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The implication of oxidative stress in human ageing and Alzheimer's disease

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THE IMPLICATION OF OXIDATIVE STRESS

IN

HUMAN AGEING AND ALZHEIMER'S DISEASE

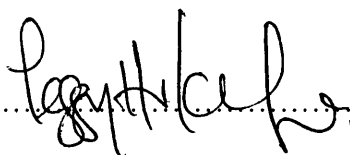Submitted by Peggy Ho Ka Yue for the degree of Ph.D.

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ABSTRACT

Throughout life, we are constantly exposed to a wide variety of extrinsic and intrinsic agents which have the potential to damage cellular biomolecules, including DNA and lipids. Imperfections in cellular defence systems in combination with other age-related changes may contribute to ageing and the development of age-related pathologies such as Alzheimer's Disease (AD).

The aim of this project was to investigate the effect of age and AD on oxidative-damage-related parameters and antioxidants in peripheral blood mononuclear cells (PBMCs) or blood plasma from three groups of volunteers: healthy individuals aged under 30, healthy individuals aged over 60 and patients diagnosed with AD of age over 60 (n = 25 in each group). Hydrogen peroxide (H_2O_2)-induced DNA damage was measured in PBMCs using the comet assay and it was found to increase with increasing concentration of H_2O_2. A higher level of DNA damage was found in the healthy younger group than in the healthy older group whilst AD was found to have no significant effect on the level of DNA damage. AD was found to have a significant effect whilst age was shown to have no significant effect on malondialdehyde (MDA) level, a biomarker for lipid peroxidation. Neither age nor AD had any effect on reduced glutathione level whilst oxidised glutathione level was found to be significantly affected by AD but not age. Neither age nor AD was shown to have any significant effect on the total antioxidant activity (TAA) in blood plasma. However, TAA measured in healthy females over 60 was found to be significantly higher than that in healthy females under 30. Attempts were also made to study the oxidative induction of heme oxygenase-1 expression using quantitative and semi-quantitative RT-PCR.

DEDICATION

To my family and Renate Woodward

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 The ageing population

Although philosophers and scientists have long been interested in the ageing process, general interest in this topic was minimal before 1960. In recent decades, however, interest in the various aspects of ageing has grown due to the realisation that not only do the elderly form an ever-increasing percentage of the population, but they also utilise a disproportionately high percent of health care costs. In 1996, the population of the United Kingdom based on mid-year estimates was 58,801,000. Of that figure, 18.14 % (10,668,000 people) were over pensionable age (i.e. men aged 65 and over and women aged 60 and over). Further statistics on the ageing population in 1996 are shown in Table 1.1.

Group	Number of people
Men aged 65 and over	3,768,000
Women aged 60 and over	6,900,000
People aged 65 and over	9,250,000
People aged 75 and over	4,192,000
People aged 85 and over	1,067,000

Table 1.1 The ageing population in the United Kingdom in 1996 (Population Trends, Summer 1998, Table 6).

In 1996, in the United Kingdom, almost two-thirds of people aged 75 and over were women and almost three-quarters of those aged 85 and over were women (Population Trends, Summer 1998, Table 6). In 1996, 5,670 women and 655 men were aged 100 and over. In 2021, it is estimated that 22,493 women and 4,386 men will be in this age group. In 1996, a man of 60 could be expected to live for another 18.5 years and a woman of the same age for 22.4 years. The number of people of pensionable age is projected to increase from 10.7 million in 1996 to 11.8 million in 2010. This will rise to 14 million by 2021 and will peak at just over 17 million. The number of people aged over 75 will have doubled by the middle of the next century, while the number aged 90 and over will have more than tripled. All statistics shown above were obtained from the 1996-based National Population Projections provided by the Government Actuary's Department. The projected change in the population of the United Kingdom is shown in Table 1.2. It is estimated that at present some 5 % of the population aged 65 and over and 20 % of the population aged 80 and over suffer from dementia (House of Commons, Hansard, 30/10/95, col, 45W and PT op cit.).

Group	Number of people		
	1996	**2001**	**2011**
People aged over 75 and over	4,193,000	4,383,000	4,516,000
People aged over 85 and over	1,069,000	1,158,000	1,281,000

Table 1.2 The projected change in the population of the United Kingdom (IBDI, Appendix 1).

Improvements in general living conditions such as better nutrition, housing, medical care and advances in medical research are responsible for the rapid growth of the ageing population. As these factors within a population approach optimum, the curve of the chance of death versus age shifts towards a limit determined by individual ageing changes associated with the environment and disease plus those formed as a result of an increasing inborn process, the ageing process.

1.2 The ageing process

It is true that as people age, they become more susceptible to a variety of diseases. Nevertheless, it is important to recognise that ageing and disease are distinctly different. So, how is ageing defined? Harman defined ageing as "the progressive accumulation of changes with time associated with or responsible for the ever increasing susceptibility to disease and death which accompany age" (Harman, 1981) while Rothstein stated that "the changes from maturity through senescence constitutes the ageing process" (Rothstein, 1982). Regardless of how ageing is defined, it is now widely accepted that the ageing effects include the following (Knight, 1995):

a) a progressive decrease in efficiency of essentially all physiological functions;

b) atrophy of most organs and tissues;

c) increased vulnerability to trauma, infections, and various immune system malfunctions;

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d) increased susceptibility to most malignant processes; and

e) decreased capacity for the body to extract oxygen from the air and transmit it to the circulatory system.

There have been numerous theories to explain the ageing process (Medvedev, 1990). This is a natural result of the very rapid progress in our knowledge of biological phenomena and the application of many new approaches, methods and techniques to ageing research. Almost every important discovery in cellular and molecular biology has stimulated a new family of theories of ageing or new advanced versions of older versions. Many of them are either out of date, too selective or they employ contradictory methods of classification. Many theories co-exist because they do not contradict each other, or because they attempt to explain different and independent forms of senescence. No one theory is generally accepted but scientists are working towards the same goal, that is to investigate means of increasing the functional life span of man while continue to work towards a consensus on the cause(s) of the ageing process.

1.3 The ageing theories

As an organism ages, its chance of death increases, so that all individuals of a given species are dead by some age, characteristic of that species. The maximum life span is around 90-115 years for humans. For the purpose of this discussion, theories to explain the mechanism of ageing are grouped under three main headings -

programmed ageing, damage accumulation ageing and the free radical theory of ageing.

1.3.1 Programmed ageing

The programmed theory of ageing proposes a purposeful sequence of events encoded into the genome. This genome-based theory suggests that although everyone is programmed individually, an internal "clock" starts at conception and runs a specific period of time. Accordingly, genes carry specific instructions that control not only growth and maturation, but also decline and death. This theory has considerable support, including that obtained from both the observational studies and laboratory research (Knight, 1995). The studies of telomerase (Blackburn, 1991; Kim *et al*, 1994; Haber, 1995; Price, 1999) have increased our understanding of programmed ageing. Progressive shortening of the ends of chromosomes, or telomeres, accompanies normal cell division and may contribute to cellular ageing. In humans, germ cells express telomerase and maintain their telomere length along with their ability to divide throughout life. In contrast, somatic cell tissues do not have telomerase activity, and they progressively lose telomere length (Haber, 1995). Patients with Hutchinson-Gilford Syndrome, the premature ageing syndrome (Section 1.3.1.1), have pronounced shortening of telomeres. These observations have led to the hypothesis that telomere length serves as a biological "clock" regulating the life span of normal cells. In a manner consistent with this model, cultured normal human fibroblasts undergo a finite number of cell divisions, after

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which they enter a state of senescence. The number of cell divisions correlates well with the initial length of the telomeres, and progressive loss of telomeres is observed in culture (Haber, 1995).

It is generally accepted that in humans there is a modest to moderate correlation in life expectancy between family members. That is, families in which centenarians exist are more likely to have other equally long-lived members than those families without centenarians. Furthermore, the difference in longevity i.e. the length of life is, on average, less in identical than with fraternal twins (Schächter *et al*, 1993). Hutchinson-Gilford Syndrome, Werner's Syndrome and Down's Syndrome are examples of disorders that are characterised by accelerated ageing and decreased life expectancy. They also provide some evidence that a few genes can have major effects on ageing.

1.3.1.1 Accelerated ageing syndromes

Hutchinson-Gilford Syndrome (Progeria) is extremely rare, affecting 1 in 4-8 million births. It is almost certainly genetic in origin being dominant rather than recessive and is considered to be a disease model of certain aspects of accelerated ageing (Berstein and Gensler, 1993). Sufferers appear normal at birth but within a few years, somatic growth slows and clinically apparent ageing features such as baldness, skin atrophy and atherosclerosis appear. Interestingly, the endocrine system apparently remains normal. They have normal to above average intelligence with a

median age of death at 12 years. Most deaths are due to heart failure. Cultured progeria fibroblasts show poorer growth and generally earlier *in vitro* senescence than normal human cells (Brown *et al*, 1978). They also exhibit decreased ability to repair single-strand breaks following γ-irradiation (Epstein *et al*, 1974).

Werner's Syndrome, considered by some gerontologists to be a model of precocious ageing, is a rare autosomal-recessive mutation with some striking differences to Hutchinson-Gilford Syndrome. Affected individuals are usually normal during childhood but stop growth during their teens. Greying and whitening of the hair occurs at an early age, the skin appears old with a scaly appearance. The patients develop early cataracts, tumors, bone demineralisation, diabetes and atherosclerosis. They show peripheral muscular atrophy, poor wound healing and poor gonad development. They usually die in their 40s from atherosclerosis-related conditions. A report by Goto *et al* (1992) indicated a genetic linkage in this syndrome to five markers on chromosome 8.

Individuals with Down's Syndrome develop certain characteristics similar to premature ageing. It was the first condition shown to be associated with a chromosomal abnormality; about 85 % have 47 chromosomes (three chromosome 21), while in the other 15 %, the extra chromosome is borne on another chromosome (translocation), usually Group D. The disorder is quite common, affecting one in every 600-800 births, increasing to one in approximately 25 if the mother is over 40 years old. The condition is commonly characterised by a variety of dermatological

features, mental retardation, congenital defects and a distinctly shortened life span. It is not understood why the life span of these individuals is shortened. However, one study reported an increase in their blood plasma lipoperoxides in the presence of increased red cell activity of superoxide dismutase and glutathione peroxidase (Kedziora *et al*, 1986). Although these findings are somewhat confusing in that the increased enzyme values would be expected to be protective, it suggests a possible relationship to the free radical theory of ageing.

These syndromes have most of the features associated with accelerated ageing, suggesting that normal human ageing can be greatly affected by simple mutations in one or both of a pair of key genes. In recent years, association studies have identified a number of loci, alleles associated with enhanced longevities in certain populations. A good example is apolipoprotein E ε2 (Apo E ε2), which is prevalent among centenarians (Schächter *et al*, 1994). ApoE ε2 is also associated with a decreased susceptibility to Alzheimer's disease, in contrast to the ε4 allele, which, when homozygous, is associated with the appearance of Alzheimer's disease, on average, about a decade earlier than that in individuals who are homozygous for the more prevalent ε3 allele (Strittmatter and Rose, 1995).

1.3.2 Damage accumulation ageing

Proteins, lipids and nucleic acids are all known to be susceptible to damage by free radicals/oxidants and other endogenous reactive agents. Oxidatively damaged proteins and lipids are subjected to both degradation and some repair reactions while DNA is usually not degraded but is subjected to numerous repair processes. There have been very few demonstrations or documentation of the general biological importance of protein changes to ageing at the cellular and organ level (Garfni, 1990; Beckman and Ames, 1998). On the other hand, damage can accumulate in DNA, particularly in non-replicating cells, and lead to declining production of mRNA with age.

1.3.2.1 The DNA damage theory of ageing

According to the DNA damage theory of ageing, first proposed by Alexander, mammalian ageing is due to the accumulation of DNA damage in somatic cells (Alexander, 1967). The idea that damage to DNA, rather than damage to other biomolecules or structures, is the underlying cause of ageing is based on the following general considerations. DNA encodes the genetic information of the cell and deterioration of its ability to be transcribed will lead to a decline in cellular function and lethality. By contrast, other macromolecules, such as RNA or proteins, can be replaced, in principle, by new synthesis if the DNA remains undamaged. In general, cells are thought to have a considerable DNA-dependent capacity for self-

renewal involving replacement and repair of worn-out components. DNA is especially vulnerable to damage because it ordinarily occurs in only two copies per diploid cell (compared with the numerous copies of most other macromolecules), and each of the many deoxyribonucleotides is a potential target of damaging agents. The DNA damage theory was preceded by the related theory that ageing is due to somatic mutation (Failla, 1958). These two theories are similar in that both attribute ageing to errors in DNA. However, there are important differences between DNA damage and mutations. DNA damage cannot be inherited over successive cell generations, whereas mutations can be inherited. Furthermore, DNA damage, in contrast to mutations, can be repaired. Because of these fundamental differences at the molecular level, the DNA damage theory of ageing has a logic that is substantially different from that of the somatic mutation theory (reviewed by Gensler and Bernstein, 1981). The relationship between DNA damage and ageing is schematically represented in Figure 1.1.

CASUAL
EVENTS

CELLULAR
ACTIVITIES

PHYSIOLOGICAL
PHENOMENA

PHYSICAL & BIOCHEMICAL
CELLULAR BARRIERS

Radiation

DNA lesions

DNA repair

Persisting
and/or
remaining lesions

Chemicals

Unaltered
DNA

Accurate
repair

Error prone
repair

DNA modifications,
rearrangements
&
mutations

Ageing
and/or
diseases
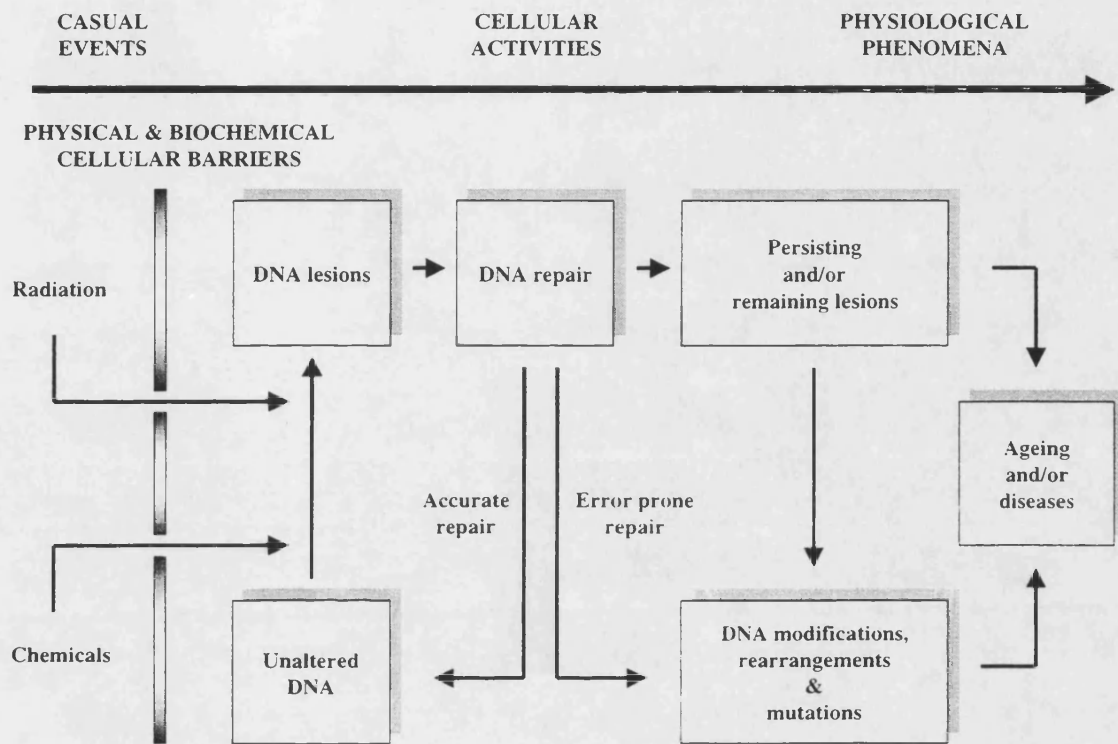
Figure 1.1 Hypothetical pathways by which induced DNA damage may contribute to the aged phenotype (Viig *et al*, 1985).

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1.3.2.2 Sources of oxidative DNA damage *in vivo*

DNA damage can be caused either by intrinsic intracellular processes such as oxidation, hydrolysis, and alkylation or by extrinsic chemicals and radiation. Agents that induce chemical change in DNA have been classified in Figure 1.2 into endogenous agents and exogenous agents. Among these possible causes of DNA damage, oxidative DNA damage caused by free radicals, produced from oxygen as a consequence of normal respiratory metabolism, appears to be a major cause of ageing in mammalians. The univalent pathway of oxygen reduction generates the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\bullet}OH$) and water (Section 1.3.3.1). It has been concluded that approximately 50 % of oxidative DNA damage is due to $^{\bullet}OH$ (Imlay and Linn, 1988).

Endogenous agents	Exogenous agents
Endogenous alkylating agents	Solar ultraviolet radiation
Formaldehyde and other aldehydes	Background ionising radiation
Reactive oxygen species	Natural mutagens in food
	Cigarette smoke
	Occupational exposure
	Pollution
	Drugs

Figure 1.2 Sources of DNA damage.

1.3.2.3 Types of Oxidative DNA damage

Oxygen radicals can interact with DNA at either the deoxyribose-phosphate backbone or at a base (Imlay and Linn, 1988). The reaction of an oxygen radical with a deoxyribose leads to fragmentation of the sugar, loss of the base to which it is bound, and strand breakage. Some of the reaction products resulting from the oxidation of DNA are thymine glycol (Section 1.3.3.2.1), methyl tartronyl urea, urea, 5-hydroxymethyluracil, 4,6-diamino-5-formamidopyrimidine and 8-hydroxy-2'-deoxyguanosine (8-OHdG). These products are removed from DNA by specific repair enzymes and excreted in urine. Indeed, their levels in human urine can be used to quantify the amount of oxidative damage. Ames and Gold (1991) estimated the total number of all types of oxidative damage to DNA per cell per day to be about 10,000 in humans and about 100,000 in rats. The difference in oxidative DNA damage incidence rates between human and rats is consistent with the proposal that oxidative DNA damage increases in proportion to species-specific basal metabolic rates (Shigenaga et al, 1989). Treatment of DNA in vitro with H_2O_2 produces DNA damages of which over 90 % are altered bases; about 2-4 % are single-strand breaks; about 0.9 % are double-strand breaks and about 0.8 % are inter-strand cross-links (Massie et al, 1972). Methods of detecting oxidative DNA damage are discussed in Chapter 3.

1.3.2.4 Cellular defence mechanisms against oxidative DNA damage

DNA damage accumulates in a cell if the rate of occurrence of DNA damage exceeds the rate of DNA repair. There appear to be three levels of defence against the DNA damage caused by active oxygen species. These levels are:

a) prevention by removal of active oxygen species before they can interact with DNA;

b) repair of DNA damage; and

c) compensation for cell loss due to lethal DNA damage through cellular redundancy and/or replication.

Cellular enzymes include superoxide dismutase which converts $O_2^{\bullet-}$ into H_2O_2, and catalase and glutathione peroxidase which convert H_2O_2 into H_2O. The removal of reactive oxygen species, however, is incomplete, allowing those that escape to damage cellular constituents. Repair of DNA damage provides another line of defence. Double-strand DNA genomes have a built-in informational redundancy because the coding sequences of the two strands are complementary. Consequently, it is possible to repair damage localised to one strand by excising the damaged sequence and replacing the lost information by copying from the complementary strand. This general process is known as excision repair (Figure 1.3). This process appears to be the principal mechanism for removing single-strand damage, which, if unrepaired, would contribute to ageing. The most well-understood pathways of

excision repair in mammalians are those initiated by specific glycosylases which remove damaged bases from the deoxyribose phosphate backbone of DNA. When the damaged base is removed from the DNA by a glycosylase, an apurinic/apyrimidine (AP) site is formed. For repair to be completed, the AP sites must be removed and replaced by an undamaged correct base, one that is complementary to the pairing partner in the undamaged strand of DNA. AP sites are removed by a process in which the DNA strand is first cleaved, either at the 3' side or the 5' side, by an AP endonuclease. Class I AP endonucleases cleave at the 3' side and Class II AP endonucleases cleave at the 5' side. After an initial incision by an AP endonuclease, exonucleases can degrade the DNA in 5' to 3' or 3' to 5' direction at the free ends created by the incision. Finally, a DNA polymerase inserts correctly paired bases in 5' to 3' direction. The final reaction in the pathway involves the formation of the last phosphodiester bond in the repaired strand by polynucleotide ligase (Demple and Harrison, 1994; Bohr *et al*, 1995; Loft and Poulsen, 1996). Another mechanism for removing single-strand damage is referred to as direct reversal. This mechanism depends on enzymes that can recognise some specific types of damage and then essentially reverse the damage-forming reaction to restore the intact sequence. This type of repair occurs with a narrow spectrum of damage that may have limited relevance to ageing. Double-strand damage is potentially important in ageing because it is difficult to repair. Excision repair is presumably ineffective in removing double-strand damage because this type of repair depends on the DNA strand opposite the damaged one being intact. Repair of double-strand damage can occur if the undamaged DNA duplex donates a single-

stranded section to the damaged DNA molecule to allow replacement of its lost information. This process is referred to as recombinational repair which may be significant in resisting ageing (Bernstein and Gensler, 1993).

Another way to cope with unrepaired DNA damage is cellular redundancy (Gensler and Berstein, 1981; Berstein and Gensler, 1993). For example, in humans, more cells are formed in the brain than are necessary for normal function. In mammals, maximum potential life span was found to be proportional to relative brain size (Hofman, 1983). The correlation of longevity with relative brain size may reflect the advantage of a reserve supply of neurons to compensate for loss of neuron function due to DNA damage. Another way for a population of cells to cope with unrepaired DNA damage is to replace lethally damaged cells by replication of undamaged ones. Examples of rapidly replicating cell populations are haemopoietic cells of the bone marrow and intestinal epithelial cells. These cells have a very large capacity for self-renewal and it appears that any cell loss due to DNA damage is compensated by the replication of those cells with little or no unrepaired DNA damage (Holmes *et al*, 1992).

FREE RADICALS

DNA → DNA with Thymine Glycol

Ligase
Polymerase
Exonuclease

Glycosylase

Nicked DNA

Damaged Base

AP endonuclease

DNA with apurinic/apyrimidinic (AP) site
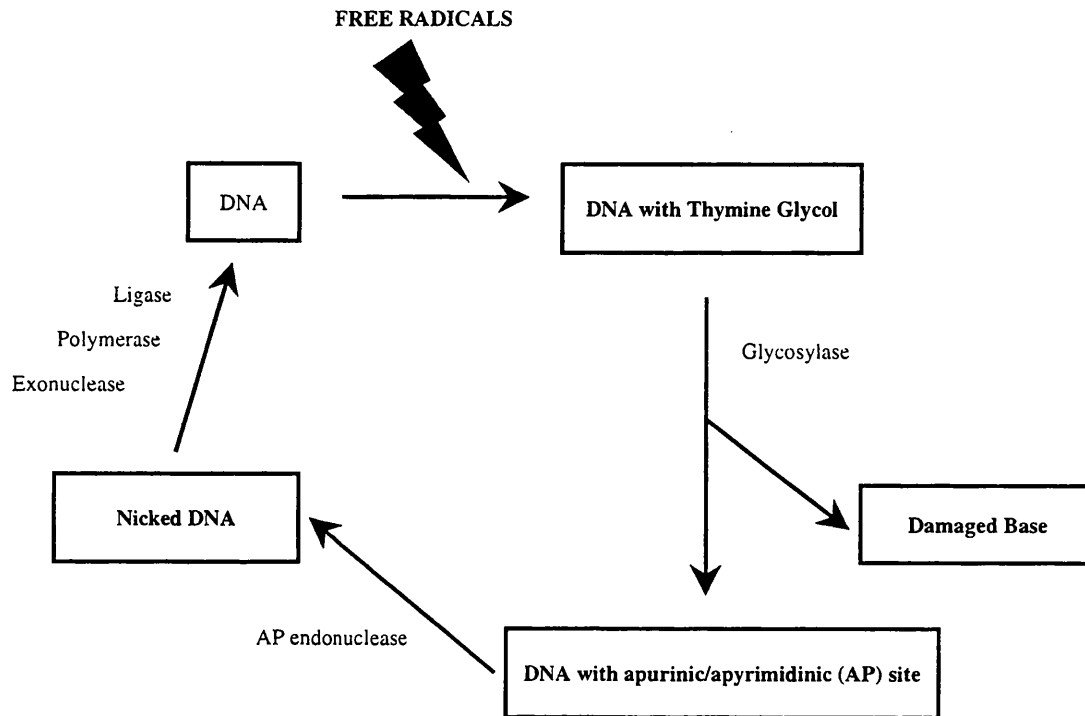
Figure 1.3 Oxidative damage to DNA and its repair. The reaction of DNA with free radicals leads to the formation of damaged base. The original base can be restored by this process known as the excision repair (Weindruch *et al*, 1993).

1.3.2.5 DNA damage accumulation and ageing

Long-lived neurons, muscle cells, and other differentiated cells either do not divide or divide slowly, and accumulate DNA damage if the rate of DNA repair is exceeded by the rate of DNA damage. The brain is comprised largely of non-dividing neurons. Here, endogenous DNA damage accumulates with age, mRNA synthesis and protein synthesis decline, cell loss occurs, tissue function is reduced, and the functional impairments directly related to the central processes of ageing occur. Thus, for the

brain, there appears to be a direct relationship between DNA damage accumulation and important features of ageing (Holmes *et al*, 1992).

Lymphocytes exist in a non-cycling state for years in humans. Consequently, lymphocytes are susceptible to the kinds of DNA damage accumulation found in other non-dividing cells. King *et al* (1997) and Piperakis *et al* (1998) presented evidence for accumulation of age-dependent DNA single-strand breaks and/or alkali labile sites in human peripheral lymphocytes. A number of different investigators have reported an increase in spontaneous chromosomal aberrations in lymphocytes with age. For instance, Fenech and Morley (1985) observed that as human beings age from newborn to 82 years, spontaneous chromosome aberrations in cultured peripheral blood lymphocytes increased by about four-fold. Other evidence suggests a relationship between DNA damage and functional decline in muscle and in the liver. This evidence is extensively reviewed by Bernstein and Gensler (1993).

1.3.3. The free radical theory of ageing

As discussed previously, the accumulation of free radical-induced oxidative damage by macromolecules may be directly related to ageing (Harman, 1992a, 1992b; Yu and Yang, 1996). The possibility that free radicals play a significant role in ageing processes and in the pathogenesis of late-life diseases continues to intrigue many gerontologists and other investigators.

At present, the most popular and widely tested ageing theory is the free radical theory of ageing (Harman, 1956, 1981, 1992a, 1992b; Beckman and Ames, 1998). Individuals are constantly exposed both to exogenous and endogenous sources of oxidants. Various antioxidant defences have evolved to combat the constant oxidant load, but free radical damage inevitably accumulates with age. This has led to the free radical theory of ageing, first proposed by Denham Harman (1956). The theory postulates that ageing is caused by free radical reactions i.e. these non-specific, essentially irreversible reactions may be involved in the production of the ageing changes associated with the environment, disease and an intrinsic ageing process. It predicts that the life span of an organism can be increased by slowing the rate of initiation of random free radical reactions and/or decreasing the chain of these reactions. The former should be achieved by decreasing ingestion of easily oxidised dietary components, calorie intake, and temperature; the latter should be achieved by increasing the concentrations of free radical inhibitors in the organism or by increasing the resistance of its constituents to free radical attack.

Oxidant attack and antioxidant defence are closely matched, but defence is less than perfect, and free radical damage accumulates with age. It was proposed that this imbalance in favour of oxidants is a cause of ageing itself (Harman 1956, 1981). Harman stated that "the ageing process may be simply the sum of random changes produced by free radical reactions" (Harman, 1992a, 1992b). Support of Harman's theory includes:

a) studies of the origin and evolution of life (Harman, 1980a);

b) studies of the effect of ionizing radiation on living organisms (Upton, 1957);

c) dietary manipulations of endogenous free radical reactions (Eriksson *et al*, 1994);

d) the plausible explanations it provides for ageing phenomena (Harman, 1980b); and

e) the growing number of studies that implicate free radical reactions in the pathogenesis of specific diseases such as cancer, arteriosclerosis and other common degenerative diseases e.g. Alzheimer's and Parkinson's diseases (Harman, 1980c; Evans *et al*, 1995; Halliwell and Gutteridge, 1989; Youdim *et al*, 1994).

Because of the popularity of free radical research, a large number of reviews have addressed various aspects of the interplay between oxidants and ageing. The phenomenon and study of ageing are incredibly diverse, encompassing organisms from rotifers to mammals and techniques from physiology to genetics. The free radical theory has been extensively reviewed by Beckman and Ames (1998).

1.3.3.1 Sources of oxidants

Biological oxidants i.e. free radicals and reactive oxygen species can derive from external sources, for example, in the skin from exposure to ultraviolet light; chemotherapy and carcinogens. They can also be produced in the form of radicals. A radical is defined as any atom or molecule with one or more unpaired electrons in

an outer valence shell (e.g. superoxide, hydroxyl and oxygen radicals). Two major

sources of endogenous radicals are mitochondria and phagocytic cells. Mitochondria

have long been recognised to generate superoxide radicals ($O_2^{\bullet-}$) during their

oxidative metabolism (Cadenas *et al*, 1977). The superoxide radical can be

damaging in itself, but it also has the potential to produce the destructive hydroxyl

radical ($^{\bullet}OH$). Superoxide radicals can dismutate to produce hydrogen peroxide

(H_2O_2) (Figure 1.4). Hydrogen peroxide is subject to Fenton chemistry (Gutteridge,

1995) in the presence of transition metal catalysts, for example, iron, to produce $^{\bullet}OH$

(Figure 1.5). Phagocytic cells also produce $O_2^{\bullet-}$ and H_2O_2 during their oxidative

burst (Ames *et al*, 1993).

Uncatalysed dismutation reactions

$$O_2^{\bullet-} + HO_2^{\bullet} + H^+ \longrightarrow H_2O_2 + O_2$$

$$HO_2^{\bullet} + HO_2^{\bullet} + H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide dismutase catalysed reaction

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$
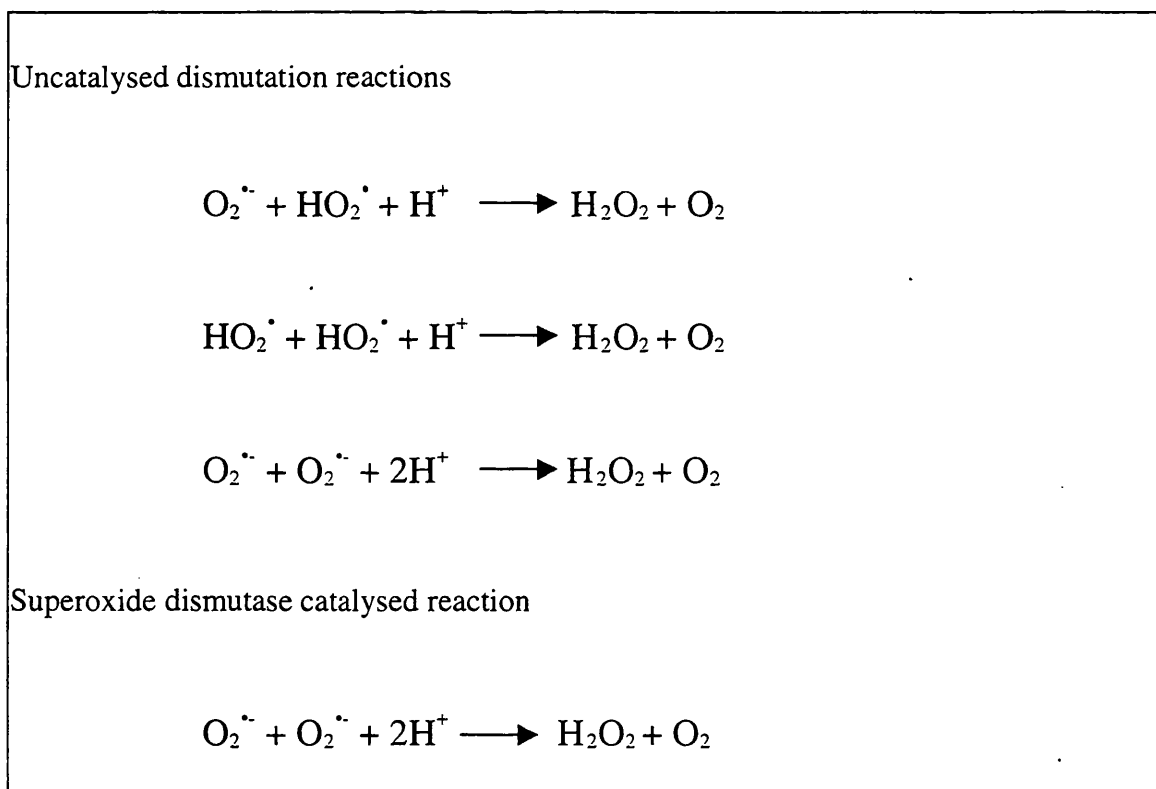
Figure 1.4 The dismutation of superoxide $O_2^{\bullet-}$ (Packer, 1995).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$

$$\text{Net: } O_2^{\cdot -} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + O_2$$
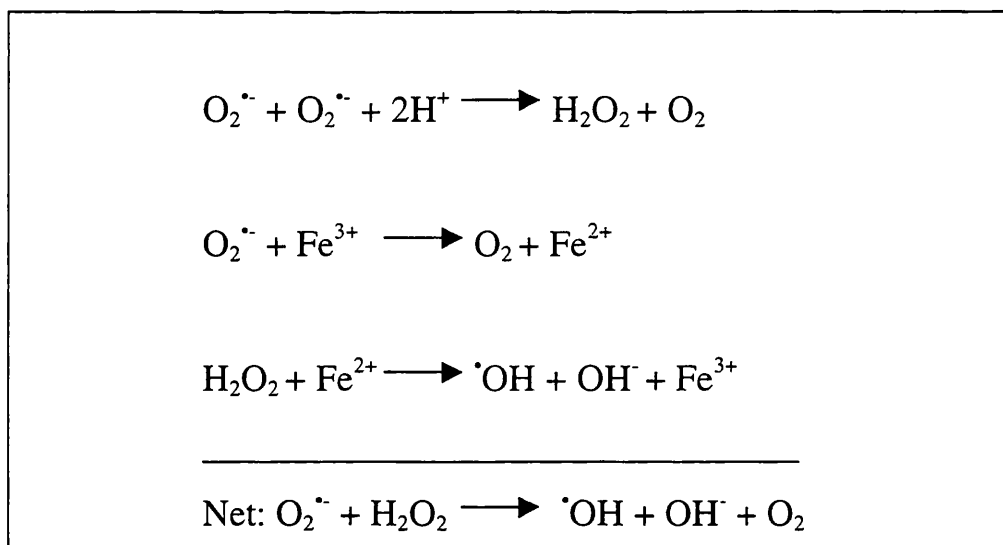
Figure 1.5 Fenton chemistry and the generation of hydroxyl radical $^{\cdot}OH$ (Packer, 1995).

