-69 years (King *et al.*, 1997). The accumulation of such damage and mutations in T cells *ex vivo*, when in critical levels, could result in failure of T cells to proliferate due to DNA damage induced cell cycle arrest; decreased rate of proliferation as a consequence of selection *in vivo* against cells carrying mutation and / or apoptosis (Barnett & Barnett, 1998). In the study reported in this chapter, no significant differences between the levels of oxidative DNA damage in untreated human peripheral blood mononuclear cells or CD4⁺ T cells *ex vivo* derived from donors of 25-30 years age group, compared to T cells derived from donors of 55-60 years age group were found. Other markers of T cell function (proliferative capacity) and integrity (GSH:GSSG ratio and total glutathione levels) investigated in this study did not reveal any significant differences between the two age groups.

There could be several reasons for the difference in the results of this study compared to the previous studies of the group. One of the differences is due to the differences in age groups used in the two studies. Age group of donors used in this study were 55-60 year age group compared to the 65-69 year age group used in the previous study by the group. Another difference could be the differences in sensitivity of the assays used. The previous study carried out by the group of Barnett revealing an increase in basal levels of DNA damage and mutations in mononuclear cells *ex vivo* with age, used sandwich enzyme linked immunosorbent assay (ELISA) technique based on the action of a monoclonal antibody (Barnett and King, 1995). The combined comet assay used in this investigation detects single stranded breaks and oxidised bases. Other studies have shown the comparison between the output of ELISA and comet assays and both assays have shown comparable results in detecting DNA damage. But one of the important advantage of comet assay over ELISA is the detection of DNA damage in individual cells and its distribution is assessed giving a pattern of the DNA damage (Hughes *et al.*, 1999).

In summary, the overall data of the current study revealed that 30µM ebselen or 5mM / 7.5mM NAC supplementation demonstrated antioxidant potential evidenced by the significant increase in proliferation capacity, significantly higher GSH:GSSG ratio and total glutathione levels, and significant decreases in levels of oxidative damage to DNA in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*. From the results of this study, it is suggested that the possible *in vivo* antioxidant potential of ebselen or NAC should be investigated alongside their potential as anti-immunosenescent interventive strategies.

CHAPTER-6

**The effects of acute / chronic
exposure to mitoQ on human
peripheral blood mononuclear cells
*ex vivo***

6.1. The effects of acute / chronic exposure to mitoQ on human peripheral blood mononuclear cells *ex vivo*

6.1.1. Introduction

Previous work from the group of Barnett has demonstrated an age related increase in DNA damage and mutations in human T lymphocytes *ex vivo* (King *et al.*, 1994; 1997; Barnett and King, 1995). This increase in DNA damage may result in cell cycle arrest or apoptosis (Barnett & Barnett, 1998; Hyland *et al.*, 2000). Recent studies have revealed that damage to mitochondria could contribute to an age related immunodeficiency and may also result in insufficient ATP production, through OXPHOS, for the function of lymphocytes (Ross *et al.*, 2002).

The important role of mitochondria in the life and death of a cell has been well established. Mitochondria are a major source of ROS, which can cause damage to cellular constituents such as DNA, RNA, proteins and lipids and contribute to cellular ageing and death. This is the basis of mitochondrial theory of ageing (Salvioli *et al.*, 2001; Ross *et al.*, 2002). Mitochondria possess a genome which has been sequenced (Anderson *et al.*, 1981; Chapter-1, Section 1.1.1.2.1). mtDNA lacks a protective histone protein and, an efficient DNA repair mechanism; this contributes to a 5-10 times faster mutation rate in mtDNA compared to the rate for nDNA (Richter, 1995).

A decline in mitochondrial bioenergetic function with age in various human tissues has been widely implicated as a major factor contributing to the onset of a wide range of diseases (Smith *et al.*, 2003; Chapter-1, Section 1.1.6.3). One of the main difficulties encountered in treating diseases caused by mitochondrial dysfunction is the inability to target bioactive molecules to mitochondria *in vivo*. This problem was solved by the development of mitoQ (Murphy, 1997; Kelso *et al.*, 2001; Chapter-1, Section 1.1.6.3; Figure 1.13 & 1.14). The lipophilic cation attached to the mitoQ enables it to penetrate the lipid bilayers and the large mitochondrial membrane potential enables mitoQ to accumulate several hundred-fold within the mitochondria. The ubiquinone derivative, then buried within the lipid core of the mitochondrial inner membrane, accepts two electrons from complex II, resulting in reduction to ubiquinol. The ubiquinol donates a hydrogen atom from its hydroxyl group to a lipid peroxy radical resulting in a decrease

in lipid peroxidation within the mitochondrial inner membrane (Takada *et al.*, 1984; Ernster *et al.*, 1992; Ingold *et al.*, 1993). The ability of the ubiquinol derivative to donate a hydrogen atom to a lipid peroxy radical enables the inhibition of lipid peroxidation caused by the leakage of superoxide from the mitochondrial respiratory chain, an important source of oxidative stress (Raha and Robinson, 2000). This mechanism helps mitoQ to protect mitochondria from oxidative damage (Kagan *et al.*, 1994; Lass and Sohal, 1998; Chapter 1, Figure 1.15).

Previous studies have demonstrated the ability of mitoQ to completely block H₂O₂ induced caspase activation and apoptotic cell death in Jurkat cells on acute exposure (Kelso *et al.*, 2001). The accumulation of caspases by H₂O₂ may result in mitochondrial oxidative stress resulting in mitochondrial membrane transition pores leading to loss of mitochondrial membrane potential that may play an important role in H₂O₂ induced apoptosis in Jurkat cells (Hampton and Orenius, 1997; Dumont *et al.*, 1999). Other studies have revealed antioxidant potential on long term exposure to mitoQ, evidenced by prevention in rise of telomere shortening in human fibroblasts (Saretzki *et al.*, 2003). In this chapter, the impact of a range of doses of mitoQ, following acute (after 3 or 6 hours of mitoQ supplementation) or chronic (after 3 weeks of mitoQ supplementation) exposure, on endogenously produced ROS or H₂O₂ induced oxidative stress was examined in human peripheral blood mononuclear cells *ex vivo*. Decyl triphenyl phosphonium cation (DTPP, a non-antioxidant mitochondria-targeted compound) and vitamin E (a lipophilic, non-mitochondria-targeted antioxidant) were used as controls in this investigation.

6.1.2. Materials and Methods

6.1.2.1. Subject selection

The human blood samples for this study were drawn from ten healthy, non-smoking males fasted overnight, five each in the age groups of 25-30 and 55-60 years. The subjects were not on any form of medication nor had they suffered any recent illness. The results of the previous studies in human peripheral blood mononuclear cells carried out by the group of Barnett were taken into consideration before choosing the two age groups of donors in this study (Barnett and King, 1995; Chapter-2, Section 2.1.3.1).

6.1.2.2. Sample collection

Human blood samples were drawn in the morning between 8.00 and 9.00 am by a licensed individual and collected in a tube containing heparin to prevent coagulation. The samples were kept on ice and taken to the laboratory within 5 minutes for separation of peripheral blood mononuclear cells.

6.1.2.3. Acute / chronic exposure to mitoQ in human peripheral blood mononuclear cells *ex vivo*

Freshly isolated human peripheral blood mononuclear cells were washed separately in phosphate buffered saline or X-Vivo 10 media (Bio Whittaker) and each of the samples were maintained separately in culture as explained in chapter-2, section 2.1.7.1. The human peripheral blood mononuclear cells from ten healthy, non-smoking males fasted overnight, five each in the age group of 25-30 years and 55-60 years were incubated separately with mitoQ or DTPP (0 μ M, 0.1 μ M, 0.25 μ M, 0.5 μ M, 1.0 μ M) or Vitamin E (Sigma) (37 μ M or 74 μ M) for 3 or 6 hours. 0-250 μ M H₂O₂ (Sigma) was exogenously applied after 3 or 6 hours of mitoQ or DTPP or vitamin E supplementation. The samples were frozen down within 15 minutes of H₂O₂ treatment. The entire H₂O₂ treatment was performed at 4°C to minimise DNA repair. The existing scientific literature was consulted in order to confirm the dose range of the antioxidants and the oxidant (explained in detail in chapter-2, Section 2.1.7). DTPP and Vitamin E were used as controls for mitoQ, in this investigation.

For chronic exposure to mitoQ, freshly isolated human peripheral blood mononuclear cells from six healthy, non-smoking males fasted overnight, three each from the age group of 25-30 years and 55-60 years were maintained in culture separately for three weeks as described in chapter-2, section 2.1.3.3. The human peripheral blood mononuclear cells were supplemented with 0-30nM mitoQ or DTPP in X-Vivo 10 and were maintained in culture for up to 3 weeks. The cells *ex vivo* were supplemented with mitoQ or DTPP on days one and four of the growth cycle. The mitoQ or DTPP supplementation were performed on both days (one and four), similar to the previous analysis investigating the impact of chronic exposure of other antioxidants in T cells *in vitro* and *ex vivo* (Hyland *et al.*, 2000; Chapter-4, Section 4.1.2.4; Chapter-5, Section 5.1.2.5). MitoQ supplementation was performed on day four in order to replenish the

culture system with the antioxidant. Previous studies were taken into consideration before choosing the dose range for chronic exposure to mitoQ and DTPP (Chapter-2, Section 2.1.7). Only DTPP was used as a control for mitoQ for this part of the investigation.

6.1.2.4. Quantification of mitochondrial membrane potential in *ex vivo* human peripheral blood mononuclear cells exposed to mitoQ

The JC-1 staining kit assay (Invitrogen) (Chapter-2, Section 2.1.8) was optimised by trying a range of cell numbers (0.5×10^6 to 2×10^6 cells/ml) for assessing the mitochondrial membrane potential in human peripheral blood mononuclear cells *ex vivo*. After optimisation of the technique, 1.5×10^6 human peripheral blood mononuclear cells were analysed using the kit. The cells were suspended in warm PBS (Oxoid Ltd) and stained with JC-1 dye at a final concentration of $2\mu\text{M}$ and were maintained at 37°C at 5% CO_2 for 30 minutes. The cells were then re-suspended in fresh warm PBS or X-Vivo 10 media and the mean fluorescent intensity of green signal (percentage of apoptotic cells) was compared to the mean fluorescent intensity of red signal (percentage of live cells). Intensity values were determined using a flow cytometer at absorption maxima of 488nm. The results were expressed as mean ratio values. Mean value represents the mean fluorescent intensity giving the highest number of counts in a flow cytometer.

The mean ratio values were calculated using the following formula:

$$\text{Mean ratio} = \frac{\text{Mean value of green signal}}{\text{Mean value of red signal}}$$

A higher mean ratio represents lower mitochondrial membrane potential due to higher levels of oxidative stress and a lower mean ratio or ratio closer to basal levels represents higher mitochondrial membrane potential due to low levels of oxidative stress.

6.1.2.5. Determination of levels of oxidative damage to DNA in human peripheral blood mononuclear cells *ex vivo* pre-treated with mitoQ / DTPP / Vitamin E

The levels of oxidative damage to DNA plus DNA single strand breaks and alkali labile sites in human peripheral blood mononuclear cells *ex vivo* were determined using the modified alkaline comet assay as previously described (Collins *et al.*, 1993; Hyland *et al.*, 2000, 2001; Duggan *et al.*, 2004; Chapter-2, Section 2.1.10). Comet assays for the samples were analysed in duplicate.

6.1.2.6. Intracellular GSH:GSSG ratio and total glutathione levels in human peripheral blood mononuclear cells *ex vivo* pre-treated with mitoQ / DTPP / Vitamin E

The ratio of the reduced and oxidised form of glutathione and total glutathione levels were assessed using a GSH:GSSG ratio assay kit (Calbiochem, UK) (Chapter-2, Section 2.1.11). The samples were analysed in duplicate.

6.1.2.7. Statistical analysis

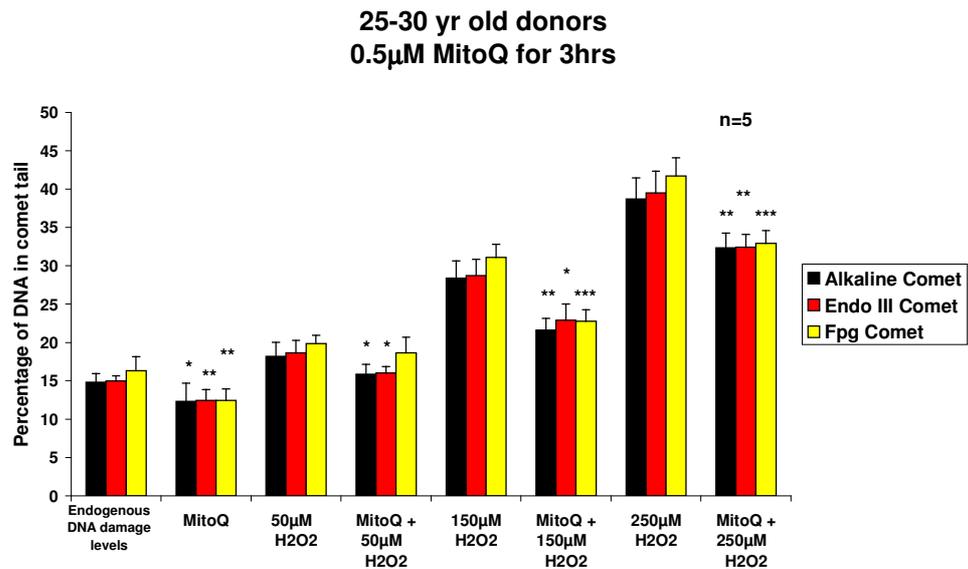
The results were tested for significance using paired two-sample Student's t-tests assuming equal variances; p values are presented as appropriate.

6.1.3. Results

6.1.3.1. Impact of mitoQ on levels of oxidative DNA damage in human peripheral blood mononuclear cells *ex vivo*

The effect of 0.5µM mitoQ (Figures 6.1A & 6.1B) on the levels of endogenous oxidative DNA damage and on DNA damage levels following treatment with H₂O₂ in peripheral blood mononuclear cells is illustrated. Control (unsupplemented) human peripheral blood mononuclear cells were grown in parallel to the mitoQ supplemented cells.

6.1A



6.1B

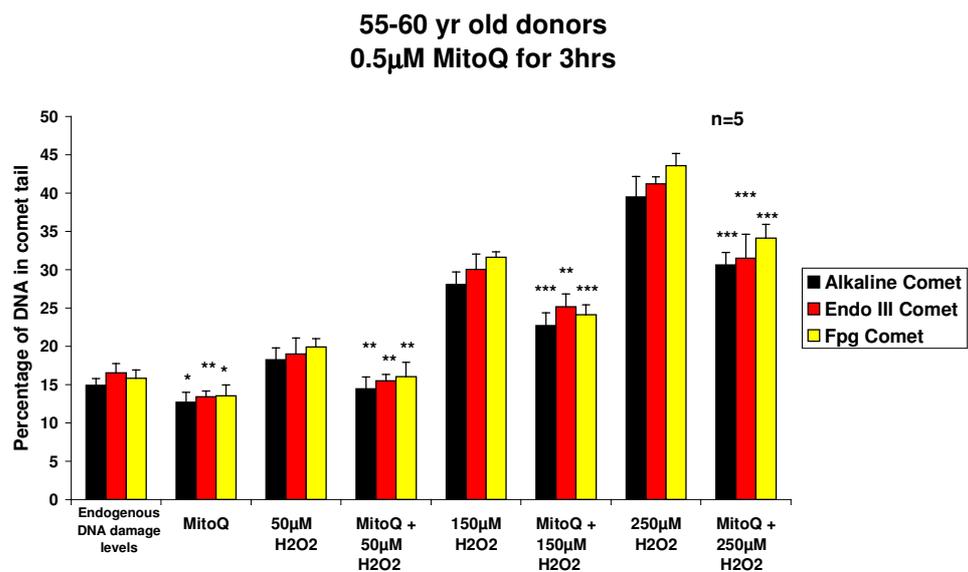


Figure 6.1 The effect of 0.5 μ M mitoQ on levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (6.1A) and 55-60 years (6.1B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

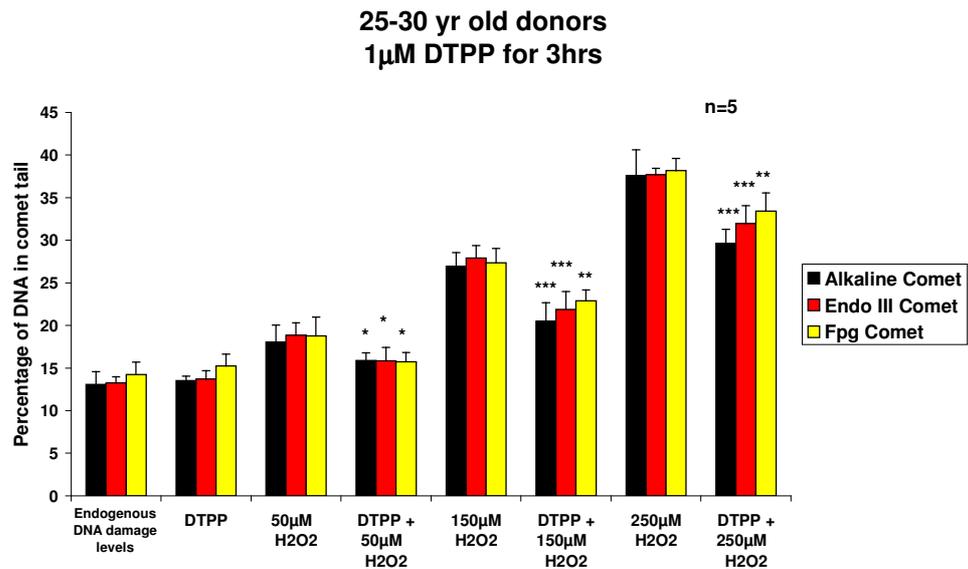
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ - Significantly lower than control levels

Figures 6.1A and 6.1B show a significant (Student's t-test, 95% confidence levels) decrease in endogenous levels of oxidative DNA damage following a 3 hour treatment with 0.5 μ M mitoQ. In addition, 0.5 μ M mitoQ treatment afforded significant protection against H₂O₂ (50 μ M-250 μ M) induced DNA damage. There was no significant difference in, the levels of oxidative DNA damage in cells derived from donors from the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. In all cases, there was a consistent increase in levels of oxidative DNA damage with increase in concentration of H₂O₂ (0-250 μ M) treatment. The levels of oxidative DNA damage observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.1A & 6.1B).

Further studies were carried out to investigate the effect of a higher dosage of mitoQ (1 μ M) on human peripheral blood mononuclear cells. The results of this investigation revealed that the antioxidant potential of 1 μ M mitoQ in peripheral blood mononuclear cells derived from donors from both age groups were not significantly different from the effect of 0.5 μ M mitoQ (Table 6.1).

In the next part of the investigation, in order to confirm the antioxidant potential of ubiquinone in mitoQ, the impact of 0.5 μ M or 1 μ M DTPP (control for mitoQ) on human peripheral blood mononuclear cells was investigated. The effect of 1 μ M DTPP (Figures 6.2A & 6.2B) on the levels of endogenous oxidative DNA damage and on DNA damage levels following treatment with H₂O₂ in peripheral blood mononuclear cells is illustrated. Control (un-supplemented) human peripheral blood mononuclear cells were grown in parallel to the DTPP supplemented peripheral blood mononuclear cells.

6.2A



6.2B

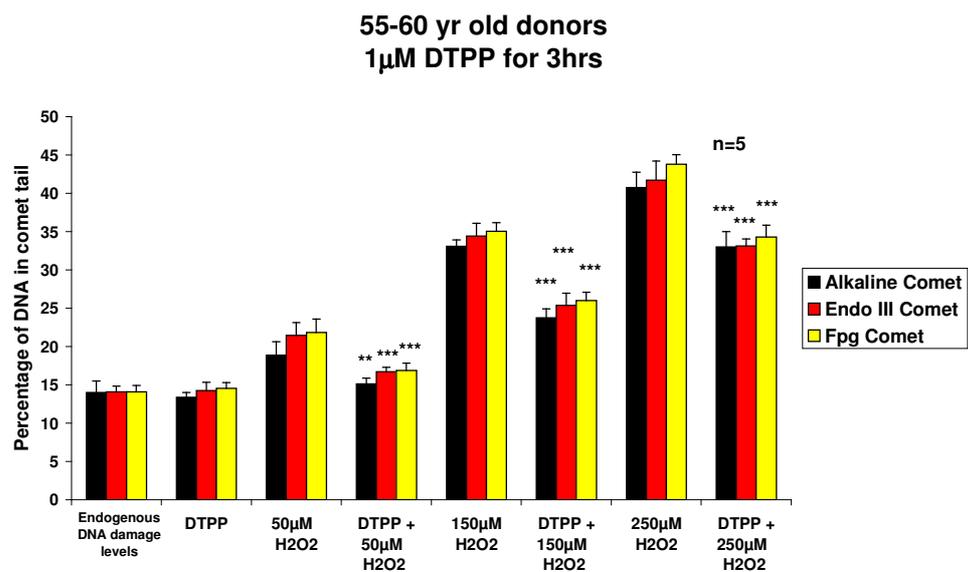


Figure 6.2 The effect of 1 μ M DTPP on levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (6.2A) and 55-60 years (6.2B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ - Significantly lower than control levels

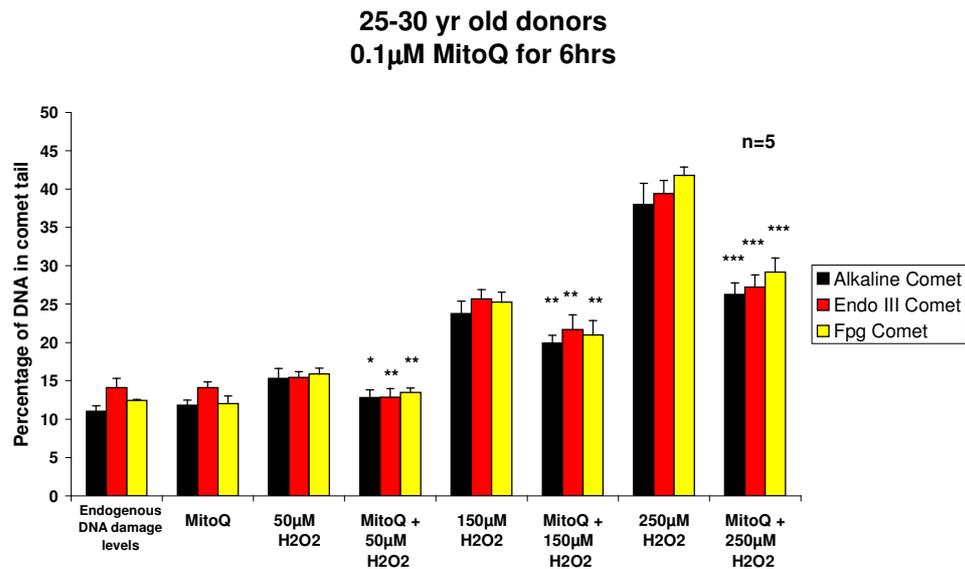
Figures 6.2A and 6.2B do not show any significant changes in endogenous levels of oxidative DNA damage following treatment with 1 μ M DTPP. However, 1 μ M DTPP treatment resulted in significant (Student's t-test, 95% confidence levels) protection against H₂O₂ induced DNA damage. There was no significant difference in, the levels of oxidative DNA damage in cells derived from donors from the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. There was a consistent increase in levels of oxidative DNA damage with increase in concentration of H₂O₂ (0-250 μ M) treatment. Furthermore, the levels of oxidative DNA damage observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.2A & 6.2B).