1.3.3.2 Targets of oxidants

Proteins, lipids and nucleic acids have all been studied for their sensitivity to oxidative modification by a wide variety of free radicals and reactive oxygen species. The superoxide radical is not particularly reactive with lipids, carbohydrates, or nucleic acids, but does exhibit limited reactivity with proteins containing transition-metal prosthetic groups, such as heme moieties (Stadtman, 1990). Such reactions result in damage to amino acids. Hydrogen peroxide is an oxidant for many biological molecules, especially those containing sulphydryl groups and iron-sulphur clusters. Hydroxyl radical is the most reactive of all the oxygen radicals and will readily oxidize proteins, lipids, carbohydrates, DNA and RNA (Halliwell and Gutteridge, 1989; Beckman and Ames, 1998).

1.3.3.2.1 Free radicals and DNA

Oxygen radicals may attack DNA at either the sugar or the base, giving rise to a large number of products. Attack at a sugar ultimately leads to sugar fragmentation, base loss, and a strand break with a terminal fragmented sugar residue. Such single-stranded DNA breaks accumulate during exposure of bacterial and mammalian cells to H_2O_2, $O_2^{\cdot-}$, γ-irradiation, or ozone (Imlay and Linn, 1988). Some of the major products from the initial reaction of the $^{\cdot}$OH with thymine, adenine and deoxyribose are shown in Figure 1.6. Once formed, these products can undergo a wide variety of reactions. In aerobic systems, one of the more significant reactions is with molecular oxygen to form organic hydroperoxides, as illustrated for the "6-yl radical" of thymine in Figure 1.7. Chemical or enzymatic reduction of the hydroperoxide leads to the production of an alcohol, for example, thymine glycol in Figure 1.7. Hydrogen peroxide can also produce ring-saturated thymines, hydroxymethyluracil, thymine fragments, and an adenine ring-opened product, presumably through oxygen radical attack at bases (Saul *et al*, 1987).
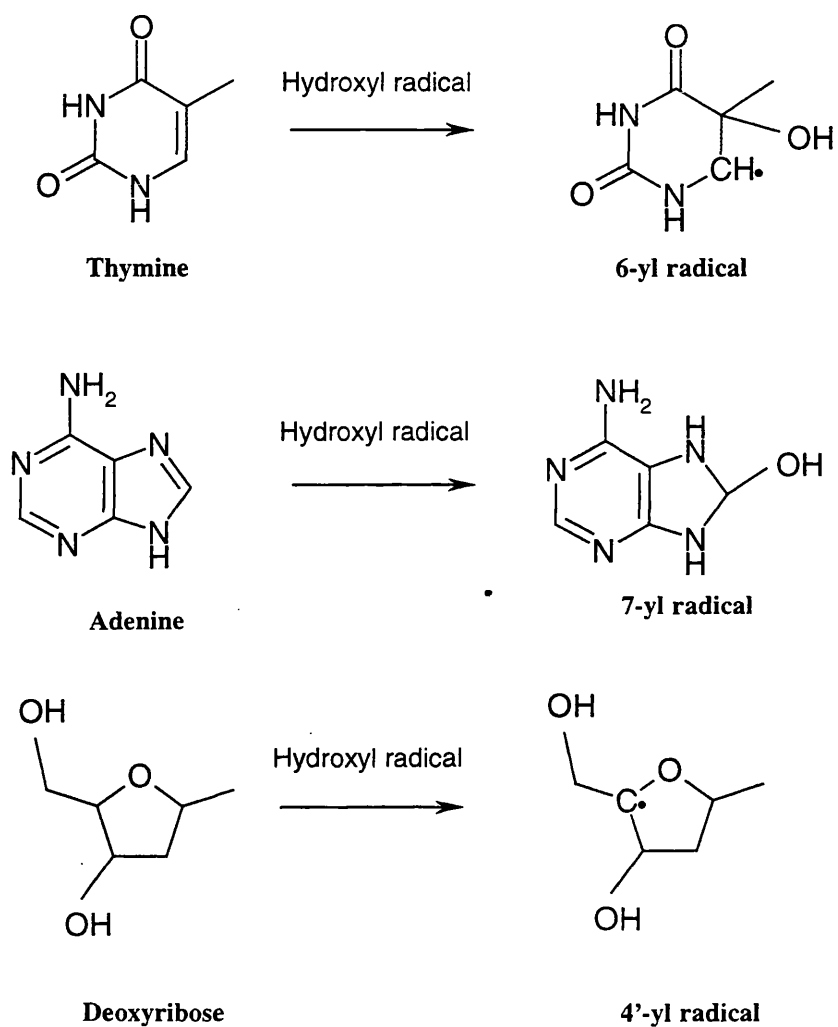
Figure 1.6 Examples of some initial products formed by reaction of hydroxyl radical with DNA components (Saul *et al*, 1987).
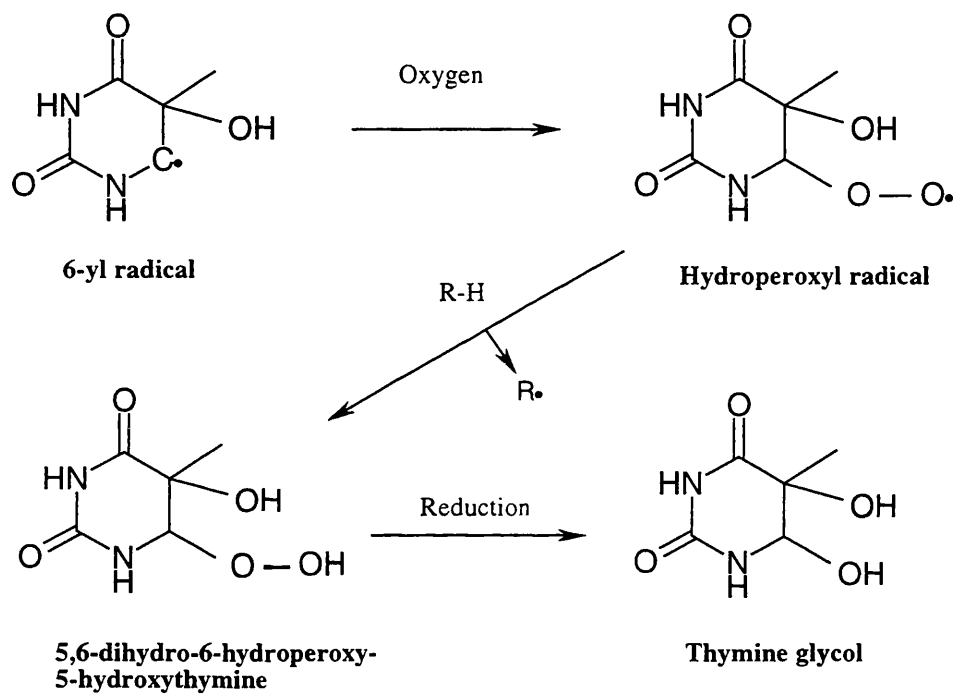
Figure 1.7 Possible mechanism of the formation of thymine glycol from 6-yl radical in aerobic cells (Saul *et al*, 1987).

1.3.3.2.2 Free radicals and lipids

The earliest research on the destruction of biological molecules by oxidants involved lipids (Halliwell and Gutteridge, 1990). Food chemists have long understood that the rancidity of fats results from peroxidative chain reactions in lipids (autoxidation). A peroxyl radical (produced when conjugated dienes in lipids react with oxygen) abstracts a hydrogen atom from the double bond of neighbouring unsaturated lipid, forming a hydroperoxide and an alkyl radical. The latter combines with oxygen to regenerate a peroxyl radical capable of initiating another round of oxidation (Yu, 1993) (Figure 1.8). Ultimately, intramolecular reactions and decomposition yield cyclic endoperoxides and unsaturated aldehydes (e.g. malondialdehyde). Such unsaturated aldehydes are reactive and may act as mutagens or enzyme inactivators. Moreover, the primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins. A number of techniques are available for measuring the rate of peroxidation of membrane lipids or fatty acids and they are discussed in Chapter 4.

FATTY ACID WITH 3 DOUBLE BONDS

- H•   Hydrogen abstraction

Molecular rearrangement

Conjugated diene

Oxygen uptake

Peroxyl radical abstracts H
from another fatty acid causing
an autocatalytic chain reaction

H•

Lipid hydroperoxide

Cyclic peroxide ⟶ Fragmentation to
aldehydes (including
malondialdehyde) and
polymerisation products

Cyclic endoperoxide
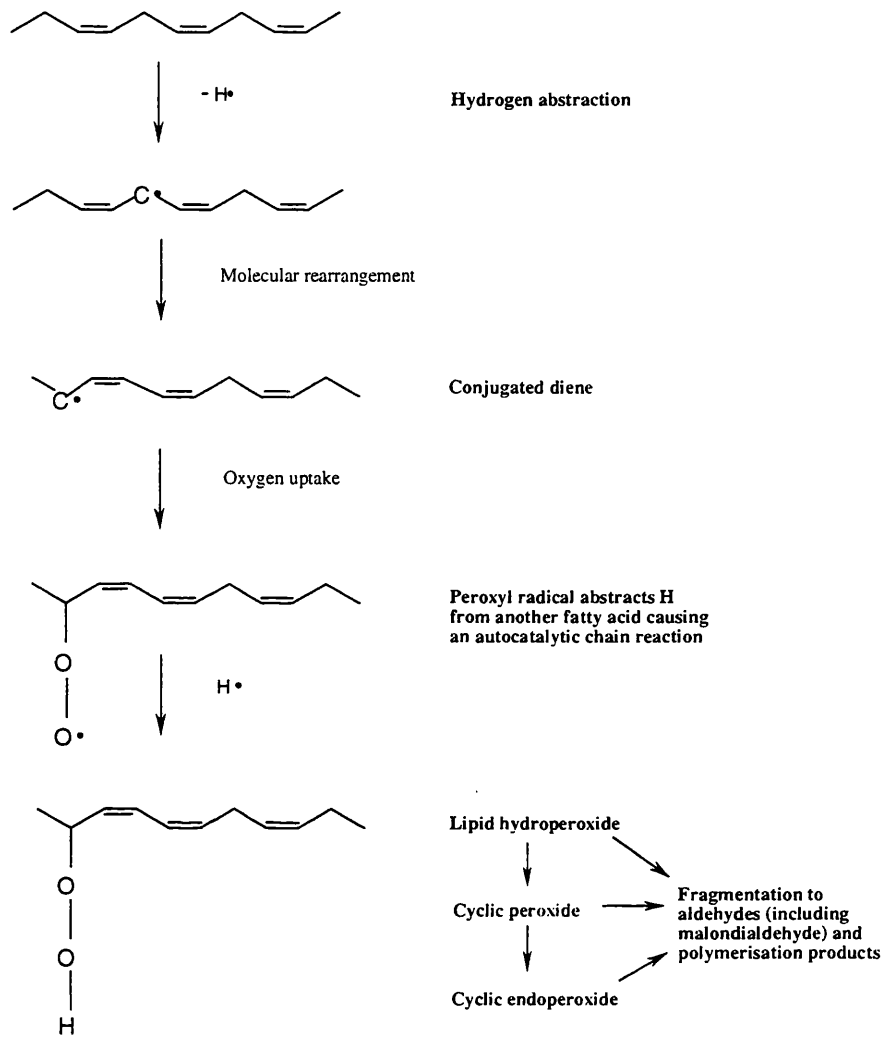
Figure 1.8 Idealised representation of the initiation and propagation reactions

of lipid peroxidation (Yu, 1993). The peroxidation of a fatty acid with three double

bonds is shown.

1.3.3.2.3 Free radicals and proteins

The oxidation of proteins is less well characterised, but several classes of damage have been documented. These include oxidation of sulphydryl groups, reduction of disulphides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalysed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation (reviewed by Halliwell and Gutteridge, 1989). A particularly intriguing development has been the realisation that a number of enzymes possessing active-site iron-sulphur clusters are acutely sensitive to inactivation by $O_2^{\cdot-}$ (Flint et al, 1993). Moreover, differences between proteins in their sensitivities to oxidative attack have been reported (Berlett et al, 1996). Differential sensitivities raise the possibility that the loss of homeostasis, a hallmark of ageing, could result from the selective oxidation of proteins.

1.3.3.3 Antioxidant defence

Because of the potential damage that can be inflicted by oxidants, antioxidant defence has developed as summarised in Figure 1.9 (Culter et al, 1995). Protection against oxidant damage occurs at different levels. To survive reactive oxygen species, cellular antioxidant defence mechanisms have evolved to prevent or repair any damage caused by oxidative stress. These include:

a) antioxidant enzymes, e.g. superoxide dismutase, glutathione peroxidase, catalase and glutathione reductase;

b) antioxidant compounds, e.g. glutathione, ferritin, ceruloplasmin, carotenoids, vitamin C, vitamin E, uric acid and bilirubin;

c) induced defence system, e.g. induction of heme oxygenase-1 expression in response to oxidative stress; and

d) damage repair enzymes e.g. phospholipase A_2.
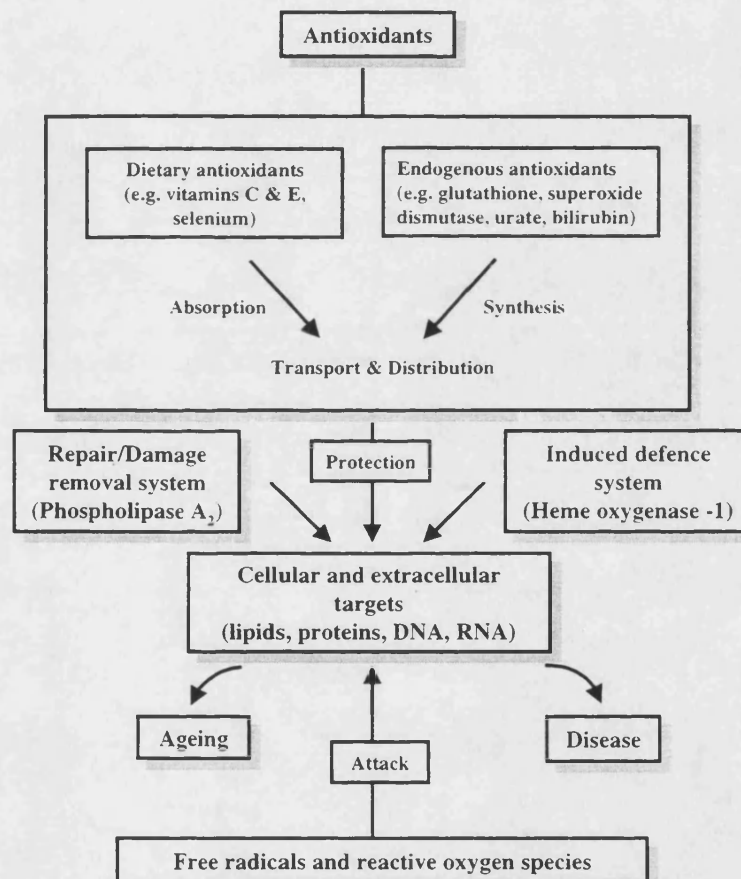
Figure 1.9 Antioxidant defence (Culter *et al*, 1995).

Oxidants can be removed by antioxidant enzymes (Halliwell and Gutteridge, 1989; Mitsuyoshi, 1993). Superoxide dismutase eliminates $O_2^{\cdot-}$ by catalysing its dismutation to H_2O_2 (Figure 1.4), which is then converted to water and oxygen by catalase or glutathione peroxidase (Figure 1.10). Glutathione peroxidase utilises the reducing power of glutathione (GSH), a tripeptide with a free sulphydryl, to detoxify H_2O_2. In the process of reducing H_2O_2, GSH is oxidised to glutathione disulphide (GSSG). Glutathione reductase then utilises NADPH to re-reduce one molecule of GSSG to two molecules of GSH, thus permitting the continuous action of glutathione peroxidase (Figure 1.10). The role of glutathione (GSH and GSSG) as an antioxidant and its measurement are discussed in Chapter 5.

Catalase:

$$H_2O_2 + H_2O_2 \longrightarrow H_2O + H_2O + O_2$$

Glutathione Peroxidase:

$$H_2O_2 + GSH + GSH \longrightarrow GSSG + H_2O + H_2O$$

Glutathione Reductase:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

GSH - reduced glutathione
GSSG - oxidised glutathione
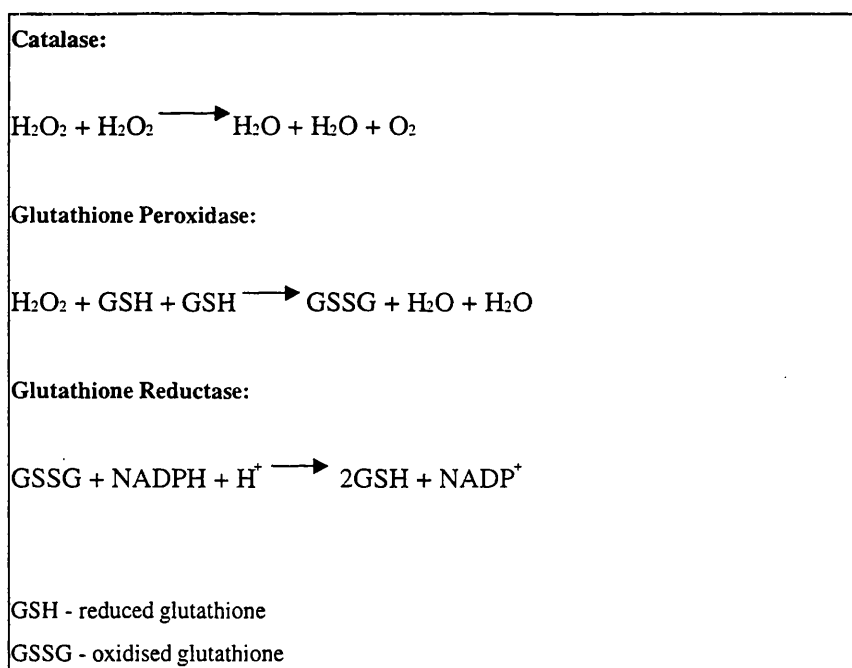
Figure 1.10 Mechanisms of antioxidant enzymes (Halliwell and Gutteridge, 1989; Mitsuyoshi, 1993).

Two non-enzymatic proteins, ferritin and ceruloplasmin, also appear to play important roles in the storage of transition metals and antioxidant defence *in vivo* (Balla *et al*, 1992). Ferritin binds iron in mammalian cells and ceruloplasmin binds copper in plasma, thereby preventing the formation of $^\bullet OH$ through metal-catalysed oxidation. Other important biological antioxidant compounds include carotenoids, vitamin C, and vitamin E, which are all present in a well-balanced healthy diet. Carotenoids are singlet oxygen scavengers. Vitamin C scavenges $O_2^{\bullet-}$ and other free radicals; and vitamin E is the major lipid-peroxidation chain-breaking antioxidant in lipid domains, such as membranes and lipoproteins. Many workers have studied the effect of dietary restriction or supplementation with dietary antioxidants on life span. Results have been mixed but encouraging (reviewed by Beckman and Ames, 1998). Other antioxidant compounds that may play a role in protecting against free radicals include uric acid (Ames *et al*, 1981) and bilirubin in plasma (Stocker *et al*, 1987). A number of techniques have been developed to measure the total antioxidant capacity of body fluids, allowing scientists to investigate the relationship between diet, oxidative stress, ageing and human disease. These techniques are discussed in Chapter 6. Some proteins can also be induced under oxidative stress and this provides an effective defence against reactive oxygen species. One such example is the enzyme heme oxygenase which plays a role in heme degradation. Its role in antioxidant defence will be discussed in Chapter 7.

Unlike defence against oxidants, which has been extensively characterised, the machinery for repairing oxidative damage is relatively unexplored. Nevertheless, it

is clear that cells can repair oxidised macromolecules. For example, phospholipase A_2 cleaves lipid peroxides from phospholipids (Pacifici and Davies, 1991); and glycosylases specifically recognise and excise oxidised bases from double-stranded DNA (Bohr and Anson, 1995).

1.3.3.3.1 Induced defence system – heme oxygenase-1 gene induction

Ageing is associated with alterations in the level of oxidative stress and changes in gene expression (Thakur *et al*, 1993). Investigations in the adult housefly have indicated that the level of oxidative stress increases during ageing (Sohal and Allen, 1986). The underlying cause for age-related increases in the level of oxidative stress appears to be both an increase in the rate of $O_2^{\cdot-}$ and H_2O_2 production and a decline in the level of antioxidant defence such as catalase activity and GSH concentration. It was reported that the ability of organisms to respond to oxidative stress by increasing their rate of GSH synthesis decreased significantly with age (Harman and Henry, 1987). This age-associated increase in oxidative stress leads to changes in gene expression. In the course of expression of genes, the first step is the transfer of genetic information encoded by DNA in the form of a highly heterogeneous nuclear RNA which undergoes processing, splicing or editing to produce a functional mature mRNA. This message travels from the nucleus to the cytoplasm where it interacts with ribosomes, amino acetyl tRNAs and other components of the translational machinery and ultimately synthesizes a specific protein.

An interesting group of genes that exhibit age- and oxidative stress-related expression is the heat shock genes (hsps). These genes and their encoded proteins (HSPs) are induced in response to stress generated by heat shock or chemical treatment (Fargnoli et al, 1990; Marini et al, 1996; Rao et al, 1999). The magnitude of this response varies with age. Studies by Fargnoli et al (1990) demonstrated an age-related reduction in cellular HSP70 expression after heat stress suggesting that the protein afforded by HSP70 induction may be impaired with ageing. Many other genes and proteins have also been shown to be inducible in response to stresses such as radiation and chemical treatment, for example, sodium arsenite and H_2O_2 in mammalian cells and tissues. These include the early response genes, c-fos, c-jun and jun-B, so called because they are all implicated in transcriptional regulation and may be responsible for the induction of many secondary DNA damage response genes; CL-100 which encodes a novel protein tyrosine phosphatase and heme oxygenase gene (Keyse, 1993).

The regulation of heme oxygenase (HO) is an example of a cellular mechanism that may protect organisms from oxidative stress. HO plays a defined and essential role in heme degradation by cleaving heme to form biliverdin and iron, with the concurrent release of carbon monoxide (CO). In mammals, biliverdin is then converted to bilirubin by the cytosolic enzyme biliverdin reductase; bilirubin is subsequently conjugated with sugars (mainly glucuronic acid) by UDP-glucuronyl transferase and then excreted into the bile (Abraham et al, 1996). Heme, a lipid-soluble and a transmissible form of iron, is a pro-oxidant in cells and its removal may

be advantageous under conditions of oxidant stress. Moreover, bilirubin has been shown to possess antioxidant properties (Stocker *et al*, 1987; Gopinathan *et al*, 1994). It has also been suggested that HO may be induced in order to break down damaged respiratory cytochromes thus facilitating turnover of these proteins following oxidant damage. In addition to its role in regulating cellular levels of heme, HO is responsible for recycling iron from senescent red blood cells and extra-hematopoietic cells such as liver.

There are two forms of HO: HO-1 and HO-2 (Maines, 1988; Abraham *et al*, 1996). They are the products of two different genes and differ in their tissue expression and regulation. The primary structures of HO-1 and a HO-2 fragment of 91 amino acid residues show only 58 % homology, but share a region with 100 % secondary structural homology. This region is believed to be the catalytic and/or heme binding site of HO-1 and HO-2. HO-2 (34,000 Daltons) is constitutively expressed whereas HO-1 (32,000 Daltons) is inducible in mammalian cells by a variety of oxidising agents or circumstances. Examples include sodium arsenite, hydrogen peroxide, agents that deplete levels of the intracellular antioxidant glutathione (Keyse and Tyrrell, 1989; Oguro *et al*, 1996) and a variety of circumstances such as heat shock and intensive endurance exercise (Niess *et al*, 1999). Studies have shown that induction of HO-1 in skin fibroblasts is of value in protection against ultraviolet (UV) light-induced oxidative stress (Vile and Tyrrell, 1993). The authors concluded that the effect of such radiation, which also promotes an increase in ferritin levels, is mediated via HO-1 release of iron and CO from endogenous heme sources. They

proposed that the increase in ferritin that follows UV light exposure and the associated HO-1 induction would decrease intracellular iron such that iron-catalysed free radical reactions would be restricted during periods of subsequent oxidative stress. Enhanced expression of the HO-1 gene is now being used in many laboratories as a marker of oxidative stress to eukaryotic cells. Various techniques have been developed to monitor its expression and these will be discussed in Chapter 7. In contrast to HO-1, the role of HO-2 in cells is not well understood; however, it is apparent that HO-2 may have an important role in epidermal cells, germ cell development and signal transduction in neural tissues (Abraham *et al*, 1996).

1.4 Ageing and disease

Harman defined ageing as "the progressive accumulation of changes with time associated with or responsible for the ever increasing susceptibility to disease and death which accompany age" (Harman, 1981). The free radical theory of ageing (Section 1.3.3) provides a plausible explanation for the relationship between disease and ageing. A disease is a combination of changes, usually forming a readily recognised pattern, that have detrimental effects on function that in some cases may lead to death. The ubiquitous free radical reactions would be expected to produce progressive adverse changes that accumulate with age throughout the body. Those changes that are common within the human population produce the normal ageing pattern. Superimposed on this normal pattern of change are patterns that differ from individual to individual owing to genetic and environmental differences that

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modulate free radical damage. The superimposed patterns of change may become progressively more discernable with time and in some individuals may eventually be recognised as diseases. It has been suggested that lowering the free radical level by any means, for example, food restriction and antioxidant intake, should decrease the probability of developing any one of the "free radical" diseases. Minimising the contributing environmental factors in the case of a specific disease should further lower the free radical level (Harman 1991). Free radicals appear to play a major role in many conditions that increase dramatically with age, for example, heart disease, cancer and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Gey, 1993; Busciglio *et al*, 1998).

1.4.1 Heart disease

The link between free radicals/oxidants and heart disease, a major killer in industrialised nations, is well established. For example, oxidation of low density lipoprotein (LDL) appears to play a key role in the development of atherosclerosis, prompting the formation and deposition of foam cells and eventual formation of atherosclerotic plaques (Gey, 1993). Although consequences of atherosclerosis, mainly coronary heart disease, are the leading cause of death in the United States, they generally do not appear until the sixth and seventh decade of life. Artherosclerotic lesions can be initiated and enhanced by substances capable of irritating the arterial wall. A possible constant source of such compounds is the reaction of molecular oxygen with the polyunsaturated compounds present in serum

and arterial wall lipids. The oxidation products, including peroxides and compounds of higher molecular weight formed through oxidative polymerisation, as well as substances arising from the reaction of intermediate lipid-free radicals with proteins and other substances, may be produced in amounts sufficient enough to contribute significantly, directly or indirectly, to atherogenesis. In addition, oxy-radicals arising from leucocytes may also serve as an important source of vessel wall irritants. Evidences supporting the role of free radicals in atherosclerosis are reviewed in detail by Harman (1992a).

1.4.2 Cancer

A link has also been proposed between free radicals and cancer (Loft and Poulsen, 1996). The theoretical basis includes both the increased rate of mitosis caused by oxidants as well as the damage that free radicals can cause to DNA (Ames *et al*, 1993, 1995). Increased cancer rates are associated with some chronic inflammatory conditions, with their resulting increased load of oxidants produced by leucocytes, and increased mitotic rates (Yu *et al*, 1991). Ionising radiation, which causes free radical production, is also a carcinogen (Pitot, 1982). Human epidemiological evidence indicates that increased consumption of fruits and vegetables, which are rich in antioxidants, leads to up to a doubling of protection against many common types of cancer (Block *et al*, 1992; Gey, 1993; Meydani, 1995). The protective effect of antioxidants is also true for heart disease. The evidence for radical involvement in

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some types of chemically induced carcinogenesis is reviewed by Harman (1992a, 1992b).

1.4.3 Neurodegenerative diseases

Free radicals have been widely implicated in the pathogenesis of a number of pathological processes in the human brain (Evans, 1993). Central nervous system tissue contains high concentrations of mitochondria and undergoes a large, constant flux of oxygen. Mitochondria inevitably produce free radicals during the process of electron transport (Packer, 1995). Cerebrospinal fluid and possibly some brain areas contain non-protein-bound iron ions, which catalyse $^{\bullet}OH$ generation through Fenton chemistry (Section 1.3.3.1). Mitochondrial DNA (mtDNA), which codes for many of the essential proteins in mitochondria, would be one of the first targets reached by $^{\bullet}OH$ and it has been shown that mtDNA undergoes such peroxidative damage (Richter *et al*, 1988). Such damage may create a vicious cycle. Damaged mtDNA codes for altered proteins, some of which are involved in electron transport. Hence the possibility of oxygen radical formation increases, leading to more oxidative damage to mtDNA, more damaged proteins, etc. However the damage does not stop at the mitochondria, but affects the whole cell, both in terms of oxidative damage itself, and in terms of lower ATP production due to a reduced population of viable mitochondria (Packer, 1995). Accumulation of such damage may give rise to various brain disorders.

1.4.3.1. Parkinson's disease

Parkinson's disease (PD) is characterised by the selective and progressive destruction of the nigrostriatal dopaminergic neurons, resulting in a deficiency of the neurotransmitter, dopamine. Clinical characteristics of PD sufferers include cardinal signs of bradykinesia, resting tremor and cogwheel-type rigidity. Evidence for the role of free radicals/oxidants in mediating this neuronal loss is mounting (Packer, 1995). Oxidative metabolism of dopamine is associated with the generation of H_2O_2 and the formation of the neurotoxin 6-hydroxydopamine. The initial discovery of increased nigral iron in Parkinsonian brains and its putative pathogenic role in causing lipid peroxidative tissue damage by the formation of $^\bullet OH$, provided initial clues to the possible pathogenic role of free radicals/oxidants (Dexter *et al*, 1989). The findings of decreased concentrations of the antioxidant GSH in the substantia nigra compared with other brain areas and the reduction of nigral GSH levels in Parkinson's disease, added further impetus to the idea of free-radical/oxidant-mediated injury (Spina and Cohen, 1989; Busciglio *et al*, 1998). In addition, the finding that the CuZnSOD gene is preferentially expressed in the substantia nigra suggests that the region is especially prone to oxidative stress (Ceballos *et al*, 1990). Moreover, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an environmental toxin, can be oxidised by monoamime oxidases (MAO) and has been shown to inhibit complex I of the mitochondrial respiratory chain, causing destruction of the substantia nigra and Parkinsonian symptoms, sometimes after a single exposure (Packer, 1995).

The concept that oxidative stress may contribute to PD has led to the rational proposal that intervention with antioxidants may be of therapeutic value in limiting the progression of the disease. Studies using vitamin E and the monoamine oxidase inhibitor deprenyl as the drug regime for PD patients demonstrated the efficacy of this regime in reducing the rate of the progression of the disease (Parkinson study group, 1989).

1.4.3.2 Alzheimer's disease

In 1907, Alois Alzheimer, a German psychiatrist and neuropathologist, described the clinical and pathological findings of a 51-year-old woman with a 4.5-year course of progressive dementia, which subsequently became recognised as a disorder bearing his name. Autopsy of this patient revealed the presence of silver positive neurofibrillary tangles (NFT), severe cerebral cortical neuron loss, amyloid angiopathy, and alterations subsequently known as the senile plaques (SP). Alzheimer's disease (AD) is a multi-neurotransmitter deficiency disease. The most consistent neurotransmitter alteration in the cerebral cortex in AD is the loss of the cholinergic markers, choline acetyltransferase and acetylchlolinesterase (Finch and Cohen, 1997; Berger, 1999). In addition, there are deficits in serotonin, noradrenaline, etc. AD results in a gradual decline in memory and intellectual function, and eventually deterioration in the ability to perform activities of daily living (Busciglio et al, 1998). The Alzheimer's Disease Society estimates that

dementia affects over 700,000 people in the United Kingdom in 1998 and 55 % of

this group are affected by AD. By the year 2000, there will be roughly 18 million

people with dementia in the world. Of these, it is thought that about 12 million

people will be AD sufferers. The well-established prevalence rates for dementia in

the United Kingdom are shown in Table 1.3.

Age	Prevalence
40-65	1 in 1,000
65-70	1 in 50
70-80	1 in 20
80+	1 in 5

Table 1.3 Prevalence rates for dementia in the United Kingdom in 1998 (The Alzheimer's Disease Society).

While numerous hypotheses have been presented, the aetiology of Alzheimer's

disease remains unknown. Epidemiological studies into the risk factors associated

with the development of the disease have confirmed that old age, a family history of

AD and the presence of Down's Syndrome are the most important risk factors

(Henderson, 1988). It also appears that both genetic and environmental influences

play an important role in the development of AD. Furthermore, there is strong

evidence that free radicals play an important role in the pathogenesis of AD.

1.4.3.2.1 Alzheimer's disease and genetics

The most exciting recent findings in AD are in the area of molecular genetics. Mutations in the amyloid precursor protein (APP) gene on chromosome 21 have been described in a modest number of patients with familial early-onset AD (St. George-Hyslop, 1993). Amyloid beta peptide (Aß), derived from the APP, is the major protein in SP and amyloid angiopathy. It has also been shown that apolipoprotein E ε4 (Apo E ε4) genotype is an important risk factor in late-onset familial and sporadic AD. Risk of AD increases and the age of onset decreases with the number of Apo E ε4 alleles. Up to 90 % of individuals homozygous for Apo E ε4 have a chance of developing AD by age 80. It has also been associated with early-onset AD and may influence the age of onset in some families with APP mutations (Roses, 1997; Ahmed et al, 1999). Apo E ε2 genotype appears to be somewhat protective for AD (Schächter et al, 1994; Strittmatter and Rose, 1995). Recently, multiple mutations have been found on gene S182 (Presenilin 1) on chromosome 14 in early-onset familial AD (Steiner et al, 1999). Another gene, E5-1 (Presenilin 2) on chromosome 1, was found to contain mutations in patients with early-onset familial AD (Markesbery, 1997).