Further studies conducted, to investigate the effect of 0.5 μ M DTPP on human peripheral blood mononuclear cells yielded results which were not significantly different from the effect of 1 μ M DTPP (Table 6.2), in case of peripheral blood mononuclear cells derived from donors from both age groups.

The effect of 6 hours of supplementation with 0.5 μ M or 1 μ M mitoQ / DTPP on the levels of endogenous oxidative DNA damage and on DNA damage levels following H₂O₂ treatment were not significantly different from the antioxidant potential after 3 hours of supplementation in cells derived from donors of both age groups (Tables 6.1 & 6.2).

The effect of 0.1 μ M mitoQ (Figures 6.3A & 6.3B) on the levels of endogenous oxidative DNA damage and on DNA damage levels following treatment with H₂O₂ in peripheral blood mononuclear cells was also investigated.

6.3A



6.3B

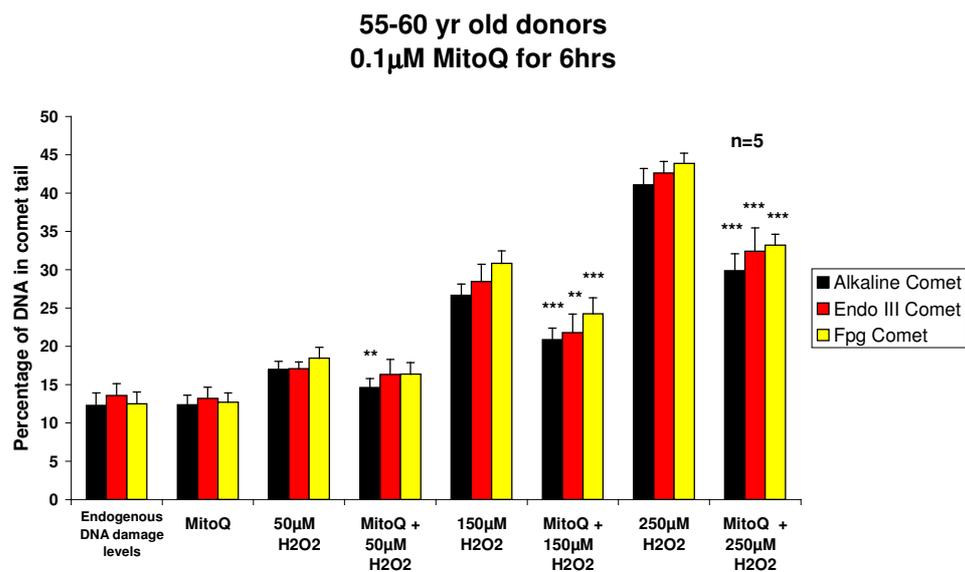


Figure 6.3 The effect of 0.1 μ M mitoQ on levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (6.3A) and 55-60 years (6.3B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

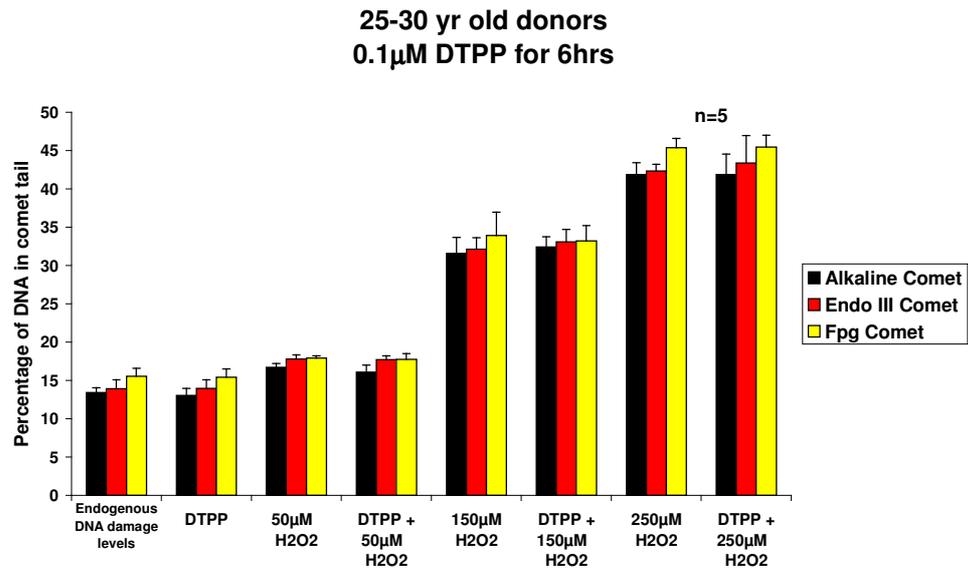
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ - Significantly lower than control levels

Figures 6.3A and 6.3B show no significant changes in endogenous levels of oxidative DNA damage in cells on treatment with 0.1 μ M mitoQ. However, 0.1 μ M mitoQ treatment resulted in significant (Student's t-test, 95% confidence levels) protection against H₂O₂ induced DNA damage. There was no significant difference in, the levels of oxidative DNA damage in cells derived from donors from the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. In all cases there was a consistent increase in levels of oxidative DNA damage with increase in concentration of H₂O₂ (0-250 μ M) treatment. The levels of oxidative DNA damage observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.3A & 6.3B).

Further studies conducted, to investigate the effect of 0.25 μ M mitoQ on human peripheral blood mononuclear cells yielded results which were not significantly different from the effect of 0.1 μ M mitoQ, in case of peripheral blood mononuclear cells derived from donors from both age groups (Table 6.1).

In the next part of the investigation, the impact of 0.1 μ M or 0.25 μ M DTPP (control for mitoQ) on human peripheral blood mononuclear cells were investigated. The effect of 0.1 μ M DTPP (Figures 6.4A & 6.4B) on the levels of endogenous oxidative DNA damage and on DNA damage levels following treatment with H₂O₂ in peripheral blood mononuclear cells is illustrated. Control (un-supplemented) human peripheral blood mononuclear cells were grown in parallel to the DTPP supplemented peripheral blood mononuclear cells.

6.4A



6.4B

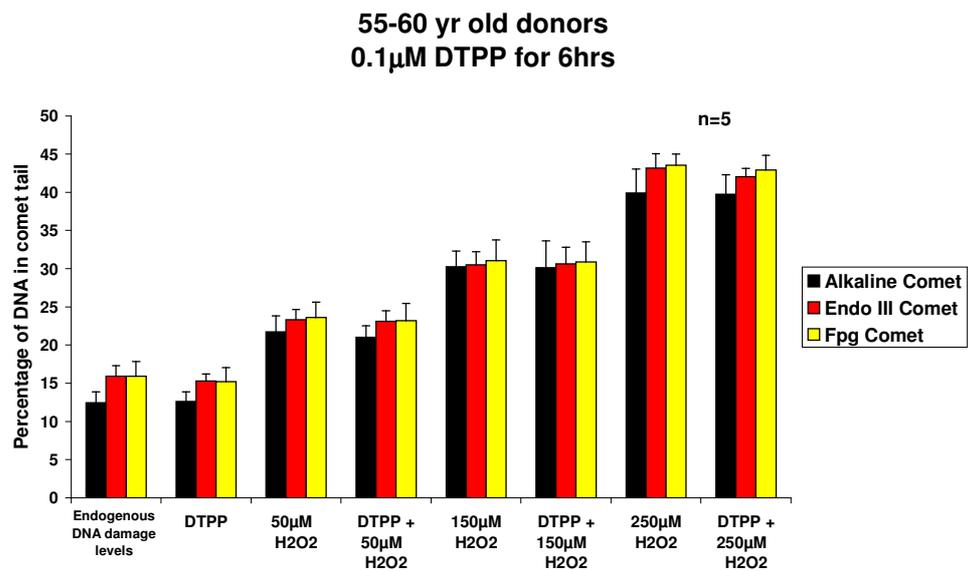


Figure 6.4 The effect of 0.1 μ M DTTP on levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (6.4A) and 55-60 years (6.4B), supplemented *ex vivo*. The bars indicate the mean \pm S.D.

Figures 6.4A and 6.4B did not reveal any significant changes in endogenous levels of oxidative DNA damage following 0.1 μ M DTPP treatment. In addition, 0.1 μ M DTPP did not result in any significant protection against H₂O₂ induced DNA damage. There was no significant difference in, the levels of oxidative DNA damage in cells derived from donors from the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. There was a consistent increase in levels of oxidative DNA damage with increase in concentration of H₂O₂ (0-250 μ M) treatment. The levels of oxidative DNA damage observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.4A & 6.4B).

The results of the investigation of the effect of 0.25 μ M DTPP on cells derived from donors of both group of ages were not significantly different from the effect of 0.1 μ M DTPP (Table 6.2).

In summary, the results of acute exposure to mitoQ (0.1 μ M, 0.25 μ M, 0.5 μ M or 1 μ M) afforded protection against H₂O₂ induced oxidative damage to DNA. In addition, 0.5 μ M or 1 μ M of mitoQ reduced levels of endogenous DNA damage in the various peripheral blood mononuclear cell samples derived from donors of both age groups in this investigation. DTPP at concentrations of 0.1 μ M or 0.25 μ M did not significantly alter the levels of oxidative DNA damage (endogenous or exogenously applied) .

Table 6.1. Summary of the results of the effect of different concentrations of mitoQ supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of mitoQ supplementation	Length of mitoQ supplementation	Endogenous DNA damage levels in mitoQ treated cells compared to untreated cells (controls)	DNA damage levels in mitoQ treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM mitoQ	3 hours	Significantly reduced	Significantly reduced
25-30	1µM mitoQ	6 hours	Significantly reduced	Significantly reduced
55-60	1µM mitoQ	3 hours	Significantly reduced	Significantly reduced
55-60	1µM mitoQ	6 hours	Significantly reduced	Significantly reduced
25-30	0.5µM mitoQ	6 hours	Significantly reduced	Significantly reduced
55-60	0.5µM mitoQ	6 hours	Significantly reduced	Significantly reduced
25-30	0.25µM mitoQ	6 hours	No significant changes	Significantly reduced
55-60	0.25µM mitoQ	6 hours	No significant changes	Significantly reduced

Table 6.2. Summary of the results of the effect of different concentrations of DTPP supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of DTPP supplementation	Length of DTPP supplementation	Endogenous DNA damage levels in DTPP treated cells compared to untreated cells (controls)	DNA damage levels in DTPP treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM DTPP	6 hours	No significant changes	Significantly reduced
55-60	1µM DTPP	6 hours	No significant changes	Significantly reduced
25-30	0.5µM DTPP	3 hours	No significant changes	Significantly reduced
25-30	0.5µM DTPP	6 hours	No significant changes	Significantly reduced
55-60	0.5µM DTPP	3 hours	No significant changes	Significantly reduced
55-60	0.5µM DTPP	6 hours	No significant changes	Significantly reduced
25-30	0.25µM DTPP	6 hours	No significant changes	No significant changes
55-60	0.25µM DTPP	6 hours	No significant changes	No significant changes

The results warranted the use of vitamin E, a lipophilic non-mitochondria targeted antioxidant as controls. The acute exposure of vitamin E at concentrations of 37µM or 74µM, on the levels of oxidative DNA damage (caused by endogenously produced and exogenously applied ROS) after 3 or 6 hours of supplementation in human peripheral blood mononuclear cells, as investigated revealed no antioxidant potential (Table 6.3).

Table 6.3. Summary of the results of the effect of different concentrations of vitamin E supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of vitamin E supplementation	Length of vitamin E supplementation	Endogenous DNA damage levels in vitamin E treated cells compared to untreated cells (controls)	DNA damage levels in vitamin E treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	37µM vitamin E	3 hours	No significant changes	No significant changes
25-30	37µM vitamin E	6 hours	No significant changes	No significant changes
55-60	37µM vitamin E	3 hours	No significant changes	No significant changes
55-60	37µM vitamin E	6 hours	No significant changes	No significant changes
25-30	74µM vitamin E	3 hours	No significant changes	No significant changes
25-30	74µM vitamin E	6 hours	No significant changes	No significant changes
55-60	74µM vitamin E	3 hours	No significant changes	No significant changes
55-60	74µM vitamin E	6 hours	No significant changes	No significant changes

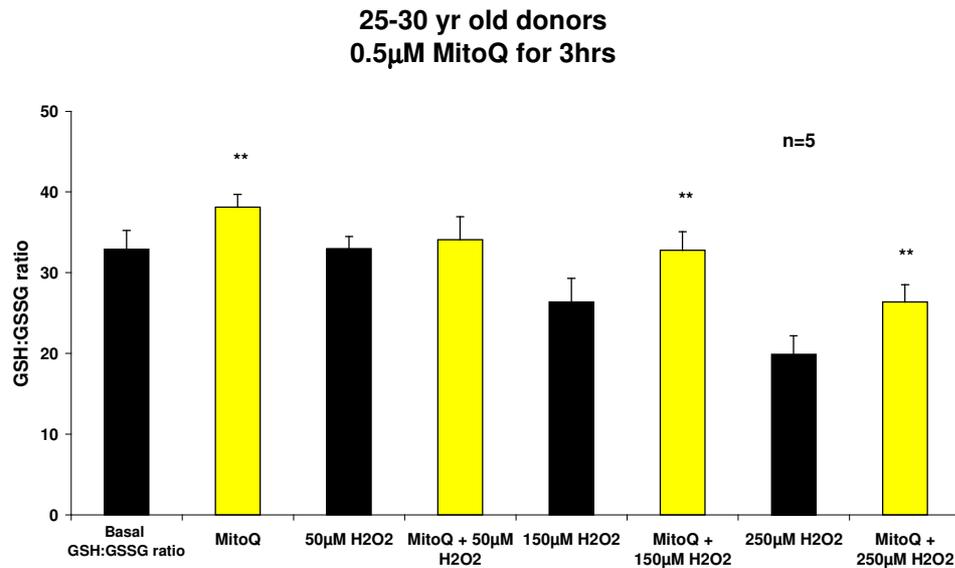
The overall summary of the effect of different concentrations of mitoQ, DTPP and vitamin E on the levels of oxidative DNA damage (endogenously produced and exogenously applied) in human peripheral blood mononuclear cells derived from donors of both age groups are illustrated in appendix-II.

Further investigations were conducted to examine the impact of chronic (up to three weeks of supplementation) exposure to mitoQ / DTPP on the levels of oxidative DNA damage in human peripheral blood mononuclear cells derived from six healthy, non-smoking males fasted overnight, three each in the age groups of 25-30 and 55-60 years. None of the concentrations of mitoQ or DTPP used (10nM, 20nM, 30nM) significantly altered the levels of oxidative DNA damage of human peripheral blood mononuclear cells *ex vivo* during their span in culture. These concentrations of mitoQ were chosen based on the existing scientific literature, showing antioxidant potential of mitoQ at these concentrations in human fibroblasts (Saretzki *et al.*, 2003).

6.1.3.2. The effect of mitoQ on intracellular GSH:GSSG ratio and total glutathione levels in human peripheral blood mononuclear cells

The effect of 0.5 μ M mitoQ on the GSH:GSSG ratio + / - H₂O₂ treatment in peripheral blood mononuclear cells derived from ten healthy, non-smoking males fasted overnight, five each in the age group of 25-30 years and 55-60 years, is illustrated (Figures 6.5A & 6.5B). Control (un-supplemented) human peripheral blood mononuclear cells were grown in parallel to the mitoQ supplemented peripheral blood mononuclear cells and the GSH:GSSG ratio was determined in both supplemented and non-supplemented cells.

6.5A



6.5B

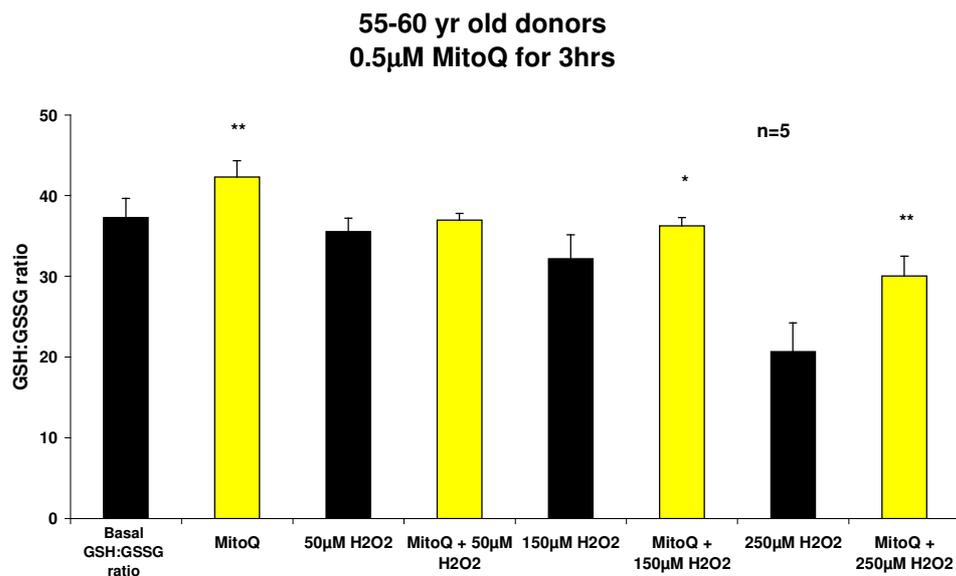


Figure 6.5 The effect of 0.5 μ M mitoQ on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age group of 25-30 years (6.5A) and 55-60 years (6.5B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

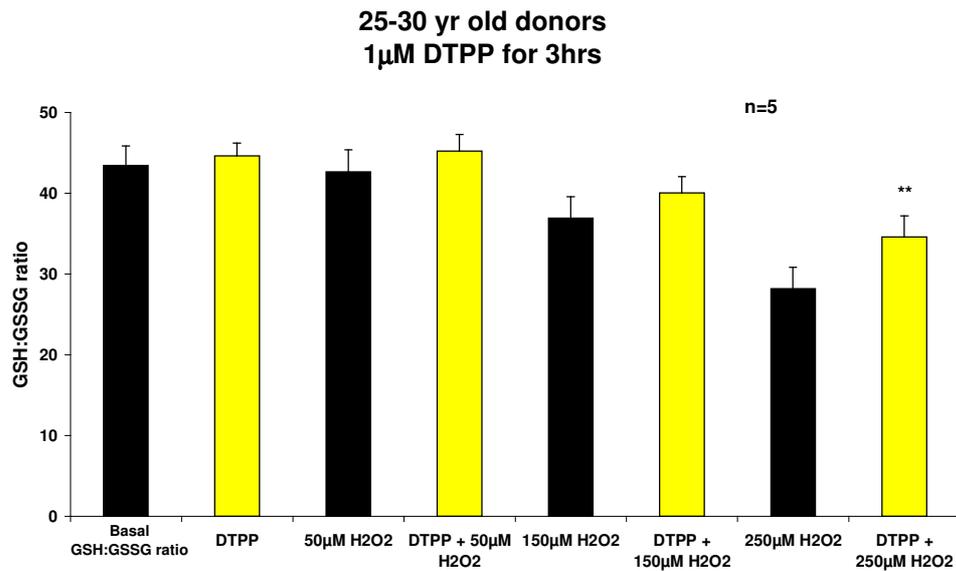
* $p < 0.05$, ** $p < 0.01$ - Significantly higher than control levels

Figures 6.5A and 6.5B show that 0.5 μ M mitoQ treatment resulted in a significantly higher GSH:GSSG ratio in peripheral blood mononuclear cells + / - H₂O₂ treatment. There was no significant difference in the levels of GSH:GSSG ratio in cells derived from donors of the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. In all cases, there was a consistent decrease in levels of GSH:GSSG ratio with increase in concentration of H₂O₂ (0-250 μ M) treatment. Furthermore, the GSH:GSSG ratios observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.5A & 6.5B).

The effect of 1 μ M mitoQ in peripheral blood mononuclear cells derived from donors from both age groups was similar to the effect of 0.5 μ M mitoQ (Table 6.4).

The effect of 1 μ M DTPP on the levels of GSH:GSSG ratio with and without treatment of H₂O₂ in peripheral blood mononuclear cells derived from donors of both age groups, is illustrated (Figures 6.6A & 6.6B). Control (un-supplemented) human peripheral blood mononuclear cells were grown in parallel to the DTPP supplemented peripheral blood mononuclear cells and the GSH:GSSG ratio was determined in both supplemented and non-supplemented cells.

6.6A



6.6B

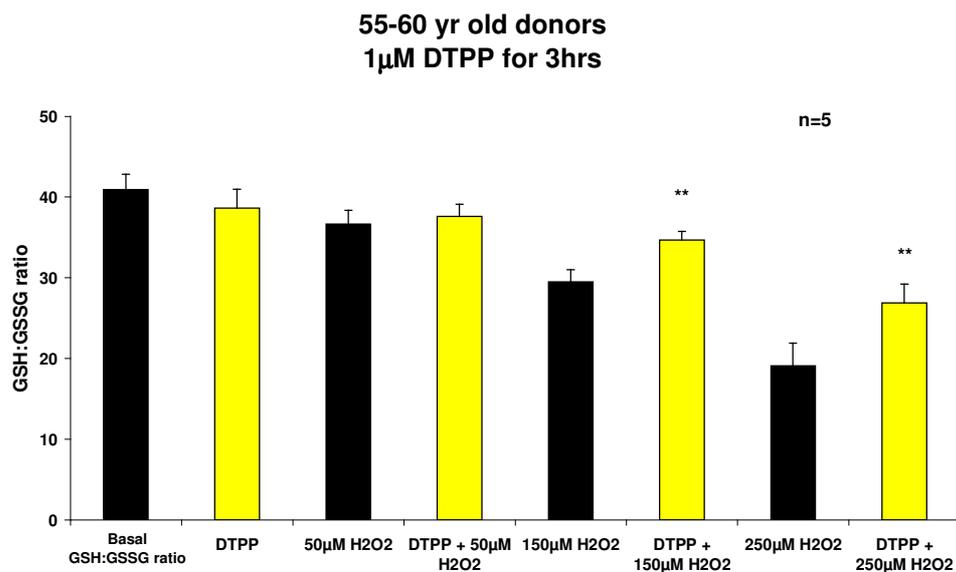


Figure 6.6 The effect of 1 μ M DTPP on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age group of 25-30 years (6.6A) and 55-60 years (6.6B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

** p<0.01 - Significantly higher than control levels

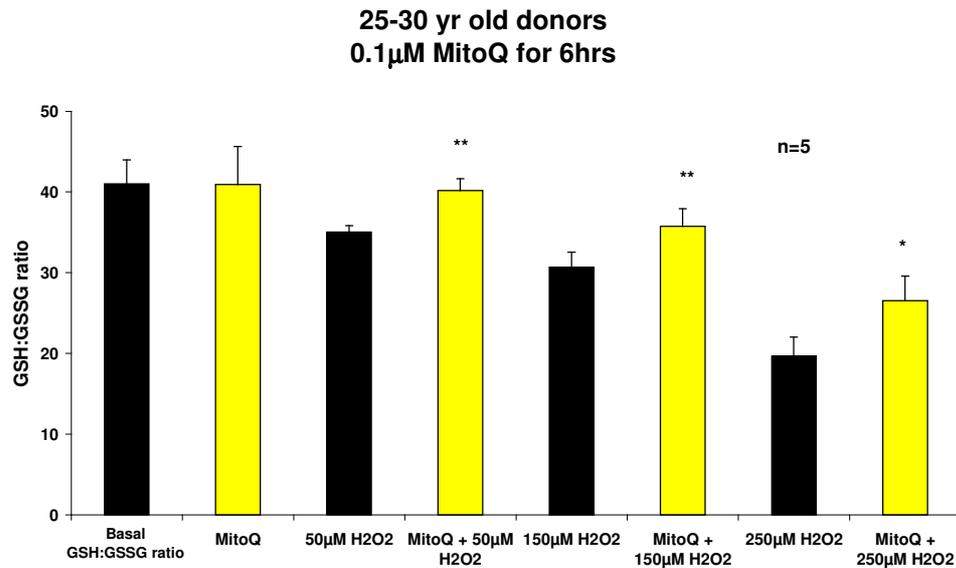
Figures 6.6A and 6.6B show that there were no significant changes in GSH:GSSG ratio (without exogenously applied ROS) on treatment with 1 μ M DTPP. However, 1 μ M DTPP treatment resulted in a significantly higher GSH:GSSG ratio in peripheral blood mononuclear cells treated with a range of doses of H₂O₂. There was no significant difference in GSH:GSSG ratio in cells derived from donors of the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. The results showed a consistent decrease in levels of GSH:GSSG ratio with increase in concentration of H₂O₂ (0-250 μ M) treatment. Furthermore, the GSH:GSSG ratios observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.6A & 6.6B).

The results of the investigation of the effect of a lower dosage of DTPP (0.5 μ M) on the GSH:GSSG ratio in human peripheral blood mononuclear cells revealed no significant differences from the results of the effect of 1 μ M DTPP in peripheral blood mononuclear cells derived from donors from both age groups (Table 6.5).

The effect of 0.5 μ M or 1 μ M mitoQ / DTPP after 6 hours of supplementation were not significantly different from the results obtained after 3 hours of supplementation in cells derived from donors of both age groups (Tables 6.4 & 6.5).

The effect of 0.1 μ M mitoQ (Figures 6.7A & 6.7B) on the levels of GSH:GSSG ratio with and without H₂O₂ in peripheral blood mononuclear cells was also investigated.

6.7A



6.7B

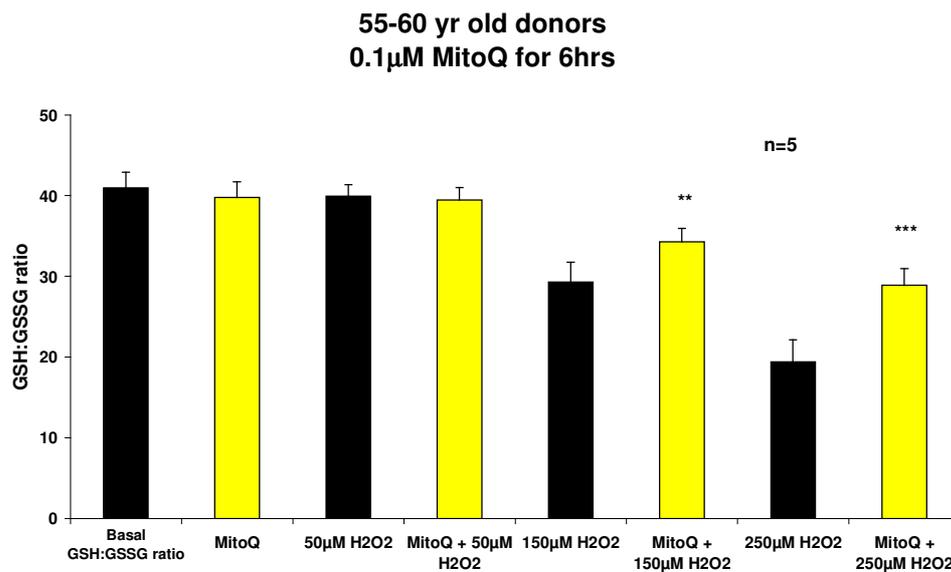


Figure 6.7 The effect of 0.1µM mitoQ on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age group of 25-30 years (6.7A) and 55-60 years (6.7B), supplemented *ex vivo*. The bars indicate the mean ± S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

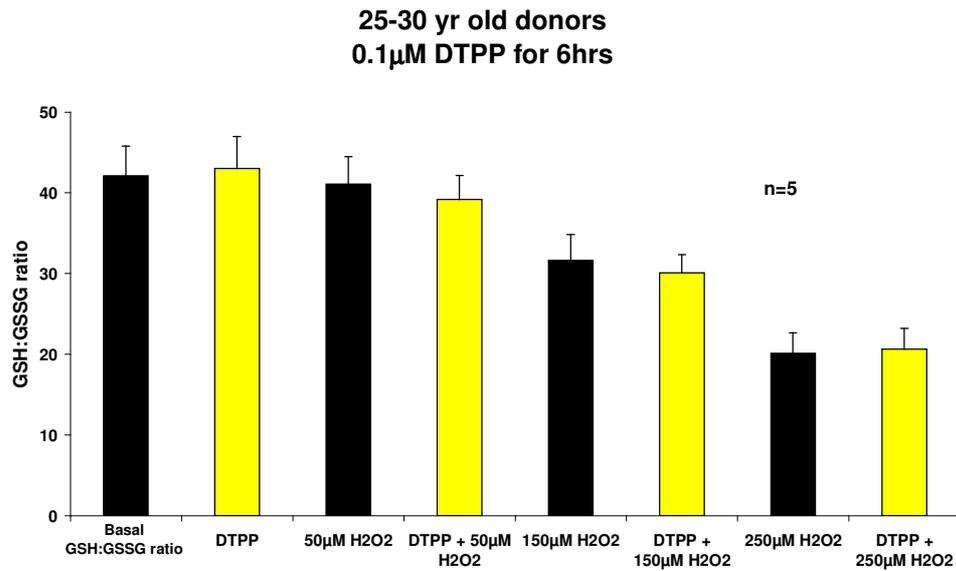
* p<0.05, ** p<0.01, *** p < 0.001 - Significantly higher than control levels

Figures 6.7A and 6.7B did not reveal any significant changes in GSH:GSSG ratio (without exogenously applied ROS) on treatment with 0.1 μ M mitoQ. However, 0.1 μ M mitoQ treatment resulted in significantly higher GSH:GSSG ratio in peripheral blood mononuclear cells treated with a range of doses of H₂O₂. There was no significant difference in the levels of GSH:GSSG ratio in cells derived from donors of the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. Furthermore, the results revealed a consistent decrease in GSH:GSSG ratio with increase in concentration of H₂O₂ (0-250 μ M) treatment. Furthermore, the GSH:GSSG ratios observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.7A & 6.7B).

Further studies investigating the effect of another dosage of mitoQ (0.25 μ M) on the GSH:GSSG ratio in human peripheral blood mononuclear cells revealed no significant differences from the results of the effect of 0.1 μ M mitoQ in peripheral blood mononuclear cells derived from donors from both age groups (Table 6.4).

In the next part of the investigation, the impact of 0.1 μ M or 0.25 μ M DTPP (control for mitoQ) on human peripheral blood mononuclear cells was investigated. The effect of 0.1 μ M DTPP (Figures 6.8A & 6.8B) on the GSH:GSSG ratio with and without H₂O₂ treatment in peripheral blood mononuclear cells is illustrated. Control (unsupplemented) human peripheral blood mononuclear cells were grown in parallel to the DTPP supplemented peripheral blood mononuclear cells.

6.8A



6.8B

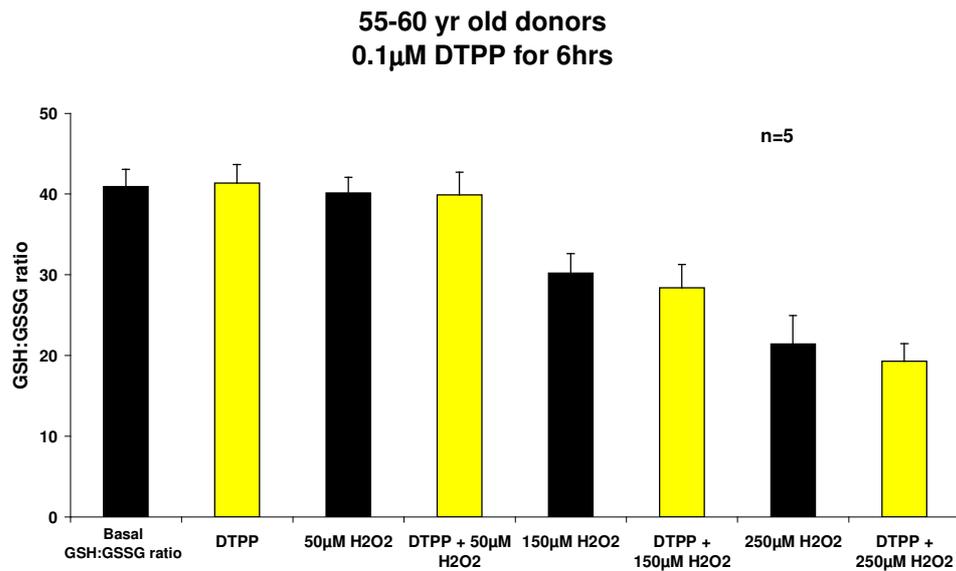


Figure 6.8 The effect of 0.1 μ M DTTP on intracellular GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age group of 25-30 years (6.8A) and 55-60 years (6.8B), supplemented *ex vivo*. The bars indicate the mean \pm S.D.

Figures 6.8A and 6.8B did not reveal any significant changes in the GSH:GSSG ratio with and without H₂O₂ treatment following 0.1µM DTPP treatment. There was no significant difference in the levels of GSH:GSSG ratio in cells derived from donors from the two age groups and the effect of H₂O₂ treatment in cells from either age group of donors. There was a consistent decrease in GSH:GSSG ratio with increase in concentration of H₂O₂ (0-250µM) treatment. Furthermore, the GSH:GSSG ratios observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.8A & 6.8B).

The results of the investigation of the effect of 0.25µM DTPP on cells derived from donors of both age groups were not significantly different from the effect of 0.1µM DTPP (Table 6.5).

In summary, the results of acute exposure to mitoQ (0.1µM, 0.25µM, 0.5µM or 1µM) revealed higher GSH:GSSG ratio in cells treated with exogenously applied ROS. In addition, 0.5µM or 1µM of mitoQ reduced the radical levels evidenced by significantly higher GSH:GSSG ratio in various peripheral blood mononuclear cell samples derived from donors of both age groups in this investigation. DTPP, at concentrations of 0.1µM or 0.25µM did not significantly alter the GSH:GSSG ratio in cells treated with and without H₂O₂.

Table 6.4. Summary of the results of the effect of different concentrations of mitoQ supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of mitoQ supplementation	Length of mitoQ supplementation	Basal GSH:GSSG ratio in mitoQ treated cells compared to untreated cells (controls)	GSH:GSSG ratio in mitoQ treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM mitoQ	3 hours	Significantly higher	Significantly higher
25-30	1µM mitoQ	6 hours	Significantly higher	Significantly higher
55-60	1µM mitoQ	3 hours	Significantly higher	Significantly higher
55-60	1µM mitoQ	6 hours	Significantly higher	Significantly higher
25-30	0.5µM mitoQ	6 hours	Significantly higher	Significantly higher
55-60	0.5µM mitoQ	6 hours	Significantly higher	Significantly higher
25-30	0.25µM mitoQ	6 hours	No significant changes	Significantly higher
55-60	0.25µM mitoQ	6 hours	No significant changes	Significantly higher

Table 6.5. Summary of the results of the effect of different concentrations of DTPP supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of DTPP supplementation	Length of DTPP supplementation	Basal GSH:GSSG ratio in DTPP treated cells compared to untreated cells (controls)	GSH:GSSG ratio in DTPP treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM DTPP	6 hours	No significant changes	Significantly higher
55-60	1µM DTPP	6 hours	No significant changes	Significantly higher
25-30	0.5µM DTPP	3 hours	No significant changes	Significantly higher
25-30	0.5µM DTPP	6 hours	No significant changes	Significantly higher
55-60	0.5µM DTPP	3 hours	No significant changes	Significantly higher
55-60	0.5µM DTPP	6 hours	No significant changes	Significantly higher
25-30	0.25µM DTPP	6 hours	No significant changes	No significant changes
55-60	0.25µM DTPP	6 hours	No significant changes	No significant changes

Furthermore, vitamin E was used as controls. The acute exposure of vitamin E at concentrations of 37µM or 74µM, on the levels of the GSH:GSSG ratio after 3 or 6 hours of supplementation in human peripheral blood mononuclear cells treated with and without H₂O₂ is revealed in table 6.6.

Table 6.6 Summary of the results of the effect of different concentrations of vitamin E supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of vitamin E supplementation	Length of vitamin E supplementation	Basal GSH:GSSG ratio in vitamin E treated cells compared to untreated cells (controls)	GSH:GSSG ratio in vitamin E treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	37µM vitamin E	3 hours	No significant changes	No significant changes
25-30	37µM vitamin E	6 hours	No significant changes	No significant changes
55-60	37µM vitamin E	3 hours	No significant changes	No significant changes
55-60	37µM vitamin E	6 hours	No significant changes	No significant changes
25-30	74µM vitamin E	3 hours	No significant changes	No significant changes
25-30	74µM vitamin E	6 hours	No significant changes	No significant changes
55-60	74µM vitamin E	3 hours	No significant changes	No significant changes
55-60	74µM vitamin E	6 hours	No significant changes	No significant changes

The results of the supplementation of vitamin E at concentrations of 37µM or 74µM, on the levels of the GSH:GSSG ratio after 3 or 6 hours of supplementation in human peripheral blood mononuclear cells treated with and without H₂O₂ revealed no significant changes compared to controls (Table 6.6).

The overall summary of the effect of different concentrations of mitoQ, DTPP and vitamin E on the GSH:GSSG ratio (basal levels and GSH:GSSG ratio after treatment of cells with exogenously applied ROS) in human peripheral blood mononuclear cells derived from donors of both age groups are illustrated in appendix-II.

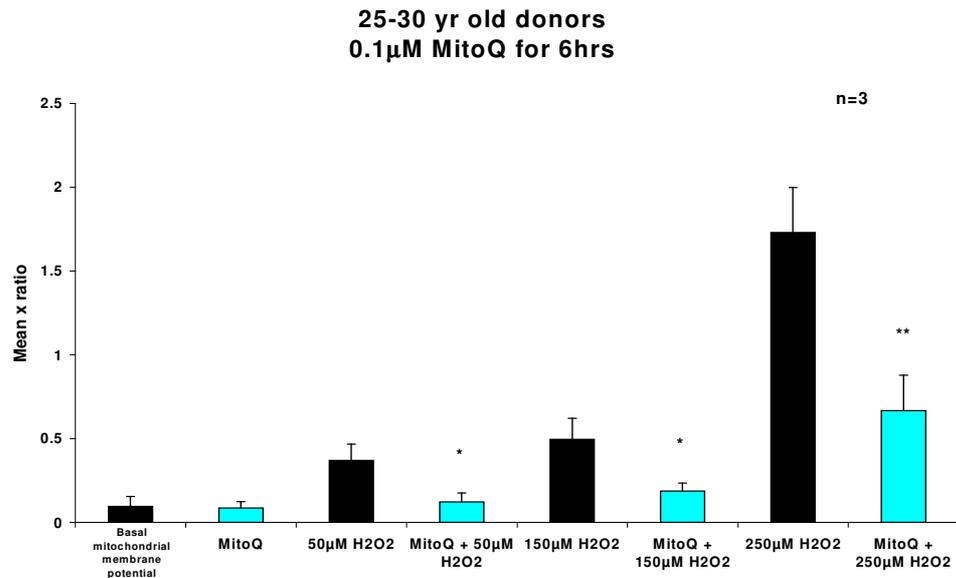
The results of the investigation of the impact of chronic (up to three weeks of supplementation) exposure to mitoQ / DTPP on the levels of GSH:GSSG ratio revealed that none of the concentrations of mitoQ or DTPP applied in this study (10nM, 20nM, 30nM) significantly altered the levels of GSH:GSSG ratio in human peripheral blood mononuclear cells *ex vivo* derived from donors of both age groups.

In terms of total glutathione levels, there were no consistent changes on supplementation with any of the concentrations of mitoQ (0.1µM, 0.25µM, 0.5µM, 1µM) or DTPP (0.1µM, 0.25µM, 0.5µM, 1µM) or vitamin E (37µM or 74µM) in human peripheral blood mononuclear cells treated with and without a range of doses of H₂O₂ in peripheral blood mononuclear cells derived from donors from both age groups.

6.1.3.3. The effect of mitoQ on mitochondrial membrane potential in human peripheral blood mononuclear cells

The effect of 0.1µM mitoQ (Figures 6.9A & 6.9B) or 0.5µM mitoQ on mitochondrial membrane potential in human peripheral blood mononuclear cells derived from six healthy, non-smoking males fasted overnight, three each in the age group of 25-30 years and 55-60 years, is illustrated. The mitochondrial membrane potential was measured with and without H₂O₂ treatment after 6 hours of supplementation with mitoQ. The mitochondrial membrane potential was determined in both supplemented and non-supplemented cells.

6.9A



6.9B

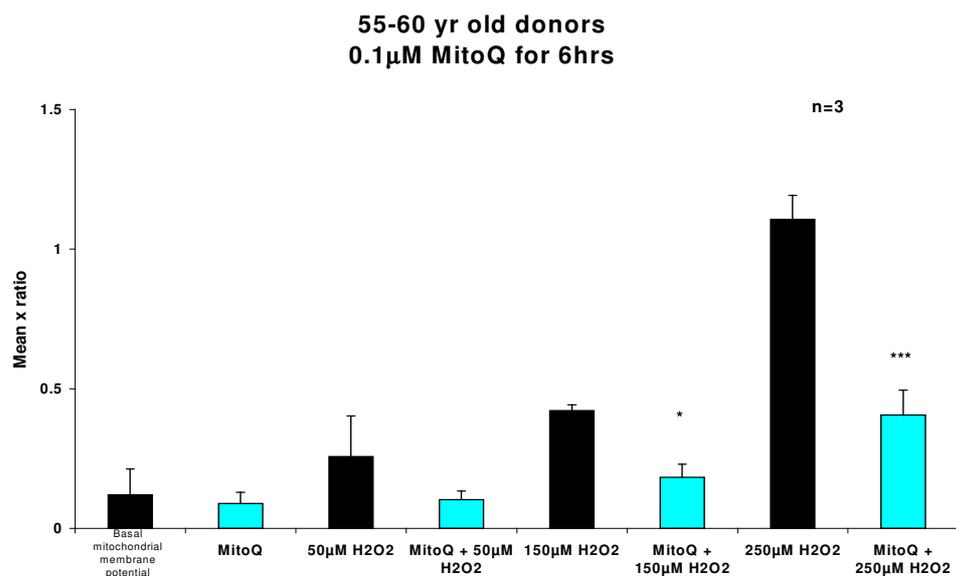


Figure 6.9 The effect of 0.1 μ M mitoQ on mitochondrial membrane potential in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years (6.9A) and 55-60 years (6.9B), supplemented *ex vivo*. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ - Significantly lower than control levels

Figures 6.9A and 6.9B indicate no significant changes in mitochondrial membrane potential on treatment with 0.1 μ M mitoQ. However, 0.1 μ M mitoQ treatment resulted in a significant increase (Student's t-test, 95% confidence levels) in mitochondrial membrane potential in peripheral blood mononuclear cells treated with a range of doses of H₂O₂. There was no significant difference in the levels of mitochondrial membrane potential in cells derived from donors of the two age groups and in the effect of H₂O₂ treatment in cells from either age group of donors. In all cases there was a consistent decrease in levels of mitochondrial membrane potential with increase in concentration of H₂O₂ (0-250 μ M). The mitochondrial membrane potential observed between human peripheral blood mononuclear cells derived from each of the five different donors in each of the above cases were not significantly different (Figures 6.9A & 6.9B).

Further studies carried out on the effect of 0.5 μ M mitoQ on the mitochondrial membrane potential in cells derived from donors of both age groups revealed similar set of results to those of 0.1 μ M mitoQ supplementation.

6.1.4. Discussion

Mitochondrial dysfunction contributes to a wide range of diseases in humans (Chapter-1, Section 1.1.6.3; Chapter-6, Section 6.1.1). The main problem in treating those diseases is the inability to target an antioxidant *in vivo* to the mitochondria. This problem was rectified by the development of mitoQ. The mechanism of action of mitoQ has been described elsewhere in this thesis (Chapter-1, Section 1.1.6.3; Chapter-6, Section 6.1.1).

Overall, the results of this investigation revealed that 0.5 μ M or 1 μ M mitoQ supplementation decreased the levels of free radicals in human peripheral blood mononuclear cell samples, from the donors of both age groups (25-30 and 55-60 year olds) evidenced by the significantly higher GSH:GSSG ratios (Figures 6.5A & 6.5B), compared to controls. Furthermore, the mitoQ supplementation decreased the levels of endogenous oxidative DNA damage (Figures 6.1A & 6.1B) in cell samples derived from both age groups. Lower concentrations of mitoQ (0.1 μ M or 0.25 μ M) did not have any impact on the GSH:GSSG ratio (Figures 6.7A & 6.7B) or on the levels of endogenous oxidative DNA damage (Figures 6.3A & 6.3B), in human peripheral blood mononuclear cell samples from the donors of both age groups.

All concentrations of mitoQ used in this investigation (0.1 μ M, 0.25 μ M, 0.5 μ M or 1 μ M) resulted in a significantly higher GSH:GSSG ratio and a significant decrease in oxidative DNA damage levels following treatment with H₂O₂ in mitoQ supplemented cell samples. The results were interesting because although mitoQ is a mitochondria targeted antioxidant, it demonstrated significant antioxidant potential in the cytosol. The antioxidant potential of 1 μ M concentration of mitoQ outside the mitochondria evidenced by their effect on exogenously applied ROS, in this investigation was in line with the results of previous findings revealing blockage of 150 μ M H₂O₂ induced caspase activation in Jurkat cells by 1 μ M mitoQ (Kelso *et al.*, 2001). The reason for the antioxidant potential outside the mitochondria may be that the mitochondria took up as much mitoQ as possible and the excess mitoQ accumulated in the cytosol with consequent radical lowering effects. Even though mitochondria are the major site for the production of ROS in cells, the results suggest that there are other sources of contributors to genomic DNA damage since endogenous oxidative DNA damage remains even at saturating concentrations of mitoQ. It could also be that the lower concentrations of mitoQ (0.1 μ M or 0.25 μ M) may be taken up by the mitochondria, but they were not sufficient to reduce free radical release from the mitochondria explaining no significant differences in endogenous DNA damage levels and the lack of changes to GSH:GSSG ratio.