1.4.3.2.2 Alzheimer's disease and the environment

While there is strong evidence for genetic influence in at least some cases of AD, the evidence for specific environmental factors is more controversial. The possible role of aluminium has been studied by a number of researchers (Markesbery, 1997; Rogers and Simon, 1999). Aluminium silicate has been found in the core of plaques and a number of epidemiological studies have suggested a weak association between exposure to aluminium in drinking water and an increased prevalence of AD (Tobiansky, 1993). At the present time, it seems that although aluminium may be neurotoxic in certain conditions, there is no conclusive evidence that aluminium is a significant risk factor in AD (Rogers and Simon, 1999). Other environmental factors suggested include infective agents such as the prion agent that is known to cause the rare form of dementia, Creutzfeld-Jacob disease; viral agents and other microbes, but there is little evidence to support this view (Tobiansky, 1993).

1.4.3.2.3 Alzheimer's disease and free radicals

As with Parkinson's disease, the pathogenic role of free radicals has also been proposed for Alzheimer's disease (Evans, 1993; Markesbery, 1997). Several processes increase free radical formation in the brain, for example, brain trauma, ageing and neurotoxin attack and they are all risk factors for AD (Figure 1.11). Brain injury results in the release of free iron and the formation of free radicals. Increased level of iron in AD brain tissue has been observed (Busciglio et al, 1998). It was

also reported that NFT observed in dementia pugilistica, which are believed to result from repeated brain trauma, are identical to those present in AD. Also, large amounts of free radical formed during reperfusion of ischaemic tissue are believed to be responsible for reperfusion injury. Amyloid angiopathy, a common feature in AD, can cause blood flow to fluctuate in very small areas of the brain. This fluctuation might result in repeated ischaemia and reperfusion with increased free radical formation and production of focal areas of neural degeneration (Volicer and Crino, 1990).
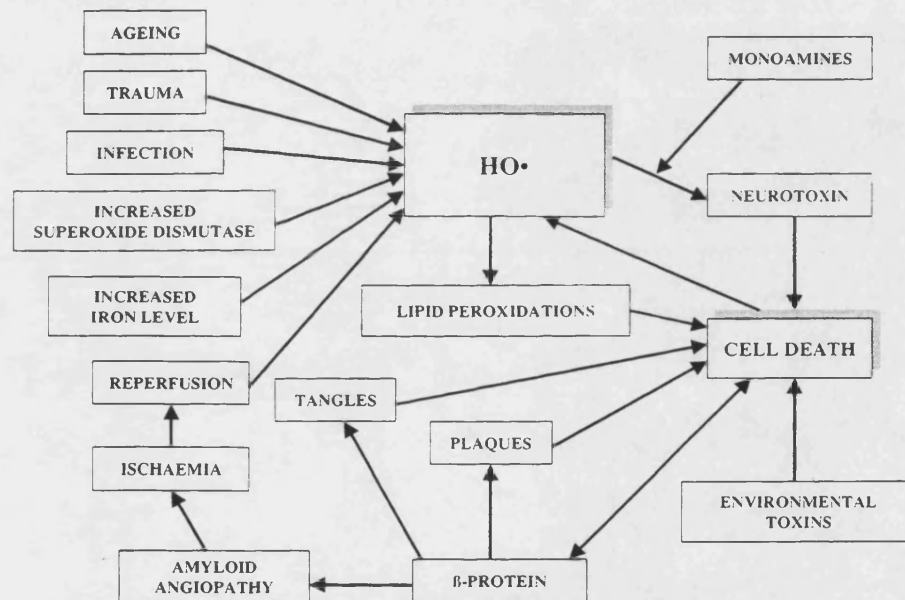
HO• - Hydroxyl radicals

Figure 1.11 Possible modes of involvement of free radicals in the pathogenesis of dementia of the Alzheimer type (Volicer and Crino, 1990).

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The contribution of genetic factors to abnormal free radical metabolism in AD is suggested by the similarity of pathological changes observed in AD and Down's Syndrome (Volicer and Crino, 1990). The gene coding for superoxide dismutase (SOD) is located in chromosome 21 and is overexpressed in Down's Syndrome because of chromosome 21 trisomy. Increased activity of the enzyme results in increased conversion of $O_2^{\cdot-}$ to H_2O_2, the substrate for the Fenton reaction (Section 1.3.3.1). This results in an increase in $^{\cdot}OH$ formation. Involvement of chromosome 21 in AD is not clear but it was reported that activity of SOD is increased in some brain regions of AD patients (Marcus et al, 1988).

Free radicals alone may also induce cell damage by damaging lipids or proteins in the brain while reactions between free radicals and neurotransmitters may lead to formation of endogenous neurotoxins such as tryptamine-4,5,-dione (4,5-DKT). Evidence indicating an enhancement of oxidative stress in AD stems from studies showing increased levels of brain cell glutathione peroxidase activities, elevated susceptibility to membrane lipid peroxidation (Marcus et al, 1998), and reduced plasma levels of the antioxidant vitamins A and E and carotenoids (Zaman et al, 1992). Co-localisation of lipofucin ("age pigment" which results from lipid peroxidation) with NFT and amyloid is further evidence of the intimate involvement of free radicals, as are the findings of an accumulation of oxidant-inactivated proteins, namely glutamine synthetase, in the brain of AD (Evans, 1993). Further evidence supporting this oxidative stress hypothesis for AD is discussed in details by Markesbery (1997) and Volicer and Crino (1990).

Until recently, the treatment of AD has been primarily to replace deficient neurotransmitter. For example, Donepizil (Aricept, Pfizer) is a cholinesterate inhibitor whose major effect is to enhance brain acetylcholine concentrations. In recent years, clinical investigations in elderly humans using supplements of various mixtures of the antioxidant nutrients, vitamins E and C, selenium and zinc have provided encourageing results in slowing the progression of AD (Evans, 1993).

1.5 Aims and objectives

Different approaches have been used to test the free radical theory of ageing and one of the most popular approaches is oxidative phenomenology (defined by Beckman and Ames, 1998), in which age-related trends in oxidative damage and antioxidant defences were studied. Biomarkers of oxidative stress were identified and analytical methods were developed for their measurement. These analytical methods have been used to compare species, genetic mutants and populations with differing life span. The aim of this project was to investigate the effect of age and AD on oxidative-damage-related parameters and antioxidants in peripheral blood mononuclear cells (PBMCs) or blood plasma from three groups of volunteers: healthy individuals aged under 30, healthy individuals aged over 60 and patients diagnosed with AD of age over 60 (n = 25 in each group). Basal and H_2O_2-induced DNA damage in PBMCs was compared in these three groups of volunteers using single cell gel electrophoresis (SCGE), also known as the comet assay and the effect of H_2O_2 on DNA damage was studied. Plasma malondialdehyde (MDA) level was assayed as an

indicator of the extent of lipid peroxidation. The antioxidant defence system was investigated by studying the levels of GSH and GSSG and the total antioxidant activity in blood plasma. To understand the role of gene expression in ageing, attempts were also made to investigate the oxidative induction of HO-1 expression in PBMCs in the three groups of volunteers using quantitative and semi-quantitative RT-PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and reagents

2.1.1 General

Freshly double-distilled deionised water (ddH$_2$O) was steam-sterilised and used to prepare all solutions (Milli-Q water purification system, Millipore, UK). Nuclease-free water (NF H$_2$O) for PCR work was obtained from Promega, UK. Phosphate buffered saline (PBS) tablets were purchased from Sigma, UK. One tablet was dissolved in 200 ml and 400 ml of ddH$_2$O to give solutions of 0.01 M and 0.005 M phosphate buffer salts, respectively (pH 7.4 at 25 °C). PBS (0.005 M) solution was only used in the total antioxidant activity assay. Hydrogen peroxide (30 % w/v) solution (Aristar, specific gravity = 1.1) was purchased from BDH, UK. This stock solution was standardised daily using 39.4 ± 0.2 M^{-1} cm^{-1} as the molar extinction coefficient for H$_2$O$_2$ at 240 nm (Aebi, 1983). For the comet assay, a 882 μM H$_2$O$_2$ stock solution was freshly prepared in RPMI 1640 medium. For the TAA assay and HO-1 assay, a 5 mM and a 100 μM H$_2$O$_2$ solution were freshly prepared in PBS, respectively.

2.1.2 Preparation of peripheral blood mononuclear cells and plasma

Histopaque-1077 *(Sigma, UK)*

Trypan blue 0.4 % solution *(Sigma, UK)*

2.1.3 Comet assay

Reagents:

Dimethyl sulfoxide (DMSO)

Ethylenediaminetetraacetic acid disodium salt (EDTA-Na$_2$)

Sodium chloride (NaCl)

Sodium hydroxide (NaOH)

Triton X-100, tris(hydroxymethyl)aminomethane base (Trizma base)

The items listed above were all purchased from Sigma (UK).

Concentrated hydrochloric acid (HCl, AnalaR) *(BDH, UK)*

Ethidium bromide 10 mg/ml solution *(Bio-Rad, UK)*

Normal melting point agarose *(Promega, UK)*

SeaPlaque low melting point (LMP) agarose *(FMC BioProducts, UK)*

Medium:

RPMI 1640 medium (1 x) without L-glutatmine and phenol red was purchased from Life Technologies (UK) as a sterile solution and stored at 4 °C.

Lysis solution:

NaCl, EDTA-Na$_2$, Trizma base and NaOH were dissolved in 500 ml ddH$_2$O to give a final concentration of 2.5 M, 100 mM, 10 mM and 300 mM, respectively and the pH was adjusted to 10.5 with NaOH. This solution was stable for up to one week when stored at room temperature. It was kept at 4 °C for one hour before use. Just before use, Triton X-100 and DMSO were added to the pre-cooled solution to give a final concentration of 1 % (v/v) and 10 % (v/v).

Electrophoresis buffer:

A 200 mM stock solution of EDTA-Na$_2$ was prepared in ddH$_2$O, filter sterilised and stored at room temperature. A 5 M NaOH stock solution was freshly prepared daily in ddH$_2$O. Two litres of buffer (300 mM NaOH and 1 mM EDTA-Na$_2$) was then freshly prepared for each run using ddH$_2$O pre-cooled to 4 °C.

Neutralisation buffer:

A 0.4 M Trizma base solution was prepared in ddH$_2$O and the pH was adjusted to 7.5 with concentrated HCl. The solution was filter sterilised and stored at room temperature.

Staining solution:

A 1 mg/ml stock solution of ethidium bromide was prepared in ddH$_2$O. This was diluted 1 in 50 to give a working solution of 20 µg/ml ethidium bromide which was stored at 4 °C in the dark.

2.1.4 Malondialdehyde measurement

Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Sigma (UK). Hydrochloric acid (AnalaR HCl, specific gravity = 1.16) was purchased from BDH (UK). Stock TCA-TBA-HCl reagent (15 % w/v TCA, 0.375 % w/v TBA, 0.25 *N* HCl) was prepared with ddH$_2$O and the solution was heated gently to assist in the dissolution of the TBA.

2.1.5 Glutathione measurement

EDTA

Oxidised glutathione (GSSG)

Reduced glutathione (GSH)

Reagent-grade absolute methanol

N-ethylmaleimide (NEM)

o-phthalaldehyde (OPT)

NaOH

Sodium phosphate

TCA

The items listed above were all purchased from Sigma (UK).

Sodium phosphate/EDTA buffer (0.1 M/5 mM, pH 8) and 0.1 N NaOH were prepared in ddH₂O. NEM (40 mM) and OPT (1 mg/ml) were prepared daily in ddH₂O and methanol, respectively. TCA (10 % w/v) was prepared in ddH₂O and stored at 4 °C until use. GSH (0.5-10 μM) and GSSG (0.25-10 μM) were prepared daily in the phosphate/EDTA buffer and 0.1 N NaOH, respectively. Both solutions were kept on ice until use.

2.1.6 Total antioxidant activity assay

Trolox:

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma, UK. A 2.5 mM Trolox standard was prepared in PBS and stored at -20 °C for up to six months. Working standards (0.25, 0.50, 0.75, 1.00, 1.50 mM) were prepared daily.

Metmyoglobin:

Myoglobin from horse heart (minimum 90 % lyophilised, essentially salt free, 0.3 % iron content) and potassium ferricyanide (99 %) were purchased from Sigma, UK. Metmyoglobin (MetMb) was purified prior to use to ensure that the iron was in the ferric (Fe^{3+}) form. This was achieved by adding a 400 μM myoglobin solution (0.0752 g of myoglobin freshly prepared in 10 ml of PBS) to an equal volume of a 578 μM potassium ferricyanide solution. After mixing, the solution was passed through a Sephadex G-25/PD-10 column (Pharmacia, UK) equilibrated with PBS. The first fraction was collected and its absorbance read at 490, 560, 580, 700 nm. The absorbance reading at 700 nm was subtracted from the readings at 490, 560, 580 nm to correct for background absorbance. Calculation of the relative proportions of the different forms of myoglobin was made by applying the Whitburn equations based on the extinction coefficients at 490, 560 and 580 nm (Whitburn *et al*, 1982):

$$[MetMb] = 146 \, A_{490} - 108 \, A_{560} + 2.1 \, A_{580}$$

$$[FerrylMb] = -62 \, A_{490} + 242 \, A_{560} - 123 \, A_{580}$$

$$[MbO_2] = 2.8 \, A_{490} - 127 \, A_{560} + 153 \, A_{580}$$

where FerrylMb was ferrylmyoglobin and MbO_2 was oxymyoglobin. MetMb prepared in this way was not suitable for use unless it was more than 94 % of the total haem species present. The MetMb prepared throughout this study was > 97 %. The purified MetMb was diluted to a concentration of 70 μM, divided into aliquots and stored at -20 °C for up to six months.

ABTS:

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) - ABTS (98 % pure) was purchased from Sigma, UK. A 5 mM ABTS solution was prepared daily in PBS and protected from light.

Pure antioxidant substances:

L-ascorbic acid (AnalaR, 99.7 %) was purchased from BDH, UK. Reduced glutathione (free acid, 98 %), uric acid (99 %) and lithium carbonate were purchased from Sigma, UK. Solutions (1.0 mM) of L-ascorbic acid and reduced glutathione

were prepared in ddH$_2$O and a 1.0 mM solution of uric acid was prepared in 0.5 g/l lithium carbonate solution that had no antioxidant activity.

2.1.7 Heme oxygenase-1 assay

Preparing plasmid DNA:

Plasmid pBsHO-1 *(provided by Professor Rex Tyrrell, Department of Pharmacy and Pharmacology, University of Bath, UK)*

Plasmid pBsHO-1/L *(provided by Dr Abdu Ahmed, Research Institute of the Care of the Elderly, St. Martin's Hospital, Bath, UK)*

Luria Bertani (LB) Medium *(all ingredients from Sigma, UK)*

Luria Bertani (LB) Agar *(all ingredients from Sigma, UK)*

Antibiotics - Ampicillin sodium salt and Tetracycline hydrochloride *(Sigma, UK)*

Wizard Plus SV Minipreps DNA Purification System *(Promega, UK)*

*Hin*d III (10 000 units/ml) and buffer (10 x) *(Promega, UK)*

*Eco*R I (HC) - 60 000 units/ml and (10 x) *(Promega, UK)*

In vitro transcription:

Riboprobe *in vitro* Transcription System using T3 RNA polymerase *(Promega, UK)*

Preparing total RNA from human peripheral blood mononuclear cells:

RPMI 1640 Medium

Foetal bovine serum

L-Glutamine 200 mM solution (100 x)

Penicillin and Streptomycin (10 000 units and 10 000 µg/ml) solution

Supplemented RPMI 1640 Medium (RPMI 1640 Medium with 10 % foetal bovine serum, 300 mg/l L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin)

All items listed above were purchased from Life Technologies (UK).

RNeasy Mini Kit *(Qiagen, UK)*

PCR and RT-PCR:

PCR buffer with 15 mM $MgCl_2$ (10 x) *(Sigma, UK)*

dNTP mix (10 mM) *(Life Technologies, UK)*

Primer P_1 and P_2 (5 µM, sequences shown in Section 2.4.7) *(Immunogen International, UK)*

JumpStart Taq DNA Polymerase (2.5 units/µl) *(Sigma, UK)*

Titan One-tube RT-PCR System *(Boehringer Mannheim, UK)*

RNasin ribonuclease inhibitor (40 units/µl) *(Promega, UK)*

First strand buffer (5 x) *(Life Technologies, UK)*

RNase-free DNase I (10 units/µl) *(Boehringer Mannheim, UK)*

EDTA *(Sigma, UK)*

Oligonucleotides 50 µM pd(T)$_{12-18}$ *(Amersham, UK)*

DTT (100 mM) *(Life Technologies, UK)*

Superscript II RNase H⁻ Reverse Transcriptase (200 units/µl) *(Life Technologies, UK)*

Expand High Fidelity PCR System *(Boehringer Mannheim, UK)*

Primer G$_1$ and G$_2$ (5 µM, sequences shown in Section 2.4.13) *(Immunogen International, UK)*

Total RNA from human skin fibroblast cell culture (FEK$_4$) *(provided by Professor Rex Tyrrell, Department of Pharmacy and Pharmacology, University of Bath, UK)*

Preparing nucleic acid:

Chloroform *(Sigma, UK)*

Ethanol (70 and 100 %) *(Sigma, UK)*

Phenol:chloroform:isoamyl alcohol (25:24:1) *(Sigma, UK)*

Sodium acetate buffer solution (3 M) *(Sigma, UK)*

Tris-EDTA buffer (100 x) *(Sigma, UK)*

Gel electrophoresis:

Normal melting point agarose *(Promega, UK)*

Tris-borate (TBE) buffer (5 x)

DNA loading buffer

Ethidium bromide solution (10 mg/ml) *(Bio-Rad, UK)*

pUC18 DNA Msp I digest (55 µg/µl) *(Sigma, UK)*

λDNA/*Eco*R I and *Hin*d III Marker (0.5 mg/ml) *(Promega, UK)*

DNA 100 bp ladder (1 µg/µl) *(Life Technologies, UK)*

Formaldehyde solution (37 %) *(Sigma, UK)*

MOPS (3-[N-morpholino]-2-hydroxypropanesulfonic acid) buffer (10 x) *(all ingredients from Sigma, UK)*

Gel running buffer (1 x) *(all ingredients from Sigma, UK)*

RNA loading buffer (5 x) *(all ingredients from Sigma, UK)*

RNA G319 marker (1 mg/ml) *(Promega, UK)*

2.2 Equipment

Disposable items:

Kartell UV grade quartz cuvettes *(Fisher, UK)**

MicroAmp 0.2 ml capped reaction tubes *(Perkin-Elmer, UK)*

Microcentrifuge 0.5 ml and 1.5 ml tubes *(Elkay, UK)*

Microcentrifuge 2 ml tubes *(Sigma, UK)*

Pasteur pipettes *(Sigma, UK)*

Petri dishes *(Sterilin, UK)*

Polystyrene cuvettes *(Scientific Laboratory Supplies, UK)**

PrecisionGlide 21G x 1.5" needles *(Becton-Dickinson, France)*

Translucent polypropylene Corning 50 ml centrifuge tubes *(Sigma, UK)*

Universal 5 ml and 30 ml containers *(Sterilin, UK)*

Vacutainer blood collection tubes (10 ml) containing EDTA *(Becton-Dickinson,*

France)

All items listed above were either obtained as sterile or sterilised after purchase

except those denoted by *.

General:

Microcentrifuge *(Beckman, UK)*

MSE Mistral 3000i centrifuge *(Fisons Scientific Equipment, UK)*

PowerPac 300 Power Supply *(Bio-Rad, UK)*

Spectronic Genesys 2 *(Life Science International Ltd., UK)*

Preparation of peripheral blood mononuclear cells and plasma:

Modified Fuchs Rosenthal 0.2 mm (1/16 mm^2) D/C rhodium-coated haemocytometer

with coverslips *(Hawsley, UK)*

Olympus BH2 light microscope *(Olympus, UK)*

Comet assay:

Borosilicate glass coverslips (Size: 22 x 22 mm; thickness: '1.5') *(BDH, UK)*

Frosted glass microscope slides (Size: 76 x 26 mm; thickness: 1.0/1.2 mm; one

frosted surface only) *(Chance Propper Ltd., UK)*

Gel electrophoresis tank from Maxi Easi-E Cast Gel system (Size: 28 x 10 x 37.5

cm) *(Hybaid, UK)*

Nikon Optiphot 2 microscope fitted with epi-fluorescene illumination (FITC filter

blocks, excitation filter at 450-490 nm, dichroic mirror at 510 nm and barrier filter at

520 nm) *(Nikon, UK)*

Trays and racks for preparing, washing and staining the slides

Workshop-made humidified airtight and lightproof container

Workshop-made slide platform accommodating a maximum of 3 x 8 slides

Zeiss Axiovert with LSM 510 laser scan *(Carl Zeiss Ltd, UK)*

Glutathione measurement:

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LS 50B Luminescence spectrometer and FL Win Lab software package *(Perkin-Elmer, UK)*

Total antioxidant activity assay:

Techne Dri-Block heater set *(Techne, UK)*

Heme oxygenase-1 assay:

Electrophoresis and gel assembly equipment *(Bio-Rad, UK)*

GeneAmp PCR System 2400 *(Perkin-Elmer, UK)*

Kodak Digital Science Electrophoresis Documentation and Analysis System 120 *(Anachem, UK)*

Ultra-Micro quartz SUPRASIL cell *(Hellma, UK)*

2.3 Human subjects

All volunteers were enrolled from the Research Institute of the Care of the Elderly (RICE), St. Martin's Hospital, Bath and the University of Bath, Bath. Thirty to forty-five millilitres of blood were collected using sterile 10 ml Vacutainers containing lithium heparin as an anticoagulant and sterile PrecisionGlide 21G x 1.5" needles.

This study was approved by the Bath Local Research Ethics Committee and written consent was obtained from each volunteer (Appendix I). Three groups of volunteers participated in this study: healthy individuals aged < 30, healthy individuals aged > 60 and patients diagnosed with Alzheimer's disease (AD) of age > 60. Twenty-five younger healthy volunteers (12 males and 13 females), 25 older healthy volunteers (7 males and 18 females) and 25 Alzheimer's patients (11 males and 14 females) were recruited. The choice of age 30 and 60 as the dividing points between young and old/AD group was arbitrary and was made to provide similar sample sizes in the three groups. Each subject was asked about his/her diet, health status, smoking habits, medications, intake of vitamins or antioxidants, minerals and other supplements; and regular exercise regimes in a self-administered questionnaire when blood was taken (Appendix II).

2.4 Methods

2.4.1 Preparation of peripheral blood mononuclear cells and plasma

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples within two hours of collection using Histopaque-1077 based on a method described by Boyum (1968). Fifteen millilitres of blood was carefully layered onto 15 ml Histopaque-1077 in a 50 ml translucent centrifuge tube, ensuring that the two layers did not mix. This was centrifuged for 30 min at 400 g after which the upper plasma layer was drawn off leaving the lymphocyte layer undisturbed at the opaque interface.

This plasma, which was essentially free of cells, was transferred to a 30 ml universal tube and stored at -70 °C for up to 6 months. The lymphocyte layer was transferred to a 50 ml translucent centrifuge tube. At least 3 volumes of PBS were added to the lymphocytes in the tube. This was mixed by gentle aspiration and centrifuged for 10 min at 250 g. The supernatant was removed and the cell pellet was suspended in 5 ml PBS. This was mixed by gentle aspiration and centrifuged for 10 min at 250 g. The supernatant was then removed. The pellet was washed again with PBS and the cell was suspended in PBS, ready for counting.

2.4.1.1 Cell counting and cell viability determination

The PBMCs were counted using a Modified Fuchs Rosenthal 0.2 mm (1/16 mm^2) D/C rhodium coated haemocytometer. The haemocytometer slide and coverslip were prepared by cleaning with 70 % ethanol. The edges of the coverslip were slightly wetted and pressed down over the slide grooves and semi-silvered counting area. The appearance of interference patterns indicated correct attachment, thereby determining the depth of the counting chamber. Ten microlitres of the cells were diluted with 90 µl of PBS and mixed thoroughly. One volume of the diluted cell suspension was mixed gently with 1 volume of 0.4 % trypan blue solution. A small amount of this mixture was transferred to the edges of the haemocytometer chamber where capillary action drew it under the coverslip. Any surplus fluid was blotted off, without drawing from under the coverslip. The haemocytometer was then transferred to the microscope stage for cell counting. Using x 40 objective and x 10 eyepiece, cells

were counted in at least 4 x 4 small squares (1 mm^2). One hundred was the minimum number of cells to be recorded. Cell numbers and cell viability were calculated as follows:

Cell count per millilitre

= average number of cells counted in each 4 x 4 subdivisions (n)

x dilution factor of cells

x dilution factor of trypan blue

÷ 0.2 mm x 1 mm x 1 mm x 10^{-3}

Therefore,

Cell count per millilitre

= n x factors x 5 x 10^3

% Cell viability

= $\dfrac{\text{total number of viable cells (unstained)}}{\text{total number of cells (stained and unstained)}}$ x   100 %

These cells were then centrifuged for 10 min at 250 g, suspended in clear RPMI 1640 medium and incubated at 37 °C under 5 % CO_2.

2.4.2 Comet assay

The method used in this study was based on that developed by Singh *et al* (1988) with some modifications. Three millilitres of 0.8 % normal agarose and 3 ml of 1.2 % LMP agarose were prepared in RPMI 1640 medium. The 0.8 % normal agarose was incubated at 45 °C and the 1.2 % LMP agarose was incubated at 37 °C.

Frosted slides were pre-warmed to 50 °C on a metal baking tray. Eighty-five microlitres of 0.8 % normal agarose solution was added onto the frosted surface of the slide and a coverslip was lowered immediately and carefully onto the slide to avoid the formation of bubbles. When the agarose had been added to all the slides, the baking tray was placed over ice at 4 °C for 30 min to allow the agarose to set. PBMCs were prepared from 30 ml of blood collected from volunteers (Section 2.4.1). Eighty microlitres of cells (4 x 10^5 cells) from the 5 x 10^6 cells/ml stock were centrifuged for 10 min at 250 g and washed twice with RPMI 1640 medium. The cells were suspended at a density of 1 x 10^6 cells/ml in different concentrations of H_2O_2 prepared in RPMI 1640 medium (0, 5, 20 and 50 μM) and incubated at 4 °C for 30 min in the dark. All subsequent steps described below were performed in dim yellow light to prevent additional DNA damage.

After the treatment period, cells were centrifuged at 4 °C for 10 min at 250 g, washed twice with RPMI 1640 medium and suspended in 100 μl of RPMI 1640 medium. One hundred microlitres of 1.2 % LMP agarose were added to the cell suspension and mixed at 37 °C. The coverslip was removed gently from each slide and 1 x 10^5 cells i.e. 50 μl of the cell suspension was loaded on top of the solidified normal agarose gel. A new coverslip was lowered onto the new LMP agarose layer as before. The same procedure was repeated for the duplicate slide. When the agarose had been added to all the slides, the tray was left on ice at 4 °C for another 15 min to set. To achieve uniformity of gel thickness which was essential to prevent the escape of DNA from cells near the surface during unwinding and electrophoresis, this

layering was carried out quickly as liquid agarose solidified rapidly on top of a cold agarose layer. The LMP agarose solidified after 15 min and the coverslips were removed. DMSO and Triton X-100 were added to the pre-cooled lysis solution (4 °C) and the slides were then placed into this solution at 4 °C for 1 h in the dark.

All subsequent steps described below were carried out at 4 °C to minimise DNA repair. Slides were removed gently from lysis solution after one hour, drained and placed on a horizontal slide platform side by side, avoiding spaces. Blank slides were placed in any empty spaces to ensure consistent geometry. The platform was placed in a gel electrophoresis tank and electrophoresis buffer was added gently to the tank to cover the slides completely but only just. The slides were left in buffer for 40 min to allow unwinding of DNA before electrophoresis. Electrophoresis was carried out at 26 V for 20 min at 300 mA in the same buffer. The current was adjusted to approximately 300 mA by raising or lowering the buffer level in the tank. After electrophoresis, slides were washed gently to remove alkali and detergents that could interfere with staining. The slides were placed on a rack, flooded slowly with the neutralising buffer and allowed to drain for 10 min. The same procedure was repeated twice. Slides were stained with 60 µl of 20 µg/ml ethidium bromide and a new coverslip was placed on each slide. Slides were placed in a humidified airtight and lightproof container at 4 °C until analysis to prevent drying of the gels, and analysed within 1-3 days.

2.4.2.1 Comet analysis

To evaluate the extent of DNA damage, 50 randomly selected cells per slide were visually analysed at 400 x magnification using a Nikon Optiphot 2 microscope fitted with epi-fluorescence illumination and pictures were taken. They were visually graded into four categories, depending on the level of DNA damage (Figure 3.1). Categories were similar to those described by Anderson *et al* (1994). A rank number ranging from 0-3 was assigned to the categories: undamaged (0), slightly damaged (1), damaged (2) and highly damaged (3). The sum of rank number i.e. the total comet score for 50 cells was recorded. A score of 0 represented totally undamaged cells and a score of 150 represented the maximum level of damage. The number of cells scored in each category was also recorded. Analysis was performed by one investigator, thus minimising variability due to subjective scoring. Cells were selected by moving from top to bottom and left to right along the slide to avoid analysis of the same comet twice. Cells were selected at random with no attempt to select particular comets other than to avoid obvious debris and any comets that were too close together for accurate image resolution. Areas close to the edge of the agarose were avoided. The identity of each slide was only revealed after scoring to avoid bias.

2.4.2.2 Experimental variability

All subjects for the following studies investigating the experimental variability of the comet assay were recruited from healthy volunteers of age > 19:

a) intra-assay variability (variation between each triplicate measurement);

b) inter-assay variability (variation between assays carried out at two different times on the same day);

c) intra-subject variability (variation between samples collected from the same subject over a period of about a month);

d) inter-subject variability (variation between individuals in each group);

e) intra-slide variability (variation between the total comet score of 50 cells obtained between six different areas on the agarose on each slide i.e. the effect of the distribution of cells on the agarose on total comet score).

2.4.3 Malondialdehyde measurement

Lipid peroxidation was measured by determining the malondialdehyde (MDA) production, using TBA (Buege and Aust, 1978). Plasma (1 ml) was mixed thoroughly with 2 ml of TCA-TBA-HCl reagent and the mixture was heated in a boiling water bath (100 °C) for 15 min. After cooling, the flocculent precipitate was removed by centrifugation for 10 min at 1000 g. The supernatant was transferred to a cuvette. The absorbance of the sample was determined at 535 nm against a blank

that contained all the reagents but the plasma. The MDA concentration of the sample was calculated using an extinction coefficient of $1.56 \times 10^5 \ M^{-1}cm^{-1}$ as shown below (Buege and Aust, 1978):

$$\text{MDA concentration (M)} = \frac{\text{Absorbance at 535 nm}}{1.56 \times 10^5 \ M^{-1}cm^{-1} \times 1 \ cm}$$

Intra-day variability (variation between assays carried out at two different times on the same day) and inter-day variability (variation between assays carried out on two separate days) were studied. Intra-assay, intra-subject and inter-subject variability were also estimated.

2.4.4 Glutathione measurement

GSH and GSSG levels were measured using a fluorometric method developed by Hu (1994). All measurements were carried out in triplicate. The LS 50B Luminescence spectrometer was set as followed: slit width at 5 nm, emission filter at 'open' position, integration time at 10 sec and the temperature at 17-24 °C.

2.4.4.1 GSH standard curve

A series of GSH solutions of known concentrations (0-10 μM) were prepared in the phosphate/EDTA buffer. GSH solution (100 μl) was mixed with 1.8 ml of the phosphate/EDTA buffer and 100 μl of 1 mg/ml OPT to give a total volume of 2 ml.

The mixture was incubated at room temperature for 15 min and was later transferred to a UV grade cuvette. Fluorescence emission at 420 nm was determined after excitation at 350 nm.

2.4.4.2 GSSG standard curve

A series of GSSG solutions of known concentration (0-10 μM) was prepared in 0.1 N NaOH. GSSG solution (100 μl) was mixed with 1.8 ml of 0.1 N NaOH and 100 μl of 1 mg/ml OPT to give a total volume of 2 ml. The mixture was incubated and analysed as described in Section 2.4.4.1.

2.4.4.3 Experimental variability

The protocols used for this validation study are described in Section 2.4.4.4 and 2.4.4.5. Intra- and inter-day, intra-assay, intra-subject and inter-subject variability were estimated.

2.4.4.4 GSH measurement

Plasma (500 μl) was added to 500 μl of cold 10 % TCA and allowed to stand on ice for 10 min and then centrifuged at 4 °C for 15 min at 3000 g. The supernatant

(200 μl) was mixed with 1.7 ml of the phosphate/EDTA buffer and 100 μl of 1 mg/ml OPT to give a total volume of 2 ml and assayed as described in Section 2.4.4.1.

2.4.4.5 GSSG measurement

Plasma (500 μl) was added to 500 μl of cold 10 % TCA and allowed to stand on ice for 10 min and then centrifuged at 4 °C for 15 min at 3000 g. The supernatant (500 μl) was incubated with 200 μl of 40 mM NEM for 30 min at room temperature. To this mixture, 4.3 ml of 0.1 N NaOH was added. This mixture (200 μl) was further mixed with 1.7 ml of 0.1 N NaOH and 100 μl of 1 mg/ml OPT to give a total volume of 2 ml and was assayed as described in Section 2.4.4.1.

2.4.5 Total antioxidant activity assay

The total antioxidant activity of blood plasma was determined using the method developed by Miller and Rice-Evans (1996). The following concentrations of reagents were used per tube: 0.84 % Trolox/plasma; 2.5 μM metmyoglobin; 150 μM ABTS; 375 μM H_2O_2 and 0.005 M PBS to a total volume of 1.0 ml. The reagents were mixed thoroughly in the order shown: Trolox/plasma, PBS, metmyoglobin and ABTS. H_2O_2 was added to this mixture, the clock was started promptly and the reactants were mixed vigorously. This mixture was immediately transferred into a cuvette that had been pre-incubated at 30 °C. The mixture was incubated at 30 °C for

200 s after which the final absorbance at 734 nm was read using a spectrophotometer. The assay was repeated three times. Timing, temperature, reagent concentrations and the choice of wavelength were established by Miller and Rice-Evans (1996) and strictly controlled throughout the assay. Solutions (1.00 mM) of L-ascorbic acid, reduced glutathione and uric acid were also analysed as before except the final concentration of H_2O_2 was decreased to 75 μM, the incubation period was increased to 6 min and the assay repeated six times. Moreover, intra- and inter-day, intra-assay, intra-subject and inter-subject variability were estimated.