The ability of mitoQ at specific concentrations to scavenge radicals, resulting in a significantly higher GSH:GSSG ratio compared to controls, is invaluable considering the role of intracellular redox status (GSH:GSSG ratio) as a mediator of apoptosis in several cell systems (Slater *et al.*, 1996; Cotgreave and Gerdes, 1998; Hall, 1999). GSH is a main determinant of intracellular redox status, and is known to become depleted before the onset of apoptosis (Beaver and Waring, 1995; Macho *et al.*, 1997; Ghibelli *et al.*, 1998). GSH is abundant in the cell (Schafer and Buettner, 2001), serving as an indicator of the cellular redox environment. The redox status of the cell is altered on oxidation of even a small amount of GSH. Oxidation of GSH results in GSSG coupled with decrease in the levels of GSH leading to the lowering of the GSH:GSSG ratio, suggested to be responsible for several human diseases (Chapter-1, Section 1.1.4.1.1). The higher GSH:GSSG ratio on mitoQ supplementation represents an increased

antioxidant status in cells that further explains the decrease in levels of oxidative DNA damage in cells on mitoQ supplementation.

DTPP is a lipophilic mitochondria targeted compound having a similar structure to mitoQ, except for the ubiquinone derivative in mitoQ. The ubiquinone derivative is responsible for the antioxidant capability of mitoQ. Rather surprisingly, DTPP (0.5 μ M or 1 μ M) significantly decreased the DNA damage levels and reduced the radical levels (evidenced by significantly higher GSH:GSSG ratio) following treatment with H₂O₂ (treatment after 3 or 6 hours of supplementation with DTPP) in peripheral blood mononuclear cell samples from both age groups. It may be that DTPP stimulated or up-regulated other cellular antioxidants although there is no scientific evidence to support this hypothesis. According to existing scientific literature, only one previous study has shown the ability of 1 μ M DTPP to reduce succinate (a complex I substrate) driven ROS in isolated bovine aortic endothelial mitochondria in rats (Malley *et al.*, 2006). But the mechanism was not explained. Lower concentrations of DTPP (0.1 μ M or 0.25 μ M) did not have any significant impact on free radical levels (GSH:GSSG ratio unchanged) and on DNA damage levels with and without H₂O₂ treatment in peripheral blood mononuclear cell samples from both age groups examined.

Recent studies revealed the ability of vitamin E at a concentration of 37 μ M to reduce toxicity in murine splenocytes (Horton *et al.*, 2007). In this investigation, the impact of 37 μ M or 74 μ M concentrations of vitamin E (a non-mitochondria targeted antioxidant) on the levels of free radicals (determining the GSH:GSSG ratio) and oxidative DNA damage, in human peripheral blood mononuclear cells, was investigated. None of the concentrations of vitamin E resulted in significant changes in the levels of oxidative DNA damage or GSH:GSSG ratio in human peripheral blood mononuclear cells treated + / - H₂O₂. Even though vitamin E at 37 μ M concentration was able to reduce toxicity in murine cells, the concentrations used in this study may not be enough to reveal an antioxidant potential or free radical scavenging potential in human peripheral blood mononuclear cells *ex vivo*. Vitamin E targets the cell membranes since it is lipophilic and is known to prevent their oxidation. The reason for the lack of effect of vitamin E supplementation in human peripheral blood mononuclear cells compared to the murine cells could be due to the differences in cell types. It could be that the concentrations of

vitamin E used in this study may have been too low to cause an effect on the markers investigated in this study and these low concentrations could have been taken up by the cell membranes. Further investigations could be carried out using a higher range of vitamin E concentrations and analyse their impact on the markers of T cell integrity used in this study. Of further interest would be to determine the ROS production for different concentrations of vitamin E supplementations using flowcytometric analysis of samples subjected to 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (DCFDA) staining.

Investigations on the impact of a range of concentrations of mitoQ / DTPP / vitamin E on total glutathione levels, a marker of T cell integrity, revealed a lack of consistent changes in levels of total glutathione on administration of any of the concentrations of mitoQ or DTPP (0.1 μ M, 0.25 μ M, 0.5 μ M, 1 μ M) or vitamin E (37 μ M or 74 μ M) in human peripheral blood mononuclear cells derived from donors of both age groups, in this investigation. This result reveals that none of these supplements in any way had an impact on glutathione pathways, and therefore the lack of significant changes in glutathione levels, in the mononuclear cells examined.

Mitochondrial membrane potential is another marker of T cell integrity that plays an important role in apoptosis (Chapter-2, Section 2.1.8). The results of this investigation indicated no significant changes in mitochondrial membrane potential in human peripheral blood mononuclear cells on treatment with 0.1 μ M or 0.5 μ M mitoQ. However, 0.1 μ M (Figures 6.9A & 6.9B) or 0.5 μ M mitoQ treatment resulted in a significant increase in mitochondrial membrane potential on treatment with a range of doses of H₂O₂ in cells derived from donors of both age groups. In the existing scientific literature only one previous study has revealed the maintenance of mitochondrial membrane potential on supplementation with 1 μ M mitoQ. This study was performed *in vitro* in isolated mitochondria in rat models where 1 μ M mitoQ treatment decreased ROS production, thereby, protecting the mitochondria from damage and resulted in maintenance of the mitochondrial membrane potential (Lowe *et al.*, 2008). Furthermore, mitoQ suppressed the release of pro-inflammatory cytokines and increased the release of anti-inflammatory cytokines from the cells in the *in vitro* study conducted in rat models.

Previous studies have revealed antioxidant potential on chronic exposure to mitoQ at concentrations of 10-20nM in MRC-5 fibroblasts evident by telomere shortening (Saretzki *et al.*, 2003). In this investigation the impact of chronic exposure of 10-30nM concentrations of mitoQ / DTPP in human peripheral blood mononuclear cells derived from donors of both age groups was investigated. None of the concentrations of mitoQ or DTPP (10nM, 20nM or 30nM) caused any significant alterations which was reflected in a lack of change to free radical levels (unchanged GSH:GSSG ratio), oxidative DNA damage levels and total glutathione levels. From the little information available in the existing scientific literature, on the effects of chronic exposure of mitoQ in cells *in vitro* and *ex vivo*, only one other recent study investigated the effects of chronic exposure of mitoQ. However, the study was performed in isolated mitochondria. The results revealed the potency of 1 μ M mitoQ after a week of supplementation to decrease oxidative stress, evidenced by a decrease in mitochondrial damage and maintenance of mitochondrial membrane potential in isolated mitochondria in human achilles tendon cells (Lowe *et al.*, 2009).

MitoQ has revealed significant antioxidant potential previously in other cell and animal models. The findings of this current investigation in human peripheral blood mononuclear cells are consistent with the earlier findings revealing the antioxidant potential of mitoQ. Although mitochondria are the major site for the production of ROS in cells, there may be other potential sources that are major contributors of genomic DNA damage. Well functioning mitochondria is a pre-requisite for human life. A decrease in the respiratory function of mitochondria in human tissues and an increase in mtDNA damage are some of the age related molecular changes observed within mitochondria affecting mitochondrial integrity and function (Hayakawa *et al.*, 1993). However, this study has shown that the use of carefully selected concentrations of mitoQ may be a viable strategy to decrease harmful levels of free radicals in human peripheral blood mononuclear cells *ex vivo*, to help manage oxidative stress levels and elements of the resultant age-related decline in T cell structure and function.

CHAPTER-7

**An investigation of the impact of
low O₂ tension on cultured human
peripheral blood mononuclear cells**

7.1. An investigation of the impact of low O₂ tension on cultured human peripheral blood mononuclear cells

7.1.1. Introduction

An investigation into the effects of reduced O₂ tension on T cells was carried out as part of collaboration with the Centre for Medical Research, Tuebingen Ageing and Tumor Immunology Group, University of Tuebingen, Germany. The work was done as part of the research project funded by The European Union 5th framework T cells in aging (T-CIA). The group of Barnett has collaborated with the Tuebingen Ageing and Tumor Immunology group, led by Professor Graham Pawelec for nearly ten years.

T cell models *ex vivo* and *in vitro* have been invaluable in immunological research, enabling the generation of huge amount of information about physiological and pathological T cell behaviours. Studies with T cell models enable the mimicking of *in vivo* conditions. Most of the experiments carried *ex vivo* and *in vitro* are with 5% CO₂, 95% air (20% O₂) (Hyland *et al.*, 2000; 2001; de Souza, 2007). The percentage of O₂ in these systems is much higher than that encountered by T cells *in vivo*. The O₂ levels in peripheral blood are much lower than in air. The levels of O₂ also depend on the size of the arteries. The smaller the arteries the lower the O₂ level. Regardless of the existing discrepancies, there is a consensus that the O₂ level experienced by T cells may vary from 1% to 10% (Caldwell *et al.*, 2001; Atkuri *et al.*, 2007).

ROS have been implicated to be responsible for the intrinsic oxidative damage in cells, ultimately playing an important role in ageing and the development of age related pathologies. Many different studies have elaborated the importance of O₂ tension to cellular senescence and lifespan (Chapter-1, summarised in section 1.1.6.4). Previous work from the group of Barnett demonstrated a shorter lifespan and reduced proliferative capacity in T cell clones derived from young and old human donors when cultured in fewer than 6% O₂ tension, compared to clones cultured fewer than 20% O₂ tension (Chapter-1, Table 1.13 & Table 1.14). The oxidative damage to DNA in T cell clones cultured at 6% O₂ tension remained low throughout their lifespan, compared to the clones cultured at 20% O₂ tension due to lower levels of ROS (Chapter-1, Figure 1.16; Duggan *et al.*, 2004). Even though ROS are pre-dominantly associated with

causing detrimental effects on cellular molecules, they do act as signals / mediators of cell division within T cells (summarized in chapter-1, Section 1.1.6.4). Limited ROS levels, fewer than 6% O₂ may have led to the reduction in proliferation in T cell clones compared to clones maintained at 20% O₂ tension. Other studies have also reported loss of T cell proliferative capacity with low O₂ levels (Atkuri *et al.*, 2007). In the study reported in this chapter the impact of low O₂ tension on human peripheral blood mononuclear cells *ex vivo* was investigated.

7.1.2. Materials and Methods

7.1.2.1. Culture of human peripheral blood mononuclear cells (work carried out by Larbi *et al.*, as part of the collaboration)

Blood samples derived from three different healthy middle aged individuals at the University Hospital Blood Bank, courtesy of Prof. D. Wernett, were processed using FicoLite-H (Linaris, Germany) to separate human peripheral blood mononuclear cells. The *ex vivo* human peripheral blood mononuclear samples were then re-suspended in serum free culture medium X-Vivo 10 at concentrations of 1-5 x 10⁶ cells/ml. The samples were left unstimulated or stimulated with anti-CD3 and anti-CD28 coated beads (Dynabeads, Karlsruhe). Stimulation of T cells were carried out to enhance their growth. Samples were prepared in duplicates, with half being placed in the hypoxia work station in 2% O₂ balanced with nitrogen and the other half in a regular incubator where cells were grown in air supplemented with 5% CO₂ for 1 to 4 days. The samples were frozen down for analysis at the end of every day (Chapter-2, Section 2.1.1.2).

7.1.2.2. Assessment of levels of oxidative DNA damage

The levels of oxidative DNA damage in human peripheral blood mononuclear samples *ex vivo* maintained for 1 to 4 days at 2% or 20% O₂ tension culture conditions were measured at NTU using the modified alkaline comet assay (Chapter-2, Section 2.1.10).

7.1.2.3. GSH:GSSG ratio

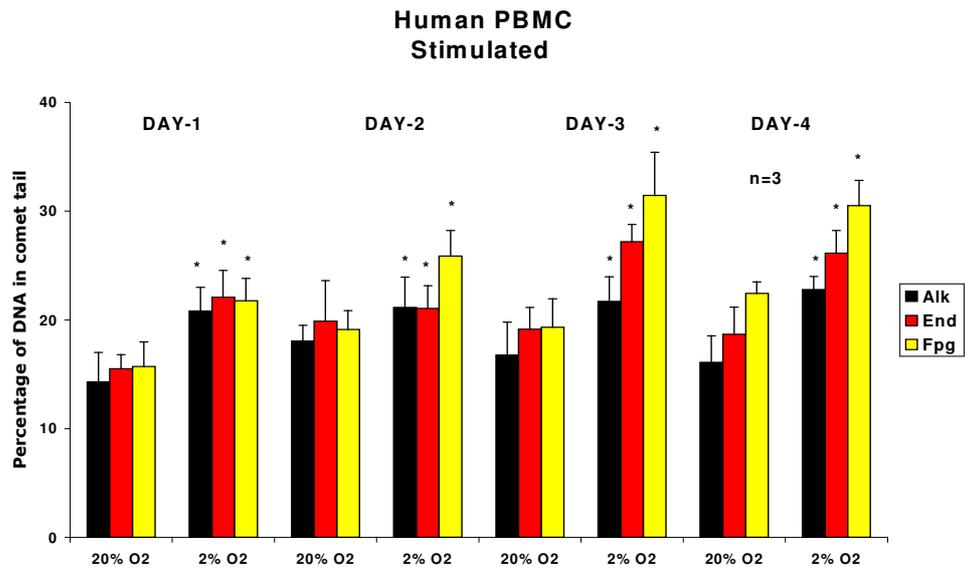
The GSH:GSSG ratio in human peripheral blood mononuclear samples *ex vivo* under both O₂ tensions was measured at NTU using the GSH:GSSG ratio assay kit (Chapter-2, Section 2.1.11).

7.1.3. Results

7.1.3.1. The effect of low O₂ tension on levels of oxidative DNA damage in human peripheral blood mononuclear cells *ex vivo*

Aliquots of unstimulated or stimulated human peripheral blood mononuclear samples *ex vivo* were taken from culture maintained at 2% or 20% O₂ tension conditions at the end of each day from day 1 to day 4. Figure 7.1A shows the oxidative DNA damage levels at the end of each day from day 1 to 4 of human peripheral blood mononuclear cells stimulated with beads. Figure 7.1B shows the oxidative DNA damage levels at the end of each day from day 1 to 4 of unstimulated human peripheral blood mononuclear cells.

7.1A



7.1B

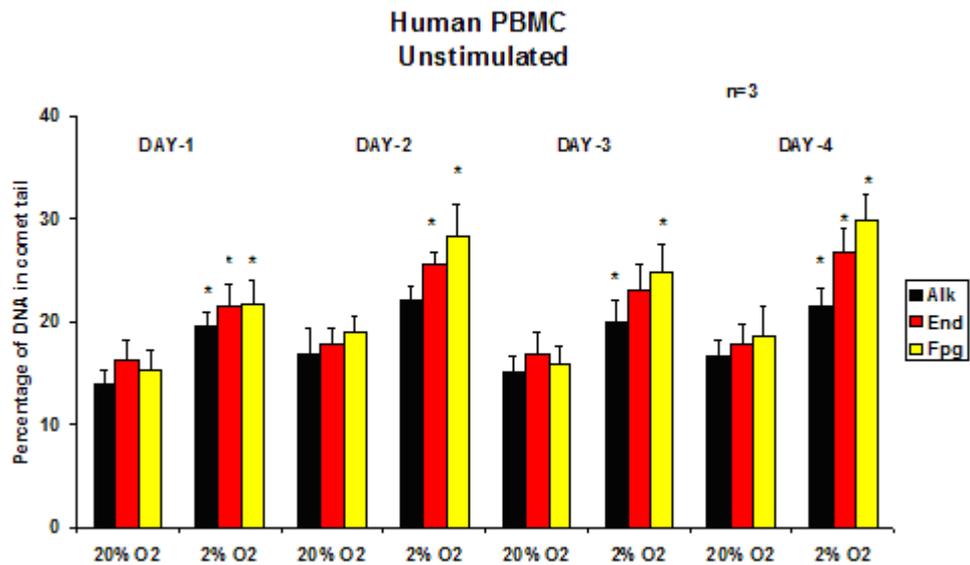


Figure 7.1 The impact of 2% or 20% O₂ tension on oxidative DNA damage levels at the end of each day, days 1 to 4 of culture, in human peripheral blood mononuclear cells derived from three different middle aged donors stimulated (A) and unstimulated (B) cells. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterisk.

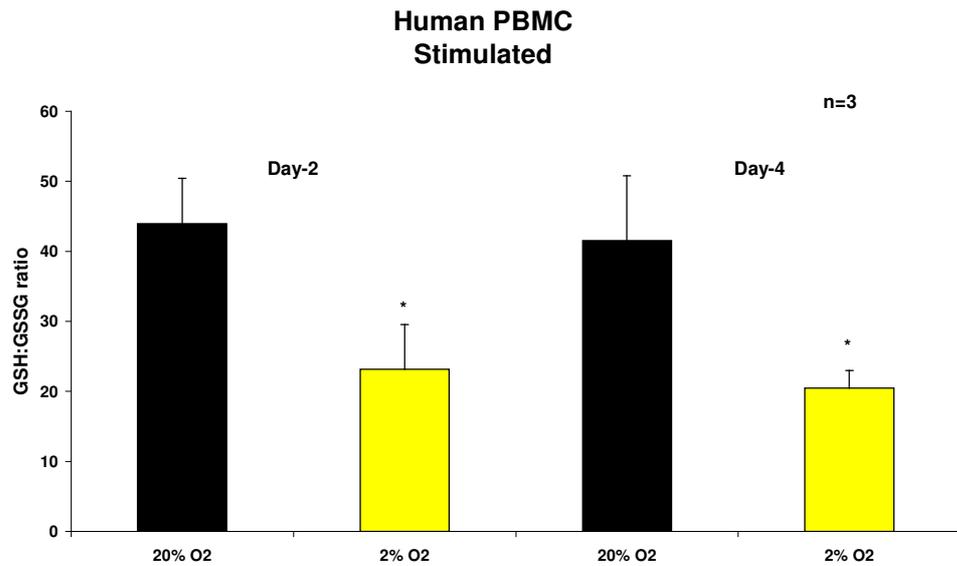
* $p < 0.05$ - Significantly higher than damage levels in samples maintained at 20% O₂.

Figure 7.1A and 7.1B shows a significantly higher level of oxidative DNA damage in human peripheral blood mononuclear cells maintained at 2% O₂ tension culture conditions compared 20% O₂ tension culture conditions. Significantly higher levels of oxidative damage to DNA were observed in stimulated (Figure 7.1A) and unstimulated samples (Figure 7.1B) maintained at 2% O₂, at the end of each day from 1 to 4. There was no significant difference in the percentage of DNA in the comet tail in unstimulated and stimulated samples for any of the culture conditions.

7.1.3.2. The effect of low O₂ tension on the GSH:GSSG ratio in human peripheral blood mononuclear cells

Aliquots of unstimulated or stimulated human peripheral blood mononuclear samples *ex vivo* were taken from culture maintained at 2% or 20% O₂ tension conditions at the end of day 2 and day 4. Figure 7.2A shows the results of the effect of 2% or 20% O₂ tension on the GSH:GSSG ratio at the end of day 2 and day 4 of human peripheral blood mononuclear cells stimulated with beads. Figure 7.2B shows the results of the effect of 2% or 20% O₂ tension on the GSH:GSSG ratio at the end of day 2 and day 4 of unstimulated human peripheral blood mononuclear cells.

7.2A



7.2B

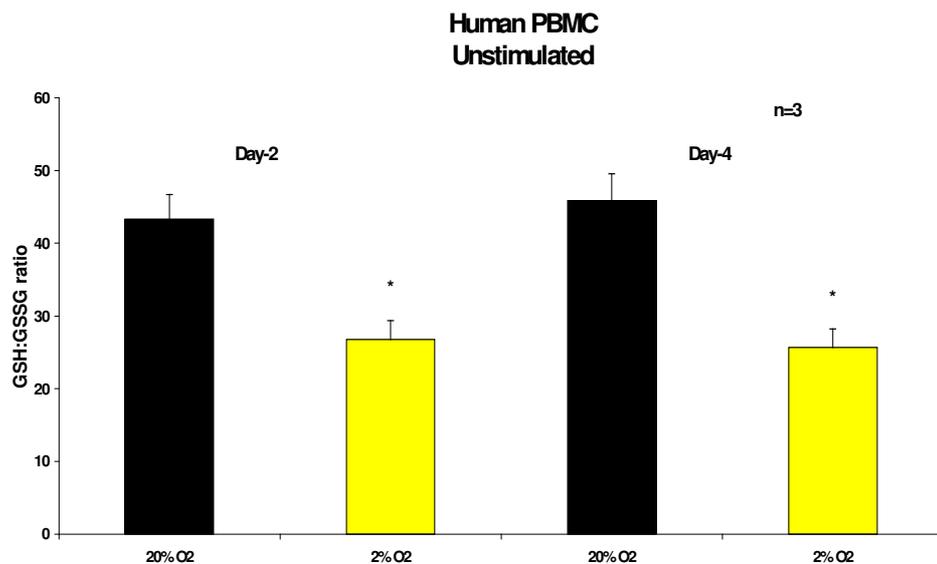


Figure 7.2 The impact of 2% or 20% O₂ tension on the GSH:GSSG ratio of human peripheral blood mononuclear cells derived from three different middle aged donors stimulated (A) and unstimulated (B) cells at the end of day 2 and day 4 in culture. The bars indicate the mean \pm S.D. Values statistically different from their controls (Student's t-test, 95% confidence level) are indicated with an asterix.

* $p < 0.05$, *** $p < 0.001$ - Significantly lower GSH:GSSG ratio compared to cells maintained at 20% O₂ tension culture conditions.

Figures 7.2A and 7.2B shows a significantly lower level of GSH:GSSG ratio in human peripheral blood mononuclear cells maintained at 2% O₂ tension culture conditions compared 20% O₂ tension culture conditions. Significantly lower level of GSH:GSSG ratio were observed in stimulated (Figure 7.2A) and unstimulated samples (Figure 7.2B) maintained at 2% O₂, at the end of days 2 and 4. There was no significant difference in the GSH:GSSG ratio in stimulated samples when compared to untreated samples in any of the culture conditions.

7.1.4. Discussion

Prior to this investigation being carried out, there was little information in the scientific literature on the impact of low O₂ tension culture conditions in human peripheral blood mononuclear cells *ex vivo* and *in vitro*. It is important to study the effect of low O₂ tension in studies investigating the effect of oxidative stress considering the fact that the intracellular O₂ level is much lower than the O₂ level in the cells maintained in an extracellular environment. Previous studies have shown that cell culture media in air contained 2% of O₂ as rapidly as 4 hours after they were transferred from air (20% O₂) to a 2% O₂ incubator proving that extracellular environment of cells rapidly equilibrated to the ambient O₂ level on transfer to low O₂ conditions (Newby *et al.*, 2005).

The results of the study reported in this chapter revealed a significantly lower levels of oxidative DNA damage and significantly higher GSH:GSSG ratio in human peripheral blood mononuclear cells (stimulated or unstimulated) maintained at 20% O₂ tension culture conditions, compared to cells maintained at 2% O₂ tension culture conditions.