2.4.5.1 Dose-response curve

Trolox standards of initial concentration 1.50, 1.00, 0.75, 0.50, 0.25 and 0 mM (buffer blank) were assayed as described above in Section 2.4.5. The percentage inhibition of absorbance at 734 nm ($A_{734\ nm}$) of standard was calculated as described in Section 2.4.5.2 and was expressed as a function of the initial concentration of Trolox to construct the dose-response curve. This curve was constructed to determine total antioxidant activity of blood plasma samples.

2.4.5.2 Calculation

A quantitative relationship exists between the percentage inhibition of absorbance at 734 nm ($A_{734\ nm}$) and the antioxidant activity of the added sample or standard (test).

inhibition of $A_{734\,nm}$ (%) $=$ $\dfrac{\text{Buffer blank } (A_{734\,nm}) - \text{Test } (A_{734\,nm})}{\text{Buffer blank } (A_{734\,nm})}$ x   100 %

Inhibition of $A_{734\,nm}$ (%) $\propto$ Antioxidant activity

The unit of antioxidant activity is the Trolox equivalent antioxidant capacity (TEAC), which is defined as the millimolar concentration of Trolox having the equivalent antioxidant capacity to a 1.00 mM solution of the substance under investigation.

2.4.6 Plasmid DNA purification

Plasmid pBsHO-1, containing the full length cDNA for HO-1 (1550 bp) inserted into the *Eco*R I site of phagemid pBluescript SK and plasmid pBsHO-1/L, containing the plasmid pBsHO-1 with a 45 bp linker inserted into its *Nco* I site, were constructed and then transformed into *Escherichia coli* XL-1 blue as described by Ahmed *et al* (1996). These transformed colonies growing on fresh LB agar plates containing

50 μg/ml ampicillin and 12.5 μg/ml tetracycline were supplied by Dr Abdu Ahmed (RICE, Bath). A single, well-isolated colony was used to inoculate 10 ml LB medium, containing the same concentrations of antibiotics. This primary culture was incubated at 37 °C overnight (12-16 h), aerated by gentle shaking. A 1-ml aliquot was then subcultured into 100 ml LB medium, containing the same concentrations of antibiotics. This secondary culture was incubated overnight at

37 °C as before. Minipreps of plasmid DNA were performed using Wizard Plus SV

Minipreps DNA Purification Systems according to the manufacturer's protocol. The

plasmid pBsHO-1/L DNA (4553 bp) and the plasmid pBsHO-1 (4508 bp) were

finally eluted in NF H_2O, quantified and evaluated using agarose gel electrophoresis

(Section 2.4.15 and 2.4.16) and stored at -20 °C until use.

2.4.7 JumpStart PCR

All reagents were added in the order shown in Table 2.1 to a 0.5 ml microcentrifuge

tube. They were mixed gently by pipetting and briefly centrifuged to collect all

solutions at the bottom of the tube. The PCR reactions were performed in 0.2 ml

capped MicroAmp reaction tubes, using GeneAmp PCR System 2400. The mixture

was heated for 30 cycles at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min,

followed by 1 cycle of 72 °C for 7 min and held at 4 °C. Samples (15 µl) of the

amplified DNA products were then evaluated by agarose gel electrophoresis (Section

2.4.16). Positive and negative control reactions were also included. To ensure the

PCR system was set up properly, DNA template which had been successfully and

consistently amplified in previous reactions was chosen to be the positive control

DNA. To control for contaminating DNA amplicons from a previous PCR, a

negative control reaction without DNA template was set up. All amplified samples

were stored at -20 °C. Primers P_1 (5' CCT TGT TGA CAC GGC CAT GAC CAC

3') and P_2 (5' AGT TAG ACC AAG GCC ACA GTG CCG 3') were used in this

PCR. These primers were designed to amplify the 1198 bp to 1440 bp region of

HO-1 cDNA (242 bp fragment), containing the *Nco* I restriction site. Since plasmid

pBsHO-1/L DNA contains the 45 bp linker inserted into its HO-1 region, its PCR

amplified products using the same set of primers should be 45 bp larger (287 bp)

than the same fragment amplified from plasmid pBsHO-1 DNA.

Reactants	Amount	Final concentration
PCR Buffer with Mg^{2+} (10 x)*	5 µl	1 x[†]
dNTP mix (10 mM)**	1 µl	200 µM each
Primer P_1 (10 µM)	3 µl	0.6 µM
Primer P_2 (10 µM)	3 µl	0.6 µM
JumpStart *Taq* DNA Polymerase (2.5 units/µl)	0.4 µl	1 unit
DNA template (pBsHO-1/L or pBsHO-1)	24 ng	-
NF H_2O	to 50 µl	-
Total volume	**50 µl**	

*This buffer contains 100 mM Tris-HCl, 500 mM KCl, 0.01 % gelatin and 15 mM

MgCl₂ (pH 8.3 at 25 °C).

**10 mM dNTP mix contains 10 mM each of dATP, dCTP, dGTP and dTTP (2'-

Deoxyadenosine, 2'-Deoxycytidine, 2'-Deoxyguanosine and 2'-Deoxythymidine

5'-Triphosphates)

[†]1.5 mM Mg^{2+}

Table 2.1 PCR using JumpStart *Taq* DNA Polymerase. Plasmid pBsHO-1/L

DNA and plasmid pBsHO-1 DNA were amplified using conditions shown above and

the cycling parameters described in Section 2.4.7.

2.4.8 Restriction digestion

To prepare for subsequent *in vitro* transcription, plasmid pBsHO-1/L DNA was linearised with *Hin*d III (5 units/µg). When setting up restriction digestion, reactants were kept on ice until use and mixed thoroughly before incubation at 37 °C for at least 1-2 h (Sambrook *et al*, 1989). After restriction digestion, the products were analysed by agarose gel electrophoresis (Section 2.4.16) to confirm complete digestion. The linearised products of plasmid pBsHO-1/L DNA were then extracted with phenol-chloroform-isoamyl alcohol, concentrated with ethanol and quantified as described in Section 2.4.15.

2.4.9 *In vitro* transcription

Plasmid pBsHO-1/L DNA was transcribed *in vitro* using T3 RNA polymerase (Riboprobe *in vitro* Transcription Systems) according to the manufacturer's instructions. Five micrograms of DNA template were used per preparation and a yield of 5-10 µg RNA/µg plasmid DNA was obtained. The components were added at room temperature since DNA can precipitate in the presence of spermidine if kept at 4 °C. The mixture was incubated at 37 °C for 2 h. Subsequently, the DNA template was degraded using RQ1 RNase-free DNase (1000 units/µl) at a concentration of 1 unit per 1 µg of DNA template. This mixture was then incubated at 37 °C for 30 min. The RNA transcripts (1697 b) were subsequently extracted with phenol-chloroform-isoamyl alcohol and concentrated with ethanol (Section 2.4.15).

The synthetic RNA was suspended in NF H_2O, quantified and stored at -70°C. When verifying RNA transcripts on a gel, RNA was diluted in RNA loading buffer and heated to 65 °C for 5 min prior to loading on an agarose gel as an alternative to a denaturing gel. Details of RNA quantification and denaturing gel electrophoresis were described in Section 2.4.15 and 2.4.16.

2.4.10 Treatment of peripheral blood mononuclear cells with hydrogen peroxide

PBMCs were isolated as described in Section 2.4.1 and then exposed to H_2O_2. The cells were treated with 100 µM H_2O_2 prepared in PBS at 37 °C for 30 min at a cell density of 1 x 10^7 cells/ml. The cells were then washed twice with PBS and incubated in supplemented RPMI 1640 medium at a cell density of 1 x 10^7 cells/ml at 37 °C for 2 h under 5 % CO_2. Using trypan blue dye (see Section 2.4.1), the % cell viability was determined before and after the treatment period; and after the 2-h incubation period.

2.4.11 Total RNA preparation from peripheral blood mononuclear cells

Total RNA was extracted from H_2O_2-treated PBMCs using the RNeasy Mini Kit according to manufacturer's protocol. Cell pellets or cell lysates were stored at -70 °C and processed soon after harvesting. RNA was eluted in NF H_2O, and stored at -70 °C until use. The isolation was carried out as quickly as possible to maintain integrity of the RNA.

2.4.12 Competitive one step "one-tube" RT-PCR

RT-PCR assays were performed for each RNA sample (pBsHO-1/L RNA and total RNA) using the Titan One-tube RT-PCR System, the primers P_1 and P_2 (Section 2.4.7) and the GeneAmp PCR System 2400. The protocol used for the RT-PCR assay is shown in Table 2.2. Reaction components for master mix 1 and master mix 2 were set up separately. Master mix 1 (25 µl) was then added to 25 µl of master mix 2. This mixture (50 µl) was then heated for 1 cycle at 50 °C for 30 min and 94 °C for 2 min; followed by 45 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 68 °C for 45 sec; followed by 1 cycle at 68 °C for 7 min and held at 4 °C. To control for contaminating DNA amplicons from a previous PCR, a negative control reaction without RNA template was set up. Another negative control reaction was also set up

where RT was eliminated by inactivating the Avian myeloblastosis virus Reverse Transcriptase (AMV RT). Master mix 2 was heated at 94 °C for 2 min before master mix 1 was added to it. This mixture was then subjected to thermal cycling as before but eliminating the RT step (50 °C for 30 min). To ensure the RT-PCR system was set up properly, an RNA template which had been successfully and consistently amplified in previous reactions was used as a positive control. Samples (15 µl) of the amplified DNA products were evaluated by agarose gel electrophoresis (Section 2.4.16). PCR products were stored at -20 °C.

Serial dilutions of pBsHO-1/L RNA (competitor) containing a fixed amount of total RNA (target) extracted from PBMCs were reverse transcribed and co-amplified (using the same set of primers) in one-tube reactions under the same conditions as described in Table 2.2. Samples (15 µl) of the amplified DNA products were then evaluated by agarose gel electrophoresis (Section 2.4.16). Data analysis using densitometry is described in detail by Ahmed *et al* (1996).

Reactants	Amount	Final concentration
Master mix 1		
DTT (100 mM)	2.5 µl	5 mM
dNTP mix (10 mM)	1 µl	200 µM
Primer P_1 (10 µM)	3 µl	0.6 µM
Primer P_2 (10 µM)	3 µl	0.6 µM
RNase inhibitor (40 units/µl)	0.25 µl	10 units
RNA template (pBsHO-1/L RNA or total RNA or both)	x µl	1 pg-1 µg
NF H_2O	to 25 µl	-
Total volume	**25 µl**	
Master mix 2		
RT-PCR buffer with Mg^{2+} (5 x)*	10 µl	1 x[†]
Titan enzyme mix**	1 µl	-
NF H_2O	14 µl	-
Total volume	**25 µl**	
Final volume (Mix 1+2)	**50 µl**	

*This buffer contains 1 ml DMSO and 7.5 mM $MgCl_2$.

**This enzyme mix contains Expand High Fidelity enzyme mix (*Taq* DNA polymerase and *Pwo* DNA polymerase) and AMV RT in a storage buffer.

[†]1.5 mM $MgCl_2$

Table 2.2 Protocol for RT-PCR using Titan One-tube RT-PCR System.

2.4.13 Optimisation of RT-PCR

Amplification of plasmid pBsHO-1/L RNA using the primers P_1 and P_2 was achieved with relative ease when compared to that of total RNA using the same set of primers. Successful performances of RT-PCR depend on a number of factors. For example, if the starting material is substantially degraded before the cDNA synthesis, then it is unreasonable to expect the RT-PCR to work. To check the integrity of the total RNA, GAPDH primers (G_1: 5' GAC ATC AAG AAG GTG GTG AA and G_2: 5' TGT CAT ACC AGG AAA TGA GC 3') were used to amplify the abundant housekeeping gene, glyceraldehyde-3-phosphate dehydrogense (GAPDH). Amplified products at 187 bp should confirm the integrity of this starting material and at the same time, the efficiency of RT-PCR. The latter can also be checked by amplifying the HO-1 gene in human cultured FEK_4 total RNA ("positive control" RNA) using the primers P_1 and P_2. Induction of HO-1 gene expression by UVA radiation, H_2O_2 and sodium arsenite in FEK_4 cells was previously demonstrated by Keyse and Tyrrell (1989). Amplification of the HO-1 gene in FEK_4 total RNA can also be used to check the suitability of primers P_1 and P_2. The final concentration of the primers in the reaction should also be optimised. RT-PCR was carried out using 1, 0.8, 0.6, 0.4, 0.2 µM of each primer during optimisation. Another factor to be considered in RT-PCR optimisation is the number of thermal cycles. Most RNA samples can be detected using 30-40 cycles of amplification. If the target RNA is rare or if only small amount of starting material is available, it may be necessary to increase the number of cycles to 45-50. This strategy was taken in this study in

amplifying the total RNA. Annealing temperature was also varied in an attempt to achieve amplification. Magnesium and dNTPs concentration may also be varied during optimisation although they were both kept constant in this study.

2.4.14 Semi-quantitative RT-PCR

While setting up competitive one step "one-tube" RT-PCR for the study of HO-1 gene expression, difficulty was encountered because the amplification of total RNA was inconsistent. A different approach was therefore required to tackle this problem. Semi-quantitative RT-PCR described here is a more basic and simpler approach because the RT and the PCR step are separated and the elimination of a competitor means that optimisation of the reaction is easier since there is only one set of RNA to be amplified. PBMCs of 13 healthy volunteers (7 from the younger group and 6 from the older group) were treated with H_2O_2 and the total RNA was isolated as described in Section 2.4.10 and 2.4.11. To ensure RNA purity, genomic DNA contamination was eliminated from total RNA by treatment with RNAase-free DNase I (10 units/μl) (Dilworth and McCarrey, 1992). The reaction was set up as shown in Table 2.3. The mixture was incubated at 37 °C for 15 min. EDTA (25 mM) was added to the reaction mixture to a final concentration of 2.5 mM and this mixture was incubated at 65 °C for 10 min to inactivate the RNase-free DNase I.

Reactants	Amount	Final concentration
Total RNA sample	2 μg	0.1 μg/μl
First strand buffer (5 x)	4 μl	1 x
RNase-free DNase I (10 units/μl)	2 μl	20 units
NF H$_2$O	to 20 μl	-
Total volume	**20 μl**	

Table 2.3 Removal of genomic DNA from total RNA by RNase-free DNase I.

For first strand cDNA synthesis, 2 μl of 50 μM oligo pd (T)$_{12-18}$ was added to 10 μl (1 μg) of the DNase-treated RNA to give a final concentration of 5 μM in the final 20 μl reaction mixture (H). This mixture was denatured at 70 °C for 10 min and chilled immediately on ice (G). The RT reaction mixture (8 μl of F) was added to 12 μl of the denatured RNA mixture (G) to give a total volume of 20 μl (H) (Table 2.4). The final 20 μl mixture (H) was reversed transcribed using Superscript II RNase H$^-$ Reverse Transcriptase at 42 °C for 60 min, 95 °C for 5 min and held at 4 °C. The newly synthesised cDNA was stored at -20 °C.

	Reactants	Volume per PCR tube (μl)	Final concentration
A	First strand buffer (5 x)	4	1 x
B	DTT (100 mM)	1	5 mM
C	dNTP (10 mM)	1	500 μM
D	RNasin ribonuclease inhibitor (40 units/μl)	1	40 units
E	Superscript II (200 units/μl)	1	200 units
F	RT mixture (A+B+C+D+E)	8	-
G	Denatured RNA mixture	12	1 μg
H	Total volume (F+G)	20	

Table 2.4 Protocol for the reverse transcription of total RNA from PBMCs using Superscript II RNase H⁻ Reverse Transcriptase.

The newly synthesised cDNA was used as a template for PCR amplification using the Expand High Fidelity System. This system is similar to the Titan One-tube RT-PCR System but no AMV RT is included in the enzyme mix. Reactants were added to a PCR reaction tube in a volume of 25 μl (Table 2.5). cDNA template was added last outside the UV-PCR hood. This mixture was then heated at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec, followed by 1 cycle at 72 °C for 7 min and held at 4 °C. The housekeeping gene, GAPDH was used as a control to estimate the amount of HO-1 mRNA per sample. GAPDH primers (G_1 and G_2) and HO-1 primers (P_1 and P_2, Section 2.4.7) were used to amplify cDNA obtained from the same total RNA. PCR amplified products by GAPDH primers and HO-1 primers were 187 bp and 242 bp long, respectively. Samples (15 μl) of the amplified DNA products were evaluated by agarose gel electrophoresis (Section 2.4.16). To check for genomic DNA contamination, a negative control was set up using DNase-treated RNA which had not been reverse transcribed as the template. To check for any other sources of DNA contamination, another negative control was set up without any template.

Reactants	Amount	Final concentration
PCR buffer with Mg^{2+} (10 x)*	2.5 µl	1 x[†]
dNTP mix (10 mM)	0.5 µl	200 µM
HO-1 **or** GAPDH primers P₁ **or** G₁ (5 µM) P₂ **or** G₂ (5 µM)	2.5 µl 2.5 µl	500 nM 500 nM
Expand High Fidelity enzyme mix**	0.125 µl	-
cDNA **or** DNase-treated RNA **or** NF H₂O	1.25 µl 10 µl 1.25 µl	5 % 1 µg -
NF H₂O	to 25 µl	-
Total volume	**25 µl**	

*This buffer contains 100 mM Tris-HCl, 500 mM KCl and 15 mM $MgCl_2$ (pH 8.3 at 20 °C).

**This enzyme mix contains *Taq* DNA polymerase and *Pwo* DNA polymerase.

[†]1.5 mM $MgCl_2$

Table 2.5 Protocol for PCR reaction using the Expand High Fidelity System.

2.4.15 Nucleic acid purification, concentration and quantification

Purification of nucleic acid and inactivation/removal of protein by phenol:chloroform:isoamyl alcohol extraction were routinely carried out in this study according to the protocol described by Sambrook *et al* (1989). The nucleic acid was recovered by precipitation with 3 M sodium acetate (pH 5.2) and ethanol. The nucleic acid pellet was suspended in NF H_2O. The concentration of nucleic acid was determined spectrophotometrically using a Ultra-Micro quartz SUPRASIL cell and a Spectronic Genesys 2 as described by Sambrook *et al* (1989). Concentrations were calculated as follows:

Concentration of DNA (µg/ml) = A_{260} x 50 µg/ml x Dilution factor

Concentration of RNA (µg/ml) = A_{260}x 40 µg/ml x Dilution factor

Concentration of oligonucleotides (µg/ml) = A_{260}x 20 µg/ml x Dilution factor

2.4.16 Gel electrophoresis

Gel electrophoresis was performed as described by Sambrook *et al* (1989). During the preparation of materials for RT-PCR assays, electrophoresis of DNA samples using a 1 % agarose gel pre-stained with ethidium bromide was used to identify any DNA fragments. Following RT-PCR, 15 µl samples of amplified products were electrophoresed through a 2 % agarose gel. For fine separation and analysis of RNA, a denaturing gel system (1 %) utilising formaldehyde as the denaturing agent, was

used (Sambrook *et al*, 1989). Documentation of gels was carried out using ultraviolet illumination and the Kodak Digital Science Electrophoresis Documentation and Analysis System.

2.5 Statistical analyses

Statistical analyses were carried out using either the parametric two-sample t test for normally distributed data or the non-parametric Mann-Whitney U test for non-normal data, employing the Minitab Release 11.12 statistical software package. *P* values < 0.05 (95 % confidence intervals) were considered significant. Mean values, standard deviations (SD) or standard error (SE) and 95th percentile reference interval (mean ± 1.96 x SD) were calculated. Variability was expressed as a coefficient of variation (CV) as shown below:

$$\text{coefficient of variation (CV)} = \frac{\text{standard deviation (SD)}}{\text{mean}} \times 100\ \%$$

The higher the CV value, the higher the variability observed. Dose-response curves were constructed using the regression-fitted line plot function in Minitab. The adjusted R-squared (R^2) value was used to assess the goodness-of-fit of the regression line. A value of 100 % indicated the existence of a perfect linear relationship between two variables. The Anderson-Darling normality test was used to study the normality of the data, utilising Minitab, where $P > 0.05$ indicated normality and $P < 0.05$ indicated that the data deviated from normality. In addition,

the effect of various factors on the collected data was studied using either one-way or

two-way analysis of variance (ANOVA), followed by the Tukey's comparison tests

for normal data; or the Kruskal-Wallis test, followed by Mann-Whitney U test for

non-normal data, utilising Minitab. Statistical analyses used throughout this study

were described in further detail by Altman (1991).

CHAPTER 3

COMET ASSAY

3.1 Introduction

Active oxygen radicals are known to induce chromosomal aberrations with high efficiency and significant oxidative DNA damage (e.g. single- and double-strand breaks) resulting from endogenous free radical attack has been suggested to contribute to ageing and age-related diseases (Section 1.3.2.1). Hydrogen peroxide, for example, is a common intermediate in a variety of oxidative stresses. It induces DNA damage leading to mutagenesis (Imlay and Linn, 1988), although it does not interact with DNA directly to produce oxidative lesions. The hydroxyl radical produced when H_2O_2 is subjected to the Fenton reaction (Section 1.3.3.1) is the reactive oxygen species responsible for DNA damage. The aim of this study was to evaluate H_2O_2-induced DNA damage in the PBMCs of all three groups of volunteers using the single cell gel electrophoresis (SCGE) technique, also known as the comet assay. The effect of age, Alzheimer's disease (AD) and gender on oxidative DNA damage of PBMCs was assessed.

3.1.1. Detection of DNA single-strand breaks

Procedures for measuring DNA strand breaks are generally based upon the principle that strand breaking agents reduce the size of the large duplex DNA molecule (Olive, 1992). In addition, DNA single- and double-strand breaks can have dramatic effects on higher-order chromatin structure because of its supercoiling and tight packaging within the nucleus. Assays that measure DNA single-strand breaks generally require unwinding of the double stranded DNA molecule for sensitive detection. Nucleoid sedimentation makes use of the fact the degree of DNA supercoiling or unwinding, reflecting strand break levels, can be measured by the rate of sedimentation (Cook and Brazell, 1975). A high pH (> 12.3) is generally utilised to facilitate denaturation, unwinding, and expression of single-strand breaks as well as DNA breaks that only become apparent after exposure to alkali i.e. alkali labile lesions. Alkaline sucrose sedimentation is based upon the assumption that larger DNA fragments sediment further in a sucrose gradient than smaller fragments (McGrath and Williams, 1966). Alkaline filter elution measures the rate of DNA elution through a filter membrane under alkaline conditions, and the amount of DNA single-strand breaks or lesions converted to single-strand breaks under alkaline conditions is estimated on the basis of the increase in DNA elution rate (Kohn, 1991). Most of these techniques require radiolabelling of DNA for sensitive detection of breaks, and they can only provide an indication of average number of breaks per cell. An exception is the *in situ* nick translation method that requires the presence of free 3′ OH groups to serve as templates for incorporation of radiolabelled nucleotide precursors. This method

gives an indication of the extent of damage in individual cells (Krause *et al*, 1993).

3.1.2 Development of the comet assay

The detection of DNA single-strand breaks in individual cells was first described by Rydberg and Johanson (1978). Cells were embedded in agarose on microscope slides and lysed under mildly alkaline conditions in order to allow a partial unwinding of the DNA. After neutralisation, the cells were stained with acridine orange and the extent of DNA quantitated. A ratio of green to red fluorescence, representing the ratio of double to single-strand DNA, respectively, was determined. In order to increase the sensitivity of damage detection, Östling and Johanson (1987) subsequently modified these procedures and included a neutral electrophoresis step. Comets formed as relaxed DNA loops or broken DNA free ends, stretched out as a tail in the direction of the anode. They observed that the extent of DNA liberated from the head of the comet during electrophoresis was a function of the dose of irradiation. The nature of comets, and the physicochemical events underlying their formation, are not fully understood or agreed upon and it has been investigated by Collins *et al* (1997a). The generally adopted SCGE technique was introduced by Singh *et al* (1988), in which the procedure of Östling and Johanson (1987) was modified by performing the electrophoresis at high pH, thus revealing the alkaline labile site and breaks. Following staining with ethidium bromide, increased extension of the DNA from the nucleus towards the anode was observed in cells with increased damage caused by either X-rays or H_2O_2. The number of studies based on

the comet assay has increased over recent years, although most use the Singh method with slight variations in different steps (McKelvey-Martin *et al*, 1993; Fairbairn *et al* 1995). The comet assay has also become a particularly valuable technique since it allows detection of intercellular differences in DNA damage and repair in virtually any eukaryote cell population that can be obtained as a single-cell suspension; it requires only extremely low cell numbers (1-10,000 cells); and results can be obtained in a single day. Apart from image analysis, which greatly facilitates and enhances the possibilities of comet measurements, the cost of performing the assay is low. To date, the comet assay has been usefully applied to screen lymphocyte samples from human populations for their susceptibility to oxidative damage, UV and ionising radiation (Singh *et al*, 1988; Olive *et al*, 1990; Green *et al*, 1995). The comet assay can also be applied in the study of radiation biology, genetic toxicology and apoptosis. Comprehensive reviews of the comet assay, its modifications and applications have been published by McKelvey-Martin *et al* (1993), Fairbairn *et al* (1995) and Collins *et al* (1997a, 1997b). A summary of DNA-damaging agents that have been used in various comet assay-based studies was also provided by McKelvey-Martin *et al* (1993). Leroy *et al* (1996) evaluated the three methods generally used to detect DNA single-strand breaks in human lymphocytes: alkaline elution, nick translation and the comet assay; the latter was demonstrated to be the most feasible, sensitive and reproducible method to monitor populations exposed to genotoxic agents.

The comet assay, based on that developed by Singh *et al* (1988), was used in this study to detect DNA strand breaks in individual human PBMCs after treatment with different concentrations of H_2O_2. The degree of DNA damage in H_2O_2-treated PBMCs was analysed from three groups of volunteers: healthy individuals under the age of 30, healthy individuals over the age of 60 and Alzheimer's patients over the age of 60; taking into the account age, gender and the concentration of H_2O_2 used.

3.2 Results

3.2.1 Human subjects

The average age of the healthy younger group, the healthy older group and the Alzheimer's group was 24 ± 3 (range: 19-29 years), 72 ± 4 (range: 62-80 years) and 78 ± 6 (range: 62-86 years), respectively. Each subject was asked about his/her diet, health status, smoking habits, medications, intake of vitamins or antioxidants, minerals and other supplements; and regular exercise regimes. This information is summarised in Table 3.1 and Appendix III. The majority of volunteers were Caucasian and non-smokers; therefore the effects of race and smoking habits were not studied. Vitamins or antioxidants taken by the volunteers included vitamin B, vitamin C (ascorbic acid), vitamin E (α-tocopherol) and multivitamins. Minerals

and other supplements taken included calcium, magnesium, iron, multi-minerals, cod

liver oil, evening primrose oil, garlic oil, halibut oil, coenzyme Q10 and gingko

biloba. The healthy volunteers were of good general health although some of the

older volunteers suffered health problems such as hiatus hernia and hypertension but

none was diagnosed with AD or taking drugs for the treatment of AD. The

Alzheimer's group included patients recruited from RICE and the diagnosis of these

patients was established in accordance with the DSM-IV (Diagnostic and Statistical

Manual of Mental Disorders-IV) criteria and NINCDS-ADRDA (National Institute

of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and

Related Disorders Association) classification for probable AD. Most of these

patients have been/are involved in clinical trials, carried out by RICE, to determine

the safety and efficacy of drugs such as donepezil, galanthamine, propentofylline or

T-588 in the treatment of AD. Some of the 25 AD volunteers were/are receiving the

active drug or placebo. In the case of volunteer number 20 in this group (Appendix

III), it was not known whether the active drug was taken since the study was double-

blinded in nature. Full details of these studies and the selective criteria of AD

patients were obtained from RICE and remained confidential. Further details of the

75 volunteers involved in this study are summarised in Appendix III.

Status of volunteers	Number of individuals (% in each group)		
	Young (age < 30) n = 25	Old (age > 60) n = 25	Alzheimer's (age > 60) n = 25
Gender			
Male	12 (48)	7 (28)	11 (44)
Female	13 (52)	18 (72)	14 (56)
Ethnic origin			
Caucasian	24 (96)	24 (96)	25 (100)
Others	1 (4)	1 (4)	None
Smoker	3 (12)	3 (12)	None
Intake of vitamin(s)/antioxidant(s)	4 (16)	7 (28)	4 (16)
Intake of mineral(s) and other supplement(s)	2 (8)	11 (44)	5 (20)
Medication(s)			
Contraceptives	5 (20)	None	None
Others	3 (12)	11 (44)	16 (64)
Trial drug(s)	None	None	18 (72)
Health problem(s)	4 (16)	13 (52)	25 (100)

Table 3.1 Summarised information on the 75 volunteers recruited for this study.

3.2.2 Experimental variability

Experiments of various kinds were used to illustrate the performance of the assay and to assess its reproducibility and validity. The PBMCs count was at least 1×10^7 cells per 1 ml of fresh blood. Cell viability, measured before and immediately after H_2O_2 treatment, always exceeded 90 %. Table 3.2 shows the variability observed in the comet assay expressed as coefficients of variation (CV). The higher the concentration of H_2O_2 used for treatment, the smaller the CV values of intra- and inter-assay, intra-subject and intra-slide variability i.e. the smaller the variability observed. The inter-subject variability expressed as CV was shown in Table 3.3. Again, the higher the concentration of H_2O_2 used for treatment, the smaller the CV values of inter-subject variability. The inter-subject variability for basal and induced DNA damage had the highest CV values amongst all other variability observed in each volunteer group.

Concentration of hydrogen peroxide (μM)	Variability expressed as mean ± standard deviation (range) coefficient of variation (CV) in %			
	Intra-assay (n = 8)	Inter-assay (n = 4)	Intra-subject (n = 5)	Intra-slide (n = 10)
0	-	-	-	66.4 ± 43.9 (34.5–179.2)
5	24.5 ± 15.4 (8.3–57.3)	25.6 ± 16.5 (14.1–49.5)	38.8 ± 21.7 (8.9–56.7)	37.4 ± 18.0 (20.9–77.9)
20	-	-	-	15.4 ± 5.1 (5.8–24.2)
50	10.6 ± 8.5 (2.2–28.5)	9.3 ± 9.1 (1.4–22.4)	15.5 ± 10.1 (4.2–31.9)	14.1 ± 6.8 (3.4–23.6)

Table 3.2 Intra- and inter-assay, intra-subject and intra-slide variability in the comet assay.

Concentration of hydrogen peroxide (μM)	Inter-subject variability expressed as coefficient of variation (CV) in %		
	Young (age < 30)	Old (age > 60)	Alzheimer's (age > 60)
0	74.0 (n = 25)	95.0 (n = 24)	101 (n = 25)
5	60.2 (n = 25)	72.1 (n = 25)	82.0 (n = 25)
20	25.4 (n = 15)	39.5 (n = 18)	62.7 (n = 24)
50	18.9 (n = 25)	21.4 (n = 25)	42.4 (n = 25)

Table 3.3 Inter-subject variability in the comet assay.

3.2.3 Scores of DNA damage in peripheral blood mononuclear cells

The comet assay was used to study oxidative DNA damage on PBMCs of all 75 volunteers. A preliminary study (n = 10) was carried out to establish the concentration range of H_2O_2 used for this work. The degree of DNA damage was quantified by visual classification of cells into four categories, which are shown in Figure 3.1. Figure 3.2 shows a typical example of the appearance of PBMCs after treatment with different concentrations of H_2O_2. Table 3.4 summarises the total comet scores of the three groups of volunteers after incubation with various concentrations of H_2O_2. Non-parametric statistical tests (Section 2.5) were used for data analysis. The mean total comet score observed in untreated PBMCs i.e. 0 μM H_2O_2 in the young was not significantly different from that in the old ($P = 0.20$). However, a significant difference was revealed in the mean total comet scores between young and old healthy volunteers after PBMCs were treated with 5, 20 or 50 μM H_2O_2 ($P = 0.039$, $P = 0.030$ and $P = 0.034$, respectively). A higher mean

total comet score i.e. a higher level of DNA damage was observed in the H_2O_2-treated PBMCs of the younger volunteers than in the older group. On the other hand, no significant difference was observed in the mean total comet scores in both untreated and H_2O_2-treated PBMCs between the old and healthy group and the Alzheimer's group ($P > 0.05$). Figure 3.3 shows the distribution patterns of DNA damage among different groups of volunteers expressed as the number of cells in the four comet categories, from 0 (undamaged) to 3 (highly damaged), as a function of H_2O_2 concentration. There was an increasing number of category 3 comets observed with increasing concentration of H_2O_2 in all cases. However, a large number of comets always remained in category 0. The frequency distribution pattern of DNA damage in response to the doses of H_2O_2 was not significantly different between the three groups ($P = 1.00$).
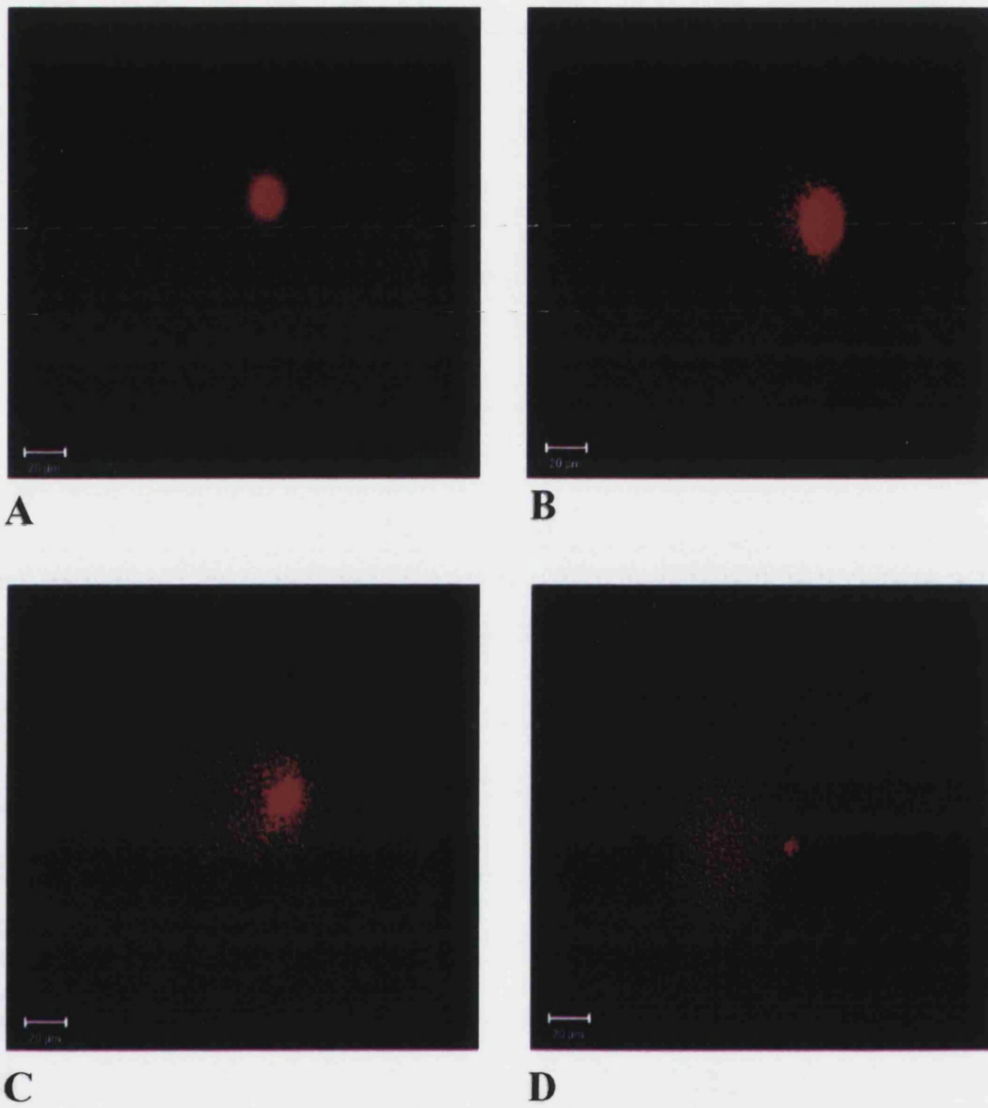
Figure 3.1 Visual categories of DNA damage. A: Category 0 (undamaged); B: Category 1 (slightly damaged); C: Category 2 (damaged); D: Category 3 (Highly damaged).
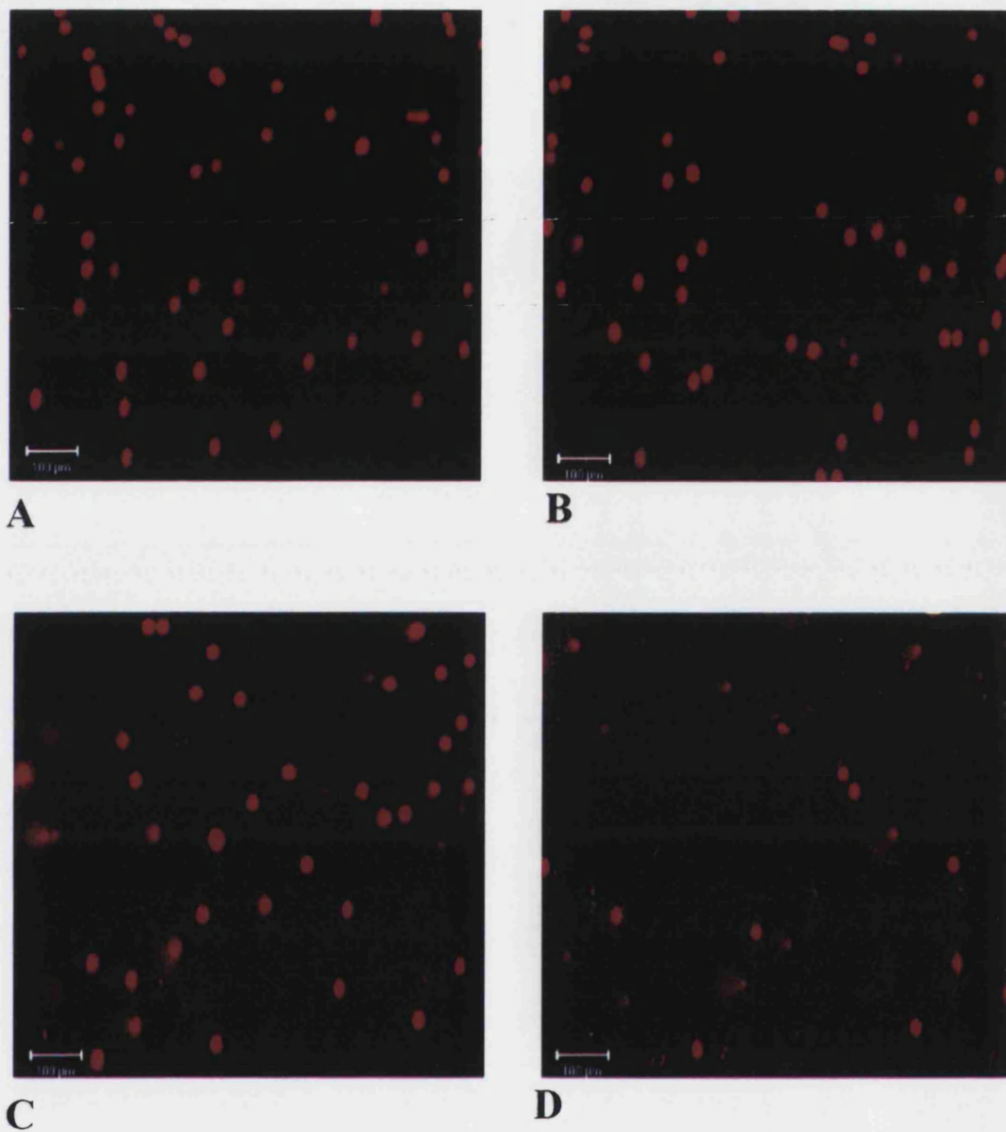
Figure 3.2 A typical example of the appearance of PBMCs after treatment with different concentrations of H₂O₂. A: 0 μM; B: 5 μM; C: 20 μM; D: 50 μM.

Concentration of hydrogen peroxide (μM)	Total comet scores expressed as mean ± standard error		
	Healthy (Age < 30)	Healthy (Age > 60)	Alzheimer's (Age > 60)
0	12.04 ± 1.78 n = 25	10.79 ± 2.09 n = 24	18.92 ± 3.81 n = 25
5	31.32 ± 3.77 n = 25	21.40 ± 3.08 n = 25	24.68 ± 4.05 n = 25
20	63.00 ± 4.13 n = 18	48.94 ± 4.56 n = 15	47.04 ± 6.02 n = 24
50	95.76 ± 3.63 n = 25	84.56 ± 3.61 n = 25	71.32 ± 6.05 n = 25

Table 3.4 Comparison of total comet scores obtained from the healthy young and old and the Alzheimer's group. PBMCs were treated with various concentrations of H_2O_2 before comet assay was carried out to study the status of oxidative DNA damage. Data shown as mean ± SE. Note that 20 μM H_2O_2 was introduced at a later stage into the experimental system, hence n < 25.
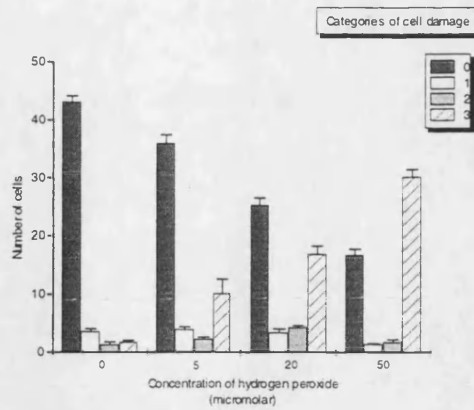
A. Young and healthy (Age < 30)
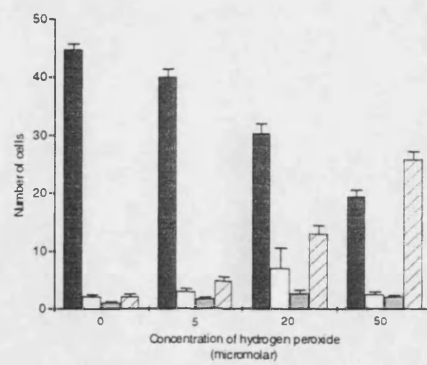
B. Old and heathy (Age > 60)
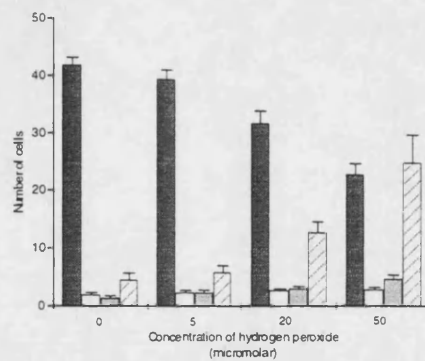
C. Alzheimer's (Age > 60)

Figure 3.3 Comparison of the distribution of DNA damage, expressed as number of cells in the four comet categories (0-3) between group A-C, after treatment with H_2O_2. Data based on 50 cells per slide and duplicate slides from 25 subjects for each group. Data expressed as mean ± SE.

3.2.4 Effect of hydrogen peroxide and gender

Figure 3.4 shows the effect of increasing concentrations of H_2O_2 on the total comet scores i.e. the extent of DNA damage in human PBMCs (n = 25 in each group) using the comet assay. A statistically significant dose-response relationship was seen with significant responses at all dose levels ($P = 0.00$). PBMCs showed dose-dependent increases in DNA damage following H_2O_2 treatments. The concentration range of H_2O_2 was established by a preliminary study (n = 10) and the results were included in the final data. Analysis was also carried out considering gender as a factor and the results were of no significance ($P > 0.05$).
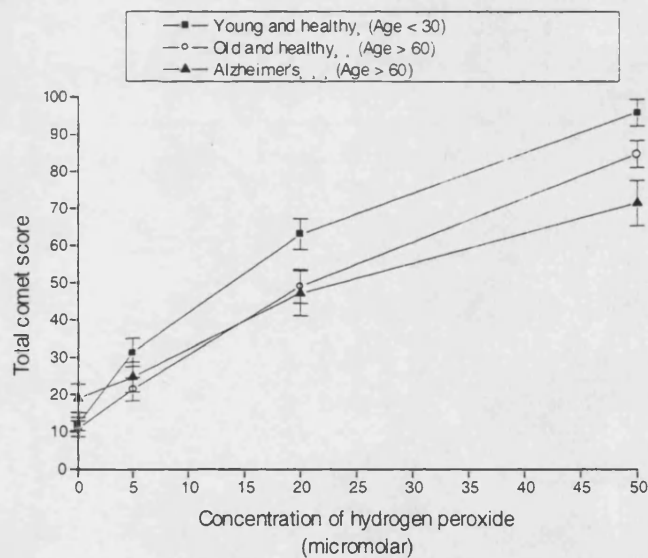
Figure 3.4 Mean dose-response curve for PBMCs from the three groups of volunteers (in duplicate) after in vitro exposure to different concentrations of H_2O_2 in the comet assay. Mean ± SE (n = 25 in each group) of total comet scores are given here and also in Table 3.4.

3.3 Discussion

3.3.1 Variability in the comet assay

The comet assay, which measures DNA strand breaks at the level of single cells, can be easily applied to human PBMCs, and therefore lends itself to human biomonitoring studies. However, before applying the assay to these studies, the variability inherent in this method has to be determined, thus allowing us to understand the power and the limitation of this assay in detecting DNA damage. Variability was expressed as a CV for this study (see Section 2.5) and that conducted by Holz et al (1995a). The findings in this work are similar to those presented by Holz et al (1995a) and Collins et al (1997b). As the concentration of H_2O_2 used for treatment increased, the CV values of intra- and inter-assay, intra- and inter-subject and intra-slide variability decreased, indicating a gradual decrease in the variability observed. The values of the intra-assay, inter-assay and intra-slide variability were lower than that of the intra- and inter-subject variability. Therefore, when standardising the comet assay, real physiological variations inherent to individuals could present a bigger problem than variations arising during the experimental procedures. CV values of inter-subject variability for basal and induced DNA damage were the highest amongst all other variability observed in each group of volunteers; with the highest CV values observed in the AD group and the lowest CV values observed in the young and healthy group. Volunteers in the young and healthy category are generally "drug-free" and "disease-free" and this may explain a

relatively low variability observed between individuals in this group. In contrast, it is inevitable that some of the volunteers from the old and healthy category suffer from health problems at some point in their lives and are currently on prescription drugs (see Table 3.1 and Appendix III). Again, most of the AD patients recruited have other health problems and all are involved in AD drug trials (see Table 3.1 and Appendix III) and this may explain the relatively high variability observed between individuals. To minimise inter-subject availability, the problem of limited availability of old and AD volunteers needed to be addressed. It is also important to take into account that age-associated changes in the structure and function of various systems within our bodies do not occur at the same rate for different individuals and substantial variability exists between people of the same age (Rowe and Kahn, 1987).

So, what are the other sources of variability? The type of cells under investigation provides the majority of variations observed in the comet assay. Virtually any eukaryotic cell can be analysed for DNA damage using the comet assay but the most commonly examined human cells are lymphocyte populations (Fairbairn et al, 1995). PBMCs, which consist mainly of lymphocytes and monocytes, are widely used in biomonitoring of DNA damage. For studies of reproducibility, these cells have the advantage that they can be obtained repeatedly from single subjects, which is not feasible with other cells. Lymphocytes and/or blood samples also have the additional advantage that they can be stored and processed later, without loss of viability or factors affecting responses. Visvardis et al (1997) evaluated the comet assay using cryopreserved lymphocytes. The induction of DNA damage, as well as the repair

capacity of γ-ray- and H_2O_2-exposed cryopreserved human lymphocytes, was found

to be the same as that of freshly isolated lymphocytes. In addition, Anderson et al

(1997) demonstrated that blood samples stored at 4 °C or room temperature for up to

four days were suitable for use in the comet assay for studies in human cells.

However, many parameters can affect the response of lymphocytes in the comet

assay. Both findings from present study and Collins et al (1997b) demonstrated

considerable intra-subject variability of comet formation with time. Such variability

may reflect handling variation or real physiological variation – for example, a

transient increase in oxidative stress resulting from an infection or a diet-induced

change in antioxidant status, which could be excluded by using cryopreserved

PBMCs instead of fresh PBMCs (Holz et al, 1995a). This observation may not be

applicable to this study because no sudden variations in diet and health status were

recorded. Some reports have pointed to a variety of possible factors that may be

responsible for differences in cell response including the age of the blood donor

(Singh et al, 1990, 1991), the physical activity of the donor (Hartmann et al, 1994)

and whether or not the donor smokes (Betti et al, 1994). In addition, cell cycle status

is likely to impose an additional level of complexity to the problem, since chromatin

structure, which is fundamental to replication and transcriptional activity, will affect

DNA during comet formation (Olive and Banáth, 1993). Sampling time was

therefore found to influence the comet test (Betti et al, 1995). Possible changes in

cellular composition should also be taken into account when assessing single-strand

breaks using PBMCs. Findings by Holz et al (1995b) indicated that monocytes have

both a lower degree of basal DNA damage and a lower susceptibility to H_2O_2-

induced DNA damage than lymphocytes isolated from the same blood sample. Despite the risk of reduced sensitivity, most investigators using the comet assay seem to prefer to use the whole leukocyte population (PBMCs) when studying induced DNA damage and basal levels of DNA damage in "peripheral lymphocytes". Fractionation of cells, during which introduction of damage to the cells is possible, is generally tedious and usually requires special equipment and access to specific antibodies. Also, there is always the question of how far to go when trying to separate different cell types.

The ability to measure DNA damage accurately also depends on several technical factors. For example, lysis conditions (i.e. salts concentration, pH, and lysis time in particular) can profoundly affect the ability to detect damage (Klaude *et al*, 1996). Washing and equilibrating the slides with the running buffer before electrophoresis were also important in avoiding problems caused by residual salt from the lysis solution. For this study, an incubation temperature of 4 °C was chosen to reduce the formation of single-strand breaks in control cells. McKelvey-Martin *et al* (1993) showed that when cells were incubated at 37 °C for 1 hr in PBS, either additional repair of pre-existing breaks or additional strand breaks occurred. Scoring can also be a source of variability in the comet assay (as shown by intra-slide variability) although measures were taken in this study to reduce the extent of such variations (see Section 2.4.2.1). One consistent difficulty with comparing results produced in different laboratories using the comet assay is the variability in the application of the technique itself. Even with the adoption of a standardised assay protocol, it may still

be necessary to tailor lysis and electrophoresis conditions to the expected level of damage. This is because the extent of DNA damage will vary depending on the agent used to induce damage, the nature of the lesions produced, the type of biological conditions the cells are exposed to and the amount of strand rejoining allowed.

Although considerable variation is seen in samples of cells collected from different individuals in the same group and from the same person on different occasions, differences between groups of individuals can reliably be measured, given only a sufficient number of individuals per group. For the determination of minimum difference and minimum sample sizes, power analysis was carried out by Holz *et al* (1995a) based on the quantification of the sources of variability as determined in their study. The minimum true difference between the groups that would be clinically valuable has to be defined and established by general consent of scientists in this area of research before the appropriate sample size can be determined by power analysis.

3.3.2 Comet image analysis

In this study, comet slides were analysed within 1-3 days of the experiment. Klaude *et al* (1996) air-dried and fixed the slides in methanol, allowing analysis at a later time. The methods of comet image analysis reported are as varied as the applications for which the assay has been used. The simplest method is to score the comets

empirically on the basis of damage extent. A series of pictures can be taken of comets with varying degrees of damage (Figure 3.1) and it is possible to allocate comets to the appropriate class of damage, provided adequate precautions are used to protect against observer bias as this study has shown. Although this method of evaluation lacks the sophistication of image analysis, it has been used successfully by Anderson *et al* (1994) and Holz *et al* (1995a) and was used in this study. Other investigators collect head diameter, comet length and comet area data either manually (McKelvey *et al*, 1990) or using commercially available image analysis systems (Green *et al*, 1995). Other parameters measured by image analysis systems and reported in various studies include tail moment, tail length, tail length/head radius and tail fluorescence (McCarthy *et al*, 1997). Collins *et al* (1997b) carried out parallel scoring by eye and image analysis of human lymphocyte comets showing a wide range of degrees of damage. A close correspondence between the visual and computer scores was observed. The manual method would therefore appear to offer a good, reliable and relatively inexpensive method of analysing comet slides. However, it is considerably slower than the image analysis method and it does not provide any information on the area or the fluorescent intensity of the comet.

3.3.3 Dose-response relationship

The mean extent of basal and H_2O_2-induced DNA damage as a function of the increasing concentration of H_2O_2 was measured by means of visual scoring of the comets. These values were presented in Figure 3.4 and show similar dose-response

relationship to those previously reported by Holz *et al* (1995a), Collins *et al* (1997a)

and Piperakis *et al* (1998). Similar dose-response relationships also existed when

other known DNA damage-inducing agents, for example, bleomycin (Anderson *et al*,

1994) and radiation (Green *et al*, 1995; Collins *et al*, 1997a) were used.

3.3.4 Factors affecting the extent of DNA damage

According to some predictions of the DNA-related theories of ageing and age-related

diseases, cells from older individuals or individuals who suffer from age-related

diseases should be expected to have increased levels of basal DNA damage, possibly

accompanied by a reduced rate of damage recognition and repair (Lindahl, 1993).

Attempts to show an increase in DNA damage with age have yielded conflicting

results. Singh *et al* (1990) and Betti *et al* (1995) both reported a non-significant

relationship with age. In the study conducted by Piperakis *et al* (1998), an increase

in basal and induced DNA damage was generally observed with increasing age. In

this study, the reverse was observed i.e. a decrease in induced DNA damage was

observed with increasing age although age did not have an effect on the basal DNA

damage. Age-associated changes in our bodies do not occur at the same rate for

different individuals and substantial variability exists for people of the same age

(Rowe and Kahn, 1987). High genomic stability and an increased resistance to

oxidative stress have been highlighted within centenarians (probably the best

example of successful ageing) (Franceschi *et al*, 1995). There appears to be evidence

for a link between lifespan, genomic stability and defence systems which protect

against genomic damage. To understand this, various genetic and defence endpoints can be analysed over a long period (age 50-75). The results of this study should highlight two extreme subsets of data, one presumably demonstrating a trend of age-related increases in genetic damage and development of pathology and the other showing individuals who remain healthy for longer. Conflicting data on changes to cellular DNA repair capacities with age have been reported and reviewed (Barnett and King, 1995). King *et al* (1997) showed that lymphocytes from individuals in the 75-80 age groups exhibited DNA repair capacities comparable to those observed for lymphocytes from subjects in the 35-39 age group. These results highlight the importance of DNA repair capacity in the maintenance of genomic stability and may explain the results obtained from this study. In contrast to the prediction of the DNA damage theory of ageing and age-related diseases, the effect of AD was not significant. This again maybe explained by the theory of genomic stability. This may also explained by an artifact effect since 72 % of the volunteers from the AD group were taking AD trial drugs which aimed to alleviate the symptoms of AD. Since clinical trial information is highly confidential, it is not possible to decide whether these drugs can interfere with the study of DNA damage in PBMCs using the comet assay. However, many studies have demonstrated the implication of oxidative stress in the pathogenesis of AD, as reviewed by Markesbery (1997). For example, in a study of AD subjects compared with control subjects, there was a significant increase in mitochondrial DNA oxidation in parietal cortex in AD (Mecocci *et al*, 1994).

In this study, DNA damage in PBMCs increased with increasing concentration of H_2O_2. The concentration range of H_2O_2 chosen for this study was widely used (Holz et al, 1995a; Collins et al, 1997b; Piperakis et al, 1998). A preliminary study (n = 10) carried out for this work demonstrated that this concentration range was sufficiently high to induce visible changes in the cells for analysis without killing the cells. H_2O_2 can penetrate the cell membrane easily and can initiate the generation of highly reactive species. In order for the reactive oxygen species to proceed to DNA damage, a sufficient concentration must be available to overwhelm the threshold of antioxidant capacity. This consists of the presence of antioxidant enzymes, scavenger molecules and the ability of cells to remove altered molecules by turnover. The observed differences in the physiological response of one group compared with the other group are possibly due to the effects of age, disease state and smoking on the formation of reactive oxygen species inside cells, and to their influence on protective cellular pathways against induced oxidative damage.

In this study, an increasing number of category 3 comets was observed with increasing concentration of H_2O_2 in all cases. However, a large number of comets always remained in category 0. Similar observations were made by Collins et al (1997a) and these may be due to the existence of subpopulations in PBMCs which respond in different extents to H_2O_2 exposure. However, the frequency distribution pattern of DNA damage in response to the doses of H_2O_2 was not significantly different between the three groups of volunteers.

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Like other work such as that conducted by Betti *et al* (1995), this study revealed that gender has no significant effect on DNA damage in PBMCs. The effect of smoking on DNA damage of PBMCs was not investigated in this work but it has been extensively studied by Betti *et al* (1995) and Piperakis *et al* (1998) who reported that smoking significantly increased the extent of DNA damage observed using the comet assay. Smoking is an important variable in inducing significant alterations to genetic material. The classes of compounds identified in tobacco e.g. aromatic hydrocarbons and amines, are all well-known sources of reactive oxygen species, such as $^{\bullet}OH$, $O_2^{\bullet-}$ and peroxides. The effect of antioxidant intake on DNA damage in PBMCs was not studied in this work although intake of non-prescribed vitamins and minerals was recorded (see Table 3.1 and Appendix III). However, it has been extensively studied by Anderson *et al* (1994), Green *et al* (1994) and Collins *et al* (1997a). The results were inconclusive although it seems that antioxidants may provide a protective effect in PBMCs against DNA damage. Further studies would be required to establish such a relationship. The restricted number of human blood donors included in the present study makes it impossible to draw firm conclusions about associations between the recorded levels of DNA damage in the PBMCs and various types of personal exposures and/or habits. Nevertheless, this work is a valuable introductory study of basal and induced DNA damage in PBMCs in human populations by the comet assay.

3.3.5 Modification of the comet assay

The comet assay used in this study was simple and economical. However, it only measured strand breaks and alkali-labile sites. Strand breaks are not a major form of damage with most genotoxins – or if they are, as in the case of ionising radiation, they are quickly rejoined by cellular repair. Strand breaks and apurine/apyrimidine (AP) sites appear as intermediates in the cellular processing of damage (Section 1.3.2.4). Thus, the results of this study gave us only some idea of the extent of oxidative DNA damage in PBMCs. Collins *et al* (1997a, 1997b) described a modification of the comet assay, by including digestion with lesion-specific endonucleases, in order to obtain specific information about oxidative DNA damage and its repair in human cells, as well as to increase the range and sensitivity of the assay. Digestion with endonuclease III and formamidopyrimidine glycosylase (FPG) was incorporated into the comet assay protocol to reveal oxidised pyrimidines and oxidised purines (mainly 8-OHdG), respectively. This modified comet assay has been applied to study the oxidative DNA damage in human disease such as insulin-dependent diabetes mellitus (Collins *et al*, 1997b), the effect of dietary antioxidants on oxidative DNA damage (Green *et al*, 1995; Collins *et al*, 1997a; Panayiotidis and Collins, 1997) and the kinetics of repair of oxidative DNA damage in human cells (Collins *et al*, 1995).

Several questions about the relationship between DNA damage and ageing are frequently asked. First, what is the functional importance of the PBMCs with high

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levels of DNA single-strand breaks and/or alkali labile sites? For example, is the DNA damage evenly distributed among all PBMCs, or limited to a functional subpopulation? Another possibility is that our subjects' immune systems are ageing at different rates and therefore their cells might display different levels of damage. A final question is whether these results apply just to PBMCs or to other types of cells. While further research will be required to assess these questions, it is clear that the comet assay has provided an important insight into basal and induced DNA damage in ageing cells and cells extracted from Alzheimer's patients.

CHAPTER 4

MALONDIALDEHYDE MEASUREMENT

4.1 Introduction

Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids producing a variety of breakdown products, including alcohols, ketones, aldehydes, and ethers (Section 1.3.3.2.2). Biological membranes are often rich in unsaturated fatty acids and bathed in an oxygen-rich metal-containing fluid. Therefore, it is not surprising that membrane lipids are susceptible to peroxidative attack. Conditions that accelerate lipid peroxidation include exposure of the microsomes to γ-irradiation, light, hyperoxia, ozone and radical initiators, such as dialuric acid. The incorporation of molecular oxygen into polyunsaturated fatty acids to yield hydroperoxides as the primary initial product is the focus of intense current interest in biomedical and pharmacological research. The basis of this interest rests with the potential role of lipid peroxidation and peroxidation products in the degeneration associated with ageing and in the pathogenesis of several clinically significant diseases (Beckman and Ames, 1998; Marcus *et al*, 1998). This increased interest demands quantitative methods which are diagnostic of the process and meet basic analytical criteria regarding accuracy, reliability, sensitivity and specificity. A number of techniques are available for measuring the rate of lipid peroxidation (Halliwell and Gutteridge, 1989). For example,

three measurable species are produced during microsomal lipid peroxidaton, including lipid hydroperoxides, lipids containing conjugated dienes and malondialdehyde (MDA). Reduction of iodide by peroxides is a convenient method for determining the amount of lipid hydroperoxides present in a membrane sample and it permits an accurate comparison of lipid peroxide levels in dissimilar lipid membranes. However, its use is limited by the fact that lipid hydroperoxides are transient species that are exposed to factors such as transition metals that catalyse their breakdown. *In vivo* and *in vitro* lipid peroxidation is accompanied by a rearrangement of the polyunsaturated fatty acid double bonds, leading to the formation of conjugated dienes, which absorb at 233 nm. Therefore, lipid peroxidation can be also measured by recording the increase in absorbance of extracted membrane at this wavelength. MDA, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. The chemistry and biology of MDA and its formation from lipid are extensively reviewed by Janero (1990). Methods for the quantitative analysis of MDA include HPLC, UV and fluorescene spectrophotometry, and the commonly used thiobarbituric acid (TBA) test (Janero, 1990). The TBA test allows simple and direct quantification of fatty peroxide-derived, TBA-reactive MDA in the form of stable, red MDA:TBA (1:2) adduct whose formation requires no sample preparation other than acidification. In acid solution, the product absorbs light at 535 nm (Buege and Aust, 1978; Uchiyama and Mihara, 1978). Most of the MDA that reacts in the TBA test was not present in the sample assayed but formed by decomposition of lipid peroxides during the acid heating stage of the TBA assay. This test has been widely employed to detect and quantify lipid peroxidation in a variety of biological

samples (Suleiman *et al*, 1996; Nielsen *et al*, 1997; Marcus *et al*, 1998; Liu *et al*, 1999). Due to its sensitivity and specificity, the TBA test was used to measure MDA levels in the three groups of volunteers recruited for this study and the effect of age, AD and gender were investigated.

4.2 Results

Lipid peroxidation was measured by determining the MDA production, using TBA (Buege and Aust, 1978). The MDA level was measured in the blood plasma of the three groups of volunteers and all measurements were carried out in triplicate.

4.2.1 Experimental variability

Experimental variability was studied and the results were summarised in Table 4.1. Inter-subject variability showed CV of 17.0 % for the healthy younger group (n = 25), 12.1 % for the healthy older group (n = 25) and 8.7 % for the AD group (n = 25).

Variability	Variability expressed as mean ± standard deviation (range) coefficient of variation (CV) in %
Intra-day (n = 3)	4.6 ± 3.4 (1.3-8.0)
Inter-day (n = 3)	34.5 ± 7.0 (26.4-38.7)
Intra-assay Age < 30 (n = 25) Age > 60 (n = 25)	5.6 ± 3.3 (0.8-13.3) 6.5 ± 4.8 (0.7-21.1)
Intra-subject (n = 5)	5.6 ± 2.9 (1.6-8.7)

Table 4.1 Intra- and inter-day, intra-assay and intra-subject variability in MDA assay.

4.2.2 Plasma malondialdehyde concentration

A two sample t-test and ANOVA were used for analysis because the data were normally distributed. This was shown by the Anderson-Darling normality test ($P = 0.57$, n = 75). The plasma MDA concentration in the blood plasma of all three volunteer groups was measured and the findings are summarised in Table 4.2.

Volunteer group	Plasma MDA concentration (μM)		Sample size (n)
	mean ± SD	Range	
All volunteers	1.32 ± 0.18	0.74-1.72	75
Healthy (age < 30)	1.28 ± 0.22	0.94-1.71	25
Healthy (age > 60)	1.26 ± 0.15	0.74-1.48	25
Alzheimer's (age > 60)	1.41 ± 0.12	1.12-1.72	25

Table 4.2 The plasma MDA concentration of the three groups of volunteers: healthy young (age < 30) and old (age > 60); and the Alzheimer's group (age > 60).

Statistical analysis revealed no significant effect of age (P = 0.20) and gender (P = 0.19) on plasma MDA level. However, the plasma MDA level of the AD group was significantly higher than that of the healthy older group (P = 0.0003).

4.3 Discussion

One of the most frequently used biomarkers of the overall lipid peroxidation level is the plasma concentration of MDA, one of several by-products of lipid peroxidation processes. Proper utilisation requires reliable reference intervals from large unselected human population and thorough evaluation of the influence

of age and gender, as well as other variations within the population. Nielsen *et al* (1997) conducted a study of plasma MDA using the TBA test (with additional HPLC as a separation step) to establish a reference interval in a sample group and to assess the possible influence of age, gender, smoking and alcohol on plasma MDA. Their findings revealed no significant effect from age but from gender with men having higher plasma MDA levels than women. Also, smokers were revealed to have a significantly higher plasma MDA level than non-smokers. Plasma MDA was also found to correlate with alcohol consumption. In this study, the effect of age, AD and gender on plasma MDA level was investigated but only AD had a significant effect on plasma MDA. This observation is similar to that reported by Marcus *et al* (1998) where there was an overall average increase in lipid peroxidation (MDA-TBA level) in all sampled regions of the AD brain. Other studies on various AD brain regions also showed an increase in oxidative stress as evidenced by elevated levels of peroxidation products (Richardson, 1993; Markesbery, 1997). Although age was revealed to have no effect on lipid peroxidation measured in this study and that by Marcus *et al* (1998), other studies have demonstrated an increase of lipid peroxidation with age (Mizuno and Ohta, 1986; Beckman and Ames, 1998), reflecting a high level of oxidative stress experienced during the ageing process as proposed by the free radical theory of ageing.

Two underlying assumptions are implicit from the widespread use of the TBA test to assess lipid peroxidation: an operative and quantitative relationship exits between lipid peroxidation and MDA; product formation during the TBA test is diagnostic of the presence and amount of fatty peroxides. Although the TBA

assay is the most frequently used method for determining the extent of membrane lipid *in vitro*, it is not suitable for *in vivo* study since MDA is readily metabolized *in vivo* and in tissue suspensions. In addition, MDA reacts with tissue components to form cross-linked lipofucin pigments, thus decreasing its intracellular concentration. MDA production during the peroxidation of microsomal membranes varies among different types of tissues, thus making it difficult to accurately compare the extent of lipid peroxidation. This is caused in part by the different amounts of polyunsaturated fatty acids present in the microsomal membranes from different tissues. Since only unsaturated fatty acids with three or more methylene-interrupted double bonds can ultimately form MDA, variation in MDA production may be a reflection of the lipid composition rather than the susceptibility to lipid peroxidation. Tissue aldehydes and sugars also react with TBA to produce a chromophore absorbing at 535 nm. Both acetaldehyde and sucrose interfere with the detection of MDA when present in millimolar quantities. Reducing the temperature in the heating step from 100 °C to 80 °C can minimise interference from sucrose present in buffers. Furthermore, the anticoagulant used during blood sampling might affect the plasma MDA level. However, EDTA-treated plasma that was used in this study was found to generate the least interference to the degree of lipid peroxidation at the time of blood sampling (Nielson *et al*, 1997). Despite these problems, the TBA assay remains a useful tool in monitoring lipid peroxidation *in vitro* owing to its sensitivity and simplicity. It is important to remember that the TBA test is by no means the only method for the assessment of lipid peroxidation. Different techniques measure different products of lipid peroxidation and to date, no single method sufficiently meets analytical standards in all applications to make it the

method of choice. · In future work, it would therefore be useful to measure different parameters using two or more methods to assess lipid peroxidation. This would provide a better understanding of the effect of oxidative damage to lipids which are involved in ageing and the pathogenesis of age-related diseases.