In addition to the two markers of T cell integrity investigated in NTU as part of this study, other markers of T cell integrity and function were also investigated as part of the overall investigation in The University of Tuebingen by the group of Professor Graham Pawelec. Cell adaptation to a low O₂ level is associated with the up-regulation of hypoxia inducible factor-1 α (HIF-1 α). HIF-1 α pathway is known to have different effects on the post-translational modification of proteins and can influence a broad range of cellular pathways by acting at the transcription level (Lukashev *et al.*, 2006). The results showed high antioxidant capacity in the culture media evidenced by an up-regulation of HIF-1 α under low O₂ tension determined by western blotting. The high antioxidant capacity observed in the culture media was not reflected in the function of T

cells. T cells had significantly lower proliferative capacity and stimulated cells were significantly more susceptible to apoptosis at low O₂ tension, compared to 20% O₂ tension. Proliferative capacity and the rate of apoptosis were investigated by carrying out carboxyfluorescein succinimidyl ester (CFSE) and Annexin staining respectively. The reason for the differences in antioxidant capacity of the media, compared to those inside the cells, could be due to the differences in molecular exchanges between the cells and the environment at different O₂ levels. The results of this investigation contradicted the earlier findings by other studies (Atkuri *et al.*, 2005) where the O₂ level did not influence the proliferative capacity of human peripheral blood mononuclear cells stimulated by phytohemagglutinin (PHA) or the cell viability in unstimulated cells.

Furthermore, investigations were carried out on the intracellular redox status (GSH:GSSG ratio) and whether the intracellular antioxidant capacity protected against oxidative damage to proteins. Reduced GSH is a low molecular weight thiol playing an important role in cellular defense system in the body scavenging peroxide radicals and reduces oxidised sites of proteins, DNA and lipids. The GSH:GSSG redox couple maintains the redox environment of the cell and GSH is abundant in the cell (Schafer and Buettner, 2001) serving as an indicator of the cellular redox environment. Redox status of the cell is hindered on oxidation of even a small amount of GSH. This oxidation results in the formation of GSSG and mixed disulfides between protein sulfhydryl groups in biological systems. It will also decrease the levels of GSH resulting in an increase in levels of GSSG and so lowering the GSH:GSSG ratio, which has been suggested to be responsible for several human diseases (Bonnefont Rousselot *et al.*, 2000; Kharb, 2000; Lang *et al.*, 2000; Mills *et al.*, 2000; Pastore *et al.*, 2001). The results showed (as shown in figures 7.2A & 7.2B) a significantly lower GSH:GSSG ratio in human peripheral blood mononuclear cells *ex vivo* maintained at low O₂ tension compared to 20% O₂ tension. Of further interest were the results of the carbonyl group formation (an index of global protein oxidation leading to oxidative damage in proteins) on intracellular proteins using oxy blot technique. This technique revealed significantly higher levels of carbonyl group formation in stimulated human peripheral blood mononuclear cells *ex vivo* maintained at low O₂ levels compared to 20% O₂ levels.

The significantly higher oxidative damage to proteins was later explained by the reduced Msr and proteasome activities using their respective enzyme assay kits. Msr A

and Msr B catalyze the reversion of methionine-S-sulfoxide and methionine-R-sulfoxide, respectively to the reduced form of methionine within proteins. Methionine can be easily oxidized into methionine sulfoxide and its reduction by methionine sulfoxide reductase (Msr) A (Yermolaieva *et al.*, 2004) and Msr B2 (Carbreiro *et al.*, 2008) could represent an efficient antioxidant system, because in proteins, the surface-exposed methionine residues can act as scavengers of a variety of oxidants (Levine *et al.*, 1996). The proteasome system has been implicated in the degradation of oxidatively modified proteins (Poppek and Grune, 2006). The proteasome is also involved in regulating protein content and degrading misfolded proteins. The loss of efficiency of intracellular protective and repair mechanisms of proteins has been implicated in cellular senescence *in vitro* (Petropoulos *et al.*, 2005). At the protein level, only a slight up-regulation of Msr and proteasome subunit expression was observed following activation under low O₂ tension. The increased carbonylation of proteins was associated with the increased amount of DNA damage under low O₂, suggesting overall increased oxidation in the presence of low O₂ tension. The increase in oxidation may be due to the inefficient Msr and proteasome activities. The results of the study of human peripheral blood mononuclear cells *ex vivo* under low O₂ tension, suggested a problem in molecule exchange between T cells and the extracellular milieu. It is possible that antioxidants present in the media are not available any longer to T cells at low O₂ because of a still unknown mechanism. It could be due to changes in membrane potential, fluidity or integrity. The increased intracellular protein and DNA damage observed under decreased O₂ may be due to free radical production resulting in changes to the respiratory chain and signalling processes. Other studies have revealed the increased free-radical cytotoxicity to Jurkat cells under lower O₂ levels (Chen *et al.*, 2008) evidenced by increased lipid peroxidation, release of cytochrome c and apoptosis under low O₂. Activated cells may experience even more free radical production, as suggested by the increased apoptotic rate, compared to resting cells, in the present study. T cells *in vivo* are normally exposed to O₂ tension conditions of less than 20%. Even though they proliferate better at 20% O₂, this does not reflect their real physiology. The results suggest a far more complicated mechanism yet to be fully unveiled.

Further investigations are being performed by the group of Pawelec in order to understand how T cell intracellular redox homeostasis is modulated at physiological O₂ level.

CHAPTER-8

General discussion

8.1. General discussion

Age-related deterioration and dysregulation of T cell function, termed 'immunosenescence', may lead to increased mortality and morbidity in humans through greater susceptibility to infections and disease. Previous research from the group of Barnett, suggested that oxidative stress may play a role in the immunosenescence process, through causing genomic instability and cell cycle delay and arrest. Previous work has demonstrated an age-related increase in oxidative DNA damage and mutations in T lymphocytes (*in vitro* T cell clones and also *ex vivo* cells) (Barnett & Barnett, 1998; Hyland *et al.*, 2000).

The aim of this research program was to investigate the effect of antioxidants on T cell function and integrity using T cell clones and peripheral blood mononuclear cells from human donors of different ages. The effect of different concentrations of the antioxidants, ebselen or NAC or mitoQ on: the life span and proliferative capacity; levels of oxidative damage to DNA; intracellular redox tone status (GSH:GSSG ratio); total glutathione levels; mitochondrial integrity and function and the effect of low O₂ tension culture conditions on human peripheral blood mononuclear cells *ex vivo* has been examined.

The monoclonal human *in vitro* T cell clone model is well established (Pawelec *et al.*, 2000) and has been pre-dominantly useful for the study of age associated changes in individual T cells over their lifespan and for the study of factors contributing to T cell senescence (Pawelec *et al.*, 2002). Previously, the *in vitro* T cell clone model has been successfully and extensively used by the group of Barnett (Barnett *et al.*, 1999; Hyland *et al.*, 2000; 2001; Duggan *et al.*, 2004; Annett *et al.*, 2004; 2005). The use of this monoclonal model enables the manipulation of immunosenescence *in vitro* which may in future lead to the manipulation of immunosenescence *in vivo* due to the similarities between T cells *in vivo* and the *in vitro* T cell clone model (Chapter-1, Section 1.1.3.8). The monoclonality of T cell clones is a distinct advantage, the changes observed in polyclonal systems may be due to the alterations in the proportions of the different cells present (Pawelec *et al.*, 2002) and thus results from polyclonal systems are difficult to interpret. For example, in a polyclonal population only the longevity of the last surviving clone from the original polyclonal population can be measured. The use of the

monoclonal *in vitro* model alongside *ex vivo* derived polyclonal cells in this study permitted a comparison of the effect of antioxidants on a range of cellular biomarkers detailed above, within both systems.

The results of previous studies gave further insight into the growth characteristics of T cell clones and revealed that all CD4⁺ T cell clones undergo apoptosis at the end of their lifespan, resulting in complete deletion of a clone within a single culture cycle (Pawelec *et al.*, 2002). The results of this current study revealed that T cell clones had considerably higher levels of growth (determined by analysing population doublings undergone) at the start of the culture compared to the later stages of their life span in culture (Appendix-I, Figures 1-12). The regression analysis data determining the relationship between PD per week and the *in vitro* age of the T cell clones (as revealed in table 3.2), suggested a decreased PD capacity with age evidenced by a significant downward slope reaching statistical significance for the complete lifespan data, in the regression analysis curve. However, after the initial peak values, the remainder of the lifespan of the clones transitioned into a steady state, demonstrating the maintenance of PD capacity throughout the lifespan of the clones. The results of this investigation were in line with the previous findings of the group of Barnett and also the group of Pawelec using human peripheral blood derived CD4⁺ T cell clones (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

All twelve CD4⁺ T cell clones examined in this investigation, grew in waves of maximal and minimal rates of proliferation variable from one to several growth cycles in length during the early part of their lifespan (as indicated in figure 3.1 and figures 1-12 in appendix-I). However, the size or length of the waves decreased with increase in wave frequency towards the later stages of their lifespan. This finding was similar to that previously reported (Hyland *et al.*, 2000). The decrease in size and length of the waves observed in T cell clones towards the later stages of their lifespan may be an indication of a frequency in cell cycle arrest due to the age-related accumulation of oxidative DNA damage in T cell clones *in vitro* (Barnett and Barnett, 1998; Hyland *et al.*, 2000; 2001; Chapter-4). Thus, if in sufficient levels, DNA damage could result in DNA damage mediated cell cycle arrest halting the proliferation of the T cells to give the cells time to repair their damage and finally high enough levels of DNA damage

may result in apoptosis (Barnett and Barnett, 1998). The increase in frequency of the waves towards the later stages of a T cell clone's lifespan data is evidence of their ability to retain their responsiveness to proliferative stimulation (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

T cells are supposed to undergo rapid clonal expansion upon antigenic stimulation to produce an effective immune response. There are several factors which may affect the ability of T cells to undergo clonal expansion. Accumulation of genetic damage within T cells is one of the factors and this may contribute to an age related decline in T cell replicative capacity through cell cycle arrest, apoptosis or selection against T cells containing mutations (Barnett and Barnett, 1998). Previous findings from the group of Barnett suggested maintenance of low levels of oxidative DNA damage *in vivo* and significantly higher plasma antioxidant capacity in Swedish nonagenarian (people between 90 and 100 years of age) subjects compared to controls (Hyland *et al.*, 2002), further emphasising the importance of a relationship between intact immune function and longevity. If oxidative damage to DNA in T cells results in a decline of function with age, it would be of benefit to identify interventions to counteract the age related increase in oxidative damage to DNA *in vivo*. Theoretically, one way to do this is to use antioxidant administration. Previous findings from the group of Barnett, successfully demonstrated the antioxidant capability of carnosine and PBN in human T cell clones. Carnosine, at physiological concentrations of 20mM significantly increased the lifespan and lowered the levels of oxidative DNA damage in T cell clones derived from a healthy young subject and which were exposed to carnosine throughout their lifespan (Hyland *et al.*, 2000). 250µM PBN increased the longevity and significantly lowered the levels of DNA damage in T cell clones compared to controls.

In this current PhD investigation, the impact of long term exposure (throughout the lifespan of clones or from the midpoint of their *in vitro* lifespan) of a range of concentrations of the *in vivo* relevant antioxidants, ebselen or NAC on markers of T cell function (proliferative capacity and lifespan) and integrity (levels of oxidative DNA damage, GSH:GSSG ratio and total glutathione levels) in human peripheral blood derived CD4⁺ T cell clones was investigated. In spite of the well documented antioxidant potential of ebselen or NAC which had been reported for several cell types (explained in detail in chapter-1, Section 1.1.6.1, 1.1.6.2; Chapter-4, Section 4.1.2.4),

the impact of ebselen or NAC in human peripheral blood derived CD4⁺ T cell clones *in vitro* had been determined.

The results of this investigation revealed that, from the range of concentrations of ebselen or NAC investigated, 30µM ebselen or 7.5mM NAC significantly extended the lifespan (cumulative PD at the end of the lifespan) of CD4⁺ T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor when supplemented from a young *in vitro* age. T cell clones derived from a healthy 26 year old donor and a healthy 45 year old donor supplemented with 30µM ebselen survived for an additional three and two weeks respectively before apoptosis, compared to non-supplemented clones. 7.5mM NAC supplementation extended the lifespan of clones for 2 additional weeks in culture before apoptosis, compared to the non-supplemented clones. Furthermore, 30µM ebselen or 7.5mM NAC significantly decreased the levels of oxidative damage to DNA and the GSH:GSSG ratio was significantly higher after 14 weeks of supplementation in both CD4⁺ T cell clones when supplemented from their young *in vitro* age, compared to non-supplemented clones. The antioxidant potential of ebselen evident by their effect on markers of T cell integrity and function investigated above is a sign of fewer radicals present in culture or the radical scavenging capacity of ebselen. In terms of NAC, cysteine amino acid present is a derivative of GSH formation explaining the increase in GSH:GSSG ratio. The significantly higher GSH:GSSG ratio on supplementation with ebselen or NAC, represents an increased antioxidant state. The increase in antioxidant status further explains the decrease in levels of oxidative DNA damage that in turn explains a lower apoptotic state explained by the extended lifespan of the clones on antioxidant supplementation. In terms of total glutathione levels, neither of the antioxidants significantly altered the total glutathione levels in either of the CD4⁺ T cell clones (detailed in results section 4.1.3.3 in chapter-4). This result reveals that none of the antioxidant supplementations had an impact on glutathione pathways in any way resulting in the lack of significant changes in glutathione levels, in the T cell clones examined.

Of further interest was the lack of a significant change in proliferative capacity or lifespan in T cell clones derived from either age groups of donors on supplementation from the midpoint of their *in vitro* age (58.7 & 63.4 Initial PD) with any of the

concentrations of ebselen or NAC. In terms of the levels of oxidative damage to DNA and the GSH:GSSG ratio, neither of the antioxidant's supplementation significantly altered the levels of DNA damage or the GSH:GSSG ratio in T cell clones, compared to non-supplemented clones, at any of the time points examined during their span in culture. This pattern of results were in line with the previous findings from the group of Barnett that revealed no significant changes in longevity in CD4⁺ T cell clones on long term culture with 20mM Carnosine from the midpoint of their *in vitro* lifespan (Hyland *et al.*, 2000). The suggested reason for the failure of carnosine to reveal its antioxidant potential may have been the high background of biomolecule damage that already existed in these T cells accumulated during earlier stages of their lifespan under conditions of 20% O₂ that may have decreased the antioxidant or free radical scavenging potential of carnosine. There may be more radicals in older T cell clones that may affect the function of mitochondria and other defense systems in the body. So the antioxidants at specific concentrations, able to reveal an antioxidant potential in T cell clones supplemented from a young *in vitro* age may not be enough to reveal an antioxidant potential in old aged T cell clones. This could be the reason for the failure of ebselen or NAC at specific concentrations to reveal an antioxidant potential in clones when supplemented from the midpoint of their lifespan. It would also be worth noting the decrease in other markers of T cell integrity with age revealed in the existing scientific literature for example: DNA repair capacity and HSPs. The DNA repair capacity in T cells *in vitro* has been shown to decline with age, revealed in previous studies by the group (Annett *et al.*, 2004; 2005; Chapter-1, Section 1.1.5.5.1 & 1.1.5.5.2). Furthermore, HSPs activity has been demonstrated to decline in response to age making the body more susceptible to stress induced damage (Favatier *et al.*, 1997; Chapter-1, Section 1.1.5.2).

Higher concentrations of ebselen (60-100µM) or NAC (10mM) in human peripheral blood derived CD4⁺ T cell clones from a healthy young or middle aged donor completely inhibited the growth of T cell clones. The reason for this inhibition could be the complete scavenging of intracellular oxygen free radicals, contributing to the inhibition of the growth of the CD4⁺ T cell clones. Although, in excessive amounts ROS can be harmful molecules, at less critical levels they have an important role in T cell signalling events such as protein tyrosine phosphorylation and activation of JNK (Pani *et al.*, 2000), which has been previously explained elsewhere in this thesis (Chapter-1,

Section 1.1.6.4). Previous studies have demonstrated a significant increase in intracellular GSH depletion with an increase in ebselen concentration in HepG₂ cells (Yang *et al.*, 2000). Ebselen has the ability to bind with GSH to form ebselen selenyl sulphide (Haenen *et al.*, 1990). Ebselen selenyl sulphide reacts with excess of GSH to form ebselen selenol intermediate that in turn reacts with peroxides (Morgenstern *et al.*, 1992). Ebselen selenol intermediate can further react with ebselen selenyl sulphide to form ebselen diselenide. Ebselen diselenide can further react with peroxides resulting in intracellular GSH depletion that leads to induction of apoptosis (Yang *et al.*, 2000) or cells succumbing to stress (Maher, 2005). In terms of NAC, supplementation with high concentrations of 10mM, may have completely scavenged the ROS which are important in less critical levels for cell proliferation and differentiation, which explains the complete inhibition of the growth of the clones at 10mM NAC concentration.

The impact of 30µM Ebselen or 7.5mM NAC revealing antioxidant potential in human peripheral blood derived CD4⁺ T cell clones *in vitro*, a monoclonal population (Chapter-4) lead to the investigation of the impact of these specific concentrations of ebselen or NAC in human peripheral blood mononuclear cells *ex vivo*, a polyclonal population (Chapter-5). Preliminary studies in human peripheral blood derived CD4⁺ T cell clones supplemented from the midpoint of their *in vitro* lifespan with 5mM NAC revealed higher lifespan compared to clones supplemented with other concentrations of NAC (Chapter-4). This factor was also taken into consideration before choosing the dose range of ebselen or NAC. Since the clones used in the previous part of the investigation in this study were human CD4⁺ T cell clones (Chapter-4), the impact of ebselen (30µM) or NAC (5mM / 7.5mM) on CD4⁺ T cells *ex vivo* were also investigated (Chapter-5) to compare the effect of the antioxidants on CD4⁺ T cells *ex vivo* and *in vitro*. In addition, the study has facilitated the investigation of the impact of age of the donors on markers of T cell integrity and function by comparing the proliferative capacity, the levels of oxidative DNA damage, GSH:GSSG ratio and total glutathione levels.

The results of this part of the investigation revealed that the supplementation of human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* derived from donors from either age groups, with 30µM ebselen or 5mM/ 7.5mM NAC resulted in a significantly higher GSH:GSSG ratio, total glutathione levels and proliferative capacity at various

time points during their span in culture compared to non-supplemented human peripheral blood mononuclear cells (detailed in results section 5.1.3 in chapter-5). The elevation of the GSH:GSSG ratio on supplementation with 30 μ M ebselen or 5mM / 7.5mM NAC is of significance considering the important role of intracellular redox status (GSH:GSSG ratio) as a mediator of apoptosis in many cell systems (Cotgreave and Gerdes, 1998; Hall, 1999; Chapter-1, Section 1.1.4.1). The significantly higher GSH:GSSG ratio represents an improved antioxidant state for the ebselen or NAC treated cells. Previous studies have demonstrated the ability of ebselen at concentrations of 1-25 μ M to stimulate cell growth, inhibit H₂O₂ induced suppression, significantly reduce LDH leakage and lipid peroxidation and significantly reduced intracellular ROS levels in HepG₂ cells in a concentration dependent manner, due to its ability to scavenge hydroxyl radicals and other ROS (Yang *et al.*, 1999). In terms of NAC, other studies investigating the effect of a range of NAC concentrations (0.5-2mM) in HeLa cells have shown that 2mM NAC increased intracellular GSH by 15% compared to controls after 24hrs of incubation (Hansen *et al.*, 2004). In HepG₂ cells, the increase in intracellular GSH level was highest after 5mM NAC supplementation for 24hrs (Yang *et al.*, 2000) (detailed in the discussion section 5.1.4 in chapter-5).

The results of this investigation also revealed a significant decrease in the levels of oxidative DNA damage at various time points in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* during their span in culture, when compared to non-supplemented peripheral blood mononuclear cells, when supplemented with either of the antioxidants. The ability of ebselen to almost completely inhibit oxidative DNA damage has been previously demonstrated in HepG₂ cells (Yang *et al.*, 1999) and HL-60 cells (Ji *et al.*, 2000) attributed to their ability to scavenge hydroxyl and peroxide radicals. Of further interest is a study in PC-12 cells where NAC supplementation (100-300 μ M) significantly lower than the concentrations used in this investigation, reduced the DNA damage compared to controls (Yan and Greene, 1998). The differences in concentration range of the same antioxidant revealing optimum free radical scavenging capacity may be due to the difference in cell types.

The significant increase in total glutathione levels on supplementation with 5mM / 7.5mM NAC demonstrates the ability of NAC to contribute glutathione to the cell

thereby supplementing the *in vivo* antioxidant status. A significant increase in total glutathione levels was also observed on supplementation with 30 μ M ebselen. Ebselen is a GPx mimetic. GPx has the ability to scavenge organic and inorganic peroxides using GSH as substrate. GSH is depleted during peroxide accumulation. Ebselen has a proven ability to scavenge hydroxyl radicals and ROS that initiates lipid peroxidation as a result of peroxide accumulation. In this study, ebselen may have scavenged the radicals present in the cell system. The fewer the radicals, the lesser will be the need for GSH to scavenge them. Since the GSH is not consumed to scavenge the radicals, levels of GSH may appear greater in the cell system on supplementation with ebselen.

The results of the study of the effect of 30 μ M ebselen or 7.5mM NAC on CD4⁺ T cell clones / peripheral blood mononuclear cells / CD4⁺ T cells *ex vivo* reveal a significant antioxidant potential against endogenously generated oxidants and those associated with the 95% air, 5% CO₂ culture conditions at various time points during their span in culture. The significantly higher GSH:GSSG ratio represents an increased antioxidant state on supplementation with either of the antioxidants. The increase in antioxidant status explains the decrease in levels of oxidative DNA damage that further explains a lower apoptotic state explained by the increase in proliferative capacity of the cells on antioxidant supplementation.

5mM or 7mM NAC supplementation had similar antioxidant potential in human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo*. Furthermore, the effects of 30 μ M ebselen or 7.5mM NAC in human CD4⁺ T cells *ex vivo* compared to the effects on human peripheral blood derived CD4⁺ T cell clones *in vitro*, were not significantly different evidenced by their effect on the markers of T cell integrity and function investigated. 5mM NAC supplementation did not reveal any significant antioxidant potential in CD4⁺ T cell clones *in vitro* (Chapter-4), unlike the antioxidant potential demonstrated in the *ex vivo* polyclonal peripheral blood mononuclear cell population and CD4⁺ T cells *ex vivo*. Human T cell clones are maintained in culture throughout their lifespan over several months. This is not the case in cells *ex vivo*. It may be that the longer span of culture *in vitro* may have altered certain aspects of the biochemical / molecular status of the cells. Under these extensive culture conditions,

antioxidant or free radical scavenging effects of NAC at 5mM concentrations may be insufficient to reveal any significant antioxidant potential in T cell clones *in vitro*.

Previous findings from the group of Barnett revealed that human peripheral blood mononuclear cells derived from donors of 65-69 years had higher levels DNA damage compared to cells derived from donors aged 35-39 years (Barnett and Barnett, 1998) as explained in chapter-5, section 5.1.4. The results of the investigation reported in this study demonstrated no significant differences between the levels of oxidative DNA damage in untreated human peripheral blood mononuclear cells or CD4⁺ T cells *ex vivo* derived from donors of 25-30 years age group, compared to T cells derived from donors of 55-60 years age group. The difference in results from the previous study could be due to the differences in sensitivity of assay used and differences in age group of the donors (explained in detail in chapter-5, Section 5.1.4).