CHAPTER 5

GLUTATHIONE MEASUREMENT

5.1 Introduction

Glutathione is an extremely important water-soluble intracellular antioxidant, found primarily in the cell cytosol (Meister and Anderson, 1983). Oxidants, in particular H_2O_2, are neutralised by conversion of reduced glutathione (GSH) to its oxidised form, GSSG, after which GSH is reformed through the glutathione peroxidase-reductase enzyme system (Section 1.3.3.3) (Halliwell and Gutteridge, 1989). GSH is also important in maintaining ascorbate levels, the reduced form of vitamin C, in the cytosol and in plasma. Ascorbate in turn maintains levels of the lipid soluble vitamin E in the cell membrane, necessary to prevent lipid peroxidation (Martensson and Meister, 1991). GSH is widely distributed in human tissues (Meister and Anderson, 1983) and participates in the protection of cells against the toxic effects of free radicals which are produced during oxidative stress and are involved in human diseases, ageing and age-related diseases such as AD (Section 1.3.3). One of the claims in the free radical theory of ageing is that there is a significant decrease in the defence mechanism towards stress in old cells relative to young cells. (Harman, 1981). This reduction results in biological damage to essential macromolecules, eventually leading to cell death (Beckman and Ames, 1998). AD has been described

as a "free radical" disease by Harman (1992), suggesting the involvement of oxidative stress in the pathogenesis of the disease.

Several methods have been devised to measure tissue levels of GSH and GSSG. The HPLC technique proposed by Fariss and Reed (1987) is universally considered as a standard method but it is time-consuming. A commonly used procedure is the enzymatic assay developed by Tietze (1969). A fluorometric method was proposed by Hissin and Hilf (1976). More recently, a simple, patented colorimetric method (GSH-400, Bioxytech, France) has also been made available. These three commonly used non HPLC-based methods were extensively evaluated for the rapid determination of glutathione in guinea-pig heart and liver by Floreani *et al* (1997). The enzymatic method was found to be the most reliable method for tissue glutathione measurements but the fluorometric method is still widely used because of its high sensitivity. For plasma glutathione measurement in humans, the enzymatic method is not sufficiently sensitive since plasma contains mainly albumin and only small amounts of GSH and a modified version of the fluorometric method developed by Hu (1994) can be used instead.

5.1.1 Fluorometric method for the determination of GSH and GSSG

A convenient method for measuring tissue glutathione using the fluorescent reagent OPT, (Hissin and Hilf, 1976) has been modified to measure plasma glutathione (Hu, 1994). This method was shown to be specific for GSH and GSSG, since the

fluorescence observed with a variety of other substances appeared to be minimal compared to that seen-for GSH and GSSG. GSH is the predominant form of total glutathione under physiological conditions. The GSSG assay utilises NEM to prevent oxidation of GSH to GSSG, removing the error that may arise from this unintentional conversion. GSH was shown to react specifically with OPT at pH 8, yielding a highly fluorescent product that could be activated at 350 nm with an emission peak at 420 nm (Cohn and Lyle, 1966). The reaction was found to be pH-dependent since the fluorescent intensity decreased below pH 8. At pH 12, the conversion of GSH to GSSG was negligible and the absorbance and emission spectra for the OPT-GSSG reaction were similar to that for GSH, namely 350 nm and 420 nm, respectively. To understand the role of glutathione in the defence mechanism against free radical attack, the effects of age, AD and gender on the plasma GSH and GSSG levels were investigated using this fluorometric technique.

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5.2 Results

5.2.1 Standard GSH and GSSG curves

In constructing GSH and GSSG standard curves (Figure 5.1 and 5.2), variations were observed. The results for standard curves derived from three sets of separately prepared stock standards permitted study of the intra-day variation (i.e. all three curves constructed in one day) and the inter-day variation (i.e. two curves constructed on two separate days) in system standardisation. A good linear relationship existed in these standard curves as indicated by high adjusted R-squared values (96.2 % - 99.4 %). The fluorescence intensity for the OPT-GSH action at pH 8 was directly related to GSH concentration and was linear in the concentration range of 0.5-10 μM (Figure 5.1). Similarly, the fluorescence intensity for the OPT-GSSG at pH 12 was observed to be linear over the concentration range of 0.25-10 μM of GSSG (Figure 5.2).
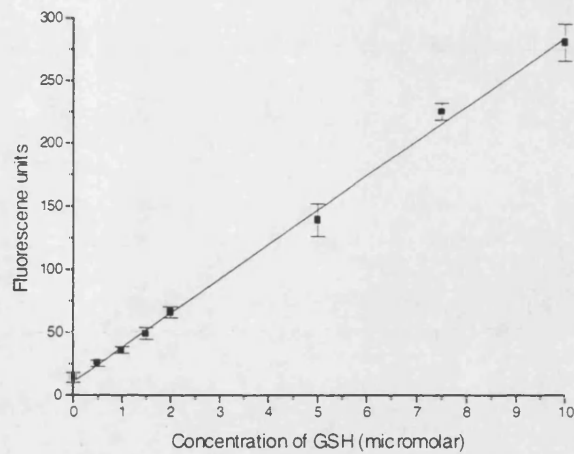
A. Intra-day
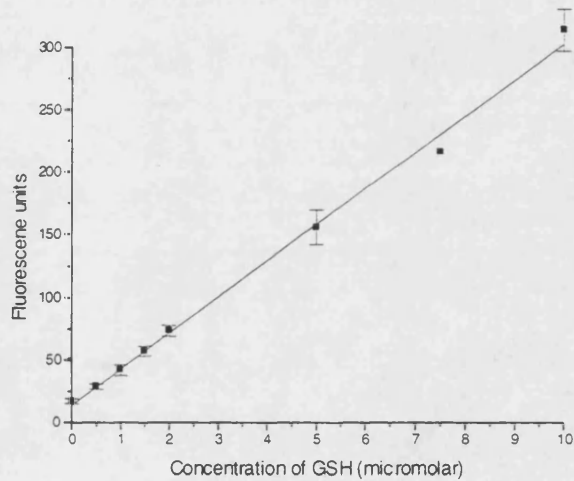
B. Inter-day

Figure 5.1 Intra-day (A) and Inter-day (B) GSH standard curves. Fluorescence intensity was measured at 420 nm (excitation was at 350 nm) and expressed as a function of the concentration of GSH. A: Intra-day curve was derived from three separate standard curves obtained within one day, R^2 (adjusted) = 99.3 %; B: Inter-day curve was derived from two separate standard curves obtained on two separate days, R^2 (adjusted) = 96.2 %.

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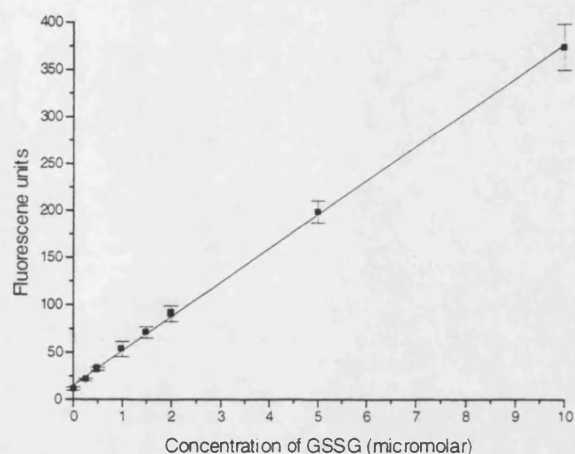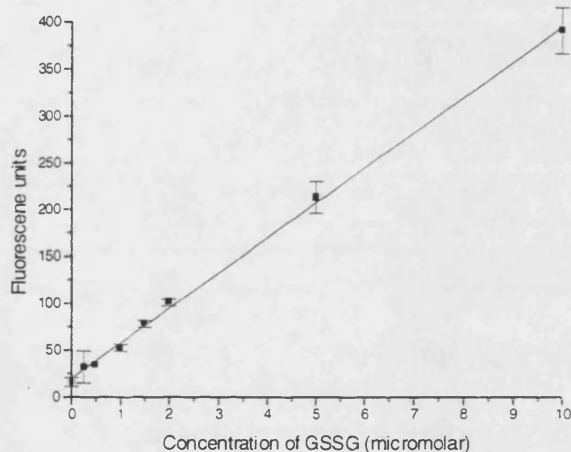
A. Intra-day

B. Inter-day

Figure 5.2 Intra-day (A) and Inter-day (B) GSSG standard curves. Fluorescence intensity was measured at 420 nm (excitation was at 350 nm) and expressed as a function of the concentration of GSSG. A: Intra-day curve was derived from three separate standard curves obtained within one day, R^2 (adjusted) = 99.4 %; B: Inter-day curve was derived from two separate standard curves obtained on two separate days, R^2 (adjusted) = 99.3 %.

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5.2.2 Experimental variability

Experimental variability was studied and the results were summarised in Table 5.1 and 5.2.

Variability	Variability expressed as mean ± standard deviation (range) coefficient of variation (CV) in %	
	GSH	GSSG
Intra-day (n = 3)	25.2 ± 11.1 (16.2-37.6)	27.8 ± 5.4 (22.6-33.3)
Inter-day (n = 4)	30.2 ± 24.1 (4.5-61.3)	14.8 ± 1.4 (13.4-16.8)
Intra-assay Healthy　　　　Age < 30	28.1 ± 14.3 (5.3-61.8) (n = 24)	11.6 ± 7.5 (2.1-27.8) (n = 25)
Age > 60 (n = 25)	24.3 ± 11.8 (4.9-44.4)	13.6 ± 12.4 (0.8-50.0)
Alzheimer's　Age > 60 (n = 25)	10.2 ± 7.8 (1.0-30.9)	3.6 ± 2.2 (0.1-9.2)
Intra-subject (n = 5)	25.5 ± 5.7 (18.6-34.0)	17.9 ± 11.6 (6.1-32.0)

Table 5.1 Intra- and inter-day, intra-assay, intra-subject variability in the GSH and GSSG assay.

Assay	Inter-subject variability expressed as coefficient of variation (CV) in %		
	Young (age < 30) n = 25	Old (age > 60) n = 25	Alzheimer's (age > 60) n = 25
GSH	46.6	48.8	68.0
GSSG	51.8	40.0	22.1

Table 5.2 Inter-subject variability in the GSH and GSSG assay.

5.2.3 Measurement of GSH and GSSG in plasma

Using this fluorometric method, GSH and GSSG levels in human blood plasma collected from three volunteer groups were measured. These values were summarised in Table 5.3. Using non-parametric statistical tests (Section 2.5), age, AD and gender were revealed to have no significant effect on the levels of GSH ($P = 0.90$, $P = 0.24$ and $P = 0.64$, respectively). However, in the male, over 60 subgroup, GSH levels in the healthy sector were found to be significantly higher than that in the AD group. For GSSG measurements, the effect of both age and gender were insignificant ($P = 0.50$ and $P = 0.32$). However, AD was revealed to have a significant effect on GSSG levels with that in the healthy older group significantly

lower than that in the AD group ($P = 0.002$). The level of GSH was found to be significantly higher than that of GSSG in all three volunteer groups ($P = 0.00$).

Age group	Mean ± SD (μM)	Range (μM)	Sample size (n)
All age group			
GSH	4.83 ± 2.61	1.44-12.7	75
GSSG	2.22 ± 0.88	0.80-4.65	75
Healthy Age < 30			
GSH	4.99 ± 2.33	2.03-11.2	25
GSSG	2.29 ± 1.18	0.82-4.65	25
Healthy Age > 60			
GSH	4.96 ± 2.42	1.44-10.8	25
GSSG	1.90 ± 0.70	0.80-3.71	25
Alzheimer's Age > 60			
GSH	4.55 ± 3.09	1.48-12.7	25
GSSG	2.48 ± 0.55	1.72-4.21	25

Table 5.3 GSH and GSSG levels in blood plasma samples measured by fluorometric method.

5.3 Discussion

In this study, the GSH and GSSG levels of the three volunteer groups were compared. The values of the intra-day, inter-day, intra-assay and intra-subject variability were lower than that of the inter-subject variability. Therefore, when standardising the GSH and GSSG assay, real physiological variations inherent to individuals could present a bigger problem than variations arising during the experimental procedures. Due to limited availability of volunteers, it was difficult to apply strict selection criteria during recruitment and therefore variations such as lifestyle, diet and intake of antioxidants may affect the findings and explain a high inter-subject variability. To minimise inter-subject availability, the problem of limited availability of old and AD volunteers needed to be addressed. GSH was the predominant form of glutathione in blood plasma. In general, age, AD and gender did not affect GSH measurements although in the male old subgroup, GSH level was higher in the healthy sector than in the AD sector. A better antioxidant defence in healthy individuals compared to that in Alzheimer's patients may explain this observation although why this difference is observed exclusively in males is not understood. Age and gender did not affect GSSG measurements but AD was found to have a significant effect on the measurements. GSSG levels in the AD group were found to be significantly higher than that in the healthy older group. The explanation for this observation may be due to the high level of oxidative stress possibly found in AD patients, with glutathione mainly in the oxidised form. Various complications arise during interpretation of data collected for any glutathione studies. One of these

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is that defence is induced in response to stress. Therefore, a higher level of GSH may indicate better protection, or alternatively, greater need for antioxidant defence due to increase in oxidant generation. On the other hand, a lower level of GSH may indicate a depletion in the antioxidant reserve following oxidative stress or it may simply be a result of a low antioxidant supply in the diet. In addition, interactions between antioxidants are complex, which aggravates the problem. Few studies have been carried out to monitor glutathione levels in humans and these findings suggest that glutathione levels may vary with several demographic and health-related attributes such as age, gender, disease, diet and diet-supplementation (Flagg *et al*, 1993; Hu *et al*, 1996; Liu and Wei, 1999). Flagg *et al* (1993) found that total plasma glutathione decreased with age in females but increased with age in males; Liu and Wei (1999) found that the level of total blood glutathione was significantly higher in young smokers than in ageing smokers. In the study conducted by Hu *et al* (1996), short-term supplements of α-tocopherol increased both plasma α-tocopherol and GSH in a time-dependent and dosage-dependent fashion. Data from human glutathione studies, including this investigation, should be interpreted with caution because several factors may affect the study results. Sample size was often small and only allowed preliminary conclusions and should be considered as a pilot project for a larger confirmatory study in the future. Furthermore, GSH and GSSG were assayed separately in some studies while a combination of these two forms was assayed in others. It is also important to note that glutathione levels have been measured in various extracts, for example, liver, blood and plasma and different levels have been detected at different sources (Ikegami *et al*, 1994). Plasma GSH

has been found to be a useful marker of a generalised increase in oxidant activity and a decrease in antioxidant defence since plasma GSH level were found to reflect other tissue GSH levels (Ikegami *et al*, 1994). In contrast, plasma GSSG levels did not reflect GSSG levels in tissue. Also, conditions for sample storage and processing can affect glutathione levels. Furthermore, it is difficult to compare data from different studies because the methods for measurement are often different. A large number of glutathione studies have been carried out in animal models and those studies that have measured age-related changes in antioxidant defence have generated conflicting results (Beckman and Ames, 1998). GSH decreased in the ageing rat brain whereas GSSG remained practically constant or dropped only slightly thus lowering the glutathione redox index during ageing (Benzi *et al*, 1988). In contrast, an inconsistent pattern of changes with age was observed in GSH levels in various tissues of gerbils (Sohal *et al*, 1995). In conclusion, glutathione levels do seem to change with age but the pattern of change has yet to be established.

Oxidative stress, meaning an imbalance between the spread of reactive oxygen species and the antioxidant defence may be involved in the pathogenesis of AD (Benzi and Moretti, 1995). Age-related GSH depletion leads to reduced protein and DNA synthesis because of its pivotal role as a reducing equivalent for the glutaredoxin system supplying electrons to ribonucleotide reductase. GSH depletion in the rat led to striking degeneration of brain mitochondria (Jain *et al*, 1991). After GSH oxidation, mitochondria are unable to export GSSG and this may be an important mechanism of neuronal derangement or death. Benzi and Moretti (1995)

suggest there may be a compensatory increase in GSH in the ageing AD brain concomitant with an even larger increase in GSSG. Data obtained for the plasma GSSG level of AD patients from this study agree with this suggestion. There is no doubt that glutathione plays an important role in the antioxidant defence mechanism against oxidative stress in ageing and AD. Further human studies are needed to establish the method of choice for glutathione measurements and to investigate the GSH and GSSG relationship with age and AD.

CHAPTER 6

TOTAL ANTIOXIDANT ACTIVITY ASSAY

6.1 Introduction

Ageing and age-related diseases are often proposed to be associated with increased oxidative stress (Section 1.4). Excess production of reactive oxygen radicals by various enzymatic and non-enzymatic processes in living organisms has been associated with the oxidation of DNA, lipids and proteins (see Section 1.3.3.2). Antioxidants, which assist cells to cope with oxidative stress by effectively quenching free radicals, have been linked to disease prevention (Gey, 1993). A broad definition of an antioxidant is "any substance that when present at low concentration compared to those of an oxidised substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1989). L-ascorbic acid, reduced glutathione, uric acids and enzymes such as glutathione peroxidase and superoxide dismutase are examples of antioxidants which protect living organisms from oxidative damage.

A number of methods to measure total antioxidant activity (TAA) in biological fluids such as plasma and serum have been reported over the last decade, reflecting the increasing interest in this area. The majority of these assays are based on the inhibition of accumulation of oxidised products and utilise fluorescent or chemiluminescent end points. The generation of free radical

species is inhibited by the addition of antioxidants, which reduces the end point

by scavenging the free radical. The radical that is generated, the end point that is

used, and the reproducibility of the generating process vary. These methods were

extensively reviewed by Miller and Rice-Evans (1996). The most widely used

TAA assay has been the TRAP assay of Wayner *et al* (1985) and its subsequent

developments (Metsa-Ketela, 1991). Briefly, peroxyl radicals are produced at a

constant rate by thermal decomposition of 2,2'-azobis-(2-amidinopropane) in the

aqueous phase. Peroxyl radical reactions are monitored by luminol-enhanced

chemiluminescence. The time for which the added test sample of plasma

extinguishes the reaction is directly proportional to the peroxyl radical-trapping

antioxidant capacity of the sample i.e. TRAP which is expressed as micromoles

of peroxyl radicals trapped by one litre of the sample. A further modification has

been the introduction of an assay for antioxidants in the lipid phase using 2,2'-

azobis-(2,4-dimethylvaleronitrile) as the source of peroxyl radicals (Metsa-Ketela

and Kirkkola, 1992). Another enhanced chemiluminescent reaction involving

horseradish peroxidase and luminol to detect antioxidants and measure

antioxidant capacity was reported by Whitehead *et al* (1992).

6.1.1 Spectrophotometric determination of TAA by inhibition of the radical cation of ABTS$^{\bullet+}$ formation

The principle behind the ABTS$^{\bullet+}$ assay system (Miller *et al*, 1993; Rice-Evans

and Miller, 1994) is the formation of the ferrylmyoglobin radical from

metmyoglobin and H_2O_2 in the presence of the peroxidase ABTS (λ_{max} 342 nm)

to produce the ABTS radical cation (ABTS$^{\bullet+}$), a blue/green chromogen with

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characteristic absorption maxima at 645, 734, and 815 nm, as well as the well-recognised maximum at 417 nm (Figure 6.1). The formation of this coloured radical cation can be suppressed by hydrogen-donating antioxidants. The extent of the suppression can be directly related to the antioxidant capacity (activity) of the sample being investigated. There are several strategies for studying the antioxidant activity of compounds using this method. These strategies are:

a) a decolourisation assay, in which the reaction between ABTS and H_2O_2 is allowed to proceed until a mixture with a stable colour is produced by the generation of a stable $ABTS^{\bullet+}$ radical. An aliquot of the antioxidant-containing sample is added and the colour lost or the colour remaining can be used as an index of TAA;

b) an inhibition assay, measuring the parameters at a fixed point in time; ABTS, MetMb and the sample are mixed and the reaction is initiated by adding H_2O_2. After a fixed time, the absorbance is read, along with a buffer blank which will have a higher absorbance value than a solution containing antioxidant(s). The percentage inhibition is determined by comparison with the blank assay (Section 2.4.5.2);

c) an inhibition assay in which the reaction rates are measured. All the reagents are added together, and the reaction is started by addition of H_2O_2. Comparisons are made using the reaction rates rather than absorbance at a fixed time point; and

d) lag time measurement. All the reagents are mixed together at time zero and the time taken for a colour to develop in steady state is monitored. The length

of the lag time before reaching the steady state in the reaction is proportional to the concentration of antioxidant in the sample.

The strategy followed in the development of the assay protocol by Miller and Rice-Evans (1994) was outlined in b) above and Figure 6.2. This procedure (automated or manual) has been applied to body fluids, physiological antioxidant compounds and radical-scavenging drugs, and an antioxidant ranking was established based on their reactivity relative to a 1.0 mmol/l Trolox standard. Assay conditions (75 μM H_2O_2 and incubation time of 6 min) in the original method (Miller *et al*, 1993) allow the slowest reaction rate to take place and is most suitable for TEAC determination of pure substances. The plasma protocol (375 μM H_2O_2 and incubation time of 200 s) is more suitable for use with complex mixtures which may have endogenous peroxidase activity (Miller and Rice-Evans, 1996). The manual procedure was used in this study to investigate the total plasma antioxidant activity of the young and old healthy volunteers and Alzheimer's patients. The TAA of known antioxidants were also compared with the Trolox standard.

ABTS

-e⁻

ABTS radical cation

-e⁻
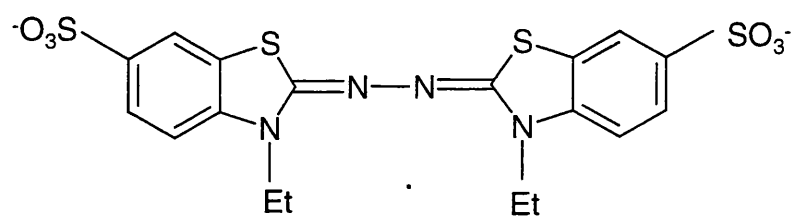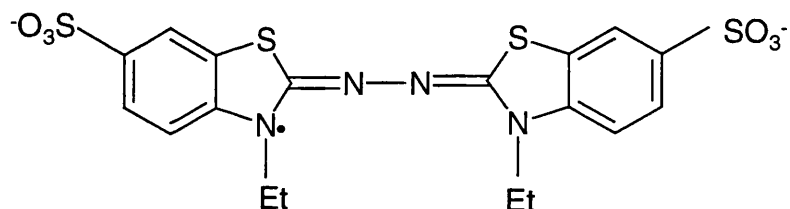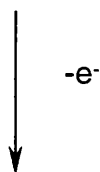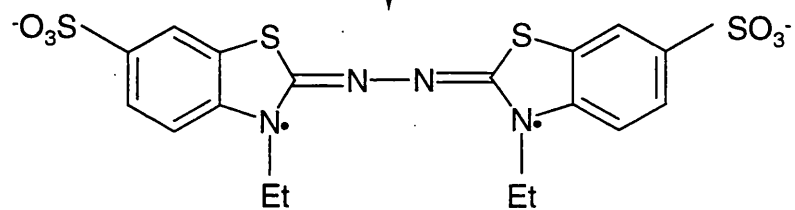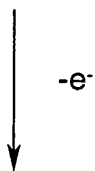
ABTS radical dication

Figure 6.1 ABTS (λ_{max} 342 nm) and its radical cation (λ_{max} 417 nm, peaks at 645, 734 and 815 nm) and dication derivatives (λ_{max} 513 nm) (Miller and Rice-Evans, 1996).

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$$HX - Fe^{III} + H_2O_2$$

MetMb

$$^{\bullet}X - [Fe^{IV} = O] \qquad ABTS$$

FerrylMb

$$ABTS^{\bullet +} + HX - Fe^{III}$$

Blue/green
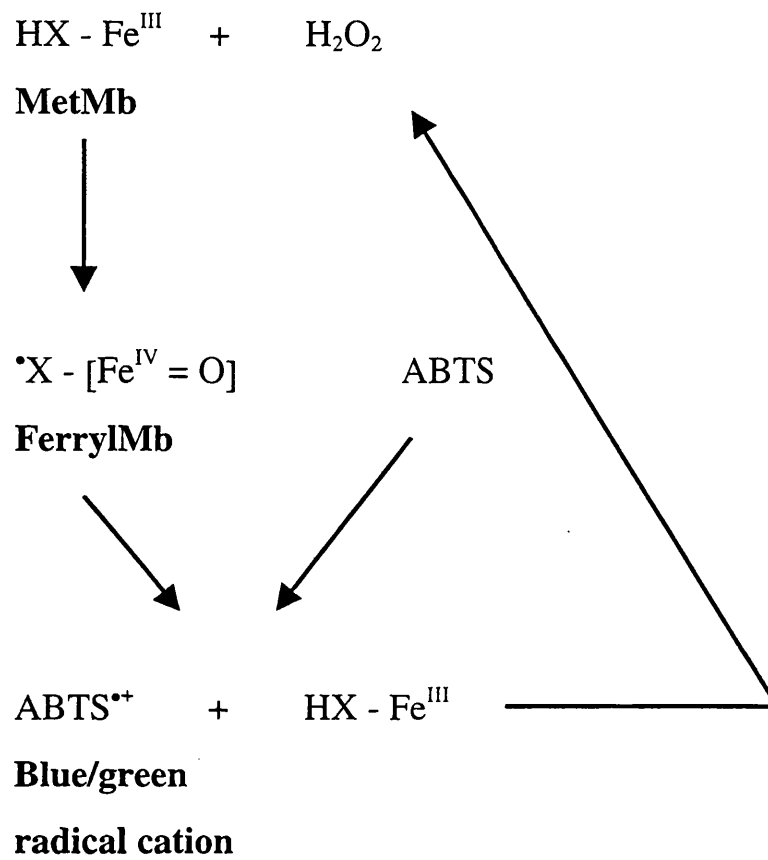
radical cation

Figure 6.2 Formation of the $ABTS^{\bullet +}$ radical cation from activated myoglobin

(Rice-Evans and Miller, 1994).

6.2 Results

The derivation of a TEAC value allows investigation of the effect of age, AD and gender on the total antioxidant capacity of human plasma collected from the three groups of volunteers.

6.2.1 Standardisation of the assay

The system was standardised using Trolox, an α-tocopherol analogue with enhanced water solubility. The dose-response curve for percentage inhibition of $A_{734\,nm}$ versus Trolox concentration was highly reproducible under the conditions described in Section 2.4.5.1. Two types of dose-response curve were constructed for this assay, with the incubation period being 200 s and 6 min, respectively. The results for dose-response curves derived from three sets of separately prepared stock standards allowed study of the intra-day variation (i.e. all three curves constructed in one day) and the inter-day variation (i.e. three curves constructed on three separate days) in system standardisation. These results are summarised in Figure 6.3 and 6.4. Regression lines were fitted and a linear relationship existed in each dose response curve (adjusted R^2 = 95.8-98.5 %).
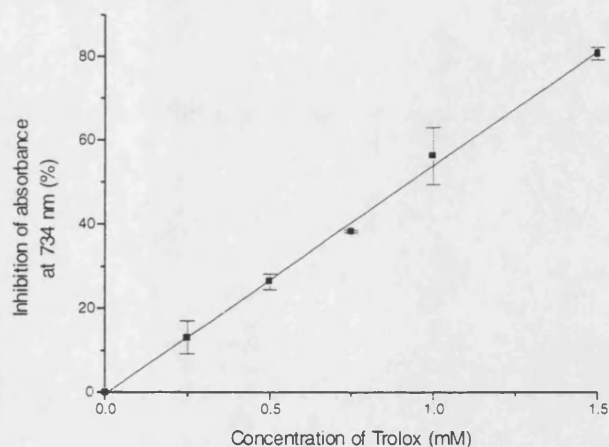
A. Intra-day
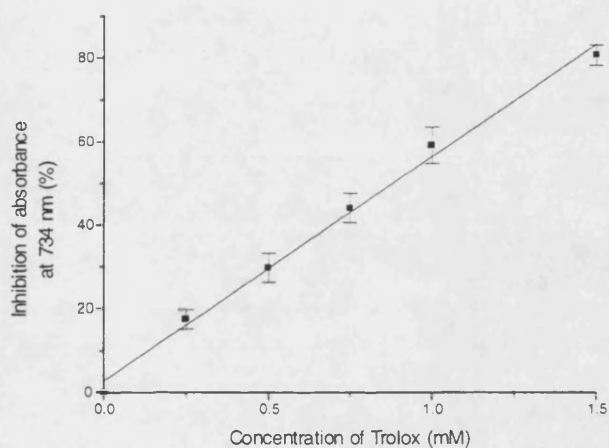
B. Inter-day

Figure 6.3 Intra-day (A) and Inter-day (B) dose-response curve (t = 200 s) for the determination of TEAC values of blood plasma samples. Percentage of inhibition of the absorbance at 734 nm was expressed as a function of the concentration of Trolox. A: Intra-day curve was derived from three separate calibration curves obtained within one day, R^2 (adj) = 98.4 %; B: Inter-day curve was derived from three separate calibration curves obtained on three separate days, R^2 (adj) = 98.5 %. The incubation period was 200 s. Mean ± SD shown (n = 3).
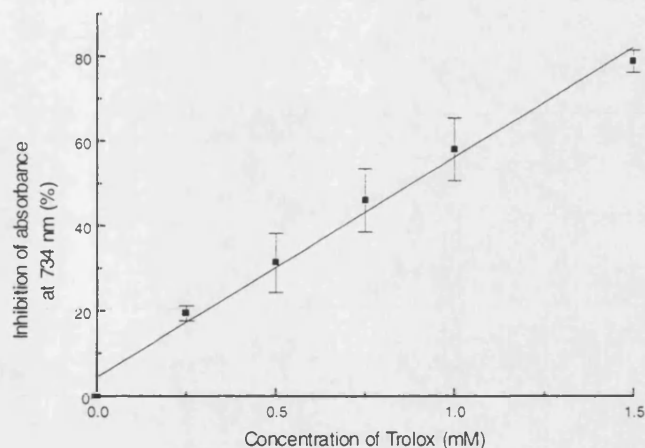
A. Intra-day
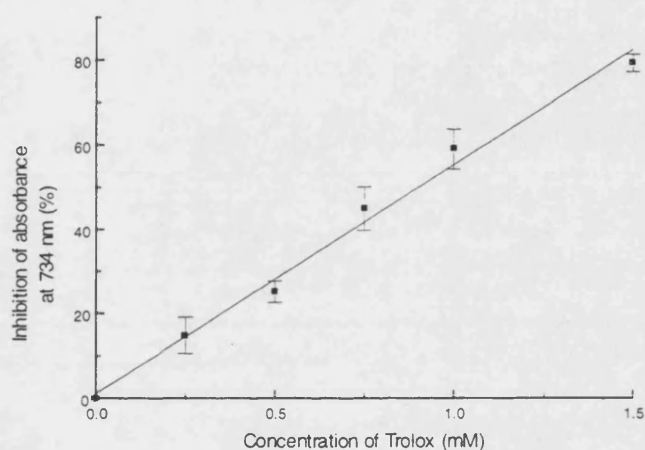
B. Inter-day

Figure 6.4 Intra-day (A) and Inter-day (B) dose-response curve (t = 6 min)
for the determination of TEAC values of pure antioxidant substances.
Percentage of inhibition of the absorbance at 734 nm was expressed as a function
of the concentration of Trolox. A: Intra-day curve was derived from three
separate calibration curves obtained within one day, R^2 (adj) = 95.8 %; B: Inter-
day curve was derived from three separate calibration curves obtained on three
separate days, R^2 (adj) = 97.7 %. The incubation period was 6 min. Mean ± SD
shown (n = 3).

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6.2.2 Experimental variability

Experimental variability was studied and the results are summarised in Table 6.1.

Inter-subject variability showed a CV of 19.2 % (n = 25) for the young, 15.6 % (n = 25) for the old and 54.1 % (n = 25) for the Alzheimer's patients.

Variability	Variability expressed as mean ± standard deviation (range) coefficient of variation (CV) in %
Intra-day (n = 12)	13.7 ± 7.8 (5.5-31.1)
Inter-day (n = 4)	7.0 ± 8.6 (1.4-19.7)
Intra-assay Healthy	
Age < 30 (n = 25)	5.3 ± 3.6 (0.9-15.3)
Age > 60 (n = 25) Alzheimer's	5.8 ± 3.3 (0.0-12.9)
Age > 60 (n = 25)	4.8 ± 3.3 (0.00-10.1)
Intra-subject (n = 5)	26.2 ± 22.2 (9.2-65.0)

Table 6.1 Intra- and inter-day, intra-assay, intra-subject variability in TEAC assay.

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6.2.3 Total antioxidant activity of plasma

The total plasma antioxidant activity of all three groups of volunteers was measured and the findings are summarised in Table 6.2.

Volunteer group	TEAC value (mM)		Sample size
	mean ± SD	Range	(n)
All volunteers	0.84 ± 0.33	0.20-1.92	75
Healthy (age < 30)	0.76 ± 0.15	0.52-1.07	25
Healthy (age > 60)	0.80 ± 0.12	0.60-1.03	25
Alzheimer's (age > 60)	0.96 ± 0.52	0.20-1.92	25

Table 6.2 Total antioxidant activity (TAA) for human plasma samples from three groups of volunteers: healthy young (age < 30) and old (age > 60); and the Alzheimer's group (age > 60). Trolox equivalent antioxidant capacity (TEAC) was used as the unit for TAA.

Non-parametric statistical tests (Section 2.5) were used to compare the results shown in Table 6.2 (data deviated from normality). No significant difference was observed between the total plasma antioxidant status of the three groups of volunteers. Neither age nor AD were revealed to have any significant effect on TEAC values of the plasma population in this study (P = 0.29 and P = 0.62, respectively). Further analysis revealed no significant effect of gender on the

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TEAC values as a whole ($P = 0.23$). However, in the healthy female group, TEAC values were found to be significantly higher in the old than in the young ($P = 0.031$).