Considering the antioxidant potential of 30µM ebselen or 7.5mM NAC in T cells *ex vivo* and *in vitro*, their free radical scavenging ability may be more pronounced under physiological O₂ conditions that will likely reveal any *in vivo* anti-immunosenescent potential. Previous work from the group of Barnett investigating the effect of low O₂ tension on several pathways involved with ROS such as JNK phosphorylation and P38 phosphorylation in T cell clones (Duggan *et al.*, 2004), generated interesting results as discussed in chapter-1, section 1.1.6.4. Other studies have revealed inhibition of H₂O₂ induced P38 MAP kinase activation and cytochrome c release by 0.3µM ebselen supplementation in HUVECs and inhibition of the H₂O₂ induced activation of JNK signalling pathway by ebselen in PC-12 cells (Yoshizumi *et al.*, 2002). JNK and P38 MAP kinase has been proven to be activated on cytotoxic insult and are often associated with apoptosis on activation (Ali *et al.*, 2004). ERK signalling pathway is known to be activated by growth factors involved in mitogenesis and is effective for cell proliferation and differentiation. Ebselen has previously been demonstrated to activate ERK pathway in rats (Baljinnayam *et al.*, 2003). On the otherhand, in PC-12 cells, NAC activated ERK pathway (Yan and Greene, 1998) and inhibited JNK activity (Park *et al.*, 1996). It will be interesting to examine the impact of ebselen or NAC on these pathways involved with cell proliferation, differentiation and apoptosis in CD4⁺ T cells *ex vivo* and CD4⁺ T cell clones *in vitro*.

The importance of mitochondria in human cell and ageing has been previously explained (Chapter-1, Section 1.1.1.2, 1.1.1.2.1). Mitochondrial dysfunction has been implicated as a major contributor to a range of diseases (Chapter-1, Section 1.1.6.3; Chapter-6, Section 6.1.1). The main drawback in treating these diseases is the inability to target an antioxidant *in vivo* to the mitochondria. This can be achieved with the use of a mitochondria targeted antioxidant mitoQ (mechanism explained in detail in chapter-1, Section 1.1.6.3; Chapter-6, Section 6.1.1). In this part of the investigation, the effect of mitoQ on certain markers of T cell integrity in human peripheral blood mononuclear cells was investigated.

The results of this investigation conducted on the effect of mitoQ in human peripheral blood mononuclear cells revealed that 0.5 μ M or 1 μ M mitoQ supplementation decreased the levels of free radicals in cell samples, from the donors of both age groups evidenced by the significantly higher GSH:GSSG ratios, compared to controls. Further results in cell samples derived from both age groups revealed the ability of mitoQ supplementation to decrease the levels of endogenous oxidative DNA damage. The results revealing the antioxidant potential of 1 μ M mitoQ was in line with the previous findings revealing that 1 μ M mitoQ blocked 150 μ M H₂O₂ induced caspase activation in Jurkat cells (Kelso *et al.*, 2001). Lower concentrations of mitoQ (0.1 μ M or 0.25 μ M) did not have any impact on the levels of free radicals (unchanged GSH:GSSG ratio) or in the levels of endogenous oxidative DNA damage, in human peripheral blood mononuclear cell samples from the donors of both age groups.

All the concentrations of mitoQ used in this investigation (0.1 μ M, 0.25 μ M, 0.5 μ M or 1 μ M) revealed an antioxidant potential against exogenously applied ROS evidenced by a significantly higher GSH:GSSG ratio and a significant decrease in oxidative DNA damage levels following treatment with H₂O₂ in mitoQ supplemented cell samples. The results were of interest considering the significant antioxidant potential of mitoQ in the cytosol, even though it is a mitochondria targeted antioxidant. The reason for the antioxidant potential outside the mitochondria may be that the mitochondria took up as much mitoQ as possible and the excess mitoQ accumulated in the cytosol with consequent radical lowering effects. The results also revealed the presence of endogenous oxidative DNA damage even at saturating concentrations of mitoQ. This

result reveals that even though mitochondria are the major site for the production of ROS in cells, there are other sources of contributors to genomic DNA damage. Another reason could be that lower concentrations of mitoQ may be taken up by the mitochondria, but were not able to reduce free radical release from the mitochondria explaining no significant differences in endogenous DNA damage levels and is reflected in the lack of changes to GSH:GSSG ratio.

The ability of mitoQ at specific concentrations to scavenge free radicals, resulting in a significantly higher GSH:GSSG ratio compared to controls, is invaluable considering the role of intracellular redox status (GSH:GSSG ratio) as a mediator of apoptosis in several cell systems (Slater *et al.*, 1996; Cotgreave and Gerdes, 1998; Hall, 1999; Chapter-1, Section 1.1.4.1). Higher GSH:GSSG ratio on mitoQ supplementation represents an increased antioxidant status in cells that further explains the decrease in levels of oxidative DNA damage in cells on mitoQ supplementation.

DTPP has a similar structure to mitoQ, except for the ubiquinone derivative in mitoQ responsible for the antioxidant potential, as explained in chapter-6. Unexpectedly, DTPP (0.5 μ M or 1 μ M) decreased the levels of free radicals (evidenced by significantly higher GSH:GSSG ratio) and significantly decreased the DNA damage levels, following treatment with H₂O₂ (treatment after 3 or 6 hours of supplementation with DTPP) in peripheral blood mononuclear cell samples from both age groups. The antioxidant potential of DTPP may be due to stimulation or up-regulation of other cellular antioxidants in the cell on DTPP supplementation, although there is no scientific evidence to support this hypothesis. In the existing scientific literature, only one previous study has revealed the ability of 1 μ M DTPP to reduce succinate (a complex I substrate) driven ROS in isolated bovine aortic endothelial mitochondria in rats (Malley *et al.*, 2006), without the mechanism being explained. Lower concentrations of DTPP (0.1 μ M or 0.25 μ M) did not have any significant impact on both the markers examined in this study, with and without H₂O₂ treatment in cell samples from both age groups examined.

Furthermore, in this investigation, the impact of 37 μ M or 74 μ M concentrations of vitamin E (a non-mitochondria targeted antioxidant) on the levels of free radicals

(determining the GSH:GSSG ratio) and oxidative DNA damage, in human peripheral blood mononuclear cells, was investigated as a control for mitoQ. Recent studies revealing the ability of vitamin E at a concentration of 37 μ M to reduce toxicity in murine splenocytes (Horton *et al.*, 2007) was taken into consideration before choosing the dose levels. None of the concentrations of vitamin E resulted in significant changes in GSH:GSSG ratio or the levels of oxidative DNA damage in human peripheral blood mononuclear cells treated + / - H₂O₂. Although vitamin E at 37 μ M concentration was able to reduce toxicity in murine cells, the concentrations used in this study may not be enough to reveal an antioxidant potential or free radical scavenging potential in human peripheral blood mononuclear cells *ex vivo*. The reason for the lack of effect of vitamin E supplementation in human peripheral blood mononuclear cells compared to the murine cells could be due to the differences in cell types. Investigations could be carried out using a broader range of vitamin E concentrations and analyse their impact on the markers of T cell integrity used, in this study. Of further interest would be determining the ROS production for different concentrations of vitamin E supplementations in CD4⁺ T cells *ex vivo* using FACS analysis of samples subjected to DCFDA staining.

The impact of a range of concentrations of mitoQ / DTPP / vitamin E on total glutathione levels, a marker of T cell integrity, was also investigated as part of this study. The results revealed a lack of consistent changes in levels of total glutathione on administration of any of the concentrations of mitoQ or DTPP (0.1 μ M, 0.25 μ M, 0.5 μ M or 1 μ M) or vitamin E (37 μ M or 74 μ M) in human peripheral blood mononuclear cells derived from donors of both age groups. This result reveals that none of these supplements in any way had an impact on glutathione pathways, and therefore the lack of significant changes in glutathione levels, in the peripheral blood mononuclear cells examined.

Impact of mitoQ on mitochondrial membrane potential, another marker of T cell integrity, playing an important role in apoptosis was also investigated (Chapter-2, Section 2.1.8). The results indicated no significant changes in mitochondrial membrane potential in human peripheral blood mononuclear cells on treatment with 0.1 μ M or 0.5 μ M mitoQ. However, 0.1 μ M or 0.5 μ M mitoQ treatment resulted in a significant increase in mitochondrial membrane potential on treatment with a range of doses of

H₂O₂ in cells derived from donors of both age groups. In the existing scientific literature there is only one other study demonstrating the effect of 1µM mitoQ on mitochondrial membrane potential. This study was performed *in vitro* in isolated mitochondria in rat models where 1µM mitoQ treatment decreased ROS production, thereby, protecting the mitochondria from damage and resulted in maintenance of the mitochondrial membrane potential (Lowes *et al.*, 2008). The results of this study also revealed that mitoQ suppressed the release of pro-inflammatory cytokines and increased the release of anti-inflammatory cytokines from the cells.

In this investigation the impact of chronic exposure of 10-30nM concentrations of mitoQ / DTPP in human peripheral blood mononuclear cells derived from donors of both age groups was also investigated. Previous studies have revealed antioxidant potential on chronic exposure to mitoQ at concentrations of 10-20nM in MRC-5 fibroblasts evident by telomere shortening (Saretzki *et al.*, 2003). The result of that study was taken into consideration before choosing the dose range in this investigation. None of the concentrations of mitoQ or DTPP (10nM, 20nM or 30nM) caused any significant alterations which was reflected in a lack of change to free radical (unchanged GSH:GSSG ratio), oxidative DNA damage and total glutathione levels. From the information in the existing scientific literature, on the effects of chronic exposure of mitoQ in cells *in vitro* and *ex vivo*, only one other recent study investigated the effects of chronic exposure of mitoQ. However, the study was performed in isolated mitochondria and the results revealed the antioxidant potency of 1µM mitoQ after a week of supplementation, evidenced by a decrease in mitochondrial damage and maintenance of mitochondrial membrane potential in isolated mitochondria in human achilles tendon cells on mitoQ supplementation (Lowes *et al.*, 2009).

Furthermore, in terms of the effect of mitoQ, the impact of the selected doses of mitoQ, on the superoxide production in the mitochondria in human peripheral blood mononuclear cells *ex vivo* could also be investigated.

Finally, the effect of reduced O₂ tension as a defense system in human peripheral blood mononuclear cells *ex vivo* was investigated (Chapter-7). There was little information in the scientific literature on the impact of low O₂ tension culture conditions in T cells *ex*

vivo and *in vitro* before the start of this investigation. The fact that intracellular O₂ level is much lower than the O₂ level in the cells maintained in an extracellular environment makes this study very important. Previous work from the group of Barnett demonstrated a shorter lifespan and reduced proliferative capacity in T cell clones derived from young and old aged human donors when cultured under 6% O₂ tension, compared to clones cultured under 20% O₂ tension (Chapter-1, Table 1.13 & Table 1.14; Chapter-7, Section 7.1.1). But the clones cultured at 6% O₂ had significantly lower levels of oxidative DNA damage compared to clones cultured at 20% O₂ tension. The results of this investigation revealed significantly higher levels of oxidative DNA damage and lower GSH:GSSG ratio in human peripheral blood mononuclear cells *ex vivo*, when cultured at 2% O₂ tension compared to cells cultured at 20% O₂ tension (Chapter-7, Section 7.1.3). The results reveal the increase in levels of free radicals at 2% O₂ (evidenced by significantly lower GSH:GSSG ratio) contributing to increase in levels of DNA damage.

Apart from the two markers of T cell integrity investigated in NTU as part of this study, other markers of T cell integrity and function were also investigated as part of the overall investigation in The University of Tuebingen by the group of Professor Graham Pawelec. The results showed high antioxidant capacity in the culture media evidenced by an up-regulation of HIF-1 α under low O₂ tension. The high antioxidant capacity observed in the culture media was interestingly not reflected in the function of T cells. It may be due to differences in molecular exchanges between the cells and the environment at different O₂ levels. T cells had significantly lower proliferative capacity and stimulated cells were significantly more susceptible to apoptosis at low O₂ tension, compared to 20% O₂ tension. The results of this investigation contradicted the earlier findings by other studies (Atkuri *et al.*, 2005) where the O₂ level did not influence the proliferative capacity of human peripheral blood mononuclear cells stimulated by phytohemagglutinin (PHA) or the cell viability in unstimulated cells. Furthermore, there was significantly higher levels of carbonyl group formation (an index of global protein oxidation leading to oxidative damage in proteins) in stimulated human peripheral blood mononuclear cells *ex vivo* maintained at low O₂ levels compared to 20% O₂ levels which was later explained by the reduced Msr and proteasome activities revealed under low O₂ tension. The results are further explained in detail in chapter-7, section 7.1.4. Previously, other studies have revealed the increased free-radical cytotoxicity to Jurkat cells under lower O₂ levels (Chen *et al.*, 2008). The increased apoptotic rate of activated

cells at 2% O₂ tension suggests an increase in free radical production in cells. Even though, T cells proliferate better at 20% O₂, this does not reflect their real physiology where they are exposed to low levels of O₂. The results suggest complicated mechanism yet to be fully explored.

8.1.1. Conclusion

The aim of this PhD programme was to investigate the effects of antioxidants (ebselen or NAC or mitoQ) or low O₂ tension on T cell integrity and function using T cell clones and human peripheral blood mononuclear cells from donors of different age groups.

The findings of this novel investigation revealed that for a limited set of conditions, 30µM ebselen or 7.5mM NAC supplementation among a range of concentrations of ebselen or NAC investigated, showed antioxidant potential, evidenced by the significant increase in lifespan; significantly higher GSH:GSSG ratio and a significant decrease in levels of oxidative damage to DNA in CD4⁺ T cell clones (a monoclonal model system), when supplemented from a young *in vitro* age. Further studies of supplementation of human peripheral blood mononuclear cells and CD4⁺ T cells *ex vivo* (both polyclonal model systems) derived from donors of both age groups, with 30µM ebselen or 5mM / 7.5mM NAC, demonstrated a significant antioxidant potential evidenced by the significant increase in proliferation capacity; significantly higher GSH:GSSG ratio and total glutathione levels, and significant decreases in levels of oxidative damage to DNA.

Furthermore selected concentrations of mitoQ, a mitochondria targeted antioxidant, revealed significant antioxidant potential in human peripheral blood mononuclear cells in this investigation. A well functioning mitochondria is a pre-requisite for human life and mitochondria dysfunction has been attributed to a wide range of diseases in humans. However, this study has shown that the use of mitoQ may be a viable strategy to increase the antioxidant defenses in human peripheral blood mononuclear cells *ex vivo* to help manage oxidative stress levels.

In terms of low O₂ tension studies, 2% O₂ tension revealed an increase in free radical production in human peripheral blood mononuclear cells *ex vivo* compared to cells

cultured at 20% O₂ tension evidenced by their effect on a range of markers of T cell function and integrity investigated.

From the results of this study revealing the antioxidant potential of carefully selected concentrations of ebselen or NAC or mitoQ, evidenced by their ability to alleviate oxidative stress, may play an important role in its use as an *in vivo* antioxidant and in potential anti-immunosenescent interventive strategies *in vivo*.

APPENDIX-I

Growth records of human CD4⁺ T cell clones

1)

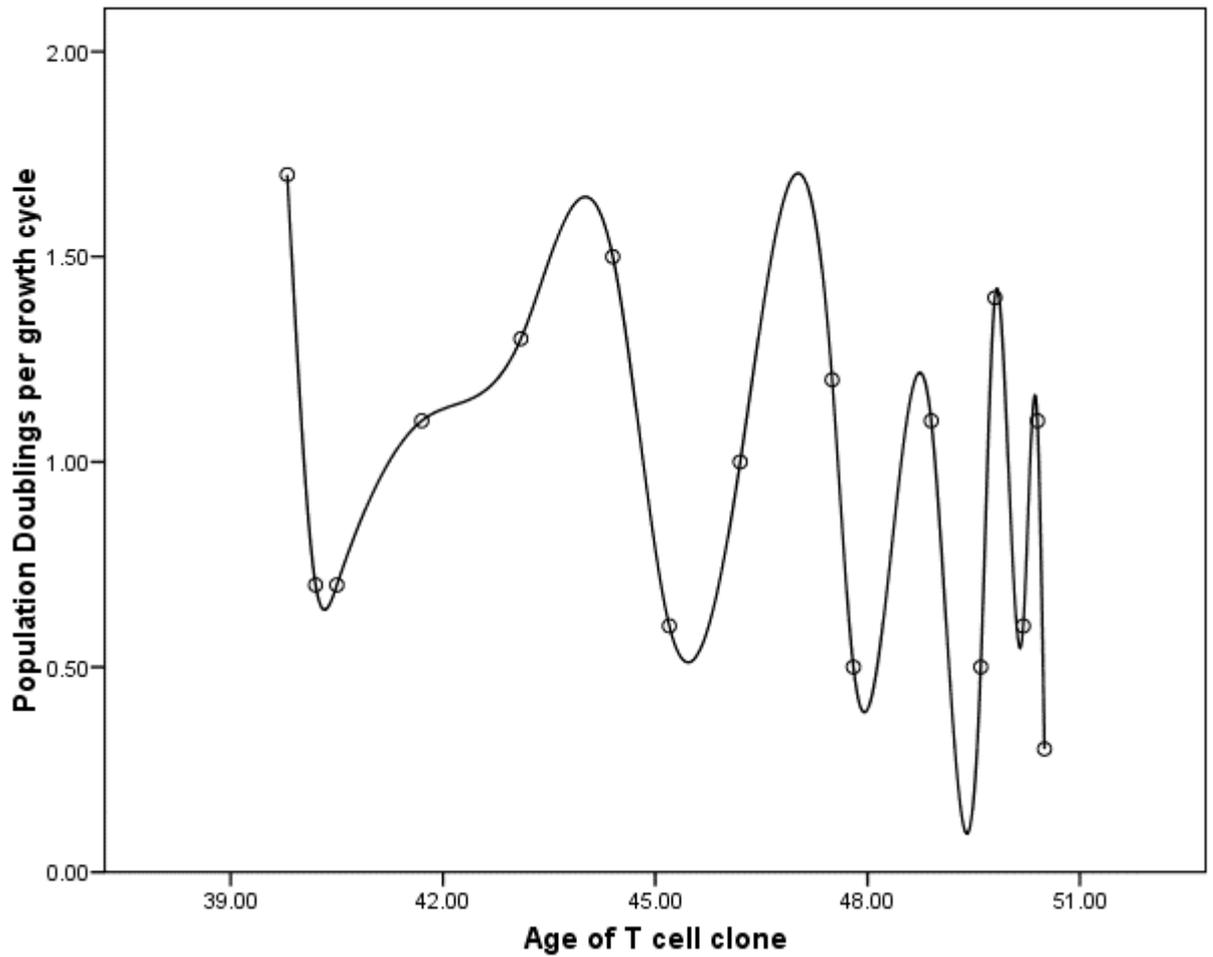


Figure 1 Proliferative capacity of 385-2a clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle - 0.8, Maximum PD achieved - 50.5

2)

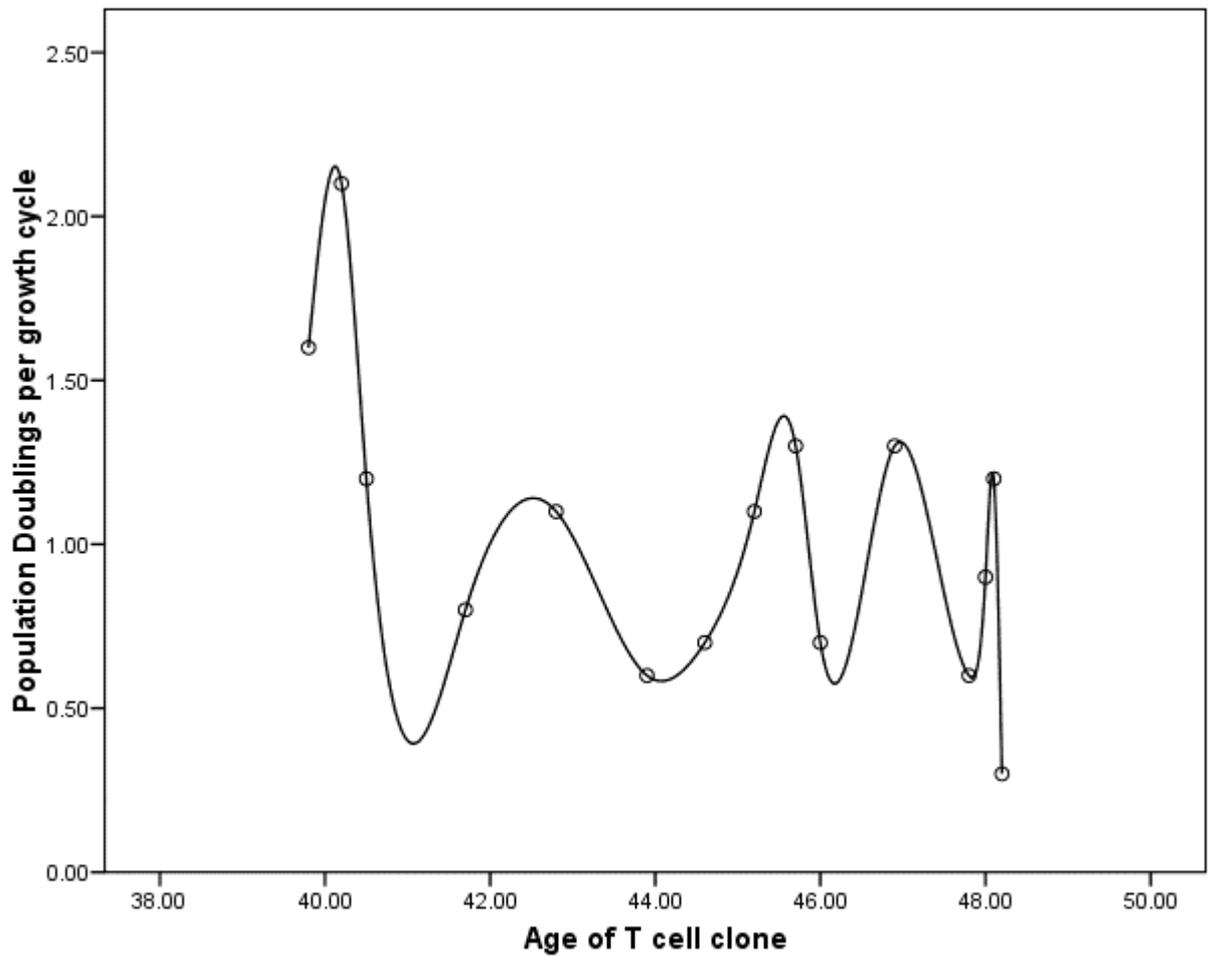


Figure 2 Proliferative capacity of 385-2b clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle - 0.7, Maximum PD achieved - 48.1

3)