6.2.4 TEAC values of pure antioxidant substances

Solutions of antioxidant substances were compared to Trolox solutions by means of this assay, and hence to one another on the basis of molar antioxidant activity. TEAC values of potential antioxidants were derived and the results are shown in Table 6.3. The table shows that the data collected from this study and that from another study were similar (Rice-Evans and Miller, 1994). Table 6.3 also shows that the plasma antioxidants ascorbate, urate and glutathione had similar antioxidant capacity to Trolox. Double distilled deionised water and lithium carbonate solution were both used in preparing the antioxidants and they both had TEAC values of 0.00 indicating that they did not influence the total antioxidant value of the sample.

Substance	Published data*			Data from this study		
	TEAC	n	SD	TEAC	n	SD
L-ascorbic acid	0.99	5	0.04	0.91	6	0.12
Reduced glutathione	0.90	3	0.03	0.94	5	0.12
Uric acid	1.02	5	0.06	1.02	6	0.14

*Rice-Evans and Miller, 1994

Table 6.3 Trolox equivalent antioxidant capacity (TEAC) values of pure antioxidant substances. TEAC is the concentration in millimolar of Trolox having the equivalent antioxidant capacity to a 1.00 mM solution of the substance under investigation.

6.3 Discussion

The derivation of a TEAC value provides a method to compare the antioxidant activity among groups of compounds, provided they are water-soluble or can be solubilised, or of body fluids, such as plasma and serum. Applying this method, the plasma TAA of 75 volunteers was monitored and the effect of age, AD and gender on the TAA status was investigated. Blood plasma was obtained for this study because of its ease of collection and its reflection of the total antioxidant capacity of the human body. According to Miller and Rice-Evans (1996), the principle antioxidants of human plasma are albumin and uric acid which account

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for 57 % of the TAA of most samples. The residual activity is referred to as the "antioxidant gap", which reflects the combined activity of ascorbic acid, α-tocopherol, bilirubin, β-carotene and other plasma antioxidants such as flavonoids. Applying the method devised by Rice-Evans and Miller (1994), ascorbate, urate and glutathione had almost the same molar equivalent antioxidant capacity as Trolox (TEAC value about 1.00). Similar findings were reported by Miller *et al* (1993). They also reported that bilirubin was a more efficient antioxidant (TEAC value: 1.50) and albumin is less active (TEAC value: 0.63) on a mole for mole basis.

The reference interval (95th percentile range) obtained from the 75 volunteers for this study was 0.19-1.49 mM. This value is lower than that quoted by Miller *et al* (1993) in their study of TAA in premature neonates using the same TEAC method. An adult reference interval (95th percentile range) of 1.32-1.60 mM was derived for their method, based on 312 normal human plasma or serum samples (information on subjects not given). This difference maybe accounted for by the difference in the selection criteria of subjects, the sample size of the two studies and the type of protocol used. We used the manual TAA protocol in this study compared with the automated TAA protocol using premixed reagent and the Cobas Bio Centrifugal analyser, carried out by Miller *et al* (1993). The automated method offers the advantages of better control of timing and temperature, the ability to process a large batch of samples at one time, simplicity and speed. However, the manual method is a cheaper alternative and with strict regulation of timing and temperature, can be used for TAA study effectively. In other studies which measure plasma or serum TAA, Wayner *et al* (1985) quoted a

reference interval of 0.81-1.12 mM for human plasma TAA using the TRAP

assay and Whitehead *et al* (1992) quoted a reference interval of 0.75-0.91 mM for

human serum TAA using the technique of enhanced chemiluminescence.

Ageing and the diseases that typically follow with increasing age are often

proposed to be involved in increased oxidative stress. In this study, neither age

nor AD was shown to have a significant effect on human plasma TAA. However,

in the healthy female subgroup, plasma TAA was found to be higher in the old

(age > 60) than in the young (age < 30). Aejmelaeus *et al* (1997) using a

chemiluminescence assay to assess TRAP in human plasma, demonstrated that

TRAP changes with age. In females, TRAP increased significantly during the life

span which is consistent with the findings of our study in TEAC. In males,

TRAP increased until age 51-74, and then significantly decreased. Aejmelaeus *et*

al (1997) suggested that these changes in TRAP in both sexes were mainly due to

the contribution of unidentified antioxidants. Uric acid concentration, determined

by HPLC, was also found to increase significantly with age in the female group,

contributing to 57 % in the increase in TRAP. Previous results indicated that

plasma uric acid concentration is clearly lower in the female than in the male

population until the sixth decade of life (Akizuki, 1982), possibly because either

17-β oestradiol partly regulates purine biosynthesis and uric acid metabolism or

tubular urate postsecretory reabsorption is lower in premenopausal women

(Mateos *et al*, 1986). Uric acid may preserve the ability of vascular endothelium

to mediate dilatation by preventing oxidative inactivation of endothelial cyclo-

oxygenase and angiotensin converting enzyme. Uric acid scavenges

peroxynitrite, the formation of which may be accelerated in ischaemic tissues.

This is why increasing uric acid concentration may be beneficial in postmenopausal women, who have lost the protecting properties of oestradiol, which is proposed to regulate arterial relaxation and prevent oxidative damage of low density lipoprotein (Aejmelaeus *et al*, 1997). It should be stressed that the interpretation of assay values can be made in different ways. High values may indicate an induced response to oxidative stress. On the other hand, low values may indicate lack of antioxidant supply in the diet e.g. ascorbate or from the endogenous sources e.g. urate, thus leaving the individual susceptible to oxidative damage during periods of stress. Alternatively, it may be seen as evidence that the antioxidant reserve has been depleted following oxidative stress. The former can be substantiated by measuring the concentration of an individual antioxidant in plasma. The latter seems to be the case with the acute falls seen following stressful events such as acute inflammatory diseases or burns (Whitehead *et al*, 1992). Oxidative stress has also been implicated in the pathogenesis of AD (Markesbery, 1997). Although the effect of AD on plasma TAA was found to be insignificant in this study, the study carried out by Zaman *et al* (1992) proved otherwise. Plasma concentrations of vitamins A and E and carotenoids were found to be significantly lower in AD patients than those in controls.

Plasma is essentially a mixture of known and unknown antioxidants and this has presented scientists with the challenge to develop an assay which not only determines the overall antioxidant capacity of plasma but also to study the nature of the antioxidant profile. Each existing assay has its own characteristics and therefore none of the assays can give a complete picture of the antioxidant profile of a compound. The TAA assay developed by Rice-Evans and Miller (1994)

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used in this work is widely used, however several pitfalls have to be considered when it is used to determine total scavenging capacity. The time course of radical formation in the presence of the compound, and the length of the lag phase clearly indicate that the antioxidant effects of the compounds are different. Therefore, consideration has to be given to the fact that the assay does not distinguish between a scavenger effect and an inhibitory effect in the rate of ABTS radical formation. This is because the absorbance is measured at a fixed time point regardless of the time course of ABTS formation. This point has also been stressed by Arnao *et al* (1996). To circumvent the effect of the compounds on radical formation, Strube *et al* (1997) used the post-addition assay in conjunction with the method devised by Rice-Evans and Miller (1994), in which the test compound was added after the ABTS radicals had formed. The decrease in absorption caused by the test compound reflected ABTS radical scavenging capacity, not inhibition of radical formation. The decrease in the slope after addition of the test compound indicated that the test compound inhibited radical formation. Strube *et al* (1997) found that Trolox acts only by scavenging ABTS radicals, while another antioxidant, quercetin displays a mix of scavenging and inhibitory effects. The TEAC value of a compound such as quercetin may depend on the time point at which absorbance was measured, due to the effect of the compound on the rate of radical formation. Since plasma contains a mixture of antioxidants, it is likely that it may display a mix of scavenging and inhibitory effects and so the post addition assay described by Strube *et al* (1997) may be useful if discrimination between these two effects is required. It should also be emphasised that the inhibitory and scavenging effects observed in *in vitro* systems such as ABTS-type assays do not necessarily reflect the *in vivo* situation.

Therefore, the physiological significance of the *in vitro* results should be confirmed by *in vivo* studies. In extrapolating the results to an *in vivo* situation, one has to ask a) if the peroxidase process mimics the process of radical formation *in vivo* and b) if the ABTS radicals resemble the radical species involved in an *in vivo* situation. Further studies are required to establish the relevance of these antioxidant measurements *in vivo*. Nevertheless, the ABTS-type assay is a useful tool in studying the effect of age and age-related disease on the total antioxidant status in the human population.

CHAPTER 7

HEME OXYGENASE-1 ASSAY

7.1 Introduction

Oxidants induce expression of the heme oxygenase-1 (HO-1) by enhancing the transcription rate and so altered expression can be monitored at a variety of levels. Direct measurement of altered transcription rates is preferable; however the run-on transcription assay usually used for this measurement is labour-intensive and subject to inter-experimental variation. One-dimensional sodium dodecyl sulphate - polyacrylamide gels are normally sufficiently sensitive to detect induction of *de novo* synthesis of the protein corresponding to HO-1, but background levels are high due to the large number of constitutive proteins in this molecular range (Marini *et al*, 1996). Western blot analysis has been used to study HO-1 activity using antibodies (Maines *et al*, 1988). Induction of HO-1 enzyme activity several hours after the initial treatment is easy to measure by a spectrophotometric assay which requires exogenous sources of biliverdin reductase (Gabis *et al*, 1996). Recently, an assay for HO (HO-1 and HO-2) activity in cultured human cells, using high performance liquid chromatography of biliverdin and bilirubin has been developed by Ryter *et al* (1998). Currently, the method of choice for gene expression analysis is the measurement of the accumulation of HO-1 mRNA (Crawford *et al*, 1994; Tyrrell and Basu-Modak, 1994).

7.1.1 Techniques for measuring mRNA accumulation

An important aspect in the determination of gene expression is measuring mRNA levels of specific genes. Classical techniques to determine mRNA levels include the transcriptional run-on assay and Northern blot analysis (Tyrrell and Basu-Modak, 1994). The transcription run-on assay is used to quantify the amount of primary RNA transcript transcribed from isolated cell nuclei, whereas northern blot hybridisation is used to assess the modulation of mRNA levels in the cell, regardless of whether the changes are due to altered transcription, stability, or transport. Tyrrell and Basu-Modak (1994) have described detailed methodology based on the Northern blot procedure to measure HO-1 mRNA levels and provided an example in which increased accumulation of HO-1 mRNA after oxidative stress by UVA radiation or H_2O_2 was suppressed by the free radical scavenging antioxidant, N-acetylcysteine. Both transcription run-on assay and Northern blot analysis have the limitation of requiring large amounts of total RNA, making their use unfeasible when limiting numbers of cells are available. With the recent advent of Reverse Transcription-Polymerase Chain Reaction (RT-PCR), the sensitivity and specificity for mRNA determination has been increased dramatically, and this technique is becoming widely used in gene expression studies involving small amounts of total RNA (Samar *et al*, 1994; Riedy *et al*, 1995).

7.1.2 PCR and RT-PCR

PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA developed by Mullis *et al* (1986) and Saiki (1989). The first stage of RT-PCR reaction is the RT reaction in which RNA from cells or tissues is reverse transcribed to complementary DNA (cDNA) by a reverse transcriptase. The newly synthesised cDNA is then used as a template for PCR, using primers designed to amplify a selected cDNA region of a specific RNA message in the presence of DNA polymerase(s). The PCR-generated product is then analyzed by agarose gel electrophoresis and typically identified on the basis of its size. The PCR product can further be validated by hybridization, restriction digestion and/or nucleotide sequencing if desired. While Northern blot analysis typically requires several micrograms of total RNA with a detection limit of approximately 10^5-10^7 molecules, RT-PCR can be performed with much lower quantities of total RNA and can detect transcripts representing as little as 6 pg of total RNA. Thus RT-PCR is extremely well suited to detect rare transcripts and transcripts in small localized tissue samples. Another unique aspect of RT-PCR is that it allows for simultaneous analysis of multiple gene transcripts (Saiki, 1989).

7.1.3 Heme oxygenase-1 assay

Because senescence is characterised by a reduced ability of an organism to maintain homeostasis in response to oxidative stress, the purpose of this study was to measure

HO-1 gene expression to determine if ageing or age-related disease affects the ability of human PBMCs to respond to the oxidative stress of H_2O_2 treatment. A competitive one-step "one-tube" RT-PCR assay for the quantification of HO-1 mRNA was developed by Ahmed *et al* (1996). Competitor RNA is an RNA sequence which contains the same primer sequences present in the target transcript and thus competes with the target for those same primers. When a competitor is used, a restriction site or a sequence is added (as in this study) or deleted so that the target sequence can be differentiated from the competitor by electrophoresis due to the difference in the size of the two RNAs (Siebert and Larrick, 1992). Following RT-PCR, the amount of products generated by the competitor and the target are compared and the initial amount of target mRNA deduced. Competitive one-step "one-tube" RT-PCR was used initially for this study but it was later replaced by semi-quantitative RT-PCR due to inconsistencies associated with the competitive technique when amplifying total RNA in preliminary studies. Semi-quantitative RT-PCR uses an ubiquitously-expressed gene such as GAPDH as a control to estimate the relative level of gene expression. The procedures used were similar to that described for competitive RT-PCR but no competitor RNA standard was included and the steps, RT and PCR were separated. The level of expression or PCR signal observed for the test gene is normalised to the PCR signal obtained for the control gene in the same sample (Chelly *et al*, 1990).

7.2 Results

7.2.1 Preparation of competitor and target RNA

Minipreps of plasmid pBsHO-1/L and pBsHO-1 were used to prepare the respective

plasmid DNAs. Since the size difference between plasmid pBsHO-1/L and

pBsHO-1 DNAs was very small (45 bp), their identification by gel agarose

electrophoresis was not possible. A PCR assay using JumpStart *Taq* DNA

Polymerase was therefore carried out to confirm their identities. This PCR assay

utilises *Taq* DNA polymerase and TaqStart antibody and aims at improving "hot

start" PCR. This PCR analysis confirmed the incorporation of the synthetic 45 bp

linker into the plasmid pBsHO-1 to give the plasmid pBsHO-1/L. The amplified

products of pBsHO-1/L and pBsHO-1 DNA were 287 bp and 242 bp, respectively

(Figure 7.1). Plasmid pBsHO-1/L DNA was then linearised with *Hin*d III in

preparation for *in vitro* transcription. Since only one *Hin*d III restriction site exists in

this plasmid, linearisation by the enzyme produced one DNA fragment of 4553 bp as

predicted (Figure 7.2). The linearised DNA template was transcribed *in vitro* and the

presence of RNA (1697 b) was confirmed by gel electrophoresis (Figure 7.3). This

RNA was used as the competitor in the competitive one-step "one-tube" RT-PCR.

To prepare the target RNA, PBMCs from volunteers were treated with H_2O_2 which

has been shown to induce HO-1 mRNA gene expression in other studies (Keyse and

Tyrrell, 1989). The aim was to use a sub-lethal concentration of H_2O_2 that did not

kill the cells. As a starting point, the cells were treated with 100 μM H_2O_2 and the %

cell viability before and after the treatment period; and after the 2-h incubation period always exceeded 95 %. Total RNA was extracted and analysed on a 1 % denaturing agarose gel. rRNA - 28S (5.1 kb) and 18S (1.9 kb) were resolved. As expected, the mRNA ran as a smear between the two rRNA bands (Figure 7.4).
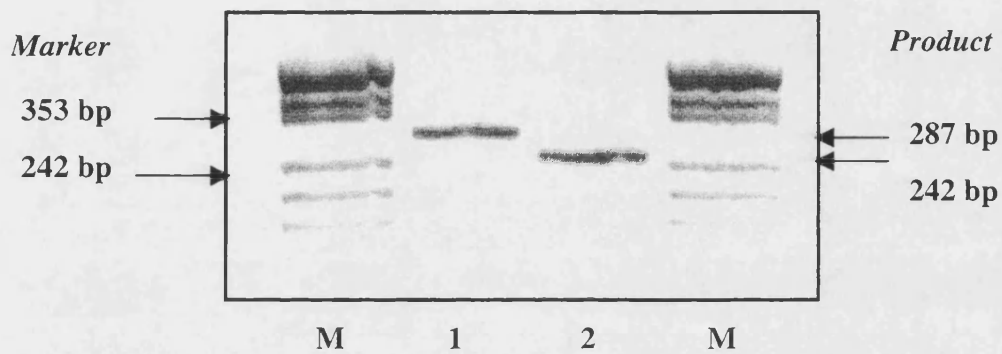
Figure 7.1 Analysis of the PCR amplified products of pBsHO-1/L (lane 1) and pBsHO-1 DNA (lane 2) on a 2 % agarose gel stained with ethidium bromide. M: 1 μg of pUC18 DNA Msp I digest (55 μg/μl) as marker.
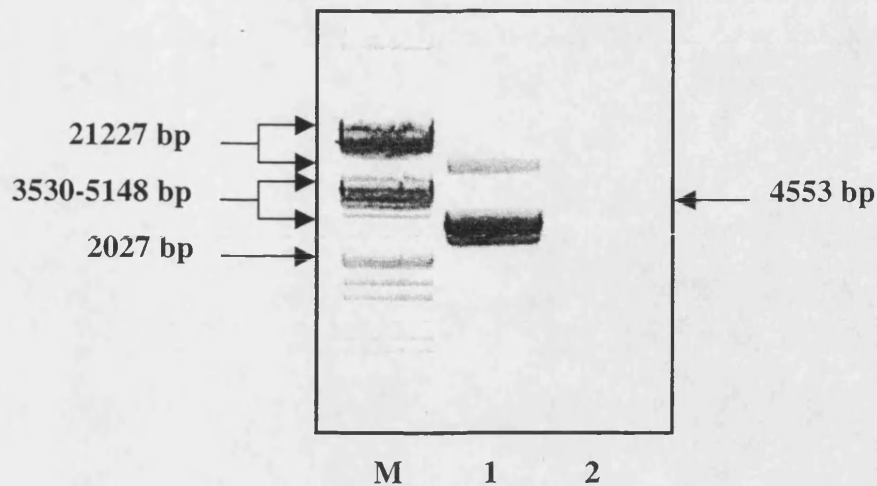
**Figure 7.2 Analysis of the *Hin*d III digestion of pBsHO-1/L DNA on a 1 %
agarose gel stained with ethidium bromide**. M: 1 µg of λDNA/*Eco*R I and *Hin*d
III Marker (0.5 mg/ml); **1:** 1 µg of undigested pBsHO-1/L DNA (circular, linear and
supercoiled DNA); **2:** 1 µg of the *Hin*d III digest product of pBsHO-1/L DNA at
4553 bp.
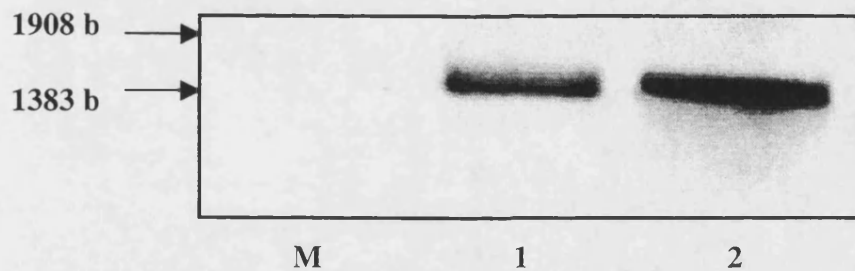
**Figure 7.3 Analysis of the RNA product after *in vitro* transcription of
pBsHO-1/L DNA on a 1 % denaturing gel stained with ethidium bromide.** M: 1
µg of G319 RNA Marker (1 mg/ml); **1:** *In vitro* transcribed product, positive control
RNA at 1584 b **2:** *In vitro* transcribed product, pBsHO-1/L RNA at 1697 b.
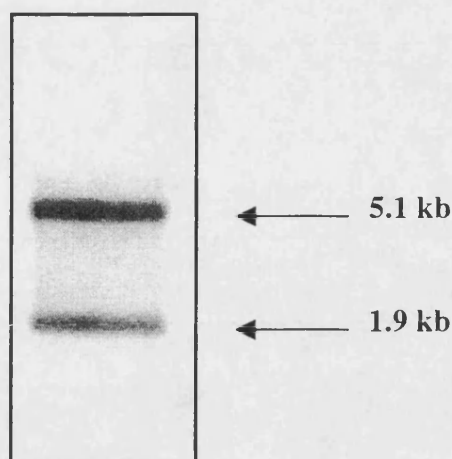
**Figure 7.4 Analysis of total RNA prepared from human PBMCs on a 1 %
denaturing gel stained with ethidium bromide.** A smear (mRNA) with two clear

ribosomal bands (28S at 5.1 kb and 18S at 1.9 kb) as seen after electrophoresis.

7.2.2 Competitive one-step "one-tube" RT-PCR

Separate RT-PCR assays were performed on pBsHO-1/L RNA and total RNA,

allowing various assay parameters to be established for both RNAs before the

competitive RT-PCR assay was carried out. Figure 7.5 shows the amplified products

of an RT-PCR assay (using the Titan One-Tube RT-PCR System and primers P_1 and

P_2) on plasmid pBsHO-1/L (1000 pg) and total RNA (1 µg) in separate tubes. The

amplified products of pBsHO-1/L RNA and total RNA were 287 bp and 242 bp,

respectively. To establish the sensitivity of the assay, five-fold dilutions of the

pBsHO-1/L RNA (130000, 26000, 5200, 1040, 208, 41.6, 8.32 pg) were prepared

and then amplified in separate tubes (Figure 7.6). Preliminary RT-PCR assays

carried out on total RNA prepared from PBMCs were not as successful as that on

pBsHO-1/L RNA and the amplification was weak and inconsistent. This problem

with amplification, combined with limited availability of total RNA, made it difficult

for further optimisation of the assay to be carried out. Nevertheless, a number of

competitive RT-PCR assays have been carried out and generally, co-amplification

was weak, inconsistent and unsatisfactory. Figure 7.7 shows an example of this co-

amplification. Two amplified products (287 bp and 242 bp) were observed on both

lanes but the signals were too weak to be used for reliable quantification.
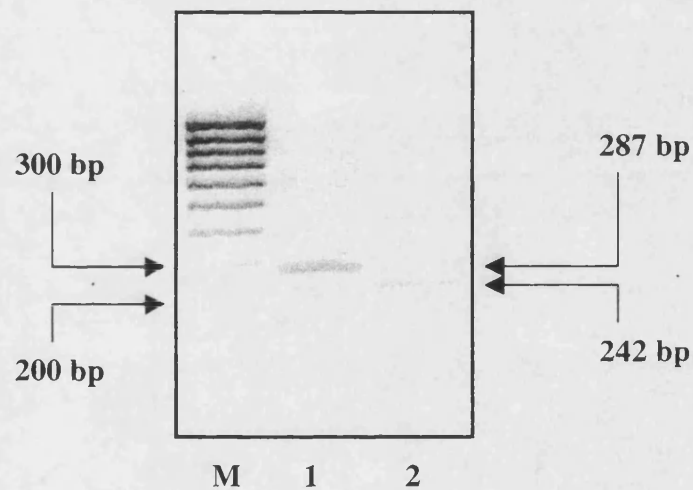
Figure 7.5 Analysis of the amplified products of an RT-PCR assay on pBsHO-1/L RNA (lane 1) and total RNA (lane 2) on a 2 % agarose gel stained with ethidium bromide. M: 1 μg of DNA 100 bp ladder (1 μg/μl).

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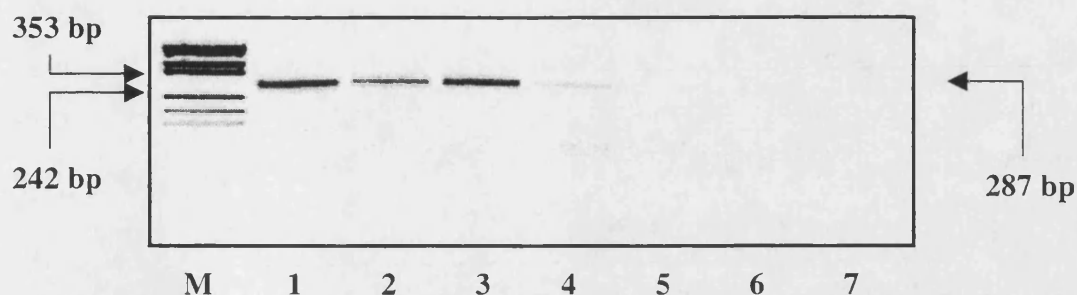
Figure 7.6 Analysis of amplified products of an RT-PCR assay, using different amount of pBsHO-1/L RNA as template on a 2 % agarose gel stained with ethidium bromide. M: 1 μg of pUC18 DNA Msp I digest (55 μg/μl); **1-7:** 15 μl of the amplified products (287 bp) from RT-PCR of pBsHO-1/L RNA. Five-fold dilutions of pBsHO-1/L RNA were used as templates: **(1)**130000 pg; **(2)** 26000 pg; **(3)** 5200 pg; **(4)** 1040 pg; **(5)** 208 pg; **(6)** 41.6 pg; **(7)** 8.32 pg.
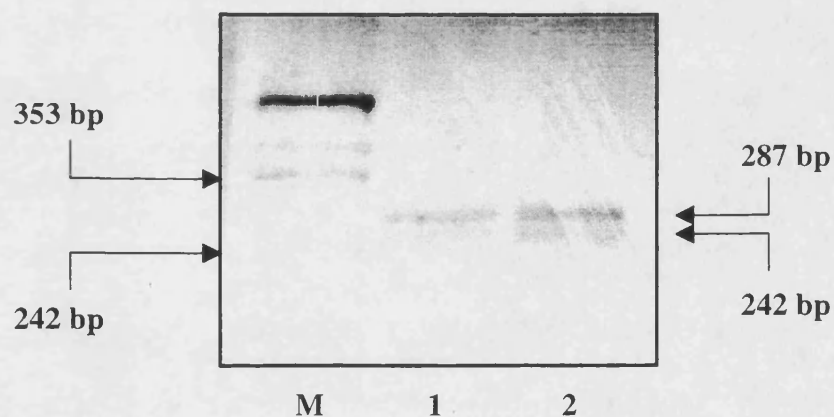
Figure 7.7 Analysis of the co-amplified products of a competitive one-step "one-tube" RT-PCR on pBsHO-1/L RNA and total RNA on a 2 % agarose gel stained with ethidium bromide. M: 1 μg of pUC18 DNA Msp digest (55 μg/μl); **1:** 15 μl of the co-amplified products after competitive RT-PCR of pBsHO-1/L RNA (1000 pg) and total RNA (1 μg), products at 287 bp and 242 bp, respectively; **2:** 15 μl of the co-amplified products after competitive RT-PCR of pBsHO-1/L RNA (200 pg) and total RNA (1 μg), products at 287 bp and 242 bp, respectively.

7.2.3 Optimisation of RT-PCR

Many attempts have been made to optimise the conditions for the HO-1 amplification of total RNA extracted from human PBMCs (e.g. varying primer concentration, the number of thermal cycles and the annealing temperature) but the amplification still failed. Was the template intact? The integrity of this template was confirmed by running an aliquot on a denaturing gel. 28S (5.1 kb) and 18S (1.9 kb) ribosomal bands were resolved and the mRNA ran as a smear between the 2 ribosomal RNA bands. Any degraded RNA generally run below the 18S band but this was not observed throughout the study (Figure 7.4). The integrity of this template was further confirmed by the successful amplification of the GAPDH gene using the G_1 and G_2 primers (product size: 187 bp). The efficiency of the RT-PCR was confirmed by this GAPDH amplification and also by the successful amplification of HO-1 gene in total RNA extracted from human FEK_4 cells (product size: 242 bp). The suitability of primers P_1 and P_2 was also confirmed by this HO-1 amplification of FEK_4 total RNA. Despite numerous attempts to optimise RT-PCR conditions such as the concentration of primers and the number of thermal cycles, HO-1 amplification of PBMCs total RNA was unsuccessful.

7.2.4 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out at a later stage of this study in an attempt to over come the difficulty encountered previously in the original RT-PCR protocol. In this protocol, GAPDH was used as a control to estimate the relative level of gene expression, the RT and PCR steps were carried out separately and the competitor RNA was eliminated. Preliminary studies were carried out on samples donated by 13 healthy volunteers. The amplification signal of total RNA was strong and all H_2O_2-treated PBMCs expressed GAPDH gene but only 4 (2 from each age group) expressed the HO-1 gene. Thus, no densitometric analyses were performed to estimate the level of HO-1 expression. Figure 7.8 shows the gel analysis of the amplified products of semi-quantitative RT-PCR on these 4 samples of total RNA. GADPH and HO-1 gene were successfully amplified in all RNA samples collected at different stages of the experiment in subject 1 (age < 30) and 4 (age > 60). Amplification of RNA extracted from PBMCs after the 30-min H_2O_2 treatment in subject 2 and amplification of RNA extracted from PBMCs after the 2-h post-treatment incubation period in subject 3 were both unsuccessful. Amplified products by GAPDH primers and HO-1 primers were 187 bp and 242 bp, respectively. Negative controls as described in Section 2.4.14 were included in all experiments.
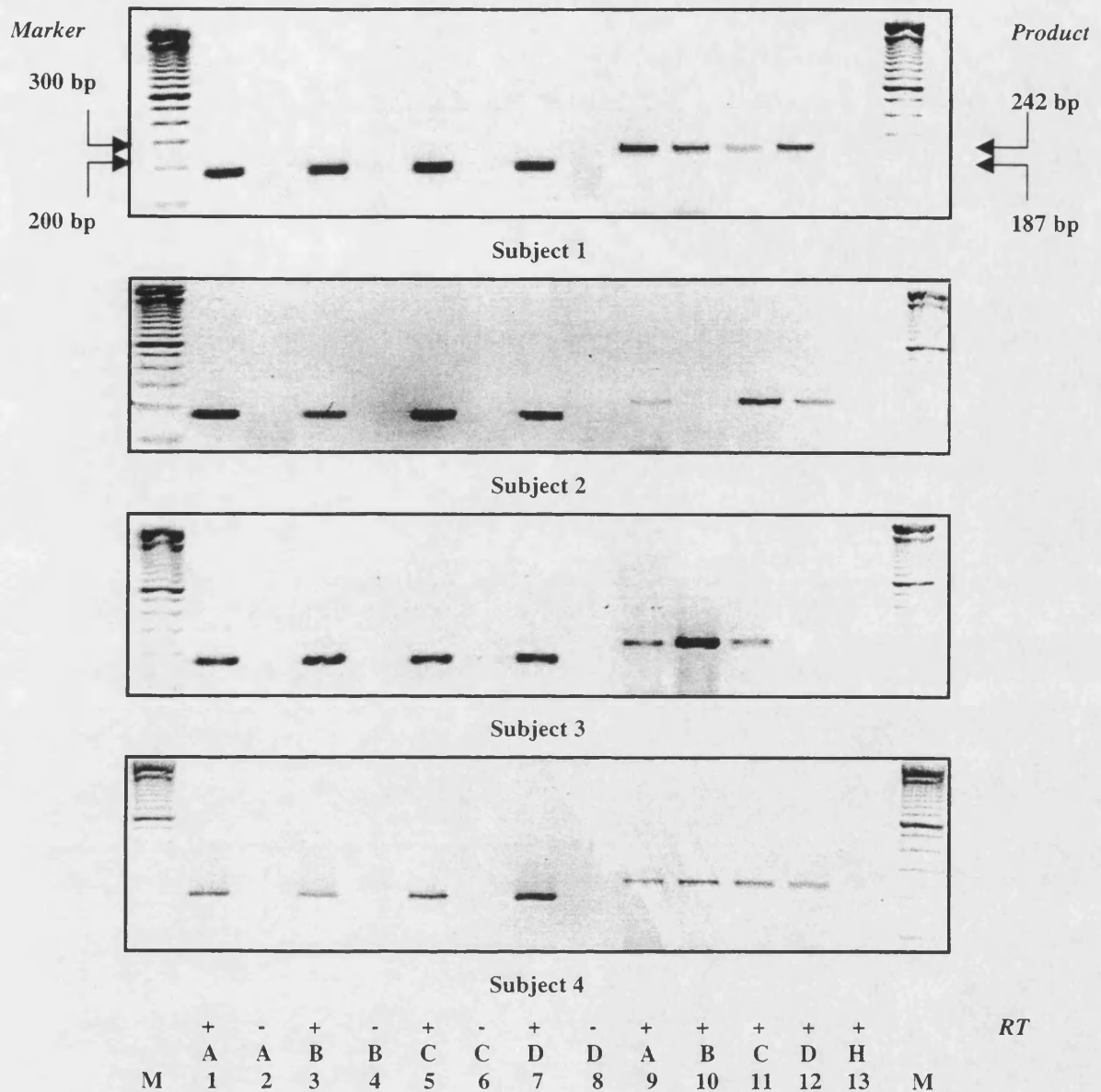
Figure 7.8 Analysis of the amplified products of semi-quantitative RT-PCR on 4 samples of total RNA (Subjects 1-4) on a 2 % agarose gel stained with ethidium bromide. All 4 sets of RNA samples collected at different stages of the experiment were either amplified with GAPDH primers (lane 1-8, 13) or HO-1 primers (lane 9-12) giving products at 187 bp and 242 bp, respectively. Negative controls included no RT step (lane 2,4,6,8) and no RNA template (lane 13). Each set of amplified products (15 µl) was loaded for lane 1-13. **M:** 1 µg of DNA 100 bp ladder (1 µg/µl); **A:** Untreated RNA, control for B; **B:** RNA extracted after 30-min H_2O_2 treatment; **C:** Untreated RNA, control for D; **D:** RNA extracted after 2-h post-treatment incubation; **H:** NF H_2O.

7.3 Discussion

Expression of the HO-1 gene is a marker of oxidative stress in mammalian cells, including that caused by UVA radiation or H_2O_2 (Keyse and Tyrrell, 1989; Applegate *et al*, 1991). It can be postulated that induction of HO-1 gene expression increases with oxidative stress and decreases with age and age-related disease, possibly due to an age-associated dysfunction in the signalling mechanism of the oxidative stress response (Section 1.3.3.3.1). In this study, RT-PCR methods were chosen to study the expression of the HO-1 gene in H_2O_2-treated human PBMCs of volunteers from different age groups. Initially, the competitive one-step RT-PCR assay for quantification of HO-1 developed by Ahmed *et al* (1996) was used. Despite numerous attempts to optimise the RT-PCR conditions, amplification of total RNA extracted from PBMCs remained weak and inconsistent. Semi-quantitative two-step RT-PCR was used later. Improved amplification was observed but the total RNA amplification remained inconsistent. So, what may be the reasons behind this difficulty in amplification?