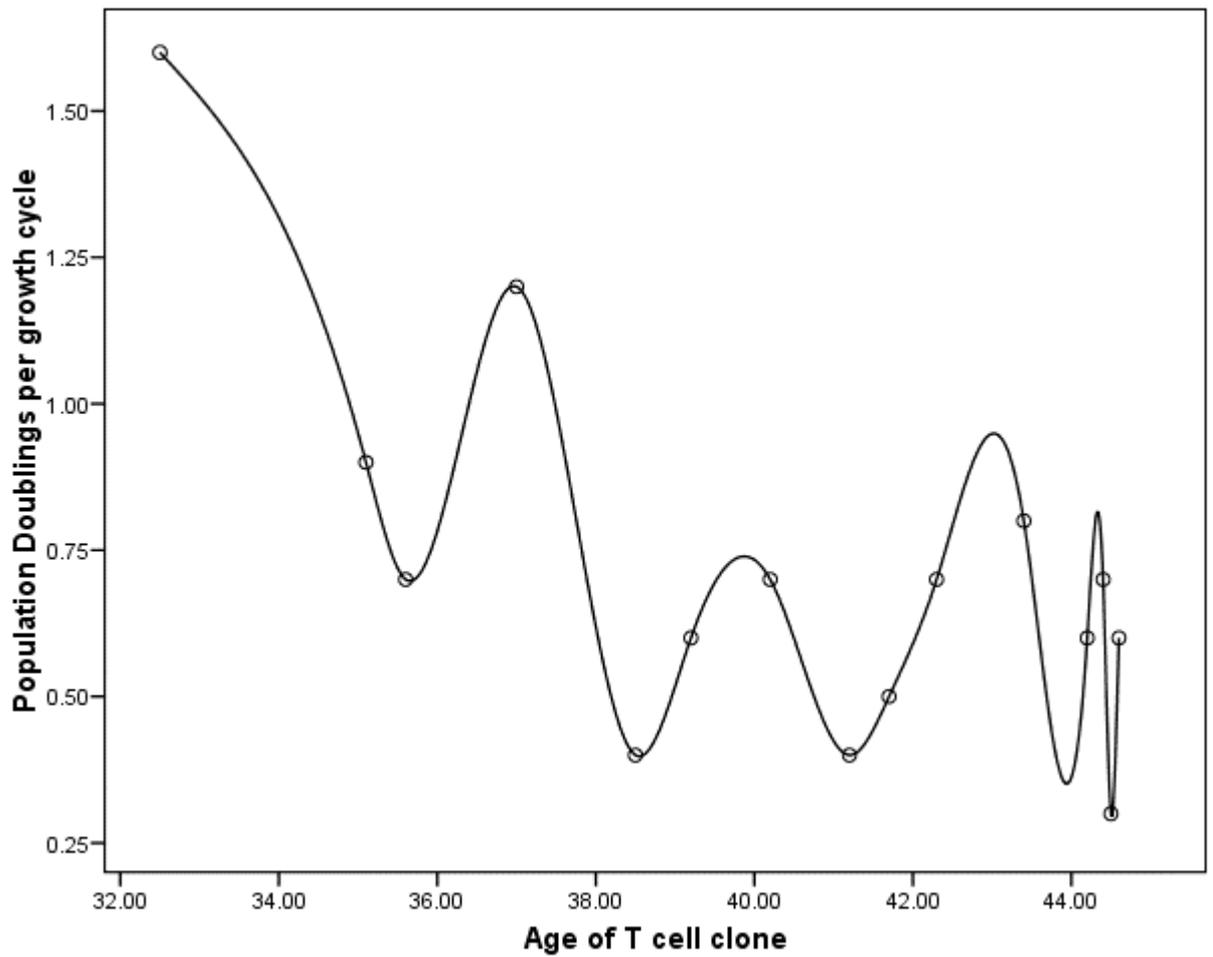


Figure 3 Proliferative capacity of 385-7a clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle - 0.9, Maximum PD achieved - 44.4

4)

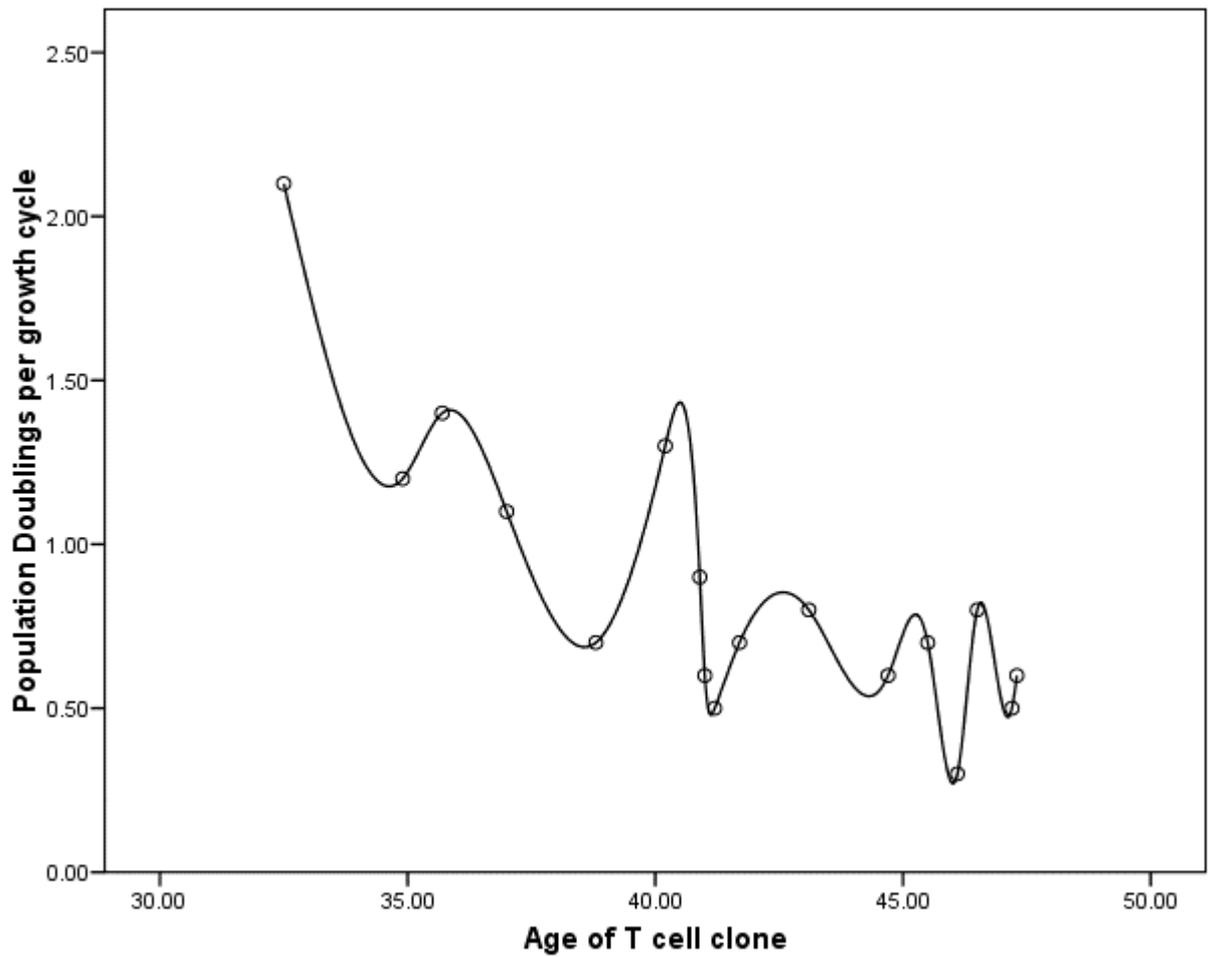


Figure 4 Proliferative capacity of 385-7b clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 1.0, Maximum PD achieved – 47.2

5)

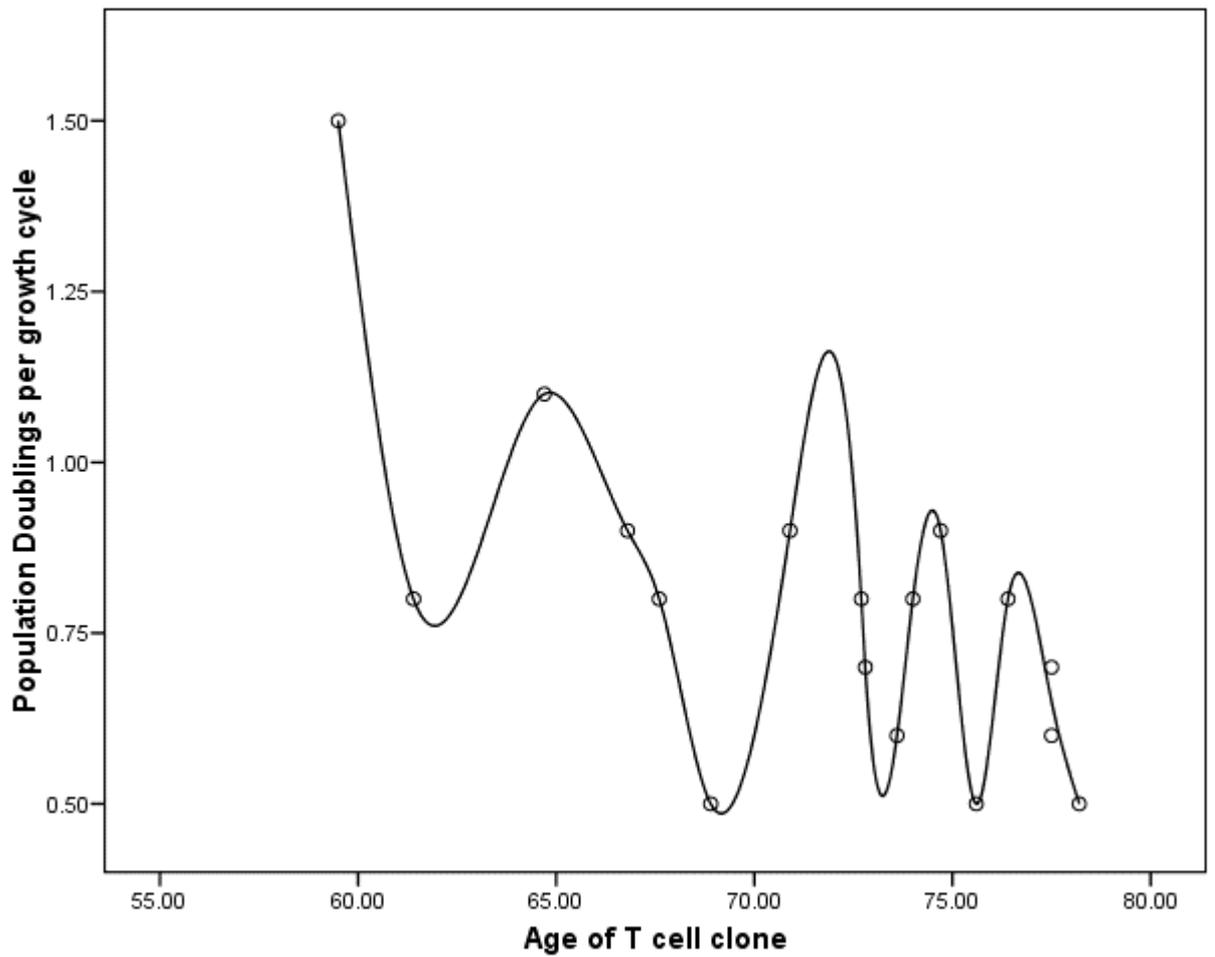


Figure 5 Proliferative capacity of 385-7c clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 1.1, Maximum PD achieved – 78.2

6)

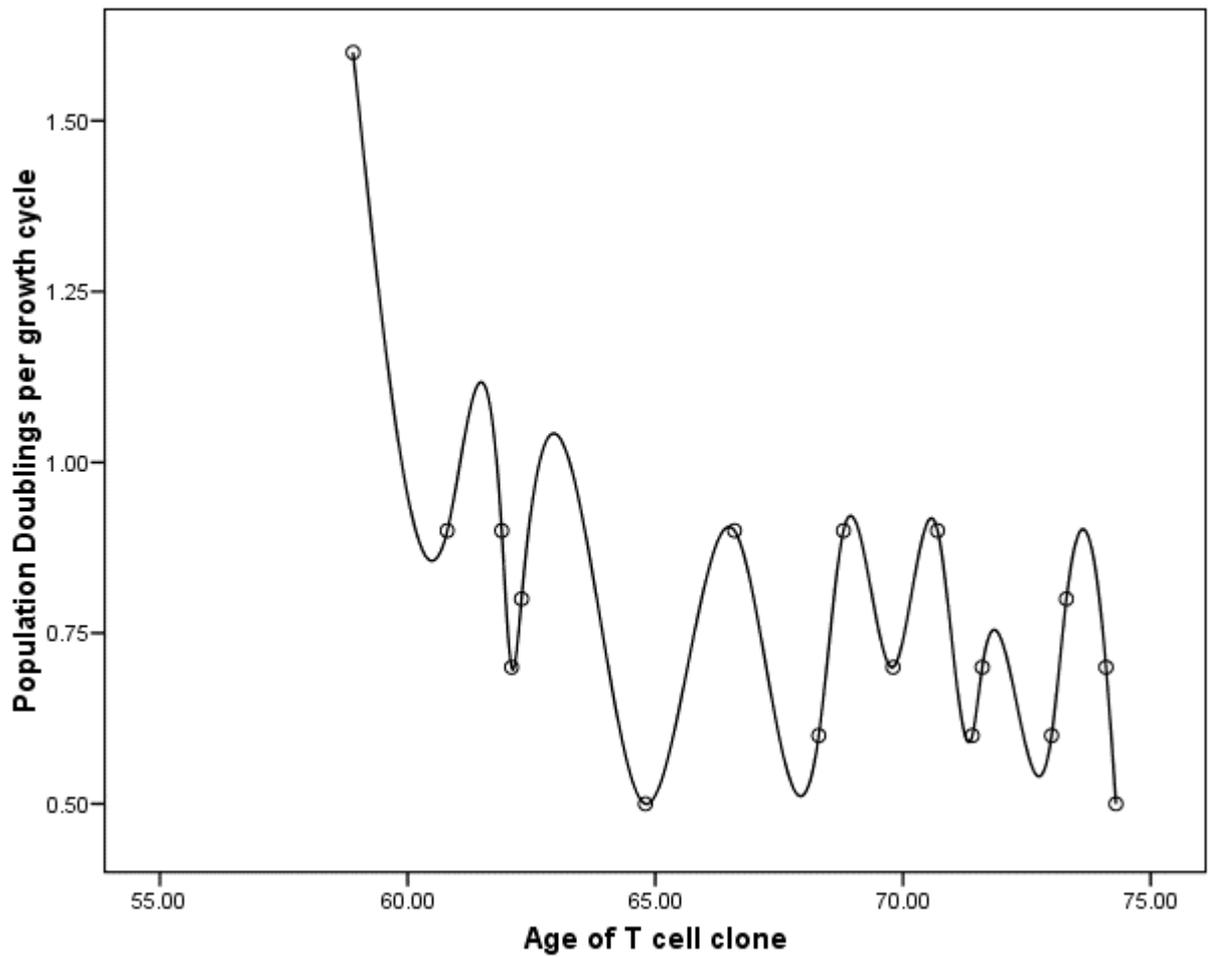


Figure 6 Proliferative capacity of 385-7d clone (derived from a 45 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.9, Maximum PD achieved – 74.4

7)

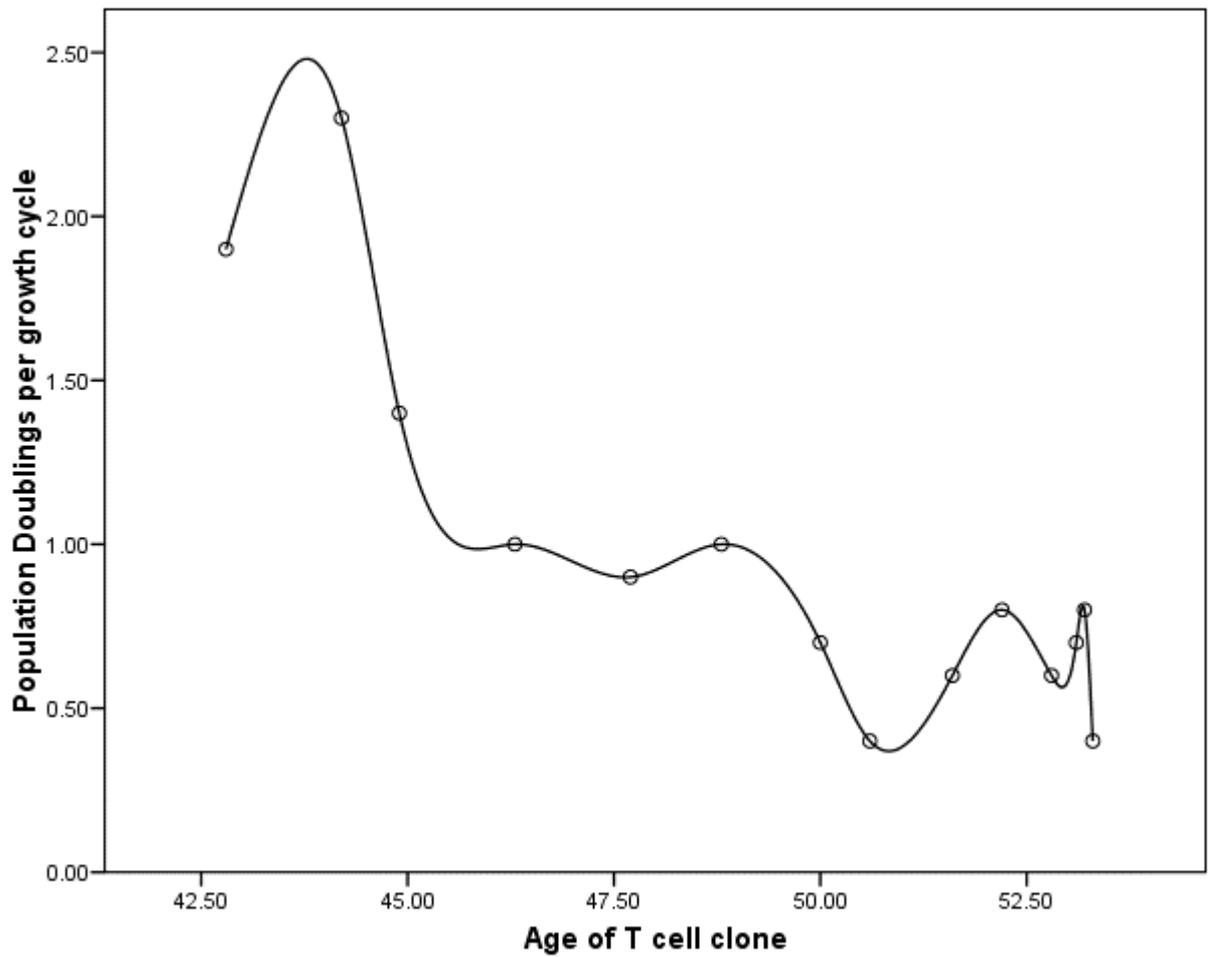


Figure 7 Proliferative capacity of 400-5a clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.9, Maximum PD achieved – 57.0

8)

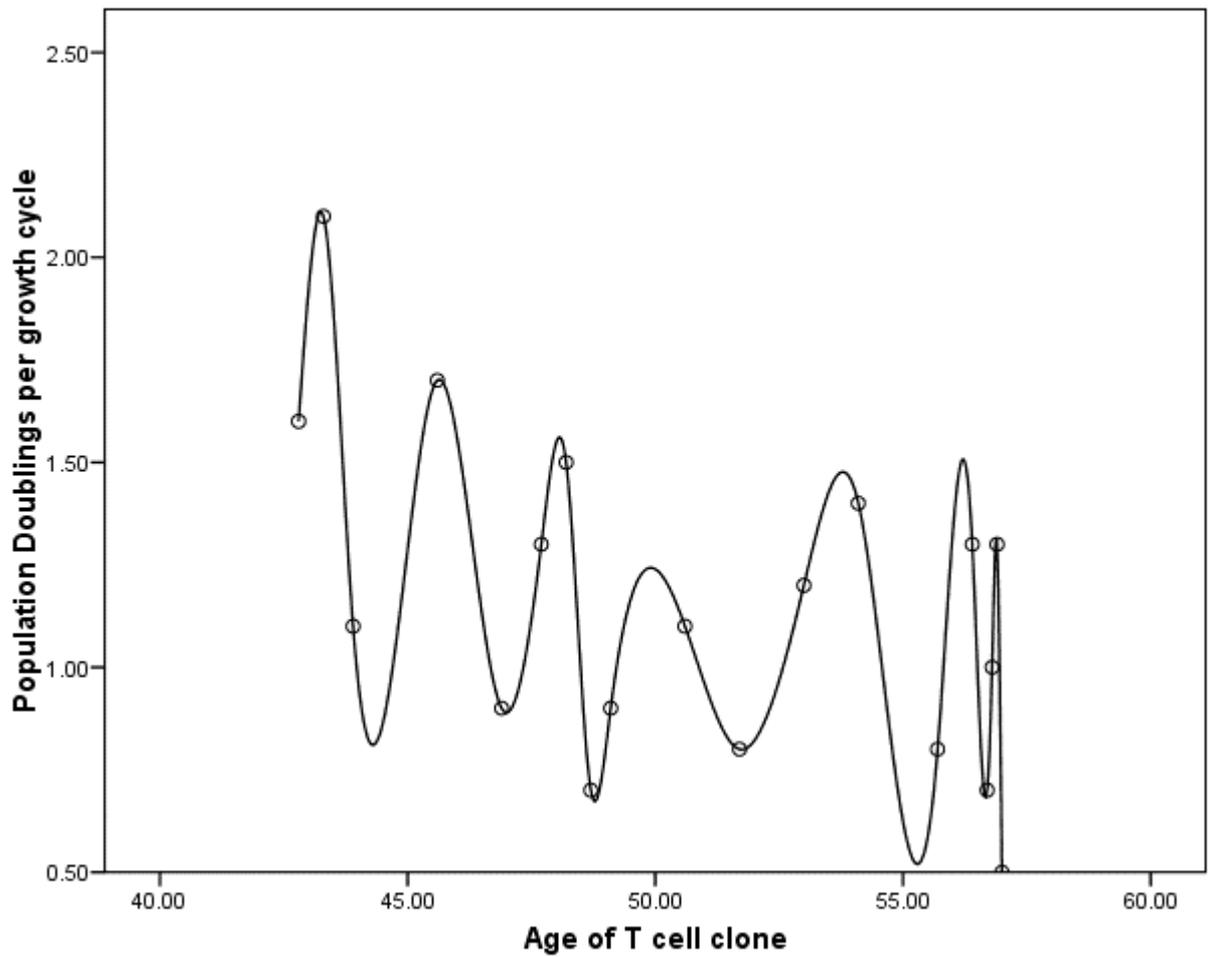


Figure 8 Proliferative capacity of 400-5b clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.8, Maximum PD achieved – 53.2

9)

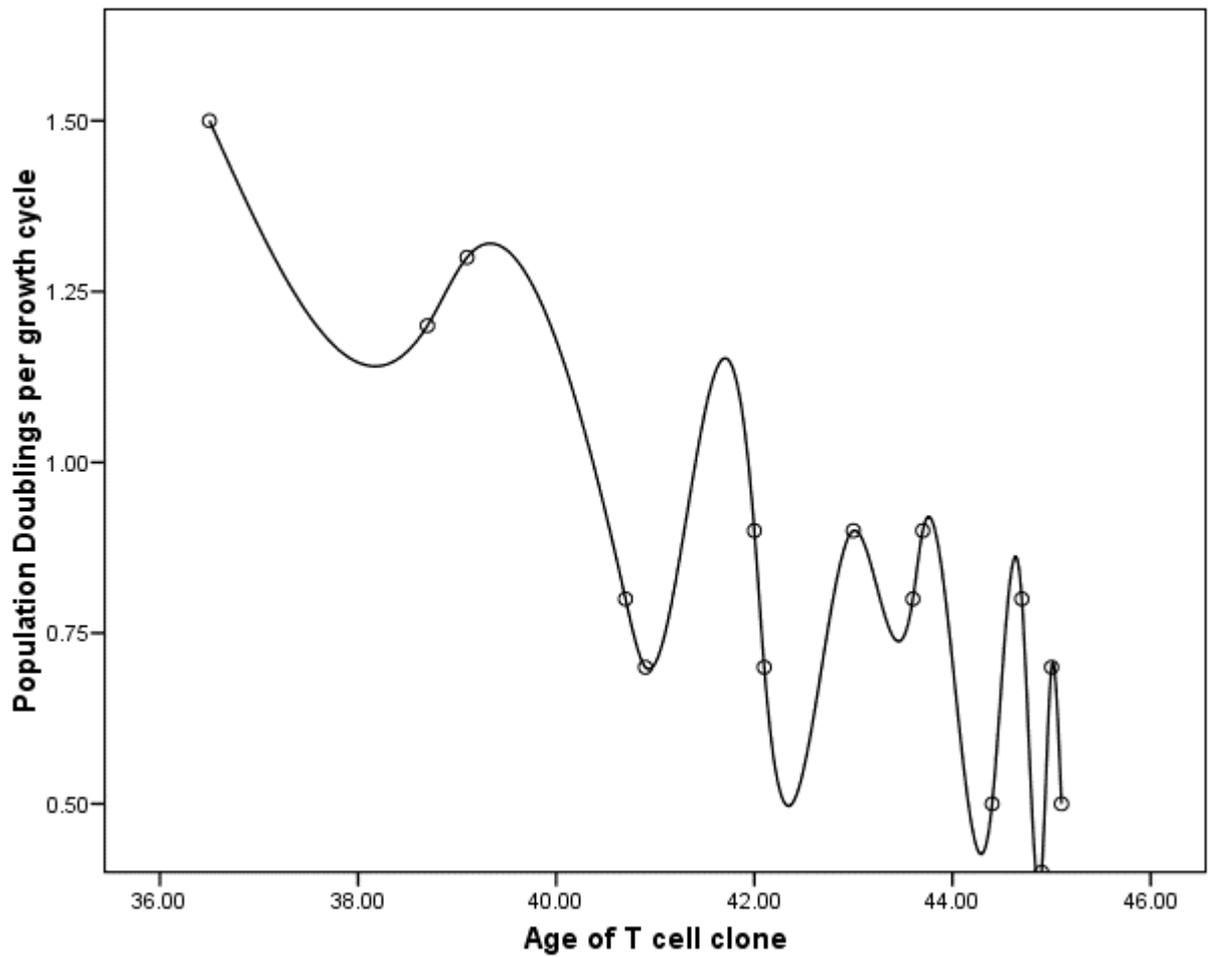


Figure 9 Proliferative capacity of 400-23a clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.7, Maximum PD achieved – 44.9

10)

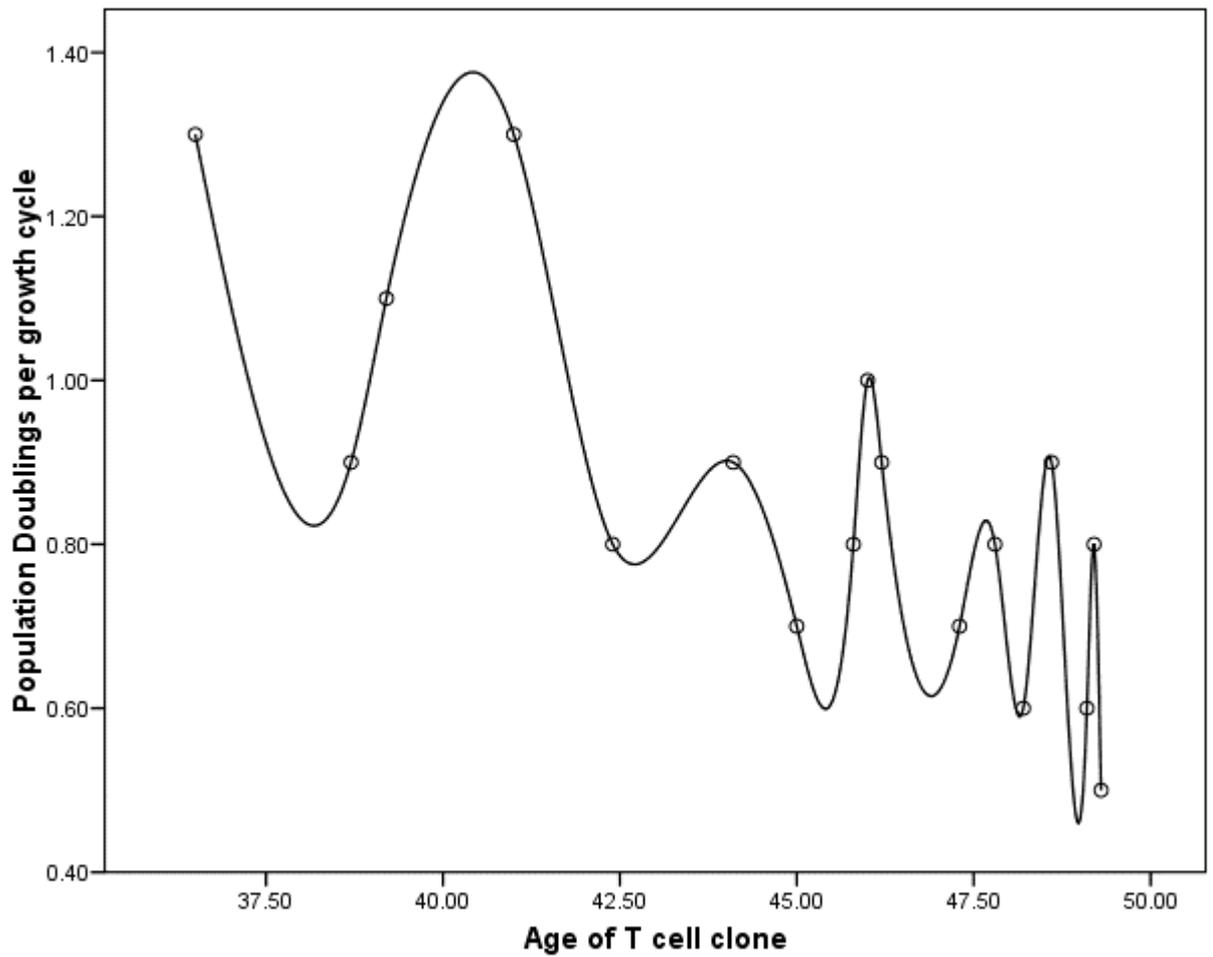


Figure 10 Proliferative capacity of 400-23b clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.9, Maximum PD achieved – 49.1

11)

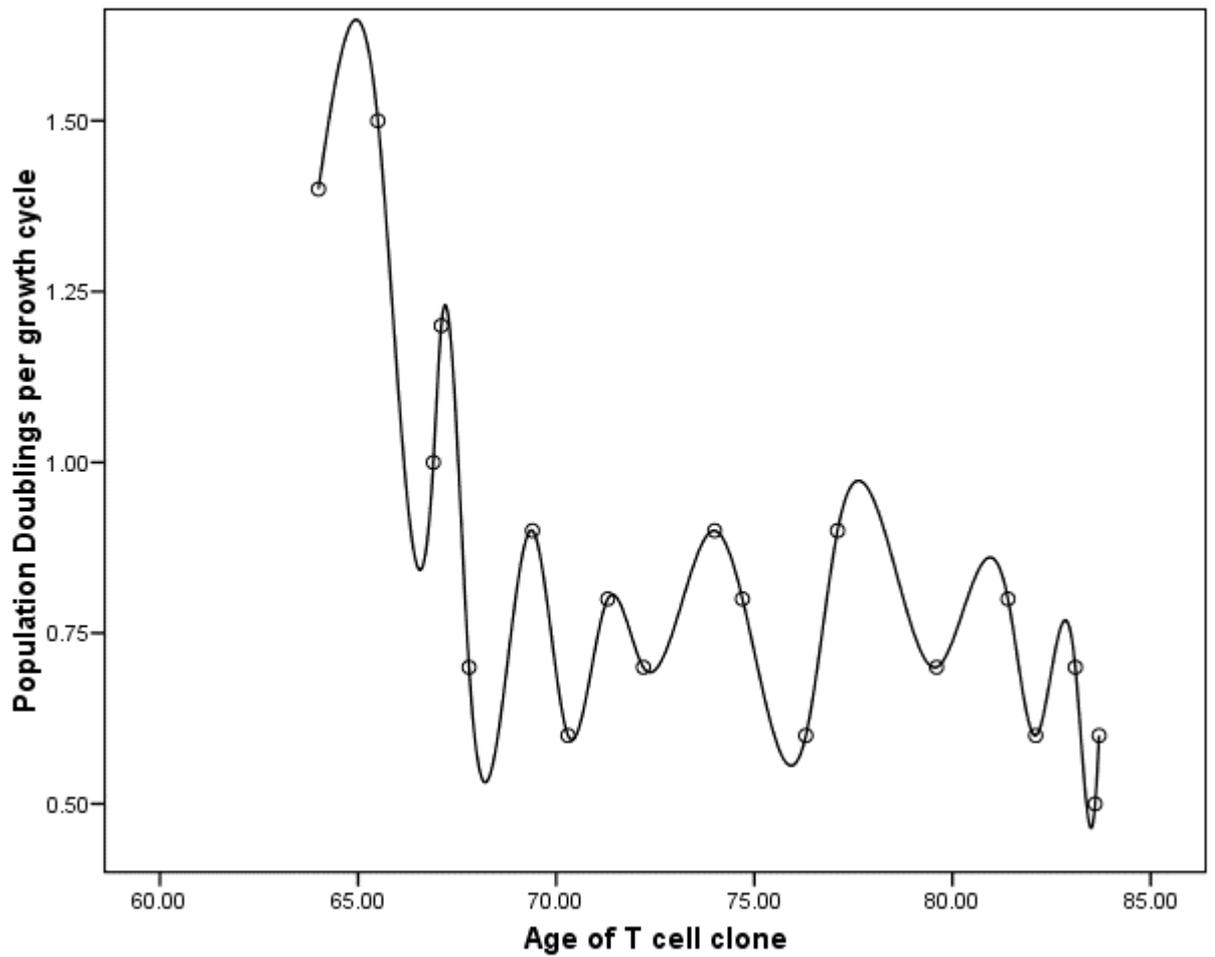


Figure 11 Proliferative capacity of 400-23c clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 1.1, Maximum PD achieved – 83.6

12)

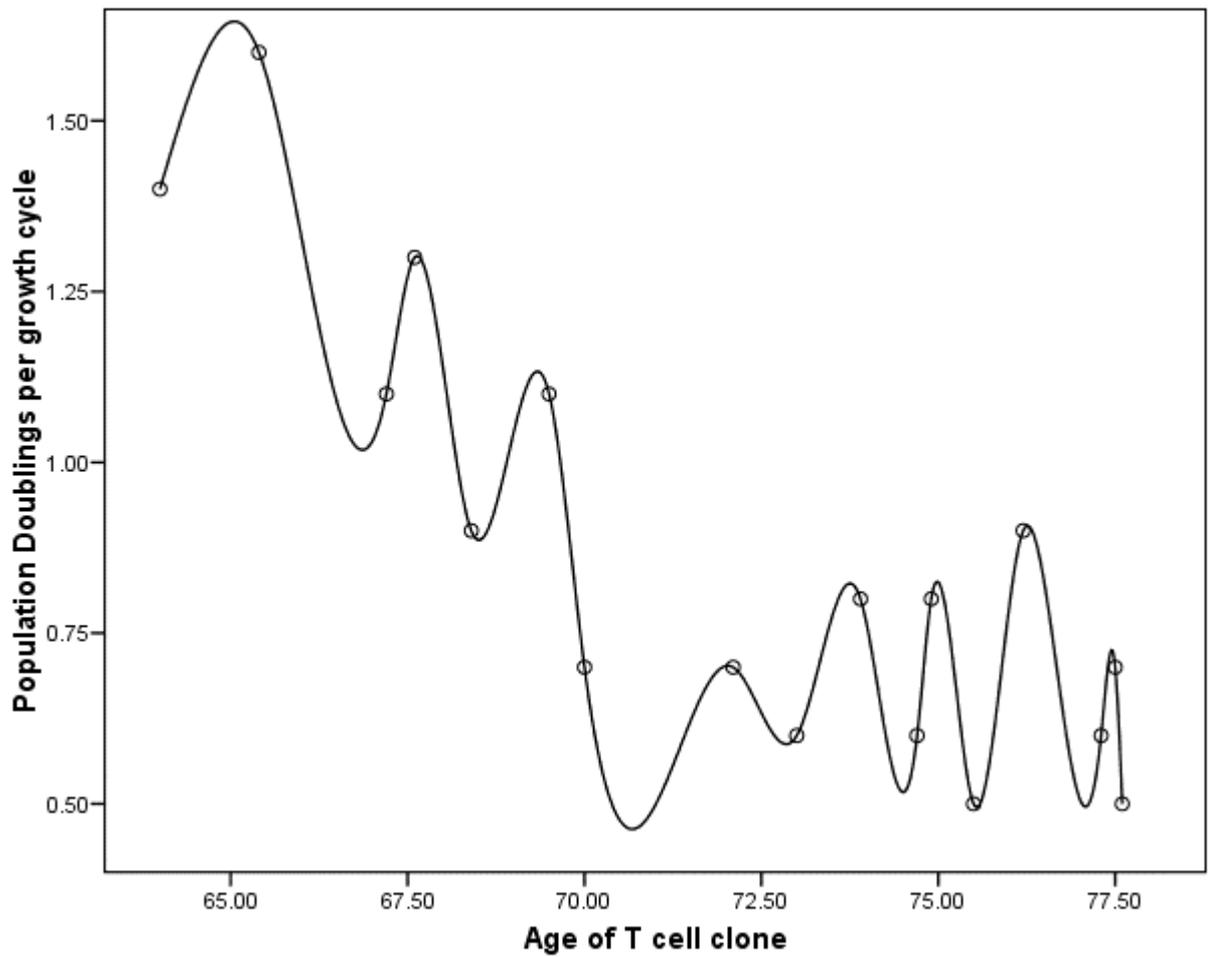


Figure 12 Proliferative capacity of 400-23d clone (derived from a 26 year old overtly healthy laboratory worker) throughout its lifespan. The points represent the PD achieved during successive seven day growth cycles and a spline curve has been used to illustrate growth rhythms. The mean PD per seven day growth cycle – 0.8, Maximum PD achieved – 77.6

As explained in chapter-3, section 3.1.3, all the twelve clones examined in this investigation, grew in waves of maximal and minimal rates of proliferation variable from one to several growth cycles in length during the early part of their lifespan (as indicated in figure 3.1 and figures 1-12 in appendix-I). But towards the later stages of their lifespan, the size or length of the waves decreased and the wave frequency increased. This type of growth of the T cell clones (as explained in detail in chapter-3, Section 3.1.3) was in line with the previous findings from the group of Barnett (Hyland *et al.*, 2000). The increase in frequency towards the later stages of a T cell clone's lifespan can be implied as their ability to retain their responsiveness to proliferative stimulation (Hyland *et al.*, 2000; Pawelec *et al.*, 2002).

APPENDIX-II

Summary tables showing the effect of acute exposure of mitoQ or DTPP or vitamin E on human peripheral blood mononuclear cells *ex vivo*

Appendix-II, Table 1. Summary of the results of the effect of different concentrations of mitoQ supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of mitoQ supplementation	Length of mitoQ supplementation	Endogenous DNA damage levels in mitoQ treated cells compared to untreated cells (controls)	DNA damage levels in mitoQ treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM mitoQ	3 hours	Significantly reduced	Significantly reduced
25-30	1µM mitoQ	6 hours	Significantly reduced	Significantly reduced
55-60	1µM mitoQ	3 hours	Significantly reduced	Significantly reduced
55-60	1µM mitoQ	6 hours	Significantly reduced	Significantly reduced
25-30	0.5µM mitoQ	3 hours	Significantly reduced	Significantly reduced
25-30	0.5µM mitoQ	6 hours	Significantly reduced	Significantly reduced
55-60	0.5µM mitoQ	3 hours	Significantly reduced	Significantly reduced
55-60	0.5µM mitoQ	6 hours	Significantly reduced	Significantly reduced
25-30	0.25µM mitoQ	6 hours	No significant changes	Significantly reduced
55-60	0.25µM mitoQ	6 hours	No significant changes	Significantly reduced
25-30	0.1µM mitoQ	6 hours	No significant changes	Significantly reduced
55-60	0.1µM mitoQ	6 hours	No significant changes	Significantly reduced

Appendix-II, Table 2. Summary of the results of the effect of different concentrations of DTTP supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of DTTP supplementation	Length of DTTP supplementation	Endogenous DNA damage levels in DTTP treated cells compared to untreated cells (controls)	DNA damage levels in DTTP treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM DTTP	3 hours	No significant changes	Significantly reduced
25-30	1µM DTTP	6 hours	No significant changes	Significantly reduced
55-60	1µM DTTP	3 hours	No significant changes	Significantly reduced
55-60	1µM DTTP	6 hours	No significant changes	Significantly reduced
25-30	0.5µM DTTP	3 hours	No significant changes	Significantly reduced
25-30	0.5µM DTTP	6 hours	No significant changes	Significantly reduced
55-60	0.5µM DTTP	3 hours	No significant changes	Significantly reduced
55-60	0.5µM DTTP	6 hours	No significant changes	Significantly reduced
25-30	0.25µM DTTP	6 hours	No significant changes	No significant changes
55-60	0.25µM DTTP	6 hours	No significant changes	No significant changes
25-30	0.1µM DTTP	6 hours	No significant changes	No significant changes
55-60	0.1µM DTTP	6 hours	No significant changes	No significant changes

Appendix-II, Table 3. Summary of the results of the effect of different concentrations of vitamin E supplementation, on the levels of oxidative DNA damage in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of vitamin E supplementation	Length of vitamin E supplementation	Endogenous DNA damage levels in vitamin E treated cells compared to untreated cells (controls)	DNA damage levels in vitamin E treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	37µM vitamin E	3 hours	No significant changes	No significant changes
25-30	37µM vitamin E	6 hours	No significant changes	No significant changes
55-60	37µM vitamin E	3 hours	No significant changes	No significant changes
55-60	37µM vitamin E	6 hours	No significant changes	No significant changes
25-30	74µM vitamin E	3 hours	No significant changes	No significant changes
25-30	74µM vitamin E	6 hours	No significant changes	No significant changes
55-60	74µM vitamin E	3 hours	No significant changes	No significant changes
55-60	74µM vitamin E	6 hours	No significant changes	No significant changes

Appendix-II, Table 4. Summary of the results of the effect of different concentrations of mitoQ supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of mitoQ supplementation	Length of mitoQ supplementation	Basal GSH:GSSG ratio in mitoQ treated cells compared to untreated cells (controls)	GSH:GSSG ratio in mitoQ treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM mitoQ	3 hours	Significantly higher	Significantly higher
25-30	1µM mitoQ	6 hours	Significantly higher	Significantly higher
55-60	1µM mitoQ	3 hours	Significantly higher	Significantly higher
55-60	1µM mitoQ	6 hours	Significantly higher	Significantly higher
25-30	0.5µM mitoQ	3 hours	Significantly higher	Significantly higher
25-30	0.5µM mitoQ	6 hours	Significantly higher	Significantly higher
55-60	0.5µM mitoQ	3 hours	Significantly higher	Significantly higher
55-60	0.5µM mitoQ	6 hours	Significantly higher	Significantly higher
25-30	0.25µM mitoQ	6 hours	No significant changes	Significantly higher
55-60	0.25µM mitoQ	6 hours	No significant changes	Significantly higher
25-30	0.1µM mitoQ	6 hours	No significant changes	Significantly higher
55-60	0.1µM mitoQ	6 hours	No significant changes	Significantly higher

Appendix-II, Table 5. Summary of the results of the effect of different concentrations of DTPP supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of DTPP supplementation	Length of DTPP supplementation	Basal GSH:GSSG ratio in DTPP treated cells compared to untreated cells (controls)	GSH:GSSG ratio in DTPP treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	1µM DTPP	3 hours	No significant changes	Significantly higher
25-30	1µM DTPP	6 hours	No significant changes	Significantly higher
55-60	1µM DTPP	3 hours	No significant changes	Significantly higher
55-60	1µM DTPP	6 hours	No significant changes	Significantly higher
25-30	0.5µM DTPP	3 hours	No significant changes	Significantly higher
25-30	0.5µM DTPP	6 hours	No significant changes	Significantly higher
55-60	0.5µM DTPP	3 hours	No significant changes	Significantly higher
55-60	0.5µM DTPP	6 hours	No significant changes	Significantly higher
25-30	0.25µM DTPP	6 hours	No significant changes	No significant changes
55-60	0.25µM DTPP	6 hours	No significant changes	No significant changes
25-30	0.1µM DTPP	6 hours	No significant changes	No significant changes
55-60	0.1µM DTPP	6 hours	No significant changes	No significant changes

Appendix-II, Table 6. Summary of the results of the effect of different concentrations of vitamin E supplementation, on the GSH:GSSG ratio in human peripheral blood mononuclear cells isolated from fresh human blood derived from ten different healthy donors, five each in the age groups of 25-30 years and 55-60 years

Donor (Age group- yr olds)	Concentration of vitamin E supplementation	Length of vitamin E supplementation	Basal GSH:GSSG ratio in vitamin E treated cells compared to untreated cells (controls)	GSH:GSSG ratio in vitamin E treated cells subjected to exogenously applied ROS (H₂O₂ treatment) compared to cells treated only with exogenously applied ROS (controls)
25-30	37µM vitamin E	3 hours	No significant changes	No significant changes
25-30	37µM vitamin E	6 hours	No significant changes	No significant changes
55-60	37µM vitamin E	3 hours	No significant changes	No significant changes
55-60	37µM vitamin E	6 hours	No significant changes	No significant changes
25-30	74µM vitamin E	3 hours	No significant changes	No significant changes
25-30	74µM vitamin E	6 hours	No significant changes	No significant changes
55-60	74µM vitamin E	3 hours	No significant changes	No significant changes
55-60	74µM vitamin E	6 hours	No significant changes	No significant changes

CHAPTER-9

References

9.1. References

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