7.3.1. Factors affecting RT-PCR performance

RNA degradation may affect the performance of RT-PCR but this was unlikely to be the source of inconsistencies in this study. PBMCs were lysed and homogenised in the presence of a highly denaturing guanidinium isothiocyanate-containing buffer which immediately inactivated RNases to ensure isolation of intact RNA. Total

RNA was further protected from degradation by the inclusion of an RNAase inhibitor in the RT-PCR. Its integrity was confirmed by the successful amplification of the GAPDH gene and the absence of degraded RNA below the 18S rRNA band after resolution on a denaturing gel. The purity of the template is essential for successful amplification. Total RNA preparations with A_{260}/A_{280} ratios of 1.8-2.0 were obtained throughout the study suggesting they were essentially free of protein contamination. RNA-isolation methods involving the use of phenol and chloroform, which can inhibit RT-PCR, were replaced by the RNeasy procedure in this study in which total RNA was isolated using membrane and microspin technology. To remove genomic DNA contamination, DNase digestion of the purified RNA with RNase-free DNase was carried out before the semi-quantitative RT-PCR.

Poor design of primers may account for low PCR performance but the suitability of HO-1 primers (P_1 and P_2) was confirmed by the successful amplification of HO-1 gene using FEK_4 total RNA. P_1 and P_2 sequences were checked repeatedly to ensure the accuracy of amplification. The potential of the formation of internal secondary structure and primer-dimers in P_1 and P_2 were minimised by avoiding sequences that were complementary within each primer or between the two primers. P_1 and P_2 primers were both 24 bases long and the G-C content were 58.3 % in both primers. These parameters were within recommendations (PCR Protocols and Reference Guide, Promega, September 1996) and these primers should permit the use of an annealing temperature high enough to enhance reaction specificity but low enough to enable hybridisation between primer and template.

Low RT-PCR efficiency may also account for the weak amplification signal although the success in the amplification of HO-1 gene in FEK$_4$ cells and GAPDH gene in PBMCs demonstrated efficient RT-PCR. Inconsistencies occurring in RT-PCR amplification may be reflected by the observation that the basal and induced level of HO-1 gene expression varies in human cells cultured from different tissues (Applegate *et al*, 1991). The abundance of basal and induced HO-1 mRNA may be too low in PBMCs (mainly lymphocytes and few monocytes) to be detected. This view is further supported by Niess *et al* (1998) in their investigation of the effect of heavy endurance exercise on the expression of HO-1 in leukocytes (PBMCs and granulocytes). They reported an almost undetectable baseline expression of

HO-1 in the lymphocytes of the trained subjects. Expression of HO-1 was induced by exercise in most of the lymphocytes, but the amount of HO-1 expressed was less compared to monocytes and granulocytes. Usually, 1 µg of cytoplasmic RNA is sufficient for amplification of rare mRNA sequences (1 - 10 copies per cell) (Kawasaki, 1990). Better amplification may occur with a higher concentration of RNA template in some cases but this method will become unfeasible since the number of PBMCs available is limited and the use of RT-PCR as a sensitive tool of detecting gene expression will be questioned. The HO-1 mRNA level in PBMCs may also vary between individuals, giving rise to inconsistent RT-PCR amplification across the populations.

RT-PCR has been widely used to study gene expression. Whilst it has many advantages over RNA blot methods, it can be difficult to obtain quantitative information. This is due to the exponential nature of the PCR amplification where small variations in amplification efficiency result in dramatic changes in product yields. Small differences in any of the factors that influence the reaction efficiency will dramatically affect the level of specific PCR products. Even when these parameters are controlled precisely, there are sometimes unexpected sample-to-sample and day-to-day variations. In addition, the amount of product generated plateaus during later stages of the reaction due to consumption of necessary components and generation of inhibitors. These characteristics of PCR can obscure differences in the initial amounts of target sequences during the course of amplification. However, these problems can be overcome by the inclusion of internal controls in the PCR (Zamorano *et al*, 1996). The advantage of the use of RNA competitors (Section 7.1.3) in competitive RT-PCR is that the difference in the efficiency of amplification between the target and competitor sequences is minimal since the same primers are used. However, RNA competitors are not infallible. RNA is susceptible to nuclease-induced degradation. Unlike total RNA extracted from PBMCs, the competitor RNA used in this study was amplified consistently. However, the co-amplification of total RNA and competitor RNA resulted in no amplification observed for the former with its signal either too low to be detected or overwhelmed by that generated from the latter.

As an alternative to competitive RT-PCR, the constitutively expressed "housekeeping" gene GAPDH was used as an internal control in the semi-quantitative RT-PCR. This gene is usually expressed at moderate levels and is easy to detect. For many experimental designs, the expression level for this gene will remain constant across the experimental samples. However, expression of GAPDH can often be affected by experimental treatments, stage of development and cell type (Spanakis *et al*, 1993). Also, due to the high abundance of this gene, its amplified products accumulate at earlier cycles than those of the target sequences, which creates difficulties when one is working under the limitation of having to set the same number of cycles for both products. In addition, primers containing different sequences were used, potentially leading to different efficiencies. Many researchers carried out co-amplification of a control gene such as β-actin and GAPDH to correct for variation in tube-to-tube amplification efficiency (Mullis *et al*, 1994). However, in view of the difficulties encountered with co-amplification in competitive RT-PCR, GAPDH was not co-amplified with the HO-1 gene in the same tube during this study, in an attempt to obtain better amplification signals.

Despite numerous attempts to optimise the conditions of RT-PCR and a change of approach from competitive to semi-quantitative RT-PCR, the amplification of total RNA from PBMCs remained unsuccessful. Better amplification of total RNA was observed when RT and PCR were performed separately in different tubes (semi-quantitative RT-PCR) in contrast to competitive RT-PCR when the two steps were performed in a single tube in the same buffer. This difference in amplification

performance may be due to a difference in RT efficiency in the two systems.

Overall, amplification signals obtained in this study were not strong enough to give a

reliable estimation of basal and induced HO-1 gene expression. Further work will be

required to assess the sources of inconsistencies of RT-PCR amplification. Further

optimisation of RT-PCR conditions such as magnesium and dNTPs concentrations

should be carried out. Primers can be redesigned manually or by using commercially

available computer program. It may be necessary to take a different approach to

measure HO-1 gene expression at the mRNA level by using poly $(A)^+$ RNA as the

template. The use of poly $(A)^+$ RNA in downstream applications offers a variety of

advantages. For cDNA synthesis and RT-PCR, the use of poly $(A)^+$ RNA ensures

lower mRNA-independent synthesis. However, the selection of poly $(A)^+$ RNA from

very small amounts of total RNA can be technically challenging. The polyadenylytic

acid tail that is added to the 3' end of the RNA during nuclear processing can be used

to isolate poly $(A)^+$ mRNA from a complex mixture of the cellular RNA and

contaminating DNA. This can be done by annealing this sequence to poly(dT)

affixed to a support and capturing the tethered RNA. It may also be necessary to use

a different technique to measure HO-1 gene expression at the mRNA level, for

example, the northern blotting technique described by Tyrrell *et al* (1994).

Alternatively, HO-1 gene expression can be assessed at the protein level using

sodium dodecyl sulphate-polyacrylamide gel electrophoresis as described by Marini

et al (1996) in their study of the induction of stress proteins in human lymphocytes.

They reported the induction of a set of eight proteins in peripheral blood

lymphocytes by exposure to heat, H_2O_2 and HO-1 was found to be up-regulated by

oxidative stress. As discussed before, the level of induction of HO-1 gene expression varies in human cells cultured from different tissues and therefore it may be necessary to consider using a different human cell type that may express HO-1 gene to a detectable level in RT-PCR. Alternatively, cultured human lymphocytes may be used to provide a constant supply of total RNA for PCR optimisation and experimentation. However, the nature of HO-1 gene expression may be altered in cultured cells. Also, sodium arsenite may be used as an alternative to H_2O_2 as the source of oxidative stress in this study. Both sodium arsenite and H_2O_2 were found to induce HO-1 mRNA accumulation in human lymphoblastoid cell lines but the former stimulated the accumulation to a greater extent (Applegate *et al*, 1991). Sodium arsenite interacts with or modifies cellular glutathione levels and the intracellular redox level strongly influences the magnitude of the inducible response. These observations strongly support the hypothesis that induction of the enzyme is a general response to oxidant stress in mammalian cells and are consistent with the possibly that glutathione plays a key role (Chapter 5). The study of the induction of HO-1 gene expression in human PBMCs is at an early stage of development and more effort will be required to provide a better understanding of the techniques and the mechanisms involved.

7.3.2 Heme oxygenase-1 induction and its relationship with age and Alzheimer's disease

As discussed in Section 1.3.3.3.1, HO is the rate-limiting enzyme in heme catabolism. Two isoenzymes of HO exist: HO-1 and HO-2; the former is inducible by various agents, a variety of circumstances such as heat shock and other forms of cellular stress and the latter is constitutively expressed. HO-1 activity is increased in whole animal tissues following treatment with its natural substrate heme, as well as various metals, xenobiotics and endocrine factors. Many cells in culture, including hemopoietic, hepatic, epithelial, endothelial cells, retinal pigmented epithelial cells and fibroblasts, respond to these agents in a similar manner i.e. by a marked increase in HO-1 activity (Basu-Modak and Tyrrell, 1993; Kutty *et al*, 1995; Gabis *et al*, 1996). Furthermore, HO-1 is a heat shock protein (HSP) induced by elevated temperature and also a stress protein induced by oxidative damage (Shibahara *et al*, 1987; Keyse and Tyrrell, 1989). It is therefore possible that the induction of HO-1 may be an essential event for some types of acute reactions and for cellular protection following injury or long term oxidative stress experienced in the process of ageing. This hypothesis implies that induction of HO-1 enables the removal of the potentially toxic molecule, heme, a lipid soluble, transmissible form of iron, as well as the generation of bilirubin and biliverdin, metabolites with antioxidant properties (Stocker *et al*, 1987). Also, the increased iron concentration produced by HO-1 activity is believed to be the cause for the increased expression of ferritin and ferritin synthesis, which serves to sequester iron, a potent oxidant for cells (Vile and Tyrrell,

1993; Tyrrell, 1997). Finally CO, a by-product of heme degradation, may mimic NO and thus also serves as an important modulator of endothelial cell function following haemorrhagic injury. CO is a powerful vasodilator; it may therefore, together with NO, counteract the vasoconstrictor properties of hemoglobin and heme (Verma *et al*, 1993). The induction of HO-1 is extremely rapid - indeed Lutton *et al* (1992) have shown that heme causes an immediate increase in HO-1 in as little as 5 min, producing a 50-fold increase in HO-1 in hepatocarcinoma cell line. Activation of the HO-1 gene by heme resulted in an increase in RNA polymerase II, as evidenced by its inhibition by α-amantitin. The rapid enhancement of HO-1 activation was not prevented by inhibitors of protein synthesis and this suggests that transcription regulation plays an important role in HO-1 gene. Lavrovsky *et al* (1994) have identified two regulatory elements in the HO-1 promoter, NF-κB and AP-2. They are important transcriptional factors mediating the role of HO-1 in the response to oxidative stress/injury and growth-differentiation processes. NF-κB can be activated in many different cell types following a challenge with primary (e.g. viruses) or secondary pathogenic stimuli (e.g. inflammatory cytokines). The activated factor then leads to a rapid induction of genes encoding defence and signalling proteins, implicating NF-κB as an immediate early mediator of immune and inflammatory responses. AP-2 exerts a crucial function in mediating the regulation of gene expression in response to a number of different signal transductions (Abraham *et al*, 1996). One of the mechanisms by which hormones, growth factors and other stimuli induce gene expression is by activating various transcription factors. This is a rapid process which frequently involves transcriptional or structural activation of the

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factor(s) and allows its presence in or transfer to the nucleus. These processes may be part of the mechanism by which various agents, including heme, increase HO-1 expression and activity. Involvement of cellular regulators such as DNA-binding elements has also been implicated in the regulation of HO-1 (Abraham *et al*, 1996).

A change in HO levels has been observed with age and in a wide range of pathophysiological conditions (Maines, 1988; Abraham *et al*, 1996). To study the possible effect of ageing on HO-1 induction, HO is differentially expressed during the development of mouse germ cells (Kurata *et al*, 1993). HO mRNA accumulates in the spermatogonia and this accumulation is maintained through meiosis to the spermatocyte stage, mainly due to high transcription rate of the HO-1 gene during the spermatogonia stage. HO protein is expressed maximally at the spermatocyte stage and then both protein and mRNA levels rapidly decline until no longer detectable in the spermatozoa. The study by Lin *et al* (1989) revealed that HO mRNA levels are high in foetal liver during prenatal maturation and reach a maximum 24-h after birth. Thereafter, the levels declined but remained above adult levels throughout the course of study. A lower expression of HO-1 observed in older models suggests a possible age-related dysfunction in the induced response of HO-1. Alternatively, it may be due to a lower pro-oxidant state of the cells reflecting an adaptation mechanism to long-term oxidative stress, a view supported by Niess *et al* (1998) who observed a down-regulation of the baseline expression of HO-1 in atheletes who are constantly exposed to oxidative stress. Very few age-related studies have been carried out on HO-1 gene expression and the results are

fragmentary and inconclusive. Similarly, as reviewed by Thakur *et al* (1993), studies measuring expression of other gene such as c-fos, elastin and hsp70 yield inconclusive results. An interesting gene whose age-related expression has been extensively studied in fibroblast culture is a special group of heat shock genes (hsps). Similar to HO-1, these genes and their encoded proteins (HSPs) are induced by living cells in response to stress generated by heat shock or chemical treatment. The magnitude of this response varies with age. Fargoli *et al* (1990) have examined the induction of expression of hsp70 gene by elevated temperature in fibroblast culture obtained from the lung or skin of young and old male rats. The magnitude of induction of hsp mRNA and HSP protein is lower in confluent primary lung and skin fibroblast cultures derived from aged animals. As mentioned above, very few age-related HO-1 gene expression studies have been carried out but the induction of

HO-1 as a defence against oxidative stress such as γ-irradiation and H_2O_2 exposure has been demonstrated in many studies (Abraham *et al*, 1996). Since oxidative stress plays an important role in ageing, this observation may lead to the hypothesis that HO-1 induction may vary with age. Furthermore, findings from Premkumar *et al* (1995) suggest a specific association between HO-1 and the neurofibrillary pathology of AD. An increased expression of HO-1 transcripts was found in the cerebral cortex and cerebral vessels from subjects with AD compared with age-matched non-AD controls. Schipper *et al* (1995) have shown that HO-1 is over-expressed in neurons and astrocytes of Alzheimer-diseased human temporal cortex and hippocampus relative to age-matched, non-demented controls. In any gene expression studies, an increase in the induction of gene or protein can be a response

to an acute or chronic oxidative stress or a result of an impairment of the defence mechanism. Further research is therefore required to understand various aspects of the expression of genes and its regulation during different stages of the life span for unravelling the basic mechanism of ageing and age-related disease such as AD.

CHAPTER EIGHT

CONCLUDING DISCUSSIONS AND FUTURE WORK

8.1 Concluding discussions

The ageing process is multifactorial in nature and as yet many of the contributing mechanisms have not been identified. Several theories of the ageing process have been proposed that attribute ageing to, amongst other mechanisms, somatic mutations and free radicals (Medvedev, 1990). The free radical theory of ageing, first proposed by Harman (1956), suggested free radicals were responsible for the ageing and death of all living things (Harman, 1992). Many endogenous reactions, including the reduction of oxygen to water and the respiratory burst of phagocytes, may lead to the production of free radicals. Exogenous sources of free radicals include ionising radiation and cigarette smoke. Free radicals can damage cellular macromolecules such as DNA and lipids with consequent disruption of cellular function. Cellular defence systems such as antioxidants and stress-induced enzymes exist to counteract such damage but these mechanisms are not perfect and mutations may still occur. It has been suggested that an accumulation of somatic cell. mutations, on their own or in combination with other age-related changes, may play a causative role in ageing and the development of age-related pathologies such as AD. An understanding of the extent of oxidative damage and the defence

mechanisms in the ageing human population is not only of immense scientific interest, it is also important in opening new avenues to age-related diseases.

This investigation is a preliminary study of oxidative-damage-related parameters and antioxidant defence in human blood as a function of age and AD. Basal and oxidant-induced DNA damage were studied in PBMCs of the three volunteer groups using the comet assay. This study and previous studies have demonstrated that DNA damage increased with increasing concentration of oxidant but conflicting results were obtained when the effect of age was investigated. Singh *et al* (1990) and Betti *et al* (1995) both reported a non-significant relationship of DNA damage with age but Piperakis *et al* (1998) observed a general increase of DNA damage with age. The findings from the present study contradicted that from Piperakis *et al* (1998) and a higher level of DNA damage was found in the H_2O_2-induced PBMCs of the healthy younger volunteers than in the healthy older group. Moreover, AD was shown to be a non-significant factor in oxidative DNA damage in PBMCs. At first glance, these results seem to contradict the hypothesis that DNA damage increases with age as proposed by the free radical theory of ageing and that free radicals may be involved in AD (Volicer *et al*, 1990). However, a high DNA repair capacity to maintain genomic stability and an increased resistance to oxidative stress found in the aged population may explain these results (Franceschi *et al*, 1995), highlighting the ability of human body to adapt to changes throughout its lifetime and the complexity of the mechanisms involved in the defence system. Following the same line of investigation, the extent of oxidative damage to lipids as a function of age and

AD was studied in blood plasma. MDA level was used as the biomarker for lipid peroxidation and the TBA test was used for its measurement. Findings from this study showed that AD had a significant effect on MDA level, an observation supported by previous studies of lipid peroxidation level in AD brain, suggesting oxidative damage may play a role in the pathogenesis of AD (Markesbery, 1997). However, age was shown to have no effect on MDA level in this study, an observation also made by Marcus et al (1998) but contradicted by others (Yu, 1993).

Following on from the study of oxidative damage to DNA and lipids as a function of age and AD, a different approach was taken and the defence system against oxidative stress was investigated. Many studies dealing with the free radical theory of ageing have focused on the possibility that antioxidant levels decrease as a function of age and age-related pathologies. On the other hand, it may simply be a result of lack of antioxidant supply in the diet. It has also been proposed that the defence system may be induced in response to oxidative stress and the antioxidant level may in fact increase with age. This makes interpretation of any antioxidant assay extremely difficult. In this study, glutathione (GSH and GSSG) level and total antioxidant activity in blood plasma were measured. Glutathione is an important antioxidant which has been widely used as a parameter in antioxidant and ageing studies (Flagg et al, 1993; Hu et al, 1996). Findings from this study suggest that neither age nor AD have any effect on GSH level. Conversely, GSSG level was found to be significantly affected by AD but not age, with GSSG level higher in the AD group than in the healthy older group. Oxidative stress has been implicated in AD and this

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may explain the accumulation of glutathione in its oxidised form (Benzi and Moretti, 1995). Other glutathione studies have generated conflicting results (Benzi *et al*, 1988; Sohal *et al*, 1995) and the pattern of change of glutathione level with age and AD is yet to be established.

Interactions between antioxidants are complex and this can create further problems in any antioxidant studies. To avoid the problems posed by assays of individual antioxidants, aggregate measures of antioxidant defence such as the TAA assay have been devised (Rice-Evans and Miller, 1994). Total antioxidant activity (TAA) in blood plasma was measured in this study to take into account the complexity of the defence mechanism. Neither age nor AD was shown to have a significant effect on TAA in general. However, TAA measured in healthy females over 60 was found to be significantly higher than that in healthy females under 30, possibly due to the fact that postmenopausal women seem to have a higher level of plasma uric acid which acts as an antioxidant (Section 6.3). This observation in females is consistent with another study by Aejmelaeus *et al* (1997). As mentioned above, interpretations of data from antioxidant studies are extremely complex and therefore an increase in TAA with age would neither contradict nor prove the free radical ageing of theory.

In recent years, increasing number of investigators have become aware that a major role of HO is not only to degrade haemoglobin from senescent red blood cells, but also to serve as a key enzyme in the modulation of oxidative stress as part of the sophisticated defence system (Abraham *et al*, 1996; Tyrrell, 1997). It has been

demonstrated that HO-1 expression can be induced in response to oxidative stress or various circumstances such as UV light-exposure (Section 1.3.3.3.1). In this study, attempts have been made to study the induction of HO-1 expression in PBMCs after oxidative stress as a function of age and AD using RT-PCR techniques. Difficulties arose during the amplification of total RNA from PBMCs and inconsistent results were obtained within and between samples. Various explanations have been suggested for these poor and inconsistent findings (Section 7.3.1). The main concern is that HO-1 mRNA may be of extremely low abundance in human PBMCs rendering its level undetectable. Moreover, the use of RT-PCR techniques for quantitative purposes is still highly debatable. There is no doubt that HO-1 is an important stress-induced enzyme in the defence system against oxidative stress and its role in ageing and AD is yet to be established.

Despite strict adherence to protocols and the implementation of various measures to avoid artifacts, it is inevitable that factors other than age and AD may have an effect on the findings obtained from this study. Individual variations exist in any investigation that involves humans as study subjects. Due to the limited access to both healthy and AD volunteers, it was impossible to set up strict selection criteria during subject recruitment. As a result, various volunteer parameters such as life style, diet and intake of prescription drugs and antioxidants could not be strictly controlled. A large proportion of elderly volunteers took antioxidants, minerals and other supplements regularly compared to the younger volunteers and this may have an overall effect on our findings, since it is believed that dietary antioxidants can

improve the defence system (Section 1.3.3.3). In addition, all AD patients recruited were involved in AD random drug trial and it was impossible to establish what drugs they were taking if any, as the trials were placebo-controlled. It is unknown whether these AD trial drugs have had any effect on the defence status of individuals.

Many studies have been carried out to explore the effect of age on both the levels of markers of oxidative damage and the basal levels of various antioxidants (Lopez-Torres *et al*, 1994; Barnett and King, 1995; Mendoza-Nuñez *et al*, 1999). Findings from these studies and the present study do not suggest a consistent pattern of change for either antioxidants or oxidative-damage-related parameters with age. In this study, a higher level of DNA damage was found in the healthy younger group. In contrast, age was found to have no effect on lipid peroxidation. Moreover, the antioxidant defence system investigated in this work was found to be unaffected by age. Similarly, oxidative damage and antioxidant defence have previously been studied in AD patients (Volicer and Crino, 1990; Zaman *et al*, 1992; Marcus *et al*, 1998). Increased peroxidation, reduced antioxidant enzyme activity and lowered antioxidant levels have been reported. Findings from the present study are consistent with these previous studies, as lipid peroxidation and GSSG level were found to be higher in AD patients. However, AD was shown to have no effect on cellular DNA damage and total antioxidant capacity in plasma. The role of HO-1 expression in ageing and AD has yet to be explored. In conclusion, findings from this study demonstrate the complexities inherent in the free radical theory of ageing, with some data supporting the theory and others contradicting it. The effect of age and AD on

various parameters which measured either oxidative damage or antioxidant status varied and more work will have to be carried out to increase our understanding of the complex relationship of age and AD with oxidative stress.

8.2 Future work

Findings from this work are informative but by no means conclusive. Nevertheless, this work has provided a valuable insight into the possible relationships of age and AD with oxidative stress. Moreover, this study has emphasised the need for accurate, reliable and sophisticated techniques for oxidative-damage and antioxidant studies. For example, the sensitivity of the comet assay used to study oxidative damage to DNA could be improved by the inclusion of a digestion step with lesion-specific endonucleases. Moreover, combining the manual comet analysis method with sophisticated image analysis system and investigating different comet parameters can improve the accuracy of the assay (Section 3.3.5). Also, it would be interesting to investigate if H_2O_2-induced DNA damage is evenly distributed among all PBMCs, or limited to a functional subpopulation by measuring levels of glutathione peroxidase or catalase in different populations of cells. For the study of lipid peroxidation, it would be beneficial to measure different parameters using two or more methods since MDA is only one of the many by-products of lipid peroxidation (Section 4.3). Similarly, combining the fluorometric assay with the more reliable HPLC assay to determine glutathione may be useful in the future (Section 5.1). It would also be helpful to explore the identity and the proportion of

antioxidants that contribute to the plasma TAA. Also, a post addition assay described by Strube *et al* (1997) can be carried out to discriminate between the scavenging and the inhibitory effect of plasma antioxidants (Section 6.3). Lastly, the use of RT-PCR techniques to measure HO-1 expression has yet to be established and it would be prudent to consider different sets of primers, alternative methods or alternative sources of starting material for future studies (Section 7.3.1). It should be emphasised that the levels of parameters measured in blood plasma and PBMCs may or may not correlate with that obtained from other areas of the body. It would therefore be useful to compare these levels in different cell types e.g. AD brain cells or in different biological fluids e.g. serum. In addition, the limited availability of PBMCs can be overcome by using a cultured cell line, for example, fibroblasts which have been extensively used as a model for studying the process of cellular senescence. Fibroblasts can be passaged in culture over many generations and they eventually reach a stage at which they remain viable but are permanently unable to replicate and such cells are considered senescent. It should also be emphasised that all our observations are made using *in vitro* systems and they do not necessary reflect *in vivo* situations. Therefore, the physiological significance of the *in vitro* results should be confirmed by *in vivo* studies. Finally, a larger population size and more stringent selective criteria will improve the significance of this type of study. The power of a hypothesis test can be used to calculate the appropriate sample size for a study or clinical trial providing that the smallest true clinically valuable difference between the treatments can be specified. It is this requirement that is somewhat artificial and difficult to define and this has to be established by general consent of

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scientists in the area of research. Data from this study form a good base for the determination of sample size for similar future studies.

Whatever the eventual consensus on the cause(s) of the ageing process, attempts to minimise free radical reaction damage in humans are likely to increase the functional life-span. It is reasonable to expect the application of the free radical theory of ageing will decrease the morbidity and mortality due to degenerative diseases and non-specific age changes and possibly also slow down ageing. Slower ageing will benefit society since the additional years of use of the skills gained over a lifetime should increase productivity. More importantly, slower ageing will help to fulfill man's natural desire for a longer healthy life.

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FREE RADICALS & AGEING STUDY

INFORMATION SHEET

RESEARCH

Free radicals are chemical entities produced in the body which can cause tissue and cell damage by attacking cell proteins, DNA and lipids by the process of oxidation. They are implicated in causing various diseases including cancers, Alzheimer's disease, etc. They are also thought to be involved in the ageing process. These entities are produced as a result of normal body function but they can also be produced when the body is exposed to sunlight, radiation and certain chemicals such as hydrogen peroxide.

The body has many defence systems, which can deactivate free radicals. Some people believe we can obtain a certain degree of protection through a healthy diet and by taking vitamin supplements. The body also has repair mechanisms to repair the damages caused by these free radicals.

We propose to study these oxidative damages and defence systems in the body in people of different age groups to see if they vary with age or disease.

HOW YOU CAN HELP

We would be grateful if you would donate a small amount of your blood (45 ml) to assist us in this research. The blood will be collected at the Research Institute for the Care of Elderly at St. Martin's Hospital, Bath. The Bath Local Ethics Committee has approved this research. Please fill in the consent form enclosed if you can help.

Your help would be very much appreciated.

Peggy Ho MRPharmS
PhD Research Student

Free radicals and ageing study consent form

Have you read the information sheet?   Yes   No

Have you had any opportunity to discuss this and ask questions?

                                         Yes   No

Have you received satisfactory answers to all your questions?

                                         Yes   No

Have you received enough information about the study?

                                       Yes   No

Do you understand that you are free to refuse to participate or change your mind at any time without giving any reason?

                                       Yes   No

Do you agree to take part in this study by donating 45 ml blood?

                                       Yes   No

VOLUNTEER

Signature: _____   Date: _____

Name: _____

WITNESS

Signature: _____   Date: _____

Name: _____

Thank you for participating in this study.

APPENDIX II QUESTIONNAIRE

QUESTIONNAIRE

Name: _____

Date of Birth: _____

Sex: _____

Ethnic Origin: _____

<u>Diet</u>:

Are you a vegetarian?   Yes   No

How many portions of vegetables do you have daily?   _____

<u>Smoking</u>:

Do you smoke?   Yes   No

If Yes, how many cigarettes do you smoke daily?   _____

<u>Exercise</u>: (1 session = 30 minutes, includes walking, gardening, aerobics, etc.)

None
Low (at least 1 session a week)
Moderate (at least 2 sessions a week)
High (at least 3 sessions a week)

<u>Medical History</u>:

Do you have any known medical condition(s)?   Yes   No

If Yes, please give details: _____

<u>Drug History</u>:

Are you currently taking any drugs (prescribed drugs or over the counter drugs such as aspirin, paracetamol, etc.)?

Yes   No

If Yes, please give details: _____

Do you take vitamins or mineral supplements regularly?   Yes   No

If Yes, please give details: _____

Would you like to participate in similar research in the future?

Yes   No

If Yes, please give us your contact details:

Address: _____

_____

Telephone: _____

All information will be treated as confidential.

Thank you for your participation in this research.

Peggy Ho MRPharmS
PhD Research Student

APPENDIX III SUMMARY OF INFORMATION ON ALL VOLUNTEERS

Abbreviations applied to Appendix III

Y – Yes   N – No

VB – Vitamin B   VC – Vitamin C   VE – Vitamin E   MV – Multivitamins

Ca – Calcium   Mg – Magnesium   MM – Multi-minerals   CLO – Cod liver oil

EPO – Evening primrose oil   GP – Garlic oil   HO – Halibut oil   Q10 – Coenzyme Q10

C – Contraceptive pills   GTN – Glyceral trinitrate   HRT – Hormone replacement therapy

NSAIDs – Non-steroidal anti-inflammatory drugs   Do – Donepezil Do_p – Placebo for Donepezil trial

Ga – Galanthamine   Pr – Propentofylline   T-588 – active drug for Toyama trial

$T-588_P$ – Placebo for Toyama trial   AD – Alzheimer's disease   MI   –   Myocardial infarction

HC – Hyperchlolesterolemia   HH – Hiatus hernia   HT – Hypertension

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health problems
1	N	N	N	N	N
2	N	N	N	N	N
3	Y	N	N	Paroxetine, illegal drug use	Depression
4	N	MV	N	N	N
5	N	N	N	N	Asthma, eczema
6	N	N	N	N	N
7	N	N	N	N	N
8	Y	N	N	N	N
9	N	N	N	Salbutamol	Asthma
10	N	N	N	N	N
11	N	N	N	Ibuprofen	N
12	N	N	N	N	N

A. Summary of information on 12 male healthy volunteers age < 30.

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health problems
13	N	N	N	N	N
14	N	MV	N	C	N
15	N	N	N	N	N
16	Y	N	N	C	Irritable bowel syndrome
17	N	VC	N	N	N
18	N	N	N	N	N
19	N	N	N	C	N
20	N	N	N	N	N
21	N	N	N	N	N
22	N	N	N	N	N
23	N	N	EPO	C	N
24	N	N	Iron	C	N
25	N	VC	N	N	N

B. Summary of information on 13 female healthy volunteers age < 30.

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health Problems
1	N	N	N	Aspirin	Angina
2	N	N	N	N	N
3	N	N	N	N	N
4	N	N	N	N	Padget's disease
5	N	MV	MM	N	Diabetic
6	N	N	N	N	N
7	N	MV	N	Perindopril	HC, HT

C. Summary of information on 7 male healthy volunteers age > 60.

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health Problems
8	N	N	N	N	N
9	N	N	CLO	N	HH
10	N	N	N	N	N
11	N	N	CLO	Terbinafine	N
12	Y	VC	CLO	N	N
13	N	N	N	Ibuprofen	Osteoarthritis, thrombosis, HH
14	N	VC	Ca, CLO	Aspirin, bendrofluazide, lorsortan	Osteoporosis, HT
15	N	N	Iron, EPO	Atenolol, bendrofluazide	Diverticular disease, HH
16	N	N	N	Aspirin	Kidney stone
17	N	N	N	N	HH
18	N	N	CLO	N	N
19	N	N	N	Analgesics, NSAIDs	Arthritis
20	N	VE	CLO	Aspirin, bendrofluazide	HT
21	N	VC, MV	Ca, Mg, GO, Q10	Aspirin, digoxin, metformin, sotalol	Diabetic, HH
22	Y	MV	HO	Thyroxine, HRT	N
23	N	N	N	N	N
24	N	N	CLO, GO	N	N
25	N	N	N	N	N

D. Summary of information on 18 female healthy volunteers age > 60.

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health problems
1	N	N	N	Ibuprofen, co-amilofruse, Do	Arithritis, AD, HT
2	N	N	N	Oxybutanin, Ga	Adeno-carcinoma, AD
3	N	N	N	Do	AD
4	N	N	N	T-588$_p$	AD
5	N	N	N	Aspirin, metoprolol, isosorbide mononitrate, Do	Angina, AD
6	N	N	N	Do	AD
7	N	N	N	T-588$_p$	AD
8	N	N	CLO	Aspirin, bendrofluazide, atenolol, T-588$_p$	AD, HT
9	N	N	N	Diuretic, quinine, temazepam, Do	AD
10	N	MV	N	Aspirin, captopril, isosorbide mononitrate, GTN	Polymalgia rheumatica, AD, MI
11	N	N	N	Beconase, T-588	AD

E. Summary of information on 11 male Alzheimer's volunteers age > 60.

Subject	Smoker	Vitamins/ antioxidants	Minerals & other supplements	Drugs	Health problems
12	N	N	N	T-588	AD
13	N	N	N	Do	AD
14	N	MV	N	Gaviscon, nizatidine, setraline, Do_p	AD, HH
15	N	N	Iron, Ca	$T-588_p$	Arthritis, spondylitis, AD
16	N	N	CLO	Aspirin, Do	AD
17	N	N	N	Terbutaline, Pr	AD
18	N	N	N	Aspirin, paracetamol, Do	Migraine, AD
19	N	MV	Ca	Paracetamol, T-588	Osteoporosis, AD
20	N	VB	N	Aspirin, omeprazole, fluoxetine, Pr? .	AD, HH
21	N	N	N	Do	AD
22	N	N	Gingko-bilboa	Quinine, Do	AD
23	N	N	N	Aspirin, atenolol, amlodipine, nitrazepam, simvastatin, Do	Angina, AD, MI, HC
24	N	N	N	Paracetamol, Do	Arthritis, AD
25	N	N	N	Do	AD

F. Summary of information on 14 female Alzheimer's volunteers age > 60.